Obstructive sleep apneas naturally occur in mice during REM sleep and are more prevalent in a mouse model of Down syndrome

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SUMMARY

ABSTRACT

Study Objectives. The use of mouse models in sleep apnea research is limited by the belief that central (CSA) but not obstructive sleep apneas (OSA) occur in rodents. With this study we wanted to develop a protocol to look for the presence of OSAs in wild-type mice and, then, to apply it to a mouse model of Down Syndrome (DS), a human pathology characterized by a high incidence of OSAs.

Methods. Nine C57Bl/6J wild-type mice were implanted with electrodes for electroencephalography (EEG), neck electromyography (nEMG), diaphragmatic activity (DIA) and then placed in a whole-body-plethysmographic (WBP) chamber for 8h during the resting (light) phase to simultaneously record sleep and breathing activity. The concomitant analysis of WBP and DIA signals allowed the discrimination between CSA and OSA. The same protocol was then applied to 12 Ts65Dn mice (a validated model of DS) and 14 euploid controls.

Results. OSAs represented about half of the apneic events recorded during rapid-eye-movement sleep (REMS) in each experimental group while almost only CSAs were found during non-REMS. Ts65Dn mice had similar rate of apneic events than euploid controls but a significantly higher occurrence of OSAs during REMS.

Conclusions. We demonstrated for the first time that mice physiologically exhibit both CSAs and OSAs and that the latter are more prevalent in the Ts65Dn mouse model of DS. These findings indicate that mice can be used as a valid tool to accelerate the comprehension of the pathophysiology of all kind of sleep apnea and for the development of new therapeutical approaches to contrast these respiratory disorders.

Keywords: apneas, mice, Down Syndrome, breathing, respiratory disorder.

INTRODUCTION

Definition of Sleep

Sleep is a natural state characterized by a reduction in voluntary motor activity, a decreased response to stimulation and stereotypic posture. It can be distinguished from other states of altered consciousness, in that it is easily reversible and self-regulating[1].

Sleep is crucial for emotional, physical and cognitive wellbeing[2] and occupies around one third of a person's life[3]. It allows the human body to recover after activity, ensuring optimal subsequent functioning[4]. Sleep deprivation is associated with cognitive impairment, mood changes and hormonal abnormalities[5–7].

From evolutionary point of view, sleep or sleep-like states are conserved across the animal species, suggesting that sleep could respond to a common purpose that contributes to animal survival. However, the function of sleep has remained largely unknown so far.

Sleep stages

Sleep in mammals is a relatively evident state, as electroencephalograms (EEG) and electromyograms (EMG) can be used to easily distinguish sleep and wakefulness (figure 1).

During the night, human sleep cycles between two distinct states, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep (when vivid dreams occur) (figures 1 and 2).

NREM sleep is further subdivided into different stages, N1, N2 and N3, that can be identified by a peculiar pattern of EEG and EMG signals. The EEG of an awake person shows neural oscillations between alpha waves (during a quiet state) and beta waves (when the person pays attention to something) (7.5–12.5 Hz and 12.5–30 Hz, respectively). When a subject falls asleep first enters NREM sleep. In stage N1, alpha activity gradually decreases and theta waves (4–8Hz) appear. During stage N2, the EEG features sleep spindles (e.g., brief bursts of high frequency [7–14 Hz] activity) and K-complexes (e.g., one cycle of slow oscillation). When the person enters stage N3, slow delta wave activity (1–4Hz) starts to dominate the EEG. Stage N3 is typically called slow wave sleep, and during this stage, the person is in the deepest sleep and the threshold for awakening is highest. Classically, stage N3 was further divided into two stages based on the intensity of the slow waves (figure 1).

REM sleep follows the series of NREM sleep; beta activity again increases, along with theta activity (figures 1 and 2). In addition, a person in REM sleep shows rapid eye movement, muscle atonia, and partial loss of thermoregulation. REM sleep state can be easily distinguished from wakefulness by the low EMG signal in spite of the similar EEG patterns. At the end of REM sleep the subject can be most easily awakened with a small disturbance. Afterwards, the cycle starts again, with the person entering stage N1 of NREM sleep. A typical cycle of NREM and REM sleep lasts approximately 1.5 h. and during a normal 8 hours-sleep night, a person gets through four or five cycles of NREM/REM sleep.

REM sleep was first reported in 1953[8]. It is associated with the phenomenon of dreaming and it is also termed paradoxical sleep, since wake-like EEG patterns and loss of muscle tone are simultaneously registered during this state. REM sleep was also discovered in cats[9,10] and this made the way for the development of anatomic, pharmacologic, physiologic, and genetic studies to elucidate the neural substrates of REM and NREM sleep. These studies provided evidence that

the brainstem pontine tegmental area and adjacent mesencephalic and medullary regions contain neurons with key roles in the generation of NREM and REM sleep[11–18].

Figure 1. Representative human EEG patterns during each sleep stage (from S. Miyazaki et al.[19])

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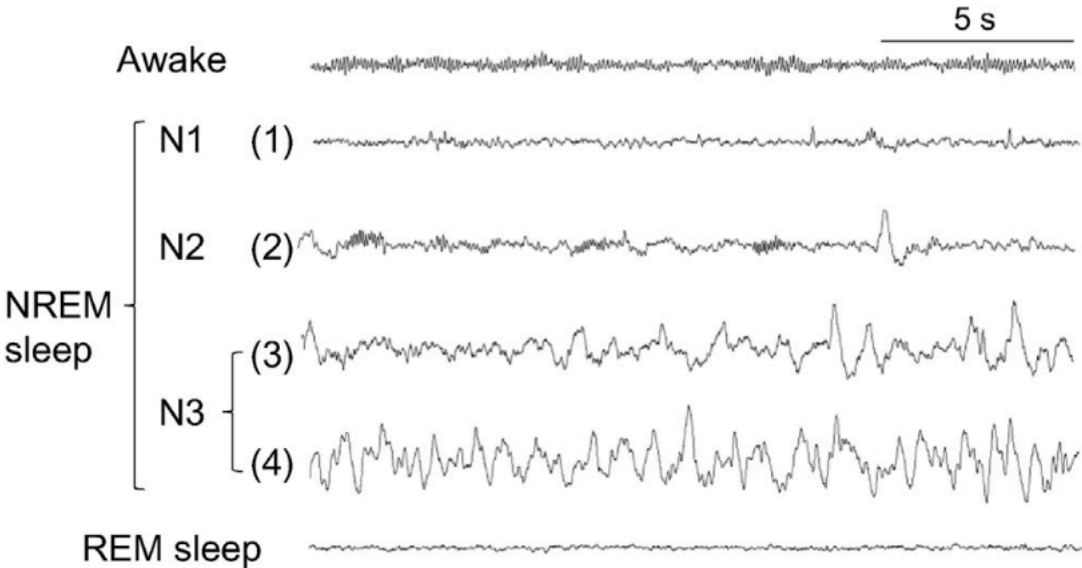
Figure 2. Typical human sleep architecture during the night (from S. Miyazaki et al.[19])
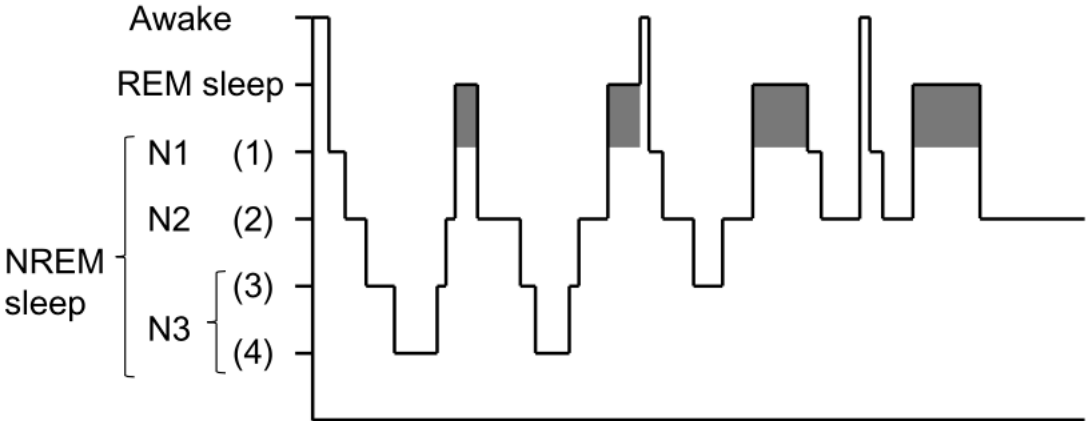
Function of Sleep

As mentioned above, the function of sleep is still poorly understood. Sleep deprivation eventually results in death in rats and in invertebrates such as fruit flies (D. Melanogaster) and roundworms (C. elegans)[20–22] but in all cases, the exact cause of the lethality is not clear.

In mammals, NREM sleep is involved in the secretion of growth hormone, synaptic plasticity, memory consolidation, and clearance of brain metabolites[23–28]. Even though slow waves that characterize NREM sleep are unique to mammals and avian species, there seems to be some similarity between the roles of human sleep and sleep-like state in invertebrates. Recent evidence on mouse brain show that dendritic spines, that are post-synaptic structures, undergo active remodeling during sleep[28–30] similar to fruit flies, in which sleep is associated with structural changes in multiple brain areas, involved in learning and memory[31,32].

The enhancement of slow wave activity during NREM sleep improves memory consolidation in mammals[24,25].

The function of REM sleep is much less understood than NREM sleep. Many studies on humans and animals investigated the role of REM sleep by means of forced awakening whenever the subject was entering REM state. Even though this procedure can reduce the amount of REM sleep, the extreme stress produced by the repeated physical stimulation entails the reduction of total sleep duration[13]. It is therefore difficult to interpret the exact roles of REM sleep on the basis of these results: many of the effects observed in these experiments could not be differentiated from the effect of stress. To overcome these problems, some studies used optogenetics and chemogenetics. In the work by Haiyashi and coworkers[13] mice brainstem neurons that negatively regulate REM sleep were either chemogenetically activated or inhibited to genetically suppress or increase REM sleep, without physical stimuli. The results showed that inhibiting or increasing REM sleep attenuates or enhances slow wave activity in the subsequent NREM sleep, respectively. This study

suggested that REM sleep is involved in regulating the cortical activity of the NREM[13] and since slow wave activity promotes learning and memory consolidation[24,33] and enhance the potentiation of synapses at cellular level[23], REM sleep might indirectly regulate memory processing in the cortex via slow wave enhancement. Moreover, during REM sleep a strong theta oscillation is observed in the hippocampus that plays a critical role in memory formation. In mice, after the inhibition of the theta oscillation during REM sleep by optogenetic silencing GABAergic neurons in the medial septum, the consolidation of what was learnt before sleeping was compromised[34]. Therefore, the occurrence of theta waves in the hippocampus during REM sleep is supposed to be of crucial importance for consolidating hippocampus-dependent memories.

Sleep deprivation eventually results in death in rats and in invertebrates such as fruit flies (D. Melanogaster) and roundworms (C. elegans)[20–22] but in all cases, the exact cause of the lethality is not clear. Specifically, in rats it results in thermoregulation impairment, high metabolic rate, skin lesions and weight loss in spite of augmented food intake. Sleep-deprived rats die within 2or 3 weeks[21,35] but the direct cause of lethality remains unclear. A study on fruit flies[22] was designed to address this issue and identified two strains that are extremely sensitive to sleep deprivation. These strains presented a mutation in either the circadian clock gene *cycle* or the heat shock induced gene *hsp83* and demonstrated an exaggerated sleep rebound after 3 h of sleep deprivation. Moreover, individuals of these strains began to die after only 10 h of sleep deprivation. These findings might provide suggestions to the fundamental function of sleep at the molecular level. In facts, the *hsp83* gene encodes a chaperone protein, and thus sleep might be required for quality management of certain proteins.

In roundworms, continuous disruption of lethargus results in lethality[20]. In addition, synaptic remodeling and pruning events in the nervous system were found to coincide with lethargus timing[36–38], suggesting that, as with mammalian NREM sleep and fruit fly sleep, neural circuit remodeling is increased during lethargus.

The study on sleep deprivation in rats that was described above, also suggested that metabolism is tightly connected with sleep, since sleep deprivation entails a higher metabolic rate and enhanced appetite[35]. This is due to the fact that hypothalamic neurons such as the orexinergic neurons and melanin-concentrating hormone-releasing neurons have double roles in the regulation of sleep and feeding[39–48]. Also in invertebrates feeding and sleep interact, as can be seen in fruit flies in which starvation suppresses sleep[49]. Sleep regulation could be an important strategy to control energy distribution and conservation through the adjustment of metabolism, reproductive behavior and feeding but more examinations and proper interpretations are needed.

Sleep regulation

The classical description of sleep regulation is based on the interaction of specific wake or sleep-active cell groups, that are responsible for the cortical grade of activity and on the crucial role played by the thalamus in sleep initiation[50].

Wakefulness requires activation of specific nuclei in the brainstem and hypothalamus to amplify cortical activity. These nuclei are situated in evolutionary old parts of the brain and can be found in all vertebrate species their counterparts can be identified also in many invertebrate species.

The waking nuclei have peculiar anatomical organization: small groups of cells with specific neurotransmitter send their axons to the cortex. When the subject is awake, an intense release of neurotransmitters by all cell types is registered to maintain cortical activity. The pathways that promote wakefulness ascend through the paramedian region of the midbrain and then split into a ventral part that innervates the basal forebrain, the hypothalamus and cortex and a dorsal part that projects into the thalamus. In normal wakefulness, the ventral pathway is crucial for the behavioral state of wakefulness, while the dorsal pathway sustains proper thalamocortical signaling and

consequently it is essential for the content of consciousness. The neurochemical systems promoting arousal and the fast cortical activity of wakefulness are shown in figure 3. From the rostral brainstem and caudal hypothalamus monoaminergic neurons (light green) directly innervate the cortex as well as many subcortical regions including the thalamus and hypothalamus. These monoaminergic regions include serotonergic neurons of the dorsal and median raphe nuclei, noradrenergic neurons of the locus coeruleus, histaminergic neurons of the tuberomammillary nucleus and dopaminergic neurons of the ventral tegmental area. Wake-promoting signals also arise from the parabrachial nucleus and cholinergic regions (dark green), including basal forebrain, the pedunculopontine and laterodorsal tegmental nuclei.

