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How to study sleep apneas in mouse models of human pathology

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ABSTRACT

Sleep apnea, the most widespread sleep-related breathing disorder (SBD), consists of recurrent episodes of breathing cessation during sleep. This condition can be classified as either central (CSA) or obstructive (OSA) sleep apnea, with the latest being the most common and toxic. Due to the complexity of living organisms, animal models and, particularly, mice still represent an essential tool for the study of SBD. In the present review we first discuss the methodological pros and cons in the use of whole-body plethysmography to coupling respiratory and sleep measurements and to characterize CSA and OSA in mice; then, we draw an updated and objective picture of the methods used so far in the study of sleep apnea in mice. Most of the studies present in the literature used intermittent hypoxia to mimic OSA in mice and to investigate consequent pathological correlates. On the contrary, few studies using genetic manipulation or high-fat diets investigated the pathogenesis or potential treatments of sleep apnea. To date, mice lacking orexins, hemeoxygenase-2, monoamine oxidase A, Phox2b or Cdkl5 can be considered validated mouse models of sleep apnea. Moreover, genetically- or diet-induced obese mice, and mice recapitulating Down syndrome were proposed as OSA models. In conclusion, our review shows that despite the growing interest in the field and the need of new therapeutical approaches, technical complexity and inter-study variability strongly limit the availability of validated mouse of sleep apnea, which are essential in biomedical research.

1. Introduction on sleep apnea

According to the 3rd edition of the International Classification of Sleep Disorders (ICSD-3) (American Academy of Sleep Medicine, 2014), sleep derangements can be divided into seven major categories: insomnia disorders, sleep-related breathing disorders (SBD), central disorders of hypersomnolence, circadian rhythm sleep-wake disorders, sleep-related movement disorders, parasomnias, and other sleep disorders. SBD refer to impaired respiration during sleep and include sleep apneas of different etiology. Among adults (30–70 years of age) in the USA, approximately 13% of men and 6% of women have moderate to severe SBD (Peppard et al., 2013). These estimates are likely to increase given the escalation of obesity and obesity-related conditions in industrialized countries (Afshin et al., 2017). Localized fat deposition promotes the occlusion of the upper airways reducing lung volume and increasing the inspiratory effort, thus, favoring SBD such as sleep apnea (Javaheri et al., 2017; Peppard et al., 2013).

1.1. Classification of sleep apnea

Sleep apnea, the major SBD, is a dangerous disorder in which breathing periodically stops for a period sufficient to disrupt sleep and frequently causes hemoglobin desaturation and hypercapnia (increased CO_2 levels). According to its characteristics, sleep apnea can be classified as either central (CSA) or obstructive sleep apnea (OSA) (Davis and O'Donnell, 2013; Sateia, 2014).

1.1.1. Central Sleep Apnea (CSA)

CSA syndromes include disorders characterized by a diminished or absent respiratory effort in an intermittent or cyclical fashion consequent to central nervous system dysfunctions. CSA occurs when there is a transient reduction in the activity of the brainstem network that generates respiratory rhythm and drives breathing, usually reflecting a reduction in the partial pressure of CO_2 below the level that is required to maintain rhythmic network activity (the apneic threshold) (Javaheri et al., 2017). Other forms (Table 1) of CSA are often associated with

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Table 1
Different forms of central sleep apneas.

CENTRAL SLEEP APNEA SYNDROMES	
Primary central sleep apnea	Idiopathic, characterized by periodic breathing stops during sleep (no respiratory effort).
Primary central sleep apnea of infancy	Central sleep apnea events in non-premature children.
Primary central sleep apnea of prematurity	Central sleep apnea events in premature children.
Central sleep apnea with Cheyne-Stokes breathing (CSA-CSB)	Repeated central sleep apneas or hypopneas alternating with a ventilatory periods with a crescendo-decrescendo pattern of flow. Respiratory pattern is characterized by cycles longer than 40 s
Central sleep apnea due to a medical disorder without Cheyne-Stokes breathing	Central sleep apnea attributable to a medical or neurological condition, without CSB pattern. Often accompanied by brainstem lesions of different origin (e.g., developmental, neoplastic or degenerative).
Central sleep apnea due to high altitude periodic breathing	Characterized by alternating periods of central sleep apnea and hyperpnea associated with recent ascent to high altitude. Respiratory pattern is characterized by cycles shorter than 40 s, often between 12 and 20 s
Central sleep apnea due to a medication or substance	Typical of users of potent long-acting opioids (e.g., methadone).
Treatment-emergent central sleep apnea	Characterized by predominantly obstructive events during a polysomnography with persistence or emergence of central sleep apnea during the administration of positive airway pressure without a backup respiratory rate, although significant resolution of obstructive events.

This table shows the different types of central sleep apneas with their definition. The table has been modified from the International Classification of Sleep Disorders-Third Edition Highlights and Modifications (Sateia, 2014)

underlying pathologies or environmental causes (Thorpy, 2017), such as Cheyne-Stokes breathing pattern or high-altitude periodic breathing (Thorpy, 2012). CSA is uncommon in the general population (Donovan and Kapur, 2016), but it is frequent in patients with heart failure, stroke, neurodegenerative disorders, and patients receiving opioids (Wang et al., 2021).

1.1.2. Obstructive Sleep Apnea (OSA)

OSA syndromes encompass events characterized by a complete or partial obstruction of the airways resulting in increased inspiratory effort and impaired ventilation (Davis and O'Donnell, 2013; Thorpy, 2012). Although this pathology is strongly associated with obesity, between 20% and 40% of OSA patients are not obese. This data suggests that other factors (such as muscle hypotonia or altered chemoreflex sensitivity) are important in the pathophysiology of this condition, possibly identifying diverse OSA phenotypes to be differentially treated (Javaheri et al., 2017). Between the 6% and 17% of the general population suffers from OSA (defined as 15 or more apnea or hypopnea events per hour (AHI)) and its prevalence reaches 49% when considering elderly population (Thorpy, 2012). OSA is therefore the most common form of SBD in occidental countries and is associated with arterial hypoxia, hypercapnia and re-oxygenation, frequent arousals with sleep fragmentation (and consequent excessive daytime sleepiness). In turn, this condition frequently leads to systemic inflammation, oxidative stress, sympathetic nervous system activation and hypercortisolemia (Javaheri et al., 2017; Maiolino et al., 2020). An elevated risk of cardiovascular morbidity and death, as well as an increased risk of metabolic and neurocognitive impairment is linked to OSA condition (Alzoubaidi and Mokhlesi, 2016; Javaheri et al., 2017; Jordan et al., 2014), whereas health consequences of CSA are still debated (Maiolino et al., 2020; Wang et al., 2021).

