Expression of hypoglossal long-term facilitation differs between substrains of Sprague-Dawley rat

D. D. FULLER, T. L. BAKER, M. BEHAN, AND G. S. MITCHELL
Department of Comparative Biosciences, University of Wisconsin, Madison, Wisconsin 53706
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Fuller, D. D., T. L. Baker, M. Behan, and G. S. Mitchell. Expression of hypoglossal long-term facilitation differs between substrains of Sprague-Dawley rat. Physiol Genomics 4: 175–181, 2001.—Long-term facilitation (LTF) is a prolonged, serotonin-dependent augmentation of respiratory motor output following episodic hypoxia. Previous observations lead us to hypothesize that LTF is subject to genetic influences and, as a result, differs between Sprague-Dawley (SD) rats from two vendors, Harlan (H) and Charles River Laboratories/Sasco (CRL/S). Using a blinded experimental design, we recorded integrated phrenic (Pphr) and hypoglossal neurograms in anesthetized, vagotomized, paralyzed, and ventilated rats. At 60 min following three 5-min hypoxic episodes (PaO2 = 40 ± 1 Torr; 5-min hyperoxic intervals), Pphr was elevated from baseline in both SD substrains (i.e., LTF; P < 0.05). Conversely, hypoglossal LTF was present in CRL/S but not H rats (P < 0.05 between substrains). Serotonin immunoreactivity within the hypoglossal nucleus was not different between H and CRL/S rats. We conclude that the expression of hypoglossal LTF differs between SD rat substrains, indicating a difference in their genetic predisposition to neural plasticity.

respiratory control; plasticity; genetics

AN APPRECIATION OF GENETIC influences on the neural control of breathing in mammals is beginning to emerge (13, 20, 31). For example, different strains, and in some cases, substrains of mice (28–30) and rats (27) exhibit variable respiratory responses to hypoxia and hypercapnia. Respiratory control is also influenced by experience or environmental factors (i.e., plasticity; Refs. 11, 19, 25). Although both genetic factors and plasticity influence respiratory motor control, there have been few, if any, studies examining how genetics influence plasticity. The hypoxic ventilatory response is subject to both genetic (9, 27–30) and experience-dependent influences (11, 19, 25). Accordingly, detailed examination of the hypoxic response in genetically distinct populations may help define genetic influences on the expression of plasticity in respiratory control.

The hypoxic ventilatory response is complex, consisting of several time-dependent mechanisms (25). A single brief exposure to hypoxia (e.g., 5 min) augments respiratory motor output (short-term hypoxic response) by at least three mechanisms: the acute response, short-term depression, and short-term potentiation (25). Following a single hypoxic exposure in anesthetized rats, respiratory burst amplitude returns gradually to prehypoxic values (2). On the other hand, exposure to repeated hypoxic episodes, separated by periods of normoxia or hyperoxia, evokes a long-lasting (≥1 h), serotonin-dependent augmentation of inspiratory motor output known as long-term facilitation (LTF) (12, 19, 25). LTF represents a form of plasticity since respiratory motor output is altered by the experience of episodic hypoxia.

Over a period of several years, our laboratory has observed considerable unexplained variability in LTF in anesthetized Sprague-Dawley (SD) rats. Originally, significant phrenic and hypoglossal (XII) LTF was consistently observed (1). In subsequent studies, phrenic LTF appeared somewhat smaller and XII LTF was not detectable (3, 17). The only apparent difference between these studies is that SD rats were initially obtained from Sasco (S) and subsequently from Harlan (H). These consistent (but uncontrolled) observations strongly suggested differences in the genetic predisposition to neural plasticity (in the form of LTF) between H and S SD rats. Other investigators have documented neuroanatomical and physiological differences between H and S SD rats (7, 8, 26, 32, 34, 35), indicating that a genetic drift has occurred between these two SD rat populations. Therefore, we speculated that genetic differences observed between H and S rats would influence components of the hypoxic response. Specifically, we hypothesized that the capacity for XII LTF expression is different between H and S SD rats. In the present study, SD rats were obtained from Harlan and Charles River Laboratories (CRL, which now maintains the S SD colonies) and directly compared using our established LTF protocol (1, 16, 17) with a blinded experimental design.

