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Acute intermittent hypoxia induced phrenic long-term facilitation despite increased SOD1 expression in a rat model of ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease characterized by motor neuron death. Since most ALS patients succumb to ventilatory failure from loss of respiratory motor neurons, any effective ALS treatment must preserve and/or restore breathing capacity. In rats over-expressing mutated superoxide dismutase-1 (SOD1^{G93A}), the capacity to increase phrenic motor output is decreased at disease end-stage, suggesting imminent ventilatory failure. Acute intermittent hypoxia (AIH) induces phrenic long-term facilitation (pLTF), a form of spinal respiratory motor plasticity with potential to restore phrenic motor output in clinical disorders that compromise breathing. Since pLTF requires NADPH oxidase activity and reactive oxygen species (ROS) formation, it is blocked by NADPH oxidase inhibition and SOD mimetics in normal rats. Thus, we hypothesized that SOD1^{G93A} (mutant; MT) rats do not express AIH-induced pLTF due to over-expression of active mutant superoxide dismutase-1. AIH-induced pLTF and hypoglossal (XII) LTF were assessed in young, pre-symptomatic and end-stage anesthetized MT rats and age-matched wild-type littermates. Contrary to predictions, pLTF and XII LTF were observed in MT rats at all ages; at end-stage, pLTF was actually enhanced. SOD1 levels were elevated in young and pre-symptomatic MT rats, yet superoxide accumulation in putative phrenic motor neurons (assessed with dihydroethidium) was unchanged; however, superoxide accumulation significantly decreased at end-stage. Thus, compensatory mechanisms appear to maintain ROS homeostasis until late in disease progression, preserving AIH-induced respiratory plasticity. Following intrathecal injections of an NADPH oxidase inhibitor (apocynin; 600μM; 12μL), pLTF was abolished in pre-symptomatic, but not end-stage MT rats, demonstrating that pLTF is NADPH oxidase dependent in pre-symptomatic, but NADPH oxidase

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AUTHOR CONTRIBUTIONS

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independent in end-stage MT rats. Mechanisms preserving/enhancing the capacity for pLTF in MT rats are not known.

Keywords

motor neuron disease; superoxide; reactive oxygen species; NADPH oxidase; phrenic motor neurons; respiratory plasticity; spinal cord; spinal plasticity

INTRODUCTION

Severe ventilatory impairment occurs in many neurological diseases, including amyotrophic lateral sclerosis (ALS) (Kiernan et al., 2011; Zinman and Cudkowicz, 2011). Although the most common cause of death in ALS patients is respiratory failure (Bourke et al., 2001; Lyall et al., 2001; Lechtzin et al., 2002), and ALS patients exhibit decreased inspiratory muscle strength (Lyall et al., 2001; Ilzecka et al., 2003; Singh et al. 2011), breathing most often remains adequate until late in disease progression. Thus, compensatory mechanisms may preserve breathing capacity despite progressive respiratory motor neuron death. Our goal is to understand mechanisms giving rise to this compensatory plasticity, and to develop ways to further preserve/restore function by enhancing plasticity (Nichols et al., 2012; Dale et al., 2014).

Genetic rodent models have been used to study ALS pathogenesis (Rosen et al., 1993; Gurney et al., 1994; Wong et al., 1995; Bruijn et al., 1997; Nagai et al., 2001; Howland et al., 2002; Wang et al., 2003), including rats over-expressing human superoxide dismutase 1 (SOD1) with mutations known to cause human familial ALS (*eg.* SOD1^{G93A} rats; Rosen et al., 1993; Gurney et al., 1994; Howland et al., 2002). SOD1^{G93A} (mutant; MT) rats retain dismutase enzymatic activity, and the mutant SOD1 causes motor neuron death through a toxic gain of function (Gurney et al., 1994). Although SOD1^{G93A} over-expressing rats undergo profound loss of phrenic and other respiratory motor neurons, they preserve breathing capacity until late in disease progression (Nichols et al., 2013a; Nichols et al., 2013b; Nichols et al., 2014). Nevertheless, MT rats lose ~50% of their capacity to generate phrenic motor output at end-stage, suggesting imminent ventilatory failure (Nichols et al., 2013a; Nichols et al., 2013b).

One strategy to improve respiratory function with ALS is to induce further respiratory plasticity, enabling spared respiratory motor neurons to compensate for the loss of others (Nichols et al., 2013a). For example, phrenic motor output may be preserved and/or restored by inducing phrenic long-term facilitation (pLTF) with acute intermittent hypoxia (AIH; Mitchell, 2007; Nichols et al., 2013a; Dale et al., 2014), a strategy successfully used to restore breathing capacity after incomplete cervical spinal injury (Lovett-Barr et al., 2012).

AIH-induced pLTF is a long-lasting increase in phrenic motor output that requires spinal serotonin receptor activation and new synthesis of brain derived neurotrophic factor (Baker-Herman and Mitchell, 2002; Baker-Herman et al., 2004). pLTF also requires reactive oxygen species (ROS) formation *via* NADPH oxidase activity (MacFarlane and Mitchell, 2008; MacFarlane et al., 2008, 2009). Because pLTF requires ROS, and SOD1^{G93A} rats

retain enzymatic activity (Gurney et al., 1994), we originally hypothesized that pLTF would not be expressed in this ALS model. To our surprise, enhanced pLTF is observed at disease end-stage (Nichols et al., 2013a). Here, we tested the hypotheses that: 1) AIH elicits pLTF in MT rats at all ages (young, pre-symptomatic and end-stage), despite diminished superoxide levels in putative phrenic motor neurons from increased SOD1 expression; and 2) NADPH oxidase is required in pre-symptomatic, but not end-stage MT rats. Our results suggest previously unknown mechanisms of ROS homeostasis within phrenic motor neurons, preserving superoxide levels and AIH-induced pLTF until symptom onset. However, distinct mechanisms must eliminate the pLTF ROS requirement, thus enabling, and even enhancing, pLTF at disease end-stage.

MATERIALS AND METHODS

Experimental groups

Experiments were performed with adult male and female SOD1^{G93A} mutant (MT) rats and age-matched wild-type (WT) littermates. Rats were assigned to the following groups: 1) young (60–90 days; MT: age 83±4 days old, weight 424±21 g; WT: age 82±3 days old, weight 407±26 g), 2) pre-symptomatic (90–130 days; MT: age 116±2 days old, weight 478±14 g; WT: age 125±2 days old, weight 511±15 g), and 3) end-stage (150–200 days; MT: age 162±3 days old, weight 393±12 g; WT: age 167±3 days old, weight 568±10 g). MT rats were considered end-stage when they reached 20% decrease from peak body mass, similar to our previous report (Nichols et al., 2013a). The Animal Care and Use Committee of the University of Wisconsin-Madison approved all experimental procedures.

Each rat group received one of two treatments: 1) acute intermittent hypoxia (AIH; PaO₂: 35–45 mmHg) or 2) sham normoxia (*ie.* time controls without AIH). AIH groups included: 1) young (n=8 MT; n=8 WT); 2) pre-symptomatic (n=9 MT; n=9 WT); and 3) end-stage (n=7 MT; n=10 WT). Time control (TC) groups included: 1) young (n=2 MT; n=2 WT); 2) pre-symptomatic (n=2 MT; n=4 WT); and 3) end-stage (n=2 MT; n=2 WT); since there were no apparent differences among TC groups, these rats were combined as a single group for statistical analyses (*ie.* MT=6 and WT=8). Limited data from the end-stage rats were published previously, specifically integrated phrenic burst amplitude at 60 min post-AIH (Nichols et al., 2013a). Additional data from these rats are reported here, including different time-points post-AIH, and other responses including phrenic burst frequency and the short-term hypoxic phrenic response. Separate rat groups received intrathecal drug delivery and are described in detail below in the neurophysiology protocol section.

Separate rat groups (without AIH) were used for analysis of phrenic motor neuron SOD1 expression (immunofluorescence) and superoxide accumulation (*via* dihydroethidium fluorescence). Young, pre-symptomatic and end-stage MT rats and WT littermates were compared for SOD1 (n=6 MT; n=4 WT per age group) and superoxide accumulation (n=5 MT; n=5 WT). Ventral spinal tissue homogenates from additional MT rats and WT littermates (without AIH) were assessed for SOD1 expression *via* immunoblots at the same time-points (young: n=5 MT and n=9 WT; pre-symptomatic: n=4 MT and n=4 WT; and end-stage: n=10 MT and n=8 WT).

Neurophysiological preparation

Experimental procedures have described previously in multiple publications (eg. Hoffman et al., 2012; Nichols et al., 2012). Briefly, rats were anesthetized with isoflurane, tracheotomized, paralyzed and ventilated (Rodent Ventilator, model 683; Harvard Apparatus, Holliston, MA, USA; tidal volume ~2.5 mL, frequency ~70–80). Isoflurane anesthesia was maintained (3.5% in 50% O₂, balance N₂) throughout surgical procedures; rats were then converted to urethane anesthesia over 15–20 minutes (1.8 g kg⁻¹, *i.v.*) while slowly withdrawing isoflurane. Adequacy of anesthesia was tested before protocols commenced, and immediately after completion, and was assessed by the lack of pressor responses or obvious respiratory neural responses to a hemostat toe pinch. After conversion to urethane anesthesia, rats were given a continuous intravenous infusion to maintain body fluid and acid-base balance; infusions (1.5–6 ml kg⁻¹ hr⁻¹) consisted of a 1:2:0.13 mixture of 6% Hetastarch (in 0.9% sodium chloride), lactated Ringer's solution, and 8.4% sodium bicarbonate.