Rapid eye movement (REM)-related OSA is a sub-type of OSA characterized by the presence of airway obstruction predominantly or exclusively during REM sleep (Mokhlesi and Punjabi, 2012). This behavioral state, which in humans represents about the 20% of the total sleep time, is characterized by the loss of tone of several skeletal muscles, such as the genioglossus, which thus facilitates the upper airways to collapse (Grace et al., 2013). REM-related OSA is estimated to constitute 14–36% of all OSA cases and to be more prevalent in females than in males (Rishi and Rishi, 2021). OSAs are reported to be greater in duration and in the magnitude of oxygen desaturation during REM sleep than during non-REM sleep (Findley et al., 1985). The literature also suggests that OSAs and hypopneas during REM sleep are more toxic than those in non-REM sleep in terms of adverse cardiometabolic and neurocognitive outcomes (Alzoubaidi and Mokhlesi, 2016; Rishi and Rishi, 2021; Varga and Mokhlesi, 2019).

1.1.3. Post-sigh and spontaneous sleep apnea in mice

For both humans and mice, sleep apnea can also be classified as post-sigh (PSA) or spontaneous, depending on the presence/absence of a preceding sigh (augmented breath). In mice, PSA occurs almost exclusively during non-REM sleep since sighs are extremely rare during REM sleep (Lo Martire et al., 2017; Nakamura et al., 2003). Moreover, PSA occurs more frequently during hypoxic conditions and less frequently during hypercapnic or hyperoxic exposure (Nakamura et al., 2003). On the other hand, spontaneous sleep apnea appears more frequently during REM sleep and its occurrence seems not to be changed by air composition (Nakamura et al., 2003). In mice lacking the progesterone receptor (Marcouiller et al., 2014), only PSA was significantly increased respect to controls, while the sole spontaneous sleep apnea was augmented in a mouse model of CDKL5 disease compared to controls (Lo Martire et al., 2017). Taken together, these data indicate that different mechanisms might be at the base of these apneic events highlighting the importance of a precise discrimination between the two apnea types. However, the categorization criteria are not standardized producing great inter-study variability and limiting the results reproducibility. For this reason, our group provided an objective analysis of PSA in mice (Bastianini et al., 2019) resulting in a data-driven definition of PSAs as events starting within 8 s from the preceding sigh. In light of the proposed definition, it must be kept in mind that studies using shorter or longer time windows in the PSAs categorization significantly underestimate or overestimate, respectively, their occurrence rate (Bastianini et al., 2019).

1.2. Animal models for the study of sleep apnea

Despite the great technical advances in tissue culture, organoids, and in silico approaches, animal models remain as essential tools in biomedical research because of the complex multilevel interactions characterizing evolved living systems. Moreover, animal models are crucial in preclinical research to confidently assess the safety and efficacy of new therapies before human trials (Baekey et al., 2009; Paterson et al., 2011). Rodents have several pros compared to other species: they are small and can be kept in small spaces; they have a short life cycle which accelerates the replicability of studies and the investigation on elderly ages; finally, they are economic. (Baekey et al., 2009; Toth and Bhargava, 2013). Furthermore, working in laboratory conditions with inbred strains dramatically reduces both genetic and environmental confounders. Historically, rats have been useful models especially in sleep (Drinkenburg et al., 2016) and breathing (Christon et al., 1996) research because their size facilitated the electrophysiological and pharmacological study of specific muscles or brain areas (Drinkenburg et al., 2016). Mice, as well, importantly helped the understanding of many basal aspects of physiology (Baekey et al., 2009; Davis and O'Donnell, 2013; Paterson et al., 2011; Toth and Bhargava, 2013). Since its DNA was completely sequenced in 2002 (Waterston et al., 2002), the

mouse has become the species of choice in biomedical research. Indeed, many genetically modified mouse strains have been developed and used to further expedite the knowledge of several pathologies. In particular, the development of genetically-modified mice reproducing specific sleep and sleep-related disorders accelerated the understanding of these diseases (Bartolucci et al., 2021; Lo Martire et al., 2017; Silvani et al., 2014) and of their comorbidities (Toth and Bhargava, 2013), such as cardiovascular (Dematteis et al., 2009) and metabolic (Jun and Polotsky, 2009) consequences.

Whole-body plethysmography (WBP) is a non-invasive and precise technique to investigate the breathing phenotype in humans and animal models. This method greatly contributed to define the neuronal network controlling the respiratory rhythm (Crone et al., 2012) and to examine several SBD (Lo Martire et al., 2017) or to examine the role of inflammation in breathing control (Giannakopoulou et al., 2019).

The present work has several aims: first, to discuss the methodological approaches for the study of respiratory activity in mice; in particular, we will discuss pros and cons in the use of WBP; second, to explore the possibility of coupling respiratory and sleep measurements; and third, to draw an updated and objective picture of the methods used so far in the study of sleep apnea. Finally, we will discuss the potential and future developments of this research approach.

2. Methods for studying sleep apnea in animal models

For the objective study of sleep apnea, it is necessary to associate a punctual measurement of respiratory activity with the concomitant survey of the animal's behavioral state (wake, non-REM sleep, REM sleep).

2.1. Detection of respiratory activity: whole-body plethysmography

A WBP apparatus consists of two chambers: the first chamber is used as reference, while the second is used to accommodate the mouse. In this latter chamber, the animal is free to spontaneously behave, and its inspirations and expirations alter the ambient pressure compared to the constant pressure recorded in the reference chamber. Pressure modifications in the mouse chamber are related to two consequences of the cyclical breathing rhythm: a) the rib cage, alternatively reducing and increasing its volume, impacts on gas compression inside the mouse chamber; b) inflow and outflow from the mouse respiratory tree influence the air humidity and temperature, thus changing the internal chamber pressure (Drorbaugh and Fenn, 1955). In other words, during inspiration, the chamber air (with a specific temperature and humidity) is drawn into the lungs, saturated with water vapor, and warmed up. These physical modifications increase the air volume and, in turn, the chamber pressure (Mortola and Frappell, 1998). Consequently, the magnitude of each pressure deflection is related to air tidal volume (TV, amount of inspired/expired air) and to physical gas modifications occurring during each breath (Stephenson and Gucciardi, 2002). Applying waveform analysis is then possible to get information on respiration rate, TV, minute volume and so on (Lim et al., 2014).