METHODS

Experiments were performed on 300- to 400-g male SD rats obtained from Harlan (rat colony 236, Madison, WI; N = 11) and Charles River Laboratories (rat colony K-62, Kingston, NY; N = 18). Both colonies are outbred (i.e., sibling and/or parent/offspring mating is prevented) and are of similar size (15,000–20,000 animals per colony). Prior experiments in our laboratory were performed on rats obtained from Sasco (1), which was subsequently purchased by
Charles River Laboratories. Charles River Laboratories has continued the Sasco SD rat colonies; thus these rats will be referred to as CRL/S. To minimize concerns regarding familial influences on LTF, H and CRL/S rats were obtained from at least four different mothers. The Animal Care and Use Committee at the University of Wisconsin-Madison approved all experimental procedures.

Experimental preparation. Anesthesia was induced with isoflurane and maintained (2.5–3.5%, \( F_{\text{IO}_2} = 0.5 \), balance \( N_2 \)) via a nose cone. The trachea was cannulated to permit mechanical ventilation with continued isoflurane anesthesia. A catheter was inserted into the femoral vein, and rats were gradually converted from isoflurane to urethane anesthesia (1.6 g/kg in distilled water). The adequacy of anesthesia was monitored throughout an experiment by observing blood pressure responses to toe pinch. To maintain fluid balance, a 5% sodium bicarbonate and lactated Ringer solution (50:50) was infused via the venous catheter at a rate of 1.7 ml-kg \(^{-1}\)-h \(^{-1}\) beginning ~1 h after urethane administration. A femoral arterial catheter enabled blood pressure measurement, monitoring of \( P_{\text{TCO}_2} \), and withdrawal of blood samples. The arterial partial pressures of \( O_2 \) (\( P_{\text{AO}_2} \)) and \( CO_2 \) (\( P_{\text{ACO}_2} \)) and pH were determined from 0.2-ml arterial blood samples (model ABL-500; Radiometer, Copenhagen, Denmark). The vagus nerves were cut bilaterally in the midcervical region, and the animals were paralyzed with pancuronium bromide (2.5 mg/kg) to prevent spontaneous breathing movements and entrainment of the neurograms with the ventilator. The end-tidal \( CO_2 \) partial pressure (\( PET_{\text{CO}_2} \)) was measured using a rapidly responding flow through \( CO_2 \) analyzer (Novametrix, model 1265) placed on the expired line of the ventilator circuit. Rectal temperature was maintained at 37–38°C using a rectal thermistor and a heated table. At the conclusion of all experiments, rats were euthanized via urethane overdose.

Nerve recordings. The phrenic and XII nerves were isolated using a dorsal approach, cut distally, desheathed, bathed in mineral oil, and placed on bipolar silver wire electrodes. Nerve signals were amplified (×10,000) and filtered (300–10,000 Hz) (model 1800; A-M Systems, Carlsborg, WA). The amplified signal was full-wave rectified and integrated (time constant = 50 ms, model MA-821RSP; CWE, Ardmore, PA) and digitized for recording on a computer (sample rate 200 Hz) using WINDAQ software (Dataq Instruments, Akron, OH).

Experimental protocol. The LTF protocol used in our laboratory has been described previously (1, 16, 17). Following conversion to urethane anesthesia, the preparation was allowed to stabilize over a 30- to 60-min period. The \( CO_2 \) apneic threshold for inspiratory nerve activity was determined by hyperventilating the rats until inspiratory nerve activity ceased, then decreasing the ventilator rate and allowing \( PET_{\text{CO}_2} \) to gradually rise until inspiratory activity reappeared. The \( PET_{\text{CO}_2} \) at which inspiratory activity resumed was noted, and \( PET_{\text{CO}_2} \) was then maintained 3 Torr above this value. In this manner, baseline neural activity was standardized relative to the \( CO_2 \) apneic threshold.

