Whole Cell Recordings From Visualized Neurons in the Inner Laminae of the Functionally Intact Spinal Cord

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Submitted 11 March 2009; accepted in final form 15 April 2009

Dyck J, Gosgnach S. Whole cell recordings from visualized neurons in the inner laminae of the functionally intact spinal cord. J Neurophysiol 102: 590–597, 2009. First published April 22, 2009; doi:10.1152/jn.00212.2009. The in vitro whole spinal cord preparation has been an invaluable tool for the study of the neural network that underlies walking because 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 that 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 constitute neural networks throughout the CNS.

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 (CPG). It was first proposed—almost a century ago (Brown 1911)—that a neural network was responsible for the specific pattern of motoneuron firing resulting in locomotor muscle synergies. 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 (for review see Kiehn 2006). Traditional methods aimed at studying the locomotor CPG have used both 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 nonmammalian 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 make the task of recording from a significant number of functionally homogeneous neurons extremely difficult.

Recently, the identification of discrete neuronal populations in the 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 (Goulding et al. 2002; Tanabe and Jessell 1996). 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 (Crone et al. 2008; Gosgnach et al. 2006; Lanuza et al. 2004; 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 rely 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. Although 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 (200–300 μm from the ventral surface of the spinal cord in the neonatal mouse), those expressing reporter proteins can neither be visualized nor targeted with a recording electrode in the intact preparation. It has thus 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 200- to 300-μm 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 hemisection 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, 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 is ideal because they both involve a complete cut of the spinal cord and substantial damage to this neural circuit.

Here we describe a method that enables visualization of neurons that express reporter proteins while leaving neuronal connectivity between the left and right sides 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 not only between different components of the CPG, but also between brain stem 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.

**METHODS**

**Animals and preparation**

All animal procedures were in accord with the Canadian Council on Animal Care and approved by the Animal Welfare Committee at the University of Alberta. Experiments were performed on 31 neonatal mice aged postnatal day 0 (P0) to P4. The mice used in these experiments resulted from a cross between the Dbx1Cre strain, which expresses the Cre recombinase in the Dbx1 locus (a gift from Dr. Martyn Goulding, Salk Institute for Biological Studies, La Jolla, CA), and the ROSA26EGFP reporter strain (Jackson Labs; Srinivas et al. 2001), which have enhanced green fluorescent protein (EGFP) cDNAs inserted into the ROSA26 locus, preceded by a loxP-flanked stop sequence. Polymerase chain reaction was used to genotype the offspring. Those that were both Cre and EGFP positive expressed EGFP in the V0 interneuronal population. This interneuronal population is located in lamina VIII throughout the rostrocaudal 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% O2–5% CO2). After evisceration, the brain stem/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 NaHCO3, 1.18 KH2PO4, 3.7 MgSO4, and 0.25 CaCl2 (pH 7.4, osmolality 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 that would be used to monitor fictive locomotor activity. A thin strip of agarose [4%, 20 × 1.75 × 1.75 mm (length × width × 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 (Fig. 1A). The strip of agarose was then glued to a second platform of 4% agarose [3 × 1 × 0.75–1 cm (length × width × height)] that was cut along its length at a ~2° angle, resulting in the caudal end of the brain stem/spinal cord being positioned higher than the rostral end (Fig. 1, B and C). 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 (Fig. 1C). Sectioning continued until the dorsal aspect of the central canal was visible using a dissecting microscope (Fig. 1D). Following sectioning, the agarose platform was carefully cut away and the preparation was situated dorsal side up on a coverslip in a Plexiglas 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...
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[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, and 2.52 CaCl2 (pH 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 (×4) objective, bipolar suction electrodes (A-M Systems) 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 5-ml syringe. Fictive locomotor activity was induced by bath application (second lumbar, i.e., L2) and extensor-related (fifth lumbar, i.e., L5) (A-M Systems) were positioned on two or three of the flexor-related ventral roots via the suction electrodes, amplified (×20,000), and band-pass filtered (100 Hz to 1 kHz) 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 an internal solution containing (in mM): 138 K-glucuronate, 10 Hepes, 0.0001 CaCl2, 0.3 GTP-Li, and 5 ATP-Mg (pH adjusted to 7.2, osmolarity 290–305 mOsm). The liquid junction potential was calculated to be about 12 mV. Membrane potential (E_m) 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) was 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 ×40 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 toward the cell of interest. Once a gigaohm 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 (R_s) and cell capacitance (C_m) were determined in voltage-clamp mode using the compensation features on the Multiclamp commander software (Axon Instruments). R_s was monitored throughout the course of each recording (if working in current-clamp mode we would periodically switch into voltage clamp to monitor R_s). Initial values of R_s were typically 10–15 MΩ. Recordings where R_s >30 MΩ were excluded from analysis. Membrane resistance (R_m) 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 >6 h after cutting the notch in the spinal cord. All whole cell and ENG data were 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 was included in the intracellular solution, the spinal cords were immediately fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) for 45 min, 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 three-dimensional reconstructions of the filled V0 interneurons were collected using Velocity (Improvision) 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- to 10-min 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 the 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 ± SD. Student’s t-tests were used to determine whether 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 IL5 burst. Phase values were determined by dividing the latency between the onset of the first IL2 burst and the following burst in rL2 or IL5 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., cobursting). The phase values were imported into MATLAB (The MathWorks) and a custom script (J. Dyck) was used to generate a polar plot and provide r values. 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 with 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., clL2) or those that occurred during the active phase of the contralateral extensor-related ventral root (i.e., cL5). The chi-square (χ²) 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 χ² distribution was used to determine significance.

