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Ventilatory impairment in the dysmyelinated Long Evans shaker (*les*) rat

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Abstract

Although respiratory complications significantly contribute to morbidity/mortality in advanced myelin disorders, little is known concerning mechanisms whereby dysmyelination impairs ventilation, or how patients compensate (i.e. plasticity). To establish a model for studies concerning mechanisms of ventilatory impairment/compensation, we tested the hypotheses that respiratory function progressively declines in a model of CNS dysmyelination, the Long Evans shaker rat (*les*). The observed impairment is associated with abnormal inspiratory neural output. Minimal myelin staining was found throughout the CNS of *les* rats, including the brainstem and cervical bulbospinal tracts. Ventilation (*via* whole-body plethysmography) and phrenic motor output were assessed in *les* and wild-type (WT) rats during baseline, hypoxia (11% O₂) and hypercapnia (7% CO₂). Hypercapnic ventilatory responses were similar in young adult *les* and WT rats (2 months old); in hypoxia, rats exhibited seizure-like activity with sustained apneas. However, 5–6 month old *les* rats exhibited decreased breathing frequencies, mean inspiratory flow (VT/TI) and ventilation (VE) during baseline and hypercapnia. Although phrenic motor output exhibited normal burst frequency and amplitude in 5–6 month old *les* rats, intra-burst activity was abnormal. In WT rats, phrenic activity was progressive and augmenting; in *les* rats, phrenic activity was decrementing with asynchronized, multi-peaked activity. Thus, although ventilatory capacity is maintained in young, dysmyelinated rats, ventilatory impairment develops with age, possibly through discoordination in respiratory motor output. This study is the first reporting age-related breathing abnormalities in a rodent dysmyelination model, and provides the foundation for mechanistic studies of respiratory insufficiency and therapeutic interventions.

Keywords

myelin basic protein; dysmyelination; phrenic motor output; ventilation; plethysmography; hypercapnia

Myelination disorders are common, affecting 0.05–0.15% of Caucasians (Noseworthy et al., 2000). Adults with demyelination disorders such as multiple sclerosis (MS) may exhibit spastic paresis, ataxia, visual loss and bladder/bowel dysfunction, depending on the specific

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lesion sites. Hereditary dysmyelination disorders usually develop in infancy or early childhood, frequently leading to loss of sight, hearing, speech and mobility, with high mortality rates in the first few years of life. An under-appreciated pathology associated with many myelin disorders is respiratory impairment (Howard et al., 1992). Indeed, respiratory impairment is a major cause of morbidity/mortality in patients with advanced MS (Redelings et al., 2006; Hirst et al., 2008).

Absence of myelin within brainstem areas involved in respiratory control is associated with abnormal respiratory rhythm generation, motor pattern formation and sensory processing (Newsom-Davis 1974; Rizvi et al., 1974; Mochizuki et al., 1988; Howard et al., 1992; Carter et al., 1994; Miller et al., 2003; O'Sullivan et al., 2008). Respiratory impairment is also seen in patients with cervical spinal myelin disorders, an area providing motor output to the diaphragm (Cooper et al., 1985; Kuwahira et al., 1990). Even chronic stable patients, or patients with relatively mild myelin disorders, demonstrate inspiratory and expiratory muscle impairment (Foglio et al., 1994; Buyse et al., 1997; Mutluay, 2005; Fry et al., 2007). Such patients can experience severe, acute symptoms, culminating in overt respiratory failure precipitated by respiratory (e.g. diaphragm, intercostal) or respiratory-related (e.g. pharyngeal) muscle dysfunction.

Despite the fundamental importance of respiratory function in myelin disorders, little is known concerning the neural mechanisms of breathing impairment or possible mechanisms whereby patients compensate for such changes (i.e. compensatory neuroplasticity). To establish a model for studies concerning mechanisms of ventilatory impairment/compensation in dysmyelination disorders, we tested the hypothesis that respiratory function progressively declines during disease in a genetic model of CNS dysmyelination, the Long Evans shaker rat (*les*). Further, we tested the hypothesis that ventilatory impairment is associated with abnormal patterns (timing) of inspiratory neural output.

