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Respiratory Plasticity Following Spinal Injury: Role of Chloride-Dependent Inhibitory Neurotransmission

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**Title and Subtitle:** Respiratory Plasticity Following Spinal Injury: Role of Chloride-Dependent Inhibitory Neurotransmission

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**Abstract:**
Our fundamental goal was to test the hypothesis that spontaneous and induced plasticity in chloride-dependent synaptic inhibition of phrenic motor neurons contributes to functional recovery from chronic cervical spinal contusion (CSC) injuries. We performed experiments to determine if CSC and repetitive acute intermittent hypoxia (rAIH) shift the NKCC1/KCC2 balance in phrenic motor neurons, degrading (CSC) and restoring (rAIH) chloride-dependent synaptic inhibition, and performed experiments to determine if spinal PKCζ plays a role in spontaneous recovery of breathing following CSC. Our analyses indicate that CSC increases membrane NKCC1, and decreases the membrane-cytosol KCC2 ratio within phrenic motor neurons, consistent with the interpretation that compensatory shifts in NKCC1/KCC2 balance in phrenic motor neurons preserves respiratory function following CSC. rAIH had no apparent effects on either protein, although the timing of rAIH delivery may not have been optimal based on newer information. We obtained conflicting results regarding the role of spinal PKCζ activity in spontaneous recovery of diaphragm EMG and breathing capacity within the first 3 days following injury; histological analysis underway will confirm if variations in the extent of injury are associated with these conflicting results.

**Keywords:** Spinal Injury, Treatment, Intermittent hypoxia, rats, spontaneous recovery, induced recovery, rAIH, PKCζ, TrkB, contusion
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>7</td>
</tr>
<tr>
<td>Conclusion</td>
<td>7</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>None</td>
</tr>
<tr>
<td>References</td>
<td>8</td>
</tr>
<tr>
<td>Appendices</td>
<td>None</td>
</tr>
</tbody>
</table>
INTRODUCTION
Our fundamental goal was to test the hypothesis that spontaneous and induced plasticity in chloride-dependent synaptic inhibition of phrenic motor neurons contributes to functional recovery from chronic cervical spinal contusion (CSC) injuries. In this project, we tested the specific hypothesis that CSC and repetitive acute intermittent hypoxia (rAIH) shift the NKCC1/KCC2 balance in phrenic motor neurons, degrading (CSC) and restoring (rAIH) chloride-dependent synaptic inhibition.

SPECIFIC AIMS
Aim 1: Test the hypothesis that midline C4 cervical spinal contusions (CSC) degrade chloride-dependent inhibitory synaptic transmission in phrenic motor neurons by an atypical PKC-dependent mechanism, contributing to spontaneous functional recovery of breathing capacity.
   a. Does CSC differentially alter membrane expression of the chloride co-transporters NKCC1 and KCC2 in the phrenic motor nucleus?
   b. Does CSC decrease GABA\(\text{A}\) receptor-induced inhibition of phrenic motor output?
   c. Is CSC-induced attenuation of chloride-dependent synaptic inhibition PKC\(\text{\varepsilon}\) dependent?
   d. Is spinal PKC\(\text{\varepsilon}\) required for spontaneous recovery of breathing capacity following CSC?

Aim 2: Test the hypothesis that repetitive acute intermittent hypoxia (rAIH) normalizes chloride-dependent inhibitory synaptic transmission in phrenic motor neurons by a TrkB-dependent mechanism.
   a. Does rAIH normalize membrane NKCC1/KCC2 expression in the phrenic motor nucleus?
   b. Does rAIH normalize GABA\(\text{A}\) receptor-induced inhibition of phrenic motor output?
   c. Does rAIH-induced normalization of chloride-dependent synaptic inhibition require TrkB activity?

OVERALL PROJECT SUMMARY
Specific Aims 1a and 2a: rAIH (repetitive acute intermittent hypoxia) normalizes shifts in membrane NKCC1 and KCC2 in the phrenic motor pool caused by C4 cervical spinal contusions (CSC).

Task 1: Complete administrative requirements (ACURO)
   Current Status: Completed.

Task 2: Quantify changes in membrane NKCC1 and KCC2 in the phrenic motor nucleus following cervical contusion injuries in rats exposed to rAIH or room air.
   Subtask 2a. Perform spinal injuries and sham surgeries
   Current Status: Completed
   Subtask 2c. Perfuse subset of rats from subtask 2b 5 wks post-surgery and quantify changes in NKCC1/KCC2 using immunofluorescence.
   Current Status: Completed
   Subtask 2d. Harvest spinal cords from a subset of rats from subtask 2b 5 wks post-surgery to quantify NKCC1/KCC2 with surface biotinylation and immunoblots.
   Current Status: Completed. Results summarized below.

