Ventilatory long-term facilitation is greater in 1- vs. 2-mo-old awake rats

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McGuire, Michelle, and Ling Lim. Ventilatory long-term facilitation is greater in 1- vs. 2-mo-old awake rats. J Appl Physiol 98: 1195–1201, 2005. First published December 10, 2004; doi:10.1152/japplphysiol.00996.2004.—Respiratory long-term facilitation (LTF) declines in middle-aged vs. adult male rats. Chronic intermittent hypoxia (CIH; 5 min 11–12% O₂/5 min air, 12 h/night, 7 nights) enhances LTF in adult rats. However, LTF in immature rats and the effect of early CIH are unevaluated. The present study compared LTF in 1- and 2-mo-old rats and examined the effect of neonatal CIH (initiated at 2 days after birth) on the LTF. Ventilatory LTF, elicited by 5 (protocol 1) or 10 (protocol 2) episodes of poikilocapnic hypoxia (5 min 12% O₂/5 min air), was measured twice by plethysmography on the same conscious rat when it was 1 and 2 mo old. In untreated (without CIH) rats, both resting ventilation (54.7 ± 0.6 vs. 43.0 ± 0.2 ml·100 g⁻¹·min⁻¹) and hypoxic ventilatory response (131 ± 4 vs. 66 ± 3% above baseline) were greater in 1- vs. 2-mo-old rats. Protocol 1 elicited LTF in 1-mo-old (12.5 ± 1.0% above baseline) but not 2-mo-old rats. Protocol 2 elicited a greater LTF in 1-mo-old (24.3 ± 0.8%) vs. 2-mo-old rats (18.2 ± 0.5%). In CIH-treated rats, protocol 1 also elicited LTF in 1-mo-old (13.1 ± 1.5%) but not 2-mo-old rats. Protocol 2 elicited LTF in both age groups, but LTF was enhanced by the CIH only in 1-mo-old rats (28.8 ± 0.9%). These results suggest that ventilatory LTF and hypoxic ventilatory response are greater in immature rats shortly before their sexual maturity and that the neonatal CIH somewhat enhances ventilatory LTF ~3 wk after CIH, but this enhancement does not last to adulthood.

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almost exclusively measured several hours after the end of CIH treatment (21, 31, 32, 40). A recent study demonstrated that CIH performed on geriatric female rats enhanced both phrenic and hypoglossal LTF, suggesting that CIH performed at old ages still enhances LTF (50). To our knowledge, the effects of CIH performed at early ages (on LTF) have not been evaluated.

The aims of the present study were thus to (1) examine the effects of young age on LTF by measuring ventilatory LTF twice in the same awake rats at the ages of 1 and 2 mo and (2) explore the possible, persistent effect of neonatal CIH (initiated at 2 days after birth) on LTF by measuring ventilatory LTF in the CIH-treated rats at 1 and 2 mo of age and comparing with those in the age-matched untreated control rats. Because younger animals tend to have more flexibility and more potential plasticity (see above), we hypothesized that (1) ventilatory LTF would be greater in 1- vs. 2-mo-old rats and (2) the neonatal CIH would persistently enhance ventilatory LTF to adulthood.

Some results have appeared in abstract form (29, 30).

METHODS

The Harvard Medical Area Standing Committee on Animals approved all experimental procedures used herein. Experiments were conducted on 28 male Sprague-Dawley rats (colony 205, Harlan, Madison, WI), which were derived from six litters and weaned 3 wk after birth.

Ventilatory and Metabolic Measurements

Ventilatory measurements were made by use of a custom-made 3-liter whole body flow-through plethysmograph (Buxco Electronics, Sharon, CT). Individual unanesthetized, unrestrained rats were placed into the precalibrated plethysmographic chamber connected via a controlled leak to a reference chamber. The gas atmosphere within the animal chamber was maintained with air flowing through the chamber at a rate of 3 l/min. A bias flow was connected to an aerosol port of the chamber to maintain the O2 concentration within the animal chamber. A custom-made computer software system (Biosystem XA, Buxco Electronics, Sharon, CT) monitored the output from the differential pressure transducer (TRD5100) connected between the animal and reference chambers. This software system provides a breath-by-breath display of the breathing frequency (f), tidal volume (VT), and VE before, during, and after the following episodic hypoxia protocols used to elicit LTF.

