The circadian system of the cockroach *Leucophaea maderae*:

The role of FMRFamide-related peptides in the circadian system

and the role of the anterior and posterior optic commissures

in the coupling of both pacemakers

(Das circadiane System der Schabe *Leucophaea maderae*:
Die Rolle von FMRFamid-ähnlichen Peptiden im circadianen System
und die Rolle der anterioren und posterioren optischen Kommissuren
bei der Kopplung der beiden Schrittmacher)

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Sandra Söhler

aus Freudenberg

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Erklärung: Eigene Beiträge, veröffentlichte und zur Veröffentlichung vorgesehene Teile der Arbeit

Laut Promotionsordnung der Philipps-Universität Marburg vom 29.11.1989 (idF. vom 12.04.2000) müssen bei den Teilen der Dissertation, die aus gemeinsamer Forschungsarbeit entstanden sind, die individuellen Leistungen des Doktoranden deutlich abgrenzbar sein. Diese Beiträge werden im Folgenden näher erläutert:

Kapitel I: Localization of leucomyosuppressin in the brain and circadian clock of the cockroach *Leucophaea maderae*

- Ausarbeitung, Durchführung und Auswertung folgender Experimente durch die Autorin: Immuncytochemische Färbungen mit dem Antiserum gegen Dromyosuppressin im Gehirn der Schabe *Leucophaea maderae*. Fertigstellung und Auswertung der in meiner Diplomarbeit begonnenen Injektionen mit Leucomyosuppressin und Hämolymphringer. Die MALDI-TOF massenspektrometrischen Untersuchungen und die Auswertung dieser Ergebnisse wurden von Dr. Susanne Neupert und Prof. Dr. Reinhard Predel, Universität Jena, durchgeführt.

- Verfassen der Veröffentlichung in Zusammenarbeit mit Prof. Dr. Monika Stengl, Prof. Dr. Reinhard Predel und Dr. Susanne Neupert.

- Veröffentlichung: Söhler et al. (2007) Cell Tissue Res 328:443-452.
  Das vorliegende Kapitel entspricht der Veröffentlichung.

Kapitel II: Examination of the role of FMRFamide-related peptides in the circadian clock of the cockroach *Leucophaea maderae*

- Ausarbeitung, Durchführung und Auswertung der immuncytochemischen Färbungen und der Injektionen mit FMRFamid und Pea-FMRFamid-7 durch die Autorin. Die Hämolymphringerinjektionen aus Kapitel I wurden auch in diesem Kapitel zur Kontrolle herangezogen. Außerdem wurden hier noch zusätzliche Injektionen durch die Autorin durchgeführt, um die Fallzahlen zu erhöhen. Die MALDI-TOF und ESI-Q-TOF massenspektrometrischen Untersuchungen und die Auswertung dieser Ergebnisse wurden von Dr. Susanne Neupert und Prof. Dr. Reinhard Predel, Universität Jena, durchgeführt.

- Verfassen der Veröffentlichung in Zusammenarbeit mit Prof. Dr. Monika Stengl, Prof. Dr. Reinhard Predel und Dr. Susanne Neupert.

- Veröffentlichung: Soehler et al. (2008) Cell Tissue Res 332:257-269.
  Das vorliegende Kapitel entspricht der Veröffentlichung.

Kapitel III: Circadian pacemaker coupling by multi-peptidergic neurons in the cockroach *Leucophaea maderae*

- Ausarbeitung und Durchführung der immuncytochemischen Experimente in Zusammenarbeit mit Dr. Thomas Reischig. Auswertung der Experimente in Zusammenarbeit mit Dr. Thomas Reischig, Universität Göttingen.

- Verfassen einer Veröffentlichung in Zusammenarbeit mit Dr. Thomas Reischig, Universität Göttingen, und Prof. Dr. Monika Stengl. Die Arbeit soll in Kürze bei Cell and Tissue Research zur Veröffentlichung eingereicht werden.
  Das vorliegende Kapitel entspricht der demnächst einzureichenden Version.

Kapitel IV: Both anterior and posterior optic commissures transmit coupling information between the pacemakers of *Leucophaea maderae*

- Ausarbeitung, Durchführung und Auswertung aller Experimente durch die Autorin.

Weiterhin ist die Verfasserin Co-Autorin der folgenden Veröffentlichung:

- Wicher D, Agricola HJ, Söhler S, Gundel M, Heinemann SH, Wollweber L, Stengl M, Derst C. (2006) Differential receptor activation by cockroach adipokinetic hormones produces differential effects on ion currents, neuronal activity, and locomotion. J Neurophysiol 95:2314-2325.

Die Abfassung der Dissertation in englischer Sprache wurde vom Dekan des Fachbereichs Biologie am 10.06.08 genehmigt.

Zusammenfassung

Alle Lebewesen unterliegen biologischen Rhythmen, d. h. biologischen Phänomenen, die sich in regelmäßigen Zeitabständen wie einem Tag, Monat oder Jahr wiederholen. Tagesperiodische Rhythmen (24 h), die sich durch Adaptation an den Tag-Nacht-Zyklus auf der Erde entwickelt haben, werden als circadiane Rhythmen bezeichnet (circa = ungefähr, dies = Tag). Viele biochemische und physiologische Prozesse sowie zahlreiche Verhaltensweisen eines Organismus sind circadianen Rhythmen unterworfen. Erzeugt werden diese circadianen Rhythmen durch innere Uhren. Dabei handelt es sich um tagesperiodisch schwingende Oszillatoren, die durch externe „Zeitgeber"-Signale wie den Licht-Dunkel-Wechsel, Temperaturänderungen, aber auch soziale Kontakte mit der Umwelt synchronisiert werden. Licht ist dabei der wichtigste Zeitgeber.

In vielen Organismen konnte die innere Uhr bereits lokalisiert werden. So befindet sich die innere Uhr der Säugetiere, der suprachiasmatische Nucleus (SCN), oberhalb der Sehnervenkreuzung im Hypothalamus. Bei Insekten befindet sie sich in den optischen Loben. Bei der Schabe *Leucophaea maderae* konnte bereits in den 60er und 70er Jahren der Sitz der inneren Uhr durch Läsionsexperimente auf die optischen Loben eingegrenzt werden. Weitere Experimente identifizierten die akzessorische Medulla, ein kleines Neuropil an der ventromedianen Seite der Medulla, als circadianes Schrittmacherzentrum der Schabe. Ebenso wie das Schrittmacherzentrum der Vertebraten enthält die akzessorische Medulla eine außergewöhnlich hohe Dichte an peptidergen Neuronen. Bisherige Ergebnisse zeigten, dass sowohl Peptide als auch klassische Neurotransmitter bei der Aufrechterhaltung, Steuerung und Synchronisation der inneren Uhren der Schabe eine bedeutende Rolle spielen. Die Identifizierung der verschiedenen Neuropeptide und die Aufklärung der Rolle, die diese Neuropeptide bei der Steuerung und Kopplung der inneren Uhr spielen, leisten daher einen wichtigen Beitrag zur Aufklärung der Funktion des Uhrwerks in der akzessorischen Medulla der Schabe *Leucophaea maderae.*

Die vorliegende Dissertation hatte vor allem zum Ziel herauszufinden, welches Peptid aus der Familie der FMRFamid-ähnlichen Peptide (FaRPs) in der akzessorischen Medulla der Schabe *Leucophaea maderae* lokalisiert ist und welche Funktion dieses in der inneren Uhr wahrnimmt. Zusätzlich sollte die Frage beantwortet werden, ob ein Mitglied der FaRPs eine Rolle bei der Kopplung der bilateralsymmetrischen Uhren spielt.

Vorhergehende immuncytochemische Untersuchungen hatten bereits ca. 30 FMRFamid-immunreaktive Neurone in der Nähe der akzessorischen Medulla lokalisiert. Jedoch konnte mit diesem Antiserum keine weitere Differenzierung der Mitglieder der FaRPs vorgenommen werden, da das Antiserum das für die Mitglieder charakteristische RFamid-Ende detektiert und somit alle

Mitglieder der Familie erkennt. Es wurde darum zunächst mit Antikörpern gegen FMRFamid (zur Verfügung gestellt von Dr. Eve Marder, Massachusetts, USA), Perisulfakinin (XGHMRFamid, zur Verfügung gestellt von Dr. Hans Agricola, Jena, Deutschland), Head peptide (XLRLRFamid, zur Verfügung gestellt von Dr. Hans Agricola, Jena, Deutschland) und Dromyosuppressin (XHVFLRFamid, zur Verfügung gestellt von Dr. Ruthann Nichols, Michigan, USA) Immuncytochemie an Gehirnschnitten von *Leucophaea maderae* durchgeführt. Diese Versuche ließen aber lediglich eine eindeutige Identifizierung eines Dromyosuppressin-immunreaktiven Neurons zu, da der Antikörper gegen die N-terminale Sequenz TDVDHV gerichtet war. Alle anderen Antikörper lieferten lediglich Hinweise auf das Vorkommen der entsprechenden Peptide in der akzessorischen Medulla. Darum wurden zusätzlich MALDI-TOF (Matrix-unterstützte Laserdesorptions-/Ionisations-Massenspektrometrie)-Experimente durchgeführt. Mittels MALDI-TOF konnte indirekt über eine Massenbestimmung das Vorkommen verschiedener Mitglieder der FaRPs in der akzessorischen Medulla nachgewiesen werden.

Neben der Fragestellung nach der Lokalisation verschiedener Mitglieder der FaRPs in der akzessorischen Medulla sollte auch der Frage nach deren Funktion in der inneren Uhr nachgegangen werden. Zu diesem Zweck wurden männlichen, im Dauerdunkel im Laufrad gehaltenen Schaben zu verschiedenen circadianen Zeiten verschiedene Mitglieder dieser Peptidfamilie in die Nähe der akzessorischen Medulla injiziert. Anschließend wurden die Phasenlage und Periodenlänge des circadianen Laufverhaltens vor und nach der Injektion verglichen. Phasenverschiebungen in Abhängigkeit von der circadianen Zeit zu der injiziert wurde, wurden dann in einer Phasen-Antwort-Kurve dargestellt.

Das dritte Projekt befasste sich mit der möglichen Beteiligung von FMRFamid-immunreaktiven Neuronen an der Kopplung der bilateralsymmetrischen Schrittmacher der Schabe. Zur Beantwortung dieser Fragestellung wurde entweder fluoreszenzmarkiertes Dextran in eine der beiden akzessorischen Medullen injiziert und das Gehirn anschließend mit FMRFamid-Antiserum immuncytochemisch markiert. Dextran wird von den Neuronen aufgenommen und nur innerhalb eines Neurons weitertransportiert. Zusätzlich wurden Backfills durchgeführt, indem eine mit Neurobiotin gefüllte Kapillare über den optischen Stil gestülpt wurde. Der optische Stil wurde vorher mit einem Schnitt durchtrennt. Anschließend wurde das Gehirn mit anti-FMRFamid- oder anti-Orcokinin-Antiserum immuncytochemisch angefärbt. Da außerdem PDH- (pigment-dispersing hormone; ein erstmalig aus Crustaceen isoliertes Neuropeptid, das in Insekten PDF = pigment-dispersing factor genannt wird) immunreaktive Neurone als Schrittmacherneurone identifiziert worden waren, wurden die Backfills zusätzlich mit PDF-Immuncytochemie kombiniert. In vorhergehenden Experimenten wurden durch Dextraninjektionen in eine akzessorische Medulla

drei Zellgruppen (MCI-III) identifiziert, die offensichtlich beide akzessorischen Medullae über die anteriore und posteriore optische Kommissur (AOC und POC) miteinander verbinden (Reischig et al. 2004). Es zeigte sich, dass drei der etwa 16 PDH-immunreaktiven Neurone pro Hemisphäre beide akzessorischen Medullen direkt miteinander verbinden. Diese Untergruppe der PDH-immunreaktiven Neurone ist daher sehr wahrscheinlich am circadianen Kopplungsweg beteiligt. Die in diesem Projekt durchgeführten Färbungen konnten zeigen, dass mindestens vier PDF-immunreaktive Neurone offensichtlich beide akzessorischen Medullae miteinander verbinden. Die zur akzessorischen Medulla gehörenden Neuronen wurden anhand ihrer Größe, Lage, morphologischen Unterschieden und Färbeintensität in sechs Gruppen eingeordnet (Reischig and Stengl 2003b). Die vier PDF-immunreaktiven Kopplungsneurone konnten der Gruppe der ventralen Neurone zugeordnet werden. Drei der vier ventralen Neurone enthalten dabei sowohl PDF als auch Orcokinin und FMRFamid. Das vierte Neuron enthält dagegen nur PDF. Außerdem zeigten die Experimente, dass offensichtlich auch Neurone aus der Gruppe der medianen Neurone beide akzessorischen Medullae miteinander verbinden.

Da die beiden akzessorischen Medullae über die anteriore und die posteriore Kommissur miteinander verbunden sind, sollte im vierten Kapitel der Frage nachgegangen werden, ob beide Kommissuren unterschiedliche Informationen übermitteln. Dazu wurde männlichen, sich im Laufrad befindenden Schaben, nachdem sie einen stabilen Aktivitätsrhythmus gezeigt hatten, die anteriore Kommissur, posteriore Kommissur oder das Zentralhirn durchtrennt. Anschließend wurden die Auswirkungen, die diese verschiedenen Eingriffe auf das Laufverhalten ausübten, ausgewertet. Die bisher durchgeführten Versuche zeigten, dass die Durchtrennung der anterioren oder posterioren optischen Kommissur keine signifikanten Unterschiede in Periodenlänge, Phasenlage und Rhythmik nach sich zog. Die Durchtrennung des Zentralhirns führte bei fast allen Tieren zur Ausbildung einer Daueraktivität.

Die Inhalte der einzelnen Kapitel der Arbeit werden im Folgenden näher zusammengefasst:

Kapitel I: Lokalisierung von Leucomyosuppressin im Gehirn und der inneren Uhr der Schabe *Leucophaea maderae*

Neuropeptide sind die vielfältigste Klasse von chemischen Botenstoffen in Invertebraten und Vertebraten. Sie fungieren entweder als Hormone oder als Neurotransmitter und Neuromediatoren. Viele dieser bioaktiven Peptide können, basierend auf ihrer genetischen Verwandtschaft oder

ähnlichen Struktur, in Gruppen zusammengefasst werden (Greenberg und Price 1983). Eine der größten Gruppen umfasst die Mitglieder der FaRPs (Orchard et al. 2001, Orchard und Lange 2006, Predel 2006). FaRPs kommen sowohl im Zentralnervensystem als auch im peripheren Nervensystem vor und erfüllen eine ganze Reihe unterschiedlicher Aufgaben (Orchard et al. 2001, Orchard und Lange 2006, Predel 2006). Die Mitglieder der FaRPs lassen sich in fünf Gruppen einteilen. Eine Gruppe umfasst die Myotropine, die entweder einen stimulatorischen oder einen inhibitorischen Einfluß auf die Darmmuskulatur haben. Die Peptide der Untergruppe der Myosuppressine haben alle die Aminosäuresequenz XDVXHXFLRF-NH$_2$ (- an den mit X bezeichneten Positionen befinden sich je nach Peptid unterschiedliche Aminosäuren -) und wirken inhibitorisch auf die Darmmuskulatur. Leucomyosuppressin (LMS, pQDVDHVFLRF-NH$_2$) wurde als erstes Peptid dieser Familie aus der Schabe *Leucophaea maderae* isoliert (Holman et al. 1986). Später durchgeführte immuncytochemische Färbungen lokalisierten LMS-immunreaktive Zellen im Zentralhirn der Schabe *Leucophaea maderae* (Meola et al. 1991). Bei der Schabe *Diploptera punctata* wurde mittels in-situ Hybridisierung LMS-mRNA auch in der Nähe der akzessorischen Medulla lokalisiert (Fusé et al. 1998). Das von Meola et al. (1991) benutzte Antiserum detektierte auch den RFamid C-Terminus. Um eine spezifische Detektion von LMS zu gewährleisten, wurde das Antiserum daher vorher mit FMRFamid-Peptid präadsorbiert. Das uns zur Verfügung stehende Antiserum war gegen die N-terminale Sequenz von Dromyosuppressin (dem Myosuppressin aus *Drosophila melanogaster*) gerichtet und wurde bereits erfolgreich zur Detektion Dromyosuppressin-immunreaktiver Neurone bei *Drosophila melanogaster* eingesetzt (McCormick und Nichols 1993, Nichols et al. 1997). Die N-terminale Sequenz von Dromyosuppressin unterscheidet sich nur in einer Aminosäure vom N-Terminus des LMS und darum war die Wahrscheinlichkeit sehr hoch, dass dieser Antikörper spezifisch LMS detektiert. Die Verwendung dieses bisher bei der Schabe noch nicht getesteten Antikörpers rechtfertigte eine Wiederholung der immuncytochemischen Experimente von Meola et al. (1991). Tatsächlich konnten mit diesem Antikörper ein bis drei LMS-immunreaktive Neurone in der Nähe der akzessorischen Medulla nachgewiesen werden. Diese LMS-immunreaktiven Neurone konnten anhand der von Reischig und Stengl (2003b) vorgeschlagenen Klassifizierung der mit der akzessorischen Medulla assoziierten Neuronengruppen der Gruppe der ventralen Neurone zugeordnet werden. Zusätzlich durchgeführte MALDI-TOF Experimente an ausgestanzten akzessorischen Medullae bestätigten das Vorhandensein von LMS in der akzessorischen Medulla. Die bereits in meiner Diplomarbeit durchgeführten Injektionsexperimente von LMS in die Nähe der akzessorischen Medulla zeigten jedoch, dass LMS keinen Einfluss auf die circadiane Laufaktivität der Schaben hat. Die Funktion von LMS in der inneren Uhr der Schabe bleibt ungeklärt.

Kapitel II: Untersuchung der Rolle von FMRFamid-ähnlichen Peptiden in der inneren Uhr der Schabe *Leucophaea maderae*

Zur weiteren Unterscheidung der ca. 30 FMRFamid-immunreaktiven Neurone in der Nähe der akzessorischen Medulla wurde mit Antiseren gegen FMRFamid (zur Verfügung gestellt von Dr. Eve Marder, Massachusetts, USA), Perisulfakinin (XGHMRFamid, zur Verfügung gestellt von Dr. Hans Agricola, Jena, Deutschland) und short Neuropeptid F (XLRLRFamid, zur Verfügung gestellt von Dr. Hans Agricola, Jena, Deutschland) Immuncytochemie an Gehirnschnitten von *Leucophaea maderae* durchgeführt. Die Auswertung der immuncytochemischen Experimente ergab die Anzahl von ca. 24 FMRFamid-immunreaktiven Neuronen in der Nähe der akzessorischen Medulla. Es wurden also etwas weniger FMRFamid-immunreaktive Neurone in der Nähe der akzessorischen Medulla detektiert als in der Studie von Petri et al. (1995, ca. 30 FMRFamid-immunreaktive Neurone). Die FMRFamid-immunreaktiven Neurone konnten aufgrund ihrer Größe, Lage und Färbeintensität in die sechs zur akzessorischen Medulla gehörenden Neuronengruppen eingeteilt werden. FMRFamid-immunreaktive Neurone wurden in der ventralen, distal frontoventralen, medianen und ventro-posterioren Neuronengruppe gefunden. Frühere Experimente lieferten Hinweise darauf, dass die Gruppe der distalen frontoventralen Neurone hauptsächlich aus lokalen Interneuronen besteht, die Gruppe der ventralen Neurone enthält dagegen Projektionsneurone, von denen einige an der Kopplung der beiden inneren Uhren beteiligt sind. Die Gruppen der medianen und der ventro-posterioren Neurone wurden bisher noch nicht genauer charakterisiert. Zusätzlich wurden noch FMRFamid-immunreaktive Neurone anterior zur akzessorischen Medulla identifiziert. Diese konnten keiner der bestehenden Gruppen zugeordnet werden und wurden darum der bislang unbeschriebenen Gruppe der anterioren Neurone zugeordnet. Die Einteilung in diese Gruppen lieferte also erste Hinweise auf eine Rolle der FMRFamid-immunreaktiven Neurone bei der Kopplung der bilateralen Schrittmacher. Anschließend sollte mit den Perisulfakinin- und den short Neuropeptid F-Antiseren eine genauere Charakterisierung der FaRPs in der akzessorischen Medulla vorgenommen werden. Mit dem anti-short Neuropeptid F-Antiserum konnten immunreaktive Neurone in der ventralen und in der anterioren Neuronengruppe detektiert werden. Hingegen detektierte das Perisulfakinin-Antiserum keine immunreaktiven Neurone in der Nähe der akzessorischen Medulla. Durch die auch in diesem Projekt angewendete Methode der Massenspektrometrie konnte ein Peptid mit der Masse 1315,9 Da detektiert werden. Diese Masse entspricht der von short Neuropeptid F aus *Periplaneta americana*. Mittels MALDI-TOF wurde also ein weiterer starker Hinweis auf das Vorkommen von short Neuropeptid F in der akzessorischen Medulla von *Leucophaea maderae* erbracht. Perisulfakinin konnte mittels MALDI-

TOF nicht nachgewiesen werden. Jedoch konnte ein weiteres Peptid mit der Teilaminosäuresequenz AVRDNFIRFamid aus der Familie der FaRPs nachgewiesen werden.

Zur Klärung der Funktion der FaRPs in der akzessorischen Medulla wurden FMRFamid und Pea-FMRFamid-7 (DRSDNFIRF-NH2) in die Nähe der akzessorischen Medulla injiziert. Die Injektionen von FMRFamid resultierten in signifikanten Phasenverzögerungen zu den circadianen Zeiten (CT) 8 und 18. Die Pea-FMRFamid-7 Injektionen führten zu signifikanten Phasenverzögerungen zu CT 4. Zusammengefasst zeigte diese Studie also, dass verschiedene Mitglieder aus der Familie der FaRPs in der inneren Uhr von *Leucophaea maderae* vorkommen. Außerdem zeigten die Injektionsexperimente, dass FaRPs eine Rolle bei der circadianen Regulation der lokomotorischen Aktivität der Schabe spielen.

Kapitel III: Kopplung der circadianen Schrittmacher der Schabe *Leucophaea maderae* durch multi-peptiderge Neurone

Läsions- und Transplantationsexperimente identifizierten die akzessorische Medulla mit ihren assoziierten pigment-dispersing hormone (PDH)-immunreaktiven Neuronen als das Schrittmacherzentrum der Schabe *Leucophaea maderae* (Nishiitsutsuji-Uwo und Pittendrigh 1968, Roberts 1974, Sokolove 1975, Page 1978, Colwell und Page 1990). Die ca. 16 PDH-immunreaktiven Neurone verzweigen sowohl in der akzessorischen Medulla als auch im optischen Lobus und im Zentralhirn (Petri et al. 1995). Das eingesetzte anti-β-PDH-Antiserum richtete sich gegen das synthetisch hergestellte *Uca pugilator* β-PDH und wurde aus Kaninchen gewonnen (Dircksen et al. 1987) und in vielen Insektenspezies zur Identifizierung PDH-ähnlicher Peptide eingesetzt. Diese PDH-ähnlichen Peptide aus Insekten wurden dann pigment-dispersing factor (PDF) genannt.

Mit Hilfe von Dextran-Injektionen in eine akzessorische Medulla und Immuncytochemie konnte gezeigt werden, dass mindestens drei der PDH-immunreaktiven Neurone beide bilateralsymmetrischen akzessorischen Medullae direkt miteinander verbinden (Reischig et al. 2004). Es konnte jedoch nicht geklärt werden, ob auch andere peptiderge Neurone wie z. B. FMRFamid- oder Asn[13]-Orcokinin-immunreaktive Neurone an der Kopplung der beiden Schrittmacher beteiligt sind. Deshalb wurden in dieser Studie Neurobiotin-Backfills mit Doppelfärbungen mit PDF- und FMRFamid-Antiseren bzw. PDF- und Asn[13]-Orcokinin-Antiseren kombiniert. Da der benutzte Antikörper gegen das *Drosophila* PDF aus Mäusen gewonnen wurde, wird hier die Bezeichnung PDF-Antikörper benutzt, und die mit diesem Antikörper

immuncytochemisch markierten Neurone werden als PDF-immunreaktive Neurone bezeichnet.

Frühere Studien konnten schon drei Gruppen kontralateral projizierender Neurone identifizieren (Reischig et al. 2004). Eine dieser Gruppen enthält die kontralateral projizierenden PDF-immunreaktiven Neurone der akzessorischen Medulla. Die in unserer Studie durchgeführten Experimente detektierten ebenfalls die drei bereits beschriebenen Gruppen kontralateral projizierender Neurone. Zusätzlich enthüllten sie eine vierte Gruppe kontralateral projizierender Neurone. Außerdem konnte gezeigt werden, dass mindestens vier PDF-immunreaktive Neurone zur kontralateralen Seite projizieren. Drei der vier PDF-immunreaktiven Neurone koexpremieren FMRFamid- und Orcokinin-Immunreaktivität und verbinden beide Schrittmacher wahrscheinlich über die anteriore optische Kommissur. Das vierte Neuron enthält nur PDF und scheint beide akzessorischen Medullae über die anteriore und posteriore optische Kommissur zu verbinden. Die Auswertung der Doppelfärbungen gegen PDF und FMRFamid bzw. PDF und Asn^{13}-Orcokinin zeigten, dass alle mittelgroßen und die meisten kleinen der zur akzessorischen Medulla gehörenden PDF-immunreaktiven Neurone zusätzlich FMRFamid und Orcokinin enthalten. Interessanterweise wurden Kolokalisationen von FMRFamid und PDF nur in wenigen Neuriten gefunden. Einige davon waren in der akzessorischen Medulla lokalisiert. PDF- und Orcokinin-Kolokalisation wurde nicht in den Neuriten gefunden. Die Neurite, in denen PDF und FMRFamid kolokalisiert waren, zeigten nie eine zusätzliche Neurobiotinfärbung. Eine mögliche Erklärung hierfür wäre differenzielle Peptidsortierung. Differenzielle Peptidsortierung könnte dazu führen, dass bestimmte Peptide nur in spezifischen Terminalen der Axone der PDF-immunreaktiven Schrittmacherneurone ausgeschüttet werden und dann als „Timing"-Signale für die Synchronisation der Schrittmacherneurone wirken.

Kapitel IV: Anteriore und posteriore optische Kommissur übertragen Kopplungsinformation zwischen den Schrittmachern von *Leucophaea maderae*

Leucophaea maderae zeigt nur dann einen stabilen Rhythmus, wenn die bilateralsymmetrischen Schrittmacher gekoppelt sind. Wie vorherige Experimente zeigten, verläuft die Kopplung über die anteriore und die posteriore optische Kommissur (Reischig und Stengl 2002). Mittels Durchtrennungen dieser Kommissuren sollte der Frage nachgegangen werden, ob beide Kommissuren gleichermaßen an der Kopplung der Schrittmacher beteiligt sind, oder ob unterschiedliche Informationen über die jeweiligen Kommissuren übertragen werden. Zu diesem Zweck wurden die anteriore optische Kommissur, die posteriore optische Kommissur oder das

komplette Zentralhirn durchtrennt. Kombiniert wurden diese Operationen mit Verhaltensversuchen zur Messung der lokomotorischen Aktivität der Schaben. Nach Etablierung eines stabilen Aktivitätsrhythmus im Laufrad im Dauerdunkel wurden die Tiere den erwähnten Operationen unterzogen und die Auswirkungen auf das Laufverhalten ausgewertet.

Durch die bisher durchgeführten Versuche konnte kein Unterschied in den übertragenen Informationen über die jeweiligen Kommissuren nachgewiesen werden. Vergleiche von Rhythmik, Phasenlage und Periodenlänge vor Durchtrennung der anterioren oder der posterioren optischen Kommissur mit der Rhythmik, Phasenlage und Periodenlänge nach der Durchtrennung der jeweiligen Kommissuren zeigten keine statistisch signifikanten Veränderungen im Laufverhalten der Schaben. Um endgültige Aussagen treffen zu können, müssen noch weitere Experimente durchgeführt werden. Die Durchtrennung des kompletten Zentralhirns führte bei fast allen Tieren zunächst zur signifikanten Steigerung der Aktivitätsdauer. Eine Erklärung wäre, dass aufgrund der Operation eine Verbindung der Schrittmacherneurone zu Neuronen im Thorax, die die Laufaktivität steuern, unterbrochen wurde. Der hemmende Einfluss der Schrittmacher wurde somit ausgeschaltet, was zu einer Daueraktivität der Tiere führte. Nach einigen Wochen kam es dann wahrscheinlich zur Regeneration einiger Fasern aus den Schrittmachern und die Verbindung von Schrittmachern zu den thorakalen Neuonen, die die lokomotorische Aktivität steuern, wurde wiederhergestellt. Dies manifestierte sich dann in einem rhythmischen Laufverhalten der Tiere. Auch hier müssen noch weitere Experimente durchgeführt werden, um eine endgültige Aussage treffen zu können.

Introduction

Circadian rhythms

Life on earth is shaped by multiple geophysical rhythms. One of the most influential is the succession of day and night. Animals and plants adapted to these temporal rhythms and developed internal timekeepers (so called circadian clocks) that anticipate the daily cycling of environmental conditions. Thus, circadian clocks provide organisms with information about the current time. The synchronization of the circadian clock with the environment (*entrainment*) happens via "Zeitgebers" such as light. Many physiological and behavioral activities are influenced by circadian rhythms. A well known circadian rhythm is the change of body temperature, which increases before waking up and decreases before falling asleep. Other important processes of our body also change in a daily manner. However, not only the molecular, cellular and physiological activities of a single organism depend on clocks. Even on the level of populations internal clocks are very important to guarantee successful social interactions between species (Engelmann 2004). The successful pollination of plants by insects, for example, depends on internal clocks in both partners. Plants open and close their flowers and maximize their nectar production daytime-dependently and bees need to visit the plants at the right time of day (Engelmann 2004). These few examples demonstrate the importance of the right "timing" for the organisms and biological clocks assure that things happen at the right time.

First incidences that endogenous clocks regulate the daily processes have already been noticed by the French naturalist Jean J. de Mairan (1729). He observed daily leaf movements of a mimosa for several days under constant darkness without external rhythmic cues. However, scientific research on biological clocks occurred not until the 20th century. Thereby was an essential observation that daily rhythms like leaf movements of plants, eclosion rhythms of insects or activity rhythms persist even under constant environmental conditions when the possibility to entrain on external time cues is missing. The period length (τ) of these *freerunning* rhythms, however, remains stable with about 24 hours. This period length which lasts about one day is called *circadian day*. One circadian day can be divided into 24 circadian hours of about one hour length, with one circadian hour being defined as the division of the internal period length (τ) by 24. Generally, nocturnal animals like cockroaches exert *freerunning* rhythms with a period length shorter than 24 hours, whereas diurnal species like flies perform rhythms that last longer than 24 hours (Pittendrigh 1960). Animals which live under light-dark cycles have to synchronize their circadian oscillators with the environmental rhythms. Consequently, they are *entrained* by the

Zeitgeberzeit (ZT) and start their activity phase every day at approximately the same time. The most important external Zeitgebers are the light-dark and temperature cycles (Aschoff 1960). The resetting of pacemaker occurs upon *phase shifts*.

To study the physiological properties of the clock it is a common practice to analyze the influences of different stimuli on amounts and directions of phase shifts in the locomotor activity of animals under constant conditions (e. g. constant darkness, DD). Circadian-driven locomotor activity rhythms are often the most obvious actions of internal clocks and can be easily recorded. Therefore, much knowledge about circadian clock properties was achieved by observing round-the-clock locomotor activity over longer time periods, e.g. for at least several days. Different methods were applied to record the locomotor activity, e. g., running-wheel (Fig. 1a), tilting cage, and photo-electric assays. Typically, results are presented in *actograms* (Figs. 1b-e). In the actogram of Fig. 1b, vertical bars plotted side-by-side represent locomotor activity. The influence of a certain stimulus at a certain time causes either no effects (Fig. 1c), slows down (phase-delaying, Fig. 1d) or accelerates (phase-advancing, Fig. 1e) the pacemaker depending on the specific phase at which the stimulus is applied. The effects of different stimuli on the phase of the pacemaker at different circadian times can be summarized in a so called phase response curve (PRC, plural PRCs, Fig. 1f).

A PRC permits to assign the amplitude and direction of a phase shift to a specific circadian time. The time difference [h] between the onset of the activity before the stimulus and the onset of the activity after the stimulus is called phase angle difference (Ψ). If the onset of the activity after a stimulus is earlier than that before the stimulus this is called *phase advance* and a later onset of the activity is called *phase delay*. The PRCs can be arranged in biphasic (Fig. 1g) and monophasic (Fig. 1h) curves, whereby the monophasic curves can be further subdivided in all delay or all advance curves. All delay curves (Fig. 1h) emerge from experiments where all stimuli cause so-called phase delays and all advance curves are the result of only phase advances after the stimuli.

By the 1960s, it was clear that a circadian clock is present in all eukaryotes and localizing the clock in complex systems became a prime target of circadian research. Distinct, dedicated circadian centers (pacemakers) were localized in insects (in the optic lobes), molluscs (in the eye), birds (in the pineal gland) and mammals (in the suprachiasmatic nucleus of the hypothalamus). Particularly insects suit well for the research on circadian clocks because they are robust, their brains are often easy accessible and many insects show a robust circadian rhythm of locomotor activity. Indeed the cockroach *Leucophaea maderae* was the first animal where a circadian pacemaker could be assigned to a particular brain region (Nishiitsutsuji-Uwo and Pittendrigh 1968, Fig. 2). Later on, surgical and electrolytic lesions and transplantation experiments narrowed down the clock location to an area in the ventral part of the optic lobe between the medulla and lobula (Roberts 1974,
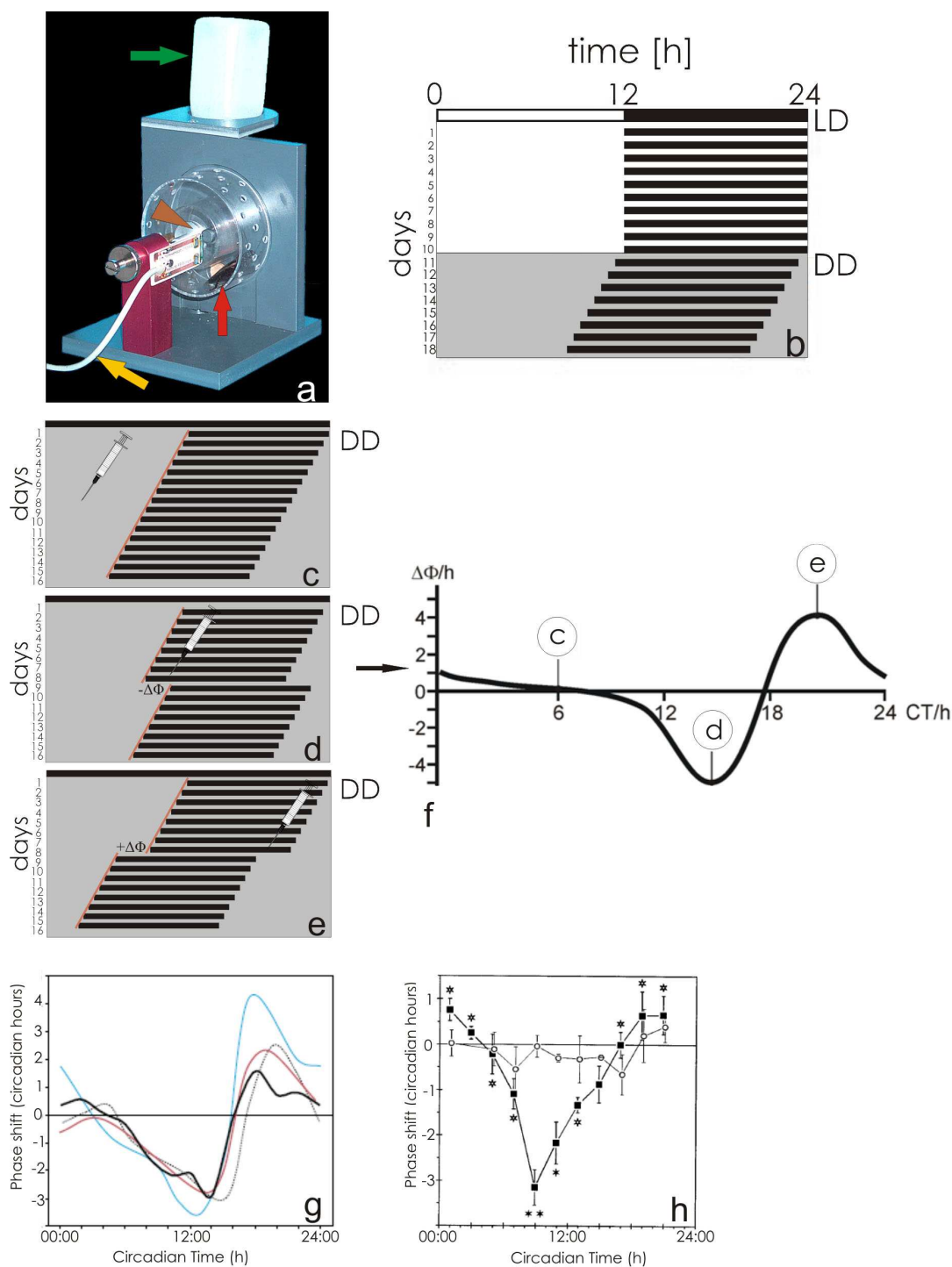
Fig. 1 a-h Schematic outline of an experimental strategy to study the influence of neuropeptides on the circadian clock in cockroaches. **a** A cockroach (*red arrow*) is placed in a running-wheel and the circadian locomotor activity is monitored; *red arrowhead*: reed relay to record rotations of the running-wheel, *yellow arrow:* connection to the computer, *green arrow:* water supply. **b** Activity phases of the cockroach are shown as *black bars* in the actogram. During the first ten days the animal is kept in a 12:12 h light-dark cycle (*LD*, light phase of Zeitgebertime 0-12 and dark phase of Zeitgebertime 12-24, represented through the *bar above the activity bars*). The following eight days the animal is kept in constant darkness (*DD*, pictured with a *grey box*). It exerts a circadian rhythm shorter than 24 h. **c-e** Cockroaches in DD receive injections with a neuropeptide at different circadian times. Dependent on the injection times (**c** CT 6, **d** CT 15, **e** CT 21), the animals react with either no phase shift (**c**), a phase delay (**d**), or a phase advance (**e**). **f** Overview of the resulting phase shifts is given by the phase response curve (*PRC*). In the PRC the resulting phase shifts are plotted against the circadian time. **g** Biphasic PRCs obtained after treating the cockroaches with light (*blue line*, Page and Barret 1989), orcokinin (*black line*, Hofer and Homberg 2006a), γ-aminobutyric acid (GABA, *red line*, Petri et al. 2002) and allatotropin (*dotted line*, Petri et al. 2002). **h** Monophasic PRC obtained after injection of pigment-dispersing hormone (*PDH closed squares, control injections open circles*, from Petri and Stengl 1997).