Body temperature was assessed with a rectal thermometer (Fisher Scientific, Pittsburgh, PA, USA) and maintained ($37.5 \pm 1^\circ\text{C}$) with a heated surgical table. To monitor end-tidal P_{CO₂} (P_{ETCO₂}), a flow-through carbon dioxide analyzer was used with sufficient response time to measure P_{ETCO₂} in rats (Capnograph, Novamatrix, Wallingford, CT). P_{ETCO₂} was maintained at ~45 mmHg throughout the surgical preparation.

Rats were vagotomized bilaterally and a polyethylene catheter (PE50 ID: 0.58mm, OD: 0.965mm; Intramedic, MD, USA) was inserted into the right femoral artery to monitor blood pressure (Gould Pressure Transducer, P23ID, USA). Blood samples were analyzed for partial pressures of O₂ (P_{O₂}) and CO₂ (P_{CO₂}) and pH using a blood gas analyzer (ABL 800, Radiometer, Westlake, OH); blood gases were assessed during baseline, the first hypoxic episode, and at 15, 30 and at 60 min post-AIH.

The left phrenic and hypoglossal nerves were isolated (dorsal approach), cut distally, desheathed and covered with a saline soaked cotton ball until placing the nerves on bipolar silver electrodes (see below). Laminectomy was performed at cervical level 2 (C₂) for all rats that received intrathecal delivery of drugs. Once rats were converted to urethane anesthesia, a minimum of 1 hour was allowed before protocols commenced.

Neurophysiology protocol

The left phrenic and hypoglossal nerves were submerged in mineral oil and placed on bipolar silver electrodes to record nerve activity. Neural signals were amplified (10,000 X), band-pass filtered (300–10,000 Hz, Model 1800, A-M Systems, Carlsborg, WA, USA), full-wave rectified and integrated (Paynter filter, time constant, 50 ms, MA-821, CWE Inc., Ardmore, PA, USA). Integrated nerve bursts were digitized (8 kHz) and analyzed using WINDAQ data acquisition system (DATAQ Instruments, Akron, OH, USA). For animals that received intrathecal drug delivery, a small incision was made in the dura and a soft silicone catheter (2 Fr; Access Technologies, Skokie, IL) was inserted caudally 3–4 mm until the tip rested approximately over the C₄ segment to deliver pre-treatment of drugs near the phrenic motor nucleus before acute intermittent hypoxia (3, 5 minute episodes of

isocapnic hypoxia (35–45 mmHg) separated by 5 minute intervals of baseline oxygen levels (partial pressure of arterial O₂ (PaO₂) 150 mmHg). After the third hypoxic episode, baseline O₂ levels were restored and then maintained for the duration of experiments. The intrathecal catheter was attached to a 50 µl Hamilton syringe filled with drug or vehicle solutions as described below. Rats were then paralyzed using pancuronium bromide for neuromuscular blockade (2.5 mg kg⁻¹, *i.v.*).

In a separate group of rats, experiments were performed to determine if NADPH oxidase is required for pLTF in pre-symptomatic (107–130 days) and end-stage (150–200 days) MT rats, and age-matched WT littermates. Vehicle (control or 10% DMSO dissolved in artificial cerebral spinal fluid, aCSF) or apocynin (12 µl 600µM; Sigma) were delivered intrathecally as described previously (MacFarlane et al., 2009). Injections were made after a 15 minute baseline was established, ~20 minutes prior to the start of the AIH protocol. “Pre-symptomatic” (PS) and “end-stage” (ES) WT rat treatment groups included: vehicle + AIH (PS n=11; ES n=9), vehicle time control (no AIH; PS n=5; ES n=4), apocynin + AIH (PS n=10; ES n=9), apocynin TC (PS n=4; ES n=4). Pre-symptomatic and end-stage MT rat treatment groups included: vehicle + AIH (PS n=8; ES n=8), vehicle time control (no AIH; PS n=4; ES n=4), apocynin + AIH (PS n=9; ES n=8), apocynin TC (PS n=4; ES n=4). Since there were no apparent differences among TC groups, all TC treated rats were grouped within WT and MT rats for statistical analyses (*i.e.* PS: MT=8 and WT=9 and ES: MT=8 and WT=8). Since pLTF, but not XII LTF, changed over disease progression, only pLTF was recorded following apocynin (or vehicle) delivery.

To begin protocols, the apneic CO₂ threshold was determined by lowering P_{ETCO₂} until nerve activity ceased for approximately one minute. The recruitment threshold was then determined by slowly increasing P_{ETCO₂} until nerve activity resumed (Bach and Mitchell, 1996). P_{ETCO₂} was raised ~2 mmHg above the recruitment threshold and ~15–20 min were allowed to establish a stable baseline. Arterial blood samples were drawn during baseline, and throughout protocols; arterial CO₂ (PaCO₂) was maintained within ± 1.5 mmHg of baseline levels by adjusting inspired CO₂ and/or ventilator rate.

Immunohistochemistry

Young, pre-symptomatic and end-stage MT and age-matched WT littermates were processed to assess phrenic motor neuron survival, SOD1 expression and/or superoxide accumulation. Rats were either euthanized with Beuthanasia (*i.p.* 0.1 µl/g; Intervet Schering-Plough Animal Health, Summit, NJ), or taken immediately after neurophysiological protocols (for phrenic motor neuron counts) and perfused with 4% paraformaldehyde in phosphate buffered saline (0.1 M PBS). After fixation, brainstems and spinal cords were excised, post-fixed overnight, and cryoprotected in sucrose at 4 °C until sinking. Transverse sections containing phrenic motor neurons (C₄–C₅; 40 µm) were cut using a freezing microtome (Leica SM 2000R, Germany).

At each age, rats were injected with 2 µM dihydroethidium (*i.p.* 20 mg/kg; DHE; Invitrogen, Grand Island, NY) to assess superoxide accumulation 60 min before rats were perfused. This technique has been characterized and validated previously (Carter et al., 1994; Zhao et al., 2005; Serrander et al., 2007; Nijmeh et al., 2010). To assess DHE fluorescence, tissues were

placed on glass slides, cover-slipped using anti-fade solution (Prolong Gold antifade reagent, Invitrogen, Oregon) and examined by a blinded investigator using an epifluorescence microscope (Nikon, Japan). DHE is a redox-sensitive probe, in which the reaction between superoxide and hydroethidine forms a two-electron oxidized product, ethidium (E+) (Carter et al., 1994; Zhao et al., 2005). E+ binds to DNA, enhancing its fluorescence (excitation = 500–530 nm and emission = 590–620 nm) (Carter et al., 1994; Zhao et al., 2005).

SOD1 was detected by washing tissues with Tris-buffered saline (TBS), 0.1% Triton X-100 in TBS (TBS-T), incubated with 5% normal goat serum for 1 hr, and then incubated with rabbit anti-SOD1 (1:500; Upstate, Lake Placid, NY) at 4°C overnight. Anti-SOD1 has been used previously (Raoul et al., 2005; Basso et al., 2006; Bao-Cutrona and Moral, 2009) and was validated and characterized in conjunction with the Human Protein Atlas (HPA) Project (HPA001401; <http://www.proteinatlas.org>). The next day, sections were washed and incubated in biotinylated secondary antibodies (1:1000; Vector Laboratories, Burlingame, CA). Conjugation with avidin-biotin complex (Vecstatin Elite ABC kit, Vector Laboratories, Burlingame, CA) was followed by visualization with 3,3'-diaminobenzidine-hydrogen peroxidase (Vector Laboratories) according to manufacturer instructions. Nissl counterstained sections were stained in 0.1% cresyl-violet for 15 min, rinsed in distilled water, dehydrated through graded alcohol (70% to 100%), cleared in xylene and cover-slipped using Eukitt (Electron Microscope Science, PA). Nissl counterstaining enabled visualization of putative phrenic motor neurons and co-localization with SOD1 label.

Quantification and analysis of photomicrographs

Sections were numbered sequentially, and every 6th section was selected for immunohistochemistry, which enabled systematic sampling. Five sections at the C₄–C₅ segmental level from each rat in each group were used for immunohistochemical analyses conducted by a blinded investigator. Location of putative phrenic motor neurons in the ventral horn was validated based on diagrams from *The Spinal Cord* (Watson et al. 2009) and extensive experience in our laboratory retrograde labeling phrenic motor neurons with cholera-toxin B fragment in normal rats (Dale-Nagle et al., 2011; Dale et al., 2012; Nichols et al., 2015). Putative phrenic motor neurons were counted as described previously (Nichols et al., 2013a; Nichols et al., 2014), where the area containing phrenic motor neurons was validated and identified as a discrete cluster of large neurons in the mediolateral C₄ ventral horn (Boulenguez et al., 2007; Mantilla, et al., 2009; Watson et al., 2009). Digital photomicrographs of immunoreactive labeling in the putative phrenic motor nucleus were taken with the 20× objective lens (Qcapture Pro 6.0, Surrey, BC, Canada), including appropriate controls (*e.g.* sections incubated without primary or secondary antibodies for SOD1 immunostaining or group of animal without DHE injection served as negative controls and each yielded no specific staining). Putative phrenic motor neuron counts were averaged across sections in each rat; statistical inferences were made across experimental groups. Densitometry for SOD1 and DHE staining was performed using NIH ImageJ software (National Institute of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij>). Images were converted to 8-bit resolution, and the threshold was set between 120–160 during all analyses. A threshold was chosen for all groups for both SOD1 and DHE in which all motor neurons were visible, but not saturated; both wild-type and mutant images were treated

identically within each group, and limit to threshold was selected in ImageJ. Optical density (OD) was measured within circumscribed putative phrenic motor neuron somata for SOD1 and DHE immunostaining, and expressed as an average OD per unit area per cell. OD was measured in all putative phrenic motor neurons per slice per animal, and then combined for analyses across groups. OD was averaged across motor neurons within each section, across sections within each rat, and then across rats within each group (to detect potential animal effects); no significant differences among rats within a group were detected, even at end stage (data not shown). OD of SOD1 and DHE immunofluorescence was expressed as a fraction of the average OD in putative phrenic motor neurons of young wild-type rats. Thus, mean OD in the control group is 1.0, with a variance reflecting rat variation in that group. The mean, normalized data presented in this paper reflect variance among rats within that experimental group (standard error).