The computer continuously monitored the output of the O2 and CO2 analyzers (Servomex Transducers Norwood, MA), which sense alternately inspired and expired gases. With the known flow rate, the measurements of the O2 and CO2 gas concentration were used to determine the VCO2 and VO2 production (VCO2). Body temperature was measured before and immediately after each experiment with a rectal thermometer.

Neonatal CIH

Mother rats and their pups were housed in normal rat cages and were given food and water ad libitum. At 2 days after birth, the cages were placed into a custom-made Plexiglas chamber (67 x 33 x 33 cm). This chamber was flushed with alternating mixtures of N2, O2, and air to achieve quasi-square wave intermittent hypoxia of 11–12% O2 for 5 min and normoxia for 5 min. It took ~30 s for the O2 level to drop to a target of 11% and also ~30 s to return to 21%. Gas mixtures were flushed at a rate sufficient to maintain the chamber CO2 levels below 0.5%. This intermittent hypoxia was repeated for 12 h per night (from 6:00 PM to 6:00 AM) for 7 consecutive nights as previously described (21, 31). The chamber was open to room air during the remaining 12 h each day. The temperature in the chamber was maintained between 20 and 22°C (which was similar to the housing facility temperature) at all times. Once the CIH treatment was finished, cages were returned to the animal housing facility and the pups were left to mature.

General Experimental Procedures

Rats were placed in the plethysmographic chamber and allowed to adapt to the chamber for ~1 h. One-month-old rats were also placed in the chamber 1 day before to acclimatize to the chamber, which had been shown to reduce rats’ movement during measurements. Baseline (resting VE) was then measured over 10 min, and VE was continuously monitored throughout the AIH stimulus protocols. During this period, rats were exposed to 5 min of poikilocapnic hypoxia (12% O2) followed by 5 min of air exposure. This cycle was repeated to achieve the episode number in the particular protocol. In the chamber, the shift from normoxia to the target hypoxia level (12% O2) took <1 min, and the shift from hypoxia to normoxia took <30 s. During these hypoxic and normoxic episodes, only the final 2 min data of each episode were averaged and included in the data analysis. The HVR is defined as an increase from baseline to hypoxic VE, normalized as a percentage of the baseline. After the last hypoxia episode, VE was measured at 15-min intervals (i.e., 15, 30, 45, and 60 min), with each value representing a 5-min average (e.g., the 15-min posthypoxia value is an average of the data collected between 15 and 20 min). Ventilatory LTF is defined as an increase from baseline in posthypoxia VE, normalized as a percentage of baseline.

Protocol 1. Rats were exposed to five episodes of 5-min poikilocapnic hypoxia (12% O2), interspersed with 5-min intervals of normoxia. VE was measured before, during, and up to 60 min after AIH to determine resting VE, HVR, and ventilatory LTF, respectively. This protocol was used to elicit ventilatory LTF in both untreated rats (i.e., animals not exposed to CIH; n = 7) and CIH-treated rats (n = 7) and was conducted twice on the same rats when they were 1 and 2 mo old. Data were collected at about the same time of day (12:00–2:00 PM) in each experiment.

Protocol 2. Rats were exposed to 10 episodes of 5-min 12% O2 with 5-min normoxic intervals, and VE was measured before, during, and up to 60 min after AIH. This protocol was also used to elicit ventilatory LTF in both untreated (n = 7) and CIH-treated rats (n = 7) and was conducted twice on the same rats at the age of 1 and 2 mo during a similar time period of day (12:00–2:00 PM). The purpose of using two stimulus protocols with different effectiveness for eliciting LTF (28) was to examine both ventilatory LTF and the stimulus intensity threshold for eliciting LTF (cf. Ref. 31).

