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Phrenic long-term facilitation following intrapleural CTB-SAP-induced respiratory motor neuron death

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Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating disease leading to progressive motor neuron degeneration and death by ventilatory failure. In a rat model of ALS (SOD1^{G93A}), phrenic long-term facilitation (pLTF) following acute intermittent hypoxia (AIH) is enhanced greater than expected at disease end-stage but the mechanism is unknown. We suggest that one trigger for this enhancement is motor neuron death itself. Intrapleural injections of cholera toxin B fragment conjugated to saporin (CTB-SAP) selectively kill respiratory motor neurons and mimic motor neuron death observed in SOD1^{G93A} rats. This CTB-SAP model allows us to study the impact of respiratory motor neuron death on breathing without many complications attendant to ALS. Here, we tested the hypothesis that phrenic motor neuron death is sufficient to enhance pLTF. pLTF was assessed in anesthetized, paralyzed and ventilated Sprague Dawley rats 7 and 28 days following bilateral intrapleural injections of: 1) CTB-SAP (25µg), or 2) unconjugated CTB and SAP (control). CTB-SAP enhanced pLTF at 7 (CTB-SAP: 162±18%, n=8 vs. Control: 63±3%; n=8; p<0.05), but not 28 days post-injection (CTB-SAP: 64±10%, n=10 vs. Control: 60±13; n=8; p>0.05). Thus, pLTF at 7 (not 28) days post-CTB-SAP closely resembles pLTF in end-stage ALS rats, suggesting that processes unique to the early period of motor neuron death enhance pLTF. This project increases our understanding of respiratory plasticity and its implications for breathing in motor neuron disease.

Keywords

respiratory motor output; ventilatory control; ALS; breathing; phrenic motor neurons; neurodegenerative disease

1. Introduction

Amyotrophic lateral sclerosis (ALS) causes paralysis from progressive motor neuron degeneration, ultimately causing death from ventilatory failure (Lechtzin et al., 2002; Lya

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et al., 2001). In a rat model of ALS (SOD1^{G93A}), signs of imminent ventilatory failure first appear as phrenic motor neuron death and decreased phrenic motor output (Nichols et al., 2013a; 2013b). Intermittent hypoxia triggers respiratory plasticity (Mitchell, 2007) and substantially restores phrenic motor output at disease end-stage in SOD1^{G93A} rats (Nichols et al., 2013a; 2015a). This functional recovery is associated with enhanced expression of phrenic long-term facilitation (pLTF). Specifically, the increase in phrenic motor output following acute intermittent hypoxia (AIH) is larger in SOD1^{G93A} rats compared to wild-type rats (Nichols et al., 2013a; 2015a). We propose that this increase, or enhanced plasticity, may be triggered by motor neuron death itself. However, degeneration is not limited to the phrenic motor nucleus and the amount and rate at which motor neuron death occurs cannot be controlled in the SOD1^{G93A} rat model. Thus, we developed a novel model of induced respiratory motor neuron death *via* intrapleural injections of cholera toxin B fragment conjugated to the ribosomal toxin, saporin (CTB-SAP; Nichols et al., 2015b); CTB-SAP has been used in other systems to cause selective motor neuron death (Lian et al., 1997; Llewellyn-Smith et al., 1999; Llewellyn-Smith et al., 2000; Lujan et al., 2010). The intrapleural model of CTB-SAP mimics aspects of the SOD1^{G93A} model including similar respiratory motor neuron death and an attenuated capacity to increase phrenic motor output (Nichols et al., 2015b). CTB-SAP treated rats also exhibit breathing deficits; however, the loss of respiratory function was not proportionate to the amount of phrenic motor neuron death, suggesting that CTB-SAP induced cell death elicits mechanisms of compensatory respiratory plasticity (Nichols et al., 2015b). Here, we tested the hypothesis that respiratory motor neuron death is a sufficient trigger to enhance AIH-induced pLTF in CTB-SAP treated rats. Our results indicate that: 1) CTB-SAP induced phrenic motor neuron death is similar as described previously (Nichols et al., 2015b); and 2) pLTF is enhanced in 7, but not 28, day CTB-SAP treated rats. Enhanced plasticity in motor neuron disease is of considerable significance given the ability to restore lost motor function with procedures as simple as a single exposure to AIH (Nichols et al., 2013a) or harnessing the underlying mechanisms of pLTF.

