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Spinal neuronal activation during locomotor-like activity enabled by epidural stimulation and 5-HT agonists in spinal rats

Paul O. Duru^{1,*}, Niranjala J.K. Tillakaratne^{1,4,*}, Jung A. Kim^{*,#}, Hui Zhong^{1,*}, Stacey M. Stauber¹, Trinh T. Pham¹, Mei S. Xiao¹, V. Reggie Edgerton^{1,2,3,4}, and Roland R. Roy^{1,4}

¹Department of Integrative Biology and Physiology, University of California, Los Angeles, Los Angeles, CA 90095

²Department of Neurobiology, University of California, Los Angeles, Los Angeles, CA 90095

³Department of Neurosurgery, University of California, Los Angeles, Los Angeles, CA 90095

⁴Brain Research Institute, University of California, Los Angeles, Los Angeles, CA 90095

Abstract

The neural networks that generate stepping in complete spinal adult rats remain poorly defined. To address this problem we used c-fos (an activity-dependent marker) to identify active interneurons and motoneurons in the lumbar spinal cord of adult spinal rats during a 30-minute bout of bipedal stepping. Spinal rats were either step trained (30 min/day, 3 days/week for 7.5 weeks) or not step-trained. Stepping was enabled by epidural stimulation and the administration of the serotonergic agonists quipazine and 8-OHDPAT. A third group of spinal rats served as untreated (no stimulation, drugs, or stepping) controls. The number of activated cholinergic central canal cluster cells and partition neurons was higher in both step-trained and non-trained than untreated rats, and higher in non-trained than step-trained rats. The latter finding suggests that daily treatment with epidural stimulation plus serotonergic agonist treatment without step training enhanced the excitability of a broader cholinergic interneuronal population than step training. The number of activated interneurons in laminae II–VI of lumbar cross sections was higher in both step-trained and non-trained than untreated rats, and highest in step-trained rats. This finding suggests that this population of interneurons was responsive to epidural stimulation plus serotonergic treatment and that load-bearing induced when stepping had an additive effect. The number of activated

Send correspondence to: Niranjala Tillakaratne, Ph.D., Department of Integrative Biology and Physiology, 621 Charles E. Young Drive, Los Angeles, CA 90095-1606, Telephone: (310) 206-2575; FAX: (310) 933-1965; nirat@lifesci.ucla.edu.

*contributed equally to the study

#Current address: Department of Exercise Science, University of Puget Sound, Tacoma, WA 98416

Role of authors:

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: RRR, NJKT, VRE, JAK, POD. Surgical procedures: RRR and HZ. Acquisition of data: POD, NJKT, JAK, HZ, SS, TP, and MSX. Analysis and interpretation of data: POD, NJKT VRE, and RRR. Drafting of the manuscript: POD NJKT, VRE, and RRR. Critical revision of the manuscript for important intellectual content: NJKT, POD, VRE, and RRR. Statistical analysis: POD. Obtained funding: RRR, VRE, and NJKT. Administrative, technical, and material support: SS, TP, MSX. Study supervision: RRR, NJKT, JAK, HZ.

Conflict of interest statement:

All authors declare that they do not have any known or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

motoneurons of all size categories was higher in the step-trained than the other two groups, reflecting a strong effect of loading on motoneuron recruitment. In general, these results indicate that the spinal networks for locomotion are similar with and without brain input.

Keywords

serotonergic agonists; central canal cluster cells; partition neurons; c-fos; RRID:AB_2106765; RRID:AB_2079751

Introduction

Several interventions have proven to be effective in modulating the spinal circuitry to recover some motor function in adult rats after a complete spinal cord transection at a mid-thoracic level. For example, epidural stimulation of the lumbosacral region of the spinal cord, administration of pharmacological agents such as the 5-HT agonists quipazine and 8-OHDPAT, and/or motor training have been effective for the recovery of bipedal stepping in spinal rats (Edgerton et al., 2012; Rossignol et al., 2011; Sławińska et al., 2014; D'Amico et al., 2014). The spinal networks associated with this recovery, however, are still largely undefined.

c-fos has been used as a marker to identify active locomotor-associated neurons within the lumbosacral spinal cord in intact (Ahn et al., 2006; Tillakaratne et al., 2014) and spinal (Ichiyama et al., 2008b; Courtine et al., 2009) rats and decerebrated cats (Dai et al., 2005; Huang et al., 2000). We have shown that treadmill locomotion activates interneurons in both the dorsal and ventral horn and motoneurons in intact rats and that the level of activation is related to the amount of loading on the hindlimbs (Tillakaratne et al., 2014). For example, we found a higher percent of activated interneurons associated with soleus and tibialis anterior muscle afferents in the majority of spinal laminae in rats stepped on a treadmill at a 25° compared to a 0° incline. We also found a close relationship between the number of Fos⁺ cholinergic interneurons around the central canal and the level of loading (increasing incline). Similarly decerebrate cats undergoing mesencephalic locomotor region-stimulated fictive locomotion showed activated cholinergic interneurons around the central canal (Huang et al., 2000). These cholinergic interneurons primarily located lateral to the central canal are thought to be involved in modulating motoneuronal excitability (Miles et al., 2007).

