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RESEARCH ARTICLE

Effect of ambient temperature on sleep breathing phenotype in mice: the role of orexins

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ABSTRACT

The loss of orexinergic neurons, which release orexins, results in narcolepsy. Orexins participate in the regulation of many physiological functions, and their role as wake-promoting molecules has been widely described. Less is known about the involvement of orexins in body temperature and respiratory regulation. The aim of this study was to investigate if orexin peptides modulate respiratory regulation as a function of ambient temperature (T_a) during different sleep stages. Respiratory phenotype of male orexin knockout (KO-ORX, N=9) and wild-type (WT, N=8) mice was studied at thermoneutrality (T_a=30°C) or during mild cold exposure (T_a=20°C) inside a whole-body plethysmography chamber. The states of wakefulness (W), non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) were scored non-invasively, using a previously validated technique. In both WT and KO-ORX mice, T_a strongly and significantly affected ventilatory period and minute ventilation values during NREMS and REMS; moreover, the occurrence rate of sleep apneas in NREMS was significantly reduced at T_a=20°C compared with T_a=30°C. Overall, there were no differences in respiratory regulation during sleep between WT and KO-ORX mice, except for sigh occurrence rate, which was significantly increased at T_a=20°C compared with T_a=30°C in WT mice, but not in KO-ORX mice. These results do not support a main role for orexin peptides in the temperature-dependent modulation of respiratory regulation during sleep. However, we showed that the occurrence rate of sleep apneas critically depends on T_a, without any significant effect of orexin peptides.

KEY WORDS: Apnea, Breathing, Mouse, Orexins/hypocretins, Sigh, Sleep

INTRODUCTION

Orexins (orexin A and B), also known as hypocretins (hypocretin 1 and 2, respectively), are neuropeptides identified in 1998 by two independent research groups (de Lecea et al., 1998; Sakurai et al., 1998). The location of orexin-containing cell bodies is restricted to the lateral hypothalamus, the perifornical area and the dorsomedial hypothalamus. However, orexinergic projections and receptors are widely distributed in the hypothalamus, thalamus, cerebral cortex, circumventricular organs, brainstem and spinal cord. Originally described as involved in sleep/arousal and feeding regulation (de Lecea et al., 1998; Sakurai et al., 1998), orexin peptides show widespread connections with many different brain regions, and can affect multiple physiological functions (Peyron et al., 1998); indeed, their loss promotes narcoleptic phenotypes in both humans (Peyron et al., 2000) and rodents (Chemelli et al., 1999; Hara et al., 2001). Moreover, orexins have also emerged as major regulatory actors of numerous biological processes, and their critical role in orchestrating behavioral and autonomic responses to environmental challenges have been clearly demonstrated (Zoccoli et al., 2011). On these bases, we hypothesized that orexins may play a role in respiratory regulation during sleep, and that this effect may be affected by temperature of the sleep environment. At present, less is known about their involvement in other regulatory systems such as body temperature and respiratory regulation.

Lateral ventricles, cisterna magna or intrathecal microinjections of orexin A increase tidal volume (V_T) and phrenic electromyographic burst amplitude. Orexin A exerts a dose-dependent decrease in ventilatory period (VP) when administered in the lateral ventricle (Zhang et al., 2005), but not when administered at the same doses in the cisterna magna (Zhang et al., 2005) or at higher doses in ventrolateral medulla or the spinal cord (Young et al., 2005). In orexin knockout (KO-ORX) mice, which have congenital orexin deficiency, VP, V_T and minute ventilation (V_E) were reported to not differ significantly from those of wild-type (WT) mice (Nakamura et al., 2007). However, it has been reported that KO-ORX mice show frequent sleep apneas, suggesting a critical role of orexins in respiratory regulation during sleep (Nakamura et al., 2007). Central and obstructive sleep apnea have been found to be associated with narcolepsy type 1 in adult patients (Chokroverty, 1986; Hoshino et al., 2019; Pataka et al., 2012; Pizza et al., 2013; Sansa et al., 2010), but these data have not been confirmed in pediatric patients with narcolepsy type 1 (Filardi et al., 2020). To date, contrasting data have also been reported on the role of orexins in body temperature regulation. Some studies indicated a possible involvement of orexins in thermogenesis (Kuwaki, 2015; Madden et al., 2012; Mohammed et al., 2016; Nakamura et al., 2003; Tupone et al., 2011), while other experiments suggested that orexins are not necessary to modulate the effects of ambient temperature (T_a) on the wake–sleep cycle or cardiovascular regulation (Lo Martire et al., 2012). In this respect, it should be noted that respiratory regulation and thermoregulation are intertwined. In particular, respiratory variables change as a function of T_a (Gordon, 1985). This modulation was verified in WT mice, in which reduced T_a was found to entail a decrease in VP values, and an increase in V_T values. However, these changes were described without taking into account the wake–sleep state (Hodges et al., 2008). WT mice exposed to cold T_a exhibit an increase in thermoregulatory energy metabolic rate during wakefulness, which is an adaptive strategy (Hodges and Richerson, 2008; Hodges et al., 2008).

