Ventilatory long-term facilitation in mice can be observed during both sleep and wake periods and depends on orexin

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Submitted 28 August 2007; accepted in final form 18 November 2007

Terada J, Nakamura A, Zhang W, Yanagisawa M, Kuriyama T, Fukuda Y, Kuwaki T. Ventilatory long-term facilitation in mice can be observed during both sleep and wake periods and depends on orexin. J Appl Physiol 104: 499–507, 2008. First published November 21, 2007; doi:10.1152/japplphysiol.00919.2007.—Respiratory long-term facilitation (LTF) is a long-lasting (>1 h) augmentation of respiratory motor output that occurs even after cessation of hypoxic stimuli, is serotonin-dependent, and is thought to prevent sleep-disordered breathing such as sleep apnea. Raphe nuclei, which modulate several physiological functions through serotonin, receive dense projections from orexin-containing neurons in the hypothalamus. We examined possible contributions of orexin to ventilatory LTF by measuring respiration in freely moving prepro-orexin knockout mice (ORX-KO) and wild-type (WT) littermates before, during, and after exposure to intermittent hypoxia (IH; 5 × 5 min at 10% O2), sustained hypoxia (SH; 25 min at 10% O2), or sham stimulation. Inspiratory data during quiet wakefulness (QW), slow wave sleep (SWS), and rapid-eye-movement sleep were separately calculated. Baseline ventilation before hypoxic stimulation and acute responses during stimulation did not differ between the ORX-KO and WT mice, although ventilation depended on vigilance state. Whereas the WT showed augmented minute ventilation (by 20.0 ± 4.5% during QW and 26.5 ± 5.3% during SWS; n = 8) for 2 h following IH, ORX-KO showed no significant increase (by −3.1 ± 4.6% during QW and 0.3 ± 5.2% during SWS; n = 8). Both genotypes showed no LTF after SH or sham stimulation. Sleep apnea indexes did not change following IH, even when LTF appeared in the WT mice. We conclude that LTF occurs during both sleep and wake periods, that orexin is necessary for eliciting LTF, and that LTF cannot prevent sleep apnea, at least in mice.

respiration; plasticity; intermittent hypoxia; behavioral state control; hypothalamus

RESPIRATORY LONG-TERM FACILITATION (LTF) is a long-lasting (>1 h) augmentation of respiratory motor output even after cessation of intermittent hypoxic stimuli (24) (see Refs. 9, 20, 25, 26, 37 for review). LTF following intermittent hypoxia (IH) has been extensively studied by measuring phrenic nerve activity in anesthetized, paralyzed, vagotomized, and artificially ventilated rats and cats (10, 14, 27, 35). Although recent studies have shown LTF in some unanesthetized awake animals including rats (21, 33) and mice (18), the magnitude and duration appear limited compared with anesthetized animals (20, 25) and even absent in humans (8). Moreover, IH has been considered a model of hypoxia during sleep apnea. Therefore, it is of interest whether LTF can be observed during naturally occurring sleep and whether the magnitude of LTF during sleep is greater than that during waking. Only meeting reports are available on this issue (29, 30), which showed LTF in rats during sleep but not in waking.

LTF relies on a serotonin-dependent central neural mechanism, because it is abolished by serotonin receptor antagonists, serotonin depletion, or serotonergic neurotoxins (9, 24). Furthermore, electrical activation of raphe pallidus or raphe obscurus elicits a degree of LTF (23). However, how IH activates serotonergic neurons is still an open question.

Two lines of evidence suggest that orexin should be a focus of attention. First, orexin-containing neurons in the hypothalamus send dense projections to the raphe nuclei (3, 32, 36), although it is unclear whether these axons terminate on serotonergic neurons. Second, a growing body of evidence shows potential contributions of orexin in respiratory regulation in addition to its multiple roles in diverse physiological functions such as sleep-wake architecture, energy homeostasis, motivation, nociception, thermoregulation, and cardiovascular regulation (13, 46, 51). For example, intracerebroventricular administration of orexin promotes ventilation (48, 49). Orexin neurons are activated by hypercapnia in vitro (45). Orexin-deficient mice show an attenuation of respiratory excitation during fight-or-flight responses (17, 50). Prepro-orexin knockout mice (ORX-KO) exhibit an attenuated hypercapnic chemoreflex during waking but not sleep periods (31); this effect can be partially retrieved by exogenous administration of orexin (7). ORX-KO also show exaggerated apnea during sleep (31). The last finding is of particular interest, because it is the first report demonstrating a possible orexin role during sleep, when orexin neuronal activity is generally slow (19, 22, 39, 47).