Figure 3. Wakefulness promoting pathways. (image taken from Scammell et al. 2017[51])
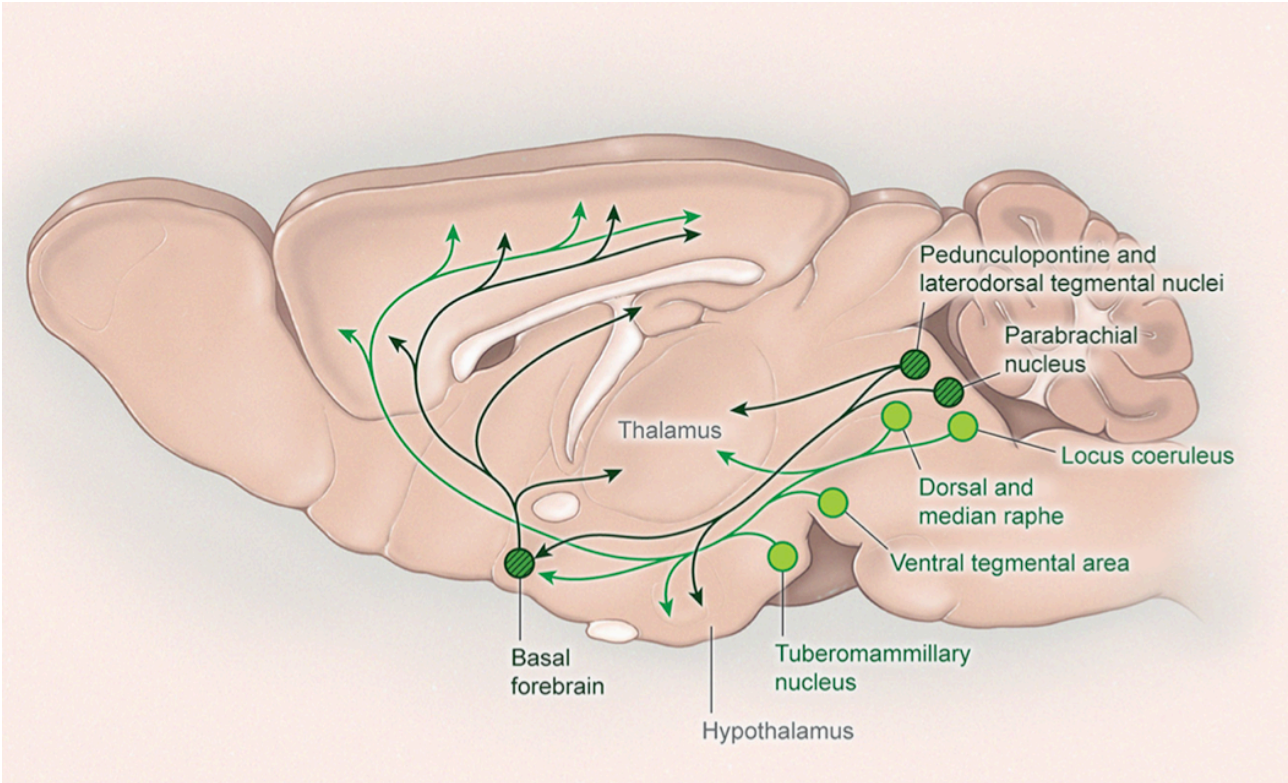
Long periods of wakefulness are generally followed by long NREM sleep periods, a homeostatic response mediated by NREM promoting substances such as Prostaglandin D2, Adenosine and cytokines (tumor necrosis factor-α and interleukin-1). The neurons promoting NREM sleep have been established to lie in the ventrolateral preoptic area and in the median preoptic nucleus. As shown in figure 4 GABAergic neurons in the ventrolateral preoptic area and median preoptic nucleus promote sleep by means of GABAergic neurons that inhibit wake-promoting neurons in brainstem and the caudal hypothalamus. The basal forebrain also contains sleep-active neurons that can promote sleep via direct projections to the cortex and projections within the basal forebrain itself. GABAergic neurons of the parafacial zone can promote sleep by inhibiting the parabrachial nucleus. The cortex contains scattered NREM sleep-active neurons that contain both GABA and neuronal nitric oxide synthase. Blue circles show NREM sleep-promoting nuclei.

Fig. 4. NREM sleep promoting pathways. (image taken from Scammell et al. 2017[51])
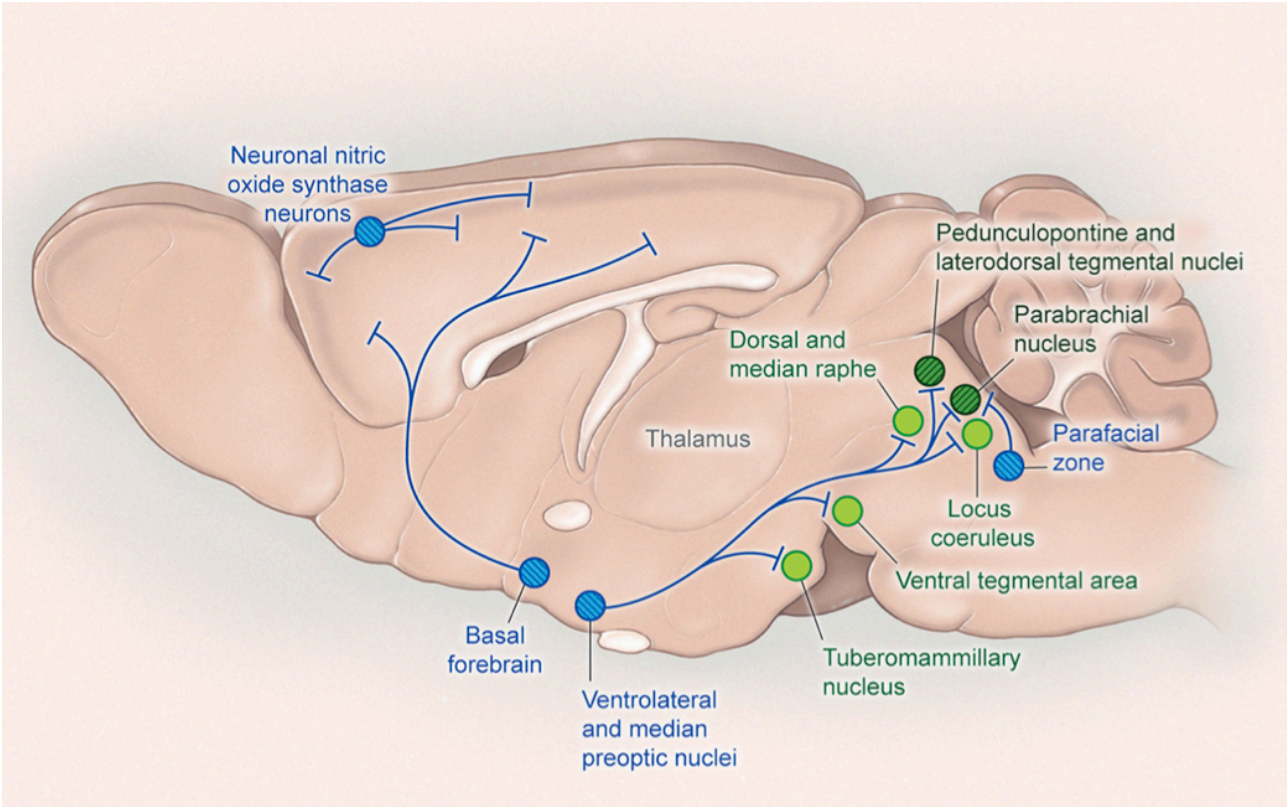
NREM sleep relies on neurons in the preoptic area (the most rostral part of the hypothalamus), in the basal forebrain, in the brainstem and in the cortex but it is still to be established how they sustain the transitions into NREM and NREM sleep itself.

For REM sleep, neural circuits lying in the pons are required. The sublaterodorsal nucleus plays a crucial role in regulating REM sleep. It is a pontine region located ventral to the caudal laterodorsal tegmental nuclei and the locus coeruleus. Glutamatergic neurons of the sublaterodorsal nucleus produce the muscle atonia of REM sleep by exciting GABAergic/glycinergic neurons in the ventromedial medulla and spinal cord that hyperpolarize motor neurons. Cholinergic neurons of the pedunculopontine and laterodorsal tegmental nuclei also promote REM sleep and are supposed to drive the fast EEG activity typical of REM sleep. During wake and NREM sleep, the sublaterodorsal nucleus is inhibited by GABAergic neurons of the ventrolateral periaqueductal gray and adjacent lateral pontine tegmentum as well as monoaminergic neurons of the locus coeruleus and raphe nuclei. During REM sleep, the ventrolateral periaqueductal gray is likely inhibited by GABAergic neurons of the sublaterodorsal nucleus and medulla. Figure 5 shows REM sleep-promoting nuclei in blue and REM sleep-suppressing nuclei in green.

Figure 5. REM sleep promoting circuits (image taken from Scammell et al. 2017[51]).
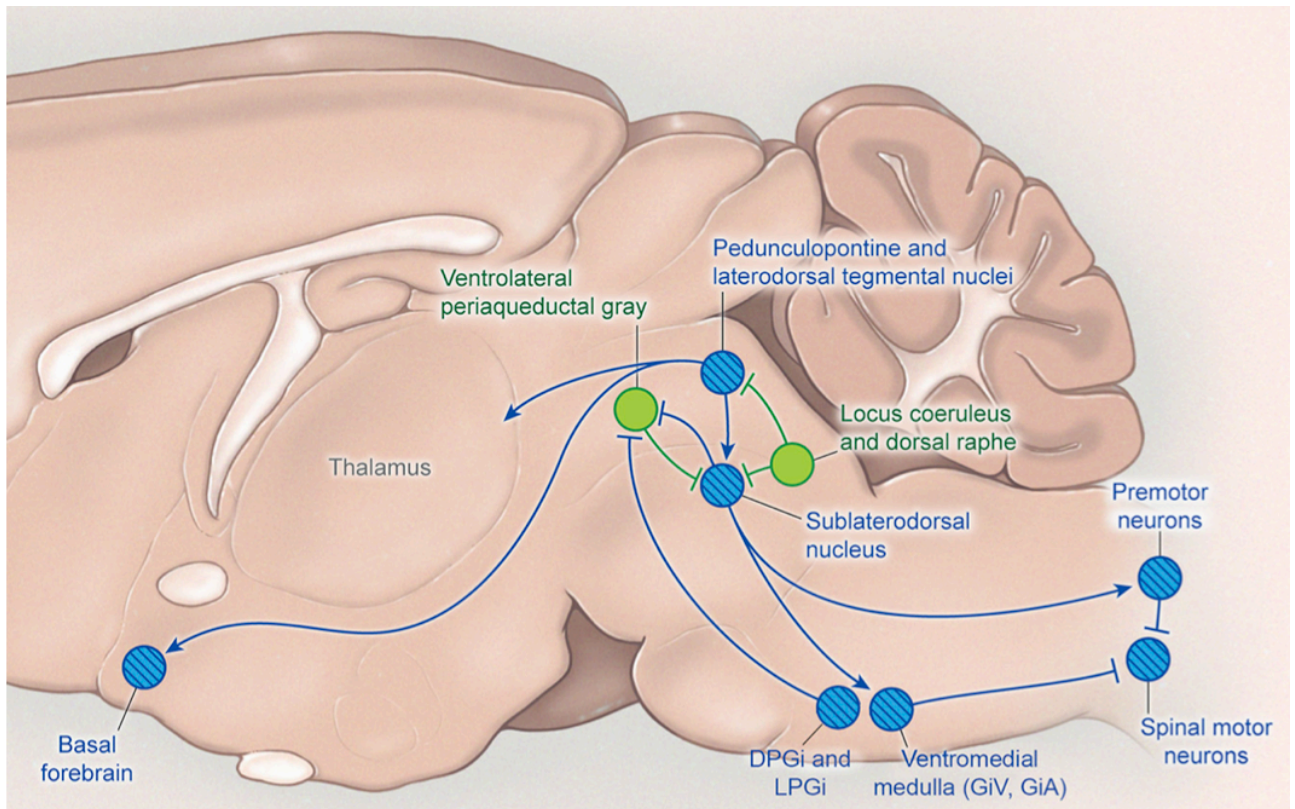
DPGi=dorsal paragigantocellular reticular nucleus; LPGi=lateral paragigantocellular nucleus; GiV=ventral gigantocellular reticular nucleus; GiA=alpha gigantocellular reticular nucleus.

In the last years, many research groups recognized a key role of the glutamatergic neurons of the sublaterodorsal nucleus in REM induction/regulation. The sublaterodorsal nucleus is supposed to provide ascending projections for EEG activation and descending projections that promote muscle atonia. During REM sleep, the neurons in the sublaterodorsal nucleus inhibit REM sleep-suppressing neurons of the ventrolateral periacqueduttal grey matter and of the adjacent lateral pontine tegmentum, but during wake and NREM sleep, the sublaterodorsal nucleus is reciprocally inhibited by the ventrolateral periacqueduttal grey matter and the adjacent lateral pontine tegmentum. The model of REM regulation is still incomplete; much has been learned about the

descending pathways controlling muscle atonia, but the ascending pathways that regulate cortical activity and dreaming remain slightly understood.

Sleep and respiratory physiology

The respiratory system is supported by a complex interaction between the central nervous system, respiratory-related motor neurons, and the muscles of respiration. Both volitional and metabolic pathways are active during wakefulness in order to determine the minute ventilation necessary to maintain eucapnia (the CO_2 level during stable breathing). With sleep onset, wakefulness stimuli are silenced, leaving metabolic demand as the primary factor of minute ventilation. Individuals with respiratory abnormalities such as restrictive or obstructive lung disease, neuromuscular weakness or anatomically small upper airway, experiment the recruitment of accessory muscles through wakefulness stimuli to maintain ventilation. With sleep onset, a marked reduction of compensatory mechanisms occurs, which can promote hypoventilation and the development of sleep-related breathing disorders. Thus, sleep represents a potentially vulnerable state for the respiratory system.

The medulla and pons have been identified as the primary central nervous system location responsible for determining respiratory drive[52]. Medullary respiratory nuclei, include 2 groups of neurons vital to breathing and referred to as the dorsal respiratory group (DRG) and the ventral respiratory group (VRG) (figure 6)[53]. The DRG is located in the nucleus tractus solitarius, an area responsible for central sensory integration of vagal afferents coming from the lungs, carotid and aortic chemoreceptors (CO_2 and O_2), central chemoreceptors (pH), and baroreceptors (blood pressure). The DRG contains predominantly inspiratory neurons. In the VRG both inspiratory and expiratory neurons are contained. Several important nuclei, or neuronal groups, are found in the

VRG, such as the Botzinger complex (expiratory neurons), the pre-Botzinger complex (inspiratory neurons), the nucleus ambiguus, the rostral retroambigualis neurons (inspiratory neurons), and the caudal retroambigualis neurons (ex- piratory neurons)[53]. The axons of bulbospinal neurons from the VRG and DRG project to spinal motor neurons that innervate the respiratory pump muscles. Important cranial motor neurons (nucleus ambiguous, contributes to the glossopharyngeal nerve; trigeminal, facial, and hypoglossal nuclei) that innervate muscles of the larynx and pharynx are in close proximity to the VRG. However, they are activated by different premotor neurons than those that drive the pump muscles, presumably because the laryngeal and pharyngeal muscles contribute to other functions, such as swallowing and phonation. Above the VRG, the pre-Botzinger complex has pacemakerlike properties that generate an underlying respiratory rhythm[54]. This complex of neurons contains mu-opioid receptors able to slow the pacemaker when stimulated, and neurokinin receptors, which can speed the pacemaker[55]. This entails important clinical implications: neuroactive medications used in the treatment of pain, such as opiates, can significantly impact ventilation. An important role in modulating respiratory activity is also played by the pons, that receives input from the pontine respiratory group (Kolliker fuse and para- brachial nuclei)[56].

