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Motoneuron Glutamatergic Receptor Expression following Recovery from Cervical Spinal Hemisection

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Abstract

Cervical spinal hemisection at C2 (SH) removes premotor drive to phrenic motoneurons located in segments C3–C5 in rats. Spontaneous recovery of ipsilateral diaphragm muscle activity is associated with increased phrenic motoneuron expression of glutamatergic N-methyl-D-aspartate (NMDA) receptors and decreased expression of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. Glutamatergic receptor expression is regulated by tropomyosin related kinase receptor subtype B (TrkB) signaling in various neuronal systems and increased TrkB receptor expression in phrenic motoneurons enhances recovery post-SH. Accordingly, we hypothesized that recovery of ipsilateral diaphragm muscle activity post-SH, whether spontaneous or enhanced by adeno-associated virus (AAV)-mediated upregulation of TrkB receptor expression, is associated with increased expression of glutamatergic NMDA receptors in phrenic motoneurons. Adult male Sprague-Dawley rats underwent diaphragm EMG electrode implantation and SH surgery. Rats were injected intrapleurally with AAV expressing TrkB or GFP at 3 weeks before SH. At 14 days post-SH, the proportion of animals displaying recovery of ipsilateral diaphragm activity increased in AAV-TrkB treated (9/9) compared to untreated (3/5) or AAV-GFP treated animals (4/10; $p < 0.027$). Phrenic motoneuron NMDA NR1 subunit mRNA expression was ~4-fold greater in AAV-TrkB vs. AAV-GFP treated SH animals ($p < 0.004$) and in animals displaying recovery vs. those not recovering ($p < 0.005$). Phrenic motoneuron AMPA GluR2 subunit mRNA expression decreased after SH and albeit increased in animals displaying recovery vs. those not recovering, levels remained lower than control. We conclude that increased phrenic motoneuron expression of glutamatergic NMDA receptors is associated with spontaneous recovery after SH and enhanced recovery following AAV-TrkB treatment.

Graphical abstract

Phrenic motoneuron NMDA NR1 subunit mRNA expression was significantly greater 14 days after unilateral spinal hemisection at C2 (SH) compared to control (*, $p < 0.001$), and in SH animals displaying recovery (Rec) of ipsilateral diaphragm activity than in non-recovered animals (Non-Rec; †, $p < 0.001$).

Keywords

neuroplasticity; neurotrophin; diaphragm muscle; phrenic motoneuron; spinal cord injury; RRID:RGD_5508397; RRID:SCR_010038; RRID:SCR_014325

Introduction

Cervical segments of the spinal cord are most commonly involved in traumatic spinal cord injury (SCI) and although injuries are frequently incomplete, cervical SCI results in significant respiratory impairment and the need for mechanical ventilation (2015). In a well-established model of incomplete cervical SCI, unilateral C2 spinal cord hemisection (SH), ipsilateral excitatory premotor drive to phrenic motoneurons is removed and the ipsilateral diaphragm muscle is inactivated. Over time following SH, there is a varying extent of spontaneous recovery of ipsilateral activity reflecting neuroplasticity and strengthening of spared excitatory drive to phrenic motoneurons (Boulenguez et al., 2007; Golder et al., 2003; Golder and Mitchell, 2005; Mantilla et al., 2013b; Miyata et al., 1995; Nantwi et al., 1999; Prakash et al., 1999; Sieck and Mantilla, 2009; Zhan et al., 1997).

Excitatory bulbospinal (premotor) respiratory drive to phrenic motoneurons is primarily glutamatergic (Greer et al., 1992; McCrimmon et al., 1989; Saji and Miura, 1990). Phrenic motoneurons express multiple excitatory glutamatergic receptor subtypes including ionotropic N-methyl-D-aspartate (NMDA) receptors and α -amino-3-hydroxy-5-

Chronic diaphragm EMG recordings

Three days before SH surgery, two pairs of electrodes wires (Cooner Wire Inc., Chatsworth, CA) were implanted into the left and right mid-costal hemidiaphragm in such a manner that an uninsulated 3 mm segment was embedded within the diaphragm, as previously described (Dow et al., 2006; Dow et al., 2009; Gransee et al., 2013; 2015; Mantilla et al., 2013a; Mantilla et al., 2013b; Mantilla et al., 2011; Martinez-Galvez et al., 2016; Trelease et al., 1982). The electrode wires were tunneled subcutaneously and externalized in the back of the animal.

In order to verify completeness of SH, absence of ipsilateral diaphragm EMG activity was confirmed under anesthesia at the time of surgery and 3 days post-SH (SH 3D). Previous studies by our group have evaluated the extent of histological damage following unilateral SH (Mantilla et al., 2014; Miyata et al., 1995; Sieck and Mantilla, 2009). The SH injury consistently results in damage to the ventral and lateral columns at C2 resulting in absence of ipsilateral diaphragm EMG activity at SH 3D. Thus, a functional outcome was used to verify completeness of SH in the present study. Tissue procurement for LCM precludes additional histological analyses. Diaphragm EMG activity was also recorded for ~ 5 min in anesthetized animals at 7, 14, and 28 days post-SH (SH 7D, SH 14D, and SH 28D, respectively). EMG signals were amplified (2000×) and band-pass filtered between 20 Hz and 1 kHz (Model 2124, DATA Inc.). Information was converted from analog to digital signals at 2 kHz using LabView data acquisition board and software (National Instruments, Austin, TX). Root mean square (RMS) EMG amplitude was calculated using a 50 ms moving window (Mantilla et al., 2013b; Mantilla et al., 2011; Mantilla et al., 2010; Seven et al., 2013; Sieck and Fournier, 1990). The entire recording was used for analysis excluding only the occasional spontaneous deep breath (defined as an inspiratory burst greater than twice eupneic RMS EMG amplitude). An animal was classified as exhibiting recovery if the diaphragm EMG signal: 1) was rhythmic (i.e., in phase with the contralateral side) and periodic (i.e., occurring on at least 90% of eupneic bursts), 2) reflected activation of more than one motor unit (identified by EMG spikes with more than one morphology consistent with more than one motor unit); and 3) had RMS EMG amplitude at least 10% of the pre-SH amplitude. Respiratory rate and duty cycle (percent of time with inspiratory activity) were calculated from contralateral diaphragm EMG recordings. As in previous studies, DIAM EMG recordings during eupnea reflected <5% variance in respiratory rate or RMS EMG amplitude.