To study the complex process of myelination and physiological (i.e. ventilatory) consequences of dysmyelination, rodents with genetic, heritable disorders have been developed (i.e. myelin mutants, Lunn et al., 1995). The *les* rat is a well-described CNS myelin mutant with an autosomal recessive mutation in the myelin basic protein gene (*mbp*) required for normal myelin development (Kwiecien et al., 1998). Initial signs appear as tremors at ~ 10–12 days and then progress to truncal ataxia and seizures at ~ 1 month of age as they fail to produce myelin in the CNS beyond the first weeks of life. With intensive rearing, *les* rats live into adulthood but die prematurely for unknown reasons.

Our results provide evidence that ventilatory capacity is normal in young *les* rats during baseline conditions and with hypercapnia, but declines progressively with age. This age-dependent ventilatory impairment may result, at least in part, via discoordination in temporal patterns of inspiratory motor output. Our study provides the foundation for mechanistic studies of respiratory insufficiency and possible therapeutic interventions in dysmyelination disorders.

Experimental Procedures

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin. Recently-weaned (postnatal day 30), male and female wild-type (WT) and dysmyelinated mutant Long Evans (*les*) rats were obtained from a breeding colony at the University of Wisconsin, or purchased from a commercial source (Harlan Laboratories, Colony 217, Indianapolis, IN) and grown to adulthood. Mutant rats were fed a commercial rodent diet compounded with

phenobarbital (87.5 mg/feeding) thrice weekly to reduce seizure-like activity. Similarly, phenobarbital was fed to WT rats to control for potential effects on breathing. In addition, we also assessed breathing in WT rats fed a standard rodent diet. As a result, data from three groups of rats were analyzed: 1) dysmyelinated, mutant *les* rats fed the phenobarbital diet (n=4; 3 females, 1 male), 2) WT Long Evans rats fed the standard rodent diet (n=6; 5 females, 1 male) and 3) WT Long Evans rats fed the phenobarbital diet (n=3; 2 females, 1 male). Since no differences due to diet were found, the data for WT rats were pooled (see Results). All rats were studied longitudinally in a whole-body plethysmograph at frequent intervals (~weekly) until subjected to terminal studies of phrenic nerve activity at ~5–6 months of age. Ventilatory data reported here are from young (2 months of age) and older (5–6 months old) adult rats since few significant differences were detected until the latter ages.

Ventilatory measurements in unanesthetized rats

At 2 months and 5–6 months of age, ventilatory responses to hypercapnia were assessed in unanesthetized, unrestrained WT (n=9) and mutant *les* (n=4) rats using a whole-body flow through plethysmograph (BUXCO Electronics, Troy, NY). This technique allows for quantitative measurement of ventilation in unanesthetized, freely-behaving animals while simultaneously controlling inspired gas concentrations. A pressure calibration signal, the plethysmograph temperature, rat body temperature, ambient and chamber pressures, and rat body mass were then used by the computer software to continuously calculate breath-by-breath ventilatory variables using the equations described by Drorbaugh and Fenn (1955) as modified by Jacky (1978). Data were rejected if there was evidence of pressure fluctuations caused by gross body movements or sniffing behavior.

Rats were weighed, body temperatures recorded, and then placed into the plethysmograph chamber (~2 liter volume) while breathing room air (21% oxygen, balance nitrogen) flushed through the chamber at ~3 liters/minute. Rats were allowed to acclimate to the chamber. Once the rats were quiet but awake, ventilatory measurements were recorded continuously for 25 minutes during baseline conditions (21% oxygen, 0% carbon dioxide, balance nitrogen), followed by 25 minutes of hypercapnia (21% oxygen, 7% carbon dioxide, balance nitrogen). Statistical analyses were performed on data collected during the last 10 minutes of each exposure period. At the conclusion of the study, rats were removed from the chambers and body temperatures recorded.

In preliminary experiments, young (1.5–2 month old) *les* and WT rats were exposed to hypoxia (11% oxygen, 0% carbon dioxide, balance nitrogen) as part of our protocol; this is a level of hypoxia easily tolerated by wild-type rats (Olson et al., 2001; McGuire et al., 2005). However, studies during hypoxia were discontinued following the death of several *les* rats during the first minutes of hypoxic exposure caused by prolonged apneas associated with seizure-like activity (see results). The finding of terminal apneas during hypoxia is reminiscent of a previous report in a different rodent model of dysmyelination (Miller et al., 2003).