Immunoblots

Fresh C₄–C₅ spinal segments from MT and age-matched WT littermates at different stages (young, pre-symptomatic and end-stage) were harvested and frozen immediately at –80°C. Frozen segments were placed dorsal-side up on a freezing microtome, and 50–100µm sections were removed until the central canal was visible. Ventral, cervical spinal segments were thawed, weighed and homogenized using a Potter-Elvehjem homogenizer in 500µL of radioimmunoprecipitation (RIPA) buffer, protease inhibitor cocktail (Roche, Indianapolis, IN, USA), and phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). Cell extracts were spun at 7,000 g for 10 min, and the supernatant was removed and aliquoted for immunoblot analysis. Aliquots were immediately analyzed or stored at –80°C for later analysis.

Total protein concentrations for each sample were measured using the bicinchoninic acid method (Pierce Biotechnologies, Inc., Rockford, IL). An equal volume of sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% β-mercaptoethanol) was added to each sample and the sample was boiled for 3 min. 25 µg of protein was electrophoresed at 120 volts (18% SDS-PAGE mini-gels, Biorad Laboratories, Hercules, CA), and transferred to a PVDF membrane (Immobilon P Transfer Membrane, Millipore Corp., Bedford, MA) at 50 mA for 2 hours. Membranes were washed (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) several times, incubated in blocking buffer (6% non-fat dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20), and then in blocking buffer containing anti-SOD1 antibody (1:1,000; Enzo Life Sciences, Inc., Farmingdale, NY) overnight at 4°C. The following day, membranes were washed several times, incubated with blocking buffer containing horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and then washed several more times. SOD1 was visualized with an enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnologies, Inc., Rockford, IL), and then quantified using densitometry (LabWorks software version 4.5, UVP BioImaging Systems, Upland, CA). GAPDH was used as a loading control for all samples. A pooled WT homogenate was loaded on each blot; data quantified across blots were normalized to this pooled WT homogenate to control for membrane variations.

Subsequently, all groups were normalized to rat SOD1 (rSOD1) expression in young WT rats to give a fractional increase in SOD1 protein expression.

Data analyses

Integrated phrenic nerve burst amplitude was averaged over 1 min bins at each time (baseline, 15, 30 and 60 min), and normalized as a percent change from baseline. Respiratory frequency was expressed as a change from baseline (bursts per min). All statistical comparisons between treatment groups for nerve burst amplitude, frequency and blood-gas values were made with a 2-way ANOVA (MT vs WT; treatment as factors) with a repeated measures design (time post-AIH: 15, 30 and 60 min). Values during successive hypoxic episodes were compared within groups using a one-way ANOVA; since no differences were detected, the three hypoxic episodes in each experiment were combined per rat and then group; hypoxic responses were compared among groups *via* 2-way ANOVA. For histology and immunoblot analyses, data were compared between treatment groups using a one-way ANOVA. With statistically significant ANOVAs, individual comparisons were made using Fisher's least significant difference *post hoc* test (SigmaPlot version 12.0; Systat Software Inc., San Jose, CA). Differences were considered significant if $p < 0.05$. Values are expressed as means $\pm$ 1 S.E.M.

RESULTS

Putative phrenic motor neuron survival decreases with age

Throughout the remaining sections, SOD1^{G93A} rats will be referred to as "mutant (MT)" rats; age-matched wild-type littermates will be referred to as "age-matched WT littermates". Although MT rats have lower cervical motor neuron counts (including phrenic motor neuron numbers) at disease end-stage (Lepore et al., 2008; Lepore et al., 2010; Lepore et al., 2011; Nichols et al., 2013a; Nichols et al., 2013b; Nichols et al., 2014), no reports are available concerning when phrenic motor neuron degeneration begins in this model. Here, we studied putative phrenic motor neuron survival in young and pre-symptomatic *versus* end-stage MT rats (Figures 1A & 1B). Putative phrenic motor neuron counts were significantly decreased in pre-symptomatic and end-stage *versus* young MT rats, and *versus* age-matched WT littermates ($p < 0.05$; Figure 1C). Putative phrenic motor neuron counts were not significantly different between young WT littermates and young MT rats, nor did phrenic motor neuron counts vary significantly with age in WT rats ($p > 0.05$; Figure 1C). End-stage SOD1^{G93A} rats had decreased putative phrenic motor neuron counts *versus* pre-symptomatic MT rats ($p < 0.05$; Figure 1C). Thus, putative phrenic motor neuron degeneration was not detectable in young MT (vs. WT) rats, but had already begun in pre-symptomatic MT rats (Figure 1C).

SOD1 expression increases with age in MT rats

As expected, SOD1 is over-expressed in MT rats, but there is a progressive increase with age. In Figures 2A–D, representative photomicrographs for SOD1 immunoreactivity in putative phrenic motor neurons from young and end-stage MT rats and WT littermates are shown; SOD1 immunolabeling is robust in MT rats, with little to no expression in WT littermates. In MT rats, SOD1 expression in surviving putative phrenic motor neurons was significantly greater in MT *versus* WT littermates, and this difference increased

progressively with age ($p < 0.05$; Figure 2E). In contrast, SOD1 expression was unchanged in WT littermates over age ($p > 0.05$; Figure 2E). Immunoblots revealed similar findings: SOD1 expression was elevated in ventral C₄–C₅ spinal homogenates at every age in MT *versus* WT littermates ($p < 0.05$; Figure 2F). SOD1 levels at end-stage were significantly greater than in young or pre-symptomatic MT rats ($p < 0.05$; Figure 2F). In contrast, SOD1 levels were unchanged over age in WT rats ($p > 0.05$; Figure 2F). Thus, we confirm SOD1 over-expression in putative phrenic motor neurons and ventral spinal homogenates of MT rats, with the greatest relative increase at disease end-stage; these findings are consistent with previous reports for other areas of the CNS (Gurney et al., 1994; Howland et al., 2002).

Blood gases and mean arterial pressures

Arterial P_{CO₂} (Pa_{CO₂}) was successfully regulated within 1.5 mmHg of its baseline value in all groups (data not shown). Thus, changes in integrated phrenic nerve burst amplitude following AIH cannot be attributed to differences in chemoreceptor feedback. Pa_{O₂} was successfully maintained within the target range for AIH (35–45 mmHg), and was above 150 mmHg at all times post-hypoxia (data not shown). Mean arterial pressure did not differ within groups, and only differed among groups when comparing AIH *versus* TC during hypoxic episodes, as expected (data not shown). Thus, differences in Pa_{CO₂}, Pa_{O₂} or blood pressure regulation among groups cannot account for differential pLTF expression.

Phrenic LTF in young and pre-symptomatic rats

In normal rats, moderate AIH (35–45 mmHg) elicits approximately 60% pLTF at 60 min post-hypoxia (Baker-Herman and Mitchell, 2002; Baker-Herman and Mitchell, 2008; Hoffman et al., 2010; MacFarlane and Mitchell, 2008; Sibigroth and Mitchell, 2011), although this value decreases in middle-aged (~1year) male rats (Zabka et al., 2001). Here, we investigated AIH-induced pLTF at several ages in MT and age-matched WT littermates (Figures 3A & 3B). AIH elicited pLTF in both young MT and age-matched WT rats at 30 and 60 min post-hypoxia ($p < 0.05$ *vs.* time controls and baseline; Figure 3C). However, there was no significant difference in pLTF between young MT and WT rats at any time post-hypoxia ($p > 0.05$; Figure 3C). As expected, there was no apparent pLTF in TC experiments in either group ($p > 0.05$; Figure 3C). Burst frequency was significantly elevated only 30 min post-AIH in young WT rats ($p < 0.05$ *vs.* baseline and WT time controls; Figure 3D). No significant differences in frequency LTF were detected at any time between young MT and WT rats ($p > 0.05$; Figure 3D).

AIH-induced pLTF was also observed in pre-symptomatic MT rats despite greater SOD1 over-expression (Figures 4A & 4B). Similar to young rats, pLTF was similar in pre-symptomatic MT and age-matched WT littermates at 30 and 60 min-post-AIH ($p < 0.05$ *vs.* TC and baseline; Figure 4C); pLTF was not significantly different in pre-symptomatic MT *versus* age-matched WT littermates at any time post-AIH ($p > 0.05$; Figure 4C). Although frequency was significantly decreased at 15 min post-AIH in pre-symptomatic WT rats *vs.* baseline and pre-symptomatic MT rats ($p < 0.05$; Figure 4D), no other significant differences were detected in burst frequency between pre-symptomatic MT *versus* age-matched WT littermates ($p > 0.05$; Figure 4D).

Enhanced pLTF in end-stage MT rats

As described previously (Nichols et al., 2013), AIH-induced pLTF was enhanced in end-stage MT *versus* age-matched WT littermates (Figures 5A & 5B). pLTF was significantly greater in MT rats ($p < 0.05$ *vs.* baseline and TC MT and WT rats) *versus* age-matched WT littermates at all times monitored ($p < 0.05$ *vs.* baseline and time control WT rats and MT rats; Figure 5C). Frequency LTF was apparent at 60 min post-AIH in end-stage WT rats *versus* baseline and TC WT rats ($p < 0.05$; Figure 5D). In end-stage MT rats, frequency was significantly elevated at 30 and 60 min post-hypoxia *versus* baseline and time control MT rats ($p < 0.05$; Figure 5D). Whereas end-stage MT rats had significantly greater pLTF 60 min post-hypoxia *versus* all other age or treatment groups ($p < 0.05$; data not shown), differences in frequency LTF were not apparent ($p > 0.05$; data not shown).

