**Title**

Cholinergic interneurons around the central canal in adult intact and spinal cord transected rats are activated in response to load-bearing stepping

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**Cholinergic interneurons around the central canal in adult intact and spinal cord transected**

**rats are activated in response to load-bearing stepping**

A thesis submitted in partial satisfaction

of the requirement for the degree Master of  Science

in Physiological Science

by

Paul Okwudiri Duru

2013

**ABSTRACT OF THE THESIS**

**Cholinergic interneurons around the central canal in adult intact and spinal cord transected**

**rats are activated in response to load-bearing stepping**

by

Paul Okwudiri Duru

Master of Science in Physiological Sciences

University of California, Los Angeles, 2013

Professor V. Reggie Edgerton, Chair

Sensory afferents to the spinal cord is the main source for therapeutic intervention and rehabilitation that can generate and coordinate complex movements even without supraspinal control. Using the activity-dependent marker c-fos, we identified cholinergic lumbar interneurons around the central canal that are activated during load bearing stepping in intact and complete spinal cord transected adult rats. We observed activation of cholinergic central canal cluster and partition neurons in intact rats after quadrapedal stepping and in spinal rats step trained bipedally for 5 weeks with epidural and pharmacological stimulation. Furthermore, increased load imposed during treadmill stepping activated more cholinergic interneurons in adult rats. Intact adult mice whose tibialis anterior (TA) hindlimb muscle was injected with transynaptic pseudorabies virus, showed activated cholinergic interneurons in the TA spinal circuitry after quadrapedal stepping. These data demonstrate the role of sensorimotor pathways and specific interneurons in the recovery after spinal cord injury.

The thesis of Paul Okwudiri Duru is approved.

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Fernando Gomez-Pinilla

David Glanzman

V. Reggie Edgerton, Chair

University of California, Los Angeles

2013

## DEDICATION


This thesis is dedicated to members of my family who have always supported me in pursuing my

educational goals.  I would like to also dedicate this thesis to the friends in my life that will

continue to give a shoulder to lean on in the upcoming years.

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**ACKNOWLEDGEMENTS**

**INTRODUCTION**

Spinal cord injury (SCI) affects many young and healthy individuals across the globe. More than 500 cases per million are reported in the United States alone [28, 30]. Due to its prevalence, researchers are focusing on ways to regain motor function after SCI with motor training and other interventional strategies such as epidural stimulation [4, 11]. Epidural stimulation of the spinal cord has been shown to be very promising in recovering some motor activity in SCI models [13, 19, 21]. Epidural stimulation in combination with pharmacological agents such as quipazine and 8- OHDPAT, facilitates treadmill stepping in complete spinal cord transected adult rats [8, 13, 20]. However, specific spinal interneurons involved in the recovery of function in SCI animals haves not been identified. C-fos has been used as a marker of active neurons to identify locomotor-associated neurons within the lumbosacral spinal cord, in intact and spinal cord transected adult rats and cats [1, 9, 21]. We have previously found that in quadrapedally stepped adult intact rats the greatest number and increase of c-fos neurons occurred in laminae IV, V, and VII, with the most significant increase in the medial aspect of lamina VII and just adjacent to lamina X in the lumbar spinal cord [1]. Fictive locomotion in decerebrate adult cats also activates neurons in the dorsal horn and lateral to the central canal [9].

The spinal cord contains cholinergic interneurons which may be involved in sensory processing and motor output [3, 18, 39]. Jordan and colleagues found that MLR-stimulated fictive locomotion in decerebrate cats activated cholinergic neurons in lamina VII and VIII [18]. Cholinergic interneurons that are primarily located lateral to the central canal are thought be involved in modulating motor neuronal excitability [34]. Retrograde labeling using cholera toxin (CTB), transynaptic labeling with pseudorabies virus (Bartha) and monosynaptic rabies virus

show that cholinergic neurons surrounding the central canal synapsing with motoneuron pools [17, 33, 44] and are phase locked with the bursting activity of motoneurons [47].

Motoneurons get cholinergic input on motoneuron cell bodies and proximal dendrites in the form of C boutons, group of large synaptic terminals [6, 32, 35]. Using various transcription factors expressed during the development of the mouse spinal cord, populations of spinal interneurons were defined genetically. V0, a subpopulation of interneurons in the ventral spinal cord, are known to send inputs to motoneurons [23]. In neonatal transgenic mice, it was shown that early V0 cholinergic interneurons (a good portion located in the medial lamina VII region) are the source of C boutons [47]. Electrophysiology studies show that these C boutons modulating the excitability of motoneurons [34].

These cholinergic modulators are classified into two types of cholinergic interneurons: central canal cluster cells and partition neurons [3, 39]. Central canal cluster cells, a group of small cholinergic cells located in lamina X have bilateral projections [44]. Partition neurons are larger than central canal cluster cells, and are located in lamina VII with projections from the central canal to the outer edge of the grey matter [3]. There are other small cholinergic neurons outside the central canal region, primarily located in lamina I-III and medial V-VI [3].

Injection of pseudo rabies virus (PRV) and rabies virus into hindlimb muscles of neonatal mice show monosynaptic connections between interneurons located lateral to the central canal and their respective motor pools in neonatal mice [7, 24, 44]. Whether these transynaptically labeled cholinergic neurons become activated after treadmill stepping has not been shown yet. Pseudorabies virus (PRV)-Bartha can be used to label motoneurons and interneurons functionally connected to motoneurons when it is injected into hindlimbs. Combining PRV with

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c-fos provides a means for identifying the interneurons providing input to the motoneurons of a given muscle performing a specific task.

Our overall aim of this study is to identify the cholinergic interneurons that are activated during load bearing locomotion in intact adult rats and complete spinal cord transected adult rats and find out if these activated neurons are functionally connected to specific motor pools. To localize activated sensory-related spinal neurons that are sensitive to load bearing, we used c-fos in combination with Cholera Toxin B (CTB) retrograde labeling from soleus (SOL, a load-bearing ankle extensor muscle) and tibialis anterior (TA, a non-load-bearing ankle flexor) in intact rats that were stepped at different loads on a treadmill. We compared the effects of stepping the rats at a $0^o$ and $25^o$ incline since increasing the incline significantly increases the loading (recruitment) of the hindlimb muscles [42]. Omori and colleagues showed that c-fos mRNA was increased in motoneurons of plantaris, a fast twitch muscle compared to motoneurons of soleus, a slow twitch muscle, in intact rats stepped at a $20^o$ incline on a treadmill. EMG activities also showed a greater increase in the plantaris than in the SOL and c-fos mRNA levels correlated with the EMG activities of these muscles [38]. However in our study we focused on c-fos expression in interneurons instead of motoneurons after load bearing stepping. Noga and colleagues used fictive locomotion preparation to investigate cells activated by supraspinal locomotor inputs and not activated by sensory feedback from hindlimbs stepping on a treadmill [37].

**SPECIFIC AIMS:**

Traumatic injuries to the central nervous system (CNS) result in devastating widespread impairments to motor, sensory and autonomic functions. With the advent of new promising neuroregenerative and plasticity-inducing interventions it is timely and imperative that

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mechanisms associated with therapeutic and rehabilitative strategies be understood in detail. The overall goal of this project is to identify and map the location of the spinal cholinergic interneurons that contribute to stepping, and to define their spatial identity and connectivity.

**Hypothesis**: *Our working hypothesis is that cholinergic interneurons around the central canal are activated during stepping in intact and spinal cord transected (spinal) animals. Furthermore, activated cholinergic interneurons are directly connected to the motoneurons of the hindlimb locomotor circuitry.*

**Specific Aim 1:** Identify spinal cholinergic interneurons that mediate the sensory input to trigger locomotor activity and examine the effects of loading on the recruitment of sensory-related cholinergic neurons in intact rats.

We imposed two loading levels on intact adult rats for 30 min by stepping on the treadmill at two incline angles, $0^o$ and $25^o$.

**Specific Aim 2:** Examine the effects of loading on the recruitment of cholinergic neurons around the central canal in spinal rats.

We imposed varying loading levels on the hindlimbs of the spinal rats during assisted locomotion on a treadmill that were step trained for 5 weeks. The final stepping bout was carried out for 30 Min at their optimum load-bearing ability. We compared the effects in three groups: susp (suspended and not stimulated), susp/stim (air stepping, suspended and stimulated, no load), stimulated, treadmill stepping (stimulated with load) spinal groups.

**Specific Aim 3:** Examine if the activated cholinergic neurons around the central canal are connected to hindlimb motoneurons.

We injected PRV-152 into TA muscle in intact mice and quadrapedally stepped these mice for 60 min. Various post-injection time points were used to get connectivity from monosynaptic to polysynaptic infection. We conducted these experiments in adult intact mice instead of adult rats to get control data since we intend to use transgenic mice with genetic markers in future studies to map activated neurons in hindlimb circuitry during various tasks.

To address the following questions, we used immunofluorescent immunohistochemistry with antibodies to c-fos and cholinergic neuronal marker choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine to identify activated spinal cholinergic interneurons around central canal in lumbar spinal cord of intact and spinal rats. We additionally used an anti-GFP to identify PRV-152 infected neurons in intact adult mice to identify activated cholinergic neurons that are connected to hindlimb circuitry.

1. Are there load bearing sensitive cholinergic interneurons activated during hindlimb stepping in intact and spinal rats?

2. Are more cholinergic partition and cluster interneurons activated in rats stepped at an incline than with no incline and non-stepped rats?

3. Are the cholinergic interneurons activated during hindlimb stepping in intact mice functionally connected to hindlimb circuitry?

## MATERIALS AND METHODS

**Experimental design**

Intact rats: adult female Sprague-Dawley rats were assigned randomly to a control (cage activity, n=3), treadmill stepping at a $0^\circ$ (n=4) or $25^\circ$ (n=4) incline group (Table 1). For three days, all rats in the trained groups were acclimated gradually to the treadmill at $0^\circ$ and $25^\circ$ inclines at 13.5 cm/s. TA and soleus in all rats were injected with retrogradely transported fluorescent tracers to

label muscle-specific motoneurons and cholera toxin B (CTB) to label muscle-specific sensory projections and motoneurons as described below. Seven days after injection, the rats in the treadmill stepping groups were subjected to a 30-min bout of quadrapedal stepping at 13.5 cm/s at either a $0^o$ or $25^o$ incline. One hour after the bout of stepping, the rats were perfused transcardially with a fixative solution of cold 4% paraformaldehyde in phosphate buffer.

Spinal rats: Seventeen spinal Adult female Sprague-Dawley rats (Table 2) were assigned to three groups: susp (no stimulation or stepping n=6), susp/stim (air stepping and stimulation n=5), and step/stim (bipedal stepping and stimulation n=6).   One week after complete spinal cord transection, rats experienced 30-min bout of epidural stimulation and bipedal stepping.  The rats are perfused one hour after stepping.

Intact mice injected with PRV: Swiss Webster mice (n=11) were grouped into four different post injection times 72, 76, 84, 90 hours (Table 3).   After PRV injection, all mice were stepped quadrapedally on a treadmill at 13.5 cm/s for one hour. The mice are perfused one hour after stepping.

**Muscle-specific motoneuron identification:** We pre-labeled selected muscle afferents and motor pools in all rats by intramuscular injection of retrogradely transported fluorescent tracers together with CTB, 1 week before conducting the terminal experiments. Under aseptic conditions, the area to be injected was exposed and rats were injected unilaterally with 2% fast blue (FB, 4 sites at 1.5 μl/site, Sigma, St. Louis, MO) plus 0.3% CTB (List Biologicals, Campbell, CA) and 2% flurogold (FG, 8 sites at 1.5 μl/site, Fluorochrome LLc, Denver, CO) plus 0.3% CTB in the soleus and tibialis anterior (TA) muscles, respectively, using a Hamilton

10 µl syringe and the wound closed and the animal was allowed to recover as described previously [25].

**Surgical procedures**

Spinal cords of Sprague-Dawley rats were completely transected and epidural electrodes were implanted, and surgical procedures and postsurgical care carried out as described before [19]. Briefly, under a combination of ketamine (100mg/kg) and xylazine (10 mg/kg) anesthesia and aseptic conditions the spinal cord was completely transected at the midthoracic level (~T7-T9). Between two partial laminectomy sites (midthoracic level and L2 region) a Teflon-coated stainless-steel wire (AS632; Cooner Wire) was passed above the dura matter of the vertebrae. The electrode is sutured to the dura matter above and below L2 (L2-S1).  The rats were allowed to recover in a warm incubator.  Bladders of rats were expressed manually three times a day for two weeks and two times a day after two weeks.  The rats were kept in individual cages.

**Bipedal stepping with Epidural stimulation**

Each trained rat experienced epidural stimulation from the L2 to S1 segment. 15 minutes before each training session the rats received an intraperitoneal injection of quipazine (0.3 mg/kg) and of 8-OHDPAT (0.15 mg/kg) subcutaneously.  Epidural stimulation was delivered at rectangular pulses with duration of 200 microseconds at 40 Hz. The rats were positioned over a treadmill in a harness that supported 10-40% their body weight.  This step up was used during bipedal locomotion.  Rats were trained 3 d/week, 20 min/session for 5 weeks, starting 7 days after electrode implantation and transection.  We had originally planned to study the effects  of a 30 min bout of locomotor activity at 4 loading levels (air stepping, and at 10%, 50% and 100% of the maximal load bearing ability of individual rats) in motor trained (5 weeks) spinal cord

injured rats. The spinal rats, however, could not perform specifically at these loads, thus we tested the rats at their optimum load-bearing ability.