The role of orexin in respiratory regulation appears to be state dependent, but this role apparently is not a tonically active influence, because basal ventilation (respiratory frequency and tidal volume) at rest in ORX-KO mice does not differ from that in wild-type (WT) littermates (31). One of these “states” may be exposure to IH. Repeated sleep apneas cause IH, and enhanced LTF may compensate for factors that predispose to sleep-disordered breathing (20). If this scenario is the case, exaggerated sleep apnea in ORX-KO may be a consequence of reduced compensation by LTF in this mouse. Therefore, we

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hypothesized that orexin might contribute to generation of LTF after IH. Sleep apnea in mice as far as we observed are thought to be the central sleep apnea (CSA) (28, 31) but not the obstructive sleep apnea (OSA) that is usually discussed in human patients. Nevertheless, we think it is worth evaluating sleep apneas in this study because there is no report examining a possible relationship between LTF and sleep apnea of either type.

The aim of the present study was threefold. First, we examined whether LTF following IH could be observed during naturally occurring sleep periods. Second, we examined whether orexin contributed to generation of LTF after IH. Third, we examined whether LTF could eliminate sleep apnea. For these purposes, we separately evaluated respiration during sleep and waking periods after IH in ORX-KO and WT mice.

METHODS

Animals. ORX-KO mice with a mixed genetic background of 129/Sv and C57BL/6 were generated as reported previously (5) and backcrossed to C57BL/6 more than nine times. The mice totally lacked orexin-A and orexin-B because both peptides are the product of one precursor, prepro-orexin. The animals were maintained in heterozygotes and crossed to obtain null mutants and WT littermates. The genotype of ORX-KO mice was identified by PCR on DNA extracted from the tail as previously reported (17). Mice used in this study were 24- to 36-wk-old male ORX-KO homozygotes (n = 8; body weight 35.9 ± 1.5 g at the time of surgery) and WT (n = 10; body weight 33.4 ± 1.0 g) mice. Body weight of ORX-KO mice did not statistically differ from that in WT controls, as has been reported (12). All mice were housed in plastic cages in a room maintained at 23–25°C, with lights on at 7:00 AM and off at 7:00 PM. Mice were provided food and water ad libitum. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Use Committee of Chiba University Graduate School of Medicine.

Simultaneous measurement of respiration and vigilance states.

Methods of surgery, recording of electroencephalography (EEG), electromyography (EMG), and respiration, and definition of vigilance states were the same as those for our previous studies (7, 28, 31) otherwise stated below. In brief, mice were surgically implanted with electrodes for EEG and EMG under isoflurane anesthesia at least 7 days before the experiments. On the experimental day, the mouse was placed into a whole body plethysmography chamber, and the electrode leads were connected to a slip ring so that the animal moved freely in the chamber. All recordings were performed between 8:00 AM and 3:00 PM. Food and water were provided in the chamber. The chamber was continuously flushed with a gas mixture at a rate of 500 ml/min using either room air or hypoxic (10% O2 balance with N2) gas mixtures. Chamber Pco2 and Po2 were continuously monitored (Respinia IH26, NEC-San-Ei-Instrument, Tokyo, Japan) at the outlet of the chamber. CO2 production was calculated from the chamber Pco2 and the flow rate and expressed as STPD.

EEG, EMG, pressure, and the animal’s movement signals were amplified and fed into a personal computer after analog-to-digital conversion (Power Lab, ADInstruments, Castle Hill, Australia). Sleep-wake architecture was determined by visual inspection of neck EMG, digitally filtered EEG (0.25–4, 4–8, and 8–30 Hz), and movement signals from the trembling sensor attached to the chamber. Vigilance state was classified into active wakefulness (AW), quiet wakefulness (QW), slow wave sleep (SWS), or rapid-eye-movement (REM) sleep for every 10 s. Time windows were set to be 10 s because our previous study showed that the minimum value of the 95% confidence limit of episode duration was 12 s in WT and ORX-KO mice (31). When multiple vigilance states were observed during the time window of 10 s, such epochs were classified as “transient state” and omitted from the later analysis (<10% of the recording time).

