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Respiratory function after selective respiratory motor neuron death from intrapleural CTB–saporin injections

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) causes progressive motor neuron degeneration, paralysis and death by ventilatory failure. In rodent ALS models: 1) breathing capacity is preserved until late in disease progression despite major respiratory motor neuron death, suggesting unknown forms of compensatory respiratory plasticity; and 2) spinal microglia become activated in association with motor neuron cell death. Here, we report a novel experimental model to study the impact of respiratory motor neuron death on compensatory responses without many complications attendant to spontaneous motor neuron disease. In specific, we used intrapleural injections of cholera toxin B fragment conjugated to saporin (CTB–SAP) to selectively kill motor neurons with access to the pleural space. Motor neuron survival, CD11b labeling (microglia), ventilatory capacity and phrenic motor output were assessed in rats 3–28 days after intrapleural injections of: 1) CTB–SAP (25 and 50 μg), or 2) unconjugated CTB and SAP (*i.e.* control; (CTB + SAP). CTB–SAP elicited dose-dependent phrenic and intercostal motor neuron death; 7 days post-25 μg CTB–SAP, motor neuron survival approximated that in end-stage ALS rats (phrenic: 36 ± 7%; intercostal: 56 ± 10% of controls; $n = 9$; $p < 0.05$). CTB–SAP caused minimal cell death in other brainstem or spinal cord regions. CTB–SAP: 1) increased CD11b fractional area in the phrenic motor nucleus, indicating microglial activation; 2) decreased breathing during maximal chemoreceptor stimulation; and 3) diminished phrenic motor output in anesthetized rats (7 days post-25 μg, CTB–SAP: 0.3 ± 0.07 V; CTB + SAP: 1.5 ± 0.3; $n = 9$; $p < 0.05$). Intrapleural CTB–SAP represents a novel, inducible model of respiratory motor neuron death and provides an opportunity to study compensation for respiratory motor neuron loss.

Introduction

Amyotrophic lateral sclerosis (ALS) causes paralysis from progressive motor neuron degeneration, ultimately causing death from ventilatory failure. Effective means of preserving and/or restoring ventilatory function in ALS are necessary to improve the quality and duration of life. During disease progression in a rat model of ALS ($SOD1^{G93A}$ over-expression), signs of imminent ventilatory failure first appear as phrenic motor neuron death and decreased phrenic motor output. At this stage of disease progression, microglial cell numbers increase throughout the spinal cord, including cervical spinal regions encompassing the phrenic motor nucleus (Nikodemova et al., 2013). Increased microglial cell number is indicative of microglial activation, although the specific microglial phenotype is not easily characterized (Colton, 2009; David and Kroner, 2011; Hanisch and Kettenmann, 2007; Nikodemova et al., 2013). Since the rate, timing and extent of respiratory motor neuron death are highly variable in genetic models of ALS, 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). This model will enable more controlled studies concerning the specific impact of respiratory motor neuron death on breathing.

CTB binds to the GM1 (Galactosyl-N-Acetylgalactosaminyl) receptor and is subsequently incorporated into motor neurons (Lian and Ho, 1997). Saporin is a ribosomal inactivating protein, disabling protein synthetic machinery and causing apoptotic cell death over hours to days (Llewellyn-Smith et al., 1999; Lujan et al., 2010). When CTB is conjugated to saporin (CTB–SAP), targeted cell types are eliminated whereas other cell types are unaffected (Llewellyn-Smith et al., 1999, 2000; Lujan et al., 2010). Once CTB–SAP reaches the targeted cell body, CTB and SAP dissociate, allowing saporin to inactivate ribosomes. When CTB–SAP is injected intrapleurally, motor neurons with access to the pleural space (*e.g.* phrenic) retrogradely transport it to the cell body, thereby killing the cell. Here, we report that intrapleural CTB–SAP injections simulate aspects of motor neuron degeneration previously observed

Abbreviations: ALS, amyotrophic lateral sclerosis; AIH, acute intermittent hypoxia; pLTF, phrenic long-term facilitation; CTB, cholera toxin B; CTB–SAP, cholera toxin B conjugated to saporin; VT, tidal volume; VT/TI, mean inspiratory flow; VE, minute ventilation; P_{ETCO2}, partial pressure of end-tidal carbon dioxide; PaCO2, partial pressure of arterial carbon dioxide; PaO_2, partial pressure of arterial oxygen.

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in a rat model of ALS, including similar respiratory motor neuron death and its effects on the capacity to increase phrenic motor output.

Materials and methods

Animals

Experiments were conducted on adult (3–4 months old) male Sprague Dawley rats (Harlan Colony 211; 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 Institutional Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin, and were in agreement with standards set forth in the National Institutes of Health Guide (NIH) for Care and Use of Laboratory Animals. The University of Wisconsin is accredited by AAALAC, and is covered by NIH Assurance (A3368-01).

Intrapleural injections

Cholera toxin B subunit conjugated to saporin (CTB–SAP; 25–50 μg dissolved in phosphate buffered saline (PBS); Advanced Targeting Systems; San Diego, CA) was administered intrapleurally to target respiratory motor neurons. 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). CTB–SAP plus extra CTB (25 or 50 μg dissolved in doubly distilled H_2O; Calbiochem; Billerica, MA; to label spared phrenic motor neurons) were 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 (25–50 μg) unconjugated to saporin (SAP, 25–50 μg dissolved in PBS; Advanced Targeting Systems; San Diego, CA) or CTB + SAP as a control to demonstrate SAP alone does not cause respiratory motor neuron death. Rats were monitored for overt signs of respiratory compromise.