A few minutes prior to episodic hypoxia, an arterial blood sample was drawn, and subsequent samples were referenced to this baseline measurement. Rats were then exposed to three 5-min episodes of hypoxia (\( F_{\text{IO}_2} = 0.12–0.14 \)), separated by 5-min hyperoxia (\( F_{\text{IO}_2} = 0.50 \)). Arterial blood was sampled during the fourth minute of the first hypoxic episode, and if \( P_{\text{AO}_2} \) was not between 35 and 45 Torr, then \( F_{\text{IO}_2} \) was adjusted accordingly for the next hypoxic episode. Phrenic and XII nerve activities were monitored for 1 h post-episodic hypoxia, during which time isocapnic conditions were maintained.
additional CRL/S rats using a nonblinded experimental design. These experiments were done to ensure that differences between H and CRL/S rats were not due to the hypo/hypocapnic response in the blinded CRL/S rats.

Phrenic and XII nerve activities and mean arterial pressure (MAP) were averaged over 30-s periods prior to the first hypoxic episode (baseline), at the conclusion of each hypoxic episode (i.e., during the final 30 s of each hypoxic episode), 15, 30, and 60 min following episodic hypoxia (i.e., coincident with arterial blood samples), and during the post-LTF hypcapnic response. Peak integrated phrenic and XII amplitudes (\( \Delta \text{Phr} \) and \( \Delta \text{XII} \)) and burst frequency (bursts/min) were assessed. \( \Delta \text{Phr} \) and \( \Delta \text{XII} \) were quantified by measuring the peak height of the integrated neurogram, and these values were expressed relative to both baseline activity and maximal hypercapnic values. This dual normalization procedure minimizes concerns regarding potential artifacts that may occur when comparing neurograms within and between experimental preparations. We present most data as a percent change from baseline, since data were qualitatively similar when expressed in either manner. Baseline neural activities are expressed as a percentage of the \( \text{CO}_2 \) response (% max).

MAP was calculated as diastolic blood pressure + (pulse pressure/3).

Table 1. \( \text{PaCO}_2 \) and \( \text{PaO}_2 \) data from the blinded protocol during baseline, the first hypoxic episode, and post-episodic hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Hypoxia</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
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<tbody>
<tr>
<td>( \text{PaCO}_2 )</td>
<td></td>
<td></td>
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<tr>
<td>H</td>
<td>46 ± 1</td>
<td>46 ± 1</td>
<td>46 ± 1</td>
<td>46 ± 1</td>
<td>46 ± 1</td>
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<tr>
<td>CRL/S</td>
<td>242 ± 7</td>
<td>207 ± 9</td>
<td>215 ± 9</td>
<td>220 ± 9</td>
<td></td>
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<tr>
<td>( \text{PaO}_2 )</td>
<td>44 ± 1</td>
<td>44 ± 1</td>
<td>44 ± 1</td>
<td>44 ± 1</td>
<td>44 ± 1</td>
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<tr>
<td></td>
<td>236 ± 8</td>
<td>230 ± 7</td>
<td>252 ± 7</td>
<td>247 ± 18</td>
<td></td>
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</table>

Values are means ± SE and are expressed in Torr. Mean arterial pressure (MAP) was lower in CRL/S rats (Table 2; see Critique of methods). Efforts to prevent MAP from decreasing post-episodic hypoxia were not always successful, and, as a result, mean MAP fell as the LTF protocol progressed; however, MAP declined to a similar extent in both H and CRL/S rats. Blood pressure.

Blood gases. Although arterial isocapnia was maintained during hypoxia in the H rats (±1 Torr), the CRL/S rats became hypocapnic during hypoxia (mean drop of 6 ± 2 Torr; \( P < 0.05 \)). Thus additional experiments were conducted on CRL/S rats in which arterial \( \text{CO}_2 \) was maintained isocapnic during hypoxia (±1 Torr). Arterial isocapnia was successfully maintained post-episodic hypoxia in both substrains (Table 1).
sodic hypoxia in both substrains (Fig. 2, bottom), but a similar increase in fXII is seen only in the CRL/S rat (Fig. 2, top). In contrast, little change in fXII post-episodic hypoxia is observed in the H rat (Fig. 2, top). Mean fXII and fPhr changes following episodic hypoxia are shown in Fig. 3. Phrenic LTF occurred in both CRL/S and H rats, and the magnitude of LTF was not significantly different between substrains. Conversely, XII LTF was significantly greater in CRL/S than H rats ($P < 0.05$); in fact, XII LTF was not significant in H rats at any time point. Because of concerns regarding hypocapnia during hypoxia (see METHODS), additional LTF experiments were conducted on CRL/S rats ($N = 5$). These animals were maintained isocapnic ($\pm 1$ Torr) during hypoxia, and the magnitude of LTF was not different ($P > 0.05$) from that presented in Fig. 3 ($\Delta$XII 60 min post-episodic hypoxia = 66 ± 9% baseline; $\Delta$Phr = 53 ± 24% baseline). Burst frequency was not significantly different from baseline at any time following episodic hypoxia in either SD substrain.

