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TrkB Gene Therapy by Adeno-Associated Virus Enhances Recovery after Cervical Spinal Cord Injury

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Abstract

Unilateral cervical spinal cord hemisection at C2 (C2SH) interrupts descending bulbospinal inputs to phrenic motoneurons, paralyzing the diaphragm muscle. Recovery after C2SH is enhanced by brain derived neurotrophic factor (BDNF) signaling via the tropomyosin-related kinase subtype B (TrkB) receptor in phrenic motoneurons. The role for gene therapy using adeno-associated virus (AAV)-mediated delivery of TrkB to phrenic motoneurons is not known. The present study determined the therapeutic efficacy of intrapleural delivery of AAV7 encoding for full-length TrkB (AAV-TrkB) to phrenic motoneurons 3 days post-C2SH. Diaphragm EMG was recorded chronically in male rats ($n = 26$) up to 21 days post-C2SH. Absent ipsilateral diaphragm EMG activity was verified 3 days post-C2SH. A greater proportion of animals displayed recovery of ipsilateral diaphragm EMG activity during eupnea by 14 and 21 days post-SH after AAV-TrkB (10/15) compared to AAV-GFP treatment (2/11; $p = 0.031$). Diaphragm EMG amplitude increased over time post-C2SH ($p < 0.001$), and by 14 days post-C2SH, AAV-TrkB treated animals displaying recovery achieved 48% of the pre-injury values compared to 27% in AAV-GFP treated animals. Phrenic motoneuron mRNA expression of glutamatergic AMPA and NMDA receptors revealed a significant, positive correlation ($r^2 = 0.82$), with increased motoneuron NMDA expression evident in animals treated with AAV-TrkB and that displayed recovery after C2SH. Overall, gene therapy using intrapleural delivery of AAV-TrkB to phrenic motoneurons is sufficient to promote recovery of diaphragm activity, adding a novel potential intervention that can be administered after upper cervical spinal cord injury to improve impaired respiratory function.

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Keywords

neurotrophin; neuroplasticity; spinal hemisection; diaphragm muscle; phrenic motoneuron; respiratory; glutamatergic; serotonergic; neurotransmitter

Introduction

Unilateral spinal cord hemisection at C2 (C2SH) interrupts descending premotor bulbospinal drive to phrenic motoneurons located between C3-C6 segments of the spinal cord in rats, resulting in transient paralysis of the ipsilateral diaphragm muscle (DIAM) (Goshgarian, et al., 1991, Mantilla, et al., 2013, Porter, 1895). Over time, spontaneous recovery of ipsilateral rhythmic DIAM activity ensues, reflecting neuroplasticity and strengthening of spared synaptic input to phrenic motoneurons (Fuller, et al., 2006, Golder, et al., 2003, Mantilla, et al., 2012, Mantilla, et al., 2013, Nantwi, et al., 1999, O'Hara and Goshgarian, 1991). This model has been widely used and provides a well validated and useful tool for investigating the mechanisms underlying recovery after incomplete upper cervical spinal cord injury (Alilain and Goshgarian, 2008, Alilain, et al., 2011, Golder and Mitchell, 2005, Mantilla, et al., 2013, Mantilla and Sieck, 2009, Rowley, et al., 2005, Sieck and Mantilla, 2009, Zhan, et al., 1997).

The importance of brain-derived neurotrophic factor (BDNF) in synaptic plasticity is well documented (Bregman, et al., 2002, Coumans, et al., 2001, Friedman, et al., 1995, Kang and Schuman, 1995, Poo, 2001, Thoenen, 1995). Recent studies highlight the role of BDNF acting through its high-affinity tropomyosin-related kinase type B (TrkB) receptor in promoting recovery of DIAM EMG activity following C2SH (Gransee, et al., 2013, Mantilla, et al., 2013, Mantilla, et al., 2014). Intrathecal administration of BDNF (Mantilla, et al., 2013), as well as increased TrkB receptor expression induced by adeno-associated virus (AAV)-mediated targeted delivery of full-length TrkB to phrenic motoneurons prior to the injury (Gransee, et al., 2013) indicate that enhancing BDNF/TrkB signaling in phrenic motoneurons promotes recovery of ipsilateral DIAM activity following C2SH. Conversely, blocking BDNF/TrkB signaling through TrkB siRNA-mediated knockdown or chemical-genetic inhibition of TrkB kinase activity abrogates spontaneous recovery after C2SH (Mantilla, et al., 2013, Mantilla, et al., 2014). At present, the therapeutic role of increasing TrkB receptor expression in phrenic motoneurons following injury has not been assessed. In particular, it is important to disambiguate the possible contribution of plasticity resulting from pre-injury AAV treatment targeting phrenic motoneurons (e.g., a conditioning effect) and/or changes in synaptic inputs to motoneurons that increase resiliency to subsequent injury. We hypothesized that targeted, viral-mediated delivery of TrkB to phrenic motoneurons is sufficient to enhance recovery of ipsilateral DIAM activity following upper cervical spinal cord injury.

Several studies indicate that expression of glutamatergic (GluR) and serotonergic (5-HTR) receptors (specifically NMDA and 5-HTR2a, respectively) increases over time after C2SH and the timing of changes in expression generally corresponds with the onset of spontaneous recovery of ipsilateral phrenic activity (Alilain and Goshgarian, 2008, Fuller, et al., 2005,

Mantilla, et al., 2012). Neurotrophins such as BDNF influence post-synaptic expression of excitatory neurotransmitter receptors (Gottschalk, et al., 1999, Kang and Schuman, 1995) and increase motoneuron excitability (Gonzalez and Collins, 1997), at least in part via altered expression of GluR (Lessmann, et al., 1994) and 5-HTR receptors (Baker-Herman, et al., 2004). Accordingly, a secondary purpose of this study was to determine phrenic motoneuron expression of GluR and 5-HTR following targeted intrapleural delivery of AAV-TrkB or AAV-GFP. We hypothesized that phrenic motoneuron expression of GluR and 5-HTR increases following targeted delivery of AAV-TrkB to phrenic motoneurons, particularly in animals displaying recovery after C2SH.