The purpose of the present study was to identify the interneurons within the spinal networks that are active during locomotion in adult, chronic spinal rats, i.e., without any supraspinal influence, and to determine if there is a load-related effect as observed in intact rats (Tillakaratne et al., 2014). We facilitated bipedal stepping in spinal rats via epidural stimulation in the lumbosacral region of the spinal cord and the administration of serotonergic agonists (Ichiyama et al., 2005, 2008a; Gerasimenko et al., 2007; Courtine et al., 2009). We determined the number and location of activated motoneurons and interneurons in transverse sections of the L3-L5 spinal cord segments, and placed emphasis on the cholinergic interneurons around the central canal because of their known function in locomotion (Huang et al., 2000; Miles et al., 2007). To parcel out the effects of locomotion

from the stimulation and drug interventions, we included a group of rats that were treated similarly to the step trained rats except that they were not stepped. We hypothesized that a larger number and somewhat unique population of motoneurons and interneurons would be activated in the step-trained compared to non-step-trained rats, reflecting the role of the afferent information emanating from the weight-bearing activity during stepping. In general, the results are consistent with this hypothesis.

Materials and Methods

Experimental Design

Nineteen adult female Sprague-Dawley rats (200-290 g body weight) were assigned randomly to one of three groups: suspended in a harness vertically such that the feet did not touch any surface (Susp: no stimulation, n=5), suspended plus epidural stimulation and administration of 5-HT agonists (see below) (Susp/Stim/Drug: stimulation/drug induced air stepping, n=5), or step-trained plus stimulation and administration of 5-HT agonists (Step-Tr/Stim/Drug: bipedal step training plus stimulation and drug, n=9). All procedures were performed according to institutional and governmental regulations, and in accordance with the guidelines set and delineated by the University of California, Los Angeles (UCLA) Chancellor's Animal Research Committee concerning the ethical use of animals.

Surgical Procedures

All survival surgical procedures were performed under aseptic conditions and with the rats deeply anesthetized using isoflurane gas administered via a facemask as needed. A partial laminectomy was performed at the T8-T9 vertebral level. A complete spinal cord transection to include the dura was performed at approximately the T8 spinal level using microscissors. Two surgeons verified the completeness of the transection by lifting the cut ends of the spinal cord and passing a glass probe through the lesion site. Gel foam was inserted into the gap created by the transection as a coagulant and to separate the cut ends of the spinal cord. For epidural electrode implantation, partial laminectomies were performed to expose the spinal cord at spinal levels L2 and S1. Two Teflon-coated stainless steel wires (AS632, Cooner Wire, USA) from a head connector were passed subcutaneously to the back incision then under the spinous processes and above the dura mater of the remaining vertebrae between the partial laminectomy sites. After removing a small portion (~1 mm notch) of the Teflon coating and exposing the conductor on the surface facing the spinal cord, the electrodes were sutured to the dura mater at the midline of the spinal cord above and below the electrode sites using 8.0 Ethilon suture (Ethicon, New Brunswick, NJ). One common ground wire (~1 cm of the Teflon removed distally) was inserted subcutaneously in the mid-back region. The wires were coiled in the back region to provide stress relief. A skin incision was made along the sagittal suture of the skull and the connective tissue and the muscles covering the skull were reflected laterally. The skull was thoroughly dried and three stainless steel screws were firmly inserted into the exposed bone. The head connector, an Omnetics circular connector (Omnetics, Minneapolis, MN, USA), was placed between the screws and rigidly affixed to the bone using dental cement.

All incision areas were irrigated liberally with warm, sterile saline solution. All surgical sites were closed in layers using 5.0 Vicryl (Ethicon, New Brunswick, NJ) for all muscle and connective tissue layers and for the skin incisions in the hindlimbs and 5.0 Ethilon for the back skin incision. All closed incision sites were cleansed thoroughly with saline solution. Analgesia was provided by buprenex (0.5–1.0 mg/kg, s.c. 3 times/day). The analgesics were initiated before completion of the surgery and continued for a minimum of 2 days. The rats were allowed to fully recover from anesthesia in an incubator. The rats were housed individually in cages that had ample CareFresh bedding, and the bladders were expressed manually 3 times daily for the first 2 weeks after surgery and 2 times daily thereafter. The hindlimbs were moved passively through a full range of motion once per day to maintain joint mobility. All of these procedures are performed routinely in our laboratory and have been described previously (Courtine et al., 2009; Gad et al., 2013).