Neurophysiological recordings in anesthetized rats

To record inspiratory motor output in normocapnia, hypercapnia, and hypoxia, WT (n=4) and mutant *les* (n=4) rats were initially anesthetized with isoflurane in 50% O₂ (balance N₂). The trachea was cannulated to permit mechanical ventilation throughout the experiment (2.0–2.5 ml, Rodent respirator model 683; Harvard Apparatus, South Natick, MA). Rats were ventilated with approximately 50% O₂ (balance N₂) throughout the surgery. Bilateral vagotomy was performed to prevent entrainment of respiratory nerve activity with the ventilator. A femoral artery was cannulated to measure arterial blood pressure (model P122,

Grass Telefactor, West Warrick, RI) and to sample arterial blood for pH and blood-gas measurements (ABL-500, Radiometer, Copenhagen, Denmark). The femoral vein was cannulated for drug and fluid administration (5 ml/kg/h, 3:1 lactated Ringers and 6% hetastarch). End-tidal PCO₂ was measured continuously using a mainstream CO₂ monitor (Capnograph, Novamatrix Medical Systems, Wallingford, CT) with sufficient response time to measure end-tidal gas composition in anesthetized rats. The left phrenic nerves were isolated *via* a dorsal approach, desheathed and submerged in mineral oil. Following surgery, the rats were converted to urethane anesthesia (1.7 g/kg iv; isoflurane was discontinued) and were paralyzed with pancuronium bromide (1 mg/kg iv).

A phrenic nerve was placed on bipolar silver recording electrodes. Nerve activity was amplified (gain, 10,000; A-M Systems, Everett, WA), band-pass filtered (100 Hz to 10 kHz), rectified, and processed with a continuous moving averager (CWE 821 filter; Paynter, Ardmore, PA; time constant, 50 ms). The signal was digitized, recorded, and analyzed using the WINDAQ data-acquisition system (DATAQ Instruments, Akron, OH). Inspired CO₂ was added and/or ventilation rate was adjusted until fictive respiratory bursts were observed in the phrenic neurogram. This generally occurred at end-tidal PCO₂ levels between 40–45 mmHg.

One hour post-surgery, the apneic and recruitment thresholds were determined. Briefly, end-tidal PCO₂ was decreased by hyperventilating the rats until phrenic inspiratory activity ceased (apneic threshold) and then slowly increased until phrenic bursting resumed (recruitment threshold). Baseline burst amplitude was set at 3 mm Hg above the recruitment threshold. End-tidal PCO₂ was monitored throughout the protocol and maintained at baseline levels.

The raw and integrated phrenic neurograms were recorded for 20–30 min after establishing baseline conditions. Following baseline measurements, rats were given 25 minutes of hypercapnia (end-tidal PCO₂ = 90 mmHg) to determine maximum respiratory activity. Rats were returned to baseline conditions and given hypoxia (11% O₂) for an additional 5–10 minutes. At the completion of the experiment, rats were euthanized with an overdose of urethane and prepared for myelin staining.

Myelin staining

At the end of neurophysiological experiments, the 5–6 month old *les* and WT rats were euthanized with an overdose of urethane and perfused via the left ventricle with 100 ml 1X PBS followed by 100 ml 4% paraformaldehyde. Tissue blocks were post-fixed in 2.5% glutaraldehyde followed by osmium, embedded in epon, and cut coronally (cervical spinal cord) or sagittally (brainstem, 1.7 mm from midline) into 1 µm-thick sections. Tissues were subsequently stained for myelin with 1% toluidine blue and 1% sodium borate for 3.5 – 4 minutes and washed with distilled water.

Data analysis

All statistical tests were conducted using SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $p < 0.05$. Data are presented as means $\pm$ SEM.

Awake ventilatory measurements—Ventilatory variables were averaged over 5 minutes bins within the last 10 minutes of normocapnia (baseline) and hypercapnia. Measurements included breathing frequency (f), inspiratory time (TI), expiratory time (TE), tidal volume (VT), minute ventilation (the product of breathing frequency and tidal volume,

$\dot{V}E$), and the mean inspiratory flow ($\dot{V}T/TI$). Breathing volumes ($\dot{V}T$, $\dot{V}E$, and $\dot{V}T/TI$) were normalized per 100 g of body mass.