Sokolove 1975, Page 1978, 1982). The data of Page (1982, 1983) confirmed that the oscillator is situated in the optic lobes and also showed that neuronal output mechanisms must convey signals from the pacemaker to specific output structures in the brain. Furthermore, the research on clocks in the different species revealed astonishing similarities. All clocks are anatomically and functionally connected with the optic system allowing synchronization with the environmental light-dark cycles. The identified clocks all possess multiple output pathways that control diverse endocrine, autonomic and behavioral functions. These findings are summarized in a general model for the organization of an internal clock (Fig. 3). This model includes an endogenous oscillator (pacemaker), which generates a self sustained circadian rhythm. Synchronization of the oscillator with the environmental time occurs upon entrainment pathways and the transfer of time information from the pacemaker to other organs occurs upon effector pathways.

While the cockroach *Leucophaea maderae* has played a major role in research on the anatomical and physiological properties of the pacemaker, the fruitfly *Drosophila melanogaster* is an important organism for studying the molecular mechanisms of the circadian clock. Identification and isolation of the clock gene period (per, Konopka and Benzer 1971) from *Drosophila* and subsequent analysis of its expression led to the first molecular model of a circadian oscillator – an
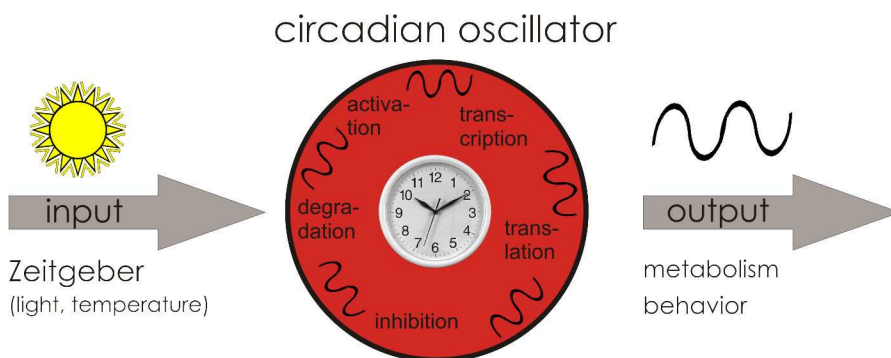

Fig. 3 General model of an internal clock with a circadian oscillator (pacemaker) that generates a circadian rhythm, input pathways that synchronize the pacemaker with environmental rhythms like the change of day and night, and output pathways that transmit the pacemaker information to the effector organs (modified from Berndt et al. 2005).

autoregulatory negative feedback loop of transcription and translation (Hall 2003). Until now, it is known that both activity and eclosion rhythms of *Drosophila* are controlled by two transcriptional feedback loops and posttranscriptional regulations (Stanewsky 2003, Hardin 2004, 2005). One of them is the period/timeless loop, in which transcription is activated by the two transcription factors CLOCK and CYCLE and repressed by PERIOD-TIMELESS. The other is the clock loop in which transcription by CLOCK-CYCLE is suppressed and induced by PERIOD-TIMELESS (Glossop et al. 1999). However, since the cockroach *Leucophaea maderae* is a favoured organism to study neurophysiological properties of circadian clocks because of its large brain which can be easily manipulated and its clear circadian activity pattern, we used this organism for our experiments. Thus in the following, the pacemaker of *Leucophaea maderae* will be described in detail.

The accessory medulla is the clock of the cockroach *Leucophaea maderae*

The optic lobes of an insect comprise a set of three consecutive, retinotopic neuropils (Fig 4a). Retinotopic mapping means that the neurons of the optic lobe neuropils form a 2D representation of the spatial distribution of the ommatidia. The outermost of these neuropils, the lamina, receives direct inputs from photoreceptors in the compound eye. The lamina and the medulla, and in turn the medulla and the lobula, the innermost neuropil, are linked by chiasmata. At the frontal medioventral edge of the medulla, a small neuropil with a conspicuous pear-shaped appearance could be localized and was named accessory medulla (AMe, plural AMae).

The AMe of the cockroach *Leucophaea maderae* was proposed as a clock locus (Fig. 4a; Homberg et al. 1991) because of several transplantation and lesion experiments (Nishiitsutsuji-Uwo and Pittendrigh 1968, Roberts 1974, Sokolove 1975, Page 1978, Colwell and Page 1990). The first experiments to locate the pacemaker of the cockroach *Leucophaea maderae* were accomplished by Nishiitsutsuji-Uwo and Pittendrigh (1968). With lesion experiments they showed that the pacemaker that generates circadian locomotor activity is positioned in the optic lobes. Subsequent microlesion experiments in the optic lobe limited the position of the clock to an area between lobula and medulla (Roberts 1974, Sokolove 1975, Page 1978). Later, Page (1982, 1983) showed that cockroaches without optic lobes restored circadian rhythmicity of locomotor activity in case they received whole optic lobes from other cockroaches.

Immunocytochemical studies with an antiserum against the crustacean peptide pigment-dispersing hormone (PDH, the substance causing PDH immunoreactivity in insects is termed
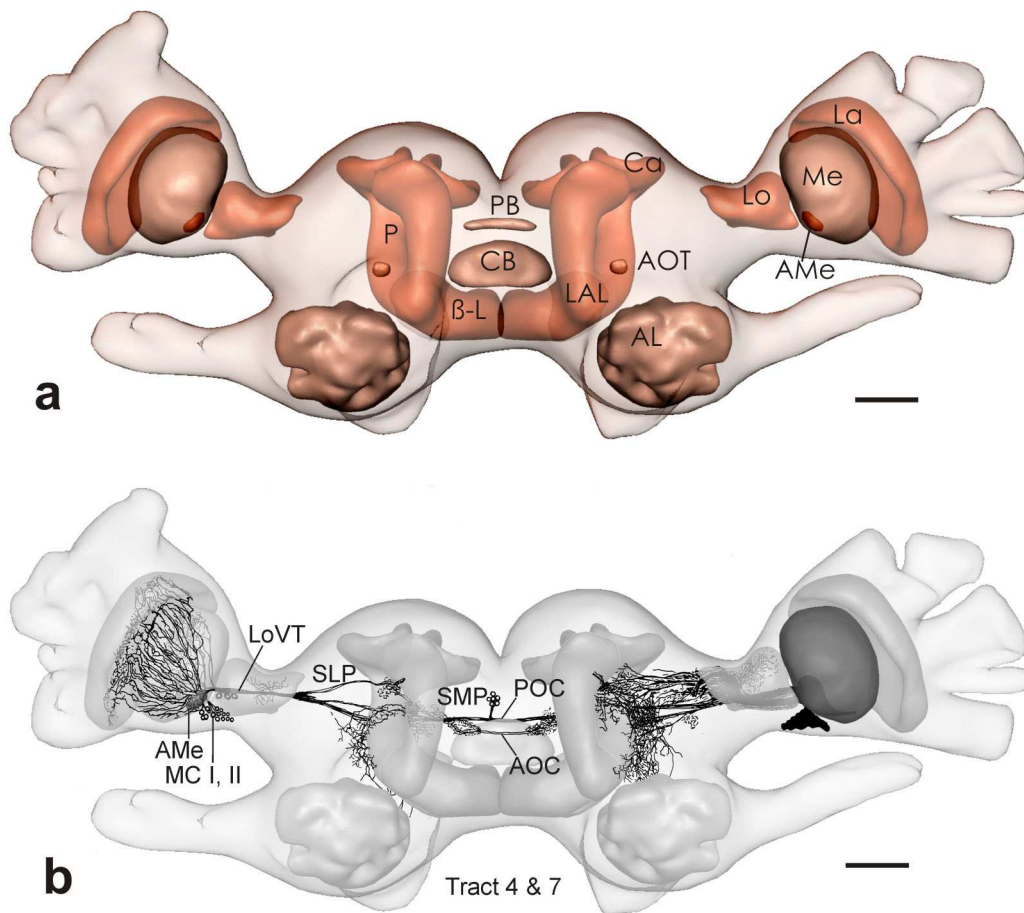
Fig. 4a Three dimensional reconstruction of the cockroach brain (modified after Reischig and Stengl 2002). *AL* antennal lobe, *AMe* accessory medulla, *AOTu* anterior optic tubercle, *ß-L* beta lobe, *Ca* Calyx, *CB* central body, *La* lamina, *LAL* lateral accessory lobe, *Lo* lobula, *Me* medulla, *P* peduncle, *PB* protocerebral bridge. **b** A combination of the reconstruction of tract 4 and 7 closely resembles the arborization pattern of the PDH-immunoreactive neurons. *AMe* accessory medulla, *AOC* anterior optic commissure, *LoVT* lobula valley tract, *POC* posterior optic commissure, *SLP* and *SMP* superior lateral and median protocerebrum. *MC I* cells project through the fan shaped anterior layer fiber system to the lamina and through the LoVT in the POC and in the AOC. *MC II* cells project in middle layers of the medulla and throught the LoVT in the POC (from Reischig and Stengl 2002). *Bars* 200 µm.

pigment-dispersing factor, PDF, Rao 2001) revealed about 16 immunoreactive neurons near the AMe with arborizations in this neuropil (Fig. 4b; Homberg et al. 1991, Reischig and Stengl 1996). The immunoreactive neurons near the AMe were termed PDF-immunoreactive (ir) medulla neurons (PDFMes). They could be further divided into the anterior (about 12 neurons) and posterior (about 4 neurons) PDFMes. The anterior PDFMes are located at the ventro-proximal medulla (Fig. 4b). These PDFMes were discussed as pacemaker neurons in orthopteroid insects, because they are located in the region where the pacemaker was suggested to reside by lesion experiments. Additionally, anterior PDFMes showed widespread axonal projections in the central brain where they might transmit pacemaker information to descending neurons. Subpopulations of the anterior PDFMes appeared to connect both optic lobes and are suggested to couple the bilateral pacemakers (Fig. 4b). Furthermore, the PDFMes have axonal ramifications over the distal surface

of the medulla to the lamina. There, PDFMes are thought to be involved in the circadian modulation of the visual system (Pyza and Meinertzhagen 1997). Indeed, by ectopic transplantation of fully differentiated AMe tissue grafts to optic lobeless cockroaches, Reischig and Stengl (2003a) provided the evidence that the AMe with its associated neurons is the pacemaker center for locomotor activity.

The structure of the accessory medulla of the cockroach *Leucophaea maderae*

The AMe of the cockroach *Leucophaea maderae* is a distinct, non-retinotopically organized neuropil, which consists of a core of nodular and internodular neuropil that is surrounded with coarse neuropil (Reischig and Stengl 1996). It extends about 90 µm over its longitudinal axis (Fig. 5a). The main input pathway into the AMe is the distal tract, which emerges in the medulla and ends in the noduli of the AMe. The lobula valley tract is the main input/output pathway that projects from the internodular and shell neuropil of the AMe to the midbrain and contralateral optic lobe (Reischig and Stengl 2003b). The segregation of the AMe in the nodular and loose neuropil is due to the differential distribution of larger output fibers and fine dendrites. Additionally, nerve terminals with different types of dense core vesicles (DCV, plural DCVs) were located in different subcompartments of the AMe (Reischig and Stengl 1996). DCVs are typical storage sites and intracellular transport vehicles for peptides in neurons (reviewed by Nässel 2002). The nodular neuropil mainly contains terminals with DCVs with a structured granular content, which are often clustered together (Reischig and Stengl 1996). The internodular and shell neuropil mainly hosts terminals with unstructured DCVs (Reischig and Stengl 1996). Probably, nodular and internodular neuropil fulfill different functions in the circadian clockwork. The ipsilateral photic input is transmitted across the distal tract and mainly ends in the nodular neuropil, whereas the contralateral photic input mainly enters the internodular neuropil via the anterior and shell neuropil (Petri et al. 2002; Reischig and Stengl 2003b). In addition, these compartments seem to constitute output regions, and the information of the pacemaker runs through the lobula valley tract to midbrain targets (Reischig and Stengl 2003b).

Anteriorly, ventrally and medially to the AMe lay about 250 AMe associated neurons, which can be assigned to six different groups by morphological criteria (Fig. 5b; Reischig and Stengl 1996, 2003b). These morphological criteria are the heterochromatin contents of the nuclei, cytoplasm staining intensity and the sizes of the nuclei and the somata. Most frontally, the distal and medial frontoventral neurons (DFVNes and MFVNes) are situated. Apparently, these two
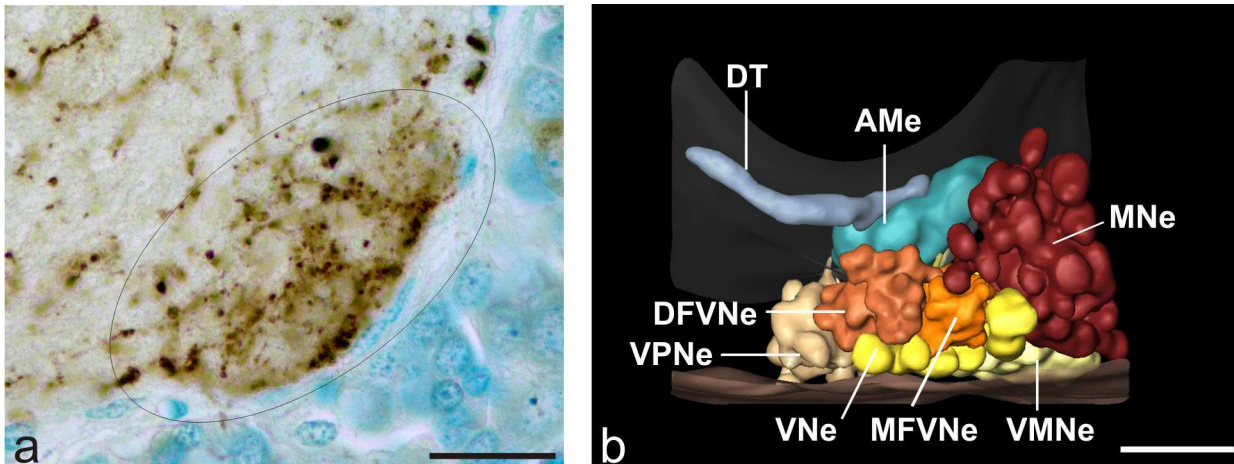
Fig. 5 a Paraffin section of the right accessory medulla (*encircled*) immunostained with an antiserum against FMRFamide. Main immunoreactivity is located in the internodular neuropil. The noduli are only sparsely invaded. *Bar* 50 µm. **b** The accessory medulla and its associated soma groups in a 3D model. *AMe* accessory medulla, *DT* distal tract, *DFVNe* and *MFVNe* distal and medial frontoventral neurons, *VMNe* and *VPNe* ventro-median and ventro-posterior neurons, *MNe* and *VNe* medial and ventral neurons (from Reischig and Stengl 2003b). *Bar* 50 µm.

groups mainly host local neurons of the AMe and medulla. In the group of the DFVNes e. g. mas-allatotropin- and leukokinin-ir neurons are located. Additionally, four PDH-ir neurons can be grouped to the DFVNes. The medial neurons (MNes) represent the largest group. This group is build of functionally and morphologically heterogeneous neurons. Many γ-aminobutyric acid (GABA)-ir neurons belong to this group. More ventrally to the MNe and separated by a glial sheath lay the ventral neurons (VNes). It could be shown that some VNes couple both AMae (Reischig et al. 2004). In this group, eight of the twelve anterior PDFMes are located. The group of the ventro-posterior neurons (VPNes) is heterogeneous like the group of the MNes (Reischig and Stengl 2003b). The last group is named ventro-medial neurons (VMNes). This group also hosts neurons which connect both AMae. Additionally, in the VMNe group light and polarization sensitive neurons are situated (Loesel and Homberg 2001). The posterior PDFMe neurons (pPDFMe) are located posterior to the AMe and associated with this neuropil as well (Petri et al. 1995, Reischig and Stengl 1996, 2003b).

Comparison of *Leucophaea* and *Drosophila* clock neurons

Since the fruitfly *Drosophila melanogaster* is also a very important organism for the research on circadian rhythms, it seems meaningful to compare the AMe and its associated neurons of *Leucophaea maderae* with that of *Drosophila melanogaster*. *Drosophila melanogaster* hosts six groups of clock neurons: three dorsal groups, which are named the dorsal neurons 1-3 (DN_1,
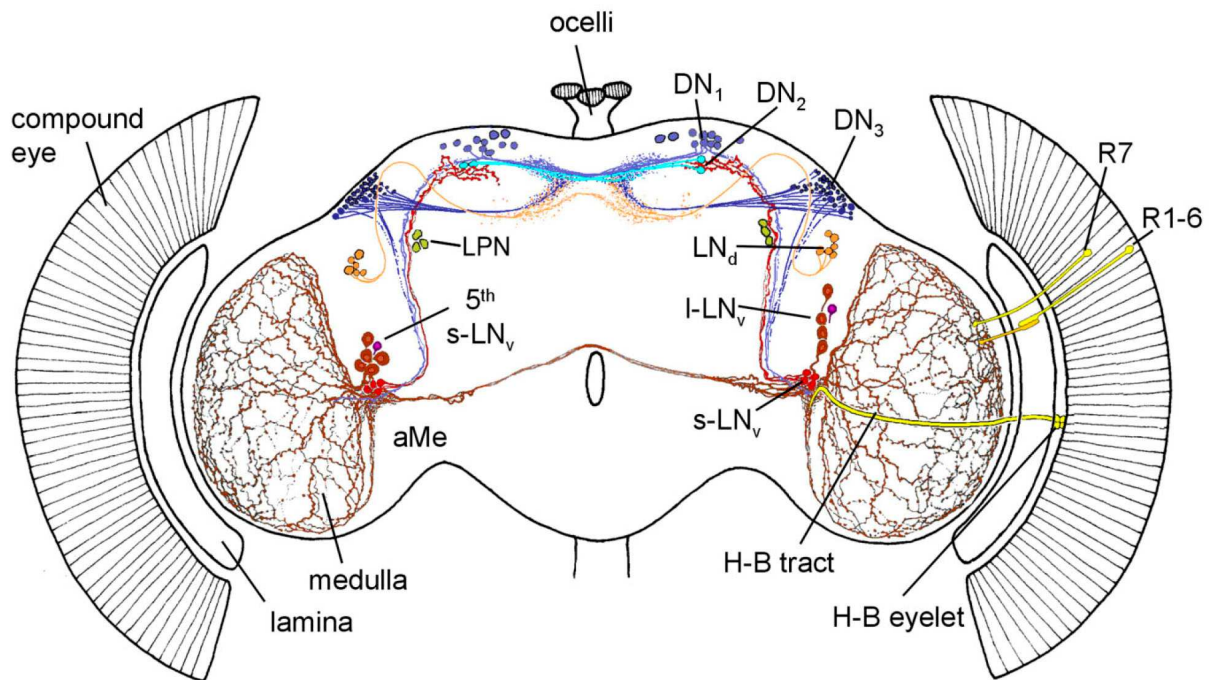
Fig. 6 Clock gene expressing neurons and their projection areas in the *Drosophila* brain. The pacemaker of *Drosophila* is composed of the dorsal neurons 1-3 (*DN₁ - middle blue*, *DN₂ - light blue*, *DN₃ - dark blue*) and the lateral neurons (*LN_d - orange*, *l-LN_v - dark red*, *s-LN_v with PDF - light red* and *s-LN_v without PDF - violet*). Furthermore, three clock gene expressing neurons are located posterior (*LPN*, lateral, posterior neurons, *green*). Apart from the l-LN$_v$, all clock gene expressing neurons send their axons into the dorsal protocerebrum. The AMe is innervated by processes of the DN₁, DN₃, s-LN$_v$ (including the 5th PDF negative one) and l-LN$_v$. The light input pathways from photoreceptor cells R1–6, and R7/8 of the compound eye and from the four Hofbauer-Bucher (*H-B*) eyelet cells are shown in *yellow* (from Helfrich-Förster et al. 2007).

DN₂, DN₃, Fig. 6), and three ventral groups, which are named the dorso-lateral neurons (LN$_{ds}$, Fig. 6) and the large and small ventro-lateral neurons (lLN$_{vs}$ and sLN$_{vs}$; Fig. 6). Whereas all six groups seem to be involved in producing normal behavioral rhythms, the lateral neurons (LNs) appear to be the most important pacemaker neurons (Ewer et al. 1992, Frisch et al. 1994, Helfrich-Förster 1998). LN$_{vs}$ appear to be most important for the generation of circadian rhythms (Helfrich-Förster 1998). Additionally, only the LN$_{vs}$ express PDF (except one sLN$_v$, the PDF-negative 5th sLN$_v$, Kaneko et al. 1997) and these neurons seem to correspond to the PDFMe of *L. maderae* (Helfrich-Förster 1995). The four PDF-positive sLN$_{vs}$ are important for the rhythmic behavior under constant conditions (Helfrich-Förster 1998, Renn et al. 1999, Blanchardon et al. 2001, Nitabach et al. 2002, Grima et al. 2004). Studies in *Leucophaea maderae* suggested that at least a subgroup of the PDFMe is responsible for transmitting the circadian information to midbrain areas and, therefore, the PDFMe appear to be important for exerting circadian locomotor activity (Homberg at al. 1991, Stengl and Homberg 1994, Petri et al. 1995). Regarding the projection patterns of the clock neurons in *Drosophila* revealed that all clock genes expressing neurons except

the lLN$_{vs}$ send their axons into the dorsal protocerebrum. The sLN$_{vs}$ and lLN$_{vs}$, LN$_{ds}$ and some of the DNs have additional projections towards the AMe (Helfrich-Förster et al. 2007). The lLN$_{vs}$ further connect both AMae via fibers in the posterior optic tract and send a network of fibers onto the surface of the medulla (Helfrich-Förster 1997, Fig. 6). Similar PDF-depending coupling pathways were proposed for *Leucophaea maderae* (Petri and Stengl 1997, Reischig et al. 2004). Possibly, lLN$_{vs}$ of *Drosophila* correspond to the VNes of *Leucophaea maderae*, which send their neurites amongst others via the posterior optic commissure to the contralateral AMe and form a network of fibers on the surface of the medulla (Reischig and Stengl 2002). Additionally, like the VNes, the lLN$_{vs}$ appear to be associated with the visual system and, therefore, may transmit information to the AMae and to the eyes (Helfrich-Förster et al. 2007). Besides the lLN$_{vs}$, the LN$_{ds}$ are discussed to exchange photic information between the two brain hemispheres (Helfrich-Förster et al. 2007). Additionally, apparently only the LN$_{ds}$ get input from the contralateral AMe, whereas the lLN$_{vs}$ receive their input only from the ipsilateral AMe (Helfrich-Förster et al. 2007). In summary, the lLN$_{vs}$ and LN$_{ds}$ appear to be morphologically well suited for the transfer of output signals to downstream neurons. Therefore, they are comparable to the PDFMe neurons of the VNe group, which were also discussed to fulfill this function (Reischig and Stengl 2003b).

Whereas the AMe of *Leucophaea maderae* has a pear-shaped appearance, the AMe of *Drosophila melanogaster* is composed of a central part and a ventral elongation area. Furthermore the AMe of *Drosophila melanogaster* lacks the bipartite organization into a core and a shell (Helfrich-Förster et al. 2007). All clock neurons which send fibers to the AMe primarily restrict their arborizations to the central part of the AMe, only dendrites from the lLN$_{vs}$ extend into the ventral elongation area of the AMe (Helfrich-Förster et al. 2007). Thus, there might be a functional difference both between the two parts of the AMe and among the different clock neurons. The same is discussed for the AMe of *Leucophaea maderae*, which is segregated into a core and a shell neuropil and both regions appeared to be involved in different functions (Reischig and Stengl 2003b).

The coupling of the bilaterally organized circadian pacemakers of the cockroach *Leucophaea maderae*

Rhythmicity in behavior and physiology is regulated by neuronal networks in which synchronization or coupling is required to produce coherent output signals. Coupling occurs among individual clock cells within a pacemaker, among functionally distinct subregions within the

pacemaker, between central and peripheral pacemakers and between the pacemakers themselves. First evidence for coupling of the pacemakers was found by lesion experiments of one of the two pacemakers of the cockroach (Page et al. 1977, Page 1978). Surgical isolation or complete excision of one optic lobe increased the free-running period of the rhythm indicating that the pacemakers interact (Page et al. 1977, Page 1978). This also corresponds to the finding that one eye is sufficient to entrain both the ipsilateral and contralateral optic lobe pacemakers (Page et al. 1977). In the cockroach *Leucophaea maderae*, the PDFMe are involved in pacemaker coupling. Reischig and Stengl (2002) identified with horseradish peroxidase backfills as well as with dextran and horseradish peroxidase injections into one AMe seven commissures that connected both optic lobes. Two of these coupled both AMae via the anterior and posterior optic commissure (AOC, POC). These two tracts resembled the arborization pattern of the PDFMe neurons (Fig. 4b). Thus, PDFMe neurons fulfill morphological properties of direct coupling neurons. Injections of PDH into the medulla of the cockroach *Leucophaea maderae* resulted in a monophasic phase response curve with phase delays during the late subjective day (Petri and Stengl 1997). This phase response curve differed considerably from the phase response curve obtained with light pulses, suggesting that PDF is not part of the light entrainment pathway. The data also indicated a role of the PDFMe in coupling and output pathways. Accordingly, tracer injections combined with PDH immunocytochemistry revealed that up to three PDFMe couple both AMae (Reischig et al 2004).

Computer simulations demonstrated that additionally to PDF, which caused phase delays in locomotor activity, another phase-advancing factor is necessary to explain all experimental findings on mutual pacemaker coupling in *Leucophaea maderae* (Petri and Stengl 2001). Since tracing studies showed that anterior AMe neurons of two clusters named MCI (4 neurons of the VNes) and MCII (35 VMNes) connect both AMae (Reischig et al. 2004), and only four of these AMe neurons produce PDF, additional neuropeptides are likely to be involved in the coupling of both AMae. By immunocytochemistry, ten neuropeptides could be localized in AMe associated neurons (Petri et al.1995, Nässel et al. 1991, 1992, 2000, Hofer and Homberg 2006a, Söhler et al. 2007, 2008). One of these neuropeptides is orcokinin, a member of the highly conserved crustacean myotropic neuropeptide family. This neuropeptide could be localized in the somata of the DFVNes, the MNes, the VNes and the VMNes (Hofer and Homberg 2006a). Furthermore, orcokinin was shown to be involved in the coupling of the AMae (Hofer and Homberg 2006a). The tracer injections and orcokinin immunocytochemistry by Hofer and Homberg (2006a) revealed about four orcokinin-ir neurons that connected both AMe; three of them belonged to the VMNe and one to the VNe. Another candidate for the participation in the coupling is a member of the family of the FMRFamide-related peptides (FaRPs). FaRPs represent a large group of neuropeptides with

diverse physiological effects on different target organs (see chapter FaRPs and their possible role in the circadian system). Next to the AMe about 24 FMRFamide-ir somata are located, which could be assigned to the somata of four of the AMe associated neuron groups. These are the DFVNes, the MNes, the VPNes and the VNes (Soehler et al. 2008). FMRFamide was found in the VNes, which are known to be involved in the coupling of both AMae (Soehler et al. 2008). Furthermore, up to six neurons showed a colocalization with PDH (Petri et al. 1995). Thus, members of the FaRPs are potential candidates for circadian coupling. Since FMRFamide-ir was detected in both the anterior and posterior optic commissure (Petri et al. 1995, Soehler et al. 2008) coupling probably depends on both PDF and FaRPs. This hypothesis was tested by neurobiotin backfills combined with FMRFamide- and PDF-immunocytochemistry (Chapter III).

The roles of neuroactive substances in the accessory medulla

Immunocytochemical studies revealed that neurons associated with the AMe express a variety of neuropeptides in an exceptional high concentration (Würden and Homberg 1995, Petri et al. 1995, 2002, Reischig and Stengl 1996, Nässel et al. 2000, Hofer and Homberg 2006a, Söhler et al. 2007, 2008). Nine different neuropeptides were so far detected in the AMe by means of immunocytochemical techniques: allatostatin, allatotropin, baratin, corazonin, FaRPs, gastrin/cholecystokinin, leucokinin, orcokinin and PDF (Petri et al. 1995; Nässel et al. 1991, 1992, 2000, Hofer and Homberg 2006b, Söhler et al. 2007, 2008). Furthermore, the neurotransmitters GABA, histamine, and serotonin were located in the AMe (Petri et al. 1995, 2002; Loesel and Homberg 1999). The function of all these neuropeptides and transmitters and their role in rhythm generation, entrainment, coupling of the pacemakers, and/or transmission of phase information to downstream effectors in the circadian system is largely unknown. Most is known about the role of PDH (Dircksen et al. 1987). PDH is an octadecapeptide first found in crustaceans where it causes pigment dispersion (Rao and Riehm 1989). Several studies on insects revealed a prominent role of PDF in the circadian clock (Petri and Stengl 1997, Renn et al. 1999, Park et al. 2000). In the fruit fly *Drosophila melanogaster*, it was shown that PDF acts as an output factor of the main pacemaker neurons that comprise the circadian clock (Renn et al. 1999, Park et al. 2000). In *Leucophaea maderae,* a dual role for PDF as output and coupling signal was proposed. Injections of PDF into the optic lobes of cockroaches monitored in a running-wheel assay resulted in a monophasic PRC with a maximum phase delay at the late subjective day (Petri and Stengl 1997). As the resulting PRC differed from that obtained with light pulses, it was assumed that PDF is not

involved in transmitting light information, but rather functions as output signal of the two pacemakers. Later, dextran tracing studies and double-labeling of commissural neurons with PDH antiserum revealed three PDH-ir neurons that interconnect both AMae (Petri 1998, Reischig and Stengl 2002). Injections of allatotropin and GABA in the vicinity of the AMe both resulted in PRCs comparable to that obtained with 6 h light pulses. This finding suggested that both substances are involved in light entrainment (Page and Barret 1989, Petri et al. 2002). Furthermore, GABA- and Mas-allatotropin immunoreactivity was mainly found in the noduli of the AMe (Petri et al. 1995, 2002). Since neurons with excitatory responses to light stimuli also innervate the noduli it was proposed that the noduli process light information (Loesel and Homberg 2001). The same is true for orcokinin. Injections of orcokinin near the AMe resulted in a PRC similar to the PRC obtained with light pulses (Hofer and Homberg 2006a). Thus, the orcokinin-ir neurons may play a role in the light entrainment of the circadian clock via the contralateral compound eye. Immunostaining and injection experiments with serotonin suggested that serotonin is not involved in light entrainment (Page 1987, Petri et al. 1995). Within the AMe serotonin immunoreactivity is concentrated in the coarse neuropil and appears to omit the noduli (Petri et al. 1995). This fits with the results from injection experiments with serotonin. The obtained PRC is different from the light-dependent PRC (Page 1987). However, other pharmacological experiments in crickets suggested an involvement of serotonin in light entrainment (Saifullah and Tomioka 2002, Yuan et al. 2005). Since serotonin-ir neurons could be assigned to local interneurons that correspond to the DFVNes and/or MFVNes of *Leucophaea maderae*, they may transmit light information from visual inputs to the pacemaker neurons (Würden and Homberg 1995, Petri et al. 1995, Homberg and Würden 1997). The role of the remaining neuropeptides detected immunocytochemically in the AMe is unclear, because physiological data are missing. Until now the four neuropeptides PDF, allatotropin, serotonin and orcokinin and the neurotransmitter GABA were injected near the AMe. While the injections of allatotropin, orcokinin and GABA resulted in biphasic PRCs which were comparable to the PRC obtained with light pulses (Petri et al. 2002, Hofer and Homberg 2006a, Fig. 1g), the PDF and serotonin injections resulted in all delay PRCs (Fig. 1h, Page 1987). Since computer models of coupled PER/TIM feedback loops indicated two antagonistic acting coupling forces to achieve the experimentally observed coupling properties (Petri and Stengl 2001), at least one other neuropeptide has to be involved in the function of the circadian clock. Injections of this so far unidentified neuropeptide should then result in an all advance PRC. Thus, another phase advance-causing factor must exist to achieve a stable period length of about 24 h. This factor might be a member of the FaRPs.

FaRPs and their possible roles in the circadian system

As described above, peptides appear to play important roles in clock function and, therefore, the identification of the peptides of the AMe and revealing their function is important for the understanding of the circadian system. Immunocytochemical studies revealed additionally to PDH-ir neurons about 30 FMRFamide-ir neurons associated with the AMe (Petri et al. 1995). The FMRFamide antiserum used in this study detected the RFamide C-terminus which is shared by all members of the FaRP family and, therefore, did not distinguish between single members of the FaRPs. FaRPs are phylogenetically diverse, but structurally similar peptides. Members of this group generally share the C-terminal RFamide while their N-terminal extensions vary in structure and length (Orchard et al. 2001, Nässel 2002, Predel 2006). FaRPs belong to the most extensively studied neuropeptides of invertebrates, and a wealth of information is available regarding structure, localization in the nervous system and physiological effects (Orchard et al. 2001, Nässel 2002, Predel 2006). The FaRP family can be divided into subfamilies including the N-terminally extended FMRFamides (among them the extended FIRFamides), myosuppressins (extended FLRFamides), sulfakinins (extended HMRFamides with a sulfated tyrosine residue) and neuropeptides F (short neuropeptides F, sNPFs, extended RLRFamides, and long NPFs, lNPFs). Three members of the FaRPs were so far identified in the cockroach *Leucophaea maderae*: leucomyosuppressin (pQDVDHVFLRFamide, Holman et al. 1986) and leucosulfakinin I and II (EQFEDY(SO3H)GHMRFamide, Nachman et al. 1986a, b). A member of the sNPFs subgroup was identified in the cockroach *Periplaneta americana* and named head peptide (Veenstra and Lambrou 1995). Since FaRPs were detected in the AMe of the cockroach *Leucophaea maderae* (Petri et al. 1995), this dissertation is aimed to get insights into the role of different members of the FaRP family in the circadian clock. Therefore, first the distribution of FMRFamide-, LMS-like-, sNPF- and perisulfakinin-ir neurons in the six distinguishable soma groups of the AMe was examined with different antisera. The anti-FMRFamide antiserum identified about 100 immunoreactive neurons in the optic lobes roughly arranged in four clusters. Two of these clusters were associated with the lamina, a group of weaker stained neurons was scattered dorsally between medulla and lobula, and one cluster was located adjacent to the AMe. Other neuropils of the optic lobes revealed FMRFamide immunoreactivity as well. The lamina showed strong staining at the proximal face and faint staining at the distal face. The medulla showed immunoreactivity in the characteristic fiber fan along the anterior surface which connects the AMe to the medulla and lamina additionally some medial layers showed immunoreactivity.

About 13 of the about 24 (23.7 ± 9.8 SD) AMe associated FMRFamide-ir neurons detected in our study (Soehler et al. 2008) could be assigned to the VNes, about nine to the DFVNes, and about two could be assigned to the VPNes and the MNes, respectively. Two immunoreactive neurons were located anterior to the neurons of the six soma groups, and hence named anterior neurons (ANes). The AMe itself expressed strong FMRFamide immunoreactivity in the anterior neuropil and in the coarse neuropil around the noduli. Except two to three noduli which showed strong staining, the noduli themselves were only sparsely, but homogenously invaded by FMRFamide-ir fibers.

Since LMS was found in the brain of *Leucophaea maderae* and LMS is a well studied member of the FaRPs, it was examined whether some of the FMRFamide-ir neurons could also synthesize LMS. Besides the fact that Meola et al. (1991) did not find LMS immunoreactivity in the optic lobes of the cockroach *Leucophaea maderae*, the employment of a new antiserum against the six aminoacids of the N-terminus of dromyosuppressin (DMS, the myosuppressin of *Drosophila melanogaster*) justified a reevaluation of the findings. The DMS antiserum allowed a clear distinction of myosuppressins from other FaRPs (McCormick and Nichols 1993). Furthermore, studies in other insect species including the cockroach *Diploptera punctata* detected LMS in the optic lobes (McCormick and Nichols 1993, Donly et al. 1996, Nichols et al. 1997, Fuse et al. 1998, Richer et al. 2000). By using this antiserum, about two LMS-like-ir neurons associated with the AMe could be detected in our study. One could be assigned to the ANes and one to the VNes. The AMe itself primarily showed LMS-like immunoreactivity in the loose and internodular neuropil. Only one nodulus showed LMS-like immunoreactivity. The medulla also expressed LMS-like immunoreactivity in the characteristic fiber fan and in one medial layer. The Pea-sNPF antiserum located about two immunoreactive neurons in the group of the VNes and about one in the group of the ANes. Inside the AMe the internodular neuropil as well as some antero-ventral noduli showed strong immunoreactivity. With the perisulfakinin antiserum no immunoreactive neurons could be detected in the optic lobes.

The results obtained with immunocytochemical techniques could partially be confirmed with a spectroscopic method named Matrix Assisted Laser Desorption/Ionisation-Time of Flight (MALDI-TOF). By using this method, we confirmed the existence of LMS and obtained strong indications for Pea-sNPF present in the AMe. Additionally, another peptide with high sequence similarity to the Pea-FMRFa-7 (DRSDNFIRFamide, firstly identified in the cockroach *Periplaneta americana,* Predel et al. 2004) could be identified in the AMe of *L. maderae*. Since a peptide homolog of the Pea-FMRFamide-7 could be identified in the thoracic perisympathetic organs of *Leucophaea maderae*, it seems likely that this peptide is present in the AMe as well.

To examine a possible circadian function of different members of the FaRPs, the tetrapeptide FMRFamide and Pea-FMRFa-7 were injected into the vicinity of the AMe. The injections revealed that these peptides were involved in the circadian control of the locomotor activity. Injections of 100 fmol FMRFamide resulted in significant phase delays at two circadian times (CT 8 and CT 18) and injections of 150 fmol Pea-FMRFa-7 resulted in a significant phase delay at CT 4. Thus, the results obtained with immunocytochemistry, mass spectrometry and bioassays indicate a role of several FaRPs in the circadian system of this cockroach. These studies are described in Chapter I and II.