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List of symbols and abbreviations

ATX3-ORX	ataxin3-orexin
KO-ORX	orexin knockout
NREMS	non-rapid eye movement sleep
REMS	rapid eye movement sleep
s.d.$_1$	short-term breath-to-breath (VP or V_T) variability
s.d.$_2$	long-term breath-to-breath (VP or V_T) variability
T_a	ambient temperature
V_E	minute ventilation
VP	ventilatory period
V_T	tidal volume
WBP	whole-body plethysmography
WT	wild-type
Δsigh	difference between sigh occurrence rate at T_a=20°C and that at T_a=30°C

This study was designed primarily to investigate if orexin peptides modulate respiratory regulation as a function of T_a in different sleep stages. We addressed this question measuring V_T, VP and V_E in KO-ORX mice exposed to mild cold stress (T_a=20°C) compared with thermoneutrality (T_a=30°C). The secondary aim of the study was to verify if the described increase in sleep apnea occurrence rate in KO-ORX is modulated by exposure to different T_a (20 versus 30°C). Based on the available evidence, we hypothesized that in the absence of orexin peptides, the effects of T_a on breathing variables would be blunted, and that the occurrence rate of sleep apneas would be increased.

MATERIALS AND METHODS

The study protocol was approved by the Bologna University ethics committee on animal experimentation and complied with the National Institutes of Health guide for the care and use of laboratory animals. All the experiments included in this study were executed non-invasively. Experiments were performed on eight male C57Bl/6J WT mice and nine congenic (≥10 generations of backcrossing) male KO-ORX mice matched for age (45.3±0.4 and 44.9±0.6 weeks, respectively). The mice were maintained at 23°C with a 12 h:12 h light:dark cycle and free access to food and water. Genotypes were assessed as previously described (Bastianini et al., 2011). The experimental protocol consisted of two recordings performed with the mouse placed inside a whole-body plethysmography (WBP) chamber (PLY4223; Buxco, Wilmington, NC, USA) flushed with air at 1.5 litres h^{-1} and exposed either to mild cold stress (T_a=20°C) or to thermoneutrality (T_a=30°C). Each animal underwent two recording sessions, one at T_a=20°C and the other at T_a=30°C. The order of the recording sessions was randomly chosen and balanced between experimental groups, leaving a 2–28 day interval between sessions. The groups were also matched for the interval between sessions (8.7±2.6 versus 10.4±3.2 days in KO-ORX versus WT mice, t_{15}=−0.412, P=0.686). Each recording session lasted for 8 h during the rest (light) period, starting at lights-on at 09:00 h. The respiratory signal was derived from the differential pressure between the mouse chamber and a second reference chamber, measured with a high-precision differential pressure transducer (DP103-06+CD223 digital transducer indicator; Validyne Engineering, Northridge, CA, USA). Differential pressure and chamber humidity and temperature were continuously recorded, digitized and stored at 128, 4 and 4 Hz, respectively. The system was calibrated with a 100 µl microsyringe (Hamilton, Reno, NV, USA) at the end of each recording. Wakefulness, non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) were scored based on inspection of the raw respiratory recordings, with a procedure validated against gold standard conventional electroencephalography and electromyography (Bastianini et al., 2017). As in previous studies with this technique (Bastianini et al., 2015; Silvani et al., 2014), quantitative analysis of breathing was restricted to stable sleep episodes ≥12 s because of the frequent occurrence of movement artefacts during wakefulness. Breath-to-breath values of VP, V_T and V_E were obtained as previously reported for each sleep state (Bastianini et al., 2017). The variability of VP and V_T was analysed with a technique originally proposed for the study of heart rate variability (Brennan et al., 2001) and already applied to respiratory physiology (Bastianini et al., 2015; Silvani et al., 2014). Briefly, the short-term (breath-to-breath) and long-term variability of VP and V_T were calculated based on Poincaré plots, in which the abscissa and ordinate of each point indicate the duration or amplitude of the Nth and (N+1)th successive breaths, respectively. In this analysis, the standard deviation of VP and V_T values around the axis oriented with the line of identity of the Poincaré plot estimates the short-term (breath-to-breath) variability of VP and V_T (s.d.$_1$), while the standard deviation of VP and V_T values around the orthogonal axis estimates long-term variability (s.d.$_2$). The mean values of VP, V_T, V_E and the s.d.$_1$ and s.d.$_2$ of VP and V_T were computed for each mouse after exclusion of the breaths with VP and/or V_T that deviated more than 3 s.d. from the respective mean value in the whole recording (Bastianini et al., 2015). These computations were thus protected from the effects of breaths with extreme values of VP and/or V_T. Finally, apneas and augmented breaths (sighs) were automatically detected as breaths with values of VP (apneas) or V_T (sighs) more than three times the average values of VP or V_T, respectively, for each mouse and sleep state, and detection accuracy was checked on raw recordings (Bastianini et al., 2015; Silvani et al., 2014). Because augmented breaths (sighs) often precede apneas during NREMS, we further categorized NREMS apneas as post-sigh apneas if they followed a sigh by ≤8 s or as spontaneous apneas if they followed a sigh by >8 s (Bastianini et al., 2019).

At the end of the experimental protocol, mice were perfused, under deep anesthesia (isoflurane 4%, inhalation route), with saline followed by 4% paraformaldehyde. Brains were cryoprotected in phosphate-buffered saline (PBS) with 20% sucrose and coronally sectioned at 30 µm using a cryostat microtome at −22.0°C. Hypothalamic sections were processed for immunoreactivity for orexin A. Briefly, free-floating sections were washed in 0.3% Triton X-100 in PBS for 30 min. After blocking for 90 min with 3% bovine serum albumin (Sigma-Aldrich, Milan, Italy) in 0.3% Triton X-100 in PBS, sections were incubated overnight at 4°C with rabbit anti-orexin A antiserum (Phoenix Pharmaceuticals, Burlingame, CA, USA) diluted 1:5000 in 0.3% Triton X-100 in PBS and 1% bovine serum albumin (BSA). Sections were then washed in 0.3% Triton X-100 in PBS for 30 min and incubated for 2 h with a Cy3-conjugated AffiniPure Donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) secondary fluorescent antibody diluted 1:200 in 0.3% Triton X-100 in PBS and 1% BSA. Sections were counterstained with Hoechst 33342 (Sigma-Aldrich) in order to label cell nuclei. Immunofluorescence images were taken with a Nikon Eclipse TE 2000-S inverted microscope (Nikon Corporation, Kawasaki, Japan) equipped with a Nikon DS-Qi2 digital camera. The same lot of primary antiserum was used for staining the brains of WT and KO-ORX mice. Control and experimental animals were processed simultaneously for staining to avoid any batch-to-batch variation.

Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA) with two- or three-way mixed design ANOVAs. In all analyses, genotype was the between-subject factor

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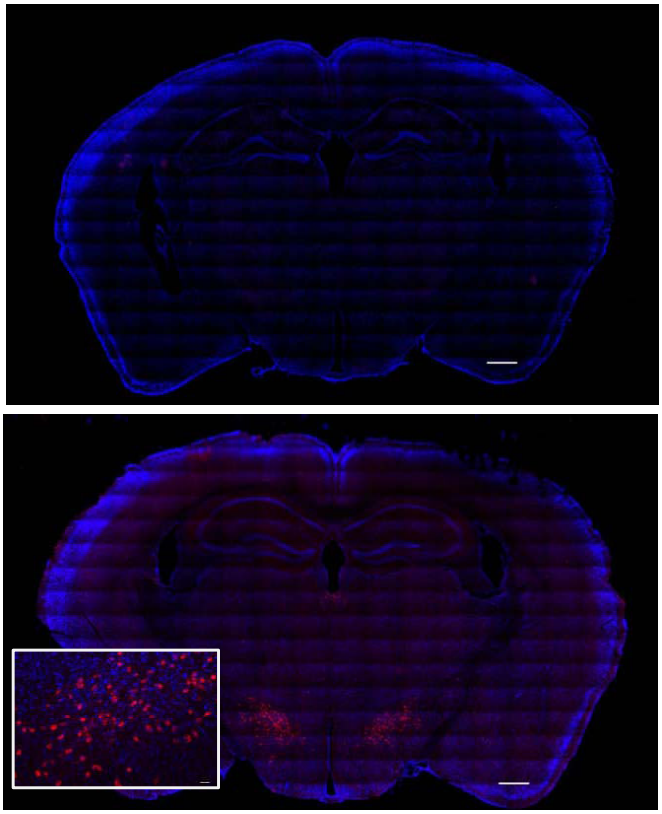

Fig. 1. Orexin A immunostaining in orexin knockout and control mice. Representative sections of orexin A immunostaining (red) with Hoechst nuclear counterstaining (blue) in orexin knockout (KO-ORX, top) and control wild-type (bottom, with higher magnification in inset) mice. Cells with red nucleus are orexin A+ cells, which are altogether lacking in KO-ORX mice, and cells with blue nucleus are Hoechst-stained cells. Scale bars, 500 μm (lower magnification) and 50 μm (higher magnification).

(two levels: KO-ORX versus WT), and T_a was a within-subject (i.e. repeated measure) factor (two levels: 20 versus 30°C). Most analyses included the sleep state as another within-subject factor (two levels: NREMS versus REMS) as appropriate. In order to limit the complexity of the interpretation of statistical analysis, for the special case of the analysis of breathing variability we replaced the sleep state factor with a variability index factor (two levels: $s.d._1$ versus $s.d._2$), and ran two separate ANOVAs for NREMS and REMS. An independent *t*-test was then applied to compare the differences between the two experimental groups, whereas a dependent *t*-test was used to compare the effect of different conditions (i.e. sleep states or T_a) on a variable that was not affected by mouse genotype (thus we considered both experimental groups as a whole). Results are shown means± s.e.m. with significance at $P<0.05$.

RESULTS

First, we verified the validity of the KO-ORX mice used in this study as a model of orexin deficiency and of body weight and wake–sleep cycle alterations related to narcolepsy type 1. The orexin A antiserum produced no staining in the brains of KO-ORX mice, whereas it clearly labelled orexin A neurons in WT brains (Fig. 1).

The body weight of KO-ORX mice before each recording was significantly higher than that of WT mice (34.2±1.2 versus 30.0± 0.7 g, $t_{12.3}=-2.962$, $P=0.012$).

The percentage of recording time spent in wakefulness, NREMS or REMS did not differ significantly between KO-ORX and WT mice (two-way ANOVA: for wakefulness $F_{1,15}=0.109$, $P=0.746$; for NREMS $F_{1,15}=0.226$, $P=0.641$; for REMS $F_{1,15}=0.353$, $P=0.561$), nor was it significantly affected by T_a (for wakefulness $F_{1,15}=0.484$, $P=0.497$; for NREMS $F_{1,15}=0.595$, $P=0.452$; for REMS $F_{1,15}=0.001$, $P=0.977$) (Table 1). However, two-way ANOVA revealed significant main effects of orexin deficiency (i.e. KO-ORX versus WT), with no significant interaction between T_a and orexin deficiency, on specific features of sleep architecture. In particular, KO-ORX mice showed significant fragmentation of wakefulness (i.e. reduced episode duration) ($F_{1,15}=7.404$, $P=0.016$) and NREMS ($F_{1,15}=5.945$, $P=0.028$) episodes, and shorter REMS latency ($F_{1,15}=19.313$, $P=0.001$) compared with WT mice, irrespective of T_a (for fragmentation of wakefulness episodes $F_{1,15}<0.001$, $P=0.991$; for fragmentation of NREMS episodes $F_{1,15}=0.126$, $P=0.728$; for REMS latency $F_{1,15}=4.534$, $P=0.0502$). Accordingly, KO-ORX and WT mice constituted distinct clusters on a scatterplot of reduced REMS latency and reduced wakefulness episode mean duration, particularly at $T_a=20$°C (Fig. 2). Overall, these results are fully consistent with previous work on KO-ORX mice (Bastianini et al., 2011; Chemelli et al., 1999), confirming the validity of the KO-ORX mice used in this study as a model of orexin deficiency and narcolepsy type 1.