Materials and Methods

Animals

Adult male Sprague Dawley rats (280–300g; n = 26) were purchased from Harlan (Indianapolis, IN). Food and water were provided *ad libitum* for the duration of the study. Anesthesia for surgical procedures was conducted with intramuscular injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). All of the experimental procedures were conducted in accordance with the Institutional Animal Care and Use Committee at Mayo Clinic, in compliance with the American Physiological Society guidelines.

Animals were randomly assigned to two groups: intrapleural injection of AAV-GFP (n = 11) or AAV-TrkB (n = 15) prior to placement of electrodes for chronic EMG recordings. DIAM EMG activity was used to verify the completeness of C2SH prior to AAV injection and to monitor recovery of rhythmic ipsilateral activity over time (7, 14 and 21 days post-C2SH). At the terminal experiment rats were euthanized by exsanguination and the spinal cord was dissected under RNase free conditions and frozen for further analysis.

Electrode Implantation

Implantation of chronic DIAM electrodes was performed in all rats at ~3 days prior to C2SH, as previously described (Dow, et al., 2006, Dow, et al., 2009, Gransee, et al., 2013, Gransee, et al., 2015, Mantilla, et al., 2013, Mantilla, et al., 2011, Trelease, et al., 1982). Briefly, a 2 mm segment of insulated stainless steel wire (AS631, Cooner Wire Inc., Chatsworth, CA) was stripped of insulation and inserted into the midcostal region of the DIAM. Two electrodes were inserted in each side and externalized for chronic EMG recordings.

Spinal Hemisection (C2SH)

Spinal hemisection was conducted as previously detailed and validated (Gransee, et al., 2013, Gransee, et al., 2015, Mantilla, et al., 2012, Mantilla, et al., 2014, Mantilla, et al., 2013, Mantilla, et al., 2007, Miyata, et al., 1995, Prakash, et al., 1999). Using a dissecting microscope under sterile conditions a dorsal C2 laminectomy was performed and only the right anterolateral spinal cord was transected at C2 with a microknife. Animals received analgesics for the first three days postoperatively, including oral acetaminophen (100–300 mg/kg) and intramuscular buprenorphine (0.1 mg/kg) as needed. Complete hemisection was

verified by absence of ipsilateral DIAM EMG activity immediately following the procedure and 3 days following C2SH.

Chronic EMG Recordings and Analysis

In anesthetized animals, DIAM EMG recordings were conducted during eupnea at various time points: prior to C2SH on day 0 and at 3, 7, 14 and 21 days post-C2SH, in accordance with previous studies (Gransee, et al., 2013, Gransee, et al., 2015, Mantilla, et al., 2013, Mantilla, et al., 2013, Mantilla, et al., 2011). Eupneic recordings were conducted for at least 2 min in lightly anesthetized animals (using ~ one-third of the surgical anesthesia dose) while the animals were supine resting on a heating pad. At day 21, EMG recordings were additionally conducted during exposure to hypoxic (10% O₂)-hypercapnic (5% CO₂) conditions for 5 min and during sustained airway occlusion for 45 s. Data acquisition was conducted using LabView software (National Instruments, Austin, TX) at a sampling frequency of 2 kHz, following filtering (20–1000 Hz bandpass) and amplification (x2000). Root mean square (RMS) DIAM EMG activity was measured using a 50 ms window as in previous studies (Mantilla, et al., 2011, Mantilla, et al., 2010). Digitized EMG signals were analyzed using MatLab 8.2 (MathWorks, Natick, MA). The mean peak RMS EMG activity was measured for eupnea (2 min), hypoxia-hypercapnia (last 30 s) and airway occlusion (last 5 s). In addition, spontaneous deep breaths (“sighs”), defined as inspiratory events with amplitude at least twice eupneic amplitude, were identified during periods of eupnea and hypoxia-hypercapnia pre-C2SH.

Recovery of ipsilateral DIAM EMG activity during eupnea was defined by the following criteria: 1) rhythmic DIAM EMG signal reflecting inspiratory activity in phase with contralateral DIAM activity across most inspiratory bursts (>90%), 2) DIAM EMG signal comprising more than one motor unit (multiple units with different waveform profiles), and 3) DIAM EMG amplitude at least 10% of pre-C2SH, consistent with previous studies (Dow, et al., 2009, Gransee, et al., 2013, Gransee, et al., 2015, Mantilla, et al., 2013, Mantilla, et al., 2013). The proportion of animals meeting criteria for recovery was determined at various time points following C2SH. In addition, peak RMS EMG amplitude was normalized to the mean peak RMS EMG pre-C2SH during eupnea for the same animal (normalized RMS EMG) in order to calculate the extent of recovery of ipsilateral DIAM EMG activity post-C2SH. Respiratory rate and duty cycle were calculated from contralateral DIAM EMG recordings at days 0 and 21 post-C2SH.

AAV Administration

AAV serotype 7 vectors encoding human GFP (AAV.CMV.PI.EGFP.WPRE.bGH) or TrkB.FL (AAV7.CMV.Flag-TrkB.WPRE.bGH) under a CMV promoter were obtained from the Vector Core at University of Pennsylvania, as previously reported (Gransee, et al., 2013). At day 3 post-C2SH, 1×10^{11} genome copies of the AAV7 vector (GFP or TrkB.FL) were administered into the right (ipsilateral) pleural space between the 7th and 8th ribs via 2 injections (50 μ l total) using a Hamilton syringe, as previously reported (Gransee, et al., 2013, Mantilla, et al., 2009). Intrapleural AAV injection results in greater than 15% efficiency in transduction of ipsilateral phrenic motoneurons with no other neurons labeled in the cervical spinal cord (Gransee, et al., 2013). Specific transduction of phrenic

motoneurons following intrapleural AAV injection was previously verified by GFP immunoreactivity, FLAG protein expression in cervical homogenate and TrkB mRNA expression in laser capture microdissected phrenic motoneurons (Gransee, et al., 2013).

