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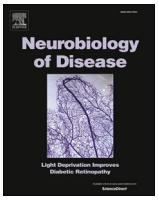

Obstructive sleep apneas naturally occur in mice during REM sleep and are highly prevalent in a mouse model of Down syndrome

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ABSTRACT

Study objectives: The use of mouse models in sleep apnea study is limited by the belief that central (CSA) but not obstructive sleep apneas (OSA) occur in rodents. We aimed to develop a protocol to investigate the presence of OSAs in wild-type mice and, then, to apply it to a validated model of Down syndrome (Ts65Dn), a human pathology characterized by a high incidence of OSAs.

Methods: In a pilot study, nine C57BL/6J wild-type mice were implanted with electrodes for electroencephalography (EEG), neck electromyography (nEMG), and diaphragmatic activity (DIA), and then placed in a whole-body-plethysmographic (WBP) chamber for 8 h during the rest (light) phase to simultaneously record sleep and breathing activity. CSA and OSA were discriminated on the basis of WBP and DIA signals recorded simultaneously. The same protocol was then applied to 12 Ts65Dn mice 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 the majority of CSAs were found during non-rapid eye movement sleep. Compared with euploid controls, Ts65Dn mice had a similar total occurrence rate of apneic events during sleep, but a significantly higher occurrence rate of OSAs during REMS, and a significantly lower occurrence rate of CSAs during NREMS.

Conclusions: Mice physiologically exhibit both CSAs and OSAs. The latter appear almost exclusively during REMS, and are highly prevalent in Ts65Dn. Mice may, thus, represent a useful model to accelerate the understanding of the pathophysiology and genetics of sleep-disordered breathing and to help the development of new therapies.

1. Introduction

Sleep apnea consists of recurrent episodes of cessation of breathing or decrease in airflow during sleep. Sleep apnea can be classified as central sleep apnea (CSA) when there is interruption of diaphragm muscle effort, or obstructive sleep apnea (OSA) when repetitive obstructions of the upper airways occur together with a continued or increased respiratory effort (Alzoubaidi and Mokhlesi, 2016; Davis and O'Donnell, 2013; Javaheri et al., 2017; Sateia, 2014; Thorpy, 2012). The prevalence of OSA in the general adult population estimated at the arbitrary cutoff of ≥15 events of apnea or hypopnea per hour ranged from 6% to 17% and reached 49% in the advanced ages (Senaratna et al., 2017). CSA is uncommon in the general population compared to OSA (Heinzer et al., 2015) but is common in specific subpopulations of patients, including those with heart failure, stroke, and during opioid administration (Maiolino et al., 2020). A high occurrence rate of OSAs is associated with increased risk of morbidity and mortality due to adverse cardiovascular events and with metabolic and neurocognitive

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impairments (Alzoubaidi and Mokhlesi, 2016; Jordan et al., 2014), whereas the adverse cardiovascular consequences of CSAs are less clear (Maiolino et al., 2020).

Despite the well-recognized clinical relevance of OSAs, genetic studies of OSAs have lagged behind those of other chronic diseases (Mukherjee et al., 2018), and better knowledge of OSA pathophysiology is needed for uncovering new treatments of both the ventilatory disorder and its multiple sequelae (Dempsey et al., 2010). Basic research on experimental animals has the potential to accelerate pathophysiological understanding owing to the relative ease of performing mechanistic studies, and mice are the mammalian species of choice for functional genomics of integrative functions such as breathing during sleep (Bogue, 2003). Recent data suggest that obese mice can show upper airway flow limitation in which airflow plateaus with increases in inspiratory effort. Obstruction is characterized by the development of inspiratory flow limitation in the presence of increased effort, whereby negative intraluminal pressure decreases the radius and, thereby, increases resistance of the collapsible oropharyngeal airways (Fleury Curado et al., 2018; Hernandez et al., 2012; White and Younes, 2012). These data raise the hypothesis that simultaneous measurements of sleep state, lung volume, and respiratory muscle activity could allow the identification of previously unrecognized OSAs also in non-obese mice, opening the way to the development of new genetic mouse models of OSAs. Consequently, the first aim of this study was to develop and validate a new technique that allows the detection of OSAs in mice based on simultaneous measurements of sleep state, lung volume, and respiratory muscle activity. This aim was pursued with experiments on wild-type mice with the C57BL/6J genetic background, which is the most widely used inbred strain, the first to have its genome sequenced, and a permissive background for maximal expression of most mutations (https://www.jax.org/strain/000664). The second aim of this study was to characterize apneic events with this new technique by applying it to the Ts65Dn, a widely-used mouse model of Down syndrome. Ts65Dn mice are characterized by a partial triplication of the chromosome 16 (Reeves et al., 1998) and exhibit various features that mimic those of Down syndrome: increased fat mass (Fructuoso et al., 2018), craniofacial dysmorphology (Richtsmeier et al., 2000), increased incidence of hypoxemia (Das et al., 2015), and sleep disturbances (Colas et al., 2008). Down syndrome, the most common human chromosomal disorder (de Graaf et al., 2015; Presson et al., 2013) is a complex condition entailing intellectual disabilities together with many other systemic dysfunctions (Capone et al., 2018; de Graaf et al., 2015), including OSA syndrome, whose prevalence is 50–100% in childhood (Hill et al., 2016; Lal et al., 2015) and nears 100% in adulthood (Trois et al., 2009). Though Ts65Dn mouse model of Down syndrome (Reeves et al., 1998) could thus represent a promising candidate as a genetic mouse model of OSAs, this feature has never been investigated in this model so far.

2. Methods

2.1. Ethical approval

The study protocol complied with the EU Directive 2010/63/EU for animal experiments and with Italian law (authorization n. 779/2017-PR 205/2019-PR). The experiments were carried out according to the guidelines of the animal welfare committee of the University of Bologna, Italy. All efforts were made to minimize animal suffering.