The analysis of respiratory variables demonstrated that in both KO-ORX and WT mice, T_a strongly and significantly affected VP and V_E values (three-way ANOVA, main effect: for VP $F_{1,15}=97.273$, $P<0.001$; for V_E $F_{1,15}=17.515$, $P=0.001$) (Fig. 3), but did not have any significant effect on V_T (ANOVA, main effect: $F_{1,15}=1.133$, $P=0.304$). In particular, the values of VP were significantly lower and those of V_E were significantly higher at $T_a=20$°C than at $T_a=30$°C, irrespective of the sleep state and orexin deficiency (temperature×sleep state interaction on VP: $F_{1,15}=2.285$, $P=0.151$; temperature×orexin deficiency interaction on VP:

Table 1. Wake–sleep architecture as a function of ambient temperature in orexin knock-out and wild-type mice

		WT		KO-ORX	
State	Measure	T_a 20°C	T_a 30°C	T_a 20°C	T_a 30°C
Wakefulness	Percentage of recording time	48.4±1.8	54.5±3.1	48.9±2.9	51.4±4.6
	Mean episode duration (s)*	410±22	446±62	301±28	338±33
NREMS	Percentage of recording time	47.3±1.5	40.8±2.7	47.1±2.7	44.2±4.1
	Mean episode duration (s)*	206±11	220±17	166±10	188±17
REMS	Percentage of recording time	4.1±0.3	4.6±0.6	3.8±0.3	4.3±0.6
	Mean episode duration (s)	126±8	133±22	100±3	117±11
	Latency (s)*	414±45	390±23	188±22	275±38

Percentage of recording time and mean episode duration of wakefulness, non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) of orexin knockout (KO-ORX) and wild-type (WT) mice exposed to different values of ambient temperature (T_a). REMS latency, time from sleep onset to the first epoch of REMS. *Main effect of genotype, $P<0.05$, ANOVA.

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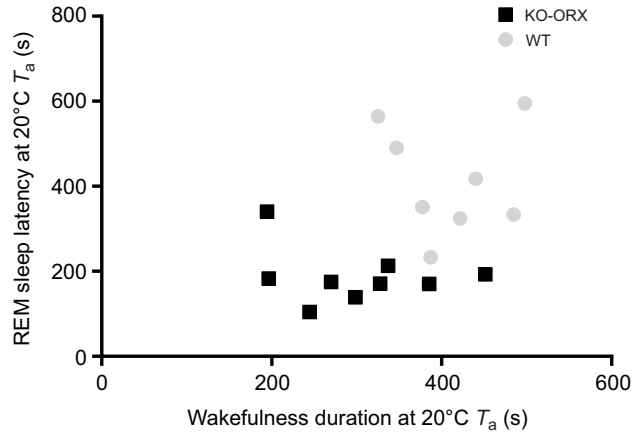

Fig. 2. Narcoleptic phenotype in orexin knockout mice. The graph shows rapid eye movement (REM) sleep latency and wakefulness episode mean duration (both at 20°C of ambient temperature, T_a) for each individual orexin knockout (KO-ORX, *N*=9, black symbols) and wild-type mouse (WT, *N*=8, grey symbols) under study. As expected, KO-ORX mice exhibited a clear narcoleptic phenotype with significantly shorter REM sleep latency and inability to sustain long wakefulness bouts, constituting a cluster clearly distinct from that of WT mice.

$F_{1,15}$=0.433, P=0.520; temperature×sleep state interaction on V_E: $F_{1,15}$=3.668, P=0.075; temperature×orexin deficiency interaction on V_E: $F_{1,15}$=0.001, P=0.980).

Three-way ANOVA did not reveal any significant main effect of orexin deficiency on sleep apnea occurrence rate ($F_{1,15}$=1.934, P=0.185), but revealed a significant interaction between T_a and sleep state ($F_{1,15}$=5.039, P=0.040) on this variable. Dependent *t*-test considering both experimental groups together confirmed that sleep apnea occurrence rate increased with T_a during NREMS (t_{16}=−4.601, P<0.001) but not during REMS (t_{16}=0.351, P=0.730) (Fig. 4). Moreover, categorization of NREMS apneas as post-sigh or spontaneous as a function of their proximity to a preceding sigh (Table 2) indicated that the occurrence rate of both post-sigh and spontaneous sleep apneas significantly increased with T_a (dependent *t*-test: t_{16}=−5.573, P<0.001 and t_{16}=−2.348, P=0.032, respectively).

As sighs occur almost exclusively during NREMS in mice (Bastianini et al., 2019), the analysis of their occurrence rate was restricted to NREMS. We found a significant interaction between orexin deficiency and T_a on sigh occurrence rate (two-way ANOVA, $F_{1,15}$=6.297, P=0.024). Sighs were significantly increased at T_a=20°C compared with T_a=30°C in WT mice (12.7±2.3 versus 8.2±1.8 episodes h^{-1}, respectively; paired *t*-test, t_7=4.111, P=0.005), but not in KO-ORX mice (7.9±1.2 versus 7.2±0.8 episodes h^{-1}, respectively; paired *t*-test, t_8=0.642, P=0.539). In order to better describe this interaction effect, for each mouse we calculated the difference between sigh occurrence rate at T_a=20°C and that at T_a=30°C (Δsigh). Δsigh was positive, and was significantly lower in KO-ORX compared with WT mice (independent *t*-test, t_{15}=2.509, P=0.024) (Fig. 4).