Since the staining with different antisera of output neurons implies a role of FaRPs in the coupling of both pacemakers, Chapter III and IV are concerned with detailed colocalization studies and corresponding behavioral assays. Backfills from one optic stalk were performed and combined with immunolabeling with anti-PDF and either anti-FMRFamide or anti-Asn[13]-orcokinin. These studies revealed that at least four VNes connect both AMae. One of these VNes belongs to the large PDFMes the others belong to the medium-sized PDFMes. All of the contralaterally projecting medium-sized PDFMe appeared to contain FMRFamide, orcokinin, and PDF. The large PDFMe probably contains only PDF. With the behavioral assays it should be clarified whether the two commissures that connect both AMae, i.e. the AOC and POC, carry the same information from one AMe to the other. Therefore, the AOC, the POC or the central brain were cut and it was examined if these transections had different effects on the locomotor activity. Comparison of rhythm, phase and period before the transections of the anterior and posterior optic commissures respectively with rhythm, phase and period after the transections resulted in no significant changes. Transection of the central brain resulted in most cases in a permanent locomotor activity.

Abbreviations

AMe(ae)	accessory medulla(e)
ANe(s)	anterior neuron(s)
AOC	anterior optic commissure
CT	circadian time
DCV(s)	dense core vesicle(s)
DD	constant darkness
DFVNes	distal frontoventral neurons
DMS	dromyosuppressin
DN(s)	dorsal neuron(s)
FaRPs	FMRFamide-related peptides

GABA	γ-aminobutyric acid
-ir	immunoreactive
lLN_{vs}/sLN_{vs}	large/small ventro-lateral neurons
LMS	leucomyosuppressin
LN_{ds}	dorso-lateral neurons
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation-Time Of Flight
MFVNes	medial frontoventral neurons
MNes	medial neurons
PDF	pigment-dispersing factor
PDFMe	PDF-ir medulla neurons
PDH	pigment-dispersing hormone
PER	PERIOD
POC	posterior optic commissure
PRC(s)	phase response curve(s)
s/lNPF(s)	short/long neuropeptide(s) F
TIM	TIMELESS
VMNes	ventro-median neurons
VNes	ventral neurons
VPNes	ventro-posterior neurons
ZT	Zeitgeberzeit

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Chapter I. Localization of leucomyosuppressin in the brain and circadian clock of the cockroach *Leucophaea maderae*

Localization of leucomyosuppressin in the brain and circadian clock of the cockroach *Leucophaea maderae*

Sandra Söhler • Susanne Neupert • Reinhard Predel •
Ruthann Nichols • Monika Stengl

Abstract The myosuppressins (X1DVX2HX3FLRFamide), which reduce the frequency of insect muscle contractions, constitute a subgroup of the FMRFamide-related peptides. In the cockroach *Leucophaea maderae*, we have examined whether leucomyosuppressin (pQDVDHVFLRFamide) is present in the accessory medulla, viz., the circadian clock, which governs circadian locomotor activity rhythms. Antisera that specifically recognize leucomyosuppressin stain one to three neurons near the accessory medulla. MALDI-TOF mass spectrometry has confirmed the presence of leucomyosuppressin in the isolated accessory medulla. Injections of 1.15 pmol leucomyosuppressin into the vicinity of the accessory medulla at various circadian times have revealed no statistically significant effects on the phase of circadian locomotor activity rhythms. This is consistent with the morphology of the myosuppressin-immunoreactive neurons, which restrict their arborizations to the circadian clock and other optic lobe neuropils. Thus, leucomyosuppressin might play a role in the circadian system other than in the control of locomotor activity rhythms.

S. Söhler · M. Stengl (✉)
Department of Biology, Animal Physiology,
Philipps University Marburg,
35032 Marburg, Germany
e-mail: stengl@staff.uni-marburg.de

S. Neupert · R. Predel
Department of General Zoology and Animal Physiology,
Friedrich Schiller University,
07743 Jena, Germany

R. Nichols
Department of Biological Chemistry,
University of Michigan Medical School,
Ann Arbor MI 48109-0606, USA

Introduction

Neuropeptides are widespread signaling molecules in the nervous system, but only little is known about their specific functions and mechanisms of actions in information processing (Nässel 2000, 2002). The family of the FMRFamide-related peptides (FaRPs) are especially abundant and occur in diverse groups (Nässel 2000; Orchard et al. 2001). They share the common C-terminal structure of -RFamide and are subgrouped into the extended FLRFamides (including the myosuppressins), the extended HMRFamides (sulfakinins), the extended FMRFamides, and the extended RFamides (including the head peptides; Nässel 2002; Orchard et al. 2001). Despite their sequence similarity, the FaRPs in *Drosophila melanogaster* are expressed by different precursor genes, indicating their different evolutionary origin and possibly their different functions (Nichols 2003; Vanden Broeck 2001).

Immunocytochemical studies with an antibody against the C-terminal sequence of FMRFamide have identified a group of about 35 immunoreactive neurons in the optic lobe of the cockroach *Leucophaea maderae*, indicating the presence of FaRPs but without identifying the specific neuropeptide subgroups (Petri et al. 1995). The FMRFamide-immunoreactive (FMRFamide-ir) neurons are associated with the accessory medulla (AMe; plural: AMae), a small neuropil at the anterior edge of the medulla (Reischig and Stengl 2003b). Lesion and transplantation studies have demonstrated that the AMe, with its associated 250 neurons arranged in

six cell clusters, is the circadian clock in the cockroach brain (Reischig and Stengl 2003b; Stengl and Homberg 1994). The circadian pacemaker center controls circadian locomotor activity rhythms via pigment-dispersing factor-ir (PDF-ir) processes in the superior lateral protocerebrum (Reischig and Stengl 2003a; Stengl and Homberg 1994). Some of these PDF-ir neurons colocalize FMRFamide-immunoreactivity, but the members of the FaRPs that they might contain remain unknown (Petri et al. 1995).

The myosuppressins were the first FaRPs identified from head extracts of the cockroach *Leucophaea maderae* (Holman et al. 1986). They are myoinhibitory peptides known to be expressed in the brain and in the gut (Fuse et al. 1998; Holman et al. 1986; Kaminski et al. 2002; Predel et al. 2001; Richer et al. 2000). Nevertheless, the function of leucomyosuppressin (LMS; pQDVDHVFLRFamide) in the central nervous system itself remains unclear.

An antiserum generated against the N-terminal amino acids of *D. melanogaster* myosuppressin, which are shared by the myosuppressin of *L. maderae* (see below) but which distinguishes myosuppressins from other FaRPs (McCormick and Nichols 1993) has been employed to search for LMS-ir neurons among the FMRF-ir neurons in the circadian system of *L. maderae*. The immunocytochemical demonstration of LMS in AMe neurons indicates a possible role of LMS in the circadian system. MALDI-TOF mass spectrometry of the isolated AMae has confirmed the presence of native LMS in the circadian clock, indicating the specificity of the LMS-antisera. Injection studies combined with running-wheel assays and immunocytochemistry have shown that LMS does not affect circadian locomotor activity rhythms daytime-dependently. This is consistent with the morphology of the LMS-ir cells, which do not share the branching pattern of PDF-ir neurons but are local optic lobe interneurons.

Materials and methods

Animals

Adult male cockroaches (*Leucophaea maderae*) were taken from laboratory colonies. They were reared under a 12:12 h light-dark (LD) photoperiod with lights on at 6 pm, at about 60% relative humidity and a temperature of 26°C. The animals were fed with dried dog food, potatoes, and water *ad libitum*.

Sample preparation for MALDI-TOF mass spectrometry

The cockroaches were anesthetized with ice water for several seconds and decapitated. Brains were dissected, and

the perineurium above the AMe was opened with iridectomy scissors. Fine glass pipettes were prepared from borosilicate glass capillaries (Harvard, UK; inner diameter: 1.17 mm) with an edged tip opening of ca. 100 μm. The capillary was connected to flexible tubing to apply negative pressure by mouth suction. The tip was placed on the surface of the optic lobe above the AMe. The location of the AMe (diameter: ~100 μm) was easily recognized by external markers (Petri and Stengl 1997). The isolated AMe was placed directly onto a stainless steel sample plate for MALDI-TOF mass spectrometry and allowed to dry at room temperature. All samples were washed with a drop of water for several seconds, air- dried once again, and covered with approximately 20 nl α-cyano-4-hydroxycinnamic acid solution dissolved in 80:20 methanol:water (v:v).

MALDI-TOF mass spectrometry

Mass spectra were obtained in the positive ion mode on a Voyager Pro-DE biospectrometry workstation (Applied Biosystems, Framingham, USA) equipped with a pulsed nitrogen laser emitting at 337 nm. The excised AMae were analyzed in reflectron mode by using a delayed extraction time of 150 ns, a 75% grid voltage, a 0.06%-0.1% guide wire voltage, and an accelerating voltage of 20 kV. Laser strength was adjusted to provide an optimal signal to noise ratio. External mass spectrum calibration was first performed by using synthetic cockroach peptides (Pea-pyrokinins 2/5; SPPFAPRLa/GGGGSGETSGMWFGPRLa).

Immunocytochemistry

Brains were dissected and fixed for 4 h in a formaldehyde/ picric acid solution (aqueous Bouins solution modified after Hollande as described by Romeis 1989), washed in clear water, dehydrated in an ethanol series, and embedded in paraffin (Paraplast plus; Sigma, Germany). Serial frontal sections (10 μm thick) were cut as ribbons, mounted on microscope slides, deparaffinized with xylene, and rehydrated through graded ethanols. LMS was detected by using a sensitive three-step peroxidase technique according to Sternberger (1979) and Reischig and Stengl (1996). The polyclonal antibody used was generated against TDVDHV, the N-terminal extension of dromyosuppressin (DMS), which distinguishes myosuppressin peptides from FaRPs (Nichols et al. 1997). LMS (pQDVDHVFLRFamide) and DMS (TDVDHVFLRFamide) differ by only one amino acid at the N-terminus. The concentrations of the anti-myosuppressin antisera used were 1:50, 1:80, and 1:100. Detection of peroxidase was carried out with 3,3´-diaminobenzidine/H_2O_2 as chromogen. To visualize non-immunoreactive tissues, the sections were counterstained in 1% methylene blue. Antibody controls consisted of tissues

incubated in the absence of myosuppressin antisera with normal rabbit serum, or with antigen-inactivated antiserum in which the antisera had been preincubated with 10 µg/ml LMS peptide, a procedure that removed all staining.

Behavioral experiments

Operations and injections

The operations and injections of the cockroaches were accomplished as in Petri and Stengl (1997). We first tested a dose of 0.0115 fmol LMS, because this was the lowest dose to cause significant phase shifts in Petri and Stengl (1997). In our first injection series, this dose resulted in no significant phase shifts, and thus we used higher doses. Since the peptide is apparently released into the hemolymph, we assumed that it might be present in higher concentrations as compared to PDF, which appeared to be released focally at the terminals of the PDF cells. Thus, we used 1.15 pmol LMS in 2 nl hemolymph-Ringer (HLR) with 10% blue food dye (McCormick, Baltimore, MD) for the phase response curve, as indicated. LMS (pQDVDHVFLRFamide, MW=1257.61) was obtained from Peninsula Laboratories. Control injections consisted of 10% blue food dye in HLR without LMS (negative controls).

Behavioral assays and data analysis

Recording of locomotor activity and the subsequent analysis of data was described previously (Reischig and Stengl 2003a). Data were evaluated from 38 of the 57 injected animals. The remaining 19 animals were excluded from further analysis because they showed little activity after injection, exhibited strong changes in the length of periodicity, or died within 1 week after operation. A high percentage of injected animals could not be evaluated because of a breakdown in the monitoring computer. Phase shifts were determined as time differences between the regression lines, before and after injection, extrapolated to the day after treatment as described in Petri and Stengl (1997).

The behavioural data were merged into 3-h time intervals, and the means and standard deviations (SD) were calculated for each bin. Changes of phases and periods in a given interval were considered to be significantly different from zero if the calculated 95% confidence interval (CI) of the respective time interval did not contain the value zero. The phase and period changes were statistically analyzed by a two-tailed Students *t*-test. Significance was taken as $P<0.05$. Statistical analyses were performed with Superior Performing Software systems (SPSS 11.0) and Excel XP (Microsoft). The phase response curve was produced with Excel.

Results

To examine whether LMS was present in the circadian pacemaker center of the cockroach, MALDI-TOF mass spectrometry was employed on excised AMae. In addition, to test whether LMS occurred in any of the approximately 35 FMRFamide-ir neurons, such as the colocalized PDF-ir cells near the AMe, anti-DMS antisera were used on paraffin sections of the cockroach brain. Injection of LMS into the vicinity of the AMe combined with running-wheel assays was used to examine whether LMS, like PDF, affects circadian locomotor activity rhythms of the cockroach.

Mass spectrometric analysis of excised AMae

A MALDI-TOF mass spectrometric analysis (mass range of 900–2500 Da) of single excised AMae from adult cockroaches revealed the occurrence of LMS ([M+H]+ of 1257.64 Da; *n*=20; Fig. 1).

Immunocytochemistry

After identification of LMS in excised AMae, immunocytochemistry was employed to determine the number and type of neurons containing LMS in the AMe. A polyclonal antiserum raised against TDVDHV, viz., the N-terminal amino acids of DMS (the myosuppressin of *D. melanogaster*), was used on paraffin-embedded sections of cockroach brain (McCormick and Nichols 1993). The six amino acids used as the antigen distinguish myosuppressin
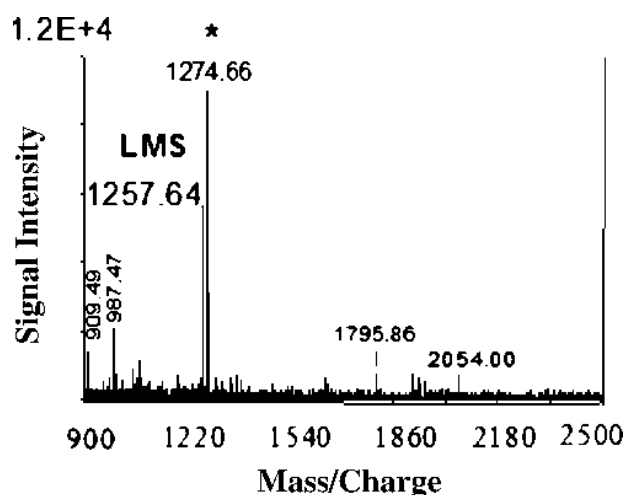

Fig. 1 MALDI-TOF mass spectrometry from a single accessory medulla of a male *L. maderae* shows the presence of leucomyosuppressin (LMS) with a mass of [M+H]+ of 1257.64 Da (*asterisk* mass of LMS, which contains a pyroglutamyl, a posttranslational modification and the cyclization of an N-terminal glutamine (QDVDHVFLRF-NH2), with an [M+H]+ of 1274.66 Da).

peptides from other FaRPs. Omission of primary antisera from the procedure abolished immunoreactivity (n=3), as did preabsortion of the diluted antibody with 10^{-4} M LMS (not shown; n=3). LMS-like immunoreactivity was detected in about 154 somata in the central brain of the cockroach (Fig. 2). LMS-like immunoreactive (LMS-lir) somata of various sizes could be observed in the anterior, lateral, and posterior cell cortex of the midbrain. In the anterior cell cortex, approximately four large neurons (about 30–40 µm in diameter) were found with undiscerned arborizations. One third of all stained neurons were concentrated in or next to the pars intercerebralis (45 ± 11, n=7; Fig. 3a–f). Approximately 12 large neurons (about 30–40 µm in diameter) in the pars intercerebralis expressed strong myosuppressin-like immunoreactivity. Axons to and from the pars intercerebralis connected this brain region with the ventral nerve cord and the retrocerebral complex via the NCC1 median bundle (Fig. 3e). Prominent staining was also visible in the tritocerebrum.

All major neuropils except the mushroom bodies showed myosuppressin-like immunoreactivity (Fig. 3a). The upper unit of the central body expressed locally restricted staining, whereas the lower unit and the protocerebral bridge were free of staining (Fig. 3a). Moreover, LMS-lir staining was seen in the anterior lip of the central body (Fig. 3f) and in commissural fibers posterior of the central body (Fig. 3b). The lateral accessory lobes (important projection areas of the neurons of the central body) expressed weak LMS-like immunoreactivity (Fig. 3a). In addition to weak staining in the antennal mechanosensory

and motor center (Fig. 3g), all glomeruli of the antennal lobes were stained via a few LMS-lir interneurons (2.7 ± 1.1, n=6; Fig. 3a). Prominent LMS-lir arborizations occurred in the superior lateral and medial protocerebrum and in the inferior lateral protocerebrum (Fig. 3a). In the ventrolateral protocerebrum, only faint staining was recognizable. Both the anterior and the posterior optic commissure showed immunoreactivity (Fig. 3c,d), with only small fibers in the anterior optic commissure.

Various neuropils in the optic lobe were innervated by sparse LMS-lir fibers apparently originating from few immunoreactive cell bodies next to the AMe (Figs. 3h–k, 4b). Immunoreactive fibers that connected the lamina and the AMe projected over the distal surface of the medulla in a fan-shaped manner (Fig. 3i–k). Furthermore, immunoreactive fibers were observed in one of the proximal medulla layers before the serpentine layer (Fig. 4b, large arrowheads). All six evaluated preparations showed at most one LMS-lir anterior neuron with its soma anterior to the lobula and, near the AMe, one ventral neuron (VNe, n=5) or two VNes (n=1) expressed LMS-like immunoreactivity in the left optic lobe (the nomenclature for anatomical orientation is used according to the longitudinal axis of the animal). In the right optic lobe, no VNes (n=1), one VNe (n=4), or three VNes (n=1) were immunoreactive (Fig. 4b). Although we could not discern whether the anterior neurons also arborized in the AMe, the LMS-lir processes of the VNes arborized mostly in the loose and internodular neuropils of the AMe (Fig. 4b, insert) and entered only one ventral nodulus (Fig. 4b, insert). The highest density of
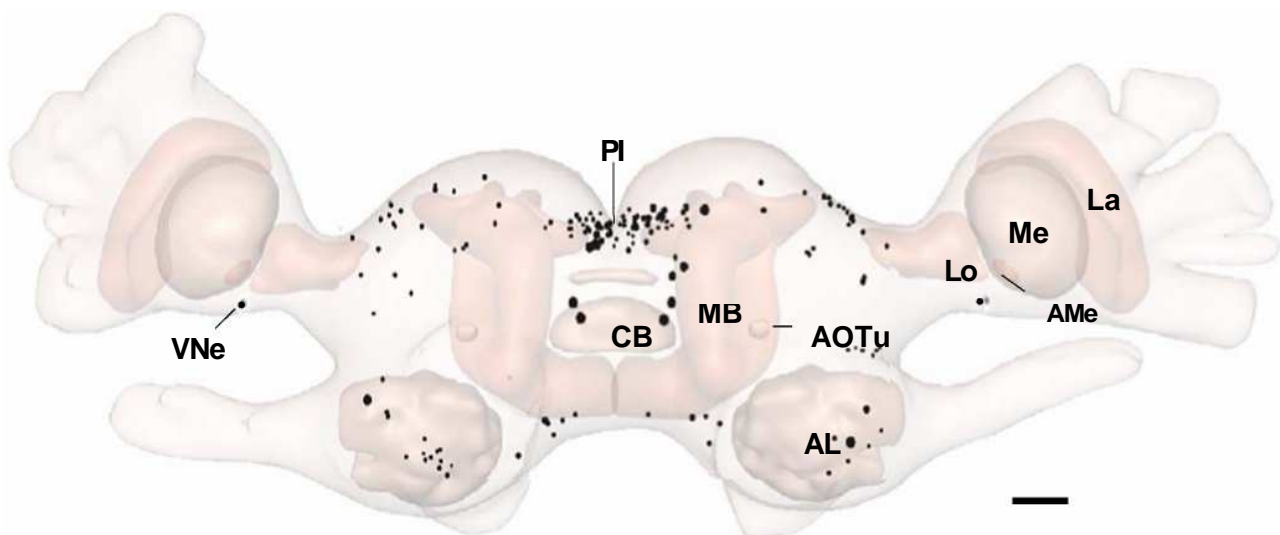
Fig. 2 LMS-like-immunoreactive (LMS-lir) cell bodies reconstructed from one brain of a male cockroach (*L. maderae*). Surface reconstruction of contours of brain structures and prominent neuropils were generously provided by T. Reischig. Only strongly immunostained somata are included. Only one immunoreactive ventral neuron (*VNe*) was found in the optic lobes near the accessory medulla (*AL* antennal lobe, *AMe* accessory medulla, *AOTu* anterior optic tubercle, *CB* central body, *La* lamina, *Lo* lobula, *MB* mushroom body, *Me* medulla, *PI* pars

Fig. 3 a–k LMS-lir cells in the brain of *L. maderae*. Frontal sections labeled with anti-DMS and detected with the peroxidase-antiperoxidase technique. **a** Overview of the left hemisphere of the central brain with immunostaining in the antennal lobe (*AL*), central body (*CB*), lateral accessory lobe (*LAL*), superior medial (*black arrowhead*), and superior lateral (*SLP*) protocerebrum. No immunoreactivity was found in the calyces (*Ca*) and peduncle (*P*) of the mushroom body (*bL* β-lobe). **b** LMS-lir in commissural fibers (*arrow*) posterior of the central body. **c,d** The anterior optic commissure (**c**, *arrow*) and posterior optic commissure (**d**, *arrow*) show immunostaining (*SMP* superior median protocerebrum, *VMP* ventro-median protocerebrum). **e** Four large neurons of about 40 µm diameter (*arrowheads*) and a group of three smaller neurons of about 20 µm (*small black arrow*) show strong LMS-like immunostaining in the pars intercerebralis. Immunoreactive fibers of the median bundle are visible (*large gray arrow*). **f** Anterior lip of the central body showing prominent staining (*arrow*). **g** Faint staining was visible in the antennal mechanosensory and motor center (*AMMC*, *arrowhead*). **h** In the optic lobe (*La* lamina, *Me* medulla), LMS-like immunoreactivity was observed in the accessory laminae (*arrowhead*). **i–k** Series of three consecutive sections showing sparse immunoreactive fibers (*arrowheads*) projecting in an anterior fan over the face of the medulla (*Me*) toward the lamina (*La*). *Bars* 100 µm.
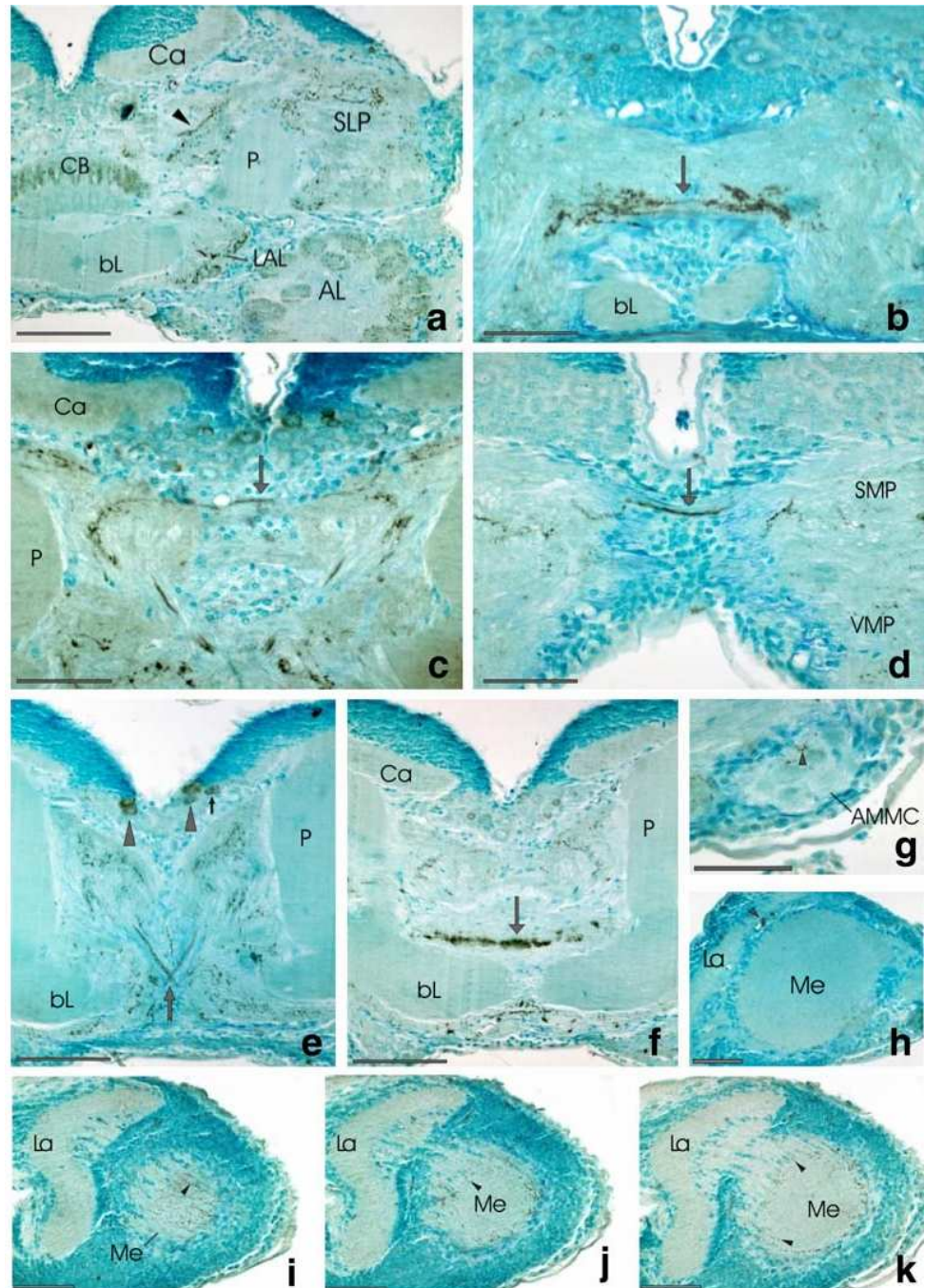
LMS-lir fibers did not occur in the AMe but rather in the anterior fiber network covering the AMe frontally (Fig. 4b).

Effects of LMS injections on the phase of the rhythm of circadian locomotor activity

By injections combined with running-wheel assays, we examined whether LMS acts as an input signal into the circadian clock, which affects locomotor rhythms. The peptide was injected in constant darkness at various circadian times (CTs) into the vicinity of the AMe (see Materials and methods). In running-wheel assays, possible time shifts in the onset of locomotor activity after the LMS injections were evaluated. As judged from the 95% CIs, LMS did not cause significant phase shifts at any CT tested (every 3 h from a CT of 0:00–24:00, $n=38$; Table 1, Fig. 6). Control injections ($n=45$) of 10% blue dye food in HLR induced no significant phase changes, except for a
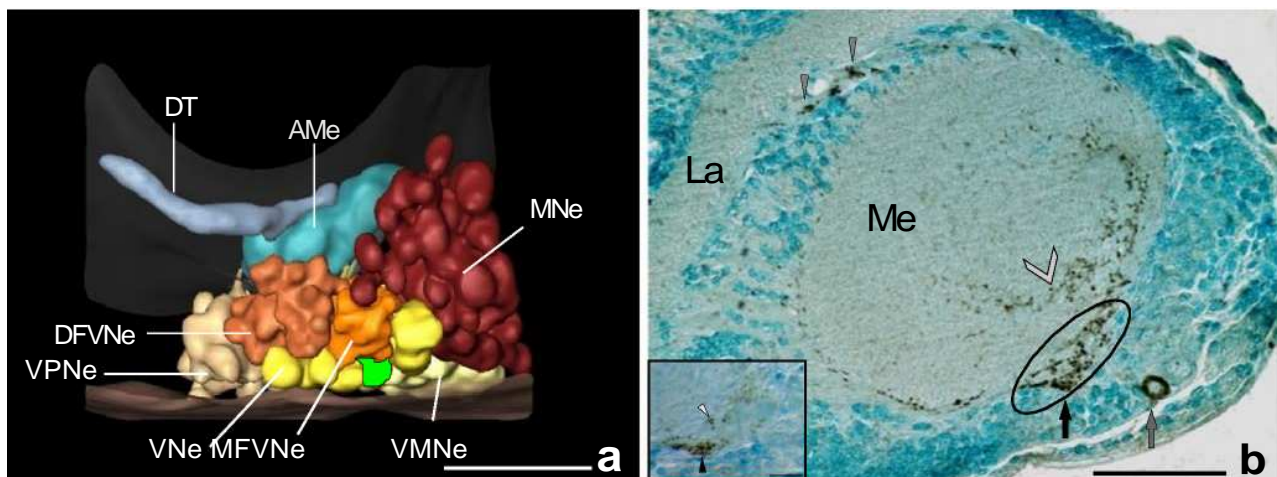
Fig. 4 a Three dimensional model of the right accessory medulla (*AMe*) with adjacent soma groups and incoming distal tract (*DT*). The neuron marked in green represents the location of the LMS-lir neuron (*DFVNe* distal group of frontoventral neurons, *MFVNe* medial group of frontoventral neurons, *MNe* medial neurons, *VMNe* ventro-medial neurons, *VNe* ventral neurons, *VPNe* ventro-posterior neurons). *Bar* 50 µm. **b** Paraffin section of the right optic lobe with one LMS-lir VNe neuron (*gray arrow*) next to the AMe (encircled). LMS-lir processes branch in the anterior neuropil (*black arrow*) and arborize in the internodular region of the AMe (*insert, white arrowhead*); additionally, immunoreactivity occurs in one visible ventral nodulus (*insert, black arrowhead*). Immunoreactive processes also invade one medulla layer proximal to the serpentine layer (*large arrowhead*). Moreover, immunoreactive fibers project toward the lamina and arborize in the accessory laminae (*gray arrowheads*). *Bar* 100 µm.

small phase delay at a CT of 09:00–12:00 (Table 1). Both the maximal phase advance (3.3 h) and the maximal phase delay (-4.8 h) of the LMS injections occurred at a CT of 09:00 (Fig. 5). At this time, we found the highest variability in the phase shifts. LMS-induced phase shifts were not statistically different from HLR control injections (Fig. 6).

Effects of LMS injections on the period of the rhythm of circadian locomotor activity

No significant changes were found in the free-running periods at any CT after the injection of LMS or HLR. On average, the mean period before the injection of LMS or HLR (23.61±0.26 h, n=72) was altered neither by LMS

(difference in period lengths before and after injection: 0.00±0.16 h, CI=[-0.06; 0.05], n=38) nor by control injections (0.01±0.15 h, CI=[-0.02; 0.08], n=34). The mean of the period before LMS injection was 23.60±0.32 (n=38) and that of the period after LMS injection was 23.58±0.27. The means of the period before and after HLR injection were 23.63±0.18 (n=34) and 23.60±0.2 (n=34), respectively. Lengthening and shortening of the periods appeared similarly frequent and were independent of the time of injection. The strongest lengthening of the period was 0.26 h, and the strongest shortening was 0.32 h. As a positive control, 150 fmol PDF was injected at a CT of 09:00 (n=3) and caused significant phase delays (not shown), as reported previously in Petri and Stengl (1997).

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Table 1 Phase shifts (in circadian hours) resulting from injections of 1.15 pmol leucomyosuppressin (*LMS*) and from control injections (*HLR* hemolymph-Ringer) at various times during the circadian cycle (*CT* circadian time). No statistically significant differences are observed following LMS injection.

CT (h)	Phase shifts (mean±SD)		95% CI (lower to upper limit)		Number	
	LMS	HLR	LMS	HLR	LMS	HLR
00:00–03:00	0.20±2.08	0.31±0.50	-4.98 to 5.37	-0.32 to 0.94	3	5
03:00–06:00	-1.13±1.33	-0.42±0.84	-4.42 to 2.16	-1.31 to 0.46	3	6
06:00–09:00	-1.56±1.69	-0.14±0.52	-4.25 to 1.13	-0.97 to 0.69	4	4
09:00–12:00	-1.24±2.55	-0.46±0.07	-3.37 to 0.89	-0.75 to -0.18[a]	8	6
12:00–15:00	-0.64±1.38	-0.64±0.89	-2.35 to 1.07	-1.74 to 0.46	5	5
15:00–18:00	0.87±1.74	0.02±0.60	-1.29 to 3.04	-0.53 to 0.58	5	7
18:00–21:00	0.01±1.89	0.21±0.85	-2.34 to 2.35	-1.14 to 1.56	5	4
21:00–24:00	-1.03±2.65	-0.05±0.86	-4.33 to 2.26	-0.77 to 0.67	5	8

[a]Phase shift significantly different from zero as judged by the 95% confidence interval (*CI*)

Fig. 5 Scatter plot of phase shifts dependent on LMS (*filled squares*) and hemolymph-Ringer (HLR; *open squares*) at various circadian times. Injections of 1.15 pmol LMS in 2 nl HLR with 10% blue food dye (*n*=38) caused maximal phase delays and phase advances at a CT of 09:00 (-4.8 and 3.3 circadian hours). At this time, considerable variance occurred in the time shifts. Control injections of 2 nl HLR with 10% blue food dye (*n*=45) caused only small phase shifts. The largest phase delay caused by a control injection occurred at a CT of 12:00 (2.1 circadian hours), and the maximal phase advance was found at a CT of 18:00 (1 circadian hour).
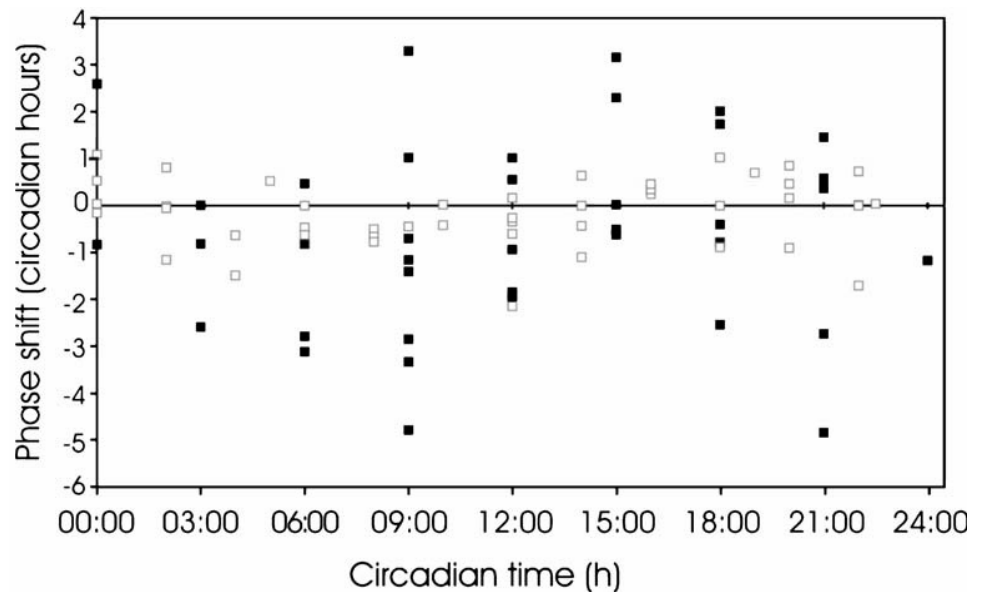
Discussion

In this study, we have examined the presence and functional role of the neuropeptide LMS in the AMe, the circadian pacemaker center of the cockroach *L. maderae*. With MALDI-TOF mass spectrometry, we have shown that LMS is present in isolated AMae. In addition, immunostaining with an antiserum raised against a myosuppressin has revealed the presence of one to three immunoreactive VNes that innervate the circadian pacemaker center of the cockroach. Injections of LMS into the vicinity of the AMe have demonstrated that this peptide is not involved in the control of circadian locomotor activity rhythms.

Specificity of the antibody

LMS shares the RFamide C-terminus with several other families of FaRPs in the cockroach *L. maderae* (Nässel 2000; Nichols et al. 1997; Orchard et al. 2001). Therefore, immunostaining with polyclonal antibodies cannot readily

Fig. 6 Phase response curves obtained in response to the injection of 1.15 pmol LMS and control injections. Data were merged into 3-h bins. LMS-dependent phase shifts (*black squares*) and phase shifts obtained from control injections (*open squares*) are plotted.
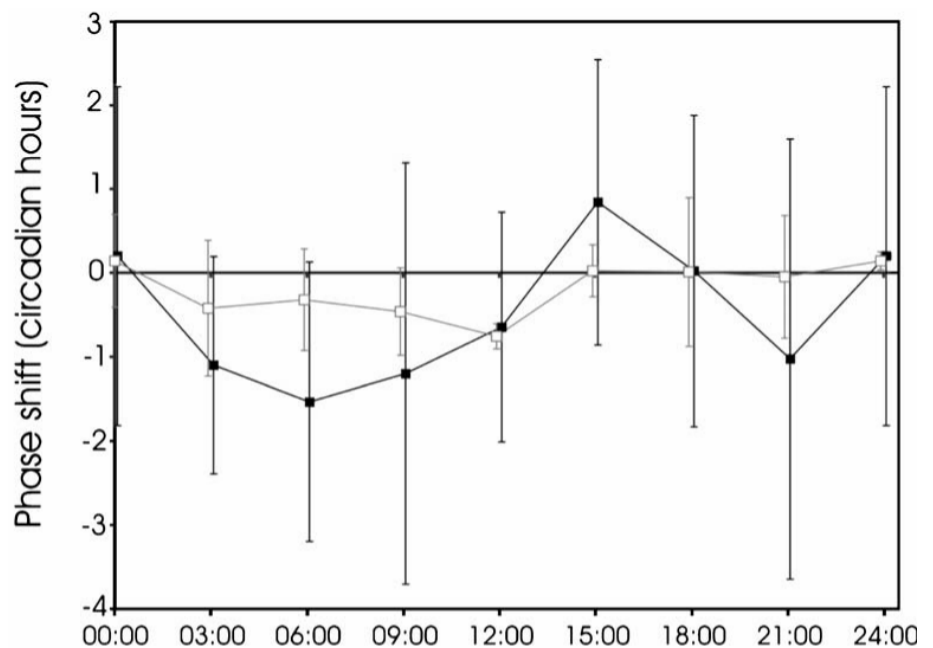
distinguish between the different FaRPs, because most of the available antisera recognize the RFamide C-terminus. Thus, to determine whether LMS is present in presumptive circadian pacemaker neurons innervating the AMe, we have employed an antiserum generated against the unique N-terminus of DMS (TDVDHV; McCormick and Nichols 1993) to avoid cross reactivity with other FaRPs. Since DMS and LMS differ only in one amino acid at the N-terminus, and since the antigen used does not contain the common C-terminal RFamide, the DMS-specific antibody probably specifically recognizes LMS, but not other FaRPs. Characterization of the antiserum supports this conclusion. Because our controls have not revealed any specific staining, and since the DMS antisera only recognizes few cells compared with a previously employed FMRFamide antiserum (Petri et al. 1995), the observed immunostaining probably represents LMS. This assumption has been confirmed by MALDI-TOF mass spectrometry, which has revealed that LMS is present in isolated AMae, despite the failure of previous immunocytochemical studies with LMS-specific polyclonal antisera to detect specific staining in the optic lobes of the cockroach (Meola et al. 1991). However, staining of the optic lobes of *D. melanogaster* has been observed with the DMS antiserum (McCormick and Nichols 1993).