2.1.1. Main limitations in WBP application

WBP allows the indirect characterization of breathing pattern of mice individually placed inside a sealed non-compliant chamber. As stated above, mouse chamber pressure deflections can be used to derive several breathing variables, however, these perturbations are of small amplitude and easy to corrupt (Stephenson and Gucciardi, 2002). Consequently, measurements of ventilation inside WBP chambers must be restricted to periods of sleep or minimal activity. In order to get proper information on TV, chamber pressure data must be recorded together with barometric pressure and chamber temperature and humidity (Drorbaugh and Fenn, 1955). The use of a calibration procedure at the end of each recording session avoids knowing the volumes of the chamber and the characteristics of the animal respiratory system. This calibration essentially consists in injecting and withdrawing known volumes of air into and from the chamber while recording the relative pressure deflections (Bastianini et al., 2017; Chapin, 1954).

2.1.2. Main advantages in WBP application

WBP has several advantages over other techniques investigating respiratory function and it is now considered the gold standard in this field. Indeed, WBP is non-invasive and it provides precise, reliable and replicable information on the breathing pattern of animal models. Moreover, WBP can be easily repeated on the same subject favoring longitudinal studies to investigate pathology progression or to test new therapies (Gennaccaro et al., 2021). Finally, it is not necessary to use anesthesia or restraining to insert a mouse inside the WBP chamber reducing animal's stress and interstudy variability (Lim et al., 2014).

2.2. Detection of wake-sleep state

Sleep studies require punctual detection of animal's behavior which, classically, is performed by the simultaneous recordings of the electroencephalogram (EEG) and the electromyogram (EMG) of neck muscles according to criteria derived from standard human classification of sleep stages (Allan Hobson, 1969; Iber et al., 2007). This technique represents the gold standard for sleep analysis but, notwithstanding its extraordinary accuracy, during the past decades some cheaper, less invasive, and possibly exploitable on large-scale methods have been developed. One of the easiest alternatives is the video monitoring of rodent's behavior inside the cage (Fisher et al., 2012) which, however, can discriminate only between wakefulness and sleep, and not between different sleep stages. Afterwards, other more sophisticated techniques have been developed. This is the case of the sensors, produced by Flores and colleagues (Flores et al., 2007) and inspired to Thoman and Tynan's studies in infants (Thoman and Tynan, 1979), that monitor the animal's movements and breathing. These tools consist of a piezoelectric material which transforms the mechanical solicitations produced by the animal's body movement into a variable differential electric potential. This, in turn, creates perturbations in the recorded electrical field. Although validated in both mice (Donohue et al., 2008; Mang et al., 2014) and rats (Topchiy et al., 2022; Vanneau et al., 2021), even this method allows the sole discrimination between wakefulness and sleep. Later, to overpass this limitation, our group proposed and validated a new technique of sleep scoring based on the different mouse breathing patterns associated to wakefulness, non-REM sleep, and REM sleep (Bastianini et al., 2017). Finally, it is worthy to mention that it has been recently developed a classification of sleep stages based on pupil dynamics in mice (Kobayashi et al., 2023), which seems to be promising, albeit used only in head-restrained conditions.

2.2.1. EEG and EMG recording

EEG/EMG recordings require the surgical implantation of specific electrodes to get information on the electrical activity of neurons and muscles. Consequently, standard EEG/EMG recordings in mice can be performed only by expert investigators with specific surgical skills and able to manage sophisticated algorithms for sleep scoring and analysis (Bastianini et al., 2014). The need of surgical implantations inevitably carries the risk of cerebral tissue damages and/or post-operative complications which may represent an unexpected or confounding variable. Therefore, taken together, all these factors might limit its use in large-scale studies.

The recording of electrical bio-signals in freely behaving rodents needs specific equipment based on tethered or telemetry systems. On one hand, tethered-based systems consist in a lightweight cable connected to 360°-rotating connectors and balanced suspension structures that prevent transmission wire's twisting during the recordings. These devices allow long continuous recordings (for days or even weeks) albeit they inevitably limit the animal's natural behavior (Bastianini et al., 2014). On the other hand, telemetry systems involve small

biocompatible transmitters, which send wireless signals to a receiver usually located under the animal cage. Notwithstanding the lack of a physical hindrance, these systems must be implanted subcutaneously, and, because of their dimensions and weight, they are usually applied in bigger rodents. Battery life is another critical point for this device which limits long-lasting recordings (Aulehner et al., 2022). However, in recent years, the advancement of technological instrumentations paved the way for the use of telemetric systems with longer battery life even in small and/or juvenile laboratory animals.

2.2.2. Sleep states discrimination from breathing pattern

As stated above, one of the non-invasive sleep assessment techniques that has demonstrated high performances in terms of agreement with the classical EEG/EMG-based system, reproducibility, and detection of REM sleep, is the one relying on behavior-dependent breathing variability (Bastianini et al., 2017). Indeed, the amplitude and frequency of breathing significantly change between wakefulness and sleep stages (Friedman et al., 2004), and these modifications can be easily recorded in freely behaving mice by the means of a WBP chamber. More specifically, the discrimination of sleep states based on the analysis of the breathing pattern relies on three components of the raw WBP trace: a) the frequency, b) the amplitude and c) the baseline. During wakefulness the baseline of the raw breathing tracing results extremely irregular with individual breaths largely obscured by animal's movements; during non-REM sleep the trace becomes steady and the breathing pattern is regular in frequency and amplitude; during REM sleep the trace baseline is still stable but breathing pattern frequency and amplitude are irregular. Consequently, the transition between wakefulness and non-REM sleep is identified when the baseline becomes steady with a regular breathing rhythm. Non-REM sleep episodes may end in the sudden onset of REM sleep (scored at the first detectable sign of breathing rhythm irregularity), or in the sudden onset of wakefulness (large and irregular baseline obscuring individual breaths). In conclusion, this non-invasive technique allows the simultaneous analysis of hypnic and respiratory variables with high reproducibility and without the need of restraining (for more details, please refer to Bastianini et al., 2017). However, some limitations must be acknowledged. First, rodents are not housed in their own cages during the recordings. This inevitably influences their natural behavior and wake/sleep cycle, even if they adapt rapidly to the new environment. Second, this method allows recordings of both juvenile and adult rodents for a maximum of 24 h (Berteotti et al., 2020; Borniger et al., 2014) due to stressing conditions and waste accumulation in the chamber. This latter limitation could be overcome by performing multiple recordings of the same mouse in a restricted time period (Bartolucci et al., 2021) or by performing longitudinal studies (Alvente et al., 2022).