Respiratory frequency (fR; breaths/min), tidal volume (VT; ml), and minute ventilation (Ve; ml·min⁻¹·g⁻¹; fR·VT/body wt) were analyzed using signal analysis software, Chart (ADInstruments). Respiratory parameters were not calculated for AW periods, because the animal’s movement might distort the plethysmographic signals. Apnea was defined as cessation of plethysmographic signals for at least two respiratory cycles (28). Apneas were classified as post-sigh, if the preceding breath was at least 100% above the average amplitude during the preceding 10 s. Apnea without a preceding sigh was defined as spontaneous apnea. Apnea occurrence index was defined as the number of apneic episodes per hour and separately calculated for each type of apnea during each stage of sleep.

Rectal temperature of each mouse was recorded immediately after the plethysmographic recording while the animal was awake. Individual measurements of rectal temperature revealed a mean value of 36.8 ± 0.2°C in ORX-KO and 36.3 ± 0.5°C in WT mice. There was no difference in rectal temperatures between ORX-KO and WT mice, and also no temperature differences in each genotype between each gas condition.

Protocols. One day before the experiment, the mouse was put into the whole body plethysmography chamber and left there for more than 6 h for acclimatization. On the experimental day, fR and Ve were measured by whole body plethysmography before, during, and after exposure to IH (5 times of 5 min of 10% O2 separated by 5 min of room air), sustained hypoxia (SH; 25-min of 10% O2), or sham stimulation (9 times of 5 min of room air; each block was separated by a transient cessation of the gas flow, mimicking the flow change between 10% O2 and room air in the IH protocol). During these hypoxic and normoxic episodes, only the final 4 min of data were averaged and analyzed. Baseline breathing was recorded for 60 min while the animals breathed room air after acclimatization to the chamber (as judged by the appearance of the sleep periods, usually ~2 h) and before the stimulation. Ventilation was recorded up to 120 min after the end of hypoxic stimulation. During this baseline and after stimulation periods, the data were averaged for 20 min. In each animal, the same protocol was repeated with one of the stimulation challenges (IH, SH, sham) in a random sequence. At least 2 days were interspersed between the experiments. In some cases, not all three stimulations were completed in one animal because of technical issues in the recordings (poor EEG or EMG).

Statistical procedure. All data were expressed as means ± SE. Differences in respiratory parameters during the prestimulus period were analyzed using two-way (genotype × vigilance state) ANOVA. Because a significant difference emerged in the respiratory parameters between vigilance states (see RESULTS), later statistical analyses were separately applied for the data set of QW, SWS, and REM. Potential differences between time points were analyzed using repeated-measures ANOVA. Differences in the respiratory parameters during the poststimulus period were analyzed using three-way (genotype × type of stimulation × vigilance state) ANOVA. When appropriate, post hoc comparisons of Student-Newman-Keuls were used. A P value of <0.05 denoted the presence of a statistically significant difference.

RESULTS

Vigilance state architecture. When the WT mice breathed room air, total waking (AW plus QW) time varied between 40 and 60%, and SWS time varied between 30 and 60%, except for the period just after SH (Fig. 1). During the stimulation period of sham and IH, the animals aroused, as evidenced by the significantly increased AW and QW time and the significantly decreased SWS and REM time. REM state completely disappeared during IH and the last half of the sham stimulation. During SH, on the other hand, a similar increase in AW time

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and reciprocal decrease in SWS time appeared only at the first 5 min after onset of stimulation. As a whole, switching of the inspired gas but not hypoxic gas per se appeared to arouse the animals. There was no obvious change of the vigilance state architecture between the prestimulation and poststimulation periods. This finding enabled us to compare respiratory parameters before and after stimulation without concern about possible indirect effects from the vigilance state architecture.

Changes in state time in response to the three types of stimulation in ORX-KO mice were very similar to those in WT mice. No significant differences between ORX-KO and WT mice were observed in any vigilance state time, although waking effects of gas switching tended to be reduced in ORX-KO mice.

**Baseline respiration.** The fR, Vt, VE, and CO2 production during normoxic baseline conditions for 60 min before the stimulation are shown in Fig. 2. In SWS periods, Vt and VE were significantly lower than those in QW periods (P < 0.05). During REM period, fR was significantly higher and Vt was significantly lower than values found in QW periods. CO2 production did not differ among vigilance states. Although fR in ORX-KO mice tended to be higher than that in WT mice, there was no statistical difference between the two genotypes.

