2013

Mapping of the Spinal Circuitry Associated with Paw Withdrawal Learning in Spinal Mice

Sophia Raefsky
sraefsky@pugetsound.edu

Mapping of the Spinal Circuitry Associated with Paw Withdrawal Learning in Spinal Mice

Sophia Raefsky

Abstract

The overall goal of this project was to identify the neural circuitry involved in paw withdrawal learning (PaWL) in complete spinal cord transected (ST) mice. Pseudorabies virus (PRV)-Bartha 152 was injected into the tibialis anterior (TA), the primary muscle involved in this learning. The use of PRV, a transsynaptic retrograde marker, allowed labeling of the TA motoneurons and its associated interneurons in the spinal cord. By combining PRV-Bartha 152 with c-fos (an activity-dependent marker) and CaMKII (a learning-associated marker), the activated motoneurons and interneurons that were associated with spinal learning were identified. Of all PRV+ labeled neurons, 21% were motoneurons and found only on the ipsilateral side of the spinal cord (same side where learning occurred). Sixty-five percent of the labeled interneurons were found on the ipsilateral side of the spinal cord and 14% were found on the contralateral side. A majority (~60%) of the interneurons and motoneurons on the ipsilateral side were activated during PaWL. Moreover, activated PRV+ interneurons that were also positive for CaMKII were mostly located in laminae VI-VII suggesting that the neural circuitry involved in PaWL occurred in these regions.

Introduction

The spinal cord has the ability to learn complex motor tasks in the absence of supraspinal input (Edgerton et al., 2004, 2008). The neuronal circuitry associated with such learning in the spinal cord, however, remains largely unidentified. By creating anatomical maps of functionally connected neurons it may be possible to gain an understanding of the pathways involved in spinal learning. Identification of these pathways can be utilized for new rehabilitative strategies, such as epidural stimulation and pharmacological treatments that could help patients regain motor function after a spinal cord injury.

In an attempt to understand the neural circuitry involved in motor tasks after spinal cord injury, we used the paw withdrawal learning (PaWL) paradigm in the hindlimbs of mice (Jindrich et al. 2009). The PaWL paradigm was first used in spinal rats (Grau et al.1998) to demonstrate learning in the spinal cord and since has been adapted for spinal mice (Jindrich et al., 2009). The master mice that received a shock in the hind leg when the leg was extended (contingent shock) learned to maintain the shocked leg in a flexed position that minimized net shock exposure. This learning was not observed in the yoked mice, who received a shock independent of their limb position.

The purpose of my project was to map the spinal circuitry involved in paw withdrawal learning in spinal cord transected mice. By using pseudorabies virus (PRV) Bartha 152, a transsynaptic retrograde marker, injected into the tibialis anterior (TA) muscle, the motoneurons and first order interneurons associated with the TA were labeled. By using c-fos, an activity dependent marker, the activated neurons were labeled. Calmodulin dependent protein kinase II (CaMKII), a marker of early learning, was used to label neurons that had learned. The activation of CaMKII induces long-term potentiation, an activity dependent strengthening of synapses believed to be an electrophysiological correlate of learning and memory (Vaynman et al. 2007). Identifying the neurons involved in learning in the spinal cord is important because it shows that the spinal circuitry is capable of re-learning certain tasks, such as flexion of the TA muscle.

The location of the neurons involved in muscle training allowed us to identify the potential neural circuitry involved in such re-learning.

Materials and Methods

Animals, ST surgery, and viral injection: Adult female Swiss Webster (n=3, master mice) were injected with 10 µl of PRV-152 from a $1.24x10^9$pfu/ml viral stock (total virus=$1.24x10^7$) using a 10 µl Hamilton syringe with an attached 30 gauge needle. PRV was injected at 5 sites (2 µl/site) in the TA muscle in order to achieve even distribution of the virus and to allow for transsynaptic retrograde transport into the spinal cord.

PaWL paradigm in spinal mice: The experimental set up used fine-wire intramuscular stimulating electrodes, a real-time video tracking system, and DC current stimulation. If the foot position of the mouse was below the 1 mm pre-determined threshold, fine-wire electrodes delivered a shock unilaterally to the TA muscle. Learning involved ankle dorsiflexion to hold the foot above the 1 mm threshold. Learning was measured behaviorally by an increase in response duration of holding the foot above the threshold line. Cameras monitored the foot position of the master and yoked mice. Only master mice were analyzed for this study. Point tracking information from the master mice was collected by one computer and was used to trigger the shock for both the master and yoked mice. The LabVIEW (Austin, TX) software collected vertical and horizontal foot position from the master mice, including the threshold level, and the number of times the master mice were stimulated (shocked).