Metabolic rate. Accompanying those ventilatory measurements, both VCO2 and VO2 were simultaneously measured before (baseline) and after the AIH stimulus protocol in each experiment. VCO2 was also measured during AIH. These measurements were also conducted twice on the same (untreated and CIH-treated) rats when they were 1 and 2 mo old. However, VO2 was not measured during AIH since our plethysmographic system had not been modified to measure this metabolic parameter during AIH at that time.

Data Analysis

Ventilatory (VE, VT, and f) and metabolic (VCO2 and VO2) parameters were measured following a strict rule, as previously described (29, 31). Briefly, these parameters were measured in rats when they were observed to be awake and in a quiet state. Data recorded when the rats were moving or appeared to be asleep (e.g., eyes closed or heads curled under their bodies) were rejected from the analysis. This rejection was done blindly; i.e., values were unknown when these data were rejected. Our criteria for data acceptance are the combination of (1) open eyes, (2) no body movement, and (3) a normal breathing pattern displayed continuously on the computer screen. Furthermore, an additional rejection algorithm was included in the computer software.
ventilatory long-term facilitation in young awake rats

result

basal hypoxia and surfactant production (ml

ventilatory long-term facilitation in 1-mo-old untreated rats (12.5 ± 1.0% above baseline; Fig. 1). This protocol also elicited both Vt and f LTF in 1-mo-old rats, with smaller magnitude and shorter duration compared with those of Ve LTF (Fig. 1B). However, protocol 1 failed to elicit LTF of Ve, Vt, or f in the same rats when they were 2 mo old (Fig. 1). These data are consistent with our laboratory’s previously reported results (28, 31), in which five episodes of 12% O2 also failed to elicit ventilatory LTF in 3- to 6-mo-old rats.

protocol 2. Ten episodes of 12% O2 elicited ventilatory LTF in both 1- and 2-mo-old untreated rats, and LTF magnitude was larger (24.3 ± 0.8 vs. 18.2 ± 0.5%), respectively; P < 10-4) and LTF duration was longer (60 vs. 45 min, respectively) in 1- vs. 2-mo-old rats (Fig. 2). Ventilatory LTF in 1-mo-old rats was a result of increases in both Vt and f, whereas LTF in 2-mo-old rats was mainly due to an increase in f (Fig. 2B).

ch effect. Ventilatory LTF, elicited by protocol 1, was similar between untreated and CIH-treated rats at both ages (both P > 0.73; Fig. 3, top), suggesting that the neonatal CIH does not enhance the protocol 1-induced LTF at either age. Ventilatory LTF, elicited by protocol 2, was greater (P < 0.002) in CIH-treated rats (28.9 ± 0.9%) vs. untreated rats (24.3 ± 0.8%) at the age of 1 mo (Fig. 3, bottom). However, this difference disappeared when the two groups of rats reached the age of 2 mo (P > 0.1; Fig. 3, bottom), indicating that the neonatal CIH treatment enhances the protocol 2-induced LTF in 1- but not 2-mo-old rats.

discussion

the present study demonstrated that baseline Ve and metabolic rate as well as hypoxic Ve and HVR (all normalized to rats’ body weights) were all greater in 1- vs. 2-mo-old untreated awake rats. Five episodes of 12% O2 elicited LTF in 1- but not 2-mo-old rats. Ten episodes of 12% O2 elicited a