Myosuppressin-like immunoreactivity in the brain of the cockroach *L. maderae*

Staining has been observed in nearly all neuropils of the cockroach brain, except for the mushroom bodies, with the DMS antiserum (McCormick and Nichols 1993) indicating that LMS is abundant in the brain and might be involved in many different functions. Because LMS-like immunoreactivity is present in the central body and in the lateral accessory lobes, which are important projection areas of the central body, LMS might be involved in navigation and motor coordination (Homberg et al. 2003a; Homberg 2004; Strauss 2002). Since LMS injections into the AMe, however, have not revealed daytime-dependent significant phase shifts in circadian locomotor rhythms, its role in motor coordination does not appear to involve its circadian control, at least not under the paradigm used here.

Almost all glomeruli of the antennal lobe are interconnected by a few LMS-lir local interneurons. These cells are located in the vicinity of the LMS-ir cells described by Meola et al. (1991), cells that seem to lose their immunoreactivity if the antiserum is preabsorbed with FMRFamide (Meola et al. 1991), indicating that they do not contain LMS, but another FaRP. The LMS-lir neurons that we have found near the antennal lobe thus differ from the LMS-ir cells described by Meola et al. (1991), because the former cells are located more posteriorly and appear to be local

interneurons of the antennal lobe. Because gamma-amino-butyric acid (GABA) has been shown to be the principal neurotransmitter of local interneurons of the antennal lobe (Homberg and Müller 1999), LMS probably acts as a co-transmitter with GABA, as shown for other peptides in other insect antennal lobe neurons (Homberg and Müller 1999). Double-labeling experiments will test this hypothesis.

Most of the stained neurons have been found in the pars intercerebralis, which is involved in controlling hormone release from the retrocerebral complex and in harboring descending neurons to locomotor control neuropils in the thorax. Our results are comparable with the number of LMS-ir neurons observed in the pars intercerebralis of *Diploptera punctata* by Donly et al. (1996) and Fuse et al. (1998) who have found, by in situ hybridization, LMS expression in 15–20 cells and, with an anti-FMRFamide antibody, approximately 30 immunoreactive neurons in the pars intercerebralis. Meola et al. (1991) have used an LMS antiserum preabsorbed for 24 h with FMRFamide and have also shown a comparable number of neurons in the pars intercerebralis of the cockroach *L. maderae*.

Myosuppressin-lir neurons in AMe

The AMe is innervated by neurons that can be grouped into six anatomically distinguishable clusters (Fig. 4a; Reischig and Stengl 2003b). Most of the neurons of these groups belong to one of four categories of neurons of the AMe. They are either photic input elements, local interneurons, output neurons, or heterolateral coupling units (Homberg et al. 2003b), and their processes appear to be differentially distributed within the AMe. Whereas the GABAergic distal tract, which apparently brings light information from the medulla into the AMe, arborizes almost exclusively in the noduli, PDF-ir outputs of the circadian pacemaker branch in the internodular and shell region of the AMe (Reischig and Stengl 2003b). With our myosuppressin antiserum, we have obtained staining in the anterior group of neurons of the AMe and in the group of the VNes. Until now, nothing has been known about the function and arborization pattern of the anterior neurons, and thus, this group is not described in the scheme of Reischig and Stengl (2003b). The anterior neurons might also project to the AMe, but this remains to be examined. The group of the VNes contains at least three neurons that connect both AMae via the anterior and posterior optic commissures (Reischig et al. 2004). Since myosuppressin-like immunoreactivity has not been observed in the lobula valley tract, which contains all cells projecting from the optic lobe to the midbrain, the LMS-lir VNes are unlikely to leave the optic lobe. This assumption is confirmed further by the lack of LMS-lir fibers in the posterior optic tubercle, a projection area of the AMe, and a possible input

pathway into the protocerebral bridge of the central complex. Therefore, the LMS-lir neurons appear to be local neurons of the optic lobe. The distribution of LMS-lir fibers in the AMe itself is largely restricted to the anterior shell and internodular neuropil, which are the presumptive input and output regions of the AMe to midbrain centers and to the ipsi- and contralateral optic lobe neuropils (Reischig and Stengl 2002). However, arborizations are also present in at least one ventral nodulus. Because the LMS-lir VNes in *L. maderae* arborize in the AMe, send fibres via the characteristic fan-shaped anterior-layer fibre system over the face of the ipsilateral medulla to the ipsilateral accessory laminae, and invade a proximal layer of the medulla, they appear to be local interneurons of specific optic lobe neuropils previously shown to be connected to the circadian clock in the AMe (Loesel and Homberg 2001; Reischig and Stengl 2003b). Their morphology closely resembles the branching pattern of local neurons of the optic lobe in the work of Loesel and Homberg (2001; their Fig. 1c, optic lobe neuron 2 [OL2] neurons). The intracellularly recorded OL2 neuron is activated by different light intensities (Loesel and Homberg 2001). Like the LMS-lir neuron, the OL2 neuron connects the AMe with a proximal layer of the medulla and has sparse processes near the proximal edge of the lamina with conspicuous arborizations in the accessory laminae. As judged from its branching pattern and its responses to light, this neuron type probably receives input in the medulla and has output regions in the accessory laminae and AMe (Loesel and Homberg 2001). Our assumption that the LMS-lir neurons contain LMS is further confirmed by our MALDI-TOF experiments but contrasts with findings by Meola et al. (1991). Possibly, the polyclonal antiserum used by Meola et al. (1991) was less specific for LMS, and thus, the limited LMS-specific staining in the optic lobe was lost during the preabsorption protocol that they employed (Meola et al. 1991).

Because we have found no indications that the LMS-lir neurons leave the optic lobe, they are unlikely to be involved in the circadian control of locomotor activity rhythms, as has been shown for the PDF-ir neurons with arborizations in the superior lateral protocerebrum (Stengl and Homberg 1994; Reischig and Stengl 2003a). This assumption is confirmed by our microinjection experiments in which no LMS-dependent daytime-dependent phase shifts have been detected in running-wheel assays. Since we did not find LMS-dependent phase shifts with 0.0115 fmol LMS, we used a higher dose of 1.15 pmol LMS for the generation of the phase response curve. Possibly, the high variability at CTs of 09:00 and 21:00 results from unspecific cross reactions of the high dose of LMS with other peptide receptors. Thus, the function of LMS in the AMe is unknown, and whether it controls other circadian outputs of

the circadian pacemaker of *L. maderae* remains to be established in other physiological assays.

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Chapter II. Examination of the role of FMRFamide-related peptides in the circadian clock of the cockroach *Leucophaea maderae*

Examination of the role of FMRFamide-related peptides in the circadian clock of the cockroach *Leucophaea maderae*

Sandra Soehler • Susanne Neupert • Reinhard Predel •
Monika Stengl

Abstract The accessory medulla, the circadian clock of the cockroach *Leucophaea maderae*, is abundant in neuropeptides. Among these neuropeptides are the FMRFamide-related peptides (FaRPs), which generally share the C-terminal RFamide. As a first step toward understanding the functional role of FaRPs in the circadian clock of the cockroach, immunocytochemistry with antisera against various FaRPs, MALDI-TOF mass spectrometry, and injections of two FaRPs combined with running-wheel assays were performed. Prominent FMRFamide-like immunoreactivity was found in maximally four soma clusters associated with the accessory medulla and in most neuropils of the protocerebrum. By MALDI-TOF mass spectrometry, various extended FMRFamides of the cockroach *L. maderae* were partially identified in thoracic perisympathetic organs, structures known to accumulate extended FMRFamides in insects. By mass match, several of these peptides were also detected in the accessory medulla. Injections of FMRFamide and Pea-FMRFa-7 (DRSDNFIRF-NH2) into the vicinity of the accessory medulla caused time-dependent phase-shifts of locomotor activity rhythms at circadian times 8, 18, and 4. Thus, our data suggest a role for the different FaRPs in the control of circadian locomotor activity rhythms in *L. maderae*.

S. Soehler · M. Stengl
Department of Biology, Animal Physiology,
Philipps University Marburg,
35032 Marburg, Germany

S. Soehler · M. Stengl
Department of Natural Sciences, Animal Physiology,
University of Kassel,
Heinrich-Plett-Strasse 40,
34132 Kassel, Germany

S. Neupert · R. Predel
Department of General Zoology and Animal Physiology,
Friedrich-Schiller-University Jena,
07743 Jena, Germany

M. Stengl (✉)
Biologie, Tierphysiologie, University of Kassel,
Heinrich-Plett-Strasse 40,
34132 Kassel, Germany
e-mail: stengl@staff.uni-marburg.de

Introduction

In the cockroach *Leucophaea maderae*, lesion and transplantation experiments have located the circadian clock, which controls locomotor activity rhythms, to the accessory medulla (AMe; plural: AMae) of the optic lobes (Stengl and Homberg 1994; Reischig and Stengl 2003a). The ~250 AMe-associated neurons form six anatomically distinguishable groups according to soma size and location (Reischig and Stengl 2003b). The AMe neurons appear to be abundant in various neuropeptides and, thus, can be further distinguished by a variety of peptide antisera (Petri et al. 1995; Hofer and Homberg 2006b). Among these neurons are pigment-dispersing factor (PDF)-like-immunoreactive (PDF-ir) neurons, which control circadian locomotor activity rhythms in this cockroach (Stengl and Homberg 1994; Reischig and Stengl 2003a). Injections of PDF into the vicinity of the AMe result in time-dependent phase delays at circadian time 9 (CT 9; Petri and Stengl 1997). Computer modeling experiments have suggested that, other than PDF, at least one other peptide must be involved in

maintaining circadian rhythms via phase advances (Petri and Stengl 2001). Approximately 35 neurons adjacent to the AMe have previously been shown to be FMRFamide-ir (Petri et al. 1995). However, whether and in what manner they belong to the various soma groups of AMe neurons remains unknown (Reischig and Stengl 2003b). In addition, which of the members of the FMRFamide-related peptides (FaRPs) are recognized by the antibody and whether any of them might phase-shift circadian locomotor activity rhythms are unsolved problems.

The FaRPs are widely distributed throughout the central and peripheral nervous system of insects and are involved in the regulation of a multitude of physiological activities (Orchard et al. 2001; Nichols 2003; Orchard and Lange 2006; Predel 2006). Members of this group generally share the C-terminal RFamide and are recognized with antibodies against FMRFamide. They include N-terminally extended FMRFamides, myosuppressin (extended FLRFamide), sulfakinins (extended HMRFamides with a sulfated tyrosine residue), short neuropeptides F (sNPFs, extended RLRFamides), and long neuropeptides F (lNPFs). These RFamides are encoded on different genes, are expressed in different neurons, and have specific receptors (Hewes and Taghert 2001; Predel 2006; Nässel and Homberg 2006; Hauser et al. 2006). Several FaRPs have been identified in various cockroaches. They include a single myosuppressin (*L. maderae*: Holman et al. 1986; *Diploptera punctata*: Donly et al. 1996; Fuse et al. 1998; *Periplaneta americana*: Predel et al. 2001), two sulfakinins (*L. maderae*: Nachman et al. 1986; *P. americana*: Veenstra 1989), a single sNPF (head peptide, *P. americana*: Veenstra and Lambrou 1995), and 24 forms of the extended FMRFamide gene (*P. americana*: Predel et al. 2004).

In this study, we focus on the role of FaRPs in the circadian clock of the cockroach. Thus, the distribution of FMRFamide-ir, sNPF-ir, and perisulfakinin (PSK)-ir neurons in the six distinguishable soma groups of the AMe and in the protocerebrum, in projection areas of AMe neurons, has been examined with various antibodies and compared with that of the previously published dromyosuppressin-ir (DMS-ir) neurons of the AMe. About 24 FMRFamide-ir neurons in various soma groups of the AMe express FMRFamide immunoreactivity. Four of these immunoreactive neurons can be further distinguished with sNPF and DMS antibodies (Söhler et al. 2007). The PSK antiserum, however, recognizes none of the neurons in the vicinity of the AMe. To determine whether different FaRPs affect circadian locomotor activity rhythms, the two available FaRPs, viz., the tetrapeptide FMRFamide and Pea-FMRFa-7 (DRSDNFIRFamide), have been injected in running-wheel assays. Mass spectrometric screening of thoracic perisympathetic organs (tPSOs), which are major release sites of extended FMRFamides in insects, have revealed partial sequences similar to extended FMRFamides of *P. americana*. One of these peptides, which has been identified in the tPSOs of *L. maderae*, shows a high sequence similarity to Pea-FMRFa-7, which has been used in our bioassays. A number of the extended FMRFamides of *Leucophaea maderae* has also been found, by mass match, in the AMe. Thus, our results obtained with immunocytochemistry, mass spectrometry, and bioassays indicate a role of several FaRPs in the circadian system of this cockroach.

Materials and methods

Animals

Adult male cockroaches (*Leucophaea maderae*) were taken from laboratory colonies. They were reared under a 12:12 h light-dark (LD) photoperiod at about 60% relative humidity and a temperature of 26°C. The animals were fed with dried dog food, potatoes, and water *ad libitum*.

Immunocytochemistry for paraffin sections

Brains were dissected and fixed for 4 h in a formaldehyde/picric acid solution (aqueous Bouin's solution modified after Hollande; Romeis 1989), washed in clear water, dehydrated in an ethanol series, and embedded in paraffin (Paraplast plus, Sigma, Germany). Serial frontal 10-μm-thick sections were cut as ribbons, mounted on microscope slides, deparaffinized with xylene, and rehydrated through graded ethanols. The brain sections were stained with antisera against FMRFamide (diluted 1:3,000; no. 671; from Dr. E. Marder, Brandeis University, Waltham, Mass.; Marder et al. 1987), sNPF (diluted 1:20,000; from Dr. H. Agricola, University of Jena), and PSK (diluted 1:100,000/80,000; from Dr. H. Agricola, University of Jena; Agricola and Bräuning 1995).

Immunoreactive cells were detected by using a sensitive three-step peroxidase technique (Sternberger 1979; Reischig and Stengl 1996). To visualize all neuropils, the sections were counterstained in 1% methylene blue. In control experiments, preincubation of the relevant primary antiserum with 8.4×10^{-4} M FMRFamide peptide or 10^{-5} M sNPF or omission of the primary antibody removed all staining. To obtain an overview of the number and location of FaRPs near the AMe, our data were compared with previously published data concerning DMS-ir somata near the AMe (Söhler et al. 2007).

Operation and injection for behavioral experiments

Operations and injections were performed under dim red light and accomplished as described in Söhler et al. (2007). Peptide

injections comprised 100 fmol FMRFamide (Bachem, Bubendorf, Switzerland) or 150 fmol Pea-FMRF-7 (DRSDNFIRFamide) in 2 nl hemolymph-Ringer (Kaissling and Thorson 1980) with 10% blue food dye (McCormick, Baltimore, Md.). These concentrations corresponded to effective doses of pigment-dispersing hormone and other peptides in previous experiments (Petri and Stengl 1997; Petri et al. 2002; Hofer and Homberg 2006a). Control injections consisted of 10% blue food dye in hemolymph-Ringer. The recording of the locomotor activity and editing of the data was described previously (Reischig and Stengl 2003a). Data were evaluated from 148 of the 245 injected animals (FMRFamide: *n*=53; Pea-FMRFa-7: *n*=42; controls: *n*=53). The remaining 97 animals were excluded from further analysis because they showed little activity after the injection, had strong changes in period lengths, or died within 1 week after the operation. Phase shifts were determined as time differences between the regression lines before and after injection, extrapolated to the day after treatment (Petri and Stengl 1997). The behavioral data were merged, for the FMRFamide injections, into 2-h time intervals. For the Pea-FMRFa-7 injections, the data were merged, from CT 0–12, into 2-h time intervals and, from CT 12–24, into 3-h time intervals. The means and standard deviations (SD) were calculated for each bin. Changes of phases and periods in a given interval were considered to be significantly different from zero if the calculated 95% confidence interval of the respective time interval did not contain the value zero. The phase and period changes were statistically analyzed by a two-tailed Students *t*-test. Outlier data were eliminated after execution of two outlier tests (Nalimov 1963; Grubbs and Beck 1972). Significance was taken as $P<0.05$. Statistical analyses were performed with Superior Performing Software Systems (SPSS 12.0) and Excel XP (Microsoft). The phase response curves were produced with Excel.

Sample preparation for MALDI-TOF mass spectrometry

Cockroaches were anesthetized with ice water for several seconds and decapitated. Brains were dissected, and the perineurium in the vicinity of the AMe was opened with ultra-fine scissors. Without enzyme treatment, pieces of the AMe were removed step by step by using an uncoated glass capillary (Hilgenberg, Malsfeld, Germany) and transferred to a stainless steel sample plate for MALDI-TOF mass spectrometry. Subsequently, any adhering insect saline (7.5 g/l NaCl, 0.2 g/l KCl, 0.2 g/l CaCl$_2$, 0.1 g/l NaHCO$_3$, pH 7.2) was removed from the sample plate by using the same capillary. Approximately 20 nl matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in methanol/water) was injected onto the dried tissue over a period of about 5 s via a nanoliter injector (World Precision Instruments, Berlin, Germany). Each preparation was air-dried again and covered with pure water for a few seconds; the water was removed by cellulose paper (Fine Science Tools, Heidelberg, Germany).

MALDI-TOF mass spectrometry

Mass spectra were acquired in positive ion mode on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, USA) equipped with a pulsed nitrogen laser emitting at 337 nm. The excised AMae were analyzed in reflectron mode by using a delayed extraction time of 150 ns, 75% grid voltage, 0.06%–0.1% guide wire voltage, and an accelerating voltage of 20 kV. Laser strength was adjusted to provide an optimal signal to noise ratio. An external mass spectrum calibration was first performed by using synthetic cockroach peptides (Pea-pyrokinins 2/5; SPPFAPRLa/GGGGSGETSGMWFGPRLa).

Sample preparation for ESI-TOF mass spectrometry

Aqueous extracts of the tPSOs were sonicated and centrifuged, and the supernatant was loaded onto an activated and equilibrated home-made microcolumn (purification capillary for electrospray spectrometry).

ESI-Q-TOF mass spectrometry

Nanoelectrospray mass spectra were acquired in the positive-ion mode by using the API Qstar Pulsar (Applied Biosystems, Applera Deutschland, Darmstadt, Germany) fitted with a Protana (Odense, Denmark) nanoelectrospray source. Typically 950–1000 V was applied as an ionspray voltage. Samples were purified by using a homemade spin column. Approximately 1–2 mm Luna C18 material (10 μm; Phenomenex, Aschaffenburg, Germany) was loaded into a 2-cm capillary column with a needle tip. Liquids were passed through the column by securing the capillary column to a purification needle holder (Proxeon Biosystems, Odense, Denmark) and by centrifugation. After the column was equilibrated in 5% formic acid, the samples were loaded and rinsed with 5% formic acid. Peptides were eluted from the column with solutions of 10%/20%/30% acetonitrile (5% formic acid) and collected into a metal-coated nanoelectrospray capillary. The purified samples were then loaded onto the source and analyzed. After determination of the m/z (mass to charge ratio) of the peptides in mass spectrometry (MS) mode, a collision energy (typically 10–40 V) was applied. The m/z of interest was selected and fragmented with the instrument in "enhance all" mode. MS/MS data were typically acquired over 5 min and manually analyzed.

Results

Immunoreactive somata in the central brain of the cockroach

The FMRFamide antiserum stained many cells in the brain and the optic lobes, whereas all other employed antisera recognized only subpopulations of the anti-FMRFamide-ir cells (Fig. 1). In the central brain of *L. maderae*, about 1100 FMRFamide-ir somata were counted, all of which were
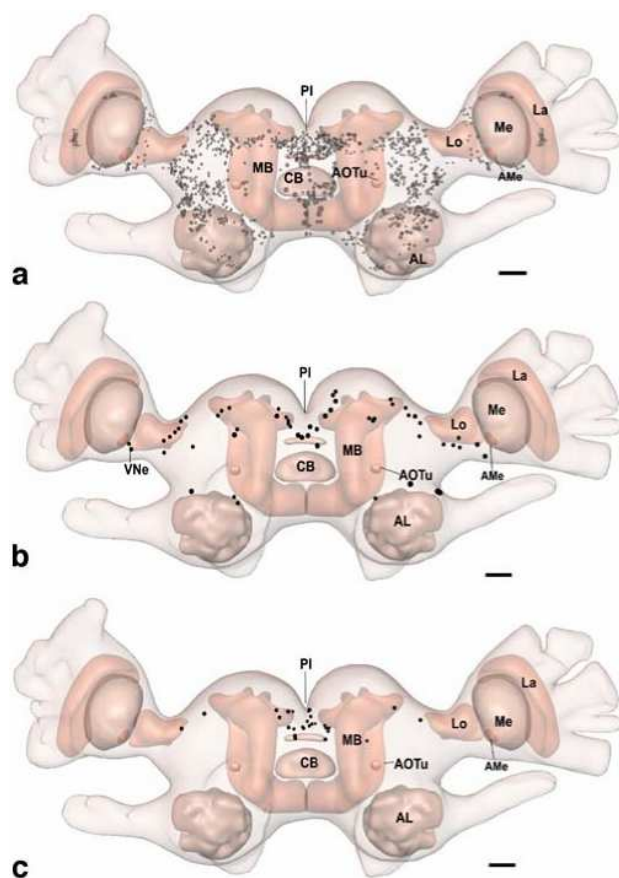

Fig. 1 FaRP-ir cell bodies in three-dimensional models of a male cockroach *L. maderae* (*AL* antennal lobe, *AMe* accessory medulla, *AOTu* anterior optic tubercle, *CB* central body, *La* lamina, *Lo* lobula, *MB* mushroom body, *Me* medulla, *PI* pars intercerebralis, *VNe* ventral neuron). Surface reconstruction of contours of brain structures and prominent neuropils was generously provided by Dr. T. Reischig (University of Göttingen, Germany; Reischig and Stengl 2002). Faintly stained somata were excluded. **a** FMRFamide-ir cell bodies. Reconstruction of stained central brain cells was from one brain and of stained cell bodies in the optic lobes was from another brain. Several FMRFa-ir cell bodies were found near the accessory medulla (*AMe*). **b** About 93 sNPF-ir cell bodies were found in the central brain and in the optic lobes of the cockroach. Most were located in the anterior neuropil of the pars intercerebralis. Near the AMe, sNPF-ir cell bodies that could be grouped to the anterior or the ventral neurons were seen. **c** About 29 PSK-ir cell bodies were found in the central brain. Most were located in the pars intercerebralis. No PSK-ir neurons were observed in the optic lobe. *Bars* 200 µm.

widely dispersed in the cell cortex (Fig. 1a). The most anterior cell cortex of the pars intercerebralis contained several large neurons under which 165 further neurons were located (Fig. 1a). Stained neurons were also observed near the calyces, near the antennal lobes, and in various other areas of the superior lateral and inferior lateral protocerebral cell cortex (Fig. 1a). Antisera against sNPF detected sNPF-like immunoreactivity in about 93 neurons throughout the central brain (Fig. 1b). Most of the neurons were located in the most superficial cell cortex of the anterior pars intercerebralis. Additionally, sNPF-ir neurons were found in the pars lateralis, near the antennal lobe, in the area of the optic stalk, near the calyces, and near the antenno-mechanosensory center (Fig. 1b). The fewest neurons were stained with the anti-PSK antiserum. PSK-like immuno-reactivity was located in about 29 neurons in the central brain (Fig. 1c). Most of these neurons were located in the most anterior cell cortex of the pars intercerebralis and in more posterior parts of the pars intercerebralis (Fig. 1c). In the posterior part of the protocerebrum, two bilaterally symmetric lateral neurons were observed in four of the five evaluated preparations (Fig. 1c).

Immunoreactivity in the optic lobes

The anti-FMRFamide antiserum used in this study identi-fied nearly 100 FMRFamide-ir neurons per optic lobe (Fig. 1a). Four main FMRFamide-ir soma groups could be distinguished in addition to some scattered somata (Fig. 1a). One group was located adjacent to the AMe (Figs. 2a, b; 3c, d), one anterior to the AMe (Fig. 3b), and two groups were associated with the lamina (Fig. 1a). Weaker-stained neurons (22 ±5.4, $n=4$ lobes) were scattered dorsally between the medulla and lobula (Fig. 2a, double arrow). Tangential neurons (27±7.4, mean±SD, $n=4$ lobes) near the ventral tip of the medulla invaded the medulla and contributed to its staining. The AMe-associated neurons (23.7±9.8, $n=8$ lobes) could be assigned to four of the six neuronal groups of the circadian pacemaker (Reischig and Stengl 2003b; Fig. 2b). About 13 (12.5± 2.8) of the FMRFamide-ir neurons associated with the AMe belonged to the ventral neurons (VNes), and about 9 (8.8± 7.5) neurons were found in the group of the distal frontoventral neurons (DFVNes; Figs. 2b, 3c). About two FMRFamide-ir neurons each belonged to the median (MNes; 2.3±1.3) and ventroposterior (VPNes; 2.4±1.6) neurons (Figs. 2b, 3c, d). At least two neurons (1.5±1.0) could not be assigned to any of the six AMe groups and were named anterior neurons (ANes) since they were located at the most anterior surface of the cell cortex associated with the AMe (Fig. 3b). The AMe expressed dense FMRFamide immunoreactivity concentrated in the anterior neuropil and in the coarse neuropil around the
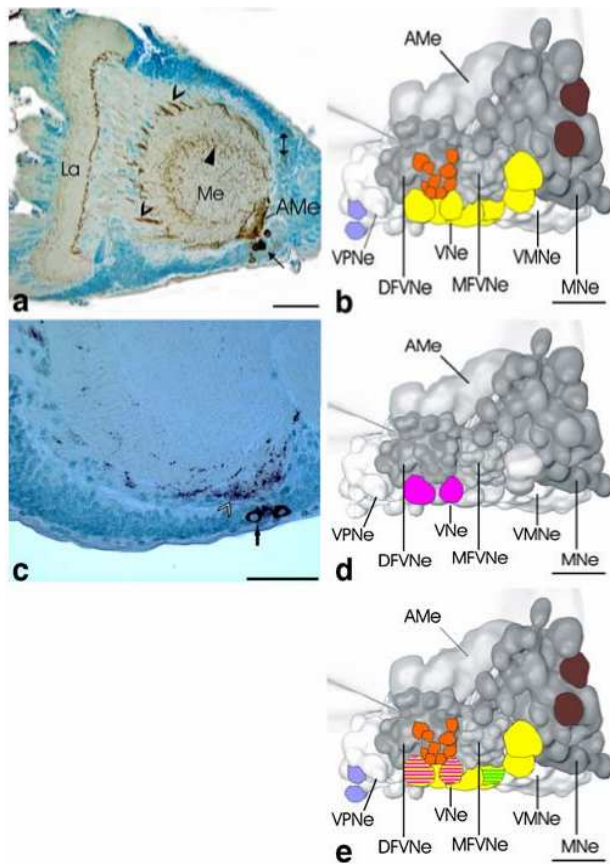
Fig. 2 Optic lobe, together with AMe neurons, immunostained with antisera against FMRFamide (**a**) and sNPF (**c**). **a** FMRFamide immunostaining in the right optic lobe. The anterior neuropil of the accessory medulla (*AMe*) with associated soma groups (*arrow*) expressed strong immunoreactivity. Additionally, weakly stained neurons were scattered dorsally between the medulla and lobula (*double arrow*). In the medulla (*Me*), a median layer (*closed arrowhead*) and fibers of the fiber fan (*open arrowheads*) connecting the medulla and lamina (*La*) were stained. The lamina expressed stronger immunoreactivity proximally than distally. **c** Paraffin section of the right optic lobe showing three sNPF-ir VNes (*arrow*). In the AMe, one nodulus (*arrowhead*) and the internodular neuropil were stained. *Bars* 100 µm. **b, d, e** Three dimensional (3D) models of the right accessory medulla (*AMe*) with adjacent soma groups (generously provided by Dr. T. Reischig, University of Göttingen, Germany; Reischig and Stengl 2003b). The cell bodies marked in *color* represent the general location of the FMRFamide-ir neurons (**b**) and of the sNPF-ir neurons (**d**). **e** Summary of the number of neurons in the six distinguishable soma groups of the AMe stained with the various antisera. Additionally to the FMRFamide and sNPF neurons, one dromyosuppressin-ir neuron (Söhler et al. 2007) is stained in *green* (*DFVNe* distal group of frontoventral neurons, *MFVNe* medial group of frontoventral neurons, *MNe* medial neurons, *VMNe* ventro-medial neurons, *VNe* ventral neurons, *VPNe* ventro-posterior neurons). *Bars* 50 µm.

noduli (Fig. 3c). Most of the noduli of the AMe were invaded only sparsely but homogeneously by immunoreactive fibers (Fig. 3d), but two to three noduli expressed strong staining. Moreover, all other neuropils of the optic lobe were invaded by FMRFamide-ir fibers. In the lamina,

stronger staining at the proximal face could be distinguished from fainter staining at the distal face (Fig. 2a). In the medulla, several medial layers showed FMRFamide immunoreactivity, additionally to the characteristic fiber fan along the anterior surface of the medulla (Fig. 2a; Petri et al. 1995). The FMRFamide-ir fiber fan connected the AMe to the medulla and lamina (Fig. 2a) and was predominantly varicose in appearance.

With the Pea-sNPF antiserum, about two sNPF-ir VNes (1.67 ± 1.51) and about 1 ANe (1.17 ± 1.21) were observed (Figs. 2c, d, 3a, e). The AMe contained strong sNPF-ir staining in the internodular neuropil and also in some
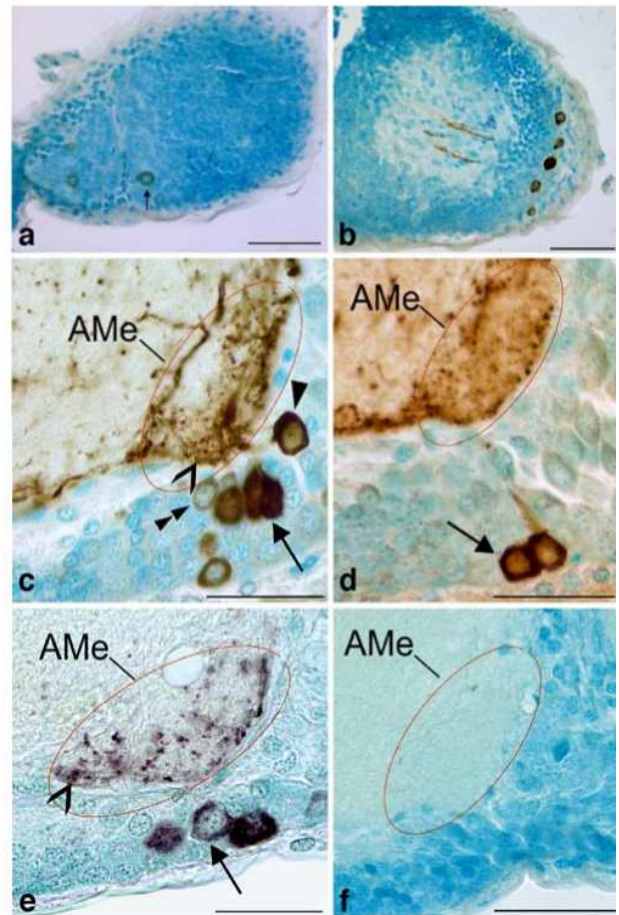
Fig. 3 FaRP-ir cells in various cells groups associated with the AMae. **a** sNPF immunoreactivity in one anterior neuron (*arrow*). **b** FMRFamide immunostaining in five anterior neurons. **c** FMRFamide-ir VNes (*arrow*), DFVNes (*double arrowhead*), and MNes (*arrowhead*) were visible next to the AMe (*delineated in red*). The anterior neuropil of the AMe expressed strong immunoreactivity. At least two noduli were strongly stained (*open arrowhead*). **d** FMRFamide-ir VPNes (*arrow*) and sparse staining in apparently all noduli of the AMe were observed. **e** The three sNPF-ir VNes (*arrow*) restricted their staining to the internodular neuropil and to one nodulus (*arrowhead*) of the AMe (*delineated in red*). **f** With the PSK antibody, no staining in the AMe (*delineated in red*) or in neurons adjacent to the AMe was obtained. *Bars* 100 µm (**a, b**), 50 µm (**c–f**).

antero-ventral noduli (Figs. 2c, 3e). In the medulla, faint sNPF-ir staining in the anterior fiber-fan and stronger immunostaining in a middle layer of the medulla were detected. The lamina expressed immunoreactivity at the proximal face. Additionally, there was staining in the lamina-organ. In some preparations, two groups of stained cells were found ventrally and dorsally of the lamina.

No PSK-ir neurons were detected in the vicinity of the AMe in six of the ten evaluated lobes (Fig. 3f). In three lobes, one neuron in each case was observed that could be grouped to the VNes (Reischig and Stengl 2003b). However, only one of these neurons was clearly identified as being above background staining, because it was observed in two consecutive 10-μm-thick sections. In two of the lobes with faintly stained VNes, one to three immunoreactive neurons anterior to the lobula were found. The AMe itself did not show PSK immunoreactivity (Fig. 3f). Among the other neuropils in the optic lobe, stained fibers were observed only in the medulla in a middle layer, but not in the fan-shaped surface. No staining was visible in the lamina.

Effects of FMRFamide, Pea-FMRFa-7, and hemolymph-Ringer injections on the phase of circadian locomotor activity rhythm

In the locomotor activity assays, the tetrapeptide FMRFamide and Pea-FMRFa-7 (DRSDNFIRFamide) were injected into male cockroaches under constant darkness (DD) before they were set back into running-wheels under DD. Pea-FMRFa-7 was chosen as an available, abundant, and physiologically active extended FMRFamide from the American cockroach *P. americana* (Predel et al. 2004). FMRFamide and Pea-FMRFa-7 were injected at different circadian times. Locomotor activities of the free-running cockroaches were recorded before and after the injection, and possible time shifts in the onset of the locomotor activity were evaluated.

Injections of 100 fmol FMRFamide resulted in significant phase delays at two CTs (CT 8 and CT 18, Fig. 4c) as judged by the 95% confidence intervals. The resulting phase delays at these two time points were also significantly different from the control injections ($P<0.05$; two tailed t-test, Table 1). In addition, the FMRFamide injections at CT 18 differed significantly from FMRFamide injections at CT 4, 5–7, 10, 14, 20, 22, and 24. The FMRFamide injections at CT 8 differed significantly from FMRFamide injections at CT 4, 5–7, 10, 14, 20, 22, and 24. The maximal phase delay that was observed in the experiment occurred after injection at CT 12 (-3.7 h), but this value differed strongly from the other values obtained after injections at this CT (Fig. 4a). At CT 18, three injections resulted in significant phase delays (-2.73±

0.39 h; Fig. 4a; Table 1). The strongest phase advance was observed after injection of FMRFamide at CT 6 (3.46 h; Fig. 4a). However, again, the other values obtained after injections at this CT were different, and the finally resulting average phase shift was close to zero. At CT 18, one injection resulted in a phase advance that could be identified as outlier according to the Nalimov test (Nalimov 1963). This value was excluded from further analysis.

Injections of 150 fmol Pea-FMRFa-7 resulted in significant phase delays at two circadian times (CT 4 and CT 8, Fig. 4d, Table 2) as judged by the 95% confidence intervals. Comparison of the phase shifts of Pea-FMRFa-7 injections with those of the control injections (Student's t-test) resulted in significant differences at CT 4. The phase delays at CT 4 were significantly different from phase shifts at CT 2, 15, 18, and 21. Maximal phase delays occurred after injection at CT 4 (-5.4 h). The highest phase advance occurred after injection at CT 15 (1.32 h; Fig. 4b). Control injections with carrier solution alone (10% blue food dye in hemolymph-Ringer; Kaissling and Thorson 1980) did not cause significant phase shifts at any CTs (Table 1).

Effects of FMRFamide, Pea-FMRF-7, and hemolymph-Ringer injections on the period of the circadian locomotor rhythm

There were no significant changes in the free-running periods at any CT before and after injection of FMRFamide. The mean period (±SD) before the injection of FMRFamide was 23.59±0.24 h (CI [0.002; 0.08], $n=52$), and the mean period after the injection of the peptide was 23.55 ±0.24 h. Of the 52 evaluated periods, we observed lengthening of the period with a maximum of 0.31 h ($n=32$) and shortening of the period with a maximum of 0.36 h ($n=19$). The 40 Pea-FMRFa-7 injections also did not cause significant changes in the free-running period. The mean period (±SD) before the Pea-FMRFa-7 was 23.57±0.22 h (CI [-0.06; 0.01], $n=40$), and after the Pea-FMRFa-7 injection was 23.60±0.23 h. We observed lengthening of the period with a maximum of 0.29 h ($n=20$) and shortening with a maximum of 0.19 h ($n=20$).

Hemolymph-Ringer injections did not significantly change the free-running period at any CT before and after the injection ($n=46$).