**PRV injection:** Adult mice received injection of the pseudo rabies virus (total~ $1-1.5 \times 10^6$) into the tibialis anterior muscle at multiple sites and after four different post injection times (72, 76, 84, 90 hrs, Table 3 and Fig.10) and went through quadrapedal treadmill stepping for 60 min and terminated 60 min later. One group was not injected with PRV to serve as a control.

**Tissue Preparation:** After treadmill stepping, animals were returned to the cage and the rats and mice in all three studies are perfused 60 minutes later. Animals were given anesthesia a few minutes before perfusion. The animals are terminated by intracardiac perfusion with 4% paraformaldehyde in phosphate buffer. After the perfusion, the spinal cords were dissected and post-fixed in 4% PF overnight. The spinal cords were then impregnated with 30% sucrose and stored at 4°C for ~2 days. Using the dorsal root ganglion of L5 as a landmark, the cords was divided into T7-T10, transection site, T13–L2, L3–L5, and L6-S2, then frozen with Neg 50 (Thermo Scientific, Hudson, NH) and were stored at minus 80°C until ready to cut. The frozen spinal cord blocks were cryostat sectioned serially at 30 μm and free-floated into wells of 96 well plates (except the transection site, which were thaw-mounted onto slides) containing 1xphosphate–buffered saline (PBS, Sigma) with 0.02% sodium azide (Sigma) and stored at 4°C. Lesion extent was carefully examined by cresyl violet staining to gauge the completeness.

**Immunohistochemisty (IHC):** Spinal cord sections containing motoneurons labeled fast blue (soleus motor pool) and fluorogold (TA motor pool) at similar segmental levels were used for immunohistochemistry. We used double labeled immunohistochemistry using anti-c-fos

(Sc7202, Santa Cruz Inc., CA) and anti-CTB (List Biologicals., CA) or anti-c-fos and anti-choline acetyltransferase (ChAT AB144P, Millipore Inc, Temecula, CA) to map activated spinal neurons associated with sensory afferents and activated cholinergic neurons around central canal during locomotion in control and experimental rats, respectively.  Triple IHC with anti-c-fos, anti-ChAT and anti-GFP (chicken Green fluorescent Protein (GFP) antibody (Aves Labs Inc., Tigard, Oregon, USA) at 1:1000) were carried out to identify activated cholinergic neurons in hindlimb circuitry.

The sections were checked under fluorescent microscope using UV filter to identify the segments containing FB and FG positive motoneurons. Sections with fluorescent labeling at the same segmental level in all rats were selected for analysis.  Rabbit anti-c-fos (SC-7202, 1:400, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat anti-CTB (1:32000, List Biological Laboratories) and goat anti-ChAT (AB144P, 1:500, Millipore, Temecula, CA),  antibodies were used to identify c-fos-positive nuclei, CTB-positive motoneurons and sensory terminals and cholinergic neurons, respectively.

The double and triple immunohistochemisty sections were processed as free floating sections [1].  Briefly, sections were transferred into Costar netwells (15 mm membrane diameter, 74 μm mesh) and rinsed for 30 min in 1xPBS and then (blocking in 3% NDS) transferred to 92-well plates, containing 200 μl/well of a mixture of *c-fos* antibody (1:400) with CTB (1:32000) or ChAT (1:500) in 1xPBS containing 0.3% Triton X100 (PBST) and incubated overnight at $4^{o}$C on an orbital shaker. Specifically for triple IHC, GFP antibody (1:1000) was also added to the mixture for the overnight incubation. Sections were then washed in 1xPBS as follows:  2 quick rinses, followed 2x5 min rinses and then 2x10 min rinses. Sections were then incubated in a mixture (200 μl/well, 96-well plate) of two secondary antibodies (anti rabbit Dylight 594 1:500

and anti goat FITC 1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for double IHC and three secondary antibodies (anti rabbit Dylight 594 1:500, anti goat Biotin 1:200, and anti chicken Alexa Fluor 1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for triple IHC for 1 hr at room temperature. The triple IHC sections (after rinsing for 30 min in 1xPBS) were then incubated in a mixture (200 μl/well, 96-well plate) containing AMCA-conjugated Streptavidin (1:200, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for an additional 1 hr at room temperature. Sections were then washed extensively as above, mounted on Fisher Superfrost slides (Fisher Scientific, Pittsburg, PA) and cover slipped with Vectorshield mounting media containing DAPI (Vector Labs, Burlingame, CA).

**Data analysis:** Spinal cord sections processed for fluorescent immunohistochemistry were examined under Zeiss (Axiophot) microscope under appropriate fluorescent filter sets. Digital images of neurons labeled with c-fos, DAPI, CTB, PRV and ChAT were acquired using Spot RT CCD Slider color camera (Diagnostics Instruments, Inc., Sterling Heights, MI) and Image Pro Plus 7, image analysis program (Media Cybernetics, Rockville, MD). Composite images of c-fos, DAPI, ChAT, CTB and GFP were created using color composite feature. C-fos+ cholinergic neurons and GFP+ neurons were tagged using the manual Tag analytical feature of Image Pro. Neurons were counted within lamina V-VII, and lamina X regions according to spinal cord atlas of adult rat and adult mouse spinal cord [46]. These neurons in rats were placed into cell three types based on specific distances from the center of the central canal. Based on studies performed by Barber and colleagues, central canal cells are located within a range of 0 to 130 microns from the center of the central canal, partition neurons are located over 130 to 600

microns lateral to the central canal and the small dorsal horn neurons are 130 to 600 dorsal to the central canal [3].

**Statistical Analysis:** All data were reported as mean ± SEM. Overall differences were be determined using analysis of variance (ANOVA), using Graph Pad Prism statistical program. Individual group differences were determined using the Turkey post hoc analysis. The criterion level for statistical significance was P< 0.05 for all analyses. In this study, the outcome was measured for each group as below: Activated cholinergic neurons around central canal, number of activated cholinergic neurons in L3-L5 (i.e., c-fos+ ChAT+) as a fraction of total ChAT+ interneurons (i.e., c-fos+ ChAT+ / /total ChAT+ INs). ChAT+ c-fos+ neurons were also compared as separate subgroups (cluster cells, partition neurons, or small dorsal horn cells) or cholinergic neurons in lamina X and Lamina VII as a combined group (i.e., ChAT+ cluster cells, partition neurons).

## RESULTS

### Cholinergic neurons in rat spinal cord

Immunostaining with ChAT antibody shows ChAT+ motoneurons in lateral and medial lamina IX and ChAT+ interneurons around central canal (CC) and in scattered in lamina III-VI of the dorsal horn (Fig. 2). Cholinergic neurons around CC consist of ChAT+ neurons in lamina X, medial V-VII, above CC in medial V/VI (Fig. 1B, 3A-C). The ChAT+ neurons lateral to the CC, consisted of two types with respect to neuron size and the distance from the central canal. For example, the smaller ChAT+ neurons are found closer to CC (~130 μm) while the larger

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ChAT+ neurons are found ~ 130 – 350 μm away from the CC. ChAT+ neurons above the central canal in lamina V-VI were similar in size to the neurons closest to CC.

Based on their studies, Phelps and colleagues categorized ChAT+ cells in lamina X as CC cluster cells, the lateral to CC in lamina VII as partition neurons and above the central canal as small dorsal horn neurons, respectively [3]. We classified and quantified ChAT+ neurons located from 0 to 130 μm from the center of the CC as central canal cluster cells, from 130 to 350 μm lateral to the CC as partition neurons and 130 to 350 μm dorsal to the CC as small dorsal horn neurons (Fig. 3D).

**Activated neurons during quadrapedal stepping in intact rats**

Activated neurons in the spinal cord during stepping in intact rats were identified as neurons expressing the c-fos protein immunolabeled by an antibody specific to c-fos (Fig. 1, 2). Compared to the Sham animals, highest c-fos labeling was in rats stepped at a $25^o$ incline (25-I) followed by those who stepped at $0^o$ incline (0-I). C-fos labeling is present prominently in the superficial lamina I-III, medial III-VI, lamina VII and around the CC in rats stepped at the $25^o$ incline. The quantification of c-fos in these rats was done by Drs. Tillakaratne, Zhong and Fujimoto and showed significant differences between groups, but the data will not be reported here. In this thesis, only the activated cholinergic neurons (i.e., c-fos+ and ChAT+) around CC are reported.

**Activation of cholinergic interneurons near the Central Canal in intact rats during quadrapedal stepping**

In the non-stepped rats (cage activity only, sham), the lamina (VII, and X) around the central canal in L3-L4 lumbar segments had significantly less activation of two types of cholinergic interneurons (central canal cluster cells and partition neurons) in comparison to stepped rats in 0-I and 25-I groups (Fig. 3A-C and 4). Percent of activation of cluster and partition cholinergic neurons around CC taken together in sham, 0-I and 25-I are 5% ±2.4 (n=147, ChAT+ neurons), 19% ±2.3 (n=192, ChAT+ neurons) and 31%±3.2 (n=302, ChAT+ neurons), respectively. Rats in the 25-I group had significantly higher percent of activation compared to both sham and 0-I group (Fig. 4).

We then compared the three types of activated cholinergic neurons separately in the three experimental groups (Fig. 5). 0-I (22±2.6, 154 ChAT+ neurons) and 25-I (33±2.2, n=217 ChAT+ neurons) rats had significantly higher percent of activation of central canal cluster cells than sham (4±2.3, n=96 ChAT+ neurons), with 25-I activating significantly more than 0-I rats. Even though 25-I group (30±10.4, n=85) showed a trend towards a higher percent of activated partition neurons than 0-I (8±3.43, n=38) and Sham (4±4.30, n=51), none of the values were statistically significant. Similarly, activated small dorsal horn neurons in both inclined groups were higher than the sham group, the values were not significant (sham, 4±3.85, n=36, 0-I, 14±7.68, n=39 and 25-I, 15±4.84, n=36).

**Activated cholinergic neurons during epidurally stimulated and pharmacologically mediated bipedal stepping in adult spinal rats**

Activated neurons in the spinal cord during stepping in spinal rats were identified as neurons expressing the c-fos protein immunolabeled by the same c-fos antibody that was used with the intact rats reported above. Compared to non-stimulated spinal rats (susp, Fig. 6A), highest c-fos labeling was in rats that were stimulated and suspended (stim/susp, Fig. 6B) followed by stimulated and stepped (stim/step, Fig. 6C). C-fos labeling is present prominently in the superficial lamina I-III, medial III-VI, lamina VII and around the CC in the 25-I rats. Similar to the intact rats, in this thesis, only the activated cholinergic neurons around CC in spinal rats are reported. Compared to susp group, susp/stim group had the highest activation of cholinergic interneurons followed by the step/stim group.

In contrast intact rats, cholinergic small dorsal horn cells above the central canal were not detected spinal rats. Percent activation in the two types of cholinergic neurons in spinal rats (i.e., both central canal cluster cells and partition neurons taken together) in L3-L5 lumbar segments were significantly lower in the susp only compared to stim/susp and stim/step spinal rats (Fig. 7A-C and 8). Furthermore, stim/susp group had significantly more activated cholinergic neurons than the stim/step group. Percent of activation cholinergic neurons in susp, stim/susp and stim/step were $10\pm3.41$ (n=162), $51\pm8.23$ (n=103) and $30\pm2.13$ (n=165), respectively. We then compared the two types of activated cholinergic neurons separately in the three experimental groups (Fig. 9). stim/susp ($50\pm6.76$, n=73) rats had significantly higher percent of activation of central canal cluster cells than the Stim/step ($27\pm4.18$, n=125), and susp only ($10\pm2.94$, n=134). Stim/Step group had significantly more activated central canal cells than susp. Even though stim/susp ($42\pm17.98$, n=30) and stim/step ($30\pm9.65$, n=42) groups of spinal rats

showed a trend towards a higher percent of activated partition neurons than susp only (10±7.37, n=28), none of the values were statistically significant.

**Pseudorabies virus (PRV)-Bartha and c-fos can be used to identify spinal neurons in functionally connected locomotor circuits**

We injected PRV-Bartha 152 into the TA muscle in adult intact mice and used PRV-GFP immunolabeling to trace functionally connected motoneurons and interneurons in hindlimb spinal circuitry (Fig. 10). We performed a series of experiments to focus on determining the optimum timeline (72, 76, 84, 90 and 96 hrs) for labeling the last order interneurons (i.e., interneurons directly connecting to the motoneuron). All mice used in these studies were stepped quadrapedally for 1 hour for c-fos activation to obtain the spatial distribution of TA-associated neurons involved in stepping.

There were few interneurons labeled after 72 hours with PRV-Bartha 152 (Fig. 10A), but after 84 and 90 hours we observed numerous interneurons near the central canal and laminae I-IV on the ipsilateral side, and a few interneurons on the contralateral side of the spinal cord (Fig.10B and C). After 90 hours, there also were a number of interneurons labeled in the L2 and L6 regions on the ipsilateral side of the spinal cord, a distance away from the TA motor pool which is primarily located in L4 (data not shown). We also observed that some of the PRV labeled cells are positive for c-fos (Fig. 11), and are localized in similar laminae in which locomotor activated neurons we have identified in our previous experiments. This combined approach can be utilized to map activated interneurons in functionally connected hindlimb networks.