Bipedal Step Training with Epidural Stimulation and Serotonergic Agonists

The rats in the Step-Tr/Stim/Drug group were stepped bipedally on a specially designed motor-driven rodent treadmill using a body weight support system (Timoszyk et al., 2002). Stepping was facilitated by bipolar epidural stimulation (40 Hz) between L2 and S1 during the entire training session and the administration of quipazine (0.3 mg/kg) and of 8-OHDPAT (0.15 mg/kg) subcutaneously 15 min prior to each training session (Courtine et al., 2009; Gad et al., 2013). The rats supported between 10–40% of their body weight during stepping. The rats were trained 3 days/week, 30 min/session for 7.5 weeks beginning 1 week post-surgery. The rats in the Susp/Stim/Drug group were treated similarly except that they were not stepped on the treadmill.

Tissue Preparation

On the last experimental day, to induce Fos activity the rats in the Step-Tr/Stim/Drug group were stepped bipedally on a treadmill for 30 min while the Susp/Stim/Drug group was suspended for 30 min. Both groups received epidural stimulation for 30 min and the serotonergic agonists were administered 15 min before starting the experimental session. The Susp group was suspended for 30 min without any stimulation or drug intervention. After the 30-min session, all rats were returned to their home cages for one hour to allow for maximum Fos expression (Morgan et al., 1987). The rats then were perfused transcardially with a fixative solution of cold 4% paraformaldehyde (PF) in phosphate buffered saline (PBS; 130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, Sigma). After the perfusion, the spinal cords were dissected and post-fixed in 4% PF overnight at 4°C. With the dorsal root ganglion of L5 as a landmark, the spinal cord was divided into T13-L2, L3-L5, and L6-S2 blocks, frozen with Neg 50 (Thermo Scientific, Hudson, NH), and stored at -80°C until ready to cut. The frozen blocks were transversely sectioned serially at 30 µm using a cryostat, placed as free-floating sections and stored in 96 well plates filled with PBS containing 0.02% sodium azide (Sigma) at 4°C. The lesion site and adjoining rostral and caudal spinal cord segments were cryosectioned (20 µm thick sections) and alternate sections were mounted on slides in a sequential fashion for histological staining. Luxol blue (myelin) and cresyl violet (neurons and glia) stains were used to verify the completeness of the spinal cord transection (Kluver and Barrera 1953).

Antibody Characterization

See Table 1 for a list of antibodies used. The c-Fos antibody recognized a single band of 62 kD MW on Western blots of PMA-treated Jurkat cells (manufacturer's datasheet), and nuclear localization in the mouse spinal cord that is similar to previous reports (González et al 2001; Rashid and Ueda, 2005). The choline acetyltransferase antibody recognized a single band of ~68-70 kD MW on Western blots of mouse brain lysate (manufacturer's datasheet). Immunostaining of motoneurons and other cholinergic neurons in mouse spinal cord was similar to that reported in previous studies (Wilson et al 2004; Marina et al 2008; Stepien et al 2010).

Immunohistochemical Analyses

Every fourth section was examined and the beginning and end of each lumbar segment was determined to select representative motoneurons at similar locations across animals in each group for immunohistochemical analysis. Choline acetyltransferase (ChAT, the synthetic enzyme of acetylcholine) was used to identify cholinergic neurons. Double-labeled immunohistochemistry with anti-c-Fos (Sc7202, Santa Cruz Inc., CA) and anti-choline acetyltransferase (ChAT AB144P, EMD Millipore Inc, Temecula, CA) was used to identify activated cholinergic neurons (ChAT⁺/Fos⁺) around the central canal and motoneurons. We counted all ChAT⁺/Fos⁺ neurons in 7-11 sections from L3-L5 from each rat (Susp (n=5), Susp/Stim/Drug (n=5) and Step-Tr/Stim/Drug n=6) to provide an adequate sampling of activated cholinergic cells (ChAT⁺/Fos⁺). For the analysis of activated motoneurons, we used two sections each from the L3-L5 spinal cord segments (n=6 sections total/rat; number of rats per group: Susp (n=4), Susp-Stim/Drug (n=5), and Step-Tr/Stim/Drug (n=10). A range of 58-109 at L3 (total 1202), 88-91 at L4 (total 1612), and 90-109 at L5 (total 1854) motoneurons per animal, and 34-48 cholinergic interneurons per animal (total 548) were analyzed. The number of Fos⁺ interneurons in each lamina was determined in 1 spinal cord section from rats in the Susp (n=4), Susp/Stim/Drug (n=5), and Step-Tr/Stim/Drug (n=10) groups.