Immunohistochemistry: The spinal cord from all mice used in the study were cut at 30-µm on a cryostat at -20°C and stored in 96-well plates containing 1X phosphate buffer saline (PBS). L4 segments were selected and analyzed because they contain the TA motoneurons. Antibodies specific to GFP (to identify GFP-PRV+ neurons), c-fos (to identify activated neurons), and CaMKII (to identify neurons associated with learning) were used for immunohistochemical analyses.

Twelve sections from each mouse were analyzed by immunohistochemistry. Spinal cord sections were rinsed in 1X PBS (2x30 seconds, 2x5 minute and 2x10 minute) to remove any impurities. The sections were then incubated in a mixture of anti-chicken GFP (1:1000), anti-rabbit c-fos (1:400), and mouse anti-CaMKII (1:500) primary antibodies in 1X PBS/0.3% triton containing 3% normal donkey serum (NDS). The sections were incubated overnight at 4° C on a shaker for 16-18 hours. Sections were rinsed in 1X PBS as described above to remove excess primary antibodies and then incubated for 60 min in a mixture of secondary antibodies against Alexa fluor anti-chicken 488 (1:500), anti-rabbit biotin (1:200), and anti-mouse biotin (1:200). Sections were again washed with 1X PBS as described above and incubated in 1X PBS/0.3% triton/3% NDS for 1 hr in AMCA strepavidin (1:200) to amplify the signal and to label CaMKII protein in blue. Following a final wash in 1X PBS sections were mounted on Fisher Super Frost Plus slides, covered with glass coverslips and sealed with clear nail polish. Sections were covered in foil during the secondary antibody and AMCA-strep

incubation steps. The slides were stored in covered slide boxes to reduce the bleaching of the fluorescent labeling and were kept in a refrigerator when not in use.

Image Analysis: The labeled spinal cord tissues were examined under epiluminescence using a Zeiss Axiophot microscope with three fluorescent filters (red-Texas red filter, green-FITC filter and blue-UV filter). Digital microscopic images were acquired using the Image Pro 7 (Media Cybernetics Inc.) software with each of the three filters separately. A composite feature in the Image Pro 7 was used to overlay individual images for each filter and composite images were created. Using the manual tag feature of Image Pro, specific labeled neurons were identified. The labeling of interneurons and motoneurons were under the following three categories: 1) PRV+ only, 2) PRV+ and c-fos+, and 3) PRV+ and c-fos+ and CaMKII+. The saved tag files of individual sections were overlaid in their respective spinal cord outlines and placed in L4 spinal cord templates with lamina drawn (Spinal Cord Atlas, Watson et al 2009).

Results

PaWL learning: All three master mice learned to hold their paw in a flexed position to minimize shock with total response durations of 15, 21 and 24 min, respectively (Fig. 1).

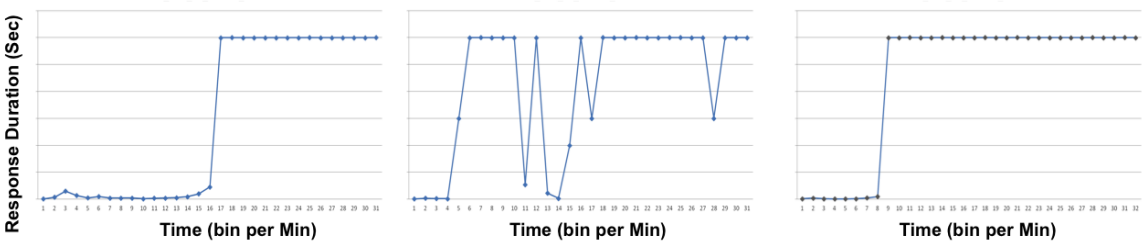

Figure 1. Mice learned to hold their paw in a flexed position to avoid shocks to the TA The three graphs show the response duration times for the 3 master mice analyzed. The master mice were given a shock to their TA muscle whenever their paw dropped below the threshold line of 1mm above their resting paw position. The three mice learned to keep their paw in a dorsiflexed position during the test, holding their paw up for 15, 21, and 24 minutes, respectively.