2.2. Mice

Experiments for the development of a new technique for the detection of OSAs in mice were performed on 9 wild-type male mice with a pure C57BL/6J genetic background (>10 generations of backcrossing). The new technique was then applied to 12 male Ts65Dn mice and 14 male euploid controls, obtained by mating B6EiC3Sn a/A-Ts(17ˆ16)65Dn females (https://www.jax.org/strain/001924) with C57BL/6JEiJ x C3H/HeSnJ (B6EiC3Sn) F1 hybrid males (https://www.jax.org/strain/001875). Founder mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Genotypes were assessed at 4 weeks of age as previously described (Reinholdt et al., 2011). All mice were housed under a 12:12-h light–dark cycle with ambient temperature set at 23 ± 1 °C and had free access to water and food (4RF21diet; Mucedola, Settimo Milanese, Italy) in the facilities of the Department of Biomedical and Neuromotor Sciences, University of Bologna, Italy.

2.3. Experimental protocol

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. Mice were also administered benzathine penicillin (3750 UI/mouse) and dihydrostreptomycin sulfate (1.5 mg/mouse) in 0.8 mL sterile saline subcutaneously to prevent infections and dehydration. The animals underwent surgery for the implantation of differential electrodes for the detection of electroencephalogram (EEG) and of neck and diaphragmatic electromyogram (nEMG and DIA). For EEG recordings, a pair of stainless-steel miniature screws (2.4 mm 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. Another pair of screws was symmetrically inserted on the other side of the skull to counterbalance the weight of the implant on the animal's head (Bastianini et al., 2014). For nEMG recordings, 2 pairs of PFA-coated stainless-steel wires were inserted in the posterior neck muscles (Bastianini et al., 2014). 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 thread was passed and then sutured to the muscles in the eighth 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 (1–2 min under isoflurane anesthesia in pure oxygen) 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. The mouse 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 with 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, representing 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 performed for 8 h 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 microsyringe (Hamilton, Reno, NV, USA) at the end 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 the 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 stored at 256 Hz, while the nEMG and DIA signals were stored at 2048 Hz. The WBP differential pressure, corresponding to the respiratory signal, was stored 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 h each) spaced by 24 h of recovery. All the variables computed during each recording session were then averaged for each mouse.

2.4. Data analysis

Offline visual scoring of wakefulness, NREMS and REMS was performed based on EEG/nEMG signals by 3 trained investigators on all consecutive 4 s epochs by means of a semi-automatic scoring program (SCOPRISM) as previously described (Bastianini et al., 2014). 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.

The values of ventilatory periods (the interval between successive breaths) and tidal volume were computed on stable artefact-free data sequences in NREMS or REMS lasting ≥12 s (i.e., at least 3 consecutive 4-s epochs) as previously described in detail (Bastianini et al., 2017; Silvani et al., 2014). Care was taken to analyze only artefact-free data sequences that were part of stable NREMS and REMS episodes, excluding periods of transition between wake-sleep states. The analysis of breathing was not performed during wakefulness due to the frequent occurrence of movement artifacts. 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 (Bastianini et al., 2019), were detected as breaths with tidal volume > 3 times the average tidal volume of each mouse in NREMS. Apneas were defined as breaths with values of ventilatory periods longer than 3 times the average values of ventilatory period of each mouse in each sleep state. The automatic identification of sighs and apneas based on these criteria was manually checked against the raw tracings for each event by an experienced investigator. Apneas were further categorized as post-sigh apneas when starting within 8 s from a sigh, or as spontaneous apneas when they started more than 8 s from the preceding sigh (Bastianini et al., 2019).

Based on the analysis of WBP and DIA during apneic events, we discriminated between CSA (concomitant absence of activity in WBP and DIA signals) and OSA (activity in the DIA signal and absence of activity in the WBP signal). The extent of airway obstruction in OSA events may vary from complete to partial, leading to the discrimination between OSA and "sub-OSA" ("sub-obstructive") events. Sub-OSA events were defined as apnea events characterized by clear-cut activity in the DIA signal concomitant with a subtle positive deflection of the respiratory WBP signal, which did not meet the criteria for automatic detection as an inspiration. In particular, the events were classified as sub-OSA if the inspiratory airflow, estimated by computing the ratio between the tidal volume and the duration of the DIA activity, showed a > 30% reduction compared with the baseline value estimated in the previous breathing event. The 30% cutoff was defined in analogy with the AASM airflow criterion for hypopnea detection in humans (Shamim-Uzzaman et al., 2018). Sub-OSAs were identified during the manual review of the raw tracings that was performed independently by two investigators for each apnea event. The identification of sub-OSAs was performed only in REMS due to the almost total absence of obstructive events during NREMS (with the single exception of 1 obstructive event recorded in a Ts65Dn mouse, see results).

For all identified apneic events (i.e., CSA, OSA, and sub-OSA events) during REMS in Ts65Dn and euploid mice, the airflow of the first breath after the breathing pause was estimated as explained above and expressed as % of the corresponding baseline value estimated during the last breath before the apneic event. For sub-OSA events, the reported airflow estimates correspond to those during the event. The purpose of this analysis was to test whether the airflow after the breathing pause differed from the baseline airflow. Moreover, DIA activity during the OSA and sub-OSA events was evaluated in terms of burst duration and amplitude, the latter expressed as the root mean square of the DIA signal, and expressed as % of the corresponding baseline values during the last breath before the event. The purpose of this analysis was to test whether the DIA activity during OSA and sub-OSA events differed in duration and amplitude from the baseline values.