**Acute responses to hypoxia during QW.** Because three stimulation challenges (sham, SH, and IH) were applied to an animal in separate days, the data presented hereafter were normalized as % of prestimulation values to eliminate possible interexperimental variation.
SH and IH, but not sham stimulation, increased all of the measured respiratory parameters (fR, VT, and V˙E) during stimulation (Fig. 3). Approximately two- to threefold increases in V˙E were observed during the five episodes of IH and the first 5–10 min of SH. During the last part of SH (15–25 min after the onset), V˙E decreased gradually to the prestimulation level, indicating negative feedback by decreased arterial PCO2. Both fR and VT contributed to the increase in V˙E during hypoxia. In response to sham stimulation, no parameter showed a significant change.

Acute hypoxic responses did not differ between WT and ORX-KO mice. For example, V˙E during SH in WT mice (70 ± 16%, n = 10) was comparable to that in ORX-KO mice (60 ± 20%, n = 6), and V˙E during IH in WT mice (132 ± 14%, n = 8) tended to be higher but was not statistically different from that in ORX-KO mice (97 ± 11%, n = 8). Integrity of baseline ventilation and acute hypoxic responses in ORX-KO mice was in accordance with our previous reports (7, 31).

Long-term responses to hypoxia during QW. As reported for other mammals, sham stimulation with room air and SH for 25 min did not induce LTF in mice (Figs. 3 and 4). On the other hand, IH (5 × 5 min) did induce LTF in the WT mice (V˙E = 20.0 ± 4.5% as an average for 120 min; Figs. 3, right, and 4). An increase in fR mainly contributed to the increase in V˙E. There was no difference in PCO2 in the chamber between pre- and post-IH periods, indicating LTF did not result from possible changes in CO2 metabolism (Fig. 4).

Whereas the WT mice showed augmented V˙E following IH, ORX-KO mice showed no significant increase in fR, VT, or V˙E (V˙E = −3.1 ± 4.6%; Figs. 3 and 4). In other words, respiratory parameters following IH in the ORX-KO mice did not differ from baseline parameters (Fig. 4). As in the WT mice, sham stimulation and SH did not affect respiratory parameters in the ORX-KO mice.

Responses to hypoxia during SWS. During SWS, a similar response to that during QW was observed. Namely, SH and IH, but not sham stimulation, increased all respiratory parameters (fR, VT, and V˙E) during stimulation in both WT mice and ORX-KO mice (Fig. 5). V˙E during SH in WT mice (75 ± 20%, n = 10) was comparable to that in ORX-KO mice (55 ± 15%, n = 6), and V˙E during IH in WT mice (182 ± 32%, n = 8) was also comparable to that found in ORX-KO mice (124 ± 23%, n = 8). IH (V˙E = 26.5 ± 5.3%) but not SH nor sham stimulation induced LTF in the WT mice (Fig. 6). In this case, an increase in VT contributed LTF for the first 20 min, and thereafter an increase in fR was the main contributor (Fig. 5, right).

As was the case during QW, ORX-KO mice did not show augmentation of respiratory parameters during SWS even after IH (V˙E = 0.3 ± 5.2%; Figs. 5 and 6). Because ORX-KO mice tended to be obese and orexin was thought to have a critical role for regulating basal metabolism, we monitored PCO2 in the chamber to assess whether possible changes in this variable related to the absence of LTF in ORX-KO mice. However, no significant differences in PCO2 could be found under basal or after hypoxic episodes in the two genotypes (Fig. 6), an observation that suggests that alterations in PCO2 do not contribute to the attenuated LTF in the ORX-KO mice.

Responses to hypoxia during REM. We could not analyze acute hypoxic response during REM periods because the animal showed virtually no REM state during the acute hypoxic stimulation in the present experimental setup (Fig. 1). Moreover, some animals did not show any REM state during the prestimulation period of 60 min. Respiratory data from such animals was omitted from further analysis. Nevertheless, a comparable occurrence of REM states after stimulation to that before stimulation (Fig. 1) enabled us to analyze long-term effects of hypoxic exposure (see supplemental Figs. 1 and 2 available online at the Journal of Applied Physiology website). Four of six WT mice showed a respiratory augmentation after IH (V˙E = 22.7 ± 7.6%), but the remaining two mice did not.
As a result, $V_E$ after IH was highly variable, and did not reach statistical significance ($V_E/9.6\%$, $n=6$; see supplemental Fig. 2 available online at the Journal of Applied Physiology website).

