



12th Biennial Meeting
Society for Research on Biological Rhythms
Program and Abstracts

SRBR

May 22–26, 2010

Sandestin Golf and Beach Resort
Destin, Florida

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Conference Arrangements



Office of Continuing Education
901 West University Avenue,
Suite 101

Urbana, IL 61801
Phone: 217-333-2880
E-mail: srbrconf@ad.uiuc.edu

SRBR Thanks Our Sponsors



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President's Welcome

The Society for Research on Biological Rhythms was founded in 1986 and 2010 marks the 24th anniversary of SRBR—a special date in circadian numerology! For this year, we look forward to another vibrant meeting continuing our tradition of leading-edge science, camaraderie, and a beautiful venue. We are the beneficiaries of months of planning and work by many people. Program Chair Ignacio Provencio and his committee have put together an outstanding set of scientific sessions, diverse in form and content. Nicolas Cermakian and his committee have organized the SRBR Trainee Professional Development Day and overseen trainee awards for research accomplishment. Frank Scheer and his committee have continued to revitalize the SRBR Web site. Just as important, Michael Nitabach and his committee on SRBR Bylaws and Incorporation have completed the task of revising the Bylaws and have paved the way for the re-incorporation of SRBR. Paul Hardin and Rebecca Prosser have taken on the task of the re-incorporation of SRBR, which is now complete. With the adoption of the revised SRBR Bylaws, the Society begins a new era as a mature entity poised for future growth in the field. We owe a great debt to the efforts of our past presidents, William Schwartz and Martha Gillette, for laying the foundation for the re-incorporation and stabilization of SRBR. Finally, I thank all the members of the Executive Committee who have worked so diligently over the last two years on behalf of the Society.

The SRBR Biennial Meeting is an opportunity for convening the Board of Directors, our standing committees on Animal Issues, chaired by Laura Smale; on ChronoHistory, chaired by Anna Wirz-Justice; and on Communications, chaired by Frank Scheer; as well as the Editorial Board of the *Journal of Biological Rhythms*, chaired by Martin Zatz. Results of the election of the new SRBR board, coordinated by Martha Gillette, chair of the Nominations Committee, will be announced at the Business Meeting on Wednesday.

Special thanks are due Michelle Chappell and Michelle Marquart at Conferences & Institutes of the University of Illinois, for their skill in organizing this event and coordinating with Sandestin. We are deeply grateful to our corporate and government sponsors for their generous support. Their names are listed at the front of this program.

I extend my thanks to all of you, presenters and participants, for sharing your ideas, energy, opinions, and wisdom to make the meeting such a success.

Best wishes for a great meeting!

Joseph S. Takahashi, President, SRBR, 2008–2010

General Information

Headquarters is at the Baytowne Conference Center, which is conveniently located within walking distance of all hotel rooms.

SRBR Information Desk and Message Center is in the Foyer of the Baytowne Conference Center main level.

The desk hours are as follows:

Friday 5/21		2:00–6:00 PM
Saturday 5/22		2:00–8:00 PM
Sunday 5/23	7:00–11:00 AM	4:00–7:00 PM
Monday 5/24	7:30–11:00 AM	4:00–6:00 PM
Tuesday 5/25	8:00–11:00 AM	4:00–6:00 PM
Wednesday 5/26	8:00–10:00 AM	

Messages can be left on the SRBR message board next to the registration desk. Meeting participants are asked to check the message board routinely for mail, notes, and telephone messages.

Hotel check-in will be at the individual properties.

Posters will be available for viewing in the Magnolia B/C/D/E rooms.

Poster numbers 1–93	Sunday, May 23, 10:00 AM–10:30 PM
Poster numbers 94–183	Monday, May 24, 10:00 AM–10:30 PM
Poster number 184–275	Tuesday, May 25, 10:00 AM–10:30 PM

Poster set-up is between 8:00 and 10:00 AM on the day of your poster session. Posters must be taken down at the conclusion of your poster session.

The Village of Baytowne Wharf—Indulge your senses at Sandestin’s charming Village of Baytowne Wharf, a picturesque pedestrian village overlooking the Choctawatchee Bay. Discover a unique collection of more than 40 specialty merchants ranging from quaint boutiques and intimate eateries to lively nightclubs, all set up against a backdrop of vibrant special events.

Special Events

Saturday, May 22

Trainee Professional Development Day • 9:00 AM–5:00 PM • Bayside & Linkside Conference Centers

The Trainee Professional Development Day is an entire day devoted to scientific and career development activities for trainees. The day consists of a keynote address, an activity consisting of one-on-one blitz discussions, and a series of workshops on various topics. The goal of the Trainee Professional Development Day is to allow the next generation of biological rhythm researchers to learn from and interact with faculty members in a more informal and intimate setting.

Trainees should attend the workshops they preselected during the registration process.

Welcome Reception • 7:00–9:00 PM, Grand Lawn

In case of inclement weather, the reception will be held in the Azalea Ballroom.

Sunday, May 23

Meet the Professors • 10:30–11:00 AM daily • *Magnolia A* • All trainees welcome to attend

(See “Program Overview” pages 11–22 for more details)

Meet the Professor Sessions are meant to provide trainees (students and postdocs) the opportunity to interact with experienced faculty members in the field and to foster scholarly conversation. Each day a number of faculty researchers (see Program Overview for a listing of professors) will make themselves available to meet with trainees, in a dedicated room of the conference center. Any trainee interested in meeting these investigators can go to *Magnolia A* and be part in this informal gathering.

Town Hall Meeting on the future of NIH grant review • 3:00–4:00 PM • *Camellia I & II*

Monday, May 24

Executive Committee Meeting • 12:30–2:30 PM • *Jasmine*

Presidential Special Symposium: Sydney Brenner, Nobel Laureate • 4:30–6:00 PM • *Azalea Ballroom*

Tuesday, May 25

JBR Editors’ Meeting • 2:00–4:00 PM • *Jasmine*

Wednesday, May 26

Business Meeting • 4:00–5:00 PM • *Azalea Ballroom*

All attendees are invited to attend

Pittendrigh/Aschoff Lecture: Michael Rosbash, Brandeis University • 5:30–6:30 PM • *Azalea Ballroom*

Cocktail Reception • 7:00 PM • *Magnolia Foyer*

Cash bar

Closing Banquet • 8:00 PM • *Magnolia Ballroom*

All guests accompanying participants to the banquet need to purchase a ticket in advance at the registration desk.

Meeting at a Glance

All events will take place in the Baytowne Conference Center, with the exception of the Trainee Professional Development Day. It will be held at the Bayside & Linkside Conference Centers.

Saturday, May 22

9:00 AM–5:00 PM **Trainee Professional Development Day** • Bayside & Linkside Conference Centers
7:00–9:00 PM **Welcome Reception** • Grand Lawn

Sunday, May 23

8:00–10:00 AM **Poster Setup (P1–P93)** • Magnolia B/C/D/E
8:30–10:30 AM **Symposium 1: Transcriptional Regulation of Circadian Clocks** • Azalea Ballroom
Symposium 2: Circadian Neural Networks • Camellia I & II
10:30–11:00 AM **Refreshment Break** • Magnolia Foyer
Meet the Professors • Magnolia A
11:00 AM–12:30 PM **Slide Sessions**
 A: S1–S6 • Azalea Ballroom
 B: S7–S12 • Camellia I & II
 C: S13–S18 • Magnolia F
12:30–4:30 PM **Free Time**
3:00–4:00 PM **Town Hall Meeting on the future of NIH grant review** • Camellia I & II
4:30–6:30 PM **Symposium 3: Circadian Clocks and Sleep** • Azalea Ballroom
Symposium 4: Entrainment of Clocks • Camellia I & II
Symposium 5: The Transcription/Translational Feedback Model in Eukaryotes and Prokaryotes • Magnolia F
8:00–10:30 PM **Poster Session I (P1–P93)** • Magnolia B/C/D/E

Monday, May 24

8:00–10:00 AM **Poster Setup (P94–P183)** • Magnolia B/C/D/E
8:30–10:30 AM **Symposium 6: Seasonal and Reproductive Rhythms** • Camellia I & II
Symposium 7: Clocks and the Immune System • Azalea Ballroom
10:30–11:00 AM **Refreshment Break** • Magnolia Foyer
Meet the Professors • Magnolia A
11:00 AM–12:30 PM **Slide Sessions**
 D: S19–S24 • Azalea Ballroom
 E: S25–S30 • Camellia I & II
 F: S31–S36 • Magnolia F
12:30–2:30 PM **Executive Committee Meeting** • Jasmine
12:30–4:30 PM **Free Time**

4:30–6:00 PM **Presidential Special Symposium: Sydney Brenner, Nobel Laureate •**
Azalea Ballroom

8:00–10:30 PM **Poster Session II (P94–P183) •** *Magnolia B/C/D/E*

Tuesday, May 25

8:00–10:00 AM **Poster Setup (P184–P274) •** *Magnolia B/C/D/E*

8:30–10:30 AM **Symposium 8: Ionic Mechanisms Underlying Circadian Oscillations •**
Camellia I & II

Symposium 9: Peripheral Circadian Clocks • *Azalea Ballroom*

10:30–11:00 AM **Refreshment Break •** *Magnolia Foyer*

Meet the Professors • *Magnolia A*

11:00 AM–12:30 PM **Slide Sessions**

G: S37–S42 • *Azalea Ballroom*

H: S43–S48 • *Magnolia F*

I: S49–S54 • *Camellia I & II*

2:00–4:00 PM **JBR Editors' Meeting •** *Jasmine*

12:30–4:30 PM **Free Time**

4:30–6:30 PM **Symposium 10: Post-translational Regulation of Circadian Clocks •**
Azalea Ballroom

Symposium 11: Rhythms in Space or Altered Gravity • *Magnolia F*

Symposium 12: Circadian Rhythms and Disease • *Camellia I & II*

8:00–10:30 PM **Poster Session III (P184–P274) •** *Magnolia B/C/D/E*

Wednesday, May 26

8:30–10:30 AM **Symposium 13: Comparative Clocks •** *Magnolia F*

Symposium 14: Interplay between Circadian Clocks and Metabolism •
Camellia I & II

Symposium 15: Systems Biology of Circadian Rhythms • *Azalea Ballroom*

10:30–11:00 AM **Refreshment Break •** *Magnolia Foyer*

Meet the Professors • *Magnolia A*

11:00 AM–12:30 PM **Slide Sessions**

J: S55–S60 • *Magnolia F*

K: S61–S66 • *Camellia I & II*

L: S67–S72 • *Azalea Ballroom*

12:30–4:00 PM **Free Time**

4:00–5:00 PM **Business Meeting •** *Azalea Ballroom*

5:30–6:30 PM **Pittendrigh/Aschoff Lecture: Michael Rosbash, Brandeis University •**
Azalea Ballroom

7:00 PM **Cocktail Reception •** *Magnolia Foyer*

8:00–11:00 PM **Closing Banquet •** *Magnolia Ballroom*

Trainee Professional Development Day

- 8:00 AM **Bus departs from Grand Sandestin, approximately every 10–15 minutes. Last bus will depart at approximately 9:00 AM.**
- 8:00 AM **Registration • Bayside Ballroom**
- 9:00–9:20 AM **Welcome and Orientation • Bayside Ballroom**
- 9:20–10:20 AM **Keynote Address • Michael Menaker, University of Virginia, Charlottesville, VA, USA • Bayside Ballroom**
Trainees should attend the workshops they preselected during the registration process.
- 10:35–11:25 AM **Session 1**
- The peer review process and how to get published • Linkside Ballroom A**
Martin Zatz, Editor, *Journal of Biological Rhythms*, Bethesda, MD, USA
This workshop will address a range of issues to enhance your chances of getting articles published in peer-reviewed journals, including: how to choose the most appropriate journal for your work, effectively write and publish data papers and review papers, what reviewers and editors are looking for, and responding to reviewer comments.
- The transition from postdoc to independent research • Linkside Ballroom B**
Megan Mahoney, University of Illinois, Urbana, IL, USA & Erik Herzog, Washington University, St. Louis, MO, USA
This workshop will address the following questions: 1) How to prepare to ensure the smoothest transition possible between postdoc and independent research positions? 2) What are the crucial steps to successfully initiate an independent research program?
- Post-doc position: the right place and environment to achieve your goals • Linkside Ballroom C**
Steven Brown, University of Zurich, SWITZERLAND & Valérie Mongrain, University of Montréal, CANADA
This workshop will discuss how to select a research laboratory and the right mentor for postdoctoral training. The importance of temporal aspects, the choice of a good scientific field, and the purpose of postdoctoral training will be addressed. Options of switching research fields (advantages and disadvantages), and doing one versus two postdocs, will also be presented.
- Genetic and molecular approaches for rhythms—Mice/Humans • Linkside Ballroom D**
Till Roenneberg, Ludwig-Maximilians-University, Munich, GERMANY
This workshop will give an overview of genetic and molecular experimental approaches to studying circadian rhythms in mice and humans. The design and approach to human studies within the circadian field will also be addressed.

Basic methodology to study human circadian rhythms • Terrace I & II

Debra Skene, University of Surrey, Guildford, UNITED KINGDOM

This workshop is aimed at those new or not familiar with human chronobiology studies, and will provide an overview of the experimental paradigms and techniques currently used to investigate circadian rhythms in humans.

11:40 AM–12:30 PM

Session 2

Basics of chronobiology • Linkside Ballroom A

Michael Menaker, University of Virginia, Charlottesville, VA, USA

Chronobiology relies on a number of fundamental concepts, many of which are unique in science. This workshop will provide the basic concepts and terminology of biological rhythms, including the notions of free-running rhythms, entrainment, temperature compensation, output pathways, masking, and so forth.

Balancing family and academic career + being a woman in science • Terrace I & II

Theresa Lee, University of Michigan, Ann Arbor, MI, USA; Mary Harrington, Smith College, Northampton, MA, USA; & Nicolas Cermakian, McGill University, Montréal, CANADA

This workshop will cover two topics: 1) finding the right balance between the requirements of an academic career and the personal aspects of life, and 2) the challenges women often face as scientists, compared to their male colleagues, concerning job recruitment, laboratory setup and running, teaching, interaction with peers, impact of personal (family) life. The speakers will briefly present their personal experience, followed by discussion with the audience.

Developing and maintaining records of research performance + interview skills • Linkside Ballroom B

David Weaver, University of Massachusetts Medical Center, Worcester, MA, USA & Kenneth Wright, University of Colorado–Boulder, Boulder, CO, USA

This workshop will cover two topics: 1) Tactics to make a good impression during a job interview and presentation to prospective employers, from the candidate and employer points of view; 2) The importance of creating and appropriately maintaining records of your research/academic performance, such as your CV.

Mathematical modeling and integration with experimental data • Linkside Ballroom C

Hans-Peter Herzel, Humboldt University, Berlin, GERMANY

This workshop will address the basics of mathematical modeling and how it can be applied to aspects of the circadian system (gene expression feedback in circadian dynamics, coupled oscillators, cell networks, etc.). Moreover, the integration of modeling with laboratory experimental approaches will be presented to highlight how models can yield testable hypotheses and in turn, how experimental data can help refine models.

Short- and long-term research program planning • *Linkside Ballroom D*

Rae Silver, Columbia University, New York, NY, USA & Frank AJL Scheer, Harvard Medical School, Boston, MA, USA

This workshop will discuss the strategies of short-term (1–5 year implementation period) and long-term (10–20 years into the future) research program planning. Aspects specific to basic rhythms research/animal models and to human chronobiology will be included in the discussion.

12:30–1:30 PM

Lunch • *Bayside Ballroom*

1:30–2:30 PM

“Positive feedback looping” (random, blitz, one-on-one meetings) • *Bayside Ballroom*

2:45–3:35 PM

Session 3

Electrophysiology • *Terrace I & II*

Stephan Michel, Leiden University Medical Center, Leiden, THE NETHERLANDS

Electrophysiological recording techniques are invaluable when studying key aspects of entrainment, synchronization, and intercellular communication of clock neurons. This workshop will provide the basics of electrophysiology, and how it can be applied to the study of circadian rhythms.

Work in the industry as an alternative career path • *Linkside Ballroom A*

Anthony Gotter, Merck Research Laboratories, West Point, PA, USA

This workshop will include an overview of working in industry following completion of your graduate/postdoc work, and a comparison of research in an industry setting versus an academic setting.

Making effective scientific presentations • *Linkside Ballroom B*

Michael Hastings, MRC Laboratory of Molecular Biology, Cambridge, UNITED KINGDOM

This workshop will cover the critical points to consider when presenting research projects, including: structure and content of the presentation, selection of the material to present, strategies for different target audiences, and appropriate use of technology.

Genetic and molecular approaches for rhythms – Mice/Humans •

Linkside Ballroom C

Till Roenneberg, Ludwig-Maximilians-University, Munich, GERMANY

This workshop will give an overview of genetic and molecular experimental approaches to study circadian rhythms in mice and humans. The design and approach to human studies within the circadian field will also be addressed.

From cyanobacteria to humans: Biological models in circadian biology •

Linkside Ballroom D

Carl Johnson, Vanderbilt University, Nashville, TN, USA ; Amita Sehgal, University of Pennsylvania, Philadelphia, PA, USA; & Paul Bartell, Pennsylvania State University, University Park, PA, USA

This workshop will provide a perspective on the use of different model organisms in circadian rhythms research, their advantages, similarities, differences, and specificities. Specific examples from each researcher's work

will highlight several model organisms, and discussion will aim at defining the importance of choosing the right models to answer biological questions in the field of chronobiology.

3:50–4:40 PM

Session 4

Genetic and molecular approaches for rhythms – Flies/*Neurospora* •

Linkside Ballroom A

Jay Dunlap, Dartmouth Medical School, Hanover, NH, USA & Amita Sehgal, University of Pennsylvania, Philadelphia, PA, USA

This workshop will introduce work on *Drosophila* and *Neurospora* circadian rhythms, and will provide an overview of the genetics and biochemistry of these model systems and the techniques used to investigate their circadian rhythms.

Imaging of luminescent and fluorescent reporter models • *Linkside Ballroom B*

David Welsh, University of California–San Diego, La Jolla, CA, USA

Over the past decade, the circadian field has taken advantage of luminescence and fluorescence imaging techniques to follow circadian rhythms in live cells, tissues, and organisms. These imaging techniques have revealed important aspects of clock mechanism and function. This workshop will describe these models and how such technology can be used in chronobiology.

Developing and maintaining records of research performance + Interview skills

• *Linkside Ballroom C*

David Weaver, University of Massachusetts Medical Center, Worcester, MA, USA & Kenneth Wright, University of Colorado–Boulder, Boulder, CO, USA

This workshop will cover two topics: 1) Tactics to make a good impression during a job interview and presentation to prospective employers, from the candidate and employer points of view; 2) The importance of creating and appropriately maintaining records of your research/academic performance, such as your CV.

Grantsmanship: dos and don'ts in grant writing • *Linkside Ballroom D*

Erik Herzog, Washington University, St. Louis, MO, USA

This workshop will discuss the important art of grantsmanship and the necessary components of successful grant writing, including effective articulation of specific aims, hypotheses, study rationale, and study design. Possible weaknesses of grant proposals from a reviewer's point of view will also be highlighted.

Networking with other researchers • *Terrace I & II*

Martha Merrow, University of Groningen, Haren, THE NETHERLANDS

This workshop will discuss approaches to communication, idea exchange, collaboration, and engaging in social interactions with other members of the scientific community.

4:50–5:00 PM

Conclusion • *Bayside Ballroom*

5:00 PM

Buses depart for Grand Sandestin

Program Overview

Saturday, May 22

7:00–9:00 PM **Opening Reception • Grand Lawn**

Sunday, May 23, 2010

8:00–10:00 AM **Poster Session Setup (P1–P93) • Magnolia B/C/D/E**

8:30–10:30 AM **Symposium 1—Transcriptional Regulation of Circadian Clocks • Azalea Ballroom**

Chair: Stacey Harmer, University of California–Davis

8:30 **The molecular mechanism of photoadaptation and light entrainment of the *Neurospora* clock**
Michael Brunner, Heidelberg University

9:00 **Novel approaches for studying circadian transcription in cells and organs**
Ueli Schibler, University of Geneva

9:30 **Molecular mechanism of the *Drosophila* clock**
Amita Sehgal, HHMI/University of Pennsylvania School of Medicine

10:00 **Identification of a new circadian component using data mining**
Stacey Harmer, University of California–Davis

Symposium 2—Circadian Neural Networks • Camellia I & II

Chair: Fernanda Ceriani, Leloir Institute Foundation-Buenos Aires

8:30 **CRYPTOCHROME is a cell autonomous neuronal blue light sensor that rapidly regulates neuronal firing rate**
Todd Holmes, University of California–Irvine

9:00 **Accessing neural connectivity in the *Drosophila* circadian clock network**
Orie Shafer, University of Michigan

9:30 **Complex electrical states of SCN neurons**
Hugh Piggins, University of Manchester

10:00 **A parallel circadian system: Making sense of olfactory clocks**
Erik Herzog, Washington University

10:30–11:00 AM **Refreshment Break • Magnolia Foyer**

Meet the Professors • Magnolia A

Steve Brown (Rodents, humans, peripheral clocks, clock genes)

Martha Gillette (Rodents, SCN, signaling, coupling peptides)

Carla Green (Rodents, clock output)

Paul Hardin (*Drosophila*, clock genes, sensory system rhythms)

Erik Herzog (Rodents, neuronal circuits, SCN)

John Hogenesch (Systems biology)

Louis Ptacek (Humans, genetics of sleep, and circadian rhythmicity)

Ueli Schibler (Rodents, clock genes, peripheral clocks)

Slide Session A • Azalea Ballroom

Chair: Martin Ralph, University of Toronto

- 11:00 **S1 • USP2, a de-ubiquitinating enzyme, directly regulates BMAL1 stability and sensitivity to early evening light**
Heather Scoma, CBNA, Medical College of Wisconsin
- 11:15 **S2 • The deubiquitinating enzyme USP2 is involved in the regulation of circadian rhythms**
Adeline Rachalski, Laboratory of Molecular Chronobiology, Douglas Mental Health Institute
- 11:30 **S3 • Circadian rhythms in astrocytes depend on intercellular interactions and connexin 43**
Luciano Marpegan, Biology, Washington University
- 11:45 **S4 • Regulation of circadian period by neuronal Agrin and the $\alpha 3$ isoform of Na^+/K^+ -ATPase (ATP1A3)**
Martin Ralph, Psychology, University of Toronto
- 12:00 **S5 • Circadian synaptic plasticity in hypocretin axons is regulated by neuronal pentraxin**
Lior Appelbaum, Stanford University
- 12:15 **S6 • Cell membranes and the *Arabidopsis* circadian clock**
Harriet McWatters, University of Oxford

Slide Session B • Camellia I & II

Chair: Frank Weber, University of Heidelberg

- 11:00 **S7 • Novel small molecules as potent enhancers and modulators of the circadian clock**
Zheng (Jake) Chen, Biochemistry & Molecular Biology, University of Texas Health Science Center at Houston
- 11:15 **S8 • Identification and characterization of inhibitors of casein kinase epsilon/delta**
James Offord, Neuroscience Research Unit, Pfizer Global Research
- 11:30 **S9 • Therapeutic rescue of disrupted circadian behavior through CK1d inhibition**
David Bechtold, University of Manchester
- 11:45 **S10 • Who's ubiquitinatin' whom: Partnering proteasomal machinery with the clock**
Jason DeBruyne, Pharmacology/ITMAT, University of Pennsylvania School of Medicine
- 12:00 **S11 • A sequence of specific phosphorylation events controls a core post-translational interval-timer of the *Drosophila* circadian clock**
Frank Weber, Biochemistry Center Heidelberg (BZH), University of Heidelberg
- 12:15 **S12 • PSEUDO-RESPONSE REGULATORS 9 (PRR9), PRR7, and PRR5 are transcriptional repressors in the *Arabidopsis* circadian clock**
Norihito Nakamichi, Plant Productivity Systems Research Group, RIKEN Plant Science Center

Slide Session C • *Magnolia F*

Chair: Nelson Chong, University of Leicester

- 11:00 **S13 • Obesity and metabolic syndrome in mice with an adipose tissue-specific deletion of *Bmal1***
Georgios Paschos, ITMAT, University of Pennsylvania
- 11:15 **S14 • The role of CREM/ICER in circadian events of the liver**
Damjana Rozman, Centre for Functional Genomics and Bio-Chips, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana
- 11:30 **S15 • Disruption of peripheral circadian timekeeping in a mouse model of Huntington's disease and its restoration by temporally scheduled feeding**
Akhilesh Reddy, Department of Clinical Neurosciences, University of Cambridge
- 11:45 **S16 • Endogenous circadian rhythm in cardiovascular biomarkers during rest and in reactivity to standardized exercise**
Frank AJL Scheer, Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School
- 12:00 **S17 • Melatonin protects isolated ventricular cardiomyocytes from high glucose induced arrhythmias**
Nelson Chong, Cardiovascular Sciences, University of Leicester
- 12:15 **S18 • Relationship of common polymorphisms in *Clock* to blood pressure and stroke outcome in man**
Madhu J. Prasai, Division of Cardiovascular and Diabetes Research, LIGHT Laboratories, University of Leeds

12:30–4:30 PM

Free Time

3:00–4:00 PM

Town Hall Meeting on the future of NIH grant review • *Camellia I & II*

4:30–6:30 PM

Symposium 3—Circadian Clocks and Sleep • *Azalea Ballroom*

Chair: Ravi Allada, Northwestern University

- 4:30 **A cell cycle gene regulates sleep in *Drosophila***
Mike Young and Dragana Rogulja, Rockefeller University
- 5:00 **Clocks and sleep: Insights of human genetics**
Louis Ptacek, University of California, San Francisco and HHMI
- 5:30 **The photic regulation of sleep**
Russell Foster, Oxford University
- 6:00 **Sleep timing and duration: Genetic and epidemiological aspects**
Till Roenneberg, Ludwig-Maximilians-Universität

Symposium 4—Entrainment of Clocks • *Camellia I & II*

Chair: Karl Obrietan, Ohio State University

- 4:30 **Temperature-controlled daily rhythms in *Drosophila***
Herman Wijnen, University of Virginia
- 5:00 **Dual role for ipRGCs in vision and circadian photoentrainment**
Samer Hattar, Johns Hopkins University
- 5:30 **Intrinsic responses of melanopsin retinal ganglion cells to light**
Michael Do, Johns Hopkins University
- 6:00 **Food: the main entraining signal for brain and peripheral oscillators**
Carolina Escobar, Universidad Nacional Autónoma de México

Symposium 5—The Transcription/Translational Feedback Model in Eukaryotes and Prokaryotes • *Magnolia F*

Chair: Paul Hardin, Texas A&M University

4:30 **Post-transcriptional and post-translational control of the *Neurospora* circadian clock**

Yi Liu, University of Texas Southwestern Medical Center

5:00 **How coupled oscillators shape the clock in the cell**

Martha Merrow, University of Groningen

5:30 **Post-translational regulation of rhythmic transcription in *Drosophila***

Paul Hardin, Texas A&M University

6:00 **The underlying oscillator: Suggestions from cyanobacteria**

Carl Johnson, Vanderbilt University

8:00–10:30 PM

Poster Session I (P1–P93) • *Magnolia B/C/D/E*

Monday, May 24, 2010

8:00–10:00 AM

Poster Session Setup (P94–P183) • *Magnolia B/C/D/E*

8:30–10:30 AM

Symposium 6—Seasonal and Reproductive Rhythms • *Camellia I & II*

Chair: David Hazlerigg, University of Aberdeen

8:30 **Circannual rhythms: Organismal approaches to elusive periodic timers**

Barbara Helm, University of Konstanz and Max Planck Institute for Ornithology

9:00 **Molecular basis of seasonal time measurement in plants**

Takato Imaizumi, University of Washington

9:30 **Regulation of seasonal body weight in the Siberian hamster**

Perry Barrett, University of Aberdeen

10:00 **Evolutionary dynamics of seasonal rhythms in *Drosophila melanogaster***

Paul Schmidt, University of Pennsylvania

Symposium 7—Clocks and the Immune System • *Azalea Ballroom*

Chair: Diego Golombek, National University of Quilmes-Buenos Aires

8:30 **The circadian control of the adaptive immune response**

Nicolas Cermakian, McGill University

9:00 **Clocks in the immune system**

Alec Davidson, Morehouse School of Medicine

9:30 **Clocks, circadian gates, and the inflammatory response**

Andrew Loudon, University of Manchester

10:00 **Ticking clocks and neuroinflammatory signaling**

Marina Bentivoglio, University of Verona

10:30–11:00 AM

Refreshment Break • *Magnolia Foyer*

Meet the Professors • *Magnolia A*

Dieter Kunz (Sleep medicine and clinical chronobiology, light, melatonin)

Francis Lévi (Chronotherapy of cancer)

Michael Menaker (Rodents, central and peripheral clocks)

Till Roenneberg (Human chronotypes, entrainment, *Neurospora*)
Paolo Sassone-Corsi (Rodents, post-translational events, transcription, epigenetics)
Rae Silver (Rodents, analysis of SCN circuits)
Joe Takahashi (Mouse genetics, clock genes)
Hiroki Ueda (Systems biology)

11:00 AM–12:30 PM

Slide Session D • Azalea Ballroom

Chair: Ruud Buijs, Instituto de Investigaciones Biomedicas

11:00 **S19 • A role for the dorsomedial hypothalamic nucleus in food anticipatory rhythms in rats**

Ralph Mistlberger, Psychology, Simon Fraser University

11:15 **S20 • The suprachiasmatic nucleus and the dorsomedial nucleus of the hypothalamus are part of a neuronal network that comprises the food entrained oscillator**

Ruud Buijs, Instituto de Investigaciones Biomedicas

11:30 **S21 • Increased food-anticipatory activity in tissue plasminogen activator knockout mice • Eric Mintz, Biological Sciences, Kent State University**

11:45 **S22 • Attenuated food anticipatory activity and abnormal circadian locomotor rhythms in *Rgs16* knockdown mice**

Naoto Hayasaka, Anatomy and Neurobiology, Kindai University

12:00 **S23 • Using automated computer vision technology to study anticipatory activity in mouse**

Andrew Steele, Biology, California Institute of Technology

12:15 **S24 • Chronic methamphetamine controls the phase and organization of peripheral oscillators**

Jennifer Mohawk, Biology, University of Virginia

Slide Session E • Camellia I & II

Chair: Derk-Jan Dijk, University of Surrey

11:00 **S25 • Physiologic indicators of sleepiness**

Chern-Pin Chua, Neuroscience & Behavioural Disorders Program, Duke-NUS Graduate Medical School

11:15 **S26 • Identification and validation of a candidate gene for wake and REM sleep**

Karrie Mrazek, Center for Sleep and Circadian Biology, Northwestern University

11:30 **S27 • The *PERIOD3* variable number tandem repeat polymorphism and human sleep timing and duration**

Derk-Jan Dijk, Surrey Sleep Research Centre, University of Surrey

11:45 **S28 • Light exposure and melatonin production in night workers**
Marie Dumont, Chronobiology Laboratory, Sacré-Coeur Hospital of Montréal

12:00 **S29 • Gender differences in rhythmic gene expression in human chronic lymphocytic leukemia cells and T-cells in nine patients: Impact of melatonin therapy on the timing of peak expression**

Georg Bjarnason, Medical Oncology, Sunnybrook Odette Cancer Centre

12:15 **S30 • Daily rhythms in human subcutaneous white adipose tissue: Comparison of lean, overweight, and type 2 diabetic subjects**
Jonathan Johnston, Faculty of Health and Medical Sciences, University of Surrey

Slide Session F • *Magnolia F*

Chair: Justin Blau, New York University

11:00 **S31 • Non-transcriptional mechanisms are competent to sustain circadian rhythms in mammalian cells**
John O'Neill, Institute of Metabolic Science, University of Cambridge

11:15 **S32 • Circadian bias of poly(A) tail length regulation**
Shihoko Kojima, Neuroscience, University of Texas Southwestern

11:30 **S33 • Transcriptome deep sequencing and the circadian regulation of alternative splicing in *Drosophila melanogaster***
Joseph Rodriguez, Brandeis University

11:45 **S34 • Translation initiation is a regulatory step in the *Drosophila* circadian clock**
Justin Blau, Biology Department, New York University

12:00 **S35 • miRNA-mediated control of circadian rhythms in *Drosophila***
Sebastian Kadener, Biological Chemistry, The Hebrew University of Jerusalem

12:15 **S36 • microRNA-mediated posttranscriptional control of mammalian clock gene expression regulates circadian oscillator performance**
Eugin Destici, Genetics, Erasmus Medical Centre

12:30–4:30 PM

Free Time

4:30–6:00 PM

Presidential Special Symposium • *Azalea Ballroom*

Chair: Joseph Takahashi, University of Texas Southwestern Medical Center
"Time Flies (in memory of Seymour Benzer)"
Sydney Brenner, Nobel Laureate

8:00–10:30 PM

Poster Session II (P94–P183) • *Magnolia B/C/D/E*

Tuesday, May 25, 2010

8:00–10:00 AM

Poster Session Setup (P184–P274) • *Magnolia B/C/D/E*

8:30–10:30 AM

Symposium 8—Ionic Mechanisms Underlying Circadian Oscillations • *Camellia I & II*

Chair: Michael Nitabach, Yale School of Medicine

8:30 **State dependence of the intracellular calcium signals generated in SCN neurons by excitatory neurotransmission**
Charles Allen, Oregon Health & Science University

9:00 **Fast delayed rectifier potassium currents: Critical for input and output of the circadian system**
Chris Colwell, University of California–Los Angeles

9:30 **Mechanisms of circadian rhythms in neuronal activity**
Andrea Meredith, University of Maryland School of Medicine

10:00 **Ionic and molecular mechanisms of SCN pacemaking**
Doug McMahon, Vanderbilt University

Symposium 9—Peripheral Circadian Clocks • Azalea Ballroom

Chair: Takashi Yoshimura, Nagoya University

8:30 **Gene targeting in monarch butterflies: Knocking out clock genes using Zinc-Finger Nucleases**

Christine Merlin, University of Massachusetts Medical School

9:00 **Analysis of mammalian PERIOD protein complexes**

Charles Weitz, Harvard Medical School

9:30 **Peripheral and central clockwork regulation by the HLH transcriptional repressor Inhibitor of DNA binding 2 (ID2)**

Giles Duffield, University of Notre Dame

10:00 **Development of circadian oscillator in mammals**

Kazuhiro Yagita, Osaka University Graduate School of Medicine

10:30–11:00 AM

Refreshment Break • Magnolia Foyer

Meet the Professors • Magnolia A

Dirk-Jan Dijk (Humans, sleep, and circadian rhythms)

Charles Czeisler (Humans, sleep and circadian rhythms)

Russell Foster (Mammals, photic input pathways)

Michael Hastings (Rodents, SCN, molecular mechanisms)

Jennifer Loros (*Neurospora*, clock genes, output)

Bill Schwartz (Rodents, circadian behavior, SCN)

Fred Turek (Rodents and humans, genetics of sleep, and rhythms)

David Weaver (Rodents, molecular mechanisms)

11:00 AM–12:30 PM

Slide Session G • Azalea Ballroom

Chair: Urs Albrecht, University of Fribourg

11:00 **S37 • Upstream Transcription Factor 1 (*USF1*) is responsible for Suppressor of Clock (*Soc*): Uncovering a hidden transcription pathway for circadian clock genes**

Kazuhiro Shimomura, Center for Functional Genomics, Northwestern University

11:15 **S38 • PAS domain interactions of *Drosophila* and mouse PERIOD proteins**

Eva Wolf, Structural Cell Biology, MPI of Biochemistry

11:30 **S39 • A mechanism for negative feedback transcriptional suppression by mammalian PERIOD proteins**

Hao Duong, Neurobiology, Harvard Medical School

11:45 **S40 • PERSuading nuclear receptors to dance the circadian rhythm**

Urs Albrecht, Medicine, University of Fribourg

12:00 **S41 • Insights on the role of Timeless in the mammalian circadian clock**

Filippo Tamanini, Genetics, Erasmus MC

12:15 **S42 • *Potorous tridactylus* CPD photolyase: A DNA repair protein with an unexpected circadian clock function?**

Ines Chaves, Genetics, Chronobiology and Health Group, Erasmus University Medical Center

Slide Session H • *Magnolia F*

Chair: Francois Rouyer, INAF, UPR3294, CNRS

- 11:00 **S43 • Decoding the logic of a circadian neural circuit**
Ben Collins, Biology, New York University
- 11:15 **S44 • The role of the dorsal neurons in free-running behavior in *Drosophila***
Stephane Dissel, Department of Genetics, University of Leicester
- 11:30 **S45 • Adult-specific electrical silencing of pacemaker neurons uncouples the molecular oscillator from circadian outputs**
Fernanda Ceriani, Laboratorio de Genetica del Comportamiento, Instituto Leloir
- 11:45 **S46 • Circadian control of Timeless degradation by a Cullin-3–based ubiquitin ligase**
Francois Rouyer, INAF, UPR3294, CNRS
- 12:00 **S47 • Odor stimuli modulate olfactory clocks and circadian behavior**
Ute Abraham, Laboratory of Chronobiology, Institute for Medical Immunology, Laboratory of Chronobiology, Charite–Universitaetsmedizin
- 12:15 **S48 • A fearful stimulus alters *per2* expression and c-fos activity in brain regions involved in fear memory**
Harry Pantazopoulos, Biology, Northeastern University

Slide Session I • *Camellia I & II*

Chair: Simon Archer, University of Surrey

- 11:00 **S49 • Modeling light adaptation in circadian clock: Prediction of the response that disturbs entrainment**
Gen Kurosawa, Theoretical Biology Laboratory, RIKEN Advanced Science Institute
- 11:15 **S50 • Neuroglobin is involved in light-induced resetting of the circadian clock**
Christian Hundahl, Clinical Biochemistry, Bispebjerg Hospital
- 11:30 **S51 • Human nonvisual responses to simultaneous presentation of short- and long wavelength light**
Victoria Revell, FHMS, University of Surrey
- 11:45 **S52 • Melanopsin-expressing neurons mediate light modulation of cognitive functions and mood-related behaviors**
Tara LeGates, Biology, Johns Hopkins University
- 12:00 **S53 • Altered retinal responses to light in *Per3*-deficient mice**
Simon Archer, Health & Medical Sciences, University of Surrey
- 12:15 **S54 • Photoperiodism in mammals: What are the long day signals?**
Sandrine Dupre, Faculty of Life Sciences, University of Manchester

12:30–4:30 PM

Free Time

4:30–6:30 PM

Symposium 10—Post-translational Regulation of Circadian Clocks •

Azalea Ballroom

Chair: Deborah Bell-Pedersen, Texas A&M

- 4:30 **Post-translational regulation in the *Arabidopsis* circadian clock**
Dave Somers, Ohio State University/Pohang University of Science and Technology
- 5:00 **Post-translational regulation of circadian clocks**
Jennifer Loros, Dartmouth Medical School
- 5:30 **Epigenetics and metabolism: the circadian clock connection**
Paolo Sassone-Corsi, University of California–Irvine
- 6:00 **The *Drosophila* PERIOD protein: How studying a single state-variable provides a window into understanding the complex role that protein phosphorylation plays in the design of circadian clocks**
Isaac Edery, Rutgers University

Symposium 11—Rhythms in Space or Altered Gravity • *Magnolia F*

Chair: Elizabeth Klerman, Harvard Medical School

- 4:30 **Rhythms in space or altered gravity**
Laura Barger, Brigham and Women's Hospital/Harvard Medical School
- 5:00 **Respiratory rhythms and sleep in space**
Kim Prisk, University of California–San Diego
- 5:30 **Gravity and the mammalian circadian clock**
Charles Fuller, University of California–Davis
- 6:00 **The effects of altered gravitational fields on the expression and stability of circadian rhythms**
Gary Wassmer, Bloomsburg University

Symposium 12—Circadian Rhythms and Disease • *Camellia I & II*

Chair: Mary Harrington, Smith College

- 4:30 **Is there light at the end of the tunnel? Circadian disruption and its effects on cancer risk**
Eva Schernhammer, Harvard Medical School
- 5:00 **Models of circadian disruption in disease**
Mary Harrington, Smith College
- 5:30 **Disrupting the circadian homeostasis of sympathetic signaling promotes tumor development in mice**
Loning Fu, Baylor College of Medicine
- 6:00 **Designing personalized cancer chronotherapeutics**
Francis Levi, INSERM U776

8:00–10:30 PM

Poster Session III (P184–P274) • *Magnolia B/C/D/E*

Wednesday, May 26, 2010

8:30–10:30 AM

Symposium 13—Comparative Clocks • *Magnolia F*

Chair: Mike Menaker, University of Virginia

8:30 **Circadian clock genes and photoperiodism in *Drosophila***

Charalambos Kyriacou, University of Leicester

9:00 **Can a Darwinian perspective direct our search for the genetic basis of photoperiodism?**

William Bradshaw & Christina Holzapfel, University of Oregon

9:30 **Impact of climate change on the phenology of plants and birds in Thoreau's Concord**

Richard Primack, Boston University

10:00 **Avian migration clocks**

Timothy Coppack, Institute of Applied Ecology

Symposium 14—Interplay between Circadian Clocks and Metabolism •

Camellia I & II

Chair: Carla Green, University of Texas Southwestern Medical Center

8:30 **Role of cell autonomous clock in insulin secretion and diabetes mellitus**

Joseph Bass, Northwestern University

9:00 **Integration of clock and metabolism through PGC-1 transcriptional coactivators**

Jiandie Lin, University of Michigan

9:30 **AMP kinase regulates the circadian clock by phosphorylation and degradation of Cryptochromes**

Katja Lamia, The Salk Institute

10:00 **Melanocortin-3 receptors and entrainment of behavior and metabolism during restricted feeding**

Andrew Butler, The Scripps Research Institute

Symposium 15—Systems Biology of Circadian Rhythms • *Azalea Ballroom*

Chair: Achim Kramer, Charite Universitätsmedizin Berlin

8:30 **Integrating molecular data into models of clock mechanisms, and outputs to metabolism and photoperiodism**

Andrew Millar, University of Edinburgh

9:00 **SEQing a more comprehensive set of clock output genes**

John Hogenesch, University of Pennsylvania School of Medicine

9:30 **Systems biology of mammalian circadian clocks: The role of delay in feedback repression**

Hiroki Ueda, RIKEN CDB

10:00 **Genes and proteins in the mammalian circadian clock**

Achim Kramer, Charité Universitätsmedizin Berlin

10:30–11:00 AM

Refreshment Break • *Magnolia Foyer*

Meet the Professors • *Magnolia A*

Vincent Cassone (Birds, mammals, Neuroendocrine pathways, melatonin)

Carl Johnson (Cyanobacteria, mammals, clock genes)

Beth Klerman (Human, circadian rhythms, sleep, mood and performance, modeling)

Charalambos Kyriacou (*Drosophila* genetics, circadian behavior)

Andrew Loudon (Mammals, circadian and circannual rhythms)

Martha Merrow (Entrainment in humans and *Neurospora*, molecular mechanisms)

Hitoshi Okamura (Mammals, clock genes)

Chuck Weitz (Mammalian clocks, mechanism, physiology)

11:00 AM–12:30 PM

Slide Session J • *Magnolia F*

Chair: Steven Brown, University of Zurich

11:00 **S55 • Timing in the immune system: The circadian clock controls T-cell function**

Erin Fortier, McGill University

11:15 **S56 • Cellular circadian clock in CD4+ T-cells and circadian T-cell immune responses**

Thomas Bollinger, Medical Microbiology and Hygiene, University of Lübeck

11:30 **S57 • The NONO protein couples senescence, cell cycle, and circadian pathways to regulate wound healing**

Steven Brown, Institute for Pharmacology and Toxicology, University of Zurich

11:45 **S58 • A circadian egg timer: Circadian influences on the timing of ovulation**

Michael Sellix, Biology, University of Virginia

12:00 **S59 • Missing neuronal links from the SCN to ovulation**

Benjamin Smarr, Neurobiology and Behavior, University of Washington

12:15 **S60 • Precision of mammalian autonomous circadian oscillators and minimal oscillator models**

Thomas D'Eysmond, Computational Systems Biology Group & Swiss Institute of Bioinformatics (SIB), Ecole Polytechnique Fédérale de Lanne (EPFL)

Slide Session K • *Camellia I & II*

Chair: Michael Hastings, Neurobiology Division, MRC Laboratory of Molecular Biology

11:00 **S61 • Cellular circadian pacemaking in the SCN of Cryptochrome-deficient mice**

Michael Hastings, Neurobiology Division, MRC Laboratory of Molecular Biology

11:15 **S62 • Paracrine signaling drives cellular pacemakers in the suprachiasmatic nucleus: Roles for Vipergic and non-Vipergic signals**

Elizabeth Maywood, Neurobiology Division, MRC Laboratory of Molecular Biology

11:30 **S63 • VIP reduces amplitude and synchrony of circadian oscillator**

Sungwon An, Biology, Washington University in St. Louis

- 11:45 **S64 • Three-dimensional mapping of phase heterogeneity in the suprachiasmatic nucleus of the mouse**
Jennifer Evans, Neuroscience, Morehouse School of Medicine
- 12:00 **S65 • Does the variability of circadian period (τ_{DD}) depend upon its value? A test using *tau* mutant, *super duper*, and *duper* hamsters**
Eric L. Bittman, University of Massachusetts
- 12:15 **S66 • The mystery of coupling between the left and right suprachiasmatic nuclei**
Stephan Michel, Molecular Cell Biology, Leiden University Medical Center

Slide Session L • Azalea Ballroom

Chair: Deborah Bell-Pedersen, Texas A&M University

- 11:00 **S67 • Circadian clock output pathways revealed by ChIP/Seq in *Neurospora***
Deborah Bell-Pedersen, Biology, Texas A&M University
- 11:15 **S68 • A surprisingly large number of CLK direct target genes in *Drosophila***
Katharine Abruzzi, Department of Biology, HHMI/Brandeis University
- 11:30 **S69 • Oscillating miRNAs and circadian rhythms in *Drosophila melanogaster***
Sadanand Vodala, Department of Biology, HHMI/Brandeis University
- 11:45 **S70 • Transcriptional regulation of clock-controlled genes**
Hans-Peter Herzel, Institute for Theoretical Biology, Humboldt University
- 12:00 **S71 • Genome-wide mapping of Bmal1 binding sites in mouse liver reveals cooperative interactions at circadian enhancers**
Guillaume Rey, Institute of Bioengineering (IBI), Ecole Polytechnique Fédérale de Lanne (EPFL)
- 12:15 **S72 • The Krüppel Like Factor KLF10 links the circadian clock to metabolism in liver**
Franck Delaunay, CNRS UMR6543, Université de Nice

12:30–4:00 PM

Free Time

4:00–5:00 PM

Business Meeting • Azalea Ballroom

5:30–6:30 PM

Pittendrigh/Aschoff Lecture • Azalea Ballroom

Introduction: **Charalambos Kyriacou**, University of Leicester

Presentation: **Michael Rosbash**, Brandeis University

7:00–8:00 PM

Cocktail Reception (cash bar) • Magnolia Foyer

8:00–11:00 PM

Closing Banquet • Magnolia Ballroom

Poster Titles

- P1 A framework for systems chronobiology: Systems Biology Software Infrastructure, SBSI™** • Andrew Millar, University of Edinburgh
- P2 Modelling circadian rhythms on transcription level of clock genes** • Anja Korencic, University of Ljubljana
- P3 Deterministic and stochastic mathematical models of the KaiABC cyanobacterial circadian oscillator** • Mark Byrne, Spring Hill College
- P4 Per/Dec interaction in the murine circadian clock** • Brid Bode, Max-Planck-Institute for Biophysical Chemistry
- P5 Uncoupling two circadian functions of mammalian cryptochrome 1** • Ines Chaves, Erasmus University Medical Center
- P6 Circadian oscillations of histone-modifications are associated with circadian transcription** • Christopher Vollmers, Salk Institute for Biological Studies
- P7 Extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated resetting of the circadian clock of the mouse embryonic brain-derived neural stem/progenitor cells** • Takahiro Moriya, Tohoku University
- P8 Orphan nuclear receptor NR2F1 directly regulates the circadian output** • Fernanda Ruiz, Baylor College of Medicine
- P9 Inhibitor of DNA binding 2 (Id2) knockout mice treated with sub-threshold discrete light pulses exhibit enhanced phase shifts and an elevated induction of mPer1 in the SCN** • Giles Duffield, University of Notre Dame
- P10 Distinct functions of reactive oxygen species in the control of circadian rhythm in *Neurospora crassa*** • Norbert Gyongyosi, Semmelweis University—WITHDRAWN
- P11 Characterization of new partners of CRYPTOCHROME, the circadian blue light photoreceptor in *Drosophila melanogaster*** • Gabriella Mazzotta, University of Padova
- P12 Gene Dosage Network Analysis identifies genome wide targets of Clock and Bmal1** • Julie Baggs, University of Pennsylvania School of Medicine
- P13 The circadian clock protein Period 1 coordinately regulates sodium transport genes** • Michelle Gumz, University of Florida
- P14 The circadian transcriptional factor Period 2 modulates cyclin B1 expression** • Carla Finkielstein, Virginia Tech
- P15 The effects of acute and chronic lithium treatments on circadian rhythm expression in human primary fibroblasts** • Sang Kil Lee, Morehouse School of Medicine
- P16 Zinc finger protein 496 is a novel target of the mouse cryptochrome-2 dependent circadian regulation** • Anand Venkataraman, University of Pennsylvania
- P17 Setting the pace of the *Neurospora* circadian clock by multiple independent FRQ phosphorylation events** • Joonseok Cha, University of Texas Southwestern Medical Center
- P18 Post-translational regulation of the *Drosophila* CLOCK protein by its interaction partner CYCLE** • Hsiu-Cheng Hung, University of Heidelberg

- P19** Deep sequencing of the circadian transcriptome in the nervous system of *Drosophila melanogaster* • Michael Hughes, Yale School of Medicine
- P20** Day, a new component of the *Drosophila* circadian clock • John Hares, University of Leicester
- P21** Expression of a novel ion channel may partially rescue evening anticipation in “arrhythmic” *per01* and *tim01* mutants • Adam Seluzicki, Northwestern University
- P22** Does a catalytically inactive DBT produce different phenotypes in different tissues, subcellular compartments or cis-mutant backgrounds? • Anandakrishnan Venkatesan, University of Missouri–Kansas City
- P23** Light and temperature control the contribution of a subset of Dorsal Neurons 1 to *Drosophila* circadian behavior • Yong Zhang, University of Massachusetts Medical School
- P24** Crustacean β -PDH I but not β -PDH II rescues circadian rhythmicity in PDF-deficient flies • Esteban Beckwith, Fundación Instituto Leloir, IIBA CONICET
- P25** Spatial regulation of GIGANTEA, a circadian clock regulator, in the cell gives a signal diversity in *Arabidopsis thaliana* • Yumi Kim, Pohang Science and Technology
- P26** Rhythms in individual cells in *Arabidopsis thaliana* • Rachel Green, Hebrew University
- P27** The intertwining of two oscillators simulates the circadian rhythm of living cyanobacteria • Stefanie Hertel, Humboldt University Berlin
- P28** Improvements in sleep during the biological day are associated with changes in thermoregulatory physiology following administration of the melatonin agonist Ramelteon • Kenneth Wright, University of Colorado
- P29** Circadian and sleep parameters differentially predict hyperactive-impulsive and inattentive symptoms in adult ADHD patients with insomnia • Karen L. Gamble, University of Alabama–Birmingham
- P30** The effects of regularizing sleep-wake schedule on the daytime mood, emotional states, and autonomic function in habitual irregular sleepers • Nana Takasu, Kyoto University
- P31** EEG markers of sleep homeostasis are affected by light and time-of-day in the mouse • Valérie Mongrain, University of Lausanne
- P32** Evaluation a non-invasive, rapid approach for the assessment of sleep in mice in response to pharmacological and environmental manipulation • Stuart Peirson, University of Oxford
- P33** Implication of hypocretinergic system in stress-induced sleep regulation • Adeline Rachalski, Université Pierre et Marie Curie-Paris 6
- P34** GABA transaminase affects sleep in *Drosophila* and is increased in sleepless mutants • Wen-Feng Chen, University of Pennsylvania
- P35** Variance of feeding time influences body weight, body fat, and gene expressions in mice • Yuta Fuse, Waseda University
- P36** Desynchronized feeding leads to weight gain in C57BL/6J but not leptin-deficient *ob/ob* mice • Deanna M. Arble, Northwestern University
- P37** Rhythmic expression of clock genes and metabolic genes in rat white adipose tissue • Rianne van der Spek, Academic Medical Center
- P38** Nocturnin, a circadian deadenylase, is necessary for normal absorption of dietary lipid • Shihoko Kojima, University of Texas Southwestern Medical Center
- P39** Pancreatic β -cell clock disruption leads to hypoinsulinemia and diabetes • Biliana Marcheva, Northwestern University

- P40 Gender and genetic differences in circadian clocks and pharmacology determine distinct chronotoxicity classes for irinotecan** • Constance Ahowesso, Université de Paris XI
- P41 Effect of a cell cycle inhibitor seliciclib on circadian timing system** • Elisabeth Filipiski, U776, INSERM
- P42 Expression of clock genes *Per1*, *Bmal*, *Clock*, *Cry1*, *Cry2* and *Rev-erb α* in Leydig tumor cells** • Adam Neumann, University of Warsaw
- P43 Intercellular coupling governs entrainment** • Adrian Granada, Humboldt University Berlin
- P44 Effect of E-boxes on central and peripheral circadian clocks in mice** • Akiko Hida, National Institute of Mental Health—WITHDRAWN
- P45 Circadian rhythms of *PER2* in mouse hepatocytes** • Mary Harrington, Smith College
- P46 Quantitative analysis of the mouse liver circadian proteome** • Charo Robles, Max Planck Institute of Biochemistry
- P47 Zebrafish retinal circadian rhythms of *per3::luc* expression *in vivo* and *in vitro*** • Ziyi Sun, Vanderbilt University
- P48 A role for VIP in the circadian regulation of the female endocrine system?** • Dawn H. Loh, University of California—Los Angeles
- P49 Altered entrainment to the day/night cycle attenuates the diurnal rhythm of corticosterone secretion** • Patricia Sollars, University of Nebraska
- P50 Lack of the circadian clock gene, *bmal1*, is associated with development of dilated cardiomyopathy in mice** • Mellani Lefta, University of Kentucky
- P51 Can timed exercise rescue diurnal and circadian rhythms of HR and clock gene expression in VIP-deficient mice?** • Analyne M. Schroeder, University of California—Los Angeles
- P52 Cell-type specific AKT signaling is regulated by circadian clock component *BMAL1* in vascular cells** • Maoxiang Zhang, Medical College of Georgia
- P53 Deciphering the pineal gland and immune-system crosstalk in chicken (*Gallus gallus*)** • Sailaja Kallur, Texas A&M University
- P54 Influence of time of day on *IL-6* and *IL-1 β* mRNA expression in LPS-stimulated equine blood** • Olga McGlynn, University College Dublin
- P55 Effects of photoperiod-driven variations in life-history on longevity and aging in the Siberian hamster, *Phodopus sungorus*** • Evan Raiewski, University of California—San Diego
- P56 Seasonal variation in equine pituitary dopamine concentrations** • Melissa Cordero, Oklahoma State University
- P57 Circadian control of migratory restlessness and the effects of food and temperature in the blackheaded bunting, *Emberiza melanocephala*** • Vinod Kumar, University of Delhi
- P58 Reduced foraging yield causes diurnality in mice** • Roelof A. Hut, University of Groningen
- P59 Diurnal and nocturnal preference in a physiologically based model of mammalian sleep** • Andrew Phillips, Harvard Medical School
- P60 Continuous alcohol exposure impairs circadian adaptation to simulated time zone travel in *C57BL/6J* mice** • S.A. Sinning, University of California—San Diego
- P61 Photoperiod and jet lag: Influence of pineal melatonin onset timing on reentrainment rate of rats** • L. Samantha Zhang, University of Michigan

- P62** Constant light disrupts circadian rhythms and suppresses melatonin, without reducing cell proliferation and cell survival in the hippocampus of adult rats • Anka Mueller, Simon Fraser University
- P63** Rhythmic gene expression in the medial prefrontal cortex of the mouse • Brooke Rakai, University of Calgary
- P64** Behavioral characterization of ePet-1 knock out mice • John Axley, Vanderbilt University
- P65** The relationship between the urinary 6-sulfatoxymelatonin rhythm and core body temperature and plasma melatonin rhythms under forced desynchrony • Joseph Hull, Harvard Medical School
- P66** Phase delay shifts and melatonin suppression to a 6.5-hour evening light exposure of moderate intensity (275 lux) in healthy older adults • Karine Scheuermaier, Harvard Medical School
- P67** Effects of blue solid-state lighting on melatonin suppression and alertness • John Hanifin, Thomas Jefferson University
- P68** Sex differences in phase angle of entrainment in humans • Sean Cain, Harvard Medical School
- P69** 24-hour rhythms of muscle strength • Leana Araujo, Universidade Federal de São Paulo
- P70** Photic entrainment in cultured chick astrocytes: Effects on clock gene expression, metabolism, and opsins • Jiffin Paulose, Texas A&M University
- P71** Phase response curve to a single 6.5-hour light pulse of short-wavelength light • Melanie Rueger, Harvard Medical School
- P72** Perinatal photoperiodic input influences organization of the circadian clock • Christopher Ciarleglio, Vanderbilt University
- P73** Consequences of different lighting conditions on melanopsin bistable photoresponse properties • Howard Cooper, INSERM and ENTPE
- P74** An unexpected contribution of rods to circadian photoentrainment at all light intensities • Cara Altimus, Johns Hopkins University
- P75** Light-induced phase shifts and fos response in the suprachiasmatic nucleus of aromatase knock-out mice • Rebecca Brockman, University of Illinois at Urbana-Champaign
- P76** Different mechanisms of adjustment to a change of the photoperiod in the suprachiasmatic and liver circadian clocks • Alena Sumova, Academy of Sciences of the Czech Republic
- P77** Structurally stable spatiotemporal organization of circadian oscillation of SCN neurons • Rae Silver, Barnard College and Columbia University
- P78** Fractal patterns of multi-unit activity of the suprachiasmatic nucleus (SCN): SCN-intrinsic and SCN-extrinsic network properties • Kun Hu, Harvard Medical School
- P79** Circadian modulation of the Cl⁻ reversal potential in the suprachiasmatic nuclei, in rats • Raul Aguilar-Roblero, Universidad Nacional Autonoma de Mexico
- P80** Ca²⁺ signaling induced by glutamate in rat SCN neurons • Tongfei Wang, University of Illinois at Urbana-Champaign
- P81** Serotonergic enhancement of photic phase shifts: Location of action of BMY7378 • Victoria Smith, University of Calgary
- P82** Effects of exogenous melatonin on the electrical activity of Per1 and non-Per1 suprachiasmatic nuclei (SCN) neurons *in vitro* • Fiona Scott, University of Manchester

- P83** Circadian rhythms of PER2 protein activity are maintained in hypothalamic regions from behaviorally arrhythmic animals with disrupted VIP-VPAC2 signaling • Clare Guilding, University of Manchester
- P84** Photic and nonphotic effects on VIP, GRP, and AVP release in the hamster SCN circadian clock • Jessica Francl, Kent State University
- P85** Little SAAS peptide expression forms a new sub-compartment in the retinorecipient suprachiasmatic nucleus of the rat • Norman Atkins, University of Illinois at Urbana-Champaign
- P86** Preliminary evidence that the food-entrainable oscillator is not exclusively responsive to food • Roberto Refinetti, University of South Carolina
- P87** Rev-erb α participates in the clockwork of food anticipation • Julien Delezie, CNRS
- P88** Absence of a serum melatonin (MT) rhythm under acutely extended darkness (DD) despite an oscillating skeletal muscle clock in the horse • Barbara Murphy, University College Dublin
- P89** Circadian rhythmicity in the response of a collision detecting interneuron in solitary and gregarious locusts • Edward Gaten, University of Leicester
- P90** A honey bee (*Apis mellifera*) light phase response curve • Nicola Ludin, University of Auckland
- P91** Genome-wide analysis of temporal expression in the coral *Acropora millepora*, the lowest multicellular animal, in the context of circadian clocks • Oren Levy, Bar Ilan University
- P92** Rhythmic behavior in krill: A role for the clock? • Ozge Ozkaya, University of Leicester
- P93** The frequency of the behavioral response in *Paramecium tetraurelia* is simultaneously modulated by both ultradian and circadian rhythms • Rachel Mialki, Indiana University of Pennsylvania
- P94** Investigating the causes of spontaneous internal desynchrony using a physiologically based sleep model • Andrew Phillips, Brigham and Women's Hospital, Harvard Medical School
- P95** Wavelet analysis reveals period wandering in circadian neurons • Kirsten Meeker, University of California–Santa Barbara
- P96** Neuronal modeling of the SCN • Daniel Forger, University of Michigan
- P97** Distinct functions of the Period genes in the mammalian circadian system: Evidence from *in vitro* and *in vivo* studies • Julie Pendergast, Vanderbilt University
- P98** Biochemical mechanism of transcriptional control of the mammalian circadian clock by cryptochrome • Rui Ye, University of North Carolina, School of Medicine
- P99** A functional genomics screen for microRNA regulators of CREB signaling • Anthony Olarerin-George, University of Pennsylvania
- P100** Functional implications for microRNAs in the intracellular and extracellular regulation of the mammalian circadian clockworks • David Earnest, Texas A&M University
- P101** Protein arginine methyl transferase 5 links the circadian clock to the regulation of alternative splicing • Marcelo Javier Yanovsky, University of Buenos Aires
- P102** The transcriptional repressor ID2 can interact with the canonical clock components CLOCK and BMAL1 and mediate inhibitory effects on mPer1 expression • Sarah Ward, University of Notre Dame
- P103** Identification of a novel *Neurospora* clock gene by deep sequencing • Randy Lambregts, Dartmouth Medical School

- P104 Large-scale analysis of clock gene rhythms in rats housed under normal lighting conditions reveals tissue-specific variability in clock machinery and output • Yuval Weigl, Concordia University**
- P105 Canonical clock genes regulate circadian rhythmicity and ATP release from cortical astrocytes *in vitro* • Christian Beaulé, Washington University**
- P106 Connexin mRNA expression changes between subjective day and night in rat main olfactory bulb • John Corthell, Florida State University**
- P107 *In vivo Neurospora* protein expression using the vvd promoter • Jennifer Hurley, Dartmouth Medical School**
- P108 Transcriptional regulation on circadian rhythm of autophagy • Di Ma, University of Michigan**
- P109 Global analysis of circadian protein stability • Katja Schellenberg, Charité–Universitätsmedizin, Berlin**
- P110 Functional loss of GSK-3 β and circadian behavior: Impact of direct inhibition of the enzyme versus modulation of its phosphorylation state • Ligia Westrich, Lundbeck Research USA**
- P111 Phosphorylation of CLOCK/NPAS2-BMAL1 complex in suppression of E-box–dependent transcription • Hikari Yoshitane, The University of Tokyo**
- P112 PERL alters *Drosophila's* circadian rhythms by modifying CLK-mediated transcription • Yue Li, Brandeis University**
- P113 Identification of dyschronic, a novel gene involved in circadian output in *Drosophila* • Kyunghee Koh, University of Pennsylvania**
- P114 Identification and analysis of a novel timeless allele that disrupts post-translational control of TIM and PER in *Drosophila* • Taichi Hara, University of Pennsylvania**
- P115 A central brain circuit processes photic input to regulate acute masking behavior in *Drosophila* • Brian Chung, Northwestern University**
- P116 New genetic determinants of temperature entrainment in *Drosophila* • Tadahiro Goda, University of Virginia**
- P117 DPP signaling contributes to set basic properties of the PDF circuit in *Drosophila* • Esteban Beckwith, Fundación Instituto Leloir, IIBA CONICET**
- P118 Circadian rhythm of *Arabidopsis* petal movement • Shinsuke Kutsuna, Yokohama City University**
- P119 Circadian transcription in the dark without cyclic kai gene expression I: Clock-gating of dark-induced transcription in *Synechococcus* • Norimune Hosokawa, Waseda University**
- P120 Circadian transcription in the dark without cyclic kai gene expression II: Damping oscillation in expression of a dark-induced gene • Tetsuhiro Hatakeyama, Waseda University**
- P121 Insight into teenage sleep patterns from the slow-developing rodent, *Octodon degus*: Pubertal hormones act on the circadian system • Megan Hagenauer, University of Michigan**
- P122 Differential effects of voluntary and induced wakefulness in arousal and reward areas in a diurnal brain • Alexandra Castillo-Ruiz, Michigan State University**
- P123 The regulation of orexin receptors by dynein light chains • Erika Belanger-Nelson, McGill University**
- P124 Per genes regulate the metabolic response to sleep loss • Jana Husse, Max Planck Institute for Biophysical Chemistry**
- P125 Circadian rhythms and sleep behavior in an annelid worm, *Lumbriculus variegatus* • Mark Zoran, Texas A&M University**

- P126 Elevated NOX-4 and oxidant stress in Bmal1-KO mice** • Ciprian Anea, Medical College of Georgia
- P127 Polymorphisms in BMAL1 (MOP3) and PER3 are associated with features of obesity in man** • Madhu J. Prasai, University of Leeds
- P128 RIP140 is a conduit for circadian control of genes involved in lipid metabolism** • Joshua Gamsby, Dartmouth College
- P129 Diet-induced obesity attenuates diurnal variation in glucose tolerance and insulin sensitivity—a possible role for the molecular clock?** • Madhu J. Prasai, University of Leeds
- P130 Mitochondria dynamics and circadian rhythms** • Karen Schmitt, Psychiatric University Clinic Basel
- P131 *In vitro* molecular chronopharmacology of anticancer drug irinotecan on human cancer cells** • Sandrine Dulong, INSERM U776
- P132 Control of cancer progression through reinforcement of host circadian physiology and rhythmic induction of tumor stress genes with circadian meal timing** • Xiao-Mei Li, INSERM U776
- P133 Circadian-independent cell mitosis in immortalized fibroblasts** • Shin Yamazaki, Vanderbilt University
- P134 Melatonin and peripheral molecular circadian rhythms** • Lucia Pagani, UPK Basel
- P135 Evaluation of qPCR reference gene stability for circadian studies in peripheral organs of different mouse strains** • Rok Kosir, Center for Functional Genomics and Bio-Chips
- P136 Circadian clock-coordinated translation in mouse liver** • Frédéric Gachon, University of Lausanne
- P137 Ontogeny of circadian rhythm in the eye of *Xenopus laevis*** • Kristen Curran, University of Wisconsin–Whitewater
- P138 The molecular clock in muscle: The phase of PER2:LUC expression is similar among striated muscles of differing function** • Gretchen Wolff, University of Kentucky
- P139 Circadian regulation of cortisol release in behaviorally split golden hamsters** • Travis Lilley, University of Washington
- P140 Immunohistological characterization of the circadian clock in cardiovascular tissues** • Ana M. Merloiu, Medical College of Georgia
- P141 Existence of an endogenous circadian blood pressure rhythm that paradoxically peaks at night** • Steven A. Shea, Harvard Medical School
- P142 Length polymorphism in the PER3 polymorphism modulates sleep structure and EEG power spectra in older subjects** • Antoine Viola, Universitäre Psychiatrische Kliniken
- P143 Chronic jet lag modulates innate immunity in peritoneal macrophages** • Oscar Castañón-Cervantes, Morehouse School of Medicine
- P144 The role of the circadian clock in the murine inflammatory response to lipopolysaccharide** • Julie Gibbs, University of Manchester
- P145 Circadian and photoperiodic clocks: Is every similarity just a coincidence?** • Joao Gestó, University of Leicester
- P146 The effect of season on inflammatory response in captive baboons** • Dianne McFarlane, Oklahoma State University
- P147 Pinealectomy abolishes clock genes rhythm in peripheral tissues in house sparrow, *Passer domesticus*** • Amit Trivedi, University of Kentucky

- P148 Circadian control of calcium utilization in birds** • Paul Bartell, Pennsylvania State University
- P149 Voluntary wheel running induces PER2 rhythms in the dentate gyrus of day-active and night-active grass rats (*Arvicanthis niloticus*)** • Antonio Nunez, Michigan State University
- P150 Period aftereffects are modulated by phase incoherence in the forced desynchronized rat** • Michael Schwartz, University of Washington
- P151 Internal desynchronization in a model of chronic jetlag in mice** • Gisele Oda, Instituto de Biociências
- P152 Differential functions of the Period genes in the regulation of mood and anxiety-related behaviors** • Jaswinder Kumar, University of Texas Southwestern Medical Center
- P153 Reward portals to the circadian clock: Mesopontine and mesocorticolimbic pathways for non-photic phase regulation** • Jessie Guinn, Kent State University
- P154 Manic-like behavior in Clock-delta19 mice is associated with molecular, cellular, and physiological dysfunction in the nucleus accumbens** • Michelle Sidor, University of Texas Southwestern Medical Center
- P155 Characterization of circadian rhythm parameters in two strains of mice with impaired responsiveness to estrogen** • Margaret Blattner, University of Illinois at Urbana-Champaign
- P156 Comparison of wrist-level and eye-level light measurements for in-hospital circadian studies** • Anisoara Jardim, University of Auckland
- P157 Light-induced melatonin suppression in humans: Effect of light duration and irradiance** • Debra J. Skene, University of Surrey
- P158 Detailed characterization of nonvisual light responses in a blind individual** • Ivan Ho Mien, Duke-NUS Graduate Medical School
- P159 Endogenous tau in free-running individuals with delayed sleep phase** • Patricia Murphy, Weill Cornell Medical College
- P160 The genetic architecture of circadian light sensitivity in *Drosophila*** • Eran Tauber, University of Leicester
- P161 Chronic ethanol attenuates photic-phase delays, impairs photic entrainment, and disrupts nocturnal locomotor activity in the mouse** • Allison Brager, Kent State University
- P162 Effects of irradiance, circadian phase, and photoperiod on light sampling behavior in Syrian hamsters** • Gena Glickman, University of California–San Diego
- P163 Experience-independent development of the hamster circadian visual system** • August Kampf-Lassin, The University of Chicago
- P164 Effect of the absence of melanopsin on the endogenous functioning of the mammalian retinal clock** • Ouria Dkhissi-Benyahya, INSERM U846
- P165 The DNA-binding activity of BMAL1 and CLOCK is affected by time-of-day and sleep pressure in the mouse brain cortex** • Valerie Mongrain, University of Lausanne
- P166 mTOR signaling couples light to entrainment of the suprachiasmatic circadian clock** • Ruifeng Cao, Ohio State University
- P167 Functional connectivity analysis in synchronized circadian networks** • Mark Freeman, Washington University
- P168 Clock-to-clock coupling of SCN and non-SCN cells by diffusible factors** • Patty Kandalepas, University of Illinois at Urbana-Champaign

- P169 Neural mechanisms mediating circadian phase resetting by activation of 5-HT7 receptors in the dorsal raphe: Role of GABAergic and glutamatergic neurotransmission** • Marilyn Duncan, University of Kentucky Medical School
- P170 Functional development of the mouse SCN *in vitro*** • Daniel Wreschnig, Northeastern University
- P171 The serotonergic innervation of the SCN is necessary for median raphe nucleus stimulation induced phase shifts** • Glenn Yamakawa, University of Calgary
- P172 Aberrant nonphotic resetting in null mutant mice reveals roles for dexras1 and the intergeniculate leaflet (IGL) in the entrainment of circadian rhythms** • Margaret M. Koletar, University of Toronto
- P173 Identification of a short, bioactive form of vasoactive intestinal peptide (VIP) in the SCN** • Jennifer Mitchell, University of Illinois at Urbana-Champaign
- P174 Impairment of the cAMP-response-element-binding protein pathway attenuates gastrin-releasing, peptide-induced phase shifts in the early-subjective night** • Roxanne Sterniczuk, University of Calgary
- P175 NeuropeptideY induces long-term suppression of action potential frequency in *Per1*-expressing neurons during the late day** • Karen L. Gamble, The University of Alabama–Birmingham
- P176 The food-entrainable oscillator does not drive anticipatory activity, but controls it as circadian manner** • Wataru Nakamura, Osaka University Graduate School of Dentistry
- P177 Food schedule induces rapid re-entrainment after a 6-hour phase advance in rats** • Manuel Angeles-Castellanos, Universidad Nacional Autónoma de México
- P178 A circadian clock is not required in an Arctic mammal** • Qing-Jun Meng, University of Manchester
- P179 General anaesthesia is a thief of time** • James Cheeseman, University of Auckland
- P180 Maternity-related plasticity in circadian rhythms of bumble bee (*Bombus terrestris*) queens** • Ada Eban-Rothschild, The Hebrew University of Jerusalem
- P181 Circadian and circatidal clocks in a marine organism** • Lin Zhang, Leicester University
- P182 Jetlag in malaria parasites: The importance of synchronizing with the host** • Harriet G. McWatters, University of Oxford
- P183-1 Circadian rhythmicity persists without transcription in a eukaryote** • Gerben van Ooijen, University of Edinburgh
- P183-2 The circadian clock influences short-time perception in mice** • Diego A. Golombek, National University of Quilmes
- P184 A mechanistic analysis of frequency demultiplication** • Christian Hong, Dartmouth Medical School
- P185 How the circadian clock can generate cellular 12-hour rhythms** • Pål Westermarck, Charite Universitaetsmedizin
- P186 Seq-ing new insights into the circadian transcriptome** • Nicholas Lahens, University of Pennsylvania School of Medicine
- P187 Methylation analyses on promoters of *mPer1*, *mPer2*, and *mCry1* during perinatal development** • Xiaodong Li, Wuhan University
- P188 A functional circadian clock in HaCaT-Keratinocytes** • Annika Schrader, Beiersdorf AG
- P189 Accelerated re-entrainment in BALB/cJ mice** • Thomas Vajtay, Rider University

- P190 A DEAD Box RNA helicase-ribonucleoprotein complex regulates transcriptional repression by mammalian PERIOD proteins** • Kiran Padmanabhan, Harvard Medical School
- P191 TRAP150, a circadian interface between CLOCK-BMAL1 and the general transcription machinery** • Laura Lande-Diner, Harvard Medical School
- P192 Untangling transcriptional networks in the *Neurospora* circadian system** • Luis F. Larrondo, Pontificia Universidad Catolica de Chile
- P193 Baculovirus photolyases are DNA repair enzymes with circadian clock regulatory function** • Magdalena Biernat, Wageningen University
- P194 Transcription factor coding of the mouse SCN** • Cassandra VanDunk, Washington University
- P195 Circadian calcium oscillations inversely correlate with rhythmic extracellular ATP accumulation in SCN cultures** • Alisa Womac, Texas A&M University
- P196 Towards the molecular mechanisms regulating circadian olfactory sensitivity in mammals** • Manjana Saleh, Institute of Medical Immunology
- P197 Circadian control of DNA excision repair by HERC2 ubiquitin ligase** • Tae-Hong Kang, University of North Carolina School of Medicine
- P198 The circadian deadenylase, Nocturnin, interacts with a novel form of KSRP** • Danielle Shingle, University of Texas Southwestern Medical Center
- P199 Sequential phosphorylation of FRQ by CKI is crucial for establishing rhythmicity in the *Neurospora* clock** • Axel Diernfellner, Biochemistry Center Heidelberg
- P200 *In vivo* analysis of phosphorylation-dependent degradation of mCRY2** • Arisa Hirano, University of Tokyo
- P201 The circadian proteome of *Drosophila melanogaster*** • Renata Azevedo, University of Leicester
- P202 Functional study of a novel ZP protein, Quasimodo, in *Drosophila* circadian clock** • Ko-Fan Chen, University of London
- P203 Molecular and functional characterization of *2mit*, an intronic gene of *D. melanogaster* timeless2 locus** • Federica Sandrelli, University of Padova
- P204 Non cell-autonomous TIMELESS degradation and phase-shifting of the *Drosophila*** • Chih-Hang Tang, Brandeis University
- P205 Live imaging evidence for cAMP signaling as a response to PDF in subsets of *Drosophila* pacemaker neurons** • Laura Duvall, Washington University
- P206 Trp channels affecting temperature entrainment of the circadian clock in *Drosophila melanogaster*** • Werner Wolfgang, University of London
- P207 Genetic architecture of circadian clock function in *Brassica rapa*** • Qiguang Xie, Dartmouth College
- P208 Role of the *Arabidopsis thaliana* circadian clock in *Pseudomonas syringae* infection** • Laura Roden, University of Cape Town
- P209 Global circadian transcription rhythms without robust kai-gene cycling in the heterocyst-forming multicellular cyanobacterium, *Anabaena* sp. PCC 7120** • Hiroko Kushige, Waseda University
- P210 Effects of partial circadian adjustment on sleep and vigilance in simulated night work** • Simon Chapdelaine, Sacre-Coeur Hospital and University of Montreal
- P211 Preliminary evidence that phase relationships between core body temperature, melatonin, and sleep are associated with the severity of global and anhedonic depression** • Brant Hasler, University of Pittsburgh School of Medicine

- P212 Sex differences in the sleep of a diurnal rodent, *Octodon degus*** • Jamie Perryman, University of Michigan
- P213 Sleep-wake Chromosome 1 QTL exploration in the mouse** • Joseph R. Owens, Northwestern University
- P214 Phase-dependent effects of orexin-2 receptor blockade mediated sleep promotion** • Jonathan Shelton, Johnson & Johnson PRDUS
- P215 Nonlinear dynamics of heart rate variability show sleep-wake homeostatic predominance during sustained wakefulness** • Antoine Viola, Universitäre Psychiatrische Kliniken
- P216 Natural genetic variation underlying sleep in *Drosophila* identified by QTL mapping** • Mobina Khericha, University of Leicester
- P217 Altered body mass regulation in male mPeriod mutant mice on high-fat diet** • Robert Dallmann, University of Zurich
- P218 Paradoxical post-exercise responses of acylated ghrelin and leptin during a simulated night shift** • Christopher Morris, Brigham and Woman's Hospital, Harvard Medical School
- P219 Rhythms in white adipose pre-adipocytes and adipocytes** • Jonathan Johnston, University of Surrey
- P220 Insulin-dependent Per2 gene expression is the first entrainment signal to the peripheral circadian clocks by food or glucose** • Yu Tahara, Waseda University
- P221 Regulation of circadian metabolic rhythms through the crosstalk between casein kinase 1 delta and PGC-1 alpha** • Siming Li, University of Michigan
- P222 Regulation of rhythmic expression of circadian and circadian-controlled genes by a chemopreventive regimen of methylselenocysteine** • Mingzhu Fang, University of Medicine and Dentistry of New Jersey
- P223 Circadian clocks in mouse tumors** • Maria Comas, Roswell Park Cancer Institute
- P224 Circadian dysfunction in mouse models of Parkinson's and Huntington's disease** • Takashi Kudo, University of California–Los Angeles
- P225 Postnatal development of peripheral tissue rhythms in mice** • Fred Davis, Northeastern University
- P226 A role for a circadian clock in the pancreas in glucose homeostasis** • Amanda Sadacca, Harvard Medical School
- P227 Circadian clock-coordinated 12-hour period rhythmic activation of the IRE1a pathway controls lipid metabolism in mouse liver** • Gaspard Cretenet, University of Lausanne
- P228 Keeping time in the eye: Clock gene dependence of mouse retinal circadian clock** • Douglas McMahon, Vanderbilt University
- P229 MYOD regulates clock gene expression in skeletal muscle** • Xiping Zhang, University of Kentucky
- P230 A role for adrenal glucocorticoids in the circadian resynchronization during jet lag** • Silke Kießling, Max-Planck-Institute for Biophysical Chemistry
- P231 Contribution of the vascular clock in remodeling: Insights through isograft arterial transplantation studies** • Bo Cheng, Medical College of Georgia
- P232 The circadian clock and hypertension** • Paramita Pati, Medical College of Georgia
- P233 Activation of thromboxane receptors plays a major role in the development of pial vasomotion and low frequency oscillations of the cerebrocortical blood flow in NO-deficiency** • Zoltan Benyo, Semmelweis University—WITHDRAWN

- P234 Cellular and molecular pathways of immune-circadian communication** • José M. Duhart, Universidad Nacional de Quilmes
- P235 Impaired immune function in behaviorally arrhythmic Siberian hamsters** • Brian Prendergast, University of Chicago
- P236 Molecular correlates of photoperiodic diapause in *Drosophila melanogaster*** • Mirko Pegoraro, University of Leicester
- P237 Circadian modulation of developmental transcription factors in the pars tuberalis governs seasonal photoperiodism in mammals** • Hugues Dardente, Aberdeen University
- P238 CLOCK Δ 19 mutants exhibit increased ethanol preference and consumption** • Angela Ozburn, University of Texas Southwestern Medical Center
- P239 Diurnal rodents: Lab versus field studies** • Noga Kronfeld-Schor, Tel Aviv University
- P240 Timed, sustained, attention-demanding performance reorganizes or dampens multiple circadian rhythms** • Theresa Lee, University of Michigan
- P241 Daily rhythms in morphine-induced behaviors** • Stephanie Perreau-Lenz, CIMH
- P242 Circadian pacemaker in “shift worker” rats: Effect of a sustained daily cognitive task on circadian gene expression and corticosterone** • Jin Yan, University of Michigan
- P243 The circadian clock differentially regulates intermediate and long-term memory** • Maximilian Michel, Florida State University
- P244 Diurnal rhythms in cFos immunoreactivity in the mesolimbic reward system** • Ricardo M. Baltazar, University of Western Ontario
- P245 Regulation of limbic circuitry and mood-related behavior by the CLOCK protein** • Colleen McClung, University of Texas Southwestern Medical Center
- P246 Molecular biomarkers for human circadian differences** • Ludmila Cuninkova, University of Zurich
- P247 Human nocturnal nonvisual responses to polychromatic white light cannot be predicted by the melanopsin photosensitivity function** • Victoria Revell, University of Surrey
- P248 Spectral sensitivity of melatonin suppression in the elderly** • Claude Gronfier, INSERM U846
- P249 Prevalence of circadian rhythm disorders in 118 blind women with and without light perception living in North America** • Erin Flynn-Evans, Harvard Medical School
- P250 Internal phase relationships in free-running individuals with delayed circadian phases** • Patricia Murphy, Weill Cornell Medical College
- P251 Identification of encephalic photoreceptors in the avian brain** • Gang Wang, University of Kentucky
- P252 Nighttime dim light exposure alters the responses of circadian system** • Dorela Shuboni, Michigan State University
- P253 Lifelong consequences of early postnatal light environment** • Maria Canal, University of Manchester
- P254 Dim and dimmer: Pupil reflex, masking, and entrainment thresholds** • Matthew Butler, Columbia University
- P255 Determining retinal photoreceptor contribution in light-dependent acute changes in body temperature** • Cara Altimus, Johns Hopkins University
- P256 CAST/EiJ mice—a unique animal model for studies in circadian entrainment, altered activity phases, masking, and sleep** • Peng Jiang, University of Kentucky

- P257 Neurochemical connections between sleep and circadian centers regulate behavioral timing** • Jennifer Arnold, University of Illinois at Urbana-Champaign, Urbana, IL, USA
- P258 Reduced NaV1.1 activity impairs SCN inter-neuronal communication, lengthens circadian period, and blocks light-induced phase shift in NaV1.1 mutant mice** • Sung Han, University of Washington
- P259 Ocular and circadian regulation of phosphorylation of extracellular regulated kinase and the NR1 NMDA receptor subunit in the hamster suprachiasmatic nucleus** • Ian C. Webb, University of Western Ontario
- P260 Interactions between the circadian clock and serotonin gene networks** • Holly Resuehr, Vanderbilt University
- P261 CRY1 and CRY2 are not necessary for circadian firing rhythms in the mouse suprachiasmatic nucleus** • Daisuke Ono, Hokkaido University Graduate School of Medicine
- P262 Aging in the circadian system: The VIP hypothesis** • Christopher Colwell, University of California–Los Angeles
- P263 Vasoactive intestinal peptide mediates photic regulation of Per1 expression in the suprachiasmatic nucleus** • Andrew Vosko, University of California–Los Angeles
- P264 Cholecystokinin (CCK) expressing neurons in the suprachiasmatic nucleus: Innervation, light responsiveness, and entrainment in CCK-deficient mice** • Jens Hannibal, Bispebjerg Hospital
- P265 Hypothalamic neuropeptides involved in the SCN control of hepatic glucose production** • Andries Kalsbeek, University of Amsterdam
- P266 Anticipation of a daily palatable snack in mice varies with behavior and snack content** • Danica Patton, Simon Fraser University
- P267 Lack of effect of phenobarbital administration on number or activation of RFRP3 neurons in the DMH of proestrous golden hamsters** • Kathleen Franklin, University of Kentucky
- P268 Genome-wide profiling of diel and circadian gene expression of the malaria vector *Anopheles gambiae*** • Samuel Rund, University of Notre Dame
- P269 General anesthesia phase shifts the honey bee circadian clock** • Eva Winnebeck, University of Auckland
- P270 There is a worm in my clock: Circadian rhythms in *C. elegans*** • Diego Golombek, National University of Quilmes
- P271 A blue-light photoreceptor is involved in circadian clock resetting in the model organism *Chlamydomonas reinhardtii*** • Jennifer Forbes-Stovall, Western Kentucky University
- P272 A hypothesis to explain the ultradian clock that controls the frequency of the behavioral response in *Paramecium*** • Robert Hinrichsen, Indiana University of Pennsylvania
- P273 The circadian clock of the marine picoeukaryote *Ostreococcus tauri*: Insight into the transcriptional regulation of CCA1 through a G box hexamer** • Sophie Sanchez-Ferandin, Université Paris 6
- P274 Suprachiasmatic nucleus organization** • Robert Y. Moore, University of Pittsburgh

Slide Session Abstracts

11:00 • S1

USP2, a de-ubiquitinating enzyme, directly regulates BMAL1 stability and sensitivity to early evening light

HEATHER SCOMA, MONICA HUMBY, JOSEPH BESHARSE

Cell Biology, Medical College of Wisconsin, Milwaukee, WI, USA

Daily entrainment to environmental light dark cycles involves adjustments in the expression of several clock components in the SCN. We have identified a highly circadian de-ubiquitinating enzyme, USP2, involved in the direct regulation of BMAL1 stability through de-ubiquitination. Mice lacking USP2 (USP2^{-/-}) are more sensitive to early evening dim light exposure and BMAL1 protein abundance in the SCN is reduced with altered phasing. USP2 is found in complexes containing several clock components, and BMAL1 stability and half-life are increased by over-expression of USP2. Furthermore, BMAL1 can be de-ubiquitinated in the presence of USP2. This identifies USP2 as a new component of the molecular clock machinery directly regulating BMAL1 stability. However, the simple regulation of BMAL1 half-life does not readily explain the entrainment phenotype of USP2^{-/-} mice. One possibility is that loss of USP2 could enhance BMAL1 transcriptional activity in keeping with the finding that BMAL1 transcriptional activation is closely coupled to its ubiquitination. Alternatively, USP2 may target other circadian clock components or other proteins within the retinohypothalamic tract. To begin testing the former we have found that PER1 stability is not directly regulated by USP2 and that loss of Per1 does not enhance sensitivity to early evening light. Nonetheless, altered expression of CLOCK/BMAL1 controlled genes, like PER1, during early evening dim light exposure may account for the increased sensitivity observed in USP2^{-/-} mice.

11:15 • S2

The deubiquitinating enzyme USP2 is involved in the regulation of circadian rhythms

DAVID DUGUAY¹, YAOMING YANG², ADELINE RACHALSKI¹, GERRY BAQUIRAN², LYDIA OUELLET²,
KAI-FLORIAN STORCH¹, SIMON WING², NICOLAS CERMAKIAN¹

¹*Department of Psychiatry, Douglas Mental Health University Institute,
McGill University, Montreal, Quebec, CANADA*

²*Department of Medicine, Polypeptide Hormone Laboratory and Health Centre
Research Institute, McGill University, Montreal, Quebec, CANADA*

Post-translational modifications of clock proteins are involved in the mechanisms governing circadian physiology. Ubiquitination by enzymes SCFFbx13 and SCFbeta-TRCP1/2 is required for the proper functioning of the clock, raising possibilities about the involvement of deubiquitinases. The gene encoding deubiquitinase USP2 is among the few genes to be rhythmically expressed in multiple tissues. Our objective is to test the possible role of USP2 in the molecular clockwork and in clock output. To this end, we have generated Usp2 knock-out (KO) mice and studied their locomotor activity rhythms in running wheels (KO, n=6; WT, n=9). The endogenous period was increased by 16 minutes in Usp2 KO mice compared to WT mice. Phase resetting after an advance of 6 hours of the light/dark cycle was slower while phase resetting after a delay of 6 hours was faster in Usp2 KO mice. The phase-response curve was altered in Usp2 KO mice: a pulse at CT14 induced a larger delay while advances after pulses at CT20 and CT22 were blunted compared to WT mice. Biochemical studies

showed that USP2 interacts with PER1, PER2, CRY1, CRY2 and BMAL1. Furthermore, ubiquitinated PER1 was found to be a target of USP2. To assess the effect of USP2 on clock gene regulation, we tested USP2 action on the transcriptional activity of CLOCK/BMAL1 and PER/CRY heterodimers on a Per1-luciferase reporter: USP2 reduces CLOCK/BMAL1-induced luciferase activity driven by Per1 promoter and increases the transcriptional repression by PER/CRY heterodimers. Consistent with this, in mouse embryo fibroblasts, PER1 and PER2 protein levels were increased in cells prepared from Usp2 KO embryo. The first circadian peak of Per2 and Rev-erb α mRNA expression after fibroblast clock synchronization was increased in Usp2 KO cells. Taken together, our results indicate a key role for USP2 in the post-translational regulation of core clock proteins.

11:30 • S3

Circadian rhythms in astrocytes depend on intercellular interactions and connexin 43

LUCIANO MARPEGAN, CONNIE TSAI, TATSIANA SIMON, ERIK HERZOG
Biology, Washington University, St. Louis, MO, USA

Evidence from in vivo and in vitro studies indicates that intercellular communication modulates the rhythmic physiology of the suprachiasmatic nuclei (SCN). Glial cell activity has been implicated, but understudied, in the circadian system. We showed previously that astroglia are competent circadian oscillators which synchronize to diffusible signals from the SCN and that vasoactive intestinal polypeptide (VIP) can coordinate rhythms in glia. Here, we studied the effects of cell-cell interactions on circadian timing in cultured astrocytes. We measured PER2::LUC bioluminescence rhythms from cultured, mouse cortical astrocytes. We found that astrocytes plated at high density (1200 cells/mm²) displayed longer periods and faster damping rates than low density cultures (100 cells/mm²; 24.7 \pm 0.1 vs. 25.9 \pm 0.1 h, $p < 0.01$). These period and damping rate differences were preserved when astrocytes at high density were cultured next to astrocytes at low density, leading us to hypothesize that glia can modulate circadian timing in neighboring glia. Since gap junctions can mediate short-range interactions between astrocytes, we tested the effects of gap junction blockers. Meclofenamic acid (150 μ M) increased the damping rate in both high and low density astroglia and decreased the period in high density cultures (25.4 \pm 0.1h to 23.6 \pm 0.3h, $p < 0.05$). Similar results were observed with Carbenoxolone (200 μ M), another potent gap junction blocker. Using immunocytochemistry and Western blots, we found connexin 43, the major component of gap junctions in astrocytes, was circadian in cultured cortical astrocytes. Expression of luciferase from a transgenic connexin 43 promoter transfected into astrocytes revealed circadian rhythms in bioluminescence. Taken together these results suggest that intercellular communication, perhaps via gap junctions, among astrocytes modulates the period and sustainability of their circadian cycling. Supported by NIMH grant 63107 and NIH, Neuroscience Blueprint Center Core Grant P30 NS057105.

11:45 • S4

Regulation of circadian period by neuronal Agrin and the $\alpha 3$ isoform of Na⁺/K⁺-ATPase (ATP1A3)

MARTIN RALPH¹, GREER KIRSHENBAUM², VINCENT BRIENZA³, JOHN RODER⁴

¹*Centre for Biological Timing and Cognition, University of Toronto, Toronto, Ontario, CANADA*

²*Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, CANADA*

³*Psychology, University of Toronto, Toronto, Ontario, CANADA*

⁴*Medical Genetics, University of Toronto, Toronto, Ontario, CANADA*

Mutations in the $\alpha 3$ subunit of Na⁺/K⁺-ATPase (ATP1A3) are associated with rapid onset Parkinsonian dystonia, epileptiform seizures, and other physical and behavioral disorders. One point mutation

(I810N, Myshkin, myk) produces significant lengthening of circadian period of locomotor behavior in myk/+ hets.—comparable to the long period Clock mutation in the mouse. But, whereas this long period phenotype (~25.5h) is reliably produced in behavior, the circadian period in vitro is not different from WT (~23.7h), measured as rhythms of Per2::luciferase activity from the suprachiasmatic nucleus (SCN), other brain regions, and peripheral organs. Based on the hypothesis that altered input to the SCN could lengthen period in vivo, we examined a null mutation of the neuronal Agrin gene (agr). Neuronal Agrin regulates Na⁺/K⁺-ATPase activity, and is an inhibitory ligand of ATP1A3. To date, its primary defined roles have been in synaptogenesis and synaptic stability at the neuromuscular junction (NMJ). While agr/+ hets also showed minor period lengthening, this mutation produced a near complete rescue of the WT phenotype in myk⁺/agr⁺ double hets. The results suggest that period lengthening results from the reduction of ATPase activity (42%), and that this can be reversed by reducing inhibitory regulation. This is the first demonstration of a functional role for Agrin in the brain, and suggests that the regulation of Na⁺/K⁺-ATPase activity at synapses in entrainment pathways may be operative in the control of rhythm generation. Supported by grants from the Natural Sciences and Engineering Research Council of Canada (MRR) and the Canadian Institutes of Health Research (JCR).

12:00 • S5

Circadian synaptic plasticity in hypocretin axons is regulated by neuronal pentraxin

**LIOR APPELBAUM¹, GORDON WANG², TOHEI YOKOGAWA³, GEMINI SKARIAH¹, STEPHEN SMITH²,
PHILIPPE MOURRAIN¹, EMMANUEL MIGNOT¹**

¹*Psychiatry and Behavioral Sciences, Stanford University, Palo Alto, CA, USA*

²*Molecular and Cellular Physiology, Stanford University, Palo Alto, CA, USA*

³*NICHHD, NIH, Bethesda, MD, USA*

Neurons exhibit rhythmic activity that ultimately affects behavior, such as sleep. Here, we use time-lapse two-photon imaging in living zebrafish of pre- and postsynaptic markers in hypocretin/orexin (HCRT) neurons to determine the dynamics of synaptic modifications during day and night. We observed circadian rhythmicity in synapse number in HCRT axons, which is independent of sleep and wake. Furthermore, we identify Neuronal Activity-Regulated Pentraxin (NARP/NPTX2/NP2), a protein implicated in AMPA receptor clustering, as a regulator of this process. In zebrafish, nptx2b is a clock-controlled gene primarily expressed in HCRT and pineal gland cells. Transgenic nptx2b arrhythmic overexpression (hcrt:NPTX2b) increases synapse number and abolishes rhythmicity in HCRT axons. Finally, hcrt:NPTX2b fish are resistant to the sleep promoting effects of melatonin. This behavioral effect, opposite to that reported in HCRT receptor mutants (hcrt^{r-/-}), is consistent with NPTX2b mediated increased activity of hypocretin circuitry. These data provide real time, in vivo evidence of circadian regulation of synaptic plasticity.