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**Activated interneurons in TA neuronal circuitry labeled by PRV-152 in intact mice.**

We were able to identify activated interneurons near the central canal on the ipsilateral and contralateral sides in lumbar enlargement segments (Fig. 12). The virus did not label all the activated interneurons around the central canal (Fig. 12B). We observed only 1-2 cholinergic interneurons labeled with PRV near the central canal for L2 (Fig. 13) and L4 (Fig. 14), that were located 100 μm dorsal and 200 μm dorsolateral to the central canal in the L2 and L4 regions respectively. Cholinergic interneurons labeled with PRV as a fraction of PRV labeled cells in all spinal lamina were 0.8% and 1.6% for L2 and L4, respectively while cholinergic neurons comprised of 3.8% and 4% when calculated as a fraction of PRV labeled cells around the central canal (Table 4). Of the 3 PRV+ ChAT+ neurons, 2 were c-fos+ (L4-(1/1) and L2-1/2). Stepped mice at 74/76, 84 and 90hr time points showed activation in PRV infected neurons (i.e., PRV+ c-fos+) (45±15.05, n=21, 72/76 hrs; 56±15.5, n=20, 84 hrs; 51±4.2, n=104, 90 hrs). In these animals, activation of cholinergic interneurons (i.e., ChAT+ c-fos+) around the central canal were 23.2±8.3, n=103, 12±6.6, 47, and 19±2.3, n=109, respectively for 72/76, 84 and 90 hrs.

We compared the activation of interneurons around the central canal for all post injection time points grouped together in three specific classes, namely, PRV+ c-fos+, ChAT+ c-fos+ and PRV+ ChAT+ c-fos+ (Fig 16). Cholinergic interneurons labeled with PRV had the highest level of activation (75±25, n=3 GFP/ChAT+ neurons) followed by PRV infected only interneurons (50±7.592, n=145 GFP+ neurons) then cholinergic interneurons (19±4.169, n=259 ChAT+ neurons). The percent activation of cholinergic interneurons in intact mice (all post- injection time points grouped together) and rats stepped on a $0^o$ incline are very similar (Fig 17, mice-19±4.169, n=259, rats-19±2.32, n=192 ChAT+ neurons).

**DISCUSSION**

*C-boutons, input to motoneurons from cholinergic cells around the central canal*

C-boutons, large cholinergic varicosities on spinal motoneuronal cell bodies and proximal dendrites were first identified four decades ago [6] and were known to be associated with postsynaptic type 2 muscarinic (m2) receptors [16]. However, the neuronal source of this input and its functional significance were not known until few years ago. Miles and colleagues found that the C boutons originated from a heterogeneous population of Dbx1^{+} interneurons located lateral to the central canal [34]. The homeobox gene Dbx1 controls the specification of a class of commissural interneurons, V0 interneurons [29]. During locomotion, the post-spike afterhyperpolarization is modulated in spinal motoneurons [5]. Miles and colleagues showed that this modulation can be produced by activity in the cholinergic interneurons near the central canal [34] by demonstrating that cholinergic inputs to motoneurons are active during drug-induced fictive locomotion. For example, the recordings from motoneurons in mouse spinal cord slices showed that cholinergic activation of m2-type muscarinic receptors increases excitability by reducing the action potential afterhyperpolarization. They suggested that by activating m2 receptors, which are found at synapses formed by C boutons, cholinergic inputs increase motoneuron excitability, thus ensuring that appropriate output is generated during motor behavior.

Zagoraiou and coworkers found that a subset of V0 neurons identified by expression of the transcription factor Pitx2 and cholinergic phenotype were located near the central canal at lumbar, thoracic, and cervical levels of the spinal cord [47]. They showed that Pitx2^{+} neurons are the major source of cholinergic input to spinal motoneurons by generating mice in which Pitx2^{+} neurons expressing a fluorescent protein and that nearly all C boutons originate from

Pitx2^{+} neurons. Even though V0C (called V0C for cholinergic) neurons do not receive direct primary afferent input, their firing is tightly phase locked with motoneuron burst activity during fictive locomotion, indicating they receive input from spinal locomotor networks [47]. Furthermore, when they silenced the cholinergic output of these neurons through elimination of ChAT inputs, it affected swimming behavior showing that its modulatory input in some motor tasks.

*Embryological origins of Central canal cluster cells and Partition cells,*

We examined cholinergic cells located around the central canal in adult rats and in mice during treadmill locomotion. In rats, according Barber, Phelps and colleagues cholinergic cells around the central canal are categorized into two types, 1) located in the immediate vicinity of the central canal (lamina X, central canal cluster cells), and 2) located lateral to the central canal (medial VII, partition neurons) [3]. In our studies, we counted the cholinergic cells around central canal in mice as one group since the location and sizes were not as distinct as in rats.

Cholinergic interneurons involved in locomotion originate from a small percentage of V0 interneurons that arise within the ventricular zone [14, 15, 26]. During rat embryological development central canal cluster cells and partition cells appear at differing time points in the developing spinal cord [2, 40]. Partition neurons are the first cholinergic interneurons developed rostrocaudally [2] and the first ChAT immonoreactive interneurons to appear and are seen outside the ventricular zone by E15 [40]. Central canal cluster cells are formed within the ventricular zone in a U shaped fashion by E17 [40] and are the second cholinergic interneurons developed [2].

Using Pitx2 in mice, Enjin and colleagues showed that a subpopulation of Pitx2^{+} cells located laterally and not immediately near the central canal were cholinergic and categorized

them as partition cells [12]. Injecting PRV-614 into tibialis anterior and gluteus muscles in adult

mice, Zagoraiou and colleagues showed these cells are directly connected to motoneurons [47].


**Activated cholinergic interneurons in adult mice are connected to hindlimb motoneurons**

*Cholinergic neurons around central canal project several segments rostrally and caudally*

We focused on interneurons in lumbar enlargement L3-L5 since a large number of

motoneurons from hindlimb muscles are located in these segments [36]. We grouped the

cholinergic neurons from L3-L5 together since cholinergic neurons project to several segments

rostral and caudal to individual hindlimb motor pools. For example, monosynaptic Rabies virus

expressing fluorescent proteins injected into quadriceps of neonatal mice show that the

cholinergic partition neurons projecting rostrally to Thoracic 11 and caudally to Lumbar 6, even

though quadriceps motoneurons are primarily located in L3 and L4 [44]. Similarly,

monosynaptic rabies virus injected into the triceps surae muscle (motoneurons primarily in L4

and rostral L5) show cholinergic interneurons in L3-L4 lumbar segments [7]. Most of these

cholinergic interneurons project ispilaterally and some contralaterally to mostly functionally

equivalent motor pools [44]. These studies provide evidence that the cholinergic interneurons

above and below motor pools have extensive connections to hindlimb motoneurons.

*Activated interneurons in TA neuronal circuitry labeled by PRV-152 in intact mice.*

The injection of Pseudo rabies virus (PRV) into the TA muscles of mice is critical in

identifying interneurons that directly synapse with the TA motoneurons in the hindlimb circuitry.

With the use of PRV as a retrograde transsynaptic marker, we were able to map the connectivity

from motoneurons to interneurons found near the central canal as well as the other laminae in

L3-L5 of mice spinal cord. Although over 50% of PRV-labeled interneurons in the TA pathway

were activated (i.e., PRV+ c-fos +) after quadrapedal stepping, the number of cholinergic neurons labeled were low (~0.8% L2 and 1.6%, L4). These numbers agree with the number of labeled cholinergic neurons using monosynaptic rabies virus, 2% and 1.5% for quadriceps and triceps surae, respectively in neonatal mice [44]. The fact that we saw only few PRV+ cholinergic neurons is not due to PRV interfering with the expression of ChAT, since we saw many motoneurons labeled with both PRV and ChAT.

The use of PRV as a retrograde transynaptic labeling marker has been useful in identifying cholinergic interneurons connected to TA motoneurons in neonatal mice [24]. Jovanovic and colleagues have used various post-injection times for labeling only the motoneurons and first order interneurons [24]. We used a similar strategy with varying post-injection times to see when cholinergic cells around central canal are labeled by PRV. We saw PRV-labeled neurons surrounding the central canal at 84 and 90 hr post injection times, but not all of them were ChAT+. Monosynaptic rabies virus is effective in identifying cholinergic interneurons that synapse with quadriceps and triceps surae hindlimb motoneurons [44], but whether these neurons are activated during a locomotor task was not carried out. Stepien and colleagues were able to identify a multitude of premotor interneurons such as cholinergic partition neurons, renshaw cells, group 1a sensory neurons, dorsal horn interneurons and serotonergic neurons that make connections with the mouse brainstem, using of monosynaptic rabies virus together with various molecular identification techniques [44]. We only identified cholinergic premotor interneurons which are infected with the PRV virus that are activated after stepping. Other types of activated neurons during stepping can be done using various interneuronal markers in future studies. Rotto-Percelay and colleagues were able to inject PRV into the medial gastrocnemius muscle of adult rats and observed PRV labeling in lumbar spinal

regions after 4 days post-injection. They showed that transynaptic labeling of PRV injected into muscles occurs only through efferent projections from the motor columns since dorsal root transections did not change the labeling patterns [41].

**Quadrapedally stepping in intact rats and mice increases activation of laminae V-VII/X cholinergic interneurons**

*Sensory Input from hindlimbs leads to activation of cholinergic interneurons*

Our results show that treadmill locomotion in adult intact and spinal rats and in intact adult mice activates cholinergic neurons around the central canal. Furthermore, we found that imposing increased loads during stepping in adult intact and spinal rats activated more of these cholinergic neurons compared to non-stepped and non-epidurally stimulated rats, respectively. Jordan and colleagues demonstrated activation of cholinergic neurons in response to fictive locomotion in adult cats correspond to partition cells in medial lamina VII [18]. In decerebrate cats mesencephalic locomotor region (MLR)- stimulated fictive locomotion showed activated cholinergic interneurons predominantly in lamina IV- X with the most activation in lamina VII in comparison to non-stimulated cats [18]. Percent activation of cholinergic neurons (including occasional ChAT+ neurons elsewhere besides lamina IV, X and VII) ranged from 1.6-2%. In intact adult rats we found about 20% and 30% cholinergic neurons around the central canal are activated due to treadmill locomotion at $0^o$ and $25^o$ incline, respectively. Sensory inputs during treadmill stepping most probably activated more cholinergic neurons around central canal (both cluster cells and partition cells) in our animals. Electrophysiological studies in the regions of activated cholinergic neurons showed that they were vigorously active during fictive locomotion in phase with ipsilateral extension, and subsequent processing for ChAT immunohistochemistry showed they projected to the contralateral side of the spinal cord [18]. They claimed that they

have identified a population of cholinergic partition cells with commissural projections that are active during the extension phase of locomotion. Stepien and colleagues also identified bilateral partition cells, projecting into functionally equivalent motor pools in neonatal mice [44]. Together with data from other groups using electrophysiology, transgenic mice expressing early cholinergic markers, and transynaptic labeling, these cholinergic neurons synapse on motoneurons and changes motoneuronal excitability [5, 7, 34, 44, 47].

Based on these data, increased activation of cholinergic neurons during stepping can lead to increased excitability in the motoneurons. We suggest that these cholinergic interneurons are activated through indirect sensory input from hindlimb muscles. Studies in cats show that group 1a and 1b afferents terminate on medial laminae V-VI. These neurons mediate the control of ankle/toe extensors and flexors in these cats by both exciting and inhibiting interneurons found in these laminae [22]. In rats group 1a afferents from the gastrocnemius muscle terminate in lamina near the central canal such as IV-V, VII and X [45]. It is well documented that during locomotion in cats Ib afferents may act as load detectors, switching between Ib extensor facilitatory and inhibitory pathways [10]. One mechanism of action for the load bearing sensory input may be that interneurons receiving the first-order information from load sensing primary afferents synapse with cholinergic interneurons located in lamina VII/X conveying second order sensory information. Injecting PRV into hindlimb muscles and detecting activated first and second order neurons associated with sensory afferents after load bearing stepping may answer this type of question.

**Epidural stimulation increases activation of lamina X/VII cholinergic interneurons in spinal rats**

Animals that were stimulated epidurally with or without bipedal step training have differing levels of neuronal activation of central canal cluster cells and partition neurons. We suggest that the sensory input from hindlimb muscles and epidural stimulation lead to activation of these cholinergic interneurons. Lavrov and colleagues have shown that stepping in spinal transected animals can be recovered only in non-deafferented hindlimbs of rats [31]. Even with the help of epidural stimulation, deafferented hindlimbs of adult rats were not able to recover stepping. The lack of sensory input and epidural stimulation in susp animals may prevent activation of cholinergic interneurons that may be involved in the hindlimb locomotion.

Courtine and colleagues used a similar training regimen for stepping, including the pharmacological agents and epidural stimulation order to activate non-functional spinal circuits in spinal adult rats [8]. They showed that in L3-L5 regions had the most activated total neurons in the susp/stim groups followed by step/stim. We saw similar trends for activated cholinergic cells around the central canal. Furthermore, our data shows step training regimen activates cholinergic neurons around the central canal in spinal rats similar to intact rats during stepping.

The lower activation of cholinergic interneurons in epidurally and pharmacological stimulated step trained rats compared to the suspended rats with similar stimulation parameters may be due to sensory input from hindlimb reinforcing specific neural circuitry, while non-trained rats have nonspecific activity. For example, Courtine and colleagues measuring EMG activities in hindlimb muscles showed that suspended stimulated spinal rats have partial co-activation of reciprocal flexor (TA) and extensor (SOL) muscles in comparison to step-trained

spinal rats with epidural and pharmacological stimulation [8]. They suggested that the spinal circuitry in these animals is being strengthened by stimulation and stepping.