**Apnea.** During quiet wake periods, the mice showed sighs and post-sigh apneas but not spontaneous apnea before hypoxic stimulation (Table 1). Hypoxic stimulation or genotype did not affect sigh and apnea indexes.

As to sleep apnea before hypoxic stimulation, post-sigh sleep apneas were observed exclusively during SWS, and spontaneous apneas were seen during both SWS and REM sleep. Spontaneous sleep apnea indexes in ORX-KO mice were significantly greater than those in WT mice, whereas post-sigh apnea indexes did not differ between the two genotypes. These observations were in accordance with our previous report (31). Although we expected decreased sleep apnea after IH in the WT mice (namely during

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**Fig. 3. Long-term responses to hypoxia during quiet wake period.** Changes of $f_R$ (top), $V_T$ (middle), and $V_E$ (bottom) as a percentage of baseline values (prehypoxia = 100%) were averaged for every 20-min period (before and after the stimulation, the scale range is 50–150%) or every 5-min period (during stimulation, the scale range is 0–400%). Note that two scale ranges are used to show time-related changes at higher resolution. WT mice ($n=7–10$) are indicated by the open symbols, and ORX-KO mice ($n=6–8$) are indicated by the closed symbols. The timing of stimulation is indicated by the arrow. Black bar under the abscissa indicates the timing when hypoxic gas (10% CO$_2$) was introduced into the chamber. Note that only intermittent hypoxia induced increases in the respiratory parameters after the stimulation in the WT but not ORX-KO mice, although both intermittent and sustained hypoxia increased respiratory parameters in both WT and ORX-KO mice. Data are means ± SE. *Significant difference compared with baseline value ($P < 0.05$).
LTF), there was no significant change in sleep apnea occurrence indexes after IH, SH, or sham stimulation. Contrary to our expectations, we found a tendency for decreased sleep apnea after IH in ORX-KO mice that lacked LTF. Taken together, these observations suggest no relationship between LTF and sleep apnea.

**DISCUSSION**

This study showed ventilatory LTF following IH during both waking or sleep periods in the mice. To the best of our knowledge, this is the first comprehensive report showing LTF during natural sleep. This study also supported our hypothesis...
that orexin is necessary for eliciting ventilatory LTF. The ORX-KO mice showed no significant ventilatory LTF, whereas the WT mice showed augmented $\dot{V}E$ during QW and SWS for 2 h following IH. There was no difference in vigilance state architecture, baseline ventilation, acute hypoxic response, and $PCO_2$ between ORX-KO and WT mice, suggesting a specific role for orexin in LTF. Contrary to our expectation, LTF did not affect the occurrence of sleep apnea.

Our first aim was to examine whether LTF could be observed during natural sleep periods in mice. In WT mice, we observed consistent LTF during SWS as well as during QW. Although the magnitude of LTF during SWS (26.5 ± 5.3%) was slightly greater than that during QW (20.0 ± 4.5%), the difference was not statistically significant ($P > 0.05$). These values were much smaller than the magnitude of LTF observed in phrenic nerve activity in anesthetized, paralyzed, vagotomized, and artificially ventilated rats (>50%) (25) and were comparable to the magnitude of LTF (20–25%) observed in unanesthetized and presumably awake (confirmed vigilance state by EEG was missing in the previous reports) rats (21, 33) and mice (18). The contribution of $f_R$ rather than $V_T$ in unanesthetized animals (25) also coincides with the current results. Taken together, poikilocapnic condition appears to blunt the magnitude of LTF in intact animals (33), and the unmasking effect of natural sleep seems to be small, if any. Larger contribution of $f_R$ rather than $V_T$ indicates a more rhythmogenic than spinal mechanism of action in intact animals.

During REM sleep, only four of six WT mice showed LTF. We examined possible effects of IH in eight WT mice, but data from two mice were omitted from the analysis because these mice showed no REM sleep during the control period of 60 min. Since appearance of REM sleep is highly variable in mice, more studies with a prolonged time schedule are needed to make conclusions about the presence of LTF during REM sleep. At present, we can safely say that LTF can be observed during “sleep,” at least in mice.

