Whole cell recordings from visualized neurons in the inner laminae of the functionally intact spinal cord.

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ABSTRACT
The in vitro whole spinal cord preparation has been an invaluable tool for the study of the neural network that underlies walking, as it provides a means of recording fictive locomotor activity following surgical and/or pharmacological manipulation. The recent use of molecular genetic techniques to identify discrete neuronal populations in the spinal cord, and subsequent studies showing some of these populations to be involved in locomotor activity, have been exciting developments that may lead to a better understanding of the structure and mechanism of function of this neural network. It would be of great benefit if the in vitro whole spinal cord preparation could be updated to allow for the direct targeting of genetically-defined neuronal populations, allowing each to be characterized physiologically and anatomically. This report describes a new technique that enables the visualization of, and targeted whole cell patch clamp recordings from, genetically-defined populations of neurons while leaving connectivity largely intact. The key feature of this technique is a small notch cut in the lumbar spinal cord which reveals cells located in the intermediate laminae while leaving the ventral portion of the spinal cord, the region containing the locomotor neural network, untouched. Whole cell patch clamp recordings demonstrate that these neurons are healthy and display large rhythmic depolarizations that are related to electroneurogram bursts recorded from ventral roots during fictive locomotion. Intracellular labeling demonstrates that this technique can also be used to map axonal projection patterns of neurons. We expect that this procedure will greatly facilitate electrophysiological and anatomical study of important neuronal populations that comprise neural networks throughout the central nervous system.
INTRODUCTION

The manner in which specific neurons are interconnected to produce physiological outputs has long been a fundamental issue for neuroscientists, particularly those studying neural circuits. One neural circuit in which connectivity has proven elusive is the mammalian locomotor central pattern generator (i.e. the locomotor CPG). It was first proposed that a neural network was responsible for the specific pattern of motoneuron firing resulting in locomotor muscle synergies almost a century ago (Brown, 1911). In the years since, there has been a great deal of study attempting to identify component interneurons of this circuit as well as patterns of connectivity (see Kiehn, 2006 for review). Traditional methods aimed at studying the locomotor CPG have employed in vivo and in vitro techniques to identify and characterize single interneurons based on their electrophysiological characteristics, and used anatomical tracing techniques to determine their projection patterns. These approaches have been effective in providing detailed network structure of locomotor circuits in non-mammalian species (Grillner, 2003; Roberts et al. 1998). Despite this, the large number of cells in the mammalian spinal cord, coupled with the fact that neurons of a similar function are intermingled with others of different functions, makes the task of recording from a significant number of functionally homogeneous neurons extremely difficult.

Recently, the identification of discrete neuronal populations in the central nervous system (CNS), via molecular genetic characterization of gene and transcription factor expression at early embryonic time points, has led to optimism that an understanding of the structure and function of the locomotor CPG is attainable (Tanabe and Jessell, 1996; Goulding et al. 2002). Since gene and transcription factor expression determine neuronal characteristics such as cell fate, channel composition, axonal projection pattern and neurotransmitter phenotype, it stands to reason that
populations of neurons with a similar genetic lineage will share many properties, and perhaps have an analogous function in locomotion (Goulding et al. 2002). Molecular genetic techniques have been used to silence, ablate, and label (via expression of reporter proteins) populations of neurons in the ventral spinal cord and show that they have specific functional roles in the production of locomotor activity (Lanuza et al. 2004; Gosgnach et al. 2006; Crone et al. 2008; Zhang et al. 2008). Despite this progress, there have been few investigations focusing on the intrinsic membrane properties of any of the genetically-defined neuronal populations. These types of studies are key to furthering our understanding of how the locomotor CPG functions since the behavioral outputs generated by the CPG are reliant on the intrinsic membrane properties of its component interneurons (Harris-Warrick, 2002).