Hypoglossal LTF and hypoxic response over age in WT and MT rats

AIH-induced hypoglossal (XII) LTF (Figures 6A & 6B) was observed in young MT and age-matched WT littermates at 30 and 60 min post-AIH $p < 0.05$ *vs.* TC and baseline; Figure 6C), but there were no significant differences between young WT *vs.* MT rats at any time post-AIH ($p > 0.05$; Figure 6C). Time control experiments without AIH did not exhibit changes in XII burst amplitude ($p > 0.05$; Figure 6C). Similarly, AIH-induced XII LTF was observed in pre-symptomatic MT and age-matched WT littermates 60 min-post-AIH ($p < 0.05$ *vs.* TC and baseline; Figure 6D); XII LTF was not significantly different in pre-symptomatic MT *versus* WT rats at any time post-AIH ($p > 0.05$; Figure 6D). In contrast to pLTF (Figure 5), AIH induced XII LTF was not enhanced in end-stage MT rats at 15, 30, or 60 min post-AIH $p < 0.05$ *vs.* baseline and TC; Figure 6E). XII LTF was not different between end-stage MT and age-matched WT littermates at any time post-AIH ($p > 0.05$; Figure 6E).

End-stage WT rats had a significantly smaller short-term hypoxic XII response, whereas end-stage MT rats had a significantly larger hypoxic XII response *versus* age-matched WT littermates ($p < 0.05$; Figure 6F). Pre-symptomatic MT rats had significantly smaller hypoxic XII responses *versus* all other MT groups, and pre-symptomatic WT rats ($p < 0.05$; Figure 6F). All AIH-treated WT and MT groups had significant hypoxic XII responses *versus* TC treated rats ($p < 0.05$; Figure 6F). Lastly, pre-symptomatic MT and age-matched WT littermates had smaller XII LTF 60 min post-AIH *versus* all other groups ($p < 0.05$; data not shown).

Superoxide accumulation in phrenic motor neurons is preserved despite increased SOD1 expression until disease end-stage

Since enzymatically active SOD1 is over-expressed, and the level of over-expression further increases with age in MT rats, we assessed superoxide accumulation rates in putative phrenic motor neurons using DHE. Despite elevated SOD1 levels in young and pre-symptomatic MT rats, DHE immunofluorescence (indicating superoxide production) was normal in putative phrenic motor neurons (Figures 7A–D). However, with further increases in SOD1 expression at disease end stage, superoxide accumulation decreased in surviving, putative phrenic motor neurons in end-stage MT rats *versus* age-matched WT littermates (Figures 7E & 7F). DHE fluorescence in surviving, putative phrenic motor neurons at end-

stage was significantly lower than in young and pre-symptomatic MT rats (both $p < 0.001$; Figure 7G). In contrast, DHE fluorescence was unchanged in WT rats at any age ($p > 0.05$; Figure 7G), and was unchanged between young MT and age-matched WT littermates ($p = 0.054$; Figure 7G). However, DHE fluorescence (*ie.* superoxide accumulation) was significantly decreased in end-stage MT *versus* age-matched WT littermates ($p < 0.001$; Figure 7G).

pLTF in end-stage MT rats is NADPH oxidase independent

Since superoxide accumulation in putative phrenic motor neurons decreases with age in MT rats (Figure 7), we assessed the requirement of NADPH oxidase for pLTF in MT rats over age. Following pre-treatment with the NADPH oxidase inhibitor apocynin, AIH-induced pLTF was blocked in pre-symptomatic MT and age-matched WT littermates *versus* vehicle treated rats. pLTF was significantly greater in pre-symptomatic WT rats treated with vehicle (*versus* baseline, apocynin and TC treated WT rats ($p < 0.05$; Figure 8A). For pre-symptomatic MT rats, pLTF was significantly greater in MT rats treated with vehicle *versus* baseline, apocynin, and TC treated MT rats ($p < 0.05$; Figure 8A). Thus, pLTF is NADPH oxidase-dependent in young and pre-symptomatic MT rats.

In contrast, apocynin had no effect on AIH induced pLTF in end-stage MT rats *versus* vehicle treated MT rats, and was still increased *versus* age-matched WT littermates. pLTF was significantly greater in end-stage WT rats treated with vehicle *versus* baseline, apocynin and TC treated WT rats ($p < 0.05$; Figure 8B). For end-stage MT rats, pLTF was significantly greater in MT rats treated with vehicle and apocynin *versus* baseline, age-matched WT littermates and TC treated MT rats ($p < 0.05$; Figure 8B). Thus, pLTF had become NADPH oxidase-independent in end-stage MT rats when superoxide accumulation was reduced.

Frequency LTF was only apparent in pre-symptomatic, not end-stage, MT rats treated with vehicle *versus* baseline, apocynin treated rats, and TC MT treated rats ($p < 0.05$; Figures 8C & 8D). Pre-symptomatic WT rats treated with vehicle also exhibited frequency LTF, but only at 60 minutes post-AIH compared to apocynin treated rats and TC treated WT rats ($p < 0.05$, Figure 8C).

Short-term hypoxic phrenic responses

For unknown reasons, the hypoxic phrenic response was significantly larger in pre-symptomatic WT rats *versus* other ages, and pre-symptomatic MT rats ($p < 0.05$; Figure 9A). End-stage MT rats had a significantly larger hypoxic phrenic response *versus* all other MT age groups, and age-matched WT littermates ($p < 0.05$; Figure 9A). Thus, the larger hypoxic phrenic response in end-stage MT rats may reflect or contribute to the mechanism enhancing pLTF since the short term hypoxic phrenic response and pLTF are positively correlated in normal rats (Fuller et al., 2000; Baker-Herman and Mitchell, 2008). As expected, all AIH-treated MT and WT groups had larger hypoxic phrenic responses *versus* TC ($p < 0.05$; Figure 9A).

Following intrathecal vehicle or apocynin treatment, the hypoxic phrenic response was significantly increased in all treated rats at both time-points. In pre-symptomatic rats, the

hypoxic phrenic response was significantly larger in all treatment groups (compared to TC treated rats ($p < 0.05$; Figure 9B). At the end-stage time point, the hypoxic phrenic response was significantly increased in all treatments groups compared to time control treated rats ($p < 0.05$; Figure 9B). The hypoxic phrenic response was also significantly increased when comparing end-stage MT rats to WT littermates and to pre-symptomatic MT rats within the same treatment group (*i.e.* vehicle *vs.* vehicle; $p < 0.05$; Figure 9B). Thus, regardless of treatment (vehicle or apocynin), the hypoxic phrenic response was still significantly increased in end-stage MT rats compared to age-matched WT littermates and similarly treated pre-symptomatic MT rats.

DISCUSSION

Although our original hypothesis was that transgenic rats over-expressing SOD1^{G93A} would exhibit reduced superoxide accumulation and impaired pLTF, this prediction was not completely supported by our results. Although we verified SOD1 over-expression in putative phrenic motor neurons, superoxide accumulation was unchanged in young and pre-symptomatic MT rats. At these ages, normal superoxide accumulation despite increased enzymatically active SOD1 expression suggests unknown mechanisms of ROS homeostasis within putative phrenic motor neurons. Continued increases in SOD1 expression at end-stage eventually surpassed the capacity for regulation, and superoxide accumulation rates decreased. Although superoxide regulation is sufficient to explain normal pLTF in young and pre-symptomatic MT rats, enhanced pLTF despite diminished superoxide accumulation at disease end-stage is difficult to explain. Enhanced pLTF with normal XII LTF at end-stage suggests that motor neuron degeneration *per se* plays a key role in the mechanisms enhancing pLTF since XII motor neurons exhibit no degeneration at this same end-stage in MT rats (Nichols et al., 2013a). These surprising results leave us with interesting questions including: 1) how is ROS formation preserved in young and pre-symptomatic MT rats despite increased enzymatically active SOD1 expression? and 2) how is pLTF expressed (and even enhanced) in end-stage MT rats in the face of decreased superoxide accumulation?

Age-dependence of pLTF and XII LTF in MT rats

The ability of MT rats to express both AIH-induced pLTF and XII LTF (Figures 3–6) is surprising since superoxide dismutase mimetics (MacFarlane and Mitchell, 2008; MacFarlane et al., 2008) and NADPH oxidase inhibitors (MacFarlane et al., 2009) abolish AIH-induced LTF in normal rats. Despite our initial prediction that over-expression of mutated, but enzymatically active SOD1^{G93A} (Gurney et al., 1994) would abolish LTF, we found evidence for a novel form of ROS homeostasis, preventing decreased superoxide accumulation rates within putative phrenic motor neurons (Figure 7), at least until our defined end-stage. The functional outcome of such ROS homeostasis is preservation of phrenic and XII LTF. Since ROS are key regulators of many kinases (activators) and phosphatases (inhibitors) (Klann 1998; Klann et al., 1998; Klann and Thiels, 1999), ROS homeostasis in the face of increased functional SOD1 may also preserve other important physiological functions. Multiple kinases and phosphatases play key roles in orchestrating

neuroplasticity, in this case respiratory motor plasticity (MacFarlane et al., 2008; Wilkerson et al., 2007).