To increase our statistical power, XII data from the present study were pooled with data collected previously in our laboratory using a nonblinded approach, which increased the number of successful XII LTF protocols in H rats to 26 (Fig. 4). With this large sample size, the small increase in fXII 60 min post-episodic hypoxia ($20 \pm 8 \%$ baseline) was significant ($P = 0.04$) but was still less than the increase seen in CRL/S rats ($P < 0.05$). *Different from baseline (i.e., time 0). †Different from corresponding H data point.

Serotonin immunoreactivity. The area occupied by serotonin immunoreactivity in the four quantified re-

![Fig. 2](image1.png)

**Fig. 2.** Representative XII and phrenic neurograms during an LTF protocol: integrated XII (fXII, top) and integrated phrenic (fPhr, bottom) amplitudes during baseline (BL), the first (of 3) hypoxic episodes (H1), 30 and 60 min following episodic hypoxia, and during the hypercapnic exposure which ended each experiment (CO2). Note the considerable augmentation of fXII (relative to baseline) following episodic hypoxia in the CRL/S rat. Conversely, fXII remained relatively constant in the H rat following episodic hypoxia.

![Fig. 3](image2.png)

**Fig. 3.** Mean long-term facilitation (LTF) of fXII (top) and fPhr (bottom) following episodic hypoxia in H (solid circles) and CRL/S (open circles) rats. Changes ($\Delta$) in peak integrated amplitude are expressed as a percentage of the baseline values. Significant LTF was seen in phrenic neurograms of both substrains ($P < 0.05$); however, XII LTF was only statistically significant in the CRL/S rats ($P < 0.05$). *Different from baseline (i.e., time 0). †Different from corresponding H data point.

![Fig. 4](image3.png)

**Fig. 4.** LTF of XII inspiratory output in H rats. Data from the current blinded experimental protocol ($N = 7$, solid bars) were pooled with nonblinded data collected previously in our laboratory ($N = 26$, open bars). This approach was taken to increase the statistical power of the analysis. The pooled data demonstrate that XII LTF at 60 min post-episodic hypoxia in H rats is statistically significant, but considerably smaller than that seen in CRL/S rats. *Different from baseline.
variations in the XII nucleus for H and CRL/S rats is presented in Fig. 5. An apparent trend for increased XII serotonin labeling in H rats was not statistically significant ($P = 0.31$). Thus there were no differences in XII serotonin immunoreactivity that could account for differential XII LTF.

**DISCUSSION**

Although differential rearing practices cannot be conclusively ruled out, our results indicate that a form of serotonin-dependent plasticity in respiratory motor control (i.e., LTF) is subject to genetic influences and that these genetic influences are specific (and perhaps unique) to XII motor output. SD rats obtained from two different commercial vendors were subjected to identical LTF protocols, and XII LTF was significantly greater in CRL/S SD than H SD rats. Conversely, phrenic LTF was not different between these substrains, although the magnitude of phrenic LTF in H rats was 30–40% greater than previous studies on H SD rats from a different colony (16, 17). Immunohistochemical analyses revealed no significant strain differences in serotonin immunoreactivity within the XII nucleus that could account for differential XII LTF.