As stated above, the DIA signal was obtained by applying 2 electrodes in contact with the abdominal surface of the diaphragmatic muscle and digitized stored at 2048 Hz. This configuration allowed us to properly record the DIA signal and, simultaneously, to detect the cardiac electrical activity. Particularly, as shown in Fig. 1, R waves of the QRS complexes could be clearly detectable on the background of the DIA signal. Consequently, to obtain information on the modulation of heart rhythm during the apneic events, we computed the time intervals between successive R wave peaks (RR interval, corresponding to the heart period, HP) during these events. For each apneic event, the difference (ΔHP) between the mean HP value during the apneic event and the mean HP value of the 10 heart beats before the event was eventually computed and analyzed. Values of heart rate (HR = 1/HP, expressed as beats per minute) were used instead of those of HP for graphical purposes for greater clarity.

2.5. 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 $p < 0.05$. Data were analyzed with two-way ANOVAs with sleep state (2 levels: NREMS or REMS) or wake-sleep episode duration (4 temporal bins: < 12 s, between 12 s and 60 s, between 60 s and 120 s, > 120 s) or apnea subtypes (2 levels: either post-sigh vs. spontaneous sleep apnea or CSA vs. OSA) and mouse genotype (2 levels: Ts65Dn vs. euploid controls) as factors. In case of significance of the two-way interaction, simple effects of the mouse genotype were assessed with independent-sample *t*-tests for each of the 4 temporal bin durations; inflation of the family-wise type 1 error rate was controlled with the False Discovery Rate procedure (Curran-Everett, 2000). To evaluate whether OSAs impacted on the modulation of heart rhythm in mice, the values of ΔHP during OSA events in Ts65Dn mice were analyzed with a one-sample *t*-test with reference value of 0. The values of ΔHP were not analyzed in euploid control mice because most of them did not show any OSA event.

3. Results

3.1. OSA detection in C57BL/6J mice

The C57BL/6J mice underwent surgery at 41.8 ± 0.5 weeks of age and the mean weight of the mice at surgery was 31.2 ± 1.6 g. These mice spent 20.1 ± 2.9%, 64.9 ± 2.1%, and 10.6 ± 1.6% of the recording time in wakefulness, NREMS, and REMS, respectively. The values of ventilatory period, tidal volume, and total occurrence rate of sighs and apneas during NREMS and REMS are reported as supplementary material (Table S1).

Our technique of simultaneous measurement of sleep state, DIA activity, and breathing activity in the WBP allowed us to discriminate and identify CSA, OSA, and sub-OSA events in C57BL/6J wild-type mice. Representative examples of raw tracings corresponding to CSA, OSA, and sub-OSA events are shown in Fig. 1. A detailed report of this categorization is reported in Table S2. Strikingly, we found that during