12:15 • S6

Cell membranes and the Arabidopsis circadian clock

ALEXANDRE MARTINIERE¹, DOMINIC CONQUEST², JOHN RUNIONS¹, HARRIET McWATTERS²

¹*School of Life Sciences, Oxford Brookes University, Oxford, UNITED KINGDOM*

²*Department of Plant Sciences, University of Oxford, Oxford, UNITED KINGDOM*

Circadian clocks are an important adaptation to life on a rotating planet and are found in all eukaryotes. The cycle of day and night is the major signal used by a clock to synchronise with its environment. However, the circadian system also integrates information from daily changes in temperature as temperature cycles can entrain the Arabidopsis clock in constant light or dark. However, no temperature receptor is known in higher plants. Our experiments have revealed that the viscosity of wild type

Arabidopsis plant cell membranes alters across the day and this response is changed in plants with mutations in fatty acid biosynthesis pathways (fatty acid desaturase (fad) mutants). We have examined the circadian phenotypes of a range of fad mutants and found a subset have temperature-sensitive circadian phenotypes and changes in temperature compensation. Hypocotyl growth and flowering time are affected in the same mutants, as is the response to blue light, suggesting that fad mutations cause pleiotropic effects in a range of light and clock pathways. This is suggestive that lipid membranes are involved in temperature sensing. Given the apparent requirement for normal lipid synthesis for temperature compensation of a fungal clock, this work will allow a comparative view of 'thermometer' function in circadian clocks.

11:00 • S7

Novel small molecules as potent enhancers and modulators of the circadian clock

ZHENG (JAKE) CHEN¹, SEUNG-HEE YOO², KYUNG IN KIM², KEON-HEE KIM¹, ETHAN BUHR³, DAVID FERSTER³, JOSEPH TAKAHASHI², STEVEN MCKNIGHT⁴

¹*Biochemistry & Molecular Biology, University of Texas Health Science Center at Houston, Houston, TX, USA*

²*Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA*

³*Neurobiology and Physiology, Northwestern University, Evanston, IL, USA*

⁴*Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA*

The circadian clock coordinates daily oscillation of essential physiological and behavioral processes; conversely, dampened amplitude and abnormal period are associated with numerous chronic diseases and ageing. The core molecular clock, present in virtually all cells of our body, has been relatively well-characterized, but clock regulatory pathways are not fully understood. To identify small molecules capable of manipulating the clock, we conducted a high-throughput screening of 200,000 diversity compounds using mPer2::lucSV reporter fibroblast cells. Validated primary hits were subjected to secondary screening using Bmal1:luc U2OS cells harboring a distinct reporter. Twenty five hit molecules were identified, including 6 analogue series and 9 lone hits. Several structurally related quinazoline and pyridopyrimidine derivatives most dramatically lengthened the period; in vitro kinase assay demonstrated their inhibitory activities of casein kinase I (CKI), highlighting a predominant role of CKI in the regulation of clock period. A number of small molecules were found to broadly enhance clock amplitude, acting through cAMP-dependent or -independent pathways. Importantly, these small molecules robustly enhanced the weakened clocks in mutant cells derived from mouse models harboring individual mutations in core clock genes. Furthermore, specific benzodiazepine and pyrazolopyrimidine derivatives were found to exert opposing clock effects in both cells and suprachiasmatic nucleus (SCN) slices, suggesting novel modes of action distinct from those of classical benzodiazepines and pyrazolopyrimidines widely used as agonists of GABAA receptors and psychoactive drugs. Taken together, these studies highlight predominant roles of CKI and cAMP signaling in the respective regulation of clock period and amplitude, and suggest novel regulatory mechanisms that can be manipulated by novel drug-like small molecules.

Identification and characterization of inhibitors of casein kinase epsilon/delta

JESSICA ADAMS¹, KATHERINE FISCHER¹, JENNIFER BRADLEY¹, DAVID GEBHARDT¹, ANGELA DORAN², KEVIN WALTON³, TRAVIS WAGER⁴, JEFFREY OHREN⁵, RAMALAKSHMI CHANDRASEKARAN⁴, SCOT MENTE⁵, VANESSA PARADIS⁴, JIANKE LI⁵, BLOSSOM SNEED¹, JAMES OFFORD³

¹Primary Pharmacology Group, Pfizer Global Research, Groton, CT, USA

²Pharmacokinetics and Drug Metabolism, Pfizer Global Research, Groton, CT, USA

³Neuroscience Research Unit, Pfizer Global Research, Groton, CT, USA

⁴Department of Chemistry, Pfizer Global Research, Groton, CT, USA

⁵Structural Biology Group, Pfizer Global Research, Groton, CT, USA

CK1 delta (CK1 δ) and CK1 epsilon (CK1 ϵ) are closely related members of a family of seven mammalian serine/threonine protein kinases previously known as casein kinases. In this study, we report the discovery and characterization of a highly selective class of inhibitors of CK1 δ/ϵ derived from a substituted pyrazolopyrimidine scaffold. Surprisingly, given the 100% predicted sequence identity between the two CK1 isoforms within 8Å of the kinase ATP-binding pocket; ATP-competitive compounds were identified that demonstrate more than 40-fold selectivity for CK1 ϵ over CK1 δ . Structural analysis suggests that the compounds' broad selectivity against the protein kinome and their CK1 ϵ isoform selectivity stems from the presence of a three-point interaction with the kinase hinge region, the ATP ribose binding pocket and in particular, from the hydrophobic spine region located near the CK1 gate-keeper residue. The discovery of these compounds now allows for the careful dissection of the roles the two CK1 isoforms in the mammalian molecular clock as well as in Wnt signaling, cancer and other diseases.

Therapeutic rescue of disrupted circadian behavior through CK1 δ inhibition

DAVID BECHTOLD¹, ELIZABETH MAYWOOD², QING-JUN MENG¹, JOHANNA CHESHAM², FRANCIS RAJAMOCHAN³, JEFFREY OHREN³, KEVIN WALTON³, TRAVIS WAGER³, MICHAEL HASTINGS², ANDREW LOUDON¹, WEI-QUN LU¹, JIAN LI¹, MARTIN SLÁDEK², JULIE GIBBS¹, SANDRINE DUPRE¹, JOHN KNAFELS³, BLOSSOM SNEED³, LAURA ZAWADZKE³

¹Faculty of Life Sciences, University of Manchester, Manchester, UNITED KINGDOM

²MRC Laboratory of Molecular Biology, University of Cambridge, Cambridge, UNITED KINGDOM

³Global Research and Development, Pfizer, Groton, CT, USA

Circadian rhythms in gene expression are dictated not only by transcriptional feedback between the core clock genes, but also through post-translational modification, cellular compartmentalization, and degradation of the clock proteins. For example, in mammals the activities of casein kinase 1 (CK1) ϵ and δ can potently modulate circadian period by regulating PER protein turnover. Using selective pharmacological inhibitors, here we demonstrate that inhibition of CK1 δ (with PF-670462), but not CK1 ϵ (with PF-4800567), dose-dependently lengthens circadian periods in behavioral activity, as well as clock gene rhythms in SCN and peripheral tissue slices derived from WT mice, confirming the dominant role played by CK1 δ in the circadian clockwork. Importantly, we go on to show that period lengthening induced by CK1 δ inhibition either *in vivo* or in SCN slice cultures is accompanied by an enhanced nuclear retention of PER protein (presumably due to impaired CK1-dependent phosphorylation and degradation of PER), and a selective extension of PER-mediated transcriptional repression. The enhancement of PER activity suggests that CK1 δ inhibition may be effective at strengthening inherently

weak (low amplitude) oscillators. This is indeed the case. First, whereas exposure of WT mice to constant light disrupts their behavioral cycles, daily administration of PF-670462 results in robust and stable 24hr entrainment. Second, daily treatment with PF-670462 rescues the disrupted behavioral rhythms of *Vipr2*^{-/-} mice housed in constant dark. Moreover, CK1 δ inhibition is also effective at rescuing clock gene oscillations in SCN slices derived from *Vipr2*^{-/-} mice. Therefore, CK1 δ modulation of PER protein localization and activity represents a novel and effective therapeutic target for strengthening of circadian rhythms. (Grant support: BBSRC, UK, and MRC, UK).

11:45 • S10

Who's ubiquitinatin' whom: Partnering proteasomal machinery with the clock

JASON DEBRUYNE, JOHN HOGENESCH

Pharmacology/ITMAT, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Ubiquitin-mediated proteasomal degradation is vital to nearly all aspects of cellular function. Deficits in this process are linked to diseases ranging from cancers to neurodegenerative disorders. Kinetic processes, such as the cell cycle and the circadian clock, require active degradation of their constituents; a protein cannot have a 24-hour abundance rhythm if its half-life is several days. Recent biochemical and genetic screens have begun to determine components of the proteasome that target mammalian clock proteins for degradation (e.g., β TrCP1/2 and Fbxl3). To approach this problem at scale, we have devised a genetic screen of more than 400 ubiquitin (E3) ligases and related proteins employing high content imaging of mammalian clock proteins. Our initial screening of PER1, CRY1, and NR1D1 (Rev-Erb α) has revealed a handful of E3 ligases capable of degrading each. Several of these genes have a circadian transcriptional rhythm in the liver with phases consistent with roles in degrading their targets in vivo. Our preliminary knockdown studies indicate that several of these enzymes are required for normal cellular oscillator function. Although preliminary, these results suggest the utility of this approach in identifying biologically relevant E3-substrate pairs. Its extension has obvious utility for many other biological pathways.

12:00 • S11

A sequence of specific phosphorylation events controls a core post-translational interval-timer of the Drosophila circadian clock

DANIELA ZORN, HSIU-CHENG HUNG, CHRISTIAN MAURER, WAI-LING CHANG, FRANK WEBER

Biochemistry Center Heidelberg, University of Heidelberg, Heidelberg, GERMANY

The circadian clock facilitates a temporal coordination of most homeostatic activities and their synchronization with the environmental cycles of day and night. The core oscillating activity of the circadian clock in *Drosophila* and mammals is formed by a heterodimer of the transcription factors CLOCK (CLK) and CYCLE (CYC). Post-translational regulation of CLK/CYC has previously been shown to be crucial for clock function and accurate timing of circadian transcription. We identified a sequence of compartment specific phosphorylation events that control the life cycle of the *Drosophila* CLK protein. Newly synthesized CLK is hypo-phosphorylated in the cytoplasm prior to nuclear import. Inside the nucleus, CLK is stabilized and converted into an intermediate phosphorylation state that correlates with trans-activation and inhibition of circadian transcription. Nuclear export promotes hyper-phosphorylation and degradation of the CLK protein. In addition to post-translational modification, we found CLK to be controlled by sub-nuclear distribution. Localization of the CLK protein in discrete sub-nuclear foci represents a storage form of the transcription factor, while homogeneous re-distribution correlates with transcriptional activation of nuclear CLK. Interestingly, we were able to identify and characterized specific consensus phosphorylation sites that control individual steps in the life cycle of the CLK protein

including hetero-dimerization with CYC, nucleo-cytoplasmic transport, transcriptional activation and inactivation, as well as degradation. These results reveal a sequence of specific phosphorylation events that target the CLK protein through its life cycle, constituting a core post-translational timing mechanism of the *Drosophila* circadian clock.

12:15 • S12

PSEUDO-RESPONSE REGULATORS 9 (PRR9), PRR7, and PRR5 are transcriptional repressors in the Arabidopsis circadian clock

NORIHITO NAKAMICHI¹, TAKATOSHI KIBA¹, ROSSANA HENRIQUES², TAKESHI MIZUNO³, NAM-HAI CHUA², HITOSHI SAKAKIBARA¹

¹*Plant Productivity Systems Research Group, RIKEN Plant Science Center, Yokohama, JAPAN*

²*Laboratory of Plant Molecular Biology, The Rockefeller University, New York, NY, USA*

³*School of Agriculture, Nagoya University, Nagoya, JAPAN*

An interlocking transcriptional-translational feedback loop of clock-associated genes is thought to be the clock's 'central oscillator' in a model plant *Arabidopsis thaliana*. Recent genetic studies have suggested that PSEUDO-RESPONSE REGULATOR 9 (PRR9), PRR7, and PRR5 act within or close to the loop, however their molecular functions remain unknown. In this study, we demonstrate that PRR9, PRR7, and PRR5 act as transcriptional repressors of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) genes. Each PRR9, PRR7, and PRR5 suppresses CCA1 and LHY promoter activities, and confers transcriptional repressor activity to a heterologous DNA binding protein (Yeast GAL4-promoter binding protein) in a transient reporter assay. Using a glucocorticoid-induced PRR5-GR (glucocorticoid receptor) construct, we found that PRR5 directly down-regulates CCA1 and LHY expression. Furthermore, PRR9, PRR7, and PRR5 associate with the CCA1 and LHY promoters in planta from morning to midnight, coinciding with the time these genes are repressed. These results suggest that the repressor activities of PRR9, PRR7 and PRR5 on the CCA1 and LHY promoter regions constitute the molecular mechanism that accounts for the role of three PRR proteins in the feedback loop of the circadian clock. We also discuss evolutionally conserved amino acid region in PRR9, PRR7, and PRR5, which is responsible for the repressor activity.

11:00 • S13

Obesity and metabolic syndrome in mice with an adipose tissue-specific deletion of Bmal1

GEORGIOS PASCHOS¹, WENLIANG SONG¹, SALAM IBRAHIM¹, TAKESHIGE KUNIEDA¹, CHRISTOPHER BRADFIELD², GARRET FITZGERALD¹

¹*ITMAT, University of Pennsylvania, Philadelphia, PA, USA*

²*School of Medicine and Public Health, University of Wisconsin, Madison, WI, USA*

Several lines of evidence suggest that energy metabolism and the circadian clock are interconnected. Previous studies of universal and tissue-specific knockout animal models of dysfunctional circadian clocks have shown changes in glucose homeostasis. To study the role of the adipose circadian clock in glucose metabolism we generated mice with an adipose tissue specific deletion of *Bmal1* (KO) and found them to be overweight compared to their littermate controls ($P < 0.05$, $n = 10$). Challenge with a high fat diet (HFD, 45% fat) reveals increased susceptibility of the KO mice to diet-induced obesity ($P < 0.01$, $n = 12$). The observed difference in body weight is attributed to increased body fat (21.9 ± 4.8 g vs. 14.6 ± 3.9 g, $P < 0.05$ in mice fed a HFD for 16 weeks) accompanied by adipocyte hypertrophy. No changes are observed in locomotor activity, energy expenditure or thermogenesis across the 24-hour cycle. Plasma leptin (12.2 ± 1.7 ng/ml vs. 7.5 ± 0.9 ng/ml, $P < 0.05$, $n = 22$) and triglycerides (101 ± 10.4 mg/dl vs. 66 ± 8.0 mg/dl, $P < 0.05$, $n = 21$) are increased in the KO mice. The KO mice show normal sensitivity

to insulin but delayed glucose clearance. Hyperinsulinemic-euglycemic clamp studies reveal reduced insulin suppression of hepatic glucose production of KO mice with no difference in the rate of glucose disposal from the circulation. These findings suggest resistance to insulin in the liver, but not in the periphery. Indeed, glucose uptake by muscle and visceral adipose tissue is normal in KO mice. In conclusion, deletion of *Bmal1* in the adipose tissue triggers a number of metabolic and systemic defects leading to abnormal glucose metabolism and obesity. This work is supported by NIH Grant RO1 HL097800.

11:15 • S14

The role of CREM/ICER in circadian events of the liver

DAMJANA ROZMAN, URSULA PROSENC, JAKA BEOVIC, ROK KOSIR

CFGBC, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, SLOVENIA

cAMP signaling participates in numerous processes in the liver, but little is known about its potential role in hepatic circadian regulation. CREB represents the major cAMP-dependent activator, while the isoform of the *Crem* gene ICER (inducible cAMP early repressor) is a cAMP-dependent repressor. Four *Icer* isoforms are transcribed from an alternative promoter of the *Crem* gene. We measured by qPCR expression of *Icerl* and *Icerlg* and found both expressed rhythmically. Additionally, a 17kDa CREM/ICER immunoreactive protein is rhythmically expressed in synchronised Hepa1-6 cells. To evaluate if CREM/ICER contribute to *Per* regulation in vivo, we measured expression of *Per1* and *Per2* in livers of *Crem*^{-/-} and w.t. mice (C57BL6x129Pas background) that were sacrificed in DD every 4h. The phase of hepatic *Per1* expression is not changed in *Crem* knockout mice but the *Per1* amplitude at CT12 is significantly lower. The peak of hepatic *Per2* expression at CT16 seems to be shifted to CT12–CT16 in *Crem*^{-/-} livers. Proximal promoters of *mPer1* and *mPer2* contain four potential CREs. To evaluate the importance of individual CREs, Hepa1 cells were transfected with mutated *mPer1* and *mPer2* reporters and overexpressed CREM τ activator and *Icerl* or *Igamma* repressors. Results indicate that more than a single CRE is involved in CREM-mediated activation or repression of *mPer* promoters. The presence of gamma exon in *Icer* seems to be important at least for the repression of *mPer1*. In conclusion, we show that *Icer* isoforms (mRNA and proteins) are rhythmically expressed in the liver. The absence of *Crem* in DD diminishes the peak amplitude of hepatic *Per1* and tends to shift the peak of *Per2* expression. Different CRE elements are responsible for CREM/ICER-mediated activation/repression of *mPer* promoters where gamma exon of *Icer* is important for the repression of *mPer1*.

11:30 • S15

Disruption of peripheral circadian timekeeping in a mouse model of Huntington's disease and its restoration by temporally scheduled feeding

ELIZABETH MAYWOOD¹, ELOISE FRAENKEL², KATHERINE McALLISTER², NIGEL WOOD², AKHILESH REDDY³,
MICHAEL HASTINGS¹, JENNIFER MORTON²

¹Neurobiology Division, MRC Laboratory of Molecular Biology, Cambridge, UNITED KINGDOM

²Department of Pharmacology, University of Cambridge, Cambridge, UNITED KINGDOM

³Department of Clinical Neurosciences, University of Cambridge, Cambridge, UNITED KINGDOM

Behavioural circadian rhythms disintegrate progressively in the R6/2 mouse model of Huntington's disease (HD), recapitulating the sleep-wake disturbance seen in HD patients. Here we show that disturbances in circadian pacemaking are not confined to the brain, but that peripheral clocks controlling circadian co-ordination of metabolic pathways are also disrupted. Notably, circadian rhythms of clock-driven genes that are key metabolic outputs in the liver are abolished. This loss of circadian metabolic programming is not a consequence of deficient pacemaking intrinsic to the liver, because when cultured in vitro, R6/2 liver slices (and other tissues) exhibit self-sustained circadian bioluminescence rhythms. Compromised metabolic cycles are therefore likely to be due to an internal desynchronisation

arising from impaired circadian signaling from the central pacemaker of the suprachiasmatic nucleus (SCN) to the peripheral clocks. Evidence for this is that in vivo there is a loss of rhythmic Cry1 and Dbp expression and a phase advanced Per2 cycle in the liver of R6/2 mice. Interestingly, the SCN-independent food-entrainable oscillator remains intact in R6/2 mice and, if invoked, can restore daily behavioral cycles and reverse some of the metabolic abnormalities seen in the liver. Disturbances of metabolism have long been thought to be important in HD. Uncoupling liver metabolism from circadian drives will reduce metabolic efficiency and cause imbalances in metabolites known to be deleterious to brain function. Thus, even subtle imbalances in liver function may exacerbate symptoms of HD, where neurological function is already compromised. Work supported by CHDI Foundation Inc. and The Royal Society (AJM), the Medical Research Council, U.K. (MHH and ESM) and the Wellcome Trust (ABR, WT083643MA).

11:45 • S16

Endogenous circadian rhythm in cardiovascular biomarkers during rest and in reactivity to standardized exercise

FRANK A. SCHEER, KUN HU, HEATHER EVONIUK, ERIN E. KELLY, ATUL MALHOTRA, MICHAEL F. HILTON, STEVEN A. SHEA

Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

The risk of adverse cardiovascular events peaks in the morning (~09:00) with a secondary peak in the evening (~20:00) and a trough at night. This pattern is generally believed to be caused by the day/night distribution of behavioral triggers (e.g., onset of activity in the morning). However, it is unknown whether the endogenous circadian system contributes to these daily fluctuations. Thus, we tested the hypotheses that the circadian system modulates autonomic, hemodynamic and hemostatic risk markers at rest and the responsiveness to physical activity, thereby increasing these cardiovascular risk markers at vulnerable times of day. 12 healthy adults were each studied throughout 240 hours while standardized rest and exercise periods were uniformly distributed across the circadian cycle. At rest, there were large circadian variations in plasma cortisol (peak-to-trough ~85% of mean, peaking at the circadian phase equivalent to ~09:00) and in circulating catecholamines (epinephrine; ~70%; norepinephrine; ~35%, both peaking during the biological daytime). Around ~20:00 there was a circadian trough in cardiac vagal tone and circadian peak in blood pressure. Sympathetic variables were consistently lowest and vagal markers consistently highest during the biological night. We detected no clear circadian effects on hemostasis (platelet count and aggregability). Notably, there was significant circadian modulation of cardiovascular reactivity/responsiveness to exercise, with greatest reduction in cardiac vagal tone around ~09:00, and peaks in catecholamine responsiveness to exercise at ~09:00 and ~21:00. Thus, the endogenous circadian system affects multiple cardiovascular risk markers and their responsiveness to exercise, with resultant circadian profiles that may contribute to the daily fluctuations in adverse cardiovascular events. Support: This work was supported by a Brigham Research Institute Fund to Sustain Research Excellence (to F.A.J.L.S.); NHLBI Grants RO1-HL76409 and K24-HL076446 (to S.A.S.); and GCRC Grant MO1-RR02635 (to Brigham and Women's Hospital).

12:00 • S17

Melatonin protects isolated ventricular cardiomyocytes from high-glucose-induced arrhythmias

NELSON CHONG, RICHARD RAINBOW

Cardiovascular Sciences, University of Leicester, Leicester, UNITED KINGDOM

Adverse cardiovascular events such as myocardial infarction, ventricular arrhythmias and sudden cardiac death occur with a day/night pattern, with high morning occurrence. Circadian disruption/

misalignment in rodents and humans can lead to cardiovascular dysfunction and diabetes. Diabetics and pre-diabetics have disrupted sleep and lower circulating melatonin. Hyperglycaemia associated with diabetes is an independent risk factor in cardiovascular disease. Sustained hyperglycaemia can cause abnormal cardiac electro-conductivity, cardiomyopathy and organ damage. The role of melatonin on cardiac function is unclear. Thus, we examined the effect of melatonin on HG induced cardiac conductivity dysfunction in vitro using patch clamp electrophysiology. Adult male rat ventricular cardiomyocytes were isolated and either treated with melatonin (10 μ M) or vehicle for one hour in media 199, prior to perfusion in Tyrode solution with either melatonin or vehicle [with normal (4 mM) or high glucose (HG, 15 mM)] during patch clamp electrophysiology recordings. Action potentials were stimulated at 1Hz and HG induced prolongation of action potential duration (APD) in myocytes from 88 ± 0.22 ms to 386 ± 36 ms ($n=51$, p 0.05). In conclusion, HG renders the cardiomyocytes "pro-arrhythmogenic" which was completely blocked by melatonin. This demonstrates that melatonin can regulate cardiac electrical conductivity in vitro and provides a novel insight in understanding the mechanisms of arrhythmias and platforms for therapeutic intervention.

12:15 • S18

Relationship of common polymorphisms in Clock to blood pressure and stroke outcome in man

MADHU J. PRASAI¹, AZHAR MAQBOOL², ROBERT WEST³, ANGELA M. CARTER¹, FATEMEH SAKHINIA¹, THOMAS MENTOR¹, PETER J. GRANT¹, ELEANOR M. SCOTT¹

¹Division of Cardiovascular and Diabetes Research, LIGHT Laboratories, University of Leeds, Leeds, UNITED KINGDOM

²Division of Cardiovascular and Neuronal Remodeling, LIGHT Laboratories, University of Leeds, Leeds, UNITED KINGDOM

³Division of Epidemiology and Biostatistics, LIGHT Laboratories, University of Leeds, Leeds, UNITED KINGDOM

Introduction: Blood pressure and presentation of acute vascular events show diurnal variation. Hypertension is known to be a major risk factor for vascular disease and animal studies have demonstrated that the molecular clock is involved in blood pressure control. We have previously shown an association between Clock polymorphisms and the metabolic syndrome in man and hypothesised that these polymorphisms were also associated with hypertension and vascular outcome in man. **Methods:** We performed genotype and haplotype analysis of 3 polymorphisms in the Clock gene in two studies: 87 patients with essential hypertension and 85 healthy controls; and 545 patients with ischemic stroke and 330 healthy controls. We looked at rs4864548, a C>T polymorphism in the Clock promoter; rs3736544, 2121G>A; and rs1801260, 3111T>C and the TGT and CGC haplotypes. **Results:** Adjusting for age, gender and BMI in a multiple regression model we found a strong association between possession of TGT haplotype and blood pressure (systolic BP $p<0.009$; diastolic BP $p<0.008$). There was no association with individual polymorphisms or haplotype and mortality after stroke. rs1801260 ($p=0.009$) and CGC haplotype ($p=0.005$) were associated with the individual having survived a previous ischemic event. **Conclusions:** This study supports our previous work and provides novel evidence that genetic variation in Clock is associated with the development of hypertension and may influence outcome after a vascular event in man.

A role for the dorsomedial hypothalamic nucleus in food anticipatory rhythms in rats

GLENN LANDRY¹, DANICA PATTON¹, MARK JAHOLKOWSKI¹, BRIANNE KENT², RALPH MISTLBERGER¹

¹Psychology, Simon Fraser University, Vancouver, British Columbia, CANADA

²Psychology, Yale University, New Haven, CT, USA

The hypothalamic dorsomedial nucleus (DMH) is a site of circadian clock gene expression inducible by daytime restricted feeding schedules that entrain food anticipatory activity rhythms (FAA). The role of the DMH in the expression of FAA is unclear. Ibotenic acid DMH lesions spare fibers of passage and attenuate FAA (Gooley et al, 2006), whereas radiofrequency DMH lesions ablate fibers of passage but do not suppress FAA (Landry et al, 2006, 2007; Moriya et al, 2009), unless the lesions extend ventrally to include the arcuate nucleus (ARC; Moriya et al, 2010). A preliminary report indicates that FAA attenuated by axon-sparing DMH lesions can be restored by suprachiasmatic nucleus (SCN) ablation (Agosta-Galvan et al, SRBR 2008). These results suggest a specialized role for the DMH as inhibitor of SCN outputs that normally oppose daytime activity in nocturnal rodents. We report additional evidence for this interpretation. Rats received bilateral DMH injections of ibotenic acid or NPY-saporin toxin and intraperitoneal transponders to measure activity and temperature. Lesions were evaluated using stains for nissl, Per1, cFos and NPY receptor. FAA to a daytime meal (ZT6-10) was attenuated in some rats, but improved when mealtime was shifted to the night (ZT21-ZT1). Greater attenuation of FAA was associated with damage that included the VMH/ARC. These results support a model in which the DMH permits daytime FAA by inhibiting rest-promoting outputs from the SCN. These SCN outputs may project through or near the DMH, such that radiofrequency ablation of this area is functionally equivalent to combined DMH-SCN ablation. SCN outputs do not inhibit activity at night, explaining improved FAA to nighttime meals in DMH-ablated rats. Clock cells necessary for FAA may be distributed widely within the basomedial hypothalamus, explaining attenuated FAA in rats with more extensive DMH-ARC damage.

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The suprachiasmatic nucleus and the dorsomedial nucleus of the hypothalamus are part of a neuronal network that comprises the food entrained oscillator

RUUD BUIJS¹, GUADALUPE ACOSTA¹, CHUN-XIA YI², CAROLINA ESCOBAR³

¹Fysiology, Instituto de Investigaciones Biomedicas, Mexico, MEXICO

²Physiology, Netherlands Institute for Neuroscience, Amsterdam, THE NETHERLANDS

³Anat, UNAM, Mexico, MEXICO

Neuronal activity in the suprachiasmatic nucleus (SCN) signals sleep in nocturnal animals. Restricting food availability to the rest phase induces food anticipatory activity (FAA); increases neuronal activity in the dorsomedial nucleus of the hypothalamus (DMH) and reduces neuronal activity of the SCN. We show that inhibition of SCN neuronal activity is the consequence of FAA induced activation of NPFF and GABA containing neurons of the DMH that inhibit neurons in the light receiving part of the SCN. Therefore DMH lesioned animals diminish their FAA, because of loss of inhibitory input to the SCN resulting in restoration of daytime SCN neuronal activity which inhibits locomotor activity. Consequently animals that lost FAA after DMH lesion recover FAA following SCN lesion. These results demonstrate that the mechanism for FAA is a complex process facilitated by a versatile neuronal network of which SCN and DMH are just two components.

Increased food-anticipatory activity in tissue plasminogen activator knockout mice

ERIC MINTZ¹, LINLEY MORELAND¹, XIANG MOU², REBECCA PROSSER²

¹*Biological Sciences, Kent State University, Kent, OH, USA*

²*Biochemistry, Cellular and Molecular Biology, The University of Tennessee–Knoxville, Knoxville, TN, USA*

Tissue plasminogen activator (tPA) is a key enzyme in the pathway responsible for the generation of mature brain-derived neurotrophic factor (BDNF). Since BDNF activity is a necessary component of the photic entrainment pathway, we sought to determine the impact of the loss of tPA on entrainment of circadian activity rhythms. Mice deficient in tPA (tPAKO) and wildtype (WT) mice from the same background strain (C57BL/6J) were housed in constant darkness for at least two weeks, then exposed to a 15-min light pulse at CT 16. No significant differences in light-induced phase shifts between genotypes were detected using light pulses of 5, 50, and 300 lux intensities. Light pulses at CT 22 also did not result in phase shifts that differed between genotypes. A reduced level of mature BDNF in the SCN was confirmed by western blot, suggesting that a compensatory mechanism developed to maintain photic responses in an environment of reduced mature BDNF concentrations. We then sought to determine whether tPAKO animals showed any deficiencies in food anticipatory activity (FAA) during scheduled food restriction in a light-dark cycle. Since tPAKO animals are deficient in long-term potentiation and memory, we hypothesized that tPAKO animals would show decreased FAA as the animals would have difficulty learning the time of food restriction. In contrast, the results showed significantly enhanced FAA in tPAKO mice, which gradually decreased over a period of about 10 days until FAA was no longer enhanced relative to controls. No differences in activity were observed during a 48-hour fast prior to the period of restricted feeding, indicating that the increased FAA in tPAKO mice was specific to the restricted-feeding paradigm and not merely fast-induced. These data support a role for a tPA-dependent pathway during adaptation to a circadian restricted feeding schedule.

Attenuated food anticipatory activity and abnormal circadian locomotor rhythms in Rgs16 knockdown mice

NAOTO HAYASAKA¹, KAZUYUKI AOKI², SEITAROH YAMAGUCHI², KAZUMASA HORIKAWA², HIROSHI IKEGAMI³, SHIGEHARU WAKANA⁴, TAKAMICHI MURAKAMI⁵, MASAAKI MIYAZAWA⁶, SHIGENOBU SHIBATA²

¹*Anatomy and Neurobiology, Kindai University, Osaka-Sayama, JAPAN*

²*Pharmacology, Waseda University, Tokyo, JAPAN*

³*Endocrinology, Metabolism and Disease, Kindai University, Osaka-Sayama, JAPAN*

⁴*Japan Mouse Clinic, RIKEN BioResource Center, Tsukuba, JAPAN*

⁵*Radiology, Kindai University, Osaka-Sayama, JAPAN*

⁶*Immunology, Kindai University, Osaka-Sayama, JAPAN*

Regulators of G protein signaling (RGS) are multi-functional protein family, which functions, in part, as GTPase-activating proteins (GAPs) for activated G protein alpha-subunits. Previous studies have demonstrated that the transcript of the Rgs16 gene encoding one of the RGS family members, exhibits circadian rhythms both in the liver and suprachiasmatic nucleus (SCN), the central circadian light-entrainable oscillator (LEO) in the hypothalamus. To study a role of RGS16 in the circadian clock in vivo, we generated two independent Rgs16 knockdown mouse lines using lentiviral vectors stably expressing short hairpin RNA (shRNA). The knockdown mice demonstrated significantly shorter period in circadian locomotor activity rhythms and reduced total activity as compared to wild-type siblings. In addition, when feeding was restricted for four hours during the daytime, food-entrainable oscillator (FEO)-operated food-anticipatory activity, i.e. elevated food-seeking behavior observed for 1-3 hours

prior to the feeding time, was significantly attenuated in the knockdown mice. Food deprivation induced the Rgs16 expression both in the liver and restricted brain regions. Thus, our present study suggests that RGS16 is involved in the regulation of circadian clock systems operated not only by LEO, but also by FEO. We propose that RGS16 controls a G protein-mediated signaling pathway (s) in the brain and/or liver, which governs both LEO and FEO through a G protein-coupled receptor(s).

12:00 • S23

Using automated computer vision technology to study anticipatory activity in mouse

CYNTHIA HSU, KEITH GUNAPALA, DANIEL CHANG, **ANDREW STEELE**

Biology, Caltech, Pasadena, CA, USA

Animals in nature show an extraordinary ability to sense time and predict future events. We are using the mouse as a model system to understand the neural basis of anticipatory behaviors. We have worked with several different models for anticipation: 1) anticipation of daily food during chronic calorie restriction 2) anticipation of water access on chronic temporally restricted water access and 3) anticipation of social/sexual activity during daily timed female access for male mice. We measure anticipatory activity using a commercial computer vision system and are able to automatically describe the fine details of home cage behavior, including, food bin entry, drinking, hanging, jumping, walking, and grooming. Recently, we have been collaborating with computer vision scientists (T. Serre, H. Jhuang, E. Garrote, and T. Poggio) to develop an open source computer vision system to describe the fine details of home cage behaviors in mice automatically. Preliminary results of this system and its application to the study of anticipatory activity will be presented.

12:15 • S24

Chronic methamphetamine controls the phase and organization of peripheral oscillators

JENNIFER MOHAWK¹, PINAR PEZUK¹, TOMOKO YOSHIKAWA², MICHAEL SELLIX¹, MICHAEL MENAKER¹

¹*Biology, University of Virginia, Charlottesville, VA, USA*

²*Chronomedicine, Hokkaido University Graduate School of Medicine, Sapporo, JAPAN*

The suprachiasmatic nucleus (SCN) orchestrates circadian synchrony throughout the organism, coordinating rhythms of locomotor activity, body temperature, and clock gene expression in individual cells and tissues. The methamphetamine-sensitive circadian oscillator (MASCO) is capable of driving locomotor activity rhythms in the absence of the SCN, but its capacity as a true pacemaker, coordinating gene expression in peripheral oscillators, has not been thoroughly examined. We tested the ability of chronic methamphetamine to influence clock gene expression in peripheral tissues of PERIOD2::LUCIFERASE mice. In SCN-intact mice, methamphetamine had a disruptive effect on circadian organization. In the presence of an entraining light:dark cycle, methamphetamine shifted the phase of PER2 expression in some, but not all, tissues, resulting in altered phase relationships. Phase delays were observed in cornea and salivary gland. In animals housed in constant darkness, methamphetamine treatment resulted in highly variable phases of peak PER2 expression in salivary glands, whereas the salivary glands of untreated mice had peak PER2 expression with a more stable phase angle relative to locomotor activity. Methamphetamine also altered the phase relationships among individual tissues. In SCN-lesioned animals, however, methamphetamine administration was able to reinstate phase synchrony, with PER2 expression in peripheral tissues peaking with a more consistent phase relative to locomotor activity onset. In these animals, methamphetamine quickly synchronized the phases of salivary glands, lungs, and kidneys. Following an extended period on methamphetamine, the phases of livers and pineal glands were also synchronized. The results support

a role for both the SCN and MASCO in the organization of circadian rhythms. In the presence of methamphetamine, these two pacemakers compete for control of peripheral tissues. We conclude that MASCO is capable of controlling components of the canonical circadian molecular clock (e.g., PER2 expression) and organizing circadian oscillators in the absence of the SCN.

11:00 • S25

Physiologic indicators of sleepiness

ERIC CHUA, IVAN HO MIEN, ISABELLE JANG, JONATHAN BOSTICK, CHRISTOPHER PACHAS, JOSHUA GOOLEY
*Neuroscience and Behavioral Disorders Program,
Duke-NUS Graduate Medical School, SINGAPORE*

Sleepiness is a major cause of occupational and automobile accidents. Hence, there is considerable interest in non-intrusive techniques for detecting sleepiness that can be incorporated into an early-warning intervention system. Heart rate variability (HRV)-based measures are potential candidates, as electrocardiograph (ECG) signals can be obtained easily through wireless means. To evaluate HRV measures as indicators of sleepiness, we compared HRV features to commonly-used percentage eye closure (PERCLOS) measures for their ability to track changes in psychomotor vigilance. In a 4-day laboratory study, young healthy male subjects (21-30 yrs) performed a 10-minute psychomotor vigilance task (PVT) every 2 hours across a 40-hour constant routine procedure. Two PERCLOS measures, P70 and P80, i.e. the proportion of time the eyes were closed at least 70 and 80 percent respectively, were obtained by automated analysis of eye tracking data. HRV measures, as recommended by the Task Force on HRV Analysis, were obtained from a Lead II ECG. These HRV and PERCLOS measures were correlated with reaction time and lapses measured during the PVT. We found that several HRV measures correlated to a similar extent to PVT outcomes as the PERCLOS measures. These results suggest that HRV measures carry information about changes in vigilance, and that a model-based approach could potentially be used to estimate changes in vigilance from HRV measures. Support: NMRC (NIG09MAY07, JJG); Duke-NUS (Block Grant, A*Star & MOE, JJG). The authors are grateful to the staff at the Chronobiology and Sleep Lab, Duke-NUS GMS, for their assistance in performing the studies.

11:15 • S26

Identification and validation of a candidate gene for wake and REM sleep

KARRIE MRAZEK¹, MARTHA VITATERNA¹, CHRISTOPHER OLKER¹, JOSHUA MILLSTEIN², ANDREW KASARSKIS², ANTHONY GOTTER³, CHRISTOPHER WINROW³, JOHN RENGER³, FRED TUREK¹

¹*Center for Sleep and Circadian Biology, Northwestern University, Evanston, IL, USA*

²*Statistical Genetics, Sage Bionetworks, Seattle, WA, USA*

³*Neuroscience Department, Merck Research Laboratories, West Point, PA, USA*

From a large-scale sleep-wake phenotype and genotype analysis of 269 adult male mice from a [C57BL/6J X (BALB/cByJ X C57BL/6J F₁)] N₂ segregating cross, we identified 52 quantitative trait loci (QTL). Included in these was a QTL on chromosome 17 for wake, rapid eye movement (REM) sleep amount, and REM bout length. Furthermore, analysis of over 40,000 expressed transcripts in three brain regions (frontal cortex, hypothalamus, and thalamus) lead to the identification of the candidate gene, Neurotensin Receptor 1 (NTSR1), with an expression QTL (eQTL) LOD score of 3.385 in the hypothalamus. The expression levels of NTSR1 significantly correlated with wake and REM amounts mapped to the chromosome 17 QTL. Neurotensin has been previously shown to have effects on anxiety and despair behaviors. We have measured these behaviors in the NTSR1 knockout mouse, and studies are currently underway to examine sleep. Preliminary data demonstrates NTSR1 knockouts have significant differences compared to the wild type controls in open field activity measures including distance traveled ($p < 0.01$), percent of time spent in the center ($p < 0.05$), and percent of

time spent in the corners ($p < 0.01$), indicating an increase in anxious behavior. Depression has been associated with characteristic sleep alterations. Despite evidence for a strong genetic component for depression and anxiety, the specific genes and associated networks remain largely unknown. We have previously demonstrated associations between anxiety and depressive-like behavior and sleep architecture and amount, and now we have demonstrated that a candidate gene in the region of a QTL for sleep characteristics known to have comorbidity in humans with depression shows the same behavioral characteristics. Taken together, these findings indicate that a combined phenotype QTL and gene expression level eQTL approach is a powerful method for identifying genes involved in the regulation of complex behaviors, including sleep and related affective disorders. Support: This research was supported by Merck & Co., Inc., and a grant from Defense Advanced Research Projects Agency (DARPA) and the Army Research Office (ARO), award number DAAD19-02-1-0038.

11:30 • S27

The PERIOD3 variable number tandem repeat polymorphism and human sleep timing and duration

DERK-JAN DIJK¹, ALPAR LAZAR¹, ANA SLAK¹, JUNE LO¹, NAYANTARA SANTHI¹, MALCOLM VON SCHANTZ¹, JOHN GROEGER², SIMON ARCHER¹

¹Surrey Sleep Research Centre, University of Surrey, Guildford, UNITED KINGDOM

²Department of Applied Psychology, University College Cork, Cork, IRELAND

A variable number tandem repeat (VNTR) polymorphism in PER3 associates with diurnal preference. The PER3 VNTR also predicts sleep structure and vulnerability to the negative effects of sleep loss on cognition during the circadian night, as assessed behaviourally and by fMRI. Whether the PER3 VNTR affects sleep duration and sleep timing is currently not known. Here, we report associations between PER3 VNTR genotype and sleep timing and sleep duration, as assessed by three questionnaires administered to subjects not previously genotyped. Five hundred eighty nine healthy participants (358 males and 231 females, mean age = 25.68, SD = 4.04, age range = 20-36 years) were genotyped as part of an ongoing study. 11% of the subjects were homozygous for the long repeat (PER3^{5/5}), 45% were homozygous for the short repeat (PER3^{4/4}), and 44% were heterozygous (PER3^{4/5}). All subjects completed the Pittsburgh Sleep Quality Index (PSQI), the British Sleep Survey (BSS), the Horne-Östberg (HÖ), and the Munich Chronotype Questionnaire (MCQ). Diurnal preference varied significantly across the three genotypes. Self-reported wake time also varied significantly across the three genotypes ($p < 0.005$ for all three questionnaires). Wake time was earlier in PER3^{5/5} compared to both PER3^{4/4} and PER3^{4/5}. Bedtime (PSQI and MCQ) also varied significantly across the three genotypes and was earlier in PER3^{5/5} compared to the other two groups. Total time in bed and sleep duration varied across the three genotypes and was shortest in PER3^{5/5}. These preliminary analyses of the effects of the PER3 VNTR in a new sample of young adults confirm that homozygosity for the long allele (PER3^{5/5}) is associated with morningness, as assessed by both the HO and MCQ. The data also show that PER3^{5/5} is associated with earlier wake time and bedtime and a shorter sleep duration. Supported by AFOSR (FA-9550-08-1-0080) and BBSRC (B/F022883/1).

11:45 • S28

Light exposure and melatonin production in night workers

MARIE DUMONT, VALÉRIE LANCTÔT, RAPHAELLE CADIEUX-VIAU

Chronobiology Laboratory, Sacre-Coeur Hospital & University of Montreal, Montreal, Quebec, CANADA

It has been suggested that decreased melatonin production, due to acute suppression of pineal melatonin secretion by light exposure during night work, may explain increased cancer risks in night

workers. In this study, light exposure and melatonin production were assessed in 17 full-time rotating shift workers (13 women, 4 men, aged 23-50 y), and compared between night and day/evening shifts. Workers collected all urine for 48 h on each shift, using different bottles for “work time”, “sleep time”, and “other”. Melatonin production was estimated by the total excretion of 6-sulphatoxymelatonin (aMT6s), expressed in ng/h and averaged over the two 24-h periods. Light exposure was measured with an ambulatory monitor worn around the neck at all time, except during sleep when the monitor was placed at bedside. For the analyses, a recording of exactly 24 h was selected in the middle of the data train. There was no difference in total aMT6s excretion between night shifts (681.7 ± 335.0 ng/h) and day/evening shifts (639.4 ± 323.2 ng/h ($p= 0.69$; $n=14$)). For light exposure, analyses are in progress and results are available for 8 subjects. Averaged light exposure measured during work time was similar for night shifts (85.0 ± 62.8 lux) and day/evening shifts (119.9 ± 106.8 lux) ($p= 0.67$). Light exposure during night work was not significantly correlated with aMT6s excretion during the same period ($p= 0.10$), but it was negatively correlated with the total excretion over the 24 h ($p< 0.05$). Light exposure during day/evening work was not related to aMT6s, excreted either during work time ($p= 0.87$) or over the 24 h ($p= 0.82$). These preliminary results show that melatonin production is not necessarily suppressed during night work, but that higher light exposure during night work may decrease total melatonin production over the 24 h.

12:00 • S29

Gender differences in rhythmic gene expression in human chronic lymphocytic leukemia cells and T-cells in 9 patients: Impact of melatonin therapy on the timing of peak expression

GEORG BJARNASON¹, DAVID SPANER¹, CATHY WANG¹, TAMI MARTINO², MARTY STRAUME³

¹*Medical Oncology, University of Toronto, Toronto, Ontario, CANADA*

²*Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, CANADA*

³*Statistics, Customized Online Biomathematical Research Applications, Charlottesville, VA, USA*

Blood samples were obtained every 4 hours over 24 hours (6 samples) from 3 male and 6 female patients with chronic lymphocytic leukemia (CLL) being followed off therapy. CLL cancer cells and normal T-Cells were separated using negative selection and density gradient centrifugation. RNA was extracted and subjected to microarray studies (Affymetrix HG_U133_Plus2 chip). The purity of the isolated samples (measured by flow cytometry) was 99% for CLL cells and 80% for T cells. For T cells, there were 12,733 and 11,632 rhythmic transcripts in males and females respectively. Only 2,968 rhythmic transcripts were common to males and females. For CLL cancer cells, there were 15,094 and 13,415 rhythmic transcripts in males and females respectively, only 6,629 of which were common. In both T-cells and CLL cancer cells, thousand of other gender specific rhythmic transcripts had peak expression at very different times in males and females. The expected rhythm in clock gene expression was maintained in both CLL cancer cells and T-Cells. When the above sampling and microarray studies were repeated after patients were treated with 5 mg of Melatonin at night for 2 months, significantly fewer rhythmic genes were detected (13,415 vs. 8,977 in female CLL cells) and the peak time of gene expression was dramatically shifted (4 AM and 4 PM vs. 1 AM in female CLL cells). The rhythmic expression of important genes seen in a patient with indolent CLL was lost in a patient with an aggressive form of CLL. Loss of rhythmicity in locomotor activity has been associated with more rapid cancer growth. The gender differences in rhythmic RNA expression have clinical implication for biomarker discovery. The effect of melatonin on gene expression was unexpected.

Daily rhythms in human subcutaneous white adipose tissue: Comparison of lean, overweight, and type 2 diabetic subjects

DANIELLA OTWAY¹, SIMONE MÄNTELE¹, SILVIA BRETSCHEIDER¹, JOHN WRIGHT¹, PAUL TRYAHURN²,
DEBRA SKENE¹, DENISE ROBERTSON¹, JONATHAN JOHNSTON¹

¹*Faculty of Health and Medical Sciences, University of Surrey, Guildford, UNITED KINGDOM*

²*School of Clinical Sciences, University of Liverpool, Liverpool, UNITED KINGDOM*

There is growing evidence for a link between circadian and adipose biology. Daily rhythms of plasma leptin and adiponectin have been reported in both rodent and human subjects. Murine adipogenesis in vitro depends upon the circadian genes *Bmal1* and *Rev-erba*. Moreover, approximately 20% of the murine white adipose tissue (WAT) transcriptome undergoes 24-hour rhythmicity. Daily mRNA rhythms are attenuated in WAT of mice given a high fat diet and in a murine model of diabetes. However, the relationship between daily rhythms and obesity/diabetes has not been investigated in humans. In this study, we recruited lean, overweight and overweight-diabetic male volunteers. Subjects maintained a prescribed daily routine prior to entering the laboratory. Following laboratory acclimation, subjects gave hourly blood and 6-hourly subcutaneous WAT samples over a 24-hour period. All clock-related genes exhibited significant temporal variation of expression in WAT biopsies. For 7 of the 8 genes, there was no significant effect of subject group on expression. These data suggest that there is minimal difference in daily WAT rhythmicity between lean, overweight and overweight-diabetic humans. Differences in murine models may therefore be primarily due to dietary and strain-dependent effects. Current work is extending our analysis with plasma hormone assays and WAT arrays.

Non-transcriptional mechanisms are competent to sustain circadian rhythms in mammalian cells

JOHN O'NEILL¹, GERBEN VAN OOIJEN², MICHAEL HASTINGS³, ANDREW MILLAR², AKHILESH REDDY¹

¹*Institute of Metabolic Science, University of Cambridge, Cambridge, UNITED KINGDOM*

²*CSBE, University of Edinburgh, Edinburgh, UNITED KINGDOM*

³*Laboratory of Molecular Biology, Medical Research Council (UK), Cambridge, UNITED KINGDOM*

In recent years, a number of ubiquitous post-translational mechanisms have been reported to contribute to circadian rhythmicity. At the same time, few transcriptional mechanisms have been shown to be absolutely indispensable to the clockwork. One interpretation is that cellular rhythmicity may be an emergent property of a distributed network, comprised of reciprocally regulating systems of signal transduction, metabolic flux and gene expression. In order to investigate whether such non-transcriptional components are sufficient to sustain rhythmicity in isolation, we employed a range of experimental platforms and approaches. We now present evidence for circadian timekeeping in the absence of transcription in eukaryotes (mammalian and non-mammalian cells). For example, using saturating concentrations of transcriptional inhibitors we observe an additional cycle of correctly phased clock protein expression. Most critically, using enucleated cells, we observe persistent circadian oscillations in post-translational modification.

Circadian bias of poly(A) tail length regulation

SHIHO KOJIMA, CARLA GREEN

Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA

A key feature of circadian clocks is their generation of rhythms in both protein and mRNA levels, resulting in widespread control of physiology and behavior. Although almost 5-10% of mRNAs are expressed rhythmically in any given tissue, growing evidence suggests that in addition to transcriptional rhythms, post-transcriptional, translational, and post-translational rhythms are also critical for the maintenance and fine-tuning of the circadian rhythms. Because poly(A) tail length is important for determining the stability and/or translatability of mRNAs, we sought to find mRNAs that exhibit circadian rhythmicity in their poly (A) tail length. We isolated mouse liver RNA every four hours and used oligo(dT) chromatography to fractionate it into short (<75nt) and long (>75nt) classes of poly(A) tail length. These RNAs were analyzed on Affymetrix Gene 1.0 ST platform, we then examined the ratio of long to short tailed RNAs at each time point. We identified almost 400 genes as “poly(A) rhythmic” and the rhythms in tail length on a sample of >40 mRNAs were validated using the Poly(A) Tail Assay. Poly(A) tail length of these genes ranged from 0-300 nt approximately, and the average peak/trough difference in poly(A) length was 92nt. Among those poly(A) rhythmic genes many did not exhibit rhythms in total steady-state expression and most of the peaks of poly(A) rhythms are 18-21 hours later (or 3-6 hours earlier) than their steady-state expression rhythms. Surprisingly, 50% of poly(A) rhythmic genes had longest poly(A) tails at late daytime (ZT6-11), even though peak phase of steady-state mRNA expression was equally distributed around the clock. These data suggest that the regulation of poly(A) length is independent from its transcription and there might be a circadian bias for control of poly(A) length.

Transcriptome deep sequencing and the circadian regulation of alternative splicing in *Drosophila melanogaster*

JOSEPH RODRIGUEZ¹, SADANAND VODALA¹, KATHARINE ABRUZZI², MICHAEL ROSBASH²

¹*National Center for Behavioral Genomics, Brandeis University, Waltham, MA, USA*

²*Howard Hughes Medical Institute, Brandeis University, Waltham, MA, USA*

Circadian rhythms are regulated by a central clock, which is entrained by external stimuli like light and temperature in many organisms. The best-studied features of the central clock are positive/negative transcriptional feed-back loops, which contain components conserved between flies and mammals. This feedback mechanism has been implicated in the oscillating expression of hundreds to thousands of genes throughout the 24 hour cycle in mammals as well as flies. Although there are indications that alternative splicing of a key clock mRNA aids in maintaining robust rhythms in flies, alternative splicing more generally is poorly documented from the circadian point of view. To look at alternative splicing and the circadian transcriptome from a genomic perspective, we used deep sequencing of head RNA to identify alternative splicing events that change in frequency across circadian time. Preliminary data has identified over 200 alternative splicing candidates that oscillate in abundance. A significant portion of these candidates occur in CLK direct target genes (see abstract from Abruzzi et al.) and are perturbed in a clock mutant. We are in the process of confirming the extent to which global alternative splicing changes across circadian time and is regulated by co-transcriptional presence of the clock transcriptional machinery. Our current experiments also address the regulatory and functional role of selected alternative splicing candidates on the circadian clock itself, some of which may feedback to alter clock function.

Translation initiation is a regulatory step in the Drosophila circadian clock

DOGUKAN MIZRAK, MARC RUBEN, DEBORAH McLAUGHLIN, FUAD CHOWDHURY, JUSTIN BLAU

Department of Biology, New York University, New York, NY, USA

We have generated whole genome expression profiles from a homogenous population of functional pacemaker neurons—the *Drosophila* larval LNvs. From this, we identified a set of genes which are more highly expressed in LNvs than in other neurons and a second set of genes that are rhythmically expressed in LNvs. Both sets include the core clock genes *per*, *tim*, *vri* and *Pdp1*, validating these data. We noticed that genes encoding proteins involved in translation initiation are over-represented in these datasets: *Thor* (*Drosophila* 4EBP), a negative regulator of translation, is expressed ~25-fold more highly in LNvs than in other neurons; and two protein kinases that regulate translation initiation have >5-fold higher RNA levels in LNvs than in other neurons. Altered expression of these kinases—*Lk6* (*Drosophila* Mnk homologue) and *Pdk1*—changes period length and/or causes arrhythmicity. Therefore we propose that translation initiation is an important and previously unstudied control point in the *Drosophila* molecular clock. Furthermore, RNAs levels of the translation initiation factors eIF4B and eIF4G are higher at dawn than at dusk in LNvs. Rhythmic production of eIF4B and eIF4G could lead to rhythms in translation in LNvs which, in turn, could contribute to the observed delay in appearance of clock proteins such as PER. However, since eIF4B/G are such general translation factors, their rhythmic expression could make overall translation rates oscillate in LNvs. To test this idea, we induced GFP RNA at different times of day in LNvs and measured GFP protein levels. We found that GFP accumulates to higher levels when induced just after dawn (high eIF4B/G) than after dusk. Therefore we propose that global translation rates in LNvs change with time. This mechanism could generate rhythms in protein abundance even for constitutively expressed mRNAs.

miRNA-mediated control of circadian rhythms in Drosophila

CHEN GAFNI¹, URI WEISSBEIN¹, RAGHU RAM², NETA ELUL¹, NIR FRIEDMAN³, ERAN MESHORER²,
SEBASTIAN KADENER¹

¹*Biological Chemistry Department, Silberman Institute of Life Sciences,
The Hebrew University of Jerusalem, Jerusalem, ISRAEL*

²*Genetics Department, Silberman Institute of Life Sciences, The
Hebrew University of Jerusalem, Jerusalem, ISRAEL*

³*Computer Sciences and Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, ISRAEL*

Most organisms use circadian (24hs) clocks to keep temporal order and anticipate daily environmental changes. In *Drosophila*, the master genes clock (CLK) and cycle (CYC) activate the circadian system by promoting rhythmic transcription of several key clock genes. Three of these target gene products, PER, TIM and CWO repress CLK-CYC mediated transcription on a daily basis. There is also an important

contribution of post-transcriptional and post-translational regulation to circadian timekeeping. In the last year, others and we have found yet another layer of regulation operating in the *Drosophila* circadian clock: translational regulation by miRNAs. More precisely, we have found that the miRNA bantam regulate *clk* through three evolutionary conserved binding sites in the *clk* 3'UTR. Importantly, we found that these sites are necessary for *Drosophila* circadian rhythms. In the present work we have expanded our findings and further characterize the regulation of *Clk* and other circadian relevant mRNAs by miRNAs. Using single cell live imaging we demonstrated that miRNA-mediated regulation decrease CLK variation and strongly increases the robustness of *Clk* gene expression. miRNA-mediated regulation not only decreases the levels of *Clk* but also sharpens CLK-CYC mediated gene expression. We hypothesize that this regulation is key for the generation of coherent and high amplitude oscillations on CLK-CYC transcriptional activity. We are currently testing if this role of miRNAs on the robustness of circadian gene expression can be extended to single neurons in the fly brain.

12:15 • S36

microRNA-mediated posttranscriptional control of mammalian clock gene expression regulates circadian oscillator performance

EUGIN DESTICI, GIJSBERTUS VAN DER HORST

Genetics, Erasmus Medical Centre, Rotterdam, THE NETHERLANDS

Circadian rhythms in gene expression are generated by a set of interlocked transcription/translation feedback loops. An important level of regulation contributing significantly to oscillator performance is imposed by postranslational modifications of most, if not all, of the core clock proteins. Recently, work from *Drosophila* has implicated another layer of control through microRNA-mediated posttranscriptional control of the *clock* gene. In mice, periodicity of circadian behavior and light resetting were shown to be influenced by two microRNAs that were suggested to regulate genes involved in neuronal excitability. To determine whether and to what extent microRNAs postranscriptionally regulate mammalian core clock protein synthesis, we performed a small microRNA over-expression screen in cultured fibroblasts using *Bmal1::luciferase* as real-time readout for circadian oscillator performance. We identified various microRNAs that, when over-expressed, shortened or lengthened circadian period and we found microRNAs that affected amplitude, and in some cases (almost) completely abolished oscillations. For several of these microRNAs, we provide evidence that they control core clock protein levels. The physiological relevance of these results is demonstrated by our finding that endogenous microRNAs also influence the circadian oscillator. Together, our results reveal a broad role for microRNAs in tuning circadian oscillator performance through control of core clock gene expression.

Upstream Transcription Factor 1 (USF1) is responsible for Suppressor of Clock (Soc): Uncovering a hidden transcription pathway for circadian clock genes

KAZUHIRO SHIMOMURA^{1,2,3,4}, PHILLIP L. LOWREY², VIVEK KUMAR^{1,4,5}, JASON CHONG², ETHAN D. BUHR², SHARON S. LOW², CHIAKI OMURA^{1,2}, DEBORAH FENNER^{1,2}, MARC RICHARDS², HEEKYUNG HONG^{1,2,4}, MARTHA H. VITATERNA^{1,2,3}, MATHEW T. PLETCHER⁶, TIM WILTSHIRE⁶, JOHN B. HOGENESCH⁷, AKIKO HIDA⁸, KAZUO MISHIMA⁸, HIROSHI KADOTANI⁹, JOSEPH S. TAKAHASHI^{1,2,3,4,5}

¹*Center for Functional Genomics, Howard Hughes Medical Institute, Northwestern University, Evanston, IL, USA*

²*Department of Neurobiology & Physiology, Howard Hughes Medical Institute, Northwestern University, Evanston, IL, USA*

³*Center for Sleep and Circadian Biology, Howard Hughes Medical Institute, Northwestern University, Evanston, IL, USA*

⁴*Howard Hughes Medical Institute, Northwestern University, Evanston, IL, USA*

⁵*Department of Neuroscience, Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center, Dallas, TX, USA*

⁶*Genomics Institute of the Novartis Research Foundation, San Diego, CA, USA*

⁷*Department of Pharmacology, Institute for Translational Medicine and Therapeutics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

⁸*Department of Psychophysiology, National Institute of Mental Health, National Center of Neurology and Psychiatry, Tokyo, JAPAN*

⁹*Unit of Human Disease Genomics, Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, JAPAN*

The Clock mutation in mice lengthens circadian free-running period. However, in some genetic backgrounds, the circadian period phenotype of Clock/+ mutant mice is significantly suppressed, providing an opportunity to identify loci that interact with Clock. Here, we demonstrate that the Suppressor of Clock (Soc) locus from the BALB/cJ genetic background is caused by Usf1. USF1 is a bHLH transcriptional activator that interacts with the same E-box elements in the Per1-3 and Cry1-2 promoters as the CLOCK:BMAL1 heterodimeric complex. Although there are no coding region polymorphisms between C57BL/6J and BALB/cJ in Usf1, there are a number of polymorphisms in its 5' regulatory region. Importantly, Usf1 transcription levels are significantly higher in BALB/cJ (Clock suppressor) than in C57BL/6J (Clock non-suppressor) mice. To establish its causality, we created transgenic mice expressing higher levels of Usf1 in the C57BL/6J Clock/+ background and showed that this suppressed the Clock allele. Furthermore, Usf1 mRNA levels correlate with the degree of behavioral suppression and with Per and Cry mRNA levels in the hypothalamus of transgenic Clock/+ mice. We propose that in certain genetic backgrounds Usf1 compensates for the Clock mutation by activating Per and Cry gene levels. Because USF1 is not subject to circadian feedback repression, a slight elevation can mask the effect of the Clock mutation. Finally, we show that a minor allele of USF1 is significantly associated with delayed sleep phase syndrome in a Japanese population. Our results demonstrate that Usf1 is responsible for suppression of the Clock mutant behavioral phenotype in mice and is a possible risk factor for human delayed sleep phase syndrome.

PAS domain interactions of Drosophila and mouse PERIOD proteins

NICOLE KUCERA¹, HENNIG SVEN¹, HOLGER STRAUSS², CAROLINE WIECZOREK¹, IRA SCHMALEN³, EVA WOLF³

¹*Structural Biology, MPI of Molecular Physiology, Dortmund, GERMANY*

²*Gesellschaft für Kolloidanalytische mbH, Nanolytics, Potsdam, GERMANY*

³*Structural Cell Biology, MPI of Biochemistry, Martinsried, GERMANY*

PERIOD proteins are central components of the Drosophila and mammalian circadian clock. The crystal structure of a Drosophila PERIOD (dPER) fragment comprising two PAS (PER-ARNT-SIM) domains (PAS-A and PAS B) and two additional C-terminal α -helices (α E and α F) revealed a dimer mediated by intermolecular interactions of PAS-A with Trp482 (PAS B) and helix α F (1). Yeast-two-hybrid experiments showed, that the free PAS-B β -sheet surface of dPER mediates interactions with TIMELESS (dTIM) (2). We now show, that dTIM also interacts with the PAS-A- β -sheet surface of dPER, but PAS-B interactions seem to be more important. The crystal structure of mouse PERIOD2 (mPER2) showed a homodimer, which is stabilized by interactions of the PAS-B β -sheet surface including Trp419 (equivalent to Trp482dPER) (2). Recently, we have also solved crystal structures of PAS domain fragments of mouse PERIOD1 and 3 (mPER1 and mPER3). mPER1 and mPER3 homodimers involve PAS-B β -sheet interactions (like mPER2) and an additional interaction surface in the PAS-A domain. We have also analysed mPER1 and mPER3 homodimer interactions in solution using analytical gel filtration and analytical ultracentrifugation. Summary: Structure-based models for dPER-dTIM interactions and a comparative structural analysis of mPER1, 2 and 3 homodimer interactions will be presented. (1) Yildiz et al, Mol Cell 2005 Jan 7; 17(1):69-82, (2) Hennig et al, PLoS Biol 2009 Apr 28;7(4):e94

A mechanism for negative feedback transcriptional suppression by mammalian PERIOD proteins

HAO DUONG¹, CHARO ROBLES², DARKO KNUTTI³, CHARLES WEITZ¹

¹*Neurobiology, Harvard Medical School, Boston, MA, USA*

²*Proteomics and Signal Transduction, Max Planck Institute for Biochemistry, Martinsried, GERMANY*

³*DSM, Nutritional Products, Basel, SWITZERLAND*

The mammalian circadian clock is built on a transcriptional negative feedback loop, but the mechanism of feedback is not known. At the center of the loop is the transcription factor CLOCK-BMAL1, which drives the expression of multiple target genes, including Per and Cry clock genes. In turn, PER and CRY proteins enter the nucleus and assemble into one or more multi-protein complexes that repress CLOCK-BMAL1 transcription by an unknown mechanism. To gain insight into PER function, we analyzed PER1 and PER2 complexes from mammalian tissues, and we found that they include the RNA-binding and transcriptional regulatory protein PSF. PSF has been shown in a few cases to inhibit transcription by recruiting the SIN3/HDAC complex, a repressive modifier of chromatin, to the promoters of certain genes. We found that PER2, PSF, and SIN3A are mutually co-immunoprecipitated from nuclear extracts. These three proteins plus CRY1 and HDAC1 exhibit a specific and coordinated circadian accumulation at E-box sites of the Per1 promoter (CLOCK-BMAL1 binding sites), with peak occupancy occurring at the beginning of the circadian phase of negative feedback transcriptional suppression. Tissues from Per1^{-/-}; Per2^{-/-} mice showed reduced enrichment of PSF, SIN3A, and HDAC1 at the Per1 promoter, and fibroblasts from which PSF was depleted showed reduced SIN3A and HDAC1 enrichment at the Per1 promoter. Depletion of either PSF or SIN3A from fibroblasts shortened the circadian period length, indicating that both proteins play a role in the clock mechanism. Furthermore, in the absence

of PER1 and PER2, the Per1 promoter showed increased acetylation of H3-K9 and H4-K5, the known histone deacetylation targets of SIN3/HDAC1. These data support a model in which the PER complex brings PSF to CLOCK-BMAL1 at the Per1 promoter, and PSF brings or recruits the SIN3/HDAC complex, which deacetylates H3 and H4 histones to repress transcription.

11:45 • S40

PERsuading nuclear receptors to dance the circadian rhythm

ISABELLE SCHMUTZ, JÜRGEN RIPPERGER, URS ALBRECHT

Medicine, University of Fribourg, Fribourg, SWITZERLAND

Mammalian circadian clocks provide a temporal framework to synchronize biological functions. To obtain robust rhythms with a periodicity of about a day, these clocks utilize molecular oscillators consisting of two interlocked feedback loops. The core loop generates rhythms by transcriptional repression via the Period (PER) and Cryptochrome (CRY) proteins, whereas the stabilizing loop establishes roughly antiphase rhythms via nuclear receptors. Nuclear receptors also govern many pathways that affect metabolism and physiology. Here we show that the core loop component PER2 can coordinate circadian output with the circadian oscillator. PER2 interacts with nuclear receptors including PPAR α and REV-ERB α and serves as a co-regulator of nuclear receptor mediated transcription. Consequently, PER2 is rhythmically bound at the promoters of nuclear receptor target genes in vivo. In this way, the circadian oscillator can modulate the expression of nuclear receptor target genes like Bmal1, Hnf1a and Glucose-6-phosphatase. The concept that PER2 may propagate clock information to metabolic pathways via nuclear receptors adds an important facet to the clock-dependent regulation of biological networks.

12:00 • S41

Insights on the role of Timeless in the mammalian circadian clock

FILIPPO TAMANINI¹, ERIK ENGELEN¹, ROEL JANSSENS¹, KAZUHIRO YAGITA², BERT VAN DER HORST¹

¹*Genetics, ErasmusMC, Rotterdam, THE NETHERLANDS*

²*Neuroscience and Cell Biology, Osaka University, Osaka, JAPAN*

The transcription/translation feedback loop mechanism underlying the generation of circadian gene expression is preserved in almost all organisms. Interestingly, the circadian clock proteins CRYPTOCHROME (CRY) and TIMELESS (TIM) are evolutionary well conserved at the amino acid level but reshaped/duplicated their functions in organisms ranging from *Drosophila* to mammals. In fact, the primary role of dCRY is as light sensor in flies, whereas mCRY1 and mCRY2 act as light-insensitive transcriptional repressors in mammals. The roles of the paralogs dTIM (clock) and Time-out (chromosome integrity/photoreception) have been elucidated in *Drosophila*. The function of mammalian mTIM in DNA replication is also well established, but its contribution to the clock mechanism is poorly understood. Here we show that RNAi knockdown of mTIM in NIH3T3 and U2OS cells expressing a reporter for real time clock recording has only minor effects on the amplitude and period of the core molecular oscillator. By deletion analysis, immunoprecipitation and immunofluorescence experiments we identified the region of binding between mTIM and mCRY1, as well as the nuclear localization signal for mTIM. Interestingly, the long isoform of mTIM (l-TIM), but not the short (s-TIM), interacts with mCRY1 and enhances the nuclear translocation of the latter in transiently transfected COS7 cells. We create the conditions to perform affinity binding experiments in cultured cells and showed that mPER2 compete with mTIM for the association with the coiled coil domain (CC) present in the C-terminal tail of mCRY1 (mPER2>mTIM). Finally, we show that l-TIM is mainly detected in cells undergoing proliferation

and expressed at normal level in tissues derived from Cry1^{-/-}Cry2^{-/-} animals. We propose that the dynamic interaction between these three proteins could represent a post-translational aspect of the mammalian clock, possibly necessary for their synchronous oscillation or adaption of the clock to external stimuli (eg. DNA damage).

12:15 • S42

Potorous tridactylus CPD photolyase: A DNA repair protein with an unexpected circadian clock function?

**INÈS CHAVES¹, MONIKA BAJEK¹, MAGDALENA BIERNAT², KARL BRAND¹, ANDRE EKER¹,
GIJSBERTUS VAN DER HORST¹**

¹*Genetics, Chronobiology and Health Group, Erasmus University
Medical Center, Rotterdam, THE NETHERLANDS*

²*Laboratory of Virology, Wageningen University, Wageningen, THE NETHERLANDS*

The cryptochrome/photolyase family of flavoproteins comprises members with distinct functions. Photolyases are DNA repair enzymes which use visible light to repair UV-induced DNA damage, whereas cryptochromes have functions that range from blue-light photoreception (e.g. plants), to circadian photoreception (e.g. *Drosophila*), to core circadian clock proteins (e.g. mammals). Despite the functional diversity, functional domains are interchangeable as we and others have shown that photolyase/cryptochrome chimeric proteins can act in the molecular clock. To further understand the functional evolution of the cryptochrome/photolyase protein family, we asked whether there is a naturally occurring photolyase that can (partially) function in the circadian clock. To address this question we investigated the effect of two different photolyases on the circadian oscillator, both in vitro and in vivo. The proteins studied were the (6-4)PP-photolyase from *Arabidopsis thaliana* and the CPD-photolyase from *Potorous tridactylus*, close and distant relatives of mammalian cryptochromes, respectively. Unexpectedly, our results show that the PtCPD-photolyase can function in the molecular oscillator, as shown by an altered free-running period of locomotor activity of transgenic mice carrying this protein. Furthermore, the PtCPD-photolyase affects the amplitude of circadian oscillation in cultured cells and is capable of inhibiting CLOCK/BMAL driven transcription in a dose-dependent manner, likely via interaction with CLOCK. We took this study further and are analyzing the capacity of the PtCPD-photolyase to rescue the circadian clock of non-oscillating cells, as well as its role in the circadian oscillator of *Potorous tridactylus*.

11:00 • S43

Decoding the logic of a circadian neural circuit

BEN COLLINS¹, ELIZABETH KANE², JUSTIN BLAU¹

¹*Biology, New York University, New York, NY, USA*

²*PhD Program, Harvard University, Cambridge, MA, USA*

Within the ~150 neurons of the *Drosophila* circadian network, LN_vs are usually regarded as the core pacemaker neurons as 1) the period of LN_vs sets the period of the other clock neurons and 2) ablation of LN_vs renders most flies arrhythmic. However, as some flies are rhythmic in the absence of LN_vs, non-LN_v clock neurons must also contribute to normal circadian behavior. In order to determine how LN_v and non-LN_v clock neurons function together to generate circadian behavior, we used the simple larval clock circuit where 9 neurons on each side of the brain control circadian rhythms in light avoidance. At the molecular level we found that the clock in LN_vs set the phase of DN1s, as in adults. Additionally, a signal from DN1s synchronizes the molecular oscillations between individual LN_vs. Thus LN_vs and DN1s are coupled oscillators, allowing time-of-day information to be shared across the circadian circuit. Next, we tested how these neurons control light avoidance rhythms. We found that larval LN_vs promote light avoidance, while DN1s inhibit light avoidance. This inhibitory signal from DN1s

is required for behavioral rhythms. Interestingly, even though LNs and DN1s have similarly phased molecular clocks, they appear to have anti-phase oscillations in electrical excitability, such that LNs are more likely to fire in the morning and DN1s in the evening. Thus the balance between excitatory and inhibitory signals changes over the course of the day, generating the observed rhythm of light avoidance. In *Drosophila* adults we found that signaling by non-LNv clock neurons 1) synchronizes s-LNvs, 2) inhibits overall locomotor activity and 3) is required for rhythmic behavior in DD. We conclude that *Drosophila* LNv and non-LNv clock neurons are coupled oscillators, releasing excitatory and inhibitory neurotransmitters at different times of day to generate complex behavioral rhythms.

11:15 • S44

The role of the dorsal neurons in free-running behavior in *Drosophila*

STEPHANE DISSEL, CELIA HANSEN, CHARALAMBOS P. KYRIACOU, EZIO ROSATO

Department of Genetics, University of Leicester, Leicester, UNITED KINGDOM

Under natural daylight and temperature cycles, analysis of wild-type PERIOD expression patterns in the brain of *Drosophila* uncovered a consistent phase advance in PER upswing in the Dorsal Neurons (DN1s and DN2s), suggesting a faster oscillation in these clock cells (Bhutani et al, submitted). In addition, clockless mutants in nature show characteristic features of rhythmicity. We have pursued these findings in the laboratory and observed that under DD, the DN2 molecular programme runs with a ~22 h faster period in wild-type. Pdf0 flies are mostly arrhythmic in DD, but the remaining 20-30% rhythmic flies show short ~22 h cycles in our hands. Using a variety of gal4 misexpression genotypes, we show that these residual short rhythms probably emanate from the DN's, revealing for the first time an input of these cells into the free-running oscillator. We also show that a constitutively active cryD mutation has several of the properties of the neuronal hyperstimulating NaChBac channel protein, and, when we boost the output of the DN's with cryD, flies show short period (~22 h) rhythms. When we drive cryD more extensively, we find that it can partially rescue rhythmicity in both Pdf0 and per01 mutants, suggesting both mutants have residual rhythmicity that can be enhanced by cryD hyperstimulation. Furthermore, we observe that under prolonged exposure to LD12:12 cycles, per01 shows all the hallmarks of short-period, DN-mediated rhythmicity. Thus it may be that hyperstimulation of per01 clock neurons by strong natural Zeitgebers in the wild, prolonged repeated LD12:12 exposure in the laboratory or by cryD overexpression, may underlie the residual endogenous rhythmicity of the mutants.

11:30 • S45

Adult-specific electrical silencing of pacemaker neurons uncouples the molecular oscillator from circadian outputs

JIMENA BERNI, EZEQUIEL ARANOVICH, ANA DEPETRIS CHAUVIN, ESTEBAN BECKWITH, M. FERNANDA CERIANI

Laboratorio de Genética del Comportamiento, Instituto Leloir, IIB-BA CONICET, Buenos Aires, ARGENTINA

Circadian rhythms regulate different aspects of physiology and behavior by means of self-sustained transcriptional feedback loops of clock genes. Over 150 neurons are implicated in circadian regulation of locomotor behavior in the fly brain but the small ventral lateral neurons (sLNvs) are clearly crucial. The preservation of molecular oscillations within the sLNvs is sufficient to command rhythmic behavior under free running conditions. The s-LNvs transmit this time information releasing a neuropeptide known as pigment dispersing factor (PDF), and likely changing synaptic partners by remodeling their axonal terminals in a circadian fashion. Electrical activity of PDF neurons is also required for

rhythmicity. Silencing PDF neurons by expressing a K⁺ channel (KIR) during the lifetime leads to behavioral arrhythmicity and blocks molecular oscillations in the sLN_vs. To gain insight into this process avoiding developmental defects we developed a new tool for temporal control of gene expression in PDF neurons. Silencing the PDF circuit only during the adult stage led to behavioral arrhythmicity as previously described. Surprisingly, once kir expression was shut down, flies recovered rhythmicity in a phase reminiscent to that of the initial training. PERIOD oscillations in the sLN_vs showed that the molecular clock remained intact through the silenced phase, supporting that arrhythmicity is a consequence of the inability of these neurons to transmit information rather than an effect on the clock. Accordingly, both the complexity of the axonal terminals as well as PDF accumulation were severely affected during the silenced phase, suggesting that changes in the s-LN_vs membrane potential impact additional processes as opposed to directly influence molecular oscillations.

11:45 • S46

Circadian control of Timeless degradation by a Cullin-3-based ubiquitin ligase

ALEXANDRE DOGNON, BRIGITTE GRIMA, ELISABETH CHÉLOT, FRANCOIS ROUYER
INAF, UPR3294, CNRS, Gif-sur-Yvette, FRANCE

In *Drosophila*, the control of Period (Per) and Timeless (Tim) protein oscillations largely depends on post-translational mechanisms. They involve both light-independent (circadian) and light-dependent pathways that stem upon ubiquitination and degradation of the clock proteins by the proteasome. The F-box-containing E3 ubiquitin ligase Slmb has been shown to be required for the circadian degradation of the Period protein. Another F-box-containing E3 ubiquitin ligase, Jetlag, participates to light-induced Tim degradation. Here, we reveal a new component of the post-translational control of Per and Tim oscillations. Deregulation of Cullin-3 induces behavioral arrhythmicity and abolition of Tim protein cycling in constant darkness but not in light-dark cycles. The circadian control of Tim protein oscillations thus requires a new ubiquitin ligase complex that is based on Cullin-3.

12:00 • S47

Odor stimuli modulate olfactory clocks and circadian behavior

UTE ABRAHAM, MANJANA SALEH, ACHIM KRAMER
*Laboratory of Chronobiology, Institute for Medical Immunology,
Charité-Universitätsmedizin, Berlin, GERMANY*

Odors report the availability or presence of essential environmental factors, for example food and predators, and are ubiquitous constituents of an organism's surroundings. Hence, it is conceivable that odor is able to influence one of the fundamental properties of most organisms: the circadian rhythm regulation. Previously, it has been shown that odors can modulate circadian rhythms in insects, hinting towards a general mechanism of odor-based rhythm regulation. Nevertheless, most studies investigating the effects of environmental factors on mammalian daily rhythms neglect variations in the olfactory environment, thereby, missing out on a potential new synchronizing stimulus. We systematically studied the effects of periodic odor presentations and present evidence that odor is a weak zeitgeber for circadian behavior. We found that periodic odor administration modulated circadian behavior in mice and led to partial re-emergence of rhythmicity in SCN-lesioned mice. In addition, we discovered a circadian clock in the olfactory epithelium, which displayed phase-dependent responses to odor stimulation *in vitro*, consistent with a phase response curve to a non-photic stimulus. These results will shed new light on the evolutionary basis of circadian regulation and will help us to understand the design of the mammalian circadian system. Furthermore, they will open up new opportunities for non-invasive pharmacological treatments of clock-related diseases.

A fearful stimulus alters per2 expression and c-fos activity in brain regions involved in fear memory

HARRY PANTAZOPOULOS, HAMID DOLATSHAD, FRED DAVIS

Biology, Northeastern University, Boston, MA, USA

Evidence demonstrates that rodents learn to associate a foot shock with time of day, indicating the formation of a fear related time-stamp memory, even in the absence of a functioning SCN. This type of memory may be regulated by circadian pacemakers outside the SCN. As a first step in testing the hypothesis that clock genes are involved in the formation of time-stamp fear memory, we exposed one group of mice to foot shock (TMT) at ZT 0 and one group at ZT 12 for 4 successive days. A separate group with no exposure to TMT was also included as a control. Animals were sacrificed one day after the last exposure to TMT, and per2 and cfos protein were quantified in the SCN, amygdala, hippocampus, and piriform cortex. Exposure to TMT had a strong effect at ZT0, decreasing per2 expression at this time point in all regions except the SCN, and reversing the normal rhythm of per2 expression in the amygdala and piriform cortex. These changes were accompanied by increased cfos expression at ZT0. In contrast, exposure to TMT at ZT 12 abolished the rhythm of per2 expression in the amygdala and piriform cortex, and had no effect on hippocampal per2 expression. In addition, increased cfos expression was only detected in the central nucleus of the amygdala. TMT exposure at either time point did not affect per2 or cfos in the SCN, or wheel-running activity, indicating that under a light-dark cycle, the SCN rhythm is very stable in the presence of repeated exposure to a fearful stimulus. Taken together, these results indicate that exposure to a fearful stimulus has a stronger effect on per2 expression and cell activity during the early subjective day than during the early subjective night, possibly to prepare cells at ZT0 to respond to a fearful stimulus.

Modeling light adaptation in circadian clock: Prediction of the response that disturbs entrainment

GEN KUROSAWA¹, KUNICHIKA TSUMOTO², TETSUYA YOSHINAGA³, KAZUYUKI AIHARA⁴

¹*Theoretical Biology Laboratory, RIKEN Advanced Science Institute, Wako-shi, JAPAN*

²*Center for Adv. Medical Engineering & Informatics, Osaka University, Osaka, JAPAN*

³*Institute of Health Biosciences, The University of Tokushima, Tokushima, JAPAN*

⁴*Institute of Industrial Science, The University of Tokyo, Tokyo, JAPAN*

Period of circadian clocks is different from 24h for many organisms. Thus, understanding the synchronization of the clocks with 24h environmental cycles is one of essential issues for circadian biology. An important step in the understanding the basis of the synchronization of circadian clocks with 24h day-night cycles has been the discovery of up-regulation of some genes by light for fungi and mammalian species. Intriguingly, up-regulation of gene in fungi stops within 1h that is called light adaptation. But why do organisms stop transcriptional response regardless of the continuation of light? We examined this question using a mathematical model based on experimental findings. Through analysis of bifurcations of periodic oscillations and phase response curves observed in a mathematical model, we showed that the entrainability of circadian oscillations under 24h light-dark cycles increases as the duration of transcriptional response by light shortens (adaptation). On the other hand, if the duration of transcriptional response under light is 12h (no adaptation) or up-regulation of gene by light occurs slowly (slow response), circadian oscillations under 24h light-dark cycles are more likely to be destabilized under light-dark. We quantified transcriptional response under

light-dark cycles by using published experimental data of the frequency gene expression in fungi to discuss the physiological relevance of our theoretical results. The present results may indicate that the functional consequence of light adaptation observed in fungi and probably also in mammals is to increase entrainability to 24h light-dark cycles in nature.

11:15 • S50

Neuroglobin is involved in light induced resetting of the circadian clock

CHRISTIAN HUNDAHL¹, ANDERS HAY-SCHMIDT², JENS FREDRIK REHFELD³, JAN FAHRENKRUG¹, JENS HANNIBAL¹

¹*Clinical Biochemistry, Bispebjerg Hospital, Copenhagen, DENMARK*

²*Department of Neuroscience and Pharmacology, The Panum Institute, Copenhagen, DENMARK*

³*Clinical Biochemistry, Rigshospitalet, Copenhagen, DENMARK*

Neuroglobin (Ngb) is a myoglobin-like (Mb) heme-globin, belonging the globin family located only in neuronal tissue of the central nervous system. Ngb has been shown to be up-regulated in and to protect neurons from hypoxic and ischemic injury, but the function of Ngb - in particular how Ngb may protect neurons - remains largely elusive. We have previously described the detailed localization, innervation and response to light of Ngb-ir neurons in the rat suprachiasmatic nucleus (SCN). In this study we characterized the Ngb expressing neuron of the mouse SCN and found that these cells represent a population of cells of which few co-store GRP but not VIP or AVP. The Ngb-containing cells receives input from NPY-containing nerve fibres of the geniculohypothalamic tract (GHT), whereas fewer received direct input from the eye or the midbrain raphe system. A subpopulation of the Ngb cells expresses the clock gene *Per1* and *Per2* and a subpopulation were light responsive evaluated by the expression of cFos at night. To further investigate a potential role of Ngb in light entrainment of the clock we have generated an Ngb-knock out mouse (KO) line. Ngb KO mice entrain to the light/dark cycle and show similar TAU as wild type mice in constant darkness. When exposed to a light pulse at early subjective night Ngb KO mice showed increased sensitivity responding with a larger phase delay compared to wild type mice. The results indicate that the Ngb is involved in photic entrainment possible via input from the GHT.

11:30 • S51

Human nonvisual responses to simultaneous presentation of short and long wavelength light

CHRISTIANA PAPAMICHAEL, DEBRA SKENE, VICTORIA REVELL

FHMS, University of Surrey, Guildford, UNITED KINGDOM

Work in humans and rodents has demonstrated that pre-exposure to long wavelength (red) light can enhance non-visual responses to short wavelength (blue) light. This potentiation is attributed to melanopsin being a bistable photopigment that can make stable associations with both 11-cis and all-trans isoforms of retinaldehyde and uses light to transition between these states. The aim of the current study was to assess human non-visual responses to simultaneous presentation of red and blue light, and assess the irradiance dependency of the response. Young healthy males ($n = 10$) aged 18-35 years (23.5 ± 3.7 yrs; mean \pm SD) were studied in 8 overnight laboratory sessions in a randomised, within-subject design. During each session participants were exposed to a 30 minute light stimulus, individually timed to occur on the rising phase of the melatonin rhythm. Subjective mood and alertness, heart rate and plasma melatonin levels were assessed at regular intervals before, during and after the light stimulus. Blue (λ_{\max} 479 nm) and red (λ_{\max} 627 nm) monochromatic lights at varying intensities were presented either alone or in combination. Repeated measures ANOVA analysis of the melatonin suppression response revealed a significant effect of irradiance ($p < 0.001$) for 479 nm light presented

alone as well as for 479 nm light presented simultaneously with 627 nm light. However, the presence of red 627 nm light did not significantly alter the melatonin suppression response to blue 479 nm light. The lack of response potentiation with simultaneous presentation of red and blue light suggests a complex dynamic between the melanopsin isoforms that requires further investigation.

11:45 • S52

Melanopsin-expressing neurons mediate light modulation of cognitive functions and mood-related behaviors

TARA LEGATES¹, CARA ALTIMUS¹, SUNGGU YANG², TODD WEBER³, ALFREDO KIRKWOOD², SAMER HATTAR⁴

¹Biology, Johns Hopkins University, Baltimore, MD, USA

²Neuroscience, Johns Hopkins University, Baltimore, MD, USA

³Biology, Rider University, Lawrenceville, NJ, USA

⁴Biology and Neuroscience, Johns Hopkins University, Baltimore, MD, USA

Many biological functions oscillate over the course of a day, and the precise timing of these rhythms depends on synchronization to the solar day. While artificial lighting allows us to utilize more hours of the day, the presence of light at night consequently disrupts light-dependent rhythms and can negatively impact human health. Recent work has shown that light can directly influence the limbic system mediating functions such as working memory and mood. To gain a better understanding of how light influences limbic functions, we housed mice in a light paradigm that provided exposure to light pulses throughout the circadian cycle. In this paradigm, mice experience light at the wrong time of the day while circadian rhythms remain intact and sleep unchanged. Mice exposed to this light environment show defects in hippocampal-dependent learning including a reduction in hippocampal long-term potentiation. They also exhibit increased anxiety- and depression-related behaviors, and administration of the antidepressant desipramine rescues the learning deficit that resulted from exposure to this light cycle. In mammals, light is transduced by photoreceptors in the retina and conveyed to the brain by retinal ganglion cells (RGCs). To determine if melanopsin-containing intrinsically photosensitive RGCs (ipRGCs) are responsible for conveying light information to areas of the brain that control learning and mood, we used a mouse line lacking these cells. ipRGCs have been shown to convey light information to modulate circadian rhythms and sleep but have not been linked to light-influenced limbic functions. Mice lacking ipRGCs elicit decreased anxiety- and depression-related behaviors when housed in a 12:12 light/dark cycle and are unaffected by exposure to light pulses throughout the circadian cycle. These findings establish that disruptive light conditions can negatively impact cognitive functions and mood and create a new role for ipRGCs within the function of the limbic system.

12:00 • S53

Altered retinal responses to light in Per3-deficient mice

SIMON ARCHER¹, DAAN VAN DER VEEN¹, JAZI AL-ENEZI², ANNETTE ALLEN², ROB LUCAS²

¹Surrey Sleep Research Centre, University of Surrey, Guildford, UNITED KINGDOM

²Faculty of Life Sciences, University of Manchester, Manchester, UNITED KINGDOM

Previously, we identified the existence of light-dependent behavioural phenotypes in PER3-deficient mice. Compared to WT, *Per3*^{-/-} mice had the same period in DD, an increased period in LL, and reduced negative masking in an ultradian LD cycle. Here, we investigated the retinal contribution to these effects by comparing pupil constriction and ERG recordings in WT and *Per3*^{-/-} mice. Dark-adapted mice were tested between CT02 and CT08 in alternating sequence. For the pupil light reflex, dark-adapted mice were exposed to 45s of light (intensity range of 5 log-units). Dark-adapted (DA) ERG recordings were

made in response to 15ms light flashes (8 log-units range). Light-adapted (LA) irradiance responses were recorded from 600 bright light flashes (0.75Hz) against a rod-saturating, white-light background. The ERG waveform was analysed for maximal values of A- and B-wave. Both WT and Per3^{-/-} show robust, intensity-dependent, pupil constriction in response to light and responses differed between light levels (p<0.001), but was not different between genotypes (p>0.05). An increase in A-B wave amplitude was seen with increasing light intensity, where Per3^{-/-} mice showed larger amplitudes than WT mice (p's<0.001). The same physiological difference was seen in the LA ERG measurements, where the Per3^{-/-} mice showed larger A-B wave amplitudes than WT (p's<0.001). Per3 has been characterised as a gene with a redundant contribution to the circadian clock. However, a polymorphism in the human PER3 gene has been associated with diurnal preference, sleep homeostasis, and cognitive performance in response to sleep loss. Recently, we demonstrated light-dependent behavioural phenotypes. Here, we have shown that PER3-deficient mice show normal pupil constriction and ERG responses. However, the ERG responses are greater in the PER3-deficient mice and indicate that a lack of PER3 has changed retinal physiology, which could indicate a fundamental neurochemical or physiological role for PER3 in the retina.

12:15 • S54

Photoperiodism in mammals: What are the long day signals?

**SANDRINE DUPRÉ¹, KATARZYNA MIEDZINSKA², CHLOE DUVAL¹, LE YU³, ROBERT GOODMAN⁴,
GERALD LINCOLN⁵, JULIAN DAVIS⁶, ALAN McNEILLY², DAVID BURT³, ANDREW LOUDON¹**

¹*Faculty of Life Sciences, University of Manchester, Manchester, UNITED KINGDOM*

²*MRC Human Reproductive Sciences Unit, The Queen's Medical
Research Institute, Edinburgh, UNITED KINGDOM*

³*The Roslin Institute, University of Edinburgh, Edinburgh, UNITED KINGDOM*

⁴*Department of Physiology and Pharmacology, West Virginia
University School of Medicine, Morgantown, WV, USA*

⁵*Reproductive and Developmental Sciences, University of Edinburgh, Edinburgh, UNITED KINGDOM*

⁶*Faculty of Medicine, University of Manchester, Manchester, UNITED KINGDOM*

It is well known that the pars tuberalis (PT) of the pituitary in mammals is involved in the seasonal regulation of hormone secretion, including prolactin (PRL). The external day length reflected by the nocturnal melatonin signal is translated in the PT into neuroendocrine signals which are yet to be fully characterised. We used a bovine cDNA array to define the molecular events in the PT associated with photoactivation of the endocrine system in sheep. Animals were placed either in short photoperiod (SP, LD 8:16) or 1, 7 or 28 days in long photoperiod (LP, LD 16:8) and culled 3h after the light onset and offset. In response to LP exposure, we observed significant expression of Eya3 in the PT, a gene recently identified as LP-activated in Japanese quail. In situ hybridizations interestingly revealed a biphasic expression of Eya3 in LP in the PT, with a peak at dawn and dusk of the photophase. We also detected an up-regulation in TAC1 expression in LP conditions in the PT. TAC1 codes for tachykinins, including the low molecular weight peptides Substance P and Neurokinin A. Immunohistochemistry in the sheep brain confirmed that both bioactive peptides are expressed in the PT and in vitro assays revealed that both NKA and a cleavage product of SP (SP1-7) have a significant stimulatory effect on PRL release from ovine pituitary dispersed cells. Analyses of NK1R, NK2R and NK3R in the sheep pituitary pars distalis revealed strong expression in the pars distalis but not in the PRL secreting cells suggesting that NKA and SP1-7 would act through intermediate cell types. These results reveal that tachykinins encoded by TAC1 in the PT could serve as the elusive long day "tuberalin" signal to drive the seasonal activity of the pituitary gland in mammals.

Timing in the immune system: The circadian clock controls T cell function

ERIN FORTIER¹, JULIE ROONEY², HUGUES DARDENTE³, NATHALIE LABRECQUE⁴, NICOLAS CERMAKIAN³

¹Neurology and Neurosurgery, McGill University, Montreal, Quebec, CANADA

²Maisonneuve-Rosemont Hospital Research Centre, University of Montreal, Montreal, Quebec, CANADA

³Psychiatry, McGill University, Montreal, Quebec, CANADA

⁴Microbiology and Immunology, University of Montreal, Montreal, Quebec, CANADA

Circadian variations have been found in the immune system, including daily rhythms in circulating numbers of leukocytes and serum concentration of cytokines. Although the circadian clock is known to control various physiological systems, very little is known about the timing of events in the immune system. We hypothesized that a clock in lymph nodes controls T cell function. The aims of this study were to identify clock gene expression in mouse lymph nodes (LNs), to investigate T cell proliferation rhythms, and to shed light on the molecular mechanisms underlying this rhythmic response. Adult WT and Clock mutant mice were entrained to a light-dark cycle and sacrificed at regular intervals over 24 hours. LNs were sampled and used to: i) Extract RNA and quantify clock gene expression by real-time PCR; ii) Measure T-cell proliferation following anti-CD3 stimulation; and iii) Examine the expression of T cell signaling proteins. Our results show that LNs exhibit rhythmic clock gene expression. T cells show a robust circadian variation in proliferation after stimulation via the T cell receptor (TCR) that is lost in Clock gene mutant mice. In addition, the tyrosine kinase ZAP70, which is immediately downstream of the TCR in the T cell activation pathway, exhibits rhythmic expression. This is the first evidence for control of the T cell response by the molecular clockwork. Our results have uncovered a novel mode of regulation of T-cell proliferation. This study linking circadian rhythms and the adaptive immune response may also provide cues for more efficient vaccination strategies.