Phrenic motoneuron transduction with AAV vectors

For analyses of phrenic motoneuron receptor expression, rats were assigned to one of five groups (Table 2): untreated control (n = 7), AAV-TrkB treated control (n = 5), untreated SH (n = 5), AAV-GFP treated SH (n = 10), and AAV-TrkB treated SH (n = 9). AAV7 encoding GFP (AAV7.CMV.eGFP.WPRE.bGH) and AAV7 encoding human TrkB (AAV7.CMV.Flag-TrkB.WPRE.bGH) were obtained from the Vector Core at University of Pennsylvania. Of note, the AAV-TrkB vector encodes both the full-length human TrkB receptor as well as the FLAG protein; however it is not a FLAG-tagged TrkB receptor fusion protein. The AAV7 serotype was selected based on its selective retrograde transduction of phrenic motoneurons (Gransee et al., 2013). Specifically, AAV-based delivery of human TrkB to phrenic

motoneurons was verified by evidence of human TrkB mRNA expression in microdissected phrenic motoneurons as well as measurements of FLAG protein expression in cervical spinal cord homogenates at 3 weeks after intrapleural injection. Three weeks prior to SH surgery, AAV-GFP or AAV-TrkB were administered via unilateral intrapleural injection (50 μ l; 1×10^{11} genome copies/side) as previously described (Gransee et al., 2013; Mantilla et al., 2009). Briefly this technique consists of an injection of AAV in the pleural space between the 7th and 8th ribs of the right chest. Animals were euthanized at 5 weeks after AAV injection. Injection with AAV did not result in any adverse effects; all animals displayed normal weight gain and behavior, compared to the untreated groups.

Retrograde labeling of phrenic motoneurons

Intrapleural injection of cholera toxin subunit B (CTB) was used to retrogradely label phrenic motoneurons (Gransee et al., 2013; 2015; Issa et al., 2010; Mantilla et al., 2012; Mantilla et al., 2009; Martinez-Galvez et al., 2016). We previously confirmed that intrapleural injection of CTB only labels phrenic motoneurons in the cervical spinal cord (Mantilla et al., 2009). Briefly, three days before the terminal experiment, 20 μ l of a 0.2% solution of Alexa Fluor 488-conjugated CTB or Alexa Fluor 555-conjugated CTB (Molecular Probes; Life Technologies, Grand Island, NY) was injected with a Hamilton syringe into the pleural space of the right and left sides of the chest between the 7th and 8th ribs.

Laser capture microdissection (LCM) of phrenic motoneurons

Laser capture microdissection (LCM) was used to obtain a pure population of phrenic motoneurons from spinal cord sections, as previously described (Gransee et al., 2013; Mantilla et al., 2012; Martinez-Galvez et al., 2016). After the animals were anesthetized and then euthanized by exsanguination, the cervical spinal cord (C2–C7) was removed and quickly frozen in liquid N₂ under RNase-free conditions. Longitudinal sections of spinal cord were cut (10 μ m), placed on pre-chilled slides (Fisher Scientific, Pittsburgh, PA) and stored at –80°C until microdissection. Slides were dehydrated in graded alcohols and xylene immediately before LCM. With Arcturus^{XT} LCM microdissection system (Applied Biosystems, Life Technologies Corp., Carlsbad, CA), it was possible to visualize retrogradely-labeled phrenic motoneurons under direct epifluorescence illumination (Figure 2) and microdissect selected motoneurons onto Capsure HS LCM caps (Life Technologies Corp.). In most cases, a single cap was used for 3–4 spinal cord sections, and an average of four caps were obtained from the right side of the spinal cord (ipsilateral to SH) for each animal. Caps were stored at –80°C until RNA extraction.

Real-time RT-PCR

Total RNA was extracted from the LCM samples using the RNeasy Micro kit (Qiagen Inc., Valencia, CA) according to manufacturer's protocol, and then pooled for all caps from the same animal. The RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis kit under standard conditions (Roche Applied Science, Indianapolis, IN) as previously described (Mantilla et al., 2012; Martinez-Galvez et al., 2016). Briefly, sample RNA (in duplicate for each sample), random hexamer primer, anchored-oligo(dT)18 primer, and RNase free sterile water were incubated at 65°C for 10 min and chilled on ice for 1 min.

A mixture of 1× Transcriptor reverse transcriptase reaction buffer, Protector RNase inhibitor, dNTP, and Transcriptor reverse transcriptase was added to each sample. Samples were incubated for 10 min at room temperature followed by 20 min at 55°C. Finally samples were heated to 85°C for 5 min and placed on ice.

For gene expression analysis, quantitative amplification of specific receptors subtypes was studied using 1× SYBR Green I Master on the LightCycler 480 (Roche) and the respective primer pair (5′ and 3′ primers) of each receptor, as previously reported (Mantilla et al., 2012). These receptors include: NMDA NR1 subunit, AMPA GluR2 subunit, mGluR1, and mGluR5. The ribosomal protein S16 (RPS16) was used as a reference gene. Uniform expression of RPS16 among experimental groups was verified. Amplification and quantitation of mRNA was performed on a LightCycler 480. The thermal cycling conditions were identical: 5-min pre-amplification incubation at 95 °C, 70 cycles of primer annealing at 60 °C for 12 s, PCR product extension at 72 °C for 18 s, and denaturing at 95 °C for 15 s. In order to verify specificity of the amplification product, a melting curve analysis was performed plotting the derivative of the melting curve fluorescence (–dF/dT) versus temperature. An appropriate single peak in the resultant melting curve and the lack of primer–dimer complexes were indicators of correct amplification products. DNA electrophoresis was previously performed to verify the specificity of the PCR product (Mantilla et al., 2012). All PCR reactions were performed in duplicate for each reverse transcription product.