Statistical comparisons were made between groups (WT *versus les* mutants; phenobarbital-fed *versus* standard-fed WT) during two treatments (baseline and hypercapnia) at two separate time points (2 months and 5–6 months). Body mass was compared between groups using a one-way ANOVA. Changes in body temperatures and ventilatory variables between groups were compared using a two-way repeated measures ANOVA (RM ANOVA) and Student-Newman-Keuls *post hoc* test.

Phrenic nerve responses in anesthetized rats—Phrenic measurements were averaged in 30 second bins (immediately preceding blood sampling) under baseline conditions and at the end of the hypercapnic and hypoxic challenges. Measurements included phrenic burst frequency, integrated phrenic burst amplitude (expressed as raw voltages), burst duration (equivalent to inspiratory time, TI), burst interval (equivalent to expiratory time, TE), phrenic minute activity (the product of burst frequency and amplitude), and the burst amplitude to inspiratory duration ratio (a neural analogue of mean inspiratory flow).

A one-way ANOVA was used to determine if the apneic or recruitment thresholds differed among WT or *les* mutant rats. Changes in measured variables between groups in normoxia, hypercapnia, and hypoxia were compared using a two-way repeated measures ANOVA and Student-Newman-Keuls *post hoc* test.

Results

Phenobarbital-fed WT and standard diet-fed WT rats

There were no significant differences (or apparent trends) in body mass, body temperature or any ventilatory variable between phenobarbital-fed ($n=3$) and standard-fed ($n=6$) WT rats at any time point (all $p > 0.05$; data not shown). Thus, data from the two groups were combined. Subsequent analyses were performed on combined WT rats ($n=9$) and mutant *les* rats ($n=4$).

Dysmyelination

To ensure dysmyelination at CNS respiratory-related sites, one *les* and one WT control rat were perfused and stained for myelin at 5–6 months of age (Fig. 1). Similar to previous investigations in *les* rats at different ages (Kwiecien et al., 1998), there is an obvious lack of myelin throughout the entire CNS of the *les* rat (Fig. 1B, D) while WT rats (Fig. 1A, C) have abundant myelin staining using this technique. Most notably, there is little evidence for myelin in the vicinity of brainstem structures involved in respiratory rhythm generation or burst pattern formation (i.e. the ventral respiratory group, Fig. 1B; McCrimmon et al., 2009) and descending bulbospinal pathways to phrenic motor neurons (Fig. 1D) of the *les* rat. However, the peripheral spinal motor nerve remains myelinated (Fig. 1D).

Body mass and temperature

There were no significant differences in body mass between WT and *les* rats at either age, although *les* rats tended to be lighter (both $p > 0.05$; Table 1). At 2 months old, there were no differences in body temperature between groups taken before and immediately after plethysmography experiments ($p > 0.05$; Table 1). However, at 5–6 months of age, body temperature was significantly lower in *les* rats immediately following experimentation (0.6°C ; $p < 0.05$; Table 1).

Ventilatory responses in young (2 month) awake rats

There were no significant baseline differences between rat groups in f , TI , VT , $\dot{V}E$, and VT/TI (all $p > 0.05$; Fig. 2A-D, F). However, TE was significantly lower in *les* rats with a significant interaction between rat group and treatment ($p < 0.05$; Fig 2E). Hypercapnia elicited similar increases in f , VT , $\dot{V}E$, and VT/TI and decreases in TI , and TE in WT and *les* groups (treatment effect: all $p < 0.05$; group effect: all $p > 0.05$; Fig. 2A-F).

During hypoxia ($FIO_2 = 11\%$) in the first few young *les* rats tested, seizure-like activity with prolonged apneas was observed, leading to cyanosis and death within 5 minutes if the rats were not rescued by returning to normoxic conditions (Fig. 3E). Thus, plethysmography experiments using hypoxia as a ventilatory stimulus were discontinued. During hypercapnia, *les* rats also showed seizure-like activity and apneas. However, the apneas were brief (~20–40 sec duration) and all rats recovered spontaneously without obvious complications (data not shown).