2.3. Sleep apneas: detection of CSA and OSA

Despite the clinical relevance, the pathophysiological study of CSA and OSA in rodents, and particularly in mice, is very limited. This gap is not only due to the technical difficulty of recording and characterizing sleep apneas in such small animals, but also to the fact that, for a long time, it was mistakenly believed that only CSAs physiologically occurred in mice (Nakamura et al., 2003). Recent studies have challenged this idea and confirmed the presence of spontaneous OSAs in wild-type mice (Bartolucci et al., 2021; Berger et al., 2019). Different approaches have been used to discriminate between CSAs and OSAs in adult mice, such as non-invasive methods to record respiratory effort (Berger et al., 2019; Fleury Curado et al., 2018a; b; Hernandez et al., 2012) and invasive methods to record respiratory muscles activity (Bartolucci et al., 2021). The use of a modified WBP chamber let Polotsky's group to find inspiratory flow limitations in the presence of increased respiratory efforts, characteristic of airway obstruction (Berger et al., 2019; Fleury Curado et al., 2018a; b; Hernandez et al., 2012). To measure the surrogate of the respiratory effort, the WBP chamber was equipped with two air bladders, with the first (sensor bladder) placed above and the second (reference bladder) placed below a rigid platform inside the WBP mouse chamber. Both bladders were inflated with air and connected to a pressure transducer. When the mouse was placed on the platform, the sensor bladder transduced the changes of the mechanical pressure produced by mouse breathing, while the reference bladder was used to remove the noising contaminating chamber pressure fluctuations (Hernandez et al., 2012). The measurement of the respiratory effort, together with sleep and other breathing parameters was successfully used to characterize sleep apneas.

As CSA is characterized by an interruption of diaphragm (DIA) muscle effort and OSA is caused by recurrent occlusions of the upper airways in the presence of respiratory efforts (Alzoubaidi and Mokhlesi, 2016; Hernandez et al., 2012), it has been raised the hypothesis that the simultaneous recording of breathing variables, sleep states and DIA activity could be used to categorize sleep apneas. On this basis, our group proposed a new experimental and analytical technique to discriminate CSA and OSA in mice placed inside the WBP chamber (Bartolucci et al., 2021). Specifically, we described how to record DIA activity by the means of a pair of stainless-steel wires inserted into the abdominal cavity to touch the lower surface of the DIA muscle. One end, closed at circle, was sutured in the eighth intercostal space to keep the electrodes in contact with the DIA, while the other end of the wires reached subcutaneously the mouse head and was fixed to the skull together with EEG/EMG electrodes for wake-sleep states detection. We finally demonstrated that the concomitant analysis of WBP, sleep states, and DIA tracings allowed to discriminate between CSA (simultaneous absence of activity in WBP and DIA signals) and OSA (activity in the DIA signal and absence of activity in the WBP signal) (Bartolucci et al., 2021). Despite the effectiveness of this technique in recognizing and discriminating between CSA and OSA, it must be acknowledged that these surgical implantations are technically challenging and can be performed only by expert investigators.

An innovative non-invasive approach combining pneumotachography and laser detection of abdominal movement was used to classify apneas in newborn mice with dysfunctional hypoglossal nuclei (Madani et al., 2021). To record breathing variables in such small animals, a 3D printed pneumotachometer was combined with a facemask, in which a bias flow (20 mL/min) was injected to prevent the accumulation of CO_2 and water. Abdominal movements were detected by using a laser profilometer pointing radially at the lateral abdominal wall. The visual inspection of pneumotachometer and the detection of the respiratory effort by laser profilometers was used to discriminate between CSA, OSA, or mixed apnea (Madani et al., 2021). However, although this technique is applicable even to newborn mice, the small size of the animal prevents precise categorization of non-REM and REM sleep.

2.4. Methodological approaches mimicking the occurrence of OSA

Hypoxia is one of the main physiological stresses elicited by OSA. This condition can be categorized as intermittent (IH) or chronic hypoxia according to the level and the duration of blood deoxygenation (fluctuating or chronically low). However, IH and chronic hypoxia definitions are not consistent in literature and, consequently, experimental protocols to produce artificial hypoxic condition differ significantly among studies (Hunyor and Cook, 2018). Particularly, IH protocols can widely vary in terms of severity, duration, and number of hypoxia/reoxygenation cycles. Moreover, different methodological approaches have been proposed to mimic the effect of OSA in mice through the exposure to IH. The most commonly used protocol is to control the composition of inspired air (modulating oxygen levels) in an airtight box (Bastianini et al., 2018). In this configuration, mice can be exposed to cycles of a hypoxic/normoxic gas from few days (Allaband et al., 2021) to several weeks (Hu et al., 2021). This approach does not require anesthesia or restraining and allows for long-lasting experiments. Alternatively, invasive approaches to intermittently obstruct the airways have been proposed: bulking agent-induced tongue enlargement

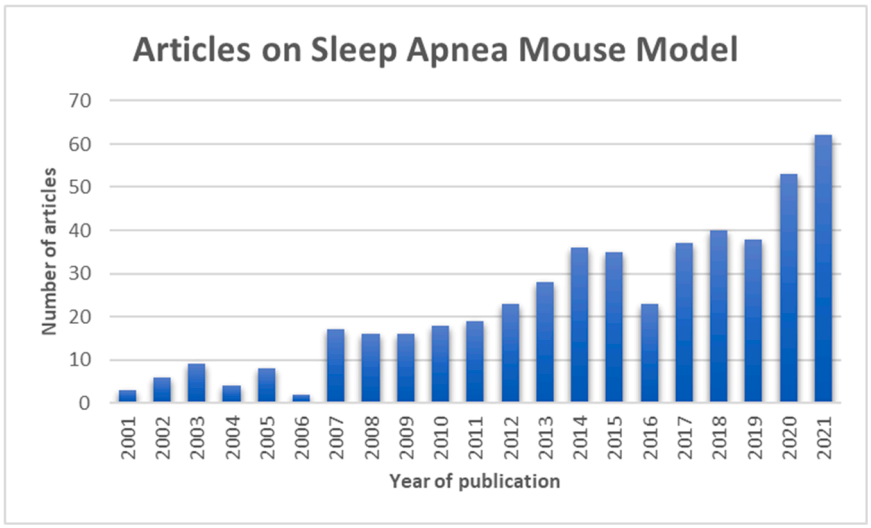

Fig. 1. Temporal trend in the number of sleep apnea studies performed on mice published from 2001 to 2021.