2. Methods

2.1 Animals

Experiments were conducted on adult (3–4 months old) male Sprague Dawley rats (Envigo Colony 217; Indianapolis, IN) maintained on a 12:12 light:dark cycle with *ad libitum* access to food and water. All procedures involving animals were approved by the Animal Care and Use Committee at the University of Missouri, and were in agreement with standards set forth in the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. The University of Missouri is accredited by AAALAC, and is covered by NIH Assurance (A3394-01).

2.2 Intrapleural injections

Intrapleural injections were done according to Mantilla and colleagues (2009) using a 50 µl Hamilton syringe and a custom needle (6mm, 23 gauge, semi-blunt to avoid puncturing of the lung). Cholera toxin B subunit conjugated to saporin (CTB-SAP; 25µg dissolved in phosphate buffered saline (PBS); Advanced Targeting Systems; San Diego, CA) was

administered intrapleurally to target respiratory motor neurons as described previously (Nichols et al., 2015b). CTB-SAP plus additional CTB (25µg dissolved in doubly distilled H₂O; Calbiochem; Billerica, MA; to label spared phrenic motor neurons) was bilaterally injected into the right and left pleural spaces (6 mm deep, fifth intercostal space) while the rats were under isoflurane anesthesia (1.5% isoflurane in 100% oxygen). Control rats received an injection of CTB (25µg) unconjugated to SAP (25µg dissolved in PBS; Advanced Targeting Systems; San Diego, CA) to demonstrate SAP alone does not cause respiratory motor neuron death and to enable comparisons for respiratory plasticity. Rats were monitored for overt signs of respiratory compromise.

2.3 In vivo neurophysiology

2.3.1 Experimental preparation—Experimental procedures were performed as described previously (Baker-Herman et al., 2004; Hoffman et al., 2010; Nichols et al., 2015a). Briefly, rats were anesthetized with isoflurane, tracheotomized, paralyzed and pump-ventilated (Small Animal SAR-1000 Ventilator; CWE, Ardmore, PA, USA; tidal volume ~2.5 mL, frequency ~70). Body temperature was assessed with a rectal thermometer (Physitemp, Clifton, NJ, USA) and maintained ($37.5 \pm 1^\circ\text{C}$) with a custom-made heated surgical table. To monitor end-tidal PCO₂ (P_{ETCO2}), a carbon dioxide analyzer with sufficient response time to measure P_{ETCO2} in rats was used (CapStar-100, CWE, Ardmore, PA). P_{ETCO2} was maintained at ~40–45 mmHg throughout the surgical preparation. Rats were bilaterally vagotomized 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 (APT300 Pressure Transducer, Harvard Apparatus, Holliston, MA, USA) and enable blood gas analysis (ABL80 Flex, Radiometer, Brea, CA). The left and right phrenic nerves were isolated (dorsal approach), cut distally, desheathed, and covered with a saline soaked cotton ball. Isoflurane anesthesia was maintained (3.5% in 50% O₂, balance N₂) throughout surgical procedures; all rats were then slowly converted to urethane anesthesia over a 20–30 minute period (1.85 g kg^{-1} , *i.v.*) while concurrently withdrawing isoflurane. After conversion to urethane, an intravenous infusion was initiated to maintain blood volume, fluid, and acid-base balance; infusions ($1.5\text{--}4 \text{ ml kg}^{-1} \text{ hr}^{-1}$) consisted of a 1:2:0.13 mixture of 6% Hetastarch (in 0.9% sodium chloride), lactated Ringer's, and 8.4% sodium bicarbonate. Once rats were converted to urethane anesthesia, a minimum of 1 hour was allowed before protocols commenced. The adequacy of anesthesia was tested before protocols began, and immediately after the protocol was complete; adequacy of anesthetic depth was assessed as the lack of pressor or respiratory neural response to a toe pinch with a hemostat (Bach and Mitchell, 1996).

2.3.2 Nerve recordings—The previously isolated left and right phrenic 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 (50 ms time constant, 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). Rats were then paralyzed with pancuronium bromide for neuromuscular

blockade to prevent spontaneous breathing efforts (2.5 mg kg^{-1} , *i.v.*; Bach and Mitchell, 1996).