Labeling of the spinal cord sections: Of all the PRV+ labeled neurons, 21% were motoneurons and found only on the ipsilateral side of the spinal cord (same side where learning occurred). Sixty-five percent of the labeled interneurons were found on the ipsilateral side of the spinal cord and 14% were found on the contralateral side. A majority (60%) of the PRV+ labeled interneurons on both the ipsilateral and contralateral side of the spinal cord were activated. About 30% of the motorneurons on the ipsilateral side were PRV+/c-fos+. There were no motorneurons labeled on the contralateral side. A majority of the interneurons on the ipsilateral side were either PRV+/c-fos+ or PRV+/ CaMKII+, whereas only around 40% were either PRV+/c-fos+ or PRV+/ CaMKII+ on the contralateral side. Approximately 30% of the motorneurons on the ipsilateral side were PRV+/c-fos+ or PRV+/CaMKII+ (Fig. 2).

Twelve spinal cord sections from one animal were analyzed in more detail. PRV+ interneurons were found in ipsilateral laminae I, II, IV-VI of the dorsal horn, VII and IX of the ventral horn, lamina X, lateral spinal nucleus (LSP) and white matter. PRV+ interneurons were found in contralateral laminae V and VI of the dorsal horn, VII and VIII of the ventral horn, and white matter. PRV+/c-fos+ (activated) interneurons were found in ipsilateral laminae I, II, IV-VI of the dorsal horn, VII of the ventral horn, lamina X, LSP and white matter. The majority of PRV+ neurons (50-100%) in these areas were activated, except in lamina VII and IX (Fig. 3).

By looking at the spinal cord template, the tripled labeled neurons (PRV+/c-fos+/CaMKII+) were located in laminae regions IV, V, VI, VII, VIII, and X. The majority of the tripled labeled neurons were located at laminae IV-VII on the ipsilateral side of the spinal cord (Fig. 4).

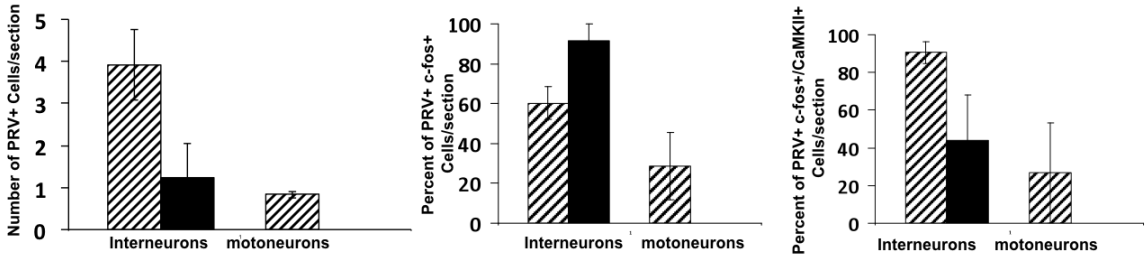

Figure 2. A majority of the PRV+ interneurons were activated
The first graph shows the majority of activated neurons per section. We found that each spinal cord section had about 4 activated neurons on the ipsilateral side and 1 activated neuron on the contralateral side. There was an average of 1 activated motoneuron on the ipsilateral side per section, and no activated motoneurons on the contralateral side. The second graph shows that out of all the neurons per section, about 60% were activated on the ipsilateral side and 90% were activated on the contralateral side. Around 30% of the labeled motoneurons were activated on the ipsilateral side. The third graph shows that around 90% of the neurons on the ipsilateral side were PRV+ and cfos+ or PRV+ and CaMKII+ and around 40% of the neurons on the contralateral side were PRV+ and cfos+ or PRV+ and CaMKII+. Around 30% of the motoneurons on the ipsilateral side were PRV+ and cfos+ or PRV+ and CaMKII+.