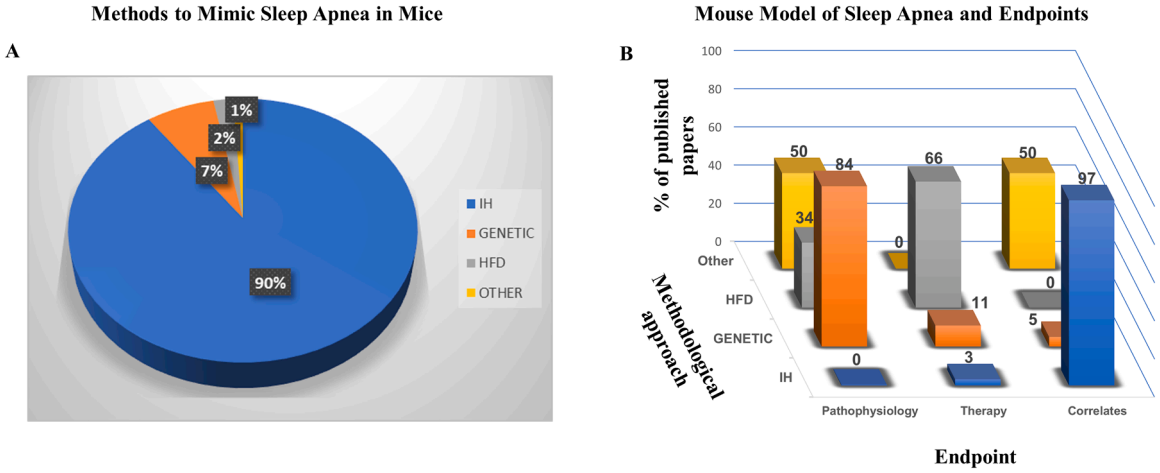

Fig. 2. Panel A shows the percentage of different methods employed to create models or to mimic sleep apnea in mice (IH: intermittent hypoxia; Genetic: genetic manipulation; HFD: high-fat diet; Other: tongue viral injections or mechanical obstruction). Panel B shows the experimental endpoints of studies conducted on the same four categories of panel A.

(Lebek et al., 2020) in mice or inflatable balloon in the trachea (Schoorlemmer et al., 2011), nasal mask (Almendros et al., 2011), and tracheostomy (Farré et al., 2003) in rats.

All these approaches mimic the brief and cyclical changes in arterial desaturation characterizing OSA patients, however, they cannot be used to investigate OSA pathogenesis, but only its pathophysiological consequences.

3. Study of sleep apnea in mice

We conducted a search in PUBMED (https://pubmed.ncbi.nlm.nih.gov/) using the keywords "Sleep apnea mouse model" and including different combinations with plural rather than singular terms. We retrieved and screened all relevant publications to review those which focused on the use of mouse models to study sleep apnea pathogenesis and/or treatment. According to our search, in the last 20 years, the growing interest in this field is confirmed by the exponential increase in the number of published papers on sleep apnea in mice (Fig. 1). Most of the studies used IH protocols and almost all of them focused on the pathological correlates (such as tumoral growth or immunological, metabolic, cardiovascular dysregulation) linked to the hypoxic conditions; on the contrary, most of the studies performed on mouse models of sleep apnea, based either on genetic manipulation or specific dietary regimen, investigated the pathogenesis or possible therapies of this condition (Fig. 2).

One of the goals of the present review was to collect updated information on mouse strains or methodological approaches to provide validated models or techniques to accelerate the knowledge on sleep apnea pathogenesis or to implement innovative treatments. Most of the studies were performed on adult male mice implanted with EEG/EMG electrodes for sleep scoring (Table 2). Exceptions are represented by Durand et al. (Durand et al., 2005) that studied newborn mice, and by Moore et al. (Moore et al., 2014) and Peng et al. (Peng et al., 2017) that also included females. Furthermore, Lo Martire et al. (Lo Martire et al., 2017) discriminated the behavioral states using the validated non-invasive method based on respiratory variability (Bastianini et al., 2017). Genetically-modified strains used to study sleep apnea include Ts65Dn mice (Down Syndrome model) (Bartolucci et al., 2021), New Zealand Obese (NZO, a model of polygenic obesity) mice (Baum et al., 2018; Kim et al., 2021), orexin knockout mice (model of narcolepsy) (Nakamura et al., 2007), mice with heterozygous mutations of the homeobox gene Phox2b (Durand et al., 2005), Cdkl5 knockout (Cdkl5-KO) mice (Lo Martire et al., 2017), mice deficient in hemeoxygenase-2 (HO-2) (Peng et al., 2017), and mice lacking monoamine oxidase A

Table 2
Characteristics of the studies performed on mouse models of sleep apnea.