Table 1. ve and metabolic rate during baseline and hypoxia (12% O2) in untreated and CIH-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Baseline Ve</th>
<th>Baseline Vco2</th>
<th>Baseline Ve/Vco2</th>
<th>Baseline Ve/Vo2</th>
<th>Baseline Vco2/Vo2</th>
<th>Hypoxic Ve</th>
<th>Hypoxic Vco2</th>
<th>Hypoxic Ve/Vco2</th>
<th>HVR % Above Baseline</th>
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<tr>
<td>1 mo</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 14)</td>
<td>54.7±0.6</td>
<td>6.2±0.5</td>
<td>5.0±0.2</td>
<td>9.4±0.5</td>
<td>11.3±0.4</td>
<td>126.2±1.7*</td>
<td>23.1±1.2*</td>
<td>130.9±4.3</td>
<td></td>
</tr>
<tr>
<td>CHI-treated (n = 14)</td>
<td>54.4±0.3</td>
<td>5.9±0.3</td>
<td>4.8±0.2</td>
<td>9.5±0.5</td>
<td>11.6±0.6</td>
<td>129.1±2.2*</td>
<td>23.4±1.5*</td>
<td>136.9±4.4</td>
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<tr>
<td>2 mo</td>
<td></td>
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<tr>
<td>Untreated (n = 14)</td>
<td>43.0±0.2†</td>
<td>2.9±0.1†</td>
<td>3.2±0.1†</td>
<td>15.1±0.7†</td>
<td>13.7±0.5†</td>
<td>71.5±1.5†</td>
<td>18.9±0.4†</td>
<td>66.2±3.3†</td>
<td></td>
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<tr>
<td>CHI-treated (n = 14)</td>
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<td>3.1±0.1†</td>
<td>3.4±0.2†</td>
<td>14.1±0.7†</td>
<td>13.3±0.7</td>
<td>70.5±1.1†</td>
<td>18.6±0.8†</td>
<td>65.7±3.2†</td>
<td></td>
</tr>
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</table>

Values are means ± SE. CHI, chronic intermittent hypoxia; Ve, minute ventilation (ml·100 g-1·min-1); Vo2, O2 consumption (ml·100 g-1·min-1); Vco2, CO2 production (ml·100 g-1·min-1); HVR, hypoxic ventilatory response to 12% O2 (% increase above baseline). All values were combination of 2 protocol groups (5 and 10 episodes of 12% O2) since there was no difference between them. *Significant difference from baseline. †Significant difference from 1-mo old rat (P < 0.05).
greater LTF in 1- vs. 2-mo-old rats. None of these parameters was different between untreated and CIH-treated rats, except that ventilatory LTF, elicited by 10 episodes of 12% O₂ hypoxia (protocol 1), was greater in CIH-treated vs. untreated rats at the age of 1 mo. Collectively, these data suggest that the stimulus intensity threshold for eliciting LTF is lower and LTF is greater in male rats shortly before their sexual maturity and that, although the neonatal CIH somewhat enhances ventilatory LTF in 1-mo-old awake rats, this enhancement has disappeared when the rats are 2 mo old.

**Methodological Consideration**

The use of barometric plethysmography to measure Vₑ and metabolic rate in awake rats provided a more natural and physiological way to investigate the effects of young age and neonatal CIH treatment on respiratory LTF, because possible confounding issues related to anesthesia, surgery, and restraint could be eliminated. In the neonatal CIH treatment, rats were placed into the controlled chamber 2 days after their birth to avoid possible interference with the putative postnatal resetting process in the hypoxic chemoreflex (15).

The present study was conducted according to within-subject as well as between-subject experimental designs. The age effect on LTF was examined in the same rats by measuring LTF when they were 1 and 2 mo old. In contrast, the CIH effect on LTF was examined in separate groups, although the comparison between untreated and CIH-treated groups was also made at both ages. This experimental design plus the strict rule

![Fig. 1](http://jap.physiology.org/)

**Fig. 1.** Different ventilatory long-term facilitation (LTF) in 1- (■) and 2-mo-old rats (□) elicited by 5 episodes of 12% O₂ hypoxia (protocol 1). **A:** minute ventilation (Vₑ) measured before [baseline (BL)] and up to 60 min after protocol 1. **B:** average LTF magnitude and duration of Vₑ, tidal volume (VT), and breathing frequency (f). LTF magnitude was calculated using the 3-value-average method (see METHODS). The time (min) on the columns in **B** is the LTF duration. Values are means ± SE. Many error bars are totally masked by the relatively bigger symbols (P < 0.05). *Significantly different from baseline. †Significantly different from the younger group (P < 0.05).