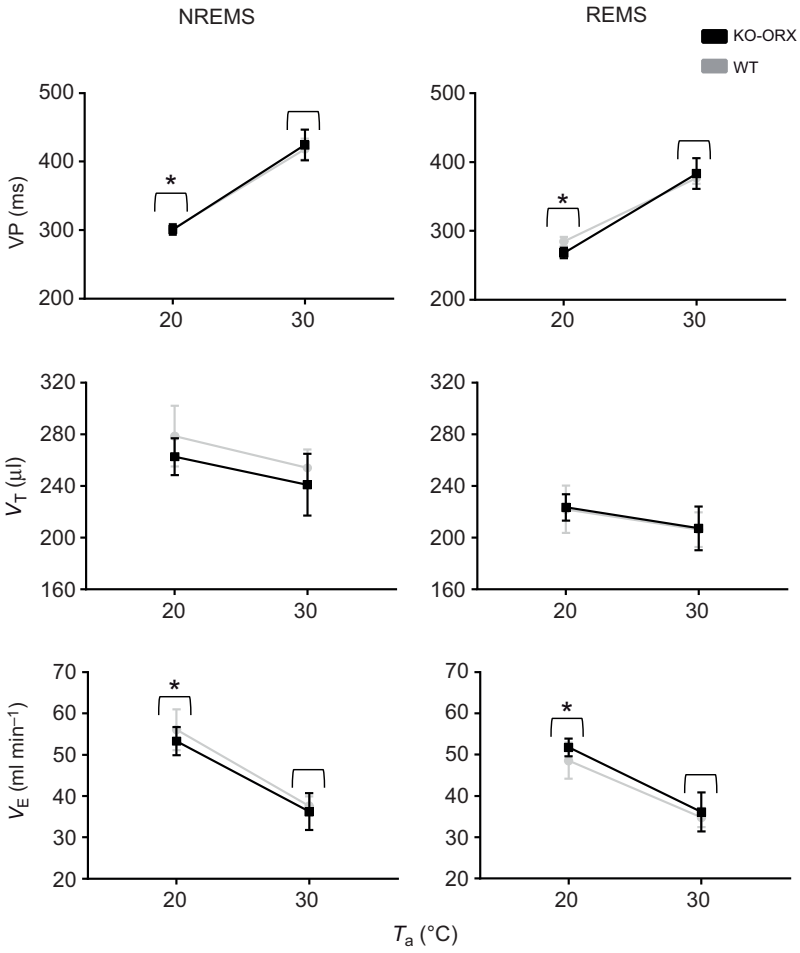

Fig. 3. Breathing phenotype in mice exposed to different ambient temperatures. Breathing variables (from top to bottom: ventilatory period, VP; tidal volume, V_T; minute ventilation, V_E) of orexin knockout (KO-ORX, *N*=9, black symbols) and wild-type mice (WT, *N*=8, grey symbols) during non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) at different values of ambient temperature (T_a). *Significant main effect of T_a (P<0.05, ANOVA).

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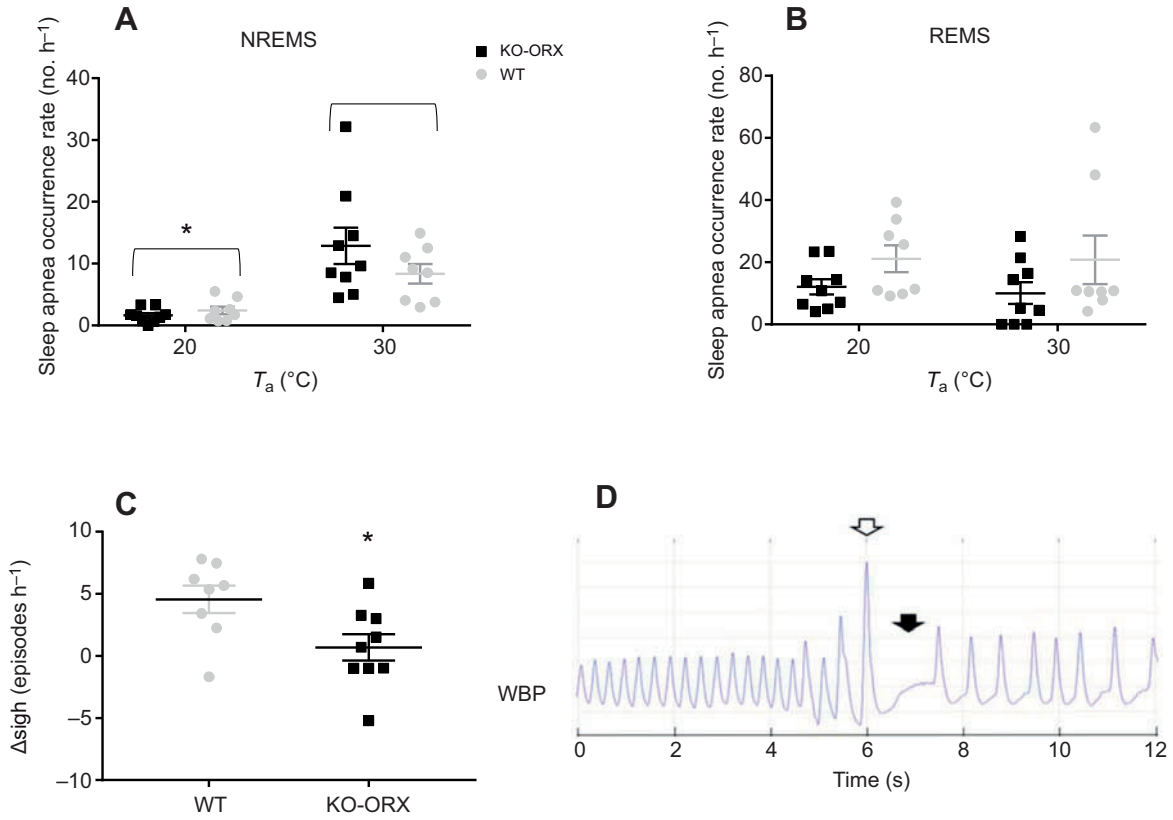