Cellular circadian clock in CD4+ T cells and circadian T cell immune responses

THOMAS BOLLINGER¹, HENRIK OSTER², ANTON LEUTZ¹, JUDIT KOVAC², BERT MAIER³, ACHIM KRAMER³, CHRISTIAN BENEDICT⁴, TANJA LANGE⁴, WERNER SOLBACH¹

¹Medical Microbiology and Hygiene, University of Luebeck, Luebeck, GERMANY

²Circadian Rhythms Group, Max Planck Institute of Biophysical Chemistry, Goettingen, GERMANY

³Laboratory of Chronobiology, Charite Universitaetsmedizin, Berlin, GERMANY

⁴Neuroendocrinology, University of Luebeck, Luebeck, GERMANY

Background: A number of immunological functions in CD4+ T cells are dependent on the circadian rhythm as we and others could previously demonstrate. Little is known about the underlying mechanisms. One possibility could be the cellular circadian clock in T cells. The circadian clock is known to control the circadian rhythm in the brain and several peripheral organs. To address the question whether T cells express a functional circadian clock we analyzed the expression of clock genes, the production of cytokines and the CD40L expression in CD4+ T cells from human volunteers. Methodology: 7 healthy young men were examined under defined conditions over 24 h in the sleep lab. Venous blood was drawn periodically every 3 h, CD4+ T cells were isolated. T cells were split: one fraction was used for the investigation of clock gene expression and the second fraction was polyclonally stimulated and analyzed applying FACS. Results: We found a strong diurnal rhythm of IFN- γ production after polyclonal stimulation. Furthermore, we found a rhythmic expression of Rev-erb α , Per3 whereas Dbp, Cry1, Per2, Rora, E4bp4, Bmal1, Clock were only in some donors rhythmic. Expression of a Bmal1-Luc reporter construct in a CD4+ T cell line revealed circadian luciferase activity in constant cell culture conditions. Conclusions: These findings demonstrate that highly purified CD4+ T cells have a strong functional circadian rhythm and that a possible underlying mechanism could be the molecular clock.

The NONO protein couples senescence, cell cycle, and circadian pathways to regulate wound healing

ELZBIETA KOWALSKA¹, PASCAL BRUEGGER¹, DOMINIK HOEGGER², JUERGEN RIPPERGER³, THORSTEN BUCH⁴, ANKE MUELLER⁵, ACHIM KRAMER⁵, CLAUDIO CONTALDO², STEVEN BROWN¹

¹*Institute of Pharmacology and Toxicology, University of Zurich, Zurich, SWITZERLAND*

²*Department of Dermatology, Universitatsspital Zurich, Zurich, SWITZERLAND*

³*Department of Biochemistry, University of Fribourg, Fribourg, SWITZERLAND*

⁴*Department of Neuroimmunology, University of Zurich, Zurich, SWITZERLAND*

⁵*Institute for Medical Immunology, Charite Universitaetsmedizin, Berlin, GERMANY*

We have identified NONO previously as a protein that binds to the circadian clock PER and CRY proteins. Using a Nono genetrapped stem cell line (Nonogt), we found a shortening in the period length of circadian transcription in Nonogt neuronally differentiated cells as well as in the free-running behavioral period of mice derived from them. In addition, an elevated cell doubling rate was observed in primary fibroblasts obtained from these mice, as well as a lower rate of cellular senescence. We trace both of these phenotypes to a loss of activation by NONO at the p16-INK4a promoter, which is bound by it in circadian fashion and which mediates circadian cell cycle division in fibroblasts. The resulting dysregulation of the cell cycle program results in defective wound healing. Paradoxically, in spite of fibroblast and keratinocyte hyperproliferation, collagen deposition and epithelialization are dramatically reduced. Based upon our data, we speculate that circadian control by NONO of the cell cycle G1 arrest checkpoint is critical to proper reorganization of epithelial structure during healing.

A circadian egg timer: Circadian influences on the timing of ovulation

MICHAEL SELLIX¹, TOMOKO YOSHIKAWA², MICHAEL MENAKER¹

¹*Biology, University of Virginia, Charlottesville, VA, USA*

²*Chronomedicine, Hokkaido University Graduate School of Medicine, Sapporo, JAPAN*

It is widely accepted that the timing of ovulation depends on the timing of luteinizing hormone (LH) secretion, itself driven by neuroendocrine releasing factors controlled by the circadian clock in the suprachiasmatic nucleus (SCN). As a consequence, a role for the ovary in this process has not been considered. We and others have demonstrated the presence of circadian clocks in the ovary and hypothesize that these clocks influence the timing of ovulation. We examined the phasic response of ovulation to exogenous LH-treatment during suppression of endogenous LH secretion in female Wistar rats and C57BL/6 mice. To suppress LH secretion, animals were injected with a specific and long-acting GnRH receptor antagonist (Cetrorelix (CET) pamoate depot; Aeterna Zentaris GmbH, Frankfurt). Rats were injected with CET on diestrus or proestrus, followed 7h later by treatment with equine LH at one of 7-8 times over an 18-21h period. On both diestrus and proestrus, rats injected during the night ovulated more often and produced more oocytes than animals injected during the day. We observed a similar response to eLH on proestrus with rats housed in constant dim light. Cycling mice housed in a 12:12 L:D cycle and treated with CET on proestrus followed by eLH also ovulated more frequently during the night and produced more oocytes than animals injected during the day. We observed a nearly identical rhythm of ovulation on proestrus with mice after release into constant darkness (DD). Our results alter the classic view that gonadotrophins provide the only timing cue for ovulation and suggest that: (1) The circadian timing of ovulation is regulated by rhythmic neural and/or non-gonadotrophic endocrine cues or (2) the molecular clock in the ovary itself, by timing the expression of target genes, regulates the timing of ovarian sensitivity to LH and thus influences the circadian rhythm of ovulation.

Missing neuronal links from the SCN to ovulation

BENJAMIN SMARR¹, HORACIO DE LA IGLESIA²

¹*Neurobiology and Behavior, University of Washington, Seattle, WA, USA*

²*Biology, University of Washington, Seattle, WA, USA*

Ovulation depends on a surge of luteinizing hormone (LH) release, which in turn relies on a gonadotropin releasing hormone (GnRH) surge. Kisspeptin (KISS) has recently emerged as the most potent activator of the GnRH neuronal network and a critical player in the generation of the LH surge, as well as in reproductive development and health. For decades it has been well established that the female-specific neuroendocrine trigger of ovulation depends both on the elevation of ovarian estrogen levels during preovulatory stages and on a circadian signal emerging from the central pacemaker within the hypothalamic suprachiasmatic nucleus (SCN). Although the involvement of the SCN in the circadian gating of the LH surge is unequivocally established, it is unclear whether and how the SCN regulates Kiss1 neuronal activity, and which specific SCN transmitters may be involved in this regulation. Our laboratory has developed a rat model of circadian desynchronization in which the stable desynchronization of circadian locomotor rhythms, melatonin and sleep stages is associated with the independent oscillations of anatomically identifiable subregions within the SCN. As this model involves only the manipulation of the light-dark (LD) cycle, it offers a unique opportunity to functionally dissect SCN output pathways in genetically and neurologically intact animals. Here we present data that the LH surge in desynchronized rats is in phase with the activity of the vasopressin-rich dorsomedial (dm) SCN. Using unilateral SCN lesions, we then show that GnRH cells fail to show their expected circadian activation (as measured by increased expression of the immediate-early gene cFos) on the side ipsilateral to the lesion and we are currently assessing a similar asymmetry on the activation of Kiss1 cells. Our data fit a model in which the activation of KISS+ cells by the vasopressinergic dmSCN in E2-primed females is responsible for the circadian gating of ovulation.

Precision of mammalian autonomous circadian oscillators and minimal oscillator models

THOMAS D'EYSMOND¹, CHARNA DIBNER², UELI SCHIBLER², FÉLIX NAEF¹

¹*Computational Systems Biology Group, Ecole Polytechnique Fédérale de Lausanne (EPFL) and Swiss Institute of Bioinformatics (SIB), Lausanne, SWITZERLAND*

²*Department of Molecular Biology & NCCR Frontiers in Genetics, University of Geneva, Geneva, SWITZERLAND*

Circadian clocks are found all across the tree of life and allow organisms to coordinate their physiological rhythms in a daily cyclic environment. Subjected to their noisy environment and stochasticity of biochemical interactions, the individual circadian oscillators at the cellular level will be characterized by a limited precision quantified by a quality factor (Q). Recent experiments showed that under conditions of reduced global transcription rate, both the period length and amplitude of molecular rhythms in individual mammalian cells decreases. These observations suggest that cells are brought closer towards a singularity point (Hopf bifurcation) as the transcription rate is reduced. Using simulations we argued that generic, weakly non-linear delayed negative loops can account for the observed period behavior (Dibner et al., 2009). Here we test whether this scenario is also consistent from the angle of the precision of the oscillations, which we quantify using the quality factor (Q). The analysis of several biochemical models for autonomous circadian oscillators predicts that the quality factor decreases as transcription is reduced, in agreement with a quantitative analysis of individual periods in single cells from the luminescence data. Using the period, amplitude, and quality of the oscillators, we summarize the different experimental conditions using generic non-linear oscillator

models (Hopf oscillators) with a minimal number of parameters. Such oscillators are tractable for computation so that we can establish a relationship relating the intrinsic noise to the quality factor in function of the reduced transcription rate. Furthermore conclusions can be drawn on the level of robustness to which circadian clocks are tuned.

11:00 • S61

Cellular circadian pacemaking in the SCN of Cryptochrome-deficient mice

ELIZABETH MAYWOOD, JOHANNA CHESHAM, MICHAEL HASTINGS

Neurobiology, MRC Laboratory of Molecular Biology, Cambridge, UNITED KINGDOM

Circadian timekeeping in the suprachiasmatic nucleus (SCN) (the principal circadian pacemaker in mammals) is driven by interlocked transcriptional/post-translational negative feedback loops, pivoted around period (*per*) and cryptochrome (*cry*) gene expression. Intra-cellular signalling by cAMP and Ca²⁺ is also essential in maintaining these loops within cells and synchrony between cells. *Cry1,2* null mice become behaviourally arrhythmic immediately on transfer to constant darkness, but it is unclear what impact this deletion has on SCN molecular pacemaking. By measuring the bioluminescence in organotypic SCN slices from 10 day old PER2:LUC:*Cry1,2*^{-/-} mice we demonstrated that despite the lack of *cry* genes the majority (ca 60%) of SCN slices do show significant rhythmicity for several days. The period of these oscillations is, however, significantly shorter than in wild-type slices (18.27±.81h vs 24.2±0.08h). This demonstrates that cryptochromes are not essential for rhythmic expression of PER2:LUC bioluminescence but are necessary for the expression of a precise 24h period. We hypothesised that cytosolic signals likely contributed to the persistent rhythms seen in *cry*-deficient SCN. A novel co-culture technique has been used to demonstrate that *Vip* and other diffusible factors can maintain intercellular communication across the SCN circuit (SRBR 2010). By placing a second SCN graft from a non-reporter mouse onto an arrhythmic host PER2:LUC:*Cry1,2*^{-/-} SCN, circadian gene expression was amplified and synchronised within 3-4 days. Therefore, even in the absence of cryptochromes, intercellular/neuropeptidergic signalling between SCN neurons is sufficiently powerful to overcome the loss of core genetic elements of the transcriptional feedback loop and sustain high-amplitude circadian gene expression. Given that *Vip* regulates cAMP, we propose that circadian cAMP-mediated signalling can rescue a transcriptionally deficient SCN clock. Work supported by Medical Research Council, U.K. Authors are grateful to JS Takahashi and G. van der Horst for provision of mutant mice.

11:15 • S62

Paracrine signaling drives cellular pacemakers in the suprachiasmatic nucleus: Roles for Vipergic and non-Vipergic signals

ELIZABETH MAYWOOD, JOHANNA CHESHAM, MICHAEL HASTINGS

Neurobiology, MRC-LMB, Cambridge, UNITED KINGDOM

Interneuronal signalling maintains and synchronises molecular time-keeping within SCN neuronal circuits. Loss of *Vip* or the VPAC2 receptor for *Vip* disrupts behavioural and SCN molecular rhythms, but whether *Vip* is the sole mediator of circuit coherence is unclear. A novel SCN co-culture technique was developed to screen for synchronising factors. PER2:LUC bioluminescence rhythms were recorded from organotypic SCN slices from 10 day old ligand-deficient mice (PER2:LUC:*Vip*^{-/-}). Once bioluminescence rhythms had damped in these "host" slices, a second SCN "graft" from a non-reporter mouse was placed on top of the host and the bioluminescence recorded for a further 10 days without medium change. This co-culture rapidly (<2 days) amplified and synchronised SCN cellular rhythms in PER2:LUC:*Vip*^{-/-} organotypic slices. Graft SCN slices with mutant circadian periods (*Fbxl3*^{aff} and *CK1e*^{tau} mutants with 28h and 20h periods respectively) drove host SCN with the genotypically specific period of the graft, confirming that the graft drives the host rhythm. Using co-culture, we then demonstrated that rhythmicity was restored in SCN lacking the *Vip* receptor (PER2:LUC:VPAC2^{-/-}),

thus showing that SCN factors other than Vip can synchronise SCN cellular pacemakers. These factors are likely to be paracrine, since placing a permeable barrier between the host and graft slices allowed host rhythms to be maintained. From this we were able to show that a diffusible factor with a molecular weight between approximately 2 and 10kDa can synchronise the cellular pacemaking in the host SCN slices. The data therefore reveal for the first time the role of Vip- and non-Vip-ergic, paracrine signalling pathways in maintaining circuit coherence of molecular pacemaking across the SCN circadian network. Work supported by Medical Research Council, U.K. Authors are grateful to JS Takahashi, CS Colwell, AJ Harmar, ASI Loudon and P Nolan for provision of mutant mouse lines.

11:30 • S63

VIP reduces amplitude and synchrony of circadian oscillator

SUNGWON AN, DANIEL GRANADOS-FUENTES, CONNIE TSAI, ERIK HERZOG

Biology, Washington University, St. Louis, MO, USA

Vasoactive intestinal polypeptide (VIP) is required to sustain and synchronize daily rhythms in locomotor behavior and in firing rate and gene expression in the suprachiasmatic nucleus (SCN). Although it released in SCN with photic stimuli and in a circadian pattern, it is not clear if or how VIP mediates entrainment among SCN cells. We applied VIP to SCN organotypic cultures from PER2::LUC mice and measured changes in circadian bioluminescence. VIP shifted the phase of SCN explants in a dose- and phase-dependent manner and daily applications entrained the SCN as predicted from the phase response curve. Surprisingly, VIP also reduced the amplitude of ensemble PER2::LUC rhythms. This amplitude reduction was maximal when VIP was applied 12 h after peak PER2 expression (CT0) with a threshold of 100 nM and saturation point of 10 μ M. Single cells in SCN explants also showed reduced synchrony and amplitude following 150 nM VIP application. These results suggest that VIP may participate in photic entrainment by shifting and reducing the amplitude of SCN rhythms. To test this possibility *in vivo*, we housed wild-type and VIP-deficient (*Vip*^{-/-}) mice in constant light (LL). *Vip*^{-/-} mice sustained their circadian amplitude compared to controls. We propose that light-induced and daily VIP release in the SCN facilitate entrainment by modulating both the phase and amplitude of individual SCN cells. Supported by T90 DA022871 and NIMH grant 63107.

11:45 • S64

Three-dimensional mapping of phase heterogeneity in the suprachiasmatic nucleus of the mouse

JENNIFER EVANS¹, TANYA LEISE², OSCAR CASTANON-CERVANTES¹, ALEC DAVIDSON¹

¹*Neuroscience, Morehouse School of Medicine, Atlanta, GA, USA*

²*Mathematics, Amherst College, Amherst, MA, USA*

The mammalian pacemaker in the suprachiasmatic nucleus (SCN) contains a population of neural oscillators capable of sustaining cell-autonomous rhythms of clock gene expression and electrical firing. A critical question for understanding SCN function is how individual oscillators are organized into a coherent tissue. Here we undertake a comprehensive analysis of rhythmic gene expression across the extent of the SCN of the adult PER2::LUC mouse using multi-position automated bioluminescence imaging and unbiased computational analyses. We demonstrate that there is phase heterogeneity across all three dimensions of the SCN that is both intrinsically regulated and extrinsically modulated

by light in a region-specific manner. Within horizontal SCN slices, phase gradients were also evident across an overlooked SCN structure that extends from the rostral pole and contains densely packed cells immunoreactive for vasopressin. The consistency of SCN spatiotemporal organization across individuals and across planes of section suggests that the precise phasing of oscillators within the SCN is a reproducible feature of the pacemaker important for its function. The present results underscore the sophistication of spatiotemporal organization within the central pacemaker and suggest new directions for exploring its basis and function.

12:00 • S65

Does the variability of circadian period (τ_{DD}) depend upon its value? A test using tau mutant, super duper, and duper hamsters

STEFANIE KRUG AND ERIC L. BITTMAN

*Department of Biology and Program in Neuroscience & Behavior,
University of Massachusetts, Amherst MA, USA*

Aschoff et al. (1971) found that the standard deviation of τ reaches a minimum at particular values. In comparing 4 rodent species, Pittendrigh and Daan (1976) argued that variability of τ_{DD} increases as its mean deviates from 24h. Recent work on mouse mutants supports this trend. Thus the clock may function most reliably when it runs at a particular speed. We evaluated this question using *duper*, a new mutation in Syrian hamsters. Unlike *tau*, *duper* amplifies phase shifting responses to light and is not a change in the coding sequence of *casein kinase 1*. τ_{DD} of hamsters expressing *duper* on a wild type background that were gestated and raised in 14L:10D was 23.11±0.04h. *Duper* hamsters born and raised in darkness had a shorter τ_{DD} (21.92±0.12 h, $p < 0.0001$). This indicates an aftereffect of rearing in DD and supports the idea that variability increases as τ_{DD} decreases below 24h. We generated 8 strains of hamsters that are homozygous or heterozygous for the *tau*, *duper*, and wild type alleles in all combinations. τ_{DD} among strains ranged from 17.92±0.07 to 24.16±0.04h. Contrary to predictions, the variability of τ_{DD} is similar in all strains, i.e., its standard deviation is unrelated to its absolute value. Crosses between *duper* hamsters and wild types indicate that the mutated *duper* allele is recessive. Variabilities of τ_{DD} of activity onsets and acrophase are similar and less than that of offsets. Females of all strains show greater variability of onset and acrophase, and of α , than males, but the variability of activity offset did not differ between sexes. α and ρ are negatively correlated, but the ρ is more strongly correlated with the preceding than the succeeding α regardless of τ_{DD} . *Duper's* effect appears to be additive on a τ_s background, but multiplicative on a τ_{ss} background. Mutations that target different components of the clock's mechanism may amplify each other but do not necessarily increase variability. Supported by NIMH RO1-70019.

The mystery of coupling between the left and right suprachiasmatic nuclei

STEPHAN MICHEL¹, ROGER MAREK², MARISKA J. VAN STEENSEL³, WILLIAM J. SCHWARTZ⁴,
JOHANNA H. MEIJER¹

¹*Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, THE NETHERLANDS*

²*The Queensland Brain Institute, University of Queensland, St Lucia, AUSTRALIA*

³*Department of Neurosurgery, University Medical Center Utrecht, Utrecht, THE NETHERLANDS*

⁴*Department of Neurology, University of Massachusetts Medical School, Worcester, MA, USA*

The autonomy of pacemaker neurons in the suprachiasmatic nucleus (SCN) is more limited than previously assumed. Coupling within the SCN seems to be crucial not only for synchronization, but even for basic rhythm generating functions. One of the pathways we know very little about is used for communication between the left and right SCN. In this study, we tested the functional significance by assessing the phase shift of circadian rhythm in electrical activity of the SCN after brief (30 min) contralateral electrical stimulation. We then used calcium imaging of mouse SCN neurons in acute brain slices combined with electrical stimulation of the contralateral SCN to characterize the localization of responding neurons. Patch clamp recording of evoked post-synaptic currents after contralateral stimulation were used to measure response delay and reversal potential. Blockers of several neurotransmitter receptors were bath-applied to describe the potential pathways used. Unilateral electrical stimulation of SCN slices at ZT 14 led to significant phase advances of the contralateral SCN rhythm (2.5 h \pm 0.9, n=7). Calcium imaging experiments as well as patch clamp recordings provided evidence for synaptic signaling dependent on action potentials. Calcium imaging results revealed that the dorsocaudal part of the SCN contains most of the neurons responding to contralateral stimulation. Patch clamp recordings showed excitatory postsynaptic responses to contralateral stimulation. Delay times indicate monosynaptic (3-5 ms) as well as multisynaptic connections (7-15 ms). Our pharmacological data suggest a role of glutamatergic transmission since application of AMPA receptor antagonist blocked calcium transients and blocked most of the postsynaptic responses in the patch clamp experiments. GABA seems to play a modulating role in inter-SCN coupling as responses were attenuated in the presence of the GABAA receptor antagonist bicuculline.

Circadian clock output pathways revealed by ChIP/Seq in Neurospora

DEBORAH BELL-PEDERSEN¹, KRISTINA SMITH², GENCER SANCAR³, TERESA LAMB¹, CHARLES GOLDSMITH¹,
ERIN BREDEWEG², CHRISTOPHER SULLIVAN⁴, MICHAEL BRUNNER³, MICHAEL FREITAG²

¹*Biology, Texas A&M University, College Station, TX, USA*

²*Biochemistry and Biophysics, Oregon State University, Corvallis, OR, USA*

³*Biochemistry, Universität Heidelberg, Heidelberg, GERMANY*

⁴*Botany and Plant Pathology, Oregon State University, Corvallis, OR, USA*

In *Neurospora crassa*, the blue light photoreceptor and PAS-domain GATA transcription factor (TF) WC-1 dimerizes with another PAS-domain GATATFWC-2 to form the White Collar Complex (WCC). The WCC, which is active in the late-night to early morning, functions as the positive element in the FRQ/WCC circadian oscillator and is necessary for light resetting of the clock. Recent evidence suggested that the WCC also signals time-of-day information through the output pathways to ultimately control the expression of a large collection of clock-controlled genes (ccgs). To understand the mechanisms by which the WCC signals through the output pathways, we identified the direct targets of the WCC using chromatin immunoprecipitation (ChIP) followed by high throughput sequencing of WC-2-bound DNA on an Illumina genome analyzer (ChIP/Seq). WC-2 was found to bind to more than 100 different gene promoters, including genes encoding transcription factors, chromatin-modifying enzymes, kinases, phosphatases, cell cycle and DNA repair proteins. These data provide direct links between the WCC

and the effector molecules in output pathways responsible for circadian rhythmicity. Experiments are currently underway to identify the direct targets of the second tier TFs, combined with mRNA/Seq in wild type versus TF knockout strains, to develop a network map of the circadian output pathways. In addition, we previously found that the clock regulates rhythms in the activity of the conserved p38 MAPK; however, the details by which components of the oscillator signal to the MAPK pathway to control activity rhythms were not known. To demonstrate the utility of this approach, using ChIP/Seq and ChIP/PCR we found that the WCC binds to the promoter of the MAPKKK in the morning resulting in rhythmic accumulation of the transcript. These data provide mechanistic insights into the connection between the clock, the MAPK output signaling pathway, and its target ccgs.

11:15 • S68

A surprisingly large number of CLK direct target genes in Drosophila

KATHARINE ABRUZZI, JEROME MENET, JOE RODRIGUEZ, JEN DESROCHERS, SASHA TKACHEV, MICHAEL ROSBASH
Biology, HHMI: Brandeis University, Waltham, MA, USA

The CLK/CYC heterodimer plays a central role in the *Drosophila* circadian clock by directly activating the expression of other core clock components like PER and TIM. Previous studies have identified only a few CLK target genes (~28) and an order of magnitude larger number of mRNAs (~200) that undergo circadian oscillations in *Drosophila* heads. Most of these cycling genes are therefore considered to be under indirect CLK/CYC control with only a small number of true target genes. To directly identify CLK target genes and to address more general aspects of circadian transcriptional regulation, we performed chromatin immunoprecipitations (ChIP) at six timepoints with antibodies recognizing CLK, PER and RNA PolIII. The genome-wide binding profiles identified >700 genes, and likely many more, to which CLK binds in a strongly oscillating manner. As described above, this is much greater than previous measurements of cycling mRNAs and especially direct CLK/CYC target genes. There are a number of explanations for the dramatic increase in the number of CLK direct targets, including 1) isoform specificity 2) tissue specificity and 3) novel and more subtle roles of CLK in gene regulation. We have evidence for all three of these possibilities. Although most identified CLK direct targets bind the transcriptional repressor PER with a delayed phase relative to CLK-binding, there are a small subset of genes that appear to bind PER without CLK. Experiments are underway to address the role of PER on these genes. Moreover, experiments are also underway to extend these approaches to mammalian chromatin, to compare and contrast CLK transcriptional regulation between *Drosophila* and mouse.

11:30 • S69

Oscillating miRNAs and circadian rhythms in Drosophila melanogaster

SADANAND VODALA, JOSEPH RODRIGUEZ, STEFAN PESCATORE, MICHAEL ROSBASH
Department of Biology, HHMI/Brandeis University, Waltham, MA, USA

Circadian rhythms (~24h) are regulated by a central clock, which is entrained by external stimuli like light and temperature. The best-studied features of central clocks are positive/negative transcriptional feedback loops, which contain mechanisms and even components conserved between flies and mammals. In contrast, translational regulation is less well studied from the circadian point of view. Yet a few recent studies, including one from our laboratory [Kadener et al., 2009], have implicated miRNAs in circadian timekeeping in mammals and flies. To further investigate this mechanism in the *Drosophila* system, we used deep sequencing of fly head RNA to identify known miRNAs that undergo circadian oscillations in level. Although most miRNAs showed no significant oscillations, there were a few exceptions with remarkably high amplitude cycling. The cycling is under clock control, as it disappears in the *per01* arrhythmic clock mutant. Six of these cycling miRNAs were derived from a single cluster of miRNAs, which are apparently synthesized as a single pri-miRNA. This suggests that the cluster is under circadian transcriptional control. It is expressed in TIM+ neurons, and its overexpression with

a tim-gal4 driver lengthens behavioral circadian period, suggesting that the targets of these miRNAs include central clock mRNAs. The data furthermore indicate that these miRNAs must have a short half-life to undergo such dramatic oscillations in level. The most intriguing speculation is that they are degraded in concert with their target mRNAs.

11:45 • S70

Transcriptional regulation of clock controlled genes

AGNES ROSAHL¹, KASIA BOZEK¹, JAN TEICHMANN¹, KATJA SCHELLENBERG², JEANNINE MAZUCH²,
BERT MAIER², ACHIM KRAMER², HANS-PETER HERZEL¹

¹*Institute for Theoretical Biology, Humboldt University, Berlin, GERMANY*

²*Laboratory of Chronobiology, Charite Universitätsmedizin, Berlin, GERMANY*

The complexity of tissue- and day-time specific regulation of thousands of clock controlled genes (CCGs) suggests that yet unknown transcriptional regulators are involved. We analyze the overrepresentation of transcription factor binding sites (TFBS) using G+C-matched controls in selected sets of sequences: (i) promoters of system-driven liver CCGs (Kornmann et al. PLoS Biology 2007), (ii) sequences with circadian DNA-binding activity from liver extracts (Reinke et al. Genes Development 2008), (iii) CCG promoters in peritoneal macrophages (Keller et al. PNAS 2009). In addition to known regulatory TFBS such as E-boxes and D-boxes we find promising candidates of additional circadian regulators. In system-driven liver CCGs, binding sites for the serum response factor (SRF) and the estrogen receptor (ER) are overrepresented. The sequences with circadian DNA-binding activities in liver extracts contain multiple high-scoring binding sites of C/EBP, E2F, TBP, IPF1, AP1, GATA and HNF-1. Promoters of macrophage CCGs have also overrepresented SRF-sites, Y-boxes, PBX-, IRF-, and FOXO-sites. Using bioluminescent recordings in cell lines we show circadian transcriptional activity for reporter constructs with E2F, GATA and C/EBP cis-regulatory motifs. Our bioinformatic analysis combined with live-cell recordings of reporter constructs helps to elucidate regulatory mechanisms of circadian rhythms in physiology, metabolism and immunology.

12:00 • S71

Genome-wide mapping of Bmal1 binding sites in mouse liver reveals cooperative interactions at circadian enhancers

GUILLAUME REY¹, FRANÇOIS CESBRON², HANS REINKE³, MICHAEL BRUNNER², FELIX NAEF¹

¹*Institute of Bioengineering (IBI), Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, SWITZERLAND*

²*Biochemistry Center, University of Heidelberg, Heidelberg, GERMANY*

³*Institut für umweltmedizinische Forschung, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, GERMANY*

The mammalian circadian clock uses interlocked negative feedback mechanisms that rely on several key transcriptional regulators. Among those, the principal activator is the heterodimeric bHLH transcription factor BMAL1/CLOCK. While the circadian clock is implicated in a variety of processes like the cell cycle or metabolism, the detailed links between circadian regulators and downstream targets have been described only in isolated cases. Using chromatin immunoprecipitation combined with deep sequencing (ChIP-seq) of BMAL1 in mouse livers at 4h time intervals, we generate a genome-wide

map of BMAL1 binding sites around the clock. At the peak of BMAL1 activity (ZT6), we identify more than 700 binding sites, most of which are not occupied at trough activity. Known circadian targets of BMAL1/CLOCK such as genes of the *Per*, *Cry*, *Dec*, *Rev-Erb*, and *Par-bZip* families are strongly bound, often at multiple sites. Functional annotation of BMAL1 target genes confirms metabolism as the major output of the circadian clock in mouse liver. Comparison with expression profiles in mouse liver shows that about a quarter of the BMAL1 targets have cyclic mRNA expression with a phase that is consistent with BMAL1/CLOCK peak activity. Interestingly, sequence analysis of the binding sites shows that BMAL1/CLOCK preferentially binds tandem E-boxes elements (E1-E2) with a spacing constraint, substantiating recent *in cellulo* and *in silico* studies. To further characterize the protein-DNA interaction between BMAL1/CLOCK and its cognate E1-E2 sequence, we performed Electromobility Shift Assay (EMSA) with nuclear extracts. We find that E1-E2 is bound by two BMAL1/CLOCK heterodimers with a cooperativity that rapidly decreases with the spacing between the two E-boxes. Transactivation assays with native and mutated sequences confirm the spacing-dependent cooperativity at E1-E2 elements. Taken together, our results refine mechanisms for BMAL1/CLOCK dependent transcription, and reveal the dynamic nature of mammalian circadian protein-DNA interactomes on a genome-wide scale.

12:15 • S72

The Krüppel Like Factor KLF10 links the circadian clock to metabolism in liver

FABIENNE GUILLAUMOND¹, ALINE GRÉCHEZ-CASSIAU¹, MALAYANNAN SUBRAMANIAM², SOPHIE BRANGOLO¹, BRIGITTA PETERI-BRÜNBACK¹, BART STAELS³, CATHERINE FIÉVET³, THOMAS SPELSBERG², FRANCK DELAUNAY¹, MICHÈLE TEBOUL¹

¹CNRS UMR6543, Université de Nice, Nice, FRANCE

²Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN, USA

³Inserm U1011, Univ Lille Nord de France, Institut Pasteur de Lille, Lille, FRANCE

The circadian timing system coordinates many aspects of mammalian physiology and behaviour in synchrony with the external light/dark cycle. These rhythms are driven by endogenous molecular clocks present in most body cells. Many clock outputs are transcriptional regulators suggesting that clock genes control primarily the physiology through indirect pathways. Here we show that Krüppel Like Factor 10, KLF10, displays a robust circadian expression pattern in wild type mouse liver but not in clock deficient *Bmal1* knockout mice. Consistently, the *Klf10* promoter recruited the BMAL1 core clock protein and was transactivated by the CLOCK:BMAL1 heterodimer through a conserved E-box response element. Profiling the liver transcriptome from *Klf10*^{-/-} mice identified 158 regulated genes with a significant enrichment for transcripts involved in lipid and carbohydrate metabolism. Importantly, approximately 56% of these metabolic genes are clock-controlled. Male *Klf10*^{-/-} mice displayed post-prandial and fasting hyperglycaemia, a phenotype accompanied by a significant time of day dependent upregulation of the gluconeogenic gene *Pepck* and an increased hepatic glucose production. Consistently, functional data showed that the proximal *Pepck* promoter is repressed directly by KLF10. *Klf10*^{-/-} females were normoglycemic, but displayed higher plasma triglycerides. Correspondingly rhythmic gene expression of components of the lipogenic pathway including *Srebp1c*, *Fas*, and *Elovl6* was altered in females. Collectively these data establish KLF10 as a circadian transcriptional regulator required that links the molecular clock to energy metabolism in liver.

Poster Abstracts

P1

A framework for systems chronobiology: Systems Biology Software Infrastructure, SBSI™

ANDREW MILLAR

Centre for Systems Biology at Edinburgh, University of Edinburgh, Edinburgh, UNITED KINGDOM

Systems biology requires an integration of data and models. Clock models are becoming more complex, and depend on increasing amounts and diversity of data, so constructing and updating models becomes more cumbersome. SBSI (www.sbsi.ed.ac.uk) is a set of open-source, modular software that aims to streamline the process of systems modelling. SBSI is a core platform of the Centre for Systems biology at Edinburgh (CSBE), with input from the PlaSMO and ROBuST projects. SBSI has been developed and tested for clock models, combining:

- A user-friendly, platform-independent client application, SBSI-Visual. Access is also possible from the command line.
- The Optimisation Framework, a tool to fit complex SBML models to timeseries data (and soon, to a custom cost functions).
- SBSI-Dispatcher, a server-based task manager to send compute jobs to High-Performance Computers, such as Edinburgh's IBM BlueGene supercomputer.
- A simple data format for timeseries data or simulations (with an online file format validation tool).
- A repository of mathematical models in XML format, such as SBML (our current version is plant-focussed, www.plasmo.ed.ac.uk). Models can also be imported into SBSI from the Biomodels database.
- A database of experimental data (in progress) that can be automatically linked to data analysis tools, such as the FFT-NLLS routine of Straume.

SBSI development is ongoing, with several major developments in the coming year. CSBE welcomes input and co-development from the chronobiology community: please contact the software development leader, Dr. Richard Adams, the lead Investigator Dr. Stephen Gilmore, or the CSBE Director, Prof. Andrew Millar.

P2

Modeling circadian rhythms on transcription level of clock genes

ANJA KORENCIC¹, MARKO GOLICNIK¹, JURE ACIMOVIC¹, ROK KOŠIR¹, MARTINA PERŠE³, DAMJANA ROZMAN²

¹*Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, SLOVENIA*

²*Center for Functional Genomics and Bio-Chips, Faculty of Medicine, University of Ljubljana, Ljubljana, SLOVENIA*

³*Institute of Pathology, Medical Experimental Center, Faculty of Medicine, University of Ljubljana, Ljubljana, SLOVENIA*

Circadian regulation of physiology is crucial for the adaptation of organisms to the day-night rhythms. It is directed by a master clock in the suprachiasmatic nuclei of the hypothalamus. Circadian oscillations

are endogenous in nature but can also be reset by environmental stimuli such as light or feeding. The central clock transduces the signal to direct expression of clock-controlled genes and can also entrain peripheral circadian oscillators such as liver. The main components of central clock are transcription factors BMAL1 and CLOCK; they activate the transcription of negative regulators PER and CRY that dimerize and inhibit transcription of CLOCK/BMAL1-controlled genes. This negative feedback loop is intertwined with a positive regulatory loop that controls rhythmic expression of Bmal1. Effects of posttranslational modifications, localization, and degradation of clock proteins are very important in building a model that is capable of showing expression profiles similar to experimental results and can be entrained by light pulses via regulation of PER synthesis. Mathematical models describing core circadian oscillators have already been published. We want to build a new predictive mathematical model of the mammalian circadian clock that is based on individual molecular reactions, is in agreement with experimental data, and contains novel genes and proteins that have not been included in any of the previous models. Parameters will be estimated from our experimental data (transcription levels of clock genes) by fitting procedure. Our model should be able to predict phase, amplitude of oscillation, and shape of time profiles of clock mRNAs.

P3

Deterministic and stochastic mathematical models of the KaiABC cyanobacterial circadian oscillator

MARK BYRNE

Physics, Spring Hill College, Mobile, AL, USA

Several mathematical models have been proposed for the in vitro KaiABC oscillator since the discovery of non-TTFL-based circadian phosphorylation oscillations in cyanobacteria. The last few years I have been working in collaboration with Carl Johnson's lab constructing various quantitative models of the KaiABC oscillator. In 2007 we published a mathematical model of the in vitro KaiABC circadian oscillator that used phase-dependent monomer exchange as a synchronization mechanism in the KaiC population (Mori et al, 2007). Since that time, new published experimental information on the KaiABC oscillator in vitro and in vivo has appeared. To investigate potential oscillatory mechanisms consistent with more recent experimental data, I have constructed both simple and more realistic versions of mass-action based ordinary differential equation models for both the in vitro and in vivo KaiABC oscillator. A phenomenological in vivo model of the system consisting of a core KaiABC phosphorylation oscillator coupled to a transcription-translation negative feedback loop (and including KaiA, KaiB and KaiC protein degradation and production) indicates how these nonlinear interactions can produce robust oscillations and set the relative phase of abundance, mRNA and phosphorylation oscillations. In terms of just the in vitro system, a more intuitive molecular stochastic matrix model that directly simulates KaiC hexamers association/dissociation kinetics (with KaiA and KaiB) and site-dependent (S431 and T432) phosphorylation and de-phosphorylation can be used to investigate the potential role of site dependence in terms of single-molecule dynamics and sustained population oscillations of KaiC phosphorylation.

P4

Per/Dec interaction in the murine circadian clock

BRID BODE¹, MORITZ J. ROSSNER², KLAUS-ARMIN NAVE², HENRIK OSTER¹

¹*Genes and Behavior, Max-Planck-Institute for Biophysical Chemistry, Göttingen, GERMANY*

²*Neurogenetics, Max-Planck-Institute for Experimental Medicine, Göttingen, GERMANY*

In both vertebrates and invertebrates, a set of interlocked transcriptional/translational feedback loops drive 24 h rhythms of physiology and behavior. In *Drosophila*, *cwo* and *period (per)* genes have synergistic functions in the negative branch of the molecular clock. *Dec1* and *Dec2* (*Bhlhe40/41*) are

the mammalian orthologs of *cwo*. To assess the interaction of Dec and Per genes in the mammalian circadian clock we analyzed locomotor activity rhythms in Per/Dec double deficient mice under different light conditions. We show that Per1 and Decs interact in both the regulation of free-running periodicity and light mediated entrainment of activity. However, the nature of this interaction varies for different experimental paradigms. In summary, our data suggest that in mice Dec genes have similar, but not identical, functions in Per feedback when compared to the fly clock.

P5

Uncoupling two circadian functions of mammalian cryptochrome 1

INES CHAVES, MONIKA BAJEK, ROMANA NIJMAN, ANTONIO CARVALHO DA SILVA, GIJSBERTUS VAN DER HORST
*Genetics, Chronobiology and Health Group, Erasmus University
Medical Center, Rotterdam, THE NETHERLANDS*

Circadian rhythms are generated by a molecular oscillator which is composed of interconnected feedback loops of transcription and translation, and are further modulated by a network of post-translational modifications. Mammalian cryptochromes have been shown to be key components of the circadian clock and function as strong repressors of CLOCK/BMAL1-driven transcription. Furthermore, the CRY proteins post-translationally regulate the sub-cellular localization and stability of the PER2 protein. We have previously identified a predicted coiled-coil domain in the C-terminus of CRY1 which is necessary for robust transcription inhibition and complex formation with the PER proteins. Here we analyse the *in vivo* effect of a CRY1 mutant protein which lacks the coiled-coil domain. Our findings suggest that, although the CRY1-dCC protein is not sufficient to drive sustained circadian oscillations, it is capable of maintaining peripheral oscillators synchronized in culture conditions. We speculate that by deleting the coiled-coil domain it is possible to uncouple two distinct functions of CRY1 in regulating circadian rhythms: core oscillator function (transcriptional feedback loop) and peripheral clock synchronization.

P6

Circadian oscillations of histone-modifications are associated with circadian transcription

CHRISTOPHER VOLLMERS¹, ROBERT SCHMITZ², JOSEPH ECKER², SATCHIN PANDA¹
*¹RBIO, Salk Institute for Biological Studies, San Diego, CA, USA
²PBIO, Salk Institute for Biological Studies, San Diego, CA, USA*

Circadian clocks help organisms to predict and adapt to regular changes in the environment caused by the rotation of the earth around its axis. Analogous, the hepatic clock mechanism helps the liver adapt to predictable changes in the energy state of the whole organism, caused by a rhythmic feeding schedule. It does so by activating transcription of its target genes at the right time of the day. In addition to the anticipation by the circadian clock, daily fluctuations in the systemic energy state cause transcription triggered by multiple metabolic pathways. We used expression profiling to dissect transcriptional programs and found that different groups of genes are driven by the hepatic circadian clock, metabolic pathways or a combination of both. Combined, these mechanisms cause thousands of transcripts to display circadian variation in their expression. Transcriptional changes of single genes controlled by the clock-mechanism are known to be accompanied by circadian rhythms in histone-modifications in their promoters. Whether these rhythms are present in genes outside of the circadian clock mechanism is unknown. Using Chromatin-IP we are answering this question by investigating select histone-modifications, thereby offering further insight into the mechanisms of the observed transcriptional changes.

Extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated resetting of the circadian clock of the mouse embryonic brain-derived neural stem/progenitor cells

TAKAHIRO MORIYA¹, TOMOKO MAEKAWA¹, HIROSHI ONOZUKA¹, TAKASHI KATURA¹, HIDENOBU OHTA², NORIMICHI NAKAHATA¹

¹*Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, JAPAN*

²*Department of Pediatrics, Tohoku University Hospital, Sendai, JAPAN*

We have previously shown that the proliferation of the mouse embryonic neural stem/progenitor cells is periodically regulated by mPER2 in a circadian fashion in vitro. In this study, we examined the molecular mechanism underlying the resetting of the circadian clock of mouse embryonic brain-derived neural stem/progenitor cells (NSPCs). NSPCs from the striatum or hippocampus of embryonic mice were expanded by neurosphere method. The treatment with either epidermal growth factor (EGF) or dexamethasone induced the transient expression of mPer1 mRNA followed by the circadian rhythm of clock-related genes in NSPCs. EGF also transiently increased the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAP kinase, c-Jun NH2-terminal kinase (JNK). The pretreatment with an inhibitor of MEK, U0126, but not inhibitors of p38 MAP kinase and JNK, blocked EGF-induced transient mPer1 expression and circadian rhythms of clock-related gene expression in NSPCs. Furthermore, we found that NSPCs exhibited the circadian proliferation upon the stimulation with EGF, but not with dexamethasone. These results suggest that extracellular signal-regulated kinase 1/2 (ERK1/2) mediates the resetting of the circadian clock governing the cell division in the mouse embryonic brain-derived NSPCs by upregulating mPer1 gene.

Orphan nuclear receptor NR2F1 directly regulates the circadian output

FERNANDA RUIZ¹, CELINA MONTEMAYOR¹, MARTIN YOUNG², FREDERICK PEREIRA¹

¹*Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA*

²*Cardiology, University of Alabama–Birmingham, Birmingham, AL, USA*

NR2F1 (aka COUP-TFI) is an orphan member of the nuclear receptor superfamily critical for development of the central and peripheral nervous systems and inner ear. In the mouse cochlea, NR2F1 controls Notch signaling to regulate precursor cell differentiation into hair cells and supporting cells. Gene expression profiling and validation by qRT-PCR from wild type and Nr2f1^{-/-} inner ears identified the core circadian gene Period1. Interestingly, many circadian gene expressions are enriched in the cochlea but no functional role is ascribed to the circadian system during development of the cochlea. Indeed, the core clock gene expressions are altered in the Nr2f1^{-/-} inner ear and brain and a complete deregulation of the circadian clock mechanism is seen in Nr2f1^{-/-} mouse embryonic fibroblasts (MEFs) and when over expressing NR2F1 in cultured cells, as compared to wild type controls. Mechanistically, NR2F1 directly occupies the Bmal1 promoter and activates its transcription rate as determined using chromatin immunoprecipitation (ChIP) and promoter-luciferase reporter assays. To further quantify NR2F1 regulation of the circadian system, we characterized a circadian gene reporter (mPER2::LUC) in the NR2F1 background. The wild type pMEFs that were not cell cycle synchronized (unsynchronized) showed cyclic expression of mPER2::LUC, with a trough at 72hrs and peak at 120hrs. NR2F1^{-/-} pMEFs showed a similar pattern but with significantly higher expression at 72 (p<0.05) and 120hrs (p<0.01) suggesting the molecular clock is altered and has a higher output in the absence of NR2FI. In cell

cycle synchronized NR2F1^{-/-} pMEFs a robust cycling of mPER2::LUC is seen with a similar period and phase as in wild type cells. However, the amplitude was increased 1.5-fold, again indicating NR2F1 regulates the molecular clock output. Cumulatively, our results implicate NR2F1 in regulating both the circadian and Notch networks during inner ear development.

P9

Inhibitor of DNA binding 2 (Id2) knockout mice treated with sub-threshold discrete light pulses exhibit enhanced phase shifts and an elevated induction of mPer1 in the SCN

GILES DUFFIELD¹, SUNG HAN², TIM HOU¹, HORACIO DE LA IGLESIA², KATHLEEN McDONALD¹,
MARICELA ROBLES-MURGUIA¹

¹Biological Sciences, University of Notre Dame, Notre Dame, IN, USA

²Biology and Program of Neurobiology, University of Washington, Seattle, WA, USA

ID2 is a rhythmically expressed HLH transcriptional repressor. Deletion of Id2 results in circadian phenotypes, highlighted by an enhanced photoentrainment response under parametric conditions (Duffield et al 2009 Curr Biol 19:297-304; Hou et al 2009 J Biol Chem 284:31735-45). By examining parametric (continuous) and nonparametric (discrete) models of photoentrainment, we have begun to explore the mechanism by which these large phase shifts and rapid entrainment are achieved in the Id2^{-/-} mouse. No differences were observed between Id2^{+/+} and Id2^{-/-} mice in 1) period lengths and frequency of arrhythmia of animals maintained in constant light (80 lux); 2) the size of accumulative phase shifts in experiments testing the photorefractory duration with a series of discrete light pulses (15 min, 250 lux) interspaced by varying time intervals (1, 1.5, 2 hr); and 3) the magnitude of phase shifts produced by discrete saturating light pulses at CT16 (30 min, 800 lux). However, treatment of mice with a single sub-saturating discrete light pulse at CT16 (4 min, 8 lux) revealed a 1.9-fold increase in the magnitude of the phase shift. Moreover, a significant elevation of light-induced mPer1 expression was observed in the Id2^{-/-} ventrolateral SCN. This correlates with our earlier finding showing a 1.8-fold larger mean phase delay with a treatment of 10 hr continuous light (250 lux), and an increase in induction of mPer1 expression in serum-stimulated Id2^{-/-} mouse embryonic fibroblasts (Ward et al 2010 SRBR Abstract). To test whether this phenotype is based on a sensitivity change at the level of the retina, we measured pupil constriction responses of mice subjected to 10 and 100 lux light. No differences between genotypes was observed at either light intensity, suggesting that the enhanced phase shift and mPer1 induction is due to changes occurring downstream of the retina/RHT. These data suggest that ID2 contributes to photoentrainment of the circadian system at the level of the SCN through regulation of the photic induction of negative elements of the core oscillator feedback loop.

P10

Distinct functions of reactive oxygen species in the control of circadian rhythm in Neurospora crassa

NORBERT GYONGYOSI, DORA NAGY, MONIKA BERCELI, AGNES SANDOR, KRISZTINA KALDI
Department of Physiology, Semmelweis University, Budapest, HUNGARY

—WITHDRAWN—

Characterization of new partners of CRYPTOCHROME, the circadian blue light photoreceptor in Drosophila melanogaster

GABRIELLA MAZZOTTA¹, ELENA CARBOGNIN¹, MOYRA MASON¹, LAURA CACCIN¹, FEDERICA SANDRELLI¹, MARIA PIA BOZZETTI², RODOLFO COSTA¹

¹*Department of Biology, University of Padova, Padova, ITALY*

²*Department of Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, Lecce, ITALY*

In *Drosophila melanogaster* CRYPTOCHROME (dCRY) is a blue light photoreceptor involved in the photic input pathway to the circadian clock. It mediates the daily resetting of the brain clock by light, a fundamental step in the generation of circadian rhythmicity. We have previously hypothesized that the activation of dCRY by light is mediated by specific “regulators” that bind the C-terminus of the protein, which has been proven to regulate the light-dependence of dCRY activity and to be a hotspot for molecular interactions. In the peripheral clocks dCRY has a fundamental role in the circadian pacemaker, where it acts as transcriptional repressor, in a light independent manner. It can be hypothesized that different regulatory elements could modulate dCRY activity in peripheral and brain oscillator cells. The research of putative partners of dCRY by co-immunoprecipitation experiments on transgenic flies overexpressing dCRY led us to the identification of BELLE, an ATP-dependent RNA helicase, known to be involved in splicing, translation and RNAi. In *Drosophila* heads the expression of this RNA-helicase shows a circadian oscillation either in LD cycles or in constant darkness (DD). Flies mutant for this gene do not exhibit the canonical rhythmic activity profile, likely due to an impairment of the circadian clock. Our data suggest that BELLE has a role in the circadian machinery of *Drosophila*, where it could act in the post-transcriptional control of circadian components.

Gene Dosage Network Analysis identifies genome wide targets of Clock and Bmal1

JULIE BAGGS, ANTHONY OLARERIN-GEORGE, JOHN HOGENESCH

Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Genetic networks confer adaptive advantages to biological systems by integrating and responding to environmental or genetic perturbation. To extend work on genetic perturbation of the clock, we performed genome wide Gene Dosage Network Analysis (GDNA). Guided by simulations, we conducted a nine point knockdown of Clock and Bmal1 in U2OS cells, then harvested RNA for analysis by quantitative RT PCR and DNA arrays. We incorporated biochemical function and regulatory element analysis in a new methodology to infer direct and indirect targets of Clock and Bmal1. Using this approach, we identified dozens of functional targets of Clock and Bmal1. In agreement with analysis of knockout mice, aside from Rev-erb-alpha and Rev-erb-beta we found few genes regulated by both factors. Instead, most genes responded to knockdown of either Clock or Bmal1, suggesting their ability to function independently. This method also indicated roles for intermediate genes in regulating transcriptional responses, and we confirmed several of these by subsequent experimentation. Finally, in proof of concept, we are performing chromatin immunoprecipitation experiments with Flag tagged version of Clock and Bmal1 and integrating these data with the quantitative transcriptional responses from GDNA. Using this approach, we are building mechanistic and functional networks describing response to genetic perturbation. These methods will be extended to other components of the circadian clock, other clocks, and to other biological systems.

The circadian clock protein Period 1 coordinately regulates sodium transport genes

MICHELLE GUMZ¹, BRIAN CAIN², CHARLES WINGO¹

¹Medicine, University of Florida, Gainesville, FL, USA

²Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, USA

The goal of the present study was to define the role of the circadian clock protein Period 1 (Per1) in the transcriptional regulation of renal epithelial transport. The renal epithelial sodium channel (ENaC) is the primary known mechanism for sodium reabsorption from the lumen of the collecting duct. We have previously shown that Per1 positively regulates expression of α ENaC (Gumz et al. JCI 2009), the aldosterone-regulated and rate-limiting subunit for channel assembly in the kidney. To determine if Per1 regulates the expression of other genes important in renal epithelial transport, we performed a microarray study using mpkCCDc14 cells as a model for the cortical collecting duct. Cells were transfected with a non-target siRNA or a Per1-specific siRNA. Following Per1 knockdown, 299 genes were differentially expressed ($p < 0.05$). Per1 knockdown led to increased mRNA expression of three known inhibitors of ENaC activity, endothelin-1, Ube2e3 and caveolin-1. The mRNA expression of Fxyd5 decreased following Per1 knockdown. Fxyd5 is a positive regulator of the Na,K-ATPase, the ion pump responsible for transporting sodium out of collecting duct cells into the bloodstream. Together these data suggest that the circadian clock protein Per1 coordinately regulates expression of genes involved in renal sodium transport.

The circadian transcriptional factor Period 2 modulates cyclin B1 expression

JIANHUA YANG, CARLA FINKIELSTEIN

Biological Sciences, Virginia Tech, Blacksburg, VA, USA

The proper timing of cell division is a major factor contributing to the regulation of normal growth and emerges as a fundamental process in the development of most cancers. The existence in most, if not all, living organisms of many behavioral and physiological events that occur at well-defined and controlled times argues for the presence of time-keeping systems that allow them to adapt to cyclic changes in environmental conditions. Disruption of circadian rhythm has recently emerged as a new potential risk factor in the development of cancer, pointing to the core gene period 2 (*per2*) as a tumor suppressor. However, it remains unclear how the circadian network regulates tumor suppression. In the present study, we use cDNA microarray analysis to identify Per2 target genes following siRNA knock-down expression of Per2 in mouse hepatocytes AML12 cells. We identified cyclin B1, a key G2/M regulator of the cell cycle, as a downstream target gene that is negatively regulated by Per2 under normal conditions. Quantitative real-time PCR confirmed our observations by showing cyclin B1 expression is enhanced under Per2 knock-down condition. Accordingly, *Bmal1* expression, a known target gene of Per2, was specifically augmented under Per2 over-expression conditions. Further experiments focused in characterizing the regulation of the human cyclin B1 promoter by Per2 using luciferase reporter assays. Results show cyclin B1 reporter activity is enhanced in siPer2 transfected cells compared to control scrambled siRNA. Promoter analysis defined the region comprising -484 to -284 of human cyclin B1 promoter containing response elements for gene trans-activation while the region between -284-1 is mainly responsible for Per2 regulation. Our results show the canonical E-box between -284-1 is not required for Per2 regulation of cyclin B1, suggesting non-canonical E-boxes might be directly responsible for Per2-mediated expression. FACS analysis revealed that siPer2 treated cells exhibit a reduced G2/M phase with correspondingly higher cyclin B1 protein levels and reduced Tyr-15-cdc2 phosphorylation compared to scrambled siRNA treated cells. Our previous results show Per2 stability is directly controlled by the endogenous levels of heme whose endogenous

levels oscillate in a 24 h cycle. Here, we evaluated whether cyclin B1 expression is altered in heme-treated cells. In contrast to its up-regulation in siPer2 treated cells, luciferase reporter assay showed a decrease in cyclin B1 promoter activity in response to heme treatment. These results may reflect a complex pattern of regulation in which Per2 acts as a sensor molecule to monitor changes in both environmental and cellular conditions that will impact cell progression.

P15

The effects of acute and chronic lithium treatments on circadian rhythm expression in human primary fibroblasts

SANG KIL LEE¹, SHAOJIA BAO¹, SUEPHY C. CHEN², CONNIE MADDOX³, MICHAEL IUVONE⁴, CHIAKI FUKUHARA¹

¹*Department of Neurobiology, Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA*

²*Department of Dermatology, Emory University School of Medicine, Atlanta, GA, USA*

³*CRC, Morehouse School of Medicine, Atlanta, GA, USA*

⁴*Department of Ophthalmology, Emory University School of Medicine, Atlanta, GA, USA*

The mood stabilizer lithium extends the period length in a variety of species, including humans. Although chronic lithium treatment within the range of therapeutic concentrations lengthens the period in intact animals and humans, acute lithium stimulation above therapeutic levels are required to show such effects in vitro. Considering the treatment strategy and the effects of lithium on mood stabilization in bipolar disorder patients, it would be of interest to test if chronic lithium treatment at the therapeutic concentration affects circadian rhythm generation. Skin biopsies were obtained from six healthy subjects. After expansion of cell cultures, Bmal1-luciferase DNA was delivered to cells that were divided into three groups. The first group was treated with 0, 1, 3, 7, or 10 mM lithium and circadian rhythms were recorded immediately. In the second group, cells were treated with 0 or 1 mM lithium for one week before rhythm recording. The last group was treated with 0 or 1 mM lithium for four weeks before recording. Although acute 7 mM and 10 mM lithium treatments significantly lengthened the period (0.93 ± 0.2 h, $P < 0.003$ and 1.68 ± 0.2 h, $P < 0.0001$, respectively), none of the other lithium treatments affected the period length. No inter-individual difference was observed at 7 or 10 mM. Since the action of lithium stimulation on the circadian rhythm expression is mediated by inhibition of glycogen synthase kinase-3beta (GSK-3beta), its phosphorylation was determined using Western blot analysis. GSK-3beta phosphorylation at Ser9 was significantly increased 6 h after 1 and 10 mM lithium stimulation; however, it was no longer affected after one and four weeks, thus it suggests that phosphorylation of GSK-3beta is not sufficient to extend the period. In conclusion, it appears that lithium treatment differentially affects GSK-3beta and circadian rhythm generation in vitro and in vivo.

P16

Zinc finger protein 496 is a novel target of the mouse cryptochrome-2 dependent circadian regulation

ANAND VENKATARAMAN, JULIE BAGGS, JOHN HOGENESCH

Pharmacology, University of Pennsylvania, Philadelphia, PA, USA

The molecular clock is made of interlocking feedback loops governed by two families of transactivators (Bmal1/2, Clock/Npas2) and two families of transrepressors (Per1/2/3 and Cry1/2). Loss of function experiments in vitro and in vivo show that mouse Cry1 and Cry2 are non-redundant and potentially regulate two independent transcriptional networks. We used a high throughput Gal4 transcription factor trap screen to identify additional transcription factor targets of Cry1 and Cry2. We identified Zfp496, a zinc finger transcriptional activator, as a potent and dose-dependent target of both Cry1 and Cry2. Using co-immunoprecipitation studies, we show that Zfp496 preferentially interacts with Cry2,

and interestingly, its transcription cycles in phase with Cry2 in mouse liver. Finally, we investigated epistatic functional interactions in U2OS cells between Zfp496 in Cry1 and Cry2. While knockdown of both Cry2 and Zfp496 did little, knockdown of Zfp496 and Cry1 resulted in arrhythmicity akin to Cry1/Cry2 knockdown. Taken together, these studies show that Zfp496 physically and genetically interacts with Cry2 to impact human clock function.

P17

Setting the pace of the Neurospora circadian clock by multiple independent FRQ phosphorylation events

CHI-TAI TANG¹, JOONSEOK CHA¹, SHAOJIE LI², CHENGZU LONG³, GUOCUN HUANG⁴, LILY LI⁵, SHE CHEN³,
YI LIU¹

¹Physiology, University of Texas Southwestern Medical Center, Dallas, TX, USA

²Institute of Microbiology, Chinese Academy of Science, Beijing, CHINA

³National Institute of Biological Sciences, Beijing, CHINA

⁴Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA

⁵Dermatology, University of Texas Southwestern Medical Center, Dallas, TX, USA

Protein phosphorylation plays essential roles in eukaryotic circadian clocks. Like PERIOD in animals, the Neurospora core circadian protein FRQ is progressively phosphorylated and becomes extensively phosphorylated before its degradation. In this study, using purified FRQ protein from Neurospora, we identified 43 in vivo FRQ phosphorylation sites by mass spectrometry analysis. In addition, we show that CK-1a and CKII are responsible for most FRQ phosphorylation events and identify an additional 33 phosphorylation sites by in vitro kinase assays. Whole-cell metabolic isotope labeling and quantitative MS analyses suggest that circadian oscillation of the FRQ phosphorylation profile is primarily due to progressive phosphorylation at the majority of these newly discovered phosphorylation sites. Furthermore, systematic mutations of the identified FRQ phosphorylation sites led to either long or short period phenotypes. These changes in circadian period are attributed to increases or decreases in FRQ stability, respectively. Together, this comprehensive study of FRQ phosphorylation reveals that regulation of FRQ stability by multiple independent phosphorylation events is a major factor that determines the period length of the clock. A model is proposed to explain how FRQ stability is regulated by multiple phosphorylation events.

P18

Post-translational regulation of the Drosophila CLOCK protein by its interaction partner CYCLE

HSIU-CHENG HUNG, DANIELA ZORN, CHRISTIAN MAURER, WAI-LING CHANG, FRANK WEBER
Biochemistry Center Heidelberg, University of Heidelberg, Heidelberg, GERMANY

Rhythmic changes in circadian transcription are driven by the oscillating activation and inhibition of the core circadian transcription factors CLOCK (CLK) and CYCLE (CYC). Although post-translational regulation of the CLK/CYC complex, particularly of CLK, has been shown to be crucial for accurate timing of circadian transcription, the mechanisms that underlay this regulation remain largely elusive. Here we investigated the contribution of the heterodimerization partner CYC to the post-translational regulation of the CLK protein in Drosophila. We found that heterodimeric complexes between CLK and CYC are not only formed inside the nucleus, where they activate circadian transcription, but also in the cytoplasm, where CYC stabilizes the CLK protein, preventing a rapid and efficient degradation of cytoplasmic CLK. Nucleo-cytoplasmic shuttling of CLK results in cytoplasmic localization of the transcription factor due to efficient nuclear export. Interaction with CYC enhances instead the nuclear accumulation of the CLK protein. In addition, CYC promotes phosphorylation of cytoplasmic CLK to

a hypo-phosphorylated state as well as hyper-phosphorylation of CLK inside the nucleus, suggesting a heterodimerization-dependent post-translational modification of the CLK protein. Together our results show that interaction with CYC is important for stabilization, nuclear accumulation and post-translational processing of the CLK protein, suggesting an important function of CYC for post-translational regulation of the CLK life cycle.

P19

Deep sequencing of the circadian transcriptome in the nervous system of *Drosophila melanogaster*

MICHAEL HUGHES, MICHAEL NITABACH

Cellular and Molecular Physiology, Yale School of Medicine, New Haven, CT, USA

The development of high-throughput sequencing technologies has dramatically improved the speed and power of genomic analyses. Applying these methods to transcriptional profiling provides significant advantages over conventional microarray analysis, especially with respect to the quantification of alternate isoforms and the identification of novel transcripts. Here we present a strategy to employ high-throughput sequencing to the analysis of the circadian transcriptome in adult *Drosophila* brains. We used the Illumina Genome Analyzer to generate over 160 million 75 base pair reads using amplified RNA from the brains of both wildtype and period-null fly brains. Bowtie/Tophat software packages aligned these reads to a reference *Drosophila* genome, resulting in the unique alignment of over 110 million reads. Quantification of transcriptional units at both the isoform- and gene-level was performed using Cufflinks software and differential expression was assessed using DegSeq and conventional statistical methods. We present the identification of candidate cycling transcripts whose RNA oscillates in phase with components of the core circadian clock, as well as candidates whose abundance is severely altered by period loss-of-function. Finally, we show progress towards applying this approach to the transcriptional profiling of purified sub-populations of circadian clock neurons.

P20

Day, a new component of the *Drosophila* circadian clock

JOHN HARES, CELIA HANSEN, STEPHANE DISSEL, EZIO ROSATO

Genetics, University of Leicester, Leicester, UNITED KINGDOM

CRYPTOCHROME (CRY) plays an important role in the circadian clock of *Drosophila*; this process is thought to be mediated via the interaction of CRY with PERIOD (PER) and TIMELESS (TIM) under light conditions. We have been working on identifying the mode of action of CRY and identifying possible interacting partners. A yeast-two-hybrid assay revealed an interaction between CRY and another uncharacterised gene, CG15803. Interestingly, the interaction of CG15803-PA with CRY was shown to occur only in darkness with no binding occurring in light conditions and hence CG15803 was re-named *day* (dark active in yeast). Transgenics flies that can upregulate or downregulate *day* were created. We found that overexpression of DAY in the PDF expressing LNvs resulted in slightly longer periods of locomotor behaviour in DD conditions. Interestingly, expression of DAY in all clock cells did not alter behaviour, suggesting that different groups of neurons respond differently to DAY. Moreover, we identified a genetic interaction between DAY and a previously described active form of CRY (CRY Δ). Downregulation of DAY in the small and large LNvs suppressed the period lengthening induced by CRY Δ expression in DD conditions. Our results suggest that DAY is a putative component of the CRY signaling cascade.

P21

Expression of a novel ion channel may partially rescue evening anticipation in “arrhythmic” per01 and tim01 mutants

ADAM SELUZICKI, BRIDGET LEAR, ROSE-ANNE MEISSNER, RAVI ALLADA

Neurobiology & Physiology, Northwestern University, Evanston, IL, USA

We initiated a study of the function of the NA ion channel in masking activity, combining the nahar mutation with per01, a putative null form of the period gene, to eliminate the potential confound of clock-driven activity. Rescuing per01nahar broadly in the circadian system with tim-Gal4>UAS-na surprisingly results in a recovery of an activity peak late in the light phase (~ZT10). This rescue is blocked by a GAL80 inhibitor of GAL4 driven by the promoter of the circadian photoreceptor cryptochrome cry-Gal80, confirming that NA activity in circadian pacemaker cells is required for this activity. This evening activity is phase advanced at 18C relative to 25C, a shift that is similar to the advance observed in wild-type evening behavior. In addition, this activity peak is also evident under 29-21C temperature cycles. NA over-expression also partially rescues an evening peak in tim01 flies, although with a more delayed phase than in per01. We hypothesize that NA over-expression increases coupling between clock neurons, partially compensating for a loss of core clock components.

P22

Does a catalytically inactive DBT produce different phenotypes in different tissues, subcellular compartments or cis-mutant backgrounds?

ANANDAKRISHNAN VENKATESAN, MICHAEL MUSKUS, EDWARD BJES, JEFFREY PRICE

School of Biological Sciences, University of Missouri–Kansas City, Kansas City, MO, USA

DBT is essential for circadian rhythms of *Drosophila* because it phosphorylates PER and targets it for degradation. In support of this view, a kinase-inactive form of DBT (DBT-K/R) expressed in all circadian clock cells produces arrhythmicity and lengthened circadian periods, with high levels of hypophosphorylated PER throughout the day. Here, we report our initial analysis of DBT-K/R with more restricted expression, or in the context of period-altering mutants of DBT. Flies expressing DBT-K/R in all the clock neurons showed only a startle response to light in LD. When DBT-K/R expression was limited to PDF-negative cells, the flies showed a dampened evening peak but a robust morning peak in LD. Targeted expression of DBT-K/R to PDF-positive cells produced a robust evening peak but a reduced morning peak in LD. These results suggest that DBT affects the morning and evening oscillators in an autonomous manner. Adult brains from these genotypes are being further analyzed for PER oscillations to determine if DBT inactivation affects cells autonomously, or nonautonomously through network interactions. In order to determine the different roles of DBT in the nucleus and the cytoplasm, mutants of DBT with preferential cytoplasmic or nuclear localization are being expressed in flies and analyzed. The DBT-S and DBT-TAU mutations produce short circadian periods, while the DBT-L mutation lengthens period. In order to address whether part of this difference may be due to different interactions with other circadian regulatory proteins rather than to mutant kinase activity, DBT-S, DBT-L and DBT-TAU mutants are being tested in a DBT-K/R protein for effects on circadian period.

Light and temperature control the contribution of a subset of Dorsal Neurons 1 to Drosophila circadian behavior

YONG ZHANG¹, YIXIAO LIU², DIANA WENTWORTH¹, PAUL HARDIN², PATRICK EMERY¹

¹Neurobiology, University of Massachusetts Medical School, Worcester, MA, USA

²Biology, Texas A&M University, College Station, TX, USA

The brain of *Drosophila melanogaster* contains ca. 150 circadian neurons functionally divided into Morning and Evening cells that control peaks in daily behavioral activity at dawn and dusk, respectively. The PDF positive small ventral Lateral Neurons (sLN_vs) promote morning behavior, while the PDF negative sLN_vs and the dorsal Lateral Neurons (LN_ds) generate evening activity. Much less is known about the ca. 120 Dorsal Neurons (DN1, 2 and 3). Using a *clk*-GAL4 driver that specifically targets a subset of DN1s, we generated mosaic *per⁰* flies with clock function restored only in these neurons. We found that the *Clk4.1M*-GAL4 positive DN1s promote only morning activity under standard (high light intensity) light:dark cycles. Surprisingly however, these circadian neurons generate a robust evening peak of activity under a temperature cycle in constant darkness. Using different light intensities and ambient temperatures, we resolved this apparent paradox. The DN1 behavioral output is under both photic and thermal regulation. High light intensity suppresses DN1-generated evening activity. Low temperature inhibits morning behavior, but it promotes evening activity under high light intensity. Thus, the *Clk4.1M*-GAL4 positive DN1s, or the neurons they target, integrate light and temperature inputs to control locomotor rhythms. Our study therefore reveals a novel mechanism contributing to the plasticity of circadian behavior.

Crustacean β -PDH I but not β -PDH II rescues circadian rhythmicity in PDF-deficient flies

ESTEBAN BECKWITH¹, YUN-WEI A. HSU², BILLIE MEDINA², HORACIO DE LA IGLESIA², M. FERNANDA CERIANI¹

¹Laboratorio de Genética del Comportamiento, Fundación Instituto Leloir, IIBA CONICET, Buenos Aires, ARGENTINA

²Department of Biology, University of Washington, Seattle, WA, USA

Species that live in the intertidal zone are exposed to the 24-h light-dark cycle and to cyclic ebb and flow of tidal waters. Accordingly, both circadian clocks and biological clocks that oscillate in synchrony with the tide, namely circatidal clocks, have been described in several intertidal organisms. Whether these two biological timing systems share common neural and molecular mechanisms remains unknown. In an effort to identify biological clock components in an intertidal decapod crustacean, the crab *Cancer productus*, we recently cloned homologs of *Drosophila* genes whose expression is essential to sustain normal circadian rhythmicity. One of them is the pigment dispersing factor (PDF) gene, expressed by eight pacemaking ventral lateral neurons in each of the fly's brain hemispheres. We have previously identified two members of the *pdf* family in *C. productus*, β -pigment-dispersing hormone I (β -PDH-I) and β -PDH II. The distribution of β -pdh I-expressing neurons in the crab's brain is similar to that of PDF in *Drosophila*. Furthermore, β -PDH I shares closer sequence homology with PDF than PDH II does, and it is expressed in neurons that also show CYCLE-like immunoreactivity in the crab's brain. Taken together, these results suggest that β -PDH I in *C. productus* may represent a functional homolog of PDF, which in *Drosophila* is a critical peptidergic signal to drive circadian outputs and to couple the circadian network. To further investigate this possibility we transformed *Drosophila pdf*-null mutants by overexpressing either β -PDH I or β -PDH II neuropeptides in the PDF

circuit. Both transformants expressed the corresponding mRNA and peptides in the PDF circuitry. Whereas the circadian locomotor activity of β -pdh I-expressing flies was virtually undistinguishable from pdf-transformed null flies, β -pdh II could only accomplish a modest rescue of some of the phenotypes associated with the loss of PDF function in the pdf01 mutant.

P25

Spatial regulation of GIGANTEA, a circadian clock regulator, in the cell gives a signal diversity in Arabidopsis thaliana

YUMI KIM¹, MIJI YEOM¹, HYUNMIN KIM¹, JEONGSIK KIM², WOE YEON KIM³, JUNHYUN LIM¹, DAVID SOMERS², HONG GIL NAM⁴

¹*Department of Life Science, Pohang Science and Technology, Pohang-shi, Kyung-buk, REPUBLIC OF KOREA*

²*Department of Plant Cellular and Molecular Biology, Plant Biotechnology Center, Ohio State University, Columbus, OH, USA*

³*Division of Applied Life Science, Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju, REPUBLIC OF KOREA*

⁴*Division of Molecular and Life Sciences, Integrative Biosciences & Biotechnology, Pohang Science and Technology, Pohang-shi, Kyung-buk, REPUBLIC OF KOREA*

Spatial regulation in cellular signaling has raised an important factor for fulfillment of complicated regulatory mechanisms. This spatial regulation also contributes for single protein could function in many signaling pathway. GIGANTEA (GI), as a multifunctional protein, has known to act in various physiological processes in Arabidopsis. However, spatial regulations of GI in cellular compartments are poorly understood. Here, we describe that GI differentially functions in the nucleus and the cytoplasm and generates separable signals to physiological aspects like hypocotyl growth, photoperiodic flowering and endogenous clock time regulation in Arabidopsis. GI regulates a hypocotyl growth and a photoperiodic flowering time mainly in the nucleus, whereas adjusts a circadian maintenance both in the nucleus and the cytosol. Although EARLY FLOWERING 4 (ELF4) interacts with GI both in the nucleus and cytosol, genetic interactions between gi-2 and elf4 varies. These variation might be come from differential biochemical interactions between GI and ELF4. Indeed, ELF4 contributes protein stabilization in the cytosol and recruit GI to the nuclear bodies in the nucleus. These biochemical interactions are likely to lead separate regulations of output. Furthermore, GI regulates both in the nucleus and cytoplasm, but nuclear GI controlled by clock seemed to participate in hypocotyl growth and photoperiodic flowering. Therefore, the spatial regulation in the cell imply that diverse signals from a single protein can elicit different physiological processes, thereby increasing the complexity of signal transduction.

P26

Rhythms in individual cells in Arabidopsis thaliana

ESTHER YAKIR¹, MIRIAM HASSIDIM¹, NAOMI MELAMED-BOOK², IDO KRON¹, DROR HILMAN¹, RACHEL GREEN¹

¹*Department of Plant and Environmental Sciences, Hebrew University, Jerusalem, ISRAEL*

²*Bio-Imaging Unit, Hebrew University, Jerusalem, ISRAEL*

The circadian system of plants regulates a wide range of rhythmic physiological and cellular output processes. The rhythms are generated by an oscillator mechanism that, in Arabidopsis, consists of interlocking feedback loops of several components including CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), TIMING OF CAB EXPRESSION 1 (TOC1) and CCA1 HIKING EXPEDITION (CHE). This CCA1/LHY/TOC loop interacts with two other loops of oscillator elements. Research carried out over the past few years has given us an increasingly detailed picture of the clock mechanism at the resolution of the whole plant, but much less is known about the specificities of the clock mechanism at the level of the individual cells that make up the plant. We have used transgenic

plants with fluorescence-tagged CCA1 to measure parameters of rhythmicity in individual cells in intact living plants. Using these transgenic plants we show that the oscillators of individual cells become desynchronized in constant conditions. Moreover, we demonstrate that there are cell-type specific differences in circadian function. We discuss our findings in the context of circadian rhythms measured in whole plants.

P27

The intertwining of two oscillators simulates the circadian rhythm of living cyanobacteria

STEFANIE HERTEL, CHRISTIAN BRETTSCHEIDER, ANNE ARNOLD, MARKUS KOLLMANN, ILKA AXMANN
Humboldt University Berlin, Institute for Theoretical Biology, Berlin, GERMANY

Many organisms coordinate their activities according to daily cycles driven by an internal clock. The simplest circadian clock ticks in cyanobacteria. It consists of just three proteins (KaiA, KaiB, KaiC) composing a post-translational oscillator (PTO). In the presence of ATP these three Kai (Japanese for cycle) proteins are able to produce robust, temperature-compensated 24-hour cycles of KaiC phosphorylation in the cell and even in a test tube (Nakajima et al., 2005, Science 308, 414-415). This unique three-protein clock is well documented for *Synechococcus elongatus* PCC 7942 where almost all genes' expression oscillates in a circadian fashion. In contrast, the basic timing mechanism in eukaryotic circadian systems is based on transcription and translation feedback loops (TTFL). Thus, the cyanobacterial core oscillator is a highly fascinating system as it is able to operate independently of transcription and translation processes (Tomita et al., 2005, Science, 307, 251-254). KaiC is the principle protein of the PTO clock combining three intrinsic enzymatic activities—autokinase, autophosphatase and ATPase—which are reversely modulated by KaiA and KaiB. However, KaiC hyperphosphorylation disrupts the PTO but does not abolish in vivo circadian gene expression. Moreover, recent findings by Kitayama et al. (2008, Genes Dev, 22, 1513-1521) suggest a dually coupled oscillatory system, in which P-KaiC oscillation is stabilized by an outer TTFL to maintain robust and precise rhythms in cyanobacteria. We have combined the inner and outer feedback loops in a unified expression-and-phosphorylation model intertwining ODEs (ordinary differential equations) for Goodwin oscillator and PTO. Taking into account gene expression, degradation and post-translational processes, we found very good agreement between model predictions and experimentally observed dynamic behavior.

P28

Improvements in sleep during the biological day are associated with changes in thermoregulatory physiology following administration of the melatonin agonist Ramelteon

RACHEL MARKWALD¹, TEOFILIO LEE-CHIONG², TINA BURKE¹, JESSE SNIDER¹, KENNETH WRIGHT¹

¹*Department of Integrative Physiology, University of Colorado, Boulder, CO, USA*

²*Division of Sleep Medicine, Nation Jewish Health Center, Denver, CO, USA*

Ramelteon is a MT1/MT2 melatonin agonist approved for the treatment of insomnia. Nighttime administration of Ramelteon is effective at shortening sleep onset latency (SOL). We examined the effectiveness of Ramelteon (8mg) for promoting sleep during the biological daytime when endogenous melatonin levels are low and examined whether changes in thermoregulatory physiology were associated with improved sleep. Fourteen healthy adults (5 females), aged (23 ± 4 yrs) participated in a randomized, double-blind, placebo-controlled, cross-over within-subjects study. Participants were studied in the laboratory on two occasions each preceded by 7 days of a ~8h sleep-wakefulness schedule. Subjects were studied under modified constant routine conditions of bed rest and dim lighting. Ramelteon and placebo were administered 2h prior to a 4h sleep opportunity in the afternoon. Core, proximal

(subclavicle) and distal (foot; T_{ft}) skin body temperatures (Vital Sense, Mini Mitter Respironics), the distal-proximal skin gradient (DPG) and polysomnography recorded sleep stage (Siesta, Compumedics) data were analyzed with repeated measures ANOVA. Daytime Ramelteon administration significantly attenuated the circadian increase of core body temperature, altered proximal skin temperature and increased the DPG (all $P < 0.05$). Stages 1 and 2 sleep and sleep efficiency were increased following Ramelteon compared to placebo (all $P < 0.05$). There were significant correlations between the DPG and SOL ($r = -0.75$, $p = 0.013$), between DPG and %stage 1 sleep ($r = -.73$, $p = .02$), between T_{ft} with SOL ($r = .68$, $p = .03$), between T_{ft} and %stage 1 sleep ($r = -.71$, $p = .02$) and between T_{ft} and %stage 3/4 sleep ($r = .69$, $p = .03$). Improvements in sleep during the biological day following Ramelteon administration were associated with thermoregulatory adjustments of skin temperature. The ability of Ramelteon to improve sleep during the biological day suggests that Ramelteon may have promise treating disturbed sleep caused by circadian misalignment (e.g., circadian sleep disorders, spaceflight).

P29

Circadian and sleep parameters differentially predict hyperactive-impulsive and inattentive symptoms in adult ADHD patients with insomnia

KAREN L. GAMBLE¹, RACHEL C. BESING², KIMBERLY H. LINDBERGH¹, RAKESHA Y. GARNER¹, KRISTIN T. AVIS³, ROBERTA S. MAY¹, RACHEL E. FARGASON¹

¹Psychiatry and Behavioral Neurobiology, University of Alabama–Birmingham, Birmingham, AL, USA

²Psychology, University of Alabama–Birmingham, Birmingham, AL, USA

³Pediatrics, Pulmonary Division, University of Alabama–Birmingham, Birmingham, AL, USA

Patients with Attention-Deficit/Hyperactivity Disorder (ADHD) often exhibit disrupted sleep and circadian rhythms, and a large percentage show signs of sleep onset insomnia (SOI) and/or delayed sleep phase syndrome. Recently, a study found that in adult ADHD patients with SOI (77.5% of total ADHD patients sampled), dim light melatonin onset and sleep onset/offset were significantly delayed compared to ADHD patients without SOI (VanVeen, M.M., et al, 2010). Here, we sought to address whether delayed sleep phase in adult ADHD patients with insomnia impacts ADHD symptoms using the first two weeks of data from an ongoing clinical trial. Measures from 25 subjects included a daily sleep diary, actigraphy (via Actigraph wrist devices), Epworth Sleepiness Scale (ESS), ADHD Rating Scale, Clinical Global Impression Scale, Pittsburgh Sleep Quality Index (PSQI), and an insomnia questionnaire. Principal components analysis was used to identify correlated circadian- and sleep-related variables. Factors explaining 88% of the variance were entered into a backwards step-wise linear regression analysis in order to identify which components significantly predicted Total ADHD Score, as well as ADHD-RS Inattentive and Hyperactive-Impulsive dimensions. Results of the Principal Components regression indicated that later midsleep times, increased sleepiness (ESS), shorter sleep duration/rest period and poor quality of sleep (PSQI) significantly predicted higher ADHD total scores (greater severity) and inattentive ADHD symptoms. These same factors as well as increased sleep latency and greater duration of night-time waking significantly predicted increased hyperactive-impulsive symptoms. Furthermore, hierarchical regression indicated that later sleep phase significantly predicted ~15% more of the variance in inattentive ADHD symptoms than that predicted by total rest duration. In summary, these results suggest that circadian rhythm and sleep disruptions are associated with more severe ADHD symptoms in ADHD-SOI patients, and these disruptions differentially contribute to inattentive and hyperactive-impulsive symptoms.

The effects of regularizing sleep-wake schedule on the daytime mood, emotional states, and autonomic function in habitual irregular sleepers

NANA TAKASU¹, WATARU NAKAMURA², YUMIKO TAKENAKA¹, MICHIKO FUJIWARA¹, MOTOMI TOICHI¹

¹Laboratory of Clinical Cognitive Neuroscience, Graduate School of Medicine, Kyoto University, Kyoto, JAPAN

²Laboratory of Oral Chronobiology, Graduate School of Dentistry, Osaka University, Suita, JAPAN

Recently, Asperger's disorder (AD), of which major symptoms are impairments of social interaction and non-verbal communication, is being gradually and widely recognized. However, affected people are still forced to live with much tension and anxiety because AD is often overlooked. Further, recently, many people including AD complain about their mental problems, and irregular sleep-wake rhythms are common among them. Based on these reports, we postulated that their mental and physiological states might be deteriorated by their irregular sleep-wake patterns which produce irregular light-dark cycles, resulting in impairments of circadian clock adjustment. Then, we investigated the effects of regularizing sleep-wake schedule on mood / emotional states and autonomic function in habitual irregular sleepers. As we predicted, regularizing sleep-wake schedule significantly decreased the daytime negative moods, such as tension-anxiety, anger-hostility and fatigue, with a significant reduction of parasympathetic activity. And these effects were long lasted even after the sleep-wake regularization was ended. The present findings suggest that the regularization of sleep-wake schedule may modulate the daytime psychophysiological functions suitable for daytime social activities.

EEG markers of sleep homeostasis are affected by light and time-of-day in the mouse

VALÉRIE MONGRAIN, YANN EMMENEGGER, PAUL FRANKEN

Center for Integrative Genomics, University of Lausanne, Lausanne, SWITZERLAND

Objectives: The increase of a need for sleep during wakefulness and its recovery during sleep is regulated by a process known as sleep homeostasis. The dynamics of this process is studied through EEG delta power (1-4 Hz) and theta power (6-9 Hz) measured during non-rapid eye movement sleep and wakefulness, respectively. Data suggest that these markers are modulated by circadian time and light. Here, we assessed the effects of time-of-day and light on the dynamics of sleep homeostasis in mice. **Methods:** Male C57BL/6J mice, equipped with EEG/EMG electrodes, were submitted to a 6h sleep deprivation (SD) by gentle handling at four different times of day: between ZT0-6, ZT6-12, ZT12-18, or ZT18-24 (ZT0=Zeitgeber time 0: lights on). One week later, animals were submitted to the exact same SD but with the light being turned off at the beginning of SD until the end of recording. Simulation analyses based on previous findings were used to predict expected levels of delta power. **Results:** We observed that the increase in theta power during SD and the rebound in delta power after SD varied greatly with the timing of the SD and light condition. Importantly, simulations showed that the level of delta power during the first 10 min of recovery sleep could not be adequately predicted for the ZT6 SD. Interestingly, when SD was performed in the dark, the simulations could appropriately predict delta power after the ZT6 SD, which no longer differed from the ZT0 SD. **Conclusion:** Our preliminary analyses suggest that, in mice, the dynamics of sleep homeostasis depend on both time-of-day and acute light exposure. These data will allow for a precise estimation of the modulating effects of light and time-of-day on hallmarks of the sleep homeostat. Research supported by University of Lausanne, FNS (3100A0-111974), and NSERC.

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Evaluation a non-invasive, rapid approach for the assessment of sleep in mice in response to pharmacological and environmental manipulation

SIMON FISHER, SOFIA GODINHO, MARK HANKINS, RUSSELL FOSTER, **STUART PEIRSON**

Nuffield Laboratory of Ophthalmology, University of Oxford, Oxford, UNITED KINGDOM

Sleep is a highly complex behavioural state, and presently the identity and full spectrum of genes underlying its regulation remain poorly defined. The current 'gold standard' method for determining sleep in mammals involves surgical implantation of electroencephalogram (EEG) electrodes; which is largely prohibitive in terms of high-throughput screening. Advances in rapid, non-invasive behavioural assays to evaluate sleep are therefore extremely valuable, particularly to support whole-genome mutagenesis and quantitative trait loci analysis. Here we describe the validation of a method using commercially available video-tracking software to assess sleep in mice based on the automated detection of immobility. Using the Bland-Altman statistical method we demonstrate that digital video analysis gave an extremely high agreement with simultaneous EEG determined sleep. Assessment of sleep using this approach across a 24 hour period when comparing it to EEG-derived sleep gave an estimated bias of just +0.48 minutes (95% confidence interval +3.41, -4.37 minutes). Furthermore we demonstrate the sensitivity of the method in that it was able to distinguish between high (5mg/kg) and low doses of zolpidem (1mg/kg) and conversely detected the wake-promoting effects of caffeine (15mg/kg). Furthermore, it was also shown to detect light-mediated sleep induction in response to a light pulse administered during the habitual wake period. This relatively inexpensive and accessible method represents a powerful primary screening tool for the assessment of sleep in mice as well as simultaneously providing the ability to investigate additional behavioral repertoires associated with changes in sleep/wake behaviour.

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Implication of hypocretinergic system in stress-induced sleep regulation

ADELINE RACHALSKI, CHLOÉ ALEXANDRE, JEAN-FRANÇOIS BERNARD, FRANÇOISE SAURINI, KLAUS-PETER LESCH, MICHEL HAMON, JOELLE ADRIEN, VÉRONIQUE FABRE

*Unité Mixte de Recherche S677, Université Pierre et Marie Curie-Paris 6,
Faculté de Médecine Pierre et Marie Curie, Paris, FRANCE*

Hypocretins (hcrt) hypothalamic neuroexcitatory peptides are known to activate brain structures involved in sleep regulation such as Raphe Nuclei (RN). Conversely, RN serotonergic neurons exert an inhibitory influence on the hypocretinergic neurons. Hcrt and serotonin play key roles in the control of sleep/wake cycle, notably through their negative influence on REM sleep. Interestingly, mutant mice deficient in serotonin transporter (5-HTT^{-/-}) exhibit alterations in homeostatic sleep regulation, with increased REM sleep at baseline compared to wild-type (WT) mice. Our study aimed to specify interaction between serotonergic and hypocretinergic systems and their involvement in sleep regulation after stress. To this goal, we used anatomical and biochemical approaches to assess the activity of hypocretinergic and serotonergic systems under basal condition and after 90 minutes of immobilisation stress in 5-HTT^{-/-} mice and their WT littermates. Moreover, sleep-wake cycles were monitored by polysomnographic recordings in parallel. Finally, the effects on sleep of pharmacological blockade of hcrt receptor 1 immediately before the stress session were analysed. Compared to WT mice, 5-HTT^{-/-} mutants exhibited an enhanced hypocretinergic neurotransmission but reduced activation of serotonergic neurons after immobilisation stress. Polysomnographic recordings showed that stress was followed by a delayed increase in REM sleep in WT mice but not in 5-HTT^{-/-} mutants. Although acute blockade of hcrt-R1 did not significantly affect vigilance states in either mouse strain under control conditions, this treatment restored the stress-induced augmentation of REM sleep in 5-HTT^{-/-} mice. Altogether, the present data show that 5-HT plays a key role in REM sleep homeostasis

after a stress challenge, and that hypocretinergic neurotransmission might underlie the disrupted sleep response to stress observed in mice with genetically-driven loss of the 5-HTT response. Finally, this study emphasizes the role of hypocretin/serotonin interactions in mediating normal sleep response to stress.

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GABA transaminase affects sleep in *Drosophila* and is increased in sleepless mutants

WEN-FENG CHEN¹, MALLORY SOWICK¹, WENYU LUO², AMITA SEHGAL²

¹Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA, USA

²Department of Neuroscience, University of Pennsylvania, Philadelphia, PA, USA

Sleep is an essential daily physiological state that is accompanied by dramatic changes in neuronal excitability and is regulated by numerous neurotransmitters. In *Drosophila melanogaster*, loss of the sleepless (*Sss*) gene product leads to a > 80% reduction in sleep amount. Sleepless encodes a glycosylphosphatidyl inositol-anchored membrane protein that interacts with the potassium ion-channel SHAKER. We used 2D-Differential in Gel Electrophoresis (2D-DIGE) to identify proteins that are altered in the brains of *sss* mutant flies and found that levels of GABA transaminase (GABAT) are enhanced in the mutants. GABAT catalyzes the turnover of GABA, which is a sleep-promoting neurotransmitter in both flies and mammals. Consistent with this role of GABA, *Drosophila* mutant for GABAT show increases in daily sleep amount, mainly in nighttime sleep. The mutants also have consolidated sleep indicated by longer sleep episodes and reduced number of sleep episodes. In addition, the sleep arousal threshold is increased in the mutants. However, locomotor activity of these flies is rhythmic and shows no difference in circadian period length when the flies are tested in freerunning (constant darkness) conditions. These results indicate that the mutation in GABAT largely affects sleep homeostasis rather than circadian clocks. Experiments are under way to determine if GABAT contributes to the sleep phenotype in *sss* mutants.

P35

Variance of feeding time influences body weight, body fat, and gene expressions in mice

YUTA FUSE, MAKIKO OTSUKA, YU TAHARA, AKIKO HIRAO, SHIGENOBU SHIBATA

Physiology and Pharmacology, Waseda University, Tokyo, JAPAN

Overeating of the dinner is generally said to lead to obesity. Recently, it is known that mammalian metabolic function is regulated by the circadian clock genes. In the present study, we examined the relationship between feeding time and body weight, body fat, and gene expressions. And we verify that the eating habits improve or prevent obesity in mice. We made an automatic device that was able to control the feeding time and food volume. Then, we defined the time of breakfast, lunch, dinner, and the midnight meal as ZT12, ZT18, ZT0, and ZT6, and the food volume of each day was limited to 3.6 g (about 70% of free feeding). We measured mice weight every four days during about two months. We used ICR mice at the term of growth and maturity. The only dinner group and the group with large amount of the dinner tended to increase weight in mice at the term of growth. The group with eating only one meal a day tended to increase the amount of visceral fat than free-feeding group. As dinner group mice had less postprandial activity, they could cause the increase of body weight. When food was given at once, all calories could not be consumed, and this feeding protocol may increase the amount of visceral fat. Therefore, abnormal eating habits could lead to obesity and metabolic syndrome.

P36

Desynchronized feeding leads to weight gain in C57BL/6J but not leptin-deficient ob/ob mice

DEANNA M. ARBLE, JOSEPH BASS, MARTHA H. VITATERNA, FRED W. TUREK

Center for Sleep & Circadian Biology, Northwestern University, Evanston, IL, USA

Recent animal studies linking energy regulation with the circadian clock have raised the possibility that the timing of caloric intake may impact weight gain independently of caloric intake and energy expenditure. For example, C57/BL6 (B6) mice fed a high fat diet (HFD) during the light phase gain more weight than those fed during the dark phase. Data from shift-workers, non-breakfast eaters, and Night-Eating Syndrome patients are also consistent with the hypothesis that the timing of feeding may be a factor in weight gain. Notably, the diurnal peak of leptin expression has been associated with meal time suggesting a possible link between behavioral feeding and metabolic processes. The present study sought to determine if leptin expression can affect body weight during a desynchronized feeding (DF) protocol. 6-week old, male leptin deficient ob/ob mice on a 12L:12D cycle were fed a HFD either during the 12L or 12D phase for 8 weeks. During this time, body weight, caloric intake, and locomotor activity were measured. Ob/ob mice were implanted with an osmotic leptin pump at either the start of DF or at week 6. In contrast to B6 mice, ob/ob mice did not gain excess weight when food was restricted to the light phase. Light-fed versus dark-fed ob/ob mice failed to show differences in body weight after 6 weeks ($62.1 \pm 1.4\text{g}$ vs. $61.3 \pm 1.6\text{g}$; $p=0.98$) suggesting a role for leptin in DF-induced weight gain. Interestingly, leptin pumps caused no differences in body weight, suggesting the importance of a leptin expression rhythm in DF-induced weight gain. These findings indicate that synchrony between circadian cues and metabolic processes play an important role in the regulation of energy balance and body weight control. A better understanding of the role of the circadian system for weight gain could have important implications for developing new therapeutic strategies for combating obesity.

P37

Rhythmic expression of clock genes and metabolic genes in rat white adipose tissue

RIANNE VAN DER SPEK, LAURA KERVEZEE, SUSANNE LA FLEUR, ERIC FLIERS, ANDRIES KALSBECK

Endocrinology and Metabolism, Academic Medical Center, Amsterdam, THE NETHERLANDS

Introduction: Recently, the existence of an intricate relationship between deregulation of circadian rhythms and the development of the metabolic syndrome has been suggested. Furthermore it has been shown that up to 20% of the genes expressed in white adipose tissue (WAT) show significant 24-hour rhythms. The mechanisms for circadian regulation of adipose metabolism are unclear at present and were the subject of this study. First, the daily expression patterns of genes involved in circadian- and metabolic pathways in perirenal white adipose tissue (prWAT) in rats were determined. Subsequently, the effects of feeding and autonomic nervous input on these daily gene expression profiles in prWAT will be investigated. **Methods:** 64 male Wistar rats were killed at 8 different time points during the light/dark cycle (12h/12h). Perirenal WAT (prWAT), subcutaneous WAT (sWAT), epididymal WAT (eWAT) and mesenteric WAT (mWAT) were dissected and gene expression analysis was performed by RT-qPCR. **Results:** All circadian genes in prWAT showed distinct rhythmicity (Per2, Cry1, Bmal1, DBP and RevErb α , $p<0.005$), with profiles that are consistent with previous observations in rats and mice. Certain genes involved in metabolic pathways showed significant daily rhythms as well (FAS, ACC1 and Glut4, $p<0.05$), while in others rhythmicity was less pronounced (LPL and HSL, $p=0.1$) or absent (ACC2 and CPT1B, $p=0.246$ and $p=0.910$, resp). Experiments investigating these rhythms in the other fat compartments are ongoing. **Discussion:** At present it is not clear which factors control the observed

rhythmic expression of genes in WAT, although body weight, food intake and circulating hormone levels (i.e. insulin) have been proposed as influencing factors. The above reported experiment will serve as a standard for ongoing and future experiments aimed to dissect the separate effects of rhythmic feeding and the autonomic nervous system on gene expression patterns in adipose tissue.

P38

Nocturnin, a circadian deadenylase, is necessary for normal absorption of dietary lipid

SHIHOKO KOJIMA¹, NICHOLAS DOURIS², XIAOYUE PAN³, ALEXANDRA LERCH-GAGGL⁴, SON Q. DUONG², M. MAHMOOD HUSSAIN³, CARLA GREEN¹

¹Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA

²Biology, University of Virginia, Charlottesville, VA, USA

³Cell Biology and Pediatrics, SUNY Downstate Medical Center, Brooklyn, NY, USA

⁴Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, WI, USA

Normal metabolic function in mammals depends on the circadian clock, which drives temporal regulation of metabolic processes. Nocturnin is a clock-regulated deadenylase that controls mRNA expression post-transcriptionally through poly-A tail removal. Mice lacking Nocturnin (Noc^{-/-} mice) are resistant to diet-induced obesity and hepatic steatosis, yet are not hyperactive nor hypophagic. Here we show that a ketogenic diet or food restriction results in severe weight loss in the Noc^{-/-} mice. Furthermore, the Noc^{-/-} mice have deficits in intestinal processing and secretion of lipids, as seen by the accumulation of large lipid droplets in the apical domains of the enterocytes, increased activity of microsomal triglyceride transfer protein (MTP) and reduced lipid uptake into the circulation. We propose that Nocturnin plays an important role in the trafficking of dietary lipid in the bowel, presumably by post-transcriptionally altering expression of rhythmic genes necessary for lipoprotein formation or secretion.

