Respiratory Physiology & Neurobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

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

Nicole L. Nichols*, Taylor A. Craig, Miles A. Tanner

Department of Biomedical Sciences, Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO, 65211, United States

ARTICLE INFO

Keywords:
Respiratory motor output
Ventilatory control
ALS
Breathing
Phrenic motor neurons
Neurodegenerative disease

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 (25 μg), or 2) un-conjugated 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.

1. Introduction

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

Abbreviations: ALS, amyotrophic lateral sclerosis; AIH, acute intermittent hypoxia; CTB, cholera toxin B; CTB-SAP, cholera toxin B conjugated to saporin; PETCO2, end-tidal PCO_2; PaO_2, partial pressure of arterial O_2; $PaCO_2$, partial pressure of arterial CO_2; pLTF, phrenic long-term facilitation

* Corresponding author at: Department of Biomedical Sciences, University of Missouri, 1600 E. Rollins St., Columbia, MO, 65211, United States.
E-mail address: nicholsn@missouri.edu (N.L. Nichols).

http://dx.doi.org/10.1016/j.resp.2017.08.003
Received 25 May 2017; Received in revised form 20 July 2017; Accepted 4 August 2017

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 et al. (2009) using a 50 µl Hamilton syringe and a custom needle (6 mm, 23 gauge, semi-blunt to avoid puncturing of the lung). Cholera toxin B subunit conjugated to saporin (CTB-SAP; 25 µg 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 (25 µg dissolved in doubly distilled H_2O; 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 (25 µg) unconjugated to SAP (25 µg 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 ± 1 °C) with a custom-made heated surgical table. To monitor end-tidal PCO_2 (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: 0.58 mm, OD: 0.965 mm; 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% O_2, balance N_2) throughout surgical procedures; all rats were then slowly converted to urethane anesthesia over a 20–30 min 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 (1.5–4 ml kg^{-1} h^{-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 h 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_{ETCO2} until nerve activity ceased for at least 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 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 ~15–20 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 (PaCO2) 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 ± 1.5 mmHg of baseline levels by adjusting inspired CO_2 and/or ventilator rate. PaO_2 was ≥ 150 mmHg during baseline, but was between 35 and 45 mmHg during the acute intermittent hypoxia (AIH) episodes (3, 5 min episodes separated by 5 min 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 0.1 M phosphate buffer saline (PBS, pH ~ 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 (C4; 40 µm) 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 1X PBS) until processed for motor neuron counting.

Cervical (C4) 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 1X 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), 1X PBS, and 0.2% Triton was added to each tissue sample, and incubated for 1 h at room temperature. Primary antibody solution was added, consisting of 5% NDS, 1X 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