**Critique of methods.** Significant hypotension was present in both H and CRL/S rats 60 min post-episodic hypoxia, with both SD substrains demonstrating a similar decline in MAP relative to baseline. Hypotension could result in increased respiratory motor output due to alterations in baroreceptor activity or brain blood flow. However, we argue that hypotension-related effects did not create, or significantly contribute to, LTF in either substrain. Our argument is based on linear regression analysis, which revealed no correla-

**Genetic influences on respiratory control.** Strong conclusions regarding genetic influences on respiratory control in humans are difficult to make, because of difficulties controlling environmental influences. However, numerous studies have documented respiratory control differences between geographically isolated human populations (for review, see Ref. 20). Moreover, monozygotic twins demonstrate greater similarity in their hypoxic ventilatory responsiveness than do dizygotic twins (9, 15). These and other observations have lead investigators to examine genetic influences on respiratory control in inbred rodent models, where genetic differences are more consistent and environmental or experimental factors can be more carefully controlled. For example, Tankersley and colleagues (28) examined hypoxic and hypercapnic ventilatory responses in eight strains of inbred mice. Substantial variability in ventilatory responses was seen between mouse strains, leading to the conclusion that genetic factors contributed to differential respiratory control. Further work showed that differences in the breathing patterns between two mouse strains could be accounted for by polymorphisms in as few as two autosomal genes (29). Moreover, it also appears that variations in the rat genome contribute to ventilatory phenotype differences among rat strains (13, 27).

Genetic differences in respiratory control also appear to exist between rat substrains, particularly within the SD strain (22–24). The SD strain originated in Madison, WI, in 1924 and was maintained as an outbred strain. Subsequently, independent, isolated colonies of SD rats have been established and maintained by different commercial vendors (i.e., SD “substrains”). The S SD line diverged from the original Madison colony in 1979, and Charles River Laboratories purchased (and continued to maintain) the Sasco colonies in 1998. The Harlan SD line continues in Madison, WI, from the original Madison SD strain. Several physiological and neuroanatomical studies indicate that genetic drift has created genetically distinct SD substrains (7, 8, 22–24, 26, 28, 34, 35). For example, SD rats obtained from Harlan (colony 205) vs. Hilltop (Scottsdale, PA) have strikingly different responses to sustained hypoxia (22). Hilltop SD rats are unable to survive exposure to simulated altitude (18,000 or 20,000 ft.) for 30–40 days, whereas Harlan SD rats exposed to the same conditions experience no mortality or morbidity. In addition, the Hilltop SD rats develop significantly greater pulmonary hypertension, hypoxemia, and polycythemia during chronic hypobaric hypoxia than do Madison SD rats (24). On the other hand, ventilatory responses to hypercapnia and hypoxia do not differ between these SD substrains (24).
Genetic predisposition for plasticity. To our knowledge, there are no previous reports concerning the genetic predisposition to plasticity in respiratory motor control. However, there are examples in other forms of neuroplasticity. Two of the most well-characterized examples of plasticity in the mammalian central nervous system are hippocampal long-term potentiation (LTP) and long-term depression (LTD). To explore the genetic basis of learning and memory, LTP and/or LTD have been compared across mouse (4, 21) and rat (10, 18) strains. These studies have shown conclusively that expression of hippocampal LTP and LTD differs among rodent strains and indicate that genetic mechanisms influence the expression of plasticity in the mammalian central nervous system.

Our laboratory has previously shown that enhanced serotonergic immunoreactivity in the phrenic motor nucleus is associated with enhanced phrenic LTF, at least following cervical sensory denervation (16). Accordingly, the greater XII LTF in CRL/S rats suggested that serotonergic innervation of XII motoneurons might be greater in CRL/S than H rats. Contrary to our expectations, there were no statistically significant differences in XII serotonergic labeling between substrains. However, these serotonergic immunohistochemical data must be viewed as somewhat inconclusive, establishing only that no obvious differences exist in serotonergic terminal density within the XII nucleus. Changes in the capacity for serotonergic modulation could involve alterations at several levels of the serotonergic nervous system, including the following: 1) changes in serotonin release and re-uptake; 2) changes in serotonin receptor density and/or subtype; and 3) alterations in intracellular signaling pathways associated with serotonin receptor activation. Until these aspects of serotonergic modulation have been explored, the detailed mechanisms underlying LTF and genetic differences in LTF will remain unclear.

The current experiments establish for the first time that different substrains of SD rat respond differentially to a standard LTF protocol. This observation suggests that genetic mechanisms influence the expression of serotonin-dependent neural plasticity. Rat strain and substrain must be considered as important variables in any study of LTF and, indeed, respiratory neural control in general.

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