Laser Capture Microdissection (LCM) of Phrenic Motoneurons

In a subset of animals ($n = 7$ per group), phrenic motoneurons were labeled retrogradely by bilateral intrapleural injection of cholera toxin subunit B (CTB) conjugated to Alexa Fluor 488 (Life Technologies, Grand Island, NY) 3 days prior to the terminal experiment, as in previous studies (Gransee, et al., 2013, Gransee, et al., 2015, Mantilla, et al., 2012, Mantilla, et al., 2013, Mantilla, et al., 2009). At the terminal experiment, the cervical spinal cord (C2-C7) was dissected and quickly frozen in liquid N_2 under RNase free conditions for LCM analysis. Spinal cord samples were cut in 10 μm thick longitudinal sections, placed on pre-chilled slides (SuperFrost, Fisher Scientific, Pittsburg, PA), and stored at $-80^\circ C$ until immediately before microdissection. Samples were dehydrated in serial alcohols followed by xylene as described in the manufacturer's protocol (Arcturus LCM, Applied Biosystems, Life Technologies Corp., Carlsbad, CA). Fluorescently labeled phrenic motoneurons were individually visualized under direct epifluorescence illumination. Labeled motoneurons from the right side of the spinal cord (ipsilateral to C2SH) were microdissected onto Capsure HS LCM caps (Arcturus) using an Arcturus XT LCM microdissection system. Retrogradely-labeled motoneurons were characteristically large neurons found in clusters in a rostrocaudal column parallel to the long axis of the cord (Mantilla, et al., 2012, Mantilla, et al., 2009). Imaging conditions were selected to minimize tissue autofluorescence. In most cases, ~4 caps (each containing motoneurons from 3–4 sections) were obtained from each animal. Caps were stored at $-80^\circ C$ until further processing.

Quantitative Real-Time PCR of Microdissected Phrenic Motoneurons

As in previous studies (Gransee, et al., 2013, Mantilla, et al., 2012), total RNA was extracted from LCM samples using RNeasy Micro kit (Qiagen Inc., Valencia, CA) with the addition of on-column DNase digestion. Reverse transcription was done using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol. All reverse transcription reactions were done in duplicate for each sample. For gene expression analysis, 1 μl of the reverse transcription reaction was added to a reaction mix containing: LightCycler 480 1 $\times$ SYBR Green I Master (Roche), and 0.5 μM of the respective primer pair. In accordance with a previous study in phrenic motoneurons (Mantilla, et al., 2012), the following receptor subtypes were studied: AMPA, NMDA, 5-HTR2a, and 5-HTR2c, with ribosomal protein S16 (RPS16) used as a reference gene. Primers used for amplification determined expression of the GluR2 and NR1 subunits of the AMPA and NMDA receptors, respectively, as previously reported (Mantilla, et al., 2012). Amplification and quantitation of mRNA was performed on a LightCycler 480 (Roche). Parameters for amplification of all products were identical: an initial 5-min pre-amplification incubation at $95^\circ C$ was followed by 60 cycles of primer annealing at $60^\circ C$ for 12 s, PCR product extension at $72^\circ C$ for 18 s and denaturing at $95^\circ C$ for 15 s. All PCR reactions were performed in duplicate for each reverse transcription product.

Following amplification, a melting curve analysis was performed to verify product specificity by identifying a single peak at the appropriate temperature. We previously verified that each transcript of interest was of the correct size using agarose gel electrophoresis of the PCR products and that these were of the correct DNA sequence (Mantilla, et al., 2012). An amplification fluorescence plot was generated for each sample using LightCycler 480 software (version 1.5, Roche). The threshold cycle (i.e., crossing point—CP, where amplification transitions from a linear to a logarithmic phase) was determined using the second derivative method. Comparison of the CP of the gene product to that of the RPS16 run concurrently permitted determination of a CP for each sample and transcript. In this context the CP reflects the relative mRNA concentration for each GluR and 5-HTR subtype transcript in each sample. An average CP was then calculated for each transcript and animal.

Statistical Analyses

Statistical comparisons were performed using JMP 10.0 (SAS Institute Inc., Cary, NC). The proportion of animals displaying recovery of rhythmic ipsilateral DIAM activity was compared across AAV-GFP and AAV-TrkB groups using a repeated measures nominal logistic test (time x AAV treatment). Mixed linear models with repeated measures (time x AAV treatment interaction) and animal as a random effect were generated for comparisons across experimental groups for extent of recovery during eupnea and ventilatory parameters (respiratory rate and duty cycle). Similarly, comparisons across DIAM motor behaviors (eupnea vs. hypoxia-hypercapnia vs. airway occlusion vs. pre-C2SH sigh) at 21 days post-C2SH and for comparisons of the relative expression of GluR and 5-HTR (average CP for each receptor per animal) were conducted using mixed linear models with animal as a random effect. When appropriate, *post hoc* comparisons were performed using Tukey-Kramer's HSD test. Secondary, subgroup analyses were conducted for animals displaying recovery of ipsilateral rhythmic DIAM EMG activity vs. not. Data are reported as mean $\pm$ SE unless otherwise specified. Significance was accepted at $p < 0.05$.