First Author and Year	Mouse Strain and Genetic Background	Methods	Study Type	Main Results
Nakamura et al. (2003)	Male 129/Sv mice	6 h of EEG/nEMG and respiratory recordings into WBP chamber. Mice were exposed to air, hypoxic, hyperoxic, and hypercapnic mixture.	Sleep Apnea Pathophysiology	Sleep apneas were frequent in normal mice, and they were classified into post-sigh and spontaneous apneas. Post-sigh apneas occurred exclusively in non-REM sleep. Hypoxia and hypercapnia, respectively, increased and decreased the occurrence of the post-sigh apnea index during non-REM sleep.
Durand et al. (2005)	Newborn mice heterozygous for Phox2b mutation and with mixed genetic background C57BL/ 6 × 129 S2/SvPas	15 min of nEMG and respiratory recordings into WBP chamber.	Sleep Apnea Pathophysiology	Newborn mutant mice showed longer sleep apneas and lower minute ventilation during REM sleep than control mice.
Nakamura et al. (2007)	Male ORX-KO with mixed genetic background 129/Sv x C57BL/6	6 h of EEG/nEMG and respiratory recordings into WBP chamber. Mice were exposed to air, hypoxic, hyperoxic, and hypercapnic mixture.	Sleep Apnea Pathophysiology	ORX-KO mice showed attenuated hypercapnic ventilatory responses and more sleep apneas than controls.
Real et al. (2007)	Male Tg(H2-IFNβ)8 transgenic mice with C3H/HeOuJ background	EEG/EOG/nEMG and respiratory recordings into WBP chamber.	Sleep Apnea Pathophysiology	Genetic lack of monoamine oxidase A increased the incidence of sleep apnea.
Real et al. (2009)	Male Tg(H2-IFNβ)8 transgenic mice with C3H/HeOuJ background	EEG/EOG/nEMG and respiratory recordings into WBP chamber. Ondansetron and Fluoxetine were administered intraperitoneally before recordings.	Sleep Apnea Treatment	Ondansetron and Fluoxetine reduced sleep apneas in mice lacking monoamine oxidase A.
Moore et al. (2014)	Male and Female C57BL/6 J mice	Intra-cerebro-ventricular injection of either OREXIN-B or Receptor 2 agonist or Receptor 2 antagonist, followed by 7 h of EEG/nEMG and respiratory recording into WBP chamber.	Sleep Apnea Pathophysiology	Orexin Receptor 2 agonist significantly reduced spontaneous sleep apneas in wild-type mice.
Lo Martire et al. (2017)	Male Cdkl5-KO mice with mixed genetic background 129/Sv x C57BL/6 J	8 h of non-invasive sleep and breathing recording into WBP chamber.	Sleep Apnea Pathophysiology	Cdkl5-KO mice showed more apneas during sleep compared to controls.
Peng et al. (2017)	HO-2 deficient mice with mixed genetic background 129/Sv x C57BL/6	6 h of EEG/nEMG and respiratory recordings into WBP chamber. A subgroup of mice was implanted only with intercostal EMG electrodes and put into WBP chamber to discriminate between CSA and OSA.	Sleep Apnea Pathophysiology	HO-2 deficient mice exhibited higher incidence of sleep apneas compared to controls. These mice displayed both CSA and OSA and might serve as preclinical model of sleep apnea.
Baum et al. (2018)	Male NZO and NZB inbred mice	24 h of EEG/EOG/EMG and respiratory recording into WBP. Mice were also instrumented with a collar oximeter.	Sleep Apnea Pathophysiology	NZO mice hyperventilated, had more spontaneous apnea, and showed desaturation compared with NZB mice.
Berger et al. (2019)	Male C57BL/6 J DIO mice	6 h of EEG/nEMG and respiratory recording into WBP chamber. DIO mice were treated with a single dose of intranasal or intraperitoneal leptin or vehicle.	Sleep Apnea Treatment	In DIO mice, intranasal leptin bypassed leptin resistance, and treated upper airway obstruction during sleep and sleep-related hypoventilation.
Freire at al. (2020)	Male C57BL/6 J DIO mice	6 h of EEG/nEMG and respiratory effort recording into WBP chamber. DIO mice received leptin or vehicle intranasally and morphine or saline intraperitoneally and subcutaneously.	Sleep apnea Treatment	Leptin attenuated morphine-induced sleep disordered breathing. Intranasal leptin may prevent opioid-induced apneas, hypoventilation, and upper-airway obstruction during sleep in patients with obesity and OSA.
Bartolucci et al. (2021)	Male C57BL/6 J and Ts65Dn mice. Ts65Dn mice have mixed genetic background C57BL/ 6JEiJ x C3H/HeSnJ	8 h of EEG/nEMG and respiratory recordings into WBP chamber. Mice were also implanted with diaphragmatic electrodes to discriminate between CSA and OSA.	Sleep Apnea Pathophysiology	Mice physiologically exhibited both CSAs and OSAs. The latter appeared almost exclusively during REM sleep and were highly prevalent in Ts65Dn mice.
Kim et al. (2021)	Male NZO and NZB inbred mice	6 h of EEG/nEMG and respiratory recording into WBP chamber before and after 8 days of systemic injections with a leptin receptor antagonist.	Sleep Apnea Pathophysiology	Systemic leptin receptor blockade did not impact on sleep apnea in NZO mice.

This table shows the principal characteristics, in terms of methodological approach and results, of the studies performed on mouse models of sleep apnea. Legend of the acronyms: Cdkl5-KO = Cdkl5 knockout; CSA = central sleep apnea; DIO = diet induced obese; EEG = electroencephalogram; EMG = electromyogram; EOG = electrooculogram; HO-2 = hemeoxygenase-2 (an enzyme responsible for the generation of endogenous carbon monoxide); nEMG = nuchal electromyogram; NZB = New Zealand Control; NZO = New Zealand Obese; ORX-KO = orexin knockout; OSA = obstructive sleep apnea; Phox2b = Paired Like Homeobox 2B (key gene in central congenital hypoventilation syndrome); REM = rapid-eye-movement; Tg(H2-IFNβ)8 = transgenic mice with increased monoamine levels due to the genetic lack of monoamine oxidase A; Ts65Dn = model of Down Syndrome; WBP = whole-body plethysmography.

(Real et al., 2009; Real et al., 2007). Diet-induced obese (DIO) mice were studied by Berger et al. (Berger et al., 2019) and Freire et al. (Freire et al., 2020). Wild-type mice have been investigated in a very limited number of studies (Moore et al., 2014; Nakamura et al., 2003). Interestingly, only 4 papers reliably discriminated between CSA and OSA either implanting specific EMG electrodes (diaphragmatic or intercostal electromyographic) (Bartolucci et al., 2021; Peng et al., 2017) or evaluating non-invasively the breathing effort during apneas (Berger et al., 2019; Freire et al., 2020).

The selected studies differed from each other in several methodological aspects (Table 3) and in the composition of mouse genetic background (Table 2), making it hard to compare their results. Notably, main sources of variability were related to the use of different criteria in defining apneas (pauses lasting twice vs. three times longer than a standard breath) and to the ambient temperature during recordings (ranging from 22° to 29°C). Finally, few studies provided a measure of hemoglobin saturation (using an oximeter), and none provided a description of a possible relationship between sleep apneas and

Table 3
Methodological differences between studies.