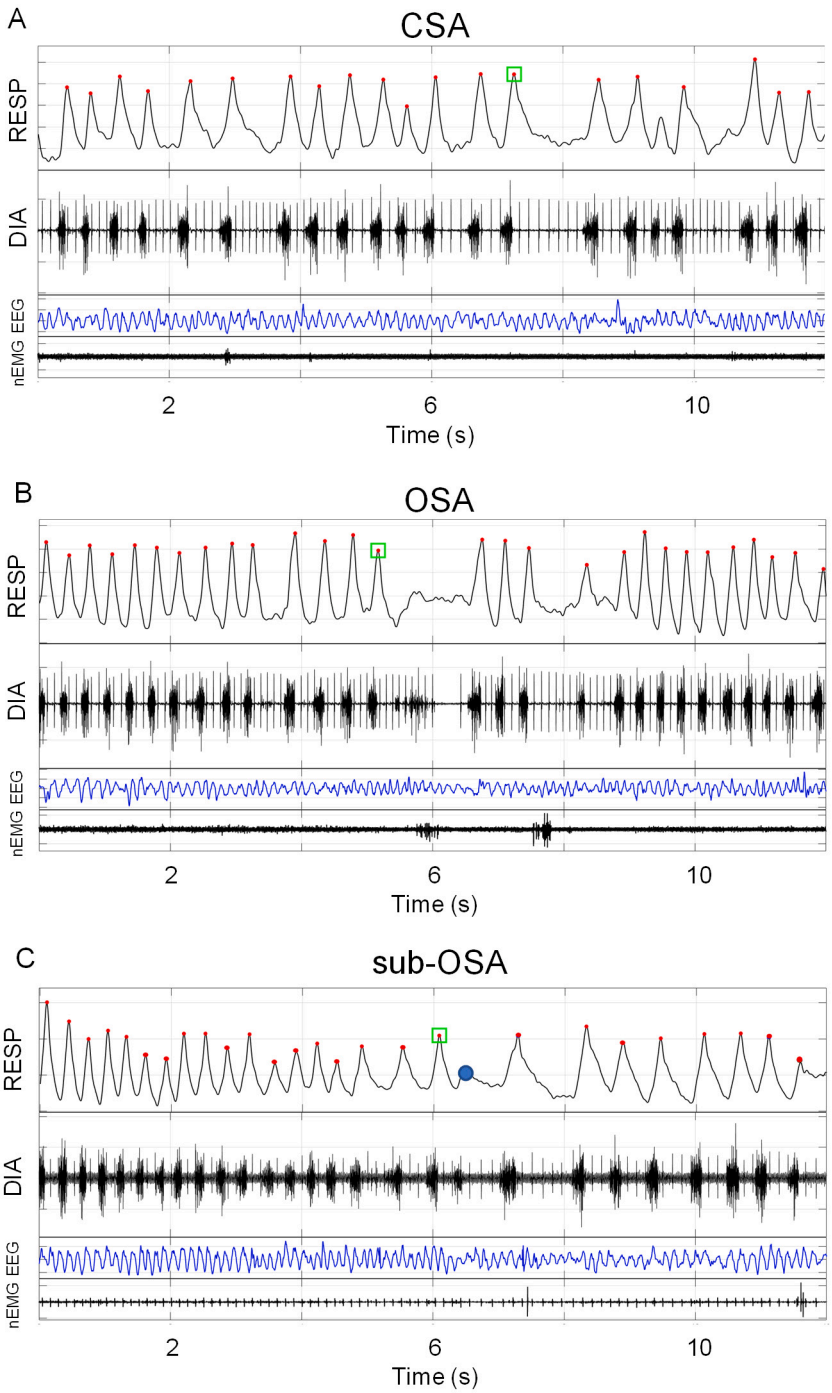

Fig. 1. Examples of raw tracings in C57BL/6J wild-type mice. REMS = rapid-eye-movement-sleep; NREMS = non-rapid-eye-movement-sleep; CSA = central sleep apnea; OSA = obstructive sleep apnea; sub-OSA = sub-obstructive sleep apnea; RESP = differential pressure recorded into the whole-body plethysmograph (which corresponds to mouse respiratory pattern); DIA = diaphragmatic electromyogram; EEG = electroencephalographic activity; nEMG = nuchal muscles electromyogram. Panels A, B, and C show raw tracings recorded during CSA, OSA, and sub-OSA, respectively. On the 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 C indicates a reduction >30% of estimated airflow compared to baseline value (computed on the last red dot before the green square) with concomitant DIA activity, consistent with a sub-OSA event classification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

REMS, almost 50% of apneas had an obstructive component, with 22.4 ± 7.7% of apneic events classified as OSAs and 20.4 ± 8.6% classified as sub-OSAs. On the other hand, apneas during NREMS were almost invariably represented by CSAs.

3.2. OSA detection in the Ts65Dn mouse model of Down syndrome

At surgery, the Ts65Dn mice had a significantly reduced body weight compared to euploid controls (25.6 ± 1.0 vs. 28.3 ± 0.6 g, respectively; *t*-test, $p = 0.043$) even though the two groups did not differ from their euploid controls in terms of age (19.5 ± 0.8 vs. 19.8 ± 0.7 weeks, respectively; t-test, $p = 0.829$).

The analysis of the time spent in each wake-sleep state did not show any significant difference between Ts65Dn mice and euploid controls (Table 1). However, the analysis of wake-sleep bout duration (Fig. 2)

Table 1
Time spent in each wake-sleep state by Ts65Dn mice and euploid control mice.

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) and rapid-eye-movement-sleep (REMS) in a mouse model of Down syndrome (Ts65Dn, $n = 12$) and their euploid controls (n = 14), which were recorded inside a whole-body plethysmograph for the first 8 h of the light period. Data are reported as mean ± SEM.

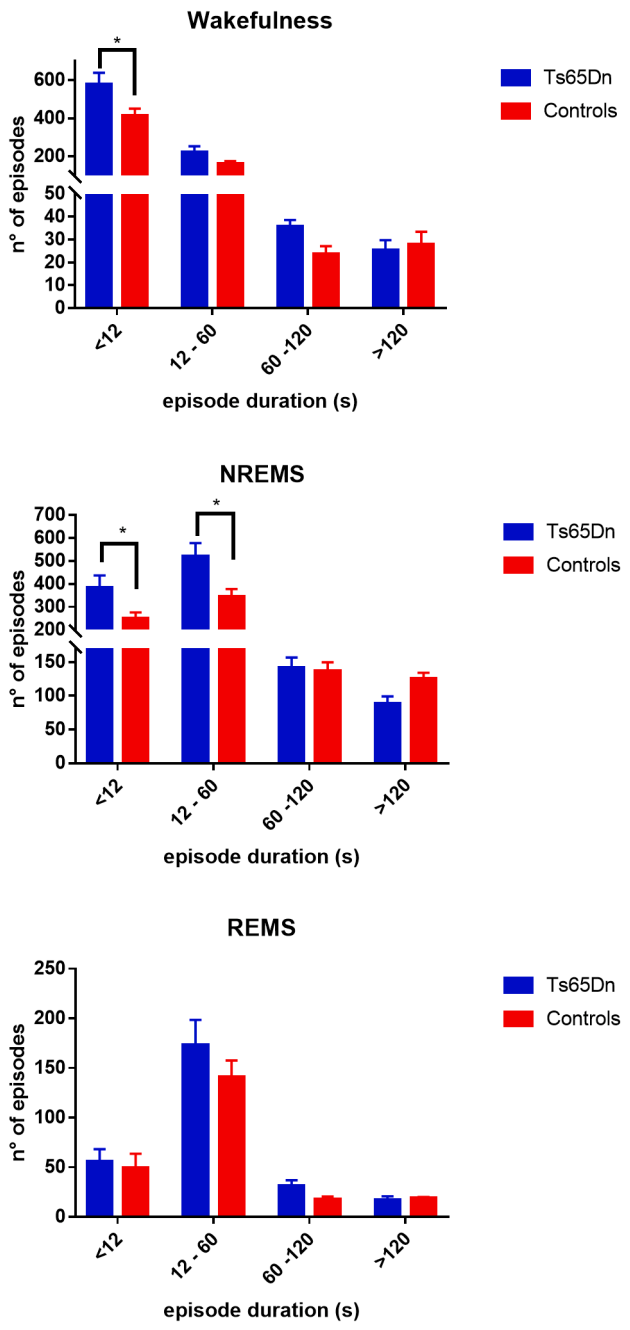

Fig. 2. Distribution of wake-sleep episodes according to their duration in a mouse model of Down syndrome and in euploid control mice.
REMS = rapid-eye-movement-sleep; NREMS = non-rapid-eye-movement-sleep; Ts65Dn = mouse model of Down syndrome. *: $p < 0.05$ Ts65Dn vs. euploid control littermates (*t*-test with false discovery rate correction).

revealed a significant interaction between mouse genotype and bout duration both for wakefulness and for NREMS, but not for REMS (two-way ANOVA, $p = 0.008$, $p = 0.001$, and $p = 0.501$, respectively). In particular, compared with euploid controls, Ts65Dn mice exhibited significantly more short bouts of wakefulness lasting <12 s and significantly more short bouts of NREMS lasting <60 s.