P39

Pancreatic β -cell clock disruption leads to hypoinsulinemia and diabetes

BILIANA MARCHEVA¹, KATHRYN MOYNIHAN RAMSEY¹, ETHAN BUHR¹, YUMIKO KOBAYASHI¹, HONG SU², LOUIS PHILIPSON³, CHRISTOPHER BRADFIELD⁴, XIAOZHONG WANG², JOSEPH TAKAHASHI⁵, JOSEPH BASS⁶, CAROLINE KO⁵, CHIAKI OMURA^{1,6}, SHELLEY MO⁷, MARTHA H. VITATERNA⁸, JAMES P. LOPEZ³, SETH D. CROSBY⁹, LELLEAN JEBAILEY¹⁰

¹Neurobiology and Physiology, Northwestern University, Evanston, IL, USA

²Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL, USA

³Department of Medicine, University of Chicago, Chicago, IL, USA

⁴McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine and Public Health, Madison, WI, USA

⁵Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA

⁶Department of Medicine, Northwestern University, Evanston, IL, USA

⁷Weinberg College of Arts and Sciences, Northwestern University, Evanston, IL, USA

⁸Center for Sleep and Circadian Biology, Northwestern University, Evanston, IL, USA

⁹Department of Genetics, Washington University School of Medicine, St. Louis, MO, USA

¹⁰GeneGo Inc., St. Joseph, MI, USA

The molecular clock maintains energy constancy by producing circadian oscillations of rate-limiting enzymes involved in tissue metabolism across the day and night. During periods of feeding, pancreatic islets secrete insulin to maintain glucose homeostasis, and while rhythmic control of insulin release is

recognized to be dysregulated in humans with diabetes, it is not known how the circadian clock may affect metabolism at the level of the pancreatic islet. Here we show that pancreatic islets possess self-sustained circadian gene and protein oscillations of main components of the circadian transcription network, such as *Per2* and *Bmal1*, *DBP* and *RevErbA*. The phase of oscillation of islet genes involved in growth, glucose metabolism, and insulin signaling is delayed in circadian mutant mice, revealing a role of the clock in synchronizing pancreatic rhythms. Genetic models of global circadian disruption in both *Clock* and *Bmal1* mutant mice reveal impaired glucose tolerance, reduced insulin secretion, and defects in size and proliferation of pancreatic islets that worsen with age. *Clock* disruption leads to transcriptome-wide defects in the expression of genes involved in islet growth, survival, and synaptic vesicle assembly. Cellular analyses showed normal levels of calcium influx and a refractory response to both cyclase activators and potassium channel agonists, indicating defective β -cell function at the very latest stage of stimulus-secretion coupling. These results demonstrate a primary role for the islet clock in mammalian glucose homeostasis, priming the β -cell to optimize insulin secretion in anticipation of the sleep-wake/ feeding-fasting cycle, and demonstrate how elimination of the clock in pancreatic islets initiates a cascade of cellular failure and organismal pathology, triggering the onset of diabetes mellitus.

P40

Gender and genetic differences in circadian clocks and pharmacology determine distinct chronotoxicity classes for irinotecan

CONSTANCE AHOWESSO¹, XIAO MEI LI¹, FRANCESCO SCOGLIONE², CATHERINE GUETIER³, ALPER OAKYAR¹, SINISA ZAMPERA⁴, SANDRINE DULONG¹, MARCO PIROVANO⁵, FRANCK DELAUNAY⁶, FRANCIS LEVI¹, S. BAREGGI², V. HOSSARD¹

¹ *Biological Rhythms and Cancers, Université de Paris XI, INSERM U776, Villejuif, FRANCE*

² *Department of Pharmacology, University of Milan, Milan, ITALY*

³ *Pathology Department, Paul Brousse Hospital, Villejuif, FRANCE*

⁴ *Helios Biosystems, Université de Paris XII, Creteil, FRANCE*

⁵ *Department of Oncology, Hospital Service, Aprilia, ITALY*

⁶ *University of Nice, Nice, FRANCE*

Background: Gender profoundly modified the circadian pattern of anticancer drug therapeutic index in cancer patients. Purpose: To identify distinct classes of chronotoxicity of topoisomerase I inhibitor irinotecan (CPT-11), according to gender and genotype in mice. CPT-11 is active against colorectal cancer, yet its severe toxicity remains unpredictable through pharmacokinetics (PK) or pharmacogenomics. Methods: Mice (270 male, 270 female) of 3 strains (C57BL/6, B6D2F1 and B6CBAF1) aged 6-8 weeks were synchronized with LD12: 12. They received a therapeutic dose of CPT-11 at ZT 3, 7, 11, 15, 19 or 23. Main toxicity endpoint was body weight loss. Additional studies (132 male, 132 female B6D2F1 or B6CBAF1) investigated hematologic, intestinal toxicities, plasma and colon PK of CPT-11 and its active metabolite SN-38, as a function of CPT-11 dosing ZT. The mRNA expression of pharmacology genes *UGT1A1*, *CES2* and *Top1* and clock genes *Per2*, *Rev-erb α* and *Bmal1* were determined q3h for 24h in liver, ileum and colon mucosae of each group using qPCR Results: Body weight loss, leukopenia, bone marrow hypoplasia and intestinal lesions varied significantly according to ZT, strain, and gender. Optimal circadian timing was ZT 11 for male B6D2F1 and ZT 15 for female B6D2F1 and male or female B6CBAF1. Plasma chronoPK of CPT-11 and SN-38 explained irinotecan chronotoxicity in female B6D2F1 only. The chronoPK of SN-38 in colon explained the chronotoxicity in the other groups. The mRNA expression patterns of pharmacology and clock genes differed significantly as a function of gender and genotype, with tissue specificity. Conclusions: The results validate the hypothesis of different classes of chronotoxicity in mice. Mathematical modelling now designs class-tailored circadian delivery schedules for the personalization of cancer chronotherapeutics.

Effect of a cell cycle inhibitor seliciclib on circadian timing system

ELISABETH FILIPSKI, JACQUES BEAU, FRANCIS LÉVI
U776, INSERM, Villejuif, FRANCE

Background: Seliciclib, a CDK and CSNK1 δ/ϵ inhibitor with anticancer properties suppressed the molecular clock in liver and induced that in tumor pending upon dosing time. Purpose: To assess the effects of seliciclib on the central pacemaker as a function of gender and genetic background in order to guide optimal circadian scheduling of this agent. Methods: Male and female B6D2F1 and B6CBAF1 mice had telemetered temperature and activity monitoring. After a week in constant darkness they received a single equitoxic dose of seliciclib: 600 mg/kg (B6D2F1) or 900 mg/kg (B6CBAF1) at one of 6 circadian times: CT3, 7, 11, 15, 19 or 23. Spectral analyses defined endogenous period τ (h) and acrophase [radians (rd)] of thermic rhythm in each mouse during the week before treatment and during the week starting 2 days after treatment. Results: Acute hypothermia in 25% of the mice depended on dosing-time, strain and sex. It was severe at ZT19 and absent at ZT3 in B6D2F1, low in ♀B6D2F1 and intermediate in B6CBAF1. Seliciclib lengthened temperature τ by 0.35 h in B6D2F1 and shortened it by 0.08 h in B6D2F1 (strain*gender, $p=0.002$). Mean phase delay was greater in B6D2F1 (1.2 ± 0.1 rd) than in B6CBAF1 (0.5 ± 0.07 rd) (strain*gender, $p = 0.001$) and least at CT3 ($p = 0.05$). Conclusions: Seliciclib modifies the endogenous period and/or the phase of circadian coordination. This could result from its proven direct inhibition of CSNK1 δ/ϵ or interactions with other enzymatic targets under study. The circadian timing system constitutes a strain- and gender-dependent toxicity target for seliciclib. Supported by the EU (STREPTEMPO, LSHG-CT-2006-037543).

Expression of clock genes *Per1*, *Bmal*, *Clock*, *Cry1*, *Cry2* and *Rev-erb α* in Leydig tumor cells

ADAM NEUMANN, JOANNA KOTWICA, LUKASZ SZEPIOLA, PIOTR BEBAS
Department of Animal Physiology, University of Warsaw, Warsaw, POLAND

The mammalian molecular circadian clock consists of a complex of transcriptional–translational feedback loops that generate rhythmic, approximate 24 hour expression patterns of core clock genes. BMAL:CLOCK heterodimer and nuclear orphan receptor REV-ERB α play major role in positive feedback loop. In turn *Per1*, *Cry1* and *Cry2* proteins are essential components of negative feedback loop of clock mechanism. Recent findings revealed that pathways critical to cell division are linked with circadian system. In addition core clock factors not only participate in transcription regulation of cell cycle related genes, but also can interact directly with the cell cycle pathway. Our aim was to characterize gene expression pattern in mice Leydig tumor cell lines: MLTC-1, I-10 compared to normal mouse Leydig cells TM3 and immortalized mouse fibroblasts NIH/3T3. Real Time RT-PCR (q RT-PCR) was assessed to determine *Per1*, *Bmal*, *Clock*, *Cry1*, *Cry2* and *Rev-erb α* mRNA levels. Study results show different expression patterns between cells lines and support hypothesis that core clock genes expression is strongly associated with testicular cancer development. Supported by MNiSzW grant no. NN303342235 to PB.

Intercellular coupling governs entrainment

**ADRIAN GRANADA¹, MARKUS HEINE², UTE ABRAHAM², PÅL WESTERMARK¹, ACHIM KRAMER²,
HANSPETER HERZEL¹**

¹*Biology, Institute for Theoretical Biology, Humboldt University Berlin, Berlin, GERMANY*

²*Institut für Medizinische Immunologie, Laboratory of Chronobiology,
Charité Universitätsmedizin Berlin, Berlin, GERMANY*

The mammalian circadian timing system is a web of interacting oscillators. Accumulating evidence suggests a hierarchical network organization where the SCN acts as a central pacemaker that orchestrates the pace for the peripheral clocks. Very little is known about systemic differences between the SCN and the peripheral clocks. Recent studies showed that at the single cell level the SCN and peripheral tissues are composed of strikingly similar cellular clocks (Liu et al., 2007, Westermarck et al., 2009). By means of entrainment range studies we identify basic mechanisms leading to central and peripheral clock differences. Here we study the entrainment of SCN and lung tissues in vitro under challenging entrainment conditions: 20 h cold-warm temperature cycles of 35.5-37°C. By following their bioluminescent reporter PER2::LUC expression we show that lung tissue can be entrained whereas SCN tissue is not entrained to the 20 h temperature cycles. Using simulations of generic amplitude-phase oscillators we show that intrinsic period, amplitude and amplitude relaxation rate determine the range of entrainment. We find that coupling between individual cells can enhance amplitude via resonance-like phenomena and that it changes amplitude relaxation rate. Our theoretical considerations explain how coupling indirectly controls the entrainment range and why the virtually uncoupled cells in lung tissue can be entrained whereas SCN tissue is not entrained. We test our predictions by reducing the cell-to-cell coupling of SCN neurons via TTX- and MDL -treatment. Indeed, we observed that compromising coupling in these ways allows entrainment of SCN slices. Thus intercellular coupling plays a major role in determining SCN and peripheral clock entrainment properties.

Effect of E-boxes on central and peripheral circadian clocks in mice

AKIKO HIDA¹, SHIN YAMAZAKI², YOSHIYUKI SAKAKI³, CARL H. JOHNSON, HAJIME TEI^{3,4}

¹*Department of Psychophysiology, National Institute of Mental Health, Tokyo, JAPAN*

²*Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA*

³*HGC, Institute of Medical Sciences, University of Tokyo, Tokyo, JAPAN*

⁴*Graduate School of Natural Science & Technology, Kanazawa University, Kanazawa, JAPAN*

—WITHDRAWN—

Circadian rhythms of PER2 in mouse hepatocytes

MARTHA LUITJE¹, CASEY GUENTHNER², PENNY MOLYNEUX¹, MARY HARRINGTON¹

¹Neuroscience, Smith College, Northampton, MA, USA

²Neuroscience, Stanford University, Palo Alto, CA, USA

Isolated suprachiasmatic nucleus (SCN) cells are capable of sustaining molecular and electrical rhythms in vitro, and intercellular communication is necessary to generate a coordinated, stable output from the SCN. Consistent with this model, SCN explants maintain rhythms for up to several weeks in vitro, while treatments that disrupt intercellular communication cause the whole-explant rhythms to damp as single-cell oscillators become desynchronized. Whether rhythms in peripheral cell types are also synchronized within a tissue remains controversial. To begin to address this question, we measured luminescence rhythms in explants of various tissues from PER2::LUCIFERASE knock-in mice. Consistent with previous reports, we observed that rhythms of SCN and liver explants damped at similar rates, while esophagus, spleen, lung and thymus damped more quickly; rhythmicity in all tissues could be reinstated by a media change. The ability of liver explants to maintain rhythms as stable as those produced by the SCN suggests that there may be a mechanism for synchronizing liver clock cells in the absence of systemic cues. To further address this possibility, we cultured primary mouse hepatocytes in the collagen gel sandwich (CGS) configuration, which has been reported to maintain cellular health and hepatocyte-specific function for several weeks. Rhythms in PER2::LUC bioluminescence from CGS hepatocyte cultures were maintained for up to 54 days, with four media changes, and were dependent on seeding density. Rhythms damped at rates intermediate between SCN and most [non-liver] peripheral tissue explants. Hepatocytes plated on a single layer of collagen did not maintain rhythms for more than a few days, consistent with previous reports. These results suggest that individual liver clock cells could be functionally coupled in vitro if culture conditions are optimized.

Quantitative analysis of the mouse liver circadian proteome

CHARO ROBLES, JUERGEN COX, MATTHIAS MANN

Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, Munich, GERMANY

Circadian clocks are endogenous and cell-autonomous oscillators that regulate rhythmicity in physiology and behavior. Metabolism is temporally coordinated in peripheral tissues by the local circadian clocks. Numerous studies have demonstrated daily transcriptional oscillations of approximately 5 to 10% of the genome in mouse organs which reflects the local coordination of physiology. However, very little is known about the global temporal behavior of the proteome in those tissues and whether oscillations of transcription translate into protein rhythmicity. By using recent advances in proteomics technology, in particular high-resolution mass spectrometry (MS) and stable isotope labeling by amino acids in cell culture (SILAC) in mice we are performing a global and quantitative analysis of the mouse liver circadian proteome. The study performed with mouse liver samples collected over two consecutive days allows to identify more than 4000 proteins and to quantify 3000 proteins. Frequency analysis of the quantified proteome shows a predominant and strong frequency corresponding to the circadian period in mice. Statistical analysis reveals that protein abundance in mouse liver oscillates daily in similar extent to the transcriptome previously described. Interestingly, we found circadian rhythmicity in a number of proteins with non-oscillating transcript which suggests defined posttranscriptional circadian regulation.

Zebrafish retinal circadian rhythms of *per3::luc* expression in vivo and in vitro

ZIYI SUN, BIN ZHAO, SHI MENG, DOUGLAS McMAHON

Biological Sciences, Vanderbilt University, Nashville, TN, USA

The zebrafish (*Danio rerio*), has gained increased interest in the study of circadian mechanisms due to its experimental advantages in genetic analysis. Most peripheral tissues in zebrafish are clock-containing and light-responsive, and can be entrained by light directly. To better understand the circadian pace-making and light entrainment machinery in the zebrafish, we examined circadian rhythms of *per3::luc* expression in cultured zebrafish retina and in whole-zebrafish eye. In this work, we first examined circadian rhythms of cultured zebrafish retina by adapting the mammalian retinal culture technique we recently reported. The luminescence rhythm from cultured retinas persisted in constant darkness for at least 10 days and up to 24 days with medium change. The rhythmic period for cultured zebrafish retinas was 27.54 ± 0.49 hour (mean \pm SD, $n=30$) for the first two cycles. Then, a phase response curve was constructed to study the light entrainment properties of cultured zebrafish retinas. 15-min light pulses (~ 500 lux) induced a maximum phase delay (~ 6 hour) at CT 16 and a maximum phase advance (~ 6 hour) at CT 19. Next, the retinal circadian rhythms were further assessed in whole-zebrafish eye in vivo. To obtain luminescent signals from the in vivo retina, an ocular microinjection technique was developed and luciferin was introduced into the vitreous space of the eye of living larval zebrafish. The luminescence rhythm from whole-zebrafish eye persisted in constant darkness for up to 7 days with a period of 24.61 ± 0.57 hour (mean \pm SD, $n=10$) for the first two cycles. In conclusion, our data demonstrate that circadian rhythms of zebrafish retina can be obtained from both in vitro and in vivo conditions. The distinct circadian properties of the retina between the cultured explant and the whole-zebrafish eye may suggest a potential interplay between the retina and other organs in vivo.

A role for VIP in the circadian regulation of the female endocrine system?

DAWN H. LOH, TAKASHI KUDO, TAKAHIRO J. NAKAMURA, SEI TATEYAMA, CHRISTOPHER S. COLWELL

IDDR, University of California–Los Angeles, Los Angeles, CA, USA

The neuropeptide vasoactive intestinal peptide (VIP) is essential for the normal functioning of a myriad of circadian-regulated processes. In male VIP-deficient mice, rhythms in glucocorticoid hormones are abolished, and the mice also display reduced testosterone and premature degeneration of seminiferous tubules, but no overt reduction in the luteinizing hormone. Intriguingly, grass rats show sex differences in the diurnal rhythm of SCN expression of *Vip* and its receptor *Vipr2* suggesting that there might also be a sex-difference in the phenotype of VIP-deficient mice. Further evidence of the involvement of VIP in circadian-regulated endocrine function in females comes from VIP antagonism studies, which find anomalies in the oestrous cycle and a reduced pro-oestrous surge of the LH, similar to the phenotype of aging female rats. We examined the effects of the loss of VIP in female mice, and found evidence of dysfunctional circadian activity, much like their male counterparts, and irregularities in oestrous cyclicity, thus making this a potential model for premature aging of the female reproductive system.

Altered entrainment to the day/night cycle attenuates the diurnal rhythm of corticosterone secretion

PATRICIA SOLLARS¹, MICHAEL WEISER², ANDREA KUDWA³, JAYNE BRAMLEY¹, MALCOLM OGILVIE¹,
ROBERT SPENCER², ROBERT HANDA⁴, GARY PICKARD¹

¹*School of Veterinary Medicine and Biomedical Sciences, University of Nebraska, Lincoln, NE, USA*

²*Psychology and Neuroscience, University of Colorado, Boulder, CO, USA*

³*Biomedical Sciences, Colorado State University, Fort Collins, CO, USA*

⁴*Basic Medical Sciences, University of Arizona, Phoenix, AZ, USA*

The suprachiasmatic nucleus (SCN) is entrained to the external environment via an input from the retina, providing stable phasing of behavioral and endocrine rhythms to the 24 h day/night cycle. Activation of presynaptic serotonin 1B (5-HT_{1B}) receptors on retinal terminals in the SCN modulates the response of the SCN to light. The response of 5-HT_{1B} receptor knockout (KO) mice to light is diminished resulting in entrainment to 23 h light:dark (L:D) cycles with activity onsets significantly delayed compared to wild type (WT) animals. Circulating corticosterone levels exhibit a robust SCN-driven daily rhythm peaking shortly before activity onset; abnormalities in rhythmic corticosterone secretion alter central nervous system and peripheral organ function. We tested the hypothesis that altered entrainment of the circadian activity rhythm to the day/night cycle affects rhythmic corticosterone secretion. Wheel-running activity and plasma corticosterone levels were monitored in WT and KO mice housed under several different lighting regimens and constant dark conditions. Mice that entrained to a 9.5L:13.5D cycle with activity onsets delayed more than 4 h after light offset exhibited a highly dampened corticosterone rhythm with peak levels reduced 50% compared to animals entrained with activity onsets less than 4 h delayed. Similar results were obtained with WT mice entrained to 8L:14D; animals with activity onsets phase delayed > 4 h had reduced peak corticosterone levels. Altered entrainment of the circadian system to the day/night cycle, manifest as large delays in activity onset relative to light offset, severely reduces the peak of the diurnal rhythm of plasma corticosterone. Corticosterone is a potent transcriptional regulator and the daily rise in circulating corticosterone levels affects rhythmic gene expression in the brain and clock gene expression in major peripheral organs. A pronounced reduction in the daily glucocorticoid rhythm could alter rhythmic gene expression contributing to metabolic pathophysiologies and/or mood disorders.

Lack of the circadian clock gene, *bmal1*, is associated with development of dilated cardiomyopathy in mice

MELLANI LEFTA, KARYN ESSER

Physiology, University of Kentucky, Lexington, KY, USA

Studies with shift workers and patients with sleep disorders have demonstrated that disruption of circadian rhythms is correlated with an increased incidence of cardiovascular pathologies. The *bmal1* knockout (*Bmal1*^{-/-}) mice are behaviorally arrhythmic, lack the diurnal variation in blood pressure and heart rate and have a shorter lifespan. Our lab has made the observation that *Bmal1*^{-/-} mice develop signs of dilated cardiomyopathy at 8 weeks of age. This is characterized by decreased ejection fraction and fractional shortening and increased left ventricular internal diameter. At the structural level, electron microscopy shows sarcomere disorganization within cardiomyocytes. We hypothesized that loss of the circadian clock gene, *bmal1*, leads to isoform shifts in the sarcomeric proteins titin and myosin heavy chain, which in turn alter cardiomyocyte mechanical function and lead to dilated cardiomyopathy. Titin isoform composition was assessed using a vertical agarose gel electrophoresis (VAGE) system. We found a down-regulation of the compliant N2BA and up-regulation of the stiff N2B

titin isoforms in the left ventricle of Bmal1^{-/-} mice compared to age matched controls, suggesting that titin-based stiffness is increased in the Bmal1^{-/-} cardiomyocytes. Myosin heavy chain isoform composition was assessed by SDS-PAGE. We did not find a difference in left ventricular myosin heavy chain protein content or isoform composition between wildtype and Bmal1^{-/-} mice. In conclusion, our data indicate that germline Bmal1^{-/-} mice develop functional and structural signs of dilated cardiomyopathy, associated with changes in titin isoform composition. Ongoing studies are directed at determining if there are additional biochemical and functional indices of cardiomyopathy at the cellular level of the Bmal1^{-/-} hearts. Future studies will determine if heart specific loss of Bmal1 leads to cardiomyopathy or whether this complex disease is due to multisystem dysfunction.

P51

Can timed exercise rescue diurnal and circadian rhythms of HR and clock gene expression in VIP-deficient mice?

ANALYNE M. SCHROEDER, DAWN H. LOH, CHRISTOPHER S. COLWELL

Psychiatry and Biobehavioral Sciences, University of California–Los Angeles, CA, USA

WT mice display robust rhythms in Heart Rate (HR) and clock gene expression in cardiac tissue under LD and DD conditions. In contrast, VIP-deficient mice exhibit abnormal HR rhythms in the LD cycle whereby the onset of HR is advanced by 4-6h. In parallel, the rhythms of clock genes Per2 and Bmal1 also show a phase advance in peak expression in the mutant heart. Under DD conditions, the VIP-deficient mice show a loss in the HR rhythm as well as disrupted clock gene expression. Importantly, we found that the presence of a running wheel is able to strengthen diurnal and circadian rhythms of HR in both WT and VIP-deficient mice. With the wheel, the mutant mice exhibited robust daily rhythms that increased at dusk. Even under DD, we continue to detect a robust rhythm of HR in VIP-deficient mice though one with a bimodal pattern and short free-running period. The presence of the wheel also rescues the rhythms in Per2 in cardiac tissue while the rhythm of Bmal1 expression continues to be abnormal. These data suggest that VIP is essential for normal rhythms in HR and clock gene expression in cardiac tissue. The addition of the running wheel counters some, but not all, of the deficits observed in VIP-deficient mice. We are interested in exploring the mechanisms by which the presence of the wheel and the resulting increase in activity, rescues rhythmicity in VIP-deficient mice. It will also be important to determine the extent to which this type of timed exercise may be able to counter the ill effects of a disrupted circadian system.

P52

Cell-type specific AKT signaling is regulated by circadian clock component BMAL1 in vascular cells

MAOXIANG ZHANG¹, DAVID FULTON², R. DANIEL RUDIC¹

¹*Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA, USA*

²*Vascular Biology Center, Medical College of Georgia, Augusta, GA, USA*

Akt signaling pathway is critical for vascular development and function in normal physiology and disease. Recent data have demonstrated that circadian clock mutant mice exhibit vascular dysfunction and impaired Akt signaling. Thus, we undertook studies to more precisely study the interaction between the circadian clock and Akt signaling. In murine aortic vascular endothelial cells (MAEC) isolated from Bmal1-KO mice, we found that phosphorylation of Akt1 at Serine 473 was decreased relative to wild type cells. In contrast, in vascular smooth muscle cells (MASMC) isolated from Bmal1-KO, the phosphorylated form of Akt1 at the 308 residue was attenuated, which was also associated with concomitant downregulation of PDK1 expression. Total expression of Akt2, most appreciated for its role in malignancies and metabolism, was decreased in Bmal1-KO MAECs, but in contrast increased in

MASMCs of the Bmal1-KO mice. Given the known role of Akt in cell viability and migration, transwell and MTT assays were conducted in subconfluent isolated vascular cells every 24 hours for 72 hours. In Bmal1-KO MAECs, cell number and migration was decreased, while in MASMCs from the Bmal1-KO mice, both migration and cell count were increased. Thus, Bmal1 inactivation induced strikingly different effects on the regulation of phosphorylated Akt and Akt isoforms in vascular endothelial cells and smooth muscle cells. These data suggest that there is a complex interplay between the circadian clock component Bmal1 and Akt in vascular cells which may in part underlie the vascular defects that occur in conditions of circadian dysfunction.

P53

Deciphering the pineal gland and immune-system crosstalk in chicken (Gallus gallus)

SAILAJA KALLUR, MICHAEL J. BAILEY

Department of Poultry Science, Texas A&M University, College Station, TX, USA

The pineal gland of chicken (*Gallus gallus*) has a large population of lymphocytes which account to upto 30% of the volume of the gland. These lymphocytes may have an important role in circadian neuro-immune interactions. Not much is known regarding the lymphocyte and pineal gland interactions, including the effects on circadian rhythms in both the immune system and the pineal gland. In this study, we examined the daily and circadian control of immunological processes in the Pineal gland. Our results indicate that circadian clock genes as well as proinflammatory cytokines including IL-6 and IL-18 exhibit rhythmic oscillations of mRNA abundance and are under control of the circadian clock. We also find that acute melatonin administration at midday induces some of the circadian clock and proinflammatory cytokines gene expression. LPS-induced systemic inflammation applied at midday versus midnight results in a differential immune response of proinflammatory cytokine induction indicating regulation by the circadian clock. Exogenous melatonin administration at midday prior to LPS stimulation enhanced TNF α , IL-6 and IL-18 induction indicating melatonin's function as a pro-inflammatory molecule in the pineal gland. These data suggest that the rhythmic properties including the differential immune response to inflammation in the chicken pineal gland are mediated by the circadian clock and melatonin hormone via localized regulation of clock gene expression.

P54

Influence of time of day on IL-6 and IL-1 β mRNA expression in LPS-stimulated equine blood

OLGA McGLYNN, JOHN BROWNE, CATRIONA BLAKE, BARBARA MURPHY

Veterinary Sciences Centre, University College Dublin, Dublin, IRELAND

The immune system demonstrates regularly recurring rhythmic variation, which has important clinical consequences and implications for medical practice. In peripheral blood, circadian rhythmicity is manifested in terms of clock gene expression, circulating levels and function of different leukocyte cell populations, cytokine production and natural killer cell function. Cytokines play a critical role in the induction and regulation of immune effector functions. The direction of an immune response may depend on the cytokine environment at time of antigen presentation. Equine viral infections are responsible for disease epidemics around the world, resulting in major economic loss to the equine industry. The currently available vaccines are poorly efficacious with immunised animals often exhibiting disease symptoms. This study aims to determine the optimum time of day for cytokine response to antigen exposure. Numerous studies have revealed that interleukin (IL) -6 is involved in the transition from innate to acquired immunity. Furthermore IL-1 β plays an important role in the innate immune response. We housed 4 healthy Thoroughbred fillies under a light/dark (LD) cycle that mimicked the natural external photoperiod (LD) and collected blood samples at 4-h intervals. 200 μ L of heparinised

blood was aliquotted into 24 well plates at each time point and stimulated with lipolysaccharide (LPS) (1µg/mL) or left un-stimulated. Plates were incubated at 37°C with 5% CO₂ for 6 h prior to harvesting by addition of PAXgene™ RNA stabilizer solution (Qiagen, Valencia, CA, USA), to immediately stabilize transcription and preserve the RNA profile. Two-way ANOVA of qPCR expression assays (SYBR® Green) reveal a significant Time X Treatment interaction ($p < .01$) and effect of LPS ($p < .0001$) on IL-6 expression. One-way ANOVA revealed significant time-of day variation on IL-1β levels ($p < .01$) in stimulated whole blood. Analysis of further cytokines is underway. These results confirm the importance of immune-circadian interaction in relation to the innate immune response.

P55

Effects of photoperiod-driven variations in life-history on longevity and aging in the Siberian hamster, Phodopus sungorus

EVAN RAIEWSKI, MICHAEL GORMAN

Psychology, University of California–San Diego, La Jolla, CA, USA

The annual cycle of changing day lengths provides predictive information to animals about energetic and biotic conditions relevant to survival and reproduction. Field and laboratory studies demonstrate marked effects on photoperiod on critical early life history traits such as the timing of sexual maturity, growth rates etc. In the Siberian hamster transitions between winter and summer phenotypes result in profoundly different physiological states—including reproductive status, body temperature, and body weight—that have been independently proposed to alter the course of aging. Although aging and longevity are known to be influenced by photoperiod in a prosimian primate, there are no comparable studies on somatic aging and longevity of rodents. Survival curves of Siberian hamsters were obtained to determine effects of photic history on lifespan, specifically, immediate versus delayed sexual maturity, and static versus simulated naturalistic photoperiods (SNPs). Additionally, longevity was assessed in hamsters under artificial, bimodal light:dark:light:dark (LDLD) cycles. Hamsters were maintained in one of six photoperiods: 1) LD8:16, 2) LD16:8, 3) SNP beginning in spring 4) SNP beginning in fall, 5) LDLD8:4:8:4, and 6) LD8:16, alternating with LD16:8 every 6 months. Preliminary results demonstrate that LDLD8:4:8:4 and static LD to have highest mortality while LD8:16 maintained lowest mortality.

P56

Seasonal variation in equine pituitary dopamine concentrations

MELISSA CORDERO¹, ALEXANDAR CHRISTOV², KENNETH HENSLEY², SALLY VIVRETTE³, DIANNE MCFARLANE¹

¹Physiological Sciences, Oklahoma State University, Center for Veterinary Health Sciences, Stillwater, OK, USA

²Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA

³Triangle Equine Mobile Veterinary Services, Cary, NC, USA

The equine pituitary pars intermedia (PI) has a seasonal pattern of hormone secretion with α-MSH reaching higher plasma concentrations in the fall. Dopamine is known to inhibit PI hormone secretion, but the mechanisms that regulate the seasonal variation in α-MSH secretion are unknown. We hypothesized that the seasonal pattern of PI hormone secretion is regulated by changes in dopaminergic output. Therefore, we expected increased concentrations of dopamine and its receptor (D2R) in pituitary and pituitary effluent samples collected in the fall, when PI output is most inhibited. To test this hypothesis, dopamine concentrations were determined by HPLC-EC in pituitary venous effluent (PitVen) and peripheral plasma collected in spring and fall from healthy horses. Dopamine concentration and D2R expression (real-time PCR) were determined in fresh frozen pituitary PI-pars nervosa tissue. Seasonal effect was assessed by t-test. PitVen and plasma dopamine concentrations were increased in the fall compared to non-fall (Paired t test, n=5. PitVen: 20.4 ± 4.7 µM dopamine/µM L-tyrosine vs. 7.5 ± 2.4

μM dopamine/ μM L-tyrosine, $P=0.05$; Plasma: $28.3 \pm 3.9 \mu\text{M}$ dopamine/ μM L-tyrosine vs. $4.1 \pm 1.7 \mu\text{M}$ dopamine/ μM L-tyrosine, $P<0.001$). Dopamine concentration and D2 receptor expression was greatest in pituitary tissue collected in the fall compared to the non-fall months (Dopamine: $278.6 \pm 49.88 \text{ nmol/mg}$ tissue, $n=5$ vs. $155.1 \pm 40.34 \text{ nmol/mg}$ tissue, $n=7$, $P=0.08$; D2R PCR relative expression: 0.34 ± 0.1 , $n=7$ vs. 0.04 ± 0.01 , $n=7$, $P=0.02$). Unexpectedly, pituitary dopamine concentrations were found to be higher in all samples collected in the fall, at the time when PI α -MSH secretion is greatest. Based on these findings, we conclude that changes in dopamine output are not regulating the seasonal changes in PI hormone secretion in the horse. Further work is needed to determine the mechanisms responsible for the seasonal modulation of PI hormone secretion.

P57

Circadian control of migratory restlessness and the effects of food and temperature in the blackheaded bunting, *Emberiza melanocephala*

JYOTI SINGH¹, ASHUTOSH RASTOGI¹, SANGEETA RANI², VINOD KUMAR¹

¹Zoology, University of Delhi, Delhi, INDIA

²Zoology, University of Lucknow, Lucknow, INDIA

Many diurnal songbirds undergo long distance nighttime migration during the autumn (breeding to wintering grounds) and spring (wintering to breeding grounds), which under captivity is reflected as the intense nighttime activity (Zugunruhe) and used as the marker of migration related laboratory investigations. This study addressed on the regulation Zugunruhe during spring migration in the Palaearctic—Indian migrant species, the blackheaded bunting (*Emberiza melanocephala*), which is less understood as yet. In particular, we investigated whether (i) the circadian clock regulated the development of Zugunruhe, and (ii) food and temperature influenced the photoperiodic induction of Zugunruhe. Buntings entrained to short day lengths (8 h light : 16 h darkness; 8L:16D) were first exposed to long day lengths for varying durations and in released into constant dim light (LLdim) in few experiments to examine the circadian rhythmicity. During the middle of day and night under long days, we measured immunohisto-chemically the expression of the c-fos and neuropeptideY in different regions of brain. The results suggested that the circadian clock regulated and redefined itself with the onset of the Zugunruhe. The period of the circadian rhythm was altered during the photosensitive and photorefractory phases of the annual cycle. However, the nighttime plasma melatonin profile at two equinox times was similar. Further, the food cycles synchronized the circadian rhythm of Zugunruhe, and the temperature influenced the photoperiodic induction of Zugunruhe. We conclude that a circadian clock regulates seasonal migrations in the blackheaded bunting, and redefines itself with the onset of migrations.

P58

Reduced foraging yield causes diurnality in mice

VIOLETTA PILORZ, ATE S. BOEREMA, ARJEN M. STRIJKSTRA, SERGE DAAN, ROELOF A. HUT

Chronobiology, University of Groningen, Groningen, THE NETHERLANDS

Nocturnal rodents show diurnal food anticipatory activity when food access is restricted to a few hours at daytime. Timed food access also results in reduced food intake, but the role of energy intake in circadian organization per se has not been described. By simulating natural food shortage in mice that work for food we show that reduced energy intake alone shifts activity from the night into the day and causes nocturnal hypothermia. Release into continuous darkness with ad libitum food, elicits immediate reversal to nocturnal activity, indicating that the classical circadian pacemaker maintained

its phase to the LD cycle. This flexibility in behavioral timing allows mice to exploit diurnal temporal niches and minimize energy expenditure under poor feeding conditions in nature. The study reveals an intimate link between metabolism and circadian organization and offers a novel, naturalistic approach to unravel the alternative circadian oscillator that controls activity and rest.

P59

Diurnal and nocturnal preference in a physiologically based model of mammalian sleep

A.J.K. PHILLIPS¹, B.D. FULCHER², P.A. ROBINSON^{3,4,5}, E.B. KLERMAN¹

¹ *Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston MA, USA*

² *Department of Physics, Oxford University, Oxford, UNITED KINGDOM*

³ *School of Physics, University of Sydney, AUSTRALIA*

⁴ *Brain Dynamics Center, Westmead Millennium Institute, Sydney Medical School – Western, University of Sydney, AUSTRALIA*

⁵ *Center for Integrated Research and Understanding of Sleep, AUSTRALIA*

The physiological basis for interspecies differences in diurnal vs. nocturnal preference remains an open problem. Experimental evidence suggests circadian physiology is fundamentally similar in nocturnal and diurnal animals, including the phase response curve to light, and the timing of neuronal firing in the suprachiasmatic nucleus (SCN). These findings imply that temporal niche is determined downstream of the SCN, most likely by how the signal is relayed to the hypothalamic sleep nuclei. We test this hypothesis in a physiologically-based model of mammalian sleep. The model includes mutually inhibitory elements of the sleep-wake switch: wake-promoting monoaminergic nuclei (MA), and the GABAergic sleep-promoting ventrolateral preoptic area (VLPO). Cholinergic and orexinergic drives to MA are modeled, while the VLPO receives homeostatic and circadian drives, using the Jewett-Kronauer model for the latter. SCN output projects to the sleep-wake switch via two pathways: (i) To the VLPO via the dorsomedial hypothalamus (DMH); (ii) To the MA via orexin. We find that changing the first pathway from net inhibitory to net excitatory results in a switch from diurnality to nocturnality. This provides a simple explanation for temporal niche plasticity in mammals such as the Nile grass rat. Additionally, this change results in an inversion of the relation between free-running period and light intensity under constant light conditions, consistent with the basic form of Aschoff's Law. Decreasing the strength of the signal conveyed by the orexinergic pathway is found to increase arousal levels at the beginning and end of the wake period as compared to those near the middle of the wake period. This pathway may therefore aid in the generation of crepuscular rhythms. Finally, we simulate SCN ablation in rats, reproducing the experimentally observed ultradian sleep pattern. These findings underline the evolutionary flexibility conferred by the circadian relay system.

P60

Continuous alcohol exposure impairs circadian adaptation to simulated time zone travel in C57BL/6J mice

S.A. SINNING, M.R. GORMAN

Psychology, University of California–San Diego, La Jolla, CA, USA

Alcohol consumption perturbs many circadian regulated systems including sleep/wake cycles, body temperature and hormone secretion. The central clock pacemaker itself may be directly affected by ethanol as both acute and chronic exposure interferes with phase-resetting in response to brief light pulses (Ruby et al., 2009; Seggio et al., 2009). Because brief light pulses produce limited phase responses, particularly for advancing pulses, the following study examined the effects of continuous alcohol vapor exposure on light induced resetting using a jet lag paradigm. Male C57BL/6J mice

were fully adapted to a 12:12 light/dark cycle. In a repeated measures, counterbalanced design, alcohol vapor inhalation (or room air as a control) commenced six hours before lights off on day 1 and ran continuously for 96 hours. Beginning on the second day of vapor exposure, the light cycle was advanced or delayed by six hours in separate experiments. At the start of lights off on the third day, animals entered constant darkness for ten days to assess the amount of photic phase resetting that had occurred. Mice were then retested in the opposite experimental condition after 14 days of re-entrainment. Phase shifts were calculated from rhythms in body temperature and locomotor activity measured telemetrically throughout the experiment. Mice receiving alcohol exposure phase shifted significantly less in the advanced light/dark cycle, but no difference was found following delays. Continuous alcohol exposure therefore interfered with synchronization to an advanced but not a delayed light/dark cycle. As phase delays are accomplished more rapidly than advances, the null result in that condition may reflect a ceiling effect.

P61

Photoperiod and jet lag: Influence of pineal melatonin onset timing on reentrainment rate of rats

L. SAMANTHA ZHANG¹, TIECHENG LIU², JIMO BORJIGIN²

¹Neuroscience Program, University of Michigan, Ann Arbor, MI, USA

²Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA

Using pineal microdialysis, we define entrainment and jet lag through the precise rhythm of nightly melatonin production in rats. Our previous work based on inter-individual differences in melatonin production suggests that there is a positive relationship between melatonin onset timing, melatonin duration, and the speed of reentrainment. In response to a delay in LD cycle, rats with earlier melatonin onset, and thus longer melatonin duration entrained faster. To further examine the factors involved in reentrainment, inbred Fisher rats with little inter-individual variation were entrained to three different photoperiods (LD16:8, LD12:12, LD8:16). Interestingly, at LD16:8, a stable daily melatonin profile could not be established, suggesting that Fisher rats were not able to completely entrain to such long photoperiods. Rats entrained to a longer photoperiod (shorter night) had significantly earlier onset along with decreased duration of melatonin production. Following a 2 h delay, we find that the melatonin profiles of rats entrained to a longer photoperiod reentrained faster to the new schedule. These results suggest that there is indeed a correlation between melatonin onset timing and speed of reentrainment. Melatonin duration on the other hand does not seem to be a factor in reentrainment rate.

P62

Constant light disrupts circadian rhythms and suppresses melatonin, without reducing cell proliferation and cell survival in the hippocampus of adult rats

ANKA MUELLER, RHIANNON MEAR, RALPH MISTLBERGER

Psychology, Simon Fraser University, Burnaby, British Columbia, CANADA

Although many hippocampal functions and properties are under circadian control, the level of influence of the circadian system on hippocampal adult neurogenesis remains to be established. Previous studies have consistently shown that REM-sleep deprivation (RSD) in combination with attenuated circadian rhythms of behavior result in diminished levels of cell proliferation in the dentate gyrus (DG) of adult rats (Mueller et al. 2008, Guzman-Marin et al. 2007). In order to differentiate the effects of RSD from circadian disorganization on hippocampal cell proliferation, we abolished circadian rhythms of behavior by subjecting male Sprague Dawley rats to constant bright light (LL) for either 4-days or 10-weeks. With a single IP injection of 5-bromo-2-deoxyuridine (BrdU, 200mg/kg) we labeled newly proliferating

cells in the granular cell layer (GCL) of the dentate gyrus (DG). The animals were sacrificed 2h later and brains were processed for BrdU immuno-histochemistry. We confirmed with EEG recordings that LL greatly reduced circadian organization of behavior. However, neither short term LL (4-days) nor long term LL (10-weeks) affected cell proliferation in the GCL of the DG. Recent studies have furthermore suggested an important role for melatonin on hippocampal cell survival but not cell proliferation (Ramírez-Rodríguez et al. 2009). It is known that LL greatly reduces physiological levels of melatonin. Therefore, we injected rats with a single dose of BrdU, subjected the animals to LL for 3 weeks and examined the brains for survival of new cells in the DG. Although the endogenous level of melatonin in LL animals was reduced by 88% in comparison to controls, the number of BrdU positive surviving cells was not significantly different. These results indicate that hippocampal neurogenesis does not require normal circadian organization of behavior or the circadian rise of endogenous melatonin, and that the antineurogenic effect of RSD is not secondary to disruption of these processes.

P63

Rhythmic gene expression in the medial prefrontal cortex of the mouse

BROOKE RAKAI¹, MICHAEL ANTLE²

¹*Psychology, University of Calgary, Calgary, Alberta, CANADA*

²*Psychology, and Physiology & Pharmacology, University of Calgary, Calgary, Alberta, CANADA*

The medial prefrontal cortex is thought to be involved in executive function and the temporal organization of behaviour. This area receives multi-synaptic innervation from the master circadian clock, and therefore many aspects of the medial prefrontal cortical function may be under circadian control. Additionally we have recently shown that lesions to the medial prefrontal cortex alter various circadian responses. There is mixed evidence concerning whether this area is itself rhythmic. Therefore we examined the expression of mPer1 and mPer2 mRNA, and PER1 protein at various times in the medial prefrontal cortex to determine if this area exhibited circadian rhythmicity. To examine mRNA levels using DIG-labeled in situ hybridization, mice were sacrificed at two time points, CT10 and CT14, which have previously been reported to exhibit the largest difference in Per1 mRNA in the cingulate cortex. Levels of mPer1 mRNA were significantly greater at CT14 than at CT10. mPer2, however, did not show the same level of significance between the two time points. To follow up these results, immunohistochemistry for PER1 protein was performed on mice perfused at CT16 and CT20 (i.e., 6 hours after the trough and peak of mPer1 expression). Results from this portion of the study will determine if the mRNA results transfer into a significant difference in the production of the PER1 protein. Our findings suggest that the medial prefrontal cortex does exhibit a circadian rhythm in mPer1 mRNA levels. Understanding the profile of clock gene expression in the medial prefrontal cortex may expand our knowledge of the circadian influence over executive function, and the temporal organization of behaviour thought to be controlled by this cortical area.

P64

Behavioral characterization of ePet-1 knock out mice

JOHN AXLEY¹, CHRIS CIARLEGLIO², EVAN DENERIS³, DOUG McMAHON¹

¹*Biological Sciences, Vanderbilt University, Nashville, TN, USA*

²*Kennedy Center for Research on Human Development, Vanderbilt University, Nashville, TN, USA*

³*Department of Neuroscience, Case Western Reserve University, Cleveland, OH, USA*

The SCN contain the master mammalian circadian clock and receive serotonergic innervation from the raphe nuclei. Serotonin acts both pre- and post-synaptically to influence SCN clock neurons.

The behavioral effects of serotonin and its influence on SCN behavioral output are not completely understood. This study attempts to determine the effect of serotonin on the underlying clock mechanism by analyzing behavioral output of an ePet-1 KO mouse. In mice devoid of ePet-1 there is a marked reduction (80-90%) in serotonin production in the neurons of the raphe. In order to observe the differences in behavioral output of ePet-1 KO mice, their activity was recorded using an infrared monitor while being run through a light entrainment paradigm with a locked or unlocked wheel and then released into DD and subjected to 30-minute light pulses to create a phase-response curve to light. SCN behavioral analysis in LD indicate ePet-1 KO mice have a significantly earlier activity onset, later activity offset and the duration of activity is longer. SCN behavioral analysis in DD indicates ePet-1 KO mice have a decrease in rhythmic power and an increase in duration of activity. Additionally, SCN behavioral analysis in DD indicates that the presence of a wheel increases rhythmic power regardless of genotype. Interestingly, ePet-1 KO mice exhibit a bimodal periodogram. Currently, mice are continuing to undergo light-pulses in the construction of a phase response curve to light. These results will add insight into serotonin's role in both behavioral and light modifications of SCN circadian output. Supported by NIH P50 MH078028.

P65

The relationship between the urinary 6-sulfatoxymelatonin rhythm and core body temperature and plasma melatonin rhythms under forced desynchrony

JOSEPH HULL¹, DERK-JAN DIJK², CHARLES CZEISLER¹, STEVEN LOCKLEY¹

¹Division of Sleep Medicine, Harvard Medical School, Boston, MA, USA

²Surrey Sleep Research Centre, University of Surrey, Guildford, UNITED KINGDOM

Core body temperature (CBT) and plasma melatonin rhythms (*p*MEL) are commonly used as markers of human circadian timing under laboratory forced desynchrony (LFD) conditions. Urinary 6-sulfatoxymelatonin (aMT6s), a major metabolite of melatonin, has also been used as a circadian marker across a range of experimental field and laboratory conditions, but has not been used under LFD conditions. We studied 11 healthy blind subjects with no light perception (3F, 8M; mean age \pm SD = 47.3 \pm 11.6 yrs; 27-68 yrs) in a LFD study employing a 28-hour 'day'. All urine voids sampled every 3-4 hours during wake and every 9-10 hours during sleep episodes. CBT was sampled every minute, and *p*MEL was sampled every 60-120 minutes. Subjects were scheduled to a 40-h constant routine (CR) protocol followed by twenty-four (n=10) or eighteen (n=1) 28-h 'days'. Circadian phase during CR and circadian period across the laboratory study were calculated by Non-Orthogonal Spectral Analysis (NOSA). The phase relationships among the three circadian markers were highly correlated (aMT6s vs. CBT: $r^2=0.98$; aMT6s vs. *p*MEL: $r^2=0.99$; *p*MEL vs. CBT: $r^2=0.98$). The timing of aMT6s peak occurred slightly earlier than CBT minimum (mean difference \pm SD, -0.28 ± 1.03 h; Student's T-test, ns) and significantly later than *p*MEL peak (0.70 ± 0.61 h; $p<0.01$). The timing of *p*MEL phase occurred significantly earlier relative to CBT phase (-0.98 ± 1.00 h; Student's T-test, $p<0.01$). The circadian period estimates among the three circadian markers were highly correlated (aMT6s vs. CBT: $r^2=0.98$; aMT6s vs. *p*MEL: $r^2=0.99$; *p*MEL vs. CBT: $r^2=0.98$). Urinary aMT6s is a reliable marker of the human circadian pacemaker. The phase of the aMT6s rhythm coincides nearer to the phase of CBT rhythm than to the *p*MEL. Sampling urinary aMT6s would be less costly and invasive compared to sampling either CBT or *p*MEL.

Phase delay shifts and melatonin suppression to a 6.5-hour evening light exposure of moderate intensity (275 lux) in healthy older adults

KARINE SCHEUERMAIER, REBECCA PULLMAN, JEANNE DUFFY

Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Age-related sleep disruption can be associated with an earlier circadian phase and a shorter phase angle of entrainment. Bright light treatments have been proposed to better align the circadian system with desired sleep timing in older people, but compliance issues have been reported. We found recently that moderate intensities of light could achieve half-maximal phase delays in older subjects. In this study, we investigated the impact of a moderate intensity light exposure on phase shifts and melatonin suppression in older subjects. **Methods:** After two weeks at home on an 8-hour sleep schedule, nine healthy subjects (age 59 ± 4.6 ; 3F) took part in an 8-day inpatient study. After 3 baseline days and a ~27h constant routine (CR) in dim light to assess initial phase, subjects were given a 6.5-hour light exposure (276 ± 7 lux, 4100K) centered in the phase-delay region of the human phase response curve against a backdrop of dim light. Phase was re-assessed in a CR after the light exposure. Melatonin was collected throughout the study and the fitted midpoint of the CR melatonin rhythm was used as the phase marker. Melatonin AUC (calculated using the trapezoidal method) during the light exposure was compared to the amount 24h earlier during the first CR to calculate suppression. **Results:** The melatonin rhythm was delayed by an average of 1.79 ± 0.64 hours (range 1.06-3.18). Melatonin suppression averaged $74.6\% \pm 19.2\%$ (range 44%-95.5%), with no significant correlation between melatonin suppression and the magnitude of the phase delay ($r=0.49$; $p=0.17$). **Conclusion:** In these very controlled laboratory conditions, light exposure averaging only 276 lux was able to significantly phase delay the melatonin rhythm in all subjects. While the subjects were likely sensitized by exposure to only dim light for the previous 2.5 days, these findings suggest that light treatments for circadian rhythm sleep disorders that use modest light levels may be effective. **Acknowledgement:** This study was funded by NIH grants R01 AG06072 (JFD), F32 AG031690 (KS) and was conducted in the Harvard Clinical and Translational Science Center funded by UL 1 RR025758.

Effects of blue solid-state lighting on melatonin suppression and alertness

JOHN HANIFIN¹, MELISSA THIESSEN¹, JOHN BALAICUIS¹, ERIN EVANS², KAT WEST¹, BENJAMIN WARFIELD¹, KATE CECIL¹, JOHN KEMP¹, MICHAEL JABLONSKI¹, MICHAEL DOWNES¹, M. JAMES¹, B. BYRNE¹, E. GERNER¹, C. PINEDA¹, D. SLINEY³, J. MAIDA⁴, C. BOWEN⁴, N. GOEL⁵, D. DINGES⁵, S. LOCKLEY², G. BRAINARD¹

¹*Department of Neurology, Thomas Jefferson University, Philadelphia, PA, USA*

²*Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*

³*United States Army Environmental Hygiene Agency, Aberdeen Proving Ground, Aberdeen, MD, USA*

⁴*Habitability & Human Factors Branch, Johnson Space Center, Houston, TX, USA*

⁵*Division of Sleep and Chronobiology, Department of Psychiatry, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

Monochromatic wavelength comparisons have indicated that photic circadian phase-shifting, melatonin suppression, and acute alerting responses are strongest in the blue portion of the spectrum. Our aim was to verify that narrow bandwidth blue solid-state lighting could evoke multiple acute physiological and neurobehavioral changes. Seventeen healthy, young subjects (mean age 25.4 ± 0.6 years, $n=8$ females) completed an ongoing three-day study in a laboratory free of time cues at Thomas Jefferson University. Subjects were limited to 4h time in bed for the night prior to admission and Night 1 timed to end at their habitual wake time. On Night 2, subjects either viewed the blue solid-state light exposure system (122 cm² blue LED array, $\lambda_{max}=475\text{nm}$, 29nm half-peak bandwidth) at 30cm for 4h beginning at their habitual sleep time to achieve a full visual field corneal exposure of $75\mu\text{W}/\text{cm}^2$

(n=9) or they remained in dim white light (<4 lux) (n=8). Subjects rated their subjective sleepiness, performed 10-minute auditory psychomotor vigilance tasks, and completed modified neurobehavioral test batteries. Polysomnography was initiated on Night 1, and Karolinska Drowsiness Tests were performed throughout the remaining wake periods. On Day 2, blood was drawn through IV catheters every 20-30 minutes and quantified for melatonin. Melatonin suppression was calculated from levels at time of "lights on" compared to 2h and 4h. Preliminary analysis showed significant suppression of plasma melatonin at 2h and 4h ($p<0.01$) for the blue light exposure group versus dim exposure. Other data will be presented at the meeting. These data will help to characterize the photoreceptor system(s) that mediate the circadian, neuroendocrine, and neurobehavioral responses to polychromatic light stimuli. Ultimately, such data may lead to the development of a flight-worthy, non-pharmacological countermeasure for acutely enhancing astronaut alertness during space missions. This work is supported by National Space Biomedical Research Institute through NASA NCC 9-58. Philips Home Healthcare Solutions provided the lighting system used in this project.

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Sex differences in phase angle of entrainment in humans

SEAN CAIN, CHRISTOPHER DENNISON, JAMIE ZEITZER, AARON GUZIK, SAT BIR KHALSA, NAYANTARA SANTHI, MARTIN SCHOEN, CHARLES CZEISLER, JEANNE DUFFY

Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Introduction: There have been inconsistent results from studies exploring sex differences in entrained circadian phase in humans. This may be because many prior studies failed to precisely control the masking effects of the rest-activity/sleep-wake cycle and the associated light-dark cycle. **Methods:** We examined circadian phase data from 56 healthy adults (18-34 yrs), matching women and men with similar wake times. All subjects had been entrained to a 16:8 light-dark cycle for at least 3 weeks. After three baseline days, they began a 27-50h constant routine, during which core body temperature (CBT) and plasma melatonin were collected. Phase estimates for CBT minimum, and dim light melatonin onset and offset were calculated. **Results:** The timing of all three phase markers were significantly earlier in the women relative to the men, although habitual sleep and wake times were not different. The relative phase relationship between sleep and circadian phase was therefore different between sexes, with sleep occurring at a significantly later circadian phase in women than men. **Conclusions:** The observed sex difference in entrained circadian phase may reflect a difference in circadian period and/or sensitivity to light. Additional research is needed to determine the biological basis for this sex difference. **Support:** Supported by NIH grants R01 MH45130 (to CAC), R01 HL080978 (to JFD), M01 RR02635 (BWH GCRC); SWC supported in part by a fellowship from the Natural Sciences and Engineering Research Council of Canada.

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24-hour rhythms of muscle strength

LEANA ARAUJO¹, EDUARDO SANTOS², BEN EDWARDS³, JIM WATERHOUSE³, ANAEL SANTOS⁴, SÉRGIO TUFIK¹, MARCO TÚLIO MELLO¹

¹*Psicobiologia, Universidade Federal de São Paulo, São Paulo, BRAZIL*

²*Physiology, Universidade Federal de São Paulo, São Paulo, BRAZIL*

³*Sports, Liverpool John Moores University, Liverpool, UNITED KINGDOM*

⁴*Veterinária, Universidade Federal de Uberlândia, Uberlândia, BRAZIL*

Physical performance varies according to the time of day. The present study investigated the influence of time of day on the parameters of muscle strength under a highly controlled protocol in eight male volunteers (age 27 ± 3.2 , weight 74.6 ± 5.33 Kg, height 174.6 ± 6.3 cm, % body fat 18 ± 6.67 and % lean mass 82 ± 6.67). After three sessions to familiarize the volunteer with the testing procedures, they were

evaluated once a week for six weeks at six different times: 02:00, 06:00, 10:00, 14:00, 18:00, 22:00 hours. The tests of muscle strength were performed as follow: isokinetic at $1.05\text{rad}\cdot\text{s}^{-1}$, isokinetic at $4.19\text{rad}\cdot\text{s}^{-1}$ and isometric, with four minutes passive rest between each test. Rectal temperature (T_{rcc}°), peak torque (PT), maximum work (MW) and the average power (AP) of the flexor (Flex) and extensor (Ext) knee muscles with a range of movement from 0° to 90° in the isokinetic mode, as well as of peak torque (PT) in maximum voluntary isometric contraction (MVIC) of knee extensors at 60° knee flexion, were measured. The T_{rcc}° presented a 24h rhythm with an acrophase (Φ) at 17:48h, mesor (Me) of 37.0°C and amplitude (A) of 0.41°C . Rhythms with a 24h period were significant ($P<0.05$) for all variables except the extensors of the knee at $1.05\text{rad}\cdot\text{s}^{-1}$ and the AP and flexor AP of the knee at $4.19\text{rad}\cdot\text{s}^{-1}$. At $1.05\text{rad}\cdot\text{s}^{-1}$: the PT of the knee extensors (17:18h, 221.1Nm, 12.0Nm), the MW of the knee extensors (16:54h, 229.3 J, 12.4 J), the MW of the knee flexors (15:36h, 143.9 J, 8.6 J) and the AP of the knee flexors (15:06h, 92.0W, 5.2W). At $4.19\text{rad}\cdot\text{s}^{-1}$, the knee extensors (17:06h, 151.6Nm, 3.7Nm) and the MW of the knee extensors (17:30h, 170.5Nm, 5.5Nm). The present study showed rhythms with a period of 24h in some indices of muscle strength performance at both speeds of movement.

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Photic entrainment in cultured chick astrocytes: Effects on clock gene expression, metabolism, and opsins

JEFFIN PAULOSE¹, VINCENT CASSONE²

¹Biology, Texas A&M University, College Station, TX, USA

²Biology, University of Kentucky, Lexington, KY, USA

Recent studies in various bird species have suggested a widespread and diverse population of photoreceptors within the brain, including the hypothalamus. The role of astrocytes in modulating neuronal communication is especially critical within the hypothalamus, where astrocyte density is highest. Our preliminary data show that embryonic chick diencephalic astrocytes express mRNAs of at least 2 opsins in addition to the canonical clock genes. Additionally, immunocytochemical analysis from our lab shows melanopsin-like staining in ependymal cells and tanycytes within the mediobasal hypothalamus. In this study, E17 astrocytes were isolated from diencephalons and cultured to 5 passages. The cells were exposed to a 12:12 LD cycle for 7 days, as previous data have shown this period of time to be required for full entrainment. Every four hours, one set of cells ($n=6$) was incubated with C142-deoxy[14C]-glucose (2DG) for one hour and the cells trypsinized for mRNA analysis of clock genes, opsins, and 2DG uptake. Parallel cultures in chambered glass coverslips were also used for immunocytochemistry. Our preliminary data show that these astrocytes express melanopsin and peropsin, as well as all of the clock genes. Furthermore, a 12:12 LD cycle is capable of driving clock gene rhythms, but not 2DG uptake. These data suggest that these opsin-expressing astrocytes are photosensitive and can use light information to entrain clock gene expression, but not 2DG uptake. This is contrast to our recently published data on melatonin entrainment of astrocytes. Along with in vivo analysis of deep-brain photoreceptors currently underway, these data contribute to our understanding of non-visual photoreception in a developmental and cell-specific context. Acknowledgement: This research is supported by NIH P01 NS39546 grant to V.M. Cassone.

Phase response curve to a single 6.5-hour light pulse of short-wavelength light

MELANIE RUEGER¹, MELISSA ST. HILAIRE¹, GEORGE BRAINARD², CHARLES CZEISLER¹, STEVEN LOCKLEY¹

¹*Division of Sleep Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*

²*Department of Neurology, Thomas Jefferson University, Philadelphia, PA, USA*

The resetting response of the human circadian pacemaker to light depends on the circadian timing of exposure and is described by a Phase Response Curve (PRC). Previous PRCs in humans have been carried out for bright white light. Given that the circadian photoreception system is most sensitive to shorter-wavelength visible light, the aim of the current study was to assess whether the PRC to a 6.5-hour exposure to monochromatic blue light (480 nm) differed substantially from the PRCs to white light constructed using the same protocol. Eighteen young (18-30 years), healthy male and female subjects were studied for 9-10 days in a time-free environment. Following three baseline days (16:8 h wake:sleep), subjects underwent an initial ~30-52-hour Constant Routine (CR) in <3 lux and, after an 8-hour sleep, were exposed to monochromatic 480 nm light (11.8 $\mu\text{W}/\text{cm}^2$) for 6.5 hours in a modified Ganzfeld dome centered in the 16-hour wake episode. The subjects' pupils were dilated 15 minutes prior to light exposure. The light timing for each subject was randomized to one of 18 circadian phases separated by 20° intervals according to habitual wake-time. After an 8-hour sleep, subjects began a second CR (~32-55 h) followed by a recovery sleep and discharge. Core Body Temperature (CBT) was measured every minute via a rectal thermistor. Data were visually inspected and non-physiological values due to probe slips and removals were excluded. The phase of CBT minimum was determined for CR1 and CR2 separately. Phase shifts were calculated for the difference in CBT-min between CR1 and CR2, plotted according to conventional criteria, and fit with a single harmonic function. Exposure to a 6.5 h pulse of 480 nm monochromatic light resets the circadian pacemaker according to a conventional Type 1 PRC with maximal delays and advances of -1.4 h and 0.9 h, respectively. This work was supported by National Institute of Mental Health Grant 5R01MH45130-19.

Perinatal photoperiodic input influences organization of the circadian clock

CHRISTOPHER CIARLEGLIO¹, JOHN AXLEY², BENJAMIN STRAUSS², KAREN GAMBLE³, DOUGLAS McMAHON²

¹*Kennedy Center, Vanderbilt University, Nashville, TN, USA*

²*Biological Sciences, Vanderbilt University, Nashville, TN, USA*

³*Psychiatry and Behavioral Neurobiology, University of Alabama–Birmingham, Birmingham, AL, USA*

Light input during development has been shown to have lasting effects on the structure of neural circuits; however, whether seasonal light cycles can affect the developmental organization of the mammalian brain's circadian clock is unknown. We now report that circadian pacemaker properties of the SCN clock can be developmentally imprinted by photoperiod, resulting in persistent changes in the gene-expression rhythms of individual clock neurons and altering the entrainment of the pacemaker to subsequent photoperiods. We developed male mice from birth to weaning (P21) in one of three light cycles—LD 8:16, LD 12:12, or LD 16:8. Mice were then subjected to one of these three photoperiods for an additional four weeks in a counter-balanced design and at ca. P50 SCN were removed and ex vivo imaging of SCN tissue and neuronal Per1::GFP rhythms were performed (N = 48 mice, 92 SCN slices, 5,538 neurons). To determine if there were persistent effects of perinatal exposure to seasonal photoperiods on the SCN clock, we analyzed SCN and neuronal properties grouped by developmental photoperiod regardless of subsequent photoperiod. SCN from long-day developed mice were imprinted with narrowed Per1::GFP rhythm waveforms due to waveform narrowing of individual SCN neurons. Neurons in the long-day developed SCN were also imprinted with shortened rhythmic period. SCN from long-day developed mice exhibited a stable timing relationship to the

nocturnal light/dark transition on both subsequent long-day and short-day light cycles, with their molecular rhythms closely tracking dusk, while SCN from short-day developed mice exhibited large changes in their timing relative to dusk in subsequent seasonal photoperiods. Our results indicate that seasonal light exposure, and furthermore maturation under a particular seasonal light regime, persistently alters the properties of the mammalian circadian clock neurons. Supported by NIH F31 MH080547 to CMC, NIH P50 MH078028 to DGM.

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Consequences of different lighting conditions on melanopsin bistable photoresponse properties

PETTERI TEIKARI¹, LUDOVIC MURE², CLAUDE GRONFIER³, DOMINIQUE DUMORTIER², HOWARD COOPER^{1,2}

¹Chronobiology, INSERM, Bron, FRANCE

²Lighting, ENTPE, Lyon, FRANCE

In bistable photopigment systems, light elicits photosensory responses and at the same time drives chromophore photoregeneration allowing restoration of photic responsiveness after photopigment bleaching. Melanopsin in the human retina has been shown to express bistable properties both in vitro and in vivo. These studies have shown that prior light exposure can modulate the amplitude of subsequent photic responses to light. Thus, a given light exposure not only triggers a response from melanopsin ganglion cells but also sets the response capacity of the system to subsequent light stimulation by determining the relative proportions of the 11-cis and all-trans retinal bound isoforms of the photopigment. The relative proportions of each "R" and "M" isoform depend on their individual absorbance spectra and the environmental light spectrum. In the present study, we modelled the effects of different light spectra on the relative responsiveness of melanopsin and on the relative degree of chromophore regeneration of the melanopsin photopigment system, based on data obtained from studies of the human pupillary light reflex in humans. Spectral templates of broadband natural and artificial light sources were applied to predict relative response amplitudes using the equilibrium spectrum of melanopsin. The difference spectrum was used to determine the outcome of the relative chromophore states of the system. The results suggest that the response to light is greater with short wavelength dominated spectra than with long wavelength dominated light spectra. Conversely, prior exposure to light sources dominated by long wavelength light increase the ability of the melanopsin system to respond to subsequent light exposures, while light sources dominated by shorter wavelength light decrease the response. The results provide a basis for understanding mechanisms underlying the effects of prior light history. Furthermore, exploiting the bistable properties of melanopsin could allow for optimization of spectral light distribution in industrial, domestic and clinical phototherapy applications by appropriate use of the potentiating effects of long wavelength light.

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An unexpected contribution of rods to circadian photoentrainment at all light intensities

CARA ALTIMUS¹, ALI GULER¹, GURPRIT LALL², CYRUS ARMAN³, NAZIA ALAM⁴, GLEN PRUSKEY⁴, ROBERT LUCAS², ALAPAKKAM SAMPATH³, SAMER HATTAR¹

¹Biology, Johns Hopkins University, Baltimore, MD, USA

²Neurobiology, University of Manchester, Manchester, UNITED KINGDOM

³Physiology and Biophysics, Keck School of Medicine, Los Angeles, CA, USA

⁴Physiology and Biophysics, Weill Cornell Medical College, White Plains, NY, USA

The presence of circadian rhythms allows the daily coordination of many physiological cycles such as alertness, hormone regulation, and metabolism. Three types of photoreceptors rods, cones and

melanopsin containing intrinsically photosensitive retinal ganglion cells (ipRGCs) are responsible for all light detection in the eye. To determine the contribution of individual outer retinal photoreceptors to circadian photoentrainment, we genetically isolated rod and cone by silencing ipRGCs and either rods or cones. When we assayed for circadian photoentrainment in mice lacking rods and melanopsin, we found minimal ability to photoentrain at high light intensities and no photoentrainment at low light intensities suggesting that cones play a minor role on their own in circadian photoentrainment. To determine the contribution of rods to circadian photoentrainment and pupillary light constriction, we silenced ipRGCs and cones. However, because rods utilize the cone cell pathway to signal their light input to retinal ganglion cells, we used several methods of cone silencing. Our first model left cones in a state of constant hyperpolarization, similar to constant light input, in the second model, cones were constantly depolarized resembling constant dark and the third mouse resulted in genetic ablation of cones to disrupt all rod input via cones. In our model with cones in a constant state of depolarization, we found that rods were able to contribute to photoentrainment at high and low light intensities. In animals with cones silenced in a state of hyperpolarization, we found that rods signal was unable to influence photoentrainment, suggesting that the cone signal was disrupting the transmission of the rod signal. Finally in the mouse lacking cones, we found that these mice were unable to photoentrain at high light intensities and gained the ability to photoentrain at low light intensities. These results shed light on how the rod signal is transmitted through the retina for circadian functions: at high light intensities, rods signal directly to cones, while at low light intensities, rods use their canonical pathway through rod bipolar cells to signal light information to the retinal ganglion cell layer.

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Light-induced phase shifts and fos response in the suprachiasmatic nucleus of aromatase knock-out mice

REBECCA BROCKMAN, MEGAN MAHONEY

Veterinary Biosciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Sleep disorders are one of the major complaints of perimenopausal women. Gonadal steroids are thought to play a role as reduced estrogen is associated with sleep disturbances and hormone replacement therapy with estrogens improves sleep quality and amount. We use aromatase cytochrome P450 knock-out mice (ArKO) to examine the relationships between estrogen, sleep, and circadian rhythms. These animals are unable to produce estradiol but they retain the ability to respond to it; ArKOs also have fragmented sleep and sleep more during the night when compared to wildtypes (WTs). We tested the hypothesis that this is due in part to a reduced responsiveness to light. Male and female WTs and ArKOs were singly housed in a 12:12 LD cycle, given phytoestrogen free food, and their activity rhythms were monitored. Animals received a 1 hour light pulse at either circadian time (CT) 4 or 16 using an Aschoff Type II protocol. In one set of animals we measured the phase change (min) of activity onset following the light pulse. A second set of animals were perfused following the pulse and brains were stained for the detection of Fos within the suprachiasmatic nucleus (SCN). This hypothalamic region contains the master circadian clock. At CT 16, ArKO females had a relatively reduced phase shift and Fos expression in the SCN when compared to WT females. No differences in Fos expression or phase shifts were apparent between males at CT 16 or in any group tested at CT 4. The results in ArKO females could be due to a reduced sensitivity to light or to a shift in the photic phase response curve. This will be examined further by testing additional time points and replacing estradiol. The results of these studies will enhance our understanding of the interactions of circadian rhythms, gender and steroid hormones.

Different mechanisms of adjustment to a change of the photoperiod in the suprachiasmatic and liver circadian clocks

SERHIY SOSNIYENKO, DANIELA PARKANOVA, HELENA ILLNEROVA, MARTIN SLADEK, ALENA SUMOVA

Department of Neurohumoral Regulations, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, CZECH REPUBLIC

Changes in photoperiod modulate the circadian system affecting the function of the central clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The aim of the study was to compare the dynamics of adjustment to a change of a long photoperiod (LD18:6) to a short photoperiod (LD6:18) of clock gene expression rhythms in the mouse SCN and in the peripheral clock in the liver, as well as of the locomotor activity rhythm. Three, five and thirteen days after the photoperiod change, daily profiles of *Per2* and *Rev-erb α* expression in the rostral, middle and caudal parts of the SCN and in the liver were determined by in situ hybridization and real time RT-PCR, respectively. The clock gene expression rhythms in the different SCN regions, desynchronized under the long photoperiod, attained gradually synchrony following transition from long to short days, mostly via advancing the expression decline. The photoperiodic modulation of the SCN was due not only to the degree of synchrony among the SCN regions but also to different waveforms of the rhythms in the individual SCN parts. In the liver, *Per2*, adjusted to the transition in a similar manner as in the SCN, i.e., by shortening of its daily expression, whereas *Rev-erb α* , as well as the locomotor activity rhythm adjusted to the transition by lengthening of the expression and activity. These data suggest that in the liver, *Per2* expression is likely modulated by the photoperiod via systemic cues emanating from the SCN while *Rev-erb α* expression mostly via photoperiodic modulation of the behavioral state. The results demonstrate different mechanisms of adjustment to a change of the photoperiod in the central SCN clock and the peripheral liver clock. Supported by the 6th Framework Project EUCLOCK 018741; Grant 305090321 and by Research Projects AV0Z 50110509 and LC554.

Structurally stable spatiotemporal organization of circadian oscillation of SCN neurons

RAE SILVER

Barnard College and Columbia University, New York City, NY, USA

BKGND: Although it is known that the cells of the suprachiasmatic nucleus (SCN) can sustain circadian oscillations in isolation, studies of isolated individual SCN neurons indicate that SCN neurons are weak, inducible and probabilistic oscillators (Webb et al. 009). These studies seem to eliminate the possibility of functionally distinct classes of SCN cells, based on some anatomical or neurochemical features. Such results make salient the question of how these individual SCN cells are organized within the tissue to produce coherent phase setting output signals to the body. **METHOD:** Using bioluminescence imaging from acute brain slices from *PER2::LUC* knockin mice expressing a reporter protein fused to firefly luciferase to monitor *PER2* expression, and parallel studies of immunostained brain sections, we assessed the spatial and temporal activation patterns of oscillations in the SCN. To identify groups of synchronously expressing neurons, coincident changes in circadian phase of bioluminescence in subsystems of neurons were identified using cluster analysis. **RESULTS:** In slices in which the amplitude of circadian oscillation was high, a daily cycle of clock gene expression involved serial

activation of specific subregions followed by a silent interval. In slices with low amplitude oscillation, the cycle to cycle variation was high, and neither stable subregions nor a silent interval were detected. Impressively, there was substantial symmetry between the left and right side of the SCN, even in slices that appeared to be asymmetrically cut in the coronal plane. In order to explore the biological import of the clusters identified in the high amplitude slices, we confirmed the co-expression of LUC and PER2 and showed that in immunostained brain sections harvested from animals killed at the start and at the peak of the circadian cycle, the spatial localization of LUC expression resembled that of the SCN regions revealed in the cluster analysis of bioluminescent slice material. **CONCLUSION:** We conclude that small groups of neurons within the SCN give rise to distinctive and identifiable subsystems and that serial activation of these groups of neurons (termed nodes) is the basis of the daily rhythm produced by the brain clock. The results suggest that the peak timing and precise duty cycle of PER2 expression within individual neurons may be determined by its location within the nucleus. The tissue, in contrast to its separated cellular component cells, produces a structurally stable and spatiotemporally organized circadian oscillation that likely reflects functionally distinct classes of cells, based on some anatomical or neurochemical features. This stands in sharp contrast to the present results showing that within an SCN slice, the pattern (phase, amplitude) of oscillation of each of the major clusters (and thus of the composite of cells within each cluster) is robust and stable across serial cycles.

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Fractal patterns of multi-unit activity of the suprachiasmatic nucleus (SCN): SCN-intrinsic and SCN-extrinsic network properties

KUN HU¹, JOHANNA MEIJER², STEVEN SHEA¹, THIJS HOUBEN², HENK TJEBBE VANDERLEEST², FRANK SCHEER¹

¹*Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*

²*Department of Molecular Cell Biology, Leiden University Medical Centre, Leiden, THE NETHERLANDS*

Many physiological processes exhibit scale-invariant/fractal properties, generating fluctuations that display similar patterns across wide time scales. These patterns are robust in healthy biological systems but can be significantly altered under pathological conditions and can be predictive of mortality. Our recent studies indicate the suprachiasmatic nucleus (SCN) not only controls many endogenous physiological rhythms at ~24 h, but also functions as a critical neural node responsible for fractal patterns in heart rate and motor activity fluctuations across a wider range of time scales from minutes to hours. Here we determined whether this scale-invariant regulation is a property intrinsic to a neuronal network within the SCN itself or it requires feedback interactions with other control nodes in the neural system. To test the hypotheses, we analyzed multi-unit activity (MUA) of the SCN: (A) *in vivo* activity of 3 freely moving adult C57BL6 mice under light-dark (LD; 12h:12h) cycles and under constant darkness (DD); and (B) *in vitro* from 4 additional mice. We showed a robust fractal pattern in the MUA of *in vivo* SCN over a wide range of time scales (~1 min–3.5 h). The long-range fractal pattern was consistent for all mice and persisted during LD and DD conditions. However, the fractal pattern was completely abolished in *in vitro* SCN activity despite the clear presence of circadian rhythms in the recordings ($p < 0.0001$). The discovery that scale-invariant MUA occurs within the *in-vivo* SCN suggests that the SCN provides a useful model system to study the minimal neuronal network required for the generation of scale invariance. However, our *in-vitro* findings also indicate that the SCN is not sufficient to impart scale invariance when isolated from the rest of the nervous system. Thus, the fractal pattern in SCN neural activity must reflect a network of feedback interactions between the SCN and other neuronal nodes.

Circadian modulation of the Cl⁻ reversal potential in the suprachiasmatic nuclei, in rats

JAVIER ALAMILLA, AZUCENA PEREZ-BURGOS, DANIEL QUINTO, RAUL AGUILAR-ROBLERO
Neurociencias, IFC, Universidad Nacional Autonoma de Mexico, MEXICO

The suprachiasmatic nucleus (SCN) is the main pacemaker involved in circadian rhythms in mammals. GABA is a prevailing neurotransmitter in the SCN and is implicated in different aspects of circadian rhythm regulation. Some debate exists whether GABA has an excitatory neurotransmitter in the SCN besides its classical inhibitory role. In this work we study the reversal potential of GABAergic postsynaptic currents at different times and SCN's regions, from neurons recorded either in perforated or in whole cell patch clamp configurations. The results indicate that in most neurons during the day GABA has a reversal potential close to -30 mV in the dorsal SCN and -60 mV in the ventral SCN; this difference reverse during the night, such that dorsal SCN neurons have a reversal potential of -60 mV, and -30 mV in the ventral region. These results confirm an excitatory role for GABA in the SCN, and further indicate both time (day vs. night) and region (dorsal vs. ventral) differences in GABA reverse potential in the SCN, which can be attributed to the Na⁺-K⁺-Cl⁻ co-transporter, since they can be abolished by adding the co-transporter blocker bumetadine.

Ca²⁺ signaling induced by glutamate in rat SCN neurons

TONGFEI WANG¹, G. GOVINDAIAH², CHARLES COX¹, MARTHA GILLETTE³

¹*Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

²*Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

³*Department of Cell & Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

Mammals' daily rhythms of physiology and behavior are synchronized to day and night by the action of the signal of environmental light on suprachiasmatic nucleus (SCN). Glutamate (Glu) is the primary neurotransmitter that conveys photic information from the retina to SCN. The effect is specific to the time of day: in early night, this signal induces phase-delay of the circadian clock, but phase-advances it in late night. Ca²⁺ plays a pivotal role in Glu signaling. Using two-photon laser microscopy and whole-cell patch-clamp recording, we studied neuronal Ca²⁺ signaling in the rat SCN brain slice. We found that in early night Glu induces a transient Ca²⁺_i increase (amplitude, 45 ± 17%, n = 22 neurons in 15 slices). This response is abolished by tetracaine, a ryanodine receptor (RyR) inhibitor. On the other hand, at CT 19 Glu induces only a small Ca²⁺_i increase (amplitude, 14 ± 6%, n = 17 neurons in 11 brain slices), which is tetracaine insensitive. Patch-clamp recording and Ca²⁺ imaging shows that neither Ca²⁺ influx through NMDA receptor nor Ca²⁺ store capacity is responsible for the differential response between early and late night. We further evaluated RyR sensitivity in the two time windows by measuring the Ca²⁺_i leak rate through RyR. The RyR-mediated Ca²⁺_i release is significantly more sensitive to excitatory stimulation in early than late night (3 fold, n=21, p<0.01). Our data demonstrate that differential Ca²⁺_i levels in SCN neurons between early and late night may set the tone for RyR activation. In early night, RyR is activatable, so Glu signaling can induce robust Ca²⁺_i release. On the other hand, in late night, the RyR is relatively insensitive, so the transient Ca²⁺_i response is weak. This study raises questions about endogenous clock processes that regulate variations in [Ca²⁺_i] in SCN neurons.

Serotonergic enhancement of photic phase shifts: Location of action of BMY7378

VICTORIA SMITH¹, RYAN JEFFERS¹, MICHAEL ANTLE²

¹*Department of Psychology, University of Calgary, Calgary, Alberta, CANADA*

²*Psychology, Physiology & Pharmacology, University of Calgary, Calgary, Alberta, CANADA*

Serotonin mixed agonists/antagonists have been shown to greatly potentiate photic phase shifts, and this potentiation has been shown to be through binding at the 5-HT_{1A} receptor. However, the precise brain region through which this potentiation of photic phase shifts occurs is still unknown. The goal of the present experiment was to examine the effects of BMY7378 on light-induced phase shifts when administered systemically compared to a local administration directly to the suprachiasmatic nucleus (SCN). Adult male hamsters, housed in constant darkness, were exposed to one of two different pretreatment conditions (systemic or local drug administration), followed by a 15-minute light pulse at one of two intensities (40 lux or 0 lux) at CT18. Those animals in the systemic pretreatment group received an i.p. injection of either BMY7378 (5mg/kg) or vehicle control (saline) at CT17.25, followed by a 15-minute light pulse at CT18. Alternatively, those animals in the local administration pretreatment group received an intra-cerebral injection of either BMY7378 (15.58 nmol/ μ L) or vehicle control 10 minutes prior to the 15-minute light pulse at CT18. Systemic pretreatment with BMY7378 resulted in a significant potentiation of the photic phase shift, however administration of BMY7378 directly to the SCN resulted in no such potentiation of the photic phase shift. Systemic administration of BMY7378 without a subsequent light pulse resulted in a significant phase shift compared to saline alone, however there was no such increased phase shift over control when BMY7378 was administered directly to the SCN without a subsequent light pulse. These results suggest that BMY7378 binding to 5-HT receptors in the SCN alone is not sufficient to potentiate photic phase shifts.

Effects of exogenous melatonin on the electrical activity of Per1 and non-Per1 suprachiasmatic nuclei (SCN) neurons in vitro

FIONA SCOTT¹, MINO BELLE¹, PHILIPPE DELAGRANGE², HUGH PIGGINS¹

¹*Faculty of Life Sciences, University of Manchester, Manchester, UNITED KINGDOM*

²*Institut de Recherches Internationales Servier, Paris, FRANCE*

The master circadian pacemaker in the suprachiasmatic nuclei (SCN) controls daily rhythms in many aspects of physiology, including secretion of the pineal gland hormone melatonin. Melatonin in turn can influence SCN timekeeping: exogenous melatonin induces phase advances in rodent behavioural rhythms and alters the activity of SCN neurons both in vivo and in vitro via high affinity G protein-coupled receptors (MT1 and MT2). However, the precise effects of melatonin on the electrical properties of individual SCN neurons are unclear. Here we investigated the acute effects of exogenous melatonin on SCN neurons in vitro in brain slices prepared from Per1::GFP transgenic mice. In current clamp mode, exogenous melatonin, at near-physiological concentrations (1 nM), hyperpolarised the majority of SCN neurons tested with no overt differences in these actions across the projected light-dark cycle. In addition, 1 nM melatonin depolarised a small number of cells. No differences were observed in melatonin's effects between Per1::GFP or non-Per1::GFP SCN neurons, however cells in the dorsal SCN were more likely to be hyperpolarised by melatonin than those tested in the ventral region. Melatonin-induced effects were blocked by the MT1/MT2 antagonist, Luzindole (1 μ M). The proportion of SCN neurons responsive to melatonin was greatly reduced in the presence of either tetrodotoxin (TTX; 500 nM) or gabazine (20 μ M) and in voltage clamp mode, 1 nM melatonin increased the frequency of GABA-mediated currents. These findings indicate that melatonin can alter neuronal excitability in the

majority of SCN neurons, regardless of whether or not they overtly express the core clock gene *Per1*. The results also suggest that melatonin acts mainly by modulating inhibitory GABAergic transmission within the SCN. This may explain why exogenous application of melatonin has heterogeneous effects on individual SCN neurons.

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Circadian rhythms of PER2 protein activity are maintained in hypothalamic regions from behaviorally arrhythmic animals with disrupted VIP-VPAC2 signaling

CLARE GUILDING, ALUN HUGHES, HUGH PIGGINS

Faculty of Life Sciences, University of Manchester, Manchester, UNITED KINGDOM

Vasoactive intestinal polypeptide and its receptor VPAC2 play important roles in the functioning of the suprachiasmatic (SCN) pacemaker. Mice lacking VPAC2 receptors (*Vipr2*^{-/-}) show altered circadian rhythms in locomotor activity, neuronal activity and clock gene expression. Further, these mice display disrupted synchrony of endogenous clock gene (*per1*) oscillations in the SCN and show altered circadian rhythms in metabolism and food intake. Within the brain however, the SCN is not the only site containing endogenous circadian oscillators, nor is it the only site of VPAC2 receptor expression. We have recently demonstrated robust endogenous circadian rhythms at both single cell and tissue levels in the arcuate (Arc) and dorsomedial nuclei (DMH) of the mediobasal hypothalamus, two areas which also express VPAC2 receptors. Consequently we determined the role of VPAC2 receptor signaling in the maintenance of endogenous molecular rhythms in these hypothalamic nuclei. We used wildtype and *Vipr2*^{-/-} mice, both on an mPer2Luc knockin background (PER2::LUC) which accurately reports PER2 protein expression. Following behavioral characterisation of mice in diurnal and circadian (constant dark) conditions, we assessed real time bioluminescence expression of PER2::LUC in adult brain slice cultures containing SCN, Arc or DMH. Surprisingly, all hypothalamic brain regions in tissue from *Vipr2*^{-/-} mice maintained circadian rhythms of PER2::LUC activity, including cultures of tissue taken from behaviorally arrhythmic animals. These results demonstrate that VPAC2 receptor signaling is not significantly involved in the maintenance of PER2 protein rhythms in the hypothalamus in vitro, and raise the possibility that the loss of VPAC2 receptor signaling differentially effects clock gene expression in the rodent brain.

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Photic and nonphotic effects on VIP, GRP, and AVP release in the hamster SCN circadian clock

JESSICA FRANCL, GAGANDEEP KAUR, J. DAVID GLASS

Biological Sciences, Kent State University, Kent, OH, USA

The circadian clock, located in the suprachiasmatic nucleus (SCN), integrates photic and nonphotic information to regulate circadian rhythmicity. Retinorecipient cells of the SCN core receive photic information via the retinohypothalamic tract and, through release of vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP), modulate arginine vasopressin (AVP)-containing pacemaker cell activity in the SCN shell. Despite this information, however, little is known concerning the release of these neuropeptides within the clock. We therefore employed microdialysis procedures to characterize this release. Male Syrian hamsters, housed under LD 14:10 or DD, received a microdialysis probe

aimed at the lateral SCN. After 48 hrs of recovery microdialysis sampling was undertaken, and each analyte was measured separately by radioimmunoassay. Under LD, VIP, GRP and AVP exhibited diurnal rhythms, with peak release occurring in the early day (211%, 176%, and 149% of the daily mean, respectively). Under DD, VIP release was arrhythmic with no discernible peak, while GRP release exhibited a broad peak extending over most of the subjective night (178% of the daily mean) and AVP closely resembled release under LD (245% of the daily mean). Effects of photic and nonphotic mediators were then studied using reverse dialysis of the SCN with NMDA and the serotonin agonist, 8-OH-DPAT, respectively. Treatment with NMDA at night increased GRP release to 202% of the baseline (p0.3). Administration of 8-OH-DPAT during the day decreased VIP and GRP release (39% and 31% of the baseline, respectively; both p0.08). These data reveal distinct diurnal patterns of neuropeptide release that, under DD, are lost (VIP), altered (GRP) or stable (AVP). Also, glutamatergic stimulation increased GRP release but had no effect on AVP, while serotonergic stimulation inhibited VIP and GRP release but did not affect AVP. NIH NS035229 to JDG.

P85

Little SAAS peptide expression forms a new sub-compartment in the retinorecipient suprachiasmatic nucleus of the rat

**NORMAN ATKINS¹, DANIEL MORGAN², TARA COMINSKI², JOHN PINTAR², JONATHAN SWEEDLER³,
MARTHA GILLETTE⁴**

¹*Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

²*Cell Biology and Neuroscience, University of Medicine and Dentistry of New Jersey, Newark, NJ, USA*

³*Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

⁴*Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

Recent technological advances have enabled functional measurements of the peptide complement in the mammalian brain. Mass spectrometry-based techniques have identified several peptides released in a circadian fashion within the rat suprachiasmatic nucleus (SCN), the mammalian circadian clock. One of these signals is little SAAS, a peptide derived from the N-terminus of the proSAAS prohormone. C-terminus proSAAS peptides have been implicated in proprotein convertase inhibition, while little SAAS function is poorly understood. When exogenously applied to the rat SCN in vitro, little SAAS evokes phase shifting. Pharmacological blockade studies suggest little SAAS functions downstream of glutamate and independent of vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP), both of which have been implicated in light-induced SCN phase shifting. In order to understand little SAAS in the context of established cytoarchitecture, we evaluated immunolocalization of little SAAS. Little SAAS-like immunoreactivity (-LIR) within mouse and rat SCN is distinct from other tested peptides. Within the SCN, little SAAS-LIR is localized to the retinorecipient region. Stereological analyses find little SAAS-LIR in 16.3% of SCN neurons, making this the third most abundant class of peptidergic neurons. Little SAAS co-localizes with some VIP and GRP neurons. Little SAAS co-localizes ~4x more frequently with GRP- than VIP-expressing neurons; however, these co-localizations account for only ~2/3 of the total number of little SAAS-LIR neurons. Little SAAS-LIR is found in perikarya, confirming endogenous SCN expression of the peptide. Confocal analyses of little SAAS-LIR find no co-expression with arginine vasopressin (AVP) throughout the parvocellular SCN. Within the vasopressinergic SCN shell, little SAAS-positive puncta juxtapose AVPergic cells. In contrast, little SAAS is observed in vasopressinergic neurons in magnocellular hypothalamic nuclei. These results suggest that little SAAS neurons comprise a new SCN sub-compartment. Supported by NIH grants T32 MH/AG-19957 (DM and TC), 1P30DA018310 (JVS) and HL67007 (MUG).

Preliminary evidence that the food-entrainable oscillator is not exclusively responsive to food

ROBERTO REFINETTI

Circadian Rhythm Laboratory, University of South Carolina, Walterboro, SC, USA

A master circadian pacemaker located in the suprachiasmatic nuclei (SCN) controls circadian rhythms in mammals. This pacemaker receives monosynaptic input from the eyes, which allows it to be entrained by environmental cycles of light and darkness. Another circadian pacemaker, whose neural substrate has not yet been identified, can control behavior in the absence of the SCN and can be entrained by environmental cycles of food availability. Because this second pacemaker is responsive to food but not to light, it has been called the food-entrainable oscillator. By contrast, the SCN pacemaker has been referred to as the light-entrainable oscillator, even though there is substantial evidence that it is responsive to food as well as to light. Interestingly, studies still ongoing in my laboratory seem to suggest that the food-entrainable oscillator is responsive not only to food but also to ambient temperature. Mice have been kept in individual cages with access to running wheels. Their rhythms of locomotor activity have been consistently shown to free-run in constant darkness and to entrain to environmental cycles of light and darkness, of food availability, and of ambient temperature. When two different environmental cycles (for example, light and environmental temperature) were presented simultaneously with different cycle lengths (for example, 23 h and 24 h), the behavior of the mice showed a dissociation into two components that seemed to reflect the separate entrainment of two pacemakers, presumably the light-entrainable oscillator and the food-entrainable oscillator. The possibility that one of the behavioral components reflected merely masking has been partially but not yet fully eliminated. Because the SCN pacemaker is preferentially entrained by light, the dissociated pattern of behavior implies that the food-entrainable oscillator can be entrained not only by a cycle of food availability but also by a cycle of ambient temperature.

Rev-erb α participates in the clockwork of food anticipation

JULIEN DELEZIE, JORGE MENDOZA, PAUL PÉVET, ETIENNE CHALLET

Neurobiology of Rhythms, Institute of Cellular and Integrative Neurosciences, CNRS, Strasbourg, FRANCE

Limiting food-access to a temporal window during the day (termed Restricted Feeding, RF), has profound synchronizing effects on circadian rhythms. A few hours before mealtime, animals display food-anticipatory activity (FAA) as well as other physiological parameters (e.g. rise in body temperature, plasma corticosterone). FAA has been proposed to be controlled by a circadian food-entrainable oscillator (FEO), independent of the main clock in the suprachiasmatic nuclei. However, the anatomical localization and molecular clockwork of the FEO are not well-defined. To study the molecular basis of the FEO, we are currently evaluating the role of the clock gene Rev-erb α in the expression of FAA. Rev-erb α mutant mice ($-/-$: homozygous) and their control littermates ($+/+$: wild-type) were maintained on a 12-12 light-dark cycle (LD) or in constant darkness (DD), with food available ad libitum (AL) except during the period of RF (food access limited to 6h). Wheel-running behavior, general activity and body temperature were recorded. The corticosterone profiles in AL and RF conditions were evaluated in both genotypes. During the scheduled RF, regardless of the lighting conditions (LD or DD), $+/+$ mice exhibited a strong FAA 1h before mealtime, illustrated by increases in wheel-running and general activities. In contrast, $-/-$ mice showed a significant reduction of both wheel-running and general activities. In addition, the rise in body temperature and the peak of corticosterone anticipating

mealtime were also diminished in –/– mice. Expression of clock and clock-controlled genes in the liver oscillator is under investigation. These results suggest that the *Rev-erb α* gene is a component of the FEO clockwork. The implication of *Rev-erb α* not only in food anticipation but also in other feeding and metabolic processes will be considered.

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Absence of a serum melatonin (MT) rhythm under acutely extended darkness (DD) despite an oscillating skeletal muscle clock in the horse

BARBARA MURPHY¹, ANN-MARIE MARTIN¹, CATRIONA BLAKE¹, JEFFREY ELLIOTT²

¹*Veterinary Sciences Centre, University College Dublin, Dublin, IRELAND*

²*Departments of Psychiatry and Psychology, University of California–San Diego, CA, USA*

The 24-h melatonin profile provides a robust marker of circadian phase and is used extensively to estimate circadian adaptation to phase shifts of the light/dark (LD) cycle in humans and rodents. In contrast to studies showing gradual adaptation of melatonin (MT) rhythms to an advanced photoperiod, we previously demonstrated that equine MT rhythms complete a 6-h LD phase advance on the first post-shift day. This suggested that the endogenous clock (SCN) may be less influential than the prevailing LD cycle in regulating melatonin secretion in the horse. These results prompted the current study to investigate whether an endogenous rhythm in equine melatonin exists in DD. As circadian variation in human athletic performance and muscle contraction strength has been reported, we also investigated circadian clock gene expression in equine skeletal muscle. Six mares were maintained in a lightproof barn under an LD cycle that mimicked the external photoperiod prior to blood sample collection at 2-h intervals for 48 h (24-h, LD; 24-h DD, respectively). Beginning at ZT0 of DD, muscle tissue was collected at 4-h intervals via punch biopsy from mid-gluteal muscles. The core clock genes (*Per1*, *Per2*, *Arntl*, *RevErb- α*), and a known clock-controlled gene (*Dbp*) were selected for qPCR using SYBR[®] Green (Applied Biosystems). Serum melatonin was measured by a commercial RIA kit (Alpco, Windham, NH). Results reveal that *Per1*, *Per2*, *Arntl*, *RevErb- α* , and *Dbp* mRNA expression varies significantly ($p < .0001$; $p < .01$; $p < .0001$; $p < .05$; $p < .01$; respectively) under DD. *Per2* and *Arntl* demonstrated antiphase expression patterns, characteristic of the clockwork mechanism. Consistent with previous studies, there was a clear 24-h rhythm in equine MT in LD ($p < .0001$). However, MT remained high throughout the 36 h of DD. Thus, while muscle function displays circadian regulation entrained by the LD cycle, 24-h variation in MT is photoperiod driven in this species.