Ventilatory responses in older (5–6 month) awake rats

Representative plethysmography traces of breathing from one older *les* (B, D) and one WT rat (A, C) are shown at baseline (normocapnia) and during hypercapnia in Figure 3, and summary data are provided in Figure 4. In normocapnia, *les* rats had significantly lower f with prolongation of TI and TE versus WT rats (all $p < 0.05$; Fig. 3A, B; Fig. 4A, C, E); baseline VT , $\dot{V}E$, and VT/TI did not differ between groups (all $p > 0.05$; Fig. 3; Fig. 4B, D, F). During hypercapnia, $\dot{V}E$ and VT/TI were significantly attenuated in *les* rats, predominantly due to prolongation of TI and TE , thereby decreasing f (all $p < 0.05$; Fig. 3C, D; Fig. 4A, C-F). These differences resulted in a significant interaction between rat group and treatment in TI and $\dot{V}E$ (both $p < 0.05$; Fig. 4C, D).

Phrenic burst activity in anesthetized rats (5–6 month old)

Expanded integrated phrenic neurograms from one *les* and one WT rat are shown in Figure 5, and summary data are provided in Figure 6. There were no significant differences between rat groups during baseline conditions in phrenic minute activity, TE , or phrenic burst amplitude/ TI (all $p > 0.05$; Fig. 6B, D-F). However, *les* rats had shorter TI versus WT rats ($p < 0.05$; Fig. 6C). Hypercapnia increased amplitude, phrenic minute activity and burst amplitude/ TI , while decreasing TI (all $p < 0.05$; Fig. 6A-D, F). However; whereas burst frequency increased in WT rats, *les* rats showed no change in frequency during hypercapnia (Fig. 6A). Although the phrenic burst amplitude at baseline tended to be lower in *les* rats, there were no statistically significant differences between groups at baseline or during hypercapnia ($p > 0.05$; Fig. 5; Fig. 6A).

When integrated phrenic bursts were amplified and examined on a shorter time scale (Fig. 5), WT rats showed a typical pattern of augmenting bursts with a single peak of activity at the end of each burst. In contrast, *les* rats exhibited disorganized activity. Bursts were asynchronized and exhibited multiple peaks within each burst. Overall, there was a decrementing discharge pattern that is not characteristic of WT rats. Due to the small population of *les* rats available for study, these subtle differences were not quantified.

During hypoxia, there were no significant differences between rat groups, as phrenic burst frequency, amplitude and minute activity all increased (treatment effect: all $p < 0.05$; group effect: all $p > 0.05$; Fig. 7A-C). However, *les* rats exhibited severe cardiovascular compromise as mean arterial blood pressure profoundly and significantly decreased during hypoxia ($p < 0.05$, Fig. 7D).

Discussion

Although ventilatory capacity is normal in young *les* rats, it becomes progressively compromised with age. In older *les* rats, ventilatory compromise is expressed largely as decreased breathing frequency and mean inspiratory flow (VT/TI). Although overall integrated inspiratory (phrenic) motor output was normal during hypercapnia in 5–6 month old rats, abnormalities in phrenic bursting pattern suggest dis-coordination of inspiratory motor output, possibly compromising the ability to generate an adequate ventilatory response to increased respiratory drive. This investigation demonstrates ventilatory insufficiency and abnormalities in the pattern of inspiratory motor output associated with CNS dysmyelination in the *les* rat. Perhaps more importantly, we have established a model to enable investigations of mechanisms underlying respiratory insufficiency in demyelinating/dysmyelinating disorders and to test the efficacy of potential therapeutic interventions.

Long Evans shaker (*les*) rat

The *les* rat has an autosomal recessive mutation in the myelin basic protein (MBP) gene (Kwiecien et al., 1998). This mutation causes an accumulation of vesicles and membranous bodies in oligodendrocytes, resulting in deficient MBP and widespread dysmyelination in all regions of the CNS (Kwiecien et al., 1998) by 8–12 weeks of age; peripheral myelin is unaffected. Despite these permanent, cytoplasmic, oligodendrocyte abnormalities, *les* rats can live into adulthood but still die prematurely. Regardless, we were able to confirm CNS dysmyelination and to repeatedly study 4 *les* rats well into adulthood, producing statistically robust data. Although sex hormones may alter ventilatory responses and respiratory neuroplasticity (for review see: Behan et al., 2003; Behan and Wenninger, 2008), it is not clear how sex hormones would affect ventilatory responses in our demyelinated rats. However, we did not observe any appreciable differences between males and females. Thus, the data from both sexes were grouped together.

