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

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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 (JPhr) and hypoglossal neurograms in anesthetized, vagotomized, paralyzed, and ventilated rats. At 60 min following three 5-min hypoxic episodes (\(PaO_2 = 40 \pm 1\) Torr; 5-min hyperoxic intervals), JPhr 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 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%, FIO₂ = 0.5, balance N₂) 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 measurements (model 2305; Gould, Cleveland, OH), and 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 CO₂ partial pressure (PETCO₂) was measured using a rapidly responding flow through CO₂ 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, and washed again in distilled water, fixed in 5% sodium thiosulfate, and stored in 0.2-ml arterial blood samples (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 CO₂ apneic threshold for inspiratory nerve activity was determined by hyperventilating the rats until inspiratory nerve activity ceased, then decreasing the ventilator rate and allowing PETCO₂ to gradually rise until inspiratory activity reappeared. The PETCO₂ at which inspiratory activity resumed was noted, and PETCO₂ was then maintained 3 Torr above this value. In this manner, baseline neural activity was standardized relative to the CO₂ apneic threshold.

Arterial blood samples were drawn at 15, 30, and 60 min post-episodic hypoxia; if Paco₂ was not within 1.5 Torr of the baseline value, then the ventilator rate and/or inspired CO₂ concentration was adjusted. At the conclusion of the experiment, hypocapnic phrenic and XII responses were established by gradually raising inspired CO₂ until PetCO₂ reached 80–90 Torr.

**Immunohistochemistry.** Additional experiments were performed on three H and three CRL/S rats to examine serotonergic immunoreactivity within the XII nucleus. These rats were not exposed to the LTF protocol. Rats were deeply anesthetized with pentobarbital sodium (1 ml, 40 mg/ml ip) and perfused with 350 ml of 4% paraformaldehyde in 0.1 M PBS. Brains were cryoprotected in 30% sucrose with 5% glycerol in PBS. Sections were cut (60 μm) and stored in a solution of 0.02% sodium azide in PBS. Every second section through the XII nucleus was reacted for serotonin. To avoid inconsistencies associated with reactions performed at different times and on different days, sections from all six rats were reacted simultaneously in a multivat to maintain consistency and to avoid contamination. Specificity of the serotonin antibody was verified by a pattern of staining consistent with rat brain serotonergic neurons, by immunoabsorption blockade with serotonin-BSA, but not by serotonin metabolites or catecholamine conjugates, and by selective staining of cryosectioned serotonin-gelatin blocks, but not serotonin metabolite or catecholamine-gelatin sections (6). A dilution series was initially run (1:250 to 1:100,000), and the final dilution used was 1:10,000.

Sections were immunoreacted according to the protocol of Behan and Brownfield (5). To intensify the reaction product, free-floating sections were incubated in 1.4% silver nitrate for 1 h at 56°C, washed in distilled water, and placed on a 0.2% gold chloride in distilled water for 10 min at room temperature, washed again in distilled water, fixed in 5% sodium thiosulfate in distilled water for 10 min, and washed again prior to mounting, dehydrating, and coverslipping. This silver-gold intensification resulted in darkly stained axons and boutons on a pale background. Controls consisted of sections reacted similarly, but with the omission of incubation with primary antibody.

Images of the XII nucleus were captured at ×20 magnification with a digital color camera (Diagnostics Instruments, Fryer, Huntley, IL). Serotonin immunoreactivity shows a nonhomogeneous distribution throughout the XII nucleus, and thus all regions of the nucleus were unilaterally sampled (dorsomedial, dorsolateral, ventromedial, and ventrolateral; areas 1–4, respectively) in every second section throughout the rostrocaudal extent using a standard sized box. Using the automatic dark object setting with ImagePro software (Media Cybernetics, Silver Spring, MD), we determined the relative area occupied by darkly stained axons and boutons in the sample box.