To begin protocols, the apneic CO_2 threshold was determined by lowering P_{ETCO_2} until nerve activity ceased for at least one minute. The recruitment threshold was then determined by slowly increasing P_{ETCO_2} until nerve activity resumed (Bach and Mitchell, 1996). P_{ETCO_2} was raised $\sim 2 \text{ mmHg}$ above the recruitment threshold to establish a level of nerve activity that is stable, repeatable and is low enough that it retains substantial capacity to increase, thus minimizing the potential for “ceiling effects, and $\sim 15\text{--}20 \text{ min}$ were allowed to establish a stable baseline activity. Arterial blood samples were analyzed for arterial partial pressures of O_2 (PaO_2) and CO_2 (PaCO_2) and were drawn during baseline, the first hypoxic episode, and at 15, 30 and at 60 min post-AIH. Arterial CO_2 (PaCO_2) was maintained within $\pm 1.5 \text{ mmHg}$ of baseline levels by adjusting inspired CO_2 and/or ventilator rate. PaO_2 was 150 mmHg during baseline, but was between $35\text{--}45 \text{ mmHg}$ during the acute intermittent hypoxia (AIH) episodes (3, 5 minute episodes separated by 5 minute intervals of baseline oxygen levels). Rats then received AIH or continuous exposure to baseline oxygen levels (sham AIH). Following the third hypoxic episode, rats were returned to baseline O_2 levels that were maintained throughout the protocol. For AIH treated rats, phrenic motor output was assessed in 7 ($n=8$) and 28 ($n=8$) day controls, and 7 ($n=8$) and 28 ($n=10$) day treated CTB-SAP rats. For sham AIH (or time controls; TC), phrenic motor output was assessed in 7 ($n=3$) and 28 ($n=3$) day control rats, and 7 ($n=3$) and 28 ($n=3$) day treated CTB-SAP rats. Since no differences existed between TC treated controls or TC treated CTB-SAP treated rats at the two time-points (*i.e.* 7 vs. 28 days), rats were combined for TC controls and TC CTB-SAP treatment.

2.4 Immunohistochemistry

Rats were processed to assess phrenic motor neuron survival at the end of neurophysiology experiments by transcardially perfusing them with 4% paraformaldehyde in 0.1M phosphate buffer saline (PBS, $\text{pH}\sim 7.4$). The spinal cord tissue was harvested and post-fixed with 4% paraformaldehyde overnight, and then cryoprotected in graded sucrose (20 and 30%) at 4°C until sinking. Transverse sections containing phrenic motor neurons (C_4 ; $40 \mu\text{m}$) were cut using a freezing microtome (Leica SM 2010R, Germany) and stored at -20°C in anti-freeze solution (30% ethylene glycol, 30% glycerol and $1\times \text{PBS}$) until processed for motor neuron counting.

Cervical (C_4) spinal cord sections (6 per rat) taken from control and CTB-SAP rats were stained using CTB immunohistochemistry for phrenic motor neuron counts. Sections were first separated, and washed with $1\times \text{PBS}$ three times for five minutes; each rat's tissue was contained in a separate well. To prevent non-specific antibody binding, blocker consisting of 5% normal donkey serum (NDS), $1\times \text{PBS}$, and 0.2% Triton was added to each tissue sample, and incubated for 1 hour at room temperature. Primary antibody solution was added, consisting of 5% NDS, $1\times \text{PBS}$, 0.1% Triton, and the antibody against cholera toxin B subunit (CTB; goat polyclonal, 1:2000, Calbiochem; Billerica, MA). Sections were incubated overnight in the primary antibody solution on a shaker at 4°C . The following day, tissues were washed three times with $1\times \text{PBS}$ (five minutes each), and then incubated in the

secondary antibody solution, which was composed of 5% NDS, 1× PBS, 0.1% Triton and the secondary antibody (donkey anti-goat Alexa-Fluor 555, 1:1000; Molecular Probes, Eugene, OR) on a shaker for two hours in the dark at room temperature. Following incubation, tissues were washed with 1× PBS using the same procedure (3 × 5min). Sections were then mounted on positively charged glass slides, covered with ProLong® Gold Antifade Reagent (Molecular Probes, Eugene, OR) to prevent quenching of fluorescence, cover-slipped and air-dried. Slides were stored at −20°C until quantification of staining was performed by a blinded investigator using a confocal microscope (Leica SPE, Germany) at 20× magnification. Sections incubated without primary or secondary antibodies served as negative controls.