Fig. 4. Temperature-dependent regulation of sleep apneas and augmented breaths. (A,B) Apnea occurrence rate during non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) in orexin knockout (KO-ORX, *N*=9, black symbols) and wild-type mice (WT, *N*=8, grey symbols) exposed to different values of ambient temperature (T_a); *significant main effect of T_a (P<0.05, ANOVA). (C) Augmented breath (sigh) occurrence rate differences between T_a=20°C and T_a=30°C (Δsigh) during NREMS in KO-ORX and WT mice; *P<0.05 versus WT, independent *t*-test. (D) Raw tracing of the whole-body plethysmography (WBP) signal of one KO-ORX mouse during NREMS at T_a=20°C. The black arrow indicates a sleep apnea; the white arrow indicates a sigh.

Sleep apneas and sighs are extreme manifestations of VP and V_T variability, respectively. We went on to analyse the overall variability of VP and V_T with a method protected from the effects of extreme values of breathing variables (see Materials and Methods). Three-way ANOVA showed a significant main effect of T_a on VP variability ($F_{1,15}$=110.909, P<0.001), whereas it did not highlight any significant effect of orexin deficiency ($F_{1,15}$=0.002, P=0.968). In particular, the short-term (breath-to-breath, s.d.$_1$) and the long-term (s.d.$_2$) variability of VP were significantly lower at T_a=20°C than at T_a=30°C both in NREMS (dependent *t*-test, considering both experimental groups together, t_{16}=−7.185, P<0.001 for s.d.$_1$ and t_{16}=−10.501, P<0.001 for s.d.$_2$) and in REMS (dependent *t*-test, considering both experimental groups together, t_{16}=−8.546, P<0.001 for s.d.$_1$ and t_{16}=−9.201, P<0.001 for s.d.$_2$) (Fig. 5). Three-way ANOVA of V_T variability failed to show significant main effects of T_a ($F_{1,15}$=3.408, P=0.085) and orexin deficiency ($F_{1,15}$=0.117, P=0.738), whereas it highlighted a significant T_a×V_T variability interaction ($F_{1,15}$=11.079, P=0.005). In particular, the long-term variability (s.d.$_2$) of V_T was significantly lower at T_a=20°C than at T_a=30°C in NREMS (dependent *t*-test, t_{16}=3.602, P=0.003) (Fig. 6).

Table 2. Non-rapid eye movement sleep apnea subtypes as a function of ambient temperature in orexin knockout and wild-type mice

		WT		KO-ORX	
State	Measure	T_a 20°C	T_a 30°C	T_a 20°C	T_a 30°C
NREMS	Post-sigh apneas (episodes h^{-1})*	0.8±0.3	3.8±0.8	0.5±0.3	5.3±1.0
	Spontaneous apneas (episodes h^{-1})*	1.1±0.4	3.3±0.8	0.8±0.2	5.1±2.5

Occurrence rate of post-sigh and spontaneous apneas during non-rapid eye movement sleep (NREMS) in orexin knockout (KO-ORX) and wild-type (WT) mice exposed to different values of ambient temperature (T_a). Sleep apneas were classified as post-sigh if they followed a sigh by ≤8 s, or as spontaneous if they followed a sigh by >8 s. ANOVA showed no significant main effect of mouse genotype on any of these variables, whereas it showed a significant (*) main effect of T_a on post-sigh and spontaneous apneas ($F_{1,15}$=31.198, P<0.001 and $F_{1,15}$=5.135, P=0.039, respectively).

DISCUSSION

We investigated if orexin peptides modulate respiratory regulation as a function of T_a. Our findings do not support a significant role for orexin peptides in the temperature-dependent modulation of breathing in different sleep states with one notable exception. Δsigh during NREMS was significantly lower in ORX-KO mice than in WT mice (Fig. 4). The mechanisms underlying apneas and sighs are still incompletely understood. Sighs may be the results of an inspiratory-augmenting reflex elicited by activation of lung and chest wall receptors in response to reduced lung compliance, or by stimulation of peripheral chemoreceptors in response to hypoxia or hypercapnia (Nakamura et al., 2003; Qureshi et al., 2009). There is evidence that orexins contribute to central chemoreflex sensitivity to carbon dioxide levels, but this contribution appears to be restricted to wakefulness (Nakamura et al., 2007), particularly in the daily active period, which corresponds to the dark period in rats and mice (Li and Nattie, 2010). This effect is thus unlikely to