The scarcity of these types of studies is, at least in part, due to the lack of an appropriate preparation for the targeting of labeled interneurons with a recording electrode. While it is feasible to visually identify and record from genetically-labeled neurons located in superficial laminae since they are visible through the surface of the spinal cord (Nishimaru et al. 2006), a major impediment to the aforementioned experiments is that the core of the locomotor CPG is located in the ventromedial aspect of the lumbar spinal cord close to the central canal (Kjaerulff and Kiehn, 1996). Due to the depth of these neurons (200μm-300μm from the ventral surface of the spinal cord in the neonatal mouse), those expressing reporter proteins cannot be visualized nor targeted with a recording electrode in the intact preparation. It has therefore been necessary to devise alternative techniques to study these neurons. Thus far two techniques have been used. The first is the spinal cord slice technique in which a 200μm-300μm coronal section of the lumbar spinal cord is cut, and labeled neurons close to the cut surface are visible and can be
recorded (Wilson et al. 2007). The second is the hemisect technique in which a midsagittal section of the spinal cord (Kiehn et al. 1996) allows labeled cells located close to the midline to be targeted for recording (Hinckley et al. 2005, Hinckley et al. 2006). Since the locomotor CPG has been shown to be distributed throughout the ventromedial aspect of the lower thoracic and lumbar spinal cord and interneurons that coordinate bilateral alternation of the CPG send axons through the ventral commissure, neither of these techniques are ideal as they both involve a complete cut of the spinal cord and substantial damage to this neural circuit.

Here we describe a method which enables the visualization of neurons that express reporter proteins while leaving neuronal connectivity between the left and right side of the spinal cord, as well as all tissue rostral to the recorded neuron, intact. Since the ventral portion of the spinal cord is unlesioned, connectivity between different components of the CPG, as well as connectivity between brainstem centers that initiate locomotor activity and the CPG, remain intact and can be mapped. Since the size and the location of the notch can vary, this technique allows for the electrophysiological and anatomical investigation of neurons and neural networks throughout the CNS.
MATERIALS AND METHODS

Animals and preparation

All animal procedures were in accord with the Canadian Council on Animal Care (CCAC) and approved by the Animal Welfare Committee at the University of Alberta. Experiments were performed on 31 neonatal mice aged postnatal day 0 (P0) - postnatal day 4 (P4). The mice used in these experiments resulted from a cross between the $Dbx1^{Cre}$ strain, which express the Cre recombinase in the $Dbx1$ locus (gift from Dr. Martyn Goulding, Salk Institute for Biological Studies, La Jolla, CA) and the $ROSA26^{EGFP}$ reporter strain (Jackson Labs; Srinivas et al. 2001) which have EGFP cDNAs inserted into the $ROSA26$ locus, preceded by a $loxP$-flanked stop sequence. PCR was used to genotype offspring. Those that were both Cre and enhanced green fluorescent protein (EGFP) positive expressed EGFP in the V0 interneuronal population. This interneuronal population is located in lamina VIII throughout the rostral-caudal extent of the spinal cord (Pierani et al. 2002) and is involved in producing appropriate left-right alternation during locomotion (Lanuza et al. 2004). Mice were anesthetized via inhalation of isoflurane (4% delivered with 95% O$_2$-5% CO$_2$). After evisceration, the brainstem-spinal cord was dissected out in a bath containing oxygenated, ice-cold dissecting artificial cerebrospinal fluid (d-aCSF) containing (in mM) 111 NaCl, 3.08 KCl, 11 glucose, 25 NaHCO$_3$, 1.18 KH$_2$PO$_4$, 3.7 MgSO$_4$, 0.25 CaCl$_2$ (pH of 7.4, osmolarity 280-300 mOsm). Next, the dorsal roots were cut away using fine microscissors to allow for easy access to the ventral roots with a suction electrode which would be used to monitor fictive locomotor activity. A thin strip of agarose (4%, 20 mm length x 1.75 mm width x 1.75 mm height) was glued (Roti Coll 1, Carl Roth) to the ventral midline of the spinal cord, along its length, taking care to avoid the ventral roots (Figure 1A). The strip of
agarose was then glued to a second platform of 4% agarose (3 cm length x 1 cm width x 0.75 cm-1 cm height) that was cut along its length at a 1-2 degree angle resulting in the caudal end of the brainstem/spinal cord being positioned higher than the rostral end (Figure 1B, 1C). The entire preparation (situated dorsal side up, ventral side glued to agarose block) was transferred to a vibratome (Leica VT1200S, Leica Microsystems) sectioning chamber containing oxygenated d-aCSF. The sectioning window of the blade was specified to span from the first (L1) to the sixth (L6) lumbar segments of the spinal cord and was lowered until it just made contact with the dorsal surface of the L6 segment. Using a sapphire etched blade (Leica Microsystems), 200–300 μm were cut away from the dorsal spinal cord in 50 μm increments at a speed of 0.10 mm/s with a blade displacement of 1.95 mm to create a notch (Figure 1C). Sectioning continued until the dorsal aspect of the central canal was visible using a dissecting microscope (Figure 1D). Following sectioning, the agarose platform was carefully cut away and the preparation was situated dorsal side up on a coverslip in a plexi-glass recording chamber and held in place via nylon threads stretched over a platinum wire flattened into a horseshoe shape.