Dose dependency of phase shifts induced by FMRFamide and Pea-FMRF-7

The FMRFamide-dependent phase delays at CT 18 were not positively correlated with the dose of FMRFamide injections. Whereas injections of 10^2 fmol peptide caused significant phase delays, injections of 10^{-2} fmol caused
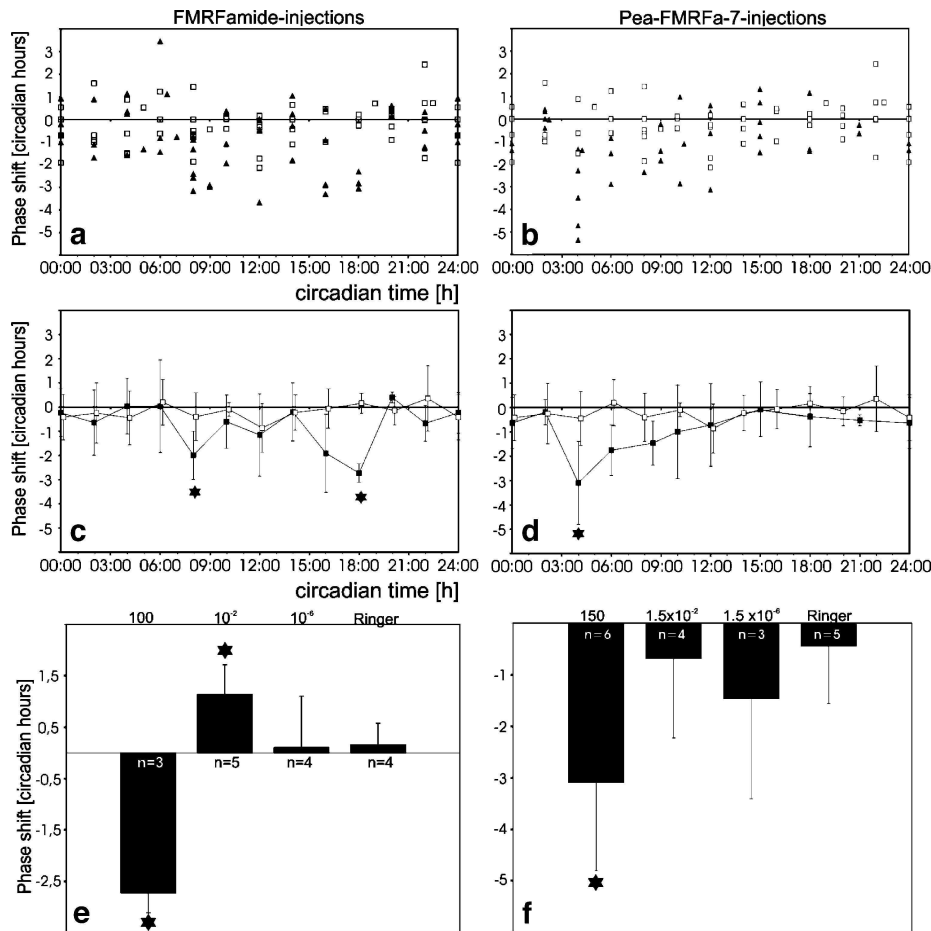
Fig. 4 Scatter plots, phase response curves, and dose-response relationship obtained after injection of FMRFamide or Pea-FMRFa 7 (DRSDNFIRFamide). **a** Scatter plot of FMRFamide-dependent (*filled triangles*) and hemolymph-Ringer-dependent (*open squares*) phase shifts at various circadian times. Injections comprised 100 fmol FMRFamide in 2 nl hemolymph-Ringer with 10% blue food dye (*n*=53). Control injections comprised 2 nl hemolymph-Ringer with 10% blue food dye (*n*=53) and were identical in **a**, **b**. **b** Scatter plot of Pea-FMRFa-7-dependent (*filled triangles*) and hemolymph-Ringer-dependent (*open squares*) phase shifts at various circadian times. Injections comprised 150 fmol Pea-FMRFa-7 in 2 nl hemolymph-Ringer with 10% blue food dye (*n*=42). **c**, **d** Phase response curves obtained in response to the injection of 100 fmol FMRFamide (**c**, mean±SD, *black squares*), control injections (**c**, **d**, mean±SD, *open squares*), and injection of 150 fmol Pea-FMRFa-7 (**d**, mean±SD, *black squares*). Data were merged into 2-h bins for the FMRFamide

injections and into 2-h (00:00–12:00) and 3-h bins (12:00–00:00) for the Pea-FMRFa-7 injections. *Stars* indicate FMRFamide- or Pea-FMRFa-7-dependent phase shifts that were significantly different (*P*< 0.05) from control injections at the same circadian time. **e** Dose-dependency of FMRFamide-induced phase shifts at CT 18. *Bars* show phase shifts resulting from injections of hemolymph-Ringer (*n*=4), 10^{-6} fmol FMRFamide (*n*=4), 10^{-2} fmol FMRFamide (*n*=5), and 100 fmol FMRFamide (*n*=3). *Stars* indicate FMRFamide-dependent phase shifts that were significantly different from control injections (*P*<0.05). **f** Dose-dependency of Pea-FMRFa-7-induced phase shifts at CT 4. *Bars* show phase shifts resulting from injections of hemolymph-Ringer (*n*=5), 1 .5x 10^{-6} fmol Pea-FMRFa-7 (*n*=3), $1.5x10^{-2}$ fmol Pea-FMRFa-7 (*n*=4), and 150 fmol Pea-FMRFa-7 (*n*=6). *Stars* indicate Pea-FMRFa-7-dependent phase shifts that were significantly different from control injections (*P*<0.05).

phase advances (1.14±0.57 h), which were significantly different from zero (CI[0.43; 1.85], *n*=5) and from control injections (Student s *t*-test). Phase shifts induced by injections of 10^{-6} fmol FMRFamide were neither significantly different from zero nor from control injections (0.11±1.0 h; CI[-1.48; 1.70], *n*=4; Fig. 4e).

Injections of 10^{-2} fmol FMRFamide at CT 8–9 caused phase shifts that were neither significantly different from

zero nor from control injections (0.27±0.64 h; CI[-0.75; 1.29], *n*=4, data not shown).

The Pea-FMRFa-7-dependent phase shifts at CT 4 were positively correlated with the dose of Pea-FMRFa-7 injections (Fig. 4f). The phase delays decreased with decreasing amounts of the injected peptide. The injections of $1.5x10^{-2}$ fmol and of $1.5x10^{-6}$ fmol Pea-FMRF-7 caused phase shifts (-0.69±1.54 and -1.47±1.95, respectively)

Table 1 Phase shifts (in circadian hours) resulting from injections of 100 fmol FMRFamide and from control (hemolymph-Ringer) injections at various times of the circadian cycle (*CT* circadian time) with statistically significant differences at CT 8 and 18 (*CI* confidence interval).

CT (h)	Phase shifts (mean±SD)		95% CI (lower to upper limit)		Number	
	FMRFamide	Hemolymph-Ringer	FMRFamide	Hemolymph-Ringer	FMRFamide	Hemolymph-Ringer
00:00	-0.24±0.84[d]	-0.42±0.95	-1.58 to 1.11	-1.59 to 0.76	4	5
02:00	-0.64±1.35	-0.25±1.24	-4.00 to 2.73	-2.22 to 1.73	3	4
04:00	0.04±1.15[d]	-0.45±1.12	-1.79 to 1.86	-1.83 to 0.94	4	5
05:00–07:00	0.04±1.91[d]	0.20±0.95	-1.97 to 2.04	-2.15 to 2.55	6	3
08:00–09:00	-1.99±1.00[a,c]	-0.40±1.00	-2.76 to 1.22[b]	-1.32 to 0.52	9	7
10:00	-0.60±1.11[d]	-0.95±0.28	-2.36 to 1.16	-0.80 to 0.61	4	3
12:00	-1.15±1.70	-0.86±1.01	-3.86 to 1.55	-2.12 to 0.39	4	5
14:00	-0.20±1.20[d]	-0.22±0.73	-2.11 to 1.71	-1.39 to 0.94	4	4
16:00	-1.91±1.62	-0.06±0.81	-3.92 to 0.10	-2.06 to 1.94	5	3
18:00	-2.73±0.39[a,c]	0.16±0.41	-3.69 to 1.77[b]	-0.50 to 0.82	3	4
20:00	0.40±0.22[d]	-0.15±0.60	-0.15 to 0.95	-1.09 to 0.80	3	4
22:00	-0.67±0.75[d]	0.36±1.35	-1.86 to 0.52	-1.06 to 1.77	4	6

[a]Phase shifts significantly different from hemolymph-Ringer injections (*P*<0.05; two-tailed *t*-test)
[b]Phase shifts significantly different from zero as judged by the 95% CI (see Materials and methods)
[c]Phase delay at CT 8 and 18 significantly different from FMRFamide-dependent phase shifts at other circadian times ([d]*P*<0.05; two-tailed *t*-test)

neither significantly different from zero nor significantly different from hemolymph-Ringer injections.

Mass spectrometric analysis of extended FMRFamides in excised AMae

Mass spectra taken from preparations of a complete AMe did not reveal clear peptide signals. Thus, the AMe was separated into different pieces, and some of these preparations yielded ion signals of unknown substances; in addition to the more prominent mass signal of leucomyosuppressin (LMS; Söhler et al. 2007). The intensity of the ion signals obtained from preparations of the AMe was not sufficient for sequence elucidation. To test the assumption that the mass spectra contained signals of hitherto unknown species-specific extended FMRFamides, the peptide complement of the tPSOs of *L. maderae* were examined (Fig. 5a). These organs are known to accumulate large amounts of extended FMRFamides in the cockroach *P. americana* (Predel et al. 2004). A first screening of the respective peptides from tPSOs by means of tandem mass spectrometry confirmed that these peptides were extended FMRFamides; a peptide with a monoisotopic mass of 1,136.6 Da [(M+H)+] (Fig. 5b) could be identified as AVRDNFIRFamide (S. Neupert and R. Predel, unpublished). A comparison of mass spectra from tPSOs with those obtained from preparations of

Table 2 Phase shifts (in circadian hours) resulting from injections of 150 fmol Pea-FMRF-7 and from control (hemolymph-Ringer) injections at various times of the circadian cycle (*CT* circadian time) with statistically significant differences at CT 4 (*CI* confidence interval).

CT (h)	Phase shifts (mean±SD)		95% CI (lower to upper limit)		Number	
	Pea-FMRFa-7	Hemolymph-Ringer	Pea-FMRFa-7	Hemolymph-Ringer	Pea-FMRFa-7	Hemolymph-Ringer
00:00	-0.63±1.05	0.42±0.95	-3.25 to 1.98	-1.59 to 0.76	7	5
02:00	-0.19±0.52[d]	0.25±1.24	-0.67 to 0.29	-2.22 to 1.73	7	4
04:00	-3.10±1.71[a,c]	0.45±1.12	-4.89 to 1.30[b]	-1.83 to 0.94	6	5
06:00	-1.75±1.04	0.20±0.95	-4.34 to 0.83	-2.15 to 2.55	3	3
08:00	-1.46±0.91	0.40±1.00	-2.90 to 0.02[b]	-1.32 to 0.52	4	7
10:00	-1.00±1.93	0.95±0.28	-5.78 to 3.79	-0.80 to 0.61	3	3
12:00	-0.72±1.70	0.86±1.01	-3.42 to 1.98	-2.12 to 0.39	4	5
15:00	-0.08±1.12[d]	0.22±0.73	-1.46 to 1.32	-1.39 to 0.94	5	4
18:00	-0.38±1.24[d]	0.16±0.41	-2.35 to 1.59	-0.50 to 0.82	4	4
21:00	-0.52±0.22[d]	0.15±0.60	-1.07 to 0.03	-1.09 to 0.80	3	4

[a]Phase shifts significantly different from hemolymph-Ringer injections (*P*<0.05; two-tailed *t*-test)
[b]Phase shifts significantly different from zero as judged by the 95% CI (see Materials and methods)
[c]Phase delay significantly different from DRSDNFIRFamide-dependent phase shifts at other circadian times ([d]*P*<0.05; two-tailed *t*-test)
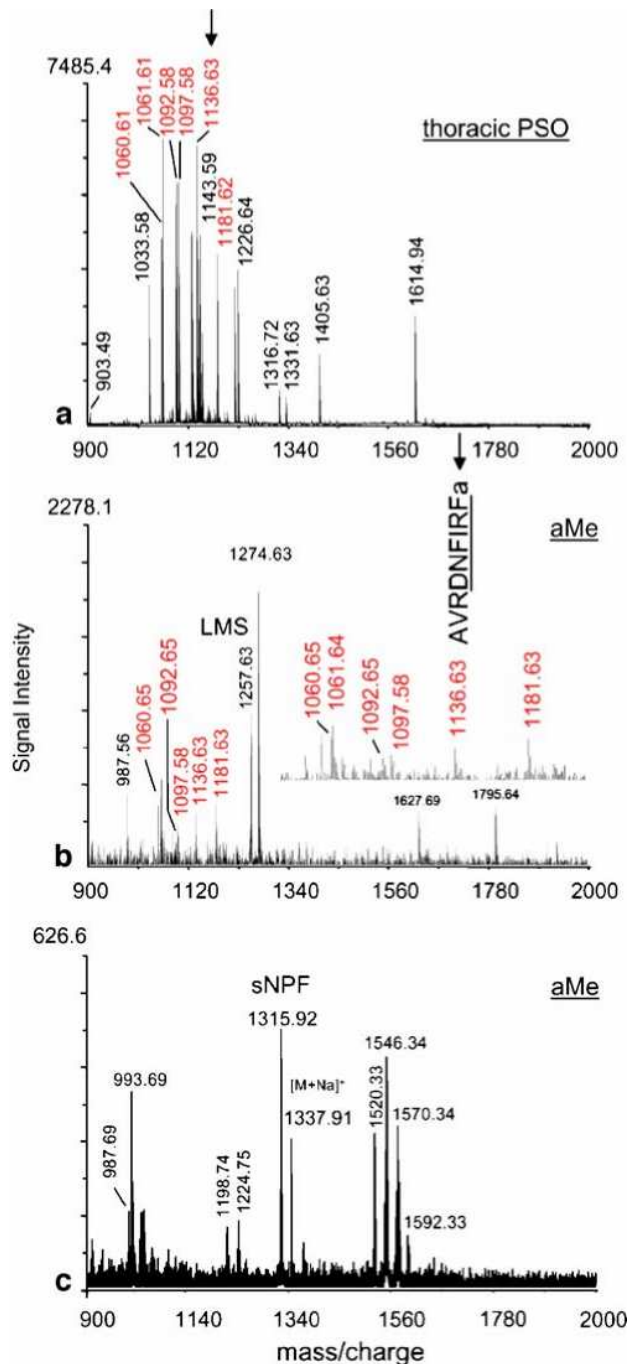
Fig. 5 a, b Comparison of mass spectra from preparations of a single thoracic perisympathetic organ (tPSO) and from the accessory medulla (*aMe*) of *Leucophaea maderae*. The tPSO spectrum appears to contain several extended FMRFamides (see Predel et al. 2004 for *P. americana*) most of which were also seen in the aMe (*red*). Enlarged in **b** Spectrum of one aMe with FMRFamide candidates shown in *red*. *Arrows* indicate the peptide at [M+H]$^+$: 1,136.63, which was identified as AVRDNFIRFamide (S. Neupert and R. Predel, unpublished). Additionally, the blocked form [M+H]$^+$: 1,257.63 and the unblocked form [M+H]$^+$: 1,274.63 of leucomyosuppressin (*LMS*) could be detected in the accessory medulla. **c** MALDI-TOF mass spectrometry showing the presence of sNPF with a mass of [M+H]+ of 1,315.92 Da.

the AMe revealed the occurrence of identical masses in both tissues (Fig. 5a,b). Additionally, we detected a peptide with a mass of 1,315.9 Da (Fig. 5c) identical with the theoretical mass of Pea-sNPF.

Discussion

This study examined the distribution and function of various FaRPs in the AMe, the circadian clock controlling circadian locomotor activity rhythms, in the cockroach *L. maderae*. Anti-FMRFamide immunocytochemistry revealed neurons in four (VNe, DFVNe, MNe, VPNe) of the six cell groups associated with the AMe. Additionally, cells in the group of ANes were immunostained. The anti-sNPF antibody and the anti-DMS antibody recognized ANes and VNes. With the PSK antibody, no immuno-reactive neurons near the AMe were detectable. Results following injections of FMRFamide and Pea-FMRFa-7 into the vicinity of the AMe suggested that various members of the FaRPs were involved in the control of circadian locomotor activity rhythms. This hypothesis was confirmed by MALDI-TOF and ESI-Q-TOF mass spectrometry, which identified FaRP candidates in the cockroach *L. maderae*.

Specificity of the antisera

Interpretation of peptide expression patterns observed by immunocytochemistry is critically dependent on the spec-ificity of the antisera used. As previously reported, the anti-FMRFamide antibodies most likely recognize most or all of the different members of FaRPs in various cockroach species (Orchard et al. 2001; Nässel and Homberg 2006; Predel 2006). In *Diploptera punctata*, for example, the FMRFamide antiserum detects, in addition to five high-performance liquid-chromatographic fractions, the LMS-containing fraction. Therefore, the anti-FMRFamide antiserum probably recognizes most FaRPs in *L. maderae* (Fuse et al. 1998). This assumption is confirmed by a comparison of anti-DMS immunoreactivity with anti-FMRFamide immunoreactivity obtained with two different anti-FMRFamide antibodies (Petri et al. 1995; Söhler et al. 2007). The DMS-ir neurons appear to be a subpopulation of the FMRFamide-ir neurons in *L. maderae*, whereas the staining patterns obtained with the various FMRFamide antibodies do not appear to be significantly different. The different numbers of FMRFamide-ir neurons detected next to the AMe seem to be attributable to differences in the dilutions of the various anti-FMRFamide antisera used (Petri et al. 1995).

The specificity of the PSK antiserum has been characterized by Agricola and Bräunig (1995) who tested the antiserum with a competitive and non-competitive enzyme-linked

immunosorbent assay and found no cross-reactions with other FaRPs at the dilutions used for immunocyto-chemistry. Additionally, immunocytochemical experiments on *Drosophila melanogaster*, the only insect species for which the differential localization of the FMRFamides, LMS, and sulfakinins has been determined, have shown that the PSK antiserum is specific for sulfakinins when used at concentrations of 1:10,000 or lower (Veenstra et al. 1995). Furthermore, East et al. (1997) have shown that neurons stained with drosulfakinin antiserum are located only in the pars intercerebralis and in four pairs of cell bodies in the posterior surface of the brain. These results, which were obtained from the cockroach *P. americana*, are comparable with our results obtained from *L. maderae* and strengthen our assumption concerning the specificity of the antiserum used.

The specificity of the Pea-sNPF antiserum has been demonstrated by preincubation of the antiserum with 10^{-5} M sNPF, which results in complete loss of the staining. Because controls do not reveal any specific staining, and since the antiserum used only recognizes a few cells compared with the FMRFamide antiserum, the observed immunostaining probably represents sNPF. This assumption has been confirmed by MALDI-TOF mass spectrometry, which has revealed the presence of sNPF in isolated AMae, whereas leucosulfakinin is not detected. However, we cannot exclude that some of the cells also contain other structurally related peptides.

FaRP immunoreactivity in the central brain

The widespread distribution of FMRFamide immuno-reactivity in the central brain of the cockroach *L. maderae* indicates a prominent role of this large peptide family in regulating neuronal and physiological activities in the cockroach. In addition to possible roles as neuromodula-tors, a neurohormonal role of FaRPs in *L. maderae* is suggested from the presence of immunoreactive somata in the pars intercerebralis and stained fibers projecting toward the retrocerebral complex. This assumption is in accordance with reports of FaRPs in neurosecretory cells and the retrocerebral complex in other insects (Meola et al. 1991; Donly et al. 1996; East et al. 1997; Fuse et al. 1998; Nässel 2000, 2002). Furthermore, FaRPs have been detected in the hemolymph from where they can reach all organs and tissues of physiological interest (Elia et al. 1993, 1995). The finding of FMRFamide-ir , PSK-ir, and sNPF-ir fibers in the superior median, superior lateral, inferior lateral, and ventrolateral protocerebrum suggests that the PSK-ir and sNPF-ir fibers are subgroups of the FMRFamide-ir fibers. PDF-ir fibers have also been observed in the same locations. Thus, in these regions, PDF and FaRPs appeared to be colocalized (Petri et al. 1995).

FaRP immunoreactivity in the circadian system

As previously shown by Petri et al. (1995) with a different anti-FMRFamide antibody, the distribution of FaRPs is highly prominent in the circadian system of the cockroach. Since, in the earlier work, the different soma groups associated with the circadian clock were not known, the assignment of the immunostaining to different functional circuits of the circadian clock was not possible. Here, we have shown that not only local interneurons of the AMe, but also output cells are immunostained, because the FMRFamide-ir neurons are located in the group of the DFVNes (about nine somata), VNes (about 13 somata), VPNes (about two somata), MNes (about two somata), and ANes (about two somata), next to the AMe. The DFVNes are assumed to be local interneurons of the AMe. Among the ~29 DFVNes are three to five small, weakly PDF-ir, medulla cells (PDFMes), four to six orcokinin-ir neurons, and about ten allatotropin-ir neurons (Reischig and Stengl 1996, 2003b; Hofer and Homberg 2006a, b). Both the orcokinin-ir and allatotropin-ir cells project into the noduli of the AMe. The noduli are involved in light-information processing, since they are densely innervated via the gamma-amino-butyric acid (GABA)-ergic distal tract, which relays light input from the medulla to the AMe (Petri et al. 1995, 2002; Reischig and Stengl 1996, 2002, 2003b). Furthermore, all substances tested that express immunoreactivity in the noduli, such as allatotropin, GABA, and orcokinin, produce light-like biphasic phase response curves (PRCs) when tested in running-wheel assays (Petri et al. 2002; Hofer and Homberg 2006b). The VNes are output neurons of the AMe and project to various targets in the central brain: the superior median proto-cerebrum, the superior lateral protocerebrum, the inferior lateral protocerebrum, and the ventrolateral protocerebrum. Among the ~24 VNes are one DMS-ir, three to six large and three to five median PDFMes, three to four allatotropin-ir neurons and GABA-ir neurons, and 16 orcokinin-ir cells (Reischig and Stengl 2002, 2003b; Petri et al. 2002; Hofer and Homberg 2006a, b; Söhler et al. 2007). Four VNe cells directly connect both bilaterally symmetric AMae, apparently as a coupling pathway (Reischig et al. 2004). Three of these cells are PDF-ir, namely the large PDFMe and two medium PDFMe cells. Because up to six of the ~12 PDF-ir PDFMe cells colocalize FMRFamide immuno-reactivity, some of the coupling cells might also be FMRFamide-ir. As one to four sNPF-ir cells and one DMS-ir cell occur among the VNes, they most probably represent a subgroup of the FMRFamide-ir VNes. It remains to be tested whether coupling VNes contain sNPF or LMS. Since no sNPF immunoreactivity, but DMS immunoreactivity, has been found in the anterior and posterior commissures, which connect both AMae, it seems

more likely that LMS acts as a coupling factor. Future colocalization studies will test this hypothesis. Lee et al. (2006) have found NPF in the dorsal lateral circadian pacemaker neurons (LNd$_s$) of the *Drosophila* brain. Therefore, NPF has been suggested to be involved in chronobiological functions. NPF might act as a neuro-modulatory substance within a subset of LNd$_s$ involved in the late-day component of locomotor activity (Lee et al. 2006). This is the first evidence for the role of NPF as an output factor that might participate in certain aspects of clock-controlled reproductive behavior. Because sNPF in the cockroach is also located in output neurons, it may play a similar role as NPF in the fly. Future injections experiments will test this hypothesis. Since both DMS-ir (Söhler et al 2007) and sNPF-ir neurons branch in the noduli, they could be involved in light-processing tasks. Among the ~36 VPNes, two are FMRFamide-ir , and four are orcokinin-ir (Hofer and Homberg 2006a, b). The function and arborization pattern of these cells is not known. At least two FMRFamide-cells belong to the MNes. The ~56 MNes represent a heterogeneous group of neurons that apparently fullfil different functions. Among them are two orcokinin-ir and two to four Mas-allatotropin-ir neurons (Reischig and Stengl 2003b; Hofer and Homberg 2006b). A subset of neurons of the group of the MNes possibly contributes to the distal tract (Homberg et al. 2003).

Functional role of the FaRPs in the circadian system

Because FMRFamide-ir neurons densely innervate the internodular and part of the nodular neuropil of the AMe, members of the FaRPs probably play a variety of roles in the circadian system. To determine at what circadian times members of this large superfamily of peptides might affect circadian locomotor activity rhythms, we have injected the most conserved FaRP sequence, viz., the tetrapeptide FMRFamide. This C-terminal structure of the last four C-terminal amino acids is most conserved between the different animal species and is expected to cross-react with diverse FaRP-receptors, whereas the N-terminal extensions vary considerably and relay receptor specificity. Further-more, methionine in position 2 is mostly tolerant to substitutions, and isoleucine and methionine are amino acids that are both nonpolar and hydrophobic (Chin et al. 1994). This suggests that the tetrapeptide FMRFamide, although not found in the cockroach *P. americana*, can bind to several different FaRP receptors. Furthermore, Cazzamali and Grimmelikhuijzen (2002) have shown that the *Drosophila* FMRFamide receptor even binds the tetrapeptide FMRFamide without any N-terminal exten-sions, and that the binding affinity for this peptide is higher than for SDNFMRFamide, suggesting that some N-terminal extensions reduce binding affinity.

The FMRFamide-dependent PRCs with significant phase delays at CT 8 and 18 most likely arise as the result of the binding of FMRFamide to different FaRP receptors. This is further supported by the finding that injection of 10^{-2} fmol FMRFamide at CT 18 causes phase advances. Thus, we hypothesize that the FMRFamide-dependent PRC represents an overlay of monophasic PRCs, with at least one FaRP acting at CT 8 and at least two acting at CT 18. To differentiate the PRC obtained from FMRFamide injections further, we have injected Pea-FMRFa-7. This peptide has been chosen because Predel et al. (2004) have shown that many FaRPs ending with FIRFamide exist in the tPSOs of *P. americana*, among them the highly prominent Pea-FMRFa-7. Because our mass spectrometric analysis of the tPSOs of *L. maderae* has revealed the occurrence of FaRPs ending with FIRFamide, Pea-FMRFa-7 probably cross-reacts with an FIRFamide-receptor of a related peptide in *L. maderae* (Pea FMRF-7 has been employed, since it closely resembles the partly sequenced Lem-FMRFa). This hypothesis has been confirmed by the Pea-FMRFa-7-dependent monophasic all-delay PRC with a significant phase delay at CT 4. Thus, an extended FIRFamide probably acts at CT 4. In contrast to the light-like biphasic PRCs obtained via injection of orcokinin, Mas-allatotropin, and GABA, the FMRFamide- and Pea-FMRFa-7-dependent PRCs suggest that FaRPs are involved in a circadian function different from light. However, the FMRFamide-dependent phase delays at CT 18 could be part of the light-entrainment pathway, because they overlap with light-type PRCs. Thus, some FaRPs possibly located in DFVNes might relay light-dependent phase delays during the early night from the contralateral eye.

Schneider and Stengl (2007) have postulated that peaks of changes in the electrical activity of AMe neurons occurring at the early day, the late day/early night and the middle of the night are correlated with a vast amount of peptide release. Interestingly, the activity peak distribution of AMe neurons correlates well with the maxima of phase responses obtained after the injections of PDF (CT 9), GABA (CT 13–15, CT 19), and serotonin (CT 7–13). With injections of FMRFamide, we have obtained maximal phase shifts at CT 8 and 18 and with Pea-FMRFa-7 at CT 4. Therefore, peptides of this family could be responsible for the maximum of activity changes during the early day, when no other substances affect locomotor activity rhythms.

Concluding remarks

We assume that several members of the FaRPs are present in the AMe. Apparently, they play different roles as non-photic and possibly also as photic inputs to the circadian system and modulate circadian locomotor activity rhythms

at, at least, three different circadian times. Future cloning of the FaRP-genes in *L. maderae* combined with single-cell mass spectrometric analysis and various physiological assays are necessary for the further characterization of this large peptide superfamily.

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Chapter III. Circadian pacemaker coupling by multi-peptidergic neurons in the cockroach *Leucophaea maderae*

Soehler S, Stengl M and Reischig T

Circadian pacemaker coupling by multi-peptidergic neurons in the cockroach *Leucophaea maderae*

Sandra Soehler • Monika Stengl • Thomas Reischig

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Abstract In the cockroach *Leucophaea maderae,* lesion and transplantation studies located its bilaterally symmetric circadian pacemakers necessary for driving circadian locomotor activity rhythms to the accessory medulla of the optic lobes. The accessory medulla is composed of a network of peptidergic neurons, among them the pigment-dispersing factor (PDF) expressing circadian pacemaker cells. In *L. maderae*, at least three of the PDF-expressing neurons directly connect both accessory medullae, apparently as a circadian coupling pathway. Here, the PDF-expressing circadian coupling pathways were examined for peptide colocalization with tracer experiments combined with double-label immunohistochemistry employing antisera against PDF, FMRFamide, and Asn^{13}-orcokinin. We found a fourth group of contralaterally projecting medulla neurons additionally to the previously known three groups, thus probably adding a new level of complexity to the pacemaker synchronization pathways. One group contained up to four contralaterally projecting PDF-expressing medulla neurons. Of them, three medium-sized PDF-neurons co-expressed FMRFamide-, and Asn^{13}-orcokinin immunoreactivity. In contrast, the fourth and largest contralaterally projecting PDF-neuron showed no further peptide colocalization. Although two third of all PDF-expressing medulla neurons were additionally FMRFamide- and orcokinin-immunoreactive, colocalization of PDF- and FMRFamide immunoreactivity was observed in only few termination sites of PDF-expressing medulla neurons, and colocalization of PDF and orcokinin immunoreactivity was never observed in terminals or optic commissures. We therefore suggest that circadian pacemaker candidates employ axonal peptide-sorting to phase control physiological processes at specific times of the day.

Keywords Circadian rhythm · Accessory medulla · Orcokinin · Pigment-dispersing hormone · FMRFamide · Cockroach, *Leucophaea maderae* (Insecta)

S. Soehler   M. Stengl
Department of Natural Sciences, Animal Physiology,
University of Kassel,
Heinrich-Plett-Strasse 40,
D-34132 Kassel, Germany

T. Reischig (✉)
Johann Friedrich Blumenbach Institute of Zoology and
Anthropology, Neurobiology,
Georg August University of Göttingen,
Berliner Str. 28,
D-37073 Göttingen, Germany
e-mail: treisch@gwdg.de

Introduction

Much research on structural, functional, and molecular properties of endogenous circadian clocks has been performed on insects. Thus, the first circadian pacemaker controlling behavioral activity patterns was detected in the optic lobes of the cockroach *Leucophaea maderae* (Nishiitsutsuji-Uwo and Pittendrigh 1968, Roberts 1974, Sokolove 1975, Page 1982). Lesion and transplantation studies located the circadian pacemaker to the anterior, ventromedial border of the medulla of the optic lobes, which is named accessory medulla (AMe, plural AMae) (Stengl and Homberg 1994, Reischig and Stengl 2003a, reviewed by Homberg et al. 2003). The AMe is formed by a set of about 250 neurons with somata close to the AMe, most of which can be classified into

several groups recognizable by morphological and immunohistochemical characters (Reischig and Stengl 2003b, see Materials and Methods). Among these is a distinct set of neurons, which expresses the neuropeptide pigment-dispersing factor (PDF), the PDF-expressing medulla neurons (PDFMe, Fig 1a).

In *Drosophila melanogaster*, homologous PDF-expressing neurons (the ventral group of the lateral neurons, LN$_{vs}$) were shown to be circadian pacemakers indispensable for maintaining circadian locomotor rhythms under constant conditions (reviewed by Helfrich-Förster 2005). Also in cockroaches and other insects the homologous PDFMe are circadian pacemaker candidates (Stengl and Homberg 1994, Singaravel et al. 2003, Reischig and Stengl 2003a, Wen and Lee 2008). Besides their role as circadian pacemakers, the PDF-expressing neurons are assumed to provide circadian timing signals as outputs from beaded terminals arborizing in large areas of the optic lobes and central protocerebrum. Moreover, it was shown in *L. maderae* and *D. melanogaster* that fibers of a subgroup of the PDF-expressing neurons of one optic lobe enter the contralateral optic lobe and also the contralateral AMe (Reischig et al. 2004, Helfrich-Förster et al. 2007). These observations suggest that contralaterally projecting PDFMe provide the neuronal pathway for mutual pacemaker coupling that was proposed by sophisticated behavioral experiments of Page et al. in *L. maderae* (Page et al. 1977, Page 1978, 1983a). This hypothesis is further supported by PDF injections into the vicinity of the AMe of *L. maderae* at different daytimes, which resulted in a monophasic phase response curve (PRC) (Petri and Stengl 1997). These experiments demonstrated that PDF phase delays locomotor activity onset only at the late day. Computer modeling based on experimental observations predicted that phase-delaying as well as phase-advancing neurons constitute mutual pacemaker coupling to explain experimental results observed so far (Petri and Stengl 2001). Thus, a search for circadian coupling pathways, which used tracer injections into the contralateral AMe combined with anti-PDF immunolabeling, identified up to four contralaterally projecting neurons at the location of the anterior PDFMe among the ventral
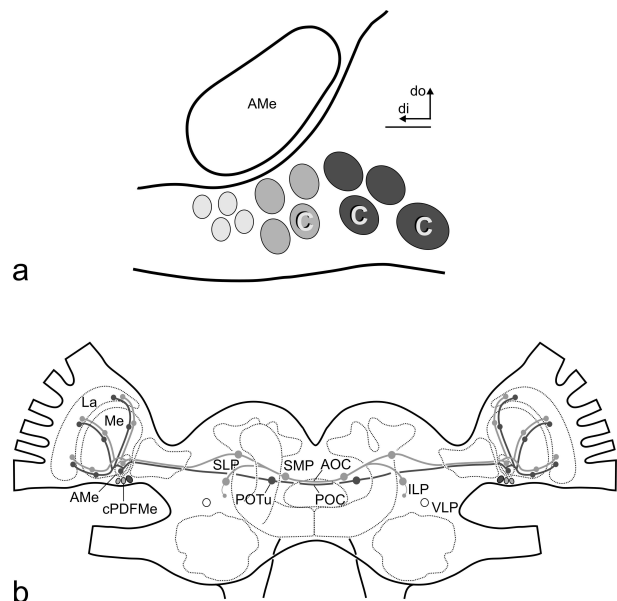

Fig. 1a Scheme of the accessory medulla (*AMe*) with its associated anterior pigment-dispersing hormone (PDH)-immunoreactive (ir) neurons (*aPDFMe*). These 12 aPDFMe could be grouped into two soma groups. The 4 small and weakly immunoreactive somata (*light grey*) belong to the distal frontoventral neurons (*DFVNe*) and the 4 middle and more intensively stained (*medium grey*) and 4 large and most intensively stained (*dark grey*) somata belong to the group of the ventral neurons (*VNe*). The group of the VNe host 3 contralateral projecting neurons (indicated by *C*). The most proximal soma projecting to the contralateral lobe is prominently larger than the others. Coordinates: *di* distal, *do* dorsal. Scheme adopted from Reischig et al. 2004. **b** Scheme of the presumptive pathways of the contralaterally projecting PDH-ir medulla cells (*cPDFMe*). The prominent large cPDFMe (*dark grey*) appears to connect both AMae via the posterior optic commissure (*POC*) and both posterior optic tubercles (*POTu*). The remaining cPDFMe (*light grey*) project via the anterior optic commissure (*AOC*) with arborizations in the anterior and proximal superior lateral protocerebra (*SLP*), the superior median protocerebra (*SMP*), and, to a lesser extend, the inferior lateral protocerebra (*ILP*). The PDH-ir fibers of the ventrolateral protocerebra (*VLP*) do not seem to belong to the contralateral AMe. Scheme provided by Dr. T. Reischig (from Reischig et al. 2004).

neurons (VNe) of the AMe. Three of these contralateral VNe neurons were shown to be PDF-immunoreactive (ir) (Fig. 1b) (Reischig et al. 2004). In addition, Hofer and Homberg (2006a) demonstrated that one of these four contralaterally projecting VNe neurons was orcokinin-ir. However, it remained unknown whether orcokinin immunoreactivity was colocalized with PDF immunoreactivity in the group of the four contralaterally projecting VNe (Hofer and Homberg 2006b).

Other neuromodulator candidates involved in a bilateral pacemaker coupling pathway are members of the FMRFamide-related peptides (FaRPs). The FaRPs share the RFamide C-terminus and are involved in the regulation of a multitude of physiological activities (Predel 2006, Orchard and Lange 2006). Since studies with antibodies against FMRFamides revealed colocalization of PDF with FMRFamide immunoreactivity in up to six PDFMe, FaRPs could be involved in the circadian coupling pathway (Petri et al. 1995). This hypothesis was supported by the finding of FMRFamide immunoreactivity in both the anterior and posterior optic commissures, which connect both AMae. Finally, injection experiments combined with locomotor activity assays showed that different FaRPs affect locomotor activity rhythms at distinct circadian times (Soehler et al. 2008). Thus, these anatomical and physiological data suggested the involvement of FaRPs in circadian pacemaker coupling of the cockroach *L. maderae*.

Here, the circadian coupling pathways were further investigated with tracer injections into one AMe combined with immunolabeling with anti-FMRFamide and backfills from one optic stalk, combined with immunolabeling with anti-PDF together with either anti-FMRFamide or anti-Asn[13]-orcokinin. We found better and more consistent tracer labeling with neurobiotin backfills compared to dextran injections, thus leading to a larger number of neurons apparently involved in pacemaker coupling. Furthermore, we demonstrated that neurons that contain at least three different neuropeptides connect both AMae with sparse colocalization of neuropeptides in their axonal terminals. In addition, further details of the neuroarchitecture of this circadian pacemaker neuropil were elucidated.

Materials and Methods

Animals

Adult male cockroaches (*Leucophaea maderae*) were taken from laboratory colonies. They were reared under 12:12 hours light-dark (LD) photoperiod at about 60% relative humidity and a temperature of 26°C. Animals were fed with dried dog food, potatoes and water *ad libitum*. Indications of position (left, right, etc.) are always referred to the animal's body axis.

Neuron classification

Frontally, medially, and ventrally to the AMe extends a group of about 250 neurons of which most appear to contribute to the AMe. According to size, position, morphological characters, and immunostaining properties these neurons can be classified into at least six main groups (Reischig and Stengl 1996, 2003b). These are the distal and medial frontoventral neurons (DFVNe and MFVNe, respectively), the medial and ventral neurons (MNe and VNe), the ventromedial neurons (VMNe), and the ventroposterior neurons (VPNe) of the AMe. The PDFMe are separated into an anterior and a posterior group (anterior PDFMe and posterior PDFMe, respectively). The anterior PDFMe further consist of three subgroups, which can be distinguished by size and intensity of anti-PDF immunolabeling: the intensively labeled large anterior PDFMe, the smaller and generally less intensively labeled medium-sized anterior PDFMe, and the rather faintly labeled small anterior PDFMe. According to the criteria of the six morphological groups, large and medium-sized anterior PDFMe belong to the VNe, while the small anterior PDFMe belong to the DFVNe. Furthermore, three groups of medulla neurons projecting to the contralateral optic lobe were found in previous works and were termed contralaterally projecting medulla neurons (MC I-III), of which at least two (MC I and MC II) definitely project to the contralateral AMe (Reischig et al. 2004). MC I are the contralaterally projecting anterior PDFMe and a non-PDF-ir VNe, MC II is identical with the VMNe. MC III is located in a posterior position similar but not identical to the posterior PDFMe.