Changes in the activity of spinal neurons or reorganization of networks among other changes may be occurring in response to spinal cord transection. Skup and colleagues showed that after complete spinal cord transection in adult rats, cholinergic boutons (identified as anti-vesicular acetylcholine transporter, VChAT terminals) were decreased on motoneurons in soleus, but not in TA [43]. The decrease in these boutons on soleus motoneurons were partially recovered after 5 weeks of quadrapedal treadmill step training, while number of VAChT-positive terminals on the TA motoneurons were increased above the control level. In our studies in rats, we examined the cholinergic activation in L3-L5, since cholinergic neurons from any particular motor pool project several segments rostrally and caudally [7, 44]. Our data from transynaptic labeling from TA muscle in intact mice showed that activated cholinergic neurons around central canal in L2 and L4 segments, showing, motoneurons in any particular hindlimb muscle can receive inputs from a wide spread propriospinal cholinergic neuronal network. Site- and time-dependent sprouting of myelinated and unmyelinated primary afferent fibers, and possibly interneurons, occurred in lumbar spinal cord after spinal cord transection [27]. Whether the increase in number of activated cholinergic neurons around the central canal in step-trained spinal rats compared to the non-trained were due to changes in sensory afferent or spinal interneurons sprouting or reestablishment of connectivity of locomotor networks through training cannot be answered, since we did not use PRV to label hindlimb circuitry.

Overall this study shows the importance of the role of sensorimotor pathways and specific interneurons in the recovery after spinal cord injury and functional-anatomical map of

spinal circuitry can be made using these techniques to place electrodes in spinal cord to induce an effective motor output during rehabilitation.

## CONCLUSIONS

We examined the effects of loading on the recruitment of sensory-related cholinergic neurons around the central canal in intact rats and showed these neurons are activated after quadrapedal stepping. Furthermore activation of these neurons is increased with stepping with increased loading imposed via increased incline of the treadmill (Specific Aim 1). In spinal rats we showed that rats trained to step bipedally with loading with epidural and pharmacological stimulation had increased activation of cholinergic neurons around the central canal compared to the non-stimulated rats, but this activation is lower than the non-trained stimulated rats (specific aim 2). Finally we examined if the activated cholinergic neurons around the central canal are connected to hindlimb motoneurons. With transynaptic labeling from PRV-152 injected into the TA muscle of intact mice we showed that these cholinergic interneurons are connected to TA motoneurons. We observed activation of PRV labeled neurons in the spinal cord after quadrapedal stepping. Furthermore, some of the PRV-labeled cholinergic interneurons around the central canal were also activated (specific aim 3).

The fact that level of activation of these cholinergic interneurons are influenced by sensory input from hindlimb muscles during load-bearing stepping and their direct connections with hindlimb motor pools gives further evidence to the role proposed by others, that these interneurons are acting as modulators of motoneuron excitability.

# TABLES

## Summary of Experimental Groups

**Table 1.  Experimental Groups of the quadrapedally stepped intact adult rats**

| Groups | Number of rats | Quadrapedal stepping | IHC for c-fos and ChAT |
|---|---|---|---|
| Sham | 3 | Cage activity | X |
| $0^o$ Incline | 4 | 30 min | X |
| $25^o$ Incline | 4 | 30 min | X |

**Table 2.  Experimental Groups of the bipedally stepped spinal cord transected adult rats**

| Groups | Number of rats | Bipedal stepping | Pharmacological treatments/epidural stimulation | IHC for c-fos and ChAT |
|---|---|---|---|---|
| Susp | 6 | None | None | X |
| Susp+stim | 5 | None | Quipazine+8-OHDPAT/epi stim | X |
| Stim+stepping | 6 | 30 min | Quipazine+8-OHDPAT/epi stim | X |

**Table 3. Experimental Groups of the PRV-152 injected adult mice**

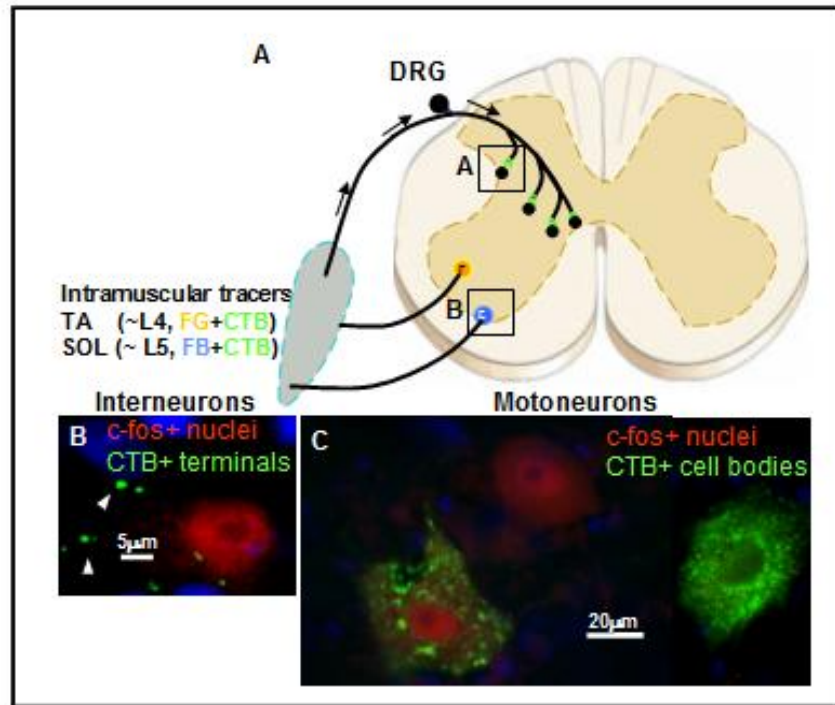| Groups | Number of rats | Quadrapedal stepping for 60 min | IHC for c-fos and ChAT | IHC for GFP |
|---|---|---|---|---|
| Control (no injection) | 5 | X | X | |
| 72 hr | 3 | X | X | X |
| 76 hr | 2 | X | X | X |
| 84 hr | 3 | X | X | X |
| 90 hr | 3 | X | X | X |

**Figure 1. Intramuscular injection scheme of retrogradely transported dyes.**

Panel A shows a schematic of retrograde labeling of various neurotracers when injected into hindlimb muscles (TA, and SOL). CTB are injected into the SOL muscle labels SOL motoneurons and primary sensory terminals while FB, labels SOL motoneurons and dorsal root ganglion cells (DRG). Similar injections into the TA muscle are done with FG instead of FB. Panel B shows an example of CTB labeled primary sensory terminals of soleus (green dots, white arrow heads), c-fos positive (red) and c-fos negative (blue, DAPI stained nuclei) interneurons. Panel C shows an example of motoneurons labeled for c-fos (red) and CTB (green) in a L5 spinal cord section from a rat in 25$^o$ incline group. It shows that one motoneuron is activated. Scale bars= 5 μM in B and 20 μM in C.
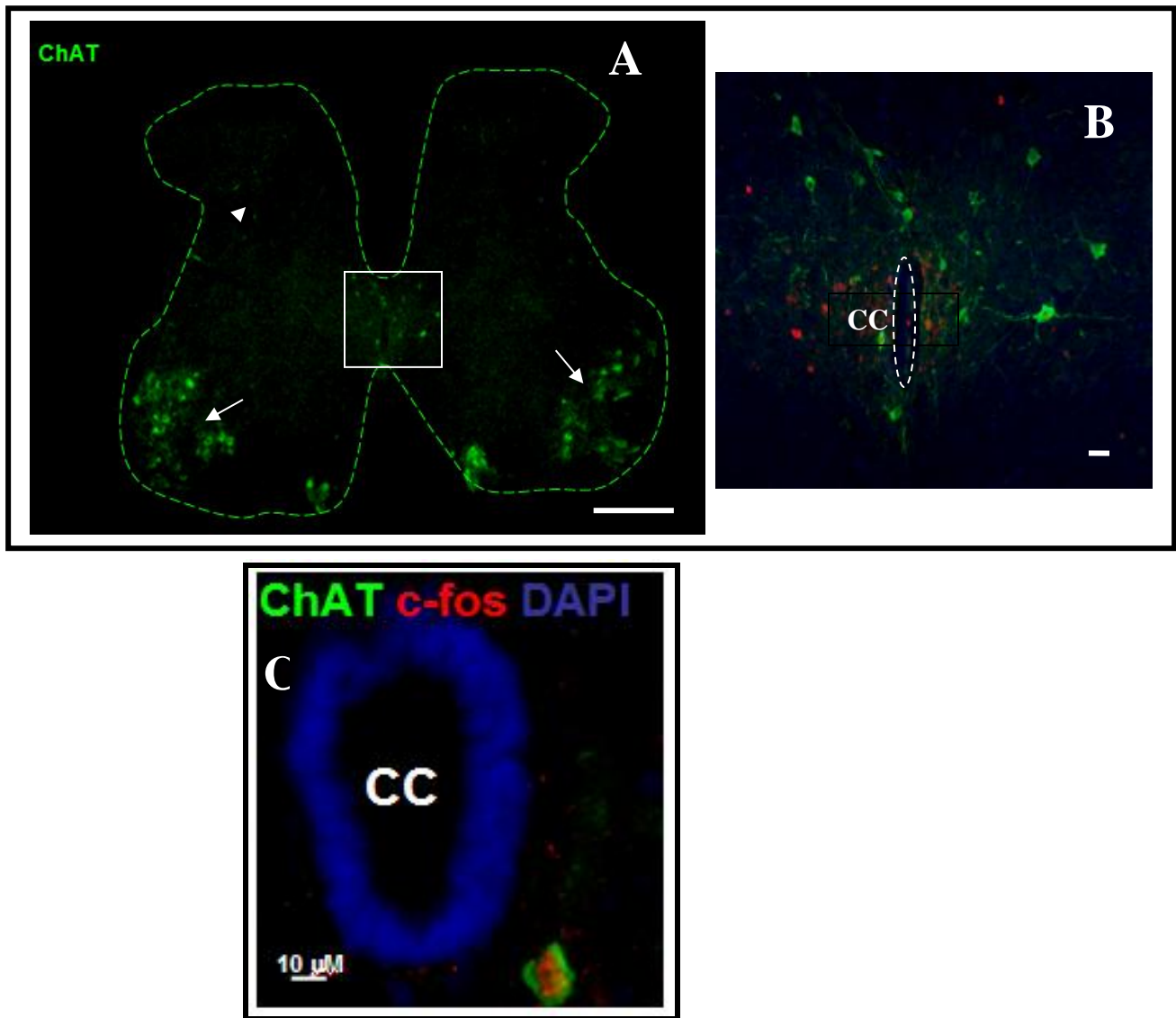
**Figure 2. Distribution of ChAT positive neurons within Lumbar region**

Immunohistochemical staining of lumbar region L4 using anti-ChAT and anti-c-fos antibodies were carried out to identify cholinergic and activated neurons. Panel A shows cholinergic interneurons (ChAT+ green cell bodies) found in the ventral horn (arrows, laminae VIII-IX), near the central canal (laminae V-VII) and scattered throughout the dorsal horn (arrowhead, laminae I-IV). Panel B represents a magnification of the area around the central canal in panel A (square box) with activated interneurons (c-fos+ red cell bodies) after quadrapedal stepping. Panel C shows an activated cholinergic neuron (c-fos+ ChAT+) near central canal. Scale bars in A=200 μM, B=25 μM and C=10 μM, respectively.
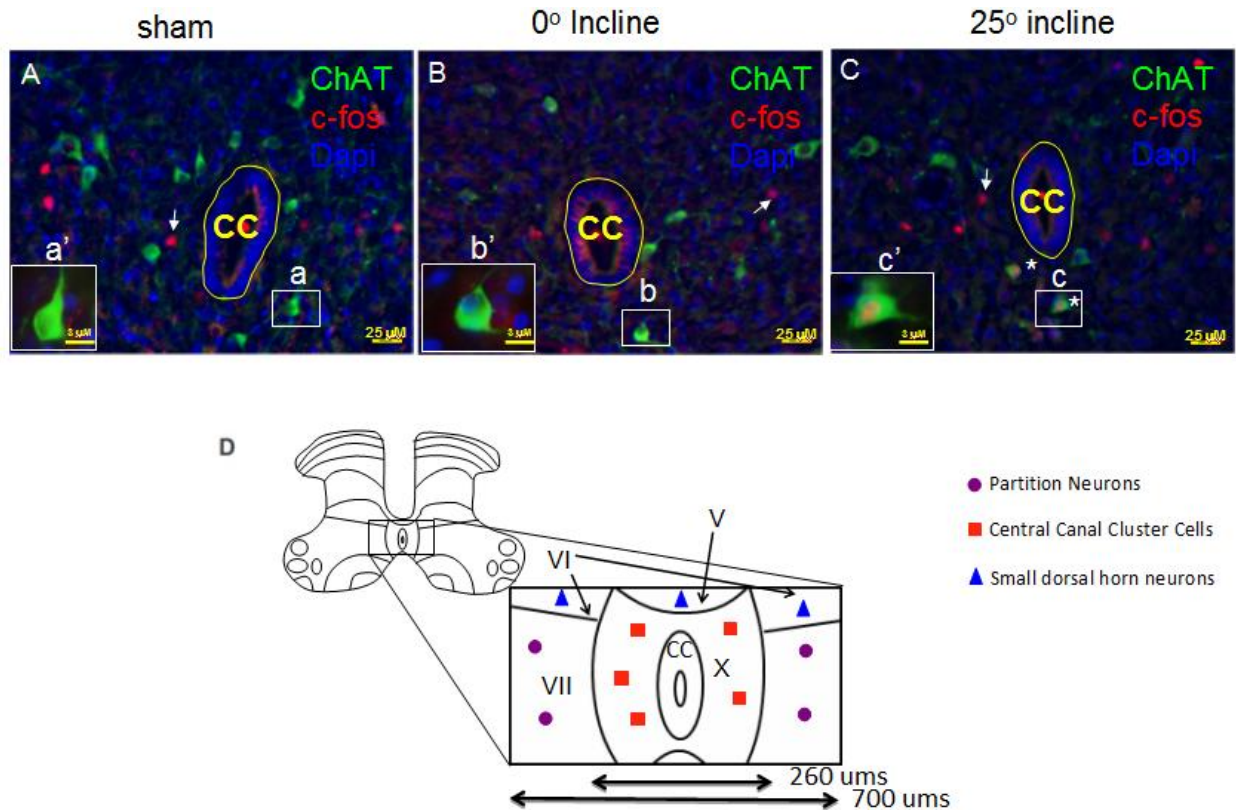
**Figure 3. Activation of lumbar cholinergic interneurons after quadrapedal treadmill stepping**.