Figure 6. Brainstem groups of neurons contributing to control respiratory drive (from Sowho et al.[57]).
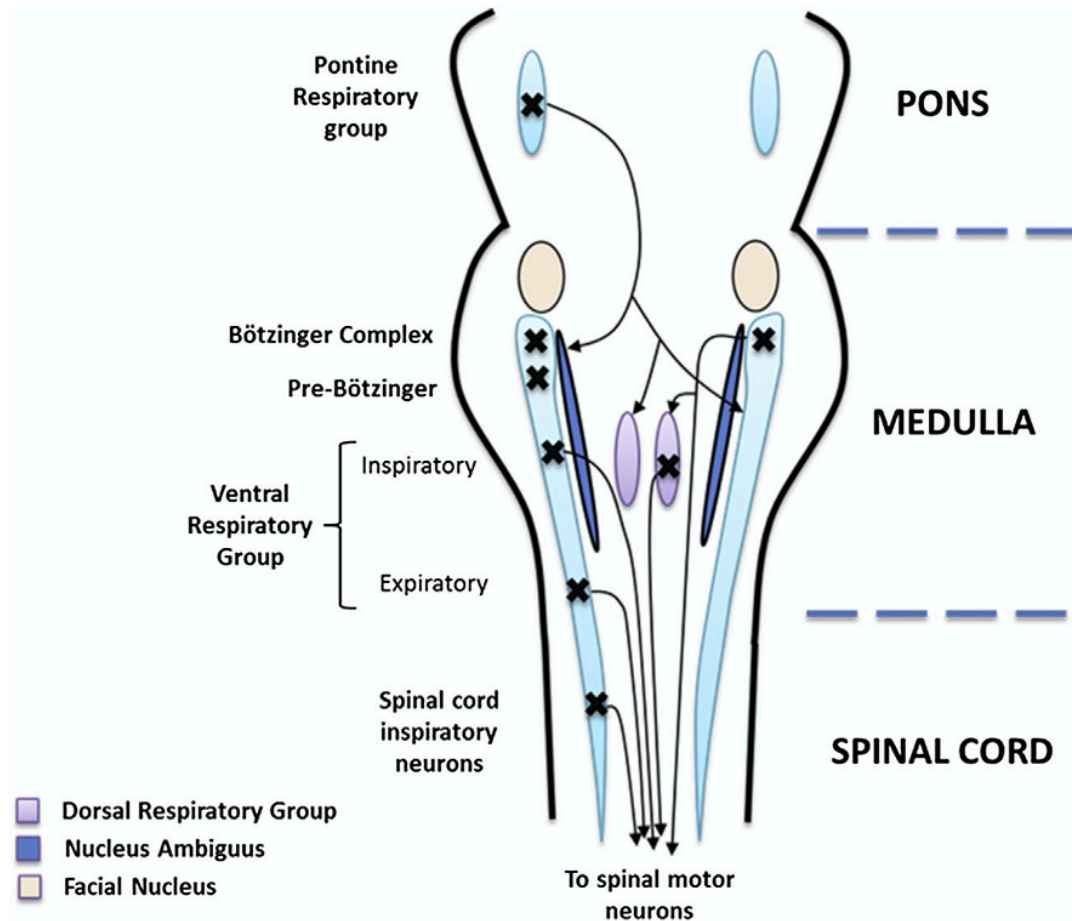
Therefore, the central respiratory generator (CRG) is composed by interactions and connections between respiratory propriobulbar neurons, premotor neurons, and motor neurons, that are responsible for producing an underlying respiratory drive (tonic activity) and respiratory rhythm (phasic activity) to the respiratory pump muscles (figure 7)[53].

Peripheral and central chemoreceptors send input to the CRG that elaborates the information and generates a proper level of tonic drive[58], The CRG generates phasic activity during inspiration: inspiratory premotor neurons from the VRG and DRG synapse with phrenic and intercostal motor

neurons. Inhibitory projections from expiratory neurons in the Botzinger complex to the brainstem and spinal cord terminate the inspiratory activity. Conversely, the control of respiratory drive to pharyngeal muscle motor neurons (e.g. the hypoglossal nerve) is not actively inhibited with expiration. The tonic activity to these motor neurons is not determined by VRG and DRG but it is sustained by inputs from the reticular formation[59]. The inspiratory activity of some pharyngeal muscle-related motor neurons depends on an underlying respiratory-related activity within the motor neuron., The reticular formation activity is withdrawn at sleep onset and leads to a reduction in tonic drive to the upper airway muscles, making the airway vulnerable to collapse[53].

Figure 7. Respiratory drive: central respiratory generators and feedback system. (From Sowho et al.[57])
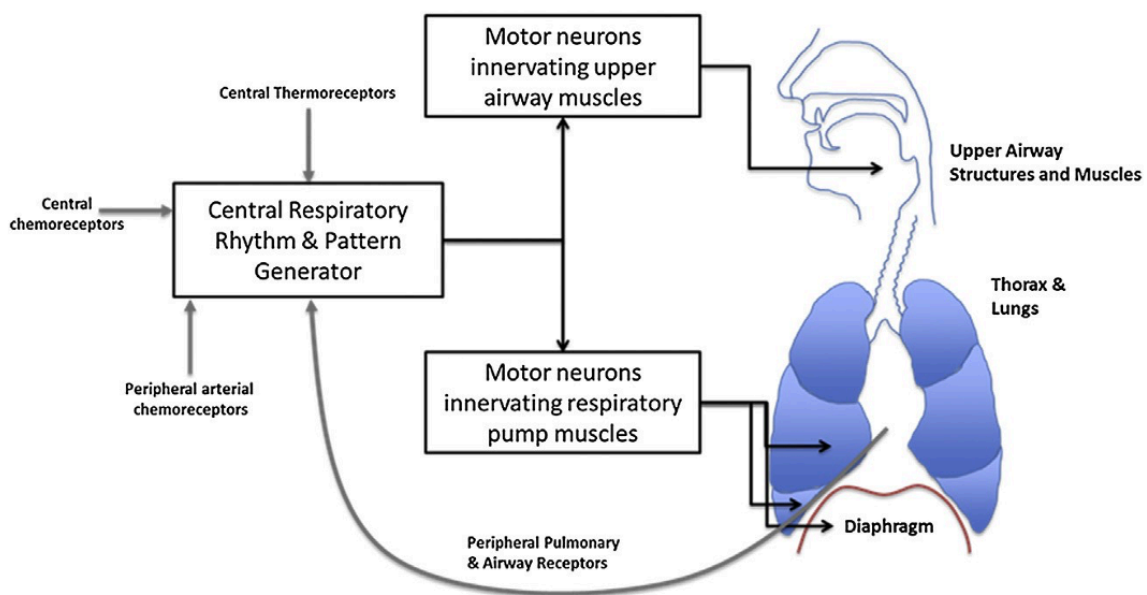
Control of ventilation

The primary purpose of the ventilatory control system is to regulate the blood gases exchange, in particular arterial CO_2 (Pa_{CO2}), within a relatively narrow range. Pa_{CO2} is determined by a hyperbolic relationship between alveolar ventilation (V_A) and metabolic CO_2 production. Assuming a constant CO_2 production and dead space, a 50% decrease in V_A would result in a doubling of the Pa_{CO2}[60]. During wakefulness, several processes are responsible for regulating ventilation and include behavioral control, the wakefulness stimulus and metabolic control[61]. At sleep onset, metabolic control becomes the primary stimulus for ventilation since behavioral control and wakefulness stimuli recede. Changes in both chemical and mechanical information arising from metabolic demand manage the ventilatory control mechanisms. The central (ventral medulla) and peripheral chemoreceptors (carotid body) are the primary sensors for chemical stimuli. With increasing hypoxemia, particularly as the Pa_{O2} falls below 60 mm Hg, the carotid body sends afferent impulses to the nucleus tractus solitarius via the glossopharyngeal and vagus nerves to increase ventilation[62]. Similarly, in case of hypercapnia or hypocapnia, linear changes in ventilation respond to central and peripheral chemoreceptors stimuli. The nucleus tractus solitarius also receives mechanical information from the thoracic cage and lungs via vagal afferent pathways responding to intrapulmonary stretch receptors, unmyelinated C fibers and irritant receptors. Stimulation of these receptors typically results in the Herring-Breuer reflex, that is the development of a shallow and rapid breathing pattern providing a negative feedback to limit overinflation of the lungs[62].

Ventilatory Control Stability (Respiratory Loop Gain Concept)

The ventilatory activity is tightly controlled by a negative feedback system to maintain the Pa_{CO2} at approximately 40 mm Hg during wakefulness. The definition of *respiratory loop gain* is used to describe the negative feedback response[61]. The ventilatory control is characterized by 3 major components: a plant, a circulatory delay and a controller (figure 8). The CO_2 inside the lungs, in the blood and in the tissues represents the respiratory plant. The change in Pa_{CO2} for a given change in ventilation ($\Delta Pa_{CO2}/\Delta V_A$) represents the plant gain. The transit time from the lungs to the central and peripheral chemoreceptors entails a circulatory delay. The central respiratory controllers respond to the consequent change in Pa_{CO2} producing a negative feedback in order to change V_A. The change in V_A for a given change in Pa_{CO2} ($\Delta V_A/\Delta Pa_{CO2}$)[60].

Figure 8. Respiratory loop gain. (From Sowho et al.[57])
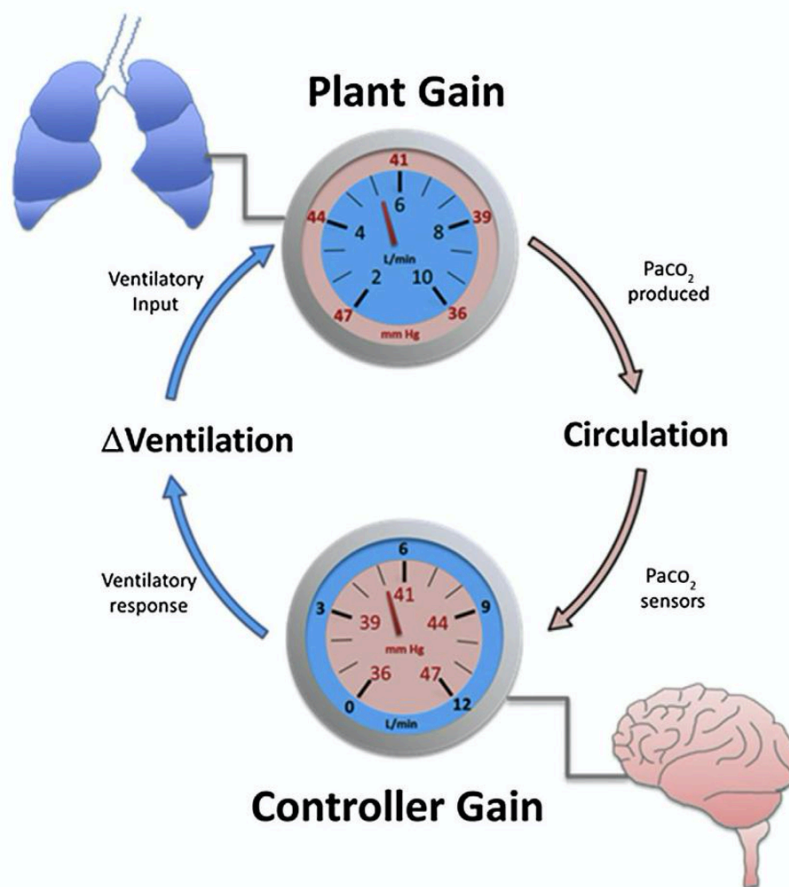
Therefore, respiratory loop gain is a measure of ventilatory stability, being a ratio of ventilatory response for a given ventilatory disturbance[61]. An example could be represented by an apnea or hypopnea (ventilatory disturbance) that causes a transient reduction in ventilatory drive entailing an increase in Pa_{CO2}. This increase, due to a circulatory delay, translates into a transitory increase in ventilatory drive (response) with consequent reduction in Pa_{CO2} that eventually returns to a steady state level. A *high loop gain* describes an instable system with frequent oscillation and difficulty in achieving a steady state. On the contrary, a *low loop gain* corresponds to a stable respiratory system[63,64].

The response to hypoxia and hypercapnia represents important elements of the controller gain.

Hypercapnic ventilatory responses (HCVR) and hypoxic ventilatory responses (HOVR) occur respectively in response to a rise in Pa_{CO2} and to a reduction in Pa_{CO2} in order to maintain homeostasis[62]. HCVR and HOVR decline during NREM and even more during REM sleep[65,66].

Sleep effects on upper and lower airway physiology

Respiration allows for gas exchange: it delivers oxygen to the circulation and eliminates carbon dioxide from the lungs.

<u>Upper airway anatomy and physiology</u>

The upper airway extends from the entrance to the nasal and oral cavities to the glottis, at the entrance of the larynx[67]. As shown in figure 9, it can be divided into 4 regions. (1) The nasopharynx, from the posterior nasal choanae to the caudal margin of the hard palate, (2) velopharynx, from the cranial to caudal margin of the soft palate, (3) oropharynx, or retroglossal segment, from the tip of the soft palate to the tip of the epiglottis, and (4) hypopharynx, ranging from the cranial margin of the epiglottis to the glottis. The upper airway responds to several functional roles such as respiration, heating and humidification of air, speech, and swallowing. To serve all these functions, the upper airway is composed by soft (24 muscles and other soft tissues), cartilaginous (thyroid cartilage, epiglottis), and bony (hard palate, mandible, and hyoid bone) tissues[68,69]. Except for the posterior vertebral column providing a kind of dorsal support, the upper airway has no bony or cartilaginous scaffold; this makes it highly deformable and prone to collapse during sleep. The most deformable region is represented by the velopharynx, followed by the

oropharinx[70–73]. The upper airway permits air movements: during inspiration the diaphragmatic contraction entails a negative intraluminal pressure but a negative pressure reflex prevents the collapse of the airway through phasic activation of dilator muscles (e.g. alae nasi and genioglossus)[74]. The CRG is likely responsible for the efficiency of this compensatory mechanism.

Figure 9. Upper airway anatomy (From Sowho et al.[57])
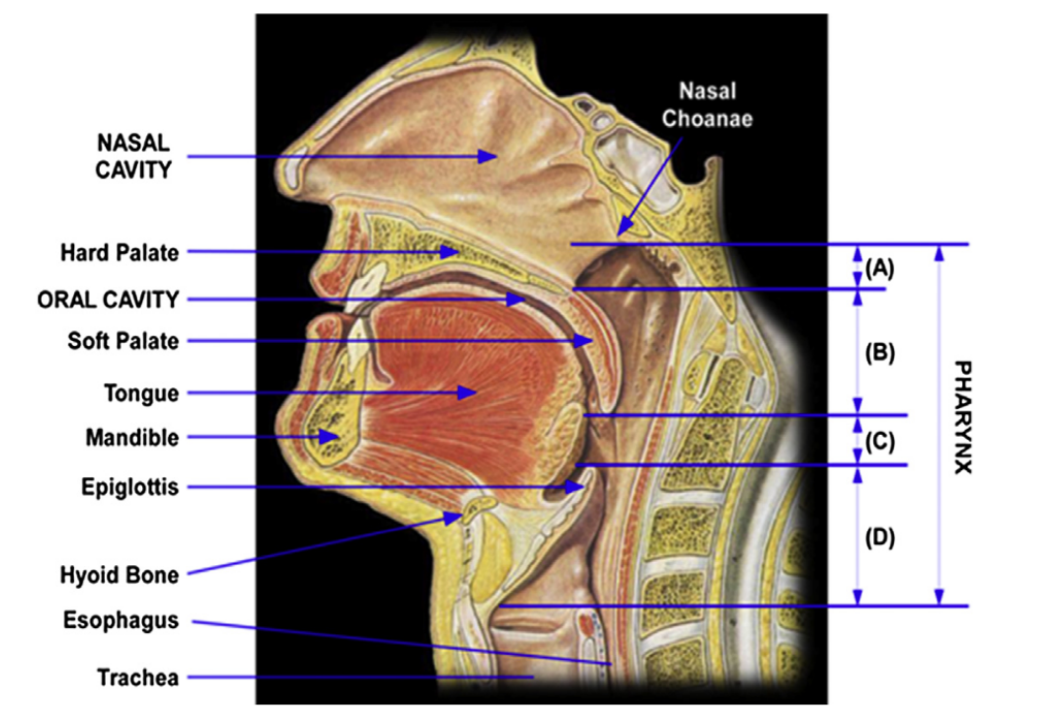
The collapse of the upper airway, especially during sleep, depends on the transmural pressure across the upper airway (intraluminal pressure - extraluminal pressure). The airflow in the upper airway follows the model of Starling resistor[75–77]: the airflow can be normal, limited or obstructed depending on the relationship between the upstream pressure (P_{us}), the critical pressure that is the collapsing pressure of the airway (P_{crit}) and the downstream pressure represented by the tracheal pressure (P_{ds}). If P_{us} and P_{ds} remain greater than P_{crit} no flow limitation occurs. If P_{ds} becomes lower

than P_{crit}, we assist to inspiratory flow limitation caused by a partial airway obstruction. A complete airway obstruction occurs when P_{crit} is higher than P_{up} and P_{ds}. P_{crit} (the collapsibility of the airway) can be increased by several factors such as increased surface tension along the pharyngeal airway[78], supine posture[79–82], reduced lung volumes[83–86].