Perhaps a greater surprise was that pLTF (but not XII LTF) is actually enhanced at disease end-stage (Figures 5 & 6), a stage where ROS homeostasis had been lost and superoxide accumulation was decreased (Figure 7). Since XII LTF was not similarly enhanced, and XII motor neurons have not yet degenerated at our defined end-stage (Nichols et al., 2013a), we suspect that pLTF enhancement arises from local processes within the phrenic motor nucleus associated with motor neuron degeneration. Possibilities include substances released from dying phrenic motor neurons that enhance pLTF in viable motor neurons, or substances released from nearby astrocytes or microglia reacting to motor neuron death. It is also possible that interneurons play a role since interneurons also degenerate in ALS (Hossaini et al., 2011; Turner and Kiernan, 2012); however, it is unknown whether interneurons are affected in the phrenic motor nucleus in this ALS model. pLTF is robust immediately post-AIH in end-stage MT rats (Figure 5); in contrast, pLTF slowly develops in other groups (significant only >30 min post-AIH; Figures 3 & 4). Thus, a second feature differentiating pLTF in MT *versus* WT littermates is the rapid onset of facilitation.

Possible mechanisms of pLTF enhancement at end-stage

In normal rats, AIH-induced pLTF is initiated by episodic metabotropic 5HT₂ receptor activation on or near phrenic motor neurons (Fuller et al., 2001; Baker-Herman and Mitchell, 2002; McFarlane and Mitchell, 2009; McFarlane et al., 2011). Subsequent to 5HT₂ receptor activation, pLTF requires protein kinase C θ activity (Devinney et al., 2015), new synthesis of brain derived neurotrophic factor (BDNF; Baker-Herman et al., 2004), activation of the high affinity BDNF receptor TrkB (Baker-Herman et al., 2004), and extracellular regulated kinase (ERK) activity (Hoffman et al., 2012). Conceivably, any of these proteins could be up regulated in end-stage MT rats, thereby over-riding diminished ROS accumulation and enhancing pLTF. Alternatively, decreased expression of protein phosphatases that constrain pLTF (Wilkerson et al., 2009; MacFarlane et al., 2008) could reduce/eliminate its ROS dependence (Wilkerson et al., 2007; MacFarlane and Mitchell, 2008; MacFarlane et al., 2009).

Another possible mechanism enhancing pLTF at end-stage is diminished cross-talk inhibition between competing pathways to phrenic motor facilitation (pMF; Dale-Nagle et al., 2010). For example, during hypoxia (particularly severe hypoxia), extracellular adenosine accumulates and activates adenosine 2A (A_{2A}) receptors, giving rise to an alternative pathway to pMF referred to as the S pathway (Dale-Nagle et al., 2010). During moderate AIH, modest adenosine accumulation elicits sub-threshold activation of the S pathway that is nevertheless sufficient to constrain the dominant Q pathway. Consequently, reducing inhibitory cross-talk with spinal A_{2A} (or 5-HT₇) receptor antagonists enhances moderate AIH-induced pLTF, similar to pLTF at disease end-stage (Hoffman et al., 2010; Nichols et al., 2012; Hoffman and Mitchell, 2013). Although possible mechanisms of diminished cross-talk inhibition between the Q and S pathways to pMF are unknown, protein kinase A activity/expression may play a critical role since protein kinase A activity underlies S-Q pathway inhibition (Hoffman and Mitchell, 2013).

When AIH consists of severe hypoxia, greater pLTF is evoked, but this form of enhanced pLTF arises predominantly from the adenosine-dependent S pathway (Nichols et al., 2012). We do not yet know if pLTF in end-stage mutant rats is dominated by the Q *versus* S pathway to pMF; thus, it is possible that the S pathway becomes predominant in end-stage SOD1^{G93A} rats.

Since pLTF correlates positively with the short-term hypoxic phrenic response (STHPR; Fuller et al., 2000; Baker-Herman and Mitchell, 2008), and the STHPR is enhanced in end-stage SOD1^{G93A} rats (Figure 9), enhanced pLTF may reflect indirect mechanisms associated with increased STHPR. Our working hypothesis is that these are independent events that, although correlated, do not reflect a causal relationship. For example, the short-term hypoxic XII response was also amplified in end-stage MT rats, yet XII LTF was not enhanced (Figures 6E & 6F). Thus, enhanced pLTF in end-stage MT rats most likely arises from mechanisms specific to pLTF.

Frequency LTF

Although AIH elicits a variable burst frequency LTF in anesthetized, vagotomized WT rats, frequency LTF is 6 fold smaller than (burst amplitude) pLTF (Baker-Herman and Mitchell, 2008). We found results in MT and WT rats at all ages consistent with previous reports (Figures 3–5 & 8). We suggested previously that amplitude and frequency LTF arise from distinct mechanisms (Mitchell et al., 2001; Baker-Herman and Mitchell, 2008).

Regulation of superoxide accumulation despite SOD1 over-expression

ROS are key regulators of neuroplasticity (Klann 1998; Klann et al., 1998; Thiels et al., 2000), including AIH-induced pLTF (MacFarlane and Mitchell, 2008; MacFarlane et al., 2008, 2009; Wilkerson et al., 2007). Thus, over-expression of enzymatically active SOD1 in SOD1^{G93A} transgenic rats was predicted to impair pLTF because of diminished ROS accumulation. Remarkably, putative phrenic motor neuron superoxide accumulation and pLTF are preserved in young and pre-symptomatic MT rats despite increased SOD1 levels (Figures 2–4 & 7), suggesting previously unrecognized compensatory mechanisms that regulate superoxide formation in respiratory motor neurons. However, through unknown mechanisms, the ROS (superoxide) dependence of pLTF is lost late in disease progression. Although NADPH oxidase activity is required for pLTF in pre-symptomatic MT rats, it is no longer required for pLTF in end-stage MT rats (Figure 8). The lack of an apocynin effect in end-stage MT rats is consistent with our previous finding that apocynin had no effect on breathing capacity in unanesthetized end-stage MT rats (Nichols et al., 2014), and by the observation that superoxide formation in end-stage MT rats is actually decreased (Figure 7). These observations further support the notion that unknown compensatory mechanisms preserve pLTF at end-stage. As mentioned, 5HT₂ receptors are necessary and sufficient for pLTF in normal rats (Fuller et al., 2001; Baker-Herman and Mitchell, 2002; McFarlane and Mitchell, 2009; McFarlane et al., 2011). However, only 5HT_{2B} (not 5HT_{2A}) receptors are NADPH oxidase dependent (MacFarlane et al., 2011). Thus, pre-symptomatic MT rats could utilize 5HT_{2B} receptors for pLTF, with a shift towards 5HT_{2A} receptor induced pLTF in end-stage MT rats.

We did not assess ROS other than superoxide, such as hydrogen peroxide or hydroxyl radicals. Both increase in the cerebrospinal fluid of MT mice (Liu et al., 1999) and may enable pLTF in MT rats. Further, since spinal nitric oxide is necessary and sufficient for pLTF in normal rats (MacFarlane et al., 2014), nitric oxide synthase upregulation with increased nitric oxide production in spared phrenic motor neurons could contribute to pLTF in MT rats. Preliminary data from our laboratory suggest that hydrogen peroxide, hydroxyl radical and nitric oxide formation/production are increased in putative phrenic motor neurons in end-stage MT rats (Satriotomo et al., 2008). At this point, we do not know the mechanisms preserving/enhancing the capacity for pLTF in end-stage MT rats.

Significance

We exploited a “flaw” in the SOD1^{G93A} transgenic rat model of ALS to demonstrate new principles, including: 1) the ability of respiratory motor neurons to compensate for increased SOD1 enzymatic activity *via* novel mechanisms of ROS homeostasis; and 2) the ability to preserve phrenic motor plasticity in the face of diminished ROS accumulation. In pre-symptomatic rats, superoxide accumulation is preserved despite increased SOD enzymatic activity, accounting for preservation of pLTF and XII LTF. However, quite unexpectedly, once superoxide accumulation decreases at end-stage, pLTF is not only expressed, but enhanced. An important implication of our findings is that superoxide accumulation is actively defended in motor neurons, thereby preserving the capacity for plasticity. The common notion of “ROS stress” needs further consideration since inadequate ROS levels pose equal problems for animals due to reduced kinase and exaggerated phosphatase activities that regulate the net phosphorylation/activation of target proteins. Diminished ROS levels shift the kinase/phosphatase balance towards net dephosphorylation, slowing/preventing key cellular functions, including neuroplasticity.

The finding that pLTF is enhanced at disease end-stage is surprising since ROS homeostasis was eventually lost from progressively increasing SOD1 over-expression (reflected in decreased superoxide accumulation). Although this observation may raise questions concerning the suitability of SOD1^{G93A} over-expressing transgenic rats as an ALS model, it points us towards recognition of previously unsuspected mechanisms that preserve the capacity for respiratory motor plasticity. Although we do not yet know its mechanism, ROS homeostasis within phrenic (and XII) motor neurons is presumably sufficient to preserve the capacity for pLTF in young and pre-symptomatic MT rats. On the other hand, once the ability to maintain ROS homeostasis has been surpassed, distinct ROS-independent mechanisms must account for enhanced pLTF in end-stage MT rats. A detailed understanding of mechanisms enhancing pLTF at disease end-stage may give new insights into the regulation of plasticity and its expression in disease. Regardless of its mechanism, our results suggest the possibility of preserving respiratory motor function in ALS with modest protocols of AIH until late in disease progression. Thus, AIH represents a novel therapeutic strategy to treat respiratory insufficiency in ALS and other clinical disorders that threaten motor neuron survival.