Immunohistochemical staining of lumbar L3-L5 sections using anti-ChAT and anti-c-fos antibodies were carried out to identify activated cholinergic interneurons surrounding the central canal in intact non-stepped (Sham, A), rats stepped for 30 min at $0^o$ incline (B) and $25^o$ incline (C). Cholinergic interneurons surrounding the central are shown in the A-C images: cholinergic neurons (ChAT positive cell bodies, green), activated (c-fos positive nuclei, red) and DAPI (nuclei, blue). Insets a, b and c are magnified in a', b' and c', respectively. D. Schematic distribution of different types of cholinergic neurons near the central canal of the adult rat lumbar spinal cord. Partition neurons (purple circles) are located in Lamina VII and are primarily found at a distance ~ 130 to 350 $\mu$M lateral to the center of the central canal. Central canal cluster cells (red squares) are located in lamina X and are found at a distance of ~0 to 130 $\mu$M from the center of the central canal. Small dorsal horn neurons (blue triangles) are located in lamina sections roughly VI and above. We quantified cholinergic neurons in lamina V and VI that are > 130 $\mu$M dorsal to the central canal. Scale bars= 25 $\mu$M in A, B and C and 3 $\mu$M in a', b' and c', respectively.
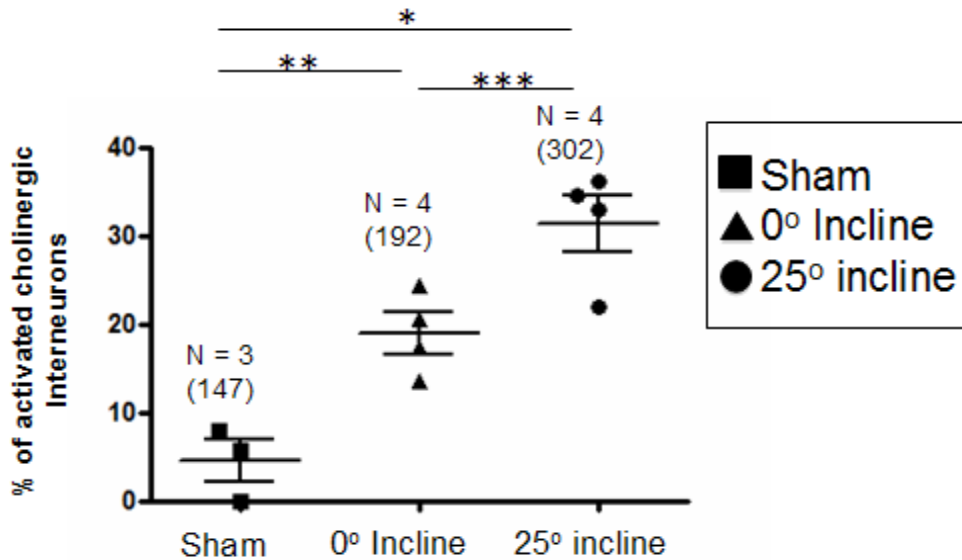
**Figure 4**. **Activated cholinergic interneurons (in lamina X and medial VII) in quadrapedally stepped intact rats**.

The scatter plot shows the activated cholinergic interneurons (i.e., c-fos+ ChAT+) as a percent of all cholinergic interneurons around central canal (ChAT+ central canal cells and partition neurons) surrounding the central canal area in L3-L5 spinal cord. Animal groups: Sham (squares), $0^o$ incline (triangles), $25^o$ Incline (circles). The asterisks indicate a p-value of $\leq 0.05$ for statistical comparisons between groups, *, sham vs. $25^o$ Incline; **, sham vs. $0^o$ incline and ***, $0^o$ incline and $25^o$ incline. Sections/group=28. The total number of analyzed cholinergic interneurons in each group is within parenthesis. Compared to sham group, $25^o$ incline group had the highest activated cholinergic interneurons followed by the $0^o$ incline group.
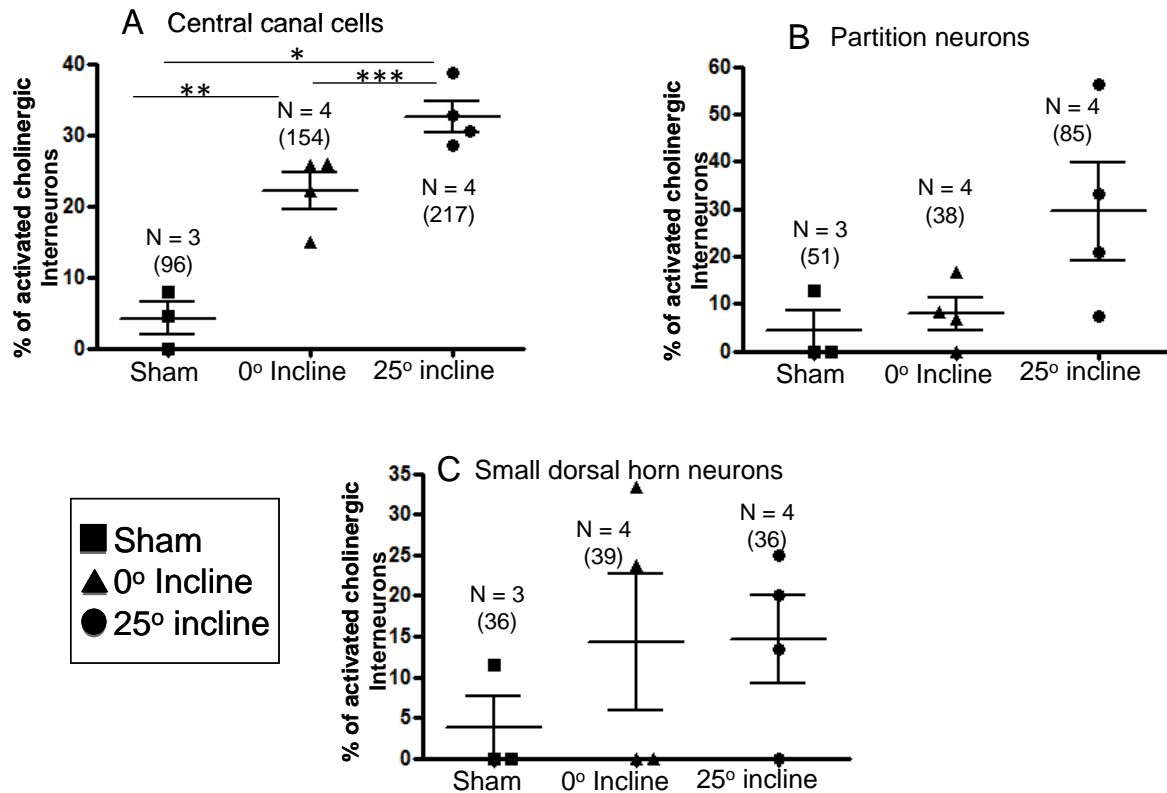
**Figure 5. Different types of activated cholinergic interneurons in quadrapedally stepped intact rats**

The scatter plots show the different types activated cholinergic interneurons (i.e., c-fos+ ChAT+) as a percent of all cholinergic interneurons (ChAT+ A. Central canal cells, B. Partition neurons, and C. Small dorsal horn neurons) surrounding the central canal area in L3-L5 spinal cord. Animal groups: Sham (squares), $0^o$ incline (triangles), $25^o$ Incline (circles). The asterisks indicate a p-value of $\leq 0.05$ for statistical comparisons between groups, *, sham vs. $25^o$ Incline; **, sham vs. $0^o$ incline and ***, $0^o$ incline and $25^o$ incline. Sections/group=28. Cells/section, =6-7. The total number of analyzed cholinergic interneurons in each group is shown within parenthesis.

Scatter plot A shows that compared to sham group, $25^o$ incline group had the highest percent of activated central canal cells followed by the $0^o$ incline group. Scatter plot B shows that $25^o$ incline group had the highest percent of activated partition neurons followed by the $0^o$ incline and sham group. Scatter plot C shows that $25^o$ incline group had the highest percent of activated small dorsal horn neurons followed by the $0^o$ incline and sham group.
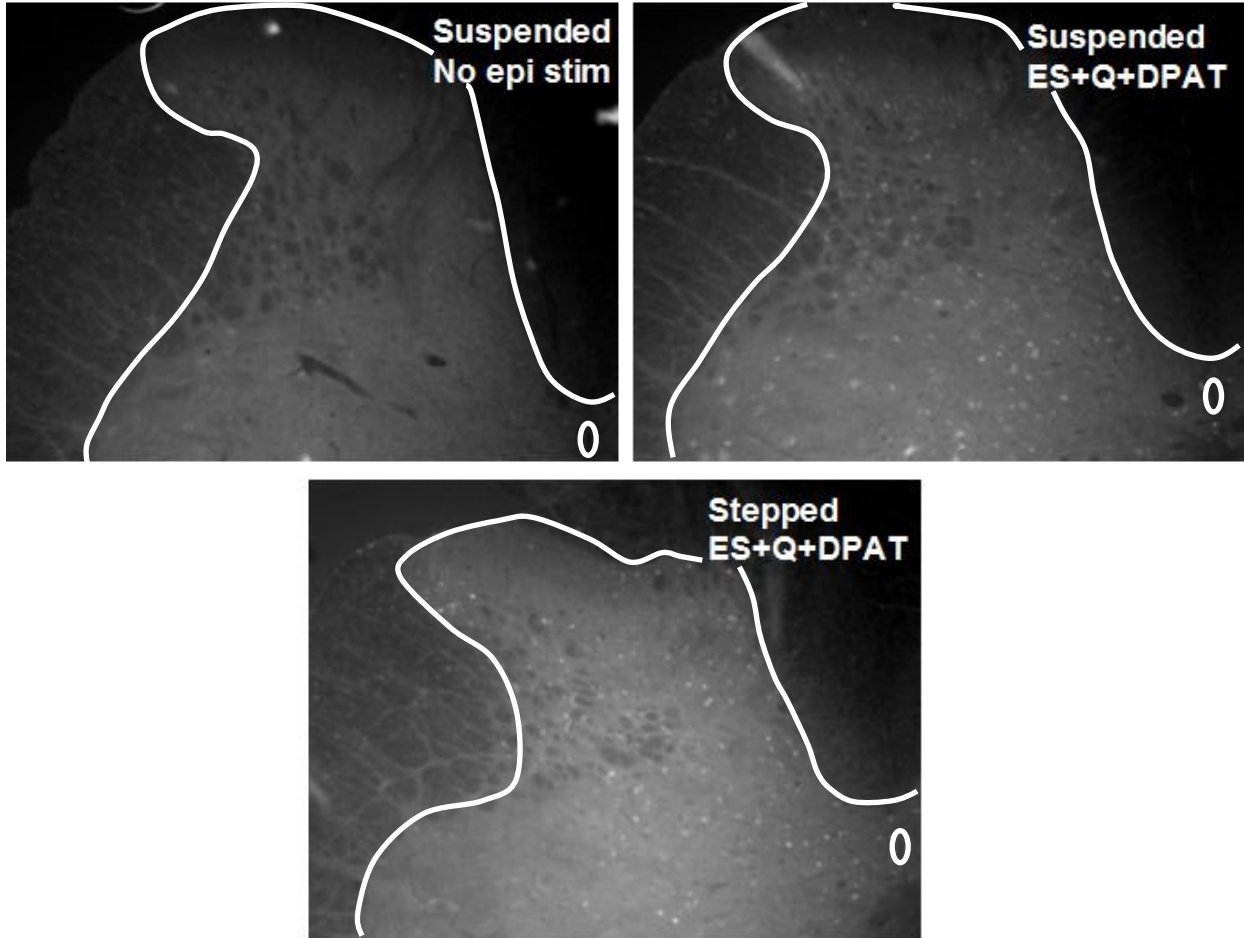
**Figure 6. Serotonergic drugs combined with epidural stimulation activate many spinal neurons.**