![Fig. 2](http://jap.physiology.org/)

**Fig. 2.** Different ventilatory LTF in 1- (■) and 2-mo-old rats (□) elicited by 10 episodes of 12% O₂ hypoxia (protocol 2). **A:** Vₑ measured before (BL) and up to 60 min after protocol 2. **B:** average LTF magnitude and duration of Vₑ, VT, and f. LTF magnitude was calculated using the 3-value-average method (see METHODS). The time (min) on the columns in **B** is the LTF duration. Values are means ± SE. Many error bars are totally masked by the relatively bigger symbols. *Significantly different from baseline (P < 0.05). †Significantly different from the younger group (P < 0.05).

![Fig. 3](http://jap.physiology.org/)

**Fig. 3.** Effect of neonatal chronic intermittent hypoxia on ventilatory LTF measured in 1- and 2-mo-old rats. **Top:** ventilatory LTF elicited by 5 episodes of 12% O₂ hypoxia (protocol 1) in the untreated (closed column) and chronic intermittent hypoxia-treated rats (open column). **Bottom:** ventilatory LTF elicited by 10 episodes of 12% O₂ hypoxia (protocol 2). LTF magnitude was calculated using the 3-value-average method (see METHODS). The time (min) on the columns is the LTF duration. Values are means ± SE. *Significantly different from baseline (P < 0.05). †Significantly different from untreated rats (P < 0.05).
Ventilatory LTF in Young Rats

One difference between 1- and 2-mo-old rats is their sexual maturity. The early puberty period of (both male and female) Sprague-Dawley rats is approximately between 33 and 40 days of age, in which sexual hormone levels are substantially increased (12). However, some Sprague-Dawley rats were observed to possess reproductive function as early as 1 mo (17). We thus speculate that the density of serotonin receptors in the region of phrenic and hypoglossal motor nuclei is higher in immature rats vs. young adult rats, and the density further declines with advancing age, or on the motoneurons is higher in immature rats vs. young adult rats, and the density further declines with advancing age, thereby eliciting a greater LTF in immature rats but a smaller/no LTF in aged rats. We previously hypothesized that CIH enhances phrenic LTF via its upregulation of serotonin recep-

Effect of Neonatal CIH on LTF

Our previous study demonstrated that the same CIH treatment performed on adult rats enhances ventilatory LTF measured at several hours after CIH and that the enhancement persists partially at 3 days but disappears at 7 days after the CIH (31). The present study showed that ventilatory LTF was greater in the neonatal CIH-treated rats at the age of 1 mo, but this CIH effect had disappeared when rats reached the age of 2 mo. These results suggest that the neonatal CIH-induced enhancing effect on LTF persists for >3 wk but <7 wk, thus a much longer persistence period than the adult-time CIH-induced one. These results, however, do not fully support our second hypothesis that the neonatal CIH persistently enhances LTF to adulthood. There is a possibility that the neonatal CIH also produced a larger enhancement in LTF than the adult CIH does and that this enhancement had waned when we conducted experiments at 1 mo of age. Our experimental design, however, was unable to test this hypothesis since we did not measure LTF until ∼3 wk after CIH.

Potential Mechanisms

It has been long known that respiratory LTF requires serotonergic mechanisms. LTF can no longer be elicited by repeated carotid sinus nerve stimulation or AIH after serotonin receptor antagonism with methysergide (4, 8). More specifically, hypoxia-induced ventilatory and phrenic LTF are eliminated by 5-HT2 receptor antagonism with ketanserin (20, 21, 32). The CIH effect on LTF also depends on serotonin receptors, although different (non-5-HT2) serotonin receptors, perhaps 5-HT6 and/or 5-HT7 subtype(s), are involved (32). LTF decreases with advancing age in male rats, e.g., both phrenic and hypoglossal LTF were significantly greater in young adult rats (3–4 mo) than in middle-aged rats (13 mo), and this age effect on LTF was mainly attributed to the decline of the serotonergic modulation of respiratory motor output in aged male rats (5, 49).