Electrophysiological Recording

The recording chamber containing the preparation was moved onto the stage of an upright microscope (Zeiss Axioskop 2 FS fitted with a GFP filter [490 nm] and infrared differential interference contrast [IR-DIC] optics) and constantly perfused with room temperature, oxygenated recording aCSF (r-aCSF) containing (in mM): 111 NaCl, 3.08 KCl, 11 glucose, 25 NaHCO₃, 1.18 KH₂PO₄, 1.25 MgSO₄, 2.52 CaCl₂ (pH of 7.4, osmolarity 280-300 mOsm). The notch that had been made in the cord was situated under the objective lens. Using brightfield and a low power (4x) objective, bipolar suction electrodes (A-M Systems Inc.) were positioned on
two or three of the flexor-related (second lumbar i.e. L2) and extensor-related (fifth lumbar i.e. L5) ventral roots on either side of the spinal cord and suction was applied with a 5cc syringe. Fictive locomotor activity was induced by bath application of 10μM 5-hydroxytryptophan (5-HT) and 5μM N-Methyl-D-Aspartate (NMDA) (both from Sigma-Aldrich). Electroneurogram (ENG) recordings were obtained from the ventral roots via the suction electrodes, amplified (20,000x) and band pass filtered (100Hz-1kHz) with custom made equipment (R&R Designs).

For patch clamp recordings, patch electrodes (tip resistance: 3–5 MΩ) were pulled from borosilicate glass (Harvard Apparatus) and filled with internal solution containing (in mM): K-gluconate, 138; Hepes, 10; CaCl2, 0.0001; GTP-Li, 0.3; ATP-Mg, 5 (pH adjusted to 7.2, osmolarity 290-305 mOsm). Liquid junction potential was calculated to be ~12mV. Em values were not corrected for the liquid junction potential since the extent to which the contents of the cells had been completely replaced with the pipette solution was unclear. In some cases either Lucifer Yellow (1%, Sigma-Aldrich) or Neurobiotin (0.2%, Vector Labs) were added to the patch electrode to allow for intracellular labeling. A micromanipulator (MPC-385, Sutter Instruments) was used to position the electrode over the notch and lower it into the tissue. GFP+ cells were identified using the 40x objective, a GFP filter, and a live image video camera (IR-1000, Dage-MTI). An IR-DIC filter was used to target these cells with a patch clamp electrode. Using a whole cell recording amplifier (Multiclamp 700B, Axon Instruments) in voltage clamp mode, a 10 mV square pulse (50 Hz) was used to monitor tip resistance as the electrode was advanced towards the cell of interest. Once a giga-ohm seal with a cell was formed, the command voltage was set to -60 mV and gentle suction was applied to break through the membrane to obtain a whole cell recording. Series resistance (Rs) and cell capacitance (Cm)
were determined in voltage-clamp mode using the compensation features on the Multiclamp commander software (Axon Instruments). Rs was monitored throughout the course of each recording (if working in current-clamp mode we would periodically switch into voltage clamp to monitor Rs). Initial values of Rs were typically 10-15 MΩ. Recordings where Rs exceed 30 MΩ were excluded from analysis. Membrane resistance (Rm) was calculated off-line by taking the inverse slope of the linear portion of the current-voltage (I-V) relationship. In some instances, a small amount of negative bias current (10-15 pA) was required to hyperpolarize the cells (to -65 mV) to prevent spontaneous firing of action potentials. Recordings from healthy neurons could be made >6h after cutting the notch in the spinal cord. All whole cell and ENG data was digitized using an analog-digital converter (Digidata 1440A, Axon Instruments) and recorded using pClamp software (Axon Instruments) on a PC. All photomicrographs of V0 neurons during recording were taken with a monochrome CCD camera (ORCA-R², Hamamatsu Photonics) fixed to the microscope used for recording. Following experiments in which Lucifer Yellow or Neurobiotin were included in the intracellular solution, the spinal cords were immediately fixed in 4% paraformaldehyde/PBS for 45 minutes, washed in PBS and moved to the stage of an inverted spinning-disk confocal microscope (IX81, Olympus) fitted with a camera (EM-CCD, Hamamatsu Photonics). Z-stack images and 3D reconstructions of the filled V0 interneurons were collected using Volocity (Improvision Inc.) software and processed using both Volocity and Photoshop (Adobe Systems) software.