As in the other mammals, we observed ventilatory LTF following IH but not SH. A difference in the acute hypoxic response appears to be the one of the reasons. During SH, $V_E$ decreased gradually to the premasking level (Figs. 3 and 4) as shown in the awake rats (33). Negative feedback by decreased arterial $PCO_2$ seems to decrease $V_E$ in the present poikilocapnic condition. On the other hand, increases in $V_E$ remained constant between five episodes of IH. As a result, $V_E$, averaged for the entire hypoxic periods of 25 min during SH (70 ± 16% for QW and 75 ± 20% for SWS) was significantly smaller than that during IH (132 ± 14% for QW and 182 ± 32% for SWS). Because the increases in $V_E$ during the first 5 min were comparable between SH and IH (Fig. 3), the differences between SH and IH could not be ascribable to interexperimental variation or repeated measurements in one animal. However, the difference in short-term hypoxic ventilatory responses between IH and SH cannot be the sole reason for the different appearance of LTF. At least in rats of isocapnic condition, there is still pattern sensitivity despite short-term ventilatory responses of the same size (33). Although the precise cellular mechanisms that differentiate the IH and SH are not clear, both the negative-feedback and the pattern-sensitive mechanisms seem to underline the different appearance of LTF after IH and SH in the present experimental condition.

### Table 1. Sigh and sleep apnea occurrence index before and after sham stimulation, sustained hypoxia, or intermittent hypoxia

<table>
<thead>
<tr>
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<th>Sham Stimulation</th>
<th>Sustained Hypoxia</th>
<th>Intermittent Hypoxia</th>
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<tr>
<td></td>
<td>WT</td>
<td>ORX-KO</td>
<td>WT</td>
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<tr>
<td>Quiet wake</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals</td>
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<td>6</td>
<td>10</td>
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<tr>
<td>Sigh, pre</td>
<td>19.2 ± 3.3</td>
<td>15.1 ± 6.0</td>
<td>20.4 ± 4.9</td>
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<tr>
<td>Sigh, post</td>
<td>12.2 ± 2.9</td>
<td>14.6 ± 4.5</td>
<td>10.4 ± 2.1</td>
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<tr>
<td>Post-sigh apnea, pre</td>
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<td>Post-sigh apnea, post</td>
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<td>ND</td>
<td>0.8 ± 0.5</td>
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<td>Spontaneous apnea, post</td>
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<td>ND</td>
<td>ND</td>
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<tr>
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Values are means ± SE. Some animals showed no rapid-eye-movement sleep during the prestimulation period of 60 min, and thus the data in such animals were excluded from this table. WT, wild-type mice; ORX-KO, prepro-orexin knockout mice; ND, not detected. There is no significant difference between the prestimulation values and the poststimulation values. *$P < 0.05$ compared with WT mice. †$P < 0.05$ compared with sham stimulation.
Post-sigh apnea index during QW periods tended to be smaller than that during SWS (Table 1) but clearly not zero in this study. In our previous study, however, we never observed any apnea during QW periods (28). Although the exact reason for this apparent discrepancy is not clear, we suspect possible contribution of the genetic background. We used WT mice of 129/Sv strain in the previous study and congenic C57BL/6 strain (>9 times backcross; 99.9%) in this study. Genetic background affects many respiratory phenotypes such as baseline respiration and chemoreflex ability (34, 42). Nevertheless, we found general agreement on sleep apnea indexes during prestimulation periods in the present study and those from our previous studies (28, 31), i.e., post-sigh apneas were observed mainly in SWS, whereas sighs and spontaneous apneas were observed during both SWS and REM sleep periods. Spontaneous apneas, but not post-sigh apneas, were significantly increased in ORX-KO. Although not examined in this study, sleep apnea in mice is thought to be CSA and not OSA both in WT and ORX-KO mice (28, 31).

Contrary to our expectation, LTF did not reduce the occurrence of post-sigh or spontaneous apneas (Table 1). This finding may result from WT mice showing only a small number of apneas even before stimulation, and therefore possible declines could not be detected. However, apnea indexes increased rather than decreased, although the difference did not reach statistical significance. In addition, apnea indexes in ORX-KO mice that lacked LTF showed a tendency to decrease after IH. It suggests that CSA may be decreased by other factor(s) than orexin or LTF after exposure to IH. There are ORX-KO mice that lacked LTF showed a tendency to decrease after IH. It suggests that CSA may be decreased by other factor(s) than orexin or LTF after exposure to IH. There are many hypoxia-respondent structures in the brain other than hypothalamus (where orexin neurons are located) and the raphe nuclei (where LTF is thought to be elicited). For example, hypoxia-inducible factor-1 is widely distributed in the brain, including hippocampus and cerebellar Purkinje cells (41). Therefore, it is difficult to speculate such factor(s) that may improve CSA. It may be of interest to point out that sustained mild hypoxia (15% \( \text{O}_2 \) for 6 h) increased CSA in our previous studies (28, 31). Thus influence of hypoxia may be pattern sensitive not only on LTF but also on the occurrence of CSA.