Authors and Year	Oximeter	Apnea Threshold (respect to baseline)	Recording Length	Recording Temperature
Nakamura et al. (2003)	No	2x	6 h	22–25 °C
Durand et al. (2005)	No	2x	15 min	Undescribed
Nakamura et al. (2007)	No	2x	6 h	22–25 °C
Real et al. (2007)	No	2x	6 h	24 °C
Real et al. (2009)	No	2x	6 h	24 °C
Moore et al. (2014)	No	2x	7 h	Undescribed
Lo Martire et al. (2017)	No	3x	7 h	25 °C
Baum et al. (2018)	Yes	2x	24 h	Undescribed
Peng et al. (2017)	No	Undescribed	6 h	Undescribed
Berger et al. (2019)	Yes	Undescribed	6 h	Undescribed
Freire at al. (2020)	No	2x	6 h	24–26 °C
Bartolucci et al. (2021)	No	3x	7 h	25 °C
Kim et al. (2021)	Yes	2x	6 h	29 °C

This table shows the main methodological differences between studies performed on mouse models of sleep apnea. These differences represent important limitations in generalizing single results.

hemoglobin desaturation. Another critical point in the use of mice in sleep apnea studies concerns the choice of the strain and the use of adequate genetic controls. Indeed, since 2004, it is known that the genetic background affects some features of breathing both during non-REM and REM sleep (Friedman et al., 2004). A few years later, it was reported that the common C57BL/6 J mouse strain had strong propensity for dysrhythmic breathing, including CSA under both hypoxic and normoxic conditions while other mouse strains did not show these alterations (BALB/c, FVB and NMRI) (Stettner et al., 2008) or had a less severe phenotype (A/J) (Yamauchi et al., 2008). Spontaneous pauses were more frequent and longer in the C57BL/6 J strain even in comparison to the B6a1 strain (which has the same genetic background of the C57BL/6 J except for the chromosome 1 taken from the A/J strain) (Yamauchi et al., 2008), confirming that even small DNA differences or allelic polymorphisms are critical in defining the respiratory pattern (Gillombardo et al., 2017).

Despite these limitations/criticisms, the use of mouse models produced several advances in mechanistic knowledge and/or treatment of sleep apnea (Table 2). Specifically, studies on sleep apnea treatment showed that the occurrence of breath pauses is reduced by a) drugs modulating the serotoninergic systems and b) by leptin administration in DIO mice. On the other hand, pathogenesis studies highlighted that sleep apnea occurrence is increased: a) when orexin system is lacking (and decreased when orexin receptors are activated); b) when specific enzymes are lacking (HO-2, Phox2b, monoamine oxidase A, and Cdkl5); and c) in specific polygenic mouse strains (NZO and Ts65Dn).

4. Results discussion

In the present review we first discussed the methodological pros and cons in the use of WBP to couple respiratory and sleep measurements and to characterize CSA and OSA in mice; then, we drew an updated and objective picture of the methods used so far in the study of sleep apnea in mice. To this regard, our literature research showed several main points of interest: a) the exponential increase of studies in the field of mouse sleep apnea; b) the large use of IH protocols to investigate OSA comorbidities in mice; and c) the limited availability of validated mouse models of sleep apnea.

OSA is more prevalent than CSA in the general adult population. In fact, according to a recent analysis (Benjafield et al., 2019), it is estimated that 936 million adults live with mild to severe OSA and 425 million with moderate to severe OSA. However, both types of apneas have important socio-economic consequences besides those related to patient's health (Alzoubaidi and Mokhlesi, 2016; Varga and Mokhlesi, 2019; Wang et al., 2021). Inasmuch, sleep apnea invariably leads to sleep loss, sleep disturbances, and reduced quality of life (Léger and Stepnowsky, 2020). Sleep plays an important role in improving cognitive performance and productivity in the workplace, highlighting how the lack of sleep leads to an increase in traffic and industrial accidents, medical errors, and loss of work productivity (Hafner et al., 2017), which, in turn, can result in huge economic losses (Léger and Stepnowsky, 2020). Therefore, sleep apnea represents a topic of great interest and the comprehension of its pathogenesis and the development of possible therapies are primary goals of research that could be more easily achieved if valid mouse models were available. The widespread interest in this field is confirmed by data of Fig. 1 showing the temporal trend in the number of sleep apnea studies performed on mice. Most of these experiments were based on the application of IH protocols (Fig. 2A), which are potentially applicable to any mouse strain to artificially create an experimental model of sleep apnea to investigate its pathological correlates (Fig. 2B). However, this methodological approach does not represent a valid tool to properly investigate sleep apnea pathogenesis or treatment.

On the contrary, the development and/or validation of mouse models of sleep apnea could concretely impact on these aspects. Unfortunately, to date only a few studies focused on this task (Table 2). We believe this discrepancy might have, at least, two possible explanations: a) mice have long been considered not suitable models to study sleep apnea; b) it is technically challenging to make all the necessary measurements to properly characterize sleep apneas in such small animals. Indeed, it is crucial that rodent studies of breathing patterns are accompanied by the simultaneous classification of sleep states to maximize the translational applicability of the data. Consequently, most of the studies in this field pointed to artificially reproduce the repetitive obstructions of airways through the administration of IH and to examine the consequences on different health endpoints (Fig. 2A). Research on sleep apnea, particularly on OSA, has also been limited by the report of several differences in the anatomy of upper airways between mice and humans (Reznik, 1990). However, Polotsky's group has repeatedly shown that mouse upper airways and hypoglossal nerve functions are similar to those of humans, opening new and interesting frontiers for sleep apnea therapies (Fleury Curado et al., 2018a; b).

The present review shows that sleep apnea studies on mouse models produced important advances on the understanding of the pathogenesis and treatment of this condition. It is interesting to note that diverse genetically-modified mouse strains have been proposed as models for sleep apnea for different reasons. Orexin-KO mice could be useful in studying the central respiratory network since orexinergic neurons project to the nucleus tractus solitarius, the pre-Bötzinger complex, and the hypoglossal and phrenic nuclei (Fung et al., 2001; Krout et al., 2003; Young et al., 2005) besides being fundamental in the wake-sleep cycle regulation (Chemelli et al., 1999). The recent finding of sleep apneas in Cdkl5-KO mice provided first evidence on the involvement of this kinase in the development of the brainstem network controlling the respiratory rhythm during sleep (Lo Martire et al., 2017), however, many more studies are needed to properly clarify its role. HO-2-KO mice could be helpful in elucidating the peripheral contribution in the genesis of sleep apnea since the protein HO-2 is expressed in the carotid bodies and has a key role in initiating the chemoreflex. Phox2b-KO mice could be useful in studying the correlation between sleep apnea and the correct