The values of ventilatory period, tidal volume, and total occurrence rate of sighs and apneas during NREMS and REMS in Ts65Dn mice and euploid controls are reported as supplementary material (Table S3). The occurrence rate of sighs during NREMS was not significantly higher in Ts65Dn mice than in euploid controls (interaction between sleep state and mouse genotype, two-way ANOVA, $p = 0.053$; Table S3). The analysis of total sleep apnea occurrence rate revealed no significant difference between Ts65Dn mice and euploid controls (two-way ANOVA, genotype main effect, $p = 0.997$; interaction between sleep state and mouse genotype, $p = 0.073$, Fig. 3A). However, a more refined analysis, performed classifying NREMS apneas according to their temporal proximity to a preceding sigh (Bastianini et al., 2019), revealed a significant interaction between mouse genotype and apnea subtype (two-way ANOVA, $p = 0.021$). In particular, during NREMS, Ts65Dn mice had significantly lower occurrence rate of spontaneous sleep apneas than euploid controls (1.25 ± 0.20 vs. 2.83 ± 0.35 events/h of NREMS, respectively; *t*-test, $p = 0.020$), while no significant difference was found concerning post-sigh sleep apnea occurrence rate (0.32 ± 0.17 vs. 0.25 ± 0.13 events/h of NREMS, respectively; t-test, $p = 0.735$).

Due to signal artifacts preventing DIA evaluation, the discrimination of CSA, OSA, and sub-OSA could be performed only in 9 Ts65Dn mice and 10 euploid controls. The results of this analysis indicated that, similarly to what occurred in C57BL/6J mice, all apneas during NREMS were categorized as CSAs, with the single exception of 1 OSA recorded in a Ts65Dn mouse. The occurrence rate of CSA during NREMS was significantly lower in Ts65Dn mice than in euploid controls (interaction between apnea type and genotype, two-way ANOVA, $p = 0.005$; *t*-test, $p = 0.006$; Fig. 3B). During REMS, the occurrence rate of apneic events with an obstructive component (i.e, OSAs and sub-OSAs) was significantly higher in Ts65Dn mice than in euploid controls (interaction between apnea type - with vs. without an obstructive component - and genotype, two-way ANOVA, $p = 0.049$; t-test, $p = 0.003$; Fig. 3C), while no difference was found concerning the occurrence rate of CSA (t-test, $p = 0.983$; Fig. 3C). A detailed report of sleep apnea categorization is reported in Table S4.

Fig. 4 graphically shows that the apneic (CSA, OSA and sub-OSA) events in NREMS and REMS were associated with slowing of heart rhythm in mice, as confirmed by the significant increase of HP during the apneic event compared to the 10-heart beats preceding it (one-sample t-test on ΔHP with reference value of 0, $p = 0.038$; Fig. 5).

Similarly, to what we did for C57BL/6J mice, we then separately analyzed the characteristics of OSAs and sub-OSAs events during REMS in Ts65Dn mice and euploid controls. The ratios of airflow estimate after the breathing pause in CSAs, OSAs, and subOSAs vs baseline and the ratios of DIA burst durations during the OSA and subOSA events vs baseline did not differ significantly between Ts65Dn mice and euploid controls during REM sleep (Table 2; *t*-tests, $p \geq 0.08$). Conversely, the ratios of DIA burst amplitude during both OSAs and sub-OSAs vs baseline were significantly higher in Ts65Dn than in euploid control mice (t-test, $p = 0.002$ and $p = 0.028$, respectively).

4. Discussion

We developed a new technique to simultaneously record the respiratory pattern and the diaphragmatic activity in sleeping mice in order to characterize and discriminate sleep apneas according to the occurrence of airway obstruction. Using this protocol we demonstrated that: a) apneas with a component of airway obstruction physiologically occur in C57Bl/6J wild-type mice almost exclusively during REMS; b) the extent of airway obstruction in these events may vary from complete to partial, leading to the discrimination between OSA and sub-OSA events; and c) the occurrence rate of apneas with an obstructive component, which include OSAs and sub-OSAs, during REMS is significantly increased in the Ts65Dn mouse model of Down syndrome, a disorder characterized by increased OSA occurrence rate in human subjects.

Even though mice are largely used as models of sleep disordered breathing, most research on mice has so far employed paradigms of intermittent hypoxia, which focus on the effects of OSA on oxygen saturation (Trzepizur et al., 2018). While the intermittent hypoxia paradigm has led to dramatic advances in our understanding of the pathophysiology of the consequences of OSA, it cannot inform on the pathophysiological mechanisms that lead to OSA, including genetic