Quantitative analyses

Using the LightCycler 480 software (version 1.5, Roche), a fluorescence plot was generated for each sample (Figure 2G). In order to determine the threshold cycle (i.e., crossing point - CP), the point where each gene product transitions from the linear phase to the logarithmic phase, the second derivative method was applied to the amplification curve. The difference in CP from the reference gene RPS16 was then calculated to determine a CP for each sample and transcript. The CP reveals relative changes in receptor mRNA expression for each glutamatergic receptor subtype following SH. RT and PCR reactions were conducted in duplicate (i.e., at least 4 measurements per transcript for each animal). Prior to statistical analyses for each glutamatergic receptor subtype, outliers were removed if CP was greater than 3 cycles away from the median CP for each animal.

Statistical analyses

All statistical evaluations were performed using JMP statistical software (JMP 10.0, SAS Institute Inc., Cary, NC). The proportion of animals displaying recovery of ipsilateral diaphragm muscle activity was compared across treatment groups using a repeated measures nominal logistic test (time × treatment). Diaphragm RMS EMG amplitude was normalized to the eupneic value before SH for the same animal and differences between groups were examined using one-way analysis of variance (ANOVA). Respiratory rate and duty cycles were compared using mixed linear models with repeated measures (time × AAV treatment) and animal as a random effect. Glutamatergic receptor subtype expression was also compared by one-way ANOVA. When applicable, post-hoc analyses were conducted using Tukey–Kramer's honestly significant difference test. The correlation between transcripts was

calculated by linear regression after calculating a Z-score for each transcript and sample in the entire population. The degree of correlation is expressed as the r^2 value, with the effectiveness of the model (difference from the mean response) determined by ANOVA. Statistical significance was established at the 0.05 level. All experimental data are presented as mean $\pm$ SE, unless otherwise specified.

Results

A total of 36 animals were used across studies. All SH animals ($n = 24$) were implanted with bilateral diaphragm EMG electrodes at 3 days before SH surgery. As in previous studies, diaphragm EMG was recorded during SH surgery and at SH 3D to confirm completeness of SH by absence of ipsilateral diaphragm EMG activity during eupnea in anesthetized animals (Gransee et al., 2013; 2015; Mantilla et al., 2013a; Mantilla et al., 2013b; Martinez-Galvez et al., 2016; Miyata et al., 1995; Prakash et al., 1999; Zhan et al., 1997). One SH animal displayed ipsilateral diaphragm eupneic EMG activity at SH 3D and was excluded.

Recovery of ipsilateral diaphragm muscle activity post-SH

Diaphragm EMG activity during eupnea was recorded up to SH 14D. Both ipsilateral and contralateral diaphragm EMG activity occurred in bursts, reflecting rhythmic inspiration in anesthetized animals (Figure 1). During a recording session, minimal variability in diaphragm RMS EMG amplitude was evident (variance $<5\%$). Over time post-SH, a subset of animals displayed spontaneous recovery of ipsilateral diaphragm EMG activity during eupnea. At SH 7D and SH 14D, 3 out of 5 untreated SH animals (60%) displayed recovery (Figure 1b). In general agreement with previous studies (Gransee et al., 2013; Martinez-Galvez et al., 2016), the proportion of animals displaying recovery of ipsilateral diaphragm muscle activity was different across SH groups (χ^2 (5, $n = 48$) = 12.61, $p = 0.027$) and was significantly higher in the AAV-TrkB treated SH group. There were no time ($p = 0.115$) or time $\times$ treatment interaction ($p = 0.147$) effects on recovery. In animals treated with AAV-TrkB, 6 out of 9 animals (67%) displayed recovery at SH 7D and all animals recovered by SH 14D. In animals treated with AAV-GFP, 4 out of 10 animals (40%) displayed recovery of ipsilateral EMG activity at SH 7D and SH 14D.

Extent of eupneic diaphragm EMG activity post-SH

Diaphragm RMS EMG amplitude was assessed to determine the extent of recovery of eupneic diaphragm EMG activity post-SH. Diaphragm RMS EMG amplitude at SH 7D and SH 14D was normalized to the amplitude before SH for the same animal. There was a statistically significant difference across treatment groups in the extent of recovery post SH ($F_{2,23} = 7.29$; $p = 0.004$). At SH 14D, RMS EMG amplitude was $32 \pm 17\%$ of the pre-SH value in the untreated SH animals (Figure 1c), including both those that displayed recovery and those that did not. If only untreated SH animals that displayed recovery are considered, RMS EMG amplitude was $53 \pm 20\%$ of the pre-SH value. Treatment with AAV-TrkB significantly increased the extent of recovery at SH 14D; diaphragm RMS EMG amplitude was $95 \pm 24\%$ of the pre-SH value in AAV-TrkB treated SH animals and was significantly different than AAV-GFP treated SH animals ($p < 0.05$). Treatment with AAV-GFP did not change the extent of recovery compared to untreated SH. At SH 14D, RMS EMG amplitude

was $12 \pm 6\%$ of the pre-SH value in AAV-GFP treated SH animals including both those that displayed recovery and those that did not. In AAV-GFP treated SH animals that displayed recovery, RMS EMG amplitude was $31 \pm 9\%$ of the pre-SH value.