Results

Outcomes after C2SH

In all animals, bilateral pairs of electrodes for chronic DIAM EMG recordings were successfully implanted. All animals recovered uneventfully from C2SH surgery without the need for mechanical ventilatory support. Following C2SH, rats displayed only limited ipsilateral forelimb deficits consistent with previous reports (Gransee, et al., 2013, Gransee, et al., 2015, Mantilla, et al., 2013, Mantilla, et al., 2013, Miyata, et al., 1995, Zhan, et al., 1997). There were no deaths in either experimental group.

Ventilatory parameters following C2SH

Respiratory rate and duty cycle were measured from contralateral DIAM EMG recordings during eupnea prior to and at day 21 post-C2SH, and also in hypoxic-hypercapnic conditions at day 21 post-C2SH (Table 1). There were no significant differences in respiratory rate during eupnea between animals treated with AAV-GFP and AAV-TrkB ($p = 0.498$) or between pre-C2SH and 21 days post-C2SH values ($p = 0.839$; interaction: $p = 0.425$),

regardless of whether animals displayed recovery of ipsilateral activity or not. During eupnea, duty cycle increased at 21 days post-C2SH vs. pre-C2SH ($p = 0.005$), but there was no AAV treatment effect ($p = 0.703$) and there was no time x AAV treatment interaction ($p = 0.086$). Respiratory rate and duty cycle were higher during hypoxia-hypercapnia conditions than during eupnea ($p < 0.001$), as expected.

Proportion of rats displaying ipsilateral recovery of DIAM activity following C2SH

In order to evaluate recovery of ipsilateral rhythmic DIAM EMG activity following C2SH, absence of eupneic DIAM EMG activity was first verified in all 26 rats at day 3 post-C2SH. Absence of ipsilateral DIAM EMG activity was interpreted as reflecting interruption of ipsilateral descending excitatory inputs to the phrenic motoneuron pool. Representative DIAM EMG recordings and RMS EMG measurements are shown in Figure 1. Following verification of complete C2SH at the 3 day time point, ipsilateral intrapleural injection of either the AAV-GFP or AAV-TrkB vector was performed according to the randomization order prescribed at electrode placement. Subsequently, DIAM EMG recordings were repeated during eupnea at 7, 14, and 21 days post-C2SH.

Over time post-C2SH, there was gradual recovery of ipsilateral DIAM activity in a subset of animals in both AAV treatment groups. Contralateral and, when present, ipsilateral DIAM EMG activity occurred in rhythmic bursts in all lightly anesthetized animals. No evidence of tonic or spastic activity was present in either the contralateral or ipsilateral DIAM EMG recordings from either AAV treatment group.

The proportion of animals displaying recovery of ipsilateral DIAM EMG activity following C2SH was higher in the AAV-TrkB group compared to the AAV-GFP group ($p = 0.031$). There were no time ($p = 0.442$) or time x AAV treatment ($p = 0.100$) effects on recovery. At 7 days post-C2SH, recovery of ipsilateral DIAM EMG activity was present in 1 out of 11 (9%) animals in the AAV-GFP group compared to 7 out of 15 (47%) animals in the AAV-TrkB group. At day 14 post-C2SH, only 2 out of 11 (18%) in the AAV-GFP group displayed recovery, while 10 out of 15 (67%) animals in the AAV-TrkB group did (Figure 2). The proportion of animals displaying recovery in each group did not change further by day 21 post-C2SH.

Extent of recovery of ipsilateral DIAM EMG activity following C2SH

To assess the extent of recovery of rhythmic DIAM EMG activity, RMS EMG amplitude was measured at 7, 14, and 21 days post-C2SH. For all time points, each DIAM side RMS EMG was normalized to the eupneic uninjured value (pre-C2SH). During eupnea, the extent of recovery of ipsilateral rhythmic DIAM EMG activity showed a time ($p < 0.001$) and a time x AAV treatment interaction ($p = 0.034$), but no main group effect ($p = 1.000$) (Figure 3). As expected, at 3 days post-C2SH there was no ipsilateral DIAM EMG activity. At day 14 post-C2SH, the AAV-TrkB group showed significantly greater normalized RMS EMG amplitude compared to the AAV-GFP group. For the animals that displayed ipsilateral recovery (2 out of 11 in the AAV-GFP group and 10 out of 15 in the AAV-TrkB group), the normalized RMS EMG amplitude was $27 \pm 13\%$ and $33 \pm 1\%$ at days 14 and 21 post-C2SH, respectively, for the AAV-GFP group, and $48 \pm 12\%$ and $34 \pm 10\%$ at days 14 and 21 post-

C2SH, respectively, for the AAV-TrkB group. The contralateral DIAM RMS EMG amplitude approximately doubled at 3 days post-SH ($p < 0.001$) in both AAV-GFP and AAV-TrkB treated animals ($96 \pm 8\%$ vs. $113 \pm 13\%$ increase, respectively) and showed no significant change throughout 21 days post-C2SH, regardless of AAV treatment ($p = 0.862$ for time $\times$ AAV treatment interaction), reflecting a sustained increase in the contralateral DIAM EMG activity for the entire experimental period.