Data Analysis

Onsets and offsets of ENG activity were selected manually during a continuous 5-10 minute window of stable fictive locomotion using Clampfit software (Axon Instruments). Measurements
of cycle period (defined as the interval between onset of burst \(n\) and burst \(n+1\)) and burst duration (defined as time between onset of burst \(n\) and offset of burst \(n\)) were determined by analysis of ENG activity of the second lumbar ventral root in the left side (IL2) or right side (rL2) of the spinal cord. All means are reported ± standard deviation (SD). Student’s t-tests were used to determine if means were significantly different. Circular statistics (Zar, 1974) were used to determine the coupling strength between L2 and L5 ventral roots. IL2 bursts occurring over the period of analysis were selected, and their phase values were calculated in reference to the onsets of each rL2 and L5 burst. Phase values were determined by dividing the latency between the onset of the first IL2 burst and the following burst in rL2 (or L5) by the cycle period. This resulted in values of 0.5 when IL2 and rL2 roots were completely out of phase (i.e. appropriate left-right alternation) and values of 1 when they were in phase (i.e. co-bursting). The phase values were imported into MATLAB (The MathWorks Inc.) and a custom script (J.Dyck) was used to generate a polar plot and provide r-values. In order to determine whether the sample \(r\) was large enough to confidently indicate a nonuniform distribution of points, Rayleigh’s test \((R=nr)\) was performed. The resulting value for \(R\) was compared to a critical values table (Zar, 1974).

To determine the significance of coupling between the firing behavior of V0 interneurons and ventral root activity during fictive locomotion, the total number of action potentials fired by a V0 cell were manually counted during 50 fictive locomotor cycles. Individual action potentials were classified as either those that occurred during the active phase of the contralateral flexor-related ventral root (i.e. cL2) or those that occurred during the active phase of the contralateral extensor-related ventral root (i.e. cL5). The Chi-square test was then used to determine whether the
interneuron preferentially fired action potentials in phase with contralateral flexor or extensor ENG activity, or whether action potential firing was evenly distributed within the two phases of fictive locomotion. A critical value of \( p<0.05 \) in the Chi-square distribution was used to determine significance.
RESULTS

In order to determine whether this preparation could be used to study neurons that comprise the locomotor CPG and the manner in which they are interconnected, it is necessary to demonstrate that after the notch is cut in the spinal cord; a) the locomotor CPG is intact and functional b) neurons located in the ventromedial region of the spinal cord are visible, can be targeted for whole cell recording and identified post-hoc in order to analyze axonal projection of those that have been filled with an intracellular tracer and c) these neurons are healthy and can fire rhythmically during fictive locomotion.