There were no differences in contralateral diaphragm RMS EMG amplitude across groups at SH 14D ($F_{2,22} = 0.06$; $p = 0.945$). Contralateral RMS EMG amplitude was $165 \pm 37\%$ of the pre-SH value in animals that did not display recovery at SH 14D ($n = 8$) and $159 \pm 23\%$ of the pre-SH value in animals that displayed recovery ($n = 15$). These results are consistent with previous reports in SH animals (Gransee et al., 2013; 2015; Mantilla et al., 2013a; Mantilla et al., 2013b; Martinez-Galvez et al., 2016).

Respiratory rate and duty cycle were measured from the contralateral hemidiaphragm EMG recordings during eupnea at pre-SH and SH 14D (Table 1). Respiratory rate was unaffected by SH and/or AAV treatment (mixed linear model; group: $p = 0.075$; treatment: $p = 0.244$; interaction: 0.275). Similarly, duty cycle was unaffected by SH and/or AAV treatment (mixed linear model; group: $p = 0.366$; treatment: $p = 0.060$; interaction: $p = 0.336$).

Laser capture microdissection of phrenic motoneurons

Retrogradely-labeled phrenic motoneurons were visualized by Alexa 488 or Alexa 555 fluorescence after intrapleural injection of fluorescently-conjugated CTB. Phrenic motoneurons were localized in a rostrocaudal column within the gray matter of cervical spinal cord segments C3–C5, as described previously (Kinkead et al., 1998; Mantilla et al., 2012; Mantilla et al., 2009; Martinez-Galvez et al., 2016; Prakash et al., 2000; Prakash et al., 1993; Zhan et al., 1989). Visual inspection of the LCM cap and the remaining spinal cord tissue confirmed complete capture of phrenic motoneurons (Figure 2). Most phrenic motoneurons on the side of the SH injury were captured ($\sim 80\%$). An average of 176 ± 13 phrenic motoneurons were captured per animal. A subset of AAV-GFP treated SH animals ($n = 5$) were retrogradely labeled with Alexa 555-conjugated CTB to estimate the AAV transduction efficiency. Phrenic motoneurons expressing both GFP and Alexa 555 fluorescence were successfully microdissected separately from phrenic motoneurons expressing only Alexa 555 fluorescence, and AAV transduction efficiency was estimated as the ratio of GFP-positive captures compared to the total number of phrenic motoneurons (CTB-positive). GFP expression was evident only in motoneurons co-labeled with CTB, indicating that intrapleural AAV-GFP transduced only phrenic motoneurons. Transduction efficiency was $16 \pm 2\%$, consistent with a previous report (Gransee et al., 2013).

Glutamatergic receptor expression in phrenic motoneurons

As in previous studies (Mantilla et al., 2012; Martinez-Galvez et al., 2016), expression of glutamatergic receptors (NMDA NR1 subunit, AMPA GluR2 subunit, mGluR1 and mGluR5), and RPS16 in microdissected phrenic motoneurons was quantified using real-time RT-PCR. A melting curve analysis for all transcripts was performed in order to verify the specificity of amplification of the corresponding primer pair for each PCR reaction, and a single peak at an appropriate temperature was identified for each. Representative amplification curves for all transcripts are shown in Figure 2.

The number of animals with detectable mRNA expression levels in each experimental group stratified by recovery post-SH is shown in Table 2. NMDA NR1 subunit was consistently found across groups (in 34 out of 36 animals examined), independent of recovery status. AMPA GluR2 subunit was consistently found in all groups except in the AAV-GFP treated SH group, and its expression was significantly more consistent in recovered compared to non-recovered animals (χ^2 (1, $n = 36$) = 4.082, $p = 0.043$). mGluR1 was found in all control animals, but was found less consistently in other groups and was not detectable in any of the AAV-GFP treated SH animals. Expression of mGluR5 was also not consistently found across groups and was undetectable in AAV-TrkB treated animals.

Phrenic motoneuron expression of glutamatergic receptors across groups is shown in Table 3. Phrenic motoneuron expression of NMDA NR1 subunit was significantly increased in all three SH groups (overall $F_{4,33} = 15.06$; $p < 0.001$). Post-hoc comparisons with Tukey-Kramer HSD revealed that NR1 subunit expression was greater in AAV-TrkB treated SH animals compared to AAV-GFP treated SH animals ($p = 0.004$). The change in CP for phrenic motoneurons in AAV-TrkB treated SH animals represents a ~4-fold increase in NMDA NR1 subunit expression relative to AAV-GFP treated SH animals. Phrenic motoneuron expression of AMPA GluR2 subunit significantly decreased (corresponding to an increased CP) in AAV-TrkB treated controls and all SH groups (overall $F_{4,27} = 17.82$; $p < 0.001$). Expression of mGluR1 remained unchanged across SH groups (overall $F_{3,15} = 4.09$; $p = 0.033$), and although it was significantly greater in AAV-TrkB treated control animals compared to untreated SH, mGluR1 expression was not consistent across groups as noted above, resulting in low animal numbers (Table 2). Phrenic motoneuron expression of mGluR5 did not change across groups ($F_{3,14} = 1.98$; $p = 0.175$).

In addition, the role of AAV treatment was assessed by examining expression of glutamatergic receptors in phrenic motoneurons expressing GFP (indicating AAV transduction) separately from those not expressing GFP in a subset of AAV-GFP treated SH animals ($n = 5$). AAV-GFP transduction had no effect on phrenic motoneuron expression of NMDA NR1 subunit ($F_{1,9} = 2.04$; $p = 0.191$), AMPA GluR2 subunit ($F_{1,9} = 0.21$; $p = 0.657$), and mGluR5 ($F_{1,5} = 1.18$; $p = 0.338$). Taken together, these results support a minimal non-specific effect of AAV transduction on phrenic motoneuron expression of glutamatergic receptors.