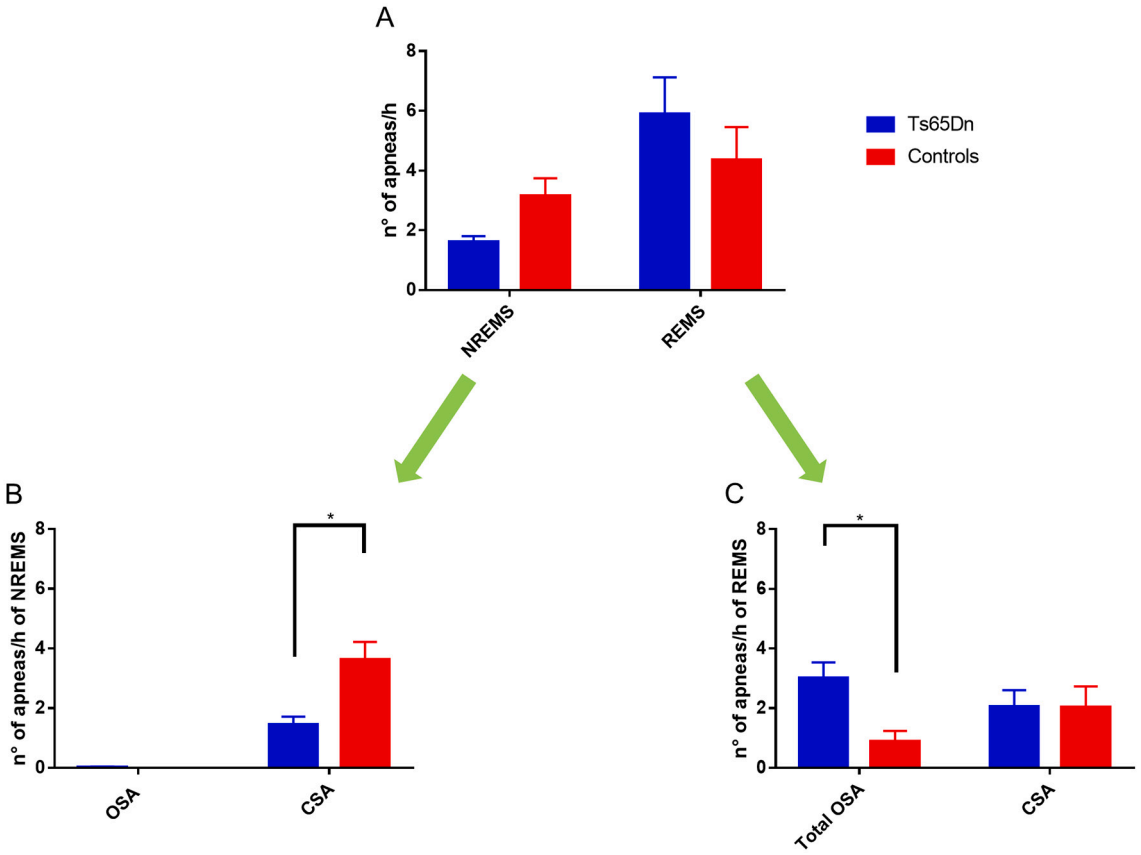

Fig. 3. Comparison of sleep apnea occurrence rate between a mouse model of Down syndrome and euploid control mice.
REMS = rapid-eye-movement-sleep; NREMS = non-rapid-eye-movement-sleep; Ts65Dn = mouse model of Down syndrome; CSA = central sleep apnea; Total OSA = obstructive sleep apnea, including both apneic events (OSAs) characterized by diaphragmatic contraction and concomitant absence of airflow and events (sub-OSAs) characterized by diaphragmatic contraction and concomitant reduction of at least 30% of estimated airflow compared to baseline value. *: $p < 0.05$ Ts65Dn vs. euploid control littermates (t-test).

predisposing factors. On the other hand, research on spontaneous apneas during sleep in mice has consistently lagged behind. The conclusions of a seminal study that reported the occurrence only of CSAs during NREMS in mice (Nakamura et al., 2003) were challenged by Polotsky's group, which highlighted inspiratory flow limitation, as assessed based on the WBP signal, as a marker of airway obstruction during sleep in mice. In particular, mouse respiratory effort was measured using a bladder placed under the mouse and equipped with a pressure transducer (Berger et al., 2019), and airway obstruction was characterized by the development of inspiratory limitation in the presence of increased effort (Berger et al., 2019). In addition, New Zealand obese (NZO/HlLtJ) mice were hypothesized to develop OSAs based on their abnormal upper airways and their tendency to sleep standing up occasionally (Brennick et al., 2011, 2009).

In the current study, by simultaneously measuring sleep state, DIA activity and lung volume, we were able to show that OSAs physiologically occur in non-obese wild-type mice of the widely studied C57Bl/6J strain and are a substantial fraction of the total sleep apneas during REMS (Table S2). We also found that similarly to what occurs in humans (Anttalainen et al., 2016; Javaheri et al., 2017), the level of obstruction during OSAs in these mice is 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, which is similar to the AASM definition of human hypopnea (Shamim-Uzzaman et al., 2018).

In humans, upper airway obstructions can occur in both NREMS and REMS, but there is an increased tendency for upper airways to collapse during REMS (Alzoubaidi and Mokhlesi, 2016). This may be due, at least in part, to decreased upper airway muscle tone (McSharry et al., 2014) caused by cholinergic muscarinic activation of G-protein-coupled inwardly rectifying potassium channels on motoneurons (Grace et al., 2013). During REMS, OSAs tend to be longer and to entail more frequent and more severe oxyhemoglobin desaturations than during NREMS (Findley et al., 1985). Severe OSA that occurs primarily during REMS is associated with higher cardiovascular risk in subjects with prevalent cardiovascular disease (Nisha Aurora et al., 2018). In humans, during OSA events there is a complex modulation of heart rhythm, with a decrease in HR (i.e., an increase in HP) in the late interapneic period, possibly reflecting the effectiveness of parasympathetic cardiac control by the chemoreceptor reflex in OSA patients during sleep (Bonsignore et al., 1997; Silvani et al., 2016). Accordingly, we found that HP significantly increased during apneic event in Ts65Dn mice (Fig. 5). This finding provides indirect evidence that the apneic events we detected and analyzed were of physiological relevance.