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Circadian rhythmicity in the response of a collision detecting interneuron in solitary and gregarious locusts

EDWARD GATEN¹, STEPHEN HUSTON², HAROLD DOWSE³, TOM MATHESON¹

¹*Biology, University of Leicester, Leicester, UNITED KINGDOM*

²*Beckman Institute, Caltech, Pasadena, CA, USA*

³*Biology and Ecology, University of Maine, Orono, ME, USA*

In this poster we describe a circadian rhythm in the response of a visual output interneuron, the descending contralateral movement detector (DCMD), of the locust *Schistocerca gregaria*, which differs in animals reared at different population densities. Locusts represent a remarkable expression of phenotypic plasticity, changing from their normal solitary form into the gregarious form that creates economically devastating swarms. They show measurable differences in their morphology, ecology, physiology and behaviour, but in relation to the current work the fact that gregarious individuals are day-active whereas cryptic solitary individuals fly after dusk is of particular interest. DCMD responds to motion and is particularly tuned to signal the approach of an object on a collision course.

It drives neurons in the thorax that participate in flight steering and escape jumping and is a crucial element in the collision avoidance strategy of the locust. The strength of DCMD's response to looming stimuli, characterised by the number of spikes and peak firing rate, varies sinusoidally with a period of approximately 24 h under constant light in both solitary and gregarious individuals. The minimum responsiveness occurs around dawn in all cases. The strongest responses occur around expected dusk in solitary animals, but up to 6 h earlier in gregarious animals. This does not occur through a simple phase shift of the curve, but is consistent with the development of a bimodal curve. Despite this, the time of peak firing relative to predicted collision remains constant, providing the insect with a reliable cue to initiate avoidance behaviours. The circadian rhythm in DCMD's response thus appears tuned to the lifestyle of the animal so that responsiveness is maximal when the animal is most active. This work provides the first evidence for a diurnal rhythm in the nervous system underlying this key behavioural difference, thus providing a neural correlate of behavioural plasticity.

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A honey bee (*Apis mellifera*) light phase response curve

NICOLA LUDIN¹, JAMES CHEESEMAN¹, CRAIG MILLAR², GUY WARMAN¹

¹Anaesthesiology, University of Auckland, Auckland, NEW ZEALAND

²School of Biological Sciences, University of Auckland, Auckland, NEW ZEALAND

Despite the honey bee (*Apis mellifera*) being the subject of extensive study in chronobiology there is no published light Phase Response Curve (PRC) for this species. We report a PRC for honey bees to one hour light pulses using an Aschoff type 1 protocol. One hundred individual pollen forager bee locomotor activity rhythms were recorded for periods of 4-7 days prior to a single one hour light pulse (1,000 lux, AquaRelle 10K Fluorescent combined with an AquaCoral Actinic 03 Fluorescent source, electronic ballast) and 4-7 days following the light pulse using infra-red light beams connected to a data acquisition system (Clocklab™). Seventy six bees survived for the duration of the experiments. Of these thirty-nine showed clear circadian rhythms in locomotor activity before and after the light pulse with an average free running period of (chi2 periodogram) of 23.7 hours (SE ± 5 min). Data from these bees were used to construct the PRC. Twenty two bees showed no clearly defined rhythmic behaviour during the experiments and 15 showed disappearance of the rhythm following light exposure. The bee PRC is a weak (type 1) PRC with a maximum advance of one hour between Ct 3 and Ct 6 and a maximum delay of three hours between Ct 9 and Ct 12. The range of entrainment is thus 22.7-26.7 hours. This phase response curve will be a useful resource for chronobiologists working on entrainment and phase shifting of honey bees. An extension of this work is to conduct concurrent light phase shift experiments with entire hives and individual bees isolated from the hive in order to ascertain whether the entire hive 'super-organism' responds in a similar manner to light as its 'sub-oscillators'.

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Genome-wide analysis of temporal expression in the coral *Acropora millepora*, the lowest multicellular animal, in the context of circadian clocks

OREN LEVY

The Mina and Everard Goodman Faculty of Life Science, Bar Ilan University, Ramat Gan, ISRAEL

Most organisms experience daily changes in their light environment. In order to anticipate these light fluctuations, organisms have evolved a molecular oscillator, known as the circadian clock, which consists of a set of clock genes that generate circadian rhythms by periodic activation of their transcription. Apart from their own regulation, clock genes regulate many genes that are engaged in a diverse range of biochemical and cellular pathways, which ultimately gives rise to a wide variety of physiological, biochemical and behavioural daily rhythms. Identifying the sequences that direct the spatial and temporal expression of genes is a significant challenge in the post-genomic era. Here,

genome-wide expression analysis, bioinformatics tools and experimental procedures were combined in order to expand our knowledge on the cellular and biochemical functions regulated by the circadian clock, in the lowest multicellular animal, the symbiotic coral *Acropora millepora*. This work presents for the first time rhythmic and clock-controlled genes, which were identified in *A. millepora*, using cDNA microarrays. The results indicate that even in a basal animal, key biological processes are hard-wired to the circadian clock, demonstrating the fundamental role of this clock in orchestrating cellular and biological processes, including chaperone activity, RNA processing, metabolic processes, cell-cycle regulation, and many transcription factors, including novel ones.

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Rhythmic behavior in krill: A role for the clock?

OZGE OZKAYA¹, EDWARD GATEN², CHARALAMBOS KYRIACOU¹, GERAINT TARLING³, EZIO ROSATO¹

¹*Genetics, University of Leicester, Leicester, UNITED KINGDOM*

²*Biology, University of Leicester, Leicester, UNITED KINGDOM*

³*Biological Sciences Division, British Antarctic Survey, Cambridge, UNITED KINGDOM*

Antarctic Krill (*Euphausia superba*) make up an estimated biomass of over 500 million tons in the Southern Ocean and represent a very important component of the food chain feeding on plankton and converting this biomass for the consumption of larger marine species like whales, seals, squids and sea birds. Krill exhibit a daily vertical migration moving closer to the surface of the ocean at night to feed and back to the oceanic floor during the day to avoid visually guided predators. It is not known how this predictable behavioural pattern is controlled but it is very likely that it is regulated by an endogenous molecular clock. The aim of this project is to identify the known clock genes in the Antarctic krill for the first time, and describe how daily migrations exhibited by this organism are controlled. Krill have been collected during January and February 2008 as part of cruise JR177 aboard the RRV James Clark Ross. All of the krill were harvested at different time points across the 24 hour cycle by target fishing from the vicinity of Signy Island (South Orkneys) at around 60° South, and the North-West of South Georgia at around 52° South. Vertical migration behaviour was investigated using an activity monitor that recorded the spatial movements of individual krill over a 10 day period, both under a light/dark cycle and in continuous darkness. Krill were also fixed or frozen for gene and protein expression studies at the University of Leicester. So far four canonical clock genes have been identified and cloned from these animals. Ongoing research is investigating the rhythmic expression of these genes throughout the 24 hour cycle.

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The frequency of the behavioral response in *Paramecium tetraurelia* is simultaneously modulated by both ultradian and circadian rhythms

RACHEL MIALKI, LYNDA JONES, ROBERT HINRICHSSEN

Biology, Indiana University of Pennsylvania, Indiana, PA, USA

The behavioral response of *Paramecium tetraurelia* is a change in the direction of swimming, which results from the re-orientation of the ciliary beat and is initiated by the generation of an action potential. It has been shown that the frequency of spontaneous behavioral responses displays an ultradian rhythm, with a periodicity of approximately 50-70 minutes. Additionally, this rhythm is disrupted by the addition of LiCl, which was shown to be caused by the inhibition of the inositol signaling pathway. Here we demonstrate that the frequency of behavioral responses are also influenced by a circadian clock; the amplitude of behavioral responses changes during a 24 hour period, while the periodicity of the ultradian clock remains unchanged during this time. The addition of LiCl also disrupts the circadian rhythm, in that it causes a lengthening of the period from 24 hours to 27.4 hours. However, while the addition of myo-inositol to cells prevents the disruption of the ultradian rhythm, it has no effect

on the disruption of the circadian rhythm. In order to determine the effect of LiCl on the circadian clock, cells were grown in the presence of indirubin, a compound known to inhibit glycogen synthase kinase 3 β ; this enzyme is also inhibited by LiCl. When cells are incubated with indirubin for several hours, the circadian rhythm is lengthened to approximately 27 hours. Therefore, the frequency of the behavioral response in *Paramecium* is influenced by two different biological rhythms, and they each are modulated by separate molecular pathways.

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Investigating the causes of spontaneous internal desynchrony using a physiologically based sleep model

ANDREW PHILLIPS, CHARLES CZEISLER, ELIZABETH KLERMAN

Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

The earliest attempts to ascertain free-running circadian period in humans used self-selected schedules. These conditions yielded unexpected results: (1) free-running periods of 25 hours, with sleep initiated at later circadian phases than during entrainment; (2) spontaneous desynchronization of sleep/wake and circadian rhythms; and (3) bicircadian rhythms. Result (1) was explained by subjects preferentially sleeping at later circadian phases, resulting in light exposure causing phase delay, although the reason for this behavior is unknown. Kronauer et al. (1982) showed that a coupled oscillator model reproduces the experimental observations, but the physiological basis for this phenomenological model is unknown. We use a physiologically-based model to investigate the mechanisms underlying these dynamics. The model includes the mutually inhibitory ventrolateral preoptic area (VLPO) and monoaminergic nuclei (MA), and circadian, homeostatic, and cholinergic/orexinergic drives. We propose that delayed sleep times indicate a wake-promoting drive of behavioral origin, associated with removal of daily schedules, and instructions given to the subject, which we model by increasing orexinergic input to MA during wake. Including this behavioral drive replicates the observed period lengthening, and delayed sleep. Additionally, weakening circadian input to VLPO causes desynchrony, with the period of the rest/activity cycle determined by the degree of orexinergic up-regulation. The model quantitatively predicts the experimental finding that when the rest/activity period lengthens, the observed circadian period shortens, and vice versa. During desynchrony, the observed period of the rest/activity cycle is driven by sleep homeostasis, yet sleep bout length maintains a circadian phase dependence. The model predicts that sleep episodes are shortest when started near the core body temperature minimum, consistent with experimental findings. The model can also display bicircadian dynamics, and correctly predicts that this regime is accessible from either a synchronized or desynchronized state. Our findings suggest that behavioral feedback plays a critical role in spontaneous internal desynchrony.

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Wavelet analysis reveals period wandering in circadian neurons

**KIRSTEN MEEKER¹, RICHARD HARANG², ALEXIS B. WEBB³, FRANCIS J. DOYLE III⁴, GUILLAUME BONNET²,
ERIK D. HERZOG³, LINDA R. PETZOLD¹**

¹*Computer Science, University of California–Santa Barbara, Santa Barbara, CA, USA*

²*Statistics and Applied Probability, University of California–Santa Barbara, Santa Barbara, CA, USA*

³*Biology, Washington University, St. Louis, MO, USA*

⁴*Chemical Engineering, University of California–Santa Barbara, Santa Barbara, CA, USA*

Period variability in circadian oscillators is a consequence of the stochastic nature of the underlying chemical processes. This variability is significant when considering the interaction of circadian neurons

in the Suprachiasmatic nucleus (SCN) and their response to external signals. We use the Morlet continuous wavelet transform to measure the period of the noisy nonstationary circadian oscillator, and demonstrate its use on data from both biological experiment and a stochastic model. Wavelet analysis of the period of individual dispersed cells shows that they sometimes cycle at longer than circadian periods, while cells in a SCN tissue slice do not exhibit these longer periods. We demonstrate that the distinct period variations observed in experiments can be reproduced in a stochastic model by an adjustment of parameter values guided by bifurcation analysis of its nonlinear dynamics. The resulting model suggests that the long periods of individual cells could be produced by switching between two oscillatory mechanisms: the positive feedback exerted by PER-CRY through CLOCK-BMAL1 on the expression of Per and Cry and the negative feedback exerted by CLOCK-BMAL1 through REV-ERB α on the expression of Bmal1. The period of individual cells are then stabilized in SCN tissue with the PER-CRY feedback producing the oscillations.

P96

Neuronal modeling of the SCN

DANIEL FORGER¹, CASEY DIEKMAN²

¹Mathematics and CCMB, University of Michigan, Ann Arbor, MI, USA

²IOE and Bioinformatics, University of Michigan, Ann Arbor, MI, USA

In mammals, the suprachiasmatic nucleus (SCN) acts as the central circadian pacemaker of the brain. I will describe recent progress in modeling the SCN as a neuronal system. Models study the interactions between: 1) ion channels in a single neuron to form complex electrical behaviors, 2) neurons within the SCN to form co-ordinated timekeeping signals and 3) the SCN itself and other brain regions. I will focus on how models are developed from biological data as well as their predictions.

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Distinct functions of the Period genes in the mammalian circadian system: Evidence from in vitro and in vivo studies

JULIE PENDERGAST, RIO FRIDAY, SHIN YAMAZAKI

Biological Sciences, Vanderbilt University, Nashville, TN, USA

To investigate the functions of the three mammalian Period (Per) genes, we examined the in vitro and in vivo phenotypes of C57BL/6J Per mutant mice. In Per1 $-/-$ mice, the Per1-luciferase rhythm was weak or absent in SCN slices in vitro, even though the free-running wheel-running activity rhythm was indistinguishable from wild-type mice. While some neurons in Per1 $-/-$ SCN explants exhibited robust circadian rhythms, others had irregular and/or low amplitude rhythms. The amplitude of the phase response curve (PRC) to light pulses was increased in Per1 $-/-$ mice compared to wild-types, an in vivo phenotype that was consistent with the weak endogenous rhythm of the Per1 $-/-$ SCN measured in vitro. We found that the Per2 $-/-$ phenotype was enhanced in vitro compared to in vivo, such that the period of Per1-luc expression in Per2 $-/-$ SCN explants was 1.5 hours shorter than in wild-type SCN, while the free-running period of wheel-running activity was only 11 minutes shorter in Per2 $-/-$ compared to wild-type mice. The PRC of Per2 $-/-$ mice was phase-advanced compared to the wild-type PRC, suggesting that the phase relationship between the circadian pacemaker and the onset of the activity rhythm in Per2 $-/-$ mice was altered. In Per3 $-/-$ mice, the free-running period of wheel-running activity, the PRC to light pulses, and the endogenous rhythm in the SCN were indistinguishable from wild-types, but the period and phase of Per1-luc expression were significantly altered in pituitary and lung explants. Based on our findings we propose that each Period gene has a distinct function: Per1 participates in coupling between cellular oscillators, Per2 is important for period determination in the SCN, and Per3 plays a role in timekeeping in peripheral oscillators.

Biochemical mechanism of transcriptional control of the mammalian circadian clock by cryptochrome

Rui YE, CHRISTOPHER SELBY, AZIZ SANCAR

*Department of Biochemistry and Biophysics, University of North Carolina,
School of Medicine, Chapel Hill, NC, USA*

The mammalian circadian clock is a biochemical oscillator which is composed primarily of Clock, Bmal, Cryptochrome and Period proteins. In this oscillator, the Clock-Bmal complex is the transcriptional activator, and the Cry and Per proteins work as repressors to repress Clock-Bmal activity. How Cry affect Clock-Bmal DNA binding capacity was investigated both in vitro and in vivo in this report. We have expressed and purified recombinant full length mammalian clock proteins, characterized the binding of Clock-Bmal heterodimer to an E box, and examined the interaction of Cry1 with this protein-DNA complex. We observed that Cry interacts with Clock-Bmal-E box tightly rather than disrupt the Clock-Bmal-E box complex in vitro. Chromatin immunoprecipitation revealed that Cry also binds to the same promoter region of circadian-controlled genes as Clock-Bmal. The absence of Cry protein in a fibroblast cell line increases the amount of Clock-Bmal1 complex on E-Box DNA in vivo. These results indicate that physical interaction between Clock, Bmal1 and Cry does not change the binding activity between Clock-Bmal1 heterodimer with naked E box DNA. However, the recruitment of Cry to the promoter changes Clock-Bmal1 protein dynamics on E-box in vivo.

A functional genomics screen for microRNA regulators of CREB signaling

ANTHONY OLARERIN-GEORGE¹, MICHAEL CONKRIGHT², JOHN HOGENESCH¹

¹Pharmacology, University of Pennsylvania, Philadelphia, PA, USA

²Cancer Biology, The Scripps Research Institute, Jupiter, FL, USA

MicroRNAs (miRNAs) are 18-23 nucleotide RNAs with broad regulatory roles. There are hundreds of miRNAs in the human genome yet only few have experimentally verified functions. In this study, we employ cell-based screening to identify miRNAs that regulate cAMP response element binding (CREB) signaling in the presence of forskolin, PMA, and calcium. We used the profiles of these miRNAs across the experimental conditions to deduce their mechanisms of action. Among the top hits were miR-132 and miR-381. miR-381 reduced CREB signaling by all three stimulants suggesting targeting of a common downstream component. On the other hand, miR-132 regulation of CREB signaling was context dependent; potentiating and inhibiting signaling in the presence of forskolin and PMA, respectively. Interestingly, miR-132, itself a CREB target gene, was previously implicated in MAPK-dependent photic resetting of the clock in the SCN. Here we show that the extracellular regulated kinases (ERKs) are direct targets of miR-132, and their knockdown is responsible for the attenuation of PMA induced CREB signaling, and consequently, MAPK mediated clock resetting.

Functional implications for microRNAs in the intracellular and extracellular regulation of the mammalian circadian clockworks

VIKRAM SHENDE¹, MARIANNA GOLDRICK², SUCHITRA RAMANI², DAVID EARNEST³

¹*Department of Biology, Texas A&M University, College Station, TX, USA*

²*Life Science Products, BIOO Scientific Corp., Austin, TX, USA*

³*Department of NExT and Biology, Texas A&M University Health Science Center, College Station, TX, USA*

MicroRNAs (miRNAs) are small, non-coding RNAs that interact with 3'UTR elements to regulate stability or translation of target mRNAs. miRNAs have been recently implicated in the regulation of a wide array of biological processes including circadian rhythms. Because rhythmicity is a prevalent property among core and regulatory elements of the circadian clock mechanism, we explored possible timekeeping function of miRNAs by first determining whether expression of specific miRNAs predicted to target clock genes oscillate in SCN cells as well as in the periphery. Real-time PCR analysis was used to examine the expression profiles of mature miRNAs that are predicted to target 3'UTR of Bmal1 mRNA because mutations in this clock gene produce behavioral arrhythmicity. Effects of miRNA overexpression on BMAL1 protein levels and 3'UTR activity of this clock gene were also analyzed. In SCN2.2 cells, levels of miR-142-3p were marked by circadian rhythmicity and overexpression of this miRNA decreased BMAL1 protein levels. It is interesting that miRNAs with Bmal1 as a predicted target were also expressed extracellularly in SCN2.2-conditioned medium and in the circulation of mice. Bimodal rhythms in the levels of miR-152, and miR-494 were observed in mouse serum. Luciferase-3'UTR reporter assays indicate that when over-expressed in NIH/3T3 fibroblasts, these miRNAs significantly repress Bmal1 3'UTR-mediated luciferase activity. Our results suggest that miRNAs may play a role in the intracellular and extracellular regulation of the circadian clockworks both within the SCN and the periphery. Supported by NIH P01-NS39546 (DE).

Protein arginine methyl transferase 5 links the circadian clock to the regulation of alternative splicing

SABRINA SANCHEZ¹, EZEQUIEL PETRILLO², ESTEBAN BECKWITH³, XU ZHANG⁴, ESTEBAN HERNANDO¹,
MATÍAS RUGNONE¹, JUSTIN BOREVITZ⁴, M. FERNANDA CERIANI³, ALBERTO KORNBLIHT²,
MARCELO JAVIER YANOVSKY¹, JUAN C. CUEVAS⁵, PALOMA MAS⁵

¹*Plant Physiology, University of Buenos Aires, Buenos Aires, ARGENTINA*

²*IFIBYNE, University of Buenos Aires, Buenos Aires, ARGENTINA*

³*Behavioral Genetics Laboratory, Fundacion Instituto Leloir, Buenos Aires, ARGENTINA*

⁴*Ecology & Evolution, University of Chicago, Chicago, IL, USA*

⁵*IBMB, Barcelona, SPAIN*

Post-transcriptional regulation is emerging as an important component of circadian networks in eukaryotic organisms, but the molecular mechanisms linking the circadian clock to the control of RNA processing are largely unknown. Here we show that Protein Arginine Methyl Transferase 5 (PRMT5), which transfers methyl groups to arginine residues present in Sm spliceosomal proteins, links the circadian clock to the control of alternative splicing in plants and flies. Mutations in *prmt5* impair multiple circadian rhythms in *Arabidopsis thaliana* and this phenotype is caused, at least in part, by a strong alteration in alternative splicing of the core-clock gene PSEUDO RESPONSE REGULATOR 9 (PRR9). Furthermore, genome wide studies show that PRMT5 contributes to regulate many pre-mRNA splicing events, and reveal an important role for PRMT5 in the control of alternative splicing. PRMT5

expression shows diurnal and circadian oscillations, suggesting it could link the circadian clock to the regulation of alternative splicing. Indeed, PRMT5 contributes to modulate rhythms in alternative splicing of RUBISCO ACTIVASE that optimize photosynthesis to daily changes in light conditions. We also show that circadian rhythms in locomotor activity are disrupted in *dart5*, a mutant affected in the *Drosophila melanogaster* PRMT5 homolog, and this is associated with a dramatic alteration in the circadian pattern of alternative splicing of the core-clock gene period (*per*). Our results indicate that the interplay between the circadian clock and the regulation of alternative splicing mediated by PRMT5 constitutes a novel mechanism that helps organisms to synchronize physiological processes with daily changes in environmental conditions.

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The transcriptional repressor ID2 can interact with the canonical clock components CLOCK and BMAL1 and mediate inhibitory effects on mPer1 expression

SARAH WARD, SHANIK FERNANDO, TIM HOU, GILES DUFFIELD

Biological Sciences, University of Notre Dame, Notre Dame, IN, USA

ID2 (Inhibitor of DNA binding 2) is a rhythmically expressed helix-loop-helix (HLH) transcriptional repressor. Deletion of *Id2* in mice results in circadian phenotypes, highlighted by disrupted locomotor activity rhythms and an enhanced photoentrainment response (Duffield et al 2009 *Curr Biol* 19:297-304; Duffield et al 2010 SRBR Abstract). ID2 can suppress the transactivation potential of the positive elements of the clock, CLOCK:BMAL1, on *mPer1* and clock-controlled gene (ccg) activity. Misregulation of ccgs is observed in *Id2*^{-/-} liver, and mutant mice exhibit associated alterations in lipid homeostasis (Hou et al 2009 *J Biol Chem* 284:31735-45). These data suggest that ID2 contributes to both input and output components of the clock, and that this may be via interaction with the bHLH clock proteins CLOCK and BMAL1. The aim of the present study was to explore this potential interaction. Co-immunoprecipitation analysis revealed the capability of ID2 to complex with both CLOCK and BMAL1 proteins, and mammalian 2-hybrid analysis revealed direct interactions between ID2, as well its related family members ID1 and ID3, with CLOCK and BMAL1. Deletion of the ID2 HLH domain rendered ID2 ineffective at inhibiting CLOCK:BMAL1 transactivation activity, suggesting that interaction between the proteins is via this protein-protein binding region. Immunofluorescence analysis of HEK293 cells revealed co-localization of ID2 with CLOCK and BMAL1 predominantly in the cytoplasm. Over-expression of both CLOCK and BMAL1 in the presence of ID2 resulted in a significant reduction in their nuclear localization, revealing that ID2 can sequester CLOCK and BMAL1 to the cytoplasm. Serum-stimulation of *Id2*^{-/-} mouse embryonic fibroblasts resulted in a 2-fold increase in induction of *mPer1* expression compared to wild type cells. These data provide the basis for a molecular mechanism through which ID2 could regulate aspects of both clock input and output through a time-of-day specific interaction with CLOCK and BMAL1.

P103

Identification of a novel Neurospora clock gene by deep sequencing

RANDY LAMBREGHTS¹, JENNIFER LOROS², JAY DUNLAP¹

¹*Genetics, Dartmouth Medical School, Hanover, NH, USA*

²*Genetics and Biochemistry, Dartmouth Medical School, Hanover, NH, USA*

Within the past few years all of the remaining clock genes identified by Feldman and colleagues in the 1970s have been cloned, with the exception of period-2 (*prd-2*), which has proven recalcitrant to traditional genetic mapping techniques. We used a novel selection-based approach to examine the

fine-structure genetic map, and uncovered a complete absence of recombination in a large region of the *prd-2* genome, co-segregating with the long period phenotype. Thus, we re-sequenced the entire genome using Solexa technology. The frequency of SNPs relative to the reference OR74A genome was surprisingly low, and did not yield any candidate mutations in the region of interest. However, paired-end analysis revealed a 300 kb inversion corresponding to the non-recombining region. This genomic rearrangement was predicted to interrupt the promoter and 5' UTR of a highly conserved, but not previously annotated, gene, tentatively named *prd-2*. Consistently, we observed that *prd-2* RNA was drastically reduced, albeit still detectable, in the mutant background. Moreover, heterokaryon knock-out strains show an increase in period length proportional to the reduction in gene dosage, while over-expression of the gene by QA-induction leads to a slightly shorter period. These results re-confirm the usefulness of deep sequencing technology in the cloning of mutations obtained from classical forward genetic screens, particularly those comprising unusual genetic rearrangements. In addition, the newly identified gene *prd-2* does not bear homology to any known clock genes, and is expected to fulfill a functionally novel role in fine-tuning period length in the WT strain. Experiments aimed at dissecting the normal function of the PRD-2 protein are ongoing.

P104

Large-scale analysis of clock gene rhythms in rats housed under normal lighting conditions reveals tissue-specific variability in clock machinery and output

YUVAL WEIGL, BARRY ROBINSON, LINE DUFRESNE, VALERIE HARBOUR, SHIMON AMIR
CSBN, Concordia University, Montréal, Québec, CANADA

In mammals, a master clock in the suprachiasmatic nucleus (SCN) times behavior and physiology by synchronizing downstream oscillators in the brain and periphery. The circadian output of the SCN is endogenous but sensitive to exogenous time cues. Hence, exposure to dominant time cue such as the light-dark cycle masks any differences in the endogenous rhythms and ensures optimal adaptation to the environment. If so, under normal conditions the coupling between the SCN clock and downstream oscillators should be conserved across tissues. To study this hypothesis we measured daily wheel running activity (WRA, a marker of SCN output) in 84 male inbred LEW/CRL rats housed under a 12h:12h light-dark cycle and assessed mRNA expression of two clock genes, *rPer2* and *rBmal1*, and one clock-controlled gene, *rDbp*, in three tissues that are synchronized by the SCN but are also sensitive to masking by non-photic time cues: olfactory bulbs (OB), liver and tail skin. Our data reveal the existence of major phase variability among tissues as a function of clock gene. Furthermore, they point to tissue and gene differences in the magnitude of expression resulting in respective variations in the overall shape of the rhythm. Moreover, variability in clock genes rhythms across tissues is greater than that of the clock output gene, *rDbp*. Finally, the coupling between the SCN and downstream oscillators vary as a function of tissue with stronger coupling seen between the SCN and both liver and OB oscillations. In conclusion, under normal lighting conditions oscillations in clock gene transcript in the liver and OB depend strongly on the SCN clock output whereas oscillations in the tail skin are influenced by other factors and are less dependent on the SCN.

Canonical clock genes regulate circadian rhythmicity and ATP release from cortical astrocytes in vitro

CHRISTIAN BEAULÉ¹, LUCIANO MARPEGAN¹, ADRIENNE SWANSTROM¹, KEVIN CHUNG¹, TATIANA SIMON¹, PHILIP HAYDON², ANDREW C. LIU³, ERIK D. HERZOG¹

¹*Department of Biology, Washington University, Saint Louis, MO, USA*

²*Department of Neuroscience, Tufts University, Boston, MA, USA*

³*Department of Biology, The University of Memphis, Memphis, TN, USA*

Circadian clocks sustain daily oscillations in gene expression, physiology and behavior by relying on intracellular transcription-translation feedback loops of canonical clock genes. Circadian clocks have been described for several mammalian cell types. For example, mammalian astrocytes in culture display circadian rhythms in the expression of the clock genes *Period1* (*Per1*) and *Period2* (*Per2*). Recently, cultured astrocytes were shown to display circadian oscillations of extracellular ATP content, suggesting that ATP release is a circadian output from astrocytes. We hypothesized that the same clock genes that generate circadian rhythms also control circadian ATP release from astrocytes. To test this hypothesis, we first characterized the molecular requirement for circadian rhythmicity in astrocytes by measuring circadian rhythms of *Bmal1::Luciferase* expression in cortical astrocytes derived from established mutant models. We found that rhythms in *Bmal1::Luciferase* required functional *Clock* and *Bmal1*, both *Per1* and *Per2*, and both *Cry1* and *Cry2* genes. In contrast, rhythms in *Bmal1::Luciferase* did not depend on the *Npas2*, *Vip*, or *Vipr2* genes. Next, we tested the effects of clock gene mutations on ATP release using real time bioluminescence reporter of extracellular ATP. We observed circadian rhythms in ATP-driven bioluminescence in wild-type astrocytes. We also found that astrocytes lacking either the *Clock*, or both *Per1* and *Per2* genes had lower ATP-driven bioluminescence than wild types. We also found that a significant proportion of the ATP depended on a calcium-mediated vesicular release. Specifically, cortical astrocytes cultures generated from two transgenic mice models interfering with vesicular release of gliotransmitters (conditional dominant-negative SNARE over expression or conditional *IP3* 5'phosphatase over expression) show significantly lower ATP release compared to wild-types. We conclude that the circadian clock in cortical astrocytes relies on the same canonical clock genes as other mammalian cells, and that rhythms in ATP release constitute one of the circadian outputs of astrocytes. Supported by NIMH grant 63107.

Connexin mRNA expression changes between subjective day and night in rat main olfactory bulb

JOHN CORTHELL, TOM BEARDSLEY, LAURA BLAKEMORE, PAUL TROMBLEY

Biological Science, Florida State University, Tallahassee, FL, USA

Gap junctions are points of electrical and chemical connection between adjacent cells; they are important throughout the brain for enhancing coincident firing between neurons. In the main olfactory bulb (MOB), gap junctions enhance coincident firing between mitral cells synapsing in the same glomerulus. This facilitates the ability of odorant 'messages' of low concentrations to sufficiently excite these cells to send information to the cortex for processing. Gap junctions are made from coupling of connexons on adjacent cells, and these connexons are formed of multiple connexin proteins. Nagy et al. found that 3 connexin proteins are expressed in the MOB: *Connexin 36* (*Cx36*), *43* (*Cx43*), and *45* (*Cx45*). Our analysis of MOB explants via real-time PCR showed that mRNA levels of the three connexin genes, as well as mRNA for a mitochondrial ribosomal protein (*Mrp12*), varied with time of day. We also examined cell cultures that had been treated with melatonin (1-Mel; 3nM) for 4 hours; they showed changes in mRNA levels of those 4 genes. We compared these data to our ICC findings, which revealed

an increase in Cx43 protein expression after treatment with melatonin. These data suggest that changes in gap junction expression may contribute to time-of-day alterations in odor processing. Future studies will include fluorescence-activated cell sorting to determine which cells specifically express which connexin mRNA, additional ICC to determine if gap junctions in the MOB are composed of duplicate connexin junctions (e.g., 43-43 connections) or heterogeneous junctions (e.g., 36-43), further analysis of circadian changes in expression and the effects of melatonin, and patch-clamp electrophysiology to elucidate the biophysical implications of changes in connexin expression.

P107

In vivo Neurospora protein expression using the vvd promoter

JENNIFER HURLEY¹, CHEN-HUI CHEN¹, JENNIFER LOROS², JAY DUNLAP³

¹*Genetics/Biochemistry, Dartmouth Medical School, Hanover, NH, USA*

²*Biochemistry, Dartmouth Medical School, Hanover, NH, USA*

³*Genetics, Dartmouth Medical School, Hanover, NH, USA*

Filamentous fungi are often studied for their use in examining a variety of eukaryotic processes. One such example is the protein processing machinery of fungi, which is able to perform all of the extensive post-translational modification needed in the complex world of eukaryotic organisms. While there are several fungal protein expression systems in place, more could be done to exploit *Neurospora crassa*. Though some promoters such as the qa-2 promoter are used in *Neurospora* to control gene expression; the qa-2 promoter is somewhat leaky and often does not induce protein expression at levels much above those seen in vivo. In order to increase and control in vivo protein expression in *Neurospora*, we are harnessing the vvd gene. Expression of vvd is suppressed in dark conditions but is strongly induced by light reaching high levels within minutes of light induction. Interestingly, the vvd promoter is also auto repressed, meaning that by deleting the vvd gene, we could further increase expression from the vvd promoter. By constructing a strain in which vvd and its promoter have been deleted, and the native promoter of a gene replaced by the vvd promoter, a system can be created in which any gene can be placed under the influence of the vvd promoter, creating a light regulated, in vivo protein over-expression system enabling phenotypic analysis and protein purification of *Neurospora* genes and potentially genes from other eukaryotic organisms.

P108

Transcriptional regulation on circadian rhythm of autophagy

DI MA, JIANDIE LIN

Cell and Developmental Biology, Life Sciences Institute, University of Michigan, Ann Arbor, MI, USA

Autophagy is a lysosome-dependent pathway for the degradation of cytoplasmic components and organelles in the cell. The biochemical pathways that initiate the formation of autophagosome, a double-membrane vesicle containing materials targeted for degradation, and its fusion with lysosome, are highly conserved during evolution. Autophagy is important for energy homeostasis in response to nutrient limitation, such as during starvation and in neonates. In addition, autophagy serves to remove protein aggregates and organelles as well as certain pathogens in the cell. Impaired autophagy has been implicated in the pathogenesis of a wide range of human diseases, such as cancer, neurodegeneration, Crohn's diseases, and type 2 diabetes. Because autophagy is an integral component of energy metabolism, we hypothesized that the activation of autophagy exhibits circadian rhythms and is regulated by the daily oscillation of nutrient and hormonal signals. Here, we found that autophagy undergoes robust circadian rhythm in mouse liver, along with cyclic activation of nutrient and energy signaling pathways. Furthermore, mRNA expression of autophagy genes is regulated by circadian clocks. Finally, we have identified C/EBP β as a novel regulator of autophagy gene expression in primary hepatocytes, which may play a key role in driving the circadian autophagy rhythm.

P109

Global analysis of circadian protein stability

KATJA SCHELLENBERG¹, HSUEH-CHI SHERRY YEN², STEPHEN J. ELLEDGE², ACHIM KRAMER¹

¹*Chronobiology, Charité–Universitätsmedizin, Berlin, GERMANY*

²*Genetics, Center for Genetics and Genomics, Boston, MA, USA*

The precise timing of gene expression and protein abundance is crucial for processes regulating mammalian physiology. We know that about ten percent of the transcriptome are under circadian control often resulting in rhythmic protein levels. Interestingly, circadian protein abundance has been also described for constitutively transcribed genes. This postulates further rhythmic regulation of post-transcriptional, translational and/or degradation processes. In fact, circadian control of protein stability has been suggested for several components of the circadian core oscillator. However, circadian protein stability at the output level has not been studied so far. Here we perform an almost genome-wide high-throughput screen exploring circadian changes of protein stabilities. To this end, a method introduced by the Elledge lab (Yen et. al, Science, 2008) is applied to screen 16.000 human open reading frames (hORFs) stably integrated into human osteosarcoma cells (U-2 OS). In a bicistronic vector each hORF is N-terminally fused to EGFP and simultaneously transcribed with a long-lived DsRed fluorescent protein. Thus, circadian alterations in the stability of the fusion proteins are reflected by changes of the ratio between EGFP and DsRed fluorescence. This is monitored with flow cytometry using synchronized cells harvested in regular 4-hour intervals. For proof-of-concept of the method we detected stability differences of the clock protein PER2 compared to two PER2 mutants exhibiting de- or increased protein half-lives. A pilot-screen covering molecular clock components assesses circadian protein stability of the core oscillator. Investigating global circadian protein stability—the so called circadian “stabilome”—might reveal a further mode of clock output regulation at an essential level of cellular functionality, the proteome.

P110

Functional loss of GSK-3 β and circadian behavior: Impact of direct inhibition of the enzyme versus modulation of its phosphorylation state

LIGIA WESTRICH, JEFFREY SPROUSE

Neuroscience, Lundbeck Research USA, Ramsey, NJ, USA

An increase in period length has long been thought to underlie the efficacy of lithium in patients with bipolar disorder, a condition frequently associated with a shortening of this parameter. At the subcellular level, GSK3 β has been regarded as lithium's primary target with a switch to the enzyme's inactive (phosphorylated) form leading to the rhythm changes. Less is known about direct inhibition of GSK3 β , although period shortening in vitro has been reported (Hirota et al., 2009). In the present study, rats treated with lithium carbonate (100 mg/kg p.o.) responded with shifts in activity onsets as a function of the time of dosing: at ZT11, a phase delay (-0.8 ± 0.2 h) was observed accompanied by an increase in phospho-GSK3 β ; at ZT5, no changes were detected in either phase (-0.2 ± 0.2 , NS) or phosphorylation state. Despite the increase in phospho-GSK3 β at ZT11, functional loss of kinase activity as judged by the degradation of the candidate substrate β -catenin was below detection limits. By contrast, the GSK3 β inhibitor TDZD-8 (30 mg/kg s.c.) yielded no change in phase with dosing near the light:dark transition (-0.4 ± 0.2 , NS) but a significant delay (-1.0 ± 0.2 h) at midday. No phase advances were detected at either dosing times. With respect to the delays, TDZD-8 significantly inhibited β -catenin degradation at ZT11, suggesting that the time of dosing and not the degree of enzyme inhibition is critical. The results indicate that modulation and inhibition of GSK3 β are not equivalent

with differences in the phase-response relationships of activity onsets and questions as to the degree of enzyme loss required for behavioral changes. As to potential clinical benefit, the equivalency of modulation vs. inhibition is unknown, but a better understanding may lie in further study of their respective actions in animal models.

P111

Phosphorylation of CLOCK/NPAS2-BMAL1 complex in suppression of E-box-dependent transcription

HIKARI YOSHITANE¹, NGOC-HIEN DU¹, TOSHIFUMI TAKAO², YOSHITAKA FUKADA¹

¹*Department of Biophysics and Biochemistry, The University of Tokyo, Tokyo, JAPAN*

²*Institute for Protein Research, Osaka University, Osaka, JAPAN*

In mammalian circadian clockwork, CLOCK-BMAL1 heterodimer activates E-box-dependent transcription, while its activity is suppressed by circadian binding with negative regulators such as PERs and CRYs. Here we found that CLOCK protein is kept mostly in the phosphorylated form throughout the day and partly hyperphosphorylated in the suppression phase of E-box-dependent transcription in the mouse liver and NIH3T3 cells. On the other hand, phosphorylation level of BMAL1 has its peak in the activation phase of E-box-dependent transcription. The difference of the phosphorylation events between CLOCK and BMAL1 indicates distinct regulatory mechanisms underlying their phosphorylation processes. Coexpression of CRY2 in NIH3T3 cells inhibited phosphorylation of CLOCK and BMAL1 proteins, whereas CIPC coexpression markedly stimulated CLOCK phosphorylation. A paralog of CLOCK, NPAS2, was also phosphorylated by CIPC in the presence of BMAL1. These data indicate that negative regulators control the phosphorylation states of CLOCK, NPAS2 and BMAL1 in the suppression phase. CLOCK-BMAL1 purified from the mouse liver was subjected to MS/MS analysis, which identified Ser38, Ser42 and Ser427 as *in vivo* phosphorylation sites of CLOCK. Ser38Asp and Ser42Asp mutations of CLOCK additively and markedly weakened the transactivation activity of CLOCK-BMAL1 with down-regulation of the nuclear amount of CLOCK and the DNA-binding activity. On the other hand, CLOCK-delta19 lacking CIPC-binding domain was far less phosphorylated and much more stabilized than wild-type CLOCK *in vivo*. Calyculin A-treatment of cultured NIH3T3 cells promoted CLOCK phosphorylation and facilitated its proteasomal degradation. Together, CLOCK phosphorylation contributes to suppression of CLOCK-BMAL1-mediated transactivation through dual regulation; inhibition of CLOCK activity and promotion of its degradation.

P112

PERL alters Drosophila's circadian rhythms by modifying CLK-mediated transcription

YUE LI, JEROME MENET, KATHARINE ABRUZZI, MICHAEL ROSBASH

Biology, HHMI, Brandeis University, Waltham, MA, USA

The period mutant allele *perL* lengthens *Drosophila* behavioral rhythms to 29 hours. Despite being identified by Konopka and Benzer almost 30 years ago, *perL*'s mechanism of action is completely unknown. In the present study, we first compared the transcriptional profile of *perL* flies to wild-type flies. Transcription of the clock gene *timeless* is dampened in *perL* flies, i.e., it has higher trough and lower peak values than in WT flies. Similar effects were observed on the transcription of a reporter gene. Consistent with the notion that these effects are due to more robust transcriptional repression, the interaction of PERL with CLK is more persistent than in wild-type flies. This may also explain why PERL is a better repressor of CLK/CYC function in S2 cells than wild-type PER, a result in the literature that we verified. During the trough of transcription in *perL* flies, we detect much more CLK bound to DNA via chromatin immunoprecipitation (ChIP) than is observed at the same trough time of wild-type

transcription. This event can explain the elevation of transcription that gives rise to higher trough levels than observed in wild-type flies. The molecular effects of PERL on CLK are more general as PERL also affects CLK post-translational modifications. Indeed, the 24hr rhythm of CLK phosphorylation is dampened in perL fly heads and PERL induces hyper-phosphorylation of CLK in S2 cells. However, it is unclear whether these effects of PERL on CLK phosphorylation occur upstream or downstream of the effects on circadian transcription. Our results taken together provide strong evidence that PERL alters circadian rhythms by affecting CLK-mediated transcription.

P113

Identification of dyschronic, a novel gene involved in circadian output in Drosophila

KYUNGHEE KOH

Neuroscience, University of Pennsylvania, Philadelphia, PA, USA

The molecular mechanisms underlying circadian oscillations of the core clock components and resetting of the clock by light have been investigated in detail, but how the clock output controls rhythmic behavior is not well understood. A forward-genetic screen for *Drosophila* circadian mutants has led to identification of a novel clock gene, *dyschronic* (*dysc*). Homozygous *dysc* mutants display arrhythmic locomotor activity in constant darkness, while heterozygous mutants exhibit wild-type circadian rhythmicity. Notably, daily oscillations of the core clock protein PERIOD (PER) in central clock cells are normal in *dysc* mutants, suggesting that the gene is involved in circadian output. The *dysc* gene maps to a region that does not include any known circadian genes. Results from ongoing experiments on mapping of the gene and detailed phenotypic characterization will be presented.

P114

Identification and analysis of a novel timeless allele that disrupts post-translational control of TIM and PER in Drosophila

TAICHI HARA, KYUNGHEE KOH, DAVID COMBS, AMITA SEHGAL

Department of Neuroscience, University of Pennsylvania, Philadelphia, PA, USA

In the course of a genetic screen, we have isolated a new *tim* allele in which a single point mutation close to the N terminus renders flies arrhythmic. In contrast to the *tim* null allele (*tim01*) that does not have a dominant phenotype, the *tim2611* heterozygotes show a long period of ~25 hours, suggesting that the mutant TIM interferes with the activity of wild type TIM. In this new *tim* mutant fly, both TIM and PER are more stable and do not cycle. In addition, the phosphorylation of PER does not cycle and PER appears to be hypophosphorylated. Moreover TIM and PER expression in the nucleus is greatly reduced in central clock cells, the ventral lateral neurons. This is the first arrhythmic mutant of *tim* in which both proteins are expressed at high levels, but largely in the cytoplasm. Consistent with fly data, PER localizes to the cytoplasm when expressed with the mutant TIM in *Drosophila* S2 cells, while wild-type TIM increases nuclear PER. In addition, the mutant TIM reduces feedback repression by PER in S2 cells. Thus, this new *tim* mutant allele provides us with a tool to identify the mechanism by which TIM affects nuclear entry of PER. Molecular analysis of the new mutant TIM indicates that the mutation affects the nuclear import and phosphorylation of TIM itself. Interestingly, we have found the mutation of a threonine near the original mutation produces the same phenotype as the new *tim* mutant in several assays. These data raise the possibility that a defect in phosphorylation is the basis of TIM dysfunction caused by the novel identified mutation, which then causes defects in PER phosphorylation and localization. TH and KK contributed equally.

A central brain circuit processes photic input to regulate acute masking behavior in Drosophila

BRIAN CHUNG¹, LUOYING ZHANG¹, YIXIAO LIU², GURUSWAMY MAHESH², ROSE-ANNE MEISSNER¹, PAUL HARDIN², RAVI ALLADA¹

¹*Neurobiology and Physiology, Northwestern University, Evanston, IL, USA*

²*Biology and Center for Biological Clocks Research, Texas A&M University, College Station, TX, USA*

Daily animal behavior is largely coordinated by endogenous timing signals from circadian clocks and environmental stimuli, such as light. In the fruit fly *Drosophila melanogaster*, the circadian system allows the animal to anticipate and synchronize rhythmic processes to the 24-hour solar cycle. However, light can also acutely enhance fly activity, a phenomenon often referred to as masking. It remains unclear how signals from both processes are integrated within central brain regions to drive timely and adaptive behavior. To address this question, we used a previously described mutant for a novel ion channel narrow abdomen (na), which lack an acute masking response and show reduced anticipatory behavior and free-running rhythms, thus providing a genetic link between photic response and circadian function. Rescue of na in ~16-20 DN1p circadian pacemaker neurons was sufficient to restore acute masking and morning anticipatory behavior. Backcrossed glass2 mutants (gl2) do not exhibit an acute masking response and also lack DN1 cells, both of which are in agreement with previous findings in gl60j mutants (Rieger et al., 2003) and na rescue data. Additionally, acute masking was significantly reduced when na rescue in DN1p was performed on a period null background (per01), suggesting a link between the circadian clock and masking behavior. na rescue of acute masking behavior in DN1p persists across multiple circadian phases under 3.5L:3.5D conditions and different temperatures. Interestingly, loss of both the visual system (GMR-hid) and the circadian clock (per01) significantly decreases the animal's ability to respond acutely to light, even though per01 or GMR-hid mutants alone do not produce any discernable effects on masking behavior. Taken together, our data reveals an important role for a highly specific group of circadian pacemaker neurons in acute light-induced activity. Future endeavors are directed at uncovering the photoreceptive substrates and visual pathways required for DN1p-mediated behavioral output.

New genetic determinants of temperature entrainment in Drosophila

TADAHIRO GODA, JAKE CURRIE, HERMAN WIJNEN

Biology, University of Virginia, Charlottesville, VA, USA

Many organisms have temperature-entrainable circadian clocks. In homeotherms clocks may be entrained to internal body temperature rhythms, while poikilotherms such as *Drosophila* can entrain to environmental temperature cycles. In previous analyses we have shown that the *Drosophila* circadian clock is remarkably sensitive to daily rhythms in relative temperature, but that wild-type flies reset their behavioral phase relatively slowly in response to daily temperature gradients. Recent experiments show that molecular rhythms of the PERIOD and TIMELESS clock proteins in fly heads are reset by a temperature cycle more quickly than rhythmic locomotor behavior, suggesting differential regulation in peripheral clocks as compared to the cells of the behavioral circadian pacemaker. Among several newly identified genetic determinants of temperature-dependent behavioral re-entrainment are mutations affecting the clock gene timeless (tim) and the the *Drosophila* Hsp90 ortholog heat shock protein 83 (hsp83). Transgenic rescue constructs for tim that restore free running rhythms but fail to produce rhythmic transcript levels specifically affect accelerated temperature-dependent resetting of locomotor behavior and a similar phenotype is observed for hsp83e6D/08445 transheterozygote mutants. We will present recent results addressing the role of tim expression as well as Hsp90 function in mediating circadian temperature entrainment.

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DPP signaling contributes to set basic properties of the PDF circuit in Drosophila

ESTEBAN BECKWITH, JIMENA BERNI, M. FERNANDA CERIANI

*Laboratorio de Genética del Comportamiento, Fundación Instituto
Leloir, IIBA CONICET, Buenos Aires, ARGENTINA*

Living organisms use circadian rhythms to maintain internal temporal order and anticipate daily environmental changes. Clocks employ self-sustained biochemical oscillators and manifest at molecular, physiological and behavioral levels. In *Drosophila*, a group of neurons expressing the Pigment Dispersing Factor (PDF) represent the “central oscillator” of the fly brain. As a result of a misexpression screen using the GAL4/UAS system we identified a fly strain that causes period lengthening of the daily activity rhythms. The transposon landed within *schurri* (*shn*), a nuclear component of the decapentaplegic (*dpp*) pathway. *shn* overexpression in the PDF circuit was necessary and sufficient to generate a 25.5h period of locomotor behavior. Interestingly, constitutive overexpression of activated receptors that initiate the DPP signalling cascade also gave rise to long period phenotypes, in agreement with those observed upon *shn* overexpression. In contrast, downregulation of the endogenous receptors also impacted circadian rhythmicity leading to arrhythmicity. A detailed analysis of PER subcellular localization showed a delayed PER nuclear entry in the mutant compared to that of wild type flies, pointing to a specific effect of *shn* deregulation on the core clock mechanism. *shn* overexpression in the PDF circuit led to a reduction in the PDF immunoreactivity at the dorsal termini, which is not due to transcriptional downregulation. Concomitantly, *shn* overexpression associated effects in the structure of the PDF circuit accompanied by a defect in the branching phenotype that leading to shorter circuits. Recently, the *dpp* pathway has been shown to operate as a retrograde signal that set synaptic properties and, in combination with other cellular clues, establish the peptidergic fate of a specific circuit in the fly brain. Ongoing experiments will explore whether this pathway is relevant in the assembly and maintenance of the PDF circuit integrity.

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Circadian rhythm of Arabidopsis petal movement

SHINSUKE KUTSUNA, SHOKO KOBAYASHI, KATSUSHI MANABE

International College of Arts and Sciences, Yokohama City University, Yokohama, JAPAN

Some flowers open in the morning. And some ones do in the evening. Linne proposed the idea of the floral clock which was made up of various kinds of flowers opening at different time each other. Recently, variation of the timing among closely related plant species in the field has been studied on aspect of the differentiation of reproductive timing. Here, we present chronobiological features of flower opening in *Arabidopsis thaliana*. The plant opens flower in the morning, but the detail is unknown. Then, we examined it under diurnal light/dark cycle. The petal began the expansion when lamp started illumination. Then, it opened completely for 3 hours in the light. We examined whether the rhythm continued in light without the daily dark periods. Observation of four days in light showed the rhythm with 23 hours period. The period was not changed in low or high temperature. In addition, the rhythm was entrained by external light/dark cycles. These properties are essential to circadian rhythm. Thus, *Arabidopsis* petal movement is under circadian clock. The chronobiological properties will allow us to know signal transduction of the movement under circadian clock.

Circadian transcription in the dark without cyclic kai gene expression I: Clock-gating of dark-induced transcription in Synechococcus

NORIMUNE HOSOKAWA, TETSUHIRO HATAKEYAMA, HIDEO IWASAKI

Department of Electrical Engineering and Bioscience, Waseda University, Tokyo, JAPAN

In the unicellular cyanobacterium *Synechococcus elongatus* PCC7942, most of gene expression is controlled by the circadian clock under continuous light (LL) conditions. When cells are transferred to continuous dark (DD) conditions from hour 12 in the light, transcription of the *kaiA* and *kaiBC* genes is rapidly suppressed to the zero level, whereas the KaiC phosphorylation cycle remains even in the presence of excess transcription/translation inhibitors (Tomita et al., 2005). DNA microarray analysis further demonstrated that expression of most of genes on the genome was also dramatically and rapidly down-regulated in the dark. Thus, we previously suggested that the clock-regulated transcription exclusively occurs in LL (Ito et al., 2009). To validate this possibility, we got interested in a minor subset of genes which was upregulated in the dark (Ito et al., 2009), and examined if such dark-induction was affected by the Kai-based clock even in the absence of *kai* gene expression. Thus, we performed DNA microarray analysis in the *kaiABC*-null mutant under DD conditions, and demonstrated that approximately 30% of dark-induced genes (4% of all genes) were regulated by the *kai* genes. Moreover, the magnitude of dark-induction in some of these genes was dependent on subjective time when the cells were transferred from the light to the dark. Thus, in contrast to our previous model, the *Synechococcus* clock regulates transcriptional outputs even in the dark.

Circadian transcription in the dark without cyclic kai gene expression II: Damping oscillation in expression of a dark-induced gene

TETSUHIRO HATAKEYAMA, NORIMUNE HOSOKAWA, HIDEO IWASAKI

Department of Electrical Engineering and Bioscience, Waseda University, Tokyo, JAPAN

In the unicellular cyanobacterium, *Synechococcus elongatus* PCC7942, most of genes show circadian expression rhythms in continuous light (LL) conditions. When cells are transferred to continuous dark (DD) conditions, expression of most genes, including the *kai* genes, is severely repressed while >10% of genes on the genome called dark-induced genes (digs) are upregulated. Because expression of digs is not highly rhythmic in LL and the clock gene expression is completely suppressed in the dark, it was previously suggested that clock-controlled transcription was nullified in the dark (Ito et al., 2009). However, in contrast to the previous model, we discovered that dark-induction of some digs was time-of-day-dependent and dramatically affected by the *kai* genes (Hosokawa, Hatakeyama and Iwasaki, an accompanying presentation). Here, we found that expression of a dark-induced gene, designated *dig2* encoding an unknown protein, showed a damping oscillation in DD. This damping oscillation disappeared in the *kaiABC*-null mutant. Furthermore, we demonstrated that the *dig2* transcription cycle was temperature-compensated. These results suggest that the *Synechococcus* clock is capable of regulating transcriptional outputs periodically in DD even when transcription/translation-feedback of the clock genes is nullified.

Insight into teenage sleep patterns from the slow-developing rodent, Octodon degus: Pubertal hormones act on the circadian system

MEGAN HAGENAUER¹, JENNIFER KU², DAVID ALTSHULER¹, SHUOQI WANG¹, THERESA LEE²

¹Neuroscience, University of Michigan, Ann Arbor, MI, USA

²Psychology, University of Michigan, Ann Arbor, MI, USA

Human teenagers are widely considered to have sleep and circadian rhythms that differ from those of adults. These differences may be, in part, due to pubertal increases in gonadal hormones, as overwhelming evidence in both humans and animal models indicates that the circadian system is hormonally-sensitive. Despite this, there is a shortage of research studies focusing on circadian rhythms during puberty in animal models. We have addressed this understudied area using the diurnal and slow-developing rodent species *Octodon degus* (degu). The circadian system in the degu continues to develop across the post-weaning and pubertal periods in a manner that is strongly sexually-differentiated. In male degus, these changes are robust, involving a switch from bimodal to unimodal activity patterns as well as a 3-5 hr magnitude phase-advance. These changes occur between the ages of P90 to P160, after the males have already developed initial secondary sex characteristics (preputial opening) but before they have completed pubertal development as indicated by penis spike growth. In contrast, females show little circadian change across the pubertal period. Pre-pubertal gonadectomy prevents circadian changes in the males, suggesting that pubertal gonadal hormones are likely to drive circadian changes in males. These behavioral changes are likely to be reflected at the level of the circadian pacemaker, as preliminary evidence indicates that *Per1* transcript rhythms in the suprachiasmatic nucleus are shifted during puberty. Paralleling behavioral data, pubertal male degus show *Per1* rhythms that are 3-6 hrs phase delayed compared to postpubertal males. These results are remarkably similar to changes we observed during puberty in the fast-developing rat and those reported for humans. Therefore, we conclude that the processes governing daily sleep and activity rhythms are still developing far into the pubertal period in many mammals.

Differential effects of voluntary and induced wakefulness in arousal and reward areas in a diurnal brain

ALEXANDRA CASTILLO-RUIZ, LAURA SMALE, ANTONIO NUNEZ

Psychology, Michigan State University, East Lansing, MI, USA

In the diurnal grass rat, access to a running wheel produces a voluntary switch in the phase preference for locomotor activity, with some individuals shifting to become predominantly active during the night (night-active, NA), while others keep their diurnal activity pattern. We found that, when compared to animals with no wheel access, NAs have increased neural activation at night in areas of the brain that mediate wakefulness and reward, such as the basal forebrain (BF), supramammillary nucleus (SUM), and anterior ventral tegmental area (aVTA) (Neuroscience 165, 337-349, 2010). In this study, we examined whether an involuntary wakefulness paradigm would result in similar patterns of neural activation in animals housed with no wheels. We assessed this question using male grass rats that were maintained awake by gentle stimulation (W group) from Zeitgeber Time (12L:12D cycle) 10 to 16 (same time point analyzed in the aforementioned study) and examined expression of Fos and/or choline acetyltransferase in three major cell groups of the BF- the medial septum (MS), and vertical and horizontal diagonal band of Broca- as well as expression of Fos and/or tyroxine hydroxylase in the SUM and the VTA. We compared these results to those obtained from an undisturbed control group (C). Different from previous results with NAs, only non-cholinergic neurons of the MS of the W group had more Fos expression than Cs. Overall these results suggest that forced and voluntary wakefulness during the night have different effects in neural systems involved in wakefulness and

reward. Because these areas project widely throughout the brain, changes in their functioning are likely to affect a broad range of neurological functions and behaviors. These observations are important for the evaluation of animal models of human shift work, some of which depend upon experimentally induced wakefulness during the animals' rest phase.

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The regulation of orexin receptors by dynein light chains

**ERIKA BELANGER-NELSON¹, DAVID DUGUAY¹, VALÉRIE MONGRAIN², ANNA BEBEN⁴, ARMEN KHATCHADOURIAN⁵,
NICOLAS CERMAKIAN³**

¹*Neurology and Neurosurgery, McGill University, Montréal, Québec, CANADA*

²*Department of Psychiatry, University of Montreal, Montréal, Québec, CANADA*

³*Department of Psychiatry, Neurology and Neurosurgery, McGill University, Montréal, Québec, CANADA*

⁴*Biochemistry, Carleton University, Ottawa, Ontario, CANADA*

⁵*Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, CANADA*

Orexins (OX-A and OX-B) are neuropeptides involved in the regulation of sleep-wake cycles, feeding and reward. These peptides act via G-protein coupled receptors, Orexin Receptors 1 and 2 (OX1R, OX2R). The daily release of orexins is under both circadian and homeostatic influence. Moreover, loss of orexins or mutations of the receptors were shown to cause the sleep disorder, narcolepsy. In aim to understand the intracellular mechanisms involved upon activation of these receptors, we have identified the dynein light chain Dynlt1 as a partner of OX1R. Our hypothesis is that Dynlt1 is important for OX1R intracellular regulation. Yeast two-hybrid assays were used to measure the interaction of OX1R and Dynlt1 and identified a motif in OX1R C-terminus, and the C-terminus of Dynlt1, as being crucial for the interaction, which was confirmed in mammalian cells by co-immunoprecipitation. Western blots on extracts of HEK293 cells expressing OX1R with or without Dynlt1, and stimulated with OX-A, showed that the ERK pathway response to OX-A is less sustained upon over-expression of Dynlt1, while down-regulation of the dynein light chain has the opposite effect (i.e. prolonged activation). This effect is not due to changes in ligand-induced internalization of the receptor, as seen by surface ELISA on HEK293 cells following OX-A stimulation and upon over-expression or down-regulation of Dynlt1. Confocal microscopy was used to visualize the intracellular localization of OX1R-GFP in HEK293 cells following OX-A stimulation. At resting conditions, OX1R is localized to the plasma membrane, and it is internalized and localizes to early endosomes following stimulation with OX-A. Preliminary results indicate that more OX1R is in endosomes upon Dynlt1 over-expression. Our work suggests a role for Dynlt1 in OX1R intracellular trafficking. This research will enhance our knowledge of proteins required for physiological orexin receptor function and thus may aid in developing pharmacological treatment for orexin-related diseases. Funding: Canadian Institutes of Health Research.

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Per genes regulate the metabolic response to sleep loss

JANA HUSSE¹, CHARLOTTE HINTZE², GREGOR EICHELE¹, HENDRIK LEHNERT³, HENRIK OSTER²

¹*Genes & Behavior, Max Planck Institute for Biophysical Chemistry, Göttingen, GERMANY*

²*Circadian Rhythms Group, Max Planck Institute for Biophysical Chemistry, Göttingen, GERMANY*

³*1st Medical Clinic, University of Lübeck, Lübeck, GERMANY*

Human and animal studies show that sleep has a strong impact on the regulation of metabolism. Lack of sleep increases food intake correlating with a deregulation of peripheral appetite-controlling factors such as leptin or ghrelin. Many metabolically relevant pathways are controlled by the circadian clock which prompted us to analyze the impact of clock function on the metabolic response to sleep restriction. We used a gentle handling approach in wild-type and congenic Per1/2 double mutant mice

that lack a functional circadian clock. Mice were sleep deprived for 6 hrs each morning (ZT0-6) on 5 consecutive days. We measured locomotor activity, body weight and food intake as well as circulating hormones and gene expression profiles in adipose tissue and liver before, during and after sleep restriction. *Per1/2* mutants showed significant alterations in the metabolic effects of sleep restriction at both behavioral and physiological levels. Our findings suggest that the circadian clock is functionally involved in the regulation of metabolic homeostasis by sleep.

P125

Circadian rhythms and sleep behavior in an annelid worm, Lumbriculus variegatus

MARK ZORAN

Department of Biology, Texas A&M University, College Station, TX, USA

The oligochaete worm, *Lumbriculus variegatus*, lives in freshwaters with its tail extended into the water column. This species-specific posture is common during daylight hours and involves long periods of inactivity. Consequently, tails are vulnerable to predatory attack and exhibit rapid escape responses. We hypothesized that, like terrestrial oligochaetes, these aquatic worms are nocturnal. The ventilatory posture facilitates gas exchange and oxygenated blood is circulated by peristaltic waves along the dorsal blood vessel. Using a photodiode-based device to monitor locomotor activity and circulatory physiology, biological rhythms in these activities were discovered that entrained to a light/dark cycle and persisted in constant conditions. Bouts of locomotor activity were significantly elevated during the dark phase. Rhythms in activity were accompanied by elevated blood vessel pulse rates at night. Interestingly, these rhythms persisted in regenerating body fragments following head and tail removal. We further hypothesized that this diurnal ventilatory behavior may represent a form of animal sleep. To test this, worms were deprived of assuming the ventilatory posture for a period of 24 hours with constant agitation and subsequent animal behavior was quantified via time-lapse video monitoring. Agitated worms exhibited an increase in ventilatory behavior in the next nocturnal phase similar to sleep rebound observed in other animals. Similar results were obtained with caffeine treatment. Finally, using real-time respirometry techniques, a rhythm in oxygen consumption was observed where animals consumed more oxygen per body mass during the interface between night and day (dawn) and consumed the least oxygen near dusk. Taken together, these results demonstrate that *Lumbriculus* is a nocturnal animal with biological rhythms in physiology and behavior, and these rhythms persist in isolated body fragments. Furthermore, this rhythmic ventilatory behavior involves a sleep-like state of sustained inactivity that rebounds following behavioral deprivation, indicating that this annelid worm sleeps during the day.

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Elevated NOX-4 and oxidant stress in Bmal1-KO mice

CIPRIAN ANEA¹, ALI M. IRFAN², DAVID STEPP², STEPHEN BLACK², R. DANIEL RUDIC¹

¹Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA, USA

²Vascular Biology, Medical College of Georgia, Augusta, GA, USA

In blood vessels, the path to disease is inextricably linked to elevations in superoxide production which impair the normal biology and signaling in vascular endothelial and smooth muscle cells. While recent discoveries have demonstrated that circadian clock dysfunction promotes detrimental processes in

blood vessels, including endothelial dysfunction, pathological remodeling, and aberrant signaling, the effect of oxidant stress in the vasculature is yet unknown. Herein, we undertook studies to examine oxidative stress in arteries of Bmal1-KO mice. Electron paramagnetic resonance (EPR) spectroscopy revealed increased levels of superoxide in aortae of Bmal1-KO mice. Localization of superoxide determined by dihydroethidium (DHE) fluorescence in aorta and common carotid arteries revealed an increase in the endothelium and medial smooth muscle layers. To determine the molecular source of increased superoxide production in Bmal1-KO mice, we assessed expression of NADPH oxidase (NOX) isoforms using real-time quantitative RT-PCR in aortae of mice. While NOX1 was not different between WT and Bmal1-KO, NOX4 expression was increased in Bmal1-KO. In remodeled vessels DHE staining was further exacerbated in Bmal1-KO mice compared to WT arteries. Furthermore, endothelial dysfunction in aortic rings of Bmal1-KO mice [assessed by the relaxant response to acetylcholine] was improved when the blood vessels were incubated in the presence of the superoxide scavenger, superoxide dismutase (SOD). These data suggest that the circadian clock may exert a significant influence in the regulation of superoxide which could be an important factor in the development and progression of pathology in arteries during vascular disease.

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Polymorphisms in BMAL1 (MOP3) and PER3 are associated with features of obesity in man

MADHU J. PRASAI¹, DANYELLA PEREIRA², SIMON ARCHER³, MALCOLM VON SCHANTZ³, SERGIO TUFIK², MARIO PEDRAZZOLI⁴, ANGELA M. CARTER¹, PETER J. GRANT¹, ELEANOR M. SCOTT¹

¹*Division of Cardiovascular and Diabetes Research, University of Leeds, Leeds, UNITED KINGDOM*

²*Psychobiology Department, Universidade Federal de Sao Paulo, Sao Paulo, BRAZIL*

³*Faculty of Health & Medical Sciences, University of Surrey, Guildford, UNITED KINGDOM*

⁴*Gerontology, Universidade Federal de Sao Paulo, Sao Paulo, BRAZIL*

Introduction: Environmental and genetic circadian clock disruption have both been linked to the development of obesity. Animal models have shown that BMAL1 is important in glucose homeostasis and adipogenesis, whilst Period is involved in feeding behaviour and sleep homeostasis. The aim of the present study was to investigate the relationship of selected polymorphisms in BMAL1 and PER3 to features of obesity and the metabolic syndrome in healthy subjects. **Methods:** We performed genotype analysis of a polymorphism in BMAL1 (rs7950226, A>G) and in PER3 (rs57875989, coding region variable number tandem repeat [VNTR]) in 537 healthy individuals from 89 different families, characterised for metabolic, atherothrombotic and inflammatory risk factors associated with insulin resistance. **Results:** The polymorphism rs7950226 in BMAL1 was associated with waist circumference ($p=0.04$) and the homeostatic model assessment (HOMA) measure of insulin resistance ($p=0.05$). Homozygosity for the longer variant of the PER3 VNTR (PER35) associated with increased plasma leptin ($p=0.007$) and hypertension ($p=0.002$). **Conclusions:** Polymorphisms in BMAL1 have recently been associated with type 2 diabetes in a family study and our data in healthy subjects support an association with insulin resistance. The PER3 VNTR has been associated with altered susceptibility to the effects of sleep deprivation. Sleep deprivation has been linked to development of obesity and hypertension and leptin levels reflect adiposity and modulate feeding behaviour. Thus, this polymorphism may influence other responses to sleep deprivation as well. Taken together these data support the hypothesis that genetic variation in the circadian clock plays a role in the development of features associated with obesity in man.

RIP140 is a conduit for circadian control of genes involved in lipid metabolism

JOSHUA GAMSBY¹, KELLY HEIM², JENNIFER DAVEY², MARY HEVER², SARAH FREEMANTLE²,
MICHAEL SPINELLA², JENNIFER LOROS¹, JAY DUNLAP¹

¹Genetics, Dartmouth College, Hanover, NH, USA

²Pharmacology and Toxicology, Dartmouth College, Hanover, NH, USA

The connection between the circadian clock and hormonal signaling is an actively studied field of Chronobiology. Investigation of how these two processes impinge upon one another provides clues as to how the clock controls various output systems such as cellular metabolism. Here we provide more insight into this connection through the characterization of a clock controlled gene (ccg), RIP140 and its potential role in metabolic output. RIP140 is a nuclear receptor ligand-dependent interacting protein that can also bind to orphan nuclear receptors, such as Rev-erb and RORs, and is induced by hormones such as retinoic acid and estrogen. RIP140 has been shown to primarily function as a co-repressor of nuclear receptor signaling and is involved in energy expenditure by suppressing genes involved in glucose and lipid metabolism. We confirm that this gene is a ccg that oscillates in phase with BMAL1 in mouse embryonic fibroblast (MEF) cells, potentially through the control of the positive arm proteins RORs and Rev-erbs. Additionally, we show that this control is potentially mediated through putative intronic ROR and Rev-Erb response elements (RREs). Furthermore, overexpression of RIP140 represses ROR-mediated transactivation of the BMAL1 promoter, and suggests a RIP140/ROR feedback loop that is responsible for RIP140's circadian oscillation. We also show that RIP140 $-/-$ MEF cells have a fully functional circadian clock, but the expression profile and potential oscillation of ccgs that are also transcriptionally controlled by RIP140 during hormonal signaling is lost. These findings suggest that RIP140 is a second order ccg that controls the homeostatic oscillation of tertiary ccgs that are involved in lipid metabolism.

Diet-induced obesity attenuates diurnal variation in glucose tolerance and insulin sensitivity—a possible role for the molecular clock?

MADHU J. PRASAI, STEPHEN B. WHEATCROFT, PETER J. GRANT, ELEANOR M. SCOTT

LIGHT Laboratories, University of Leeds, Leeds, UNITED KINGDOM

Introduction: There is a known diurnal rhythm of glucose metabolism with enhancement of insulin sensitivity at the onset of the active period. Obesity is recognised to induce insulin resistance and impaired glucose handling. Recent studies have indicated a role for the molecular clock in the pathogenesis of obesity and in regulating normal diurnal glucose metabolism. In a mouse model of diet-induced obesity we postulated a link between disruption of core clock gene cycling in metabolically active tissues and dampening of diurnal variation in glucose and insulin tolerance testing (GTT and ITT). **Methods:** C57BL6/J mice were fed a high fat diet or control chow diet for 10 weeks. Intraperitoneal GTT and ITT were performed at ZT2 and ZT14. RNA was extracted from epididymal fat and liver samples at ZT2, ZT8, ZT14 and ZT20 and analysed by quantitative PCR for Bmal-1, Clock and Per2 expression. **Results:** Body mass and epididymal fat pad mass were significantly different between the two groups ($p < 0.0001$). GTT: overall glucose tolerance calculated by area under the curve (AUC) showed no significant diurnal variation in either obese or lean mice but there was a higher 30 minute glucose peak at ZT2 compared to ZT14, which difference was less pronounced in obese mice ($p < 0.03$). ITT: overall insulin sensitivity was enhanced at ZT14 compared to ZT2 in both obese and lean mice (AUC: $p < 0.02$ and $p = 0.0004$ respectively). The variation in 60 minute glucose nadir at the two time

points was attenuated in obese compared to lean animals ($p < 0.004$). Quantitative PCR: there was significant blunting of core clock gene cycling in epididymal fat but not in liver. Conclusions: Diet-induced obesity is associated with blunting of diurnal variation in GTT and ITT which may be related to disruption of core clock gene cycling in metabolically sensitive tissues.

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Mitochondria dynamics and circadian rhythms

KAREN SCHMITT¹, LUCIA PAGANI¹, STEVEN BROWN², STEPHEN FRANK³, ANNE ECKERT¹

¹*Neurobiology Laboratory, Psychiatric University Clinic Basel, Basel, SWITZERLAND*

²*Institute of Pharmacology and Toxicology, University of Zurich, Zurich, SWITZERLAND*

³*Department of Neuropathology, Institute of Pathology, Basel, SWITZERLAND*

Circadian rhythms are needed to coordinate a variety of physiological processes such as metabolism in order to anticipate the periodical changes of the external environment. Mitochondria are recognized to play a pivotal role in cell survival and death by regulation of both energy metabolism and apoptotic pathways. In mitochondria, metabolism (i.e. ROS production) and cellular defence (i.e. ROS scavengers induced by melatonin) mechanisms seem to be coordinated by the circadian clock. Several evidences indicate that control and maintenance of functional mitochondria require a tightly regulated equilibrium between opposing mitochondrial fusion and fission activities. Nevertheless, the link between mitochondrial dynamics and circadian rhythms remains unknown at the present. In our study, we addressed the question of whether the biological clock could be involved in the modulation of mitochondrial dynamics. For this purpose, we are currently investigating the expression levels of mitochondrial fusion (MFN1, MFN2, and OPA1) and fission (DNML1, Fis1) genes in human primary skin fibroblasts around the clock. Acknowledgements: This work was supported by grants from the Swiss National Science Foundation (#31000_122572) and Synapses Foundation.

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In vitro molecular chronopharmacology of anticancer drug irinotecan on human cancer cells

SANDRINE DULONG¹, ANNABELLE BALLESTA², LISA HENNING¹, CHADI ABBARA³, JEAN CLAIRAMBAULT², FRANCIS LÉVI¹

¹*U776, INSERM, Villejuif, FRANCE*

²*U776, INRIA, Rocquencourt, FRANCE*

³*U776, AP-HP, Villejuif, FRANCE*

Irinotecan is a topoisomerase I (Top1) inhibitor which is active against human colorectal cancer and can display severe toxicities in patients. Circadian timing in mice and in cancer patients can modify irinotecan toxicity. Here, we investigate irinotecan chronopharmacology in human Caco-2 cells in order to determine the critical parameter values required for the design of optimal drug delivery schedules in individual patients.

Methods: The circadian clocks of Caco-2 were synchronized with 2 h-serum shock, so as to systematically map the temporal relations between circadian clocks (Per2, Bmal1 and Rev-erb α) and irinotecan transport (Abcb1; Abcc1, Abcc2, Abcg2), bioactivation and detoxification (CES2, UGT1A1) and molecular target (TOPO1). We determine mRNA and protein expressions with qPCR and Western blots on cells sampled every 4 h for 48 h in 3 experiments. Synchronized cells were also exposed to irinotecan at different CT, and apoptosis was measured. Results: Rhythmic mRNA expression of clock genes Rev-erb α , Bmal1 and Per2 was demonstrated ($p < 0.001$), with mean period length of 26.6 ± 0.3 h. The acrophase ϕ of Rev-erb α occurred at 10.4 h and that of Bmal1 was located at 17.0 h, a finding which supports their known reciprocal regulations. Rhythmic mRNA expression was significantly

validated for Abcb1 ($\phi=16.1$ h), Abcc1 ($\phi=10.3$ h), Abcc2 ($\phi=13.6$ h), Abcg2 ($\phi=13.6$ h), Ugt1a1 ($\phi=14.7$ h), and Top1 ($\phi=15.6$ h) but not for Ces2. Irinotecan-induced apoptosis was enhanced in cells exposed at CT14, when Top1 was highest and least at CT2, when Top1 was lowest. Conclusion: Synchronized human Caco-2 cells constitute an in vitro model for quantitative investigation of multiple cellular and molecular mechanisms of anticancer drug chronopharmacology. Supported by the EU (STREPTEMPO, LSHG-CT-2006-037543).

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Control of cancer progression through reinforcement of host circadian physiology and rhythmic induction of tumor stress genes with circadian meal timing

XIAO-MEI LI

Rythmes Biologiques et Cancers, INSERM U776, Villejuif, FRANCE

Background Circadian disruption accelerates experimental and clinical cancer progression. Purpose To slow down cancer progression through the reinforcement of host circadian physiology and/or tumor molecular clocks with meal timing (MT). Methods Mice bearing Glasgow osteosarcoma (GOS) or pancreatic adenocarcinoma (P03) were synchronized with LD12:12. They were fed ad libitum or with MT for 4 or 6h during light or darkness with normal diet. The circadian timing system was assessed through 1/ telemetered rest-activity and body temperature and 2/circadian liver and tumor mRNA expression using qPCR for clock genes (Rev-erb β , Per2 and Bmal1) and clock-controlled temperature-sensitive stress genes (Hspa8 and Cirbp). Results: In GOS-bearing mice, MT during darkness doubled the circadian amplitude in host body temperature and reduced tumor growth by ~30% as compared to ad libitum. MT during light tripled circadian amplitude of temperature and reduced tumor growth by 62%. In P03-bearing mice, MT during light nearly doubled the circadian amplitude of body temperature and halved tumor growth as compared to ad libitum. While MT during light phase-advanced the rhythms in clock gene transcription by 8-12h in liver, the tumor clock gene patterns remained arrhythmic both on ad libitum and on MT. A circadian transcriptome study in tumor was complemented with qPCR. Results revealed that MT induced strong rhythmic transcription of Hspa8 and Cirbp, two regulators of cell cycle and apoptosis. The MT-induced peaks of Hspa8 and Cirbp expressions in the clock-defective tumor respectively corresponded to peak and trough body temperature. Conclusions The reinforcement of the host circadian timing system with MT induced 24-h rhythmic expression of relevant genes in clock-deficient tumors, which translated into cancer inhibition. This finding supports a critical role of the circadian amplitude in host core body temperature for cancer control.

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Circadian-independent cell mitosis in immortalized fibroblasts

MIJUNG YEOM¹, JULIE PENDERGAST¹, YOSHIHIRO OHMIYA², SHIN YAMAZAKI¹

¹*Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA*

²*Research Institute of Genome-Based Biofactory, National Institute of Advance Industrial Science and Technology, Sapporo, JAPAN*

Two prominent timekeeping systems, the cell cycle which controls cell division, and the circadian system which controls 24-hour rhythms of physiology and behavior, are found in nearly all living organisms. A distinct feature of circadian rhythms is that they are temperature compensated such that the period of the rhythm remains constant (~24 hours) at different ambient temperatures. Even though the speed of cell division, or growth rate, is highly temperature-dependent, the cell mitosis rhythm is temperature compensated. Twenty-four hour fluctuations in cell division have also been observed in numerous species, suggesting that the circadian system is regulating the timing of cell

division. We tested whether the cell cycle rhythm was coupled to the circadian system in immortalized rat-1 fibroblasts by monitoring cell cycle gene promoter-driven luciferase activity. We found that there was no consistent phase relationship between the circadian and cell cycles and the cell cycle rhythm was not temperature compensated in rat-1 fibroblasts. These data suggest that the circadian system does not regulate the cell mitosis rhythm in rat-1 fibroblasts. These findings are inconsistent with numerous studies that suggest that cell mitosis is regulated by the circadian system in mammalian tissues *in vivo*. To account for this discrepancy, we propose two possibilities: (i) there is no direct coupling between the circadian rhythm and cell cycle, but the timing of cell mitosis is synchronized with the rhythmic host environment, or (ii) coupling between the circadian rhythm and cell cycle exists in normal cells but it is disconnected in immortalized cells.

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Melatonin and peripheral molecular circadian rhythms

LUCIA PAGANI¹, FIDES MEIER¹, CHRISTIAN CAJOCHEN², STEVEN A. BROWN³, ANNE ECKERT¹

¹*Neurobiology Laboratory for Brain Aging and Mental Health, UPK Basel, Basel, SWITZERLAND*

²*Center of Chronobiology, UPK Basel, Basel, SWITZERLAND*

³*Department of Pharmacology and Toxicology, University of Zurich, Zurich, SWITZERLAND*

The neurohormone melatonin (Mel) is synthesized and secreted by the pineal gland during the dark period. Mel is the best marker for human circadian rhythms, however, its physiological function is still unclear. To investigate the effects of Mel as a chronobiotic substance, we characterized the period length and amplitude of the circadian rhythms of human skin fibroblasts infected with an engineered lentiviral circadian reporter (mBmal-1::luc) in the presence of different concentrations of Mel. The treatment of fibroblasts with Mel did not increase the synchronization of the cells. Cells that have been treated with a rather low concentration of Mel (5 uM) showed a dampening of the amplitude similar to cells treated with vehicle, while, fibroblasts treated with a high concentration of Mel (500 uM) showed a higher amplitude dampening. To better understand the role of Mel on circadian rhythms, cells were measured with different concentrations of Mel (from 5 uM up to 500 uM). Low Mel concentrations (5 uM and 10 uM) did not influence the circadian period length, whereas 50 uM and higher Mel concentrations reduced the circadian period length of peripheral oscillators up to 4.23 % \pm 1.5 (P < 0.01). To investigate the pathway through which Mel acts on the circadian rhythms, cells were treated with Mel and Gi-inhibitor or PKC inhibitor. The influence of Mel on the fibroblasts period length is not inhibited in the presence of PTX but is inhibited in the presence of chelerythrine. From this study we can conclude that Mel is not able to synchronize peripheral oscillators, but it seems able to shorten the circadian period length via Gq-PKC pathway, which may reflect an immediate phase advance and thus its chronobiotic properties.

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Evaluation of qPCR reference gene stability for circadian studies in peripheral organs of different mouse strains

ROK KOSIR¹, JURE ACIMOVIC², ANJA KORENCIC², MARKO GOLICNIK², MARTINA PERSE³, MARTINA FINK⁴, DAMJANA ROZMAN¹

¹*Center for Functional Genomics and Bio-Chips, Faculty of Medicine, Ljubljana, SLOVENIA*

²*Institute of Biochemistry, Faculty of Medicine, Ljubljana, SLOVENIA*

³*Institute of Pathology, Faculty of Medicine, Ljubljana, SLOVENIA*

⁴*Department of Haematology, University Medical Center Ljubljana, Ljubljana, SLOVENIA*

Selection of reference genes is a crucial step of qRT-PCR that ensures accurate data analysis. Recent studies discuss differences in reference gene stability between species and tissues, but none has taken

into consideration circadian experiments and potential differences between mouse strains. The aim of this work is to evaluate the circadian stability of candidate reference genes in two frequently used mouse strains, the inbred C57BL6 and mixed strain C57BL6x129Pas. 33 C57BL6 mice and 96 mice with mixed background (51 wild type and 45 Crem^{-/-}) have been included in our study. Livers and adrenals were taken from 4-5 animals per time point every 4 h in a 24 h period. Expression of 10 candidate reference genes was measured by qPCR and their stability evaluated by geNorm, BestKeeper and NormFinder. In all tested cases, identical ranking of genes was obtained irrespective of the algorithm used. Important differences in reference gene expression were detected when comparing livers of C57BL6 and C57BL6x129Pas mice, but not livers of mixed background w.t. and Crem^{-/-}, nor the adrenals. C57BL6x129Pas generally exhibits a higher stability of reference genes compared to C57BL6. Ppib, 18sRNA and Rplp0 are the best reference genes for circadian studies in the adrenal irrespective of the mouse strain. Rplp0, Hprt1 and Utp6c are most stable in C57BL6x129Pas but, surprisingly, the least stable in C57BL6. Best normalization genes for liver circadian experiments in C57BL6 are Hmbs, Ppib and Eif2a. Our data show for the first time quantitative differences in expression of potential liver and adrenal reference genes in circadian performed experiments in the two mouse backgrounds. We show that the best normalization genes for liver circadian studies in C57BL6x129Pas mice are indeed the worst selection for C57BL6. This should be taken into consideration to avoid false conclusions in interpreting the circadian qPCR expression data.

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Circadian clock-coordinated translation in mouse liver

GASPARD CRETENET¹, FELIX NAEF², FRÉDÉRIC GACHON¹

¹*Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, SWITZERLAND*

²*School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, Lausanne, SWITZERLAND*

The mammalian circadian clock plays a fundamental role in the liver by regulating fatty acid, glucose and xenobiotic metabolism. Impairment of this rhythm has been shown to lead to diverse pathologies including metabolic syndrome. At present, it is supposed that the circadian clock regulates metabolism mostly by regulating the expression of liver enzymes at the transcriptional level. We have now collected evidence that post-transcriptional regulations play also an important role in this regulation. Particularly, recent results from our laboratory show that the circadian clock can synchronize mRNA translation in mouse liver through rhythmic activation of the Target Of Rapamycin Complex 1 (TORC1). Based on this unexpected observation, we identified rhythmically translated mouse liver mRNAs in a genome-wide fashion by applying microarray technology for the analysis of polysomal RNAs. This study allows the identifications of hundreds of rhythmically translated proteins with constant expression of their mRNAs. If these mRNAs coding for proteins involved in different pathways are translated at different phases, we found a strong enrichment for proteins involved in protein synthesis whose translation start at ZT8, before the onset of the feeding period of the mice. It thus seems that the circadian clock allows the anticipation of massive protein synthesis that take place when high amount of amino acids coming from food are available by stimulating the translation of the protein synthesis apparatus before the onset of the feeding period.

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Ontogeny of circadian rhythm in the eye of *Xenopus laevis*

KRISTEN CURRAN¹, LACMBOUH ADE¹, MICHAEL ZUBER²

¹*Biological Sciences, University of Wisconsin–Whitewater, Whitewater, WI, USA*

²*Ophthalmology and Biochemistry and Molecular Biology, SUNY
Upstate Medical University, Syracuse, NY, USA*

Our lab has been analyzing the timing of the onset of circadian rhythm in various organs of the developing *Xenopus* embryo, such as the eye. Our long term goal is to understand the development of the ability of organs peripheral to the suprachiasmatic nucleus (SCN) to synchronize with the external environment. Eyes were isolated at ZT 1, 5, 9, 13,17, and 21 from embryos maintained in a 24 hour LD cycle. At stage 38 (54 hpf) isolated eyes did not show obvious rhythmic expression of xBmal1, but significantly higher levels of xNocturnin were present at night as analyzed by qRT-PCR. These results and others indicate that time of day dependent expression of xBmal1 and xNocturnin is present in the developing eye by stage 40/41 (66 hpf). Experiments are ongoing to test whether stage 40/41 eyes can maintain rhythmic gene expression in constant darkness. We are also investigating whether interactions between the developing eye and other tissues of the head (brain, sensorial placodes, pineal gland) is necessary for maturation of circadian rhythm in the eye. In a pilot experiment, we transplanted the anterior neural plate of stage 14/15 embryos into the flank of similarly staged embryos. The embryos were aged to stage 45+ in a 24 LD cycle. The transplants formed eyes on the side of the embryo. Transplanted and endogenous eyes displayed a time of day dependent expression of xNocturnin. A caveat to this experiment is that the transplanted tissue also formed other tissues of the head (otic vesicle, neural tissue). Future experiments will test whether transplanted animal cap ectoderm injected with a suite of transcription factors that induce eye only can also display rhythmic gene expression when developing away from the tissues of the head.

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The molecular clock in muscle: The phase of PER2:LUC expression is similar among striated muscles of differing function

GRETCHEN WOLFF, KARYN ESSER

Physiology, University of Kentucky, Lexington, KY, USA

The molecular clock consists of a group of genes that utilize transcriptional and translational feedback loops to create a rhythm in gene expression which cycles approximately once per day (20-24hours). Molecular clocks have been identified in most tissues in the mammalian system. Although it is one of the most abundant tissues in mammals, little is known about what synchronizes the molecular clock in muscle. Skeletal muscles have very diverse phenotype and function throughout the body. They differ in their speed of contraction or fiber type (fast vs. slow) and their range in metabolism from highly oxidative to primarily glycolytic. With such diversity among the skeletal muscles and so little known about the molecular clock across different muscles we initially asked whether the molecular clocks in these different muscles were synchronized. Using muscle tissue collected from the PERIOD2::LUCIFERASE mice preliminary data suggests that the molecular clocks in skeletal muscle are in the same phase. Four muscles were collected for bioluminescence measurements. The soleus is primarily a mixed fibertype, highly oxidative hindlimb muscle that contributes to postural control. The extensor digitorum longus (EDL) has a fast fibertype and is more reliant on glycolysis for metabolism and is used for spreading of the toes. The diaphragm is the primary respiratory muscle that is in constant use throughout the day and night. It is a mixed muscle that is highly oxidative. Lastly we used explants from the left ventricle of the heart as a striated muscle with chronic functional demands. The peak bioluminescence measured in hours relative to the L:D cycle 23.6 ± 1.6 (soleus), 24.1 ± 0.3 (edl), 23.9 ± 0.8 (diaphragm), and 23.4 ± 0.2 (heart) (Avg \pm StDev). Dispersed single fibers

from the flexor digitorum brevis muscle also displayed a peak bioluminescence at 21.5 hours. These preliminary data suggest that the molecular clocks across different muscles are in phase with each other. Traditionally these peripheral tissues have been viewed as “slaves” to the suprachiasmatic nucleus (SCN) of the hypothalamus, which synchronize clocks in the peripheral tissues to the light-dark cycle. However, studies have identified other non-photic cues, such as feeding, that may synchronize certain peripheral clocks independently of the SCN. Future studies will focus on trying to define the cues that are critical for maintaining synchronization across skeletal muscles in the organism.

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Circadian regulation of cortisol release in behaviorally split golden hamsters

TRAVIS LILLEY, CHERYL WOTUS, HORACIO DE LA IGLESIA
Biology, University of Washington, Seattle, WA, USA

In mammals, the master circadian clock is located within the hypothalamic suprachiasmatic nucleus (SCN). Evidence suggests the SCN is composed of a heterogeneous population of single-cell neuronal oscillators with functionally discrete cellular subpopulations that could potentially regulate different rhythmic outputs via specific neural and humoral factors. One critical SCN output is the regulation of cortisol (CORT) release, which shows a circadian oscillation peaking near the onset of locomotor activity. CORT is a steroid secreted by the adrenal gland, functioning as a hormonal signal to mobilize energy reserves throughout the body, as well as, a potentially important endogenous signal by which the SCN coordinates the phase of numerous slave oscillators. Additionally, plasma CORT rapidly increases in response to physiological or emotional stress which is mediated by the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland. Recent evidence suggests circadian CORT release is potentially regulated in part by a multisynaptic sympathetic pathway from the SCN to the adrenal gland, independent of ACTH release. In constant light, ~60% of hamsters will behaviorally “split” into 2 bouts of locomotor activity ~12 h apart, mirroring antiphase oscillations of the bilaterally paired SCN. In this study, hamsters were housed in constant light and wheel-running activity was monitored. Split and un-split hamsters received jugular catheters and serial blood samples were collected for 24 h. Plasma CORT and ACTH concentrations were assayed by radioimmunoassay. Un-split hamsters exhibit a single peak of CORT and ACTH whereas, split hamsters exhibited 2 peaks of CORT. Collectively, the split hamster represents an elegant model to characterize the pathways mediating circadian CORT release and a framework to study corticosteroid-dependent synchronization of peripheral tissues.

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Immunohistological characterization of the circadian clock in cardiovascular tissues

ANA M. MERLOIU, CIPRIAN B. ANEA, R. DANIEL RUDIC
Pharmacology & Toxicology, Medical College of Georgia, Augusta, GA, USA

The circadian clock is a unique molecular pathway that controls 24-hour cycles pervasive in the physiology of organisms including the cardiovascular system of mammals. While recent discoveries have demonstrated the presence of an oscillating circadian clock in blood vessels, cell-specific characterization of the circadian components within cardiovascular system has not been well characterized. Herein, we undertook experiments to determine the tissue and cell-specific expression of circadian clock components in arteries, veins, heart, and lung. Expression patterns were determined by immunohistochemistry for Bmal1, Clock, Npas2, Per1, Cry1, Rev-erb?, Ror1, CK1-? and EPAS in mouse (femoral artery, femoral vein, aorta, common carotid artery, lung and heart), and human (saphenous vein) samples. Bmal1, Clock, Per1, and Cry1 were strongly present throughout endothelial, smooth muscle, cardiac myocytes, and alveoli, with a predominant nuclear localization for Bmal1, Clock and

Cry1 whereas Per1 was predominantly found in cytoplasm. While there was only weak staining for Npas2 and Rev-erb α in mouse vasculature, Npas2 and Rev-erb α exhibited increased nuclear and cytoplasmic staining in mouse lung and the human saphenous vein. Ror1 and Per1 seemed to have a strong nuclear localization in lung tissue, but in heart were found more intensely in the cytoplasm. Nuclear Cry1 staining was strikingly more prominent in lung and heart compared to the other vascular tissues. In the human saphenous vein Rev-erb α (nuclear) and EPAS exhibited most robust positive staining in comparison to mouse tissues. These studies may reveal insight into cell and tissue specific clock signaling/dynamics in the cardiovascular system.

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Existence of an endogenous circadian blood pressure rhythm that paradoxically peaks at night

STEVEN A. SHEA, MICHAEL F. HILTON, KUN HU, FRANK A. SCHEER

Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

The risk of adverse cardiovascular events has a day/night pattern with a primary morning peak (~06:00-12:00) and a secondary evening peak (~18:00-22:00), possibly related to blood pressure changes at those times. Thus, we determined whether or not there exists an endogenous circadian rhythm of blood pressure (BP). In 32 normotensive adults (19 men), we repeatedly assessed BP across three complimentary, multi-day, in-laboratory protocols in which behavioral and environmental influences were controlled and/or uniformly distributed across the circadian cycle. The protocols were: (i) 38-h Constant Routine, including continuous wakefulness, semi-recumbency, and 2-hourly isocaloric snacks; (ii) 196-h Forced Desynchrony with seven 28-h sleep/wake cycles; and (iii) 240-h Forced Desynchrony with twelve 20-h sleep/wake cycles. Circadian phase was derived from core body temperature (0° , temperature minimum; ~05:00 in these subjects). Each protocol revealed significant circadian rhythms in systolic and diastolic BP, with almost identical rhythm amplitudes and phase relationships among protocols. The average peak-to-trough amplitudes were 3-6 mmHg for systolic BP and 2-3 mmHg for diastolic BP (always $P < 0.05$). All six peaks (systolic and diastolic BP in three protocols) occurred at 240° , equivalent to ~21:00. These data demonstrate—consistently across three separate circadian protocols—an endogenous circadian rhythm in blood pressure, thus independent of changes in sleep, posture, activity or light exposure. Surprisingly, the lowest BP occurred across the circadian phases corresponding to the peak of adverse cardiovascular events. Thus, the circadian BP profile is unlikely to be involved in the morning peak in cardiovascular events but could be involved in the secondary evening peak. Further studies are required to determine whether the circadian blood pressure rhythm is amplified and/or shifted in populations vulnerable for adverse cardiovascular events. Supported by R01-HL64815, K24-HL76446 and GCRC M01 RR02635.