RESULTS

To determine whether this preparation could be used to study neurons that constitute 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: 1) the locomotor CPG is intact and functional; 2) neurons located in the ventromedial region of the spinal cord are visible, can be targeted for whole cell recording, and identified post hoc to analyze axonal projection of those that have been filled with an intracellular tracer; and 3) 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 and the timing of fictive locomotor activity were compared. Normal fictive locomotor activity in the neonatal mouse in 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 about 4 s.
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. 1995; Cowley and Schmidt 1997). NMDA (5 µM) and 5-HT (10 µM) 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., L2, rL2) and between flexor- and extensor-related ventral roots on the same side of the spinal cord (i.e., L2, L5) was observed (Fig. 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 from 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 coordinates left–right alternation (Lanuza et al. 2004). Positioning the ×40 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

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**FIG. 2.** Electroneurograms (left) recorded from the 2nd lumbar (L2, flexor-related) and 5th lumbar (L5, extensor-related) ventral roots on the left and right sides 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 constitute 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).
tracers passively diffused into the neuron and allowed for post hoc anatomical analysis (Fig. 3, A and B). In the absence of 5-HT and NMDA (r-aCSF alone), 33 neurons were recorded using the preparation described to assess intrinsic membrane properties. The mean membrane potential of these cells was $-47.6 \pm 3.6$ mV and mean spike height was $62.7 \pm 10.2$ mV. Of those in which membrane resistance was calculated ($n = 16$) the mean value was $757.6 \pm 342$ MΩ. The effect of an intracellular current ramp was a linear increase and decrease of the firing rate with current injection (Fig. 3C). Collectively, these data provide support that the neurons located under the notch in the spinal cord were healthy because 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 identification of intrinsic cell properties in healthy neurons and determination of the specific activity of a neuron in relation to ipsilateral and contralateral fictive locomotor activity—thus an essential step in proving the utility of this technique 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 Fig. 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 Fig. 4 is clearly locomotor related because it depolarizes and fires action potentials in synchrony with the contralateral L2 ventral root (Fig. 4, B and C). Analysis of the data using the $\chi^2$ 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, although 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 6 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 6 caudally located cells, 5 were rhythmically active. Four fired preferentially out of phase and one 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 constitute the locomotor CPG (i.e., the effect of sensory feedback on locomotor outputs). We propose that the technique described herein 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 (Figs. 2 and 4). Rhythmic bursts can be recorded from labeled interneurons and are related to ENG activity recorded from ventral roots (Fig. 4). Addition of fluorescent tracer to the recording electrode allows for the morphological study of labeled neuronal populations (Fig. 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 (Crone et al. 2008; Gosgnach et al. 2006; Lanuza et al. 2004; Zhang et al. 2008). To this point, studies investigating membrane properties and connectivity of these genetically defined neuronal populations have been sparse. 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 constitute 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 are 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 (Brownstone and Wilson 2008; Wilson et al. 2005, 2007). This makes examining these neurons particularly intriguing, in that 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 used the spinal cord slice technique where a thick (200–300 μm) 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 rostrocaudal 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 brain stem.

An alternative approach used to study the Hb9 interneurons has been to perform a midsagittal section of the spinal cord,
situating the preparation in a recording chamber with the cut region (and thus the Hb9 interneurons) exposed to the microscope objective and made accessible with a recording electrode using GFP and IR-DIC optics (Hinckley et al. 2005, 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 important, 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 with studies in the intact mouse spinal cord using similar concentrations of 5-HT and NMDA. Furthermore, fictive locomotion can be evoked in hemisected spinal cords only about 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 here improves on both of these techniques because 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 because it allows for mapping of axonal projections in the rostrocaudal, dorsoventral, and mediolateral 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 of analyzing 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 constitute neural circuits and enables identification of not only electrophysiological properties but also 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.

ACKNOWLEDGMENTS

We thank Dr. Martyn Goulding for the Dbx1tm mouse strain, Dr. Guillermo Lanuza for advice with the mouse work, Drs. John Greer and Kevin Carlin for comments on an earlier version of this manuscript, Dr. Nicholas Touret (University of Alberta, Department of Biochemistry) and C. Carson (Quorum Technologies) for providing assistance with microscopy and imaging, and K. Wong and F. Olsen for providing technical assistance.

GRANTS

This research was supported by Canadian Institutes for Health Research Grant MOP86470, Alberta Heritage Foundation for Medical Research, March of Dimes Birth Defect Foundation Grant 5-FY07-648, and University of Alberta Faculty of Medicine and Dentistry.

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