Clinical studies have shown significant dose-relationships between OSA occurrence rate during REMS and arterial hypertension, whereas that was not the case for OSA occurrence rate during NREMS (Mokhlesi et al., 2014). Our findings on mice may pave the way to the use of mouse models of OSAs during REMS to accelerate understanding of OSA pathophysiology, genetics, and cardiovascular consequences.

We applied the same experimental protocol that we developed on C57Bl/6J mice to the Ts65Dn mouse model of Down syndrome, in order to test the hypothesis of an increased occurrence rate of airway obstructions during sleep, as it happens in the human subjects affected by this condition (Trois et al., 2009; Hill et al., 2016; Lal et al., 2015). Life

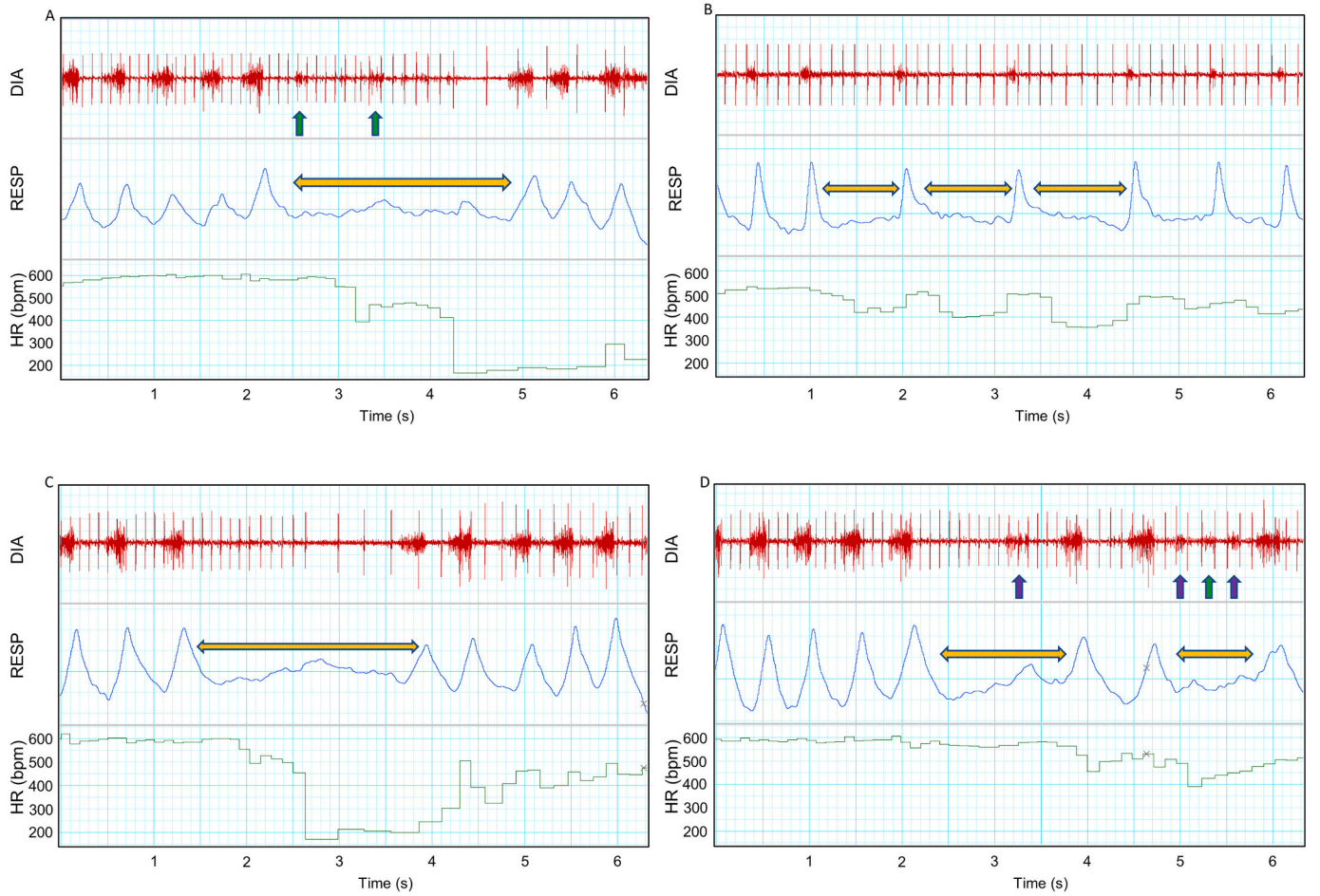

Fig. 4. Examples of raw tracings in a mouse model of Down syndrome.
DIA = diaphragmatic electromyogram; RESP = differential pressure recorded inside the whole-body plethysmograph (which corresponds to mouse respiratory pattern); HR = heart rate (bpm); REMS = rapid-eye-movement-sleep; NREMS = non-rapid-eye-movement-sleep; CSA = central sleep apnea; OSA = obstructive sleep apnea; sub-OSA = sub-obstructive sleep apnea.
Panels A, B, C and D show raw tracings recorded during an OSA in REMS, a CSA in NREMS, a CSA in REMS and a sub-OSA in REMS, respectively.
On the RESP signal, orange arrows indicate apnea events. On the DIA signal, green arrows indicate OSA events and the purple arrow indicates a sub-OSA event. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