In order to evaluate correlations within animals in the expression of glutamatergic receptors, Z-scores for each transcript and sample were determined using the entire animal population (all 5 experimental groups). Correlations were analyzed using pairwise comparisons, and the only receptor pair that displayed a significant correlation across groups was AMPA GluR2 subunit -NMDA NR1 subunit (Figure 3). Control animals were clustered in the upper left quadrant, reflecting relatively lower NMDA NR1 subunit expression with higher AMPA GluR2 subunit expression in this group. In addition, there was a significant, positive correlation between AMPA GluR2 and NMDA NR1 subunit expression in SH animals ($F_{1,17} = 11.51$; $p = 0.004$; slope = 0.55; $r^2 = 0.42$). Lastly, AAV-TrkB treated SH animals clustered in the right quadrants, reflecting higher relative expression of NR1 subunit.

The extent of ipsilateral diaphragm activity at SH 14D (expressed as percent of pre-SH amplitude) was compared to glutamatergic receptor expression for each SH animal (Figure 4). There was a significant correlation between diaphragm activity post-SH and NMDA NR1 subunit CP ($F_{1,22} = 6.19$; $p = 0.021$; slope = 18.6; $r^2 = 0.23$), indicating that animals with greater extent of recovery of diaphragm activity had higher NMDA NR1 subunit expression (corresponding to a higher CP). There was no significant correlation between diaphragm activity and AMPA GluR2 subunit CP ($F_{1,17} = 0.57$; $p = 0.461$; slope = 8.9; $r^2 = 0.03$).

In an additional analysis of glutamatergic receptor expression, animals across all SH experimental groups were grouped by recovery of rhythmic diaphragm EMG activity at SH 14D and compared to control animals. Phrenic motoneuron mRNA expression of NMDA NR1 subunit ($F_{2,28} = 24.91$; $p < 0.001$) and AMPA GluR2 subunit ($F_{2,22} = 32.48$; $p < 0.001$) was affected by SH and by evidence of recovery after SH (Figure 4). Phrenic motoneuron NR1 subunit mRNA expression was significantly greater in both non-recovered and recovered SH animals compared to control. In addition, NR1 subunit expression was greater in SH animals displaying recovery compared to non-recovered animals. Phrenic motoneuron GluR2 subunit mRNA expression was significantly less in both non-recovered and recovered SH animals compared to control. SH animals displaying recovery had significantly greater GluR2 subunit expression compared to non-recovered animals.

Discussion

The results of this study support the importance of glutamatergic receptor expression in recovery of ipsilateral rhythmic diaphragm muscle activity after SH in models of spontaneous and TrkB-mediated enhanced recovery. In animals displaying recovery of diaphragm activity, NMDA NR1 subunit mRNA expression in phrenic motoneurons increased ~4-fold compared to animals in which recovery was not present. Ipsilateral diaphragm activity post-SH was also significantly correlated to NMDA NR1 expression in phrenic motoneurons. In contrast, phrenic motoneuron AMPA GluR2 subunit mRNA expression decreased after SH and albeit increased in animals displaying recovery vs. those not recovering, levels remained lower than control and no correlation to diaphragm activity was evident. We conclude that recovery of diaphragm muscle activity following incomplete cervical SCI is associated with increased phrenic motoneuron expression of glutamatergic NMDA receptors.

Glutamatergic receptor subunit mRNA expression in phrenic motoneurons post-SH

Excitatory bulbospinal (premotor) respiratory drive to phrenic motoneurons is primarily glutamatergic (Greer et al., 1992; McCrimmon et al., 1989; Saji and Miura, 1990). Phrenic motoneurons express multiple excitatory glutamatergic receptor subtypes including ionotropic NMDA and AMPA receptors as well as metabotropic mGluR1 and mGluR5 (Chitravanshi and Sapru, 1996; Dong and Feldman, 1999; Robinson and Ellenberger, 1997). Expression of mRNA for all of these receptors was confirmed in laser capture microdissected phrenic motoneurons (present study and (Mantilla et al., 2012)). Both NMDA and AMPA receptors located in the phrenic motor nucleus play an important role in

neurotransmission of inspiratory drive (Chitravanshi and Sapru, 1996; Pierrefiche et al., 1994; Robinson and Ellenberger, 1997).

In the present study, we found that phrenic motoneuron expression of NMDA NR1 subunit mRNA increased in those animals displaying recovery of diaphragm muscle activity, both spontaneous and enhanced by AAV-mediated increase in TrkB expression, compared to animals that did not display recovery at SH 14D as well as uninjured controls. In addition, there was a significant relationship between diaphragm muscle activity and NMDA NR1 subunit expression, such that animals with higher extent of recovery post-SH had higher NMDA NR1 subunit expression. The NR1 subunit was chosen in the present study because it is mandatory to all NMDA receptors (Paoletti et al., 2013; Traynelis et al., 2010). A contribution of NMDA receptor signaling in phrenic motoneurons to recovery post-SH was also suggested by changes in NMDA receptor mRNA and protein expression in cervical spinal cord homogenates (Alilain and Goshgarian, 2008), by quantitative NMDA receptor mRNA measurements in retrogradely-labeled motoneurons (Mantilla et al., 2012), and by NMDA receptor immunohistochemical staining in putative phrenic motoneurons (Alilain and Goshgarian, 2008).

Similar to NMDA NR1 subunit, we found that phrenic motoneuron expression of AMPA GluR2 subunit mRNA increased in animals displaying recovery compared to animals that did not display recovery at SH 14D. Unlike the NMDA NR1 subunit, there was no relationship between diaphragm muscle activity and AMPA GluR2 subunit expression post-SH. Importantly, GluR2 subunit mRNA expression remained less in all SH groups compared to uninjured control. This is consistent with previous studies where AMPA GluR2 receptor expression decreased in spinal cord at levels below the injury (but not just motoneurons) after SH (Alilain and Goshgarian, 2008). At present, the ability to measure motoneuron excitability in a motoneuron pool *in vivo* remains limited. Nonetheless, it is likely that an increase in NMDA receptor expression at motoneurons, even with a decrease in AMPA receptor, will increase excitability due to longer-duration excitatory postsynaptic currents and slower receptor desensitization kinetics (Rekling et al., 2000).