2.5 Phrenic motor neuron counts

LAS-AF program software and confocal microscopy (Leica SPE) were used to take photomicrographs (20×) of phrenic motor neurons in C₄ spinal cord tissues. Location of the phrenic motor neurons in the ventral horn were determined and counted based on diagrams from *The Spinal Cord* (Watson et al. 2009). Specifically, phrenic motor neurons are defined as the cluster of neurons medio-lateral of the cervical ventral horn (Boulenguez et al., 2007; Mantilla et al., 2009). Surviving phrenic motor neurons were counted as described previously (Nichols et al., 2015b), and were characterized as those exhibiting a CTB (+) stain, an identifiable cell body and nucleus. Right and left CTB (+) motor neurons from all rats were counted by a blinded investigator, entered into Microsoft Excel, and averaged across sections in each rat to enable statistical comparisons. Phrenic motor neuron survival was assessed in all control and CTB-SAP treated rats. There were no differences in phrenic motor neuron survival for TC treated animals vs. AIH treated animals, and there were no differences for controls regardless of time-point; thus, TC and AIH treated animals were combined together and all controls were grouped together. The number of phrenic motor neurons in the C₄ segment was extrapolated from the 6 sections (length of the entire phrenic motor nucleus is ~2000µm; 40µm sections), as described previously (Nichols et al., 2013a, 2015a, 2015b, 2017).

2.6 Data and Statistical analyses

Integrated phrenic nerve burst amplitudes and frequency were averaged over 1 min bins during baseline, 15, 30 and 60 min post-AIH. Phrenic nerve burst amplitude is the voltage of the integrated signal, expressed as a percent change from baseline. Nerve burst frequency was expressed as an absolute change from baseline (bursts/min). Statistical comparisons between treatment groups for AIH studies (amplitude, frequency, PaCO₂, PaO₂, and MAP) were done using a two-way ANOVA with a repeated measures design. Since no differences were detected among successive hypoxic exposures within groups (data not shown), comparisons of the short-term hypoxic phrenic response were made using one-way ANOVA of phrenic burst amplitude during the fifth minute of hypoxic episodes averaged from all three episodes. A one-way ANOVA was also used when directly comparing phrenic nerve burst amplitudes and frequency across groups at 60 min post-hypoxia, and for the histological analyses. When significant ANOVA differences were detected, individual comparisons were made with Fisher's least significant difference *post hoc* test (Sigma Plot

version 13.0; Systat Software Inc., San Jose, CA, USA). Differences between the groups were considered significant if $p < 0.05$; all values are expressed as means $\pm$ 1 S.E.M.

3. Results

3.1 CTB-SAP induced phrenic motor neuron death

Here, we verified phrenic motor neuron loss following CTB-SAP to assure that the amount of motor neuron death was similar to our prior report (Nichols et al., 2015b). As shown previously, intrapleural injections of CTB-SAP selectively kills phrenic motor neurons (Figure 1). Representative photomicrographs of CTB labeled phrenic motor neurons from the C₄ spinal ventral horn from a control, 7 day CTB-SAP treated rat, and a 28 day CTB-SAP treated rat are depicted in Figure 1 (A–C). Phrenic motor neuron numbers were decreased in all CTB-SAP treated *vs.* control rats, where control rats had 219 ± 18 neurons (Fig. 1D; $p < 0.05$). Average phrenic motor neuron survival counts following CTB-SAP injections were 92 ± 11 neurons for 7 day treated rats and 55 ± 19 for 28 day treated rats (Fig. 1D; both $p < 0.05$ *vs.* control; $p > 0.05$ when comparing CTB-SAP treated groups). Thus, the amount of CTB-SAP induced phrenic motor neuron death is consistent with our previous report (Nichols et al., 2015b).

3.2 Hypoxia response in CTB-SAP treated rats

The short-term hypoxic phrenic response (represented as a percent change from baseline) was significantly larger in CTB-SAP treated rats *vs.* controls ($p < 0.05$; Figure 2). Average and individual responses are depicted in Figure 2, while representative short-term hypoxic phrenic responses are depicted in Figures 3A, 3B, 4A, and 4B. As expected, controls and CTB-SAP treated rats exhibited a significantly greater hypoxia response *vs.* TC treated rats ($p < 0.05$; Figure 2). There were no differences observed between the 7 and 28 day time-points for controls and CTB-SAP treated rats ($p > 0.05$; Figure 2). Enhanced hypoxic phrenic responses have been observed previously in end-stage ALS rats (Nichols et al., 2015a). Thus, the larger hypoxic phrenic response observed here in CTB-SAP rats is consistent with observations in end-stage ALS rats.