For immunohistochemistry, sections were processed as free-floating sections (Ahn et al., 2006). Briefly, sections were transferred into Costar netwells (15 mm membrane diameter, 74 µm mesh) and rinsed for 30 min in 1xPBS. The sections then were transferred to 24-well plates containing 200 µl/well of a mixture of c-fos antibody (1:400) with ChAT (1:500) in 1xPBS containing 0.3% Triton X100 (PBST) and incubated overnight at 4°C on an orbital shaker. Sections were washed in 1xPBS as follows: 2 quick rinses, followed 2×5 min rinses, and 2×10 min rinses. Sections then were incubated in a mixture (200 µl/well, 24-well plate) of secondary antibodies (anti-rabbit Dylight 594 1:500 and anti-goat FITC 1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hr at room temperature. Sections were washed extensively as above, mounted on Fisher Superfrost slides (Fisher Scientific, Pittsburg, PA) and coverslipped with Vectashield mounting media containing DAPI (Vector Labs, Burlingame, CA).

Identification of Cholinergic Interneurons

The staining characteristics of the cholinergic interneurons around the central canal have been described previously (Tillakaratne et al., 2014). Cholinergic interneurons around the

central canal and medial lamina VII were classified as central canal cluster cells, partition neurons, or small dorsal horn neurons (Barber et al., 1984; Phelps et al., 1984). Central canal cluster cells are a group of small cholinergic cells located in lamina X. Partition neurons are larger than the central canal cluster cells and are located in lamina VII, with projections from the central canal to the outer edge of the grey matter. Small dorsal horn neurons are located in laminae V/VI and above the central canal. Therefore, we identified neurons located in lamina X and less than 130 μm from the center of the central canal as central canal cluster cells and neurons located in lamina VII and 130-350 μm lateral to the center of the central canal as partition neurons. Cholinergic interneurons in laminae V/VI and >130 μm dorsal to the central canal were considered to be small dorsal horn neurons (Fig. 1A).

Data Analyses

Spinal cord sections processed for fluorescent immunohistochemistry were examined under Zeiss (Axiophot) microscope under appropriate fluorescent filter sets. Digital images of neurons labeled with Fos, DAPI, and ChAT were acquired using a Spot RT CCD Slider color camera (Diagnostics Instruments, Inc., Sterling Heights, MI) and the Image Pro Plus 7 image analysis program (Media Cybernetics, Rockville, MD). Composite images of Fos, DAPI, and ChAT were created using color composite feature of Image Pro. Soma sizes of motoneurons were measured by manually outlining individual motoneurons using Image Pro. Neurons of the following types were tagged using the manual tag analytical feature of Image Pro Plus: 1) activated cholinergic interneurons (Fos⁺ nuclei and cell bodies labeled by ChAT: ChAT⁺/Fos⁺; Fig. 1C and D, insets), 2) non-activated cholinergic interneurons (ChAT⁺/Fos⁻; Fig. 1B, inset), 3) activated (Fos⁺) interneurons in laminae I-VI; Fig. 3A-C), 4) activated motoneurons (ChAT⁺/Fos⁺; Fig. 4B-C, arrows), and 5) non-activated motoneurons (ChAT⁺/Fos⁻; Fig. 4A-C). Laminae and motor column borders were drawn onto spinal cord sections according to the spinal cord atlas of the adult rat with tagged neurons (Watson et al., 2009) and the numbers of activated interneurons in individual spinal cord laminae were counted. The number of activated cholinergic neurons around the central canal in L3-L5 (i.e., ChAT⁺/Fos⁺) was calculated as a percent of total ChAT⁺ interneurons (i.e., ChAT⁺/Fos⁺ divided by the total number of ChAT⁺ interneurons). The number of ChAT⁺/Fos⁺ neurons is reported for all neurons combined and for cluster cell, partition neurons, or small dorsal horn cell sub-groups (Fig. 2). The percent of total activated motoneurons per soma size bin was calculated by dividing the number of Fos⁺ motoneurons in that bin divided by the total number of motoneurons across all bins.