Differences in the relative distribution of glutamatergic receptors may also contribute to recovery following SCI. For instance, retrogradely-labeled phrenic motoneurons displayed immunoreactivity for the NMDA NR1 subunit at the somata but not dendrites (Robinson and Ellenberger, 1997). In contrast, almost all retrogradely-labeled phrenic motoneurons were immunoreactive for AMPA GluR2 in both the somata and dendrites (Robinson and Ellenberger, 1997). Accordingly, a change of glutamatergic signaling to the motoneuron somata would be expected to contribute to increased motoneuron discharge rates in response to similar synaptic inputs.

Future studies could elucidate changes in phrenic motoneuron expression of other glutamatergic receptor subunits. For instance, expression of post-synaptic NMDA receptors with varying subunit composition also regulates motoneuron excitability (Traynelis et al., 2010). In particular, NMDA NR2 subunits display varying sensitivity to Mg^{2+} blockade as well as deactivation kinetics (Glasgow et al., 2015; Siegler Retchless et al., 2012). Similarly, AMPA receptor function may differ in receptors lacking the GluR2 subunit. Thus, a

systematic assessment of motoneuron glutamatergic receptor expression including various subunit types, and their relative distribution along the motoneuron dendritic tree would further the understanding of the role of glutamatergic signaling to enhanced functional recovery following SCI.

BDNF/TrkB signaling in recovery following SCI

Previous reports indicate that BDNF/TrkB signaling enhances spontaneous recovery post-SH (Gransee et al., 2013; 2015; Mantilla et al., 2013a; Martinez-Galvez et al., 2016) and regulates NMDA receptor expression in other neuronal systems. Brain-derived neurotrophic factor (BDNF) signaling through TrkB plays an important role in recovery following SCI (Bregman et al., 2002; Coumans et al., 2001; Friedman et al., 1995; Gill et al., 2015). Recovery of ipsilateral diaphragm muscle activity after SH was enhanced by delivery of exogenous BDNF via either intrathecal infusion (Mantilla et al., 2013a) or intraspinal transplantation of mesenchymal stem cells engineered to release BDNF (Gransee et al., 2015). Conversely, recovery post-SH was prevented by delivery of a TrkB-Fc fusion-protein to the region of the phrenic motoneuron pool that binds endogenous BDNF. Furthermore, recovery post-SH was blunted by siRNA-mediated down regulation of TrkB receptor expression in phrenic motoneurons (Mantilla et al., 2013a), or selective inhibition of TrkB kinase activity using a chemical-genetic approach in *TrkB^{F616A}* mice (Mantilla et al., 2014). Finally, selective retrograde transduction of phrenic motoneurons using intrapleural AAV7 encoding TrkB enhanced recovery of ipsilateral diaphragm muscle activity post-SH (Gransee et al., 2013; Martinez-Galvez et al., 2016). With the goal of evaluating shared changes in glutamatergic receptor expression between animals with spontaneous and TrkB-mediated enhanced recovery, the present study used the regimen of pre-treatment with AAV-TrkB in order to provide the most robust recovery of diaphragm activity after SH. Our previous study confirmed AAV delivery to phrenic motoneurons using an AAV encoding for both the full-length human TrkB receptor as well as the FLAG protein by: 1) evidence of human TrkB mRNA expression in microdissected phrenic motoneurons and 2) measurements of FLAG protein expression in cervical spinal cord homogenates (Gransee et al., 2013). Tissue procurement for LCM precluded such analyses in the present study, however, the functional effects of AAV-TrkB treatment in the present study replicated those in our previous validation study where AAV-TrkB administered 3 weeks before SH also resulted in recovery of diaphragm activity in 100% of animals at SH 14D (Gransee et al., 2013). In contrast, 67% of animals displayed recovery after AAV-TrkB is administered at 3 days after SH (Martinez-Galvez et al., 2016), consistent with a more delayed increase in motoneuron TrkB expression with the later administration. In further support that the enhancement of spontaneous recovery mediated by AAV-TrkB treatment is due to increased TrkB expression in phrenic motoneurons rather than non-specific effects of AAV treatment, intrapleural injection of AAV-GFP did not enhance recovery. Thus, the present studies optimized assessment of the role of increased BDNF/TrkB signaling at phrenic motoneurons in regulating motoneuron glutamatergic receptor expression.

Regulation of glutamatergic receptor expression by BDNF/TrkB signaling

There is evidence that BDNF/TrkB signaling regulates both pre- and post-synaptic plasticity in various neuronal systems, including the spinal cord, and acts as a bi-directional modulator

of excitatory synaptic transmission (Carvalho et al., 2008). Pre-synaptically, BDNF enables persistent glutamate release during action potentials, thereby enhancing synaptic responses and enabling an increase in docking of synaptic vesicles to the plasma membrane in hippocampal slices (Jovanovic et al., 2000; Tyler and Pozzo-Miller, 2001). Post-synaptically, BDNF/TrkB signaling regulates phosphorylation of NMDA or AMPA receptor subunits contributing to long-term potentiation of synaptic transmission in synaptosomes (Lin et al., 1998; Suen et al., 1997) and hippocampal neurons (Caldeira et al., 2007; Kang and Schuman, 1995; Lessmann et al., 1994). In general agreement, the present study demonstrates that when recovery of diaphragm muscle activity is enhanced by AAV-mediated upregulation of TrkB receptor expression in phrenic motoneurons, both NMDA NR1 subunit and AMPA GluR2 subunit mRNA expression also increase, suggesting an association with increased BDNF/TrkB signaling. Based on these studies, it appears that post-synaptic mechanisms evoked by BDNF/TrkB signaling are sufficient to enhance recovery and may be mediated by regulating excitatory NMDA and AMPA receptor glutamatergic neurotransmission in phrenic motoneurons post-SH.