To assess whether the locomotor CPG was intact and functional, ENGs were recorded from lumbar ventral roots during pharmacologically-induced fictive locomotion in unlesioned spinal cords (n=7) as well as spinal cords in which a notch had been cut in the dorsal region of the L1-L6 segments (n=8). Coordination between the flexor and extensor related ventral roots as well as timing of fictive locomotor activity were compared. Normal fictive locomotor activity in the neonatal mouse in vitro spinal cord preparation is characterized by rhythmic alternation of ENG activity between ipsilateral L2 (flexor related) and L5 (extensor related) ventral roots as well as alternation between contralateral L2 ventral roots and contralateral L5 ventral roots with a cycle period of approximately 4 seconds (Kullander et al. 2003; Lanuza et al. 2004; Gosgnach et al. 2006). This preparation is routinely used to study the locomotor CPG and has been shown to produce rhythmic flexor and extensor outputs similar to those underlying locomotion in the adult despite the fact that both flexor and extensor motor units course through common lumbar ventral roots (Cowley and Schmidt 1994). Lesions to the locomotor CPG result in aberrant coordination of the ENG activity and/or increases in both cycle period and burst duration (Cazalets et al...
NMDA (5μM) and 5-HT (10μM) were applied to the bath containing both unlesioned and notched spinal cords to elicit fictive locomotor activity. In both groups, appropriate alternation of ENG activity between flexor-related ventral roots on opposite sides of the spinal cord (i.e. IL2, rL2) and between flexor and extensor-related ventral roots on the same side of the spinal cord (i.e. IL2, IL5) was observed (Figure 2). In addition, mean cycle period (unlesioned 4.12 ± 0.69 s, n=7, notch 3.98 ± 0.71 s, n=7, p<0.05, t-test) and mean burst duration (unlesioned 1.9 ± 0.56 s, n=7, notch 2.10 ± 0.32 s, n=8, p<0.05, t-test) in the notch group were not significantly different than those evoked using the same drug concentrations in the unlesioned spinal cord. These results provide evidence that this preparation can be used to assess the function of the locomotor CPG since the outputs do not differ significantly from those in the unlesioned spinal cord. This result is expected since the notch does not extend into the ventral portion of the spinal cord, the region in which the locomotor CPG resides.

The next step in demonstrating the utility of this preparation was showing that labeled neurons in the intermediate nucleus of the spinal cord were clearly visible below the notch, that these cells were healthy, and that whole cell recordings could be made from them. To this end, transgenic mice were used that expressed green fluorescent protein (GFP) in all cells that express the transcription factor Dbx1 (i.e. the V0 interneuron population). The V0 interneurons are located primarily in lamina VIII, close to the central canal (Pierani et al. 2002) and are thought to be a key component of the locomotor CPG that coordinate left-right alternation (Lanuza et al. 2004). Positioning the 40x objective over the notch that had been cut in the spinal cord allowed for identification of V0 neurons by GFP expression. Once a cell was confirmed to be GFP+, an IR-DIC filter was used and the neuron was targeted for patch clamp recording. Figure 3A illustrates
the appearance of a GFP+ V0 cell using a GFP and IR-DIC filter. In some cases, Lucifer Yellow (or Neurobiotin) was included with the intracellular solution in the recording pipette. During recording, the tracer passively diffused into the neuron and allowed for post hoc anatomical analysis (Figure 3A, 3B). In the absence of 5-HT and NMDA (r-aCSF alone), 33 neurons were recorded using the preparation described in order to assess intrinsic membrane properties. The mean membrane potential of these cells was -47.6 ±3.6 mV, mean spike height 62.7 ±10.2 mV. Of those in which membrane resistance was calculated (n=16) the mean value was 757.6 ±342 MΩ. The effect of an intracellular current ramp was a linear increase and decrease of the firing rate with current injection (Figure 3C). Collectively, this data provides support that the neurons located under the notch in the spinal cord were healthy, as intrinsic properties did not differ substantially from ventromedially located interneurons recorded from the unlesioned mouse spinal cord (Zhong et al. 2006).