3.3 Enhanced pLTF in 7, not 28, day CTB-SAP treated rats

Representative neurograms depicting the average pLTF (*i.e.* progressive increase in phrenic activity following the last episode of hypoxia) that is induced *via* AIH is shown in Figure 3 for a control (Figure 3A) and 7 day CTB-SAP treated rat (Figure 3B). Phrenic amplitude was significantly greater in controls and 7 day CTB-SAP treated rats following AIH *vs.* baseline and TC treated rats, which indicates pLTF ($p < 0.05$; Figure 3C). AIH induced pLTF was also enhanced in 7 day CTB-SAP treated rats *vs.* controls ($p < 0.05$; Figure 3), consistent with what has been observed in end-stage SOD1^{G93A} rats (Nichols et al., 2013a; 2015a). Changes in phrenic frequency were not different among groups after 7 days ($p > 0.05$; Figure 3D).

In contrast, AIH induced pLTF was still present, but not enhanced, in 28 day CTB-SAP treated rats *vs.* controls ($p > 0.05$; Figure 4). Typical phrenic activity before and after AIH is depicted in the representative neurograms in Figure 4 for a control (Figure 4A) and 28 day

CTB-SAP treated rat (Figure 4B). Phrenic amplitude was significantly greater in controls and 28 day CTB-SAP treated rats *vs.* TC treated rats and baseline, indicating pLTF ($p < 0.05$; Figure 4C). AIH-induced frequency LTF was also not exhibited in any group after 28 days ($p > 0.05$; Figure 4D). An overall comparison of all groups is depicted in Figure 5 for pLTF (including both average and individual pLTF) and phrenic frequency. When comparing the two time-points (7 *vs.* 28 day), 7 day CTB-SAP treated rats exhibit significantly larger pLTF at 60 min post-hypoxia *vs.* all other groups ($p < 0.05$; Figure 5A). There were no differences in phrenic frequency among the different treatment groups (Figure 5C).

3.4 Blood gases and mean arterial pressures

Arterial PCO_2 (PaCO_2) was successfully regulated within 1.5 mmHg of its baseline value in all groups (Table 1). Thus, changes in integrated phrenic nerve burst amplitude following AIH cannot be attributed to differences in chemoreceptor feedback. PaO_2 was successfully maintained within the target range for AIH (35–45 mmHg), and was above 150 mmHg at all times post-hypoxia (Table 1). As expected, hypoxia significantly decreased mean arterial pressure, where mean arterial pressure was significantly different among groups when comparing AIH *versus* TC during hypoxic episodes ($p < 0.05$; Table 1). Thus, differences in PaCO_2 , PaO_2 or blood pressure regulation among groups cannot account for differential pLTF expression.

4. Discussion

4.1 CTB-SAP mimics aspects of the ALS rat model

Respiratory failure is often the cause of death in ALS, which is associated with significant motor neuron loss. We hypothesize that surviving phrenic motor neurons “fight back” and somehow retain breathing capacity until they can no longer compensate. The lack of an appropriate model has been a critical barrier in studying this “fighting back” phenomenon and in developing therapies to improve ventilation during motor neuron loss. Thus, we developed a unique model of respiratory motor neuron death by injecting cholera toxin B conjugated to saporin (CTB-SAP) into the intrapleural space. This novel model is advantageous because cell death is targeted selectively by injection location, and the magnitude of its effects are stable and dose dependent (Nichols et al., 2015b). Key aspects of ALS that are mimicked in this unique CTB-SAP model include similar phrenic motor neuron death (~60%; Figure 1) and decreased phrenic motor output, as compared to a rat model of ALS ($\text{SOD1}^{\text{G93A}}$; Nichols et al., 2013a; 2013b; 2015b). In this study, we found that another aspect is also similar, but not at all time-points following CTB-SAP. Specifically, pLTF is enhanced in 7 day CTB-SAP treated rats (Figures 3 and 5A), similar to end-stage $\text{SOD1}^{\text{G93A}}$ rats. However, pLTF is no longer enhanced in 28 day CTB-SAP treated rats (Figures 4 and 5A).