At day 21 post-C2SH, DIAM EMG activity was examined during additional ventilatory and non-ventilatory behaviors subjecting animals to hypoxia (10% O₂)-hypercapnia (5% CO₂) conditions and sustained airway occlusion. On average, ipsilateral DIAM RMS EMG activity was significantly different across behaviors ($p < 0.001$), and across both AAV treatment groups, DIAM RMS EMG activity increased from eupnea to hypoxia-hypercapnia (4.5-fold) and to airway occlusion (10-fold) at day 21 post-C2SH. The proportion of animals displaying ipsilateral rhythmic DIAM EMG activity at day 21 post-C2SH varied depending on the behavior examined. The proportion of animals displaying ipsilateral DIAM EMG activity during hypoxia-hypercapnia was 64% (7 out of 11) in the AAV-GFP group compared to 93% (14 out of 15) in the AAV-TrkB group ($p = 0.070$). Normalized to eupneic DIAM RMS EMG activity pre-C2SH, RMS EMG amplitude during hypoxia-hypercapnia conditions was $61 \pm 17\%$ and $82 \pm 20\%$ for the AAV-GFP and AAV-TrkB, respectively, with no difference between AAV treatment groups ($p = 0.468$). When only animals displaying ipsilateral DIAM EMG activity during hypoxia-hypercapnia were considered, normalized RMS EMG amplitude was $95 \pm 15\%$ and $87 \pm 21\%$ of the eupneic for the AAV-GFP and AAV-TrkB groups, respectively. All animals displayed ipsilateral DIAM EMG activity during airway occlusion and normalized RMS EMG amplitude was $148 \pm 35\%$ in the AAV-GFP group vs. $171 \pm 28\%$ in the AAV-TrkB ($p = 0.610$). Regardless of AAV treatment, ipsilateral DIAM EMG activity did not achieve normalized values observed during pre-C2SH sighs ($\sim 250\%$ of eupnea), reflecting the limited extent of recovery post-C2SH in both treatment groups. In agreement, there were no significant differences between contralateral DIAM EMG activities in both AAV treatment groups during hypoxia-hypercapnia and airway occlusion ($p = 0.330$).

Glutamatergic and serotonergic receptor expression in phrenic motoneurons

Real-time RT-PCR was used to quantify the expression of glutamatergic (AMPA, NMDA) and serotonergic (5-HT_{2a}, 5-HT_{2c}) receptors in addition to the housekeeping RPS16 gene in laser capture microdissected phrenic motoneurons at day 21 post-C2SH. As expected, labeled motoneurons were clearly visualized in a craniocaudal column within cervical spinal cord segments C3-C5. All visualized motoneurons were captured and the completeness of capture was confirmed by visual inspection of the remaining tissue, as in previous studies (Gransee, et al., 2013, Mantilla, et al., 2012). Melting curve analysis verified the specificity of amplification of the corresponding primer pairs (Figure 4). Across animals in both experimental groups, mRNA expression of the RPS16 transcript was consistent (mean CP: 32.64 ± 0.38), also in agreement with our previous report (Mantilla, et al., 2012). Phrenic motoneuron mRNA expression for the AMPA, NMDA, 5-HT_{2a}, and 5-HT_{2c} receptors (as measured by the CP relative to RPS16) was not significantly different across treatment groups ($p = 0.261$, Table 2).

Correlations between mRNA expression for AMPA vs. NMDA and 5-HTR2a vs. 5-HTR2c at 21 days post C2SH were determined by comparing the Z-scores in the entire population for each transcript and sample (Figure 5). For glutamatergic receptor mRNA expression, there was a significant, positive, strong correlation between AMPA and NMDA expression (slope = 0.60; $r^2 = 0.82$; $p < 0.001$) across AAV-treated groups. Clustering of animals that received AAV-TrkB and displayed recovery after C2SH is evident in the upper right quadrant of Figure 5A, reflecting increased relative co-expression of AMPA and NMDA receptors (3/5 animals). Animals that did not show recovery after C2SH present a decreased relative NMDA expression (6/7 animals) regardless of AAV-treatment group (Figure 5B). There was no significant correlation between the relative expression of 5-HTR2a and 5-HTR2c receptors across treatment groups or recovery status ($p = 0.743$).

Discussion

The present study supports the therapeutic role for increased full-length TrkB expression in phrenic motoneurons in enhancing recovery of rhythmic DIAM activity following unilateral upper cervical spinal cord injury. Selective targeting of phrenic motoneurons below the level of injury was sufficient to enhance recovery during eupnea. The proportion of animals displaying ipsilateral DIAM EMG activity during eupnea was greater following intrapleural AAV-TrkB treatment compared to AAV-GFP in a model of unilateral injury in which lack of ipsilateral activity was verified 3 days post-injury using chronic DIAM EMG recordings. The extent of recovery observed in the present study was limited, however. Normalized DIAM RMS EMG amplitude was ~50% of ipsilateral pre-injury levels in animals displaying recovery. In addition, there was no change in contralateral DIAM EMG activity (which was roughly doubled following C2SH). The increase in contralateral DIAM EMG activity likely reflects increased neural drive to phrenic motoneurons since similar changes are also seen following acute unilateral DIAM denervation (Gill, et al., 2015). Furthermore, ipsilateral DIAM RMS EMG activity during hypoxia-hypercapnia and airway occlusion did not achieve normalized values observed during pre-C2SH sighs (~60% of maximum transdiaphragmatic pressure; (Mantilla, et al., 2010, Seven, et al., 2014), regardless of AAV treatment.

Targeted and selective transduction of phrenic motoneurons was previously documented using intrapleural delivery of AAV7-TrkB (Gransee, et al., 2013). Treatment 3 weeks before C2SH surgery enhanced recovery of ipsilateral phrenic activity at 14 days post-C2SH. The proportion of animals displaying ipsilateral DIAM EMG activity was 100% with pre-treatment compared to 67% with treatment 3 days post-SH (present study). The extent of recovery (peak RMS EMG amplitude) was 73% of the pre-C2SH eupnea value with pre-treatment vs. 32% in the present study. These results indicate that increased TrkB expression in phrenic motoneurons is sufficient to enhance spontaneous recovery following an incomplete cervical spinal cord injury. The importance of TrkB signaling (presumably via the full-length TrkB receptor) to spontaneous recovery following C2SH was previously documented by 1) a chemical-genetic approach using 1NMPP1-induced inhibition of TrkB kinase activity in *TrkB^{F616A}* mice (Mantilla, et al., 2014) and 2) siRNA-mediated knockdown of TrkB receptor expression in phrenic motoneurons (Mantilla, et al., 2013). When all of these results are taken together, it is evident that TrkB signaling in phrenic

motoneurons is not only sufficient but necessary to enhance both the proportion of animals that display recovery post-C2SH and the extent of such recovery. The present study now documents that increasing TrkB signaling in phrenic motoneurons is effective even when performed as a therapeutic intervention following C2SH. Importantly, administering AAV-TrkB through intrapleural injection is a practical and minimally-invasive approach that is clinically feasible.