Sham surgeries and left lateral C3 contusion injuries (CSC; 135 kD) were successfully performed on 24 rats; these rats were divided into the following groups: a) normoxia + sham surgery; b) normoxia + CSC; c) rAIH + sham surgery; and d) rAIH + CSC. rAIH was initiated one week-post CSC, and consisted of 10 AIH episodes per day, 3x per week for 4 weeks). After immunofluorescence for KCC1/NKCC2, confocal z-stacks of cholera-toxin back-labeled phrenic motor neurons were made ipsilateral and contralateral to injury. A semi-automated quantification algorithm implemented in MATLAB (developed “in house”) was used to assess membrane and cytosolic NKCC1 and KCC2 immunofluorescence in Cholera toxin B fragment labeled phrenic motor neurons. A three-way MANOVA and Tukey's post hoc test were performed. We found: a) NKCC1 increased in cell membranes and decreased in the cytosol of phrenic motor neurons after CSC, both ipsi- and contralateral to injury (p<0.05), b) membrane KCC2 non-significantly decreased following injury (p = 0.07); membrane to cytosolic ratio decreased post-CSC (p<0.05). rAIH had no significant effects on NKCC1 or KCC2 (p>0.05). Collectively, these data are consistent with the hypothesis that inhibitory neurotransmission is decreased following CSC. Although the findings per rAIH were clear and negative, new information leads us to suspect
that the timing of rAIH exposures (post-injury) was not optimal. Thus, we leave open the possibility of such effects in rats with more chronic spinal injuries, once serotonergic innervation of the phrenic motor nucleus has been restored. Such new experiments were not possible within the time frame or budget of this award.

**Specific Aims 1b and 2b**: rAIH restores CSC-induced loss of GABA<sub>A</sub> receptor-induced inhibition of phrenic motor output.

**Task 3**: Quantify GABA<sub>A</sub> receptor-induced inhibition of phrenic motor output with pressure microinjections of muscimol following cervical injuries in rats exposed to rAIH or room air.

- **Subtask 3a**: Perform cervical contusion injuries and sham surgeries.  
  *Current Status*: Completed.
- **Subtask 3b**: Expose rats to rAIH or room air beginning 1-week post-surgery.  
  *Current Status*: Completed.
- **Subtask 3c**: Quantify phrenic responses to pressure microinjections of muscimol into phrenic nucleus 5 wks post-surgery.  
  *Current Status*: Completed. Results summarized below.

Rats were urethane-anesthetized, ventilated, paralyzed and the phrenic nerve isolated for recording. Muscimol (GABA<sub>A</sub> receptor agonist) was injected into the intrathecal space of the cervical spinal cord to assess the potency of chloride-dependent synaptic inhibition in rats 5 weeks post- CSC or sham surgery, with and without rAIH (n=8-9 per group, 4 groups; 34 rats total; Figure 2). Contrary to our hypothesis, CSC may have actually enhanced inhibitory neurotransmission, and rAIH had no additional affect. We question whether the technique used (i.e., intrathecal injections) were sensitive enough to detect differences in inhibitory neurotransmission in the phrenic motor pool.

**Figure 1**: Immunofluorescence of NKCC1 (red), KCC2 (green) in C4 phrenic motor neurons in normoxia and rAIH treated rats with and without CSC. All micrographs are at 100X magnification. KCC2 is highly localized on phrenic motor neuron (indicated by small white asterisk; CtB was turned off in these micrographs to emphasize target proteins) membranes. CSC reduced KCC2 membrane expression, which was marginally significantly different from sham rats (p=0.07). NKCC1 significantly increased in the membrane, and decreased in the cytosol, following CSC (p<0.05). rAIH had no additional effect on KCC2 or NKCC1.

**Figure 2**: Phrenic burst amplitude ipsilateral to sham or CSC injury 60 min following intrathecal muscimol (7.5 mM). In all groups, phrenic burst amplitude decreases following muscimol injections, suggesting strong GABA<sub>A</sub> receptor-mediated inhibition. Rats receiving CSC had a increased phrenic responses to muscimol, suggesting that CSC enhances inhibitory neurotransmission, which is contrary to our hypothesis.
Subtask 2d: Histological verification of injury for all rats in task 3.

Current Status: Analysis underway. We will histologically describe the CSC injury in rats studied in Subtask 2b and 2c to determine if variability in the extent of injury accounts for variability in NKCC1/KCC2 expression and the electrophysiological response to GABA\textsubscript{A} agonists.