Statistical Analyses

Mean comparisons of the percentages and number of Fos⁺ cholinergic neurons, interneurons and motoneurons as well as the number of activated interneurons based on lamina location among the three groups were carried out using variations of the ANOVA model. For Figures 2 (Fos⁺ cholinergic interneurons) and 6A-D (motoneurons), mean percentages were compared using the non-constant variance analysis of variance (ANOVA) model. Normality was evaluated using quantile-quantile plots of the residuals. The constant variance assumption was evaluated using plots of the residuals vs. the means. We determined that the data had a Gaussian distribution but variances were not constant across groups. We therefore used the non-constant variance model allowing for separate variance estimates within each

group. For Figure 3D (number of Fos⁺ interneurons) and Figure 5A (number of Fos⁺ motoneurons), the data on the original scale were skewed but the data on the log scale followed a Gaussian distribution. Therefore, means were compared using the analysis of variance ANOVA model on log-transformed data. For the outcome of Figure 3E, we could not use a parametric model because the number of activated interneurons based on lamina location did not follow a Gaussian distribution on either the original or any transformed scale. Therefore, a non-parametric repeated measures ANOVA model with re-sampling was used to compare mean counts by group and location. Standard errors of the means were estimated empirically using 1000 re-samples of the data under this model.

To compare the mean percentage of activated motoneurons among the three groups based on motoneuron size we used a logistic model. Linearity between size and percent activation on the log odds scale was evaluated by fitting splines under the logistic model with respect to intervention (Fig. 5B). Based on this, we determined that size had a nonlinear, non-monotone relationship with (log odds) activation for most of the groups. Moreover, the shape of the relationship was not the same across all groups. We therefore modeled the relationship using size categories (bins) in steps of 400 μm^2 (<500, 500-900, 900-1300, 1300-1700). The cell counts for motoneurons above 1700 μm^2 were negligible and therefore were not used in generating the logistics model. The criterion level for statistical significance was $P < 0.05$ for all analyses.

Results

Activation levels of cholinergic interneurons around the central canal

Three types of cholinergic interneurons (central canal cluster cells, partition neurons and small dorsal horn cells) around the central canal were identified based on which laminae they were located in and their distance from the central canal (Fig. 1A) Examples of non-activated and activated cholinergic interneurons are shown in Figure 1B, and Figure 1C and D, respectively (magnified in the lower left of each panel). The percent of total activated cholinergic interneurons (Fig. 2A) and central canal cluster cells (Fig. 2B) was higher in the Susp/Stim/Drug and Step-Tr/Stim/Drug groups than the Susp group and higher in the Susp/Stim/Drug than Step-Tr/Stim/Drug group. A similar pattern was observed for the partition neurons except that there was no difference between the two stimulated groups (Fig. 2C). Approximately 50% of the central canal cluster cells and 54% of the partition neurons were activated in the Susp/Stim/Drug (Fig. 2B and C). In contrast, 23% and 40% of the central canal cluster cells and partition neurons, respectively, were activated in step-trained rats. No differences were observed among groups for the small dorsal horn neurons (Fig. 2D).

Activation levels and laminar distribution of interneurons in the dorsal horn

Schematic representations of the laminar distribution of Fos⁺ interneurons in the dorsal horn of the Susp, Susp/Stim/Drug, and Step-Tr/Stim/Drug groups are shown in Figure 3A, B, and C, respectively. The overall number of Fos⁺ cells in the dorsal horn was higher in the two stimulated groups compared to the Susp group (Fig. 3D). This pattern also was observed in laminae II-V (Fig. 3E). The highest number of activated interneurons was in lamina V and

was approximately three-fold and four-fold greater in the Susp/Stim/Drug, and Step-Tr/Stim/Drug groups, respectively, than in the Susp group.

Activation levels of motoneurons

Examples of the identification of non-activated and activated motoneurons in each group are shown in Figure 4. The total number of Fos⁺ motoneurons was higher in the Step-Tr/Stim/Drug group than in the other two groups (Fig. 5A). The percent of total motoneurons activated was higher for each soma size bin in the Step-Tr/Stim/Drug group than in the other two groups (Fig. 5B). In addition, these values were higher for motoneurons < 500 μm^2 and between 500-900 μm^2 in the Susp/Stim/Drug than Susp group. A comparison of the total number and size distribution of Fos⁺ and Fos⁻ motoneurons across groups is shown in Figure 5C-E. Note that these distributions were bimodal in all groups with peaks at around 500-700 μm^2 and 1100 and 1500 μm^2 . The percent of activated motoneurons per cross section was higher in all four motor columns studied in the Step-Tr/Stim/Drug than Susp group and higher in motor column 3 in the Step-Tr/Stim/Drug than Susp/Stim/Drug group (Fig. 6).