The specific mechanism(s) underlying how increased TrkB receptor expression regulates glutamatergic receptor expression (NMDA receptor and AMPA receptor) in motoneurons is not presently known and needs to be further investigated. Although BDNF/TrkB signaling may exert acute effects on glutamatergic neurotransmission (e.g., via NMDA receptor phosphorylation (Crozier et al., 2008; Slack and Thompson, 2002)), changes in NMDA receptor mRNA expression (as in the present study) likely reflect long term changes in synaptic efficacy. Whether post-synaptic changes in Ca^{2+} signaling via NMDA receptors (as in hippocampus (Lin et al., 1998) or postnatal spinal cord (Arvanian and Mendell, 2001)) are necessary for the expression of synaptic plasticity and enhanced neurotransmission at phrenic motoneurons is unclear. Furthermore, it is not clear that NMDA receptor upregulation post-SH follows the same mechanism(s) as that induced by AAV-TrkB treatment. Importantly, increased NMDA NR1 subunit mRNA expression in AAV-TrkB treated SH animals is due to increased TrkB rather than effects of AAV, since expression of NR1 was the same in phrenic motoneurons transduced with AAV-GFP compared to those that were not transduced. Regardless, the present study supports manipulating neurotrophic signaling in motoneurons as sufficient to produce sustained enhancement of recovery following incomplete SCI.

Expression of glutamatergic receptor mRNA was assessed using real-time RT-PCR measurements in identified phrenic motoneurons sampled using LCM. This strategy has been used previously to document the time course of changes following SH (Mantilla et al., 2012). After intrapleural AAV-GFP treatment, there is evidence of GFP in 16% of retrogradely-labeled motoneurons (present study and Gransee et al., 2013). In order to ensure inclusion of a sufficiently large number of transduced motoneurons, we sampled as many motoneurons as possible from the ipsilateral spinal cord. On average, 176 ± 13 motoneurons were captured per animal in the present study, representing a large proportion of the total number of phrenic motoneurons present on one side of the cervical spinal cord (224 ± 21 phrenic motoneurons/side in Sprague-Dawley rats; Mantilla et al., 2009). It is worth noting that changes in NMDA and AMPA receptor expression were present in microdissected phrenic motoneurons after AAV-TrkB treatment. Due to the large size of the

TrkB gene, packaging of a large reporter gene (e.g., GFP) was not technically possible; thus, it was not feasible to distinguish which phrenic motoneurons were transduced by AAV-TrkB and selectively sample those motoneurons using LCM. Regardless, even if few motoneurons overexpress human TrkB, they contribute to a robust change in glutamate receptor mRNA expression in the entire pool of motoneurons. These measurements highlight the strength of the approach using LCM compared to other methods that evaluate receptor expression (e.g., western blot or immunofluorescence). Quantitative analyses of mRNA expression in microdissected motoneurons may indeed provide a much more sensitive assessment of viral transduction.

Conclusion

The results of this study show that NMDA receptor-mediated glutamatergic neurotransmission plays an important role in spontaneous recovery after SCI as well as the enhancement of recovery mediated by BDNF/TrkB signaling in phrenic motoneurons. Our results link the recovery of ipsilateral diaphragm muscle activity post-SH to an increase in phrenic motoneuron NMDA NR1 subunit expression that can be enhanced by increasing TrkB signaling in phrenic motoneurons and posit a new role for NMDA receptor neurotransmission in mediating BDNF/TrkB signaling effects on recovery. Understanding the concurrent effects of increased BDNF/TrkB signaling and altered phrenic motoneuron neurotransmitter receptor expression after SCI provides important insight for the identification and development of appropriate pharmacological targets to enhance recovery of diaphragm muscle activity after injury.

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Figure 1.

Ipsilateral diaphragm EMG activity during eupnea post-SH. **A.** Representative raw diaphragm EMG recordings and root mean square (RMS) EMG tracings post-SH. Untreated SH animals (SH; $n = 5$), AAV-GFP treated SH animals (SH + AAV-GFP; $n = 10$), and AAV-TrkB treated SH animals (SH + AAV-TrkB; $n = 9$) were chronically monitored using EMG recordings obtained via implanted diaphragm electrodes. EMG was recorded prior to (Pre-SH) and at 3 days (SH 3D) and 14 days post-SH (SH 14D). **B.** Proportion of animals displaying recovery of ipsilateral diaphragm EMG activity during eupnea post-SH. The criteria for recovery are detailed in Methods section. Diaphragm EMG activity was absent at SH 3D in all animals, confirming complete interruption of descending ipsilateral drive to phrenic motoneurons and diaphragm muscle paralysis. At SH 7D, the proportion of animals recovered was the same across groups ($p = 0.490$). By SH 14D, all of the AAV-TrkB treated SH animals displayed recovery, compared to 4 out of 10 AAV-GFP treated SH animals and 3 out of 5 untreated SH animals (*, $p = 0.027$). **C.** Extent of recovery of ipsilateral diaphragm EMG activity at SH 14D. In untreated SH and AAV-GFP treated SH animals, RMS EMG amplitude was reduced at SH 14D compared to pre-SH. Treatment with AAV-TrkB increased the extent of diaphragm activity post-SH compared to AAV-GFP treated SH (*, $p = 0.004$).

Figure 2.

Real-time RT-PCR was used to quantify the expression of glutamatergic receptors (NMDA NR1 subunit, AMPA GluR2 subunit, mGluR1 and mGluR5) and ribosomal protein S16 (RPS16) in microdissected phrenic motoneurons. **A.** Representative photomicrographs showing phrenic motoneurons retrogradely labeled by intrapleural injection of Alexa 488-conjugated cholera toxin subunit B. **B.** Visual inspection of the LCM cap confirms selective capture of phrenic motoneurons. **C.** Confirmation of complete capture of phrenic motoneurons by lack of Alexa 488 fluorescence in the selected section of spinal cord tissue.