Primary antisera

The monoclonal anti-*Drosophila*-PDF antibody was generated by Dr. Justin Blau (New York University, USA) and purchased at Developmental Studies Hybridoma Bank

(DSHB) at the University of Iowa, USA. B-Lymphocytes were raised in cell culture, and pure supernatant was used for immunostaining at a working dilution of 1:5. The anti-β-PDH antiserum (#3B3) was a gift from Heinrich Dircksen, Stockholm, and was raised in rabbits against synthetic *Uca pugilator* β-PDH (Dircksen et al. 1987) This antiserum was widely used to detect PDF expressing neurons in many insect species and recently systematically characterized by Honda et al. (2006), while PDF was isolated in *L. maderae* by Hamasaka et al. (2005). The anti-β-PDH antiserum was used at a working dilution of 1:15,000 to perform specificity control of the monoclonal anti-*Drosophila*-PDF antibody in *L. maderae*. For this, double-labeling was performed with the anti-*Drosophila*-PDF antibody and the anti-β-PDH antiserum. Procedures, buffers, and incubation times were the same as described below. The antibodies were detected with goat anti-mouse Cy2 and goat anti-rabbit Alexa 633. Both antibodies labeled identical structures with the above mentioned working dilutions (data not shown). The anti-FMRFamide antiserum (# 671) was a gift from Dr. E. Marder, Massachusetts, USA (Marder et al. 1987), and was raised in rabbits against the C-terminal amino acid sequence FMRFamide. Hence, the antiserum is supposed to detect all members of the large FaRP family. We used the antiserum at a working dilution of 1:4,000. The anti-orcokinin antiserum (kind gift from Heinrich Dircksen) was raised in rabbits against the tridecapeptide Asn[13]-orcokinin of the crayfish *Orconectes limosus*, and was used at a working dilution of 1:1,000. The specificity of the Asn[13]-orcokinin antiserum on cockroach brain sections was demonstrated by Hofer et al. (2005), who identified orcokinin-related peptides in *Schistocerca gregaria* and *L. maderae*, which were recognized by the anti-Asn[13]-orcokinin antiserum.

Injection of dextran

The operations and injections of the cockroaches were accomplished as described by Reischig and Stengl (2002). About 5 nl 10% rhodamine-dextran solution (dextran conjugated
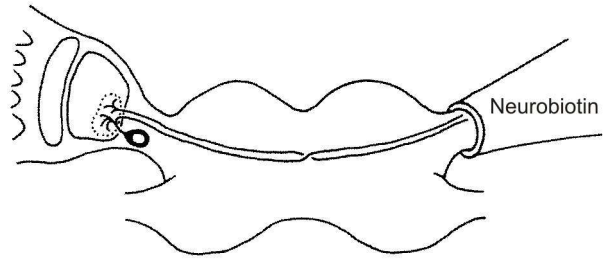

Fig. 2 Scheme of the cockroach brain with proposed direct coupling pathways connecting both accessory medullae. For the neurobiotin backfills one optic stalk was cut and the neurobiotin filled suction pipette was slipped over the cut optic stalk (from Reischig and Stengl 2002).

with the fluorescent dye rhodamine, 3,000 MW, lysine fixable, Molecular Probes Inc., USA 0.1 µg/µl in hemolymph-ringer; Kaissling and Thorson 1980) was injected into the left AMe. After the injection the head capsule was closed with wax. The animals were kept overnight in a box to allow intracellular transport of the dye.

Backfill with neurobiotin

The neurobiotin backfills were accomplished as reported by Reischig and Stengl (2002, Fig. 2). Animals were anesthetized with CO_2 and fixed in a mounting device with their back down, thus enabling a direct view onto the frontal side of the head. The whole operation was performed while maintaining anesthetization by means of continuous aerating of the animal with CO_2. A small window was cut into the head capsule to expose the left optic lobe. The application pipette was filled with 1-2 µl of solution of 5% neurobiotin in distilled water. The left optic stalk was cut and the application pipette with a tip opening of 300-400 µm was slipped over the stump of the optic stalk. The pipette was then fixed with modeling clay and the operation field was closed with Vaseline. The animals were stored overnight in a box at 4°C to allow intracellular transport of the dye.

Immunocytochemistry for backfilled and injected animals

The next day after the operation, the brains were removed from the head capsules and fixed for four hours in 4% paraformaldehyde/

7.5% saturated picric acid in sodium phosphate buffer (PB, 0.1 M, pH 7,4) at room temperature. The fixed brains were embedded in gelatin/albumin (4.8% gelatin and 12% ovalbumin in demineralized water) and postfixed overnight in 4% paraformaldehyde in PB in the refrigerator. Then, the brains were sectioned with a vibrating blade microtome (Leica, Nussloch, Germany) in frontohorizontal slices at 50-60 μm thickness. The sections were washed with Tris-buffered saline (TBS: 0.1 M Tris-HCL/0.3 M NaCl, pH 7.4) containing 0.1% Triton X-100 (TrX) three times for 10 minutes, and preincubated in TBS with 0.5% TrX and 5% normal goat serum (NGS, DAKO, Hamburg, Germany) for one hour. Two antisera were applied simultaneously: either monoclonal anti-PDF with anti-FMRFamide or monoclonal anti-PDF with anti-orcokinin (see 'primary antisera' for dilutions) in TBS containing 0.5% TrX and 1% NGS. The sections were incubated in the antisera cocktails for 3–4 days in the refrigerator and then washed in TBS containing 0.5% TrX for 3 times for about 40 minutes. After that, the sections were incubated with a solution containing FITC-conjugated streptavidin (1:100) for detection of the neurobiotin, Cy2-conjugated goat anti mouse antiserum (1:300) for detection of anti-PDF, and Alexa 633-conjugated goat anti rabbit antiserum (1:300) for detection of anti-FMRFamide and anti-orcokinin, respectively (all probes from Dianova, Hamburg, Germany). The substances were diluted in TBS containing 0.5% TrX and 1% NGS for 2 h. The sections were then thoroughly rinsed with PB and cleared with Glycerol/PB 1:1 for at least 30 minutes. Finally, the sections were mounted on microscope slides in anatomical order, and coverslipped in Glycerol/PB 1:1.

Evaluation and visualization

The preparations were examined with a Leica confocal laser scanning microscope TCS SP2 equipped with an acusto-optical beam splitter for separation of excitation and emission light, and with a variable detection filtering system (spectrophotometer) for arbitrary selection of spectral intervals of emission light.
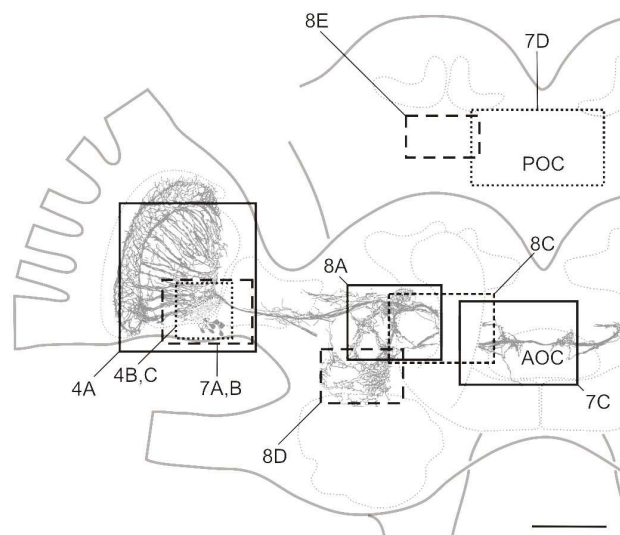

Fig. 3 Scheme of a semi brain of the cockroach to demonstrate the scanning sites of the confocal laser scan images presented in Figs. **4**, **5**, **6**, **7**, and **8**. The scheme shows the PDH-ir neuron system contralaterally to the backfilled optic lobe. *AOC* anterior optic commissure, *POC* posterior optic commissure. *Bar* 200 μm.

Most scans were performed using a Leica HC PL apochromate 20x/0.7 dry lens, but also a HCX PL apochromate 40x/1.25 oil immersion lens was used for high resolution scans of neuronal fibers. To exclude crosstalk artifacts all three channels were scanned sequentially, and the detection ranges were separated as far as possible (FITC: excitation with 488 nm line of argon laser, detection between 455 and 530 nm, Cy3: excitation with 543 nm line of helium/neon laser, detection between 490 and 610 nm, Alexa 633: excitation with 633 nm line of helium/neon laser, detection between 680 and 800 nm). All specimen with recognizable backfill staining (n = 25) were scanned. There, we scanned at least every section where the right AMe, the anterior and posterior PDFMe, and backfilled neurons of the AMe were present (Fig. 3, z-distance of single optical sections 2 μm). In some specimen, central termination areas of PDF-ir neurons and PDF-ir commissures were additionally scanned with the 40x optic and a z-resolution of 0.5–1 μm (Fig. 3). Data evaluation was done on a graphics computer workstation. For neuron counting, every soma was identified individually through and between image stacks to prevent counting artifacts resulting from the pure evaluation of neuron profiles.
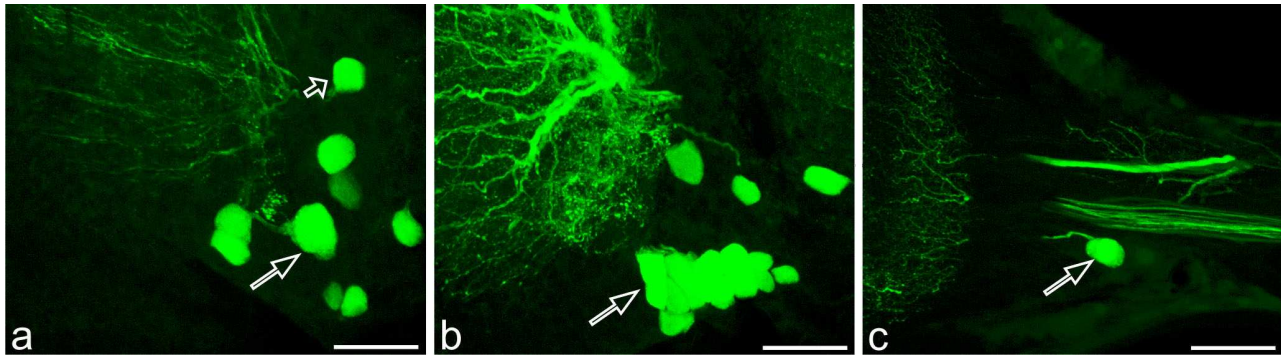
Fig. 4 Confocal laser scan images obtained from vibratome sections of the accessory medulla (*AMe*). Neurobiotin is shown in green. **a** Neurobiotin filled neurons of the groups of the VNe (= *MC I, large arrow*) and MNe (= *MC IV, small arrow*) are visible. **b** In the group of the VMNe neurons (= *MC II, arrow*) all neurons are filled with neurobiotin. **c** One neurobiotin filled neuron in the group of the posterior neurons (= *MC III, arrow*) is visible. *Bars 50* µm.

Results

To identify all neurons coupling both AMae and to clarify, if, next to PDF, the peptides orcokinin and FMRFamide are involved in the coupling of both AMae, neurobiotin backfills from one optic stalk were performed. Additionally, injections of dextran into one AMe and comparison of the contralaterally projecting neurons with previous studies (Reischig and Stengl 2002, Reischig et al. 2004) should differentiate the AMae coupling neurons from neurons that couple both optic lobes but not the AMae. The backfill experiments were combined with immuno-histochemistry with antisera either against PDF and FMRFamide (n = 11), or against PDF and orcokinin (n = 14). Labeled neuronal somata of the AMe contralaterally to the backfilled side, corresponding fiber projections in the contralateral optic lobe and central brain, as well as commissural fibers were detected with confocal laser scanning microscopy. Additionally, one AMe in 72 animals was injected with rhodamine dextran and stained with anti-FMRFamide antiserum. The neurobiotin backfills generally revealed crisper and more reproducible labeling compared to similar experiments employing rhodamine dextran in former works (Reischig and Stengl 2002, Reischig et al. 2004), and in the injection experiments of this work. In total, more backfilled neurons were counted, and a new group of backfilled neurons emerged that was not visible with rhodamine dextran backfills or injections (Fig 4). Also, contralaterally projecting medium-sized PDFMe were found that were additionally immunoreactive against both the anti-FMRFamide and anti-orcokinin antisera.

Anti-FMRFamide antiserum and anti-orcokinin antiserum labeled small and medium-sized PDFMe

Immunocytochemical labelings in the AMe of the cockroach with antisera against FMRFamide and orcokinin were described previously, and also colocalization with anti-PDF staining was reported (Petri et al. 1995, Hofer and Homberg 2006b, Soehler et al. 2008). Here, additional results make a reevaluation of the previously published labeling patterns necessary. A new monoclonal anti-PDF antibody (Cyran et al. 2005) was employed for the first time in the cockroach. To determine whether this monoclonal antibody, raised in mice against *Drosophila*-PDF, labeled the same structures as the well-known anti-*Uca*-β-PDH antiserum of Dircksen et al. (1987), double labeling experiments with both antisera were employed. The staining pattern obtained with both antisera completely overlapped (data not shown). Within the anterior PDFMe, the organization in strongly labeled large, weaker labeled medium-sized, and even weaker labeled small PDFMe was generally apparent. However, the *Drosophila* anti-PDF antibody revealed higher background and weaker specific labeling. Thus, a high concentration of antibody solution was required and a longer incubation time of at least two

days for satisfying tissue penetration. The difference in labeling intensity between large and medium-sized anterior PDFMe appeared larger than with the anti-β-PDH antiserum, thus facilitating discrimination between these groups, but hampering the discrimination between medium-sized and small anterior PDFMe (for cell counts see table 1). Double labelings with anti-FMRFamide and anti-*Drosophila*-PDF revealed colocalizing immunoreactivities in a subgroup of the PDFMe, which was far more consistent than in the work of Petri et al. (1995). Invariably, all small and all medium-sized anterior PDFMe showed additionally FMRFamide immuno-reactivity, while the large PDFMe never did (Fig. 5a$_{1-3}$). Consequently a clear distinction between the PDFMe subgroups was possible. Evaluating the posterior PDFMe revealed that all small posterior PDFMe were additionally FMRFamide-ir, while large posterior PDFMe were not (Fig 5b$_{1-3}$), except one case. Cell counting (table 1) revealed an interesting result: The sum of non-FMRFamide-ir anterior and non-FMRFamide posterior PDFMe nearly always resulted in a total of six neurons. In cases where no non-FMRFamide-ir PDFMe were found among the posterior PDFMe, six of them were found among the anterior PDFMe. Therefore, we assume that *L. maderae* actually

possesses six anterior large PDFMe, some of which can be shifted posteriorly by a developmental process (see discussion). In fiber projections, co-localization of PDF and FMRFamide immunoreactivity was found only in three areas of the brain, namely in the AMe (Fig. 5c$_{1-3}$), in a dorsal part of the superior lateral protocerebrum (dSLP, Fig. 6a$_{1-4}$), and in the posterior optic tubercle (POTu, Fig. 6b$_{1-4}$). Contrary to previous studies (Reischig and Stengl 1996, 2003a) on the AMe, PDF-ir fibers with varicosities were present in addition to the shell neuropil (including the anterior neuropil) also in the nodular neuropil. The PDF-ir varicosities of the nodular neuropil exhibited far lower labeling intensity and were generally more irregularly shaped than the strongly labeled PDF-ir varicosities of shell and internodular neuropil (Fig. 5c$_{1-3}$). Only the weakly labeled nodular PDF-ir projections showed additional FMRFamide immuno-reactivity. Additionally, the AMe was densely invaded by FMRFamide-ir fibers without additional PDF immunoreactivity. In the dSLP only PDF/FMRFamide-ir fibers occurred and no fibers with PDF-immunoreactivity alone. Many fibers with FMRFamide immuno-reactivity alone could also be detected in the dSLP. The origin of these fibers was unclear. The POTu host PDF/FMRFa-ir fibers, fibers

Table 1 Mean numbers of anterior and posterior PDFMe

	large anterior PDFMe	medium-sized anterior PDFMe	small anterior PDFMe	large posterior PDFMe	small posterior PDFMe
Total of all experiments ($n = 25$)	4.2 ± 1.6; 25	3.6 ± 1.0; 24	3.9 ± 1.8; 18	1.7 ± 1.7; 22	2.4 ± 1.3; 22
PDF + FMRFamid immunoreactivity ($n = 11$)	0 ± 0; 11	3.9 ± 0.9; 10	4.4 ± 1.3; 10	0.1 ± 0.3; 9 [1]	1.8 ± 1.5; 9 [2]
PDF + Asn[13]-orcokinin immunoreactivity ($n = 14$)	0 ± 0; 14	4.1 ± 1.1; 10	2.5 ± 0.6; 4 [3]	0 ± 0; 13	0.1 ± 0.3; 13 [4]

The table displays the total quantities of neuron somata in the five PDFMe subgroups over all backfill experiments, and with colocalizing FMRFamide and orcokinin immunoreactivity, respectively. Quantities are given as means ± standard deviation; the numbers after the semicolons report the number of evaluated animals (*n*). Where the *n* is lower than the total amount of animals, the count was not undertaken due to missing sections or, in most cases, due to low staining intensity that did not allow unambiguous identification.

[1] In only one case one large posterior PDFMe was FMRFamide-ir.

[2] In only one case one small posterior PDFMe was not FMRFamide-ir. In four cases, 1–2 small FMRFamid-ir somata were additionally found amongst the PDFMe that were not PDF-ir.

[3] Low *n* due to difficulties in labeling procedure (see text).

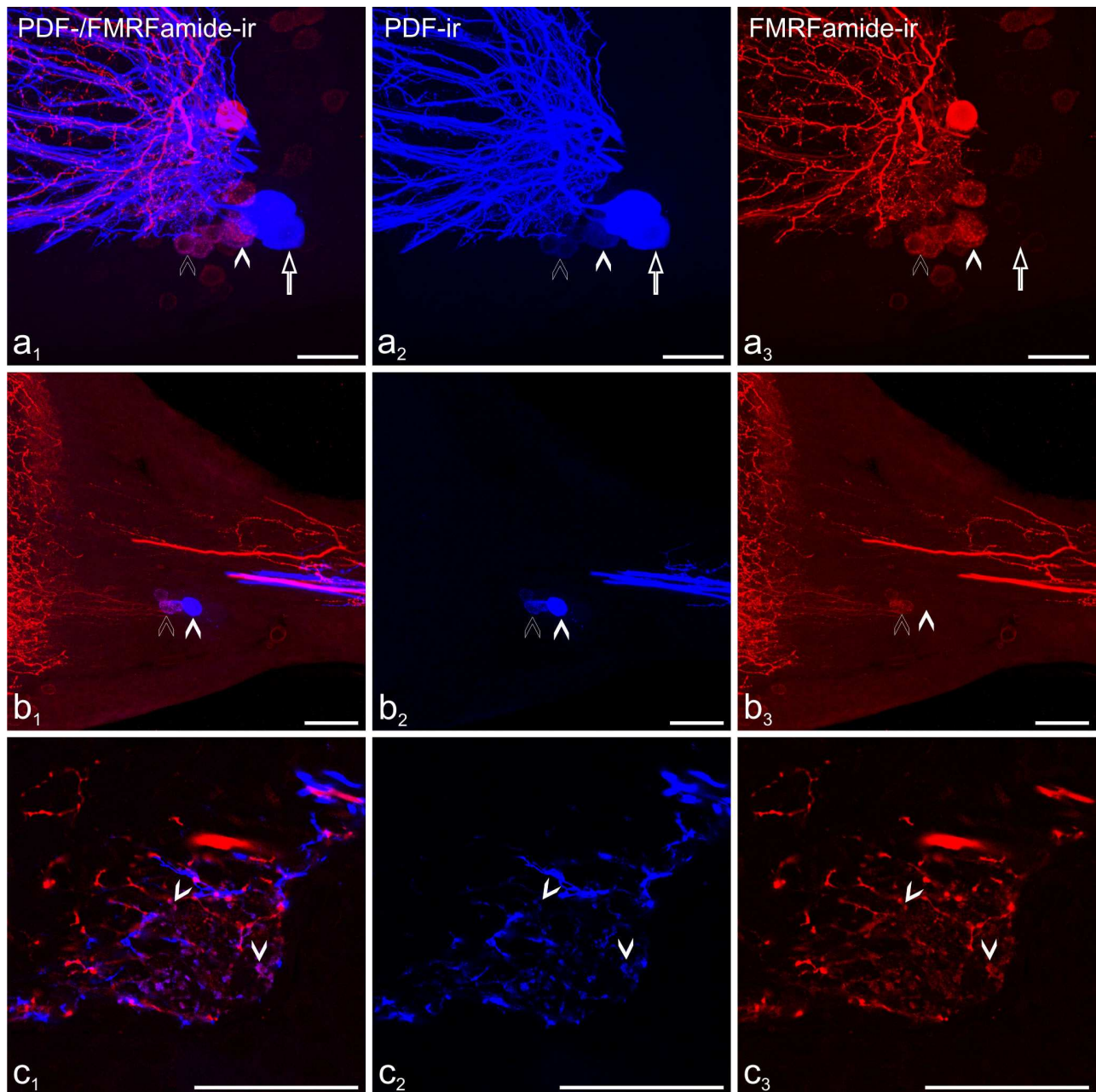[4] In only one case one small posterior PDFMe was orcokinin-ir.

Fig. 5 a-c Confocal laser scan images obtained from vibratome sections of the accessory medulla (*AMe*). PDF-ir is shown in blue and FMRFamide-ir is shown in red. The respective overlay images in the left column show colocalization. **a₁₋₄** Neurons of the small (*open arrowhead*), medium-sized (*closed arrowhead*) and large aPDFMe (*arrow*) are visible. **b₁₋₃** Of the posterior neurons (= MC III, *arrow*) near the lobula valley tract the small PDFMe neurons showed PDF- and FMRFamide-ir (*open arrowhead*) whereas the large showed only PDF-ir staining (*closed arrowhead*). **c₁₋₃** Fibers with colocalized immunoreactivities are visible in the AMe (*arrowheads*). *Bars* 50 μm.

which express only PDF immunoreactivity, and fibers with only FMRFamide immunoreactivity. Like in the dSLP the origin of the FMRFamide-ir fibers was unclear. As was shown previously, FMRFamide immunoreactivity was also found in other neuron groups of the AMe next to the small and medium-sized PDFMe, namely the MNe, the VPNe, the DFVNe including non-PDF-ir and PDF-ir neurons and the VNe

including non-PDF-ir and PDF-ir neurons. Cell counts generally corresponded to the previous work of Soehler et al. (2008) (table 2). In the VNe more non-PDF-ir FMRFamide-ir neurons than PDF/FMRFamide-ir colabeled neurons were found. Double labelings with anti-Asn[13]-orcokinin and anti-*Drosophila*-PDF revealed results which showed similarities to the anti-FMRFamide labelings (Fig. 7). Generally, in the
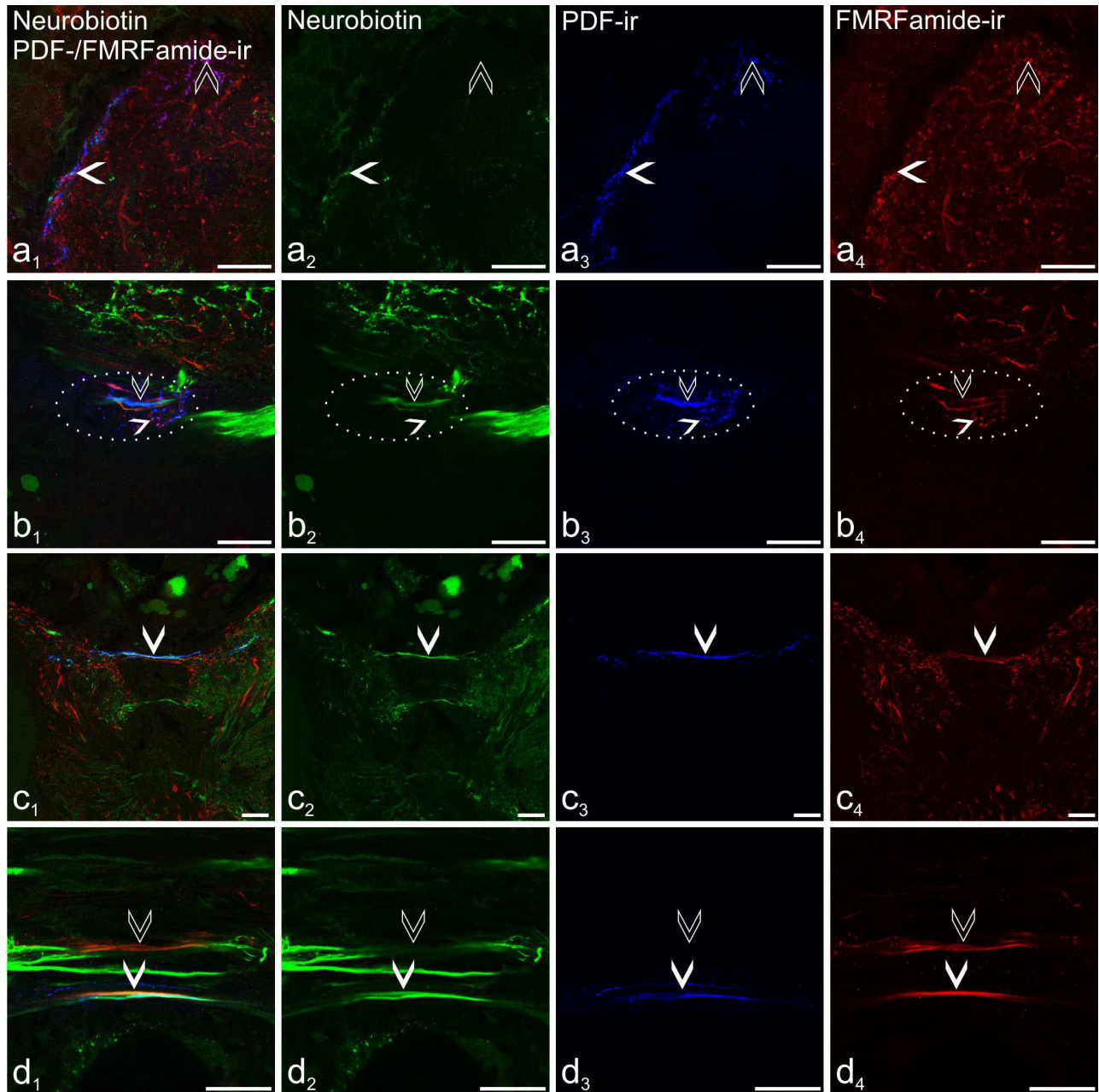
Fig. 6 Confocal laser scan images of typical projection areas of the PDFMe in the central brain after backfill of neurobiotin in the left optic stalk. Neurobiotin is shown in green, PDF-ir is shown in blue and FMRFamide-ir is shown in red. The respective overlay images in the left column show colocalization. a_{1-4} In the region of the dorsal superior lateral protocerebrum (*SLP*) no triple labeled fibers was detectable. At the distal rim of the SLP fibers hosting neurobiotin and PDF were located (*closed arrowhead*). Additionally at the dorsal part of the SLP, fibers which show colocalization of PDF and FMRFamide were visible (open arrowhead). b_{1-4} In the area of the posterior optic tubercle (*POTu*, *dotted line*), a fiber which showed colocalization of PDF and neurobiotin was visible (*open arrowhead*). Additionally, there were varicosities with colocalization of PDF and FMRFamide (*closed arrowhead*). c_{1-4} Only in a fiber of the superior median protocerebrum (*SMP*) triple labeling of neurobiotin, PDF and FMRFamide was visible (*arrowhead*), whereas the FMRFamide-ir was only very weak. d_{1-4} In the anterior optic commissure (*arrowhead*), fibers only double-labeled with neurobiotin and PDF were visible. The FMRFamide-ir fibers (d_4) were situated behind the neurobiotin and PDF colocalized fibers. e_{1-4} In the area of the posterior optic commissure, many fibers cross the midline of the central brain. However, no colocalized fibers of neurobiotin, PDF and FMRFamide were visible in the posterior optic commissure (*open arrowhead*). A fiber beneath the posterior optic commissure showed triple labeling of neurobiotin, PDF and FMRFamide (*close arrowhead*). *Bars* 50 µm.

anti-orcokinin labelings, the background staining was relatively high. In some preparations this resulted in ambiguous labeling. This mainly affected the DFVNe and
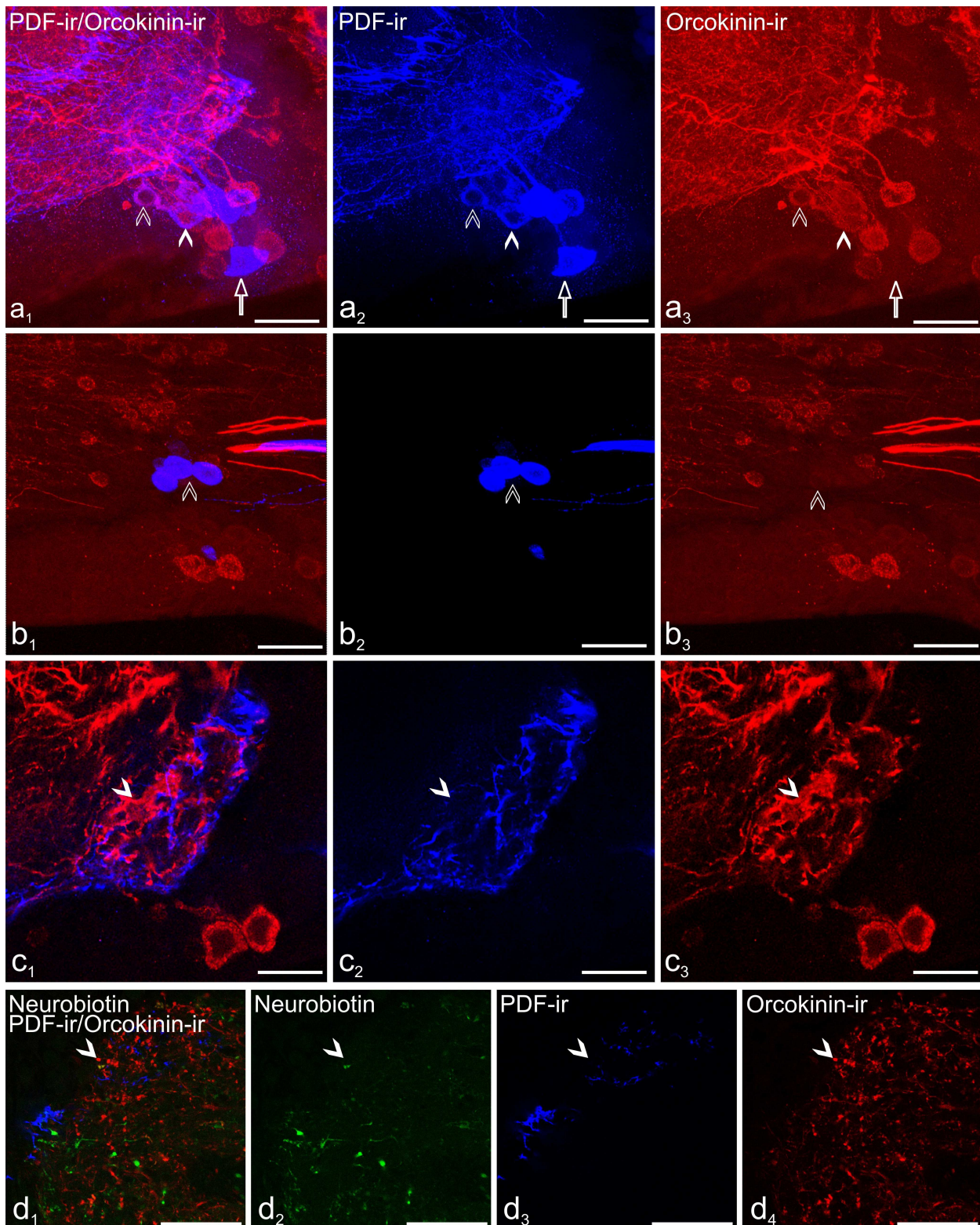
Fig. 7 Confocal laser scan images obtained from vibratome sections of the accessory medulla (*AMe*). PDF-ir is shown in blue and orcokinin-ir is shown in red. The respective overlay images in the left column show colocalization. **a_{1-4}** Neurons of the small (*open arrowhead*), medium-sized (*closed arrowhead*) and large aPDFMe (*arrow*) are visible. **b_{1-3}** Of the posterior neurons (= MC III, *arrow*) near the lobula valley tract the small ones showed PDF- and only in one case additionally orcokinin-ir whereas the large showed only PDF-ir staining (*open arrowhead*). **c_{1-3}** Most orcokinin-ir fibers were not found in the anterior, but in the distal shell neuropil between AMe and medulla (**c3**, *arrowhead*). In no case colocalization was observed in PDF-ir fiber terminals in the AMe (*arrowheads*). **d_{1-4}** In the region of the dorsal superior lateral protocerebrum (*SLP*) no colocalization was observed in PDF-ir fiber terminals. Neurobiotin labelled fibers were visible and also orcokinin-ir fibers were abundant in these areas (*closed arrowhead*). *Bars* 50 µm.

VNe, where orcokinin-ir labeling was generally of lower intensity, with the lowest in the DFVNe. Therefore, only preparations with at least three visible orcokinin-ir DFVNe were considered for cell counting. As in the anti-FMRFamide immunolabelings, the large anterior PDFMe where completely devoid of any additional orcokinin-ir labeling (Fig $7a_{1-3}$) (see table 1 for numbers). The medium-sized anterior PDFMe were always additionally orcokinin-ir. In the small PDFMe this was often, but not always the case. Within the small posterior PDFMe, only in one preparation an orcokinin-ir neuron was found. Large posterior PDFMe never were orcokinin-ir, thus delivering further support that they in fact are large anterior PDFMe (Fig. $7b_{1-3}$). Regarding the fiber projections in no case PDF/orcokinin colocalization was observed in the AMe (Fig. $7c_{1-3}$), the dSLP (Fig $7d_{1-3}$), and the POTu; (data not shown). However, orcokinin-ir fibers were abundant in these areas. Within the AMe, varicose orcokinin-ir fibers were concentrated in the shell neuropil. Contrarily to PDF-ir fibers, most orcokinin-ir fibers were not found in the anterior, but in the distal shell neuropil, which is the transition zone between AMe and medulla (Fig. $7c_3$). Also in the core neuropil orcokinin-ir fibers were present, mainly in the internodular neuropil and posterior parts of the nodular neuropil. Diffuse orcokinin immunoreactivity was present throughout the nodular neuropil. Orcokinin-ir fiber distribution in other parts of the brain corresponded to the descriptions of Hofer et al. (2005). Other neuron groups of the AMe with orcokinin immuno-reactivity were the MNe, the VPNe, and the VMNe (table 2), and corresponded in position, and largely also in numbers, to the findings of Hofer and Homberg (2006b). Within the VNe and the DFVNe non-PDF-ir and PDF-ir neurons were orcokinin-ir (compare tables 1 and 2). Total numbers of orcokinin-ir VNe and DFVNe in this study were either lower (VNe) or higher (DFVNe) compared to the numbers of Hofer and Homberg 2006b (there: VNe 16, DFVNe 6 neurons).

Tracer backfills and injections identify neurons with projections in the contralateral AMe

In this study, 11 backfills from one cut optic stalk with neurobiotin combined with anti-PDF and anti-FMRFamide antisera, and 14 backfills with anti-PDF and anti-orcokinin antisera were successfully performed. The backfills revealed similar results as shown by Reischig et al. (2004) with rhodamine-dextran as tracer. However, the neurobiotin labeling was crisper, and the medium and maximal numbers of counted neurons among the PDFMe was higher. In addition, backfilled neurons appeared in the MNe group of the AMe, which were not observed before (Fig. 4a). Due to the proposed relevance of PDFMe for mutual pacemaker synchronization, the evaluations concentrated on these neurons. For calculation of the mean

Table 2 Mean numbers of somata in AMe neuron groups labeled with anti-FMRFamide and anti-Asn[13]-orcokinin in the backfill experiments

	VNe [1]	DFVNe	MNe	VPNe	VMNe
FMRFamid-ir ($n = 11$)	10.6 ± 2.2; 11 [2]	8.9 ± 4.1; 11[3]	4.3 ± 0.5; 4	2.8 ± 1.0; 4	0 ± 0; 11
Orcokinin-ir ($n = 14$)	7.8 ± 1.1; 10 [2, 5]	10.6 ± 3.7; 10 [4, 5]	2.4 ± 0.8; 7	3.9 ± 1.6; 7	3.3 ± 0.5; 7

Numbers are given $\pm$ standard deviation; the numbers after the semicolons report the number of evaluated animals. Only one AMe per animal was evaluated, since the contralateral optic lobe was cut prior immunocytochemistry. Only for the VNe and DFVNe groups all animals were evaluated.
[1] For nomenclature of neuron groups see Materials and Methods.
[2] Including the medium-sized PDF, which were all FMRFamide- or orcokinin-ir.
[3] Including the small PDFMe, which were all FMRFamide-ir.
[4] Including those members of the small PDFMe that were orcokinin-ir.
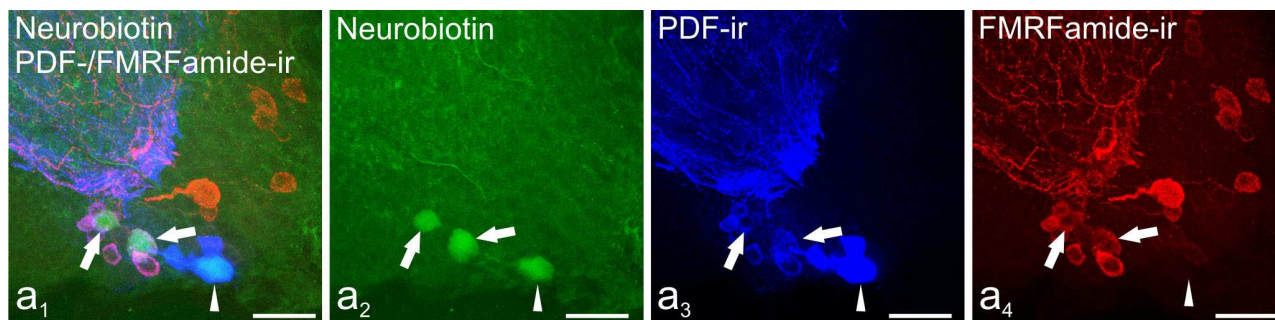[5] Counted only in preparations were at least three orcokinin-ir DFVNe were visible.