Although body mass was not different between rat groups, *les* rats tended to be smaller than their age-matched WT controls, an effect especially evident at 5–6 months of age. To account for any influence of body mass on respiratory volumes, respiratory volume data were analyzed with and without normalization to body mass. Since there were no differences in our conclusions using these approaches, normalized data are presented here.

Respiratory compromise in *les* rats

Although this is the first report of respiratory insufficiency in the *les* rat, respiratory dysfunction has been implicated in the death of the myelin-deficient (*md*) rat which has a mutation in the proteolipid protein gene (*plp*) required for myelination (Lunn et al., 1995). For example, hypoxic ventilatory responses were depressed in *md* rats at 21–24 days of age, and in fact several died during hypoxic exposures (Miller et al., 2003). However, hypercapnic responses appeared to be normal in these same rats (Miller et al., 2003). The authors concluded that rats died from abnormal central processing of chemo-afferent neural inputs arising from the peripheral chemoreceptors due to mutations in the *plp* gene. These data are consistent with our investigation in that young (2 month old) *les* rats have minimal ventilatory impairment during hypercapnia, but exhibited profound seizure-like activity in hypoxia with loss of consciousness, apneas and subsequent death. Based on our limited observations, it is unclear whether profound respiratory depression resulted in seizure-like activity and death or if the seizures precipitated the apneic events. Thus, we did not investigate hypoxic ventilatory responses further in awake *les* rats and were able to rear and study them well into adulthood which was not possible in the *md* rat.

Although young *les* rats exhibit similar hypercapnic ventilatory responses to WT rats, diminished hypercapnic ventilatory responses were apparent by 5–6 months of age. In the older rats, TI and TE were prolonged, reducing breathing frequency and the overall ventilatory response to hypercapnia. However, similar alterations in respiratory timing were not seen in phrenic nerve recordings from anesthetized, vagotomized *les* rats. Anesthesia and vagotomy remove important peripheral and central sources of afferent input to brainstem respiratory neurons and, thus, may alter respiratory rhythm generation and pattern formation. In contrast, these sensory pathways were intact in the unanesthetized plethysmography experiments, possibly accounting for the difference in experimental results between conditions. On the other hand, cortical demyelination is associated with desynchronization of electrical activity and impaired corticomotor function with associated fatigue and heightened perception of effort required for motor activity in humans (Petajan and White, 2000; Perretti et al., 2004; Liepert et al., 2005; Thickbroom et al., 2005, 2006; for review see: Zwartz et al., 2008). Although we cannot comment on perception in our awake *les* rats (Banzett et al., 2008), it is possible that dysmyelination of the motor cortex or the cortical integrative pathways may be associated with the mechanisms underlying respiratory impairment during plethysmography experiments in the older *les* rats. Abnormalities in the diaphragm muscle or the neuromuscular junction were not investigated in our study and cannot be ruled out as contributors to the respiratory deficiencies seen. Indeed, in rodent models of peripheral dys- and demyelination, neuromuscular synapses are dysfunctional (Yin et al., 2004; Court et al., 2008). However, two points argue against this possibility: 1) there is no peripheral dysmyelination in the *les* rat; and 2) the predominant ventilatory control abnormality was expressed in respiratory frequency whereas neuromuscular abnormalities would affect primarily tidal volume.

Although timing parameters did not differ between rat groups in the anesthetized preparation, the phrenic burst amplitude, a reflection of overall electrical activity in the compound action potentials, tended to be lower (not significant) and asynchronous in *les* rats. This finding is consistent with reports of abnormal compound action potentials in other areas of the CNS in *les* rats (e.g. dorsal spinal columns; Eftekharpour et al., 2005). Since compound action potentials depend on several factors (e.g. axon number, size, excitability, conduction velocity and synchrony of arrival to the recording site), it is possible that dis-coordination of phrenic output in *les* rats may result from asynchronous conduction velocities and, in some cases, conduction failure within bulbospinal projections to phrenic motor neurons (Leocani et al., 2001; Nashmi and Fehlings, 2001; Eftekharpour et al., 2005). A consequence of such dis-coordination may be impaired diaphragmatic (or inspiratory muscle) contraction, and reduced mean inspiratory flow. However, the specific effects of dysmyelination and asynchrony in respiratory-related areas such as the brainstem and cervical spinal cord are not clear in this study since *les* rats have generalized CNS dysmyelination.