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REFERENCES

- Bach KB, Mitchell GS. Hypoxia-induced long-term facilitation of respiratory activity is serotonin dependent. *Respir Physiol*. 1996; 104:251–260. [PubMed: 8893371]
- Baker-Herman TL, Fuller DD, Bavis RW, Zabka AG, Golder FJ, Doperalski NJ, Johnson RA, Watters JJ, Mitchell GS. BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. *Nat Neurosci*. 2004; 7:48–55. [PubMed: 14699417]
- Baker-Herman TL, Mitchell GS. Phrenic long-term facilitation requires spinal serotonin receptor activation and protein synthesis. *J Neurosci*. 2002; 22:6239–6246. [PubMed: 12122082]
- Baker-Herman TL, Mitchell GS. Determinants of frequency long-term facilitation following acute intermittent hypoxia in vagotomized rats. *Respir Physiol Neurobiol*. 2008; 162:8–17. [PubMed: 18450525]
- Bao-Cutrona M, Moral P. Unexpected expression pattern of tetracycline-regulated transgenes in mice. *Genetics*. 2009; 181(4):1687–1691. [PubMed: 19204377]
- Basso M, Massignan T, Samengo G, Cheroni C, De Biasi S, Salmons M, Bendotti C, Bonetto V. Insoluble mutant SOD1 is partly oligoubiquitinated in amyotrophic lateral sclerosis mice. *J Biol Chem*. 2006; 281(44):33325–33335. [PubMed: 16943203]
- Boulenguez P, Gestreau C, Vinit S, Stamegna JC, Kastner A, Gauthier P. Specific and artifactual labeling in the rat spinal cord and medulla after injection of monosynaptic retrograde tracers into the diaphragm. *Neurosci Lett*. 2007; 417(2):206–211. [PubMed: 17412505]
- Bourke SC, Shaw PJ, Gibson GJ. Respiratory function vs. sleep-disordered breathing as predictors of QOL in ALS. *Neurology*. 2001; 57:2040–2044. [PubMed: 11739823]
- Bruijn LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron*. 1997; 18(2):327–338. [PubMed: 9052802]
- Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukoc Biol*. 1994; 55(2):253–258. [PubMed: 8301222]
- Dale EA, Ben Mabrouk F, Mitchell GS. Unexpected benefits of intermittent hypoxia: enhanced respiratory and nonrespiratory motor function. *Physiology*. 2014; 29(1):39–48. [PubMed: 24382870]
- Dale EA, Satriotomo I, Mitchell GS. Cervical spinal erythropoietin induces phrenic motor facilitation via extracellular signal-regulated protein kinase and Akt signaling. *J. Neurosci*. 2012; 32(17):5973–5983. [PubMed: 22539857]
- Dale-Nagle EA, Hoffman MS, MacFarlane PM, Mitchell GS. Multiple pathways to long-lasting phrenic motor facilitation. *Adv Exp Med Biol*. 2010; 669:225–230. [PubMed: 20217354]
- Dale-Nagle EA, Satriotomo I, Mitchell GS. Spinal vascular endothelial growth factor induces phrenic motor facilitation via extracellular signal-regulated kinase and Akt signaling. *J. Neurosci*. 2011; 31(21):7682–7690. [PubMed: 21613481]
- Devinney MJ, Fields DP, Huxtable AG, Peterson TJ, Dale EA, Mitchell GS. Phrenic long-term facilitation requires PKC θ activity within phrenic motor neurons. *J Neurosci*. 2015; 35(21):8107–8117. [PubMed: 26019328]
- Fuller DD, Bach KB, Baker TL, Kinkad R, Mitchell GS. Long term facilitation of phrenic motor output. *Respir Physiol*. 2000; 121(2–3):135–146. [PubMed: 10963770]
- Fuller DD, Zabka A, Baker TL, Mitchell GS. Physiological and Genomic Consequences of Intermittent Hypoxia: Selected Contribution: Phrenic long-term facilitation requires 5-HT receptor

- activation during but not following episodic hypoxia. *J Appl Physiol.* 2001; 90:2001–2006. [PubMed: 11299296]
- Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliando J, Hentati A, Kwon YW, Deng HX, Chen W, Zhai P, Sufit RL, Siddique T. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science.* 1994; 264(5166):1772–1775. [PubMed: 8209258]
- Hoffman MS, Golder FJ, Mahamed S, Mitchell GS. Spinal adenosine 2A receptor inhibition enhances phrenic long term facilitation following acute intermittent hypoxia. *J Physiol.* 2010; 588:255–266. [PubMed: 19900961]
- Hoffman MS, Mitchell GS. Spinal 5-HT7 receptors and protein kinase A constrain intermittent hypoxia-induced phrenic long-term facilitation. *Neuroscience.* 2013; 250:632–643. [PubMed: 23850591]
- Hoffman MS, Nichols NL, MacFarlane PM, Mitchell GS. Phrenic long-term facilitation after acute intermittent hypoxia requires spinal ERK activation but not TrkB synthesis. *J Appl Physiol.* 2012; 113(8):1184–1193. [PubMed: 22961271]
- Hossaini M, Cardona Cano S, van Dis V, Haasdijk ED, Hoogenraad CC, Holstege JC, Jaarsma D. Spinal inhibitory interneuron pathology follows motor neuron degeneration independent of glial mutant superoxide dismutase 1 expression in SOD1-ALS mice. *J Neuropathol Exp Neurol.* 2011; 70(8):662–677. [PubMed: 21760539]
- Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, Erickson J, Kulik J, DeVito L, Psaltis G, DeGennaro LJ, Cleveland DW, Rothstein JD. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci U S A.* 2002; 99(3):1604–1609. [PubMed: 11818550]
- Ilzecka J, Stelmasiak Z, Balicka G. Respiratory function in amyotrophic lateral sclerosis. *Neurol Sci.* 2003; 24:288–289. [PubMed: 14658052]
- Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, Burrell JR, Zoing MC. Amyotrophic lateral sclerosis. *Lancet.* 2011; 377(9769):942–955. [PubMed: 21296405]
- Klann E. Cell-permeable scavengers of superoxide prevent long-term potentiation in hippocampal area CA1. *J Neurophysiol.* 1998; 80:452–457. [PubMed: 9658063]
- Klann E, Roberson ED, Knapp LT, Sweatt JD. A role for superoxide in protein kinase C activation and induction of long-term potentiation. *J Biol Chem.* 1998; 273(8):4516–4522. [PubMed: 9468506]
- Klann E, Thiels E. Modulation of protein kinases and protein phosphatases by reactive oxygen species: implications for hippocampal synaptic plasticity. *Prog Neuropsychopharmacol Biol Psychiatry.* 1999; 23:359–376. [PubMed: 10378223]
- Lechtzin N, Rothstein J, Clawson L, Diette G, Wiener CM. Amyotrophic lateral sclerosis: evaluation and treatment of respiratory impairment. *Amyotrophic Lateral Sclerosis.* 2002; 3:5–13. [PubMed: 12061943]
- Lepore AC, O'Donnell J, Kim AS, Williams T, Tuteja A, Rao MS, Kelley LL, Campanelli JT, Maragakis NJ. Human glial-restricted progenitor transplantation into cervical spinal cord of the SOD1 mouse model of ALS. *PLoS One.* 2011; 6(10):e25968. [PubMed: 21998733]
- Lepore AC, Rauck B, Dejea C, Pardo AC, Rao MS, Rothstein JD. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nat Neurosci.* 2008; 11(11):1294–1301. [PubMed: 18931666]
- Lepore AC, Tolmie C, O'Donnell J, Wright MC, Dejea C, Rauck B, Hoke A, Ignagni AR, Onders RP, Maragakis NJ. Peripheral hyperstimulation alters site of disease onset and course in SOD1 rats. *Neurobiol Dis.* 2010; 39(3):252–264. [PubMed: 20381620]
- Liu D, Wen J, Liu J, Li L. The roles of free radicals in amyotrophic lateral sclerosis: reactive oxygen species and elevated oxidation of protein, DNA, and membrane phospholipids. *FASEB J.* 1999; 13:2318–2328. [PubMed: 10593879]
- Lovett-Barr MR, Satriotomo I, Muir G, Wilkerson JER, Hoffman MS, Vinit S, Mitchell GS. Repetitive intermittent hypoxia induces respiratory and somatic motor recovery following chronic cervical spinal injury. *J Neurosci.* 2012; 32:3591–3600. [PubMed: 22423083]
- Lyall RA, Donaldson N, Polkey MI, Leigh PN, Moxham J. Respiratory muscle strength and ventilatory failure in amyotrophic lateral sclerosis. *Brain.* 2001; 124(10):110–118.