D. In a subset of AAV-GFP treated SH animals, phrenic motoneurons were retrogradely labeled with Alexa 555-conjugated CTB. **E.** Phrenic motoneurons from (D) that also expressed GFP (indicated by arrows) were used to estimate transduction efficiency of AAV-GFP. **F.** Low magnification image of LCM cap shows column of phrenic motoneurons captured from spinal cord sections. Bar, 100 μm in **A–E**; 500 μm in **F**. **G.** Representative amplification curves for each transcript across all experimental groups are shown. Groups include control (CTL), AAV-TrkB treated control (AAV-TrkB), untreated SH (SH), AAV-GFP treated SH (SH + AAV-GFP), and AAV-TrkB treated SH (SH + AAV-TrkB). Quantification of receptor expression was performed using the second derivative method for crossing-point (CP) analyses. Expression level (CP) for each sample and transcript was found by the difference between crossing points for receptor transcript and RPS16.

Figure 3.

Correlation between phrenic motoneuron expression of NMDA NR1 subunit and AMPA GluR2 subunit following SH. Each point represents the Z-score for transcript expression for an individual animal, which was calculated from the mean CP across PCR reactions for that animal and overall mean and standard deviation for all animals from all groups. Filled symbols denote animals that display recovery at any point beyond 3 days post-SH. Correlated changes in expression (increase or decrease) across experimental groups are evident by clustering within a quadrant (right upper quadrant represents increased expression for both transcripts; left lower quadrant, decreased expression). Only the SH, SH + AAV-GFP and SH + AAV-TrkB groups are shown for the sake of clarity (see text).

Figure 4.

Relationship between phrenic motoneuron expression of glutamatergic receptors and diaphragm activity following SH. **A.** There was a significant correlation between diaphragm activity post-SH and NMDA NR1 subunit Δ CP ($p = 0.021$), indicating that animals with greater extent of recovery of diaphragm activity had higher NMDA NR1 subunit mRNA expression (corresponding to a higher Δ CP). There was no significant correlation between diaphragm activity and AMPA GluR2 subunit mRNA expression ($p = 0.461$). Filled symbols denote animals that display recovery at any point beyond 3 days post-SH. **B.** Phrenic motoneuron NMDA NR1 subunit mRNA expression was significantly greater in SH animals compared to control (*, $p < 0.001$), and NMDA NR1 subunit expression was greater in SH

animals displaying recovery (Rec) than in non-recovered animals (Non-Rec; †, $p < 0.001$). Phrenic motoneuron AMPA GluR2 subunit mRNA expression was significantly less in SH animals compared to control. SH animals displaying recovery had significantly greater AMPA GluR2 subunit expression compared to non-recovered animals.

Table 1

Ventilatory parameters during eupnea across experimental groups before and after unilateral cervical spinal cord hemisection at C₂ (SH)

Time	Ventilatory Parameter	Experimental Group		
		SH (n = 5)	SH + AAV-GFP (n = 10)	SH + AAV-TrkB (n = 9)
Pre-SH	Respiratory rate (min ⁻¹)	73 ± 4	78 ± 4	78 ± 5
	Duty cycle (%)	30 ± 3	40 ± 4	33 ± 3
SH 14D	Respiratory rate (min ⁻¹)	80 ± 4	78 ± 4	90 ± 4
	Duty cycle (%)	37 ± 3	40 ± 2	32 ± 2

Data presented as mean ± SE. Duty cycle was calculated as the percent of total time corresponding to inspiration. There were no differences in respiratory rate or duty cycle between experimental groups or time.

Table 2

Distribution of animals used for analyses of glutamatergic receptor expression in control and at 14 days following spinal cord hemisection at C₂ (SH), with or without intrapleural AAV-GFP or AAV-TrkB treatment.

Group	n	Recovery	NMDA NR1		AMPA GluR2		mGluR1		mGluR5	
			Rec	Non	Rec	Non	Rec	Non	Rec	Non
Control	7	-	6	-	5	-	7	-	4	-
AAV-TrkB	5	-	5	-	5	-	3	-	0	-
SH	5	3/5	3	2	3	2	0	2	3	2
SH + AAV-GFP	10	4/10	4	5	2	2	0	0	1	2
SH + AAV-TrkB	9	9/9	9	-	9	-	4	-	3	-

The number of animals (n) in each group is indicated, as well as the number of animals that recovered in SH groups (- : not applicable). Animals were divided into recovered (Rec) and non-recovered (Non) groups based on evidence of rhythmic ipsilateral diaphragm muscle activity (see Methods). For each transcript, the number of animals in which the transcript was detected by RT-PCR is indicated and may differ from the total number of animals in the group (n). In particular, mGluR1 and mGluR5 receptor mRNA expression was not consistently detectable in all animals.

Glutamatergic receptor expression in microdissected phrenic motoneurons in control and at 14 days following spinal cord hemisection at C₂ (SH), with or without intrapleural AAV-GFP or AAV-TrkB treatment.

Table 3

Transcript	Control	AAV-TrkB	SH	SH + AAV-GFP	SH + AAV-TrkB
NMDA NR1 subunit	11.6 ± 0.6	9.6 ± 0.8	7.6 ± 0.4 [*]	8.9 ± 0.4 [*]	6.5 ± 0.4 ^{*,†,‡}
AMPA GluR2 subunit	7.6 ± 0.3	13.4 ± 0.5 [*]	12.5 ± 0.4 [*]	11.6 ± 0.8 ^{*,†}	11.4 ± 0.5 [*]
mGluR1	11.3 ± 3.1	0.8 ± 1.1	18.1 ± 2.1 [†]	-	14.8 ± 1.7
mGluR5	9.9 ± 0.5	-	12.5 ± 0.7	11.2 ± 0.6	10.3 ± 1.6

For each transcript, data are mean ± SE of CP across animals.

^{*} Significantly different than Control;

[†] Significantly different than AAV-TrkB;

[‡] Significantly different than SH + AAV-GFP.