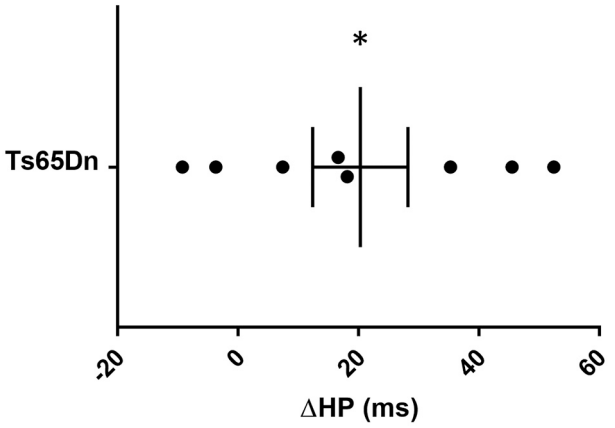

Fig. 5. Heart rhythm modulation during apneic event in a mouse model of Down syndrome.
ΔHP = difference in the heart period between the apneic event and the 10 heart beats that precede the apneic event; each black dot represents a single Ts65Dn mouse. The middle line indicates the mean value of ΔHP and the external lines indicate SEM. *: $p < 0.05$ vs. reference value of zero (one-sample t-test).

expectancy of patients with Down syndrome has more than doubled in the last 40 years, underscoring the importance of research aimed at improving the quality of life of such patients (Presson et al., 2013). In subjects with Down syndrome, OSA is highly prevalent particularly during REMS (Ferri et al., 1997) and leads to hypoxemia, cognitive deficits, arterial hypertension and sleep fragmentation (Ridore et al., 2017). Interestingly, we found evidence of sleep fragmentation in Ts65Dn mice in this study (Fig. 2), confirming previous results (Colas et al., 2008). Our data also verified, for the first time, the hypothesis that Ts65Dn mice have increased incidence of obstructive events during REMS (Fig. 3) and could thus represent a promising candidate as a genetic mouse model of OSAs. In this respect, it would be of interest in future experiments to test whether our results depend, at least in part, on diaphragm muscle weakness in Ts65Dn mice, which have shown weakness of the soleus muscle in previous work (Cowley et al., 2012). We were not able to evaluate muscle weakness using DIA recordings, whose calibration would be technically challenging due to variable impedance of the recording electrodes. However, by analyzing the ratio of DIA burst amplitude during both OSA and sub-OSA events vs baseline, we were able to show that DIA burst amplitude during the obstructive event was significantly higher relative to that of the breath before the apneic event in Ts65Dn than in euploid control mice. It would also be of interest to apply the technique of the present study to Dp(16)1Yey mice,

Table 2
Characteristics of apneas during REMS in Ts65Dn mice and euploid control mice.

	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	116 ± 13	114 ± 25	53 ± 11	52 ± 10
DIA burst amplitude (%)	/	/	127 ± 17*	52 ± 7	90 ± 11*	45 ± 11
DIA burst duration (%)	/	/	72 ± 12	53 ± 12	94 ± 14	56 ± 9

Values of estimated airflow and of amplitude and duration of the diaphragmatic electromyogram (DIA) bursts 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 in euploid control mice. For sub-OSA events, the reported airflow estimates correspond to those during the event. For CSA and OSA, which both entail complete absence of airflow, the reported airflow estimates correspond to those in the first post-apneic breath. DIA bursts were absent during CSA, leading to missing estimates of their amplitude and duration. Airflow estimates and DIA burst amplitude and duration were all expressed as % of the corresponding values in the breath that preceded the CSA, OSA, or sub-OSA. Data are means ± SEM. *, $p < 0.05$ vs. euploid controls.

which have been recently found to recapitulate the craniofacial abnormalities and upper airway obstruction that create an OSA-prone environment in patients with Down syndrome (Takahashi et al., 2020). These data could provide important insight on the pathophysiological mechanisms of OSA in Down syndrome.

Unexpectedly, we found that during NREMS, Ts65Dn mice showed lower CSA occurrence rate than euploid controls (Fig. 3). The occurrence of CSA has been well described in patients with Down syndrome, but this condition is usually mild compared to OSA (Fan et al., 2017; Lal et al., 2015; Simpson et al., 2018; Thottam et al., 2015). Since CSA occurrence rate increases with age in patients with Down syndrome (Ferri et al., 1997), it is possible that Ts65Dn mice older than those we studied here would have exhibited relatively more CSA events. This hypothesis will have to be tested in future work. On the other hand, CSAs in patients with Down syndrome were reported to occur mainly after sighs and to reflect immaturity of the peripheral chemoreceptor reflex control (Ferri et al., 1997). In contrast, our data showed that in Ts65Dn mice, CSAs were mainly unrelated to sigh appearance. This discrepancy may depend on the mouse genetic background, as suggested by our observation of prevalent spontaneous CSA over post-sigh CSA not only in Ts65Dn mice, but also in their euploid counterparts, both with DBA/2J genetic background. Conversely, we previously reported that post-sigh CSAs prevail over spontaneous CSAs during NREMS in mice with 129/Sv genetic background (Lo Martire et al., 2017).

Finally, some limitations of the present study must be acknowledged. The surgery for proper implantation of DIA electrodes is technically challenging, and respiratory bursts in the DIA signal were clearly discernible against background noise only in 19 out of 26 mice under study. We identified sub-OSA events based on an indirect estimation of mean airflow of breaths that had escaped automatic detection due to their low amplitude, and on a 30% threshold reduction of this airflow compared with baseline, based on criteria for the definition of hypopnea in human subjects (Shamim-Uzzaman et al., 2018). However, the whole procedure was based on raw tracing analysis by consensus between two researchers. Another limitation of the present study is that we did not attempt to further categorize apneic events as mixed apneas, which should be the focus of future work.

In conclusion, data of the present experiments indicate that mice physiologically exhibit not only sleep apneas of central origin, but also events with a partial or total obstructive component similar to human OSA, particularly during REMS. Strikingly, we demonstrated that these obstructive events are more prevalent in a mouse model of Down syndrome, which is known to entail increased OSA occurrence in humans. Thus, we propose that mice can be studied to accelerate the understanding of the pathophysiology and genetics of sleep apneas and development of new therapeutical approaches to contrast sleep-disordered breathing and its adverse health consequences.

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All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Appendix A. Supplementary data

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