Discussion

Decreased activation of cholinergic central canal cluster cells and partition neurons in step-trained spinal rats

The lower percentage of activated cholinergic interneurons around the central canal in Step-Tr/Stim/Drug than Susp/Stim/Drug rats could reflect a pruning effect of training on the activation of interneurons associated with the task, as has been reported for an overall decrease in neuronal activation with motor training (Ichiyama et al., 2008; Courtine et al., 2009). A decrease in the activation of these cholinergic interneurons in step-trained rats also could be due to an increase in upstream inhibitory influences indirectly through the control of pre-synaptic inhibition of primary afferents. Electrophysiological studies have demonstrated that cholinergic interneurons surrounding the central canal do not receive primary afferent input (Zagoraoui et al., 2009), but they may receive oligosynaptic sensory input (Witts et al., 2014) as well as rhythmic input from locomotor central pattern generators (Jordan et al., 2014; Witts et al., 2014). Fink and colleagues (2014) demonstrated that a subset of GABAergic (GAD₆₅) interneurons provide pre-synaptic inhibitory control over sensory input involved in performing skilled limb movements. We have shown that bilateral step or stand training alter GAD₆₇ mRNA expression in the ventral and dorsal horn neurons in spinal cats (Tillakaratne et al., 2002). In addition, down-conditioning of the soleus H-reflex increases GABAergic input to soleus motoneurons in intact rats (Wang et al., 2006). All of these data are consistent with there being less neuronal activation in performing a given task after training.

The administration of serotonergic agonists during the training sessions may have contributed to the group differences observed in the activation of these cholinergic cells. For example, bath application of 5-HT increased neuronal excitability of lamina X neurons in neonatal spinal cord preparations using c-fos-EGFP mice (Dai et al., 2009). Furthermore, *in vivo* electrophysiological properties of interneurons in lamina X showed phasic, tonic, or single spike activity in response to depolarizing step currents after a swimming task. Using

whole cell clamp recordings within the lumbar region of the neonatal rat spinal cord and pharmacological stimulation of the ventral roots, Kiehn and colleagues (1996) found that 88% of the spinal interneurons surrounding the central canal receive both excitatory and inhibitory input. These inputs are known to drive the rhythmic activity of the spinal interneurons and some of these interneurons have oscillating membrane potentials in phase with ventral root activity. These data suggest a heterogeneous population of neurons were being activated. Takeoka et al. (2009) observed 5-HT positive axons apposing central canal cluster cells and partition neurons in the lumbar segments below the lesion 7-8 months after a complete mid-thoracic spinal cord transection in adult rats. Step training, however, had no effect on the number of 5-HT appositions to these cholinergic interneurons surrounding the central canal. Electrophysiological studies have shown that activation of 5-HT₂ and 5-HT₃ receptors via administration of 5-HT or 5-HT agonists results in depolarization of rat dorsal root ganglion cells from the caudal lumbar region (Todorovic and Anderson, 1990). Many neurons near the central canal in decerebrate adult cats responding to electrical stimulation of sacral dorsal roots had 5-HT positive contacts (Honda and Lee, 1985). Combined, these results provide a basis for using caution when interpreting results associated with the levels of activation of interneurons, as if the interneurons are functionally homologous.

Sensory input associated with weight-bearing stepping activates hindlimb motoneuronal pools

In a recent study we compared the number of Fos⁺ motoneurons in both the soleus and tibialis anterior motor pools in uninjured cage control rats and rats that were stepped on a treadmill for 30 min at either a 0° or 25° (Tillakaratne et al., 2014). There was a progressive load-related increase in the number of activated motoneurons in both motor pools: 25° incline > 0° incline > cage control. The present results are consistent with this pattern of activation, i.e., the total number and the percent of activated motoneurons relative to the total number of motoneurons in each soma size bin for spinal segments L3-L5 was higher in the Step-Tr/Stim/Drug group than in the other two groups. In addition, these values were, in general, higher in the Susp/Stim/Drug than Susp group, most likely reflecting the enhanced movement of the limbs (and thus increased sensory input to the spinal cord) enabled by the epidural stimulation. Furthermore, the group differences in the proportion of Fos⁺ motoneurons less than 900 µm² vs. those between 900-1500 µm² suggest that there was an increasing proportion of Fos expression among the larger motoneurons in rats that were stepping with stimulation compared to the other two groups.

Effects of serotonergic agonists on the activation of motoneurons

Quipazine and 8-OHDPAT, serotonergic agonists, are routinely used to enhance stepping ability in spinal rats (Courtine et al., 2009; Gerasimenko et al., 2007; Ichiyama et al., 2008a, b; Slawinska et al., 2014). Quipazine also has been shown to induce non-locomotor and locomotor type movements in paraplegic mice (Landry et al., 2004; Fong et al., 2005). Spinal rats trained to step with quipazine and 8-OHDPAT administration plus epidural stimulation show improvement in hindlimb inter-limb coordination, weight-bearing ability, muscle activation (based on EMG recordings), and kinematics (Courtine et al., 2009; Gerasimenko et al., 2007). Previous studies on step training of spinal rats with epidural/pharmacological stimulation examined only the EMG activity in various hindlimb muscles,

but these data do not address the issue of selectivity of the activation of motoneurons with respect to their soma size or topology as performed in the present study.