Immunohistochemical staining of lumbar region L4 using anti-c-fos antibody were carried out to identify activated neurons in the dorsal horn, and central canal area of suspended only adult rats (A, no stepping or epidural stimulation), suspended plus epidurally stimulated (ES) spinal animals (B) and bipedally stepped and epidurally stimulated animals (C). All stimulated animals (B and C) received pharmacologically injections of quipaizine (Q) and 8-OHDPAT (DPAT).
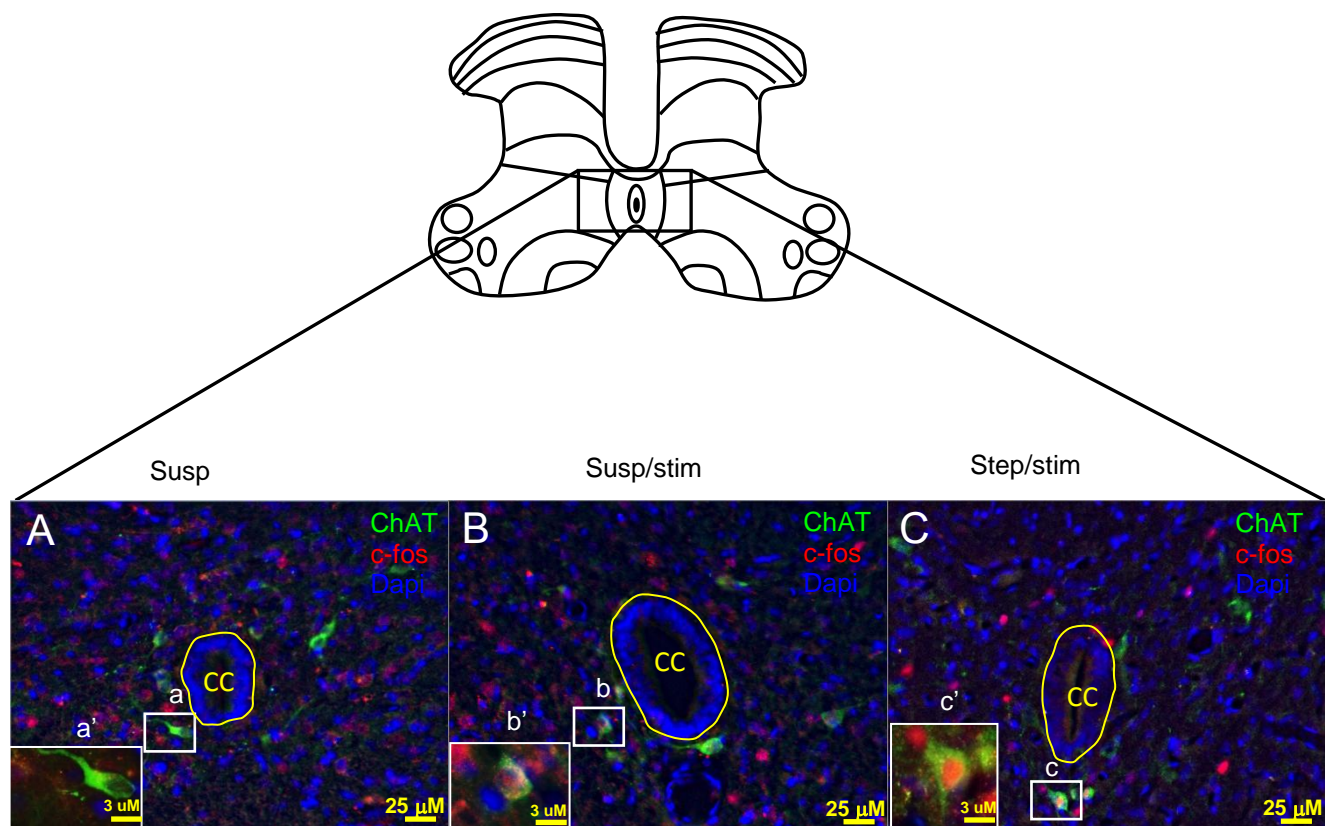
**Figure 7. Activation of Lumbar Cholinergic Interneurons after epidural stimulation**

Immunohistochemical staining of lumbar L3-L5 sections using anti-ChAT and anti-c-fos antibodies were carried out to identify activated Cholinergic interneurons surrounding the Central Canal in spinal rats that were suspended in a harness, but non-stimulated (Susp, A), suspended and epidural stimulated (Susp/stim, B) and epidural stimulated and stepped (Step/stim, C). Cholinergic interneurons surrounding the central are shown in the A-C images: Cholinergic neurons (ChAT positive cell bodies, green), activated (c-fos positive nuclei, red) and DAPI (nuclei, blue). Insets a, b and c are magnified in a', b' and c', respectively. Scale bars= 25 μM in A, B and C and 3 μM in a', b' and c', respectively.
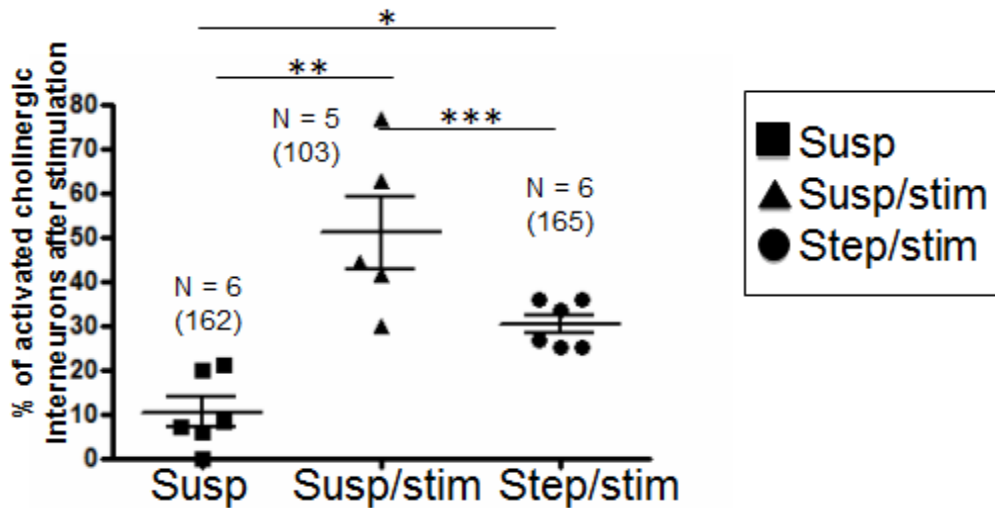
**Figure 8. Activated cholinergic interneurons after Bipedal stepping and epidural stimulation in adult spinal rats**

The scatter plot shows the activated cholinergic interneurons (i.e., c-fos+ ChAT+) as a percent of all cholinergic interneurons after epidural stimulation (ChAT+ central canal cells, and partition neurons) surrounding the central canal area in L3-L5 spinal cord. Animal groups: Susp (squares), Susp/stim (triangles), Step/stim (circles). The total number of analyzed cholinergic interneurons in each group is shown within parenthesis. The asterisks indicate a p-value of $\leq 0.05$ for statistical comparisons between groups, *, Susp vs. step/stim, **, Susp vs. susp/stim, ***, susp/stim vs. step/stim. Sections/group=24. Compared to Susp group, Susp/stim group had the highest activation of cholinergic interneurons followed by the Step/stim group.
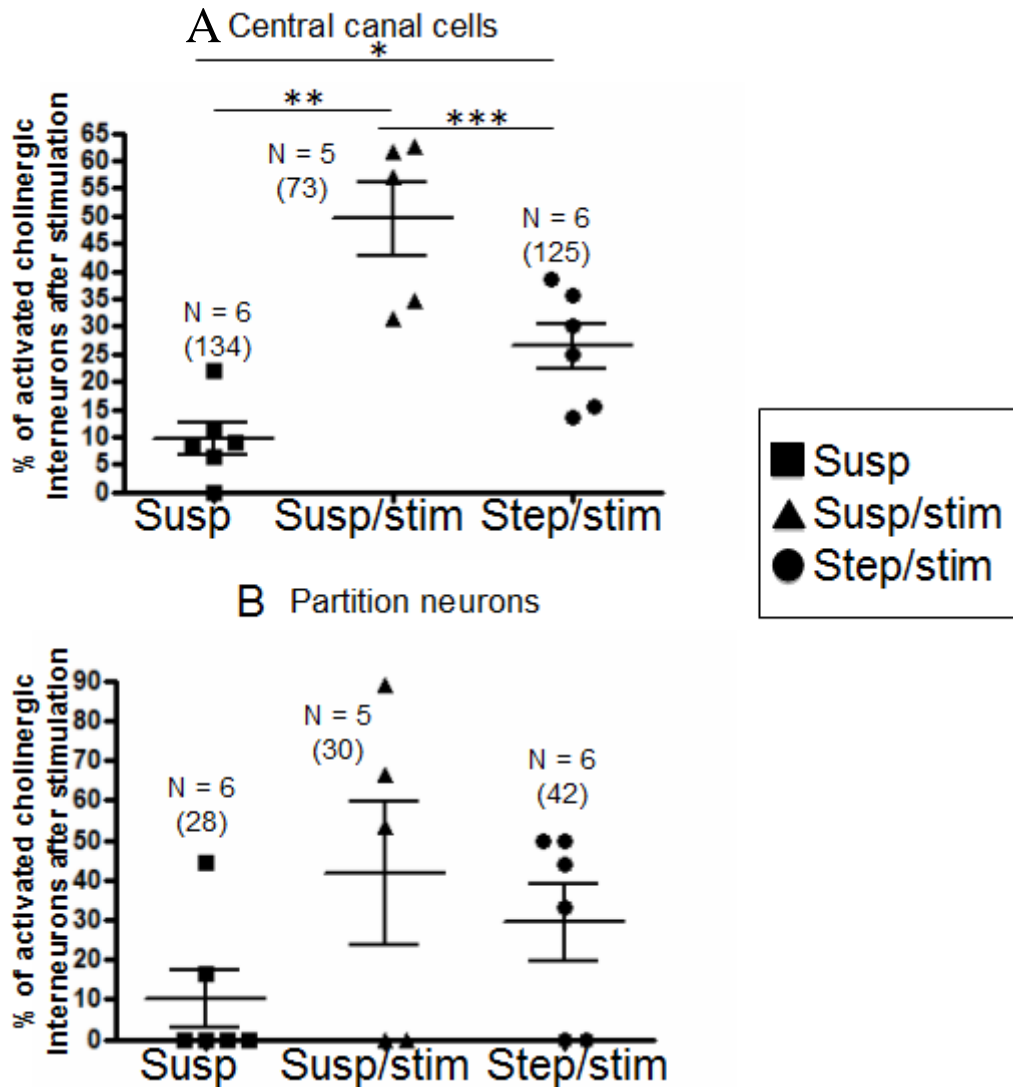
**Figure 9. Different types of Activated Cholinergic interneurons after bipedal stepping and epidural stimulation in spinal rats**

The scatter plots show the activated cholinergic interneurons (i.e., c-fos+ ChAT+) as a percent of all cholinergic interneurons after epidural stimulation (ChAT+ A. Central canal cells, and B. Partition neurons) surrounding the central canal area in L3-L5 spinal cord. Animal groups: Susp (squares), Susp/stim (triangles), Step/stim (circles). The total number of analyzed cholinergic interneurons in each group is shown within parenthesis. The asterisks indicate a p-value of $\leq$ 0.05 for statistical comparisons between groups, *, Susp vs. step/stim, **, Susp vs. susp/stim, ***, susp/stim vs. step/stim. Sections/group=24. Compared to Susp group, Susp/stim group had the highest activation of central canal cluster cells (A) and partition neurons (B) followed by the Step/stim group.
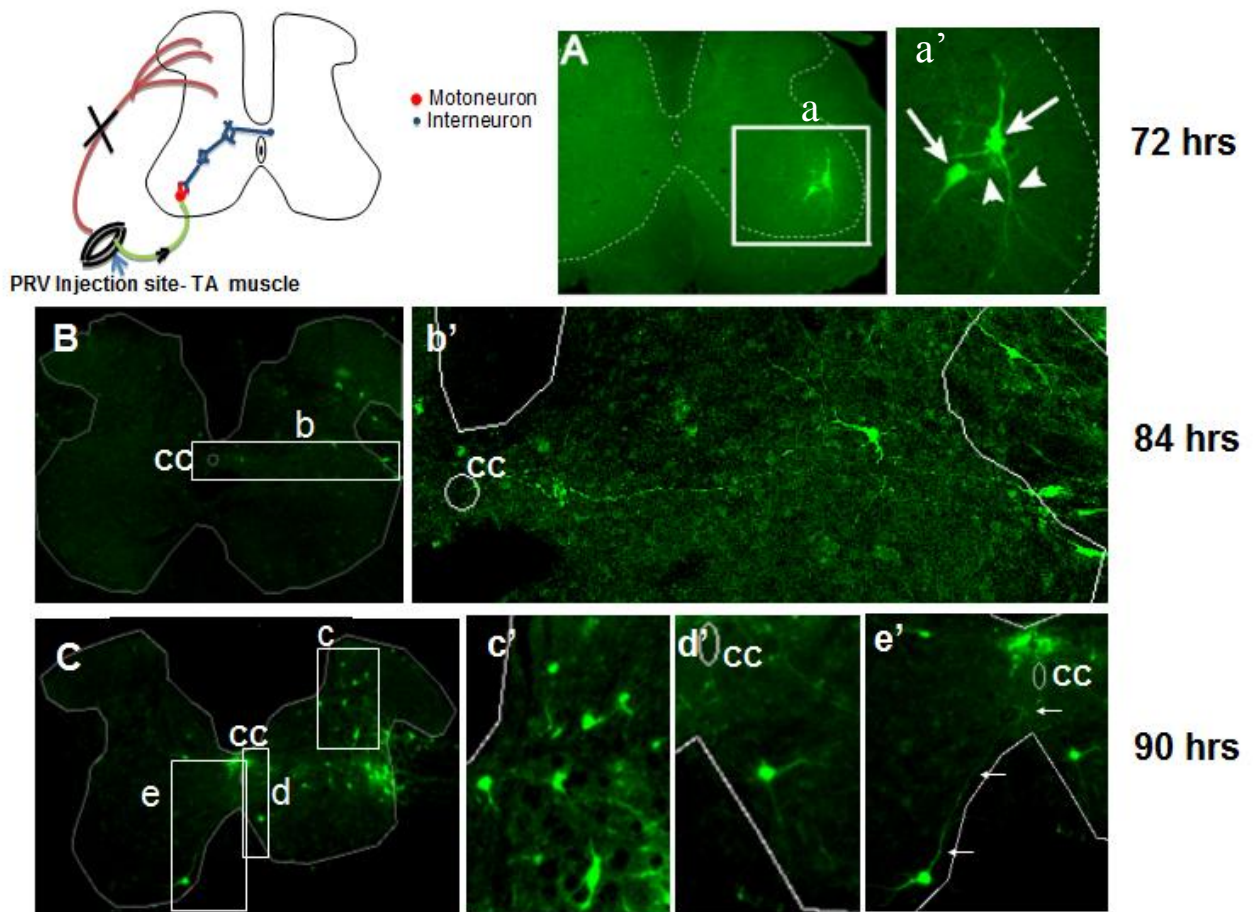
**Figure 10. PRV-Bartha (152) labeling in intact adult mice after different post-injection times.**