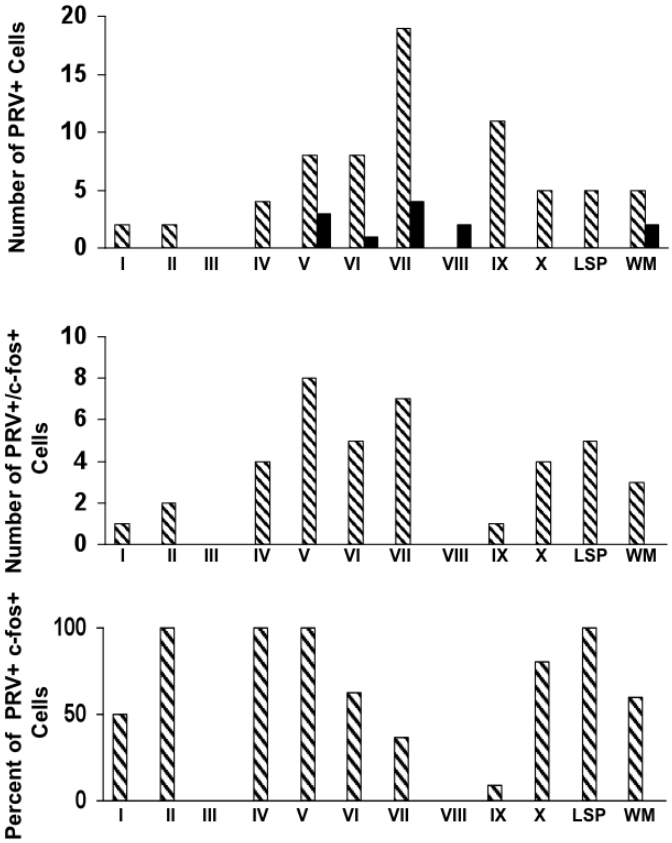

Figure 3. PRV+ interneuron activation based on lamina
We analyzed one mouse in more detail (12 spinal cord sections). The bars with the slanted lines represent the ipsilateral side of the spinal cord and the solid black bars represent the contralateral side of the spinal cord. The first graph shows the distribution of PRV+ interneurons based on lamina in the spinal cord. There were more PRV+ interneurons on the ipsilateral side than the contralateral side in every lamina. The second graph shows the number of activated interneurons per lamina. There are no activated neurons on the contralateral side. The third graph shows the percent of activated interneurons per lamina. Laminae II, IV, V, VI, X, lateral spinal nucleus (LSP), and white matter (WM) all had a majority of their interneurons activated.