At sleep onset, a great number of changes in the upper airway predispose the soft tissues to collapse: upper airway muscles activity decreases[87–89] with consequent decrease in upper airway compliance, the lumen size of the airway becomes smaller and the upper airway resistance increases[90,91]. This might result in sleep disordered breathing in some individuals. Moreover, we assist to a reduction of the pharyngeal neurocompensatory reflexes[92–94] (triggered by negative intraluminal pressure) and of ventilatory motor output (due to the loss of the wakefulness stimuli)[95,96].

Respiration can be described as a complex interplay between the central nervous system, respiratory motor neurons, auxiliary components and respiratory muscles. The sleep onset entails mild hypoventilation in normal subjects while in patients with chronic lung problems or different neuromuscular impairments or with diseases involving muscle tone, it can represent a vulnerable state.

BACKGROUND OF THE CURRENT STUDY

Sleep apnea represents a highly prevalent disorder that consists in recurrent episodes of cessation of breathing or decrease in airflow for 10 seconds or more while sleeping. It can be classified as central sleep apnea (CSA) when there is interruption of effort from respiratory pump muscles, or obstructive sleep apnea (OSA) when repetitive obstructions of the upper airways occur together with a continued or increased respiratory effort[97–101]. These breathing abnormalities during sleep are associated with increased risk of morbidity and mortality for cardiovascular events, and with metabolic and neurocognitive impairments[101,102]. The prevalence of moderate to severe (≥15 events per hour) in the general population is 23.4% in women and 49.7% in men; more specifically the 12.9% of men and 5.9% of women aged ≥ 40 resulted affected by OSAs[103]. However, several pathophysiological aspects of the disease are yet to be clarified, and methodologically sound clinical trials are needed to rise the quality of evidence. Basic research on experimental animals represents an important instrument to improve both diagnostic and therapeutic clinical practice[39,104]. Several studies have used mice as either natural models or experimentally-induced models of sleep apnea but the research remains focused on the genesis and modulation of CSA since to date, apneas in mice were anecdotally described as exclusively central events[105]. Although recent data suggest that obese mice can show upper airway flow limitation[106,107], it is still unclear whether clinically relevant OSAs occur in sleeping mice since no research focused on the analysis of the inspiratory muscles activity during episodes of decreased/absent airflow. Therefore, accurate classification of apneas by means of simultaneous evaluation of sleep state, breathing stability and respiratory muscles activity could allow to identify OSAs in mice, providing new useful animal models for studying apneas pathophysiology and develop targeted therapies.

In particular, the identification of OSAs in mice could allow to deepen the knowledge of the pathophysiological mechanisms underlying them in mouse models of human diseases

characterized by a high prevalence of OSAs, such as Down syndrome. Down syndrome, the most common human genetic pathology[108,109], is a complex condition entailing intellectual disabilities together with many other systemic dysfunctions[108,110] including OSAs. Among Down syndrome patients, the high prevalence of OSA (50-100% in childhood[111,112], nearing the 100% in adulthood[113]) is linked to anatomic abnormalities such as macroglossia, adenotonsillar hypertrophy and midface hypoplasia, together with associated systemic conditions (muscle hypotonia, hypothyroidism, gastroesophageal reflux etc.)[114,115] and represents a debilitating condition increasing the risk of hypoxemia, cognitive deficits and sleep fragmentation, further worsening the quality of life of these patients. Specifically, non-restorative sleep implies excessive daytime sleepiness and worsens the tendency to develop obesity, diabetes, hypertension, and cognitive impairment[116]. Among the different mouse models of Down syndrome[117], the Ts65Dn is one of the most used in research laboratories since it resembles many aspects of human pathology. In particular, Ts65Dn[118] mice are characterized by a partial triplication of the chromosome 16, containing approximately 55% of genes orthologues to genes on human chromosome 21. This model recapitulates different features of Down syndrome: increased fat mass[119], craniofacial dysmorphology[120], increased incidence of hypoxemia[121] and sleep disturbances[122]. All these aspects strongly support the hypothesis that Ts65Dn mice might present high prevalence of OSAs, like human patients, even if no direct prove has been produced so far. Consequently, the present study has 2 main aims: a) to develop a new technique that allows the potential detection of OSAs in mice; b) to establish through this new approach whether the Ts65Dn mouse model of Down syndrome exhibits a higher occurrence of OSAs, thereby replicating the human condition.

METHODS

The study protocol was approved by..

The present investigation is composed by two studies. The first was performed on wild-type male mice (with a pure C57BL/6J genetic background). The second study was performed on Ts65Dn (mouse model of Down syndrome) mice and euploid littermate controls obtained by mating B6EiC3Sn a/A-Ts(17^16)65Dn females with C57BL/6JEiJ x C3H/HeSnJ (B6EiC3Sn) F1 hybrid males. This parental generation was provided by Jackson Laboratories (Bar Harbor, ME, USA). The following experimental protocol was applied to all the mice included in the present manuscript.

Experimental protocol

The mouse colonies were housed in a standard laboratory environment at ($23 \pm 1°C$) with a 12-hour light-dark cycle and free access to food and water. All surgical procedures were performed under isoflurane anesthesia (2% in pure oxygen) with intra-operative analgesia (Carprofen 0.1 mg subcutaneously, Pfizer Italy, Latina, Italy) to minimize discomfort. Dihydrostreptomycin sulfate (1.5 mg/mouse) in 800 μL saline was administered subcutaneously at the end of surgery to prevent infections. The animals underwent surgery for the implantation of 3 differential electrodes for the detection of electroencephalogram (EEG), nuchal and diaphragmatic electromyogram (nEMG and DIA) activity. For EEG recordings, a pair of stainless-steel miniature screws (2.4mm length, Plastic One, Roanoke, VA, USA) soldered to a multi-stranded PFA-coated stainless-steel wire (KF Technology srl, Roma, Italy), were implanted into the frontal and parietal bones through burr holes and positioned in contact with the dura mater, while the head was immobilized with a stereotaxic frame. A couple of the same screws were symmetrically inserted on the other side of

the skull to balance the weight on the animal's head[123]. For nEMG recordings, 2 pairs of PFA-coated stainless steel wires were inserted in the posterior neck muscles[124]. For DIA recordings, 2 pairs of PFA-coated stainless-steel wires were inserted into the abdominal cavity and put in contact with the abdominal surface of the diaphragmatic muscle. Each of these wires ended with a circular uninsulated tip through which a 5-0 silk was passed and then sutured in the 8th intercostal space to keep the electrodes in contact with the diaphragm. The other extremity of the wires was tunneled subcutaneously to the mouse head, soldered to a socket and then fixed to the skull together with the sockets of the EEG and nEMG electrodes by means of dental cement (RelyX Unicem, 3M ESPE, Segrate, Italy), and dental acrylic (Respal NF, SPD, Mulazzano, Italy). After 7 days of postoperative recovery, the animals were slightly anesthetized to plug a lightweight cable for the acquisition of EEG, nEMG and DIA signals and then inserted in a whole-body plethysmograph (WBP, PLY4223, Buxco, Wilmington, NC, USA). The WBP consisted of 2 chambers: the first chamber was used as reference while the second chamber was used to accommodate the mouse. This chamber had an internal volume of 0.97 L and was equipped with a rotating electrical swivel (SL6C/SB, Plastics One, Roanoke, VA USA) to prevent twisting of the mouse wire tether, and probes to measure chamber temperature and humidity (PC52- 4-SX-T3 sensor, Rense Instruments, Rowley, MA, USA). The differential pressure between the chamber which contained the mouse and the reference chamber, which represents the mouse respiratory signal, was measured with a high-precision differential pressure transducer (DP103–06 + CD223 digital transducer indicator; Validyne Engineering, Northridge, CA, USA). Recordings were always performed for 8 hours starting at lights on. The EEG, nEMG, DIA and respiratory signals were continuously recorded together with WBP chamber humidity and temperature. The WBP system was calibrated dynamically with a 100 µL micro-syringe (Hamilton, Reno, NV, USA) at the termination of each recording session. The EEG, nEMG and DIA signals were acquired via cable transmission, amplified, and filtered (EEG, 0.3–100 Hz; EMG and DIA, 100–1000 Hz) using 7P511J amplifiers

(Grass, West Warwick, RI, USA) and digitized, together with mouse respiratory signal, with a PCI-6224 board (National Instruments, Austin, TX, USA) operated by software written in the laboratory using Labview (National Instruments, Austin, TX, USA). The EEG signal was digitalized at 258 Hz, while the nEMG and DIA signals were stored at 2048 Hz. The WBP differential pressure was digitalized at 1024 Hz, while WBP temperature and humidity were stored at 4 Hz together with all the above-mentioned signals. Each mouse underwent 2 recording sessions (8 hour each) spaced by 24h of rest. All the variables computed during each recording session were than averaged within each mouse.

Data Analysis

Offline visual scoring of wakefulness, non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS) based on EEG/nEMG signals was performed by 3 trained investigators on all consecutive 4 s epochs by means of a semi-automatic scoring program (SCOPRISM) as previously described[124]. Wakefulness was scored when the nEMG tone was high and the EEG was at low voltage with possible δ (0.5-4 Hz) and θ (6-9 Hz) frequency components. NREMS was scored when the nEMG tone was lower than in wakefulness and the EEG was at high voltage with prominent δ frequency components. REMS was scored when the nEMG indicated muscle atonia with occasional muscle twitches and the EEG was at low voltage with predominant θ frequency components.

Concerning respiratory variables, the values of ventilatory period (the interval between successive breaths, VP) and tidal volume (TV) were computed on stable NREMS and REMS episodes lasting ≥12 s (i.e., at least 3 consecutive 4-s epochs) as previously described in detail[125,126]. The analysis of breathing was not conducted during wakefulness for the frequent occurrence of movement

artefacts. During sleep periods, breaths were identified automatically from the upward (+) deflection peak of WBP pressure. Errors in breath detection as well as pressure artifacts (e.g., due to opening and closing of the room door) were manually excluded from subsequent analyses. Augmented breaths (sighs), which in mice occur almost exclusively during NREMS[127], were detected as breaths with TV > 3 times the average TV in NREMS of each mouse. Apneas were defined as the cessation of WBP pressure signals longer than 3 times the average VP value of each mouse in each sleep state. Apneas were further categorized as post-sigh apneas when starting within 8s from a sigh, or as spontaneous apneas when they started more than 8s from the preceding sigh[128].

Matching the WBP and DIA data in correspondence of each apneic event, we discriminated between CSA (concomitant absence of activity on WBP and DIA signals) and OSA (DIA contraction, no activity on WBP signal). Given that the severity of airways obstruction could vary between cases, as it happens also in humans, we decided to distinguish "sub-OSA" events, characterized by DIA contraction concomitant to an airflow (computed as the ratio between the TV and the duration of the correspondent DIA contraction) reduction higher than 30% compared to baseline airflow, and to properly call "OSA", those events characterized by DIA contraction concomitant to a complete absence of airflow activity (i.e. isoelectric WBP signal).

Finally, a further analysis was performed to characterize the patterns of airflow and DIA activity in terms of burst duration and amplitude during the apneas. The airflow of the post-apnea event was expressed as the % of the airflow exhibited during the breath preceding the apnea (used as arbitrary baseline). The purpose of this analysis was to verify if the post-apnea airflow was significantly different in amplitude from the baseline. Moreover, DIA activity during the obstructive events was evaluated in terms of burst duration and amplitude (expressed as the root

mean square of the DIA signal) and, again, expressed as % compared to the baseline event (the one preceding the apneic event).

Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS Inc, Chicago, IL, USA) results are shown as mean ± SEM with statistical significance set at $p < 0.05$.

In the first experiment of the present study, we did not apply any statistics since only one experimental group was involved. On the contrary, in the second experiment we compared 2 groups of mice. Consequently, we performed 2-ways ANOVAs with sleep state (2 levels: NREMS or REMS) or wake-sleep episode duration (4 temporal bins: <12s, between 12 and 60s, between 60 and 120s, >120s) or apnea subtypes (2 levels: either post-sigh Vs. spontaneous sleep apnea or CSA Vs. OSA) and genotype (2 levels: Ts65Dn and euploid controls) as factors. In case of significance of the 2-way interaction, simple effects of the mouse group were assessed with independent-sample t-tests. As stated, the analysis concerning the wake-sleep episode duration was performed on 4 different temporal bins thus, to prevent type I error, we applied the False Discovery Rate method as correction for multiple comparisons.

RESULTS

OSAs detection in C57BL/6J mice

The investigation was performed on 9 wild-type male mice (with a pure C57BL/6J genetic background) that underwent surgery at 41.8 ± 0.5 weeks of age. The mean weight of the sample at surgery was 31.2 ± 1.6g. The wake-sleep cycle and breathing profiles of these mice are provided as supplementary material (Table S1). The discrimination between CSA and OSA events was performed on all the mice during NREMS but only on 7 mice during REMS since 2 mice did not exhibit any apneic event in the latter sleep state. The results of these analyses (Figure 10) indicated that almost all the apneas during NREMS were classified as CSA ($98.3 \pm 1.2\%$) while only half of the apneic events during REMS were classified as CSAs ($57.2 \pm 10.8\%$). In specific, the $22.4 \pm 7.7\%$ of apneic events during REMS were classified as OSAs while the $20.4 \pm 8.6\%$ as sub-OSAs (Figure 10).

Table S1. Wake-sleep cycle and breathing profiles of wild-type mice.

Wake-sleep cycle profile		**Wakefulness (%)**	20.1 ± 2.9
		NREMS (%)	64.9 ± 2.1
		REMS (%)	10.6 ± 1.6
Breathing profile during	**NREMS**	**VP (ms)**	379 ± 9
		TV (µl)	218 ± 14
		Sighs (events/h)	14.3 ± 1.3
		All apneas (events/h)	5.9 ± 1.6
		Post-sigh apneas (events/h)	0.5 ± 0.2
		Spontaneous apneas (events/h)	5.2 ± 1.4
Breathing profile during	**REMS**	**VP (ms)**	361 ± 10
		TV (µl)	190 ± 11
		All apneas (events/h)	3.0 ± 0.7

This table shows a series of sleep and respiratory variables recorded in 9 wild-type mice (with a C57BL/6J genetic background) while kept into a whole-body plethysmograph for the first 8h of the light period. NREMS = non rapid-eye-movement sleep; REMS = rapid-eye-movement sleep; VP = ventilatory period; TV = tidal volume; sighs = augmented breath with TV > 3 times the average value recorded during NREMS. The total number of apneas were further categorized, during NREMS, as post-sigh apneas when starting within 8s from a sigh, or as spontaneous apneas when they started more than 8s from the preceding sigh. All the data are reported as mean ± SEM.