Immunohistochemical staining of lumbar L3-L5 sections using anti-GFP antibody was carried out to identify PRV infected neurons after different post injection times, 72 hrs (A), 84 hrs (B) 90 hrs (C). Post injection time 72 hrs shows infected motoneurons but very few infected interneurons, on ipsilateral side. (B-C) Post injection times 84-90 hrs show more infected ipsilateral interneurons. Interneurons on the contralateral side are labeled at 90 hr post injection time point. Insets a, b, c, d and e are magnified in a', b', c', d' and e', respectively.
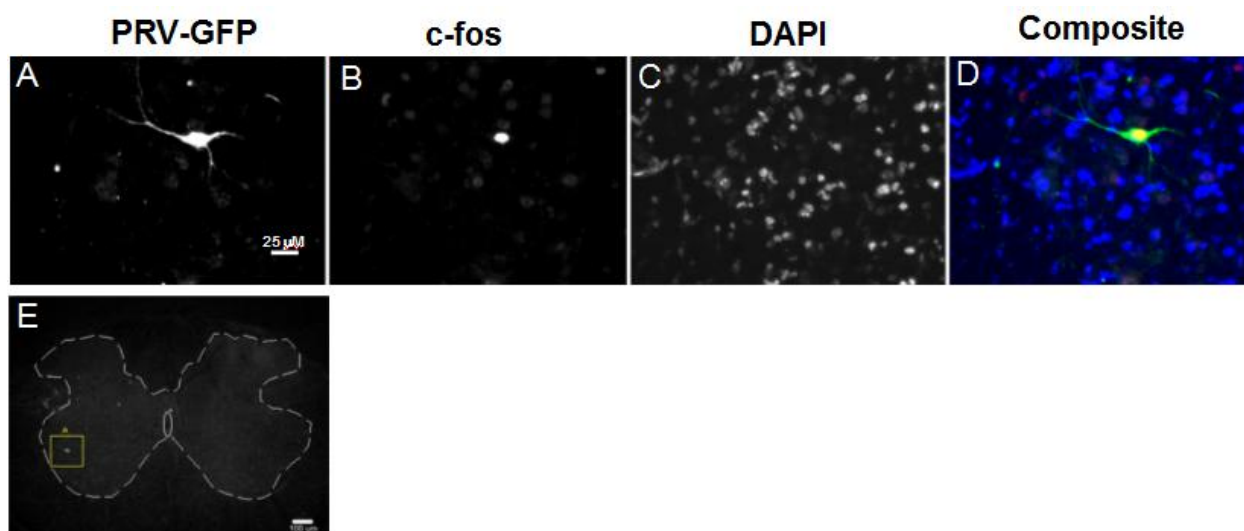
**Figure 11. Immunohistochemistry of Mouse lumbar region at PRV post injection time 84 hrs.**

Immunohistochemical staining of lumbar sections using anti-GFP (A) and anti-c-fos antibodies (B) were carried out to identify activated PRV infected interneurons in intact mice stepped on a treadmill at a $0^o$ incline. C. DAPI staining is used to label interneuron nucleus. D. PRV infected neurons (GFP positive cell bodies, green), activated (c-fos positive nuclei, red) and DAPI (nuclei, blue). E. Low magnification of A showing the entire lumbar section. Scale bar = 25 μM for A-D and 100 μM for E.
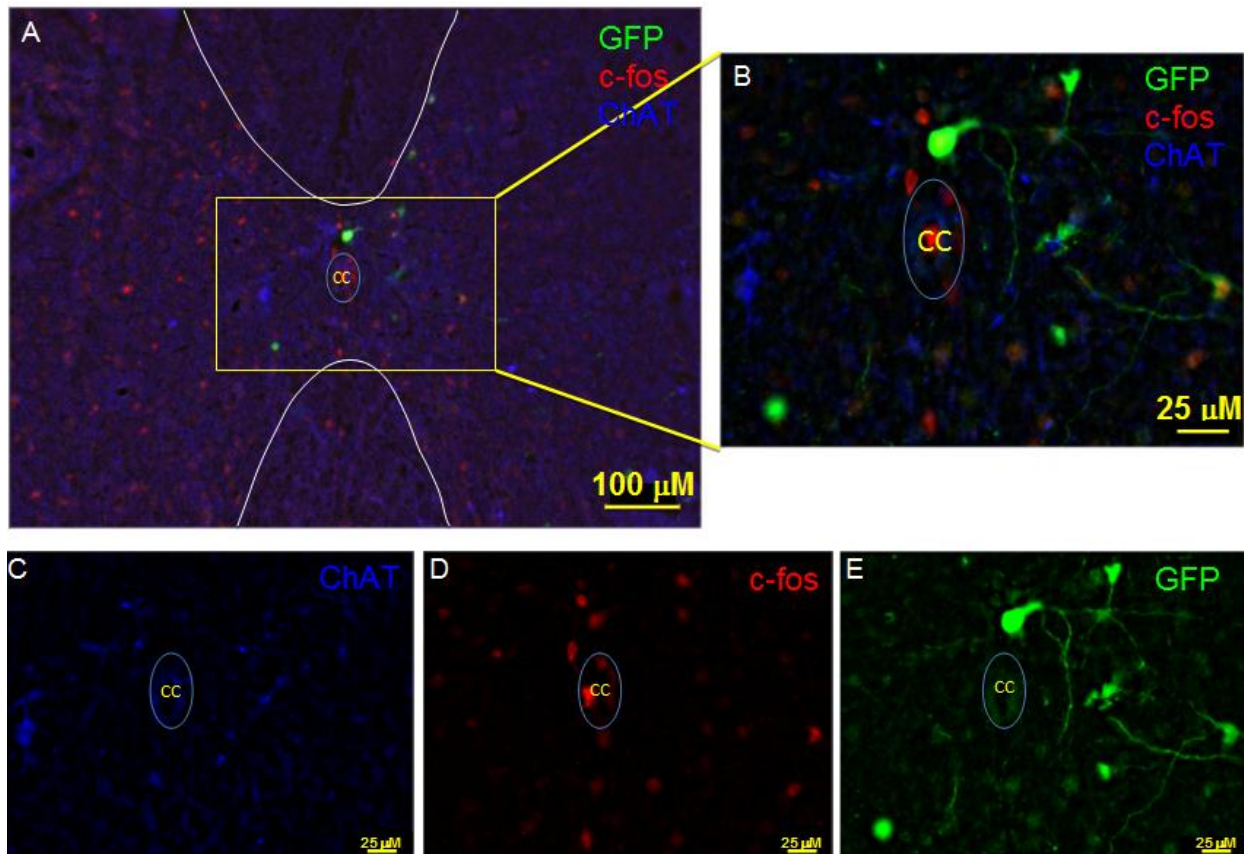
**Figure 12. Activation of PRV infected cholinergic neurons in intact mice.**

Triple immunohistochemical staining of lumbar L3-L5 sections using anti-ChAT, anti-GFP and anti-c-fos antibodies were carried out to identify activated Cholinergic and PRV infected interneurons surrounding the central canal in intact mice stepped at a $0^o$ incline. These interneurons surrounding the central are shown in the A-E images: Cholinergic neurons (ChAT positive cell bodies, blue), activated (c-fos positive nuclei, red) and PRV (GFP positive cell bodies, green).

Panel A shows a low magnification image of a mouse lumbar section showing activated, cholinergic and PRV infected interneurons. Panel B shows a magnified image of the area around the central canal marked by a rectangle in A. Panels C-E shows images of cholinergic (C), activated neurons (D), and PRV infected interneurons (E) around the central canal respectively. Scale bar= 100 μM for A and 25 μM for B-E.
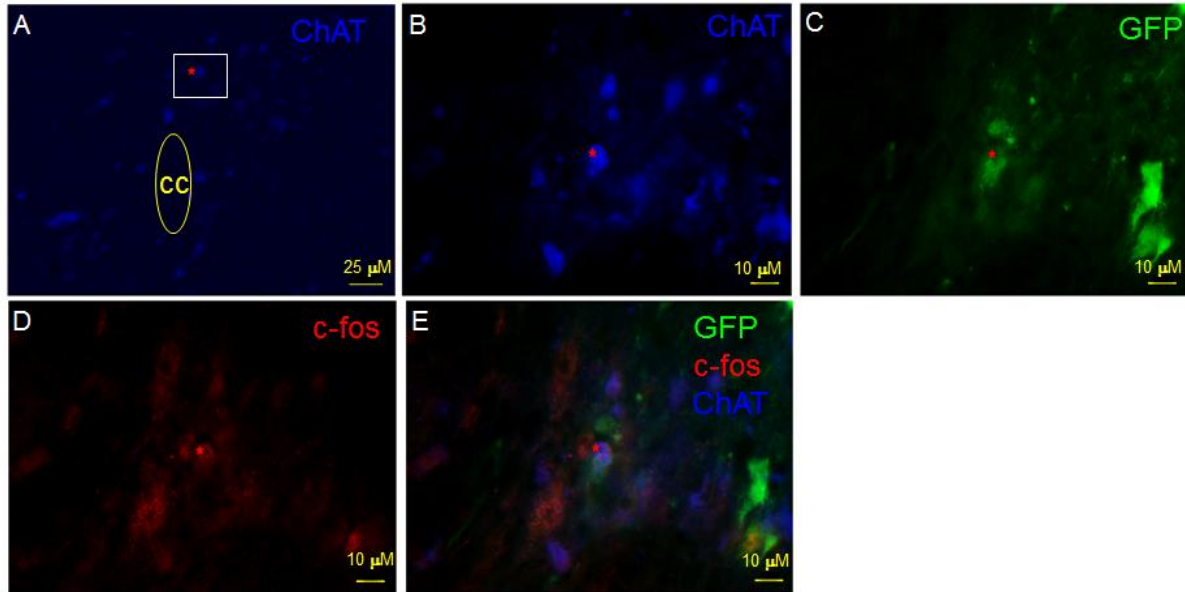
**Figure 13. Activated cholinergic neurons in TA spinal circuitry in lumbar 2 segment.**

Triple immunohistochemical staining of lumbar L2 sections using anti-ChAT, anti-GFP and anti-c-fos antibodies were carried out to identify activated cholinergic and PRV infected interneurons surrounding the central canal in intact mice stepped on a treadmill at a $0^o$ incline. Presence of triple-labeled neurons around the central canal (I.e., ChAT+ GFP+ c-fos+), shows that TA motoneurons are functionally connected to these cholinergic cells in L2 lumbar segment and get activated during stepping. These interneurons surrounding the central canal are shown in the A-E images: Cholinergic neurons (ChAT positive cell bodies, blue), activated (c-fos positive nuclei, red) and PRV (GFP positive cell bodies, green).