The key to this technique, and what we believe provides an advantage over previous methods, is that it enables visualization and targeting of labeled neurons for patch clamp recording while leaving the core of the locomotor CPG intact. This allows for the identification of intrinsic cell properties in healthy neurons, and the determination of the specific activity of a neuron in relation to ipsilateral and contralateral fictive locomotor activity. An essential step in proving the utility of this technique therefore, was to demonstrate that recordings could be made simultaneously from both a labeled neuron and from lumbar ventral roots during fictive locomotion. Twenty-one V0 interneurons were recorded during fictive locomotion. A representative is illustrated in Figure 4. In this example, ENG recordings were made from the left L2 and L5 ventral roots while also recording from a neuron located in the L3 segment on the
contralateral side of the spinal cord. The health of the locomotor CPG is illustrated by the large (10 mV) depolarizing bursts and action potentials (50 mV amplitude) recorded from the neuron after application of NMDA and 5-HT, as well as the alternation and cycle period of the ENGs recorded from the contralateral L2 and L5 ventral roots. The neuron illustrated in Figure 4 is clearly locomotor-related as it depolarizes and fires action potentials in synchrony with the contralateral L2 ventral root (Figure 4B, 4C). Analysis of the data using the Chi-square test demonstrates that 16 of the 21 V0 neurons recorded in the presence of 5-HT and NMDA fired rhythmic bursts of action potentials during fictive locomotion. The remaining 5 neurons fired action potentials, however they were not preferentially active during either flexion or extension (i.e. not rhythmically active). Fifteen of the 21 neurons were located in the rostral segments of the lumbar spinal cord (L2 or L3, primarily flexor-related) and six were located in the caudal segment of the lumbar spinal cord (L5, primarily extensor-related). Of the 15 rostrally located cells, 11 were rhythmically active. Nine fired preferentially out of phase, and 2 fired preferentially in phase, with contralateral flexor activity. Of the six caudally located cells, 5 were rhythmically active. Four fired preferentially out of phase, and 1 fired preferentially in phase, with contralateral extensor activity. Since V0 cells are a mixed population of interneurons that project commissurally (Pierani et al. 2002) and are primarily inhibitory (Lanuza et al. 2004) these results are consistent with previous reports suggesting that V0 neurons coordinate left-right alternation during locomotion by inhibiting contralateral motoneurons (Lanuza et al. 2004).
DISCUSSION

This report describes a new protocol for performing whole cell patch-clamp recordings from genetically-labeled interneurons in the intermediate lamina of the embryonic/neonatal rodent spinal cord while leaving the locomotor CPG functionally intact. It is difficult to imagine any preparation enabling the visualization of interneurons located in the ventral spinal cord that allows for the study of all aspects of neurons that comprise the locomotor CPG (i.e. the effect of sensory feedback on locomotor outputs). We propose that the technique described within is the ideal method to use for analysis of neurons that comprise distributed neural circuits such as the locomotor CPG, since labeled cells are able to be visually identified and targeted with a patch clamp electrode, enabling healthy neurons to be recorded from while simultaneously monitoring robust network activity.

In the present study, the validity of this technique is demonstrated via recordings from genetically identified interneurons located in lamina VIII of the spinal cord. These cells have membrane properties (i.e. membrane potential, spike height, response to an injected current ramp) similar to those observed in the unlesioned spinal cord. In addition, cycle period and coordination of fictive locomotor activity are the same as in an unlesioned spinal cord (Figures 2, 4). Rhythmic bursts can be recorded from labeled interneurons and are related to ENG activity recorded from ventral roots (Figure 4). Addition of fluorescent tracer to the recording electrode allows for the morphological study of labeled neuronal populations (Figure 3B) and can be used to map axonal projection patterns.
Since the initial demonstration that the mammalian CPG could be divided into interneuronal populations based on transcription factor expression, a handful of studies have investigated whether these populations are rhythmically active during locomotion and, if so, their specific role in generating locomotor outputs (Lanuza et al. 2004; Gosgnach et al. 2006; Crone et al. 2008; Zhang et al. 2008). To this point, there have been a paucity of studies investigating membrane properties and connectivity of these genetically-defined neuronal populations. It follows that a better understanding of the mechanism of function of the locomotor CPG relies on our ability to characterize the membrane properties of the cells that comprise this neural network. In addition, identification of connectivity patterns between these populations is crucial if we are to understand how these component neurons interconnect to form a functional circuit.