Fig. 8 Confocal laser scan images obtained from vibratome sections of the accessory medulla (*AMe*). Neurobiotin is shown in green, PDF-ir is shown in blue and FMRFamide-ir is shown in red. The respective overlay images in the left column show colocalization. a_{1-4} Neurons of the medium-sized and large aPDFMe are visible. Of the three neurobiotin filled neurons, two could be grouped to the medium-sized aPDFMe (*arrows*). The other neurobiotin filled neuron, located most closely to the central brain, could be grouped to the large aPDFMe (*arrowhead*). This neuron hosts only neurobiotin and PDF. *Bars* 50 μm.

numbers of backfilled neurons in a given neuronal group or subgroup, only those preparations were considered which contained at least one labeled neuron per group (see discussion). The large and medium-sized PDFMe were identified according to the presence or absence of additional immunoreactivity (see above). The mean number of neurobiotin-labeled PDFMe over 24 experiments was 2.9 ± 1.0 (mean ± SD, maximal counts 4 neurons in six cases). Of these, maximally 1 neuron, which was visible in 20 experiments, belonged to the large PDFMe, and this was generally the largest and most proximal PDF-ir neuron (Fig. 4a). In one case, this neuron was located among the posterior PDFMe. All other neurobiotin-labeled PDFMe belonged to the medium-sized PDFMe, with a mean number of 2.0 ± 0.7 (mean ± SD, n = 20, max. counts: 3 neurons in five cases). In four experiments PDFMe were not discriminated into large and medium-sized due to low orcokinin immunoreactivity with high background. When the experiments with anti-FMRFamide and anti-orcokinin were evaluated separately (Figs. 8, 9), the distribution of backfilled neurons in large and medium-sized PDFMe was similar in both experimental series (table 3). This implies that despite of one large PDFMe, up to 3 medium-sized PDFMe that simultaneously express FMRFamide and orcokinin immunoreactivity project to the contralateral optic lobe. No backfilled neuron was found among the VNe that was FMRFamide- or orcokinin-ir and lacked PDF-ir. In six cases, backfilled VNe were found that

were not immunolabeled at all (table 3, in one case these were 4 neurons, in another case these were 2, and in four cases 1 neuron). As in previous studies (Reischig and Stengl 2002, Reischig et al. 2004), backfilled and contra-laterally projecting VNe were assigned to the group I of medulla cells projecting to the contralateral optic lobe (MC I). The MC II (Fig. 4 b) and MC III (Fig. 4c) neurons could be observed in every experiment. MC II neurons corresponded to the VMNe group of the AMe, while MC III neurons were mostly situated in close vicinity to the posterior PDFMe. Since these neurons were also consistently labeled with rhodamine-dextran in previous experiments (Reischig and Stengl 2002), their numbers were not further evaluated here. Among the contralaterally projecting MC II/VMNe were about 3 orcokinin-ir neurons (table 2), as were described by Hofer and Homberg (2006a, b). Remarkably, the neurobiotin labeling intensity of MC II and III somata was nearly always higher than that of MC I neurons. With the neurobiotin backfills from optic lobe stumps, a new group of labeled neurons emerged that was never observed in the rhodamine-dextran backfills (Reischig and Stengl 2002, Reischig et al. 2004). These neurons (Fig. 4a) appeared among the MNe group with a mean number of 2.2 ± 1.2 SD (n = 18, max. counts: 5 neurons in two cases). According to the existing nomenclature for medulla neurons projecting to the contralateral optic lobe they were called MC IV. The MC IV never showed FMRFamide- or orcokinin-ir.
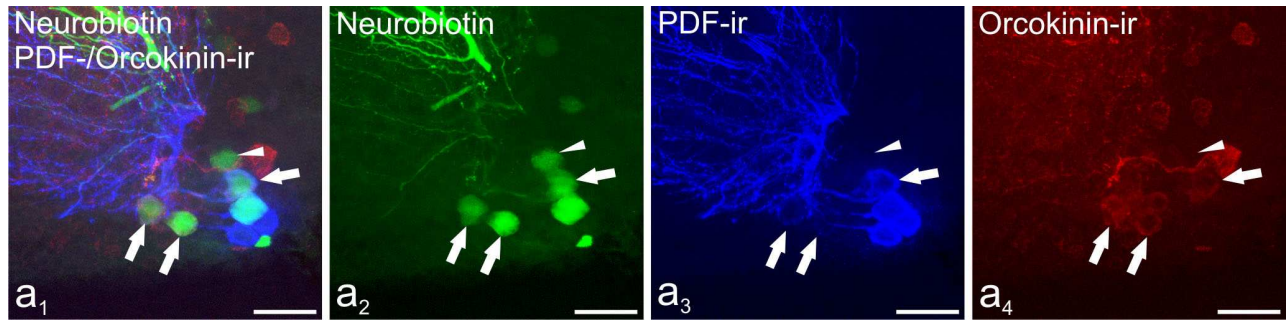
Fig. 9 Confocal laser scan images obtained from vibratome sections of the accessory medulla (*AMe*). Neurobiotin is shown in green, PDF-ir is shown in blue and orcokinin-ir is shown in red. The respective overlay images in the left column show colocalization. **a_{1-4}** Neurons of the medium-sized aPDFMe are visible (*arrows*). The other neurobiotin filled neuron, could be grouped to the large MNe neurons (*arrowhead*). This neuron hosts only neurobiotin. *Bars* 50 µm.

Within the AMe neurobiotin was found in fibers in the internodular core neuropil, and posterior parts of the nodular core neuropil (Fig. 4b). In the anterior and proximal shell neuropil and internodular core neuropil, they were often additionally PDF-ir, and generally belonged to the strongly labeled type of PDF-ir fibers. Many neurobiotin-labeled fibers of the distal shell neuropil and posterior nodular core neuropil were additionally orcokinin-ir. No neurobiotin-labeled fibers were found that showed additional colocalizing FMRFamide or PDF and FMRFamide or PDF and orcokinin immunoreactivity. In the brain hemisphere contralaterally to the backfilled side, neurobiotin-labeled PDF-ir fibers without additional FMRFamide or orcokinin immunoreactivity were found in all projection areas of the PDF-ir neuron system, namely the superior median protocerebrum (SMP), the SLP, inferior

lateral protocerebrum (ILP), and the POTu (Fig. 6). Astonishingly, as in the AMe, the PDF/FMRFamide-ir fibers of the dorsal SLP and the POTu did not show additional neurobiotin labeling. Also in PDF-ir commissural fibers, the evaluation of neurobiotin and immunolabeling colocalizations revealed enigmatic results. PDF-ir fibers with additional neurobiotin labeling were found in the anterior and posterior optic commissures (AOC and POC, respectively), but never additional FMRFamide- (Figs. 6c_{1-4}, d_{1-4}) or orcokinin-ir (data not shown) co-labeling was found in these fibers. However, non-PDF-ir backfilled fibers that were FMRFamide-ir were found in the posterior optic commissure paralleling the PDF-ir fibers. In no case all PDF-ir commissural fibers were labeled with neurobiotin, even in preparations where the maximal count of four backfilled PDFMe were reached. Therefore and

Table 3 Mean numbers of ventral neurons (*VNe*) of the AMe backfilled from the contralateral optic lobe

	Contralateral VNe with PDF immunoreactivity alone	Contralateral VNe with PDF and second immunoreactivity	Contralateral VNe without PDF and FMRFamid or orcokinin immunoreactivity
PDF + FMRFamide (n=11)	1.0 ± 0.0; 1; 9	1.9 ± 0.6; 3; 10	1.8 ± 1.3; 4; 5 [1]
PDF + orcokinin (n=14)	1.0 ± 0.0; 1; 7 [2]	2.1 ± 0.9; 3; 10 [2]	1.0 ± 0.0; 1; 1

Numbers are given ± standard deviation; the numbers after the first semicolon is the highest count in the respective group, the number after the second semicolon reports the number of animals contributing to the mean value. For calculating the means, only those experiments were considered where at least one neuron was found in the respective group (see discussion).
[1] High medium number is the result from one preparation with 4 labeled neurons; would be 1.2 ± 0.4; 2; 5 without this specimen.
[2] Preparations with low orcokinin-ir labeling intensity were not counted.

since the fibers in the respective commissures could not clearly be separated the fibers in the commissures could not be counted. Additionally to the backfill experiments, injections of rhodamine-dextran into one AMe were performed and combined with anti-FMRFamide immunolabeling (n = 72). Regularly, neurons of the MC I and MC II groups were labeled with rhodamine-dextran in the contralateral AMe. Since in these preparations only a combination of rhodamine-dextran and FMRFamide labeling was performed no clear assignment of the labeled neurons to the PDFMe was possible. Additionally, numbers of preparations with successful labeling of the MC I were considerably lower than in the neurobiotin backfills (n = 14), and also numbers of labeled neurons did never reach the maximal counts achieved in the backfill experiments. Therefore, these preparations were not evaluated numerically. However, in six cases rhodamine-dextran labeled MC I neurons were detected that were additionally FMRFamide-ir (max counts = one). Additionally 9.46 ± 6.69 (mean ± SD, n = 26) rhodamine-dextran labeled VMNe could be counted. In none of the experiments the new MC IV group was detected with dextran injections.

Discussion

To determine which neuronal groups and pathways form direct connections between both optic lobes and both AMae as candidates for mutual pacemaker synchronization in the cockroach *L. maderae*, we performed backfill labeling with neurobiotin from the stump of one sectioned optic lobe, as well as dextran injections into one AMe. The backfills were combined with double immunolabeling against PDF and FMRFamide, or PDF and orcokinin. The injection experiments were combined with immunolabeling against FMRFamide. In the AMe contralaterally to the backfilled side PDFMe, FMRFamide-ir, orcokinin-ir neurons as well as backfilled neurons in the VNe and MNe groups were quantitatively evaluated. The backfills revealed in contrast to former publications that in the MC I cell group at least

four PDFMe neurons project to the contralateral optic lobe, in addition to the occasional findings of contralateral projecting MC I neurons that never stained with the antibodies employed. One of the contralateral PDFMe neurons was the largest PDFMe which never colocalized FMRFamide and/or orcokinin immunoreactivity. The other 3 PDFMe cells belong to the medium-sized subgroup of the PDFMe and always colocalize FMRFamide and orcokinin immunoreactivity in addition to PDF immuno-reactivity. A new fourth group of contralaterally optic lobe projecting medulla neurons was identified, the MC IV cells which belonged to the MNe group of the AMe. The double immunolabelings revealed for the first time that all medium-sized and small PDFMe were additionally FMRFamide-ir, and all medium-sized and most small PDFMe were additionally orcokinin-ir. The large anterior PDFMe did not show further immunolabeling. The small posterior PDFMe were always FMRFamide-ir, but were in most cases devoid of additional orcokinin-ir immunolabeling. Intriguingly, double-immunolabeling of neurites was not observed pointing to peptide sorting in processes of cells which contain different peptides in the soma. Thus, differential release of neuromodulating peptide cocktails might be an important mechanism in circadian pacemaker coupling.

Neuron classification and developmental plasticity in the AMe

In neuroanatomy, differentiation of neurons in groups and subgroups is indispensable for a characterization of neuronal circuits. It is mostly assumed that common morphological characters and positions imply functional similarities in neurons. In the AMe of the cockroach, the PDFMe neurons were classified into three subgroups by relative position, soma size, and intensity of PDF-ir immunolabeling, thus implying more morphological and functional commonalities within each subgroup than among them. The identification of these subgroups is reproducible in most animals and for most single neurons, and neuron counting in different animals revealed similar results, however with certain variations (Reischig and Stengl 2003b). Nevertheless, in some animals,

neuron positions diverged considerably from the "standard situation" that is depicted in the scheme of Fig. 10a. Large PDFMe were observed to reside among the normally more posterior VMNe neurons, or, intensely labeled PDFMe that obviously belong to the large PDFMe were smaller than the largest medium-sized PDFMe. Hence, for some individual neurons the correct assignment may be uncertain and even may lead to discordant results if it is done by different researchers. Here, a clear distinction of the large versus other PDFMe was possible for the first time, because large PDFMe never were immunoreactive to one of the other peptide antisera used herein. Furthermore, this distinction revealed an interesting developmental aspect. It clarified that the sum of large anterior and large posterior PDFMe was exactly 6, forming one group of neurons with common developmental origin. It appears that the neuron group is pulled apart while the optic lobe is growing during development, and it may be decided by chance which of the individual neurons is shifted posteriorly. This view is supported by the fact that posterior PDFMe were not yet described in any other insect, although PDF-ir neurons were demonstrated in more than 50 insect species (e.g. Homberg et al. 1991, Sehadová et al. 2003, Závodská et al. 2003). Thus, the existence of a posterior PDFMe group appears to be a peculiarity in *L. maderae*. We therefore now assume no functional differences between anterior and posterior PDFMe. These results indicate firstly that neurons with quite different positions may have a common developmental origin, and secondly that position alone is not always a good criterion for neuron grouping. This shift to a posterior position appears to happen rather rarely to the medium-sized and small anterior PDFMe neurons. Further, small anterior and small posterior PDFMes differ with respect to additional immunoreactivities. While all of them were labeled by the anti-FMRFamide antiserum, orcokinin immunoreactivity was mostly (with only one exception) found in the small anterior, but not in the small posterior PDFMe. Although lack of clear orcokinin-ir in parts of the anterior PDFMe might result from insufficient immunolabeling, at least the one orcokinin-ir small posterior
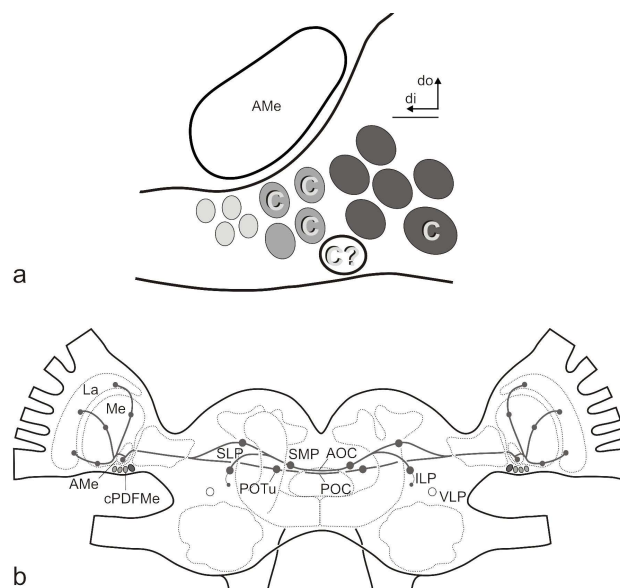

Fig. 10 a Scheme of the contralateral projecting PDFMe (indicated by *C*) obtained after the backfill experiments. The group of the VNe host 3 contralateral projecting medium-sized PDFMe (*cPDFMe*, light grey) which contain PDF, FMRFamide and orcokinin. Also one of the large PDFMe (dark grey, consisting of 6 neurons) project to the contralateral accessory medulla (*AMe*). Occasional contralateral projecting MC I neurons were found that never stained with the antibodies employed (white) **b** Scheme of the pacemaker coupling pathway of *L. maderae*. The large PDFMe appears to connect both AMae via the anterior and posterior optic commissure (*AOC, POC*). *La* Lamina, *Me* Medulla, *ILP/SLP* inferior/superior lateral protocerebra, *SMP* superior median protocerebra, *VLP* ventrolateral protocerebra Scheme provided by Dr. T. Reischig (from Reischig et al. 2004).

PDFMe indicates a certain dynamic in the distribution of small PDFMe between the anterior and posterior position. This is further indicated by the fact that small posterior PDFMe were not found in all animals. While the recognition of the large PDFMe is greatly facilitated by the lack of both FMRFamide and orcokinin immunoreactivity, the differentiation between small and medium-sized anterior PDFMe still relies on differences in staining intensity and soma size. These weaker criteria and the fact that small PDFMe are not always identifiable owing to weak and noisy immunolabeling might be the reason for the larger variations in cell counts compared to the large PDFMe. There, the cell number turned out to be very constant, and future observations with better criteria for neuron differentiation might reveal if this is also the case for the other PDFMe.

Multiple peptide immunoreactivities in single presumptive pacemaker neurons: distinct outputs from one cell by peptide sorting?

In the present work we demonstrated immunoreactivities against up to three peptide antisera in a subset of AMe-neurons, namely the small and medium-sized anterior PDFMe. Even more, in a current study immunoreactivity for an antiserum against the newly discovered neuropeptide baratin (Nässel et al. 2000) was observed in all medium-sized and at least a part of the anterior small PDFMe (T. Reischig, unpublished). Thus, at least four different neuropeptides could be expressed in single circadian pacemaker cells. The main drawback for the assumption of multiple peptide expression is that so far it is mostly based on histological antibody labelings only. However, several studies have shown in other species that neurons homologous to the cockroach's PDFMe indeed express PDF (Park and Hall 1998, Chuman et al. 2002, Matsushima et al. 2004, Honda et al. 2006). Furthermore, the PDFMe neurons as well as the PDF peptide itself are very conserved throughout the insects (Matsushima et al. 2004). Finally, in brains of *L. maderae* PDF was demonstrated by mass spectroscopy (Hamasaka et al. 2005). Therefore, very likely the anti-PDF antibody indeed recognized solely the PDF-peptide in PDFMe neurons. This is more complex for the anti-FMRFamide antiserum. The peptide FMRFamide was originally isolated from a bivalve mollusk (Price and Greenberg 1977). Since then, more than 100 neuropeptides were isolated from central and periphery nervous systems of invertebrates and vertebrates that generally share the C-terminal amidated RF (Arg-Phe-NH$_2$) sequence (reviewed by Orchard et al. 2001, Nässel 2002, Mercier et al. 2003, Orchard and Lange 2006). These include the N-terminally extended FMRFamides and FL/IRFamides, the short and long extended neuropeptides F (sNPFs and lNPFs, respectively), the myosuppressins (extended FLRFamides such as LMS), and the sulfakinins (extended HMRFamides with a sulfated tyrosine residue). These peptides are commonly referred to as FMRF-related peptides (FaRPs), although these families are structurally different

and are not further related to one another (hence, the term FMRFamide-like peptides, FLPs, is often used). The FMRFamide antibody used in our study is known to label a variety of FaRPs in invertebrates (Orchard et al. 2001, Nässel and Homberg 2006, Predel 2006). It was also used in *L. maderae* to distinguish AMe neurons (Soehler et al. 2008). In this work, the presence of several FaRPs in neurons of the AMe was demonstrated by mass spectroscopy, and injections of two members of the FaRP family into the optic lobe of free-running cockroaches resulted in time-dependent phase shifts of locomotor activity. Therefore, it is likely that AMe-neurons labeled by the FMRFamide antiserum indeed express peptides of the FaRP family, including the small and medium-sized PDFMe. The third antiserum used herein was raised against the crustacean neuropeptide Asn^{13}-orcokinin, a member of the orcokinin peptide family (Bungart et al. 1994). In several hemimetabolous insects, this antiserum labeled various neurons throughout the brain including the AMe of *L. maderae* (Hofer et al. 2005, Hofer and Homberg 2006b). Furthermore, orcokinin-related peptides were directly identified in the cockroaches *L. maderae* and *Blatella germanica*, and in the locust *Locusta migratoria* (Pascual et al. 2004, Hofer et al. 2005). As PDF and FaRPs mentioned above, Asn^{13}-orcokinin was injected at different circadian times into the optic lobes of free-running cockroaches, and caused light-like phase response curves. Therefore, also for the anti-orcokinin antiserum it is probable that it labeled neurons expressing orcokinin-related neuropeptides in the AMe, including parts of the small and all medium-sized PDFMe. Since colocalizations as well as single labelings were obtained for all antibodies employed the double labelings appear to be specific. In summary, the immunohistochemical evidences indicate that at least three different neuropeptides are expressed in single AMe neurons. Future studies with mass spectroscopy of single identified neurons will challenge this hypothesis. Whereas co-expression of two neuropeptides in single neurons was already demonstrated (Pollak et al. 2005), to our knowledge, co-expression of three or more neuropeptides were not shown before. Furthermore, whereas co-expression is mostly

observed among peptides that are transcribed from one gene and undergo subsequent cleavage and postprocessing in the Golgi apparatus and secretory vesicles, this appears to be not the case for PDF, FaRPs, and orcokinin-related peptides. Interestingly, PDF/FMRFamid-ir colocalization in neuronal terminals was observed only in few areas (AMe, dSLP, POTu), and never for PDF/orcokinin immunoreactivity. Moreover, colocalizations of peptide immunoreactivities were never observed in the commissural fibers, although PDFMe neurons with additional FMRFamide and orcokinin immunoreactivities were shown to project contralaterally. This confirms previous observations (Petri et al. 1995, Hofer and Homberg 2006a) and suggests that either peptide concentrations in the terminals are below the detection threshold, or that peptide sorting occurs in the small and medium-sized PDFMe neurons. The first might be the case for the less reliable, often weak orcokinin-labeling, while the latter might apply to the FMRFamid labeling. Peptide sorting was already demonstrated, e.g. by Sossin et al. (1990) in bag cells of *Aplysia californica*. In their study the authors showed that the prohormone of the egg laying hormone is sorted into distinct vesicle classes and that these distinct vesicles are localized to separate processes by unknown mechanism. Future studies have to challenge the hypothesis of multiple peptide coexpression and sorting in the PDFMe.

Significance of the backfill experiments

Previous studies showed that neurobiotin, an amino derivative of biotin, as well as dextran and horseradish peroxidase (HRP) function well as neuronal tracers to reveal pathways connecting both optic lobes (Reischig and Stengl 2002, Reischig et al. 2004). However, compared to the dextran injections and the former HRP injections, the labeling with neurobiotin was more consistent, stronger, and more reproducible. Thus, in this study now up to four PDFMe could be labeled compared to the maximally three PDFMe found before, and secondly, with the labeled MNe neurons a new fourth group emerged as candidate for a circadian coupling pathway. However, while the backfills labeled all fibers traversing the optic stalk, the dextran injections are more specifically labeling direct connections between both AMae since small dye droplets were positioned quite precisely in the injected contralateral AMe. Since results concerning the MC I, II, and III neurons were very similar in both the injection and backfill experiments here and from previous studies we conclude that all of the maximally four contralateral PDFMe neurons project not only to the contralateral optic lobe, but also to the contralateral AMe. Future single cell injections will challenge our hypothesis that four PDFMe project to the contralateral AMe, namely the largest PDFMe next to three medium-sized PDFMe neurons. The neurobiotin injections demonstrated for the first time that at least five MC IV neurons connect both optic lobes. While at least some of the MC IV neurons project into the ipsilateral AMe it remains to be examined whether they connect both AMae. This cell group could not be revealed with the rhodamine-dextran injections into the contralateral AMe or previous backfills of the stump of the optic lobe with rhodamine-dextran (Reischig and Stengl 2002). However, since the rhodamine-dextran injection studies were more variable due to the difficulty to precisely inject into the contralateral AMe they cannot exclude the possibility of a direct coupling pathway of MC IV cells. Because injections of neurobiotin into one AMe never achieved successful labelings we mostly concentrated on backfill studies from the optic stalk.

Anatomical properties of circadian coupling pathways

This study falsified the former hypothesis that additional FMRFamide- and/or orcokinin-ir VNe neurons form coupling pathways next to the PDF-ir neurons, because all contralaterally projecting VNe neurons which were labeled by anti-FMRFamide- and anti-orcokinin antisera were also PDF-ir. It still remains to be examined which neuromodulators are located in the 1–4 non-PDF-ir MC I VNe somata. The contralaterally projecting VMNe neurons (identical with MC II) generally form a

compact cluster posteriorly to the VNe neurons. However, because single neurons of the VMNe neurons were sometimes displaced to the MC I cluster, it is possible that the 1-4 non-PDF-ir MC I VNe somata are indeed displaced VMNes. Thus, it is possible that the MC I cells only consist of 4 VNe neurons, namely the contralaterally projecting PDFMe (Fig. 10). From this and former studies we hypothesize that in the cockroach the only bilateral coupling pathway connecting both AMae and projecting in a fan-like manner over the distal surface of the medulla is formed by the 4 contralaterally projecting PDFMe. In contrast, conflicting results describe the branching pattern of the contralaterally projecting large LNvs in *Drosophila* (Helfrich-Förster et al. 2007, Park and Griffith 2006). Thus, further studies need to elucidate the circadian coupling pathways in the different insect species. A question that is still open considers the exact pathways and termination sites of the contralaterally projecting PDFMe. These neurons pass the brain midline via the AOC and POC. Furthermore, in the brain half contralaterally to the backfilled side neurobiotin/PDF-ir co-labeled fibers were observed in the AMe, in a fanshaped arborization pattern through the distal layer of the medulla and in the proximal lamina of the contralateral optic lobe. In SMP, SLP, ILP, and POTu, neurobiotin/PDF-ir colabeled fibers occurred in most brain termination sites of the PDF-ir neuron system. Interestingly, in no case neurobiotin/PDF-ir co-labeled fibers were found that were additionally FMRFamide- or orcokinin-ir, not even in the AMe. The most parsimonious conclusion would be that all of the neurobiotin/PDF-ir co-labeled fibers originate from the largest contralaterally projecting PDFMe. In this case, neurites of this neuron would travel through both the AOC and POC and would innervate most arborization sites of the PDF-ir neuron system in the ipsi- and contralateral brain hemispheres of the cockroach. This hypothesis is supported by the fact that all neurobiotin-labeled PDF-ir terminals in the contralateral SLP, the SMP, ILP, POTu, and in the distal fiber-fan and shell neuropil of the AMe in contralateral optic lobe are strongly labeled with large and round varicosities. Our experiments cannot resolve where the termination sites of the remaining medium-sized contralateral PDFMe are. We assume that they contribute to the PDF-ir fibers in the AOC, because there are only two PDF-ir fibers in the posterior optic commissure, possibly originating from the two largest PDFMe per hemisphere. However, in the AOC no FMRFamide or orcokinin immunoreactivity colocalized with PDF and neurobiotin was visible. Possibly, the colocalized peptide concentrations were below the detection threshold, or, within the same PDF-ir cell the different colocalized peptides were sorted into different processes (see above). Thus, it can be only stated that small and/or medium-sized PDFMe terminate in the AMe, dSLP, and POTu due to PDF/FMRFamide-ir colocalization found there. We must further consider that neurobiotin might not be distributed equally in all terminals of every labeled neuron, since this tracer appears to be generally transported retrogradelly and thus fills only axonal endings but no dendritic regions in backfilled neurons (Heinrich et al. 1998). This could be the reason why neurobiotin-labeling was never visible in PDF/FMRFamide-ir fibers of the dSLP and the POTu. Even more, in some cases neurobiotin labeling was not at all visible in any of the PDF-ir commissural fibers, although somata were successfully labeled.

Mutual pacemaker synchronization: bi- or multi-modal coupling pathways?

Behavioral and anatomical studies postulated a neuronal coupling pathway between both bilaterally symmetric circadian pacemakers in the cockroach (Roth and Sokolove 1975, Page 1978, Page 1983a, b). Since the PDF-ir neuron systems were discovered in insects, these neurons are discussed to provide a part of this coupling pathway (Homberg et al. 1991). Stengl and Homberg (1994) discovered that the resulting circadian period length correlated with the number of regenerated commissural PDF-ir fibers in cockroaches that experienced bilateral transection of the optic stalks. In contrast to cockroaches, in crickets the bilateral pacemakers can be easily de-coupled in behavioral experiments and these animals possess only few or no commissural PDF-ir

fibers (Page et al. 1977, Wiedenmann and Loher 1984, Stengl 1995, Ushirogawa et al. 1997). Our study together with previous works (Reischig and Stengl 2002, Reischig et al. 2004) revealed four different neuron groups that are in question to provide mutual coupling pathways between the AMae of either side in the optic lobes of the cockroach *L. maderae*. The first group are the MC I with four PDFMe, which project through AOC and POC, and include the largest of the large PDFMe and three medium-sized PDFMe. In electro-physiological studies, neurons resembling PDFMe with commissural projections responded at most weakly to light stimuli (Loesel and Homberg 2001). Thus, PDFMe do not appear to transmit light information. Phase response curves obtained with extracellular injections of Uca-β-PDH next to one AMe confirm that PDF-ir neurons provide a non-photic input into the AMe, possibly as circadian coupling pathway (Petri and Stengl 1997). Computer simulations predicted that the physiological data can only be modeled via circadian coupling pathways providing both phase delays and advances to the AMe (Petri and Stengl 2001). Therefore, either one coupling neuron provides both, an advancing and a delaying signal, or at least two different parallel coupling pathways exist. Immunohistochemical observations suggested parallel coupling pathways: PDF-, FMRFamide- and orcokinin-ir contralateral AMe neurons (Petri et al. 1995, Petri and Stengl 2001, Hofer and Homberg 2006a). Here, we showed that a separate pathway does not exist for FMRFamide- and orcokinin-ir neurons. However, since most contralateral PDFMe appear to express three or more modulatory neuropeptides a single coupling neuron could provide the advancing and the delaying signal from different processes with sorted neuropeptides. Future studies will test this hypothesis. The second contralateral pathway of the AMe is provided by the 35 MC II neurons, which belong to the VMNe group (Reischig and Stengl 2002, Reischig et al. 2004). This appears to be the group of cells that were suggested previously to from a contralateral pathway in the cockroach (Roth and Sokolove 1975). The VMNe neurons project solely via the POC to the contralateral side, where their neurites generally were located more dorsally than the commissural fibers of MC I. It has been shown by intracellular single cell labelings in cockroaches, crickets, and locusts that some but not all neurons of this group project into the ipsi- and contralateral AMe (Labhart and Petzold 1993, Homberg and Würden 1997, Loesel and Homberg 2001, Yukizane et al. 2002). In the cockroach, the MC II neurons contain about three contralaterally projecting orcokinin-ir neurons (Hofer and Homberg 2006a, b), as was confirmed in this study. The MC II neurons are especially well investigated in crickets, where they were called medulla bilateral neurons (MBNs) (reviewed by Tomioka and Abdelsalam 2004). In the cricket *G. bimaculatus* up to 25 MBN were counted (Yukizane et al. 2002). In all investigated animals, the MC II/VMNe/MBN group form tangential arborizations in middle layers, but never in the fiber fan, of both medullae via the POC (Labhart and Petzold 1993, Homberg and Würden 1997, Loesel and Homberg 2001, Yukizane et al. 2002). In electrophysiological observations, VMNe neurons often showed regular spontaneous spiking activity and were light sensitive in an intensity-dependent manner (Homberg and Würden 1997, Loesel and Homberg 2001, Yukizane et al. 2002). MBNs of *G. bimaculatus* showed a clear circadian rhythm in their response behavior to light, which could be influenced in diametrically opposed manner by application of serotonin and PDF (Saifullah and Tomioka 2002, 2003a, b). Therefore, these neurons are discussed as a pathway transferring light information to the contralateral optic lobe to maintain stable phase angle relationships between the bilaterally symmetric circadian pacemakers (reviewed by Tomioka and Abdelsalam 2004). Very interestingly, a subgroup of the MBNs reacted to e-vector changes of polarized light (Labhart and Petzold 1993, Homberg and Würden 1997, Loesel and Homberg 2001). Orientation after polarized sky light of insects largely relies on precise information about current daytime, which might be one cause for the intercalation of the AMe as putative circadian pacemaker in the polarization vision pathway. In summary, MC I and MC II both appear to couple the

bilateral AMae, but subserve different functions. While the PDFMe of the MC I group appear to form circadian coupling pathways via the AOC and POC, the VMNe group appears to provide contralateral light information to the AMe via the POC.

A third and a fourth coupling pathway could be provided by the MC III and the newly discovered MC IV cells, respectively. However, so far we do not know anything about the projection patterns of these neurons, and we even do not know whether they indeed connect both AMae. Therefore, the mutual circadian synchronization pathway of the cockroach might be more complex than we know at the time, and awaits further investigation.

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Chapter IV. Both anterior and posterior optic commissures transmit coupling information between the pacemakers of *Leucophaea maderae*

Soehler S

Both anterior and posterior optic commissures transmit coupling information between the pacemakers of *Leucophaea maderae*

Sandra Soehler

Abstract Circadian pacemakers have been localized in the nervous system of several bilaterally symmetric animals. The circadian pacemaker of the cockroach *Leucophaea maderae* could be localized to the accessory medulla with associated pigment-dispersing hormone-immunoreactive (PDH-ir) neurons in the optic lobes at the ventromedian edge of the medulla. To exert stable rhythms with a common period, the bilateral accessory medullae have to be coupled. Lesion and backfill experiments demonstrated that PDH-ir neurons which send processes into the anterior and posterior optic commissures couple both accessory medullae. Here, we examine whether the anterior and posterior optic commissures transmit coupling information between both pacemakers. The anterior optic commissure, the posterior optic commissure, or the complete central brain were transected and then it was investigated by running-wheel assays whether these operations alter circadian locomotor activity. The experiments revealed that transection of the anterior and posterior optic commissures neither influenced the rhythmicity of the locomotor activity nor the period length of locomotor activity rhythm. However, transections of the posterior optic commissure caused comparably stronger phase delays than transections of the anterior optic commissure. Transections of the central brain typically caused permanent arrhythmic activity of the cockroaches which lasted for several weeks. Therefore, coupling circadian information is

redundantly encoded in more than one optic commissure. However, the posterior optic commissure appears to carry more advancing information as compared to the anterior optic commissure.

Keywords Pacemaker coupling · Circadian rhythm · Accessory medulla · Commissure Transection · Cockroach, *Leucophaea maderae* (Insecta)

S. Soehler (✉)
Department of Natural Sciences, Animal Physiology,
University of Kassel,
Heinrich-Plett-Strasse 40,
D-34132 Kassel, Germany
e-mail: ssoehler@web.de

Introduction

The rotation of the earth around its axis causes a light and dark cycle in a 24 hour fashion. Consequently, most organisms on earth developed a circadian system to anticipate the daily changes in their environment. In the cockroach *Leucophaea maderae*, the pacemaker that controls locomotor activity could be localized to the optic lobes (Nishiitsutsuji-Uwo and Pittendrigh 1968, Page 1982). Both optic lobes contain a small neuropil at the ventromedian edge of the medulla, the accessory medulla (AMe), which is innervated by a set of associated pigment-dispersing hormone immunoreactive (PDH-ir) neurons (Homberg et al. 1991). Transplantation of the AMe with associated PDH-ir neurons restored circadian rhythmicity in optic lobe ablated cockroaches (Reischig and Stengl 2003a). Thus, in the cockroach, the bilateral accessory medullae (AMae) with their adjacent PDH-ir neurons are circadian pacemakers controlling locomotor activity rhythms. Since one optic lobe is sufficient to maintain locomotor rhythmicity, each cockroach possesses two potentially autonomous pacemakers. However, removal of one optic lobe resulted in an

increase in the period length of locomotor activity rhythm (Page et al. 1977) suggesting that the bilaterally symmetric pacemakers are mutually coupled. Roth and Sokolove (1975) reported that non-identified cells in the optic lobes of *Leucophaea maderae* have axonal projections in the contralateral optic lobe. About 20 years later Stengl and Homberg (1994) showed by lesion experiments that the two bilaterally symmetric pacemakers of *Leucophaea maderae* are strongly coupled, contrary to the weak pacemaker coupling in crickets (Wiedenmann 1983, Tomioka et al. 1991, Tomioka 1993, Ushirogawa et al. 1997). Closer analysis of the lesion experiments and the morphology of PDH-ir neurons in both species revealed a correlation between the number of PDH-ir commissures and the coupling strength (Homberg et al. 1991, Stengl and Homberg 1994). Therefore, PDH-ir neurons appear to be involved in the coupling of the circadian pacemakers. A comprehensive morphological analysis of coupling pathways identified seven types of commissures connecting both optic lobes. Of these 7 fiber tracts only tracts 3, 4 and 7 connect both AMae (Reischig and Stengl 2002). Tract 3 runs from one optic lobe through the lobula valley tract (LoVT) to the ipsilateral superior lateral protocerebrum (SLP). There, tract 3 loops around the α-lobe of the ipsilateral mushroom body and proceeds via the anterior optic commissure (AOC) to the contralateral α-lobe, SLP and optic lobe. Tract 4 projects from one optic lobe through the LoVT to the ipsilateral SLP and superior median protocerebrum (SMP) and from there via the AOC, ventrally to tract 3, to the contralateral SMP, SLP, and optic lobe. Tract 7 runs from the optic lobe via the LoVT to several regions in the ipsilateral posterior protocerebrum and from there via the posterior optic commissure (POC) to several regions in the contralateral posterior protocerebrum and the contralateral optic lobe (Reischig and Stengl 2002). Two of the mentioned tracts, tract 4 and 7, show a similar arborization pattern as that of PDH-ir neurons, which were suggested to be involved in the coupling of the bilateral AMae. Because both commissures AOC and POC appeared to be necessary for circadian coupling, we here examined whether both commissures

transmit different information from one pacemaker to the other. Therefore, the AOC, the POC or the complete central brain of cockroaches were transected and free-running locomotor activity was monitored in running-wheel assays. Operation dependent changes in the amplitude, duration, period length or phase shifts of locomotor activity rhythms were evaluated.

Materials and Methods

Animals

Adult male cockroaches (*Leucophaea maderae*) were taken from laboratory colonies. They were reared under a 12:12 h light-dark (LD) photoperiod, at about 60% relative humidity and a temperature of 26°C. Animals were fed with dried dog food, potatoes and water *ad libitum*.

Behavioral assays and data analysis

Experimental animals were taken from laboratory colonies, set in running-wheels (Wiedenmann 1977), and constantly provided with dry rat pellets and water. Each running-wheel was equipped with a magnetic reed switch and one revolution of the running-wheel resulted in one impulse. Impulses were counted by a computer in 1 minute intervals. The complete equipment was kept at 28°C and 60% relative humidity in constant darkness (DD). Acquired data were plotted in double plot activity histograms. The free-running period τ and potentially occurring phase shifts were determined by converting the raw data into ASCII format, merging them into 30 min intervals and analyzing them with Chrono II software (provided by Till Roenneberg; Roenneberg and Morse 1993). The Chi-square periodograms and the rhythm detector plots were calculated with Tempus 1.6 (Reischig and Stengl 2003a). Recording of locomotor activity and subsequent data analysis were performed as described previously (Reischig and Stengl 2003a). Cockroaches, which showed a stable rhythm for at least ten days were used for surgery. All manipulations were done at room

temperature under dim red light. Animals were anesthetized with CO_2 and then mounted in metal tubes. Depending on the kind of operation, transection of the AOC, POC and complete central brain, respectively, a small window was cut into specific areas of the head capsule (Fig. 1). The cuticle within the window was removed, tracheae and fat body were pushed aside to expose the central brain, and the transections of the commissures were accomplished. To avoid regeneration of the commissures, a small piece of parafilm was placed into the cut. The removed cuticle piece was waxed back and the cockroach was put into the running-wheel. The sham operation consisted of opening the head capsule at the same location as for the AOC transection (Fig. 1), removing the fat body and subsequent closing of the head capsule with wax. To determine if operated cockroaches regain rhythmicity in their locomotor activity, they were kept in the running-wheels for up to 93 days. By the time a cockroach strongly increased or decreased its activity it was sacrificed and the brain used for immunocytochemistry. In total 67 cockroaches were operated. Data of locomotor activity could be analyzed from 33 of the 67 operated cockroaches (10 actograms of AOC transected, 11 actograms of POC transected and 12 actograms of central brain transected). Five animals received a sham operation, but their actograms could not be analyzed. The brains of 6 AOC transected, 7 POC transected, and 7 central brain transected cockroaches could be analyzed with immunocytochemistry. Because we focused on long term analysis of locomotor activity many operated animals died unexpectedly during the running-wheel assays. Changes in locomotor activity were statistically evaluated with t-tests. Significance was taken as $P<0.05$. Statistical analyses were performed with Superior Performing Software Systems (SPSS 11.5) and Excel XP (Microsoft).