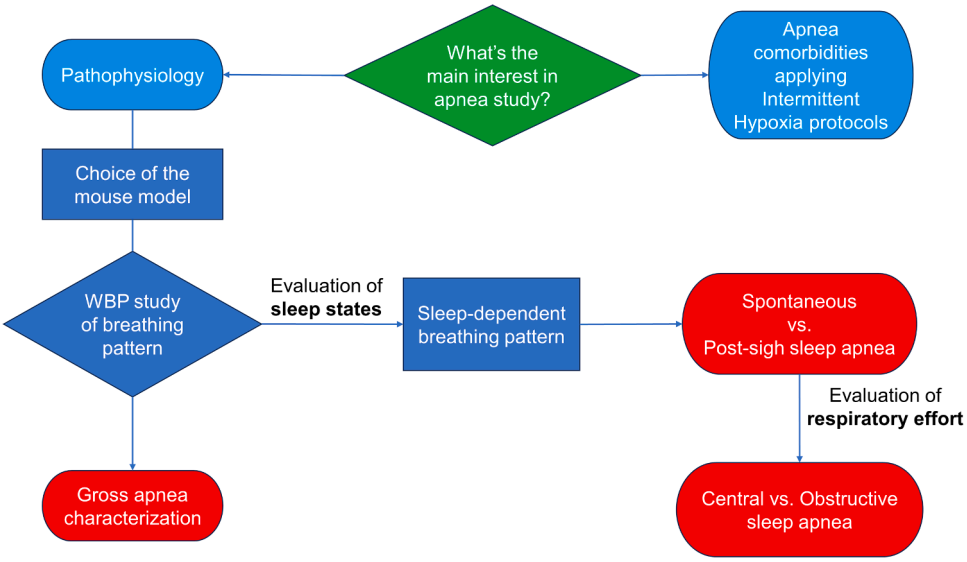

Fig. 3. Flow-chart of the best practices according to the goals of future experiments.

development of both visceral afferent projections and the central respiratory network. Indeed, Phox2b gene encodes a protein fundamental for the correct differentiation of peripheral afferent visceral pathways from the carotid body to the nucleus tractus solitarius and its alteration leads to the central congenital hypoventilation syndrome (Dauger et al., 2003). Mice deficient for the monoamine oxidase A enzyme may allow to better understand the contribution of monoamines in central sleep breathing regulation and, particularly, in OSA pathophysiology. For instance, the monoamine serotonin is known to be implicated in the control of ventilation and its antagonists have beneficial effects in some OSA patients (Kraiczi et al., 1999). Finally, NZO and Ts65Dn mice are polygenic models of OSA which could be effective in determining major genetic, neuroanatomical, and physiological traits leading to this condition (Bartolucci et al., 2021; Baum et al., 2018; Kim et al., 2021).

It is, however, evident that some methodological limitations complicate the overall interpretation of the results (Table 3). In our opinion, main limitations concern: a) the lack of data on hemoglobin desaturation during sleep apneas, b) the application of different criteria to define sleep apneas, and c) the variability in recording temperature. Collar oximeters are useful to continuously detect hemoglobin desaturation in anesthetized or awaked rodents; however, the use of such devices is limited in the studies we selected (Baum et al., 2018; Berger et al., 2019; Kim et al., 2021). This is, at least in part, due to the black coat of the most used mouse strain (C57BL/6 J) that interferes with the infrared detection system, thus limiting its reliability. Of course, it is possible to shave the application area (neck) but, in our experience, this procedure only partially solves the problem, while possibly changing animal's thermoregulation. A second point of attention concerns the variability in the sleep apnea definition, an issue we have already highlighted (Bastianini et al., 2019), due to the lack of a consensus definition for rodents. Again, it must be remarked that ambient temperature deeply affects several physiological/pathological aspects including metabolic rate, TV, ventilatory period, and sleep apnea occurrence (Berteotti et al., 2020). Consequently, it should be mandatory to carefully check the ambient temperature (and possibly body temperature as well) during plethysmographic recordings and to include this information in scientific articles. Finally, since genetic background modulates breathing pattern and sleep apnea (Gillombardo et al., 2017; Stettner et al., 2008; Yamauchi et al., 2008) attention must be paid also in choosing genetic models and the appropriate controls.

5. Conclusions and future directions

Overall, our review highlights the great progresses and interest in sleep apnea research showing that, thanks to technical and conceptual advancements, mice can be now considered valid models to accelerate the comprehension and the treatment of this condition. Despite that, the availability of validated mouse models of sleep apnea is still poor due to technical complexity and inter-study variability of this kind of research. The experiments over the next few years should produce a more complete pathophysiological characterization of these models and work to provide others. It is also necessary to check the presence of a possible gender-effect in mouse models, which has already been described in humans (Rishi and Rishi, 2021), and to reduce the methodological variability seen so far. The use of suitable animal models will make possible to test innovative therapeutic approaches. Currently, many symptomatic patients with moderate or severe OSA are effectively treated with continuous positive airway pressure (CPAP), but the adherence to therapy is low, especially in the long term (Gambino et al., 2022). Mandibular advancement devices and hypoglossal nerve stimulation are recent alternative therapies, which have yet to be consolidated. The development of validated CSA and OSA mouse models will allow to test new genetically-advanced therapeutical approaches (Fleury Curado et al., 2017). In particular, the potential of optogenetics and chemogenetics techniques able to modulate neurons in a type-, temporal-, and region-specific manner could be exploited (Doyle et al., 2021).

Finally, we summarized the information produced by the present review with a flow-chart representing the best practices according to the goals of future experiments (Fig. 3). Investigators who are mainly interested in studying apnea comorbidities in mice should opt for the application of IH protocols; on the other hand, when the aim is to study apnea pathophysiology several steps should be considered. First, researchers should choose the appropriate mouse strain (checking for the genetic background and the correct control group) to be studied with WBP. When the project purpose is beyond the gross counting of apnea, then the wake-sleep cycle must be analyzed by implanting EEG/EMG electrodes or, alternatively, by evaluating the mouse breathing pattern variability. At this point, it will be possible to reliably study the mouse sleep breathing pattern, including sleep apneas and their discrimination into spontaneous and PSA. Finally, when the goal is to specifically study CSA or OSA, investigators also need to concomitantly detect respiratory effort or respiratory muscle activity.

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CRediT authorship contribution statement

Alvente Sara: Writing – review & editing, Writing – original draft. **Matteoli Gabriele:** Writing – review & editing, Writing – original draft. **Miglioranza Elena:** Writing – review & editing. **Bastianini Stefano:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Zoccoli Giovanna:** Writing – review & editing.

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none.

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No data was used for the research described in the article.

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