Figure 10. Percentual distribution of central and obstructive apneas across sleep states and examples of raw tracings in wild-type mice.
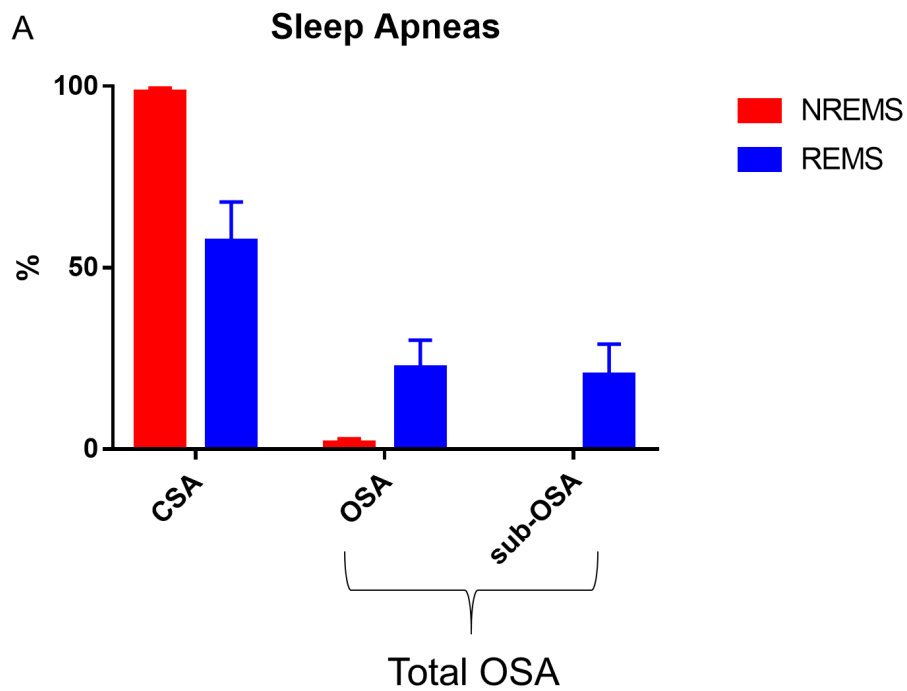
Panel A shows the different percentual distribution of CSAs, OSAs and sub-OSAs during NREMS and REMS in a group of C57BL/6j wild-type mice. The amount of total obstructive events (total-OSA) includes either those apneic events (OSA) characterized by DIA contraction and concomitant absence of airflow (isoelectric RESP signal) or those events (sub-OSA) characterized by DIA contraction and concomitant reduction of at least 30% of airflow compared to baseline value (the one computed on the breath preceding the apnea).

B sub-OSA

C CSA
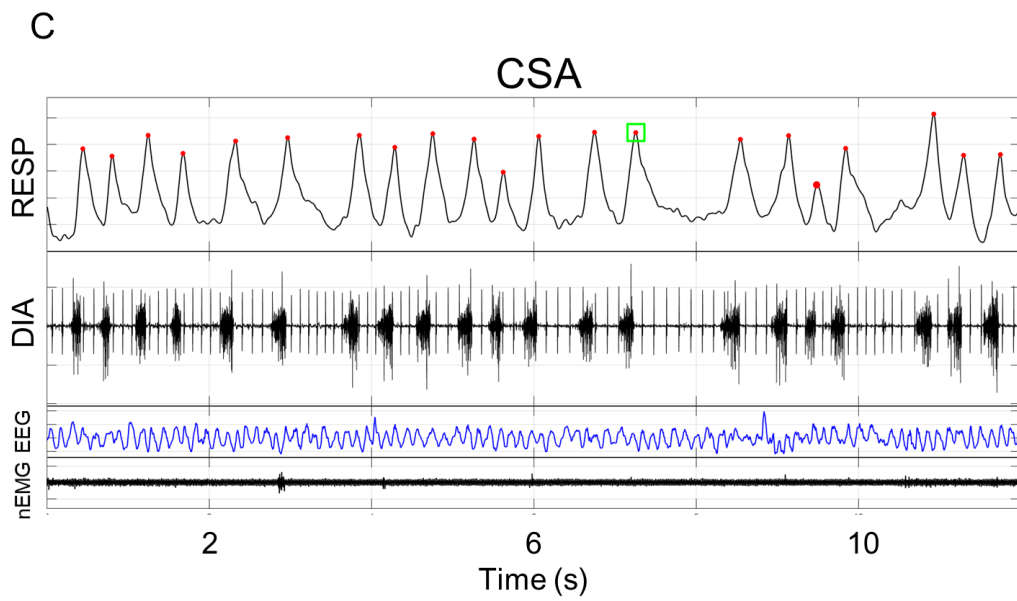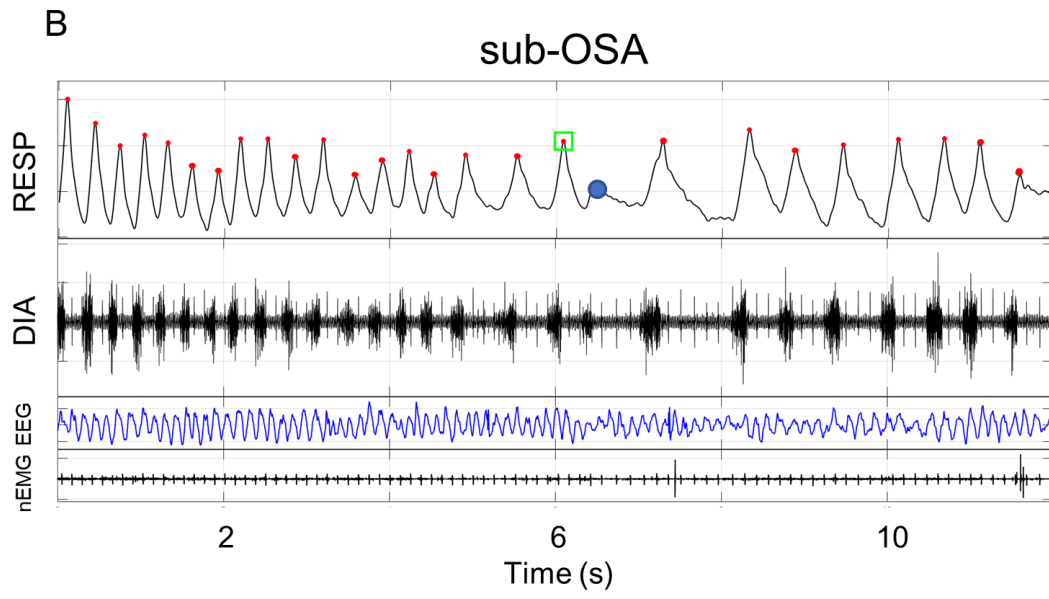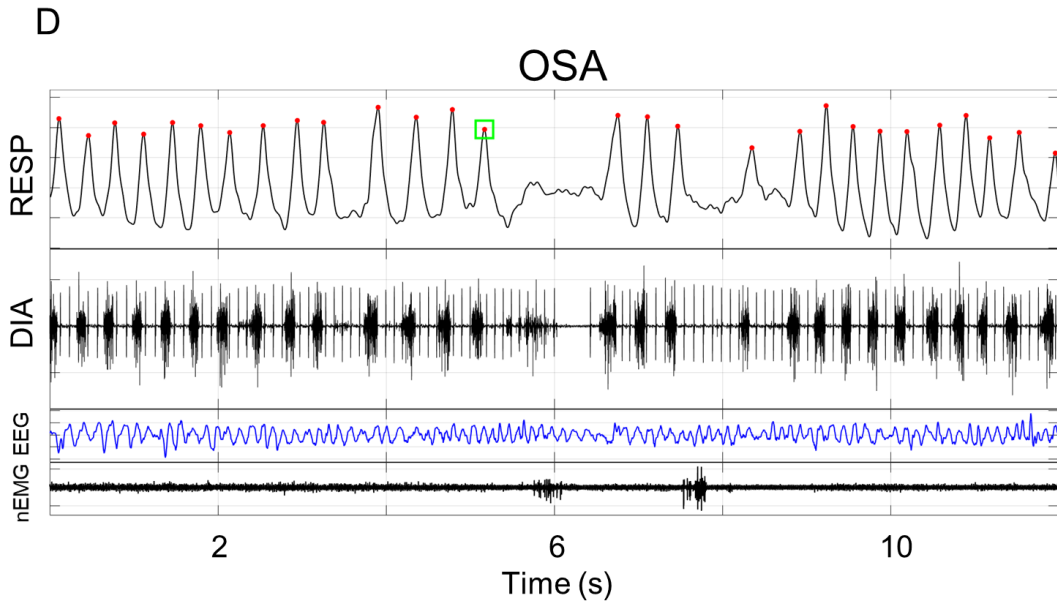
Panel B, C and D show raw tracings recorded during sub-OSA, CSA and OSA, respectively. On RESP signal, red dots indicate the peak of each inspiratory act while green squares indicate the beginning of the apneic event. The blue dot in panel B shows a peak with a reduction >30% compared to baseline value (computed on the last red dot before the green square) with concomitant DIA contraction and, consequently, this event was classified as sub-OSA.

REMS = rapid-eye-movement sleep; NREMS = non rapid-eye-movement sleep; CSA = central sleep apnea; OSA = obstructive sleep apnea; sub-OSA = sub-obstructive sleep apnea; RESP = differential pressure recorded into the whole-body plethysmograph (which corresponds to mouse respiratory pattern); DIA = diaphragmatic electromyogram; EEG = electroencephalographic activity; nEMG = nuchal muscles electromyogram.

OSA detection in Ts65Dn mouse model of Down syndrome

The investigation was performed on 12 male Ts65Dn mice and 14 male euploid controls (both with a DBA/2J genetic background). At surgery, the average age and weight of the mice were, respectively, 19.5 ± 0.8 weeks and 25.6 ± 1.0g for Ts65Dn and 19.8 ± 0.7 weeks and 28.3 ± 0.6g for their euploid controls. The 2 groups did not differ in terms of age at surgery (independent t-test, $p = 0.829$), however, Ts65Dn resulted significantly leaner than control mice (independent t-test, $p = 0.043$).

The analysis of the time spent in each wake-sleep state did not show any significant difference between Ts65Dn and euploid controls (Table 1). Therefore, we conducted a specific analysis aiming at assessing the presence of wake-sleep cycle fragmentation. Figure 11, indeed, shows the number of wakefulness, NREMS and REMS episodes as a function of their duration. Two-way ANOVAs indicated a significant interaction effect between mouse's genotype and bin duration both during wakefulness ($p = 0.008$) and NREMS ($p = 0.001$) but not during REMS ($p = 0.501$). The subsequent corrected multiple comparisons showed that Ts65Dn mice exhibited significantly more episodes of short duration both during wakefulness (lasting less than < 12s) and NREMS (lasting less than < 60s) compared to controls.

Table 1. Wake-sleep cycle and breathing profiles of Ts65Dn mice and euploid controls.

Experimental Group	Wakefulness (%)	NREMS (%)	REMS (%)
Ts65Dn (n=12)	22.6 ± 1.3	59.5 ± 2.6	13.2 ± 1.9
Controls (n=14)	18.6 ± 1.9	64.8 ± 2.5	10.7 ± 1.9

This table shows the percentage of time spent in wakefulness, non rapid-eye-movement sleep (NREMS) or rapid-eye-movement sleep (REMS) of a mouse model of Down syndrome (Ts65Dn, n = 12) and their euploid controls (n = 14) while kept into a whole-body plethysmograph for the first 8h of the light period. Data are reported as mean ± SEM.

Figure 11. Distribution of wake-sleep episodes according to their duration in a mouse model of Down syndrome and control mice.
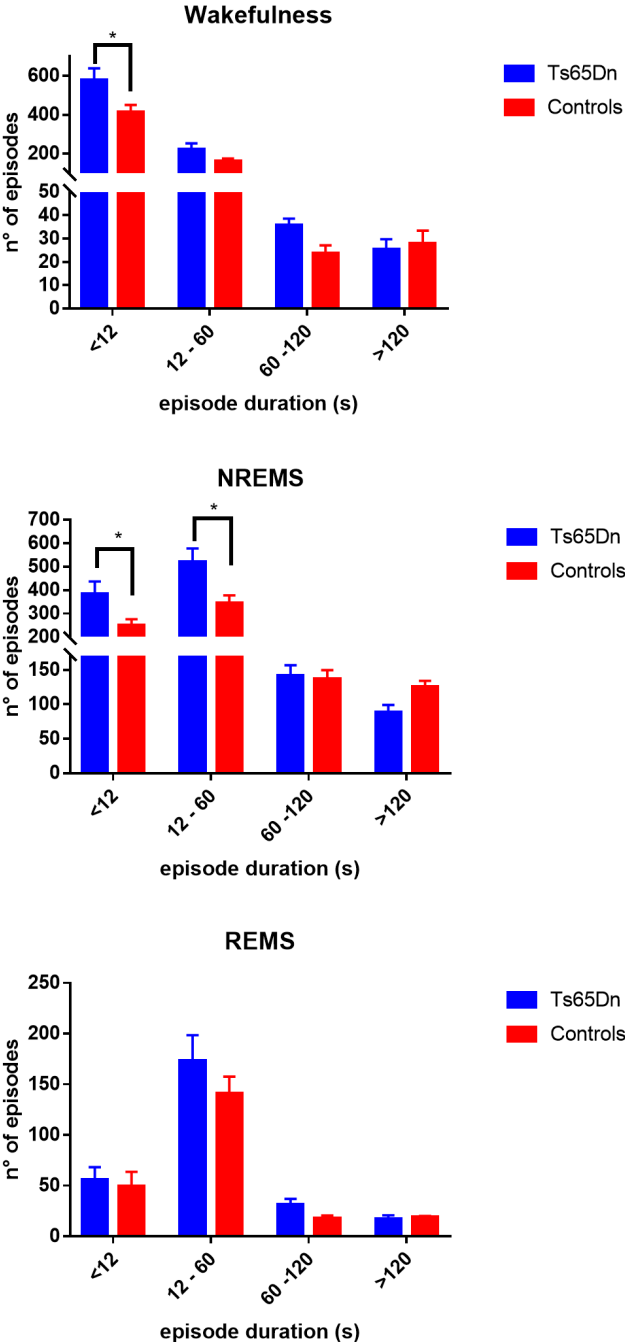
REMS = rapid-eye-movement sleep; NREMS = non rapid-eye-movement sleep; Ts65Dn = mouse model of Down syndrome. *: $p < 0.05$ Ts65Dn vs. euploid littermates (t-test with false discovery rate correction)

Concerning the global analysis of sleep apnea occurrence during NREMS and REMS (Figure 12A), no difference was found between experimental groups (two-way ANOVA interaction effect between sleep state and genotype, $p = 0.073$). However, classifying NREMS apneas according to their proximity to a preceding sigh[128], we found a significant interaction effect between mouse genotype and apnea subtype (Two-way ANOVA, $p = 0.020$). The subsequent independent t-tests indicated that Ts65Dn had significantly ($p = 0.020$) lower occurrence rate of spontaneous sleep apneas (1.25 ± 0.20 events/h of NREMS) compared to controls (2.10 ± 0.35 events/h of NREMS) while no difference was found concerning post-sigh sleep apnea occurrence (0.32 ± 0.17 and 0.28 ± 0.10 events/h of NREMS, respectively for Ts65Dn and euploid controls).