4.2 Hypoxic responses do not explain differential pLTF in CTB-SAP treated rats

The magnitude of the hypoxic phrenic response has previously been shown to be positively correlated with the level of pLTF in normal rats (Fuller et al., 2000; Baker-Herman and Mitchell, 2008). However, here we observe that short-term hypoxic responses were significantly larger in both 7 and 28 day CTB-SAP treated rats *vs.* controls (Figure 2), but

pLTF was only enhanced in 7 day CTB-SAP treated rats (Figures 3 and 5A). Since the short-term hypoxic phrenic responses were not different from each other in CTB-SAP rats (Figure 2), this suggests that the neural circuit that responds to hypoxia to increase breathing is intact (Feldman et al., 2009; Mitchell et al., 2009), and is not affected in this CTB-SAP model of induced respiratory motor neuron death. Furthermore, this suggests that the difference in AIH-induced pLTF following 7 and 28 days of CTB-SAP is indeed due to differences at the location of the phrenic motor nucleus such as: 1) differences in the amount of motor neuron death that is induced (~60% for 7 days *vs.* ~75% for 28 days; Figure 1); or 2) the underlying mechanism of pLTF that is required.

4.3 Why is pLTF differentially expressed in the CTB-SAP model?

Phrenic motor neuron death itself does appear to trigger enhanced pLTF after 7 days of CTB-SAP. In contrast, pLTF returns to normal after 28 days of CTB-SAP. Phrenic motor neuron death is relatively stable once it is induced by CTB-SAP (*i.e.* comparison of 7 and 28 day CTB-SAP treated rats indicate no statistical difference in phrenic motor neuron death; Nichols et al., 2015b; Figure 1), and is similar to phrenic motor neuron death that has been observed in end-stage SOD1^{G93A} rats (Nichols et al., 2013a; 2013b; 2015a; 2017). Thus, differential phrenic motor neuron death alone (Figure 1) does not account for the differential pLTF that is exhibited following 7 and 28 days of CTB-SAP (Figures 3–5). It is likely that there are different mechanisms responsible for AIH-induced pLTF following 7 *versus* 28 days of CTB-SAP. However, the mechanism(s) that underlies phrenic plasticity following acute (7 days) and chronic (28 days) phrenic motor neuron death in CTB-SAP treated rats remains unknown.

Respiratory plasticity can be induced pharmacologically through G_q (5HT_{2A/B} and α -1; MacFarlane et al., 2011; Neverova et al. 2007) or G_s (5HT₇ and A_{2A}; Golder et al., 2008; Hoffman and Mitchell, 2011) protein-coupled metabotropic receptors (Dale-Nagle et al., 2010). Thus, there are multiple pathways that can induce respiratory plasticity, which are referred to as the G_q and G_s pathways. Moderate AIH-induced pLTF requires 5HT₂ receptors (Kinkead et al., 1999; Fuller et al. 2001), Brain Derived Neurotrophic Factor (BDNF; Baker-Herman et al., 2004), and ERK activation (Hoffman et al., 2012 (*i.e.* G_q-pathway dependent)). In contrast, the G_s pathway requires new synthesis of the high affinity BDNF receptor tyrosine kinase, TrkB, and PI3 kinase/Akt activity (Golder et al., 2008; Hoffman and Mitchell, 2011). This latter pathway either constrains pLTF during moderate AIH, or elicits enhanced A_{2A} receptor dependent pLTF in response to severe AIH (Hoffman et al., 2010; Nichols et al., 2012). Since 7 day CTB-SAP treated rats exhibit enhanced pLTF (Figures 2 and 4A), it is possible that the underlying mechanism: 1) is similar to that utilized in SOD1^{G93A} rats (BDNF synthesis and MEK/ERK dependent pLTF; Nichols et al., 2017); 2) switches to the A_{2A} receptor dependent pathway to express enhanced pLTF; or 3) requires both the G_q and G_s pathways as has been observed following chronic intermittent hypoxia (Ling et al., 2001; McGuire et al., 2004). In contrast, pLTF is still present but enhancement is lost in 28 day CTB-SAP treated rats (Figures 4 and 5A), and it remains unknown if pLTF simply returns to normal and requires the G_q and/or the G_s pathway or if pLTF is being constrained by peripheral and/or local influences such as inflammation. Inflammation has been shown to abolish pLTF in naïve rats (Huxtable et al., 2011; Vinit et al., 2011; Huxtable

et al., 2013; Huxtable et al., 2015), and microglial number is increased in the CTB-SAP model (Nichols et al., 2015b); thus, it is possible that inflammation in the face of chronic phrenic motor neuron death constrains pLTF.