Immunocytochemistry for paraffin sections

Brains were dissected and fixed for 4 hours in a formaldehyde/picric acid solution (aqueous Bouin´s solution modified after Hollande (Romeis 1989), washed in clear water,
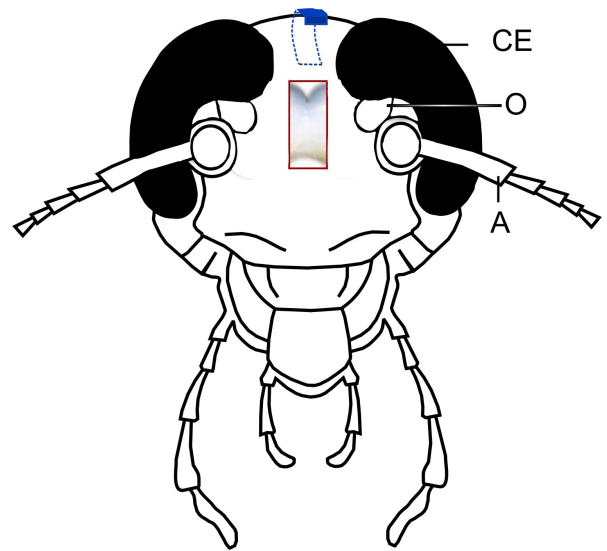

Fig. 1 Scheme of the head of the cockroach *Leucophaea maderae*. The red rectangle marks the window which was cut into the head capsule for the transection of the AOC and the central brain. The blue rectangle marks the window which was cut for the transection of the POC. *A* antenna, *CE* complex eye, *O* ocellus. Modified after Hofer (2004).

dehydrated in an ethanol series and embedded in paraffin (Paraplast plus, Sigma, Germany). Serial frontal 10 µm sections were cut as ribbons, mounted on microscope slides, deparaffinized with xylene and rehydrated through graded ethanols. Brains were stained with antisera against pigment-dispersing hormone (anti-*Uca*-β-PDH-antiserum, 1:3000, Dircksen et al. 1987) that clearly labels the AOC and POC. Furthermore, PDH-ir neurons near the AMe have been shown to be involved in the coupling of the pacemakers (Reischig et al. 2004). Immunoreactive cells were detected by using a 3-step peroxidase technique (Sternberger 1979, Reischig and Stengl 1996). To visualize the neuropils of the brain, the sections were counterstained with 1% methylene blue. In total, the brains of 20 of the 67 operated animals could be analyzed by immunocytochemistry. The remaining cockroaches died during the running-wheel assays.

Results

To examine whether the AOC and POC transmit the same information from one

pacemaker to the other we performed different transections combined with running-wheel assays. After the transections of the AOC, the POC, or the central brain different parameters of the locomotor activity rhythms were evaluated: transection-dependent changes of activity intensity and duration, appearance of a split rhythm, changes of the phase and period length. Subsequent staining of the brains were performed to investigate the success of the specific transections.

Altogether 67 animals were operated: 11 obtained AOC transection, 17 obtained POC transection, 34 obtained transection of the complete central brain and 5 obtained sham operation. Of the operated cockroaches the locomotor activity of 10 AOC -, 11 POC - and 12 central brain dissected animals could be evaluated. The sham operated animals could not be evaluated due to a computer breakdown during the running-wheel assays. The brains of 6 AOC-, 7 POC-, and 7 complete central brain transected cockroaches could be analyzed immunocytochemically.

Analysis of the locomotor activity

First, the effects of the different transections on the locomotor activity were evaluated. Among the 33 evaluated actograms 10 actograms were obtained from AOC dissected cockroaches, 11 from POC, and 12 of central brain transected cockroaches. All of these 33 animals showed stable locomotor activity rhythms for at least 10 days before operation (Figs. 2a, 4a, 6a). After operation most cockroaches with an AOC or POC transection regained locomotor rhythmicity (Figs. 2a, d; 4a, d), whereas most cockroaches which received a transection of the central brain developed arrhythmic locomotor activity (Fig. 6a, c). After AOC transection, 9 out of 10 evaluated cockroaches restored locomotor rhythmicity within 17 - 31 days (table 1). One cockroach showed a very weak activity without rebuilding a clear rhythm after AOC transection. Following POC transection locomotor rhythmicity was restored in 9 out of 11 cockroaches within 13 - 37 days (table 1). Two of these 9 animals showed long stretches of permanent locomotor activity before

recovering rhythmicity. The remaining two cockroaches exhibited no clear rhythm for 17 days after POC transection. Of the 12 cockroaches which obtained a transection of the central brain 7 exhibited permanent locomotor activity immediately or within 14 days after operation (Fig. 6a). Three cockroaches showed arrhythmic locomotor activity and two lacked locomotor activity for several weeks after operation. Three of the 12 evaluated cockroaches recovered rhythmicity, one after 27 days, and the other two after 61 days (Fig. 6a-d, table 1). The remaining cockroaches did not regain locomotor rhythmicity until they died [within 30 days (n = 4), within 30 – 60 days (n = 2), and ≥ 60 days (n = 3), respectively].

For the evaluation of the quantity and duration of the locomotor activity 6 POC, 8 AOC, and 8 central brain dissected cockroaches were analyzed. The other cockroaches were excluded from the analysis, because they showed very long durations of inactivity after operation or due to computer problems. To determine changes in the quantity of the locomotor activity the means of running-wheel revolutions before the operation were compared those after the operation over 9 - 11 days. In the cases of both the AOC and POC transections 50% of the transected cockroaches exhibited an increase and 50% a decrease of the overall locomotor activity (n = 4 for the AOC transections, n = 3 for the POC transections). Central brain transection led to a decrease in locomotor activity in 87.5% (n = 7) of the operated cockroaches, and to an increase in 12.5% (n = 1) of the operated cockroaches. Statistical comparison of the intensity of locomotor activity before and after the transections with a paired t-test resulted in no significant differences ($p > 0.05$). For the analysis of changes in the duration of locomotor activity the mean locomotor activity in multiple 30 min bins (maximal 48 per day) with two and more running-wheel revolutions over a certain number of days (9 days for central brain transected, 10 days for POC transected, 11 days for AOC transected cockroaches) before the operation was compared to the mean of bins with two and more running-wheel revolutions over the same number of days after the surgery.
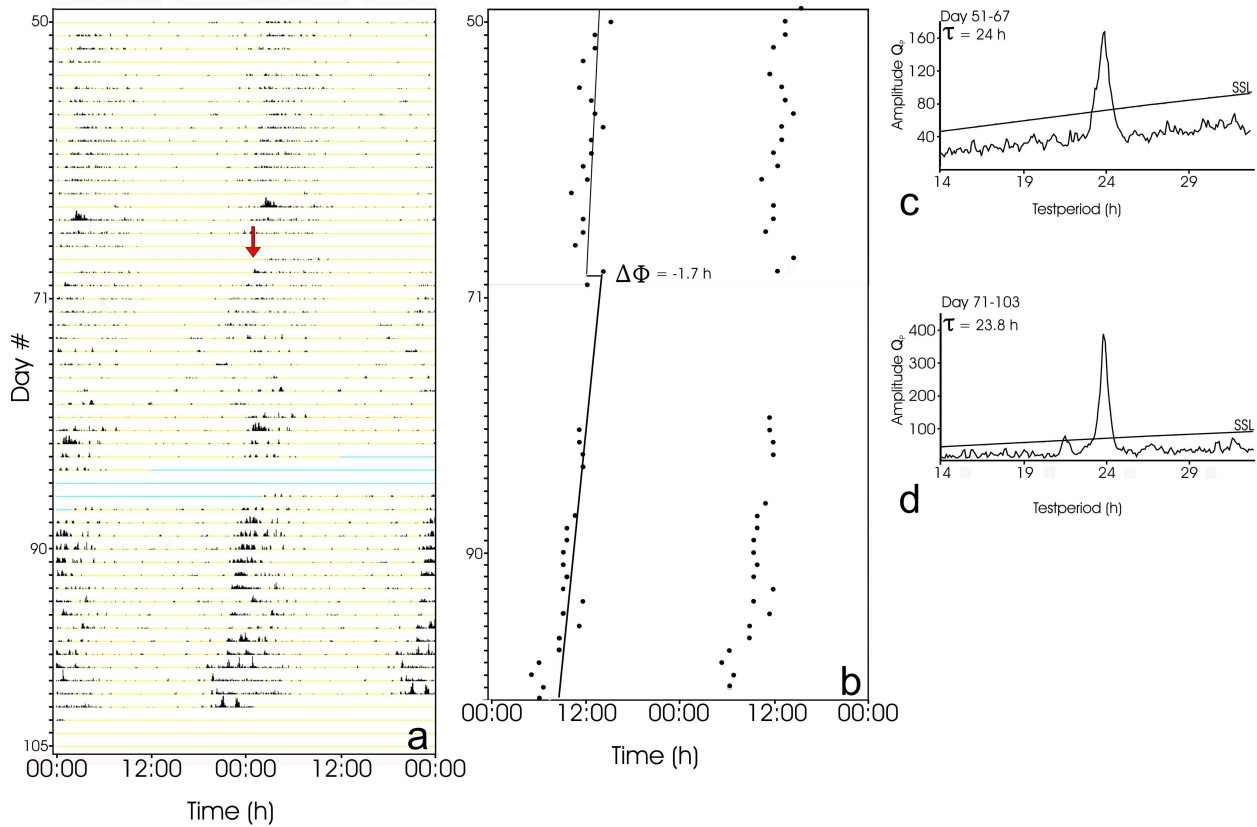
Fig. 2 a Running-wheel activity of an AOC-transected cockroach kept in constant darkness. The cockroach exerted a stable rhythm before and after the AOC transection (operation day is marked with red arrow). Blue lines mark periods without recording of locomotor activity. **b** Regression analysis through consecutive activity onsets revealed a phase delay of 1.7 h after the AOC transection. **c-d** Chi-square periodograms of day 51-67 (**c**) and 71-103 (**d**) confirmed the rhythmicity before and after the transection. The period length was 24 h before the transection (**c**) and 23.8 h after the transection (**d**). (*SSL*= Sokolove significance line = chi-square for p = 0.01).
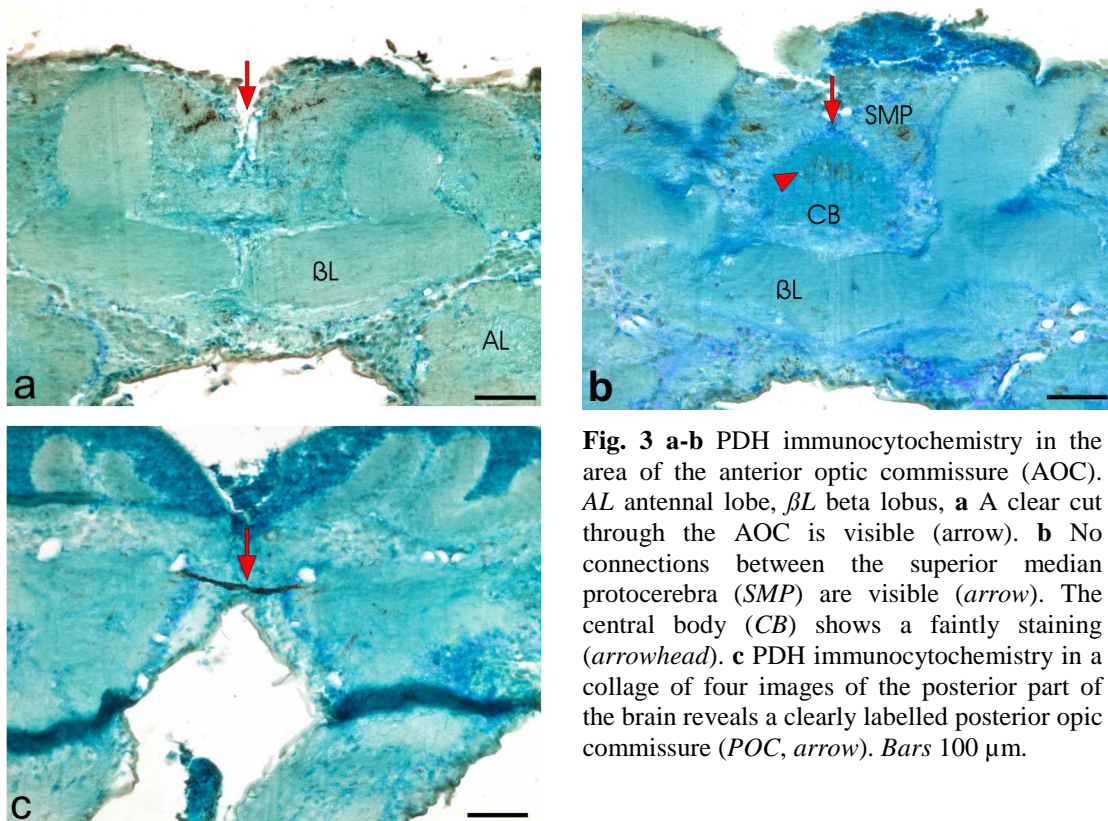
Fig. 3 a-b PDH immunocytochemistry in the area of the anterior optic commissure (AOC). *AL* antennal lobe, *ßL* beta lobus, **a** A clear cut through the AOC is visible (arrow). **b** No connections between the superior median protocerebra (*SMP*) are visible (*arrow*). The central body (*CB*) shows a faintly staining (*arrowhead*). **c** PDH immunocytochemistry in a collage of four images of the posterior part of the brain reveals a clearly labelled posterior opic commissure (*POC, arrow*). *Bars* 100 µm.
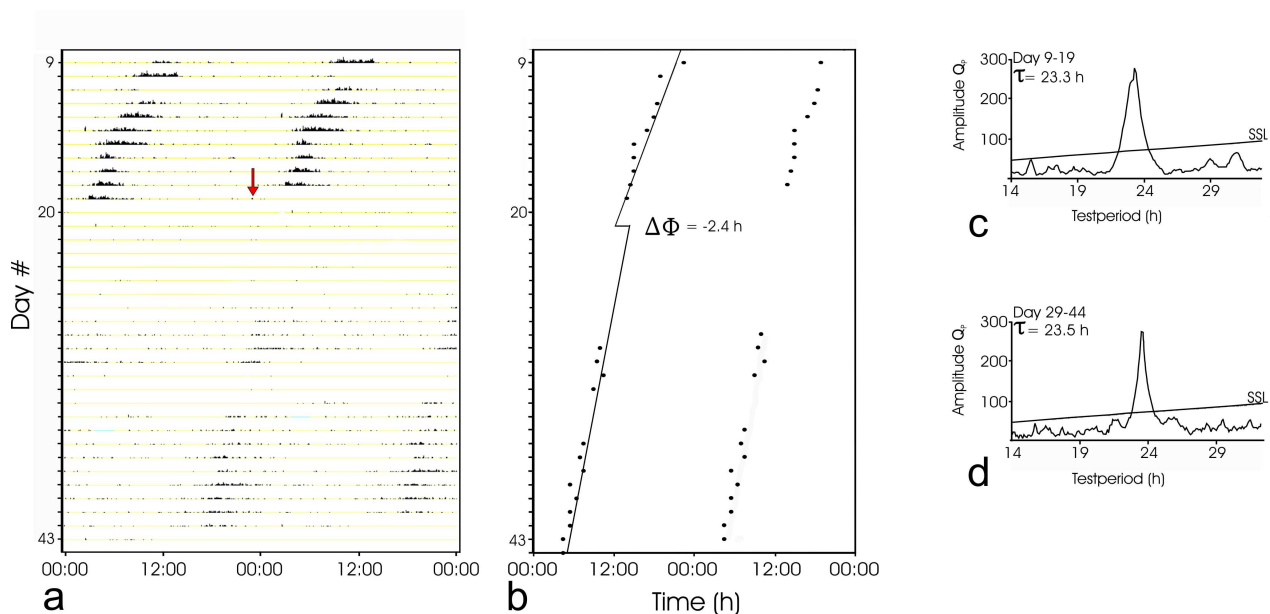
Fig. 4 a Running-wheel activity of a POC-transected cockroach kept in constant darkness. The cockroach showed a stable locomotor rhythm before and after the POC transection (operation day is marked with red arrow). **b** Regression analysis through consecutive activity onsets revealed a phase delay of 2.4 h after the POC transection. **c-d** Chi-square periodograms of day 9-19 (**c**) and 29-44 (**d**) confirmed the rhythmicity before and after the transection. The cockroach expressed a rhythm with a period length of 23.3 h before operation (**b**) and a period length of 23.5 h after the operation (**d**) (*SSL*= Sokolove significance line = chi-square for p = 0.01).
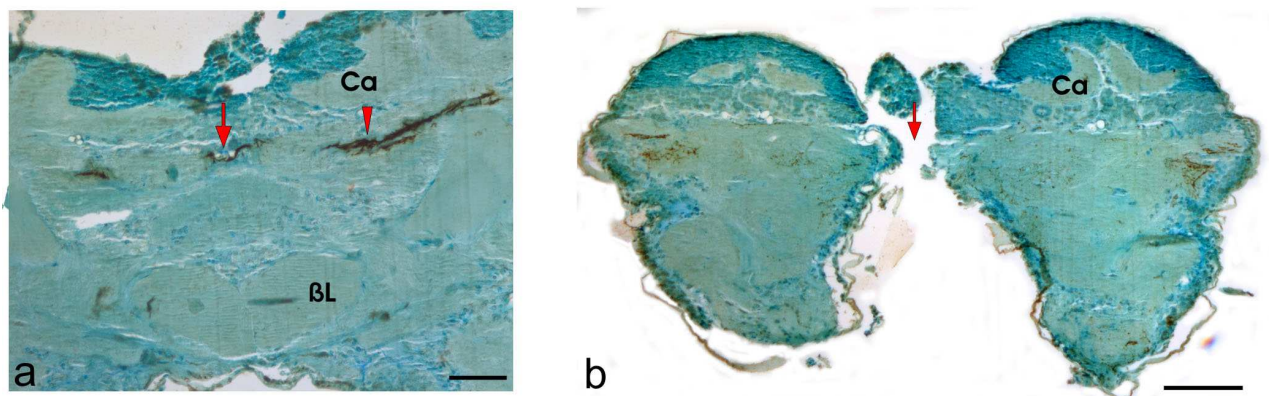
Fig. 5 a-b PDH immunocytochemistry in the area of the anterior optic commissure (*AOC*, **a**) and posterior optic commissure (*POC*, **b**). **a** The AOC (arrow) and staining in the SMP (*arrowhead*) is visible. **b** A clear cut through the POC is visible (*arrow*). *ßL* beta lobus, *Ca* calyces. *Bars* 100 µm.

This evaluation revealed in 87.5% (n = 7) of the central brain transected animals, in 62.5% (n = 5) of the AOC transected animals and in 50% (n = 3) of the POC transected animals an increase of the duration of the activity. The statistical comparison of the means of 30 min bins before the transection with the same number of means after the transection with a paired t-test resulted in significant differences between the duration of activity before and after the central brain transections (*P*<0.05, Fig. 8).

Effects of the transections on the period of the circadian locomotor activity rhythm

After transection of the AOC or POC no significant changes were found in the free-running period. The mean period before the AOC transection was 23.55 ± 0.31 h (mean ± SD, n = 8, table 1) and the mean period after the AOC transection was 23.54 ± 0.29 h (mean ± SD, n = 8, table 1). Of the eight evaluated AOC transections we observed lengthening of the
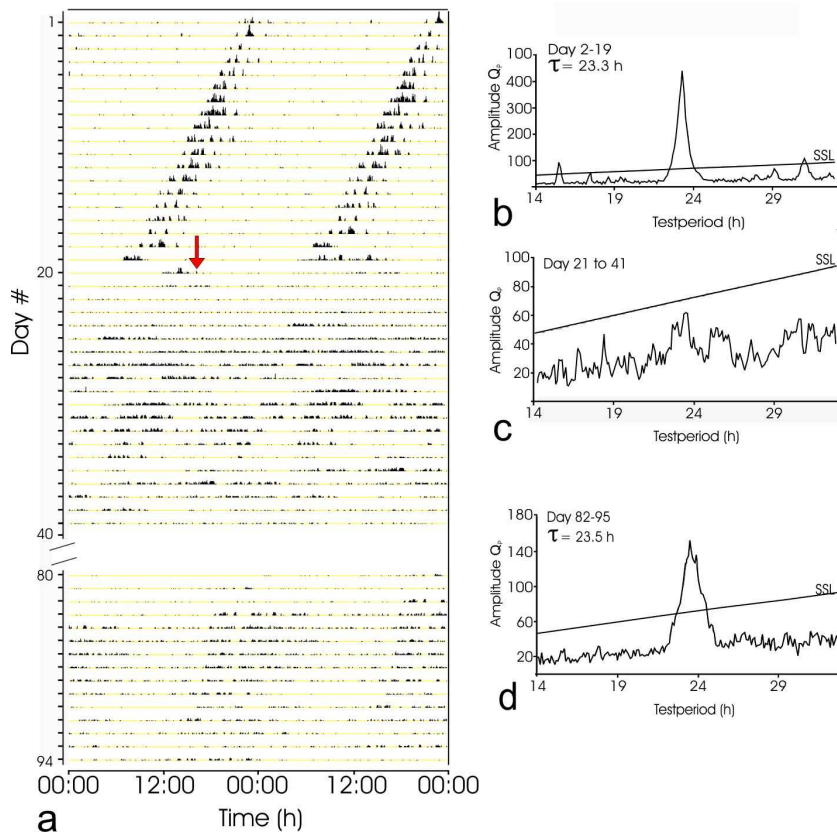
Fig. 6 a Running-wheel activity of a whole-brain-transected cockroach kept in constant darkness. The cockroach showed a stable rhythm before transection of the brain (marked with red arrow). After the operation no rhythm was detectable, but from day 83 on the animal regained rhythmicity. **b-d** Chi-square periodograms of day 2-19 (**b**), 21-41 (**c**) and 82-95 (**d**). The period length was 23.3 h before and 23.5 h after the operation (**b** and **d**). From day 21-82 the cockroach is arrhythmic (**c**).
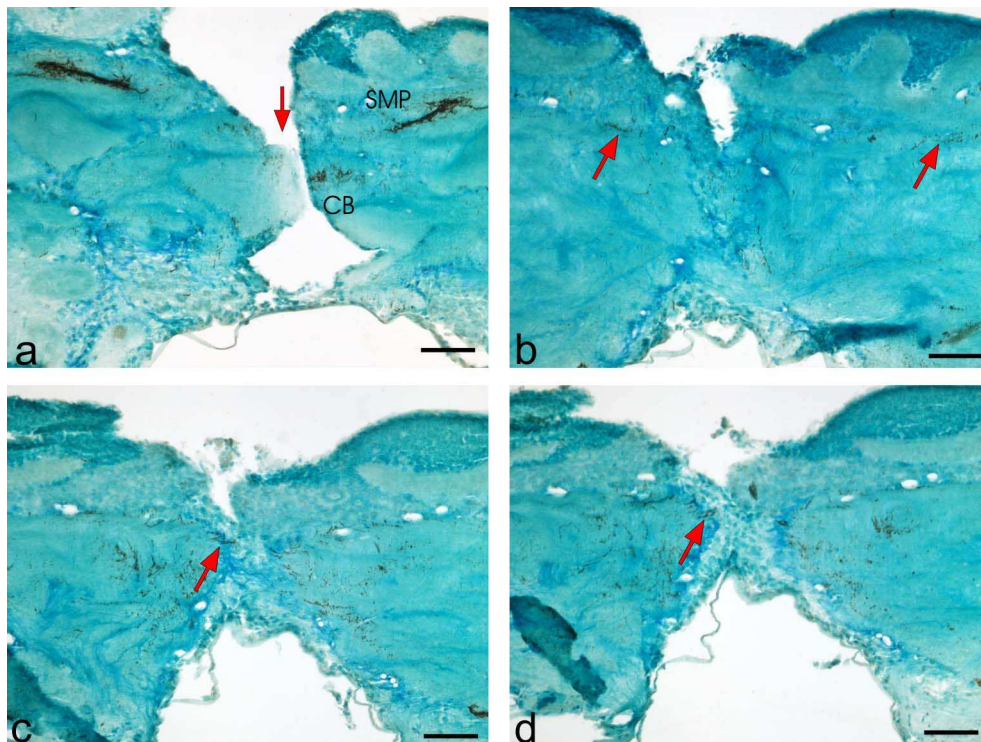

Fig. 7 a-d PDH immunoreactivity in the central brain of a cockroach with a central brain transection. **a** the central brain is almost completely cut (arrow). PDH immunocytochemistry is in the superior median protocerebrum (*SMP*) as well as in the central body (*CB*) visible. **b** Section posterior to **a** shows only small PDH-ir fibers (*arrows*). The brain hemispheres appeared to have fused again. **c-d** POC transection on two consecutive sections. Only parts of the POC are visible in **c** and **d** (*arrows*). *Bars* 100 µm.

period (n = 4) with a maximum value of 0.18 h and shortening (n = 4) with a maximum of 0.3 h (Figs. 2c, d). Two animals showed very low activity levels after operation and were not further evaluated. The POC transections also caused no significant changes in the free-running period. The mean period before the POC transection was 23.39 ± 0.2 h (mean ± SD, n = 11, table 1) and the mean period after the POC transection was 23.27 ± 0.16 h (mean ± SD, n = 10, table 1). We observed lengthening (n = 3) with a maximum of 0.37 h (Figs. 4c, d; 0.2 h), shortening (n = 6) with a maximum of 0.43 h or no effect (n = 1) on the period length. One animal could not be evaluated because it was arrhythmic after surgery. Transections of the central brains caused in most cases permanent locomotor activity after the operation. Out of three cockroaches which became rhythmic after long stretches of continuous activity, one showed a by 0.2 h lengthened period (Figs. 6b-d) and two showed a by a maximum of 0.6 h shortened period. In the other animals the changes in the period lengths could not be determined.

Effects of the transections on the phase of the circadian locomotor activity rhythm

After AOC transection, eight cockroaches showed phase delays (0.79 ± 0.43, mean ± SD, table 1) with a maximum of 1.7 h (Fig. 2b). Two cockroaches, however, showed phase advances (2.21 ± 1.15, mean ± SD, table 1) with a maximum of 3.02 h. After POC transections, six cockroaches showed phase delays (Fig. 4b; 3.11 ± 2.65, mean ± SD, table 1) with a maximum of 6.29 h and two showed phase advances (2.26 ± 1.63, mean ± SD, n = 2, table 1) with a maximum of 3.41 h. In three cases, the phase shift could not be calculated because the activity onset after the POC transection was very irregular. AOC and POC transection resulted in comparable phase advances, but in different phase delays. Altogether, the transection experiments showed that the transection of either the AOC or the POC alone did not significantly alter the period length or the rhythmicity of the locomotor activity rhythm. The transection of the POC, however, caused stronger phase delays
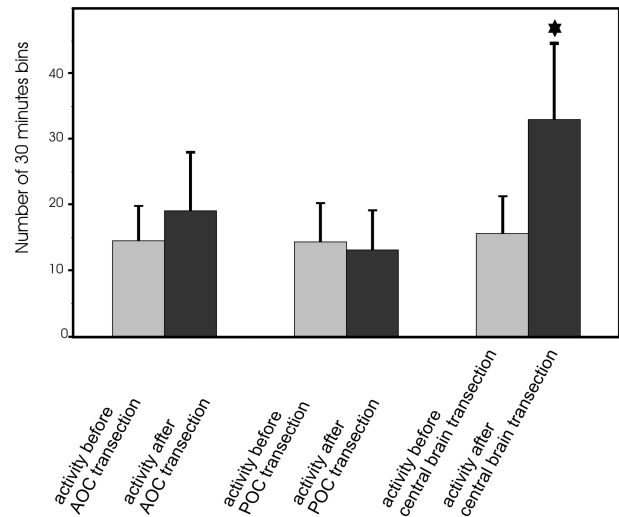

Fig. 8 Locomotor activity of cockroaches before and after AOC, POC, or central brain transection. Bars show the number of 30 min bins during which the cockroaches showed locomotor activity.

compared to the phase delays caused by the AOC transections. The transection of the central brain caused in most cases permanent locomotor activity which was significantly different to the activity intensity before the surgery.

Immunoreactivity in the AOC and POC commissure

By using anti-PDH-antiserum the AOC as well as the POC could be clearly identified. In two of the six evaluated AOC transections the AOC remained intact. In three brains, the AOC was completely transected and in one brain only parts of the AOC showed anti-PDH-immunoreactivity, and a connection between the superior median protocerebra could not be found (Fig. 3a, b). Only in one case the staining of the POC transected brains showed a clear cut of the POC (Fig. 5b). In three brains some fine fibers were visible which could be the result of either a failure in cutting the commissure or, more likely, the reinervating of cutted fibers. In the remaining three brains the POC was still intact. After the transections of the central brains in five of the seven evaluated cockroaches, no AOC and no POC were detectable. In two of these five brains it seemed that the brain hemispheres were grown together again (Fig. 7b-d). The remaining three brains showed an almost

Table 1 Mean and standard deviations for the time to regain rhythmicity, changes of period length and phase shifts after the various transections.

Treatment	Time to regain rhythmicity (mean ± SD)	Period length before surgery (mean ± SD)	Period length after surgery (mean ± SD)	Phase delay (mean ± SD)	Phase advance (mean ± SD)
AOC transection	10.25 ± 9.91 days n = 8	23.55 ± 0.31 h n = 8	23.54 ± 0.29 h n = 8	0.79 ± 0.43 h n = 8	2.21 ± 1.15 h n = 2
POC transection	10.67 ± 11.35 days n = 9	23.39 ± 0.2 h n = 11	23.27 ± 0.16 h n = 11	3.11 ± 2.65 h n = 6	2.26 ± 1.63 h n = 2
Central brain transection	51 ± 20.88 days n = 3	23.4 ± 0.1 h n = 2	23.4 h n = 2	not determined	not determined

complete transection of the brain (Fig. 7a). One preparation was not evaluable and in one preparation the POC was still intact. Comparison of the staining of the transected brains with that of untreated brains revealed that the main targets of PDH-ir fibers in the central brain [the SMP and SLP and the ventrolateral and inferior lateral protocerebrum] were still visible in the transected brains. The cockroaches which received a transection of the central brain displayed a fainter staining in all of these regions. In two cockroaches, two PDH-ir neurons, located near the calyces, were visible.

Discussion

In running-wheel assays, it was examined whether transection of the AOC and POC affects the locomotor activity of cockroaches differently. To determine whether the transections were successfully the brains of the monitored cockroaches were removed and stained with an anti-PDH-anitserum. The present study revealed that transection of the POC caused stronger phase delays than transection of the AOC. Other parameters of locomotor activity did not differ between the respective animals. Transection of the complete central brain however, caused permanent locomotor activity with a statistically higher overall activity after operation.

Both AOC and POC transmit coupling information between bilateral pacemakers

To exert a well synchronized circadian behavior the bilaterally paired pacemakers in the insect brain have to be mutually coupled. While coupling in crickets is relatively weak, the cockroach *Leucophaea maderae* exerted strong coupling (Page et al. 1977, Wiedenmann and Loher 1984, Ushirogawa et al. 1997). Previous experiments also showed that each single optic lobe is sufficient to drive locomotor rhythmicity (Sokolove 1975, Page et al. 1977, Wen and Lee 2000). Since the period of the locomotor activity rhythm driven by one pacemaker is slightly longer than that driven by the mutually coupled pacemakers (Page et al. 1977) the bilateral pacemakers are supposed to interact via phase advances. In our study both lengthening and shortening of the period after transection of AOC or POC could be observed. Since changes in the period length did not significantly differ between AOC and POC transected cockroaches, information for maintaining a stable period appear to be transmitted by the AOC and POC in paralell. Since transections of the POC resulted in stronger phase delays than transections of the AOC the POC may transmit more advancing information than the AOC. However, to investigate the specific role of the AOC and POC in coupling, both commissures need to be transected. It is known that neurons of four

different AMe groups are involved in the coupling of both pacemakers (Reischig and Stengl 2002, Reischig et al. 2004, Chapter III). In addition it was shown that neurons of the ventromedian group of the AMe are light-sensitive (Loesel 1999, Loesel and Homberg 1998, 2001). These neurons project exclusively through the POC to the contralateral lobe and appear to transmit light information between the bilateral pacemakers. Since our behavioral assays were performed in DD no information upon the transport of light information could be obtained. In contrast to the ventromedian neurons, neurons of the ventral group do not respond to light stimuli (Loesel and Homberg 2001). They project through both the AOC and POC to the contralateral AMe (Reischig et al. 2004). Thus, ventral neurons may transmit phase information between the pacemakers. In honeybees, the AOC contains fibers of unidirectional, motion-sensitive neurons (Hertel et al. 1987), and hence the AOC of cockroaches may also carry information from motion sensitive VNes to the contralateral pacemaker. Computer simulations on the coupling of two oscillators showed that both phase delays and advances together are necessary to create a stable locomotor period length (Petri and Stengl 2001). This also fits with the finding that injection of neuropeptides into the AMe often induces biphasic PRCs similar to that obtained after donation of light pulses (Page and Barret 1989, Petri et al. 2002, Hofer and Homberg 2006). Since PDH injections in the vicinity of the AMe resulted in significant phase delays PDF is unlikely to act as an advancing factor in the POC (Petri and Stengl 1997). Members of the FMRFamide-related peptides (FaRPs) are also located in coupling neurons and a peptide of this family may be involved in the coupling of the bilateral pacemakers. However, FMRFamide and Pea-FMRFamide-7 injections into the AMe of cockroaches resulted in significant phase delays in locomotor activity (Soehler et al. 2008) and, therefore, these peptides are not the missing phase advances causing factors. The same applies to leucomyosuppressin, another member of the FaRPS. Injections of leucomyosuppressin into the AMe of cockroaches did not induce changes of locomotor activity (Söhler et al. 2007). Thus,

another - yet unidentified - member of the large FaRP family may cause the phase advances which are necessary for creating a stable locomotor period length. Further experiments will test this hypothesis. Since immunocytochemical data could not be obtained for most of the cockroaches in our experiments a clear correlation of POC transection and phase delays could not be demonstrated. Nevertheless, the actograms of all POC, AOC or central brain transected cockroaches were comparable inside each group. Further experiments are needed to investigate the role of the AOC and POC for the transmission of coupling information.

Output pathways of locomotor pacemakers

Already 1983, Page assumed that the coupling pathways and output pathways by which the pacemakers drive the activity rhythm are distinct. Innervation of the SMP and SLP appeared to be particularly important for the coupling between the bilateral pacemakers and central brain neuropils which control locomotor activity. Accordingly, Reischig and Stengl (2003a) showed that all optic lobeless cockroaches which regained rhythmicity after transplantation of AMe showed reinnervation of the SMP and SLP by PDH-ir fibers. From the SMP and SLP the pacemaker information controlling loco-motor activity is transmitted to the sub-oesophageal and thoracic ganglia that control leg muscle activity. Wen and Lee (2000) suggested that the hyperactivity of cockroaches with cut optic tracts is due to the disruption of the output pathway of the pacemakers to the motor control center in the thoracic ganglia. Based upon the work of Colwell and Page (1990) and others (Roeder 1967, Pearson and Iles 1970, Burrows 1996). Wen and Lee (2000) concluded that central pacemakers provide an output temporal signal that inhibits the descending excitatory output of the suboeso-phageal ganglion to motor control centers in the thoracic ganglion. This corresponds to our finding that transection of the central brain typically caused permanent locomotor activity of cockroaches. Three out of 12 cockroaches with a complete brain transection regained locomotor rhythmicity. These cockroaches might

have rebuilt a neuronal connection between a pacemaker and the descending excitatory drive. However, like in the case of the cockroaches with AOC and POC transections, an interpretation of the obtained results is difficult and further behavioral and immunocytochemical data are needed. Nevertheless, our results suggest that the AOC and POC transmit different information and that a connection between the pacemakers to the SMP and SLP is necessary to create rhythmic locomotor behavior.

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Erkärung

Ich versichere, dass ich meine Dissertation

The circadian system of the cockroach *Leucophaea maderae*: The role of FMRFamide-related peptides in the circadian system and the role of the anterior and posterior optic commissures in the coupling of both pacemakers

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den

(Sandra Söhler)

Curriculum vitae

Sandra Söhler

address: FB Biologie, Tierphysiologie
Karl-von-Frisch Straße
35032 Marburg, Germany
phone: +49 6421 28 26944
email: ssoehler@web.de

Personal

Date and place of birth: 02.01.1971, Hüttental, now Siegen (Germany)

Marital status: married, one child

CV

2002 – present	Doctoral thesis, Philipps-Universität Marburg, Germany
2001	Diploma, Philipps-Universität Marburg, Germany; Title of diploma thesis: Analysis of the circadian coupling pathway of the cockroach *Leucophaea maderae*. The role of a member of the peptide family of the FMRFamides for the function of the circadian clock.
1999/2000	free semester because of birth of my son
1994 – 2001	Undergraduate, Philipps-Universität Marburg, Germany
1991 – 1994	Education for medical secretary
1987 – 1991	Evangelisches Gymnasium Siegen, Germany Academic appointment
1981 – 1987	Realschule Freudenberg

Academic Appointment

2001 – 2002	Research assistant, Philipps-Universität Marburg, Germany

Teaching

Tutor in undergraduate courses:
- Animal physiology (chronobiology)
- Neurobiology

Publications

Reviewed articles:

- Wicher D, Agricola HJ, Soehler S, Gundel M, Heinemann SH, Wollweber L, Stengl M, Derst C (2006)
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Marburg, November