The percentage of motoneurons activated was higher in all four motor columns studied in the Step-Tr/Stim/Drug than Susp group. In addition, this percentage was higher in the ventromedial motor column in the Step-Tr/Stim/Drug than Susp/Stim/Drug group. The ventromedial motor column contains more motoneurons from the L3-L5 spinal cord segments that innervate the hindlimb (Nicolopoulos-Stournaras et al., 1983). Thus this latter difference most likely reflects the contribution of stepping-associated sensory input activating the motoneurons in this motor column. The percent of total motoneurons activated was approximately two-fold greater in the spinal Step-Tr/Stim/Drug rats (~46%) than that reported for intact rats (~20%) (Ahn et al., 2006). Furthermore, the Susp/Stim/Drug group receiving serotonergic agonists showed considerable (~19%) motoneuronal activation. This finding is consistent with the report of step-like movements with alternate recruitment of extensor and flexor motor pools (based on EMG) in spinal rats treated similarly to the Susp/Stim/Drug group in the present study (Courtine et al., 2009). It appears that serotonergic agents induce a general, net increase of motoneuron excitability. Serotonergic agonists have been shown to act on 5-HT₂ receptors to increase the persistent sodium inward current and repetitive firing in sacral motoneurons of chronic spinal rats (Harvey et al., 2006). Quipazine, a 5-HT₂ agonist, also may increase the excitability of the lumbar motoneurons via a similar mechanism. Additionally, constitutively active 5-HT₂ receptors assist in the reestablishment of calcium-mediated persistent inward currents after spinal cord injury (D'Amico et al., 2012). These inward calcium currents help motoneurons sustain muscle contraction and recovery of locomotor function after injury (Murray et al., 2010). Although we did not identify Fos⁺ neurons associated with 5-HT boutons in the present study, activation of motoneurons also could be due to 5-HT associated interneurons. For example, Noga et al. (2009) identified serotonergic innervated interneurons in the thoraco-lumbar region that were activated after fictive locomotion in mesencephalic locomotor region-stimulated decerebrated cats. They found 80-100% of locomotor-activated interneurons co-localized with serotonergic boutons. In the L3-L5 region, 5-HT₇ receptors (80-100%) had the greatest percent of co-localization with Fos⁺ interneurons followed by 5-HT_{2A} (60-80%) and 5-HT_{1A} receptors (35-80%), respectively. Thus, the observed motoneuron activation patterns are likely to have reflected the interactions of the serotonergic agonists and the sensory input from the hindlimb movements.

We did not step the non-trained spinal rats on the last session as we have done previously (Ichiyama et al., 2008b, and Courtine et al., 2009). Thus we cannot determine if the non-trained rats had more nonspecific activation than the step-trained group. Furthermore, since the Susp/Stim/Drug and Step-Tr/Stim/Drug groups both received epidural stimulation and serotonergic agonists but differed in weight-bearing conditions and training status, whether the differences in activation between the groups in the present study are due to the step training and/or suspension/weight-bearing conditions cannot be distinguished. Essentially, however, the Susp/Stim/Drug group was stimulated for the same amount of time as the Step-Tr/Stim/Drug group. Thus the comparison of these two groups illustrates the combined effect of step-related proprioception and training.

In summary, we observed a lower percentage of Fos⁺ cholinergic interneurons around the central canal in Step-Tr/Stim/Drug than Susp/Stim/Drug rats, suggesting that step training reduces the activation of some types of spinal interneurons. This finding is consistent with earlier studies reporting a lower percentage of Fos⁺ neurons in step-trained than non-trained adult spinal rats (Ichiyama et al., 2008b; Courtine et al., 2009). Combined these results suggest that step training enhances the efficacy of more selective spinal sensorimotor pathways associated with the spinal locomotion. In addition, we observed a higher percentage of Fos⁺ motoneurons in the Step-Tr/Stim/Drug than Susp/Stim/Drug rats, most likely reflecting a greater recruitment of motoneurons due to increased loading on the hindlimbs during stepping. These data are consistent with our earlier report of a load-related increase in motoneuron recruitment in intact adult rats (Tillakaratne et al., 2014). The similarity in the responses of spinal and intact rats to specific afferent inputs indicates that the spinal networks can function behaviorally similar to that observed when there is input from the brain. These data also could be of importance clinically in that they suggest specific anatomical areas within the gray matter that could be useful targets for selective neuromodulation either electrically or pharmacologically.