Due to signal artifacts preventing DIA evaluation, the discrimination between CSA and OSA was performed only in 9 Ts65Dn mice and 10 controls. This analysis indicated that in NREMS, same as for the C57BL/6J group, basically all the apneas were identified as CSAs (except for 1 obstructive event recorded during 1 session in a Ts65Dn mouse). In this case (Figure 12B), we found that the occurrence rate of CSA during NREMS was significantly lower in Ts65Dn mice than in controls (independent t-test, $p < 0.001$). On the contrary, during REMS (Figure 12C), the occurrence rate of all the obstructive events (OSAs and sub-OSAs) resulted significantly higher in Ts65Dn mice than controls (two-way ANOVA, interaction effect for apnea type and genotype, $p = 0.049$; subsequent independent t-test, $p = 0.011$).

Figure 12. Comparison of sleep apnea occurrence between a mouse model of Down syndrome and euploid controls.
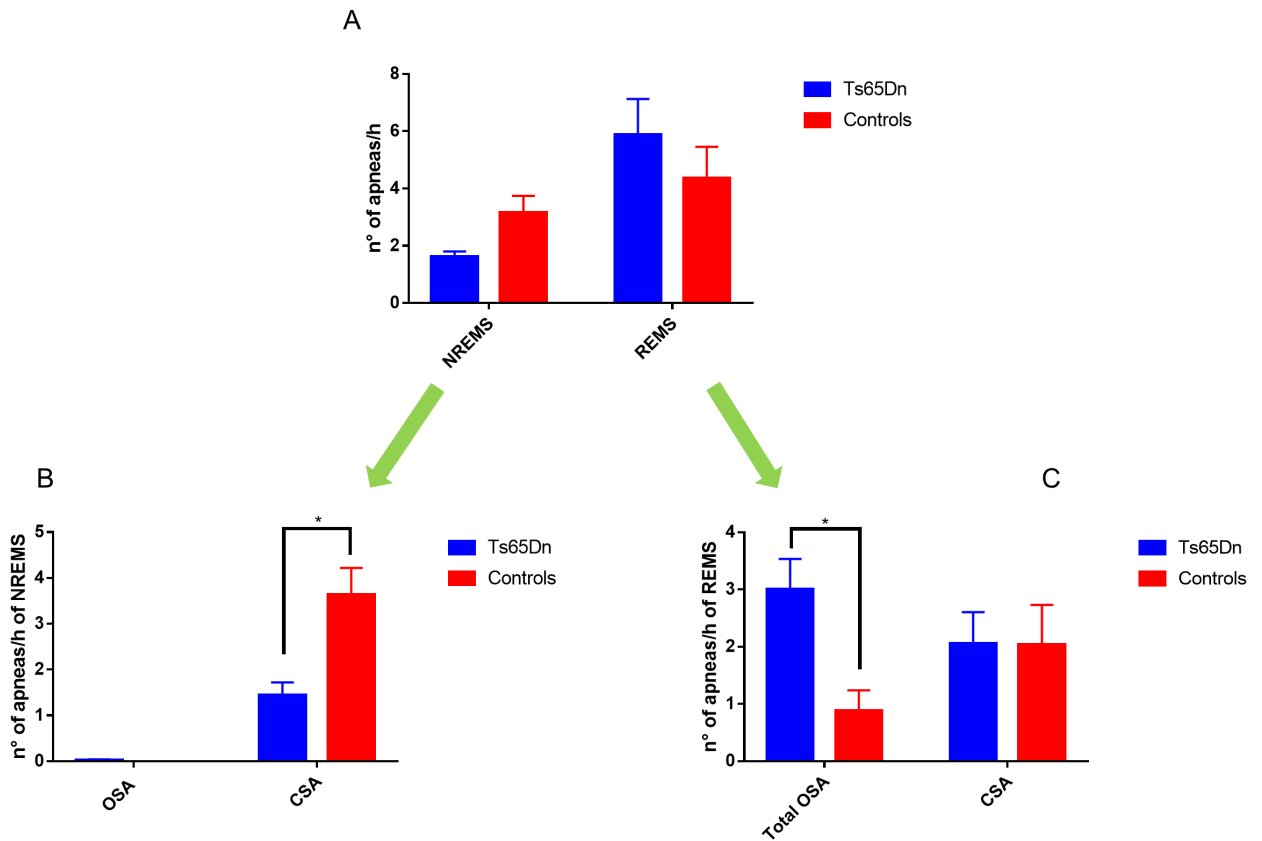

REMS = rapid-eye-movement sleep; NREMS = non rapid-eye-movement sleep; Ts65Dn = mouse model of Down syndrome. CSA = central sleep apnea; Total OSA = obstructive sleep apnea which includes either those apneic events (OSAs) characterized by diaphragmatic contraction and concomitant absence of airflow or those events (sub-OSAs) characterized by diaphragmatic contraction and concomitant reduction of at least 30% of airflow compared to baseline value. *: p < 0.05 Ts65Dn vs. euploid littermates (t-test).

The results of the quantitative analysis on the airflow parameters and on the DIA bursts in REMS for each group are reported in Table 2. No significant difference between groups in the airflow parameters was registered as far as CSA and OSA events are concerned. Similarly, the duration of DIA burst during obstructive events did not differ between groups, whereas, the amplitude of DIA activity during both OSA and sub-OSA events resulted significantly higher in Ts65Dn group than in controls ($p = 0.002$ for OSA and $p = 0.028$ for sub-OSA).

Table 2. Characterization of REMS apneas in Ts65Dn mice and euploid controls.

	CSA		OSA		SUB-OSA	
	Ts65Dn (n = 6)	Controls (n = 5)	Ts65Dn (n = 9)	Controls (n = 4)	Ts65Dn (n = 6)	Controls (n = 4)
Airflow (%)	99 ± 16	81 ± 6	118 ± 19	114 ±24	53 ± 11	52 ± 10
Diaphragm amplitude (%)	/	/	127 ± 17*	52 ± 7	90 ± 11*	45 ± 11
Diaphragmatic duration (%)	/	/	72 ± 12	53 ± 12	94 ± 14	56 ± 9

This table shows the values of airflow, amplitude and duration of the diaphragmatic activity during central sleep apneas (CSAs), obstructive sleep apneas (OSAs) and sub-obstructive sleep apneas (SUB-OSAs) recorded during rapid-eye-movement sleep (REMS) in a mouse model of Down syndrome (Ts65Dn) and their euploid controls. For CSA and OSA (both characterized by the complete absence of airflow activity during the apneic event), the airflow values refer to the post-apneic event and are expressed as % compared to the airflow of the event preceding the apnea. For SUB-OSA, the airflow values refer to the event presenting the partial (> 30% compared to baseline) obstruction and are expressed as % compared to the airflow of the event preceding the apnea. Amplitude and duration of the diaphragmatic activity refer to the bursts occurred during the obstructions (thus they could not be computed for CSA) and, again, expressed as % compared to the values of the event preceding the apnea. All the values are express as mean ± SEM. *, $p < 0.05$ Vs. controls.

DISCUSSION

In the present experiment, we developed a new method to simultaneously record the respiratory pattern and the diaphragmatic activity in sleeping mice in order to characterize and discriminate sleep apneas according to their central or obstructive origin. Using this protocol we demonstrated that: a) OSAs physiologically occur in wild-type mice almost exclusively during REMS; b) total OSAs (which include complete OSAs and sub-OSAs) during REMS resulted significantly more frequent in a mouse model (Ts65Dn) of Down syndrome compared to euploid controls.

Even though mice are largely used as models of sleep disordered breathing, no specific investigation on the presence of CSAs or OSAs in these rodents have been performed so far. Indeed, the widespread belief that all sleep apneas in mice are of central origin (CSA) is actually based on 1 investigation showing the anecdotical presence of CSAs during NREMS in few mice[129]. In the present study, we first performed an exhaustive investigation of the presence of CSAs and OSAs during either NREMS or REMS in a group of wild-type mice and then applied the same approach to evaluate whether the prevalence of OSAs is increased in a mouse model of Down syndrome, similarly to what happen in human patients[116]. In particular, for the first time, we showed that OSAs physiologically occurred in wild-type mice (Figure 10). We also realized that similar to what occurs in humans[100,130], the level of obstruction during OSAs in mice is highly variable. Consequently, we included in the counting of total OSAs both those events characterized by the complete absence of airflow with concomitant diaphragmatic contraction (i.e. complete OSAs) and those events with only a partial (>30%) reduction of the airflow (analogously to human hypopnea definition[131]). Moreover, we highlighted how obstructive events almost exclusively occurred during REMS. This remark may explain the discrepancy of our results with respect to those reported by Nakamura et al., which did not document OSAs in their pioneering study[129]. In humans, upper airway obstructions can occur in both NREMS and REMS. However, there is an

increased tendency for upper airway to collapse during REMS due to the decreased genioglossus muscle tone secondary to the cholinergic-mediated inhibition of the hypoglossal nerve[101]. The clinical significance of REMS OSA is still controversial[101,132] even if it is now well established that, compared with NREMS, REMS is associated with greater sympathetic activity and cardiovascular instability both in normal subjects[133] and in patients with OSAs[134]. Indeed, obstructive apneas and hypopneas during REMS lead to greater degrees of hypoxemia and higher levels of sympathetic activity, compared with events in NREMS[134]. Thus, REM-related events could impose greater cardiometabolic risk than events during NREMS[132]. Considering this evidence, our novel finding concerning the occurrence of OSAs during REMS might represent a valid tool for future studies aiming at elucidating the pathophysiology of this condition and its possible long-term cardiometabolic consequences.

Next, we applied the same experimental protocol to a mouse model of Down syndrome to check for the possible increased occurrence of OSAs, mimicking what happens in human patients. Despite the numerous risk factors associated with this pathology[115], in the last 40 years life expectancy of Down syndrome patients has more than doubled making critical those research studies focused at improving patient quality's life[109]. Among the most debilitating condition affecting Down syndrome patients, OSA has a prominent role being highly prevalent[116,135,136], particularly during REMS[137], and increasing the occurrence of hypoxemia, cognitive deficits, arterial hypertension and sleep fragmentation[138]. Ts65Dn is one the most diffused mouse model of Down syndrome[118] resembling many aspects of human pathology[119–121] including also sleep fragmentation[122], as we confirmed in the present study (Figure 11). However, no direct investigation on the presence of sleep apneas was earlier performed on this model. Data of the present study confirmed, for the first time, the hypothesis that Ts65Dn mice have increased incidence of OSAs during REMS (Figure 12) similar to what occurs in human patients[116,135–137]. As it also happens in humans[139], these obstructive events are characterized, in both experimental

groups, by an increased amplitude of diaphragmatic activity which is not accompanied by a ventilatory act (OSA), or which corresponds to a consistent reduction of airflow (sub-OSA) (Table 2). Unlike what happens in human patients, during NREMS Ts65Dn mice showed lower CSA occurrence rate than controls (Figure 12). However, even if CSAs occur in Down syndrome patients[136,137,140,141], this condition is usually classified as mild and has a marginal role compared to OSA occurrence[136,140–142]. Since it has been reported that CSAs correlate with age in Down syndrome patients[137], it is possible that older Ts65Dn mice exhibit relatively more CSA events than younger Ts65Dn mice and controls. This hypothesis will have to be tested in subsequent studies. CSAs in Down syndrome patients were also reported to mainly occur after sighs[137]. In contrast, our data showed that in mice, CSAs were mainly classified as spontaneous and unrelated to sigh appearance. Sighs physiologically occur in infants; thus, the correlation between CSAs and sighs have been suggested to indicate an immature control of the peripheral chemoreceptor reflex in Down syndrome patients[137]. The discrepancy between clinical and basic research data could be due to a different species-specific pathophysiology of this particular aspect. Alternatively, it could depend on the modulation exerted by mouse genetic background, as suggested by the observation that in our study also mice of control group (with a DBA/2J genetic background) exhibited a higher prevalence of spontaneous rather than post-sigh apneas. On the contrary, mice with different genetic background (129/Sv or C57BL/6J)[143] showed a completely inverted distribution.

Finally, some limitations of the present study must be acknowledged: A) due to the complexity of surgical technique for the implantation of DIA electrodes, DIA signal was satisfactory only in 19 over 26 subjects while in 7 animals the background noise overwhelmed it. B) We arbitrarily chose to define sub-OSAs those events with an airflow reduction of at least 30% compared to the baseline. We decided to use this cutoff following the human criteria for the definition of hypopnea[131]; however, it must be remarked that no consensus have been yet established for mice. C) We could not directly measure mouse airflow, rather we computed it as the ratio between TV

and the corresponding duration of DIA activity. A direct measurement of airflow would be more precisely, however, to our knowledge, this cannot be performed non-invasively in unrestrained mice[144].

CONCLUSIONS

Data of the present experiment indicate that mice physiologically exhibit sleep apneas not only of central origin, but also of obstructive origin (particularly during REMS); we also demonstrated that OSAs are more prevalent in the Ts65Dn mouse model of Down syndrome, suggesting that this model, by replicating the human condition, may be exploited to study sleep apnea in DS. Thus, we propose that mice can be used as a valid tool to accelerate the comprehension of the pathophysiology of all kind of sleep apnea (CSA and OSA) and for the development of new therapeutical approaches to contrast these respiratory disorders.

REFERENCES

1. Fuller PM, Fuller CA. Genetic evidence for a neurovestibular influence on the mammalian circadian pacemaker. *J Biol Rhythms*. 2006;21(3):177-184. doi:10.1177/0748730406288148

2. Hirshkowitz M, Whiton K, Albert SM, et al. National sleep foundation's sleep time duration recommendations: Methodology and results summary. *Sleep Heal*. 2015;1(1):40-43. doi:10.1016/j.sleh.2014.12.010

3. Lee KA. An overview of sleep and common sleep problems. *ANNA J*. 1997;24(6). https://pubmed.ncbi.nlm.nih.gov/9444102/. Accessed December 7, 2020.

4. Vyazovskiy V V., Delogu A. NREM and REM sleep: Complementary roles in recovery after wakefulness. *Neuroscientist*. 2014;20(3):203-219. doi:10.1177/1073858413518152

5. Dinges DF, Pack F, Williams K, et al. Cumulative sleepiness, mood disturbance, and psychomotor vigilance performance decrements during a week of sleep restricted to 4-5 hours per night. *Sleep*. 1997;20(4):267-277. doi:10.1093/sleep/20.4.267

6. Ferrara M, De Gennaro L. How much sleep do we need? *Sleep Med Rev*. 2001;5(2):155-179. doi:10.1053/smrv.2000.0138

7. Carskadon M, Dement W. Normal Human Sleep: an overview. *Princ Pr Sleep Med*. 2005;4:13-23.

8. Aserinsky E, Kleitman N. Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. *Science (80-)*. 1953;118(3062):273-274. doi:10.1126/science.118.3062.273

9. Dement W. The occurence of low voltage, fast, electroencephalogram patterns during behavioral sleep in the cat. *Electroencephalogr Clin Neurophysiol*. 1958;10(2):291-296. doi:10.1016/0013-4694(58)90037-3

10. JOUVET M, MICHEL F, COURJON J. On a stage of rapid cerebral electrical activity in the course of physiological sleep. *C R Seances Soc Biol Fil*. 1959;153:1024-1028. https://pubmed.ncbi.nlm.nih.gov/14408003/. Accessed December 7, 2020.