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Length polymorphism in the PER3 polymorphism modulates sleep structure and EEG power spectra in older subjects

ANTOINE VIOLA¹, SARAH CHELLAPPA¹, SIMON ARCHER², DERK-JAN DIJK², CHRISTIAN CAJOCHEN¹

¹*Centre for Chronobiology Basel, Universitäre Psychiatrische Kliniken, UPK, Basel, SWITZERLAND*

²*SSRC, University of Surrey, Guildford, UNITED KINGDOM*

A variable number tandem repeat polymorphism of the clock gene PER3 predicts specific EEG power spectra differences in NREM sleep, REM sleep and wakefulness in young subjects. Whether these effects of this polymorphism are also observed in older subjects remains unknown. Here we investigate whether baseline sleep structure can differ between older subjects homozygous for the longer (PER35/5) and for the shorter (PER34/4) allele of the gene PERIOD3. In this ongoing project, healthy volunteers were

selected exclusively on the basis of their PER3 genotype. A total of 168 healthy older men and women (55-75y) have been genotyped for the PER3 polymorphism, comprising subjects homozygous PER35/5, PER34/4 and heterozygous PER34/5. Forty older subjects completed sleep analysis by actigraphy and sleep diaries to characterize habitual sleep and wake timings during three consecutive weeks. Currently, 27 subjects, out of which 10 subjects are PER35/5 and 10 are PER34/4, have completed the in-lab part of the study, under constant routine conditions. Only 7 PER34/5 subjects completed the constant routine so far, and thus were not included in the analysis. Our preliminary analyses of actiwatch data indicate significant differences between the two genotypes, such that PER35/5 subjects exhibited earlier increased activity count in the morning hours. Homozygosity PER35/5 had a considerable effect on baseline sleep structure, with significantly lower sleep efficiency and a tendency for more wakefulness. No differences in REM sleep were observed. On the other hand, NREM sleep in PER35/5 subjects was significantly associated with high frontal EEG power density in the delta range (0.75-1.5Hz) and low frontal EEG activity in the spindle range (10.25-13Hz) ($p < 0.05$). PER35/5 subjects exhibit NREM sleep EEG frequency-specific changes, with higher delta activity, and reduced low frequency sleep spindles, which suggests that this PER3 polymorphism modulates baseline sleep structure and EEG power spectra during sleep also in older people.

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Chronic jet lag modulates innate immunity in peritoneal macrophages

**OSCAR CASTAÑÓN-CERVANTES¹, MINGWEI WU¹, J. CHRISTOPHER EHLEN¹, KETEMA PAUL¹,
MICHAEL MENAKER², ANDREW GEWIRTZ³, ALEC DAVIDSON¹**

¹*Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA, USA*

²*Department of Biology, University of Virginia, Charlottesville, VA, USA*

³*Department of Pathology, Emory University, Atlanta, GA, USA*

Modulation of innate immunity by the circadian system may play an important role in the ontogeny and prevalence of diseases associated with circadian disruption. Human shift work and frequent international travel (jet lag) result in chronic circadian misalignment, and lead to a higher risk of cancer, strokes, and other inflammation-related pathologies. We have developed a mouse model of chronic jet lag in which a 6h phase advance of the light/dark cycle applied once per week during four weeks results in a pathological response to bacterial lipopolysaccharide (LPS) reflecting a dysregulated immune response leading to profound hypothermia and death associated with high levels of proinflammatory cytokines. The magnified response to LPS requires repeated phase shifts, and is not due to sleep loss. When cultured in the presence of LPS, peritoneal macrophages harvested from mice exposed to chronic jet lag released higher levels of IL-6 than those from control unshifted mice, indicating that these cells represent a target for circadian disruption.

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The role of the circadian clock in the murine inflammatory response to lipopolysaccharide

JULIE GIBBS¹, STEPHEN BEESLEY¹, STUART FARROW², DAVID RAY³, ANDREW LOUDON¹

¹*Faculty of Life Sciences, University of Manchester, Manchester, UNITED KINGDOM*

²*Respiratory CEDD, GSK, Stevenage, UNITED KINGDOM*

³*Faculty of Medicine, University of Manchester, Manchester, UNITED KINGDOM*

Several inflammatory diseases exhibit a circadian element to their symptoms. For example, rheumatoid arthritis patients report diurnal variations in their symptoms, experiencing greater joint pain, stiffness and functional disability in the mornings. In contrast, some asthma patients experience night-time exacerbations (nocturnal asthma) which can be attributed, in part, to diurnal variations in lung

physiology, but also increased bronchial responsiveness at night. The nature of these links between the inflammatory response and the circadian clock are unclear. Here we focus on the relationship between the circadian clock and the inflammatory response using a mouse model. Wild-type mice were administered an intraperitoneal injection of 1mg/kg lipopolysaccharide (LPS; a component of the gram negative bacterial cell wall) or saline (control) either at CT0 or CT12. 4 hours after inflammatory challenge, mice were sacrificed, blood was collected and lung tissue harvested. Q-PCR analysis of RNA extracted from liver samples showed that administration of LPS significantly affected expression levels of clock genes. *Per2* and *rev-erb alpha* levels were significantly reduced after LPS, whilst *bmal1* expression was significantly increased. These data suggest that inflammatory challenge with LPS directly affects the molecular clock. Analysis of levels in cytokines and chemokines in serum samples revealed that of the 22 assayed, 13 cytokines/chemokines were responsive to LPS. Five showed significant differences between CT0 and CT12 treatment: IL-6, IL-12(p40), CCL2 (MCP-1), CXCL1 (KC) and CCL5 (RANTES). All five showed statistically significant increases in levels released after challenge at CT12 compared to CT0. None of the cytokines assayed showed significant day/night differences in vehicle treated animals. These results indicate that elements of the inflammatory response are gated by the circadian clock. Further experiments using transgenic mice are underway to assess the involvement of specific clock genes in this gating phenomenon.

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Circadian and photoperiodic clocks: Is every similarity just a coincidence?

JOAO GESTO, ZAINAB AHMED, ERAN TAUBER, CHARALAMBOS KYRIACOU

Genetics, University of Leicester, Leicester, UNITED KINGDOM

Photoperiodism comprises a number of clock-related phenomena that allows organisms to distinguish the long days (or short nights) of summer from the short days (or long nights) of autumn and winter, and thereby obtain calendar information from the environment. Unlike the circadian system, very little is known regarding the molecular genetics of photoperiodism in *Drosophila melanogaster*, which is observed as an adult ovarian diapause. Interestingly, there is evidence both supporting and refuting the hypothesis that both the circadian and photoperiodic clocks share similar molecules to operate the timing mechanism. In this study, natural variants of the clock gene *timeless* (*tim*) were raised across a range of temperatures (8°C, 10°C, 12°C, 14°C) and two different photoperiods, representing summer (LD 16:8) and winter (LD 8:16). It was observed that flies carrying the newly derived allele *Is-tim* show higher diapause levels than those carrying the ancestral one, *s-tim*. Interestingly, both genotypes responded differently to photoperiod in a temperature-dependent way, raising the possibility of *tim* involvement in the photoperiodic pacemaker. To investigate any further implications of the circadian clock in photoperiodism, we generated a collection of flies carrying clock mutations in the same congenic background, derived from an isofemale line collected in Houten (Holland). Surprisingly, our results suggest that canonical clock mutations cannot only alter diapause levels, but also attenuate the photoperiodic effect. Although we consider the possibility of pleiotropic effects exerted individually by each of the candidate genes tested, we believe that this is not the case since most if not all of them contribute to shape the seasonal phenotype. Therefore, the data presented here corroborate the hypothesis that the circadian clock system underlies the photoperiodic response in *D. melanogaster*.

The effect of season on inflammatory response in captive baboons

DIANNE McFARLANE¹, ROMAN WOLF², KRISTEN McDANIEL¹, GARY WHITE²

¹Physiological Sciences, Oklahoma State University, Stillwater, OK, USA

²Comparative Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

The intensity of immune response has been shown to vary with season in humans, rodents, birds, amphibians and fish. Little is known about the effect of season on immune reactivity in non-human primates. Seasonal variation in immune response could have profound implications in experimental design and data interpretation, as well as general herd health. We hypothesized that baboons housed in outdoor corrals would experience seasonal fluctuations in inflammatory response whereas animals maintained indoors under a constant schedule of 12 hours artificial light: 12 hours dark would not. To test this hypothesis, 24 healthy, 2-4 year old male baboons were divided into two groups; half were housed in outdoor corrals, the other half indoors under 12 hrs per day of artificial lighting. After 3 months of acclimation, blood samples were collected in Mar, Jun, Sept and Dec. Serum C-reactive protein and IL-6 concentration were measured by ELISA, serum cortisol by radioimmunoassay. Peripheral blood mononuclear cells (PBMC) were stimulated with lipopolysaccharide (LPS) for 4 hours; TNF- α and IL-6 release were measured by ELISA. The effect of season on inflammatory mediators was assessed by repeated measures one-way ANOVA (IL-6, sCRP, cortisol) or Friedman test (sIL-6, TNF- α) with Bonferroni's or Dunn's post-hoc correction for multiple comparisons. Baboons housed outdoors had a greater sCRP concentration ($P < 0.01$) and increased PBMC cytokine release (TNF- α , $P < 0.001$; IL-6, $P < 0.01$) following immune stimulation in Jun and Sep compared to Mar and Dec. Serum cortisol and IL-6 concentration did not show a seasonal pattern. Indoor baboons had a similar cytokine response to LPS (TNF- α and IL-6, $P < 0.0001$), however CRP did not differ by season. Captive baboons have an increased inflammatory response in Jun and Sept, therefore season must be considered when designing immune studies in baboons. Further work is needed to determine the specific environmental factors responsible for this seasonal effect.

Pinealectomy abolishes clock genes rhythm in peripheral tissues in house sparrow, *Passer domesticus*

AMIT TRIVEDI, GANG WANG, CLIFFORD HARPOLE, VINCENT CASSONE

Biology, University of Kentucky, Lexington, Kentucky, USA

Avian circadian organization is complex and involves the interactions of at least three components: the pineal gland, suprachiasmatic nuclei (SCN) and retina. In addition, clocks have been localized in many peripheral tissues (e.g., the liver, lungs, kidneys, heart and gonads). However, the role of central avian circadian structures in synchronizing peripheral oscillators in avian species is largely unknown. Our current understanding in mammals is that many peripheral clocks are controlled or synchronized by the SCN through neural and blood-borne signalling factors that oscillate in a circadian fashion. To understand these interactions in the house sparrow (*Passer domesticus*), we measured 2-deoxy[14C]-glucose (2DG) uptake and mRNA expression of the clock genes in the brain areas and the peripheral organs in pinealectomized or sham operated birds. In Japanese quails, where constant melatonin administration abolishes and/or disrupts the expression of behavioural rhythms and the rhythmic melatonin administration entrains feeding rhythms, no effect of the hormone can be found on the expression of the clock genes in the mSCN. Similarly, we found that pinealectomy had no effect on expression of clock genes in peripheral tissues of pinealectomized sparrows on the day they are transferred from LD to DD. However, the rhythm was lost for most clock genes in most peripheral tissues after 10 days in DD when the birds were behaviourally arrhythmic. In the house sparrow, at

least, the pineal gland serves to coordinate peripheral rhythms. Currently we are analyzing brain structures of these same birds to understand the effect of pinealectomy on rhythms of 2DG uptake and of the expression of the clock genes mRNA and protein. Acknowledgement: This research is supported by NIH P01 NS39546 grant to V.M. Cassone.

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Circadian control of calcium utilization in birds

PAUL BARTELL, ELIZABETH EBERT-ZAVOS, MARIA HORVAT-GORDON

Poultry Science, Pennsylvania State University, University Park, PA, USA

Avian eggs possess a shell composed of primarily calcium carbonate. The egg shell matrix is deposited while the developing egg is within a specialized structure called the shell gland. Because of the high demand for calcium, the daily turnover rate of calcium in egg laying birds is roughly equivalent to 10% of the bird's total calcium stores. As such, birds are continuously under calcium stress during their reproductive period; the effects of this stress, over time, can lead to weakened egg shells or osteoporosis and other degenerative bone diseases. Birds have developed a specialized type of woven bone called medullary bone to function as a labile source of calcium as a mechanism to cope with this extreme demand to mobilize calcium. Together with the shell gland and the duodenum (the main location of calcium uptake in the digestive system), the regulation of calcium utilization and homeostasis for the purpose of forming an egg shell is accomplished. Because birds lay one egg per day under the direct control of a circadian clock within the ovary, we hypothesized that a system of interlocked circadian clocks controls calcium homeostasis in birds during their reproductive period. To demonstrate this, we investigated circadian clocks within the duodenum, the medullary bone, and the shell gland. We found that a functional molecular circadian clock exists in these three structures. Our data also demonstrate that circadian clocks in these structures regulate the transcription of genes whose products can be directly used to regulate calcium homeostasis. Furthermore, our findings suggest that circadian clocks in the respective structures also regulate the transmission and reception of information about calcium demand in order to function as a system to regulate calcium utilization in egg laying birds.

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Voluntary wheel running induces PER2 rhythms in the dentate gyrus of day-active and night-active grass rats (Arvicanthis niloticus)

CHIDAMBARAM RAMANATHAN, ADAM STOWIE, LAURA SMALE, ANTONIO NUNEZ

Department of Psychology, Neuroscience Program, Michigan State University, East Lansing, MI, USA

The clock genes that were first identified in the suprachiasmatic nucleus (SCN) are also rhythmically expressed in various brain regions and peripheral organs. Our work with grass rats shows that the phase of many extra-SCN oscillators is reversed when diurnal and nocturnal species are compared. Although grass rats are clearly diurnal, a subset of these animals shifts to a night-active pattern (NA) when given access to running wheels, while the rest continue to be day-active (DA). We used these animals to examine the nature of the plasticity of oscillators within the hippocampus and of their coupling to the light dark cycle. In this study, grass rats were maintained in a 12:12 light/dark cycle [lights on as Zeitgeber Time (ZT) 0] with access to running wheels, and classified as DA or NA on the basis of their activity rhythms. Animals were perfused at one of six ZTs (2, 6, 10, 14, 18, or 22) and brain sections processed for immunocytochemistry to examine PER1/2 expression in the dentate gyrus (DG). PER1 and PER2 were rhythmically expressed in the DG, but the rhythms for DA and NA animals were 12 hours out phase; both proteins peaked in the late light period in DA grass rats and in the late dark period in NA grass rats. In animals without wheels, there is a rhythm of PER1 expression in the DG,

with a phase identical to that of DA animals, but PER2 production is minimal and arrhythmic. Voluntary exercise increases neurogenesis in the DG and PER2 appears to be involved in regulating this process. Thus, the induction of a PER2 rhythm by voluntary exercise seen here may represent a mechanism by which exercise, modulated by the circadian system, induces neurogenesis in the DG.

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Period aftereffects are modulated by phase incoherence in the forced desynchronized rat

MICHAEL SCHWARTZ¹, SHIN YAMAZAKI², HORACIO DE LA IGLESIA¹

¹*Department of Biology, University of Washington, Seattle, WA, USA*

²*Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA*

Rats housed in a 22-hour light-dark (LD) cycle exhibit two distinct circadian activity bouts simultaneously: one is entrained to the LD cycle and a second dissociated bout maintains a period greater than 24 hours. These two activity bouts are associated with independent oscillations in clock gene expression in the ventrolateral (vl-) and dorsomedial (dm-) SCN, respectively. Previously, we showed that light pulses only induced phase shifts of locomotor activity when the vl- and dmSCN were phase-aligned at the time of stimulation, suggesting that the dmSCN is a major determinant of SCN output. Here, we further explored this question by examining resynchronization following exposure to LD22. Wistar rats were desynchronized in LD22, and were then released into DD at maximal phase-alignment or misalignment as determined by locomotor activity. Activity rhythms rapidly resynchronized upon release into DD; however, the period of the fused rhythm was longer in rats released from misaligned than aligned phases. We then asked whether the period changes observed in vivo were present in the SCN neuronal clock ex-vivo. Per1-luc rats were desynchronized in LD22, then sacrificed at maximal phase alignment or misalignment; a control group was housed in LD24. SCN explants were prepared 30 minutes before lights-off and imaged for seven days. The period of both misaligned and aligned phase explants was longer than that of LD24 controls. Together, these data are consistent with a model of pacemaker organization in which a light-sensitive vlSCN entrains a second, light-insensitive, dmSCN oscillator; exposure to short LD cycles induces period lengthening in the dmSCN, reflected in the long period aftereffects observed ex vivo and, to a lesser extent, in vivo.

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Internal desynchronization in a model of chronic jetlag in mice

LEANDRO CASIRAGHI¹, GISELE ODA², W. OTTO FRIESEN³, DIEGO GOLOMBEK¹

¹*Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Buenos Aires, ARGENTINA*

²*Departamento de Fisiologia, Instituto de Biociências, São Paulo, BRAZIL*

³*Department of Biology, University of Virginia, Charlottesville, VA, USA*

The effects of an abrupt phase shift of the light/dark cycle on the internal synchrony of the circadian system are well known in the chronobiological literature. Frequent phase shifts probably have a much more disruptive effect on the circadian system; this “chronic jet-lag” state has been shown to affect survival and morbidity. Here we present activity data of mice under one particular experimental chronic jet-lag protocol which sheds light and provides a new direction for analyzing this complex state under a concrete mathematical basis. Locomotor activity of C57 mice (n=15) was recorded by IT motion detectors in individual cages under a 12:12 LD cycle, after which they were subjected to a chronic phase-shifting of the LD cycle consisting in a 6h advance of lights onset every 2 days, caused by a shortening of the dark phase previous to the advance. Mice were kept under this chronic jet-lag schedule from 30 to 60 days and were then released into DD. Under the chronic jet-lag condition, 10/15 (67%) of animals showed two distinct activity components: one with a short period of 21.02 ± 0.06 h and another with a long period of 24.9 ± 0.15 h. After being released into DD, animals displayed an

intermediate activity period of 24.15 ± 0.23 h. Computer simulations were performed with coupled Pavlidis oscillators forced by 24h pulses that were shifted variously with respect to size and frequency. Frequent phase shift schedules can be globally interpreted as a new Zeitgeber which elicits relative coordination or even internal dissociation between circadian components. Mathematical modeling provides a conceptual framework for understanding different levels of desynchrony of the circadian system under chronic jet lag schedule and for evaluating the size of disruption which is ensued by the combining roles of phase shifts' size and frequency. Support: UNQ/ANPCyT/CONICET/NIH/FAPESP.

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Differential functions of the Period genes in the regulation of mood and anxiety-related behaviors

JASWINDER KUMAR, AMI GRAHAM, SHARI BIRNBAUM, ALICE ANN SPURGIN, VAISHNAV KRISHNAN, ELIZABETH GORDON, COLLEEN McCLUNG

Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX, USA

It has been suggested for some time that circadian rhythm abnormalities underlie the development of multiple psychiatric disorders. However, it is unclear how disruptions in individual circadian genes might regulate mood and anxiety. Here we examined the influence of the Period genes on measures of activity, mood, and anxiety-related behavior. We utilized mice lacking mPer1, mPer2, mPer3, or both mPer1 and mPer2 (mPer1;mPer2). We found that mice lacking mPer1 or mPer2 individually do not have consistent behavioral abnormalities in measures of activity, anxiety, or mood. However, mice lacking both mPer1 and mPer2 have lowered levels of depression-like behavior and have an increase in anxiety-related behavior. Mice lacking mPer3 also have a large decrease in depression-like behavior, but are normal in measures of anxiety. Interestingly, wild type mice exposed to chronic social defeat stress have a significant and lasting decrease in mPer3 expression in the nucleus accumbens only if they develop strong depression-related behavior following the defeat. Furthermore, chronic antidepressant treatment leads to large increases in all three mPer genes. Taken together, these results implicate the Per genes in the control of mood and anxiety-related behaviors and demonstrates the specificity of action of individual circadian genes in these behaviors.

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Reward portals to the circadian clock: Mesopontine and mesocorticolimbic pathways for non-photic phase regulation

JESSIE GUINN, ALLISON BRAGER, DAVID GLASS

Biological Sciences, Kent State University, Kent, OH, USA

There is evidence that the IGL receives clock-resetting input from brain systems that register the reinforcing effects of natural and drug-related reward. Reward stimuli act on a neural network consisting of the mesolimbic dopamine pathway (including dopaminergic neurons in the ventral tegmental area [VTA]) and its projection to the mesopontine system (including cholinergic neurons of the pedunclopontine tegmentum [PPT] and the laterodorsal tegmentum [LDT]). The IGL receives cholinergic input from the mesopontine system, and cholinergic stimulation of the IGL induces nonphotic phase-shifts. Linkage between reward and circadian pathways is reflected behaviorally in the reinforcing nature of non-photic stimuli that reset circadian clock timing, including exercise and sex. This study was undertaken to explore a regulatory role of the reward network in circadian timekeeping. In experiment 1, the phase-resetting effect of VTA activation was examined in hamsters using 1 hr electrical VTA stimulation at ZT 6-7, immediately followed by release to DD (Aschoff type II procedure). This stimulation produced phase-advance shifts of 57 ± 30 min (range; 23-148 min). In experiment 2, the effects of voluntary wheel-running (a reinforcing phase-resetting stimulus) at ZT 6-7 on the activity of VTA, PPT and LDT cells was estimated by Fos expression. This treatment increased the

number of Fos-immunoreactive cells in these areas by 454+46%, 491+95% and 582+100%, respectively vs. nonrunning controls (all $p < 0.05$). In experiment 3, chronotypic effects of chronic VTA dopaminergic/glutamatergic stimulation were studied using bilateral constant-release acamprosate microimplants in C57BL/6 mice. These VTA implants markedly enhanced rhythm amplitude and increased activity bout duration under LD. Under DD, tau was shortened vs. blank implant controls (23.1 vs. 23.7, respectively; $p < 0.05$). These observations, together with those from other groups, suggest a mechanism whereby tegmental reward centers activated by reinforcing non-photic stimuli modulate circadian timekeeping via mesopontine cholinergic input to the IGL. NIH grant NS35229 to JDG

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Manic-like behavior in Clock-delta19 mice is associated with molecular, cellular, and physiological dysfunction in the nucleus accumbens

MICHELLE SIDOR¹, KAFUI DZIRASA², LAURENT COQUE¹, MIGUEL NICOLELIS², COLLEEN McCLUNG¹

¹Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX, USA

²Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC, USA

There is accumulating evidence to suggest a role for altered circadian rhythms in the pathophysiology of bipolar disorder. Indeed, previous work in our lab has shown that mice with a point mutation in the circadian gene, Clock (Clock-delta19), display manic-like behaviors that are attenuated by lithium treatment. Our goal is to understand the biological basis of this behavioral phenotype and explore the mechanisms by which lithium exerts its antimanic-like actions. Alterations in mesolimbic circuitry, a region implicated in reward and mood regulation, were examined at a cellular level by assessing both neuronal morphology and levels of excitatory receptor expression, and at a systems level through electrophysiological recordings. By Sholl analysis we found that Clock-delta19 mice have an increase in dendritic length and complexity within the nucleus accumbens (NAc) compared with wild-type littermates. Furthermore, this phenotype was reversed by chronic lithium treatment in Clock-delta19 mice. Interestingly, lithium exerted the opposite effect in wild-types by increasing dendritic complexity to Clock-delta19 levels. This was accompanied by decreased protein levels of the glutamate receptor subunit, GluR1 and Ser-845 phosphorylated GluR1 in the NAc of Clock-delta19 mice, with no changes in NMDAR1 or 2B subunits. To explore how these cellular changes may impact neuronal function on a systems level, electrophysiological recordings from multiple mesolimbic regions of free-moving mice were conducted. Clock-delta19 mice displayed altered phase synchronization in the VTA-NAc circuit as revealed by altered NAc neuronal entrainment that was rescued by lithium. Surprisingly, chronic lithium treatment disrupted neural phase signalling in wild-type littermates. Overall these results suggest that altered NAc neuronal structure and function are associated with aberrant mesolimbic circuit communication and may ultimately underlie the manic-like behavioral profile of Clock-delta19 mice. These results lend insight into the biological underpinnings of bipolar disorder and unveil potential mechanisms by which lithium acts as a mood stabilizer.

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Characterization of circadian rhythm parameters in two strains of mice with impaired responsiveness to estrogen

MARGARET BLATTNER¹, MEGAN MAHONEY²

¹Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL, USA

²Veterinary Biosciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Estrogen influences the expression of circadian rhythms, including free running period, phase angle, and photic phase response curves, yet the mechanisms underlying these effects are unknown. We want to develop a model to examine the interactions between estrogen and circadian rhythms using

estrogen receptor alpha knock-out mice (ERKO) and non-classical estrogen receptor knock-in animals (NERKI). ERKO animals are unable to respond to estrogen at the estrogen receptor alpha subtype, but still produce estrogen and have active beta-type estrogen receptors. NERKI animals lack a classical estrogen response element but can respond to estrogen via non-classical pathways. We compared ERKO, NERKI and wildtype (males and females) with respect to free running period in constant darkness and the ratio of daily activity in the light compared to dark portion of the photoperiod. Preliminary data indicates there is a significant effect of genotype on tau ($P=0.003$) and NERKIs have a shorter tau than ERKOs. The light:dark ratio of activity/rest is significantly affected by genotype ($P=0.003$) and ERKO and NERKI animals have significantly more activity in the light relative to dark than do wildtype animals. We are currently characterizing the photic phase response in these animals and predict that the ability to respond to estrogen will influence the strength of the phase shift. These models will contribute to our understanding of estrogenic interactions with circadian rhythms. These data may help elucidate the mechanisms underlying disrupted rhythms and sleep wake cycles observed in peri-menopausal women and provide insight for therapeutics.

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Comparison of wrist-level and eye-level light measurements for in-hospital circadian studies

ANISOARA JARDIM¹, MATTHEW PAWLEY¹, MIRJAM GUESGEN¹, JAMES CHEESEMAN¹, CHRISTOPHER STEELE², ALAN MERRY¹, GUY WARMAN¹

¹*Anaesthesiology, School of Medicine, University of Auckland, Auckland, NEW ZEALAND*

²*Military & Emergency Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, USA*

The accurate assessment of light levels in the hospital environment is essential to understand how hospitalization may impact patients' circadian entrainment. While wrist-level light measurement is commonplace, there is a paucity of data available to indicate whether levels measured in this manner provide a valid estimate of light entering the eye. We conducted a study on 12 post-operative cardiac patients in which we continuously measured light exposure for between one and six post-operative days in the intensive care unit (ICU) and cardiac ward (WD) using two different devices: a wrist-worn Actiwatch-L (Philips Respironics) and a head-worn daysimeter (Rensselaer Polytechnic Institute, N.Y.).

	Daysimeter (Lx)		Actiwatch (Lx)	
	ICU	WD	ICU	WD
Minimum light levels	0.34	0.11	0	0
Maximum light levels	1068	12778	1961	6602
Average daytime light levels (SE)	122 (146.6)	120 (142.9)	72 (65.2)	69 (63.6)

Light levels (Lux) as measured in the intensive care unit and cardiac ward.

Correlation between light measured by the two devices was variable, with a range of R^2 of 0.37–0.9. Forty two percent of patients had an $R^2 > 0.8$ and 17% an $R^2 < 0.5$. The agreement between the devices is strongly influenced by patient location. We believe wrist-level light measurements provide a reliable estimate of eye-level light exposure only when patients remain in the ward or ICU. The main limitation of wrist worn light meters appears to be when patients leave the ward during the day at which time they considerably under-estimate eye-level light exposure.

Light-induced melatonin suppression in humans: Effect of light duration and irradiance

CHRISTIANA PAPAMICHAEL, DEBRA J. SKENE, VICTORIA REVELL

Centre of Chronobiology, University of Surrey, Guildford, UNITED KINGDOM

Light-induced melatonin suppression is irradiance-dependent but the effect of the duration of a light stimulus on the response is less well understood. The aim of the current study was to assess the dependency of the melatonin suppression response and its dynamics on light irradiance, total photon content and light duration.

Young healthy males ($n = 9$) aged 18-35 years (24.7 ± 5.3 yrs; mean \pm SD) were studied in 6 overnight laboratory sessions in a randomised, within-subject design. Each session included a monochromatic light stimulus (λ_{max} 479 nm) of 10, 20 or 30 min duration with light individually timed to occur on the rising phase of the melatonin rhythm. Subjective mood and alertness, heart rate and plasma melatonin levels were assessed at regular intervals before, during and after the light stimulus. Light irradiances were adjusted to administer the same total number of photons (5.4×10^{13} photons/cm²) in 10, 20 and 30 min stimuli or the same photon flux (3×10^{13} photons/cm²/sec) for 10, 20 and 30 min. Melatonin suppression at +10, +20 and +30 min from the start of the light stimulus, as well as the maximum suppression obtained, was calculated. For the photon flux-matched light stimuli (3×10^{13} photons/cm²/sec) the 10 min stimulus induced significantly lower melatonin suppression than the 20 and 30 min stimuli. For the total photon-matched light stimuli (5.4×10^{13} photons/cm²) melatonin suppression did not differ significantly amongst the three stimuli (10, 20 and 30 min). However, assessment of the time course of the response demonstrated that the most sustained melatonin suppression occurred with the 30 min stimulus. These preliminary findings suggest that the magnitude of the melatonin suppression response in humans is dynamic and dependent upon an interaction between the duration and irradiance of a light stimulus.

Detailed characterization of nonvisual light responses in a blind individual

JOSHUA GOOLEY¹, IVAN HO MIEN¹, JOSEPH HULL², ELIZA VAN REEN², ERIC CHUA¹, CATHERINE HANLEY², CHARLES CZEISLER², STEVEN LOCKLEY²

¹*Neuroscience and Behavioral Disorders Program, Duke-NUS Graduate Medical School, Singapore, SINGAPORE*

²*Division of Sleep Medicine, Harvard Medical School, Boston, MA, USA*

Melanopsin-containing retinal ganglion cells mediate non-visual light responses including circadian entrainment, melatonin suppression, and the pupillary light reflex. Characterizing the intrinsic light responses of melanopsin cells in vivo represents a significant challenge, however, because these cells also receive input from visual photoreceptors in normally-sighted individuals. To address this confound, we examined non-visual light responses in a blind individual lacking a functional outer retina. Consistent with the short-wavelength sensitivity of melanopsin cells reported in other species, phase-shift and melatonin suppression responses to 6.5 h of narrow-bandwidth 460-nm blue light were much greater compared to exposure to an equal photon density exposure to 555-nm green light. Also, the pupillary light reflex showed peak spectral sensitivity to short-wavelength light in the 480 to 490-nm range. In the blind subject, the pupils responded sluggishly to light, with no detectable responses below 11 log photons/cm²/s. After reaching a steady-state response, the magnitude of pupillary responses was constant for as long as the light stimulus was presented (for at least 40 min), whereas sighted subjects showed a gradual recovery of pupillary constriction toward the dark-adapted state, even in the presence of continuous light. Our results suggest that compared to the three-cone visual system, human melanopsin exhibits short-wavelength sensitivity, decreased overall sensitivity,

slow kinetics, and sustained activation in response to long-duration light exposures. These findings may have important implications for light therapy-based applications that target activation of the melanopsin signaling pathway. Support: NMRC (NIG09may007, JJG); Duke-NUS (Block Grant, A*STAR & MOE, JJG; NCCAM AT002129, SWL), NIMH (MH045130, CAC); NIH/NHLBI (T32 HL07901).

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Endogenous tau in free-running individuals with delayed sleep phase

MICHELE STANCHINA, JOELLE SCHLANG, PATRICIA MURPHY, SCOTT CAMPBELL

Laboratory of Human Chronobiology, Weill Cornell Medical College, White Plains, NY, USA

It has been proposed that Delayed Sleep Phase Disorder (DSPD), in which nighttime sleep propensity is delayed relative to desired clock time, may be the expression of an abnormally long endogenous period (τ). Here we compare τ during a free-run protocol in subjects with delayed sleep phase versus controls, and examine the relationship between circadian phase during entrainment and free-running τ . Polysomnography (PSG) and core body temperature were recorded continuously for 4 entrainment (ENT) and 14 free-run (FR) days. Bedtimes (BT) and waketimes (WT) were self-selected on ENT1-2 and assigned on ENT3-4. Entrained phase was obtained by fitting a 9th order polynomial curve to temperature data (t_{min}). The mean interval between PSG WTs in FR was used to calculate τ . Five subjects ($40 \pm 11y$; 3F) with ENT $t_{min} > 0700h$ ($7:44 \pm 2:37h$) and preferred BT of $> 0200h$ and WT $> 1000h$ were categorized as Delayed (D); 6 subjects ($40 \pm 10y$) with $t_{min} < 0700h$ ($3:43 \pm 46h$) and normal BT/WTs served as Controls (C). Differences in τ were analyzed using unpaired t-tests. Correlations assessed the relationship between t_{min} in ENT and FR τ for all subjects. FR τ was significantly longer ($t=4.32$, $p=.0019$) in D (25.16 ± 0.51 ; range 24.50-25.79) than in C (24.11 ± 0.29 ; range 23.80-24.62). Additionally, there was a strong correlation between phase in ENT and τ in FR ($r=.865$, $p<.001$). These results support the hypothesis that DSPD is due, at least in part, to a long endogenous circadian period. This long τ manifests in abnormally entrained phase, perhaps reflecting an altered phase response curve. The robust correlation between phase in ENT and FR τ suggests the possibility of predicting τ without the burden of completing long FR protocols. Analysis of "molecular τ " from cultured fibroblasts from these subjects will further examine the relationship between ENT phase and FR τ and potentially enhance our understanding of DSPD etiology.

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The genetic architecture of circadian light sensitivity in Drosophila

ADEOLU ADEWOYE¹, ANNE GENISSEL², SERGEY V. NUZHIDIN², ERAN TAUBER¹

¹*Genetics, University of Leicester, Leicester, UNITED KINGDOM*

²*Molecular and Computational Biology, University of Southern California, Los Angeles, CA, USA*

Previous studies revealed that wild populations of *Drosophila* vary significantly in their circadian photic sensitivity, and that this trait co-varies with latitude. This phenotypic variation presumably reflects adaptation of the clock to the seasonal change of day-length. Yet, the genetic variation underlying this phenotypic variation is largely unknown. Here, we used a set of 123 recombinant inbred lines generated from two parental lines collected in California to carry a quantitative trait loci (QTL) mapping of circadian light sensitivity. We have employed the 'anchored-PRC' protocol using a light pulse at ZT15 and measured the magnitude of phase shift, which typically generates phase delays. The light response of flies was highly variable, translating to a substantial broad-sense Heritability of 30%. Multiple interval mapping revealed four significant Delay QTLs. The QTLs accounted for 7-12% of the variation. The size of the genomic region intervals containing these QTL ranged from 1,400 kb to 7,000 kb and each QTL spans from 154 to 700 genes. The major QTL for Delay on the second chromosome co-localize with *timeless*, and further genotyping indicated that this polymorphism, which we have previously studied in European populations, may also contribute to the observed QTL. To narrow

down the QTL intervals we have also carried a genome-wide expression study using microarrays. We have identified 224 genes whose transcripts show a significant change in expression ($p < 0.01$). 24 of these genes reside within the provisional QTLs that we have identified. The gene Thor for example was significantly down-regulated after a light pulse, and is located in the middle of the major QTL on the 2nd chromosome. Overall, this global expression study allowed us identifying new candidate genes involved in photic entrainment of the clock, and offer candidate genes that may harbour adaptive genetic variation for this trait.

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Chronic ethanol attenuates photic-phase delays, impairs photic entrainment, and disrupts nocturnal locomotor activity in the mouse

ALLISON BRAGER¹, CHRISTINA RUBY¹, REBECCA PROSSER², J. DAVID GLASS¹

¹*Biological Sciences, Kent State University, Kent, OH, USA*

²*Cellular and Molecular Biology and Biochemistry, University of Tennessee, Knoxville, TN, USA*

We have reported that chronic ethanol disrupts circadian phase-resetting in the hamster. Here we extend this work to explore chronic ethanol effects on photic phase-resetting, photic entrainment, and daily locomotor activity patterns in the C57BL/6J mouse ($n=7/\text{group}$). First, microdialysis was used to characterize circadian patterns of ethanol uptake in the suprachiasmatic nucleus (SCN) of mice drinking 10% or 15% ethanol as their sole fluid. Second, the effects of chronic ethanol intake and withdrawal on photic phase-delays were assessed. Third, the effects of chronic ethanol intake on entrainment to a weak photic zeitgeber (1 min pulse of 25 lux light per day) were assessed to minimize masking actions of light that could obscure ethanol effects on pacemaker activity. Peak ethanol levels in the SCN occurred during the dark phase (12 mM for 10%, 20-30 mM for 15%), coinciding largely with the phase-delaying portion of the photic PRC. Light-induced delays of 1.5 ± 0.2 hr were dose-dependently inhibited by 10% and 15% ethanol intake (1.0 ± 0.1 hr and 0.7 ± 0.1 hr, respectively; both $p < 0.01$) and its withdrawal (0.9 ± 0.1 hr and 0.8 ± 0.1 hr, respectively; both $p < 0.02$). Ethanol did not affect re-entrainment to a shifted light cycle, but greatly reduced the initial period of robust nocturnal activity (63 ± 10 min vs. 185 ± 15 min for water; $p < 0.05$) and reduced alpha (11.4 ± 0.2 hr vs. 12.3 ± 0.1 hr, $p < 0.05$). Thus, chronic ethanol intake and withdrawal markedly impair photic phase-resetting, affect entrainment and disrupt the temporal structure of nocturnal locomotor activity. These results suggest direct action(s) of ethanol on the SCN clock that could underlie alcoholism. NIH grant AA015948 to RAP and JDG.

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Effects of irradiance, circadian phase, and photoperiod on light sampling behavior in Syrian hamsters

GENA GLICKMAN, DIVYA AHUJA, MICHAEL GORMAN

Department of Psychology, University of California–San Diego, La Jolla, CA, USA

A variety of factors has been shown to influence circadian response to light, including characteristics of the light source (e.g. duration, wavelength and intensity), the circadian phase in which the pulse is administered and prior photoperiodic history. Often such determinations have been made via studying the effects of carefully controlled lighting parameters on dependent measures of activity rhythms. Radiometric measurements are typically standardized by identifying photic intensity at a particular position relative to the light source. However, unless animals are maintained within a set-up that restricts movement and gaze behavior, the precise number of photons that actually hit the retina could vary substantially. In this study, light sampling behavior was examined during a 15-minute, 480 nm narrow-band light pulse in male Syrian hamsters ($n=16$) as a function of light pulse intensity (0.03 and 68.03 $\mu\text{W}/\text{cm}^2$), circadian phase of administration (LD: ZT14 and ZT19; SD: ZT14 and ZT22)

and prior photoperiodic history (LD14:10 (LD) or LD10:14 (SD)). Animals were individually housed and video-recorded for the duration of each light pulse. Subsequently, the proportion of time spent in various regions of the cage was determined, and integrated photon counts were calculated. ANOVA was used to assess differences in mean total exposure levels between groups (i.e. photoperiod) and changes with intensity/circadian phase (repeated measures). Preliminary data to date suggest a trend for increased total photic exposure later versus earlier in the subjective night. Thus far, no photoperiod or intensity differences have been identified. Further evidence of light sampling as a function of physical properties of the light source and timing of administration may provide practical insight into the ways in which volitional behaviors shape the ability of light to influence the circadian system. Support: NICHD-36460.

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Experience-independent development of the hamster circadian visual system

AUGUST KAMPF-LASSIN, JENNY WEI, JEROME GALANG, BRIAN PRENDERGAST

Psychology, The University of Chicago, Chicago, IL, USA

Experience-dependent functional plasticity is a hallmark of the primary visual system, but it is not known if analogous mechanisms govern development of the circadian visual system. These experiments investigated molecular, anatomical, and behavioral consequences of complete monocular light deprivation during extended intervals of postnatal development in Syrian hamsters. Hamsters were raised in constant darkness and opaque contact lenses were applied shortly after eye opening and prior to the instantiation of a light-dark cycle. In adulthood, previously-occluded eyes were challenged with visual stimuli. Whereas image-formation and motion-detection were markedly impaired by monocular occlusion, neither entrainment to a light-dark cycle, nor phase-resetting responses to shifts in the light-dark cycle were affected by prior monocular deprivation. Cholera toxin subunit-B fluorescent tract-tracing revealed that, in monocularly-deprived hamsters, the density of fibers projecting from the retina to the suprachiasmatic nucleus (SCN) was comparable in occluded and exposed eyes. However, prior long-term monocular deprivation attenuated light-induced c-Fos expression in the SCN. Thus, in contrast to the thalamocortical projections of the primary visual system, retinohypothalamic projections terminating in the SCN develop into normal adult patterns and mediate circadian responses to light largely independent of light experience during development.

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Effect of the absence of melanopsin on the endogenous functioning of the mammalian retinal clock

OURIA DKHISSI-BENYAHYA, CHRISTINE COUTANSON, HOWARD M. COOPER

Chronobiology, INSERM U846, Bron, FRANCE

The mammalian retina contains an endogenous pacemaker that regulates retinal physiology and adjusts the temporal phase of the central circadian timing system with environmental time. This entrainment process involves rods, cones and melanopsin-expressing retinal ganglion cells. In contrast with non-mammalian retinas, in which a circadian clock has been identified in photoreceptors, the location of the retinal clock in mammals is still controversial. In addition, the impact of specific photoreceptor degeneration on the molecular machinery of the endogenous retinal clock is unknown. The experimental strategy is based on the separation of the retina into inner (inner nuclear and ganglion cell layers) and outer (cones and rods) compartments using laser microdissection and real time RT-PCR. We investigated clock (mPer1-2-3, mClock, mBmal1, mCry1-2, mReverb?) and clock-controlled gene (mDbp, mE4bp4) expression in these two retinal compartments during the 24hr cycle at six circadian times in the wild-type mouse. We next evaluated the impact of the absence of melanopsin on the endogenous functioning of the retinal clock by using the Opn4^{-/-} transgenic mouse

model. We find that 1) All clock genes are expressed rhythmically in both inner and outer regions of the wild-type retina (except *mBmal1* in the inner compartment), but with different temporal phases. This suggests the existence of two independent oscillators or a main oscillator driving a second with a certain delay. 2) The absence of melanopsin leads to a dysfunction of the clock mechanism in the inner retina, characterized by an alteration in amplitude of the expression of certain clock genes. Our results suggest that the absence of a specific photoreceptor can contribute to a dysfunction of the retinal clock. Because circadian organization is widespread in the retina and controls fundamental pathways, disruption of circadian organization in the retina could potentially have a major impact on retinal functions such as gene cycling and photopigment regeneration.

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The DNA-binding activity of BMAL1 and CLOCK is affected by time-of-day and sleep pressure in the mouse brain cortex

VALERIE MONGRAIN, FRANCESCO LA SPADA, THOMAS CURIE, PAUL FRANKEN

Center for Integrative Genomics, University of Lausanne, Lausanne, SWITZERLAND

Introduction: We and others showed that clock genes are involved in sleep homeostasis. Indeed, mutations in some clock genes modify the markers of sleep homeostasis and increased sleep pressure using sleep deprivation (SD) alters the expression of clock genes in the forebrain. We tested the hypothesis that SD changes clock gene expression in the cerebral cortex by modifying the binding of core-clock transcription factors to the cis-regulatory sequences of target genes. Methods: Experiment 1: To assess if the DNA-binding activity of CLOCK and BMAL1 to clock genes change according to time-of-day, we performed chromatin immunoprecipitation (ChIP) on C57BL/6J mouse cortex extracts collected at ZT0, -6, -12 and -18 (ZT0=Zeitgeber time 0: lights on). DNA enrichment of E- or E'-box containing sequences was measured by qPCR. Experiment 2: C57BL/6J mice were sleep deprived from ZT0 to -6 by gentle handling and cortical extracts were submitted to ChIP and qPCR. Results: We observed that BMAL1 binds to *Per1*, *Per2*, *Cry1*, and *Dbp* genes in a time-of-day dependent manner ($p < 0.05$), reaching maximum values around ZT6-12. In contrast, the binding of CLOCK to these genes did not vary with time-of-day. SD significantly decreased the DNA-binding of CLOCK to *Per2* ($p < 0.01$) while for BMAL1, only a trend for a decrease was observed for both *Per2* and *Dbp* ($p = 0.1$). Discussion: The changes in the expression of some clock genes with sleep pressure, notably that of *Per2*, could result from changes in the DNA-binding activity of the core clock proteins CLOCK and BMAL1. Ongoing experiments investigate the functional binding of NPAS2 in relation to sleep homeostasis. Research supported by University of Lausanne, FNS (3100A0-111974), and NSERC.

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mTOR signaling couples light to entrainment of the suprachiasmatic circadian clock

RUIFENG CAO, AIQING LI, HEEYEON CHO, BOYOUNG LEE, KARL OBRIETAN

Department of Neuroscience, Ohio State University, Columbus, OH, USA

Inducible gene expression appears to be a critical event in light entrainment of the mammalian suprachiasmatic nucleus (SCN) clock. Recently, we found that photic stimulation leads to robust activation of the mammalian target of rapamycin (mTOR) signaling pathway, in the SCN (*Mol Cell Neurosci.* 2008, 38:312). Given that mTOR is a key regulator of inducible mRNA translation, these data raised the prospect that inducible mTOR-evoked protein expression contributes to light-actuated clock entrainment. Here we continued to investigate the role of this pathway in the clock resetting process. To this end, we report that the *in vivo* abrogation mTOR triggered a significant attenuation of the phase-delaying effect of an early night light pulse, whereas mTOR inhibition during the late night facilitated the phase-advancing effect of light. To assess the role of mTOR signaling within the

context of molecular clock, the effects of mTOR inhibition on light-induced expression of PERIOD1 and PERIOD2 proteins were examined. At both the early and late night time points, abrogation of mTOR signaling led to a significant attenuation of light-evoked PERIOD protein expression. We also provide data showing that light-induced mTOR activation leads to expression of mRNAs with a 5'-TOP tracts. A subset of these mRNAs encode for translational machinery include ribosomal proteins and elongation factors, which have been shown to enhance mRNA translation efficiency. Collectively, these findings indicate that mTOR functions as a potent regulator of light-evoked protein translation and SCN clock entrainment.

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Functional connectivity analysis in synchronized circadian networks

MARK FREEMAN¹, REBECCA KROCK¹, SARA ATON², ERIK HERZOG¹

¹*Biology, Washington University, Saint Louis, MO, USA*

²*Neuroscience, University of Pennsylvania, Philadelphia, PA, USA*

The suprachiasmatic nucleus (SCN) is required for near-24 hour physiological and behavioral rhythms in mammals. Individual SCN neurons have circadian rhythms in firing rate and clock gene expression. Circadian synchrony between neurons is critical for coherent output from the SCN, and thus for behavioral rhythmicity. How these neurons remain synchronous is unclear. Here we study the functional connectivity within SCN networks to determine the degree of connectivity as well as the types of communication important for circadian synchrony. By cross-correlating spike trains of hundreds of pairs of neurons recorded on multielectrode arrays, we found evidence for significant communication between neurons. These correlations peaked within 10 ms of firing by a reference neuron, and could be classified as excitatory or inhibitory based on the polarity of the cross-correlation. We found the strength of correlations to oscillate over the day, and overall connectivity only reached 3–4 % of that expected by all-to-all coupling, suggesting that fast synaptic communication is relatively rare in synchronized networks. We found that blockade of GABA signaling with bicuculline or picrotoxin decreased the number of inhibitory correlations by 90 % on average and decreased the number of excitatory correlations by 0–50% (n=4 cultures). Neither bicuculline nor picrotoxin affected circadian synchrony in SCN slices as measured by firing rate or Period2::Luciferase (PER2::LUC) bioluminescence rhythms. Preliminary experiments showed that blocking ionotropic glutamate receptors with APV and CNQX did not eliminate positive correlations and had no effect on PER2::LUC rhythms in SCN slices. These results indicate that we can probe connectivity in circadian networks and measure the degree of fast synaptic communication. Our results demonstrate that a subset of correlations is mediated by GABA; however, these connections are not necessary for circadian synchrony. The role of these correlations remains to be determined. Supported by NIMH grant 63107.

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Clock-to-clock coupling of SCN and non-SCN cells by diffusible factors

**PATTY KANDALEPAS¹, JENNIFER MITCHELL¹, KAREN WEIS¹, HARRY ROSENBERG², NATHAN HATCHER³,
NORMAN ATKINS, JR.⁴, JOHNATHAN SWEEDLER³, MARTHA GILLETTE¹**

¹*Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

²*Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

³*Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

⁴*Neuroscience, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

The suprachiasmatic nucleus (SCN) is the body's central circadian clock and is involved in synchronizing a myriad of peripheral clocks in unknown ways. Loss of coordination between the central circadian clock in the hypothalamic SCN and non-SCN clocks has been implicated in increased incidences of metabolic disorders, cardiovascular disease, cancer, and sleep disorders. Studies of central nervous

system and peripheral cells and tissues cultured in isolation have revealed that in the SCN's absence, cellular rhythms continue, but they become desynchronized within the population as phase and period properties change. Such SCN-entrainable targets include the paraventricular nucleus (PVN) and cortical glia from brain, as well as liver, lung, muscle, kidney and tail fibroblasts from the periphery. With decoupling, the various tissues and cells lose temporal coherence, as well as appropriate alignment to the daily cycle of sleep and wakefulness. Little is known about what couples an organism's circadian clocks, except that many can be entrained by diffusible factor(s). Here we show that organotypic cultures of SCN brain slices are capable of synchronizing and entraining rhythms in heart fibroblasts and cortical glia, when separated in co-culture by a MilliCell-CM membrane. Induction of circadian rhythms is assessed via expression of clock gene transcripts. Phasing of the peaks of Per2 and Bmal1 expression in non-SCN brain and peripheral cells is dependent on the phase of the SCN, as determined from spontaneous peak electrical activity of SCN brain slices prepared from alternate lighting schedules. Based on the results of these SCN:fibroblast/glia co-cultures, we have undertaken peptidomic analysis of the releasate from SCN cultures. We have identified a number of known and unknown peptides released from the SCN that provide a basis for discovering candidate coupling factors. Supported by PHS grants HL092571 ARRA and P30 DA 018310.

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Neural mechanisms mediating circadian phase resetting by activation of 5-HT7 receptors in the dorsal raphe: Role of GABAergic and glutamatergic neurotransmission

MARILYN DUNCAN, MATTHEW CONGLETON, THOMAS ROGERS, KELSEY LEWIS

Department of Anatomy and Neurobiology, University of Kentucky Medical School, Lexington, KY, USA

Activation of 5-HT7 receptors in the hamster dorsal raphe nucleus (DRN) during midsubjective day induces a circadian phase advance, mimicking other nonphotic signals. Additionally, activation of DRN 5-HT7 receptors in vitro inhibits [3H]glutamate release (Harsing et al, 2005). Blockade of DRN 5-HT7 receptors or activation of GABAA receptors with muscimol blocks novel wheel-induced phase shifts (Glass et al, 2003). Based on these findings, we tested the hypothesis that GABAergic and/or glutamatergic neurons mediate phase shifts induced by activation of DRN 5-HT7 receptors. Male hamsters were fitted with guide cannulae aimed at the DRN, housed in cages with running wheels, and exposed to 14 h light (L):10 h dark (D). In Expt. 1, hamsters were microinjected with muscimol (484 μ M) or vehicle 15 minutes before microinjections with DPAT (30 μ M) at ZT6 and transferred to constant darkness (10 days). Phase shifts were calculated and the animals were re-exposed to 14L:10D. The procedure was repeated to give each animal the alternate pretreatment. Expt. 2 tested the effect of DRN pretreatment with NMDA (glutamate receptor agonist, 100 μ M) or vehicle before DPAT at ZT 6. Other experiments tested the effects of DRN microinjections of muscimol, bicuculline (GABAA receptor antagonist, 680 μ M), NMDA, or vehicle alone. The microinjection sites were verified histologically after each study. Phase shifts (mean+S.E.M., h) in muscimol/DPAT-microinjected hamsters (1.02+0.30) were not different ($P=0.11$) from those in vehicle/DPAT-microinjected hamsters (1.34+0.30), while those in NMDA/DPAT-microinjected hamsters (0.67+0.17) were smaller ($P<0.05$) than those in vehicle/DPAT-microinjected hamsters (0.97+0.10). DRN microinjections of bicuculline, but not muscimol or NMDA, induced phase advances and arousal ($P<0.05$). These findings suggest that the neural pathway mediating DRN 5-HT7 receptor induction of phase advances involves decreased glutamatergic but not GABAergic neurotransmission, and furthermore, that inhibition of DRN GABAergic neurotransmission is sufficient to induce a phase advance. Support: NIH AG13418 (MJD).

Functional development of the mouse SCN in vitro

DANIEL WRESCHNIG, HAMID DOLATSHAD, FRED DAVIS

Department of Biology, Northeastern University, Boston, MA, USA

While the bulk of cell division in the mouse suprachiasmatic nucleus (SCN) is complete by embryonic day 15 (E15), evidence for circadian activity in SCN during prenatal development is sparse. The presence or absence of circadian activity has been examined in two ways: by detection of mRNA for key circadian regulatory genes using in situ hybridization (in vivo) and in one study (and at one age) by a bioluminescence reporter of *per1* promoter activity in rats (ex vivo). Results in vivo are inconsistent and the fetus is potentially influenced by maternal rhythms. In the present study we determined the age at which the mouse SCN is first able to express autonomous circadian rhythms. Explants of mouse anterior hypothalamus containing SCN and expressing a *per2::luciferase* transgene were harvested between E13 and E18 and recorded in a Lumicycle. By E15, SCN traces were visually and statistically distinguishable from arrhythmic cortex, having rhythms that persisted for at least seven days prior to media change. Subsequent embryonic days showed a progressive increase in the significance of circadian period by X^2 periodogram, while cortex never expressed a rhythm. Though E18 explants were robustly rhythmic, E14 explants usually did not express rhythms after 4 days, indicating that normal development is altered in culture. Occasionally, however, rhythm-like behavior spontaneously emerged in E14 explants after several days, suggesting that circadian rhythms in developing SCN explants may initiate in culture. Although the period expressed by E18 explants was relatively stable for 7 days in vitro, explants at earlier ages tended toward more variability across time and samples. This raises the possibility that changes affecting period occur during a critical time during prenatal development, and the presence of rhythmicity as early as E15 suggests that embryonic SCN cells are already coupled in some capacity prior to birth.

The serotonergic innervation of the SCN is necessary for median raphe nucleus stimulation induced phase shifts

GLENN YAMAKAWA, MICHAEL ANTLE

Psychology, University of Calgary, Calgary, Alberta, CANADA

The suprachiasmatic nucleus is shifted in a manner different than light by a group of stimuli collectively referred to as non-photic. Typically, exposure to non-photic stimuli during the mid-subjective day results in large phase advances, with smaller phase delays occurring during the subjective night. These stimuli include confinement to a novel wheel, sleep deprivation by gentle handling and dark pulses. Serotonin levels in the SCN have been shown to increase sharply in response to these stimuli. The median raphe sends a dense, partially serotonergic projection to the SCN. Electrical stimulation of the median or dorsal raphe nuclei results in phase shifts that follow the non-photic phase response curve. We sought to determine if the serotonergic projection to the SCN was necessary for phase advances induced by electrical activation of the median raphe. An alternative explanation would be that activation of the non-serotonergic part of the raphe projection to the SCN is responsible for these phase shifts. Animals received either an injection of vehicle or 5,7-dihydroxytryptamine into the SCN to lesion the serotonergic input. Next, electrical stimulation of the median raphe was conducted from circadian time 4 in the activity rhythm. Those animals receiving a vehicle injection showed phase advances to midday electrical stimulation of the raphe. Those animals receiving a lesion of the serotonin input into the SCN showed no phase shifts, or slight phase delays to electrical stimulation of the raphe. This indicates that the serotonergic input into the SCN is necessary for raphe induced non-photic phase shifting.

Aberrant nonphotic resetting in null mutant mice reveals roles for dexas1 and the intergeniculate leaflet (IGL) in the entrainment of circadian rhythms

MARGARET M. KOLETAR, MARTIN R. RALPH

Centre for Biological Timing and Cognition, University of Toronto, Toronto, Ontario, CANADA

The loss of dexas1 in gene-targeted mice impairs circadian entrainment to light cycles and produces complex changes to phase-dependent resetting responses to light. Here we describe greatly enhanced and phase specific nonphotic responses induced by arousal in dexas1^{-/-} mice that can account for some of the altered responses to light. In constant conditions, mutant mice exhibited large arousal-induced phase shifts throughout the subjective day, and significant phase advances in the late subjective night where arousal has been reported to have little effect in mice. Bilateral lesions of the intergeniculate leaflet (IGL) eliminated both nonphotic as well as the light-induced resetting of circadian locomotor rhythms during the subjective day. In addition, the lesions reduced arousal- and light-induced shifts in the subjective night. The expression of FOS-like protein in the dorsomedial suprachiasmatic nucleus (SCN shell) was not significantly affected by either photic or nonphotic stimulation in the subjective day, in either genotype. However, background expression of daytime cFOS was associated with home cage condition (cage size, animals/cage, environmental enrichment). The results suggest that enhanced or unexpected daytime phase shifts may be due to nonphotic mechanism input from the IGL whether induced by light or arousal signals, and that changes in SCN FOS expression are not necessarily related to clock phase resetting.

Identification of a short, bioactive form of vasoactive intestinal peptide (VIP) in the SCN

JENNIFER MITCHELL¹, JI EUN LEE², NORMAN ATKINS¹, SHIFANG REN², NEIL KELLEHER²,
JONATHAN SWEEDLER², MARTHA GILLETTE¹

¹*Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

²*Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

Vasoactive intestinal peptide (VIP) is thought to be the second most abundant peptide in the SCN, after arginine vasopressin (AVP). VIP signaling through the VPAC2 receptor mediates inter-cellular communication and circadian synchrony. Immunohistochemistry (IHC) has localized VIP expression to a subset of cells within the SCN core of several rodent species. This dense aggregation of VIPergic cells is innervated by the retinal hypothalamic tract (RHT) and is activated by light. Exogenous VIP is an effective signal in phase-shifting the clock in a light-like manner. Identification of VIP has been indirect, by IHC utilizing antibodies generated against the entire 28-amino acid sequence of the VIP peptide. Such antibodies recognize alterations in the full-length form, as well. In contrast to antibody-based approaches, mass spectrometry offers definitive identification of the precise forms of peptides without prior knowledge of peptide identity. We analyzed the peptides released from the SCN in response to stimulating the RHT in a horizontal brain slice from rat by use of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS), and detected a shortened form of VIP corresponding to 13 amino acids from the N'-terminus (VIPs), but not the full-length peptide. Identity of the VIPs peptide was confirmed from the analysis of SCN tissue extract by use of Fourier-transform mass spectrometry (FTMS). (Lee JE et al. 2010, PMID 19955084). The anticipated full-length forms of other predicted SCN peptides, including AVP, gastrin-releasing peptide (GRP) and somatostatin, were

detected. This truncated form of VIP is biologically active, inducing phase shift in the spontaneous electrical rhythm comparable to that of full-length VIP (Reed HE et al. 2001, PMID 11207820). These results suggest that a shortened form of VIP, present within and released from the SCN, may play an essential role in circadian signaling. Funded by HL092571 ARRA and P30 DA 018310.

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Impairment of the cAMP-response-element-binding protein pathway attenuates gastrin-releasing, peptide-induced phase shifts in the early-subjective night

ROXANNE STERNICZUK, MARY MCGILLIVRAY, MICHAEL ANTLE

¹Psychology, University of Calgary, Calgary, Alberta, CANADA

Gastrin-releasing peptide (GRP) has been shown to play an important role in the circadian photic signaling pathway by mimicking the effects of light through wheel-running activity and c-fos expression within the SCN. When an animal is exposed to light at night, both cAMP-response-element-binding protein (CREB) and mitogen-activated protein kinase (MAPK) pathways are activated in the SCN. GRP has been shown to activate the MAPK pathway in a small subset of cells within the dorsolateral SCN, and impairment of this pathway in turn prevents GRP-induced phase shifts. What remains unclear is whether activation of CREB is required to produce GRP-induced behavioral phase shifts in the hamster. Based on previous findings, it was hypothesized that GRP activates the CREB pathway to induce behavioral photic-like phase shifts, and interfering with this pathway should prevent GRP-induced phase shifts. Hamsters received a pretreatment of saline or KT 5720, an inhibitor of CREB phosphorylation, 30 minutes prior to a microinjection of GRP at CT 13 (n=10) or 22 (n=13). Blocking CREB activation significantly attenuated GRP-induced phase shifts in the early-subjective night (p=0.02), but not in the late-subjective night. These findings indicate that CREB activation is necessary for GRP to exert its maximal phase shifting effective on the circadian system in the early-subjective night only. CREB activation does not appear to be a necessary component of the GRP phase shifting pathway in the late-subjective night. (Supported by NSERC).

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Neuropeptide Y induces long-term suppression of action potential frequency in Per1-expressing neurons during the late day

KAREN L. GAMBLE

Psychiatry and Behavioral Neurobiology, The University of Alabama–Birmingham, Birmingham, AL, USA

During the day, novelty-induced exercise, arousal, foot shock and rewarding brain stimulation advance circadian rhythm phase. The “nonphotic” pathway to the brain’s primary pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus, is at least partially mediated by neuropeptide y (NPY), which down-regulates Period (Per) gene expression, inhibits neuronal activity, and induces a phase advance. One previous study suggested that NPY-induced inhibition is not phase-specific (Gribkoff, et al, 1998). However, longer-term suppression of neuronal activity (hours not minutes) may be. Therefore, the present study sought to determine whether day-time NPY application would result in persistent suppression of action potential frequency, and whether suppression of electrical activity is correlated with Per1-gene activity. Because neuronal activity was measured during the late day, loose-patch, extracellular recordings were first used to demonstrate that Per1-fluorescent SCN neurons from Per1::GFP reporter mice exhibit a moderate action potential frequency at this phase. Out of 18 cells recorded between ZT 6.4 and ZT 9.4, 100% were spiking, and action potential frequencies ranged from 1.25 to 7.52 Hz with a mean of 4.3 ± 1.7 (SD). In addition, there was a significant correlation between fluorescence intensity and spike rate (Pearson correlation, $R = 0.454$, $p < 0.05$).

Next, organotypic hypothalamic slices were cultured and treated with NPY (2.3 μ M) or vehicle for one hour beginning at ZT 6 and washed out at ZT 7 upon transfer to the recording chamber. Two to three hours after wash-out, spike rates of NPY-treated Per1::GFP cells were significantly suppressed compared to controls, suggesting that the suppressive effects of NPY are long-lasting. Altogether, these results demonstrate that neuronal activity is significantly predicted by Per1 promoter activation as reported by Per1::GFP even during the late day. Furthermore, day-time application of NPY inhibits the action potential frequency of these neurons, and this suppression persists for several hours. Supported by NIH Grant GM086683.

P176

The food-entrainable oscillator does not drive anticipatory activity, but controls it as circadian manner

WATARU NAKAMURA, NANA TAKASU

Laboratory of Oral Chronobiology, Osaka University Graduate School of Dentistry, Osaka, JAPAN

In mammals, "the master clock" in the suprachiasmatic nucleus (SCN) of the hypothalamus controls most circadian rhythms in physiology and behavior. Recent discovery of molecular machineries of circadian oscillation enables us to examine the genetically manipulated, molecular-clock-deficient mice and use of these animal models has enhanced our understanding regarding the biological clocks. It has been reported that prefeeding locomotor activity under temporal restricted feeding condition exhibits the defining characteristic of a circadian rhythm. The prefeeding activity, so-called food anticipatory activity (FAA), is driven by distinct mechanism from the SCN. Despite extensive studies performed by lesion strategy, the anatomic location of the food-entrainable oscillator has not been determined. Furthermore, recent studies using the molecular-clock deficient mice reveal that the known circadian clock is dispensable for appearance of the FAA. Thus, examining the FAA alone is not sufficient and further examination for circadian oscillatory properties of the FAA rhythm is essential for understanding the food-entrainable circadian rhythms. In the present study, we have examined the oscillatory properties of the FAA in BMAL1-deficient mice. During food deprivation following a restricted feeding regimen, BMAL1-deficient mice did not show distinguishable FAA. Instead, robust ultradian behavioral activity was induced in constant darkness. Although wild type mice showed the limit of entrainable range in T-cycle regimen for restricted feeding, BMAL1-deficient mice showed FAAs for 15h interval. Here we propose the necessity of conceptual change for the output of the FAA.

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Food schedule induces rapid re-entrainment after a 6-hour phase advance in rats

MANUEL ANGELES-CASTELLANOS, CAROLINA ESCOBAR

Anatomy, Faculty of Medicine, Universidad Nacional Autónoma de México, MEXICO

Rapid travel across several time zones results in a conflict between the new cycle of light and dark and the previously entrained cycle of the internal circadian clock, this phenomenon known as jet lag is characterized by, appetite loss, fatigue, disturbed sleep and performance deficit; and internal desynchronize. We investigated the rates of re-entrainment of locomotor activity and core body temperature rhythms following a 6-h phase advance of the LD cycle, evaluating the effect of food restriction or melatonin administration in the prevention of Jet-lag in rats. Methods: rats were maintained under 12-12 light-dark conditions with water and food ad libitum for 2 weeks, and were then exposed to a 6h phase advance. During the 5 days prior to the phase shift a group of rats (N=16) was submitted to restricted food or a subcutaneous melatonin (1mg/kg b. w.) injection scheduled at the new expected ZT12 phase (defined as lights off). A second group (N=16) received the treatment simultaneously and for the first 5 days after the phase advance at the new ZT12. Results: The transitory cycles necessary

for re-entrainment, in the control group took 11 and 7 days to reach stable phase in activity and temperature respectively. The food entrained group took 5- 6 days, and the melatonin group took 9-7 days. When food and melatonin were scheduled simultaneous to the light shift, food restriction reduced transitory cycles to 2-3 days, and melatonin reduced them to 2-5 days. Conclusion:

melatonin administration proved to be efficient for a faster re-entrainment; however scheduled food showed to be a better strategy specially when shifting meal time simultaneous to the light shift. We may presume that food entrained peripheral oscillators facilitate the task of the SCN to couple peripheral functions to the new LD cycle. GRANTS: DGAPA-PAPIT IN205809, CONACyT 82462.

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A circadian clock is not required in an Arctic mammal

QING-JUN MENG¹, WEI-QUN LU¹, NICHOLAS TYLER², KARL-ARNE STOKKAN³, ANDREW LOUDON¹

¹*Faculty of Life Sciences, University of Manchester, Manchester, UNITED KINGDOM*

²*Centre for Sámi Studies, University of Tromsø, Tromsø, NORWAY*

³*Department of Arctic and Marine Biology, University of Tromsø, Tromsø, NORWAY*

Seasonally breeding mammals use the annual change in the photoperiod cycle to drive rhythmic nocturnal melatonin signals from the pineal gland, providing a critical cue to time seasonal reproduction. Paradoxically, species resident at high latitudes achieve tight regulation of the temporal pattern of growth and reproduction despite the absence of photoperiodic information for most of the year. In this study, we show that the melatonin rhythm of reindeer (*Rangifer tarandus*) is acutely responsive to the light-dark cycle but not to circadian phase and also that two key clock genes monitored in reindeer fibroblast cells display little, if any, circadian rhythmicity. The molecular clockwork which normally drives cellular circadian rhythms is evidently weak or even absent in this species and instead, melatonin-mediated seasonal timing may be driven directly by photic information received at a limited time of year, specific to the equinoxes.

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General anaesthesia is a thief of time

JAMES CHEESEMAN¹, CRAIG MILLAR², LISA FENDALL², JAMES SLEIGH¹, MARK GOODWIN³, MAT PAWLEY¹, KONSTANTIN LEHMANN⁴, RANDOLF MENZEL⁴, GUY WARMAN¹

¹*Department of Anaesthesiology, University of Auckland, Auckland, NEW ZEALAND*

²*School of Biological Sciences, University of Auckland, Auckland, NEW ZEALAND*

³*Apiculture Research Unit, The New Zealand Institute for Plant & Food Research Limited, Hamilton, NEW ZEALAND*

⁴*Institute of Biology-Neurobiology, Free University of Berlin, Berlin, GERMANY*

We have examined the effect of general anaesthesia (GA) on time compensated behaviour in the honey bee *Apis mellifera* to test whether anaesthesia affects the perception of the passage of time. Anaesthesia is poorly understood, but recent evidence suggests it shares common neurological mechanisms with natural sleep. GAs act on the same brain centres controlling sleep, and sleep debt is recovered during anaesthesia. However, anaesthesia can disrupt sleep and patients often emerge from anaesthesia confused about how much time has passed. In order to test the effects of anaesthesia on time perception we have utilised two classical time-dependent behaviours of the honey bee: 1) time-compensated-sun-compass-orientation and 2) food-anticipatory-behaviour. Orientation of control bees and bees anaesthetised for six hours (2% isoflurane) was recorded by observing vanishing bearings and by tracking flight paths using harmonic radar. Following a six hour anaesthetic bees behave as though no time has passed since their anaesthetic began, showing an anticlockwise shift of vanishing bearings (mean 79.5°, 95%CI 53.9°, 105.2°) compared to controls (mean 166.3°, 95%CI 154.5°, 178.1°)

in the Southern hemisphere, and a clockwise shift in the flight paths of test bees (mean 16.6° 95%CI 1.3°, 31.8°) compared with controls (mean 328.7° 95% CI (324.9°, 332.5°) in the Northern hemisphere. Following anaesthesia of whole hives for six hours the timing of food-anticipatory-behaviour is delayed by approximately 3.5 hours. Importantly this effect persisted for several days despite the presence of light-dark cycles. Together these results strongly suggest that time perception is stopped during anaesthesia and that the effect of this time suspension endures for days. The underlying mechanism by which this occurs appears to be a phase shift of the circadian clock (see abstract by Winnebeck et al.). More broadly our results suggest that post-operative patient management may need to account for what is in effect drug-induced jet lag.

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Maternity-related plasticity in circadian rhythms of bumble bee (*Bombus terrestris*) queens

ADA EBAN-ROTHSCHILD, SELMA BELLUCI, GUY BLOCH

Department of Evolution, Systematics and Ecology, The Hebrew University of Jerusalem, Jerusalem, ISRAEL

Worker bees and ants naturally switch between activity with and without circadian rhythms along with their role in the division of labour that organizes their societies. This socially-modulated plasticity contrasts with evidence for increased pathologies and deterioration in performance in animals induced to be active with no circadian rhythms. Because it has been suggested that sibling brood care evolved from maternal behaviour, we tested whether bumble bee (*Bombus terrestris*) queens that care alone for their first batches of offspring, are also capable of around-the-clock activity. We monitored locomotor activity of queens at various life cycle stages, and of queens for which we manipulated the presence of brood. We found that gynes typically emerged with no circadian rhythms, but later in life showed robust rhythms. Mating and diapause did not affect the expression of circadian rhythms, but colony-founding queens with brood showed no, or only attenuated circadian rhythms. By contrast, queens for which we removed the brood or that lost it for other reasons, switched to activity with strong circadian rhythms. This remarkable plasticity in queens is consistent with the hypothesis that task related plasticity in the circadian system of workers evolved from maternity-related plasticity in the circadian clock.

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Circadian and circatidal clocks in a marine organism

LIN ZHANG¹, DAVID WILCOCKSON², SAMIRA PERERA¹, JOE O'GRADY², MICHAEL HASTINGS³,
SIMON WEBSTER⁴, CHARALAMBOS KYRIACOU¹

¹*Genetics, Leicester University, Leicester, UNITED KINGDOM*

²*Institute of Biological and Environmental Sciences, Aberystwyth University, Aberystwyth, UNITED KINGDOM*

³*Division of Neurobiology, MRC Laboratory of Molecular Biology, Cambridge, UNITED KINGDOM*

⁴*The School of Biological Sciences, University of Wales Bangor, Bangor, UNITED KINGDOM*

Many marine organisms synchronise their behaviour and physiology to predict daily cycles in light/dark and high/low tides. This adaptation is governed by endogenous clocks, yet little is known about the genetic and molecular basis of the circadian and circatidal clocks in these organisms. We have been investigating the intertidal crustacean isopod, *Eurydice pulchra*, which displays circatidal (~12.4 h) swimming activity and circadian (~24 h) rhythms of chromatophore dispersion under constant laboratory conditions. We have isolated full-length canonical circadian gene orthologues, and examined their expression patterns under various lighting conditions. Luciferase based transcription assays in *Drosophila* embryonic cell lines reveal that the negative elements of the circadian clock in these organisms is probably PER-CRY, rather than PER-TIM based. We have also transformed clock

genes from *Eurydice* into *Drosophila* per-null hosts and have observed correlations between the effectiveness of homo- and heterospecific per genes in their rescue of circadian behaviour, and their effectiveness as negative regulators in the transcriptional assays. Beach-caught and laboratory entrained *Eurydice* express circadian rhythms of chromatophore dispersal and tidal swimming rhythms. We show that under LL, this circadian phenotype is disrupted, whilst tidal swimming behaviour is unaffected, suggesting some independence of the two oscillators, at least at the level of light input pathways. We are currently developing RNAi to assess the contributions of the canonical clock genes to both circadian and tidal phenotypes.

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Jetlag in malaria parasites: The importance of synchronizing with the host

AIDAN J. O'DONNELL¹, PETRA SCHNEIDER¹, SARAH E. REECE¹, HARRIET G. McWATTERS²

¹*School of Biological Sciences, University of Edinburgh, Edinburgh, UNITED KINGDOM*

²*Department of Plant Sciences, University of Oxford, Oxford, UNITED KINGDOM*

A cornerstone of circadian biology is the expectation that clocks maximise fitness by coordinating individuals with their environment. Despite this, there have been few demonstrations of the adaptive value of circadian rhythms in eukaryotes. The cyclical nature of malaria infections has long been noted, with the cell cycle of all species being a multiple of 24 h, implying that is either controlled by an endogenous clock or responding directly to the rhythm of day and night or entrained to the host circadian rhythm. We investigated the effect of inducing 'jetlag' in the rodent malaria *Plasmodium chabaudi* by creating infections temporally mismatched to the host rhythm and testing the effects on parasite growth and transmission. We found that jetlagged parasites paid fitness costs in terms of lower in-host replication and between host transmission. However, parasites minimise these costs by adjusting the timing of their cell cycle from the original photoperiodic regime to that of their new hosts. Our novel approach reveals the role of biological rhythms in the evolution of host-parasite interactions. Understanding the interactions between host and parasite circadian rhythms could offer new insight into controlling infectious diseases, such as malaria.

P183-1

Circadian rhythmicity persists without transcription in a eukaryote

GERBEN VAN OOIJEN¹, JOHN O'NEILL¹, CARL TROEIN², LAURA DIXON², FLORENCE CORELLOU³, FRANÇOIS-YVES BOUGET³, ANDREW MILLAR⁴

¹*Centre for Systems Biology at Edinburgh, University of Edinburgh, Edinburgh, UNITED KINGDOM*

²*School of Biological Sciences, University of Edinburgh, Edinburgh, UNITED KINGDOM*

³*Laboratoire d'Océanographie Microbienne, Université de Paris 06, Banyuls s/mer, FRANCE*

⁴*Centre for Systems Biology at Edinburgh, University of Edinburgh, Edinburgh, UNITED KINGDOM*

Mechanistic models of cellular circadian clocks rely heavily on networks of transcriptional feedback loops, whereby rhythmic expression of clock gene products regulate their own expression and also that of myriad clock-controlled output genes. However, additional, post-translational mechanisms have recently been shown to contribute to timing mechanisms in a range of organisms, with the result that it is presently unclear whether timed regulation of transcription is indeed necessary to sustain the eukaryotic cellular clock. We present the first example in a eukaryote of time keeping that cannot be entirely accounted for by transcriptional feedback mechanisms, using the novel circadian model organism *Ostreococcus tauri*. This unicellular pico-eukaryotic alga possesses a naturally reduced circadian clock that shares many features with higher eukaryotes, including a central negative feedback loop between the morning- and evening-expressed genes CCA1 and TOC1 similar to plant clocks. We have demonstrated that a mathematical model describing the circadian clock as a single feedback loop captures all features of clock gene expression under different lighting conditions and in genetic

mutants. However, experimentally we observed significant windows of insensitivity to transcriptional or translational inhibition in the circadian cycle, and furthermore, phase information is conserved for >24 hours in the absence of transcription, ruling out the possibility that transcriptional feedback loops are the sole carriers of circadian rhythms. We ultimately show that the pharmacological sensitivity of the timing mechanism to inhibitors of various highly conserved post-translational mechanisms is shared between a green alga and mammalian cells, presenting *Ostreococcus tauri* as the first platform for efficient chemical biology on intact organisms in the green lineage. A common, post-translational clock mechanism in eukaryotes forms a new paradigm for circadian research that will have a deep impact in clock research.

P183-2

The circadian clock influences short-time perception in mice

PATRICIA V. AGOSTINO, MICAELA DO NASCIMENTO, IVANA L. BUSSI, DIEGO A. GOLOMBEK

Department of Science and Technology, National University of Quilmes, Bernal, ARGENTINA

Temporal perception is fundamental to environmental adaptation in humans and other animals. To deal with timing, organisms have developed multiple systems that are active over a wide range of magnitude, the most important being circadian timing, interval timing and millisecond timing. The circadian pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and is driven by a self-sustaining oscillator with a period near to 24 h. Time estimation in the second-to-minutes range—known as interval timing—involves the interaction of the basal ganglia and the prefrontal cortex. Several studies have reported that short-time perception is not independent of the influence of the circadian pacemaker. In this work we tested the hypothesis that interval timing in mice is sensitive to circadian modulations. Animals were trained following the peak-interval (PI) procedure. Briefly, mice were trained in three consecutive phases—pre-training, fixed interval training and peak interval training. Mice were trained in the middle of their diurnal phase (ZT 5-7) or their nocturnal phase (ZT 17-19). Results show significant differences in the estimation of 24-second intervals at different times of day, being more accurate the group trained at night. Interval timing was also studied in animals under constant light (LL) conditions, which abolish circadian activity. Mice under LL conditions were unable to acquire temporal control in the peak interval procedure. Moreover, short time estimation in animals subjected to circadian desynchronizations, such as jet-lag, was also affected. Taken together, our results indicate that short-time estimation is modulated by the circadian clock. We are currently studying the estimation of time throughout the circadian cycle, as well as the role of the SCN on the regulation of dopamine levels in the striatum. We also aim to develop theoretical models which simulate the mechanisms involved in the link between circadian and interval timing.

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A mechanistic analysis of frequency demultiplication

CHRISTIAN HONG¹, ANTONIO PREQUEIRO¹, LUIS LARRONDO², JENNIFER LOROS³, JAY DUNLAP¹

¹*Genetics, Dartmouth Medical School, Hanover, NH, USA*

²*Departamento de Genética Molecular y Microbiología, Pontificia Universidad Católica de Chile, Santiago, CHILE*

³*Biochemistry, Dartmouth Medical School, Hanover, NH, USA*

Entrainment to environmental cycles is a defining property of circadian rhythms, and entrainment of these rhythms by cycles that repeat twice or more often per day is also a characteristic of circadian rhythms. Although first reported nearly a century ago, this property of subharmonic entrainment, known as frequency demultiplication, has not been intensively studied and is poorly understood in theoretical and molecular terms. To better understand this phenomenon, we developed a mathematical model and tested it using the well understood circadian system that controls developmental patterning

in *Neurospora crassa*. In a core feedback loop of this circadian oscillator, the protein product of the frequency (*frq*) gene (FRQ) feeds back to depress the activity of its activator, WCC, prior to FRQ becoming phosphorylated and turning over. In addition to this self-regulation, expression of *frq* and FRQ is known to increase with temperature. We use temperature demultiplication conditions to study molecular profiles of FRQ and verify the hypotheses from our mathematical model.

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How the circadian clock can generate cellular 12-hour rhythms

PÁL WESTERMARK, HANSPETER HERZEL

Institute for Theoretical Biology, Charite Universitaetsmedizin, Berlin, GERMANY

Recently, abundant 12 h periodicities in gene expression and key metabolic processes have been discovered in mouse (Hughes et al., PLoS Genet 5, 2009; Ramsey et al., Science 324, 2009; Cretenet et al., Cell Metab 11, 2010). Basic trigonometry dictates that any such rhythm cannot simply be explained as a superposition of two phase-shifted sinusoidal (or near-sinusoidal) circadian species or processes. Here, we describe how the 12 h rhythms in gene expression and in protein concentrations can be generated by the cellular core circadian clock. We take an inventory of possible biochemical mechanisms, and it turns out that higher harmonics naturally arise in dimerization processes (such as Per/Cry complex formation), as well as in models of gene expression where two transcription factors bind to separate motifs (e.g. E-boxes, E'-boxes, and ROR elements). Surprisingly, the formal principles that govern ultradian harmonics, also apply to much slower rhythms: circannual rhythms and the decoding of seasonal photoperiodism in the pars tuberalis (Lincoln et al., PNAS 99, 2002). With a clear understanding of the generation of harmonics, we may advance towards circadian spectroscopy: the exploitation of experimentally measured waveforms to identify underlying mechanisms.

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Seq-ing new insights into the circadian transcriptome

NICHOLAS LAHENS¹, MICHAEL HUGHES², HEE-KYUNG HONG³, GREGORY GRANT⁴, ANGEL PIZARRO⁵, MARK CONSUGAR⁶, ERIC PIERCE⁶, BRIAN GREGORY⁷, JOSEPH TAKAHASHI³, JOHN HOGENESCH¹

¹*Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

²*Cellular & Molecular Physiology, Yale School of Medicine, New Haven, CT, USA*

³*Neurobiology & Physiology, Northwestern University, Evanston, IL, USA*

⁴*Penn Center for Bioinformatics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

⁵*Institute for Translational Medicine and Therapeutics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

⁶*F.M. Kirby Center for Molecular Ophthalmology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

⁷*Biology, University of Pennsylvania School of Arts & Sciences, Philadelphia, PA, USA*

Circadian rhythms in biology and behavior are thought to arise from a cascade of events beginning with transcription. To define output regulators, most circadian studies have used DNA microarrays to quantify mRNA levels over time. However, these studies typically only look at one or a few transcripts per gene and ignore noncoding RNA expression altogether. To address these limitations, we developed a hybrid approach using DNA exon microarrays (exon arrays) to quantify expression levels of all exons and paired-end high-throughput RNA sequencing (RNA-seq) to characterize the structure of mRNAs. We profiled mouse liver every two hours for two circadian cycles using exon arrays. Statistical analysis showed outstanding correlation with previous studies. In addition, this analysis revealed hundreds of transcripts with one or more cycling exons that were not detected as cycling at the whole transcript level. In parallel we developed a paired-end 115bp RNA-seq protocol. We applied this protocol to wildtype mouse livers sampled every six hours. After struggling with informatics tools to analyze this

data, we developed and applied a novel data analysis pipeline capable of accurately mapping gapped sequence reads to a reference genome, which unambiguously mapped 85% of our sequence data. We show several use cases where this information can complement exon array data including finding evidence for cycling transcription within introns. Our hybrid approach is revealing novel aspects of circadian regulation of gene expression.

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Methylation analyses on promoters of mPer1, mPer2, and mCry1 during perinatal development

YAOTING JI, YUE QIN, HONGBING SHU, XIAODONG LI

College of Life Sciences, Wuhan University, Wuhan, CHINA

Recent studies revealed dramatic changes in circadian clock genes' expression during the perinatal period. In this study, we characterized DNA methylation for three clock genes mPer1, mPer2 and mCry1 at their selected promoter regions during development. Results for the suprachiasmatic nucleus (SCN) and liver (at embryonic day 19, postnatal day 1 and postnatal day 7) were compared to those of sperm. Few methylations were detected for the mPer2 and mCry1 promoters. The 3rd E-box region of the mPer1 promoter exhibited methylation only in sperm. Significant demethylation was observed in the 4th E-box region of the mPer1 promoter between E19 and P1 in the SCN but not in liver tissue. This demethylation state was maintained at P7 for the SCN. Luciferase reporter assays using in vitro methylated promoters revealed an inhibitory effect of promoter methylations on mPer1 expression. The results suggested that epigenetic mechanisms such as DNA methylation might contribute to the developmental expression of clock genes.

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A functional circadian clock in HaCaT-Keratinocytes

FLORIAN SPOERL¹, ACHIM KRAMER², KATJA SCHELLENBERG², THOMAS BLATT¹, HORST WENCK¹,
ANNIKA SCHRADER¹

¹*R&D, Beiersdorf AG, Hamburg, GERMANY*

²*Laboratory of Chronobiology, Institute for Medical Immunology,
Charité Universitätsmedizin, Berlin, GERMANY*

Circadian rhythms govern a variety of physiological processes in most organisms including mammals and have recently been proposed to regulate essential skin functions. However, on a molecular level the link between the circadian clock and skin functions remains poorly understood. This study set out to establish Human Adult Low Calcium Temperature Keratinocytes (HaCaT) as a model system to study circadian gene expression as well as rhythmic cell growth in epidermal cells. A robust circadian phenotype could be observed by oscillating Bmal1 reporter activity over a period of 6 days after dexamethasone synchronization. Temperature entrainment (37°C/ 33°C cycles) also induced rhythmic Bmal1 expression with oscillations persisting after release into constant conditions. Furthermore, other components of the circadian clock (Per3, Rev-Erb α) as well as a clock output gene (the transcription factor Dbp) showed circadian expression profiles. When cells were temperature entrained we found significantly higher amplitudes in clock gene expression compared to dexamethasone synchronization alone. This observation identifies temperature as a potential Zeitgeber for epidermal keratinocytes. In order to monitor rhythmic cell growth as a possible physiological output of the circadian clock in vitro, real-time cell analysis (RTCA), a novel impedance-based method was employed. When HaCaT cells

were cultivated under low-serum conditions impedance measurements revealed a highly rhythmic (cosine fitting with $R^2 = 0.993$) modulation of cell growth). To our knowledge this is the first method allowing online monitoring of rhythmic cell growth to a high degree of accuracy. This study combines two approaches of real-time analysis: (i) circadian gene expression and (ii) rhythmic cell growth as a physiological output of the circadian clock. We believe this method feasible to facilitate research into the interplay between the circadian clock and cell cycle regulation.

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Accelerated re-entrainment in BALB/cJ mice

THOMAS VAJTAY, TODD WEBER

Biology, Rider University, Lawrenceville, NJ, USA

Recent work by our group demonstrated that BALB/cJ mice re-entrain to large (6-8 hour) advances of the light-dark cycle within 1-2 days, as compared to the 4-5 days required for C57BL/6J mice. However, acute exposures to light (15 minutes at 200-500 lux) during late subjective nighttime do not yield exaggerated phase advances in BALB/cJ mice. Likewise, 6-hour exposures to light during late subjective nighttime corresponding to the early onset of light during a six-hour advance of the light-dark cycle do not yield exaggerated phase shifts. These results suggest that the magnitude of light-induced phase shifts in this strain of mice is not merely dependent on discrete light exposure at phase-sensitive points of the cycle, but rather an integration of as-yet-unspecified salient features of the light exposure (e.g., duration, intensity). We continue to evaluate the circadian system in this strain of mice to determine the extent to which it conforms to current models of entrainment.

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A DEAD Box RNA helicase-ribonucleoprotein complex regulates transcriptional repression by mammalian PERIOD proteins

KIRAN PADMANABHAN¹, MARIA ROBLES², CHARLES WEITZ¹

¹*Neurobiology, Harvard Medical School, Boston, MA, USA*

²*Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, Munich, GERMANY*

The mammalian circadian clock consists of a molecular feedback loop in which the heterodimeric transcription factors CLOCK and BMAL1 drives the expression of PERs and CRYs, which in turn assemble into one or more multiprotein complexes to inhibit the action of CLOCK-BMAL1. To decipher how the principal components of the negative feedback loop function, we biochemically isolated PER1 and PER2 complexes from livers of mice harboring a FLAG-HA PER transgene by tandem-affinity purification and analyzed them by MALDI-TOF mass spectrometry. PER complexes include a group of RNA-binding proteins that fall into broad functional classes of chromatin binding, transcription and pre-mRNA processing. Of these, we investigated the role of a DEAD box RNA helicase within the negative feedback loop. In standard transcription assays, the helicase negatively regulates BMAL1-CLOCK mediated transcription, whereas siRNA-mediated depletion displays a period-shortening phenotype in oscillating fibroblasts. Density gradient centrifugation analysis of liver nuclear extracts suggests that the helicase and associated RNA binding proteins assemble in a ~1 MDa complex with PERs. Further biochemical analyses indicate that PER may recruit this pre-assembled ribonucleoprotein complex to regulate RNA Polymerase II function.

TRAP150, a circadian interface between CLOCK-BMAL1 and the general transcription machinery

CYRIL BOYAULT¹, LAURA LANDE-DINER², CHARO ROBLES³, DARKO KNUTTI⁴, CHARLES WEITZ²

¹*Differentiation and Cellular Transformation, Albert Bonniot, La Tronche, FRANCE*

²*Neurobiology, Harvard Medical School, Boston, MA, USA*

³*Proteomics and Signal Transduction, Max Planck Institute for Biochemistry, Martinsried, GERMANY*

⁴*DSM Nutritional Products, Basel, SWITZERLAND*

The mammalian circadian clock is built on a transcriptional feedback loop in which the heterodimeric transcription factor CLOCK-BMAL1 drives expression of its inhibitors, PER and CRY proteins. Despite the central importance of CLOCK-BMAL1 in the circadian clock, surprisingly little is known about its mechanism of action as a transcription factor. To obtain new insights into CLOCK-BMAL1 function, we analyzed BMAL1 protein complexes from mammalian fibroblasts, and we found that they include TRAP150, a known transcriptional co-activator for certain transcription factors. TRAP150 appears to be an accessory subunit of the Mediator, a large protein complex that integrates signals from DNA-bound factors and correspondingly influences the formation of the RNA Polymerase II pre-initiation complex at the start site of transcription. TRAP150 and BMAL1 co-immunoprecipitated from mammalian tissue extracts, and TRAP150 mRNA and protein in the liver showed a circadian oscillation peaking at circadian time 4-8 hours, the circadian phase of active CLOCK-BMAL1 transcription. In transcriptional reporter assays, TRAP150 strongly enhanced the activity of CLOCK-BMAL1 but not that of MYOD-E12 or beta-CATENIN, suggesting considerable selectivity as a transcriptional co-activator for CLOCK-BMAL1. Depletion of TRAP150 from fibroblasts caused a decrease in the steady-state concentration of Per1 pre-mRNA, a canonical CLOCK-BMAL1 target gene, but not Gapdh pre-mRNA, indicating a selective action in CLOCK-BMAL1 transcriptional co-activation. Furthermore, depletion of TRAP150 from fibroblasts caused a significant lengthening of circadian period, indicating that TRAP150 plays a role in the clock oscillatory mechanism, consistent with that of a CLOCK-BMAL1 co-activator. Thus TRAP150 appears to be a circadian-regulated transcriptional co-activator that acts as an interface between CLOCK-BMAL1 and the general transcription machinery.

Untangling transcriptional networks in the Neurospora circadian system

ALVARO GONZALEZ-VOGEL¹, ALEJANDRO MONTENEGRO-MONTERO¹, PAULO CANESSA¹, CHEN HUI-CHEN², JENNIFER J. LOROS², JAY C. DUNLAP², LUIS F. LARRONDO¹

¹*Genetica Molecular y Microbiologia, Pontificia Universidad Catolica de Chile, Santiago, CHILE*

²*Genetics, Dartmouth Medical School, Hanover, NH, USA*

The *Neurospora crassa* circadian oscillator is composed of an elegant transcriptional translation negative feedback loop in which WCC, a heterodimeric transcriptional complex (composed of White Collar-1 and White Collar-2) activates the expression of frequency (frq). As FRQ starts to accumulate, it gets progressively phosphorylated and by interacting with FRH (FRQ-interacting RNA helicase) can promote the phosphorylation and inactivation of the WCC. Thus, frq expression is shut down, the existing FRQ is further phosphorylated and degraded, and a new cycle of transcription-translation is then ready to begin. These robust rhythms can be phenotypically observed as a strong pattern of conidial banding (spore formation), with a periodicity of 22.5 hours. Molecularly, they can be also followed by a fully codon-optimized real-time luciferase reporter. By using this system we have started to develop a high-throughput screening platform to identify new players affecting the core oscillator, as well as components involved in passing the rhythm from the latter to an extensive cohort of genes subjected to circadian control. Thus, we have already identified interesting candidates affecting either the core oscillator or the output pathways. In the latter group we have identified at least one

transcription factor that regulates the expression of some ccgs, and that potentially represents a direct link between WCC (core oscillator) and the downstream output machinery. By these and other strategies we expect to start unveiling the main components involved in the circadian control of gene expression in *Neurospora*. Funding: FONDECYT 1090513, CRP-ICGEB CHI09-02.

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Baculovirus photolyases are DNA repair enzymes with circadian clock regulatory function

MAGDALENA BIERNAT¹, ANDRÉ EKER², MONIQUE VAN OERS¹, JUST VLAK¹, GIJSBERTUS VAN DER HORST², CHAVES INÊS²

¹*Laboratory of Virology, Wageningen University, Wageningen, THE NETHERLANDS*

²*Department of Genetics, Erasmus University Medical Center, Rotterdam, THE NETHERLANDS*

The cryptochrome/photolyase family (CPF) encodes photosensitive proteins that exhibit either photoreceptor or DNA repair activity. Photolyases and cryptochromes are structurally conserved, but display distinct functions. Photolyases use visible light to repair UV-induced DNA damage. Cryptochromes, on the other hand, function as blue-light receptors, circadian photoreceptors and transcriptional repressors controlling the molecular circadian clock. Recently, we have obtained evidence that this functional divergence is not so univocal anymore. *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) possesses two photolyase genes, designated *phr1* and *phr2*. It has been reported that the *phr2* gene encodes an active enzyme with DNA repair activity towards UV-induced cyclobutane pyrimidine dimers. The *phr2* gene can rescue bacteria lacking a UV repair system, whereas the *phr1* gene product does not have this ability. Here we demonstrate that PHR2 as well as PHR1, when overexpressed in NIH3T3 cells affect the amplitude of circadian oscillations, suggesting that these proteins may interact with, and thus function, in the mammalian molecular clock. Indeed, PHR2 is capable of inhibiting CLOCK/BMAL1-driven transcription of E-box promoter containing clock genes, a function normally restricted to cryptochrome proteins. Whether the baculoviral photolyases have a circadian role in ChchNPV-infected *C. chalcites* larvae remains to be studied. This observation may be highly relevant for a further understanding of the evolution of cryptochrome and photolyase functions, as well as for a better understanding of behavioral changes in insect virus-host pathosystems.

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Transcription factor coding of the mouse SCN

CASSANDRA VANDUNK, PAUL GRAY

Anatomy and Neurobiology, Washington University, St Louis, MO, USA

The suprachiasmatic nuclei (SCN) are distinct bilateral nuclei consisting of heterogeneous populations of neurons that interact to produce behavioral rhythms on a 24-hour time scale. A great deal is known about clock function and expression of specific neuropeptides and clock-related genes within the mature SCN, but the transcription factors (TFs) involved in the initial specification and maturation of SCN neurons are essentially unknown. This is most striking during early developmental time periods where current descriptions of the progenitor domain rely purely on anatomical landmarks. From an in depth analysis of a genome-scale transcription factor expression screen we identify ~20 transcription factors present relatively specifically within the early postnatal mouse SCN including *Lhx1*, *RORa* (*Nr1f1*) and *Six3*. Further analysis of these TFs at sequential stages revealed distinct profiles within the developing SCN. Interestingly, we find *Six3*, a homeobox gene sequentially important for head, retina, and forebrain development, is present in postnatal and embryonic SCN as well as within the premitotic proliferative region from which the SCN is derived. Using the CreLoxP system we determined loss of *Six3* after retina formation completely eliminates SCN formation. Using *Six3* as a genetic marker, we find the SCN progenitor region lacks SHH expression but does express *Frz5* suggesting a unique

signaling environment shapes the early SCN. The patterns of gene expression within the progenitor region, as well as the phenotype of the conditional Six3 knock out mice suggest the SCN is more closely related to the telencephalon than diencephalon. Overall, we propose that understanding the molecular development of the SCN will provide insight into SCN anatomy, organization, evolution, as well as circadian behavior.