Possible mechanisms

Les rats exhibit altered phrenic motor output, respiratory timing and reduced mean inspiratory flow (VT/TI) in *les* rats. Although the true mechanistic basis of ventilatory compromise remains unclear at this time, novel insights are gained as phrenic burst frequency and amplitude in anesthetized and vagotomized rats are essentially normal. The contrasts between ventilation in unanesthetized, and phrenic activity in anesthetized *les* rats suggests two likely sites of dysfunction: 1) alterations in supra-pontine inputs to brainstem respiratory neurons and 2) alterations in vagal afferent processing within the brainstem. The former is consistent with impaired corticomotor function associated with demyelination in MS patients (Thickbroom et al., 2005). The involvement of vagal afferent neurons, and their primary site of termination in the nucleus of the solitary tract (NTS) in modulating breathing

timing are well known (Kubin et al., 2006). Vagal involvement in pattern differences is consistent with the interpretation of Miller and colleagues (2003), who concluded that *md* rats exhibit abnormal ventilatory function due to abnormal integrative functions in brainstem respiratory centers, particularly in the nucleus of the solitary tract. Since *les* rats exhibit generalized dysmyelination, it is likely that lesions within both sites contribute in some way to ventilatory deficiencies and abnormal phrenic motor output. Another site of potential impairment is in descending, bulbospinal pathways to respiratory motor neurons however, such impairment is more likely to be expressed as limited ability to generate tidal volumes *versus* abnormal control of breathing frequency. Although the precise mechanisms underlying altered respiratory control in *les* rats are not yet known, this study provides the foundation for future mechanistic studies of respiratory insufficiency and therapeutic interventions in myelin disorders.

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List of Abbreviations

<i>les</i>	Long Evans shaker rat
<i>md</i>	myelin-deficient rat
<i>mbp</i>	myelin basic protein
<i>plp</i>	proteolipid protein
WT	wild-type
MS	multiple sclerosis
<i>f</i>	breathing frequency
TI	inspiratory time
TE	expiratory time
VT	tidal volume
VE	minute ventilation (the product of breathing frequency and tidal volume)
VT/TI	mean inspiratory flow
PCO ₂	partial pressure of carbon dioxide
FIO ₂	inspiratory fraction of oxygen
FICO ₂	inspiratory fraction of carbon dioxide

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Figure 1.

Myelin staining (20X magnification) of the brainstem (A, B) and spinal cord segment C4 (C, D) taken from a 5–6 month old wild-type (WT, left panels) and mutant (*les*, right panels) rat. Insets are taken at 200X magnification. A sagittal section taken from the caudal ventrolateral brainstem shows prominent myelin labeling in the WT rat (A) with no detectable myelin staining in the *les* rat (B). These brainstem areas contain respiratory neurons (e.g., ventral respiratory column). Coronal sections of the cervical spinal cord segment C4 also confirm the absence of myelin in the *les* rat (D) whereas axons from the WT rat are heavily myelinated (C). Note that the peripheral motor nerve of the *les* rat remains myelinated (dark arrow, D). These segments include descending bulbospinal pathways to inspiratory motor neurons, associated with the phrenic nerve/diaphragm.

Figure 2.

Baseline ventilation (normocapnia) and hypercapnic ($FICO_2 = 7\%$) ventilatory responses in **2 month old** WT (open circles, ○) and *les* (closed circles, ●) rats. In hypercapnia, WT and *les* rats exhibited similar changes in ventilation from baseline including increased breathing frequency, V_T , $\dot{V}_E$, and V_T/T_I with corresponding decreases in T_I and T_E (RM ANOVA, * $p < 0.05$ from baseline; $p > 0.05$ between groups; A-F). However, *les* rats had significantly lower T_E at baseline, resulting in an overall interaction between treatment and rat group (RM ANOVA, # $p < 0.05$ between rat groups, † $p < 0.05$ interaction; E).

Figure 3.

Representative breathing traces in 5–6 month old WT (A, C) and *les* rats (B, D) during baseline and hypercapnia (FICO₂ = 7%). Breathing frequency is slower, with longer values of TI and TE at baseline and during hypercapnia in *les* rats (B, D). However, tidal volume (normalized to body mass) do not differ between groups. Summary data are shown in Figure 4.