The inset in A is magnified in images B-E to show cholinergic (B), PRV infected (C), and activated interneurons (D). Panel E shows the composite image for B-D. Scale bar = 25 μM for A and 10 μM for B-E.
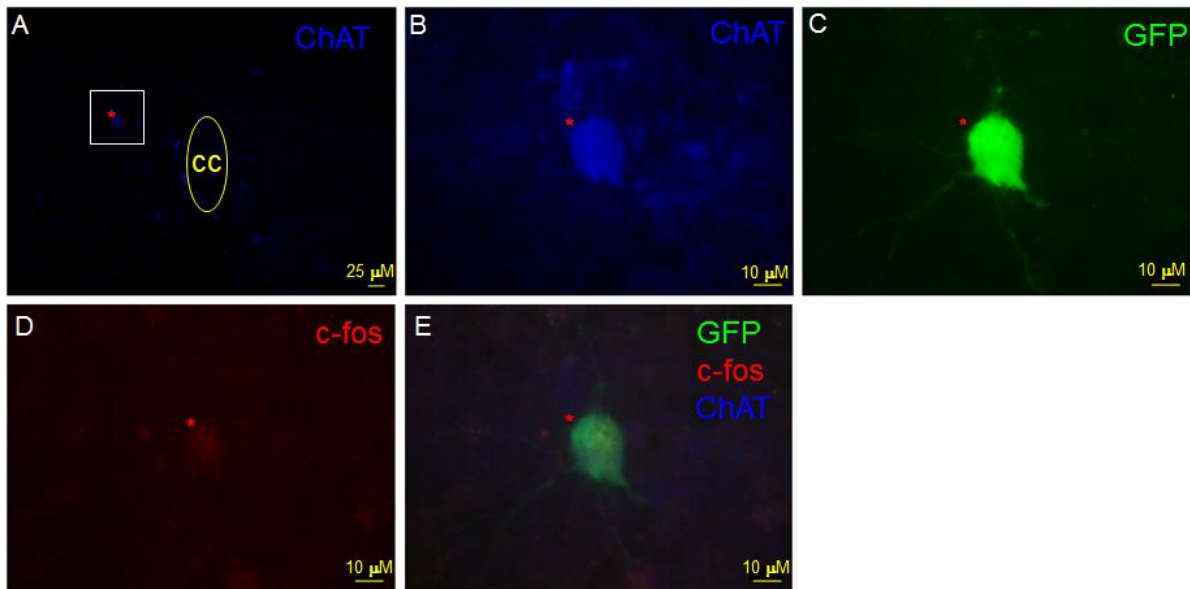
**Figure 14. Activated cholinergic neurons in TA circuitry in lumbar 4 segment.**

Triple immunohistochemical staining of lumbar L4 sections using anti-ChAT, anti-GFP and anti-c-fos antibodies were carried out to identify activated cholinergic and PRV infected interneurons surrounding the central canal in intact mice stepped on a treadmill at a $0^o$ incline. Presence of triple-labeled neurons around the central canal (I.e., ChAT+ GFP+ c-fos+), shows that TA motoneurons are functionally connected to these cholinergic cells in L4 lumbar segment and get activated during stepping. These interneurons surrounding the central canal are shown in the A-E images: Cholinergic neurons (ChAT positive cell bodies, blue), activated (c-fos positive nuclei, red) and PRV (GFP positive cell bodies, green).

The inset in A is magnified in images B-E to show cholinergic (B), PRV infected (C), and activated interneurons (D). Panel E shows the composite image for B-D. Scale bar = 25 µM for A and 10 µM for B-E.
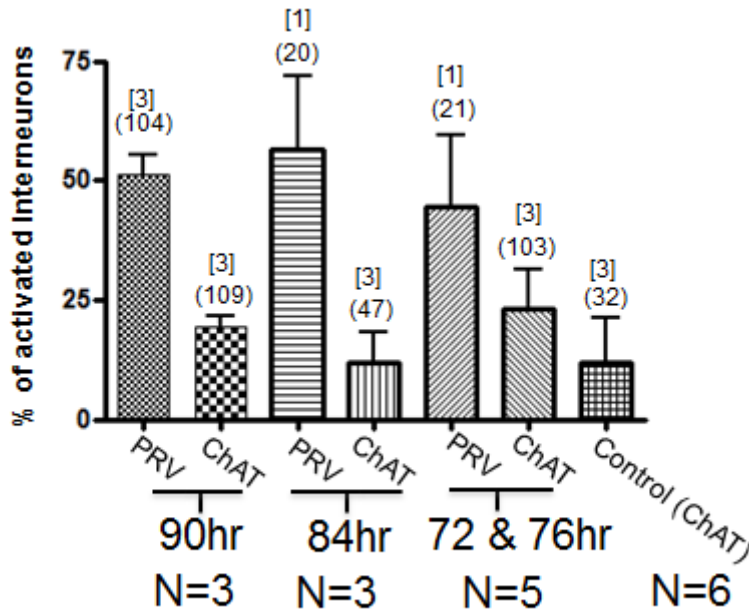
**Figure 15. Activated PRV infected/cholinergic interneurons in intact mice.**

The Bar graphs show the activated cholinergic (i.e., c-fos+ ChAT+) and PRV infected (c-fos+ GFP+) interneurons as a percent of total interneurons within their respective class surrounding the central canal area in L3-L5 spinal cord. The animal groups consisted of different post-injection times for PRV (90hr, 84hr, 76 hr, and 72hr). The number of PRV+ c-fos+ interneurons in these four time points were not statistically significant from each other. Therefore, we pooled the data from all points for calculations in Fig. 16. Similarly, all time points had similar levels of percent activation for cholinergic interneurons (i.e., ChAT+ c-fos+). The control mice were not injected with the PRV, and therefore only have values for activated cholinergic interneurons. The numbers in parenthesis are the total neurons analyzed, while numbers in brackets are average number of class specific positive (ChAT or GFP) neurons/section.
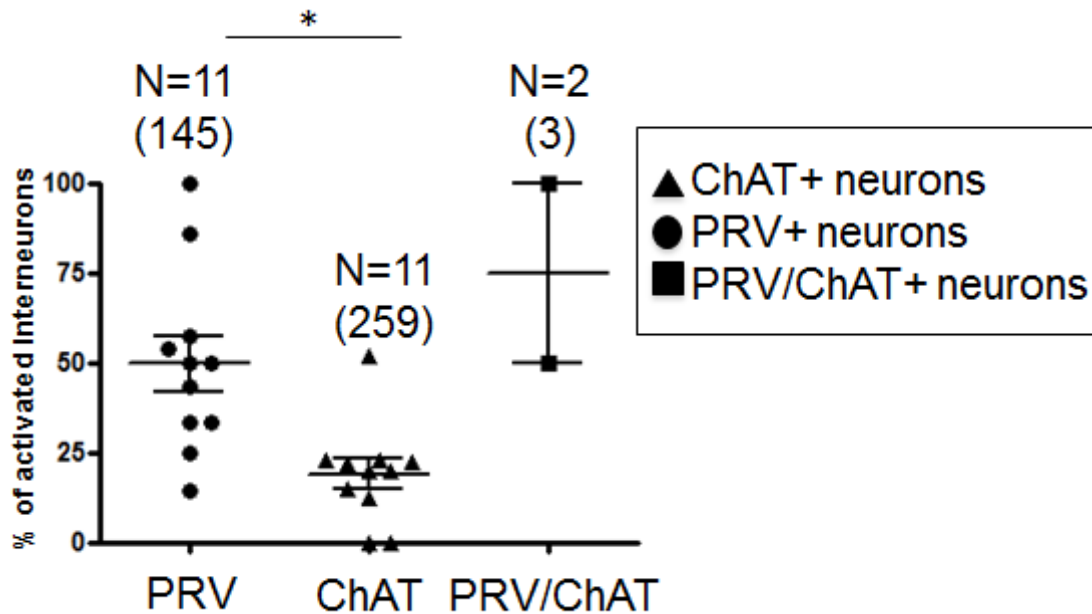
**Figure. 16. Different types of activated interneurons in intact mice.**

The scatter plot shows activated cholinergic (i.e., c-fos+ ChAT+), PRV infected (c-fos+ GFP+) and PRV/cholinergic (c-fos+ ChAT+/GFP+) interneurons as a percent of total interneurons within their respective class surrounding the central canal area in L3-L5 spinal cord for mice stepped on a treadmill at a 0° incline. The total number of analyzed interneurons in each group is shown within parenthesis. About 50% of the PRV infected cells (n=145) around the central canal are activated and are non-cholinergic. There were only 3 double labeled (PRV+/ChAT+) interneurons in the sections analyzed.
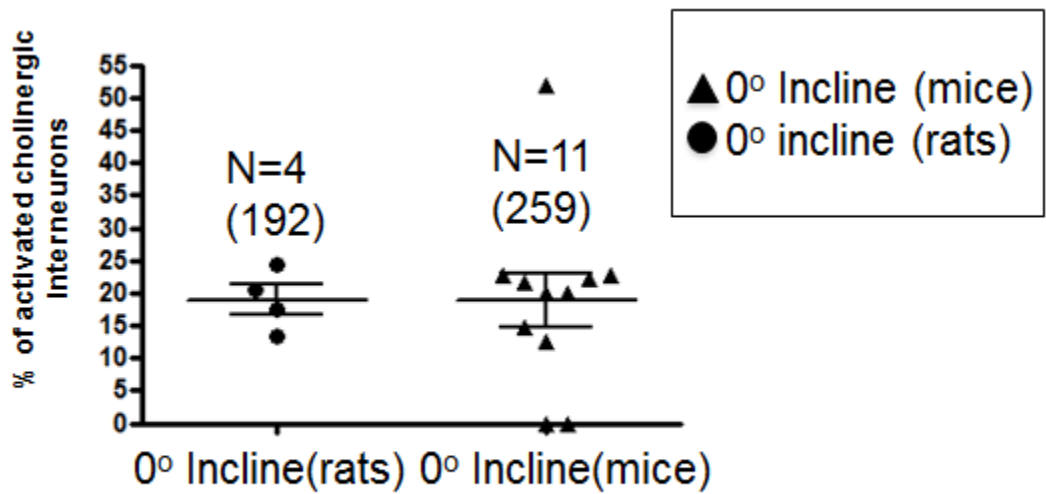
**Figure 17. Activated Cholinergic neurons in quadrapedally stepped intact rats and mice**

The scatter plot shows the activated cholinergic (i.e., c-fos+ ChAT+) interneurons as a percent of total ChAT+ interneurons surrounding the central canal area in L3-L5 spinal cord for mice and rats stepped on a treadmill at a $0^o$ incline. The activation of cholinergic interneurons around the central canal after quadrapedal stepping is similar in both intact mice and rats.

**Figure 18. Schematic view of PRV Pathway**.

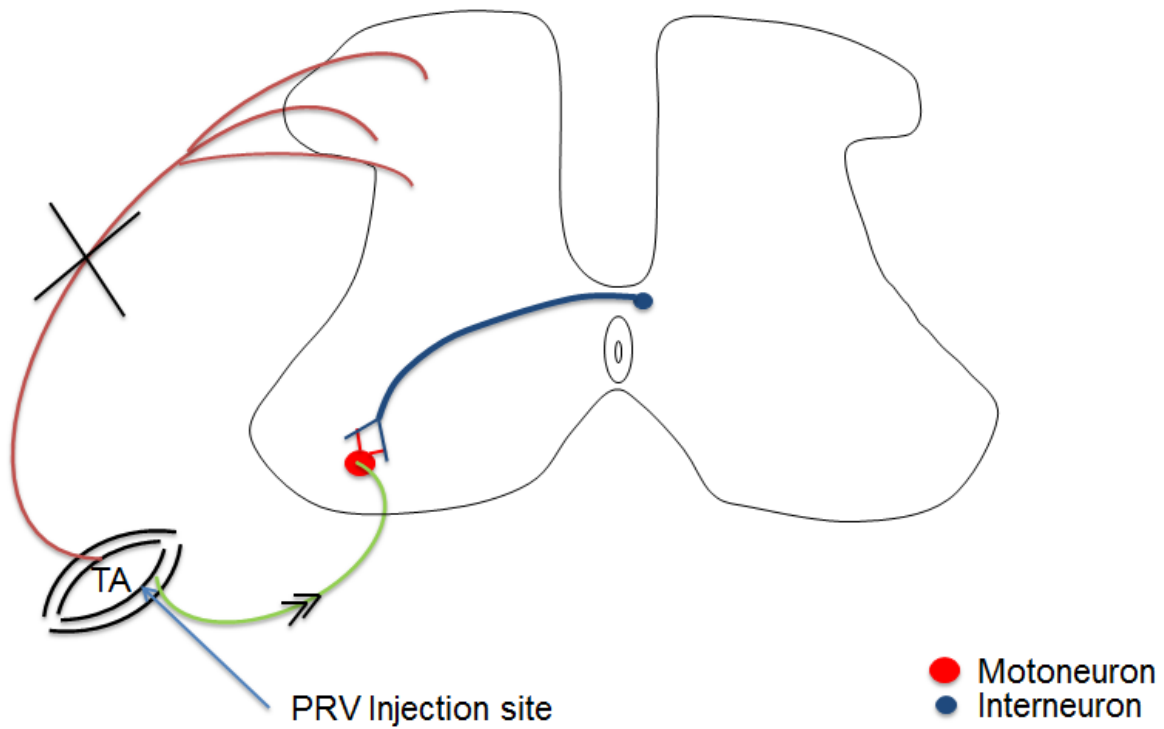The Pseudo Rabies virus (PRV)-152 with green fluorescent protein was injected into the Tibialis Anterior (TA) muscle of adult intact mice.  The virus is transported from the muscle to the TA motoneurons through its efferent projections.   Virus then travels from motoneurons to rostral, caudal, bilaterally located spinal interneurons via retrograde transynaptic transport.

**Table 4. Summary of activated cholinergic interneurons infected with PRV.**

| Lumbar Segment | % of PRV positive cholinergic interneurons | % of PRV positive central canal cholinergic interneurons | % of Activation of PRV positive cholinergic interneurons |
|---|---|---|---|
| L2 | 0.8%    (2/228) | 3.8%    (2/53) | 50%   (1/2) |
| L4 | 1.6%    (1/64) | 4%    (1/25) | 100%  (1/1) |

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