4.4 Significance

There are currently no approved treatment options to significantly preserve/restore breathing capacity in patients with ALS. Thus, understanding why pLTF is enhanced acutely or is constrained chronically and the underlying mechanism(s) for pLTF in this model of respiratory motor neuron death may allow us to harness mechanisms of respiratory motor plasticity to increase/preserve the function of surviving motor neurons. This has broad impact and suggests that motor neuron death may induce plasticity *via* multiple signaling pathways. This is important because not all patients respond the same way to therapy and pharmacological intervention, and we know that patients in general do not all present with similar symptoms; disease progression is variable. Furthermore, the CTB-SAP model may help us understand plasticity and its underlying mechanisms in other disorders involving the loss of motor neurons.

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Abbreviations

ALS	amyotrophic lateral sclerosis
AIH	acute intermittent hypoxia
CTB	cholera toxin B
CTB-SAP	cholera toxin B conjugated to saporin
P_{ETCO_2}	end-tidal P_{CO_2}
PaO₂	partial pressure of arterial O ₂
PaCO₂	partial pressure of arterial CO ₂
pLTF	phrenic long-term facilitation

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Highlights

1. Intrapleural CTB-SAP mimics aspects of ALS.
2. CTB-SAP is used to study the impact of respiratory motor neuron death.
3. Seven days of CTB-SAP enhances respiratory plasticity.

Figure 1.

Phrenic motor neuron counts in controls and CTB-SAP treated rats after 7 and 28 days. A–C. Photomicrographs depicting representative CTB stained sections from the C₄ spinal ventral horn from a control rat (A), 7 day treated rat (B), and a 28 day treated rat (C). Notice the number and shape of healthy, pyramidal-shaped CTB(+) labeled phrenic motor neurons in the control rat (A) *versus* the lack of surviving phrenic motor neurons after CTB-SAP treatment (B and C). D. Phrenic motor neuron survival in controls (white bar) and rats injected with CTB-SAP at 7 or 28 days post-injection (gray bars). Phrenic motor neuron survival is significantly decreased in CTB-SAP treated rats *versus* control rats (*; $p < 0.05$), but was not different when comparing 7 and 28 day CTB-SAP treated rats ($p > 0.05$). Means $\pm$ 1 SEM. Scale bar at $20\times = 50\ \mu\text{m}$.

Figure 2.

Average (A; controls = white bars; CTB-SAP treated rats = gray bars) and individual (B) phrenic responses during hypoxia (percentage change from baseline) in controls and CTB-SAP treated rats after 7 or 28 days. Since phrenic burst amplitude was not different among hypoxic episodes within groups ($p > 0.05$; data not shown), all three episodes were grouped for comparisons across treatment groups. Time control (TC) treated animals for controls and CTB-SAP treated rats were not different for the two time-points, so they were grouped together for each treatment group. CTB-SAP treated rats had a significantly greater hypoxic phrenic response *vs.* their time-matched controls (#). Both AIH treated controls and CTB-SAP treated rats had a larger phrenic burst amplitude *vs.* respective TC treated rats (*). Although there is one outlier for the short-term hypoxic response as depicted for the individual responses (B), the statistical differences remain the same whether it is included or not. In addition, pLTF for this same animal is not an outlier; thus, we chose to include all animals in our statistical comparisons. Values are means $\pm$ SEM and all significant differences are $p < 0.05$.

Figure 3.

pLTF in 7 day CTB-SAP treated rats. A & B: Representative traces of compressed, integrated phrenic nerve activity before and after AIH for a 7 day control treated rat (A) and a CTB-SAP treated rat (B). White, dashed line in each trace indicates baseline. AIH elicits enhanced pLTF in the 7 day CTB-SAP treated rat where the control rat exhibited typical pLTF. C & D: Phrenic burst amplitude (percentage change from baseline) and frequency (bursts/min change from baseline) in 7 day controls (black diamonds with black solid line) and CTB-SAP treated rats (black triangles with black dashed line) compared to TC treated animals (gray squares with gray dashed line for CTB-SAP TC and gray circles with gray solid line for control TC). pLTF is significantly increased in controls and CTB-SAP treated rats *vs.* baseline (#) and time controls (*), indicating pLTF. pLTF was significantly greater in CTB-SAP treated animals *vs.* controls (+). Frequency was unaffected in all treatment groups. Values are means $\pm$ SEM and all significant differences are $p < 0.05$.