- MacFarlane PM, Mitchell GS. Respiratory long-term facilitation following intermittent hypoxia requires reactive oxygen species formation. *Neuroscience*. 2008; 35:1269–1272.
- MacFarlane PM, Satriotomo I, Windelborn JA, Mitchell GS. NADPH oxidase activity is necessary for acute intermittent hypoxia-induced phrenic long-term facilitation. *J Physiol*. 2009; 587:1931–1942. [PubMed: 19237427]
- MacFarlane PM, Vinit S, Mitchell GS. Serotonin 2A and 2B receptor-induced phrenic motor facilitation: differential requirement for spinal NADPH oxidase activity. *Neuroscience*. 2011; 178:45–55. [PubMed: 21223996]
- MacFarlane PM, Vinit S, Mitchell GS. Spinal nNOS regulates phrenic motor facilitation by a 5-HT_{2B} receptor- and NADPH oxidase-dependent mechanism. *Neuroscience*. 2014; 269:67–78. [PubMed: 24680940]
- MacFarlane PM, Wilkerson JE, Lovett-Barr MR, Mitchell GS. Reactive oxygen species and respiratory plasticity following intermittent hypoxia. *Respir Physiol Neurobiol*. 2008; 164:263–271. [PubMed: 18692605]
- Mantilla CB, Zhan WZ, Sieck GC. Retrograde labeling of phrenic motoneurons by intrapleural injection. *J Neurosci Methods*. 2009; 182(2):244–249. [PubMed: 19559048]
- Mitchell GS. Respiratory plasticity following intermittent hypoxia: a guide for novel therapeutic approaches to ventilatory control disorders. In: Gaultier, C., editor. *Genetic basis for respiratory control disorders*. New York: Springer; 2007. p. 291–306.
- Mitchell GS, Baker TL, Nanda SA, Fuller DD, Zabka AG, Hodgeman BA, Bavis RW, Mack KJ, Olson EB Jr. Invited review: intermittent hypoxia and respiratory plasticity. *J Appl Physiol*. 2001; 90:2466–2475. [PubMed: 11356815]
- Nagai M, Aoki M, Miyoshi I, Kato M, Pasinelli P, Kasai N, Brown RH Jr, Itoyama Y. Rats expressing human cytosolic copper-zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. *J Neurosci*. 2001; 21(23):9246–9254. [PubMed: 11717358]
- Nichols NL, Dale EA, Mitchell GS. Severe acute intermittent hypoxia elicits phrenic long-term facilitation by a novel adenosine-dependent mechanism. *J Appl Physiol*. 2012; 112(10):1678–88. [PubMed: 22403346]
- Nichols NL, Gowing G, Satriotomo I, Nashold LJ, Dale EA, Suzuki M, Avalos P, Mulcrone PL, McHugh J, Svendsen CN, Mitchell GS. Intermittent hypoxia and stem cell implants preserve breathing capacity in a rodent model of amyotrophic lateral sclerosis. *Am J Respir Crit Care Med*. 2013a; 187(5):535–542. [PubMed: 23220913]
- Nichols NL, Johnson RA, Satriotomo I, Mitchell GS. Neither serotonin nor adenosine-dependent mechanisms preserve ventilatory capacity in ALS rats. *Respir Physiol Neurobiol*. 2014; 197:19–28. [PubMed: 24681328]
- Nichols NL, Van Dyke J, Nashold L, Satriotomo I, Suzuki M, Mitchell GS. Ventilatory control in ALS. *Respir Physiol Neurobiol*. 2013b; 189(2):429–437. [PubMed: 23692930]
- Nichols NL, Vinit S, Bauernschmidt L, Mitchell GS. Respiratory function after selective respiratory motor neuron death from intrapleural CTB-saporin injections. *Exp Neurol*. 2015; 267:18–29. [PubMed: 25476493]
- Nijmeh J, Moldobaeva A, Wagner EM. Role of ROS in ischemia-induced lung angiogenesis. *Am J Physiol Lung Cell Mol Physiol*. 2010; 299(4):L535–L541. [PubMed: 20693319]
- Raoul C, Abbas-Terki T, Bensadoun JC, Guillot S, Haase G, Szulc J, Henderson CE, Aebischer P. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nature Med*. 2005; 11(4):423–428. [PubMed: 15768028]
- Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*. 1993; 362:59–62. [PubMed: 8446170]
- Satriotomo I, Harrigan DJ, Mitchell GS. Increased peroxide, hydroxyl-radical and nitric oxide, but decreased superoxide in the phrenic motor nucleus of ALS mutant rats. *Soc Neurosci Abstr*. 2008; 49:16.

- Serrander L, Cartier L, Bedard K, Banfi B, Lardy B, Plastre O, Sienkiewicz A, Fórró L, Schlegel W, Krause KH. NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. *Biochem J*. 2007; 406(1):105–114. [PubMed: 17501721]
- Sibigtroth CM, Mitchell GS. Carotid chemoafferent activity is not necessary for all phrenic long-term facilitation following acute intermittent hypoxia. *Resp Phys Neurobiol*. 2011; 176:73–79.
- Singh D, Verma R, Garg RK, Singh MK, Shukla R, Verma SK. Assessment of respiratory functions by spirometry and phrenic nerve studies in patients of amyotrophic lateral sclerosis. *J Neurol Sci*. 2011; 306(1–2):76–81. [PubMed: 21496826]
- Thiels E, Urban NN, Gonzalez-Burgos GR, Kanterewicz BI, Barrionuevo G, Chu CT, Ourv TD, Klann E. Impairment of long-term potentiation and associative memory in mice that overexpress extracellular superoxide dismutase. *J Neurosci*. 2000; 20:7631–7639. [PubMed: 11027223]
- Turner MR, Kiernan MC. Does interneuronal dysfunction contribute to neurodegeneration in amyotrophic lateral sclerosis? *Amyotroph Lateral Scler*. 2012; 13(3):245–250. [PubMed: 22424125]
- Wang J, Slunt H, Gonzales V, Fromholt D, Coonfield M, Copeland NG, Jenkins NA, Borchelt DR. Copper-binding-site-null SOD1 causes ALS in transgenic mice: aggregates of non-native SOD1 delineate a common feature. *Hum Mol Genet*. 2003; 12(21):2753–2764. [PubMed: 12966034]
- Watson, C.; Paxinos, G.; Kayalioglu, G. *The Spinal Cord: A Christopher and Dana Reeve Foundation Text and Atlas*. London: Academic Press; 2009.
- Wilkerson JE, MacFarlane PM, Hoffman MS, Mitchell GS. Respiratory plasticity following intermittent hypoxia: Roles of protein phosphatases and reactive oxygen species. *Biochem Soc Trans*. 2007; 35(Pt5):1269–1272. [PubMed: 17956327]
- Wilkerson JE, Mitchell GS. Daily intermittent hypoxia augments spinal BDNF levels, ERK phosphorylation and respiratory long-term facilitation. *Exp Neurol*. 2009; 217(1):116–23. [PubMed: 19416672]
- Wong PC, Pardo CA, Borchelt DR, Lee MK, Copeland NG, Jenkins NA, Sisodia SS, Cleveland DW, Price DL. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron*. 1995; 14:1105–1116. [PubMed: 7605627]
- Zabka AG, Behan M, Mitchell GS. Long term facilitation of respiratory motor output decreases with age in male rats. *J Physiol*. 2001; 531(2):509–514. [PubMed: 11230522]
- Zhao H, Joseph J, Fales HM, Sokoloski EA, Levine RL, Vasquez-Vivar J, Kalyanaraman B. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc Natl Acad Sci U S A*. 2005; 102(16):5727–5732. [PubMed: 15824309]
- Zinman L, Cudkowicz M. Emerging targets and treatments in amyotrophic lateral sclerosis. *Lancet Neurol*. 2011; 10(5):481–490. [PubMed: 21511200]

Highlights

1. pLTF and XII LTF is observed in SOD1^{G93A} rats.
2. pLTF is NADPH oxidase-dependent in pre-symptomatic SOD1^{G93A} rats.
3. pLTF is NADPH oxidase-independent in end-stage SOD1^{G93A} rats.

Figure 1.

Putative phrenic motor neuron survival decreases with disease progression in $SOD1^{G93A}$ (MT) rats. *A & B:* Nissl staining (blue) reveals large, putative phrenic motor neurons in an end-stage MT (*B*) and wild-type littermate (WT; *A*). The putative phrenic motor nucleus is circumscribed in each panel, and is shown in higher magnification in panel insets. *C:* Number of surviving, putative phrenic motor neurons in young, pre-symptomatic (PS) and end-stage (ES) MT (filled triangles) and WT rats (filled circles). Putative phrenic motor neuron survival significantly decreased in PS and ES MT *versus* young MT rats (# = $p < 0.05$) and *versus* age-matched WT littermates (* = $p < 0.05$). Survival was also

significantly decreased in ES MT *versus* PS MT rats ($+ = p < 0.05$). Scale bars are 200 μm and 40 μm (higher magnification). Values are means $\pm$ 1 SEM.

Figure 2.

SOD1 expression over age in putative phrenic motor neurons. A–D: Representative SOD1 immunostaining in C₄ putative phrenic motor neurons from young (A & B) and end-stage (ES: C & D) SOD1^{G93A} (MT) and wild-type (WT) littermates; SOD1 immunostaining is brown and neurons are blue (counterstained with cresyl-violet staining). The putative phrenic motor nucleus is circumscribed in each panel, and is shown at higher magnification in panel insets. SOD1 labeling is present in the presumptive phrenic motor nucleus in MT rats. E: Optical density (fold control) representing SOD1 expression in putative phrenic motor neurons was performed on young, pre-symptomatic (PS) and end-stage (ES) MT (filled triangles) and age-matched WT littermates (filled circles). SOD1 significantly increased with age in surviving, putative phrenic motor neurons in MT rats ($p < 0.05$; young and PS MT *versus* ES MT rats), and was increased *versus* WT littermates at each time-point ($p < 0.05$). SOD1 expression in putative phrenic motor neurons was unchanged in WT rats over age ($p > 0.05$). Values are means $\pm$ 1 SEM. * = $p < 0.05$ *versus* WT, # = $p < 0.05$ *versus* young MT, + = $p < 0.05$ *versus* PS MT. Scale bars are 400 μ m (low magnification) and 40 μ m (high magnification). F The inset is a representative immunoblot for SOD1 expression in

MT and WT ES rat. Human SOD1 (hSOD1) is heavily expressed in MT *versus* WT rat, whereas rat SOD1 (rSOD1) is similar in both. Immunoblot analysis (fold control) for SOD1 expression in the C₄ ventral spinal cord from MT and WT young, PS and ES rats; ventral C₄ SOD1 significantly increased with age in MT rats, and was significantly greater than WT littermates at each time-point. SOD1 expression was unchanged in WT rats over age ($p>0.05$). Values are means $\pm$ 1 SEM. * = $p<0.001$ *versus* WT, # = $p<0.001$ *versus* young MT rats, + = $p<0.05$ *versus* PS MT.