Task 4: Analyze data and draft manuscript describing the effect of C4 cervical spinal contusions (CSC) on NKCC1/KCC2 balance and GABAA receptor-induced inhibition of phrenic motor output.

Current Status: Data analyzed, manuscript in preparation for submission to peer reviewed journal.

Specific Aim 1c: CSC-induced attenuation of chloride-dependent synaptic inhibition requires PKC\(\text{\textsubscript{\text{\v{z}}}}\).

Task 5: Quantify changes in membrane NKCC1 and KCC2 after CSC in rats with and without PKC\(\text{\textsubscript{\text{\v{z}}}}\) knockdown in phrenic motor neurons.

Current Status: Since CSC did not attenuate chloride-dependent synaptic inhibition (Task 3, subtask 3c, Figure 2), we did not complete this task.

Task 6: Quantify GABAA receptor-induced inhibition of phrenic motor output following cervical contusion injuries in rats with PKC\(\text{\textsubscript{\text{\v{z}}}}\) knockdown in phrenic motor neurons.

Current Status: Since CSC did not attenuate chloride-dependent synaptic inhibition (Task 3, subtask 3c, Figure 2), we did not complete this task.

Task 7: Quantify ventilation following cervical contusion in rats with PKC\(\text{\textsubscript{\text{\v{z}}}}\) knockdown in phrenic motor neurons.

Subtask 7a: Begin intrapleural siRNA injections 3 days prior to surgery.

Current Status: Completed

Subtask 7b: Perform contusion injuries and sham surgeries.

Current Status: Completed

Subtask 7c: Perform plethysmography 3 days post-CSC

Current Status: Completed. Results summarized below.

We investigated the impact of spinal PKC\(\text{\textsubscript{\text{\v{z}}}}\) down-regulation on spontaneous recovery of diaphragm activity following CSC. Six rats were implanted with radio-telemetric electrodes in the right and left hemidiaphragm to measure diaphragm EMG activity before and 3 days following CSC in rats receiving intrapleural injections of non-targeting siRNAs or siRNAs targeting PKC\(\text{\textsubscript{\text{\v{z}}}}\). Tidal volume and breathing frequency were also measured in a plethysmograph before and 3 days following CSC in rats receiving intrapleural injections of non-targeting siRNAs or siRNAs targeting PKC\(\text{\textsubscript{\text{\v{z}}}}\) (Figures 3 & 4). Preliminary data suggest that during maximum chemoreceptor stimulation: 1) a spontaneous recovery of diaphragm EMG activity is apparent within 3 days post-CSC (n=3), 2) spinal PKC\(\text{\textsubscript{\text{\v{z}}}}\) downregulation blocks spontaneous diaphragm recovery post-CSC (n=2).

Figure 3. Diaphragm EMG activity under maximum chemoreceptor stimulation 3 days following cervical spinal contusion (CSC) in rats receiving control injections of a non-targeting siRNA (NTsiRNA) or siRNAs targeting PKC\(\text{\textsubscript{\text{\v{z}}}}\) (siPKC\(\text{\textsubscript{\text{\v{z}}}}\)). Rats injected with NTsiRNA exhibited a full recovery of diaphragm EMG activity 3 days following CSC (n=3). Rats injected with siPKC\(\text{\textsubscript{\text{\v{z}}}}\) exhibited impaired diaphragm EMG activity 3 days following CSC (n=2), suggesting that spontaneous recovery of diaphragm activity post-CSC requires spinal PKC\(\text{\textsubscript{\text{\v{z}}}}\).

Figure 4. Tidal volume in normoxia 3 days following cervical spinal contusion (CSC) in rats receiving control injections of a non-targeting siRNA (NTsiRNA) or siRNAs targeting PKC\(\text{\textsubscript{\text{\v{z}}}}\) (siPKC\(\text{\textsubscript{\text{\v{z}}}}\)). Rats injected with NTsiRNA exhibited a full recovery of tidal volume 3 days following CSC (n=3). Rats injected with siPKC\(\text{\textsubscript{\text{\v{z}}}}\) exhibited impaired tidal volume 3 days following CSC (n=2), suggesting that spontaneous recovery of breathing post-CSC requires spinal PKC\(\text{\textsubscript{\text{\v{z}}}}\).
Given these promising results, we performed a second series of injuries with and without spinal PKCζ knockdown. To our surprise, the results we obtained were opposite to the first series. We measured breathing (using plethysmography) and diaphragm EMG activity in 5 additional rats 1-3 days following CSC with and without PKCζ inhibition (2 rats with CSC and 3 rats with CSC + PKCζ inhibition). In one of our sham rats, the diaphragm EMG battery failed prior to the final data collection, and we were not be able to include responses from this rat in data analysis. In these groups, midline CSC impaired diaphragm EMG activity (~80% reduction from pre-injury levels, bilaterally). However, to our surprise, inhibition of PKCζ actually improved diaphragm EMG activity following CSC (~130% from pre-injury levels). We are currently documenting the extent of the injury and will evaluate our data at that time. We suspect that variable CSC injuries could have caused the conflicting results observed in series 1 and 2.