Plethysmography

A sub-set of rats were placed in a whole-body flow-through plethysmograph (BUXCO Electronics, Troy, NY) 7 and 28 days following intrapleural injection. This technique allows quantitative measurement of ventilation in freely-behaving rats with altered inspired gas concentrations. The system was calibrated, the rats weighed, and their body temperature was measured with a rectal thermometer (Type T Thermocouple Thermometer, Model 600-1020, Barnant Company, Barrington, IL) before being placed in the plethysmograph chamber (~2 L volume). Ventilation was assessed in control rats (CTB + SAP; 25 μg CTB + 25 μg SAP for 7 and 28 days; n = 3 each; with no differences between 7 and 28 days, control rats were grouped for analysis) and CTB–SAP (25 μg for 7 and 28 days: n = 3 each) treated rats. The rats acclimated to the chamber while breathing room air (21% O_2, balance N_2; flushed at ~3 L/min). Recording commenced once the rats were quiet but awake; ventilatory measurements were made during baseline conditions, before exposing them to 5% hypercapnia (21% O_2, 5% CO_2, balance N_2; 15 min), 7% hypercapnia (21% O_2, 7% CO_2, balance N_2; 15 min) and an hypoxic + hypercapnic gas mixture (10.5% O_2/7% CO_2; 15 min). A pressure calibration signal, plethysmograph temperature, rat body temperature, ambient and chamber pressures, and rat body mass were used to calculate breath-by-breath tidal volume (VT; Drorbaugh and Fenn, 1955; Jacky, 1978), respiratory frequency, mean inspiratory flow (VT/TI) and minute ventilation $\left(\dot{V}\,E\right)$. VT, $\dot{V}$ E, and VT/TI are reported normalized to body mass (per 100 g). Data were rejected if there was evidence of pressure fluctuations caused by gross body movements or sniffing behavior. At the conclusion of the study, rats were removed from the chambers and their body temperatures recorded.

Neurophysiological experiments

Rats were prepared as described previously (Hoffman et al., 2012; Nichols et al., 2012). Rats were induced and maintained with isoflurane (3.5% in 50% O_2, balance N_2) throughout surgical procedures. Rats were trachotomized, pump ventilated, (Rodent Ventilator, model 683; Harvard Apparatus, Holliston, MA, USA; tidal volume = 2.5 mL), and bilaterally vagotomized. 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 (Gould Pressure Transducer, P23ID, USA) and blood gases (ABL 800, Radiometer, Westlake, OH). A rectal thermistor (Fisher Scientific, Pittsburgh, PA, USA) was used to monitor body temperature, which was maintained (37.5 ± 1 °C) with a heated surgical table. To monitor end-tidal PCO_2 (P_{ETCO2}), a flow-through carbon dioxide analyzer was used with sufficient response time to measure P_{ETCO2} in rats (Capnogard, Novametrix, Wallingford, CT, USA). P_{ETCO2} was maintained at ~45 mm Hg throughout surgery. Phrenic nerves were isolated (dorsal approach), cut distally, desheathed and covered with a saline soaked cotton ball until protocols commenced. Once surgery was complete, rats were converted to urethane anesthesia over 20–30 min (1.8 g kg^{-1}, *i.v.*). The adequacy of anesthesia was tested before protocols commenced, 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; Hoffman et al., 2012; Nichols et al., 2012). Once rats were converted to urethane, a minimum of 1 h was allowed before experiments commenced. Rats were given continuous intravenous infusions (1.5–6 mL kg^{-1} h^{-1}) of a 1:2:0.13 mixture of 6% hetastarch in 0.9% sodium chloride, lactated Ringer's solution, and 8.4% sodium bicarbonate to maintain blood volume, fluid and acid–base balance.

Experimental protocol

Phrenic nerve activity was recorded with bipolar silver electrodes, amplified (10,000×), band-pass filtered (300–10,000 Hz, Model 1800, A-M Systems, Carlsborg, WA, USA), 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). Rats were then paralyzed using 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 threshold was determined by lowering P_{ETCO2} until nerve activity ceased for approximately 1 min. The recruitment threshold was then determined by slowly increasing the P_{ETCO2} until nerve activity resumed (Bach and Mitchell, 1996). P_{ETCO2} was raised to ~2 mm Hg above the recruitment threshold and approximately 15–20 min were allowed to establish stable neural activity (*i.e.* baseline). Blood samples were analyzed for arterial partial pressures of O_2 (PaO_2) and CO_2 (PaCO2), and were assessed during baseline, the two hypercapnic challenges and the hypercapnic + hypoxic episode. PaO_2 was ≥150 mm Hg during baseline, and during hypercapnic challenges, but was between 35–45 mm Hg during hypercapnia + hypoxia. Following ~15–20 min of baseline, PaCO2 was set 20 mm Hg above baseline for 5 min and then PaCO2 was set 40 mm Hg above baseline for 5 min by adjusting inspired CO_2. Following the final 5 minute hypercapnic + hypoxic episode (PaCO2 was set 40 mm Hg above baseline and PaO_2 was between 35–45 mm Hg), rats were returned to baseline inspired O_2 and CO_2 levels. Phrenic motor output was assessed in control (25 μg CTB + 25 μg SAP for 3 (n = 2), 7 (n = 5) and 28 (n = 3) days; 50 μg CTB + 50 μg SAP for 14 days, n = 3; since there were no differences between control groups, all control rats were grouped for analysis) and CTB–SAP (25 μg for 3 (n = 4), 7 (n = 9) and 28 (n = 7) days; 50 μg for 7 (n = 3) and 14 (n = 3) days) treated rats.