**Analysises.** The initial experiments were done with a blinded design such that the identity of the rats (H vs. CRL/S) was unknown to the investigator during data collection and analysis. However, it was subsequently noted that the CRL/S rats were significantly more hypertensive and hypocapnic relative to baseline during hypoxia (Tables 1 and 2). The blinded experimental design made it impossible to predict which animals would be hypertensive during hypoxia, making it difficult to regulate hypoxic Paco₂ in the CRL/S rats. Thus the treatment groups experienced slightly (but significantly) different conditions during hypoxia. Accordingly, we conducted identical experimental protocols on five
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>
</tr>
</thead>
<tbody>
<tr>
<td>H ( \text{PaCO}_2 )</td>
<td>46 ± 1</td>
<td>46 ± 1</td>
<td>46 ± 1</td>
<td>46 ± 1</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>H ( \text{PaO}_2 )</td>
<td>242 ± 7</td>
<td>207 ± 9</td>
<td>215 ± 9</td>
<td>220 ± 9</td>
<td></td>
</tr>
<tr>
<td>CRL/S ( \text{PaCO}_2 )</td>
<td>44 ± 1</td>
<td>44 ± 1</td>
<td>44 ± 1</td>
<td>44 ± 1</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>CRL/S ( \text{PaO}_2 )</td>
<td>256 ± 8</td>
<td>250 ± 7</td>
<td>252 ± 7</td>
<td>247 ± 18</td>
<td></td>
</tr>
</tbody>
</table>


Table 2. Mean arterial pressure 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>
</tr>
</thead>
<tbody>
<tr>
<td>H ( \text{MAP} )</td>
<td>133 ± 7</td>
<td>111 ± 10</td>
<td>129 ± 6</td>
<td>119 ± 6</td>
<td>104 ± 8*</td>
</tr>
<tr>
<td>CRL/S ( \text{MAP} )</td>
<td>107 ± 6</td>
<td>68 ± 7</td>
<td>108 ± 7</td>
<td>99 ± 7</td>
<td>90 ± 9*</td>
</tr>
</tbody>
</table>

Values are means ± SE and are expressed in Torr. Mean arterial pressure in CRL/S rats was significantly lower than in H rats (\( P = 0.009 \)). *Different from baseline.

Blood pressure. MAP decreased to a similar extent during each hypoxic episode; data from the first hypoxic episode are presented in Table 2 for H and CRL/S rats. Declines in MAP during hypoxia are typical in anesthetized rats (1, 17). MAP was lower in CRL/S rats at all time points (Table 2; \( P < 0.05 \)). 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 (Table 2; see Critique of methods).

Apneic threshold. The end-tidal \( \text{CO}_2 \) apneic threshold was not different between H (43 ± 1 Torr) and CRL/S (42 ± 1 Torr) rats (\( P > 0.05 \)).

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).

Fig. 1. Mean changes in integrated XII (XII) and integrated phrenic (\( \text{JPhr} \)) amplitudes (% baseline) during the first three hypoxic episodes in Harlan (H) and Charles River Laboratories/Sasco (CRL/S) rats. Two groups of CRL/S rats are presented, as follows: those studied with a blinded experimental design (B) in which arterial \( \text{PCO}_2 \) became hypocapnic during hypoxia; and those studied with a nonblinded experimental design (NB), in which arterial \( \text{PCO}_2 \) was maintained isocapnic during hypoxia (see METHODS). *Different from corresponding response in phrenic amplitude.
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 (± 1 Torr) during hypoxia, and the magnitude of LTF was not different (P > 0.05) from that presented in Fig. 3 (Δ/XII 60 min post-episodic hypoxia = 66 ± 9% baseline; Δ/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 ± 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-
Differential serotonin expression in the XII nucleus could account for differential XII motor output. Serotonergic immunoreactivity was quantified in each area throughout the overall, ventromedial, and ventrolateral, respectively, and total serotonergic immunoreactivity was divided into 4 separate areas for analysis (areas 1–4; dorsomedial, dorsolateral, ventromedial, and ventrolateral, respectively), and total serotonin labeling to be lower in CRL/S rats was not statistically significant ($P = 0.31$). 5-HT, serotonin.

**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 substrain differences in serotonergic 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 correlation between the change in MAP (from baseline) and $f$XII and $f$Phr post-episodic hypoxia ($f$XII, $R^2 = 0.02$, $P = 0.77$; for $f$Phr, $R^2 = 0.19$, $P = 0.16$) or CRL/S ($f$XII, $R^2 = 0.00$, $P = 0.90$; for $f$Phr, $R^2 = 0.21$, $P = 0.37$) rats. Moreover, sustained declines in MAP in the range reported here have minimal impact on ventilatory output in rats (1, 33).

**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, 32, 34, 35). For example, SD rats obtained from Harlan (colony 205) vs. Hilltop (Scottsdale, PA) are 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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