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Circadian calcium oscillations inversely correlate with rhythmic extracellular ATP accumulation in SCN cultures

ALISA WOMAC, JEFF BURKEEN, DAVID EARNEST, MARK ZORAN

Department of Biology, Texas A&M University, College Station, TX, USA

Extracellular ATP is accumulated rhythmically in the suprachiasmatic nucleus (SCN) of the rat with peak ATP levels occurring in late night and persisting in constant darkness. Resting calcium in the rat SCN is high during the day and low at night. The SCN is composed of multiple neuronal cell types and a high density of astrocytes. Since the SCN exhibits circadian rhythms in ATP accumulation and ATP release from astrocytes is thought to be dependent on calcium excitability, we have hypothesized that the peak levels of extracellular ATP accumulation should correlate with the cytosolic calcium oscillations in SCN-derived astrocytes. Resting calcium concentrations were estimated for immortalized SCN2.2 cell cultures using Fura-2 ratiometric imaging. Simultaneously, extracellular accumulation of ATP was monitored in media samples with luciferin/luciferase luminometry. Resting calcium was estimated lowest (100-150 nM) when extracellular ATP accumulation was high. Conversely, cytosolic calcium levels were elevated two-fold (200-250 nM) when extracellular ATP was low. Therefore, resting calcium levels oscillate with a 24-hour periodicity in SCN2.2 cells and this astrocytic calcium oscillation is inversely correlated with rhythmic extracellular ATP accumulation. Since ATP is produced in mitochondria and calcium signaling is important to mitochondrial function, we investigated whether peak and trough levels of extracellular ATP correlated with differing levels of mitochondrial intracellular calcium. At the ATP peak, mitochondrial Rhod-2 fluorescence intensity was significantly higher than that detected when extracellular ATP accumulation was lower (trough time point). Thus, cytoplasmic calcium was highest at the ATP trough time point, when mitochondrial calcium was lowest, and cytoplasmic calcium was lowest when mitochondrial calcium and ATP accumulation were high. Therefore, circadian shifts in intracellular calcium handling may be mechanistically linked to metabolic function of mitochondria in SCN2.2 cells. Taken together, these studies suggest that calcium signaling might drive clock-controlled ATP accumulation rhythms.

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Towards the molecular mechanisms regulating circadian olfactory sensitivity in mammals

MANJANA SALEH, ACHIM KRAMER, UTE ABRAHAM

Laboratory of Chronobiology, Institute of Medical Immunology, Charite, Berlin, GERMANY

Olfactory sensitivity is modulated in a circadian manner: i.e. the activities of olfactory neurons respond in a time-of-day-dependent manner to odor presentations in insects and mice. Are these rhythms in olfactory sensitivity functionally linked to the circadian clock? In insects, G protein-coupled receptor kinase 2 is a mediator of rhythmic olfactory responses linking circadian clock output to rhythmic olfactory receptor accumulation in the antennae. Here we The aim of our project is to investigate whether similar mechanisms are realized in the mammalian system. The mammalian olfactory bulbs have been demonstrated to express SCN-independent circadian rhythms in vivo and in vitro. Our data show that also the mouse olfactory epithelium displays rhythmic clock gene expression indicating the presence of several circadian clocks in the olfactory system. The functions of these olfactory clocks

are not yet known, but it is conceivable that they are involved in the circadian regulation of olfactory sensitivity. Candidates for rhythmic regulation of olfactory sensitivity are being evaluated and validated for rhythmic expression on the mRNA and protein level. We will present preliminary results at the meeting. Furthermore, a primary olfactory epithelial cell line has been established and is currently tested for cell-autonomous molecular rhythms. Employing genetic perturbations in this cell line will enable us to study the molecular mechanisms underlying the circadian control of olfactory regulation in vitro. Ultimately, the characterization of the link between the circadian clock and odor perception will help to uncover how local circadian clocks can regulate physiological parameters.

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Circadian control of DNA excision repair by HERC2 ubiquitin ligase

TAE-HONG KANG, LAURA LINDSEY-BOLTZ, JOYCE REARDON, AZIZ SANCAR

Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, NC, USA

Cisplatin is one of the most commonly used anticancer drugs. It kills cancer cells by damaging their DNA, and hence cellular DNA repair capacity is an important determinant of its efficacy. Here, we investigated the repair of cisplatin-induced DNA damage in mouse liver and testis tissue extracts prepared at regular intervals over the course of a day. We find that the XPA protein, which plays an essential role in repair of cisplatin damage by nucleotide excision repair, exhibits circadian oscillation in the liver but not in testis. Consequently, removal of cisplatin adducts in liver extracts, but not in testis extracts, exhibits a circadian pattern with zenith at ~5 pm and nadir at ~5 am. Furthermore, we find that the circadian oscillation of XPA is achieved both by regulation of transcription by the core circadian clock proteins including cryptochrome and by regulation at the posttranslational level by the HERC2 ubiquitin ligase. These findings may be used as a guide for timing of cisplatin chemotherapy.

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The circadian deadenylase, Nocturnin, interacts with a novel form of KSRP

DANIELLE SHINGLE¹, SHUANG NIU², CARLA GREEN¹

¹*Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA*

²*Pediatrics, Stanford Medical School, Palo Alto, CA, USA*

Nocturnin is a circadian deadenylase involved in regulating the expression of mRNA transcripts. Aside from its catalytic pocket, Nocturnin's protein structure contains no known mRNA binding domains. Therefore we hypothesize that Nocturnin recognizes its target mRNAs through interactions with other RNA binding proteins. We have reported that KSRP, one such RNA binding protein known to recruit degradation machinery to mRNAs, interacts with Nocturnin. However, experiments to validate this interaction revealed that a smaller 60kD form of KSRP (KSRP60) binds Nocturnin rather than the known 75kD form. Cellular fractionation and immunohistochemistry experiments were used to examine the localization of KSRP60 and Nocturnin. We performed antibody mapping with two KSRP antibodies targeting different areas of the protein, and RNase treated cell lysates prior to immunoprecipitation with Nocturnin to further define whether KSRP60 is a truncation mutant or closely related protein. Lastly, we treated HEK293 cells with siRNAs targeting different areas of the KSRP mRNA sequence to see if the region targeted would alter their effects on the expression of KSRP60. The data show that KSRP60 and Nocturnin do co-localize in the cytoplasm, and this interaction is RNA dependent. Antibody mapping suggested that KSRP60 is missing a sequence in the N-terminal domain of KSRP. However, none of the KSRP-specific siRNAs were able to knockdown expression of KSRP60 while KSRP protein levels were significantly decreased. Our data suggest that KSRP60 is not a truncated form of KSRP, but may be a protein closely related in sequence. KSRP60 is one binding partner of Nocturnin that could serve as a link between the deadenylase activity and target mRNA.

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Sequential phosphorylation of FRQ by CKI is crucial for establishing rhythmicity in the Neurospora clock

AXEL DIERNFELLNER, CHRISTINA QUERFURTH, MICHAEL BRUNNER

AG Brunner, Biochemistry Center Heidelberg, Heidelberg, GERMANY

The Neurospora clock protein FREQUENCY (FRQ) is rhythmically expressed under the control of the transcription factor WHITE COLLAR COMPLEX (WCC). FRQ is in complex with CASEIN KINASE I (CKI) and modulates the activity of the WCC by facilitating its phosphorylation via CKI. Additionally CKI phosphorylates FRQ, which is crucial for its maturation and subsequent degradation. Thus, abundance levels and phosphorylation state of FRQ are rhythmic and determine the circadian activity profile of the WCC. Here we show that two sites in FRQ are required to bind CK1. Mutation of either site abolishes CKI binding and results in accumulation of hypophosphorylated and hyperstable FRQ. We also show that the fate of FRQ is determined by sequential phosphorylation of distinct regions in the N-terminal and central portion of the protein.

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In vivo analysis of phosphorylation-dependent degradation of mCRY2

ARISA HIRANO, NOBUHIRO KURABAYASHI, YOSHITAKA FUKADA

Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Tokyo, JAPAN

Cryptochrome proteins are critical players for molecular oscillations in the circadian clocks of central and peripheral tissues in mammals. CRY2 is phosphorylated at Ser557 located in the unique C-terminal region in the mouse SCN and liver, in both of which Ser557-phosphorylation form accumulated in parallel with CRY2 protein (Kurabayashi et al., Chronobiol. Int., 2006; Harada et al., JBC, 2005). The priming phosphorylation of mCRY2 at Ser557 by DYRK1A allows subsequent phosphorylation at Ser553 by GSK3 β , resulting in proteasomal degradation of CRY2. To reveal the physiological importance of Ser557 phosphorylation, we examined the effect of S557A mutation on the circadian clock function in vivo. First, we performed functional characterization of endogenous S557A-CRY2 by examining its cellular localization and interaction with other clock proteins. S557A-CRY2 accumulated in the nucleus and interacted with PER2, CLOCK and BMAL1 to a degree similar to that observed for wild-type CRY2 at ZT18. Together with the previous study showing that S557A-CRY2 normally inhibited the CLOCK-BMAL1-dependent transactivation (Sanada et al., Genes to Cells, 2004), it is indicated that S557A-CRY2 is functional in vivo. Next, we analyzed the effect of S557A mutation on expression rhythms of the clock genes in the fibroblast cells. Interestingly, we found that S557A mutation of CRY2 decreased mRNA expression level of Cry2, Per2 and Dbp over the day. Especially, Cry2 mRNA level was dramatically downregulated. On the other hand, the protein level of mutant CRY2 at both peak and trough time showed no change when compared to that of wild-type CRY2. These results suggest that S557A-CRY2 is expressed stably in the fibroblast cells and that CRY2 regulates its mRNA expression to maintain circadian fluctuation of CRY2 protein in a range of appropriate level.

The circadian proteome of *Drosophila melanogaster*

RENATA AZEVEDO¹, KAREN SMITH¹, KATHRYN LILLEY², MICHAEL HASTINGS³, CHARALAMBOS KYRIACOU¹

¹*Genetics, University of Leicester, Leicester, UNITED KINGDOM*

²*Biochemistry, University of Cambridge, Cambridge, UNITED KINGDOM*

³*Laboratory of Molecular Biology, MRC, Cambridge, UNITED KINGDOM*

Locomotor activity in *Drosophila melanogaster* represents a robust behavioural rhythm that can be used to study the circadian clock. This clock is located in the lateral and dorsal neurons of the fly and in the suprachiasmatic nuclei (SCN) of the hypothalamus in the mammal, and the underlying circadian molecular mechanisms in these species are conserved. Recently, the cycling proteome has been studied in mouse liver and SCN. To characterize the circadian proteome from *Drosophila melanogaster* heads we used three different strategies. The first employs a comparative approach with the mammalian circadian SCN proteome, the second uses a tap-tagging design which is used to screen the fly proteome and the third uses a yeast two-hybrid screen. Behavioural analysis and expression studies of candidate proteins, using transgenes to disrupt and silence some of these factors, have revealed two novel genes involved in glutamate metabolism that may possibly contribute to the circadian mechanism by affecting light input, one synaptic gene that may be involved in clock neuron communication or in the clock pacemaker and a component of the wingless pathway.

Functional study of a novel ZP protein, *Quasimodo*, in *Drosophila* circadian clock

KO-FAN CHEN¹, NICOLAI PESCHEL², RALF STANEWSKY¹

¹*School of Biological and Chemical Sciences, Queen Mary, University of London, London, UNITED KINGDOM*

²*Biozentrum Am Hubland, Universität Würzburg, Würzburg, GERMANY*

Compared to the central clock, the in- and output pathways of *Drosophila*'s circadian clock remain poorly investigated. So-called clock-controlled genes (ccgs) are thought to link the clock information to physiological and behavioural output. Previously, we successfully identified a novel ccg, CG13432, or *quasimodo* (*qsm*) via enhancer trap methodology (Stempfl et al., 2002). We now started to characterize *qsm*'s role in the circadian clock. Unlike wild type flies, abnormal robust rhythmic behaviour in constant light was observed after reducing *qsm* levels in all clock gene expressing cells by RNAi. In agreement with the behavioural rhythms, we also observed abnormal continuous oscillations of PER and TIM proteins in peripheral clocks and subsets of clock neurons in *qsm*RNAi flies in constant light. This suggests that *qsm* is involved in the light input pathway of the clock. Surprisingly, our preliminary data also suggest that *qsm*RNAi-mediated LL rhythmicity and its effects on TIM and PER are only partially dependant on CRY and CRY positive neurons. This indicates that *qsm* might be part of a CRY-independent pathway controlling light-induced TIM degradation and clock resetting. Further investigations are currently being performed to reveal the detailed relationship between CRY- and *Qsm*-directed light input pathways.

Molecular and functional characterization of 2mit, an intronic gene of D. melanogaster timeless2 locus

FRANCESCA BAGGIO, CLARA BENNA, MOYRA MASON, SILVIO TOSATTO, RODOLFO COSTA, FEDERICA SANDRELLI
Biology, University of Padova, Padova, ITALY

Drosophila melanogaster 2mit is a host gene of timeless2 (tim2) locus. 2mit is embedded in intron 11 and transcribed in the opposite direction compared to tim2. Its conceptual translation reveals a putative transmembrane protein characterized by a leucine-rich repeat (LRR) motif, a conserved domain involved in protein-protein interactions. 2mit is expressed from the earliest stages of development: in situ hybridization performed in embryos showed that it is prevalently transcribed in the developing Central Nervous System (CNS) and in the medium gut. In adult heads, 2mit mRNA levels show a significant circadian oscillation under 12:12 LD regimes, with higher levels of expression at the end of the night. In adult brains 2mit mRNA localizes at the level of central complex and mushroom bodies, structures involved in the control of locomotor activity and memory. No expression has been detected in the canonical circadian clock neurons. Preliminary results indicate that 2mit mutant adult flies, characterized by a ~50% 2mit mRNA decrement, exhibit a free running period of about 26h in DD. Moreover, similar results were obtained in flies with a specific 2mit knock-down (KD) driven in the whole central nervous system, suggesting a possible function for 2mit in circadian behavior. In order to explore the putative role of 2mit in the *Drosophila* circadian clock, the PER protein accumulation kinetics in 2mit mutant adult brains is currently under investigation.

Non cell-autonomous TIMELESS degradation and phase-shifting of the Drosophila

CHIH-HANG TANG, ERICA HINTEREGGER, YUHUA SHANG, MICHAEL ROSBASH
Biology, Brandeis University, Waltham, MA, USA

Light and heat are major circadian entrainment signals. The response to light is better studied, and a well-accepted model posits that photon capture by the CRYPTOCHROME (CRY) leads to intracellular JETLAG (JET)-mediated TIMELESS (TIM) degradation. This results in cell-autonomous phase shifting of clock protein accumulation and clock gene transcription. TIM degradation then causes delay or advance of the *Drosophila* circadian clock in the early or late night, respectively. This cell-autonomous view is based on multiple lines of evidence, most notably the rapid and robust TIM degradation after a light pulse. However, the model has never been systematically examined within the ca. 150 *Drosophila* circadian neurons in the early night when phase delays are maximal. To this end, we used immunohistochemistry to assay light-mediated TIM degradation in this early night-delay zone. Remarkably, TIM was light-insensitive in the key pacemaker neurons (s-LNvs) after a light pulse causing maximal phase delays at ZT15. We could convert TIM to light-sensitive by overexpressing JET as well as CRY only within these pacemaker neurons, although JET overexpression there would not rescue the behavioral deficit of jet mutants. Our data indicate that TIM degradation within the s-LNvs is neither necessary nor sufficient for delay-phase shifts; other circadian neurons appear to sense light and send signals to the pacemaker s-LNvs to effect phase delays, i.e., circadian entrainment is a network property. More generally, photosensitive neurons apparently function at different times, with some neurons (DNs, LNds or E-cells) sensing an illumination change in the early night and others (l-LNvs) serving as dawn photoreceptors. Finally, our results suggest that the canonical cell-autonomous molecular model of phase shifting might need modification. To clarify how phase shifting might occur by signaling, we are currently engaged in molecular experiments investigating other possible roles of CRY and TIM in light-induced phase shifts and entrainment.

Live imaging evidence for cAMP signaling as a response to PDF in subsets of Drosophila pacemaker neurons

Laura Duvall, Paul Taghert

Anatomy and Neurobiology, Washington University, St. Louis, MO, USA

The neuropeptide PDF (Pigment Dispersing Factor) supports circadian functions in part by synchronizing different subsets of pacemaker neurons in *Drosophila*. PDF is expressed in about 10% of the 150 principle pacemaker neurons in the fly brain. The PDF receptor belongs to the B1 subfamily of class II G-protein coupled receptors, members of which increase cAMP levels through stimulation of adenylate cyclase. We study downstream signaling associated with the PDF response in pacemaker neurons in *Drosophila* using a genetically encoded FRET (Florescence Resonance Energy Transfer) sensor (Epac1-camps) to detect changes in cyclic nucleotides. Using pharmacological and genetic methods to alter specific components of signaling pathways, the effect on PDF response was determined by loss of FRET signal. While the sensor detects changes in both cAMP and cGMP levels, genetic evidence suggests that the PDF response is mediated by cAMP in both PDF producing and non-PDF pacemaker neurons *in vivo*. The adenylate cyclase mutant rutabaga showed only slight reductions, suggesting the possible involvement of other adenylate cyclase isoforms. RNAi induced knockdown of all known adenylate cyclase isoforms alters PDF responses in the case of three and further suggests that non-rutabaga adenylate cyclases contribute to PDF signaling in both PDF and non-PDF cells. These results suggest that the relative contributions of individual adenylate cyclases differ between PDF and non-PDF cells. To date, these results support the conclusion that that signaling downstream of the PDF receptor involves cAMP but that it diverges between pacemaker subgroups.

Trp channels affecting temperature entrainment of the circadian clock in Drosophila melanogaster

Werner Wolfgang, Carla Gentile, Alekos Simoni, Ralf Stanewsky

Biological and Chemical Sciences, Queen Mary, University of London, London, UNITED KINGDOM

Light and temperature are important Zeitgebers (ZT) for resetting the circadian clock. So far, two mutants, *norpA* and *nocte* (Glaser and Stanewsky 2005; Sehadova, Glaser et al. 2009) that are known to affect clock synchronisation to temperature cycles. Monitoring of adult locomotor activity and bioluminescence cycling of Period-luciferase fusion proteins in these mutants suggest that the temperature signal is perceived by peripheral chordotonal organs, which in turn instruct clock neurons in the brain. Unlike brain autonomous synchronisation to light:dark cycles, brains can not be entrained by temperature cycles (Sehadova, Glaser et al. 2009). Three lines of evidence support a role for Trp-(transient receptor potential) channels in temperature entrainment: First, *norpA* encodes phospholipase C, which is required to transduce light signals from rhodopsins to Trp-channels Trp and Trp-like resulting in the depolarization of *Drosophila* photoreceptors. Second, other Trp-channels respond to stimuli from the exterior environment such as touch, pain, hygrosensation/osmolarity and more importantly low/high temperature [selected review (Venkatachalam and Montell 2007)]. Third, our preliminary data suggest the involvement of at least three Trp-channels in clock synchronisation to temperature cycles. Mutants of one type of channel do not entrain to 12:12hr cycles between temperature minima/maxima of 16/25°C, 20/25°C or 25/29°C in constant light (LL), whereas mutants of another channel only entrain the two higher temperature cycles in LL. The third channel mutant also fails to synchronize to the lower temperature cycle in constant light. In addition, downregulation of this channel in all clock neurons disturbs the cyclic expression of a Period-luciferase fusion protein in adult flies during temperature cycles. We suggest one or several of the 13 Trp-channel genes in *Drosophila* may be required for temperature sensing as for temperature preference, but also as an

instructive signal for the intrinsic clock network in resetting daily molecular and behavioural cycling. Our survey of Trp-channels in *Drosophila* will lead to a better understanding circadian entrainment to temperature in *Drosophila*. Glaser, F.T. and R. Stanewsky (2005). Temperature Synchronization of the *Drosophila* Circadian Clock. *Current Biology* 15(15): 1352-1363. Sehadova, H., F.T. Glaser, et al. (2009). Temperature Entrainment of *Drosophila*'s Circadian Clock Involves the Gene *nocte* and Signaling from Peripheral Sensory Tissues to the Brain. *Neuron* 64(2): 251-266. Venkatachalam, K. and C. Montell (2007). TRP Channels. *Annual Review of Biochemistry* 76(1): 387-417.

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Genetic architecture of circadian clock function in Brassica rapa

QIGUANG XIE¹, PING LOU¹, XIAODONG XU¹, CHRISTINE E. EDWARDS¹, CYNTHIA WEINIG²,
C. ROBERTSON McCLUNG¹

¹*Department of Biological Sciences, Dartmouth College, Hanover, NH, USA*

²*Department of Botany, University of Wyoming, Laramie, WY, USA*

To date, efforts to elucidate the plant circadian clock mechanism have emphasized *Arabidopsis thaliana*. We have used the circadian rhythm in cotyledon movement to extend this study to the crop plant, *Brassica rapa*. We have identified Quantitative Trait Loci (QTL) for period, amplitude and temperature compensation of the circadian rhythm in leaf movement, as well as for a number of morphometric parameters, including flowering time, size of floral organs, and hypocotyl length, in a set of Recombinant Inbred Lines (RILs) derived from two diverse parents, R500 and IMB211. We describe our efforts to identify the gene responsible for the QTL for period length identified on Chr. A9. One candidate, GIGANTEA (GI), identified as a clock component in *Arabidopsis*, maps to that region. GI is polymorphic between R500 and IMB211 and the inheritance of the two alleles is consistent with the R500 allele conferring shorter period than the IMB211 allele. We have introduced the two *B. rapa* GI alleles into the *Arabidopsis* gi-201 mutant, which lacks GI activity. The *B. rapa* IMB211 GI allele rescues the short period phenotype of gi-201 whereas the R500 allele further shortens the period, which is consistent with their relative effects on period in *B. rapa*. Both alleles rescue the late flowering phenotype of gi-201. We have identified several putative null gi alleles in the *B. rapa* TILLING collection at the John Innes Institute and will use these to test the effects of the two *B. rapa* GI alleles on period length in *B. rapa*.

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Role of the Arabidopsis thaliana circadian clock in Pseudomonas syringae infection

LAURA RODEN, VAIBHAV BHARDWAJ, LINDSAY PETERSEN, ROBERT INGLE

Molecular and Cell Biology, University of Cape Town, Cape Town, SOUTH AFRICA

Plant responses to the environmental signals and stresses are modulated by the circadian clock. The importance of light on the outcome of plant-pathogen interactions is becoming increasingly apparent. Light and temporal regulation of the immune response may allow plants to respond effectively to particular pathogen challenges. We have results that indicate that the time of day that plants are challenged with virulent *Pseudomonas syringae* pv tomato DC3000 (Pst DC3000) influences the outcome of the interaction, and that plants with a functional circadian clock are more resistant to Pst DC3000 infection than an arrhythmic mutant. Infections were carried out in constant light and bacterial growth assays were performed at 48 hr and 72 hr post infection. Wild-type Columbia A. *thaliana* had the lowest bacterial numbers when infected with Pst DC3000 at CT 2, and highest when infected in the subjective night, at CT 18. These results suggest that plants were more resistant to

infection during the subjective day than night, and that this variation may be due to the circadian clock. The arrhythmic CIRCADIAN CLOCK ASSOCIATED 1 overexpressing line (CCA1-ox) were 10-fold more susceptible to Pst DC3000 infection than wild-type plants, regardless of time of day, indicating that a functional circadian clock was necessary for a full basal defence response.

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Global circadian transcription rhythms without robust kai-gene cycling in the heterocyst-forming multicellular cyanobacterium, Anabaena sp. PCC 7120

HIROKO KUSHIGE, MASAKI MATSUOKA, HIDEYUKI KUGENUMA, HIDEO IWASAKI
Electrical Engineering and Bioscience, Waseda University, Tokyo, JAPAN

The filamentous cyanobacterium, *Anabaena* sp. PCC 7120, provides an excellent model system to analyze circadian functions which cannot be addressed in unicellular species. *Anabaena* fixes nitrogen in specialized cells called heterocysts, which differentiate from oxygenic vegetative cells at regular intervals along the filaments under conditions of nitrogen deprivation. Thus, it is one of the simplest multicellular organisms which show both pattern formation with cell differentiation and circadian rhythms. We are interested in following questions. i) Is there any difference in the kai-gene functions between *Synechococcus* and *Anabaena*? Note that KaiA homologs in filamentous species lack two-third of the protein that are well conserved among unicellular species. ii) Is there any difference between the clock systems in vegetative cells and heterocysts? iii) Are clocks in neighboring cells synchronized to each other? iv) Is heterocyst differentiation/patterning modified by the clock? As an initial step to address these questions, we performed DNA microarray analysis to reveal the genome-wide circadian expression profile under the nitrogen-containing condition. Surprisingly, expression of the kai genes showed very low-amplitude rhythms, while we found ~600 clock-controlled genes in *Anabaena*. In contrast, in *Synechococcus* expression of the kaiBC operon shows the highest amplitude cycle. Moreover, although clock-controlled genes in *Synechococcus* peaked exclusively at subjective dawn and dusk, that in *Anabaena* peaked more widely throughout the circadian cycle. Thus, there is a striking difference in circadian outputs as well as the clock gene expression profile between the two species. Further genetic and biochemical analyses on the *Anabaena* clock will be also reported.

P210

Effects of partial circadian adjustment on sleep and vigilance in simulated night work

SIMON CHAPDELAINE, JEAN PAQUET, MARIE DUMONT

Chronobiology Laboratory, Sacre-Coeur Hospital & University of Montreal, Montréal, Québec, CANADA

Healthy subjects (15 M/ 23W; 20-35 y.) were studied in a laboratory simulation of night work, including 2 day shifts followed by 4 consecutive night shifts (00:00 h—08:00 h). Three daytime profiles of light exposure were tested, designed to produce a phase delay (Delay group), a phase advance (Advance group), or to maintain a stable circadian phase (Stable group). Day sleep was scheduled from 0900–1700 h in both Delay and Stable groups, and from 1400–2200 h in the Advance group. Circadian phase was assessed with salivary DLMO on the first day shift (D1) and on the 4th night shift (D6). Mood, subjective alertness (VAS, KSS), and psychomotor performance (PVT) were assessed during day and night work, and sleep was recorded by polysomnography (PSG). Phase delays measured in the Delay (4.1 ± 1.3 h) and Stable (1.7 ± 1.6 h) groups were significantly larger in the Delay group ($p < 0.05$). There was no group difference in alertness, mood or performance on the 4th night shift. However,

there were significant correlations between absolute phase shifts and both subjective alertness (VAS: $r=0.47$, $p=0.006$; KSS: $r=-0.39$; $p=0.027$) and mood ($r=0.57$; $p=0.001$). Partial circadian adjustment had no influence on daytime sleep quality but showed positive effects on subjective alertness and mood during night work.

P211

Preliminary evidence that phase relationships between core body temperature, melatonin, and sleep are associated with the severity of global and anhedonic depression

BRANT HASLER, DANIEL BUYSSE, DAVID KUPFER, ANNE GERMAIN

Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Theory and empirical evidence link misalignment between the timing of sleep and the endogenous circadian pacemaker to depressive symptomatology in mood disorders. In addition, accumulating evidence supports associations between the circadian system and appetitive processes including reward responsiveness. We sought to determine if the respective phase angles between dim light melatonin onset (DLMO), core body temperature (CBT) minimum, and mid-sleep differed between healthy controls and individuals with major depressive disorder (MDD), and if these phase angles correlated with the severity of global depression and/or anhedonic depression. Thirteen unmedicated adults with MDD, and 13 healthy controls underwent overnight studies that were conducted between 1900 and one hour after wake-up time under dim light conditions, and which included polysomnography, CBT measurement (every 30 sec via rectal thermistor) and blood sampling for melatonin measurement (every 15 min). CBT minimum was visually-determined. Plasma melatonin concentrations were measured via RIA; DLMOs were assessed using a 10 pg/ml threshold. Three phase angles were calculated between DLMO, the CBT minimum, and PSG-based mid-sleep. Finally, severity of depression was assessed using the 17-item Hamilton Depression Rating Scale (HAM-D) and an anhedonia subscale from the Beck Depression Inventory (BDI-Anhedonia). None of the phase angles differed between the groups, but the two groups showed at least trend-level differences in the within-group variability of all three phase angles ($F=7.63$, $p=.01$; $F=3.60$, $p=.07$; $F=3.10$, $p=.09$), with greater variability among the depressed individuals. Finally, the depressed group's midsleep-CBTmin and DLMO-CBTmin phase angles were positively correlated with HAM-D scores ($\rho=0.67$, $p<.05$; $\rho=0.82$, $p<.01$) and the BDI-Anhedonia subscale ($\rho=.62$, $p<.05$; $\rho=.83$, $p<.01$), but not the total BDI score. Misalignment between sleep, CBT, and/or melatonin may be linked to depressive symptomatology in non-seasonal depression, with relatively specificity for anhedonic depression. Although the findings are preliminary, phase-angle heterogeneity may indicate the presence of multiple depressive phenotypes.

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Sex differences in the sleep of a diurnal rodent, Octodon degus

JAMIE PERRYMAN¹, MEGHAN HEWLETT², MARK OPP³, THERESA LEE⁴

¹*Neuroscience, University of Michigan, Ann Arbor, MI, USA*

²*Psychology, University of Michigan, Ann Arbor, MI, USA*

³*Neuroscience, Physiology, Anesthesiology, University of Michigan, Ann Arbor, MI, USA*

⁴*Neuroscience, Psychology, University of Michigan, Ann Arbor, MI, USA*

Very little work has examined electrophysiological sleep in diurnal rodents, and none has examined females. Behavioral sleep studies, as well as activity in the field and laboratory have demonstrated that degus prefer a diurnal niche. This study used electroencephalograph (EEG), brain temperature, and locomotor activity to investigate the homeostatic and circadian components of sleep in the diurnal rodent, Octodon degus. Seven male and female degus were implanted with screw electrodes and a

brain thermistor to collect electrophysiological sleep data. In the baseline study, twenty-four hours of data were collected and analyzed. Rapid eye movement (REM) sleep levels did not differ across day-night conditions or between male and females. Non-rapid eye movement (NREM) sleep consolidation in males was higher across the night (dark phase) than during the day (light phase), but there was no significant day/night difference for females. Males displayed more NREM sleep and consolidation during the night, which was consistent with prior studies utilizing locomotor activity and behavioral sleep. However, while female degus exhibited more overall NREM sleep than their male counterparts, sleep levels did not differ significantly between the day and night, demonstrating sexually dimorphic characteristics of sleep patterns within this diurnal rodent. Following a 6 h sleep deprivation during the dark period, both males and females demonstrated significantly increased amounts of NREM sleep during recovery. Males exhibited increased NREM sleep and delta power immediately, while females displayed a similar rebound response the following day. Degus have increased activity around the light/dark transitions, and this crepuscular activity was preserved across the sexes and after the deprivation condition. This suggests that during light/dark transitions, the circadian drive for arousal appears more powerful than the homeostatic sleep drive. These results may provide some insight into how these processes interact in a diurnal mammal.

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Sleep-wake Chromosome 1 QTL exploration in the mouse

JOSEPH R. OWENS¹, KAZUHIRO SHIMOMURA¹, PERIN KOTHARI¹, CHRISTOPHER OLKER¹, JOSH MILLSTEIN², CHRISTOPHER J. WINROW³, ANDREW KASARSKIS², JOHN RENGER³, FRED W. TUREK¹

¹*Department of Neurobiology and Physiology, Northwestern University, Evanston, IL, USA*

²*Statistical Genetics Department, Sage Bionetworks, Seattle, WA, USA*

³*Neuroscience Department, Merck Research Laboratories, West Point, PA, USA*

There is a strong genetic basis that governs sleep and wake behaviors. Despite years of study on the neuro-physiology and pharmacology regulating the sleep/wake cycle, the genetic basis of sleep-wake traits remains to be determined. In a recent mapping study involving 269-second generation progeny from a B6 x CBy backcross (N2), we found a highly significant cluster of quantitative trait loci (QTL) on the distal region of Chromosome 1 that was associated with both sleep-wake fragmentation and sleep-wake architecture traits. This QTL cluster was found to significantly overlap with QTL previously identified in two independent genome wide studies of circadian behavior. Using congenic and gene-trap approaches, the role this locus plays in regulating the sleep-wake cycle was investigated. Baseline sleep and recovery sleep as a response to sleep deprivation were tested in order to evaluate the unchallenged and challenged responses of the sleep-wake cycle at this locus. Preliminary analysis of baseline sleep indicates a significant effect on sleep fragmentation traits as well as duration of NREM sleep by this locus. The significance of these findings for sleep genetics and sleep disease patho-physiology will be discussed.

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Phase-dependent effects of orexin-2 receptor blockade mediated sleep promotion

JONATHAN SHELTON, SUJIN YUN, LEAH ALUISIO, IAN FRASER, PASCAL BONAVENTURE, CURT DVORAK, NICHOLAS CARRUTHERS, TIMOTHY LOVENBERG, CHRISTINE DUGOVIC

Neuroscience, Johnson & Johnson PRDUS, San Diego, CA, USA

Orexin peptides display a robust diurnal rhythm and help modulate sleep/wake states by activating Orexin-1 (Ox-1R) and Orexin-2 (Ox-2R) receptors in accordance with the circadian and homeostatic control of sleep. Our group has demonstrated that the pharmacological blockade of Ox-2R exerts potent sleep promotion. To examine potential phase dependency, an Ox-2R antagonist was administered

either during the sleep or active phase and various sleep parameters were examined. Experiments were performed in rats implanted with telemetry devices for recording EEG/EMG signals. Animals received a selective OX-2R antagonist (30 mg/kg, s.c.) during either the light (sleep) or dark (active) phase. Polysomnographic waveforms were recorded and scored in 10-s intervals to identify states of vigilance. Micro-dialysis was performed in specific brain regions to measure various neurotransmitters. When injected during the light or dark phase, the compound reduced latency to NREM and an increase in NREM duration which lasted longer during the dark phase (~8hrs.) than light (~2hrs.). Interestingly, the increase during the light phase was due to a prolongation in NREM bouts whereas rats treated during the dark phase showed an increase in NREM bout frequency. The sleep promoting effects during the light phase were paralleled by a transient decrease in acetylcholine while the sleep effects during the dark phase were the result of a sustained decrease in histamine release from the hypothalamus. In summary, while exhibiting potent sleep promoting effects (i.e. initiation and elongation) following blockade of the Ox-2R in rats, the duration of this phenomenon was severely truncated during the light phase versus dark. This discrepancy may be attributed to the sustained suppression of histamine release from the hypothalamus during the dark phase.

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Nonlinear dynamics of heart rate variability show sleep-wake homeostatic predominance during sustained wakefulness

ANTOINE VIOLA¹, N. MONTANO², S.L. CHELLAPPA³, A. PORTA², C. CAJOCHEN⁴, D.J. DIJK⁵

¹*Centre for Chronobiology, Universitäre Psychiatrische Kliniken, UPK, Basel, SWITZERLAND*

²*Clinical Sciences, Internal Medicine II, L. Sacco, University of Milan, Milan, ITALY*

³*The CAPES Foundation, Ministry of Education of Brazil, Brasilia-DF, BRAZIL*

⁴*Centre for Chronobiology, University of Basel, Basel, SWITZERLAND*

⁵*Surrey Sleep Research Centre, University of Surrey, Guildford, UNITED KINGDOM*

Circadian rhythmicity in heart rate is clearly established while it remains controversial for indices of the autonomic nervous system (ANS). The objective of the current investigation was to characterize variations on ANS during extended wakefulness. The ECG of 12 young healthy subjects were recorded continuously during 40-h constant routine protocol. Spectral analysis of the extracted cardiac signal was performed together with a non linear analysis (symbolic analysis; SA). In SA, RR intervals are concerted into sequence of symbols and subsequent construction of series of patterns with three symbols, out of which four patterns could be identified: 0V (no-variation), 1V (one-variation), 2LV (two-like-variations) and 2UV (two-unlike-variations). The first pattern indicates sympathetic activity, while the last indicates parasympathetic modulation. Heart rate (HR) underwent a clear circadian pattern, with nadir at 03:42±00:53. Similar to HR, the absolute spectral indices show a sinusoidal model rhythm. The total power (an index of global variability) showed circadian rhythmicity with an acrophase at 06:46±00:50, very low-frequency had an acrophase at 06:23±00:44, low frequency (LF), had an acrophase at 05:47±01:12 and high frequency (HF) had an acrophase at 06:30±01:51. However, a different sigmoid profile was observed in the normalized data, expressed by LF/(LF+HF) ratio. Before 07:05±01:21h (±20h of sustained wakefulness), LF/(LF+HF) remained stable. Afterwards, this index revealed a significant increase, and remained significantly at a higher level throughout the rest of the protocol, indicating increased sympathetic activity during sustained wakefulness. Symbolic analysis confirmed this finding and showed a clearer sleep loss effect, indicated by a striking increase in 0V pattern (06:24h±00:47h) and decrease in 2UV pattern (06:16h±00:42h), during the same time window. The absence of circadian variation in ANS and the clear circadian modulation in HR suggest an intrinsic cardiac circadian control of the heart. The sigmoid pattern of the ANS most likely reflects the influence of the increase in sleep homeostatic pressure during sustained wakefulness.

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Natural genetic variation underlying sleep in Drosophila identified by QTL mapping

MOBINA KHERICHA¹, ANNE GENISSEL², SERGEY NUZHIDIN², ERAN TAUBER¹

¹*Genetics, University of Leicester, Leicester, UNITED KINGDOM*

²*Molecular & Computational Biology, University of Southern California, Los Angeles, CA, USA*

During the recent years, *Drosophila* has been extensively used as a model organism to understand the genetic of sleep. Numbers of mutagenesis screens and global profiling by microarray have been carried, providing new candidate genes important for sleep regulation. However, less is known about genetic variation that may be segregating in wild populations and contributes to phenotypic variation in sleep that would reflect adaptive behavior in various environments. We have used a set of 189 recombinant inbred lines (RIL), generated from two parental lines obtained from a wild population in California, to map Quantitative Trait Loci (QTL) that affect variation in sleep. Sleep was measured over a period of four days under 12:12 LD cycle and significant variation, indicating substantial heritability, was associated with each of the three variables measured: (i) daily accumulated sleep, (ii) the median bout of sleep (iii) the diurnal vs. nocturnal sleep ratio. Using composite interval mapping we have identified number of significant QTLs associated with each of the sleep parameters. Using deficiency strains that span the QTL intervals, we employed complementation tests to narrow down the QTL intervals. This analysis, combined with published microarray expression data, generated a few candidate genes that are likely to bear the causative variations (e.g. SNPs) that generate the variation in sleep.

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Altered body mass regulation in male mPeriod mutant mice on high-fat diet

ROBERT DALLMANN¹, DAVID WEAVER²

¹*Pharmacology and Toxicology, University of Zurich, Zurich, SWITZERLAND*

²*Neurobiology, University of Massachusetts Medical School, Worcester, MA, USA*

The circadian clock orchestrates most physiological processes in mammals. Disruption of circadian rhythms appears to contribute to the development of obesity and metabolic syndrome. The Period genes *mPer1* and *mPer2* but not *mPer3* are essential for core clock function in mice. To assess the impact of *mPer* genes on body mass regulation, *mPer* mutant and control mice were fed a high-fat diet. Here we report that male *mPer1/2/3* triple deficient mice gain significantly more body mass than wild-type controls on high fat diet. Surprisingly, *mPer3* single deficient animals mimicked this phenotype suggesting a previously unrecognized role for *mPer3* in body mass regulation.

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Paradoxical post-exercise responses of acylated ghrelin and leptin during a simulated night shift

**CHRISTOPHER MORRIS¹, SARAH FULLICK², WARREN GREGSON², NEIL CLARKE², DOMINIC DORAN²,
DON MACLAREN², GREG ATKINSON²**

¹*Division of Sleep Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*

²*Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, UNITED KINGDOM*

In the US, ~10% of employees undertake night work, which is a significant predictor of body mass gain, possibly because responses to activity and eating are altered at night. It is known that the appetite-

related hormone, acylated ghrelin, is suppressed after an acute bout of exercise during the day, but no researcher has explored whether evening exercise alters acylated ghrelin and other appetite-related outcomes during a subsequent night shift. Six healthy men (mean±SD: age 30±8 yrs, body mass index 23.1±1.1 kg/m²) completed two crossover trials (control and exercise) in random order. Participants fasted from 10:00 h, consumed a test meal at 18:00 h, and then cycled at 50% peak oxygen uptake or rested between 19:00-20:00 h. Participants then completed light activities during a simulated night shift which ended at 05:00 h. Two small isocaloric meals were consumed at 22:00 and 02:00 h. Venous blood samples were drawn via an intravenous catheter at 1 h intervals between 19:00-05:00 h for the determination of acylated ghrelin, leptin, insulin, glucose, triglyceride, and non-esterified fatty acids concentrations. Perceived hunger and wrist actimetry were also recorded. During the simulated night shift, mean±SD acylated ghrelin concentration was 86.5±40.8 pg/ml following exercise compared with 71.7±37.7 pg/ml without prior exercise ($p=0.015$). Throughout the night shift, leptin concentration was 263±242 pg/ml following exercise compared with 187±221 pg/ml without prior exercise ($p=0.017$). Mean levels of insulin, triglyceride, non-esterified fatty acids, and wrist actimetry level were also higher during the night shift that followed exercise ($p<0.05$). These data indicate that prior exercise increases acylated ghrelin and leptin concentrations during a subsequent simulated night shift. These findings differ from the known effects of exercise on acylated ghrelin and leptin during the day, and therefore have implications for energy balance during night work.

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Rhythms in white adipose pre-adipocytes and adipocytes

SIMONE MÄNTELE¹, ARIEL POLIANDRI², ROGER WHITE², MALCOLM PARKER², DEBRA SKENE¹,
JONATHAN JOHNSTON¹

¹Faculty of Health and Medical Sciences, University of Surrey, Guildford, UNITED KINGDOM

²Faculty of Medicine, Imperial College, London, UNITED KINGDOM

Many genes exhibit circadian rhythmicity in murine white adipose tissue (WAT). However, due to the presence of multiple cell types in WAT, it is unclear whether a circadian clock resides in the key metabolic and endocrine cells, the adipocytes. We recently investigated circadian rhythms in murine pre-adipocytes and adipocytes derived from the immortalised 3T3-L1 cell line. Surprisingly, we were only able to detect temporal variation in the expression of 50% of clock genes analysed. In this study, we have utilised cells derived from IMMORTOTM mouse pre-adipocytes, termed IMWAT cells. These cells conditionally express the SV40T-antigen and provide an experimental system that more closely models primary cells than the 3T3-L1 line. Populations of IMWAT pre-adipocytes and adipocytes were synchronised by serum pulse and harvested every 3-hours across a 51-hour period. Expression of the clock genes *Per2*, *Dbp*, *Rev-erb?*, *Per1*, *Cry1*, and *Bmal1* was then measured using TaqMan qRT-PCR. Circadian rhythmicity of mRNA expression was observed for all clock genes in both pre-adipocytes and adipocytes. Cosinor analysis revealed that the phase of rhythms was advanced by about 5-hours in adipocytes compared to pre-adipocytes. Our results therefore show for the first time a molecular clock prevalent in primary-like murine adipose cells. Moreover, the different phasing of gene expression relative to the serum pulse indicates differences in the entrainment of circadian rhythmicity in pre-adipocyte and adipocyte cells.

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Insulin-dependent Per2 gene expression is the first entrainment signal to the peripheral circadian clocks by food or glucose

YU TAHARA, MAKIKO OTSUKA, YUTA FUSE, AKIKO HIRAO, SHIGENOBU SHIBATA

Physiology and Pharmacology, Waseda University, Tokyo, JAPAN

As a best synchronizer, feeding can entrain the peripheral clocks within 2-3 days (Hirao et al., 2009). However, the entrainment mechanisms by food intake remain mysterious. Using RT-PCR analysis, we visualized 16 “core clock” or “clock related” gene expression rhythms in the mouse liver during phase advancing condition over two days under daytime feeding schedule. Here we showed that the pattern of entrainment of peripheral clock genes by food was divided into three groups in the difference of their phase-advancing speed. The first group, such as Bmal1, Dbp is phase-advanced about 3 hrs by 24 hrs fasting. The second group, such as Per2, Rev-erva, and Ror?, is phase-advanced immediately after re-feeding. Actually, we clearly observed the up-regulated Per2 gene expression 2 hrs after re-feeding. The last group, such as Npas2, is phase-advanced on the next day after second re-feeding, suggesting that these kind of genes may be downstream genes after food entrainment. Thus, the acute food entrainment of peripheral circadian clocks needs both “fasting-induced phase advance” and “food-induced phase advance”. Corresponding with such criteria we focused on Per2 gene, because Per2 was up-regulated after fasting and re-feeding. The increase of Per2 expression could be confirmed by the glucose injection instead of food, and showed the insulin-dependent manner in the liver and muscle, but not small intestine when streptozotocin-induced diabetic mice were examined. Taken together, our data suggest that food signal directly transfer to the peripheral tissue through glucose metabolic pathway.

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Regulation of circadian metabolic rhythms through the crosstalk between casein kinase 1 delta and PGC-1 alpha

SIMING LI, XIAOWEI CHEN, ALAN SALTIEL, JIANDIE LIN

Life Sciences Institute, University of Michigan, Ann Arbor, MI, USA

Circadian clock coordinates behavior and physiology in mammals in response to light and feeding cycles. Perturbed clock function has been implicated in sleep disorders and it is associated with increased cardiovascular risk. In rodents, disruption of clock function leads to obesity and impaired glucose homeostasis whereas high-fat diet is able to alter clock gene expression. These results suggest that energy homeostasis is tightly linked to the biological timing system. The physiological and molecular mechanisms that integrate these processes, however, remain poorly defined. PGC-1alpha is a transcriptional coactivator that regulates glucose, lipid and mitochondrial energy metabolism. We have recently demonstrated that PGC-1alpha also modulates clock gene expression and that rhythmic PGC-1alpha; activation is essential for normal circadian rhythms of locomotor activity and metabolic rate in vivo. We have shown that PGC-1alpha is phosphorylated by casein kinase 1 delta (CK1delta), a core component of the molecular clock. In addition, CK1delta regulates gluconeogenic gene expression in hepatocytes by suppressing PGC-1alpha transcriptional activity. These results suggest a role of CK1 delta in modulating hepatic circadian glucose metabolism.

Regulation of rhythmic expression of circadian and circadian-controlled genes by a chemopreventive regimen of methylselenocysteine

MINGZHU FANG¹, XUN ZHANG², HELMUT ZARBL¹

¹*Environmental Occupational Medicine, University of Medicine and Dentistry of New Jersey, Piscataway, NJ, USA*

²*Department of Environmental and Occupational Health, University of Washington, Seattle, WA, USA*

Epidemiological and animal studies indicate that disruption of circadian rhythm increases the risk of breast cancer. Previously, we reported that methylselenocysteine (MSC) increases the rhythmic expression of circadian genes at a chemopreventive dose in mammary tissues of N-nitroso-N-methylurea (NMU)-treated pubescent Fisher 344 rats. We report herein that a single carcinogenic dose of NMU disrupts the rhythmic expression of most of core circadian genes (including Per2 and Rev-Erb α) and hormone receptor genes (MTNR1A, ER α , and ER β). In contrast, MSC significantly enhanced the level and/or reset the phase of circadian expression of these genes. Our sequence analyses demonstrated that these genes harbor E-box motifs of Bmal1 in their promoter region. However, either NMU or MSC had no impact on the rhythmic transcription levels of circadian regulatory genes, including Arntl (a homologue of Bmal1), Clock, and Npas2. Moreover, neither carcinogen exposure nor dietary MSC significantly altered plasma melatonin levels, excluding the possibility that either treatment had an effect on light-mediated melatonin secretion. Our in vitro study revealed that MSC can increase cellular histone acetylation levels; and our animal study also demonstrated that dietary MSC increased the binding of acetylated histone 3 on the E-box 1 of Per2 promoter region in mammary gland of rats. These results suggest that MSC can promote circadian expression of genes essential to normal mammary cell growth and differentiation through epigenetic regulation. (Supported by NIEHS grants, U19ES011387, P30ES007033, and P30ES005022).

Circadian clocks in mouse tumors

MARIA COMAS, MARINA ANTOCH

Molecular and Cellular Biology Department, Roswell Park Cancer Institute, Buffalo, NY, USA

Cancer therapy, survival rate and secondary effects can be improved by applying the treatment at the right time of day. In order to succeed with chronotherapy in cancer, a fundamental question to ask is whether the circadian clock in tumors is functional and if so, whether it is synchronized to other tissues. Tumors may be: 1. Synchronized in phases of expression of clock and clock-controlled genes similar to other tissues under the regulation of the SCN; 2. They may display different phase of rhythmicity if they respond to non-SCN-generated signals similar to liver, which may be synchronized by food availability independent of the SCN; or 3. They may lose their potential to be synchronized either as a result of tumor progression or a cause of malignancy. To discriminate between these possibilities, we are investigating core clock gene expression in tumors in vivo using transgenic mouse Per2-Luciferase knock-in mice crossed to tumor-prone p53^{-/-} mice. This novel mouse model carries the p53^{-/-} mutation resulting in a high rate of spontaneous tumorigenesis from the 4th month of age. Importantly, it also allows in vivo imaging of the circadian clock by using a core clock gene (Per2) fused to the luciferase reporter gene which demonstrates robust rhythmicity in multiple tissues. We are monitoring the circadian rhythm of mPer2 expression in the liver and thymus tissues as well as in lymphomas and sarcomas that the mice develop using the Xenogen sensitive IVIS Imaging System. We also analyze the temporal expression profiles of other core clock genes (Bmal1, Clock, Per1, Cry1, and Cry2) in tumor biopsies using real-time RT-PCR. Our results clearly show that both normal tissues

(liver and thymus) and spontaneously developed sarcomas express a robust circadian oscillation in PER2::Luciferase expression with a peak phase that is slightly different between the normal tissues and tumors (ZT14 and ZT18 respectively). This work is supported by NIH grants CA102522 and GM075226 (to M.P.A.)

P224

Circadian dysfunction in mouse models of Parkinson's and Huntington's disease

TAKASHI KUDO, DAWN LOH, TAKAHIRO NAKAMURA, GENE BLOCK, CHRISTOPHER COLWELL

Psychiatry and Biobehavioral Sciences, University of California–Los Angeles, Los Angeles, CA, USA

Many patients with Parkinson's disease (PD) and Huntington's disease (HD) exhibit disturbances in their daily cycle of sleep and wake as part of their symptoms. These patients have difficulty sleeping at night and staying awake during the day. This dysfunction in timing is not causal to their disorder yet these symptoms have a major impact on the quality of life of the patient population and on the family members who care for the PD and HD patients. In these studies, we take advantage of transgenic and knock-in mice to determine whether mouse models of PD and HD exhibit deficits in their basic circadian rhythms of wheel-running activity. No single mouse model can be expected to recapitulate all aspects of the human disease. We would like to know to what extent circadian dysfunction is a common feature of the other mouse models. Therefore, we examined wheel running activity of alpha-synuclein over-expressing (ASO) mice that are the most common model of PD as well as three models of HD including the R6/2, CAG 140, and BAC-HD mice under LD and DD conditions. Because diseases progress with aging, we also considered the aging effects. The ASO, R6/2, and BAC-HD mouse showed clear circadian deficits including lower amplitude, fragmented rhythms and activity deficiency by 3 months of age. This is before the onset of motor symptoms characteristic of these diseases. These findings strengthen the view that the circadian deficits are a fundamental part of the disease. By developing a mechanistic understanding of how PD and HD impact the circadian timing system, we may be able to develop new therapies and disease management strategies for PD and HD patients.

P225

Postnatal development of peripheral tissue rhythms in mice

ANDREW CARY, HAMID DOLATSHAD, DANIEL WRESCHNIG, HARRY PANTAZOPOULOS, FRED DAVIS

Biology, Northeastern University, Boston, MA, USA

Circadian regulatory cycles (CRCs) are widespread in mammalian cells and tissues and are prominent features of many cellular processes such as the cell cycle and energy metabolism. The extent to which CRCs are active and coupled to cellular pathways during development is not well understood. To address this, we surveyed the expression of key circadian regulatory genes in whole embryos and tissues during prenatal development in mice. Using real-time PCR we found little evidence for CRCs during prenatal development. We therefore extended our survey to include postnatal ages. We measured mRNA levels for *bmal1* and *per2* between postnatal (PN) days 5 and 30 in C57Bl6 mice. An initial low resolution survey indicated that rhythms began in liver and heart between PN15 and PN20. Closer examination indicated rhythm onset between E17 and E20 in liver, heart, kidney, spleen, and muscle. Complete circadian profiles (samples every 4 hours) on PNs 15 and 20 confirmed that a rhythms had not been missed the earlier age. Interestingly, preliminary results indicate that rhythms in PER2 staining in brain regions outside the SCN also develop between PN15 and PN20. Our results suggest that there is a coordinated onset of CRCs in tissues throughout the body relatively late in development. In vivo measurements using PCR contrast with reports (including our own) of in vitro rhythms measured at earlier ages from tissues with bioluminescent reporters. To address this, we compared in vivo and in vitro *per2* expression from the same liver samples collected on E5 and E30.

We again found that rhythms in per2 mRNA or PER2 protein are not seen in PN5 samples collected in vivo even though rhythms are expressed in vitro at both ages. The rather abrupt and coordinated onset of peripheral tissue rhythms throughout the body suggests that a critical systemic circadian signal develops between two and three weeks of age.

P226

A role for a circadian clock in the pancreas in glucose homeostasis

AMANDA SADACCA¹, KATJA LAMIA², ANDREW DE LEMOS¹, CHARLES WEITZ¹

¹*Neurobiology, Harvard Medical School, Boston, MA, USA*

²*Molecular and Developmental Biology, The Salk Institute for Biological Studies, La Jolla, CA, USA*

Mice lacking circadian clock function in all tissues, such as *Bmal1*^{-/-} mice, have abnormal energy homeostasis, including glucose intolerance. Because of the loss of the suprachiasmatic nucleus (SCN) clock, such mice have abnormal feeding behavior and rest-activity patterns, making it unclear whether the defective energy homeostasis is a consequence of abnormal behavior or of the loss of a non-SCN clock important for energy metabolism. The pancreas plays a central role in glucose homeostasis, and it possesses an autonomous circadian clock. To determine if the *Bmal1* gene in the pancreas is important for glucose homeostasis, we used the *Pdx1*-Cre line to generate mice in which *Bmal1* was deleted from the pancreas (*Panc-Bmal1*^{-/-} mice). *Panc-Bmal1*^{-/-} mice fully recapitulate the glucose intolerance observed in *Bmal1*^{-/-} mice, despite exhibiting normal circadian rhythms of feeding behavior and locomotor activity. Like *Bmal1*^{-/-} mice, *Panc-Bmal1*^{-/-} mice show reduced insulin secretion in response to a bolus of glucose, but a normal clearance of glucose after insulin administration. These results strongly suggest that a circadian clock within the pancreas is important for glucose-sensing and/or insulin production or release in pancreatic β -cells, and that dysregulated behavior, as observed in *Bmal1*^{-/-} mice, does not contribute significantly to the glucose intolerance phenotype. The abnormal glucose tolerance of *Panc-Bmal1*^{-/-} mice still shows circadian modulation similar to that seen in control mice, indicating that a clock outside the pancreas contributes to the circadian regulation of glucose tolerance. While examining the widely-used *Pdx1*-Cre line, we found that this line produced Cre recombinase activity in the pancreas, as expected, however we also observed activity in the posterior and dorsal hypothalamus, but not in the SCN. Our results strongly suggest that a circadian clock in the pancreas is important for glucose homeostasis, but we cannot exclude a contribution from non-SCN clocks in the hypothalamus.

P227

Circadian clock-coordinated 12-hour period rhythmic activation of the IRE1 α pathway controls lipid metabolism in mouse liver

GASPARD CRETENET¹, MIKAEL LE CLECH², FREDERIC GACHON¹

¹*Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, SWITZERLAND*

²*Institut de Génétique Humaine, UPR 1142, Montpellier, FRANCE*

The mammalian circadian clock plays a fundamental role in the liver by regulating fatty acid, glucose, and xenobiotic metabolism. Impairment of this rhythm has been shown to lead to diverse pathologies, including metabolic syndrome. Currently, it is supposed that the circadian clock regulates metabolism mostly by regulating expression of liver enzymes at the transcriptional level. Here we show that the circadian clock also controls hepatic metabolism by synchronizing a secondary 12 hr period rhythm characterized by rhythmic activation of the IRE1 α pathway in the endoplasmic reticulum. The absence of circadian clock perturbs this secondary clock and provokes deregulation of endoplasmic reticulum-

localized enzymes. This leads to impaired lipid metabolism, resulting in aberrant activation of the sterol-regulated SREBP transcription factors. The resulting aberrant circadian lipid metabolism in mice devoid of the circadian clock could be involved in the appearance of the associated metabolic syndrome.

P228

Keeping time in the eye: Clock gene dependence of mouse retinal circadian clock

DOUGLAS McMAHON, GUOXIANG RUAN

Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA

Mammalian circadian rhythms are generated by an autoregulatory network of “clock genes” that are expressed in a wide variety of tissues and cells. The effects of clock gene deletion on clock function have been found to be tissue-specific, with the highly coupled central neural clock (suprachiasmatic nucleus, SCN) being more resistant to genetic disruption than peripheral tissue clocks in which there is little evidence of cellular coupling. Here we tested the effects of genetic deletion of canonical clock genes on the function of the retinal biological clock in vitro. Knockout mice for *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, and *Clock* (gifts of D. Weaver, A. Sancar, and S. Reppert) were crossed with luciferase reporter strains and molecular circadian rhythms were measured in retinal whole-mounts as in Ruan et al., 2008. Retinas from *Per1*^{-/-}, *Cry1*^{-/-}, and *Clock*^{-/-} mice were arrhythmic or showed severely disrupted gene cycling, whereas retinas from *Per2*^{-/-}, *Per3*^{-/-} and *Cry2*^{-/-} mice were robustly rhythmic. In addition, *Cry1* gene dosage on a *Cry2* KO background affected retinal freerunning period with *Cry1*^{+/-}*Cry2*^{-/-} exhibiting a lengthened period of ca. 27 hours compared with ca. 25 hours for *Cry1*^{+/+}*Cry2*^{-/-}. The clock gene dependence of the retinal molecular circadian clock is unique among tissues tested to date. Whereas *Per1*, *Cry1* and *Clock* are individually dispensable for SCN rhythmicity, their deletion disrupts the retinal clock, similar to peripheral tissue clocks, despite the neural origin of the retinal circadian clock. These data suggest that the retinal circadian clock may lack the compensatory mechanisms of the SCN neural clock and that it may be more vulnerable to genetic perturbation than the central clock. Supported by NEI R01 EY15815 to DGM.

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MYOD regulates clock gene expression in skeletal muscle

XIPING ZHANG¹, YI CAO², ZIZHEN YAO³, ROBERT GENTLEMAN³, STEPHEN TAPSCOTT², KARYN ESSER¹

¹Physiology, University of Kentucky, Lexington, KY, USA

²Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

³Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

The molecular Clock exists in both central and peripheral tissues and has been shown to regulate a variety of physiological and biochemical processes. While the core clock genes are shared across all tissues, results from studies of the circadian transcriptomes has found that most of the genes expressed in a circadian pattern are unique to the tissue. We recently identified that *MyoD*, a master muscle lineage determination factor, as a clock controlled gene. In this study we performed ChIP-SEQ using a MYOD Ab with nuclei from mouse adult skeletal muscle to determine the extent of MYOD binding. The results show that MYOD binds within the promoter region (-2000 to +1000) of many core clock genes, including *Bmal1*, *Clock*, *Per1*, *per2*, *Cry1*, *Cry2* and *Rev-erb a*. Reporter constructs were made for these clock genes promoters based on the ChIP-SEQ results. Preliminary results from transfection studies demonstrate that overexpression of MYOD activates *Per1* reporter in C2C12 cells. Next we found that knockdown of *MyoD* using siRNA leads to down-regulation of *Per1* mRNA in C2C12 cells.

These findings are consistent with a model in which MYOD, a skeletal muscle specific transcription factor, contributes to the expression of Per1 in muscle. These findings also provide insight into potential mechanisms whereby tissue specific transcription factors may synergize with core clock factors to elicit tissue specific circadian transcriptome.

P230

A role for adrenal glucocorticoids in the circadian resynchronization during jet lag

SILKE KIESSLING¹, GREGOR EICHELE¹, HENRIK OSTER²

¹*Genes and Behavior, Max-Planck-Institute for Biophysical Chemistry, Goettingen, GERMANY*

²*Genes and Behavior: Circadian Rhythms, Max-Planck-Institute for Biophysical Chemistry, Goettingen, GERMANY*

The term jet lag comprises a range of psycho- and physiopathological symptoms that arise from temporal misalignment of the endogenous circadian clock with external time. Repeated jet lag exposure, encountered by business travelers and airline personnel as well as shift workers, has been correlated with immune deficiency, mood disorders, elevated cancer risk and anatomical anomalies of the forebrain. Here we characterized the molecular response of the murine circadian system in an established experimental paradigm for jet lag. Unexpectedly, strong heterogeneity of entrainment kinetics was found not only between different organs, but also within the molecular clockwork of each tissue. Manipulation of the adrenal circadian clock, in particular phase-shifting of adrenal glucocorticoid rhythms, regulates the speed of behavioral re-entrainment. This key role of adrenal glucocorticoid phasing for resetting of the circadian system provides a novel mechanism-based approach for the therapy of jet lag and jet lag-associated diseases.

P231

Contribution of the vascular clock in remodeling: Insights through isograft arterial transplantation studies

Bo CHENG, CIPRIAN B. ANEA, ANA M. MERLOIU, R. DANIEL RUDIC

Pharmacology & Toxicology, Medical College of Georgia, Augusta, GA, USA

Recently, we have described that global disruption of Bmal1, an essential component of the circadian clock impairs the response to flow-induced vascular remodeling. However, it remains unclear whether these impairments emanate from a defect in central or vascular clock function. Herein, we developed a model of isograft arterial transplantation in the mouse to determine the role of the vascular and central clock in vascular remodeling. The thoracic aorta of donor wild type (WT) mice was end-to-end anastomosed into the common carotid artery of same strain, recipient WT mice (WT:WT transplants) and blood flow was restored in the new hybrid vessel. After 4 weeks, mice were perfusion fixed and the grafted aorta was isolated and processed for morphometry while the recipient native aorta was also isolated as control. WT:WT transplants exhibited inward remodeling ($544 \pm 24.4 \mu\text{m}$) and wall hyperplasia ($63.4 \pm 4.49 \mu\text{m}$) relative to the lumen diameter ($718 \pm 9.38 \mu\text{m}$) and wall thickness ($24.6 \pm 0.53 \mu\text{m}$) of the native aorta. Thus, in WT:WT isografts, adaptive remodeling of the aorta occurred that approached the dimension of the native common carotid artery. We then extended our studies to study the graft remodeling response in mice with targeted disruption of Bmal1 (Bmal1-KO) by grafting either the

WT aorta into the common carotid artery of Bmal1-KO (WT:Bmal1-KO) or the Bmal1-KO aorta into the common carotid artery of WT mice (Bmal1-KO:WT). Inward remodeling of WT:Bmal1-KO grafts ($565\pm 5.33\ \mu\text{m}$) was comparable to WT:WT grafts, but in Bmal1-KO:WT grafts, narrowing of lumen diameter was exacerbated ($470\pm 42.1\ \mu\text{m}$). Wall thickening in WT:Bmal1-KO grafts ($35.4\pm 1.72\ \mu\text{m}$) was less than WT:WT grafts while Bmal1-KO :WT aortic transplants exhibited pathological remodeling evidenced as a significant increase in wall thickness ($114\pm 38.2\ \mu\text{m}$) and the development of neointimal formation that was absent in the other transplant groups. These data suggest that the circadian clock intrinsic to the vasculature is critical for vascular remodeling.

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The circadian clock and hypertension

PARAMITA PATI, CIPRIAN ANEA, R. DANIEL RUDIC

Pharmacology & Toxicology, Medical College of Georgia, Augusta, GA, USA

Recent data has demonstrated the importance of the circadian clock in blood pressure control and rhythm. However, it remains unclear how and if the circadian clock conditions the response to experimental hypertension. In order to determine whether mutation of the circadian clock exacerbates blood pressure elevation during experimental hypertension, we undertook studies to examine the hypertensive response to pressor agents in mice with targeted disruption of Bmal1 (Bmal1-KO mice), using radiotelemetry to monitor blood pressure and activity. At baseline, Bmal1-KO mice exhibited lower blood pressures than WT mice and rhythmic oscillation of blood pressure that was observed in WT mice was abolished in Bmal1-KO mice. Administration of L-nitro arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, at a concentration of 1 g/L in drinking water, caused an increase in blood pressure in WT mice after 7 days, while rhythmic blood pressure remained intact. In contrast, Bmal1-KO mice showed a greater increase in blood pressure and lacked rhythmicity. Further, we compared mean arterial blood pressure, phase of the blood pressure curve, heart rate, and locomotor activity profiles, pre- and post-L-NAME treatment in WT versus Bmal1-KO. These studies may reveal a novel molecular mechanism that influences hypertension—the circadian clock.

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Activation of thromboxane receptors plays a major role in the development of pial vasomotion and low frequency oscillations of the cerebrocortical blood flow in NO-deficiency

**BELA HORVATH¹, GABOR LENZSER¹, ANDRAS IRING¹, BALAZS BENYO², PETER HERMAN³, ZSOMBOR LACZA¹,
PETER SANDOR¹, ZOLTAN BENYO¹**

*¹Institute of Human Physiology and Clinical Experimental Research,
Semmelweis University, Budapest, HUNGARY*

*²Department of Control Engineering and Information Technology, Budapest
University of Technology and Economics, Budapest, HUNGARY*

³Magnetic Resonance Research Center, Yale University, New Haven, CT, USA

—WITHDRAWN—

P234

Cellular and molecular pathways of immune-circadian communication

JOSÉ M. DUHART, M. JULIANA LEONE, NATALIA PALADINO, DIEGO A. GOLOMBEK

Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Bernal, ARGENTINA

Background. Bidirectional interactions between the immune and the circadian systems have been under intensive study in recent years. We have previously reported that peripheral immune stimuli are capable of altering behavioral circadian outputs such as locomotor activity rhythms. We are now assessing which are the molecular and cellular mediators between the immune system and the mammalian biological clock. Results. Previous results from our laboratory showed that low dose administration of LPS at CT-15 induced photic-like phase delays in wheel running rhythms. We now show that this response is mediated by TNF-alpha, since i.c.v. administration of soluble TNF-alpha receptor (but not IL-1 β antagonism) prior to LPS stimulation inhibited phase shifts. Moreover, TLR4 (Toll-Like Receptor 4)-null mice exhibited significantly smaller phase delays as well as absence of inhibition of wheel-running activity and c-Fos and Per1 induction in the PVN after LPS stimulation, confirming a role for this receptor in the effects of immune stimuli on the circadian clock. Since we have previously reported that SCN astroglia is an interface in immune-circadian modulation, we also analyzed the effects of cytokine administration on clock gene expression of these cells, and found that TNF-alpha reduces per1-luc expression in SCN astrocytes. Also, conditioned media from immune-challenged SCN astrocytes affected per1-luc expression in NIH-3T3 cells. Conclusion. We have characterized some of the pathways that mediate the effects of immune stimuli on the mammalian circadian clock. We show that the molecular pathways involve the LPS receptor TLR4 and, at least at central level, the action of TNF-alpha. Moreover, we found new evidences that place astrocytes as mediators of the immune-circadian communication. Supported by ANPCyT, CONICET and UNQ.

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Impaired immune function in behaviorally arrhythmic Siberian hamsters

NORMAN RUBY¹, PRIYESH PATEL², BRIAN PRENDERGAST²

¹*Biology, Stanford University, Stanford, CA, USA*

²*Psychology and Neurobiology, University of Chicago, Chicago, IL, USA*

Circadian clocks entrain to the external environment, but also mediate synchrony of the internal milieu, ensuring temporal organization among countless biochemical processes. Among these processes in the immune system are daily changes in the number and distribution of circulating white blood cells, alterations in adaptive immune function, and changes in the magnitude of innate inflammatory responses. Epidemiological data suggest that the stability of the circadian system plays a critical role in normal immune function. We therefore examined immune responses in circadian arrhythmic animals. Siberian hamsters were rendered behaviorally arrhythmic via noninvasive light treatments, and multiple aspects of immune function were examined. Circadian rhythms in blood leukocyte concentrations were absent in arrhythmic hamsters, but were sustained within a normal range. Behavioral, thermoregulatory, and metabolic responses to a simulated bacterial infection were all suppressed in arrhythmic hamsters relative to entrained controls, suggesting decrements in innate immune function. Delayed type hypersensitivity skin inflammatory responses were also significantly attenuated in arrhythmic hamsters which indicates impairments in memory T cell dependent adaptive immune function. These findings indicate that circadian temporal order is essential to normal immune function.

Molecular correlates of photoperiodic diapause in *Drosophila melanogaster*

MIRKO PEGORARO, CHARALAMBOS P. KYRIACOU, ERAN TAUBER
Genetics, University of Leicester, Leicester, UNITED KINGDOM

In temperate regions, many insects including *Drosophila* detect the rapid shortening of the day during the autumn and initiate their diapause programme that allows them to survive the winter. In *Drosophila*, diapause is manifested as an arrest of ovarian development in the females. The molecular basis underlying this seasonal response, presumably driven by a photoperiodic timer, is largely unknown. Here we report a global expression profiling we have been carried using microarrays with RNA extracted from female heads. We have identified 246 transcripts differentially expressed in heads of diapausing females. A few biological functions were significantly enriched among these genes, including protein metabolism, peptide signalling, ion binding, and immune defence. We have also identified 528 genes showing differential expression in females maintained in different day-length. Over-represented processes in this list included amino-acid metabolism, kinase activity, and stress (heat-shock) response. The gene Hsp70 which was previously implicated in insect diapause was at the top of the list. The circadian-clock gene *vri* was also differentially expressed and may serve as a candidate link between the circadian and the photoperiodic clocks. We have also used microarrays to compare the expression of micro-RNA (miRNA) in flies exposed to long and short photoperiods and found six miRNA (*dme-mir-2b*, *dme-mir-11*, *dme-mir-274*, *dme-mir-184*, *dme-mir-285*) that show a significant differential expression ($p < 0.01$). Analysis of the gene ontologies of top genes targeted by these miRNA revealed significant enrichment of process such as cell development and regulation, cell junction assembly and oogenesis. Interestingly, one of the targeted genes is *Atp α* , which was previously associated with response to abiotic stimuli. There were number of genes that were targeted by more than one miRNA, but their function in diapause is yet to be verified. Overall, these results suggest that miRNA may have an important regulatory role in photoperiodic induction.

Circadian modulation of developmental transcription factors in the pars tuberalis governs seasonal photoperiodism in mammals

HUGUES DARDENTE¹, CATHY WYSE¹, SANDRINE DUPRE², MIKE BIRNIE¹, GABI WAGNER¹, ANDREW LOUDON²,
 GERALD LINCOLN³, DAVID HAZLERIGG¹

¹*School of Biological Sciences, Aberdeen University, Aberdeen, UNITED KINGDOM*

²*Faculty of Life Sciences, Manchester University, Manchester, UNITED KINGDOM*

³*Queen's Medical Research Institute, Edinburgh University, Edinburgh, UNITED KINGDOM*

In mammals, circadian production of melatonin links the daily light dark cycle to day length dependent seasonal biology. Melatonin acts by controlling thyrotropin (TSH) production by the pars tuberalis (PT) of the pituitary, which then acts locally on TSH receptor expressing cells in the hypothalamus. We hypothesised that developmental transcription factors known to control pituitary cell fate might be crucial to the process of transducing the circadian melatonin signal into long term changes in PT cell phenotype. Consistent with this, a microarray screen for genes which responded rapidly to day length extension in the PT of Soay sheep revealed effects on the expression of the clock controlled transcription factor thyrotroph embryonic factor (*Tef*), and on the developmental regulator eyes absent 3 (*Eya3*). Additionally, *Six1*, a member of the sine oculis gene family, known to act with *Eya3* during development showed highly PT-enriched expression. Detailed profiling of the temporal expression of these factors revealed acute sensitivity to photoperiod such that under long days they peak synchronously, with heightened amplitude in the early light phase preceding peak β TSH induction. The β TSH promoter contains a conserved D-box, which binds and responds preferentially to TEF. Through this site, SIX1

exerted potentiation of TEF action, and this was dramatically enhanced by EYA3. Hence coordinated phase and amplitude control of Tef, Eya3 and Six1 expression provide a mechanism to upregulate PT β TSH expression in long days. Therefore pituitary developmental factors link the circadian system to light dependent seasonal biology.

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CLOCK Δ 19 mutants exhibit increased ethanol preference and consumption

ANGELA OZBURN, ELIZABETH GORDON, COLLEEN McCLUNG

Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX, USA

Recent studies point to a link between abnormal or disrupted circadian rhythms and drug addiction. Additionally, studies identify roles for circadian genes in the regulation of drug sensitivity and reward. Mice with a dominant negative mutation in the *CLOCK* gene (Clock Δ 19 mutant mice) show an increased sensitivity to cocaine. To extend these studies, we assessed whether the core molecular *CLOCK* gene has a role in alcohol intake (using the continuous access two bottle choice paradigm) and reward (using ethanol-induced conditioned place preference -CPP). We measured ethanol preference and consumption in Clock Δ 19 and wild-type mice (n=10-11/genotype). Escalating ethanol concentrations were offered versus water for two days each, starting with 3% ethanol (v/v in tap water) and continuing with 3% increases up to 21% ethanol. Clock Δ 19 mutant mice exhibited increased ethanol preference and consumption as compared to wild-type mice (preference: genotype x concentration interaction— $p < 0.05$, main effect of genotype— $p < 0.05$, main effect of concentration— $p < 0.001$, consumption: genotype x concentration interaction— $p < 0.01$, main effect of genotype— $p < 0.05$, main effect of concentration— $p < 0.001$). CPP was assessed using 2g/kg ethanol. Both Clock Δ 19 and wild-type mice (n=15/genotype) developed ethanol-induced CPP to a similar extent (test x genotype interaction— $p = 0.08$, main effect of test— $p < 0.01$), suggesting both genotypes exhibit a similar sensitivity to the rewarding properties of ethanol. Studies generally show that very few genetically modified mice exhibit increased alcohol intake. In addition to Clock Δ 19 mutants, mice with a null mutation in the circadian gene *Per2* also exhibit increased alcohol intake (Perreau-Lenz et al., 2009). These results suggest an important role for *CLOCK* in alcohol intake. Future studies include further characterization of ethanol-related behaviors in mice with mutations in circadian genes. Supported by grants from NIDA (T32-DA7290, 1-R01-DA023988-01A2, and 5-K01DA019541-05).

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Diurnal rodents: Lab versus field studies

NOGA KRONFELD-SCHOR¹, ORLY BARAK¹, ROTEM COHEN¹, LAURA SMALE²

¹*Zoology, Tel Aviv University, Tel Aviv, ISRAEL*

²*Psychology, Zoology, and Neuroscience Program, Michigan State University, East Lansing, MI, USA*

In recent years there is a growing interest in diurnal rodents: the need to understand the mechanisms controlling activity patterns is becoming apparent. Activity patterns and the mechanisms controlling them are of great significance for diverse fields of science. Moreover, it raises questions about the extent to which findings from nocturnal research animals can be generalized to humans. Interestingly, the more diurnal rodent species are studied, the more complex the answers to these questions appear to be. Here we compare results we obtained from two rodent species that are described as diurnal in the literature, but whose activity patterns vary considerably between natural and laboratory conditions (*Acomys russatus* and *Psammomys obesus*), and compare them to other diurnal rodents. A growing number of diurnal species appear to be nocturnal under laboratory conditions (or at least under specific laboratory conditions, e.g., when they can use a running wheel). Some of these species show other traits typical for nocturnal rodents as well, such as masking response to light. The relatively variable and plastic patterns seen in these diurnal rodents compared to nocturnal ones

may reflect the fact that diurnality is the derived condition in this group. Adaptation to this niche may not be as complete and committed as those of nocturnal rodents to theirs, as nocturnality appears to represent an ancestral condition that goes back to the origins of mammals. Independent evolutionary transitions to a diurnal pattern of adaptation to the day-night cycle have occurred within both closely and distantly related taxa, raising the possibility that the neural mechanism sustaining it could vary from species to species. Moreover, it is becoming apparent that diurnality is not a precise term, and it may reflect diverse patterns of adaptation to the day- night-cycle that have evolved under varying conditions in response to a range of selection pressures.

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Timed, sustained, attention-demanding performance reorganizes or dampens multiple circadian rhythms

**THERESA LEE¹, HOWARD GRITTON², GIOVANNA PAOLONE³, JIN YAN³, WILLEMJNTJE HOOGERWERF⁴,
MARTIN SARTER¹**

¹*Psychology & Neuroscience Program, University of Michigan, Ann Arbor, MI, USA*

²*Neuroscience Program, University of Michigan, Ann Arbor, MI, USA*

³*Psychology, University of Michigan, Ann Arbor, MI, USA*

⁴*Gastroenterology, University of Michigan, Ann Arbor, MI, USA*

Gritton et al (2009) reported that rats practicing daily a sustained attention task (SAT) at ZT4 exhibit inverted activity patterns, such that the majority of activity as measured by movement occurs during the light phase. This shift was not apparent in animals trained under several other control conditions, including an operantly similar simple reaction task that requires less cognitive vigilance performed at the same time of day. Here we report that the SAT performance also alters circadian running wheel activity at ZT4 in a similar manner. Furthermore, the SAT performance causes phase advances (diurnality) at ZT4, a smaller phase advance at ZT10 and small phase delays at ZT16. Core body temperatures (T_b) are consistent with running wheel records for training at ZT10 and ZT16. However, at ZT4 the T_b is no longer synchronized with running wheel records, and contain elements of both daily training and LD effects. Acetylcholine (ACh) release in the prefrontal cortex, as measured via microdialysis, anticipates and remains entrained for at least 3 days after the last training session at ZT4. Entrainment to the time of daily training also occurs at ZT16, but the anticipatory rise is absent. In contrast, an operant schedule of reinforcement devoid of explicit demands on cognitive processes (FI-9) did not shift activity at ZT4 also did not entrain ACh release. Animals practicing at ZT4 the SAT, but not the FI-9, exhibit dampened food intake, stool output, and corticosterone rhythms in addition to the noted changes in activity rhythms and ACh release. Finally, the *per2* rhythm in the colon also had a dampened level of expression. These data suggest that attention-demanding tasks may act to attenuate SCN-control of peripheral oscillators.

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Daily rhythms in morphine-induced behaviors

STEPHANIE PERREAU-LENZ, ANITA C. HANSSON, RAINER SPANAGEL
Psychopharmacology, CIMH, Mannheim, GERMANY

The involvement of biological rhythms and biological clock components in the development of dependence to drugs of abuse is currently of great interest. In the present study, we aimed at measuring the daily rhythms of morphine-induced conditioned place preference (CPP) and behavior sensitization, indicating respectively the reinforcement properties of that drug and the neurobiological changes underlying the development of dependence towards it. We therefore assessed the expression of CPP at four different time-points (ZT4, ZT10, ZT16 and ZT22) in four groups of mice. During conditioning

mice received alternatively saline or morphine injections (10 mg/kg; i.p.) and were exposed to one of two different floor-types in the CPP boxes. Thereafter, mice were put in the box with both floor-types available, and the time spent on each floor was recorded. The preference for the morphine-paired floor was the lowest for the group of mice conditioned at ZT4. Interestingly, this follows an inverse pattern from the one observed with cocaine or amphetamine, suggesting that not all rewarding stimuli follow the same rhythm of reinforcement. The morphine-induced increase of locomotor activity (behavioral sensitization) was assessed at the four time-points during the conditioning sessions of CPP, and its daily rhythm was further confirmed in classical open fields at ZT10 and ZT22. A clear rhythm in behavior sensitization could be measured with a nadir at ZT10 and a peak at ZT22. This pattern also different from the ones observed for cocaine- and amphetamine-induced sensitization suggests that different neurobiological changes are involved. To further understand the neurobiological mechanisms underlying the development of dependence to morphine, we will then compare the meso-cortico-limbic expression of clock genes in saline-treated, as well as in acutely and chronically morphine-treated mice when the sensitization is the lowest (ZT10) and the highest (ZT22).

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Circadian pacemaker in “shift worker” rats: Effect of a sustained daily cognitive task on circadian gene expression and corticosterone

JIN YAN¹, GIOVANNA PAOLONE¹, MEGAN HAGENAUER², HOWARD GRITTON², THERESA LEE¹

¹*Department of Psychology, University of Michigan, Ann Arbor, MI, USA*

²*Neuroscience Program, University of Michigan, Ann Arbor, MI, USA*

Previous studies have demonstrated that daily performance of sustained attention tasks (SATs) for a water reward during the light phase causes nocturnal rats to entrain to a diurnal activity pattern. In addition, attentional performance of rats during the light phase was significantly lowered when compared to performance during the dark phase. We hypothesized that the shift to a diurnal activity pattern observed in animals trained during the light phase was due to desynchronization or dampening of the SCN and peripheral oscillators. To test this hypothesis, we trained rats at times ZT4 and ZT16, and handled a group of non-performing animals randomly as a control. ZT4 and ZT16 trained animals were water deprived prior to SAT performance and were given 20 minutes of water after completing the task; food was provided ad libitum. Wheel running actogram data replicated previous observations that ZT4 trained animals were significantly diurnal. Preliminary corticosterone data suggests that day/night differences are attenuated in ZT4 trained animals as compared to ZT16 trained animals, suggesting that peripheral oscillators outside of the brain are dampened in this animal model of “shift work”. To observe effects on SCN and non-SCN oscillators in the brain, tissue was collected from ZT4 and ZT16 trained animals and control animals at times ZT2 and ZT14. Per2 expression was chosen as a measure of circadian oscillation and levels were measured via radioactive in situ hybridization. Results of the in situ hybridization will be presented at the conference.

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The circadian clock differentially regulates intermediate and long-term memory

MAXIMILIAN MICHEL, CHARITY L. GREEN, JACOB GARDNER, LISA C. LYONS

Biological Science, Florida State University, Tallahassee, FL, USA

Identification of the processes through which memory may be modulated is a key issue in unraveling the mechanisms through which learning occurs. We have used the marine mollusk *Aplysia californica* to investigate how the endogenous circadian clock modulates the formation of memory in vivo. *Aplysia* exhibit circadian rhythms in long-term, but not short-term, associative learning (Lyons et al.,

2006). In the current research, we investigated whether the circadian clock regulated intermediate-term memory using an operant learning paradigm, learning that food is inedible. During training the animal's failed swallowing attempts become associated with a specific seaweed resulting in memory formation. Intermediate memory (ITM), evident at 4 hours post-training, is temporally and mechanistically distinct from short and long-term memory. Under constant conditions, we found strong circadian regulation of ITM with peak memory levels exhibited by animals trained during the early subjective day at CT 3. Animals trained during the late subjective day or during the night exhibited little or no ITM. Importantly, animals maintained under light-dark cycles exhibited a diurnal rhythm with similar phase and amplitude to the circadian rhythm. This suggests that extended time in constant darkness was not the underlying reason for the suppression of memory during the late subjective day and night. We confirmed these results using a spaced training paradigm for the induction of ITM with similar rhythms observed in LD and DD conditions. Initial investigations into the mechanisms underlying this memory blockade found that inhibition of Protein Phosphatase 1 rescued circadian suppression of ITM during the late subjective day, but did not rescue memory at night. Thus, circadian regulation of intermediate-term memory occurs through multiple phase specific mechanisms that appear mechanistically divergent from the modulatory mechanisms governing circadian regulation of long-term memory. This research was supported by NIMH Grant MH081012 to L.C.L.

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Diurnal rhythms in cFos immunoreactivity in the mesolimbic reward system

RICARDO M. BALTAZAR, IAN C. WEBB, LIQUE M. COOLEN, MICHAEL N. LEHMAN

Anatomy and Cell Biology, University of Western Ontario, London, Ontario, CANADA

Recent findings suggest that the circadian timing system exerts an important influence on responses to rewarding stimuli. In particular, sensitivity to both natural rewards and drugs of abuse is strongly influenced by time of day (Abarca et al., 2002; Webb et al. 2009). Daily fluctuations in reward responsiveness appear correlated with oscillations in mesolimbic dopaminergic activity, as protein levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, are expressed rhythmically within the ventral tegmental area (VTA) and nucleus accumbens (NAcc) (Webb et al. 2009). To further explore potential mechanisms for the circadian control of reward, rhythmic expression of the neural activity marker cFos was examined across the day in reward-related brain regions. Male adult Sprague Dawley rats were sacrificed at 6 times during the day (zeitgeber (ZT) times: 2-6-10-14-18-22) and brains were analyzed for cFos, and TH immunoreactivity (ir) in the VTA, using peroxidase labelling techniques. In the VTA, numbers of cFos-ir cells varied diurnally, with a peak at ZT18 and a nadir at ZT 10. A similar rhythm was observed in cFos-ir TH neurons. However, TH-ir neurons accounted for less than 1% of all activated cells in the VTA and a rhythm in number of TH-ir cells was not detected. Rhythms of cFos-ir in other reward-associated brain regions were comparable to the VTA. The number of cFos-ir cells in the NAcc core and shell, and subregions of the prefrontal cortex, varied diurnally, with peaks at ZT18 and nadirs at ZT10. In contrast, no diurnal rhythm of cFos-ir was observed in the central and basolateral amygdala, or dentate gyrus. Thus, it appears that the circadian regulation of reward processing may be correlated with rhythms in baseline neural activity of the mesolimbic reward system. The specific neural mediators responsible for conferring rhythmicity upon this system remain to be determined.

Regulation of limbic circuitry and mood-related behavior by the CLOCK protein

SHIBANI MUKHERJEE¹, LAURENT COQUE¹, SADE SPENCER¹, RACHEL AREY¹, JUN-LI CAO¹, DONALD COOPER¹, MIGUEL NICOLELIS², KAFUI DZIRASA², COLLEEN McCLUNG¹

¹*Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX, USA*

²*Psychiatry, Duke University, Durham, NC, USA*

Circadian rhythms are highly disrupted in individuals with psychiatric disorders such as major depression and bipolar disorder. However, the exact role of circadian genes in mood regulation remains unclear. Previously, we reported that mice with a mutation in the Clock gene (Clock-Delta19) have a complete behavioral profile that is strikingly similar to human mania. Moreover, the mood stabilizing drug, lithium, restored the majority of their behavioral responses to wild type levels. Further investigation of these mice found that they have an increase in dopaminergic activity in the ventral tegmental area (VTA) and that CLOCK expression in the VTA is crucial for mood regulation. Here we will present our most recent data which demonstrates that CLOCK is involved in the synchronization of limbic structures in the brain, and that disruption in this synchrony is central to the development of many of the features of bipolar disorder. Indeed, treatment with lithium restores proper synchronization specifically in the Clock-Delta19 mice. Furthermore, we have identified some of the key molecular and cellular changes that underlie the disrupted structural coherence among limbic regions and abnormal dopaminergic activity in the Clock-Delta19 mice. Many of these changes are also reversed with chronic lithium treatment. Taken together, our data supports a central role for the CLOCK protein in the timing of activity in multiple mood-related regions of the brain, and that disruption in this timing and subsequent circuit level desynchronization likely contributes to the development of bipolar disorder.

Molecular biomarkers for human circadian differences

LUDMILA CUNINKOVA, ERMANNO MORRIGGI, STEVEN BROWN

Institute of Pharmacology and Toxicology, University of Zurich, Zurich, SWITZERLAND

Daily rhythms in mammalian physiology and behavior are generated and synchronized to environmental light by a master clock located in the suprachiasmatic nucleus of the brain hypothalamus. Neurons within the SCN, as well as most other cells in the body, contain an intracellular molecular clock driven by an autoregulatory transcriptional and translational feedback loop. Previously, we have been successful in using lentiviral reporters for clocks in peripheral cells such as skin fibroblasts as quantitative models for those in the SCN: differences in circadian behavior among different individuals are mirrored by changes in fibroblast clock reporter properties. The entrainment of the circadian clock, accomplished primarily by light in mammals, induces not just the activation of the clock genes but also other transcription factors such as CREB and ELK-1 and various phosphatases and kinases, including the mitogen-activated protein kinase family. The main goal of this study is to look at differences in the expression patterns of these and other major signal transduction pathways among different human individuals, and to correlate them with properties of circadian input pathways, such as phase-shifting. We developed a lentivirus-based reporter system that allows us to measure expression profiles for chosen cellular pathways directly in human primary fibroblast cells. Preliminary tests with 12 subjects showed that pathway-specific transcriptional activation has individual amplitudes and kinetic patterns among individuals. We are currently correlating these pathway-specific properties with light- and chemical-dependent phaseshifting of the circadian oscillator. Our results suggest that we will be able to use our cellular assays as quantitative traits to map the genes that modify the amplitude and kinetics of chosen signaling pathways, including that of the circadian clock, in human populations. In addition, our reporters could serve as valuable biomarkers for prediction of patient response to many classes of drugs.

Human nocturnal nonvisual responses to polychromatic white light cannot be predicted by the melanopsin photosensitivity function

VICTORIA REVELL¹, DANIEL BARRETT¹, LUC SCHLANGEN², DEBRA SKENE¹

¹*FHMS, University of Surrey, Guildford, UNITED KINGDOM*

²*Philips Lighting, Eindhoven, THE NETHERLANDS*

The blue light sensitivity of human non-visual responses is attributed to melanopsin but whether the photosensitivity function of melanopsin is the sole factor in determining the efficacy of light sources in driving non-visual responses remains to be established. Previously we have demonstrated that the melatonin suppression response to a high pressure mercury lamp is greater than predicted by a melanopsin photosensitivity function with λ_{\max} 480 nm. In the current study monochromatic (λ_{\max} 437, 479 and 532 nm) and polychromatic white (colour temperature: 4000 K and 17000 K) light stimuli were photon matched for their predicted ability to stimulate melanopsin, and their capacity to influence nocturnal melatonin levels, auditory reaction time and subjective alertness and mood was assessed. A within-subject study design was used and participants were young, healthy males aged 18–35 years (23.6 ± 3.6 yrs; mean \pm SD; $n = 12$). The in-laboratory sessions included an individually timed 30 min light stimulus on the rising limb of the melatonin profile and at regular intervals relative to the light stimulus blood samples were taken, subjective mood and alertness were recorded and an auditory reaction time task was performed. Repeated measures ANOVA analysis revealed that significantly lower melatonin suppression was observed with the 4000 K and 17 000 K polychromatic light conditions compared to the monochromatic light conditions ($p < 0.01$). The findings, in accordance with previous work, demonstrate that a melanopsin photosensitivity function with λ_{\max} 480 nm cannot predict the melatonin suppression response to polychromatic light and highlight the importance of the spectrum of the polychromatic light source in determining response magnitude.

Spectral sensitivity of melatonin suppression in the elderly

RAYMOND NAJJAR¹, BRUNO CLAUSTRAT¹, PHILIPPE DENIS², HOWARD M. COOPER¹, CLAUDE GRONFIER¹

¹*Department of Chronobiology, INSERM, U846, Bron, FRANCE*

²*Department of Ophthalmology, HCL/HEH, Lyon, FRANCE*

Aging is associated with changes in sleep structure and circadian rhythmicity. Between 40-70% of aged subjects complain about sleep and alertness disorders. These alterations may result from an inappropriate entrainment of the circadian clock due to impaired photic input. A decreased sensitivity of the circadian system to light has been shown in the elderly (Duffy et al. 2007) in particular in the short wavelength range (Herlevic et al. 2005). These data, however, remain insufficient for determining the precise origin of the diminished photic sensitivity of the circadian system. The aim of this study is to investigate whether age-related changes in circadian sensitivity to light are related to an increase in ocular lens density. Eight participants (55 to 63 years old) were exposed to 60-min monochromatic light sessions at nine different wavelengths (420–620 nm) of equal photon density (3.16×10^{13} photons/cm²/sec). Plasma melatonin suppression, as an index of circadian sensitivity to light, was compared across light treatments. Photic sensitivity spectra were then established for each subject. Assessment of lens density was done using a validated psychophysical technique that eliminates the effects of inaccurate refractive correction and misalignment, and avoids the effects of pupil size on measurements (Norren 1974). Compared to young subjects, our preliminary results in the elderly show a specific decrease in circadian sensitivity to short wavelength light (< 500 nm) but no change of sensitivity for long wavelengths. Measurements of lens density show a comparable relative decrease in transmittance of the ocular crystalline lens in the aged for the same range of short wavelengths. Taken together,

our results suggest that a decreased transmittance of the ocular lens is a major factor of the reduced circadian sensitivity to light observed with age, and may contribute to impaired sleep and circadian rhythms in the elderly. Work supported by GIS-Longevity, IP-FP6-EUCLOCK (018741), Rhone-Alpes Cible. RN is supported by Ministère de l'Enseignement Supérieur et de la Recherche Français.

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Prevalence of circadian rhythm disorders in 118 blind women with and without light perception living in North America

ERIN FLYNN-EVANS¹, DEBRA SKENE², STEVEN LOCKLEY¹

¹*Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*

²*Centre for Chronobiology, University of Surrey, Guildford, UNITED KINGDOM*

Prior research has demonstrated that most blind people with no perception of light (NPL) have either non-entrained or abnormally phased circadian rhythms, while most visually impaired subjects with light perception (LP) exhibit normal entrainment. Those with NPL often lack the ability to detect light to entrain the suprachiasmatic nuclei (SCN) clock appropriately, resulting in abnormally phased or non-entrained circadian rhythm type. Although subjects with LP presumably retain the ganglion cells necessary to send light information to the SCN clock, this population also reports a high prevalence of sleep disturbance. The aims of the present investigation were to identify the prevalence of circadian rhythm disorders in 118 blind women, with the further aim of examining how eye disease may relate to circadian rhythm type. Subjects (LP n=78, NPL n=40) completed an eight-week field study including daily sleep diaries, wrist actigraphy and sequential 4-8 hourly urine collections over 48h on 2-3 occasions separated by at least two weeks. Circadian type was determined objectively using cosinor derived 6-sulfatoxymelatonin acrophase and its rate of change over time, calculated circadian period, and degree of sleep disturbance. Among subjects with NPL, 40% were classified as non-entrained (n=16), 33% were normally phased (n=13), 25% were classified as abnormally phased (n=10) with one subject unclassified. Among subjects with LP, 49% were classified as normally entrained (n=38), 17% were abnormally phased (n=13) and five were unclassified. Just over a quarter of the LP subjects (28%), appeared to have non-entrained circadian rhythms (n=22). Bilateral enucleation (67%) and Retinopathy of Prematurity (67%) were the eye conditions that were most closely associated with non-entrained or abnormally phased rhythms in NPL subjects. Retinitis Pigmentosa (41%) and glaucoma (71%) were most commonly related to abnormal circadian rhythms in LP subjects. These findings suggest that the etiology of blindness in addition to light perception status may be related to an individual's ability to process a circadian light signal.