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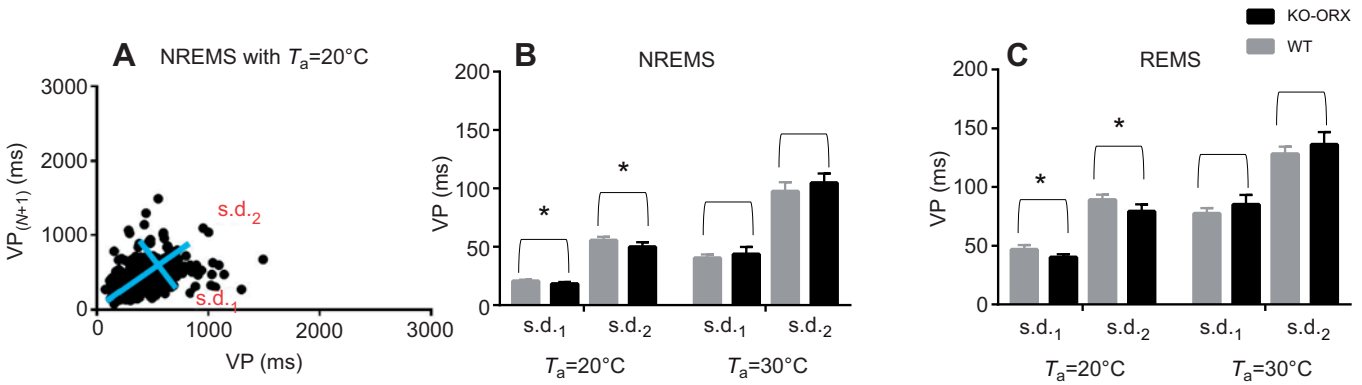

Fig. 5. Variability of ventilatory period during sleep. (A) Representative Poincaré plots of ventilatory period (VP) of each breath (*N*) versus the following breath (*N*+1) during non-rapid eye movements sleep (NREMS) in a representative orexin knockout (KO-ORX) mouse recorded at ambient temperature (T_a) of 20°C. (B,C) Values (means±s.e.m.) of indexes of the short-term (s.d.$_1$) and long-term (s.d.$_2$) variability of VP, which quantify variability along the axes highlighted graphically in panel A, for NREM sleep and REM sleep, respectively. WT, *N*=8; KO-ORX, *n*=9. *Significant main effect of T_a (*P*<0.05, ANOVA).

explain our findings concerning sigh occurrence rate during sleep in the light (rest) period. This raises the hypothesis that orexins are also involved in the integration of afferent signals from chest wall receptors.

However, these experiments showed for the first time that the occurrence rate of sleep apneas critically depends on T_a, being exacerbated at thermoneutrality (T_a=30°C) and reduced by mild cold exposure (T_a=20°C), without any significant effect of orexin peptides.

The conclusion that effects of T_a on breathing do not significantly depend on orexin peptides is in line with previous results showing that in orexin-ataxin3 transgenic mice with genetic ablation of orexin neurons (ATX3-ORX) the effects of T_a on cardiovascular regulation during different sleep states was preserved compared with WT controls (Lo Martire et al., 2012). Nevertheless, more experiments are needed to understand whether different co-transmitters, released by orexin neurons together with orexin peptides (Bonnavion et al., 2016), play a role in the adaptive breathing responses to different values of T_a. Ablation of orexin neurons in ATX3-ORX mice or rats resulted in intolerance to cold exposure (T_a=5°C) (Mohammed et al., 2016; Takahashi et al., 2013), suggesting the importance of these neurons in driving the cold defence responses. In particular, in ATX3-ORX mice but not in ORX-KO mice, abdominal temperature fell rapidly and reached the endpoint of 30°C within 50–150 min of cold exposure (Takahashi et al., 2013). These data suggest that orexin co-transmitters, rather than orexin peptides, participate in the homeostatic response to cold exposure.

In our experiments, T_a did not modulate the percentage of recording time spent in the different states of the wake–sleep cycle (Table 1). This result is at variance with our previous results on ATX3-ORX and WT mice (Lo Martire et al., 2012). However, some differences between these experiments must be acknowledged. First, in this study we recorded non-instrumented mice with a pure C57BL/6J background, whereas we obtained our previous data (Lo Martire et al., 2012) on chronically instrumented mice with a hybrid genetic background (75% C57BL/6J and 25% DBA/2J). Moreover, during this study we recorded mice inside a WBP chamber for only 8 h during the light (resting) period, and in the previous study we recorded freely moving mice for 48 h at each of two values of T_a (Lo Martire et al., 2012). Thirdly, in this study we changed the value of T_a acutely at the beginning of each recording session inside a WBP chamber, whereas in the previous study the values of T_a were changed 24 h before each recording session in the home cage (Lo Martire et al., 2012).

We found that when KO-ORX and WT mice were exposed to mild cold stress (T_a=20°C), they coped with this challenge by increasing breathing rate and V_E relative to the exposure to T_a=30°C (Fig. 3). This strategy has already been described in WT mice