Benefits of increasing BDNF/TrkB signaling following spinal cord injury may include increased survival of axotomized neurons (Koliatsos, et al., 1993, Sendtner, et al., 1992, Yan, et al., 1993), increased growth of motor axons (Boyd and Gordon, 2003, Novikova, et al., 2002), and promotion of myelination (Han, et al., 2015, Lang, et al., 2008, McTigue, et al., 1998), all of which primarily involve presynaptic drive to phrenic motoneurons. However, postsynaptic neuroplasticity within motoneurons likely also contributes to strengthening of spared synaptic inputs since motoneuron specific effects appear necessary for recovery. In studies in which increased BDNF/TrkB signaling in phrenic motoneurons was achieved by chronic intrathecal BDNF delivery (Mantilla, et al., 2013) or intraspinal transplantation of BDNF producing mesenchymal stem cells (MSCs) in the vicinity of the phrenic motoneuron pool (Gransee, et al., 2015), all animals displayed ipsilateral rhythmic DIAM EMG activity and increased peak RMS EMG amplitude compared to the corresponding control groups (~70% of pre-C2SH eupneic values). The importance of targeted strategies to increase BDNF/TrkB signaling in motoneurons (e.g., using intrapleural AAV) is highlighted by undesirable CNS effects of BDNF (independent of therapeutic delivery) on autonomic and sensory systems (Bregman, et al., 1997, Iarikov, et al., 2007, Weishaupt, et al., 2012). Thus, cell-specific therapies such as those achieved by gene therapy targeting motoneurons for full-length TrkB expression are paramount and may even supplement low-dose delivery of BDNF.

At the present time, there is limited information regarding possible therapeutic time windows for different interventions following spinal cord injury. Previous studies document the importance of timing for spinal decompression following traumatic spinal cord injury (c.f., (Furlan, et al., 2011). Based on a systematic review of randomized clinical trials, high dose methylprednisolone steroid therapy shows efficacy when administered within 8 hours (Bracken, 2012). In a series of studies using the C2SH model of unilateral cervical spinal cord injury, enhanced recovery of ipsilateral rhythmic DIAM activity was evident following: 1) chronic intrathecal BDNF infusion between days 3 and 14 post-C2SH (Mantilla, et al., 2013); 2) intraspinal transplantation of BDNF-expressing MSCs at the time of C2SH (Gransee, et al., 2015); 3) AAV-TrkB injection 3 weeks prior to C2SH with stable TrkB expression 3–10 weeks post-treatment (Gransee, et al., 2013); and 4) AAV-TrkB injection 3 days post-C2SH (present study), with peak expression expected by 10–14 days post-injection (Peel, et al., 1997). Increased neurotrophin expression, including BDNF, is evident in several models of spinal cord injury (Widenfalk, et al., 2001). Indeed, at levels below a T9 contusion injury, the number of cells expressing BDNF mRNA increases in Rexed lamina IV, V and VII of the spinal cord gray matter, compared to the uninjured cord where expression is restricted to few cells in lamina VII. Intraspinally BDNF availability, however, is likely limited since intrathecal or intraspinal delivery of BDNF or BDNF-producing MSC enhance recovery (Boyce, et al., 2012, Gransee, et al., 2015, Mantilla, et al., 2013).

Transducing macrophage/microglia or other glial cells in the spinal cord injury site may be pursued to increase local BDNF (Petrosyan, et al., 2014). Importantly, increasing phrenic motoneuron BDNF/TrkB signaling within a period spanning days 3 to 14 post-C2SH appears necessary for recovery of DIAM activity. Future studies should directly examine such therapeutic time window in order to define the role of increasing motoneuron BDNF/TrkB signaling for use in preclinical studies.

Limited transduction efficiency could explain the lack of enhanced recovery of motor behaviors requiring higher levels of force generation by the DIAM. The extent of recovery across forceful behaviors (e.g., airway occlusion) measured by DIAM RMS EMG was not different across AAV treatment groups, achieving only ~160% of pre-injury eupnea (weighted average across groups). Improving the efficiency of motoneuron transduction may enhance recovery by increasing motoneuron recruitment to levels needed for respiratory behaviors requiring higher levels of force generation (i.e., ~220% of eupnea during airway occlusion in uninjured rats; (Gill, et al., 2015, Mantilla, et al., 2010, Seven, et al., 2014). Recent studies using alternate AAV serotypes reported targeted delivery to motoneurons with transduction efficiency near 30% (ElMallah, et al., Elmallah, et al., 2014), approximately doubling our reported value for AAV7 (Gransee, et al., 2013) but with loss of selectivity. In conditions where selective targeting of phrenic motoneurons may not be needed, AAV serotypes different from AAV7 (e.g., AAV2 or AAV9) provide promising alternatives to enhance transduction efficiency.

Descending excitatory inputs to phrenic motoneurons project primarily from the ipsilateral rostral ventral medulla and more sparse, but spared contralateral projections are thought to mediate spontaneous recovery post-C2SH. Previous studies support the use of a functional outcome, i.e., absence of ipsilateral diaphragm EMG activity at 3 days post-C2SH, to evaluate the completeness of hemisection (Miyata, et al., 1995, Sieck and Mantilla, 2009). In rats the period of spinal shock after spinal cord transection (evidenced by anal areflexia) is reportedly limited to 24 hours (Holmes, et al., 1998). We assessed recovery of ipsilateral DIAM activity up to 21 days post-C2SH, and results are consistent with a previous study where no further recovery was observed after 14 days post-C2SH and at least up to 6 weeks post-C2SH (Mantilla, et al., 2013). Future studies should address whether increasing BDNF/TrkB signaling in phrenic motoneurons during this later period post-C2SH can promote neuroplasticity of spared spinal pathways in order to enhance recovery of ipsilateral DIAM activity. Strengthening glutamatergic (Kang and Schuman, 1995, Lessmann, et al., 1994) or serotonergic (Baker-Herman, et al., 2004, Murray, et al., 2010, Zhou, et al., 2001) synaptic connections constitute possible mechanisms by which BDNF/TrkB signaling can enhance phrenic motoneuron activity ipsilateral to a C2SH injury.