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Significance Statement

We identified neurons within the spinal cord networks that are activated during assisted stepping in paraplegic rats. We stimulated the spinal cord and administered a drug to help the rats step. One group was trained to step and another not trained. We observed a lower percentage of activated neurons in specific spinal cord regions in trained than non-trained rats after a one-hour stepping bout, suggesting that step training reduces activation of some types of spinal neurons. This observation indicates that training makes the spinal networks more efficient and suggests a “learning” phenomenon in the spinal cord without any brain input.

Figure 1.

A schematic showing the distribution of different types of cholinergic interneurons located near the central canal (CC) (A). Partition neurons (purple) are located in lamina VII. Central canal cluster cells (orange) are located in lamina X. Small dorsal horn neurons (red) are located in laminae V and VI. Spinal cord cross-sections at L4 showing activated (cell body green and nucleus red) cholinergic interneurons around the CC for a representative rat from the Susp (B), Susp/Stim/Drug (C), and Step-Tr/Stim/Drug (D) groups. Box in B and boxes in C, and D identify non activated and activated cholinergic interneurons, respectively (magnified in the lower left of each panel). Green, ChAT⁺; red, Fos⁺; blue, nuclei stained with DAPI. Scales in (B) same for (C) and (D).

Figure 2. Percentages of all cholinergic interneurons (IN) (**A**), central canal cluster cells (**B**), partition neurons (**C**), and small dorsal horn neurons (**D**) that were Fos⁺ are shown for the Susp, Susp/Stim/Drug, and Step-Tr/Stim/Drug groups. Each symbol represents an individual rat. The horizontal lines indicate mean values. **P* < 0.05 vs. Susp; #*P* < 0.05 vs. Susp/Stim/Drug.

Figure 3.

Schematic representations of Fos expression within laminae I-VI and X for an L4 spinal cord section are shown for the Susp (A), Susp/Stim/Drug (B), and Step-Tr/Stim/Drug (C) groups. The total numbers of Fos⁺ interneurons (IN) within the dorsal horn per section are shown for each group (D). Each symbol represents an individual animal. The horizontal lines indicate the mean values. Laminar distributions (mean ± SEM) of activated IN within the dorsal horn are shown for each group (E). * $P < 0.05$ vs. Susp.

Figure 4.

Cross-sections at L4 showing non-activated (cell body green) and activated (cell body green and nucleus red; arrows in (B) and (C)) motoneurons located in the ventral horn for a representative rat from the Susp (A), Susp/Stim/Drug (B), and Step-Tr/Stim/Drug (C) groups. Green, ChAT⁺; red, Fos⁺; blue, nuclei stained with DAPI. A schematic of the location of the motoneurons (red box) are shown in panels (A)-(C) (D). Scale in (A) same for (B) and (C).

Figure 5.

Total numbers of Fos⁺ motoneurons (Mn) per animal in the L3-L5 segments for the Susp, Susp/Stim/Drug, Step-Tr/Stim/Drug groups are shown (A). The horizontal lines indicate mean values. Each symbol represents an individual animal. The percent activated motoneurons relative to the total number of motoneurons for each size bin for each group is shown (B). The distributions of soma sizes for the total number of motoneurons (Fos⁺ and Fos⁻) per animal for the Susp, Susp/Stim/Drug, and Step-Tr/Stim/Drug groups are shown in panels (C), (D), and (E), respectively. * $P < 0.05$ vs. Susp; # $P < 0.05$ vs. Susp/Stim/Drug.

Figure 6.

The percentages of activated motoneurons (Mn) within each motor column (MC) per tissue section for the L3-L5 segments are shown in panels (A)-(D) for each group. The horizontal lines indicate mean values. Each symbol represents an individual animal. A schematic of the location of the motor columns located within the ventral horn (E). * $P < 0.05$ vs. Susp; # $P < 0.05$ vs. Susp/Stim/Drug.

Table 1

Table of Primary Antibodies Used

Antigen	Description of Immunogen	Source, Host Species, Cat. #, RRID	Concentration Used
c-Fos (H-125)	Epitope corresponding to amino acids 210-335 mapping at the C-terminus of c-Fos of human origin	Santa Cruz Biotechnology, rabbit polyclonal, Cat# sc-7202, RRID:AB_2106765	0.5 µg/ul (IF) (1:400)
Choline Acetyltransferase	Affinity Purified Human placental enzyme	EMD Millipore, goat polyclonal, Cat# AB144P, RRID:AB_2079751	1:500 (IF)

IF-Immunofluorescence histochemistry