Figure 3.

pLTF is normal in young $SOD1^{G93A}$ (MT) and WT littermates. *A & B*: Representative traces of compressed, integrated phrenic nerve activity before and after AIH in young WT (*A*) and MT rats (*B*). The dashed line in each trace represents baseline. *C & D*: Phrenic burst amplitude (percent change from baseline; *C*) and frequency (change from baseline; bursts/min; *D*) in young WT (filled circles) and MT (filled triangles) rats after AIH, and time control (without AIH) WT (open circles) and MT rats (open triangles). pLTF is significant in young WT and MT rats at 30 and 60 min post-AIH *versus* baseline (*), and at 60 min *versus* TC rats (#) (all $p < 0.05$; *C*). Small frequency LTF in young WT rats is observed at 30 min post-AIH *versus* baseline (*) and time control WT rats (#) (all $p < 0.05$; *D*). Values are means $\pm$ 1 SEM.

Figure 4.

pLTF is normal in pre-symptomatic SOD1^{G93A} (MT) and WT littermates. *A & B:* Representative traces of compressed, integrated phrenic nerve activity before and after AIH in pre-symptomatic WT (PS WT; *A*) and MT rats (PS MT; *B*); white, dashed lines represent baseline. *C & D:* Phrenic burst amplitude (percent change from baseline; *C*) and frequency (change from baseline; bursts/min; *D*) in PS WT (filled circles) and MT (filled triangles) rats post-AIH *versus* time control WT (open circles) and MT rats (open triangles). pLTF is significant in PS WT and MT rats at 30 and 60 min post-AIH *versus* baseline (*), and at 60 min post-AIH *versus* time controls (#) (all $p < 0.05$; *C*). Nerve burst frequency in PS WT rats is significantly lower at 15 min post-AIH *versus* baseline (*) and PS MT rats (+) (all $p < 0.05$; *D*). Values are means $\pm$ 1 SEM.

Figure 5.

pLTF is enhanced in end-stage in SOD1^{G93A} (MT) rats. *A & B*: Representative traces of integrated phrenic nerve activity before and after AIH in end-stage WT (ES WT; *A*) and MT rats (ES MT; *B*); white, dashed line indicates baseline. Neurograms are from different rats than those published previously for these rat groups. *C & D*: Phrenic burst amplitude (percentage change from baseline; *C*) and frequency (change from baseline; bursts/min; *D*) in ES WT (filled circles) and MT (filled triangles) rats after AIH *versus* time control WT (open circles) and MT rats (open triangles). pLTF is significantly increased in ES MT rats at 15, 30 and 60 min post-AIH *versus* baseline (*), MT time controls (#), and WT littermates (+) (all $p < 0.05$; *C*). In ES WT rats, pLTF is significant at 30 and 60 min post-AIH *versus* baseline (*), and at 60 min *versus* time control WT rats (#) (all $p < 0.05$; *C*). Small frequency LTF is seen in ES WT rats only at 60 min post-AIH *versus* baseline (*) and time control rats (#) (all $p < 0.05$; *D*). In ES MT rats, frequency LTF is significant only at 30 and 60 min post-AIH *versus* baseline (*) and time control rats (#) (all $p < 0.05$; *D*). Values are means $\pm$ 1 SEM.

Figure 6.

XII LTF and short-term XII hypoxic response *versus* age in SOD1^{G93A} (MT) and WT littermates. *A & B*: Representative traces of integrated XII neurograms before and after AIH in end-stage wild-type (ES WT; *A*) and MT rats (ES MT; *B*). White, dashed line in each trace indicates baseline. *C–E*: XII burst amplitude post-AIH (percent change from baseline) in WT (filled circles) and MT (filled triangles) rats *versus* time control WT (open circles) and MT rats (open triangles) for young (*C*), pre-symptomatic (PS; *D*) and ES rats (*E*). XII LTF is significant in young WT and MT rats at 30 and 60 min post-AIH *versus* baseline (*),

and at 60 min post-AIH *versus* TC rats (#) (all $p < 0.05$; C). XII LTF is significant in PS WT and MT rats after AIH at 60 min post-AIH *versus* baseline (*) and time control rats (#) (all $p < 0.05$; D). XII LTF is significantly increased in ES WT and MT rats at 15, 30 and 60 min post-AIH *versus* baseline (*), and at 30 and 60 min *versus* time control rats (#) (all $p < 0.05$; E). F: Hypoxic XII response over age in MT and WT littermates; white bars represent WT and black bars represent MT rats for young, pre-symptomatic (PS), end-stage (ES) and time-control (TC). ES WT rats had a significantly smaller hypoxic XII response than young WT rats ($p < 0.05$). PS MT rats had a significantly smaller hypoxic XII response *versus* all other MT groups and PS WT rats ($p < 0.05$). ES MT rats had a larger hypoxic XII responses *versus* ES WT rats ($p < 0.05$). All WT and MT groups treated with AIH had significantly larger hypoxic XII responses than time control rats ($p < 0.05$). # = $p < 0.05$ *versus* ES MT rats, + = $p < 0.05$ *versus* TC rats, @ = $p < 0.05$ *versus* ES WT rats and \$ = $p < 0.05$ *versus* PS MT rats. Values are means $\pm$ 1 S.E.M.

Figure 7.

Superoxide accumulation over age in SOD1^{G93A} (MT) and WT littermates. A–F: Photomicrographs of DHE (superoxide marker; stained in red) fluorescence in putative C₄ phrenic motor neurons from young (A & B), pre-symptomatic (PS; C & D), and end-stage (ES; E & F) MT (B, D & F) and WT littermates (A, C & E). Superoxide accumulates in presumptive phrenic motor neurons in young and PS WT and MT rats (A–D). At ES, superoxide accumulation is diminished in surviving motor neurons in MT rats (F) *versus* WT littermates (E) and all other rat groups (A–D). G: Optical density (fold control) of DHE fluorescence in young, PS, and ES MT (filled triangles) and WT littermates (filled circles). Superoxide accumulation revealed by timed DHE fluorescence was significantly larger in surviving, putative phrenic motor neurons in ES WT littermates, young and PS MT rats compared to ES MT rats (* = $p < 0.001$). Superoxide accumulation was unchanged in putative phrenic motor neurons in WT rats over age ($p > 0.05$). Scale bar in F = 50 μ m. Values are means $\pm$ 1 SEM.

Figure 8.

NADPH oxidase is required for pLTF in pre-symptomatic, not end-stage, $SOD1^{G93A}$ (MT) rats. *A & B*: Phrenic burst amplitude (percentage change from baseline) in pre-symptomatic (PS; *A*) and end-stage (ES; *B*) wild-type (WT) and MT rats after vehicle (WT + Veh: black, filled circles; MT + Veh: black, filled triangles), apocynin (WT + Apo: gray, filled circles; MT + Apo: gray, filled triangles) and time-control (-AIH) treatment (WT -AIH: open circles; MT -AIH: open triangles). pLTF is significantly increased in PS WT and MT vehicle treated rats at 15, 30 and 60 min post-AIH *versus* baseline (*), Apo treated rats (#) and time-control treated rats (*) (all $p < 0.05$; *A*). pLTF is significantly increased in ES WT vehicle treated rats at 30 and 60 min post-AIH *versus* baseline and time control treated rats (*), and at all time points when compared to Apo treated WT rats (#) (all $p < 0.05$; *B*). In ES MT Veh and Apo treated rats, pLTF is significant at 15, 30 and 60 min post-AIH *versus* baseline and time control treated rats(*), and wild-type littermates (\$) (all $p < 0.05$; *B*). *C & D*: Frequency (change from baseline; bursts/min) in PS (*C*) and ES (*D*) WT and MT rats after Veh, Apo, and time-control treatment. Small frequency LTF is seen in PS MT Veh treated rats at 15, 30 and 60 min post-AIH *versus* apocynin treated MT rats (#), but only at 30 and 60 min post-AIH when compared to baseline and time control rats (*; $p < 0.05$; *C*). PS MT Veh treated rats also exhibited larger frequency LTF compared to WT littermates (\$;

p<0.05; C). Frequency LTF exhibited by PS WT Veh treated rats was only observed at 60 minutes post-AIH compared to apocynin treated WT rats (#) and TC treated WT rats (*;p<0.05; C). There were no significant differences observed in any ES group (D). Values are means $\pm$ 1 SEM.

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Figure 9.

Short-term hypoxic phrenic response *versus* age in SOD1^{G93A} (MT) and WT littermates. Since phrenic responses were not different across hypoxic episodes within groups ($p > 0.05$; data not shown), responses during the three episodes were combined in each rat and then compared across age and treatment groups. A: Short-term hypoxic phrenic responses were compared in young, pre-symptomatic (PS), end-stage (ES) and time-control (TC) wild-type (WT) rats (white bars) and MT rats (black bars). Short-term hypoxic phrenic responses were significantly larger in PS WT rats *versus* other WT groups and PS MT rats (*; $p < 0.05$). ES

MT rats had a significantly larger hypoxic phrenic response *versus* all other MT groups and ES WT rats (#; $p < 0.05$). AIH treated WT and MT groups had significantly larger short-term hypoxic phrenic responses *versus* TC rats (+; $p < 0.05$). *B*: Short-term hypoxic phrenic responses were compared in PS and ES WT and MT rats following vehicle (Veh), Apocynin (Apo) or TC treatment. The short-term hypoxic phrenic response was significantly larger in all ES and PS WT and MT treated rats *versus* TC treated rats (+; $p < 0.05$). ES MT rats had significantly larger hypoxic phrenic responses compared to ES WT littermates (\$), and similarly treated PS rats (#; all $p < 0.05$). Values are means $\pm$ 1 S.E.M.