Recovery of ipsilateral DIAM activity post-SH is associated with changes in expression of excitatory glutamatergic (Alilain and Goshgarian, 2008) and neuromodulatory serotonergic receptors (Fuller, et al., 2005, Murray, et al., 2010). Decreased expression of the AMPA subunit receptor GluR2 was evident 16 weeks post-C2SH in the ipsilateral ventral cervical spinal cord region containing the phrenic motoneuron pool using Western blot analyses, and in retrogradely-labeled phrenic motoneurons using immunohistochemical analyses (Alilain and Goshgarian, 2008). Using similar techniques, increased GluR1 and NMDA receptor

subunit NR2A expression were evident as early as 6 weeks post-C2SH. Using Western blot analyses of the ipsilateral C4-C5 ventral horn gray matter and immunohistochemistry in putative phrenic motoneurons, increased expression of serotonergic 5-HT_{2a} receptors was evident as early as 2 weeks post-C2SH (Fuller, et al., 2005). These results are consistent with an important role for increased serotonergic signaling in phrenic motoneurons in spontaneous functional recovery post-C2SH and are in agreement with mRNA measurements in microdissected phrenic motoneurons (Mantilla, et al., 2012). Indeed, increased NMDA receptor subunit NR1 and 5-HT_{2a} expression in phrenic motoneurons (compared to uninjured controls) was associated with the time course of spontaneous recovery of ipsilateral rhythmic DIAM activity post-C2SH (Mantilla, et al., 2012). Based on these previous studies, we explored whether changes in motoneuron GluR and 5-HT_{2a} expression were associated with the enhanced recovery displayed by animals treated with AAV-TrkB. In the present study, no differences in the expression of GluR or 5-HT_{2a} between AAV-TrkB and AAV-GFP treated animals were found. However, animals displaying recovery of DIAM activity following intrapleural AAV-TrkB treatment showed increased relative co-expression of AMPA and NMDA receptors, but no change in the relative expression of 5-HT_{2a} and 5-HT_{2c} receptors. Of notice, our technique may be limited by the proportion of motoneurons effectively transduced by AAV (~15%; Gransee, et al., 2013) given that expression of GluR and 5-HT_{2a} mRNA was quantified in all labeled phrenic motoneurons. Receptor mRNA expression may also change over time following C2SH (Mantilla, et al., 2012) and in the present study, analyses were only conducted at day 21 post-C2SH. In conclusion, the present study demonstrates that intrapleural AAV mediated delivery of TrkB to phrenic motoneurons administered post-C2SH promotes recovery of rhythmic ipsilateral DIAM EMG activity during eupneic breathing. These novel findings support selective targeting of phrenic motoneurons below the level of injury as a promising therapeutic strategy for spinal cord injury.

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Highlights

- Spontaneous recovery of ipsilateral diaphragm activity was evident post-C2 hemisection
- AAV-TrkB treatment 3 days post-hemisection enhanced recovery of diaphragm activity
- AAV-TrkB increased the proportion of animals displaying eupneic activity vs AAV-GFP
- AAV-TrkB treatment increased the extent of recovery (eupneic EMG amplitude)
- Diaphragm activity during higher force behaviors was not enhanced by AAV treatment

Figure 1.

Representative raw and root mean square (RMS) diaphragm (DIAM) EMG tracings from two lightly anesthetized rats from each AAV treatment group obtained pre and post (3, 14, and 21 days) unilateral spinal hemisection at C2 (C2SH). C2SH interrupts ipsilateral descending drive to phrenic motoneurons causing ipsilateral DIAM muscle paralysis. None out of 26 rats displayed ipsilateral DIAM EMG activity on day 3 post-C2SH, verifying the completeness of the C2SH surgery. Rats randomized to receive AAV-GFP or AAV-TrkB were treated on day 3 post-C2SH. Recovery of ipsilateral DIAM EMG activity was evident in most of the AAV-TrkB treated rats, while most rats treated with AAV-GFP did not display recovery up to 21 days post-C2SH.

Figure 2.

Proportion of animals displaying recovery of ipsilateral DIAM EMG activity post-C2SH. Chronic DIAM EMG recordings during eupnea were used to determine the proportion of animals displaying recovery of rhythmic ipsilateral DIAM EMG activity post-C2SH. The full criteria used for the classification of recovery are detailed in the Materials and Methods section. At 7 days post-C2SH, 1 of 11 (9%) animals with AAV-GFP treatment displayed recovery, compared to 7 of 15 (47%) animals treated with AAV-TrkB. By 14 days post-C2SH, 2/11 (18%) of the AAV-GFP treated animals displayed functional recovery compared to 10/15 (67%) of AAV-TrkB treated animals. Data analyzed by a repeated nominal logistic test (AAV treatment, $p = 0.031$; there were no time ($p = 0.442$) or time x AAV treatment ($p = 0.100$) interaction effects). *Significantly different than AAV-GFP group on the same day.

Figure 3.