Although both CSA and OSA may cause IH and, hence, respiratory LTF, in turn, LTF may prevent successive occurrences of OSA but not (or to a lesser extent) CSA. LTF is expressed not only in motor outputs innervating respiratory pump muscles but also hypoglossal motor neurons that innervate the upper airway (1, 11). This observation suggests a prominent role of LTF in OSA. However, we found that the main contributor to increased \( \dot{V}t \) during LTF was \( \dot{V}T \) but not \( \dot{V}r \) in this study. If LTF was expressed mainly in hypoglossal motoneurons to decrease airway resistance, then we should observe an increase of \( \dot{V}r \) rather than \( \dot{V}T \). An alternative interpretation of the current results may be that LTF cannot promote breathing stability in such a way to prevent sleep apnea but only to compensate decreased arterial \( \text{PO}_2 \) in a wider time range than breath-by-breath respiratory control. However, we cannot deny the possibility that chronic IH (daily exposure to IH) may prevent sleep apnea.

Our hypothesis was that orexin might contribute to expression of LTF because LTF relies on serotonin-containing neurons in the raphe nuclei (9, 23, 24), and orexin-containing neurons in the hypothalamus send dense projections to the raphe nuclei (3). Current results in ORX-KO mice are in line with our hypothesis. However, important questions remain to be solved before making conclusive remarks. First, it is still an open question whether hypoxia activates orexin-containing neurons in the hypothalamus, although hypoxia activates some neurons in the hypothalamus (2) and hypercapnia activates orexinergic neurons, at least in vitro (45). Second, evidence for orexin-induced release of serotonin from the raphe nuclei is still lacking. Third, early reports using vagotomized and midcollicular decerebrated cats showed that LTF was well preserved without the hypothalamus; at least activity of the phrenic nerve was measured with electrical stimulation of the carotid sinus nerve (10, 24). These results apparently are at odds with our hypothesis. However, there is a report suggesting facilitatory influences of the diencephalon and inhibitory influence of the cerebrum on the hypoxic response, resulting in a null influence by midcollicular decerebration (43). Moreover, removal of the cerebellar vermis eliminates phrenic LTF (14), suggesting the importance of supra-pontine structures in LTF. These apparent discrepancies should be resolved by further experimentation. Fourth, apart from serotonin and orexin, nitric oxide (18), oxygen radicals (35), and hypoxia-inducible factor 1 (40) have been proposed to contribute generation of LTF. Although these hypotheses are not necessarily mutually exclusive, precise relationships should be determined for better understanding.

We suspect a possible involvement of orexin deficiency in some cases of human apnea. Human narcolepsy is thought to be caused by degeneration of orexin-containing neurons (44), and narcolepsy patients have a high incidence of sleep apnea (6). Some OSA patients show low levels of plasma orexin (4, 38), although an opposite result (15) and no change in CSF orexin (16) have also been reported. Because human sleep apnea is characterized by chronic IH, lack of LTF may enhance the incidence of sleep apnea, although acute IH in this study did not increase sleep apnea in ORX-KO mice.

In conclusion, this study supports our hypothesis that orexin is necessary for eliciting ventilatory LTF. Orexin deficiency may have clinical relevance to the etiology of some forms of sleep apnea. This study also showed, for the first time, ventilatory LTF following IH during both waking and sleep periods in WT mice. This observation supports the concept that IH can be used as a model of sleep apnea-associated decrease of oxygen supply during sleep. LTF did not affect the occurrence of CSA, at least in this experimental setup.

ACKNOWLEDGMENTS

We thank Dr. R. M. Harper at UCLA for valuable discussion and help in editing the manuscript.

GRANTS

Part of the study was supported by a Grant-in-Aid for Scientific Research (17590183, 18590203, 18790533) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grants from the Smoking Research Foundation and Mitsui Life Social Welfare Foundation.

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