The studies that have been performed thus far have focused primarily on the Hb9 interneurons, which are intermingled with the V0 population, are located close to the central canal, farthest from the surface of the spinal cord and thus least accessible with a recording electrode. It has been hypothesized that this population is an integral component of the locomotor CPG that may play a role in initiating locomotor activity (Wilson et al. 2005; 2007, Brownstone and Wilson 2008). This makes examining these neurons particularly intriguing as identification of their cellular properties and axonal projection pattern could serve as a means of identifying how components of the locomotor CPG are interconnected. The Hb9 interneurons can be visually identified in the Hb9:EGFP mouse, where EGFP expression is driven by the Hb9 promoter. Thus far, two approaches have been used to target these neurons for intracellular study. The first has employed the spinal cord slice technique where a thick (200-300μm) coronal section of the spinal cord is cut and cells expressing fluorescent markers close to the cut surface can be
visualized using a microscope with a GFP filter and IR-DIC optics (Wilson et al. 2005). This technique was used to demonstrate that the *Hb9* interneurons display endogenous bursting and led the authors to hypothesize that these cells play a role in activation of the locomotor CPG (Wilson et al. 2007). Despite these exciting experiments, testing this hypothesis directly has proven difficult due to technical limitations. Determining whether these neurons are a component of the locomotor CPG, and if so the specific role that they play, is arduous using this preparation since few, if any ventral roots are present and thus it is difficult to compare the bursting pattern of these neurons to ipsilateral and contralateral fictive locomotor activity. The spinal cord slice preparation is particularly poorly suited for the study of axonal morphology since cutting a slice from the spinal cord removes network connectivity in the rostral-caudal plane. It is therefore not possible to identify synaptic inputs to, or outputs from, these neurons if they originate or terminate more than a few hundred microns from the soma. In addition, this eliminates the possibility of determining whether this population receives synaptic input from the locomotor command centers located in the brainstem.

An alternative approach used to study the *Hb9* interneurons has been to perform a midsagittal section of the spinal cord, situate the preparation in a recording chamber with the cut region (and thus the *Hb9* interneurons) exposed to the microscope objective and accessible with a recording electrode using GFP and IR-DIC optics (Hinckley et al. 2005, Hinckley et al. 2006). In this case electroneurogram (ENG) recordings of ventral root activity with a suction electrode allow an assessment of pharmacologically-evoked fictive locomotor outputs. Studies using this approach have shown the *Hb9* interneurons to be rhythmically active in phase with the ipsilateral ventral root in the same segment (Hinckley et al. 2006). Like the spinal cord slice technique, however,
this technique has key limitations. Perhaps most importantly hemisecting the spinal cord
removes one half of the CPG and severs all commissural interneurons. This results in a fictive
locomotor pattern that is aberrant, displaying extremely slow bursting in ENGs recorded from
the ventral roots when compared to studies in the intact mouse spinal cord using similar
concentrations of 5-HT and NMDA. Furthermore, fictive locomotion can only be evoked in
hemisected spinal cords approximately 50% of the time (Ziskind-Conhaim et al. 2008). Also, as
is the case with the spinal cord slice preparation, since a substantial portion of the lumbar spinal
cord has been removed, it stands to reason that this technique is insufficient for an extensive
assessment of connectivity, particularly any neurons with commissural axons.