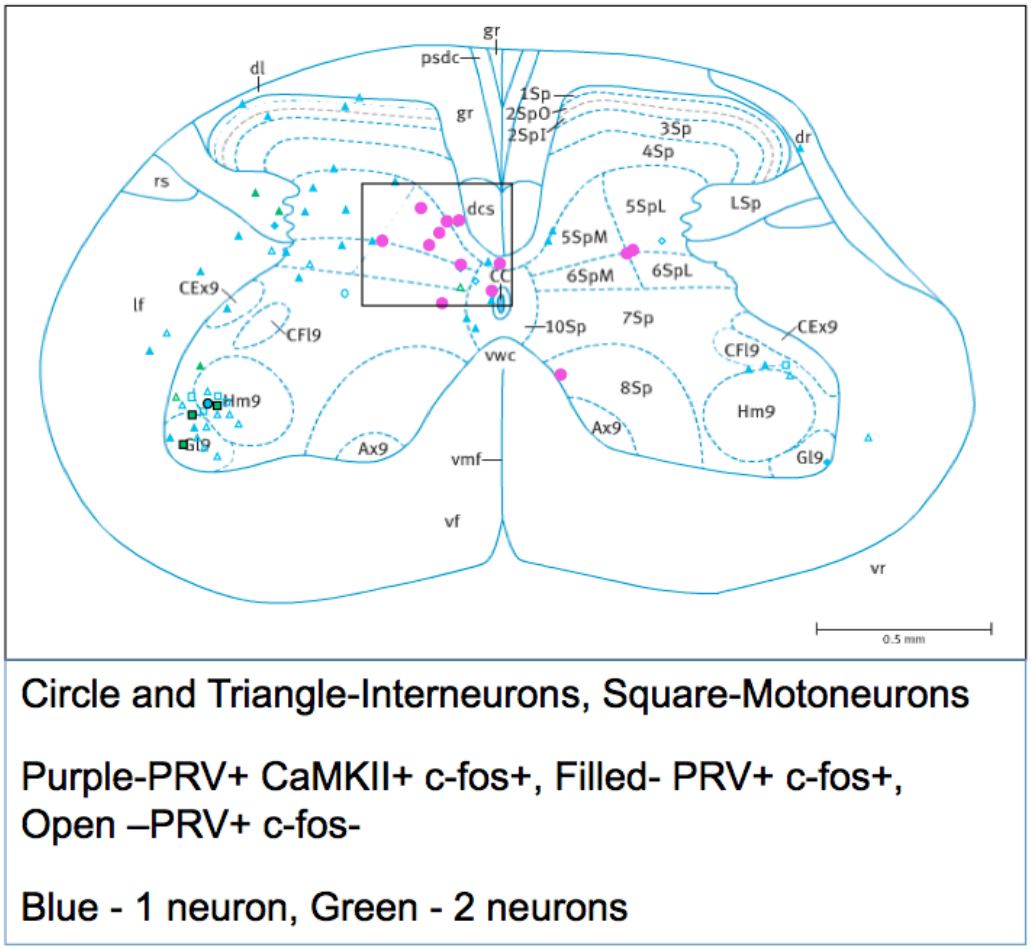

Figure 4. Lamina distribution of labeled neurons on a spinal cord template
This figure shows the outline of a L4 mouse spinal cord. The purple dots refer to the tripled labeled interneurons (PRV+/c-fos+/CaMKII+). The neurons are located in laminae IV, V, VI, VII, VIII, and X. The box represents the area where a majority of the triple labeled internurons are located.

Discussion

CaMKII is involved in the process of early learning and has been known to induce long term potentiation, which is an activity dependent strengthening of synapses that is known to be correlated to learning and memory (Vaynman et al. 2007). In this study, we not only identified neurons with CaMKII, but also labeled the neurons that were activated (c-fos) and linked to the TA muscle (PRV). Therefore, the triple labeled neurons represented those neurons that were most likely part of the neural circuitry involved in the flexion that occurred during PaWL training. These triple labeled neurons were mostly located in the dorsal horn on the ipsilateral side of the spinal cord. Because a majority of the CaMKII interneurons were found more medially in laminae IV-VII (dorsal horn), these laminae could be a potential location of where learning occurs, at least for the TA muscle. A majority of the CaMKII labeled neurons were located on the ipsilateral side of the spinal cord or the side where the PaWL training took place. These findings

demonstrated that most learning occurred on the side of the spinal cord where the muscle was activated. In conclusion, we can use the locations and patterns of triple labeled neurons to identify potential neural circuitry involved in muscle re-learning in spinal cord transected mice.

Future Directions

In the future, similar studies can replicate this study by analyzing more mice to further confirm and specify the neural circuitry with greater accuracy. Neurons can also be further classified using immunohistochemistry for other learning-associated markers such as brain derived growth factor (BDNF), CREB, and AMPA receptors. Additional neuronal markers such as GABAergic (GAD_{67}), cholinergic (ChAT) and Renshaw cells (calbindin) can be used to further classify which neurotransmitters are involved in certain tasks. This additional information can further elucidate our understanding of how learning occurs in the spinal cord and provide potential targets for designing rehabilitative strategies.

References

1) Jindrich DL, Joseph MS, Otoshi CK, Wei RY, Zhong H, Roy RR, Tillakaratne NJ, Edgerton VR. (2009) Spinal learning in the adult mouse using the Horridge paradigm. *J Neurosci Methods* 182:250-4.

2) Jovanovic K, Pastor AM, O'Donovan MJ. (2010) The use of PRV-Bartha to define premotor inputs to lumbar motor neurons in the neonatal spinal cord of the mouse. *PLoS ONE* 5: e11743.

3) Grau JW, Barstow DG, Joynes RL. (1998) Instrumental learning within the spinal cord: I. Behavioral properties. *Behav Neurosci.* 112:1366-86.

4) Edgerton VR, Tillakaratne NJ, Bigbee AJ, de Leon RD, Roy RR. (2004) Plasticity of the spinal neural circuitry after injury. *Annu Rev Neurosci.* 27:145-67.

5) Edgerton VR, Roy RR. (2008) Robotic training and spinal cord plasticity. *Brain Res Bull* 78:4-12.

6) Vaynman S, Ying Z, Gomez-Pinilla F. (2007) The select action of hippocampal calcium calmodulin protein kinase II in mediating exercise-enhanced cognitive function. *Neuroscience* 144 (3): 825-33.