Figure 4.

pLTF in 28 day CTB-SAP treated rats. A & B: Representative traces of compressed, integrated phrenic nerve activity before and after AIH for a 28 day control treated rat (A) and a CTB-SAP treated rat (B). White, dashed line in each trace indicates baseline. AIH elicits normal pLTF in the control and 28 day CTB-SAP treated rat. C & D: Phrenic burst amplitude (percentage change from baseline) and frequency (bursts/min change from baseline) in 28 day controls (black diamonds with black solid line) and CTB-SAP treated rats (black triangles with black dashed line) compared to TC treated animals (gray squares with gray dashed line for CTB-SAP TC and gray circles with gray solid line for control TC). pLTF is significantly increased in controls and CTB-SAP treated rats *vs.* baseline (#) and time controls (*), indicating pLTF. Frequency was unaffected in all treatment groups. Values are means $\pm$ SEM and all significant differences are $p < 0.05$.

Figure 5.

Direct comparison of the change in phrenic burst amplitude (percent baseline; average in A and individual in B) and frequency (bursts/min; C) in controls, CTB-SAP treated rats, and time-control (TC) treated rats for 7 and 28 days at 60 min post-hypoxia where white bars represent control treated animals and gray bars represent CTB-SAP treated animals. Phrenic amplitude was significantly increased for all groups *vs.* TC treated rats (*), and was significantly enhanced in 7 day CTB-SAP treated rats *vs.* all other groups (#). Frequency was unaffected in all treatment groups. Values are $\pm$ SEM and all significant differences are $p < 0.05$.

Table 1

Arterial PCO₂, PO₂ and mean arterial pressure (MAP) during baseline, the first hypoxic episode, and 60 minutes following acute intermittent hypoxia (AIH) in control and CTB-SAP treated rats for 7 and 28 days. Data are for rats with AIH or without AIH (-AIH); these rats were treated as time controls; TC)

	P _a CO ₂ (mmHg)			P _a O ₂ (mmHg)			MAP (mmHg)		
	baseline	hypoxia	60 min	baseline	hypoxia	60 min	baseline	hypoxia	60 min
Experimental Groups									
Control 7	48.5 ± 1.3	48.2 ± 1.5	48.7 ± 1.1	290 ± 6 ^a	36.6 ± 1 ^d	268 ± 11 ^a	116 ± 5 ^a	63 ± 7 ^d	103 ± 7 ^a
CTB-SAP 7	52.9 ± 1.1	52.4 ± 1.1	52.3 ± 1.4	288 ± 9 ^a	36.4 ± 1 ^e	245 ± 13 ^{abc}	111 ± 8 ^a	64 ± 9 ^e	99 ± 9 ^a
Control 28	48.7 ± 0.9	48.4 ± 1.1	49.1 ± 0.8	298 ± 10 ^a	36.2 ± 1 ^d	240 ± 21 ^{abc}	114 ± 4 ^a	74 ± 6 ^d	103 ± 8 ^a
CTB-SAP 28	50.3 ± 2.2	50.5 ± 1.9	50.4 ± 2.5	305 ± 20 ^a	41 ± 1 ^e	280 ± 20 ^a	118 ± 8 ^a	77 ± 7 ^e	102 ± 5 ^{ab}
TC Control	48.7 ± 1.3	49.3 ± 1.4	48.4 ± 1.3	285 ± 4	289 ± 5	264 ± 3	122 ± 1	121 ± 1	115 ± 5
TC CTB-SAP	52.3 ± 0.6	52.3 ± 0.4	52.5 ± 0.5	298 ± 10	294 ± 11	266 ± 6	120 ± 3	111 ± 6	120 ± 2

Significant differences within individual study groups are indicated as follows: significant difference from hypoxia (^d) and significant difference from 60 min (^b). Significant differences across study groups are indicated as follows: significant difference from CTB-SAP 28 (^c), significant difference from TC Control (^d), and significant difference from TC CTB-SAP (^e). Values are expressed as means ± 1 S.E.M. Differences were considered significant if p<0.05.