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Internal phase relationships in free-running individuals with delayed circadian phases

JOELLE SCHLANG, MICHELE STANCHINA, PATRICIA MURPHY, SCOTT CAMPBELL

Chronobiology, Weill Cornell Medical College, White Plains, NY, USA

Delayed Sleep Phase Disorder (DSPD) results from a misalignment between the internal clock and external environment, suggesting that alignment of internal and external rhythms will alleviate DSPD symptoms. However, recent evidence indicates that there is also an altered phase relationship amongst internal rhythms in DSPD under entrained conditions. Thus, advancing sleep timing to coincide with a socially desired sleep schedule may leave residual internal phase misalignments which could be disruptive to sleep. Here we compare the phase angle (Θ) between sleep timing and body temperature rhythms during temporal isolation in delayed versus non-delayed individuals. Polysomnography (PSG) and temperature data were collected continuously from 4 delayed (D) and 5 control (C) subjects

for 4 days in entrainment (ENT) followed by 14 days in freerun (FR) conditions. D reported preferred bedtime >0200h and waketime >1000h and a temperature nadir (9th order polynomial fit; T_{min}) >0700h during ENT. C met none of these criteria. The $\Theta<$ was calculated as the interval from PSG sleep offset to T_{min} for each subject's major sleep episodes during FR. Average T_{min} in C during ENT was 0400h \pm 24m compared with 0922h \pm 115m in D ($p<.05$). C had a mean $\Theta<$ of 3.80 ± 0.40 . D had a mean $\Theta<$ of 5.84 ± 0.47 ($t=3.38$, $p<.01$). Of D, 75% had greater $\Theta<$ than the median (4.72), as opposed to only 20% of C. These results provide support for the notion that under FR conditions D have altered $\Theta<$ between sleep timing and temp rhythms relative to C. A larger sample size will clarify whether $\Theta<$ differences in D are robust. Further analysis of PSG will be examine if altered $\Theta<$ are associated with disturbances in the quality and/or architecture of nighttime sleep.

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Identification of encephalic photoreceptors in the avian brain

GANG WANG, VINCENT CASSONE

Biology, University of Kentucky, Lexington, KY, USA

In contrast to the situation in mammals, there are multiple photoreceptors in avian system, including retinal, pineal and encephalic photoreceptors. Although encephalic photoreceptors have been known to function in circadian entrainment and in mediating photoperiodic responses, their molecular bases and exact locations within the brain are still largely unknown. Recently, several potential candidate photopigments have been suggested, including rhodopsin, melanopsin and VA opsin. However, the distributions of these photopigments are not consistent in the brain. Rhodopsin-like cells were found in lateral septum and mediobasal hypothalamus. VA opsin-like cell bodies were found in preoptic area and paraventricular nucleus with extensive projection of fibers into the median eminence. There is no evidence of melanopsin-like protein in the deep brain, while in situ hybridization study showed mRNA expression in optic tectum and cerebellum. In the present study, we examined the expression of rhodopsin and melanopsin immunocytochemically in avian brain. Both rhodopsin-like and melanopsin-like immunoreactivities have been identified in the mediobasal hypothalamus. Rhodopsin-like cell bodies appear to be neuronal and located in the tuberal area with a discrete band of fiber staining in the median eminence, while melanopsin-like staining is found in the ependymal cells and tanycytes lining the third ventricular with fibers extending to the adjacent tuberal area. Currently, the immunocytochemical results are being validating by in situ mRNA hybridization. In conclusion, our results suggest that similar to both retinae and pineal, the encephalic photoreceptors may contain multiple types of photopigment, which associate with different phototransduction pathways. The photoperiodic response may need the synergy of these different photopigments and their associated signaling pathways. Acknowledgement: This research is supported by NIH P01 NS39546 grant to V.M. Cassone.

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Nighttime dim light exposure alters the responses of circadian system

DORELA SHUBONI¹, LILY YAN²

¹Psychology, Michigan State University, East Lansing, MI, USA

²Psychology, Neuroscience Program, Michigan State University, East Lansing, MI, USA

The daily cycle of light and darkness is the most salient entraining factor for the circadian system. However, in modern society, with steadily increasing light pollution, the darkness is vanishing at night. The impact of exposure to dim light at night on ecology and human physiology is just being recognized. In the present study, we tested the possible detrimental effects of dim light exposure on the brain clock and on overt rhythms, using CD1 mice housed in light/dim light (L:dimL, 300lux:20lux) or light/dark (L:D, 300lux:1lux) conditions. The behavioral responses were assessed by wheel-running

activities. Compared to the LD group, the L:dimL group showed increased daytime activity, a higher day/night-activity ratio and smaller phase angles of activity onset. After being released into DD, the L:dimL group displayed shorter free-running period. Furthermore, following a light pulse at ZT16 (30min, 300lux), the phase shifting responses were also smaller in the L:dimL group. Next, we examined clock genes *Per1* and *Per2* to determine whether the altered behavioral responses in the L:dimL group might have arisen from changes within the SCN. Under entrained condition, there was no difference in *PER1* at the peak time between the two groups, but at the trough time, there was an increase in *PER1* in the L:dimL group, indicating a decreased amplitude of the *PER1* rhythm. After the light pulse, the induction of *Per1/Per2* genes was also attenuated in the SCN of L:dimL group. The results indicate that nighttime dim light exposure can alter both behavioral and neuronal responses of the circadian system, and suggest that the circadian perturbation is one of the mechanisms underlying the adverse effects in ecology and human physiology caused by light pollution.

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Lifelong consequences of early postnatal light environment

MARIA CANAL, LAURA SMITH

Faculty of Life Sciences, University of Manchester, Manchester, UNITED KINGDOM

Postnatal experience during circadian system development is critical for the establishment of the synchronisation of the pup's circadian rhythms to the environment. Environmental light during development has long-term effects on circadian behaviour; however, the long-lasting effects of early light experience on the brain master clock are still unclear. Here we reared three groups of mice under constant light (LL), constant darkness (DD) or light-dark cycles (LD) during the first 3 neonatal weeks. On postnatal day 21 (P21), all mice were weaned and transferred first to LL for 2 weeks, and then to LD until the end of the experiment. Locomotor activity was recorded throughout the experiment using infrared activity meters. At P50, brains were collected and arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and glial fibrillary acidic protein (GFAP) protein expression in the suprachiasmatic nuclei (SCN) of the hypothalamus was investigated. In LL, LL-reared mice showed shorter free-running period and higher amplitude in the circadian rhythm of locomotor activity, compared to LD- and DD-reared mice. In LD, LL-reared mice also showed higher amplitude activity rhythm, with an earlier acrophase, and higher activity levels during the active phase. In the SCN, LL-reared mice had lower levels of AVP and VIP immunoreactivity, compared to LD- and DD-reared mice. Although GFAP-immunoreactivity was also lowest in the LL-reared group, the number of GFAP-positive cells was highest in this group, suggesting structural remodelling of SCN astrocytes by early light experience. Postnatal light environment has long-term effects on the expression of the mouse locomotor activity rhythm, and also on neuronal and glial cell populations of the SCN. We argue that these neurochemical and structural alterations may affect clock function, which may in turn modify animal's behaviour.

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Dim and dimmer: Pupil reflex, masking, and entrainment thresholds

MATTHEW BUTLER, RAE SILVER

Psychology, Columbia University, New York, NY, USA

Background. The circadian system receives photic input from both classical photoreceptors and intrinsically photosensitive retinal ganglion cells (ipRGC). Identifying the circadian system's sensitivity to light of different wavelengths has been important for specifying the underlying photoreceptors. Threshold information is also important in determining optimal light sources for animal husbandry

during circadian studies. Most studies of photic sensitivity of the circadian system have relied on phase shifting as a measure. Whether phase shifting response thresholds accurately determine thresholds of entrainment to light:dark cycles is not known. Methods. To determine the thresholds of circadian entrainment and the responsible photoreceptors, mice (C57Bl/6) were given access to running wheels and housed in blue (470nm), green (525nm), or red light (630nm). Irradiance was stepped down approximately every 2 weeks together with a 2 h advance of the light-dark cycle. The lowest irradiance that entrained mice was defined as the threshold of entrainment. These thresholds were compared to those for masking in 3.5h:3.5h light:dark conditions and for the pupillary light reflex. Results. Mice entrained to both blue and green light with thresholds on the order of 1 nW/cm²; the threshold for blue and green was ~2.5 log units lower than for red light. The threshold for entrainment was ~4 log units (10,000-fold) lower than for either masking or pupil constriction. Conclusion. The results indicate that entrainment depends on rod and cone mediated responses rather than on ipRGC photoreception, based on the relative sensitivities to the three wavelengths. The results also highlight a practical problem. The circadian system of mice is sensitive to dim red light at irradiances (< 1 lux) often used to facilitate animal care and maintenance, and thus may be sufficient to phase shift or entrain mice. Restated, constant dim red light is not equivalent to constant darkness.

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Determining retinal photoreceptor contribution in light-dependent acute changes in body temperature

CARA ALTIMUS, ALAN RUPP, SAMER HATTAR

Biology, Johns Hopkins University, Baltimore, MD, USA

In mammals, retinal photoreception drives the alignment of circadian rhythms to the solar day, influences sleep and alertness and regulates pupillary light constriction. All light effects on these physiological functions can be accounted for by 3 photoreceptor types: rods, cones, and melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs). To understand how light influences other hypothalamic functions and which retinal photoreceptors contribute to these functions, we assayed acute changes in body temperature in response to a 3-hour light pulse administered from ZT 14-17. In wild type mice, we found that the 3-hour light pulse sustained a decrease in body temperature of 0.6°C. However, melanopsin knockout (*Opn4*^{-/-}) mice showed no change in body temperature in response to this 3-hour light pulse suggesting that the presence of melanopsin is necessary for this response. To determine if melanopsin phototransduction is sufficient to induce acute changes in body temperature, we used animals that lack both rod and cone transducins, which keep these classical photoreceptors in a depolarized state, resembling constant dark conditions. We show that melanopsin signaling is sufficient to induce body temperature changes. However, we previously showed that the state of the cone influences circadian photoentrainment in animals with rods as the only functional photoreceptors. We, therefore, sought to determine whether the state of the cone could also influence melanopsin signaling. We silenced the cones by using a CNG channel knockout, which maintains cones in a state of hyperpolarization, resembling saturating light conditions. In these melanopsin only animals (*Gnat1*^{-/-}; *Cnga3*^{-/-}), we found that the light pulse leads to an attenuated response compared to that of the melanopsin only where cones are silenced using the cone transducin knockout (*Gnat1*^{-/-}; *Gnat2*^{-/-}). Together, these data suggest that cone hyperpolarization opposes the intrinsic light response of melanopsin neurons. Our data suggests that melanopsin is necessary for acute reduction in body temperature and that cone activation opposes the melanopsin signal.

CAST/EiJ mice—a unique animal model for studies in circadian entrainment, altered activity phases, masking, and sleep

PENG JIANG¹, MARTIN STRIZ¹, JONATHAN WISOR², BRUCE O'HARA¹

¹*Department of Biology, University of Kentucky, Lexington, KY, USA*

²*VCAPP, Sleep and Performance Research Center, Washington State University, Spokane, WA, USA*

CAST/EiJ (CE) is a wild-derived inbred mouse line that is genetically divergent from most of the common inbred strains. In this study, we evaluated several circadian and sleep traits in CE mice under a wide range of conditions. When entrained, CE mice initiated activity about 3 hours before dark, while all B6 mice displayed activity onsets at dark as typical in mice. This was observed in both wheel-running activity and in sleep-wake scored by a piezoelectric system. The endogenous circadian period in CE mice was modestly shorter than B6 mice, however, it was not correlated with advanced phase within the CE strain. The photic phase-shifting responses were phase lagged by roughly 3 hours, suggesting that the early runner phenotype is produced by altered coupling between different circadian oscillators, or between the master clock and its output. Moreover, light-induced masking in CE mice was impaired in concert with the advanced phase angle to maintain robust activity in the preferred circadian phase. This, coupled with other unusual circadian traits in CE mice and wild *Mus musculus castaneus* from which CE mice are derived, raise the possibility of positive selection for altered chronotypes in these populations. In addition to variations in circadian phenotypes, CE mice also exhibited reduced sleep time by 5-10% compared to B6. Lastly, we have continued to examine a quantitative trait locus (QTL) we identified on chromosome 18 that strongly influences activity onset and phase angle of entrainment in CEx(CExB6) backcross populations. Here, we show that this QTL is further confirmed and refined using two strains of congenic GTM mice, thus allowing future fine-mapping and gene identification. Our data suggest that CE mice may serve as a novel animal model, which may be utilized to identify genetic factors that affect multiple circadian and sleep traits.

Neurochemical connections between sleep and circadian centers regulate behavioral timing

JENNIFER ARNOLD¹, SABRA ABBOTT¹, HAI MIAO², NOBUTOSHI OTA², CHRISTINE CECALA², QING CHANG³,
KEN MORRIS³, PAUL GOLD³, JONATHAN SWEEDLER², MARTHA GILLETTE⁴

¹*Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

²*Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

³*Neuroscience, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

⁴*Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

The sleep-wake cycle is controlled by two mechanisms. One of these is homeostatic, communicating tiredness, and the other is circadian, communicating an approximately 24-hour state. Together, these two processes align sleep properly according to need and the appropriate time of the day-night cycle. Homeostatic sleep centers in the brain have known neural connections to the central clock, the suprachiasmatic nucleus (SCN), but the function of these connections has been unknown. Combining stimulation of specific pontine homeostatic sleep regions with analysis of SCN microdialysate by HPLC and capillary electrophoresis, we detect significant neurotransmitter release at the SCN. We measure changes in acetylcholine and/or glutamate at the SCN when stimulation parameters are varied. These stimulation parameters cause phase resetting of behavioral rhythms that are characteristic of each neurotransmitter. Current work focuses on establishing the specificity of these stimulations to pontine

cholinergic regions, as well as examining the release of serotonin, a third neurotransmitter implicated in sleep regulation. These findings demonstrate modes of communication between sleep centers and the circadian clock that have behavioral consequences, implying a dynamic interplay between brain regions that regulate vigilance states.

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Reduced NaV1.1 activity impairs SCN inter-neuronal communication, lengthens circadian period, and blocks light-induced phase shift in NaV1.1 mutant mice

SUNG HAN, FRANK YU, MICHAEL SCHWARTZ, MARTHA BOSMA, WILLIAM CATTERALL, HORACIO DE LA IGLESIA
Program in Neurobiology & Behavior, University of Washington, Seattle, WA, USA

Sodium-dependent action potentials are thought to be essential for the synchronization of clock neurons within the suprachiasmatic nucleus (SCN). Here we report that mice carrying a heterozygous loss-of-function mutation of the voltage-gated sodium channel type 1.1 (NaV1.1) have longer circadian period than their wildtype littermates. NaV1.1 heterozygote mutant mice (scn1a+/-) also show a lack of light-induced phase shift. In contrast to these impaired photic responses, scn1a+/- mice have normal negative masking behavior by nocturnal light exposure. Analysis of light-induced gene expression in response to nocturnal light pulses revealed that although scn1a+/- mice show normal induction of c-fos and mPer1 mRNA within the ventral SCN, they lack increased gene expression within the dorsal SCN, which is characteristic in their wildtype littermates. Intracellular calcium imaging in SCN slices confirmed that electrical stimulation of optic chiasm elicits increased calcium responses in ventral and dorsal neurons of wildtype SCNs but elicits increased calcium responses only in ventral SCN neurons of scn1a+/- mice. Our data indicate that the impaired intercellular communication, results from reduced NaV1.1 activity leads to changes in the period of the master circadian pacemaker. Our results also suggests that this reduced channel activity prevents behavioral phase shifts in response to light by interfering with the communication between ventral and dorsal SCN neurons without impairing the ability of the retina to relay photic information to the SCN.

P259

Ocular and circadian regulation of phosphorylation of extracellular regulated kinase and the NR1 NMDA receptor subunit in the hamster suprachiasmatic nucleus

IAN C. WEBB, RICARDO M. BALTAZAR, LIQUE M. COOLEN, MICHAEL N. LEHMAN
Anatomy and Cell Biology, University of Western Ontario, London, Ontario, CANADA

Phosphorylation of extracellular regulated kinase (pERK) is rhythmic in both the suprachiasmatic core and shell with the former region peaking during the subjective night and the latter showing a peak during the day (Obrietan et al., 1998; Coogan & Piggins, 2003). Previous work has shown that the rhythm of pERK in the SCN core is eliminated with enucleation while that in the shell persists in the absence of the eye (Lee et al., 2003), yet the nature of the ocular signal driving core pERK rhythmicity remains to be identified. Here, we test the hypothesis that the phosphorylation of ERK in the SCN core is associated with NMDA receptor activation. To this end, a dual-label immunofluorescence approach was utilized to examine the extent of colocalization of pERK and the phosphorylated form of the NR1 (pNR1) NMDA receptor subunit in the SCN across the circadian day. The influence of enucleation on ERK and NR1 phosphorylation rhythms also was examined. The observed rhythms in SCN pERK levels were consistent with those described previously. A rhythm in SCN pNR1 was evident with peak levels in the shell observed in the late day and across the night, and a peak in the core coinciding with that seen for pERK. In addition, virtually all pERK positive neurons in both regions of the SCN were found to colocalize with pNR1. Enucleation attenuated the peak of both pERK and pNR1 in the core without

influencing that in the shell. Thus, the current data indicate a close association between rhythms in activation of ERK and NR1 in the SCN. Moreover, these data suggest that ERK phosphorylation in the SCN core, but not in the shell, is mediated by glutamate release from retinal efferents transduced via NMDA receptor binding.

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Interactions between the circadian clock and serotonin gene networks

HOLLY RESUEHR¹, JOHN AXLEY¹, GREGG ALLEN², EVAN DENERIS³, DOUGLAS McMAHON¹

¹*Biological Sciences, Vanderbilt University, Nashville, TN, USA*

²*Neuroscience and Experimental Therapeutics, Texas A&M University, College Station, TX, USA*

³*Neuroscience, Case Western Reserve University, Cleveland, OH, USA*

The SCN receives direct serotonergic input from the midbrain raphe nuclei. It has been shown that modifications of serotonergic signaling to the SCN have been linked to various disorders including depression and SAD (seasonal affective disorder). The circadian and serotonergic gene networks have the potential to interact, as elements of the serotonin (5-HT) network (receptors) are expressed in the SCN and elements of the clock gene network are expressed in the raphe nuclei. To examine how these two gene networks are interplaying we first examined what affect serotonin has on *per1* rhythms in the SCN. Using *ePet1* knockout mice, which lack 5-HT in the raphe nuclei, we looked at the SCN *per1::luc* rhythms compared to wild type. By measuring the luminescence it was determined that the SCN *ePet-1* knockout mice displayed disparate period lengths when compared to wild types. To further our understanding of how these two gene networks are related we performed a microarray study comparing gene expression in the SCN of *pet-1* knockout mice and wild type mice at two different time points (ZT 10 and ZT 22). It was found that at ZT 10 the 5-HT deficient mice had significantly upregulated genes in the circadian rhythm pathway, this was postvalidated using qRT-PCR. The lack of serotonin impacted not only the mammalian circadian rhythm pathway, but also many other genes in various biological pathways including: i.e. asthma, nicotinate and nicotinamide metabolism, primary immunodeficiency, tyrosine metabolism, etc.... Taken together these data show that the serotonergic gene network does in fact influence gene expression in the mammalian circadian clock and that these two gene networks appear to be closely interrelated. Supported by NIH P50 MH 078028.

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CRY1 and CRY2 are not necessary for circadian firing rhythms in the mouse suprachiasmatic nucleus

DAISUKE ONO, SATO HONMA, KEN-ICHI HONMA

Department of Physiology, Hokkaido University Graduate School of Medicine, Sapporo, JAPAN

Cry1 and *Cry2* double deficient (*Cry1*^{-/-}/*Cry2*^{-/-}) mice become behaviorally aperiodic immediately after exposure to DD. However, they exhibit 24h periodicity in their behavior under LD with the activity onset often precedes the dark onset. Such pre-dark activities are not explained by the light masking hypothesis, but suggest oscillatory entrainment. Since the previous observation did not exclude the possibility of rhythm generation in single SCN neurons of *Cry1*^{-/-}/*Cry2*^{-/-}, we examined the spontaneous discharges of cultured SCN neurons using a multi-electrode array dish (MED). A coronal brain slice was obtained from a 2-5 day-old pup of *Cry1*^{-/-}/*Cry2*^{-/-} and wild type mice, and a paired SCN was placed on a MED probe. After culturing for 1-3 weeks, spontaneous firing from the cultured SCN was recorded for 7-18 days. The number of spikes was counted every 1 min. Circadian rhythm and its period was evaluated by a chi-square periodogram. In wild type mice, significant circadian rhythm was detected in their firing rate from all electrodes in the SCN slice. Surprisingly, the SCN neurons from *Cry1*^{-/-}/*Cry2*^{-/-} mice also exhibited circadian rhythms which were comparable with the

controls. Although the phases of neuronal rhythms were almost identical among electrodes from a single SCN, their periods were not stable. They showed occasional dramatic change in their circadian periods. These results suggest that the circadian oscillation exists in cultured SCN of *Cry1^{-/-}/Cry2^{-/-}* mice, but its periodicity is unstable.

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Aging in the circadian system: The VIP hypothesis

CHRISTOPHER COLWELL¹, TAKAHIRO NAKAMURA², TAKASHI KUDO¹, DAWN LOH¹, GENE BLOCK¹

¹*Psychiatry, University of California–Los Angeles, Los Angeles, CA, USA*

²*Pharmaceutical Sciences, Teikyo Heisei University, Ichihara, JAPAN*

Disruptions in sleep/wake cycles including decreased amplitude of rhythmic behaviors and fragmentation of the sleep episodes, are commonly associated with aging in humans and other mammals. While there are undoubtedly many factors contributing to these changes, a body of literature is emerging that is consistent with the hypothesis that an age-related decline in VIP expression in the SCN may be a key element responsible. In the present study, we have carried out behavioral analysis of aging in C57 mice and see clear evidence of fragmentation of the activity/rest cycle, increased activity during the day and reduced light-response of the circadian system. Qualitatively, young adult VIP-deficient mice show each of same behavioral traits although the deficits are more severe in the mutants. We also have obtained in vivo multiunit (MUA) recordings from the SCN of freely-moving young and middle aged mice. Importantly, the amplitude of day-night difference in MUA was significantly reduced in the older mice. While we do not yet have the same in vivo data from VIP-deficient mice, the neural activity rhythms are clearly disrupted in the VIP-deficient mice measured in vitro. Finally, we have immunohistochemistry data indicating that VIP expression is reduced in aged mice and that this loss of expression is greatest in the ventral SCN region. This compliments previous studies that have found evidence for an age-related decline in VIP expression in hamsters and rats. Therefore, while not yet compelling, the behavioral, physiological, and anatomical evidence is consistent with the hypothesis that an age-related decline in VIP in the SCN could underlie the impact of aging on the sleep/wake cycle. Besides testing this hypothesis, future experiments will focus on identification of possible behavioral interventions that may delay these age-related changes. It is worth noting the sleep problems are rampant in the aging US population and the use of hypnotics has become the norm for those over 65 year old.

P263

Vasoactive intestinal peptide mediates photic regulation of Per1 expression in the suprachiasmatic nucleus

ANDREW VOSKO¹, JOANNA DRAGICH², TAKASHI KUDO¹, ELLEN CARPENTER¹, CHRISTOPHER COLWELL¹

¹*Psychiatry and Biobehavioral Sciences, University of California–Los Angeles, Los Angeles, CA, USA*

²*Neurology, Columbia University, New York, NY, USA*

Previously, we have shown that mice deficient in vasoactive intestinal peptide (VIP) exhibit specific deficits in the behavioral response of their circadian system to light. In this study, we investigated how the photic regulation of the molecular clock within the SCN is altered by the loss of this peptide. During the subjective day, light exposure increased the levels of the “clock gene” Period1 (Per1) in the SCN of VIP-deficient mice, but not in wild type (WT) mice. During this phase, baseline levels of Per1 were reduced in the VIP-deficient mice compared to untreated controls. In contrast, during the subjective night, the magnitude of the light-induction of Per1 was significantly reduced in VIP-deficient mice when compared to WT mice. Baseline levels of Per1 in the night were not altered by the loss of this peptide. The differences in Per1 induction appear to be due, in part, by different spatiotemporal induction patterns between WT and VIP-deficient mice. In WT mice, Per1 message levels are robustly

increased by 30 minutes following a light pulse, and after 120 minutes levels fall back to baseline. In the absence of VIP, the initial induction of Per1 is intact, but the signal has a shorter lived time course of expression compared to WT mice, specifically in the ventral aspect of the SCN. These data may help explain the pathway by which VIP regulates photic resetting of the circadian system.

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Cholecystokinin (CCK) expressing neurons in the suprachiasmatic nucleus: Innervation, light responsiveness, and entrainment in CCK-deficient mice

JENS HANNIBAL¹, CHRISTIAN HUNDAHL², JAN FAHRENKRUG¹, JENS FREDRIK REHFELD³,
LENNART FRIIS-HANSEN³

¹*Clinical Biochemistry, Bispebjerg Hospital, Copenhagen, DENMARK*

²*Department of Neuroscience and Pharmacology, The Panum Institute, Copenhagen, DENMARK*

³*Clinical Biochemistry, Rigshospitalet, Copenhagen, DENMARK*

The suprachiasmatic nucleus (SCN) is the principal pacemaker driving circadian rhythms of physiology and behaviour. Neurons within the SCN express both classical and neuropeptide transmitters which regulate clock functions. Cholecystokinin (CCK) is a potent neurotransmitter expressed in neurons of the mammalian SCN, but its role in circadian timing is not known. In the present study, CCK was demonstrated in a distinct population of neurons located in the shell region of the SCN and in few cells in the core region. The CCK neurons did not express vasopressin or vasoactive intestinal peptide, but CCK containing processes make synaptic contacts with both groups of neurons. The CCK neurons received no direct input from the three major pathways to the SCN, and the CCK neurons were not light responsive evaluated by induction of cFOS, and did not express the core clock protein, PER1. Accordingly, CCK deficient mice show normal entrainment, had similar tau, light induced phase shift and negative masking behaviour as wild type animals. In conclusion, CCK signalling in the SCN seems not to be involved directly in light induced resetting of the clock, neither does CCK regulate the core clock function. The expression of CCK in a subpopulation of neurons neither belonging to the VIP nor AVP cells but with synaptic contact to both cell types suggest that the CCK neurons are interneurons involved in non-photoc entrainment of the clock and/or that CCK via projections to the hypothalamus participates in circadian regulation of feeding.

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Hypothalamic neuropeptides involved in the SCN control of hepatic glucose production

ANDRIES KALSBEK, CHUN-XIA YI, ERIC FLIERS

Endocrinology and Metabolism, Academic Medical Center (AMC), University of Amsterdam (UvA), Amsterdam, THE NETHERLANDS

The hypothalamic paraventricular nucleus (PVN) is an important target area of biological clock output as it harbors the neuro-endocrine neurons that control peripheral hormones such as corticosterone and thyroid-stimulating hormone, as well as the pre-autonomic neurons that control the sympathetic and parasympathetic branches of the autonomic nervous system (ANS). The master biological clock, located in the hypothalamic suprachiasmatic nuclei (SCN), plays an essential role in maintaining daily blood glucose concentrations. Using local intra-hypothalamic administration of GABA and glutamate receptor (ant)agonists we previously demonstrated how changes in ANS activity contribute to the daily control of plasma glucose and plasma insulin concentrations. Selective hepatic denervations evidenced that the ANS is also an important gateway for the SCN to transmit the (phase-shifting) effects of light to the glucoregulatory and clock gene machinery of the liver. Finally, using ICV administration of neuropeptides and/or their (ant)agonists we were able to delineate how the SCN may also "use" the

hypothalamic neuropeptide systems to control the daily plasma glucose rhythms. For instance, VIP-containing SCN outputs and the orexin-containing neurons in the lateral hypothalamus are important molecular links to modulate hepatic glucose production. On the other hand, vasopressinergic SCN-projections and oxytocin-containing PVN neurons do not seem to be involved in the control of daily plasma glucose rhythms. At present we are investigating the possible role of the hypothalamic PACAP system and pineal melatonin release as a molecular link between the biological clock and hepatic rhythms in glucose metabolism.

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Anticipation of a daily palatable snack in mice varies with behavior and snack content

DANICA PATTON¹, CYNTHIA HSU², RALPH MISTLBERGER¹, ANDREW STEELE²

¹*Psychology, Simon Fraser University, Burnaby, British Columbia, CANADA*

²*Biology, California Institute of Technology, Pasadena, CA, USA*

Food anticipatory activity (FAA) in rodents exhibits formal properties consistent with control by a food-entrainable circadian oscillator (FEO). Distinguishing between FEO-based and alternative mechanisms for FAA requires evidence for persistence of FAA rhythms during fasting. While adult rats readily tolerate 3-5 days without food, mice are more susceptible to hyperactivity and severe weight loss, potentially compromising the use of genetically modified mice to elucidate the molecular basis of FEOs. An alternative strategy is to provide a palatable snack on a background of ad-lib access to regular chow. Rats anticipate a daily snack without caloric restriction, but data from mouse are lacking. We report here results on adult male C57BL/6J mice. In one laboratory (DFP & REM), 24 mice housed in running wheel cages in LD 14:10 received a daily snack (1.5g fruit crunchies or chocolate) at ZT4 (8-h before lights-off) for up to 79 days. Remarkably, mice ate the snacks, but did not anticipate snacktime when chow was available ad-libitum, even in cases (n=8) where food anticipation was first established by conventional restricted feeding. One of 8 mice showed anticipatory running to snacktime when chow was available at night in a reduced amount. Motion sensors revealed in a subset of mice weak pre-snack locomotor activity in the absence of wheel running, suggesting behavior-specific anticipation. In another laboratory (CH & ADS), 24 mice entrained to LD 13:11 were housed in cages without wheels, and recorded using an automated behavior recognition system (HomeCageScan 3.0). Mice received chow ad-lib, and palatable snacks (0.9 g of chocolate or high fat pellets) at ZT10 (2h before lights-off). Mice did not anticipate chocolate in either total activity or food-bin activity, but did anticipate high-fat pellets in food-bin activity. Snack anticipation in mice is weak by contrast with rats, and varies with behavioral endpoint and food type or caloric content.

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Lack of effect of phenobarbital administration on number or activation of RFRP3 neurons in the DMH of proestrous golden hamsters

KATHLEEN FRANKLIN¹, CLINTON PILLOW¹, SANDRA LEGAN², LANCE KRIEGSFELD³, MARILYN DUNCAN¹

¹*Anatomy and Neurobiology, University of Kentucky, Lexington, KY, USA*

²*Physiology, University of Kentucky, Lexington, KY, USA*

³*Psychology and Helen Wills Neuroscience Institute, University of California–Berkeley, CA, USA*

Phenobarbital administered to female hamsters at the onset of the early afternoon critical period (ZT5) on proestrus delays the preovulatory luteinizing hormone (LH) surge, which is under circadian control, for 24 h. Although the LH surge is stimulated by GnRH neurons in the medial preoptic area (MPOA), LH release may also be controlled by neurons in the dorsomedial hypothalamus (DMH) that synthesize and release a gonadotropin inhibitory hormone, RFamide-related peptide 3 (RFRP3). In hamsters,

the number of RFRP3 expressing neurons, as well as the percentage of RFRP3 neurons expressing Fos, decreases significantly at the average time of the peak of the LH surge (~ZT9), suggesting that inhibition of RFRP3 secretion may be required for GnRH neuronal activation (Gibson, et al, 2008). We hypothesized that administration of phenobarbital at ZT5 on proestrus would prevent the decrease in the number and activation of RFRP3-expressing neurons, as well as block the LH surge. Hamsters were injected with vehicle or phenobarbital at ZT5 and anesthetized and perfused transcordially at times when mean plasma LH levels are basal (ZT6) or maximal (ZT9). Blood samples were collected just before perfusion and were used for determination of plasma LH levels by radioimmunoassay. As predicted, LH levels at ZT9 were significantly higher in vehicle-injected hamsters than in phenobarbital-injected hamsters. However, neither the number of RFRP3-immunoreactive neurons (mean±S.E.M. veh: 500±60, drug: 360±23) nor the percentage of RFRP3-immunoreactive neurons co-expressing Fos (mean±S.E.M. veh: 14±2.2, drug: 9.6±2.5) was significantly different between the ZT9 phenobarbital and vehicle groups. These data demonstrate that barbiturate blockade of the LH surge is not associated with activation of RFRP3 neurons or an increase in the number of immunopositive RFRP3 neurons, and suggest that the mechanism whereby barbiturates block the LH surge is not mediated by inhibitory output from RFRP3 neurons to GnRH neurons. Support: R01 NS05528.

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Genome-wide profiling of diel and circadian gene expression of the malaria vector *Anopheles gambiae*

SAMUEL RUND, TIM HOU, SARAH WARD, FRANK COLLINS, GILES DUFFIELD

Biological Sciences and Eck Institute for Global Health, University of Notre Dame, Notre Dame, IN, USA

Anopheles gambiae, the primary African malarial mosquito, exhibits numerous behaviors that are under diel and circadian control including locomotor activity, swarming, mating, host seeking, egg laying and sugar feeding. However, little work has been performed to elucidate the molecular basis for these daily rhythms. To study how gene expression is globally regulated by diel and circadian mechanisms we have undertaken a DNA microarray analysis of *A. gambiae* head and bodies under 12:12 light:dark cycle (LD) and constant dark (DD) conditions. Adult mated but non-blood fed female mosquito heads and bodies were collected every 4 hr to generate 48 hr RNA time courses, and samples were processed with Affymetrix full genome microarrays. Using the COSOPT cosine-wave fitting algorithm (MMC- $\beta < 0.1$; Panda et al Cell 2002 109:307-20) we have identified 1059 rhythmic transcripts with a 20-28 hr period length in the head and 464 in the body under LD conditions, which represents 8.5% and 3.7% of the entire *A. gambiae* genome, respectively. Under free-running conditions, we have identified 646 rhythmic transcripts in the head and 333 in the body with a 18.5-26.5 hr period length (median period length in DD was 21.8 hr). A sub-set of 392 head and 139 body genes were rhythmically expressed under both LD and DD conditions, including the canonical clock components period, timeless, cryptochrome 2 and cycle. Observed rhythmic genes cover such diverse biological processes as olfaction, neurotransmission, vision, signal transduction, metabolic detoxification and immunity. Significant homology exists between *Anopheles* and *Drosophila* genomes. We utilize bioinformatic tools designed for *Drosophila*, and compare homologous clock controlled genes identified in the two Dipteran species. We describe our efforts to match observed circadian rhythms in physiology and behavior to patterns of gene expression. We propose that this work will lead to insights into novel mosquito control strategies through the identification of circadian susceptibilities, as well targets for the disruption of critical behavioral rhythms.

General anesthesia phase shifts the honey bee circadian clock

EVA WINNEBECK¹, JAMES CHEESEMAN¹, CRAIG MILLAR², MARK GOODWIN³, RANDOLF MENZEL⁴,
JAMES SLEIGH¹, GUY WARMAN¹

¹*Anaesthesiology, University of Auckland, Auckland, NEW ZEALAND*

²*School of Biological Sciences, University of Auckland, Auckland, NEW ZEALAND*

³*Ruakura Apicultural Research Unit, New Zealand Institute for Plant and Food Research Ltd., Hamilton, NEW ZEALAND*

⁴*Institute of Biology and Neurobiology, Free University Berlin, Berlin, GERMANY*

Experiments our group are conducting to determine the effect of general anesthesia on time perception clearly indicate that extended anesthesia halts the perception of time in honey bees (*Apis mellifera*) (see also abstract of Cheeseman et al.). On waking from a six hour anesthetic, bees behave as though no time has passed since their anesthetic began. Here we seek to address the behavioral and molecular mechanisms underlying these findings by measuring the effect of an extended anesthetic on the circadian clock of honey bees. Locomotor activity of five bee hives (6000-10000 individuals each) maintained in constant dim light (<2 lux) was recorded before and after a six hour general anesthetic with the inhalational agent isoflurane (2%). Anesthesia timed to begin at circadian time (Ct) 1-2 results in a mean phase delay of 4.2 hours (range 2.5-6 hours). Interestingly, the same pulse administered during the subjective night (starting a Ct 14) (n=1) does not elicit a phase delay, and may even cause a modest phase advance (approx. 2 hours). Preliminary analysis of the influence of anesthesia on key clock genes (measured by real-time qPCR analysis of brain RNA) suggests that it alters the expression of period, cryptochrome and clock. The effect of anesthesia on the phase and amplitude of expression rhythms of these genes will be presented. These findings demonstrate that a commonly used inhalational anesthetic agent phase shifts the circadian clock of honey bees, and that the magnitude and direction of the phase shift may depend on the circadian time of administration. If these phase-shifting effects persist in the presence of other Zeitgeber, these findings may have important implications for post-operative sleep disruption in humans.

There is a worm in my clock: Circadian rhythms in C. elegans

MARÍA LAURA MIGLIORI¹, ANDRES ROMANOWSKI¹, MARÍA EUGENIA GOYA¹, SERGIO SIMONETTA²,
DIEGO GOLOMBEK¹

¹*Science and Technology, National University of Quilmes, Bernal, ARGENTINA*

²*Molecular and Cellular Biology, Leloir Institute, Buenos Aires, ARGENTINA*

C. elegans is a model organism widely used in various areas of research but not well characterized in chronobiological studies, including three fundamental questions: 1) Does *C. elegans* have rhythms? We designed an automated device to track individual nematodes and demonstrated the existence of circadian activity rhythms, periods were found to be of 24.2±0.4 h (LD) and 23.9±0.4 h (DD), and were affected by a mutation in *lin-42*, a period homolog. Food consumption rate was shown to be rhythmic, peaking in the evening (ANOVA, p<0.0001); moreover, there is a 24h periodicity governing the ultradian defecation rhythm (ANOVA, p<0.0001). O₂ consumption was found to peak at night (ANOVA, p<0.0001). 2) Why does it need rhythms? *C. elegans* is a soil dwelling nematode subjected to daily changes in environmental stressors. Being able to predict when it will need to defend itself against these agents confers an adaptative advantage. We studied stress tolerance to abiotic and biotic stressors. We found rhythmic stress tolerance patterns for oxidative and osmotic stress, peaking at daytime and nighttime, respectively. Expression of stress-related genes was determined by qRealTime-PCR: *gpdh-1* and *gpx* showed a significant diurnal variation. When exposed to *P. fluorescens* or *P. aeruginosa* (two soil occurring bacteria that kill *C. elegans*), we found lower tolerance during nighttime. 3) How are

circadian rhythms entrained? Light and temperature are the main zeitgebers for circadian clocks. *C. elegans* can be entrained to both LD cycles and Tt cycles. Since *C. elegans* lacks a specific light-sensing organ we are trying to determine if the nematodes sense light. Our data shows a phototaxis index of 0.698 ± 0.096 towards the green wavelength of light. In summary, our results show that several different circadian outputs can be recorded in *C. elegans*, and the circadian rhythm of locomotor activity can be entrained to environmental signals.

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A blue-light photoreceptor is involved in circadian clock resetting in the model organism *Chlamydomonas reinhardtii*

JENNIFER FORBES-STOVALL, JONATHAN HOWTON, SIGRID JACOBSHAGEN

Biology, Western Kentucky University, Bowling Green, KY, USA

The unicellular, eukaryotic green alga *Chlamydomonas reinhardtii* has long served as a model organism for circadian clock research. It shows several well-characterized circadian rhythms of behavior. The measurement of its circadian rhythm of phototaxis (swimming towards light) has been automated. Based on this rhythm, an action spectrum for the resetting of the circadian clock by light could be obtained in a former study. It surprisingly revealed that cultures kept in constant darkness did not respond to pulses of blue light. This might have been due to the particular strain of *C. reinhardtii* used in the study, which was the cell wall-deficient strain CW15. When using the same basic experimental set-up with a wild-type strain, we now found that blue light is effective. There are two photoreceptors reported for *C. reinhardtii* that are able to absorb in the blue region of the light spectrum: a cryptochrome and a phototropin. We are currently in the process of creating RNA interference strains with reduced amounts of the cryptochrome protein. It will allow us to test these strains for reduced abilities to shift their phase upon blue-light pulses and therefore to determine whether cryptochrome is involved in circadian clock resetting in this organism.

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A hypothesis to explain the ultradian clock that controls the frequency of the behavioral response in *Paramecium*

ROBERT HINRICHSSEN, JESSICA BYERLY, HEATHER RININGER

Biology, Indiana University of Pennsylvania, Indiana, PA, USA

Paramecium tetraurelia displays a behavioral response to environmental stimuli that involves changes in the direction and speed of swimming. The frequency of the backward swimming response displays an ultradian rhythm with a period of approximately 50 minutes. However, growing cells in the presence of LiCl perturbs the ultradian rhythm; the periodicity is reduced to approximately 11 minutes. 10 mM myo-inositol was found to inhibit the effect of LiCl, which indicates *Paramecium* possesses an endogenous ultradian clock that is influenced by the inositol signaling pathway. Since the production of IP₃ results in the release of calcium from intracellular stores, it was necessary to determine the effects of an increase in intracellular Ca²⁺. We applied A23187 to the cells, as well as inhibited the SERCA gene by RNAi; in both cases the ultradian rhythm was completely eliminated. Next, in order to determine if changes in cellular membrane potential were responsible for the change in the frequency of the behavioral response, cells were artificially clamped at various membrane potentials using extracellular KCl; as the membrane potential became more positive, the periodicity of the ultradian rhythm decreased. Finally, since the guanylyl cyclase protein in *Paramecium* is stimulated by depolarization, and cGMP is known to inhibit the activity of phospholipase C (PLC), we artificially increased the intracellular concentration of cGMP by adding 8-Br-cGMP or theophylline to the cells.

In both cases, the increase in intracellular cGMP resulted in the reduction of the periodicity of the ultradian rhythm, in a manner similar to that seen with LiCl. These data have been used to formulate a hypothesis which explains, at the molecular level, the approximate 50 minute ultradian rhythm that controls the frequency of the behavioral response in *Paramecium*.

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The circadian clock of the marine picoeukaryote *Ostreococcus tauri*: Insight into the transcriptional regulation of CCA1 through a G box hexamer

SOPHIE SANCHEZ-FERANDIN, FRÉDÉRIC SANCHEZ, FRANÇOIS-YVES BOUGET

Observatoire Océanologique de Banyuls sur mer, Université Paris 6, Banyuls sur mer, FRANCE

Described as the smallest free-living eukaryote, the unicellular alga *Ostreococcus tauri* belongs to the Prasinophyceae group which emerges at the basis of the green lineage. The understanding of circadian clock architecture in this picoeukaryote is of particular interest as it may help to improve our knowledge about circadian clocks in complex multicellular organisms. Functional genomics approaches including stable genetic transformation were developed to follow the expression of genes of interest in *O. tauri* living cells, using the luciferase gene reporter system, as well as gene function through overexpression/knockdown strategies. Our team recently showed that the *O. tauri* clock consists of positive and negative elements homologous to those known in *Arabidopsis thaliana* (TOC1 and CCA1) and that many of features (such as circadian regulation of TOC1 and CCA1) are comparable to those of higher plants (Corellou et al., 2009; Djouani-Tahri et al., 2010). However though simple, it is likely the clockwork relies on more than a simple CCA1/TOC1 loop. We identified a G box hexamer in the CCA1 promoter, which is the binding site for basic Helix-Loop-Helix (bHLH) transcription factors found in many circadian regulated genes from animals to plants and fungi. We will present our recent experimental results of the single copy bHLH protein of *O. tauri*, for which we performed both functional and evolutionary analyses. We will also illustrate the role of the G box in the circadian control of CCA1 expression. Corellou F, Schwartz C., Motta, J.-P., Djouani-Tahri E. B., Sanchez F, and Bouget F.-Y., 2009. Clocks in the Green Lineage: Comparative Functional Analysis of the Circadian Architecture of the Picoeukaryote *Ostreococcus*. *The Plant Cell*, 21:3436-3449. Djouani-Tahri E. B., Motta J. P., Bouget F.-Y. and Corellou F, 2010. Insights into the regulation of the core clock component TOC1 in the green picoeukaryote *Ostreococcus*. *Plant Signalling and behaviour* (in press).

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Suprachiasmatic nucleus organization

ROBERT Y. MOORE

Departments of Neurology and Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA

The suprachiasmatic nucleus (SCN), a component of the circadian timing system, is a distinctive feature of the chiasmatal hypothalamus in all mammals. The nucleus has two subdivisions, designated core and shell (Moore, 1996). The core is located in the ventral portion of the nucleus, above the optic chiasm and the shell surrounds it. All SCN neurons are GABA-producing but core and shell neurons contain differing co-localized peptides. Core neurons in all mammals studied contain either gastrin releasing peptide or vasoactive intestinal polypeptide whereas shell neurons contain arginine vasopressin. In the rat, these neuronal groups identified by immunohistochemistry, comprise at least 75% of the population identified by Nissl staining (Moore et al, 2002). However, in other mammals, other peptides have been identified as well as calcium binding and other proteins. Differences among mammals in peptide production in SCN neurons most likely represent specialized adaptations to the local environments. The distinction between core and shell is also maintained in patterns of afferent

and efferent connections of the SCN (Watts and Swanson, 1987; Moga and Moore, 1997). Within the circadian timing system, the retinohypothalamic tract projects predominantly to the core as does the secondary visual projection from the intergeniculate leaflet of the lateral geniculate nucleus. However, RHT projections also innervate many areas also receiving SCN projections. Afferents from other forebrain areas typically innervate the shell and a surrounding subdivision of the subparaventricular zone, the perisuprachiasmatic nucleus. These observations suggest that, like many other systems, the circadian timing system is characterized by feed-forward and feedback connections.

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Participants

(as of April 23, 2010)

Abraham, Ute
Charite–Universitaetsmedizin
ute.abraham@charite.de

Abruzzi, Katharine
Howard Hughes Medical Institute
Brandeis University
katea@brandeis.edu

Adams, Dan
Philips Lighting
dan.adams@philips.com

Adams, Kandis
Morehouse School of Medicine
kadams@msm.edu

Affinati, Alison
Northwestern University
affinati@northwestern.edu

Aguilar-Roblero, Raul
Universidad Nacional Autonoma de
Mexico
raguilar@ifc.unam.mx

Ahowesso, Constance
INSERM U776
constance.ahowesso@inserm.fr

Albrecht, Urs
University of Fribourg
urs.albrecht@unifr.ch

Allada, Ravi
Northwestern University
r-allada@northwestern.edu

Allen, Charles
Oregon Health & Science University
allenc@ohsu.edu

Altimus, Cara
Johns Hopkins University
caltimus@jhu.edu

An, Sungwon
Washington University in St Louis
san@artsci.wustl.edu

Andrade, Francisco
University of Kentucky
paco.andrade@uky.edu

Anea, Ciprian
Medical College of Georgia
canea@mcg.edu

Angeles-Castellanos, Manuel
Universidad Nacional Autónoma de
México
atatu3@hotmail.com

Antle, Michael
University of Calgary
antlem@ucalgary.ca

Antoch, Marina
Roswell Park Cancer Institute
marina.antoch@roswellpark.org

Appelbaum, Lior
Stanford University
liora@stanford.edu

Araujo, Leana
Universidade Federal de São Paulo
leanaaraujo@yahoo.com.br

Arble, Deanna
Northwestern University
d-arble@northwestern.edu

Archer, Simon
University of Surrey
simon.archer@surrey.ac.uk

Arnold, Jennifer
University of Illinois
jarnol4@illinois.edu

Atkins, Norman
Northwestern University
natkins@northwestern.edu

Axley, John
Vanderbilt University
john.c.axley@vanderbilt.edu

Azevedo, Renata
University of Leicester
ra119@le.ac.uk

Baggs, Julie
UPenn School of Medicine
jbaggs@mail.med.upenn.edu

Baltazar, Ricardo
University of Western Ontario
rbaltaza@uwo.ca

Barger, Laura
Brigham and Women's Hospital
Harvard Medical School
lkbarger@hms.harvard.edu

Barrett, Perry
University of Aberdeen
p.barrett@abdn.ac.uk

Bartell, Paul
Penn State University
pab43@psu.edu

Beaule, Christian
Washington University–Saint Louis
cbeaule@mac.com

Bechtold, David
University of Manchester
david.bechtold@manchester.ac.uk

Beckwith, Esteban
Fundación Instituto Leloir, IIBA
CONICET
ebeckwith@leloir.org.ar

Belanger-Nelson, Erika
McGill University
erika.belanger-nelson@mail.mcgill.ca

Belden, William
Rutgers University
belden@aesop.rutgers.edu

Bell-Pedersen, Deborah
Texas A&M University
dpedersen@mail.bio.tamu.edu

Bentivoglio, Marina
University of Verona
marina.bentivoglio@univr.it

Bersot, Ross
ReSet Therapeutics, Inc.
ross@resettherapeutics.com

Besharse, Joseph
Medical College of Wisconsin
jbeshars@mcw.edu

Besing, Rachel
University of Alabama–Birmingham
rcbesing@gmail.com

Biernat, Magdalena
Wageningen University
magdalena.biernat@wur.nl

Bittman, Eric
University of Massachusetts
elb@bio.umass.edu

Bjarnason, Georg
Sunnybrook Odette Cancer Centre
georg.bjarnason@sri.utoronto.ca

Blattner, Margaret
University of Illinois at Urbana-
Champaign
mblattn2@illinois.edu

Blau, Justin
New York University
justin.blau@nyu.edu

Bloch, Guy
Hebrew University of Jerusalem
bloch@vms.huji.ac.il

Bode, Brid
Max-Planck-Institute for Biophysical
Chemistry
brid.bode@mpibpc.mpg.de

Bollinger, Thomas
University of Lübeck
thomas.bollinger@uk-sh.de

Bradshaw, William
University of Oregon
mosquito@uoregon.edu

Brager, Allison
Kent State University
abrager@kent.edu

Brockman, Rebecca
University of Illinois at Urbana-
Champaign
brockma2@illinois.edu

Brown, Steven
University of Zurich
steven.brown@pharma.uzh.ch

Brunner, Michael
Heidelberg University
*michael.brunner@bzh.uni-
heidelberg.de*

Buchin, Margo
Stanford Photonics, Inc.
margo@stanfordphotonics.com

Buijs, Ruud
Instituto de Investigaciones
Biomedicas
ruudbuijs@gmail.com

Butler, Andrew
The Scripps Research Institute
abutler@scripps.edu

Butler, Matthew
Columbia University
mb3024@columbia.edu

Byrne, Mark
Spring Hill College
mbyrne@shc.edu

Cain, Sean
Harvard Medical School
swcain@rics.bwh.harvard.edu

Cajochen, Christian
Centre for Chronobiology
christian.cajochen@upkbs.ch

Canal, Maria
University of Manchester
maria.canal@manchester.ac.uk

Cao, Ruifeng
Ohio State University
cao.67@buckeyemail.osu.edu

Castañón-Cervantes, Oscar
Morehouse School of Medicine
ocastanon-cervantes@msm.edu

Castillo-Ruiz, Alexandra
Michigan State University
castil71@msu.edu

Ceriani, M Fernanda
Instituto Leloir
fceriani@leloir.org.ar

Cermakian, Nicolas
McGill University
nicolas.cermakian@mcgill.ca

Cha, Joonseok
University of Texas Southwestern
Medical Center
joonseok.cha@utsouthwestern.edu

Chapdelaine Trépanie, Simon
University of Montreal
simon_c_trepanier@hotmail.com

Chaves, Ines
Erasmus University Medical Center
i.chaves@erasmusmc.nl

Cheeseman, James
University of Auckland
j.cheeseman@auckland.ac.nz

Chen, Ko-fan
Queen Mary, University of London
kofan.chen@gmail.com

Chen, Wen-Feng
Howard Hughes Medical Institute
University of Pennsylvania
chenwe@mail.med.upenn.edu

Chen, Zheng (Jake)
University of Texas Health Science
Center at Houston
zheng.chen.1@uth.tmc.edu

Cheng, Bo
Medical College of Georgia
bcheng@mcg.edu

Chiu, Joanna
Rutgers University
chiu@cabm.rutgers.edu

Chong, Nelson
University of Leicester
nc69@le.ac.uk

Chu, Adrienne
McGill University
adrienne.chu@mail.mcgill.ca

Chua, Chern-Pin
Duke-NUS Graduate Medical School
chua@duke-nus.edu.sg

Chung, Brian
Northwestern University
bychung@u.northwestern.edu

Ciarleglio, Christopher
Vanderbilt University
c.ciarleglio@vanderbilt.edu

Collins, Ben
New York University
bc772@nyu.edu

Colwell, Christopher
University of California–Los Angeles
ccolwell@mednet.ucla.edu

Comas Soberats, Maria
Roswell Park Cancer Institute
maria.comas-soberats@roswellpark.org

Cooper, Howard
INSERM
howard.cooper@inserm.fr

Coppack, Timothy
Institute of Applied Ecology,
Germany
coppack@ifaoe.de

Cordero, Melissa
Oklahoma State University
melcordr@gmail.com

Corthell, John
Florida State University
corthell@neuro.fsu.edu

Costa, Rodolfo
University of Padova
costa@civ.bio.unipd.it

Cretenet, Gaspard
University of Lausanne
gaspard.cretenet@unil.ch

Cuninkova, Ludmila
University of Zurich
cuninkova@pharma.uzh.ch

Curran, Kristen
University of Wisconsin–Whitewater
currank@uww.edu

Czeisler, Charles
Brigham and Women's Hospital
Harvard Medical School
cacadmin@rics.bwh.harvard.edu

Dallmann, Robert
University of Zurich
robert@dallmanns.de

Dardente, Hugues
Aberdeen University
h.dardente@abdn.ac.uk

Davidson, Alec
Morehouse School of Medicine
adavidson@msm.edu

Davis, Fred
Northeastern University
f.davis@neu.edu

De Groot, Marleen
University of Texas Southwestern
pero588@gmail.com

De la Iglesia, Horacio
University of Washington
hioraci@d.u.washington.edu

DeBruyne, Jason
University of Pennsylvania School
of Medicine
debruyne.jason@gmail.com

Delaunay, Franck
Université de Nice
Franck.Delaunay@unice.fr

DeLemos, Andrew
Massachusetts General Hospital
adelemos@partners.org

Delezie, Julien
Institut des Neurosciences
Cellulaires et Intégratives
julien.delezie@inci-cnrs.unistra.fr

Destici, Eugin
Erasmus Medical Centre
e.destici@erasmusmc.nl

D'Eysmond, Thomas
Ecole Polytechnique Fédérale de
Lausanne (EPFL)
thomas.deysmond@epfl.ch

Diernfellner, Axel
Biochemistry Center Heidelberg
*axel.diernfellner@bzh.uni-
heidelberg.de*

Dijk, Derk-Jan
University of Surrey
d.j.dijk@surrey.ac.uk

Dissel, Stephane
University of Leicester
sd71@le.ac.uk

Dkhissi-Benyahya, Ouria
INSERM U846
ouria.benyahya@inserm.fr

Do, Michael Tri
Johns Hopkins University School of
Medicine
mdo@jhmi.edu

Duffield, Giles
University of Notre Dame
duffield.2@nd.edu

Duhart, Jose
Universidad Nacional de Quilmes
joseduhart@gmail.com

Dulong, Sandrine
INSERM
sandrine.dulong@inserm.fr

Dumont, Marie
Sacre-Coeur Hospital of Montreal
marie.dumont@umontreal.ca

Duncan, Marilyn
University of Kentucky Medical
School
marilyn.duncan@uky.edu

Dunlap, Jay
Dartmouth Medical School
jay.c.dunlap@dartmouth.edu

Duong, Hao
Harvard Medical School
hao_duong@hms.harvard.edu

Dupre, Sandrine
University of Manchester
sandrine.dupre@manchester.ac.uk

Duvall, Laura
Washington University
lbduvall@wustl.edu

Earnest, David
Texas A&M University
dearnest@tamu.edu

Edery, Isaac
Rutgers University
edery@cabm.rutgers.edu

Elliott, Jeff
University of California–San Diego
jelliott@ucsd.edu

Emery, Patrick
University of Massachusetts
Medical School
patrick.emery@umassmed.edu

Escobar, Carolina
Facultad de Medicina UNAM
escocarolina@gmail.com

Esser, Karyn
University of Kentucky
karyn.esser@uky.edu

Evans, Erin
Brigham and Women's Hospital
eevans@rics.bwh.harvard.edu

Evans, Jennifer
Morehouse School of Medicine
jevans@msm.edu

Fan, Junmei
University of Texas Southwestern
Medical Center
biofjm@gmail.com

Fang, Mingzhu
University of Medicine and
Dentistry of New Jersey
fang@eohsi.rutgers.edu

Ferguson, Sally
University of South Australia
sally.ferguson@unisa.edu.au

Filipski, Elisabeth
INSERM U776
elisabeth.filipski@inserm.fr

Finkelstein, Carla
Virginia Polytechnic Institute and
State University
finkielc@vt.edu

Forger, Daniel
University of Michigan
forger@umich.edu

Fortier, Erin
McGill University
erin.fortier@mail.mcgill.ca

Foster, Russell
University of Oxford
russell.foster@eye.ox.ac.uk

Francl, Jessica
Kent State University
jmfrancl@kent.edu

Franklin, Kathy
University of Kentucky
kathleen.franklin@uky.edu

Freeman, Mark
Washington University–St Louis
freemanma@msnotes.wustl.edu

Fu, Loning
Baylor College of Medicine
loningf@bcm.tmc.edu

Fu, Ying-Hui
University of California–San
Francisco
yinghfu@gmail.com

Fuse, Yuta
Waseda University
fuse.yuta-1115@ruri.waseda.jp

Gachon, Frédéric
University of Lausanne
frederic.gachon@unil.ch

Gamble, Karen
University of Alabama–Birmingham
klgamble@uab.edu

Gamsby, Joshua
Dartmouth Medical School
joshua.gamsby@dartmouth.edu

Gardner, Jacob
Florida State University
jsg06@fsu.edu

Gaten, Ted
University of Leicester
gat@le.ac.uk

Gesto, Joao
University of Leicester
jg151@le.ac.uk

Gibbs, Julie
University of Manchester
julie.gibbs@manchester.ac.uk

Gillette, Martha
University of Illinois at Urbana-
Champaign
mgillett@illinois.edu

Glass, J. David
Kent State University
jglass@kent.edu

Glickman, Gena
University of California–San Diego
glickman@ucsd.edu

Goda, Tadahiro
University of Virginia
tg8j@virginia.edu

Golombek, Diego
Universidad Nacional de Quilmes
dgolombek@unq.edu.ar

Gooley, Joshua
Duke-NUS Graduate Medical School
joshua.gooley@duke-nus.edu.sg

Gorman, Michael
University of California–San Diego
mgorman@ucsd.edu

Gotter, Anthony
Merck Research Labs
anthony_gotter@merck.com

Granada, Adrián
Institute for Theoretical Biology
a.granada@biologie.hu-berlin.de

Gray, Paul
Washington University School of
Medicine
pgray@pcg.wustl.edu

Green, Carla
University of Texas Southwestern
Medical Center
carla.green@utsouthwestern.edu

Green, Rachel
Hebrew University
rgreen@vms.huji.ac.il

Gronfier, Claude
INSERM
claud.gronfier@inserm.fr

Guilding, Clare
University of Manchester
clare.guilding@manchester.ac.uk

Guinn, Jessie
Kent State University
jguinn1@kent.edu

Gumz, Michelle
University of Florida
michelle.gumz@medicine.ufl.edu

Guo, Fang
Howard Hughes Medical Institute
Brandeis University
guofang@brandeis.edu

Hagenauer, Megan
University of Michigan
hagenaue@umich.edu

Hahn, Constanze
Institute for Cognitive Neuroscience
constanze.s.hahn@rub.de

Han, Sung
University of Washington
sunghan@uw.edu

Hanifin, John
Thomas Jefferson University
john.hanifin@jefferson.edu

Hannibal, Jens
Bispebjerg Hospital
j.hannibal@dadlnet.dk

Hansen, Celia
University of Leicester
cnh2@le.ac.uk

Hara, Taichi
University of Pennsylvania
tahtah000@gmail.com

Hardin, Paul
Texas A&M University
phardin@mail.bio.tamu.edu

Hares, John
University of Leicester
jmh56@le.ac.uk

Harmer, Stacey
University of California–Davis
slharmer@ucdavis.edu

Harrington, Mary
Smith College
mharring@smith.edu

Harrison, Elizabeth
University of California–San Diego
emharrison@ucsd.edu

Hasler, Brant
University of Pittsburgh School of
Medicine
haslerbp@upmc.edu

Hastings, Michael
MRC Laboratory of Molecular
Biology
mha@mrc-lmb.cam.ac.uk

Hatakeyama, Tetsuhiro
Waseda University
tetsuhiro.hatakeyama@gmail.com

Hattar, Samer
Johns Hopkins University
shattar@jhu.edu

Hayasaka, Naoto
Kindai University School of
Medicine
hayasaka@med.kindai.ac.jp

Hazlerigg, David
University of Aberdeen
d.hazlerigg@abdn.ac.uk

Helm, Barbara
University of Konstanz and
Max Planck Institute for Ornithology
helm@orn.mpg.de

Hertel, Stefanie
Humboldt University, Berlin
stefanie.hertel@biologie.hu-berlin.de

Herzel, Hans-Peter
Humboldt University
h.herzel@biologie.hu-berlin.de

Herzel, Lydia
Harvard Medical School
l.herzel@student.uni-tuebingen.de

Herzog, Erik
Washington University
herzog@wustl.edu

Hinrichsen, Robert
Indiana University of Pennsylvania
bhinrich@iup.edu

Hirano, Arisa
University of Tokyo
ss086315@mail.ecc.u-tokyo.ac.jp

Ho Mien, Ivan
Duke-NUS Graduate Medical School
g0801849@nus.edu.sg

Hogenesch, John
University of Pennsylvania School
of Medicine
hogenesch@mail.med.upenn.edu

Holmes, Todd
University of California–Irvine
tholmes@uci.edu

Hong, Chris
Dartmouth Medical School
ickhongvt@gmail.com

Hu, Kun
Brigham and Women's Hospital
Harvard Medical School
khu@bics.bwh.harvard.edu

Huang, Guocun
University of Texas Southwestern
Medical Center
guocun.huang@utsouthwestern.edu

Hughes, Michael
Yale School of Medicine
michael.hughes@yale.edu

Hull, Joseph
Harvard Medical School
jhull@rics.bwh.harvard.edu

Hundahl, Christian
Bispebjerg Hospital
c.hundahl@gmail.com

Hung, Hsiu-Cheng
University of Heidelberg
*hsiu-cheng.hung@bzh.uni-
heidelberg.de*

Hur, Seongkwon
University of Texas Southwestern
Medical Center
skhuh78@gmail.com

Hurley, Jennifer
Dartmouth Medical School
jennifer.hurley@dartmouth.edu

Husse, Jana
Max Planck Institute for Biophysical
Chemistry
jhusse@gwdg.de

Hut, Roelof A.
University of Groningen
r.a.hut@rug.nl

Imaizumi, Takato
University of Washington
takato@uw.edu

Izumo, Mariko
University of Texas Southwestern
Medical Center
mariko.izumo@utsouthwestern.edu

Jacobshagen, Sigrid
Western Kentucky University
sigrid.jacobshagen@wku.edu

Jardim, Anisoara
University of Auckland
a.jardim@auckland.ac.nz

Jiang, Peng
University of Kentucky
pengjiang@uky.edu

Johnson, Carl
Vanderbilt University
carl.h.johnson@vanderbilt.edu

Johnston, Jonathan
University of Surrey
j.johnston@surrey.ac.uk

Jouffe, Céline
University of Lausanne
celine.jouffe@unil.ch

Kadener, Sebastian
The Hebrew University of Jerusalem
skadener@cc.huji.ac.il

Kallur, Sailaja
Texas A& M University
sailaja1978@tamu.edu

Kalsbeek, Andries
University of Amsterdam
a.kalsbeek@amc.uva.nl

Kampf-Lassin, August
The University of Chicago
august@uchicago.edu

Kandalepas, Patty
University of Illinois at Urbana-
Champaign
kandalps@illinois.edu

Kang, Jae-Eun
Washington University
jaeeun.kang08@gmail.com

Kang, Tae-Hong
University of North Carolina–Chapel
Hill
taehong_kang@med.unc.edu

Khericha, Mobina
University of Leicester
mk159@le.ac.uk

Kießling, Silke
Max Planck Institute for Biophysical
Chemistry
skiessl@gwdg.de

Kim, Eun Young
Ajou University School of Medicine
ekim@ajou.ac.kr

Kim, Kyungin
University of Texas Southwestern
kiki7454@gmail.com

Kim, Yumi
Pohang Science and Technology
yumi@postech.ac.kr

Klerman, Elizabeth
Brigham and Women's Hospital
Harvard Medical School
ebklerman@hms.harvard.edu

Koh, Kyunghee
University of Pennsylvania
kkoh@mail.med.upenn.edu

Koike, Nobuya
University of Texas Southwestern
Medical Center
nobuya.koike@utsouthwestern.edu

Kojima, Shihoko
University of Texas Southwestern
shihoko.kojima@utsouthwestern.edu

Koletar, Margaret
University of Toronto
koletar@psych.utoronto.ca

Korenčič, Anja
University of Ljubljana
anja.korencic@mf.uni-lj.si

Kosir, Rok
University of Ljubljana
rok.kosir@mf.uni-lj.si

Kostansek, Joseph
Florida State University
jak06d@fsu.edu

Kramer, Achim
Charite Universitätsmedizin Berlin
achim.kramer@charite.de

Kronfeld-Schor, Noga
Tel Aviv University
nogaks@tauex.tau.ac.il

Kudo, Takashi
University of California–Los Angeles
tkudo@mednet.ucla.edu

Kumar, Jaswinder
University of Texas Southwestern
Medical Center
jaswinder.kumar@utsouthwestern.edu

Kumar, Vinod
Delhi University
drvkumar11@yahoo.com

Kuntzweiler, Theresa
Sanofi-Aventis Pharmaceuticas
theresa.kuntzweiler@sanofi-aventis.com

Kurosawa, Gen
RIKEN Advanced Science Institute
g.kurosawa@riken.jp

Kushige, Hiroko
Waseda University
hiroko.kushige@gmail.com

Kutsuna, Shinsuke
Yokohama City University
kutsuna@yokohama-cu.ac.jp

Kyriacou, Charalambos
University of Leicester
cpk@leicester.ac.uk

Lahens, Nicholas
University of Pennsylvania School
of Medicine
nif@mail.med.upenn.edu

Lambreghts, Randy
Dartmouth Medical School
randy.lambreghts@dartmouth.edu

Lamia, Katja
The Salk Institute
klamia@salk.edu

Lande-Diner, Laura
Harvard Medical School
laura_lande-diner@hms.harvard.edu

Laposky, Aaron
NIH
laposkya@mail.nih.gov

Larrondo, Luis
Pontificia Universidad Catolica de
Chile
llarrond@bio.puc.cl

Lavedan, Christian
Vanda Pharmaceuticals
christian.lavedan@vandapharma.com

Lee, Ivan
University of Texas Southwestern
Medical Center
ivan.lee@utsouthwestern.edu

Lee, Sang kil
Morehouse School of Medicine
slee@msm.edu

Lee, Theresa
University of Michigan
terrilee@umich.edu

Lefta, Mellani
University of Kentucky
mleft2@uky.edu

Legan, Sandra
University of Kentucky
sjlegan@uky.edu

LeGates, Tara
Johns Hopkins University
tlegates@gmail.com

Lehman, Michael
The University of Western Ontario
michael.lehman@schulich.uwo.ca

Leise, Tanya
Amherst College
tleise@amherst.edu

LeSauter, Joseph
Barnard College / Columbia
University
jlesauter@barnard.edu

Levi, Francis
INSERM U776
francis.levi@inserm.fr

Levy, Oren
Bar Ilan University
levyor@mail.biu.ac.il

Li, Siming
University of Michigan
simingli@umich.edu

LI, Xiao-Mei
INSERM U776
xiao-mei.li@inserm.fr

Li, Yan
University of Texas Southwestern
Medical Center
bioyanli@gmail.com

Li, Yue
Brandeis University
liyue@brandeis.edu

Lilley, Travis
University of Washington
tlilley@u.washington.edu

Lin, Jiandie
University of Michigan
jdlin@umich.edu

Liu, Yi
University of Texas Southwestern
Medical Center
yi.liu@utsouthwestern.edu

Lockley, Steven
Brigham and Women's Hospital
steven_lockley@hms.harvard.edu

Loh, Dawn
University of California–Los Angeles
hloh@mednet.ucla.edu

Loros, Jennifer
Dartmouth Medical School
jennifer.loros@dartmouth.edu

Loudon, Andrew
University of Manchester
andrew.loudon@manchester.ac.uk

Ludin, Nicola
University of Auckland
nfra030@aucklanduni.ac.nz

Lyons, Lisa
Florida State University
lyons@bio.fsu.edu

Ma, Di
University of Michigan
dimapku@umich.edu

Ma, Peijun
Vanderbilt University
peijun.ma@vanderbilt.edu

Mahoney, Megan
University of Illinois at Urbana-
Champaign
mmm1@illinois.edu

Marcheva, Biliana
Northwestern University
b-marcheva@northwestern.edu

Marpegan, Luciano
Washington University
marpegan@biology2.wustl.edu

Martin-Fairey, Carmel
Michigan State University
martinf3@msu.edu

Maury, Eleonore
Northwestern University
e-maury@northwestern.edu

Maywood, Elizabeth
MRC Laboratory of Molecular
Biology
emaywood@mrc-lmb.cam.ac.uk

Mazzotta, Gabriella
University of Padova
gabriella.mazzotta@unipd.it

McCarthy, Ellena
Yale School of Medicine
ellena.mccarthy@yale.edu

McClung, C. Robertson
Dartmouth College
mcllung@dartmouth.edu

McClung, Colleen
University of Texas Southwestern
Medical Center
colleen.mcclung@utsouthwestern.edu

McFarlane, Dianne
Oklahoma State University
diannem@okstate.edu

McGlynn, Olga
University College Dublin
olga.mcglynn@ucdconnect.ie

McMahon, Douglas
Vanderbilt University
douglas.g.mcmahon@vanderbilt.edu

McMinn, Julie
Orphagen Pharmaceuticals
julie.mcmmin@orphagen.com

McWatters, Harriet
University of Oxford
harriet.mcwatters@plants.ox.ac.uk

Meeker, Kirsten
University of California–Santa
Barbara
kmeeker@cs.ucsb.edu

Menaker, Michael
University of Virginia
mm7e@virginia.edu

Menet, Jerome
Brandeis University
menet@brandeis.edu

Meng, Qing-Jun
University of Manchester
qing-jun.meng@manchester.ac.uk

Meredith, Andrea
University of Maryland School of
Medicine
ameredith@som.umaryland.edu

Merlin, Christine
University of Massachusetts
Medical School
christine.merlin@umassmed.edu

Merloiu, Ana
Medical College of Georgia
amerloiu@mcg.edu

Merrow, Martha
University of Groningen
m.merrow@rug.nl

Meyer-Bernstein, Beth
College of Charleston
meyerbernsteine@cofc.edu

Mialki, Rachel
Indiana University of Pennsylvania
gvfp@iup.edu

Michel, Max
Florida State University
maxmichel@neuro.fsu.edu

Michel, Stephan
Leiden University Medical Center
s.michel@lumc.nl

Millar, Andrew
University of Edinburgh
andrew.millar@ed.ac.uk

Millsaps, Brianna
Florida State University
bcm04c@fsu.edu

Mintz, Eric
Kent State University
emintz@kent.edu

Mistlberger, Ralph
Simon Fraser University
mistlber@sfu.ca

Mitchell, Jennifer
University of Illinois at Urbana-
Champaign
mitchll3@illinois.edu

Mohawk, Jennifer
University of Virginia
jmohawk@virginia.edu

Moldavan, Mykhaylo
Oregon Health Sciences University
moldavan@ohsu.edu

Mongrain, Valérie
Hôpital du Sacré-Coeur de Montréal
valerie.mongrain@umontreal.ca

Moore, David
Rider University
mooreda@rider.edu

Moore, Robert
University of Pittsburgh
rym@pitt.edu

Mori, Tetsuya
Vanderbilt University
tetsuya.mori@vanderbilt.edu

Morin, Larry
Stony Brook Medical Center
lawrence.morin@stonybrook.edu

Moriya, Takahiro
Tohoku University
moriya@mail.pharm.tohoku.ac.jp

Morris, Christopher
Brigham and Women's Hospital
cjmorris@partners.org

Mrazek, Karrie
Northwestern University
k-mrazek@northwestern.edu

Mueller, Anka
Simon Fraser University
amueller@sfu.ca

Murphy, Barbara
University College Dublin
barbara.murphy@ucd.ie

Murphy, Patricia
Weill Cornell Medical College
pjmurphy@med.cornell.edu

Nakamichi, Norihito
RIKEN Plant Science Center
nnakamichi@psc.riken.jp

Nakamura, Wataru
Osaka University Graduate School
of Dentistry
wataru@dent.osaka-u.ac.jp

Neumann, Adam
University of Warsaw
aneumann@biol.uw.edu.pl

Nitabach, Michael
Yale School of Medicine
michael.nitabach@yale.edu

Noguchi, Takako University of California–San Diego <i>tnoguchi@ucsd.edu</i>	Padmanabhan, Kiran Harvard Medical School <i>kiran_padmanabhan@hms.harvard.edu</i>	Perreau-Lenz, Stephanie California Institute for Mental Health <i>stephanie.perreau-lenz@zi-mannheim.de</i>
Norimune, Hosokawa Waseda University <i>norimune.hosokawa@gmail.com</i>	Pagani, Lucia UPK Basel <i>lucia.pagani@upkbs.ch</i>	Perryman, Jamie University of Michigan <i>pjamie@umich.edu</i>
Nunez, Antonio Michigan State University <i>nunez@msu.edu</i>	Pantazopoulos, Harry Northeastern University <i>hpantazo@mclean.harvard.edu</i>	Pezuk, Pinar University of Virginia <i>pp2v@virginia.edu</i>
Oda, Gisele Insitudo de Biociências <i>gaoda@ib.usp.br</i>	Partch, Carrie University of Texas Southwestern Medical Center <i>carrie.partch@utsouthwestern.edu</i>	Phillips, Andrew Brigham and Women's Hospital Harvard Medical School <i>ajphillips@partners.org</i>
Offord, James Pfizer Global Research and Development <i>james.offord@pfizer.com</i>	Paschos, Georgios University of Pennsylvania <i>gpaschos@mail.med.upenn.edu</i>	Pickard, Gary University of Nebraska <i>gpickard2@unl.edu</i>
Ohta, Hidenobu Tohoku University Hospital <i>hideohta@mail.tains.tohoku.ac.jp</i>	Pati, Paramita Medical College of Georgia <i>ppati@students.mcg.edu</i>	Piggins, Hugh University of Manchester <i>hugh.piggins@manchester.ac.uk</i>
Okamura, Hitoshi Kyoto University <i>okamurah@pharm.kyoto-u.ac.jp</i>	Patton, Danica Simon Fraser University <i>dfp@sfu.ca</i>	Prasai, MJ University of Leeds <i>h.prasai@leeds.ac.uk</i>
Olarerin-George, Anthony University of Pennsylvania <i>olarerin@gmail.com</i>	Paul, Matthew University of Massachusetts Medical School <i>matthew.paul@umassmed.edu</i>	Prendergast, Brian University of Chicago <i>prendergast@uchicago.edu</i>
O'Neill, John University of Cambridge <i>jso22@medschl.cam.ac.uk</i>	Paulose, Jiffin University of Kentucky <i>j.paulose@uky.edu</i>	Prewit, Kelly University of Texas Southwestern <i>kellyprewit@gmail.com</i>
Ono, Daisuke Hokkaido University Graduate School of Medicine <i>dai-ono@med.hokudai.ac.jp</i>	Pawley, Matthew University of Auckland <i>m.pawley@auckland.ac.nz</i>	Price, Jeffrey University of Missouri–Kansas City <i>pricejl@umkc.edu</i>
Owens, Joseph Northwestern University <i>jro@northwestern.edu</i>	Pegoraro, Mirko University of Leicester <i>mp248@le.ac.uk</i>	Prisk, G. Kim University of California–San Diego <i>kprisk@ucsd.edu</i>
Ozburn, Angela University of Texas Southwestern Medical Center <i>ozburna@gmail.com</i>	Peirson, Stuart University of Oxford <i>stuart.peirson@eye.ox.ac.uk</i>	Prosser, Rebecca University of Tennessee–Knoxville <i>rprosser@utk.edu</i>
Ozkaya, Ozge University of Leicester <i>o.ozkaya@talk21.com</i>	Pendergast, Julie Vanderbilt University <i>julie.pendergast@vanderbilt.edu</i>	Provencio, Ignacio University of Virginia <i>ip7m@virginia.edu</i>
		Provost, Nicolas GENFIT SA <i>nicolas.provost@genfit.com</i>

Ptacek, Louis
Howard Hughes Medical Institute
University of California–San
Francisco
ljp@ucsf.edu

Qin, Ximing
Vanderbilt University
ximing.qin@vanderbilt.edu

Rachalski, Adeline
Douglas Mental Health Institute
rachalskiadeline@orange.fr

Raiewski, Evan
University of California–San Diego
eraiewski@ucsd.edu

Rakai, Brooke
University of Calgary
bdrakai@ucalgary.ca

Ralph, Martin
University of Toronto
ralph@psych.utoronto.ca

Reddy, Akhilesh
University of Cambridge
abr20@cam.ac.uk

Refinetti, Roberto
University of South Carolina
refinetti@circadian.org

Resuehr, Holly
Vanderbilt University
holly.e.resuehr@vanderbilt.edu

Revell, Victoria
University of Surrey
v.revell@surrey.ac.uk

Rey, Guillaume
Ecole Polytechnique Federale de
Lausanne
guillaume.rey@epfl.ch

Robertson, Brian
Vanderbilt University
brian.robertson@vanderbilt.edu

Robles, Maria
Max Planck Institute for
Biochemistry
robles@biochem.mpg.de

Roden, Laura
University of Cape Town
Laura.rodén@uct.ac.za

Rodriguez, Joseph
Brandeis University
jrodriguez@brandeis.edu

Roenneberg, Till
University of Munch
roenneberg@lmu.de

Rogulja, Dragana
Rockefeller University
drogulja@rockefeller.edu

Rosato, Ezio
University of Leicester
er6@le.ac.uk

Rosbash, Michael
Howard Hughes Medical Institute
Brandeis University
rosbash@brandeis.edu

Rouyer, Francois
Centre National de la Recherche
Scientifique
rouyer@inaf.cnrs-gif.fr

Rozman, Damjana
University of Ljubljana
damjana.rozman@mf.uni-lj.si

Rueger, Melanie
Brigham and Women's Hospital
mrueger@rics.bwh.harvard.edu

Ruiz, Fernanda
Baylor College of Medicine
frruiz@bcm.tmc.edu

Rund, Samuel
University of Notre Dame
srund@nd.edu

Sadacca, Amanda
Harvard Medical School
sadacca@fas.harvard.edu

Saleh, Manjana
Institute of Medical Immunology,
Charite
manjana.saleh@charite.de

Sanchez-Ferandin, Sophie
Université Paris 6
sophie.sanchez1@obs-banyuls.fr

Sassone-Corsi, Paolo
University of California–Irvine
psc@uci.edu

Scheer, Frank AJL
Brigham and Women's Hospital
Harvard Medical School
fscheer@rics.bwh.harvard.edu

Schellenberg, Katja
Charité–Universitätsmedizin, Berlin
katja.schellenberg@charite.de

Scheuermaier, Karine
Brigham and Women's Hospital
Harvard Medical School
kscheuermaier@rics.bwh.harvard.edu

Schibler, Ueli
University of Geneva
ueli.schibler@unige.ch

Schiesari, Luca
University of Padova
luca.schiesari@studenti.unipd.it

Schlangen, Luc
Philips Lighting
luc.schlangen@philips.com

Schmidt, Paul
University of Pennsylvania
schmidtp@sas.upenn.edu

Schmitt, Karen
Psychiatric University Clinic Basel
karen.schmitt@upkbs.ch

Schrader, Annika
Beiersdorf AG
annika.schrader@beiersdorf.com

Schroeder, Analyne
University of California–Los Angeles
aschroeder@ucla.edu

Schwartz, Michael
University of Maryland School of
Medicine
mschw009@umaryland.edu

Schwartz, William
University of Massachusetts
Medical School
william.schwartz@umassmed.edu

Scoma, Heather
Medical College of Wisconsin
hscoma@mcw.edu

Scott, Eleanor
University of Leeds
e.m.scott@leeds.ac.uk

Scott, Fiona
University of Manchester
fiona.scott@manchester.ac.uk

Sehgal, Amita
Howard Hughes Medical Institute
University of Pennsylvania School
of Medicine
amita@mail.med.upenn.edu

Sellix, Michael
University of Virginia
ms4va@virginia.edu

Seluzicki, Adam
Northwestern University
a-seluzicki@northwestern.edu

Shafer, Ori
University of Michigan
oshafer@umich.edu

Shan, Yongli
University of Texas Southwestern
Medical Center
shan.yongli@gmail.com

Shelton, Jonathan
Johnson & Johnson
jshelto5@its.jnj.com

Shi, Shu-qun
Vanderbilt University
shuqun.shi@vanderbilt.edu

Shimomura, Kazuhiro
Northwestern University
shimomurakazuhiro@gmail.com

Shingle, Danielle
University of Texas Southwestern
danielle.shingle@utsouthwestern.edu

Shuboni, Dorela
Michigan State University
shubonid@msu.edu

Sidor, Michelle
University of Texas Southwestern
Medical Center
michelle.sidor@utsouthwestern.edu

Silver, Rae
Columbia University
qr@columbia.edu

Simon, Tatiana
Washington University–St. Louis
simont@biology2.wustl.edu

Sinning, Mandy
University of California–San Diego
mandysinning@gmail.com

Skene, Debra
University of Surrey
d.skene@surrey.ac.uk

Smale, Laura
Michigan State University
smale@msu.edu

Smarr, Benjamin
University of Washington
smarrb@u.washington.edu

Smith, Victoria
University of Calgary
smithvm@ucalgary.ca

Sollars, Patricia
University of Nebraska–Lincoln
psollars2@unl.edu

Somers, David
Ohio State University and
Pohang University of Science and
Technology
somers.24@osu.edu

Sprouse, Jeffrey
Lundbeck Research USA
jspr@lundbeck.com

Steele, Andrew
California Institute of Technology
steelea@caltech.edu

Sterniczuk, Roxanne
University of Calgary
rsterniczuk@shaw.ca

Storch, Florian
McGill University
florian.storch@mcgill.ca

Stovall, Jennifer
Western Kentucky University
jennifer.forbes@wku.edu

Stubblefield, Jeremy
University of Texas Southwestern
Medical Center
jeremy.stubblefield@utsouthwestern.edu

Summa, Keith
Northwestern University
ksumma@northwestern.edu

Sumova, Alena
Academy of Science of the Czech
Republic
sumova@biomed.cas.cz

Sun, Ziyi
Vanderbilt University
ziyi.sun@vanderbilt.edu

Symul, Laura
Ecole Polytechnique Federale de
Lausanne
laura.symul@epfl.ch

Taghert, Paul
Washington University Medical
School
taghertp@pcg.wustl.edu

Tahara, Yu
Waseda University
yu_tahara@ruri.waseda.jp

Takahashi, Joseph
University of Texas Southwestern
Medical Center
joseph.takahashi@utsouthwestern.edu

Takasu, Nana
Osaka University Graduate School
of Dentistry
takasu@hs.med.kyoto-u.ac.jp

Tamanini, Filippo
Erasmus Medical Center
f.tamanini@erasmusmc.nl

Tamayo, Alfred
Harvard Medical School
alfred_tamayo@hms.harvard.edu

Tang, Chih-Hang
Brandeis University
tangc@brandeis.edu

Tauber, Eran
University of Leicester
etauber@eudoramail.com

Trivedi, Amit
University of Kentucky
amit_9trivedi@yahoo.com

Turek, Fred
Northwestern University
fturek@northwestern.edu

Ueda, Hiroki
RIKEN CDB
uedah-ky@umin.ac.jp

Vajtay, Thomas
Rider University
vajtayt@rider.edu

Van der Spek, Rianne
Academic Medical Center
r.d.vanderspek@amc.nl

Van Ooijen, Gerben
Centre for Systems Biology at
Edinburgh
gerben.vanooijen@ed.ac.uk

VanDunk, Cassandra
Washington University–St Louis
cmvandun@artsci.wustl.edu

Vanin, Stefano
University of Padova
stefano.vanin@unipd.it

Venkataraman, Anand
University of Pennsylvania
anandven@mail.med.upenn.edu

Venkatesan, Anandkrishnan
University of Missouri–Kansas City
avhd3@umkc.edu

Viola, Antoine
Universitäre Psychiatrische Kliniken
antoine.viola@upkbs.ch

Vodala, Sadanand
Howard Hughes Medical Institute
Brandeis University
saddu@brandeis.edu

Vollmers, Christopher
Salk Institute for Biological Studies
vollmers@salk.edu

Vosko, Andrew
University of California–Los Angeles
avosko@ucla.edu

Wang, Bin
Dartmouth Medical School
bin.wang@dartmouth.edu

Wang, Gang
University of Kentucky
gwa225@uky.edu

Wang, Laura
University of Virginia
law5r@virginia.edu

Wang, Tongfei
University of Illinois at Urbana-
Champaign
twang20@uiuc.edu

Wang, Wei
Brigham and Women's Hospital
Harvard Medical School
wwang4@partners.org

Ward, Sarah
University of Notre Dame
sward2@nd.edu

Warman, Guy
University of Auckland
g.warman@auckland.ac.nz

Wassmer, Gary
Bloomsburg University of
Pennsylvania
gwassmer@bloomu.edu

Weaver, David
University of Massachusetts
Medical School
david.weaver@umassmed.edu

Webb, Ian
University of Western Ontario
iwebb22@uwo.ca

Weber, Frank
University of Heidelberg
frank.weber@bzh.uni-heidelberg.de

Weber, Todd
Rider University
tweber@rider.edu

Weigl, Yuval
Concordia University
yweigl@gmail.com

Weis, Karen
University of Illinois at Urbana-
Champaign
kweis1@illinois.edu

Weitz, Charles
Harvard Medical School
cweitz@hms.harvard.edu

Welsh, David
University of California–San Diego
welshdk@ucsd.edu

Westermark, Pål
Charite Universitaetsmedizin and
Humboldt University Berlin
p.westermark@biologie.hu-berlin.de

Westrich, Ligia
Lundbeck Research, USA
lwes@lundbeck.com

Wijnen, Herman
University of Virginia
hw9u@virginia.edu

Wingo, Charles
University of Florida
charles.wingo@medicine.ufl.edu

Winnebeck, Eva
University of Auckland
e.winnebeck@auckland.ac.nz

Wolf, Eva
MPI of Biochemistry
ewolf@biochem.mpg.de

Wolff, Gretchen
University of Kentucky
gretchen.wolff@uky.edu

Wolfgang, Werner
Queen Mary University of London
w.wolfgang@qmul.ac.uk

Womac, Alisa
Texas A&M University
awomac@mail.bio.tamu.edu

Wreschnig, Daniel
Northeastern University
wreschnig.d@neu.edu

Wright, Kenneth
University of Colorado
kenneth.wright@colorado.edu

Xie, Qiguang
Dartmouth College
qiguang.xie@dartmouth.edu

Xiong, Jing
Vanderbilt University
jing.xiong@vanderbilt.edu

Xu, Xiaodong
Dartmouth College
xiaodong.xu@dartmouth.edu

Xu, Yao
Vanderbilt University
yao.xu@vanderbilt.edu

Xu, Ying
Nanjing University
yingxutavel@gmail.com

Yagita, Kazuhiro
Osaka University Graduate School
of Medicine
kyagita@anat1.med.osaka-u.ac.jp

Yamakawa, Glenn
University of Calgary
gryamaka@ucalgary.ca

Yamazaki, Shin
Vanderbilt University
shin.yamazaki@vanderbilt.edu

Yan, Jin
University of Michigan
jyn@umich.edu

Yan, Lily
Michigan State University
yanl@msu.edu

Yanovsky, Marcelo
University of Buenos Aires
yanovsky@agro.uba.ar

Ye, Rui
University of North Carolina–Chapel
Hill
rye@email.unc.edu

Yildirim, Evrim
Rutgers University
evrim@eden.rutgers.edu

Yoo, Seung-Hee
University of Texas Southwestern
Medical Center
sally_yoo@yahoo.com

Yoshimura, Takashi
Nagoya University
takashiy@agr.nagoya-u.ac.jp

Yoshitane, Hikari
The University of Tokyo
stane@mail.ecc.u-tokyo.ac.jp

Young, Michael
Rockefeller University
young@rockefeller.edu

Zatz, Martin
Journal of Biological Rhythms
edjbr@earthlink.net

Zhang, L. Samantha
University of Michigan
samzhang@umich.edu

Zhang, Lin
Leicester University
lz8@le.ac.uk

Zhang, Maoxiang
Medical College of Gerogia
mazhang@mcg.edu

Zhang, Xiping
University of Kentucky
xzhan6@uky.edu

Zhang, Yong
University of Massachusetts
Medical School
yong.zhang@umassmed.edu

Zhang, Yunfei
Vanderbilt University
yunfei.zhang@vanderbilt.edu

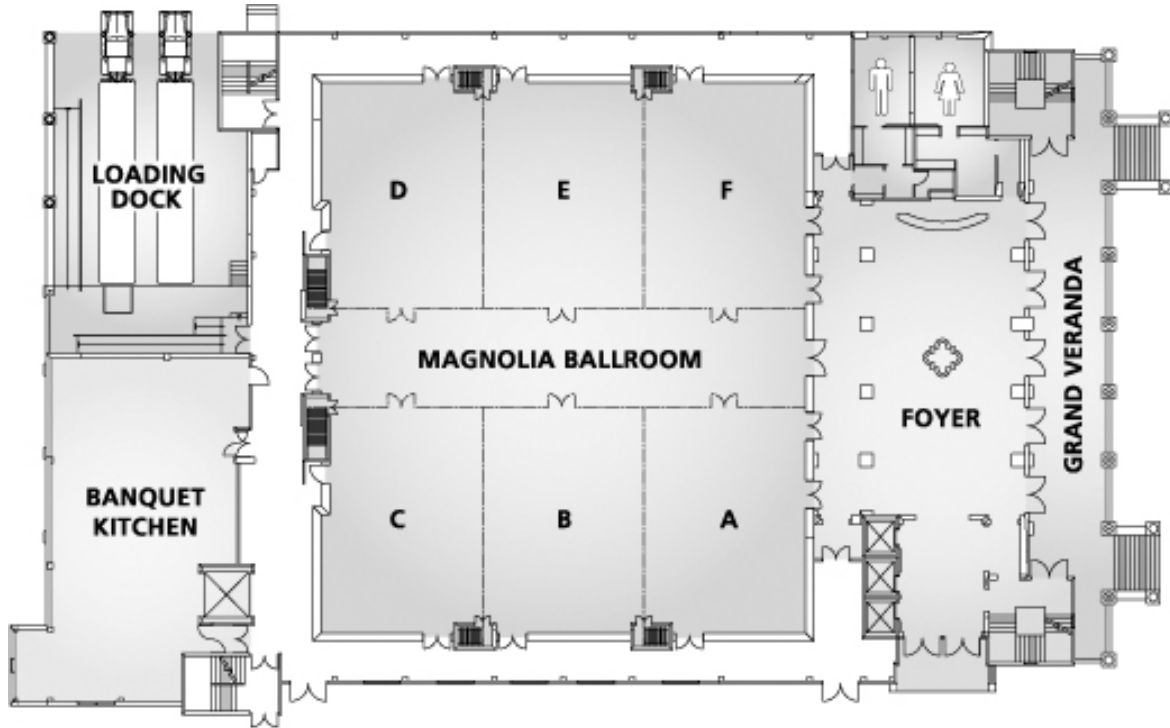
Zhu, Haisun
Thomas Jefferson University
haisun.zhu@jefferson.edu

Zoran, Mark
Texas A&M University
zoran@mail.bio.tamu.edu

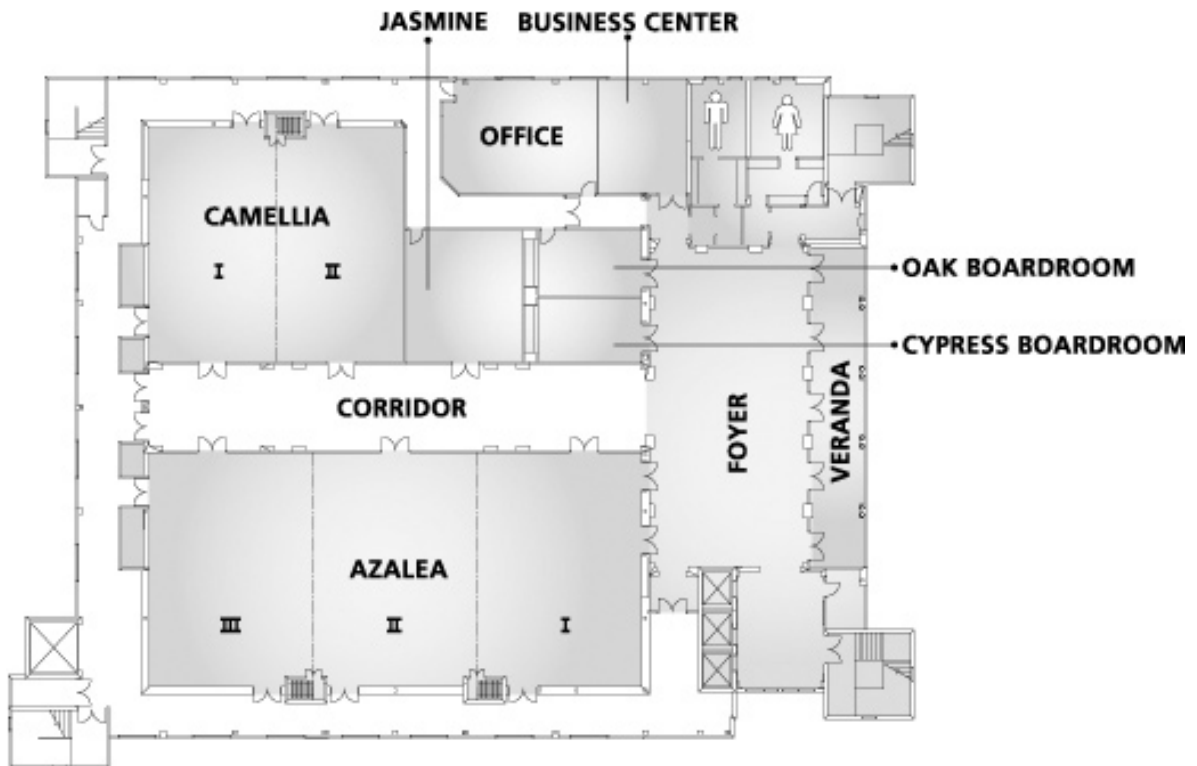
Notes

A series of horizontal dotted lines for taking notes.

Maps



THE BAYTOWNE CONFERENCE CENTER - MAIN LEVEL



THE BAYTOWNE CONFERENCE CENTER - SECOND LEVEL