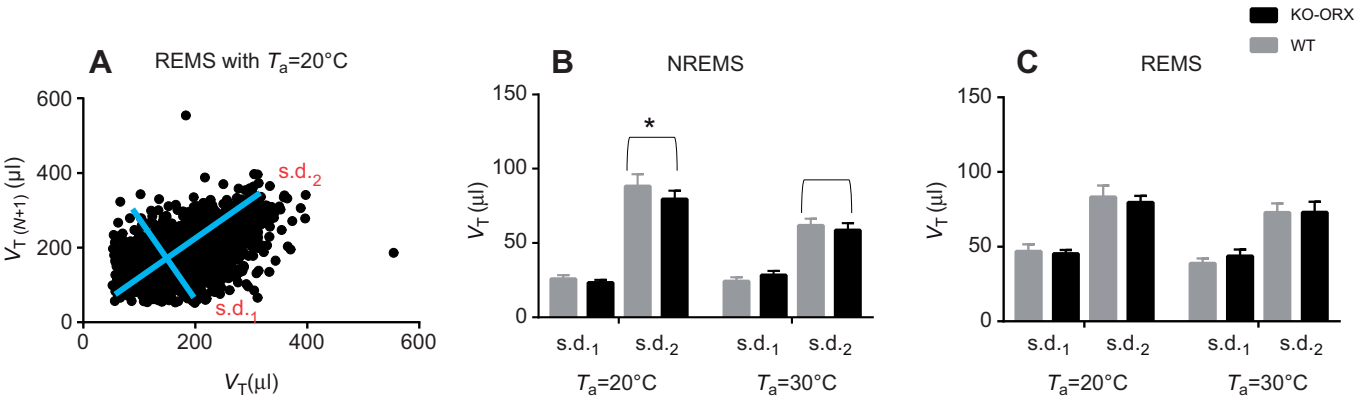

Fig. 6. Variability of tidal volume during sleep. (A) Representative Poincaré plots of tidal volume (V_T) of each breath (*N*) versus the following breath (*N*+1) during rapid eye movement sleep (REMS) in a representative orexin knockout (KO-ORX) mouse recorded at ambient temperature (T_a) of 20°C. (B,C) Values (means±s.e.m.) of indexes of the short-term (s.d.$_1$) and long-term (s.d.$_2$) variability of V_T, which quantify variability along the axes highlighted in panel A, for NREM sleep and REM sleep, respectively. WT, *N*=8; KO-ORX, *N*=9. *Significant main effect of T_a (*P*<0.05, ANOVA).

(Hodges and Richerson, 2008; Hodges et al., 2008), but only during wakefulness, with no available data during sleep. Moreover, no data on breathing adaptive response to T_a changes have been reported so far in narcoleptic KO-ORX mice lacking orexin peptides.

In our experiments, KO-ORX mice did not exhibit a higher sleep apnea occurrence rate compared with WT controls (Fig. 4; Table 2). Thus, our data challenge the view that the loss of orexin peptides is sufficient to increase sleep apnea rate in mice (Nakamura et al., 2007), but they are in broad agreement with recent findings on pediatric children with narcolepsy type 1 (Filardi et al., 2020). Stress related to chronic instrumentation in previous reports (Nakamura et al., 2007), compared with our own experiments using intact mice, might help to explain mouse differences, as the occurrence rate of sleep apneas increases with corticosterone levels (Ren et al., 2012).

A new and interesting finding of our study is that NREMS apneas were less frequent in conditions of mild cold exposure (T_a=20°C) than at thermoneutrality (T_a=30°C) (Fig. 4). This was an unexpected but robust finding as it was observed both in KO-ORX and WT mice and it concerned both post-sigh and spontaneous sleep apneas (Table 2). It is worth noting that this phenomenon is related to a specific interaction between thermoregulatory responses and NREMS state. Indeed, while we observed similar T_a-dependent increases in the mean value and in the variability of VP during both NREMS and REMS (Figs 3 and 5), the T_a-dependent increase in sleep apnea occurrence rate was restricted to NREMS (Fig. 4; Table 2). This finding is also of practical interest, as it highlights the critical importance of controlling for T_a when phenotyping mice for sleep apneas, similar to what has long been recommended when phenotyping mice for cardiovascular variables (Swoap et al., 2004).

Congenital loss of orexin peptides in KO-ORX mice may lead to developmental compensation. However, at least the behavioural phenotype (Chemelli et al., 1999) and the sleep-related blood pressure control (Bastianini et al., 2011) of KO-ORX mice strikingly resemble those of patients with narcolepsy type 1, who have a near-complete loss of orexin neurons (Berteotti and Silvani, 2018). Our conclusion that orexin peptides are not necessary for most aspects of breathing control as a function of T_a, with the notable exception of sigh occurrence rate, would therefore not be trivial if confirmed. However, the physiological effects of orexin peptides result from binding to two orexin receptors. Importantly, orexin peptides may exert different effects depending on the orexin receptor type (cf. Kakizaki et al., 2019; Willie et al., 2003). Thus, our conclusion is compatible with contrasting effects of activation of the two orexin receptor types on breathing as a function of T_a, and does not imply that orexins have no effect at all on breathing. To make that implication, it would be necessary to perform pharmacological studies targeting orexin action on each of the two orexin receptors.

Our study has a few limitations. We studied male mice to facilitate comparison with previous work on breathing in KO-ORX mice, which also was performed on male mice only (Nakamura et al., 2003, 2007). Nevertheless, the fact that we did not include female mice in the study is a limitation of our work. We have not measured the thermoneutral zone of the particular mice under study, and we are not aware of previous data published particularly on KO-ORX mice. For reference, the thermoneutral zone for C57BL/6J mice at approximatively 3 months of age lies between 29 and 31°C (Ganeshan and Chawla, 2017). Our sample size (N=8/9 per group) compares favourably with that of previous work on sleep apneas in KO-ORX mice (Nakamura et al., 2007; N=5 per group). Nevertheless, we did not perform a statistical power analysis to estimate our type II statistical error rate.

In conclusion, our findings do not support a role for orexin peptides in the temperature-dependent modulation of respiratory regulation in different sleep states. Orexin peptides only modulated the sigh occurrence rate during NREMS. However, the occurrence of sleep apneas critically depends on T_a, being exacerbated at thermoneutrality and reduced by mild cold exposure, without any significant effect of the lack of orexin peptides.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.S., G.Z.; Methodology: A.S., G.Z.; Formal analysis: C.B., V.L.M., S.A., S.B., G.M.; Investigation: C.B., V.L.M., S.A., S.B., G.M.; Writing - original draft: C.B., V.L.M.; Writing - review & editing: C.B., V.L.M., S.A., S.B., A.S., G.Z.; Visualization: C.B., V.L.M.; Supervision: G.Z.; Funding acquisition: C.B., A.S., G.Z.

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