We speculate that the LTF difference between the two age groups in the present study can also be accounted for by the alteration of serotonergic mechanisms with advancing age. One remarkable feature of aging is a gradual decline of key neurotransmitter receptor density (37, 45, 46), which is responsible for many functional deficits in older humans and animals (37, 41, 45). For example, the density of 5-HT2 receptors declines substantially with aging in both human and rodents (36). In the cortex of rats, the density of 5-HT2 receptors also decreases from 1 to 7 mo of age and remains at this level to 12 mo (17). We thus speculate that the density of serotonin receptors in the region of phrenic and hypoglossal motor nuclei or on the motoneurons is higher in immature rats vs. young adult rats, and the density further declines with advancing age, thereby eliciting a greater LTF in immature rats but a smaller/no LTF in aged rats. We previously hypothesized that CIH enhances phrenic LTF via its upregulation of serotonin recep-
tors on phrenic motoneurons (32). Similarly, the early postnatal CIH might induce a relatively larger and more persistent upregulation of serotonin receptors, thereby causing a greater and more persistent LTF enhancement. It should be noted, however, that these mechanistic discussions are mostly speculative and await more direct, experimental verification.

Recent evidence suggests that sex hormones may also play an important role in LTF, and it has been hypothesized that the diminished LTF in aged male rats may result from decreases in androgen levels with advancing age (49). Gonadectomy in male adult rats has been reported to produce a decrease in testosterone levels associated with a reduction in LTF magnitude, which can be restored by subsequent testosterone replacement (6). However, it is unlikely that the androgen level difference can explain the greater LTF in immature rats in the present study because 1-mo-old male rats (before their puberty period) presumably have lower testosterone levels than 2-mo-old rats, and testosterone appears to play a facilitative role for LTF (6).

Significance

LTF saturates, such that more episodes of stimulation cause no further increase in LTF magnitude (8, 28). The CIH effect on LTF also seems to saturate and appears to be similar when different protocols (e.g., different durations of hypoxic episode and normoxic interval, during daytime or nighttime) are used (McGuire M and Ling L, unpublished observations). The AIH and CIH protocols that mimic hypoxic events in OSA patients were also reported to elicit similar phrenic LTF and the CIH effect on LTF (40). Thus, although the AIH and CIH protocols in the present study were not explicitly designed to simulate OSA events, our results may still have some clinical implication. LTF of ventilation and genioglossal electromyographic activity was not elicited in normal humans during wakefulness (18, 27), but it was elicited in those with inspiratory flow limitation (e.g., snorers and OSA patients) during non-rapid eye movement sleep, mainly as a persistent decrease in upper airway resistance, an indication of upper airway dilatation due to motor output LTF of dilator muscles (1, 2, 3, 43). Ventilatory LTF was also elicited in some patients (2, 3, 43) but was abolished after eliminating the flow limitation by continuous positive airway pressure (3). Thus LTF may temporarily stabilize breathing and upper airway patency in OSA patients after repeated apneas/hypopneas, and CIH may somewhat increase and prolong this beneficial effect since CIH enhances LTF (21, 31) and reduces the stimulus intensity threshold for eliciting LTF (31).

Prevalence of OSA is considerably higher in old/geriatric vs. adult population (25, 48). LTF of hypoglossal nerve activity, which innervates the major upper airway dilator muscle genioglossus, was absent in middle-aged male rats (49). It has thus been speculated that the age-related loss of LTF in upper airway dilator muscle activity may contribute to the increase in OSA prevalence with aging (5, 49). The present study demonstrated that both LTF and the CIH effect on LTF are greater and/or last longer in immature vs. adult male rats. Although caution must be used when extrapolating animal data to human subjects, our data suggest the possibility that the young age-related enhancement of LTF and/or the CIH effect on LTF may somewhat contribute to the relatively less prevalent OSA in the immature population, such as middle school children and younger teenagers (cf. Ref. 26).

GRANTS

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