Figure 3E shows a young (2 month old) *les* rat during hypoxia (FIO₂ = 11%). The left side of the trace shows a normal breathing pattern with mild disturbances in the otherwise steady respiratory pattern due to rat movements. Dark arrow indicates seizure-like activity in the *les* rat followed by a prolonged period of apnea. Rats were unable to recover from these apneic periods and subsequently died unless rescued with ventilatory support and returned to normoxia.

Figure 4.

Baseline ventilation (normocapnia) and hypercapnic ($\text{FICO}_2 = 7\%$) ventilatory responses in **5–6 month old** WT (open circles, $\circ$) and *les* (closed circles, $\bullet$) rats. Similar to 2 month old rats, hypercapnia increased breathing frequency, VT, VE, and VT/TI with corresponding decreases in TI and TE (RM ANOVA, $*p < 0.05$ from baseline; $p > 0.05$ between groups; A-F). However, *les* rats had significantly slower breathing frequencies, with prolonged TI and TE, at baseline and during hypercapnia *versus* WT rats; VT did not differ between groups (RM ANOVA, $\#p < 0.05$ between rat groups, A-C, E). VE and VT/TI were diminished during hypercapnia in *les* rats due to differences in timing variables (RM ANOVA, $\#p < 0.05$ between rat groups, D, F); statistically significant interactions between group and treatment were found in TI and VE (RM ANOVA, $\dagger p < 0.05$ interaction, C, D). Thus, ventilatory responses to hypercapnia are impaired in *les* rats when compared to WT rats at 5–6 months of age. When not apparent, error bars are within data points.

Figure 5. Expanded phrenic neurograms of an individual burst (breath) from a 5–6 month old WT (top) and *les* (bottom) rat during baseline. WT rats show a typical, incrementing neural discharge with a single peak. In contrast, *les* rats frequently displayed multi-peaked bursts with incrementing/decrementing neural activity and low instantaneous voltages.

Figure 6.

Phrenic burst activity during hypercapnia in 5–6 month old WT (open circles, ○) and *les* (closed circles, ●) rats. Hypercapnia increased phrenic burst amplitude, minute phrenic activity and amplitude/TI, while decreasing TI to a similar extent in both *les* and WT rat groups (RM ANOVA, $*p < 0.05$ from baseline; $p > 0.05$ between groups; B, C-F). Phrenic burst frequency increased overall in hypercapnia (RM ANOVA, $*p < 0.05$ from baseline; A). *Les* rats had significantly shorter TI compared to WT rats at baseline, but not during hypercapnia (RM ANOVA, $\#p < 0.05$ between rat groups, C). Thus, although unanesthetized hypercapnic ventilatory responses are impaired in *les* rats, hypercapnic phrenic responses are not.

Figure 7.

Phrenic burst activity and mean arterial blood pressure during hypoxia ($\text{FIO}_2 = 11\%$) in 5–6 month old WT (open circles, ○) and *les* (closed circles, ●) rats. Hypoxia significantly increased phrenic burst frequency, amplitude and minute activity to a similar extent in both *les* and WT rats (RM ANOVA, $*p < 0.05$ from baseline; $p > 0.05$ between groups; A-C). In contrast, overall mean arterial pressure significantly decreased during hypoxia, primarily as a result of a profound decrease in *les* rats (RM ANOVA, $*p < 0.05$ from baseline; $\#p < 0.05$ between rat groups; D).

Table 1

Body mass and temperature for unanesthetized wild-type and *les* rats pre- and post-plethysmography experimentation.

	<u>WT controls</u>	<u>lesmutants</u>
Body mass (g)		
2 months	229.0 ± 16.0	192.8 ± 3.0
5–6 months	336.9 ± 27.0	292.3 ± 7.3
Body temperature (°C)		
2 months		
Pre-study	36.9 ± 0.3	36.3 ± 0.4
Post-study	36.7 ± 0.2	36.1 ± 0.3
5–6 months		
Pre-study	37.6 ± 0.2	37.2 ± 0.2
Post-study	37.6 ± 0.2	36.6 ± 0.2*

Body mass is similar between WT and *les* rats at both time points. However, body temperature is significantly lower in *les* rats post-study (* $p < 0.05$). Values are means ± SE for WT (n=9) and *les* (n=4) rats.