11. Boissard R, Gervasoni D, Schmidt MH, Barbagli B, Fort P, Luppi PH. The rat ponto-medullary network responsible for paradoxical sleep onset and maintenance: A combined microinjection and functional neuroanatomical study. *Eur J Neurosci*. 2002;16(10):1959-1973. doi:10.1046/j.1460-9568.2002.02257.x

12. Crochet S, Onoe H, Sakai K. A potent non-monoaminergic paradoxical sleep inhibitory system: A reverse microdialysis and single-unit recording study. *Eur J Neurosci*. 2006;24(5):1404-1412. doi:10.1111/j.1460-9568.2006.04995.x

13. Hayashi Y, Kashiwagi M, Yasuda K, et al. Cells of a common developmental origin regulate REM/non-REM sleep and wakefulness in mice. *Science (80-)*. 2015;350(6263):957-961. doi:10.1126/science.aad1023

14. Lu J, Sherman D, Devor M, Saper CB. A putative flip-flop switch for control of REM sleep. *Nature*. 2006;441(7093):589-594. doi:10.1038/nature04767

15. Sakai K, Crochet S, Onoe H. Pontine structures and mechanisms involved in the generation of paradoxical (REM) sleep. *Arch Ital Biol*. 2001;139(1-2):93-107. doi:10.4449/aib.v139i1.207

16. Saper CB, Fuller PM, Pedersen NP, Lu J, Scammell TE. Sleep State Switching. *Neuron*. 2010;68(6):1023-1042. doi:10.1016/j.neuron.2010.11.032

17. Vanni-Mercier G, Sakai K, Lin JS, Jouvet M. Mapping of cholinoceptive brainstem structures responsible for the generation of paradoxical sleep in the cat. *Arch Ital Biol*. 1989;127(3):133-164. doi:10.4449/aib.v127i3.939

18. Weber F, Chung S, Beier KT, Xu M, Luo L, Dan Y. Control of REM sleep by ventral medulla GABAergic neurons. *Nature*. 2015;526(7573):435-438. doi:10.1038/nature14979

19.   Miyazaki S, Liu CY, Hayashi Y. Sleep in vertebrate and invertebrate animals, and insights into the function and evolution of sleep. *Neurosci Res*. 2017;118:3-12. doi:10.1016/j.neures.2017.04.017

20.   Driver RJ, Lamb AL, Wyner AJ, Raizen DM. DAF-16/FOXO regulates homeostasis of essential sleep-like behavior during larval transitions in C. elegans. *Curr Biol*. 2013;23(6):501-506. doi:10.1016/j.cub.2013.02.009

21.   Rechtschaffen A, Bergmann BM. Sleep deprivation in the rat: An update of the 1989 paper. *Sleep*. 2002;25(1):18-24. doi:10.1093/sleep/25.1.18

22.   Shaw PJ, Tortoni G, Greenspan RJ, Robinson DF. Stress response genes protect against lethal effects of sleep deprivation in Drosophila. *Nature*. 2002;417(6886):287-291. doi:10.1038/417287a

23.   Chauvette S, Seigneur J, Timofeev I. Sleep Oscillations in the Thalamocortical System Induce Long-Term Neuronal Plasticity. *Neuron*. 2012;75(6):1105-1113. doi:10.1016/j.neuron.2012.08.034

24.   Marshall L, Helgadóttir H, Mölle M, Born J. Boosting slow oscillations during sleep potentiates memory. *Nature*. 2006;444(7119):610-613. doi:10.1038/nature05278

25.   Rasch B, Büchel C, Gais S, Born J. Odor cues during slow-wave sleep prompt declarative memory consolidation. *Science (80-)*. 2007;315(5817):1426-1429. doi:10.1126/science.1138581

26.   Takahashi Y, Kipnis DM, Daughaday WH. Growth hormone secretion during sleep. *J Clin Invest*. 1968;47(9):2079-2090. doi:10.1172/JCI105893

27.   Xie L, Kang H, Xu Q, et al. Sleep drives metabolite clearance from the adult brain. *Science (80-)*. 2013;342(6156):373-377. doi:10.1126/science.1241224

28.   Yang G, Lai CSW, Cichon J, Ma L, Li W, Gan WB. Sleep promotes branch-specific formation of dendritic spines after learning. *Science (80-)*. 2014;344(6188):1173-1178.

doi:10.1126/science.1249098

29.   Maret S, Faraguna U, Nelson AB, Cirelli C, Tononi G. Sleep and waking modulate spine turnover in the adolescent mouse cortex. *Nat Neurosci*. 2011;14(11):1418-1420. doi:10.1038/nn.2934

30.   Yang G, Gan WB. Sleep contributes to dendritic spine formation and elimination in the developing mouse somatosensory cortex. *Dev Neurobiol*. 2012;72(11):1391-1398. doi:10.1002/dneu.20996

31.   Bushey D, Tononi G, Cirelli C. Sleep and synaptic homeostasis: Structural evidence in Drosophila. *Science (80-)*. 2011;332(6037):1576-1581. doi:10.1126/science.1202839

32.   Donlea JM, Thimgan MS, Suzuki Y, Gottschalk L, Shaw PJ. Inducing sleep by remote control facilitates memory consolidation in Drosophila. *Science (80-)*. 2011;332(6037):1571-1576. doi:10.1126/science.1202249

33.   Miyamoto D, Hirai D, Fung CCA, et al. Top-down cortical input during NREM sleep consolidates perceptual memory. *Science (80-)*. 2016;352(6291):1315-1318. doi:10.1126/science.aaf0902

34.   Boyce R, Glasgow SD, Williams S, Adamantidis A. Sleep research: Causal evidence for the role of REM sleep theta rhythm in contextual memory consolidation. *Science (80-)*. 2016;352(6287):812-816. doi:10.1126/science.aad5252

35.   Rechtschaffen A, Bergmann BM, Everson CA, Kushida CA, Gilliland MA. Sleep deprivation in the rat: X. Integration and discussion of the findings. *Sleep*. 1989;12(1):68-87. doi:10.1093/sleep/12.1.68

36.   Hallam SJ, Jin Y. lin-14 regulates the timing of synaptic remodelling in Caenorhabditis elegans. *Nature*. 1998;395(6697):78-82. doi:10.1038/25757

37.   Hayashi Y, Hirotsu T, Iwata R, et al. A trophic role for Wnt-Ror kinase signaling during developmental pruning in Caenorhabditis elegans. *Nat Neurosci*. 2009;12(8):981-987.

doi:10.1038/nn.2347

38.   White JG, Albertson DG, Anness MAR. Connectivity changes in a class of motoneurone during the development of a nematode. *Nature*. 1978;271(5647):764-766. doi:10.1038/271764a0

39.   Chemelli RM, Willie JT, Sinton CM, et al. Narcolepsy in orexin knockout mice: Molecular genetics of sleep regulation. *Cell*. 1999;98(4):437-451. doi:10.1016/S0092-8674(00)81973-X

40.   Hassani OK, Lee MG, Jones BE. Melanin-concentrating hormone neurons discharge in a reciprocal manner to orexin neurons across the sleep-wake cycle. *Proc Natl Acad Sci U S A*. 2009;106(7):2418-2422. doi:10.1073/pnas.0811400106

41.   Jego S, Glasgow SD, Herrera CG, et al. Optogenetic identification of a rapid eye movement sleep modulatory circuit in the hypothalamus. *Nat Neurosci*. 2013;16(11):1637-1643. doi:10.1038/nn.3522

42.   Konadhode RR, Pelluru D, Blanco-Centurion C, et al. Optogenetic stimulation of MCH neurons increases sleep. *J Neurosci*. 2013;33(25):10257-10263. doi:10.1523/JNEUROSCI.1225-13.2013

43.   Modirrousta M, Mainville L, Jones BE. Orexin and MCH neurons express c-Fos differently after sleep deprivation vs. recovery and bear different adrenergic receptors. *Eur J Neurosci*. 2005;21(10):2807-2816. doi:10.1111/j.1460-9568.2005.04104.x

44.   Qu D, Ludwig DS, Gammeltoft S, et al. A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature*. 1996;380(6571):243-247. doi:10.1038/380243a0

45.   Sakurai T, Amemiya A, Ishii M, et al. Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell*. 1998;92(4):573-585. doi:10.1016/S0092-8674(00)80949-6

46. Szentirmai E, Krueger JM. Central administration of neuropeptide Y induces wakefulness in rats. *Am J Physiol - Regul Integr Comp Physiol*. 2006;291(2). doi:10.1152/ajpregu.00919.2005

47. Tsunematsu T, Ueno T, Tabuchi S, et al. Optogenetic manipulation of activity and temporally controlled cell-specific ablation reveal a role for MCH neurons in sleep/wake regulation. *J Neurosci*. 2014;34(20):6896-6909. doi:10.1523/JNEUROSCI.5344-13.2014

48. Verret L, Goutagny R, Fort P, et al. A role of melanin-concentrating hormone producing neurons in the central regulation of paradoxical sleep. *BMC Neurosci*. 2003;4. doi:10.1186/1471-2202-4-19

49. Keene AC, Duboué ER, McDonald DM, et al. Clock and cycle limit starvation-induced sleep loss in drosophila. *Curr Biol*. 2010;20(13):1209-1215. doi:10.1016/j.cub.2010.05.029

50. Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW. Control of sleep and wakefulness. *Physiol Rev*. 2012;92(3):1087-1187. doi:10.1152/physrev.00032.2011

51. Scammell TE, Arrigoni E, Lipton JO. Neural Circuitry of Wakefulness and Sleep. *Neuron*. 2017;93(4):747-765. doi:10.1016/j.neuron.2017.01.014

52. RA M, AJ B. Neural regulation of respiration. *Am Rev Respir Dis*. 1975;111(2). doi:10.1164/ARRD.1975.111.2.206

53. Horner R. Respiratory Physiology: central neural control of respiratory neurons and motoneurons during sleep. In: Kryger, MH, Roth, T, Dement W, ed. *Principles and Practice of Sleep Medicine*. St Louis (MO): Elsevier Saunders; 2011:237-249.

54. Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL. Pre-Bötzinger complex: A brainstem region that may generate respiratory rhythm in mammals. *Science (80-)*. 1991;254(5032):726-729. doi:10.1126/science.1683005

55. Feldman JL, Del Negro CA. Looking for inspiration: New perspectives on respiratory rhythm. *Nat Rev Neurosci*. 2006;7(3):232-242. doi:10.1038/nrn1871

56. Smith JC, Abdala APL, Rybak IA, Paton JFR. Structural and functional architecture of respiratory networks in the mammalian brainstem. *Philos Trans R Soc B Biol Sci*. 2009;364(1529):2577-2587. doi:10.1098/rstb.2009.0081

57. Sowho M, Amatoury J, Kirkness JP, Patil SP. Sleep and respiratory physiology in adults. *Clin Chest Med*. 2014;35(3):469-481. doi:10.1016/j.ccm.2014.06.002

58. Mitchell GS. Back to the future: Carbon dioxide chemoreceptors in the mammalian brain. *Nat Neurosci*. 2004;7(12):1288-1290. doi:10.1038/nn1204-1288

59. Orem J, Lovering AT, Dunin-Barkowski W, Vidruk EH. Tonic Activity in the Respiratory System in Wakefulness, NREM and REM Sleep. *Sleep*. 2002;25(5):488-496. doi:10.1093/sleep/25.5.488

60. White DP. Pathogenesis of obstructive and central sleep apnea. *Am J Respir Crit Care Med*. 2005;172(11):1363-1370. doi:10.1164/rccm.200412-1631SO

61. Wellmann, A White D. Central sleep apnea and periodic breathing. In: Kryger, MH Roth, T Dement W, ed. *Principles and Practice of Sleep Medicine*. St Louis (MO): Elsevier Saunders; 2011:1140-1152.

62. Douglas N. Respiratory Physiology. In: Kryger, MH Roth, T Dement W, ed. *Principles and Practice of Sleep Medicine*. St Louis (MO): Elsevier Saunders; 2011:250-258.

63. Verbraecken JA, De Backer WA. Upper airway mechanics. *Respiration*. 2009;78(2):121-133. doi:10.1159/000222508

64. Khoo MCK. Determinants of ventilatory instability and variability. In: *Respiration Physiology*. Vol 122. Respir Physiol; 2000:167-182. doi:10.1016/S0034-5687(00)00157-2

65. Kara T, Narkiewicz K, Somers VK. Chemoreflexes - Physiology and clinical implications. In: *Acta Physiologica Scandinavica*. Vol 177. Acta Physiol Scand; 2003:377-384. doi:10.1046/j.1365-201X.2003.01083.x

66. Smith CA, Nakayama H, Dempsey JA. The essential role of carotid body chemoreceptors

in sleep apnea. *Can J Physiol Pharmacol*. 2003;81(8):774-779. doi:10.1139/y03-056

67.   Donner MW, Bosnia JF, Robertson DL. Anatomy and physiology of the pharynx. *Gastrointest Radiol*. 1985;10(1):197-212. doi:10.1007/BF01893103

68.   Strohl KP, Fouke JM. Dilating forces on the upper airway of anesthetized dogs. *J Appl Physiol*. 1985;58(2):452-458. doi:10.1152/jappl.1985.58.2.452

69.   Olsen GN, Weiman DS. Assessment of Exercise Oxygen Consumption. *Ann Thorac Surg*. 1988;46(4):483-484. doi:10.1016/S0003-4975(10)64676-0

70.   Isono S, Remmers JE, Tanaka A, Sho Y, Nishino T. Static Properties of the Passive Pharynx in Sleep Apnea. *Sleep*. 1996;19(10 SUPPL.):75-177. doi:10.1093/sleep/19.suppl_10.175

71.   Isono S, Remmers JE, Tanaka A, Sho Y, Sato J, Nishino T. Anatomy of pharynx in patients with obstructive sleep apnea and in normal subjects. *J Appl Physiol*. 1997;82(4):1319-1326. doi:10.1152/jappl.1997.82.4.1319

72.   Ciscar MA, Juan G, Martínez V, et al. Magnetic resonance imaging of the pharynx in OSA patients and healthy subjects. *Eur Respir J*. 2001;17(1):79-86. doi:10.1183/09031936.01.17100790

73.   Ryan B. Pneumothorax: Assessment and diagnostic testing. *J Cardiovasc Nurs*. 2005;20(4):251-253. doi:10.1097/00005082-200507000-00009

74.   Horner RL. Motor control of the pharyngeal musculature and implications for the pathogenesis of obstructive sleep apnea. *Sleep*. 1996;19(10):827-853. doi:10.1093/sleep/19.10.827

75.   Patil SP, Schneider H, Schwartz AR, Smith PL. Adult obstructive sleep apnea: Pathophysiology and diagnosis. *Chest*. 2007;132(1):325-337. doi:10.1378/chest.07-0040

76.   Smith PL, Wise RA, Gold AR, Schwartz AR, Permutt S. Upper airway pressure-flow relationships in obstructive sleep apnea. *J Appl Physiol*. 1988;64(2):789-795. doi:10.1152/jappl.1988.64.2.789

77. Amatoury J, Kairaitis K, Wheatley JR, Bilston LE, Amis TC. Onset of airflow limitation in a collapsible tube model: Impact of surrounding pressure, longitudinal strain, and wall folding geometry. *J Appl Physiol.* 2010;109(5):1467-1475. doi:10.1152/japplphysiol.00096.2010

78. Kirkness JP, Madronio M, Stavrinou R, Wheatley JR, Amis TC. Relationship between surface tension of upper airway lining liquid and upper airway collapsibility during sleep in obstructive sleep apnea hypopnea syndrome. *J Appl Physiol.* 2003;95(5):1761-1766. doi:10.1152/japplphysiol.00488.2003

79. Boudewyns A, Punjabi N, Van de Heyning PH, et al. Abbreviated method for assessing upper airway function in obstructive sleep apnea. *Chest.* 2000;118(4):1031-1041. doi:10.1378/chest.118.4.1031