Amplitude of ipsilateral and contralateral DIAM EMG activity during eupnea and across behaviors in lightly anesthetized rats pre-C2SH, 3, 7, 14, and 21 days post-C2SH. At each time point, DIAM EMG activity was measured as the mean peak root mean squared (RMS) value normalized to the pre-C2SH value during eupnea on each side for each rat. Following AAV treatment, there was a time effect on DIAM RMS EMG amplitude bilaterally ($p < 0.001$), and a time x AAV treatment interaction ipsilateral to C2SH ($p = 0.034$). For the contralateral DIAM, there was no AAV treatment ($p = 1.000$) or time x AAV treatment interaction ($p = 0.862$) effects. *Significantly different than AAV-GFP on the same day for the same DIAM side. †Significantly different than pre-C2SH for the same AAV treatment for the same DIAM side. ‡Significantly different than value at day 3 post-C2SH for same AAV treatment for the same DIAM side.

Figure 4.

A) Micrographs of retrogradely-labeled phrenic motoneurons for laser capture microdissection (LCM). Top: Fluorescent imaging of phrenic motoneurons that were labeled by intrapleural injection of Alexa 488-conjugated cholera toxin subunit B. Bottom: Lack of Alexa 488 fluorescence in section of spinal cord tissue after complete capture of phrenic motoneurons. Scale bar, 100 μ m. B) Expression of glutamatergic (AMPA and NMDA) and serotonergic (5-HTR2a and 5-HTR2c) receptors in microdissected phrenic motoneurons from C2SH rats following AAV-TrkB or AAV-GFP treatment. Receptor mRNA expression was quantified using real-time RT-PCR relative to ribosomal protein S16 (RPS16). Representative melting (top panels) and amplification curves (bottom panels) for each glutamatergic and serotonergic receptor and RPS16 mRNA transcript are shown for both AAV treatment groups (AAV-GFP: $\square$; AAV-TrkB: $\triangle$). Specificity of amplification of the corresponding primer pairs is evident by the single melting peak and was also verified by including control reactions that did not include the template ($\diamond$). The second derivative method for crossing-point (CP) analyses was used for quantification of receptor expression (see Materials and Methods for details). The expression levels (Δ CP) for each sample and transcript was calculated by taking the difference between the crossing points for each receptor transcript and RPS16.

Figure 5.

Correlation between glutamatergic (AMPA vs. NMDA, A) and serotonergic (5-HTR2a vs. 5-HTR2c, B) receptor expression in phrenic motoneurons at 21 days post-C2SH. Transcript expression was normalized by calculating a Z-score for each animal using the mean CP and overall mean and SD for all animals. Each point represents the Z-score for an individual animal, stratified by evidence of recovery at 21 days post-C2SH. Correlated expression results in grouping of points within a quadrant (left lower quadrant represents decreased expression for both transcripts; right upper quadrant, increased expression). The expression of AMPA vs NMDA displayed a significant, positive, strong correlation across AAV-treated groups (slope = 0.60; $r^2 = 0.82$; $p < 0.001$).

Table 1

Ventilatory parameters across experimental AAV groups before and after unilateral cervical spinal cord hemisection at C2 (C2SH)

BEHAVIOR	VENTILATORY PARAMETER	EXPERIMENTAL GROUP			
		AAV-GFP (n = 11)		AAV-TrkB (n = 15)	
		Recovered (n = 2)	Not recovered (n = 9)	Recovered (n = 10)	Not recovered (n = 5)
Eupnea Pre-C2SH	Respiratory rate (min^{-1})	68 $\pm$ 2	87 $\pm$ 5	82 $\pm$ 4	73 $\pm$ 10
	Duty cycle	30 $\pm$ 1%	41 $\pm$ 3%	38 $\pm$ 2%	38 $\pm$ 4%
Eupnea Day 21 **	Respiratory rate (min^{-1})	80 $\pm$ 14	82 $\pm$ 7	81 $\pm$ 4	85 $\pm$ 4
	Duty cycle	60 $\pm$ 9%	39 $\pm$ 2%	45 $\pm$ 3%	52 $\pm$ 5%
Hypoxia-Hypercapnia Day 21 **	Respiratory rate (min^{-1})	108 $\pm$ 9	101 $\pm$ 5	104 $\pm$ 3	101 $\pm$ 9
	Duty cycle	59 $\pm$ 2%	52 $\pm$ 3%	59 $\pm$ 3%	57 $\pm$ 4%

Data presented as mean $\pm$ SE. Data are clustered into recovered and not recovered groups according to evidence of ipsilateral diaphragm (DIam) EMG activity at day 21 following C2SH. Duty cycle was calculated as the percent of total time corresponding to inspiration. During eupnea, there were no differences between AAV treatment groups or time, regardless of evidence of recovery. Overall, duty cycle during eupnea increased at 21 days post-C2SH vs. pre-C2SH ($p = 0.005$), but there was no significant treatment effect. Respiratory rates and duty cycles increased during hypoxia-hypercapnia in comparison to eupnea (

p < 0.001).

Table 2

Expression of glutamatergic and serotonergic receptors in phrenic motoneurons

TRANSCRIPT	EXPERIMENTAL GROUP			
	AAV-GFP		AAV-TrkB	
	n	CP	n	CP
AMPA	(7/7)	12.31 ± 0.45	(7/7)	11.67 ± 0.45
NMDA	(7/7)	8.89 ± 0.45	(7/7)	8.10 ± 0.50
5-HTR2a	(4/6)	18.73 ± 0.59	(5/6)	17.49 ± 1.19
5-HTR2c	(5/7)	7.85 ± 0.75	(6/7)	7.25 ± 0.19

Data presented as mean ± SE and represent the difference in threshold cycle between the transcript of interest and the reference transcript RPS16. Quantitative real-time RT-PCR was performed on laser capture microdissected phrenic motoneurons ipsilateral to injury 21 days post-C2SH. The numbers in parenthesis indicate the number of animals in which transcript was detected by RT-PCR out of the total number of animals available for analysis in each group. Note that mRNA was only available for 6 animals in each group for analyses of the 5-HTR2a transcript.