Subtask 7d: Histological verification of injury for all rats in task 7.
Current Status: Analysis underway.

Task 8: Draft manuscript concerning role of PKCζ in spontaneous shifts in NKCC1/KCC2 balance, degraded synaptic inhibition of phrenic motor output and spontaneous functional recovery of breathing following cervical contusions.
Current Status: Awaiting final results from histological analysis

Specific Aim 2c: Test the hypothesis that rAIH-induced normalization of chloride-dependent synaptic inhibition requires TrkB activity.
Current Status: Since rAIH had no effect on NKCC1/KCC2 balance or inhibitory neurotransmission (Task 3, Figures 1 and 2), we did not complete this task.

KEY ACCOMPLISHMENTS TO DATE
Our work resulted in two major findings. First, similar to lumbar motor neurons (Boulenguez et al., 2010; Cramer et al., 2008), NKCC1/KCC2 balance in phrenic motor neurons shifts following spinal injury in way expected to enhance cell excitability. Unfortunately, electrophysiological analysis did not support degraded inhibitory neurotransmission and, in fact, suggested the opposite; we suspect that this result is due to delivering the GABA_A agonist intrathecally versus directly into the phrenic motor pool. Although our hypothesis that rAIH normalizes inhibitory neurotransmission (while maintaining enhanced excitatory neurotransmission) was not verified, we suspect that the timing of rAIH treatment in our studies was not optimal. Indeed, recent data from the Mitchell laboratory suggests that in the weeks following spinal injury, plasticity following rAIH shifts from a predominately adenosinergic to a predominately serotonergic mechanism, with the mid-point in this transition between weeks 2 and 6. Thus, in future studies, rAIH treatment should be delayed to better correspond with the time of maximal serotonergic function. Regardless, our data are consistent with the hypothesis that following spinal injury, an endogenous compensatory mechanism is elicited in phrenic motor neurons that may act to preserve respiratory function.

A second major finding is that spinal PKCζ activity may underlie the spontaneous recovery of diaphragm EMG activity and breathing capacity that naturally develops in some human patients and rodent models following spinal cord injury (Goshgarian, 2003; Raineteau and Schwab, 2001). Indeed, we found that rats with a knockdown of spinal PKCζ lacked the normal compensatory increases in diaphragm EMG activity and breathing capacity following CSC; unfortunately, these results were not upheld when we attempted to repeat this finding. We are currently documenting the extent of injury to determine if variable severity of injury might account for these conflicting responses.

Our work on this project has resulted in two abstracts (listed below), and a manuscript describing the impact of CSC on NKCC1/KCC2 balance is currently being drafted.

PROBLEMS
Dr. Mitchell’s departure from the University of Wisconsin to the University of Florida put us behind schedule last year, leading us to request the no cost extension.

CONCLUSIONS
We made good progress in accordance with our experimental plan, although we experienced delays related to Dr. Mitchell’s move to University of Florida and unexpected experimental findings. We completed our analyses
of GABA receptor-induced phrenic inhibition and phrenic motor neuron NKCC1/KCC2 expression following CSC. rAIH had little, if any, impact on our results. This lack of effect may be due to the timing of rAIH administration due to recent discoveries in the Mitchell laboratory pertaining to shifts in the mechanism of rAIH effects on phrenic motor neurons with time post cervical spinal injury. We also obtained conflicting data suggesting that spinal PKCζ activity is necessary for spontaneous recovery of phrenic motor output post-CSC; we are currently determining if variability in injury severity can account for these conflicting findings. These results may be significant for patients with spinal injury since they suggest a new cellular target for future therapeutic development (PKCζ). Our research directly targets the goal of improving breathing capacity and/or coordination in patients with cervical SCI.

PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:


INVENTIONS, PATENTS AND LICENSES: None

REPORTABLE OUTCOMES: None, pending completion of our studies.

OTHER ACHIEVEMENTS: None

REFERENCES:


APPENDICES: None