80. Penzel T, Möller M, Becker HF, Knaack L, Peter JH. Effect of sleep position and sleep stage on the collapsibility of the upper airways in patients with sleep apnea. *Sleep.* 2001;24(1):90-95. doi:10.1093/sleep/24.1.90

81. Isono S, Tanaka A, Nishino T. Lateral position decreases collapsibility of the passive pharynx in patients with obstructive sleep apnea. *Anesthesiology.* 2002;97(4):780-785. doi:10.1097/00000542-200210000-00006

82. Tagaito Y, Isono S, Tanaka A, Ishikawa T, Nishino T. Sitting posture decreases collapsibility of the passive pharynx in anesthetized paralyzed patients with obstructive sleep apnea. *Anesthesiology.* 2010;113(4):812-818. doi:10.1097/ALN.0b013e3181f1b834

83. Begle RL, Skatrud JB. Hyperinflation and expiratory muscle recruitment during NREM sleep in humans. *Respir Physiol.* 1990;82(1):47-63. doi:10.1016/0034-5687(90)90023-R

84. Stanchina ML, Malhotra A, Fogel RB, et al. The influence of lung volume on pharyngeal mechanics, collapsibility, and genioglossus muscle activation during sleep. *Sleep.* 2003;26(7):851-856. doi:10.1093/sleep/26.7.851

85.   Tagaito Y, Isono S, Remmers JE, Tanaka A, Nishino T. Lung volume and collapsibility of the passive pharynx in patients with sleep-disordered breathing. *J Appl Physiol*. 2007;103(4):1379-1385. doi:10.1152/japplphysiol.00026.2007

86.   Squier SB, Patil SP, Schneider H, Kirkness JP, Smith PL, Schwartz AR. Effect of end-expiratory lung volume on upper airway collapsibility in sleeping men and women. *J Appl Physiol*. 2010;109(4):977-985. doi:10.1152/japplphysiol.00080.2010

87.   Wheatley JR, White DP, Edelman N, Horner R, Berger A, Sullivan C. The influence of sleep on pharyngeal reflexes. In: *Sleep*. Vol 16. Associated Professional Sleep Societies,LLC; 1993. doi:10.1093/sleep/16.suppl_8.s87

88.   Mezzanotte WS, Tangel DJ, White DP. Influence of sleep onset on upper-airway muscle activity in apnea patients versus normal controls. *Am J Respir Crit Care Med*. 1996;153(6 I):1880-1887. doi:10.1164/ajrccm.153.6.8665050

89.   Fogel RB, Trinder J, White DP, et al. The effect of sleep onset on upper airway muscle activity in patients with sleep apnoea versus controls. *J Physiol*. 2005;564(2):549-562. doi:10.1113/jphysiol.2005.083659

90.   Tangel DJ, Mezzanotte WS, White DP. Influence of sleep on tensor palatini EMG and upper airway resistance in normal men. *J Appl Physiol*. 1991;70(6):2574-2581. doi:10.1152/jappl.1991.70.6.2574

91.   Wiegand L, Zwillich CW, Wiegand D, White DP. Changes in upper airway muscle activation and ventilation during phasic REM sleep in normal men. *J Appl Physiol*. 1991;71(2):488-497. doi:10.1152/jappl.1991.71.2.488

92.   O'Donnell CP, Schaub CD, Haines AS, et al. Leptin prevents respiratory depression in obesity. *Am J Respir Crit Care Med*. 1999;159(5 I):1477-1484. doi:10.1164/ajrccm.159.5.9809025

93.   Eckert DJ, Jordan AS, Merchia P, Malhotra A. Central sleep apnea: Pathophysiology and

treatment. *Chest*. 2007;131(2):595-607. doi:10.1378/chest.06.2287

94. Mezzanotte WS, Tangel DJ, White DP. Mechanisms of control of alae nasi muscle activity. *J Appl Physiol*. 1992;72(3):925-933. doi:10.1152/jappl.1992.72.3.925

95. Douglas NJ. Control of ventilation during sleep. *Clin Chest Med*. 1985;6(4):563-575. https://pubmed.ncbi.nlm.nih.gov/3853477/. Accessed December 13, 2020.

96. Henkel J, Holthaus M. Classical resonances in quantum mechanics. *Phys Rev A*. 1992;45(3):1978-1986. doi:10.1103/PhysRevA.45.1978

97. Sateia MJ. International classification of sleep disorders-third edition highlights and modifications. *Chest*. 2014;146(5):1387-1394. doi:10.1378/chest.14-0970

98. Thorpy MJ. Classification of Sleep Disorders. *Neurotherapeutics*. 2012;9(4):687-701. doi:10.1007/s13311-012-0145-6

99. Davis EM, O'Donnell CP. Rodent models of sleep apnea. *Respir Physiol Neurobiol*. 2013;188(3):355-361. doi:10.1016/j.resp.2013.05.022

100. Javaheri S, Barbe F, Campos-Rodriguez F, et al. Sleep Apnea: Types, Mechanisms, and Clinical Cardiovascular Consequences. *J Am Coll Cardiol*. 2017;69(7):841-858. doi:10.1016/j.jacc.2016.11.069

101. Alzoubaidi M, Mokhlesi B. Obstructive sleep apnea during rapid eye movement sleep: Clinical relevance and therapeutic implications. *Curr Opin Pulm Med*. 2016;22(6):545-554. doi:10.1097/MCP.0000000000000319

102. Jordan AS, McSharry DG, Malhotra A. Adult obstructive sleep apnoea. *Lancet*. 2014;383(9918):736-747. doi:10.1016/S0140-6736(13)60734-5

103. Heinzer R, Marti-Soler H, Haba-Rubio J. Prevalence of sleep apnoea syndrome in the middle to old age general population. *Lancet Respir Med*. 2016;4(2):e5-e6. doi:10.1016/S2213-2600(16)00006-0

104. Mignot E. Sleep, sleep disorders and hypocretin (orexin). *Sleep Med*. 2004;5(SUPPL. 1).

doi:10.1016/S1389-9457(04)90001-9

105. Nakamura A, Fukuda Y, Kuwaki T. Sleep apnea and effect of chemostimulation on breathing instability in mice. *J Appl Physiol*. 2003;94(2):525-532. doi:10.1152/japplphysiol.00226.2002

106. Hernandez AB, Kirkness JP, Smith PL, et al. Novel whole body plethysmography system for the continuous characterization of sleep and breathing in a mouse. *J Appl Physiol*. 2012;112(4):671-680. doi:10.1152/japplphysiol.00818.2011

107. Fleury Curado T, Pho H, Berger S, et al. Sleep-disordered breathing in C57BL/6J mice with diet-induced obesity. *Sleep*. 2018;41(8). doi:10.1093/sleep/zsy089

108. de Graaf G, Buckley F, Skotko BG. Estimates of the live births, natural losses, and elective terminations with Down syndrome in the United States. *Am J Med Genet Part A*. 2015;167(4):756-767. doi:10.1002/ajmg.a.37001

109. Presson AP, Partyka G, Jensen KM, et al. Current estimate of down syndrome population prevalence in the United States. *J Pediatr*. 2013;163(4):1163-1168. doi:10.1016/j.jpeds.2013.06.013

110. Capone GT, Chicoine B, Bulova P, et al. Co-occurring medical conditions in adults with Down syndrome: A systematic review toward the development of health care guidelines. *Am J Med Genet Part A*. 2018;176(1):116-133. doi:10.1002/ajmg.a.38512

111. Hill CM, Evans HJ, Elphick H, et al. Prevalence and predictors of obstructive sleep apnoea in young children with Down syndrome. *Sleep Med*. 2016;27-28:99-106. doi:10.1016/j.sleep.2016.10.001

112. Lal C, White DR, Joseph JE, Van Bakergem K, LaRosa A. Sleep-disordered breathing in down syndrome. *Chest*. 2015;147(2):570-579. doi:10.1378/chest.14-0266

113. Trois MS, Capone GT, Lutz JA, et al. Obstructive sleep apnea in adults with Down syndrome. *J Clin Sleep Med*. 2009;5(4):317-323. doi:10.5664/jcsm.27541

114. Chin CJ, Khami MM, Husein M. A general review of the otolaryngologic manifestations of Down Syndrome. *Int J Pediatr Otorhinolaryngol*. 2014;78(6):899-904. doi:10.1016/j.ijporl.2014.03.012

115. Capone GT, Chicoine B, Bulova P, et al. Co-occurring medical conditions in adults with Down syndrome: A systematic review toward the development of health care guidelines. *Am J Med Genet Part A*. 2018;176(1):116-133. doi:10.1002/ajmg.a.38512

116. Trois MS, Capone GT, Lutz JA, et al. Obstructive sleep apnea in adults with Down syndrome. *J Clin Sleep Med*. 2009;5(4):317-323. doi:10.5664/jcsm.27541

117. Gupta M, Dhanasekaran AR, Gardiner KJ. Mouse models of Down syndrome: gene content and consequences. *Mamm Genome*. 2016;27(11-12):538-555. doi:10.1007/s00335-016-9661-8

118. Reeves RH, Rue E, Yu J, Kao F Ten. STCH maps to mouse chromosome 16, extending the conserved synteny with human chromosome 21. *Genomics*. 1998;49(1):156-157. doi:10.1006/geno.1998.5215

119. Fructuoso M, Rachdi L, Philippe E, et al. Increased levels of inflammatory plasma markers and obesity risk in a mouse model of Down syndrome. *Free Radic Biol Med*. 2018;114:122-130. doi:10.1016/j.freeradbiomed.2017.09.021

120. Richtsmeier JT, Baxter LL, Reeves RH. Parallels of craniofacial maldevelopment in Down syndrome and Ts65Dn mice. *Dev Dyn*. 2000;217(2):137-145. doi:10.1002/(SICI)1097-0177(200002)217:2<137::AID-DVDY1>3.0.CO;2-N

121. Das D, Medina B, Baktir MA, et al. Increased incidence of intermittent hypoxemia in the Ts65Dn mouse model of Down syndrome. *Neurosci Lett*. 2015;604:91-96. doi:10.1016/j.neulet.2015.07.040

122. Colas D, Valletta JS, Takimoto-Kimura R, et al. Sleep and EEG features in genetic models of Down syndrome. *Neurobiol Dis*. 2008;30(1):1-7. doi:10.1016/j.nbd.2007.07.014

123. Bastianini S, Berteotti C, Gabrielli A, et al. SCOPRISM: A new algorithm for automatic sleep scoring in mice. *J Neurosci Methods*. 2014;235:277-284. doi:10.1016/j.jneumeth.2014.07.018

124. Bastianini S, Berteotti C, Gabrielli A, et al. SCOPRISM: A new algorithm for automatic sleep scoring in mice. *J Neurosci Methods*. 2014;235:277-284. doi:10.1016/j.jneumeth.2014.07.018

125. Silvani A, Berteotti C, Bastianini S, et al. Cardiorespiratory anomalies in mice lacking CB1 cannabinoid receptors. *PLoS One*. 2014;9(6). doi:10.1371/journal.pone.0100536

126. Bastianini S, Alvente S, Berteotti C, et al. Accurate discrimination of the wake-sleep states of mice using non-invasive whole-body plethysmography. *Sci Rep*. 2017;7. doi:10.1038/srep41698

127. Bastianini S, Alvente S, Berteotti C, et al. Post-sigh sleep apneas in mice: Systematic review and data-driven definition. *J Sleep Res*. 2019;28(6). doi:10.1111/jsr.12845

128. Bastianini S, Alvente S, Berteotti C, et al. Post-sigh sleep apneas in mice: Systematic review and data-driven definition. *J Sleep Res*. 2019;28(6). doi:10.1111/jsr.12845

129. Nakamura A, Fukuda Y, Kuwaki T. Sleep apnea and effect of chemostimulation on breathing instability in mice. *J Appl Physiol*. 2003;94(2):525-532. doi:10.1152/japplphysiol.00226.2002

130. Anttalainen U, Tenhunen M, Rimpilä V, et al. Prolonged partial upper airway obstruction during sleep – an underdiagnosed phenotype of sleep-disordered breathing. *Eur Clin Respir J*. 2016;3(1):31806. doi:10.3402/ecrj.v3.31806

131. Budhiraja R, Javaheri S, Parthasarathy S, Berry RB, Quan SF. The association between obstructive sleep apnea characterized by a minimum 3 percent oxygen desaturation or arousal hypopnea definition and hypertension. *J Clin Sleep Med*. 2019;15(9):1261-1270. doi:10.5664/jcsm.7916

132. Mokhlesi B, Punjabi NM. "REM-related" Obstructive Sleep Apnea: An Epiphenomenon or a Clinically Important Entity? *Sleep*. 2012;35(1):5-7. doi:10.5665/sleep.1570

133. Silvani A, Dampney RAL. Central control of cardiovascular function during sleep. *Am J Physiol - Hear Circ Physiol*. 2013;305(12). doi:10.1152/ajpheart.00554.2013

134. Findley LJ, Wilhoit SC, Suratt RM. Apnea duration and hypoxemia during REM sleep in patients with obstructive sleep apnea. *Chest*. 1985;87(4):432-436. doi:10.1378/chest.87.4.432

135. Hill CM, Evans HJ, Elphick H, et al. Prevalence and predictors of obstructive sleep apnoea in young children with Down syndrome. *Sleep Med*. 2016;27-28:99-106. doi:10.1016/j.sleep.2016.10.001

136. Lal C, White DR, Joseph JE, Van Bakergem K, LaRosa A. Sleep-disordered breathing in down syndrome. *Chest*. 2015;147(2):570-579. doi:10.1378/chest.14-0266

137. Ferri R, Curzi-Dascalova L, Del Gracco S, Elia M, Musumeci SA, Stefanini MC. Respiratory patterns during sleep in Down's syndrome: Importance of central apnoeas. *J Sleep Res*. 1997;6(2):134-141. doi:10.1046/j.1365-2869.1997.00030.x

138. Ridore S, Debbarma S, Nazir R, Bennett DS, Sedky K. Obstructive Sleep Apnea in Individuals with Down Syndrome: A Meta-Analytic Literature Review. *J Sleep Sleep Disord Res*. 2017;1(2):1-15. doi:10.14302/issn.2574-4518.jsdr-17-1754

139. Luo YM, Wu HD, Tang J, et al. Neural respiratory drive during apnoeic events in obstructive sleep apnoea. *Eur Respir J*. 2008;31(3):650-657. doi:10.1183/09031936.00049907

140. Fan Z, Ahn M, Roth H, Li L, Vaughn B. Sleep Apnea and Hypoventilation in Patients with Down Syndrome: Analysis of 144 Polysomnogram Studies. *Children*. 2017;4(7):55. doi:10.3390/children4070055

141. Thottam PJ, Choi S, Simons JP, Kitsko DJ. Effect of Adenotonsillectomy on Central and

Obstructive Sleep Apnea in Children with Down Syndrome. *Otolaryngol - Head Neck Surg (United States)*. 2015;153(4):644-648. doi:10.1177/0194599815587877

142. Simpson R, Oyekan AA, Ehsan Z, Ingram DG. Obstructive sleep apnea in patients with down syndrome: Current perspectives. *Nat Sci Sleep*. 2018;10:287-293. doi:10.2147/NSS.S154723

143. Lo Martire V, Alvente S, Bastianini S, et al. CDKL5 deficiency entails sleep apneas in mice. *J Sleep Res*. 2017;26(4). doi:10.1111/jsr.12512

144. Grimaud J, Venkatesh X, Murthy N. How to monitor breathing in laboratory rodents: a review of the current methods. *J Neurophysiol*. 2018;120:624-632. doi:10.1152/jn.00708.2017.-Accurately