The method described in this report improves on both of these techniques as it allows for the
recording of intrinsic membrane properties from neurons while leaving the locomotor CPG
functionally intact. In addition, since the ventral portion of the spinal cord is untouched, network
connectivity remains largely undisturbed and extensive mapping of the axonal projections from
labeled neuronal populations can be performed by adding neuronal tracers such as Lucifer
Yellow to the recording pipette. The preparation is particularly well suited to this task as it
allows for mapping of axonal projections in the rostral-caudal, dorsal-ventral, and medial-lateral
planes. Intracellular labeling experiments will allow for the identification of downstream targets
of the genetically-identified neuronal populations and show promise for providing detailed
information on how specific populations are interconnected to produce locomotor activity. In
addition to enabling mapping of connectivity within the spinal cord, this technique can
potentially be used for the study of connections between locomotor command centers in the
midbrain (Matsuyama et al. 2004) and the locomotor CPG, since the descending tract in which these commands travel to the spinal cord (the ventrolateral funiculus) remains intact.

This preparation is particularly beneficial at the current time due to the plethora of recent work demonstrating that molecular strategies, in concert with classical electrophysiological and anatomical approaches provide a powerful means to analyze the structure and function of neural circuits. By varying the location and size of the notch, this method allows for easy access to neurons in all regions of the spinal cord while minimizing tissue damage. It thus provides the best way to access genetically-labeled neurons that comprise neural circuits, and enables identification of electrophysiological properties as well as neuronal connectivity. These are essential requirements if we are to understand how neural circuits, such as the locomotor CPG, are activated and how they generate rhythmic outputs.
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FIGURE LEGENDS

Figure 1. A-C Schematic of preparation. A. Thin strip of agarose is attached to ventral midline of spinal cord taking care to avoid ventral roots. B. Agarose strip is attached to angled agarose block with caudal end higher than rostral end and placed in the sectioning tray of a vibratome. Dorsal surface of the cord is exposed to vibratome blade. C. Notch is cut with vibratome and spans from L1-L6 segments. Lower panel is an enlargement of region within dashed box. Circles represent labeled neurons located near the central canal, close to the cut surface. D. Coronal cryostat section cut from notched region of spinal cord. Central canal encircled by dashed oval. GFP+ cells (white) are located in lamina VIII

Figure 2. Electroneurograms (left) recorded from the L2 (flexor-related) and L5 (extensor-related) ventral roots on the left and right side of an unlesioned spinal cord (A) and a spinal cord with a notch cut on its dorsal surface (B). Ipsilateral alternation of flexor-related and extensor-related ventral roots and the contralateral flexor-related ventral roots is unchanged in the lesioned spinal cord. Appropriate coordination is illustrated in the circular plots in which points are clustered around 0.5 and $r$-values are close to 1. $R$ values indicate that the points do not comprise a random distribution. Bar graphs to the right demonstrate cycle period and burst duration do not differ significantly between the unlesioned and notched preparations (Bars indicate SD).

Figure 3. A. IR-DIC image (left), fluorescent image showing GFP expression (middle) of a recorded V0 neuron located just to the right of the central canal (c.c). Soma is indicated by black arrowhead. White arrowhead indicates another V0 neuron located close by. Right panel illustrates the same cell as it is being filled with Lucifer Yellow. Note the axon leaving the soma
(white arrows) **B.** Collapsed Z-stack reconstruction of the neuron in panel A. **C.** Firing behavior of a neuron located below the notch in response to a 40pA current ramp applied over 25s. Note the linear increase and decrease of firing frequency.

**Figure 4.** **A.** Suction electrodes attached to L2 and L5 ventral roots record flexor-related and extensor-related ENGs respectively. Patch clamp electrode (PC) records from a neuron located contralaterally in lamina VIII of the L3 spinal segment. **B.** Fictive locomotion occurs in the presence of 5μM NMDA 10μM 5-HT. Upper trace recorded from the IC electrode shows the neuron located below the notch has a membrane potential (Em) close to -50 mV, is rhythmically active and fires action potentials in phase with flexor activity (L2) on the contralateral side of the spinal cord. **C.** Percentage of total number of action potentials (APs) occurring in the interneuron (illustrated in panel B) during each of ten equal windows of a normalized fictive locomotor cycle period. All action potentials during 50 consecutive cycle periods were analyzed. The horizontal bar below the x-axis (average burst duration ± SD) depicts the portion of the step cycle in which the cL2 ventral root was active.
A

B

C

n=50 cycles

Locomotor phase

% of Total APs