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# Using an In Vitro Spinal Cord-Hindlimb Rat Model to Address the Role of Sensory Feedback in Spinally Generated Locomotion

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Understanding how sensory feedback can be used to facilitate locomotion is vital for designing effective locomotor rehabilitation strategies for spinal cord injury. Here we describe an in vitro spinal cord-hindlimb preparation (SCHP) that can be used to answer clinically relevant questions about the interaction of sensory feedback and spinal locomotor circuitry. The SCHP, composed of the exposed rodent spinal cord with hindlimbs intact, combines the neural accessibility and manipulability of classic in vitro isolated cord preparations with intact sensory feedback and many of the behavioral analysis techniques used in the clinic, such as kinematics, treadmill walking, and electromyography. Using the SCHP, we demonstrate the importance of task-appropriate limb loading and posture for establishing intact rat-like kinematic trajectories and muscle activation patterns. We also show that applying stepping-like movements to the limbs, similar to those applied during physical therapist-assisted or robot-guided body weight-support treadmill training, can reinforce weak neurochemically induced locomotion or even initiate locomotion in the presence of subthreshold neurochemical concentrations. Finally, we present the first intracellular recordings from spinal interneurons during unrestrained hindlimb locomotion, demonstrating the capacity of the SCHP for directly assessing neural function during locomotion and in response to mechanosensory perturbations. As shown here, the SCHP allows for simultaneous behavioral and neuronal measurements, enabling us to relate behavioral outcomes to underlying neural mechanisms. In the future, the SCHP promises to advance our understanding of the neural mechanisms underlying therapeutic interventions and to provide a tractable platform for testing neural interfaces, rehabilitation techniques, and neurochemical agents. **Key words:** electromyography, intracellular recordings, loading, locomotion, sensory feedback

The spinal cord alone contains sufficient circuitry to produce the rhythmic motor patterns that underlie locomotion. This circuitry, termed the *central pattern generator* (CPG), has been demonstrated in numerous animal models as well as in humans.<sup>1-5</sup> After spinal cord injury, portions of this circuitry often remain intact, but damage to descending systems prevents injured persons from utilizing this circuitry. With damage to descending systems, sensory feedback is the major remaining input available for accessing and controlling residual circuitry in the spinal cord for locomotion and mobility. Thus, understanding how sensory feedback functions during locomotion and how it can be used to facilitate locomotion is vital to designing effective locomotor rehabilitation strategies.

Although seemingly far from human rehabilitation work, in vitro preparations, such as the isolated rodent spinal cord, have greatly enhanced our understanding of spinal locomotor circuitry. In the isolated spinal cord maintained in vitro, the CPG can be readily activated by neurochemicals or electrical

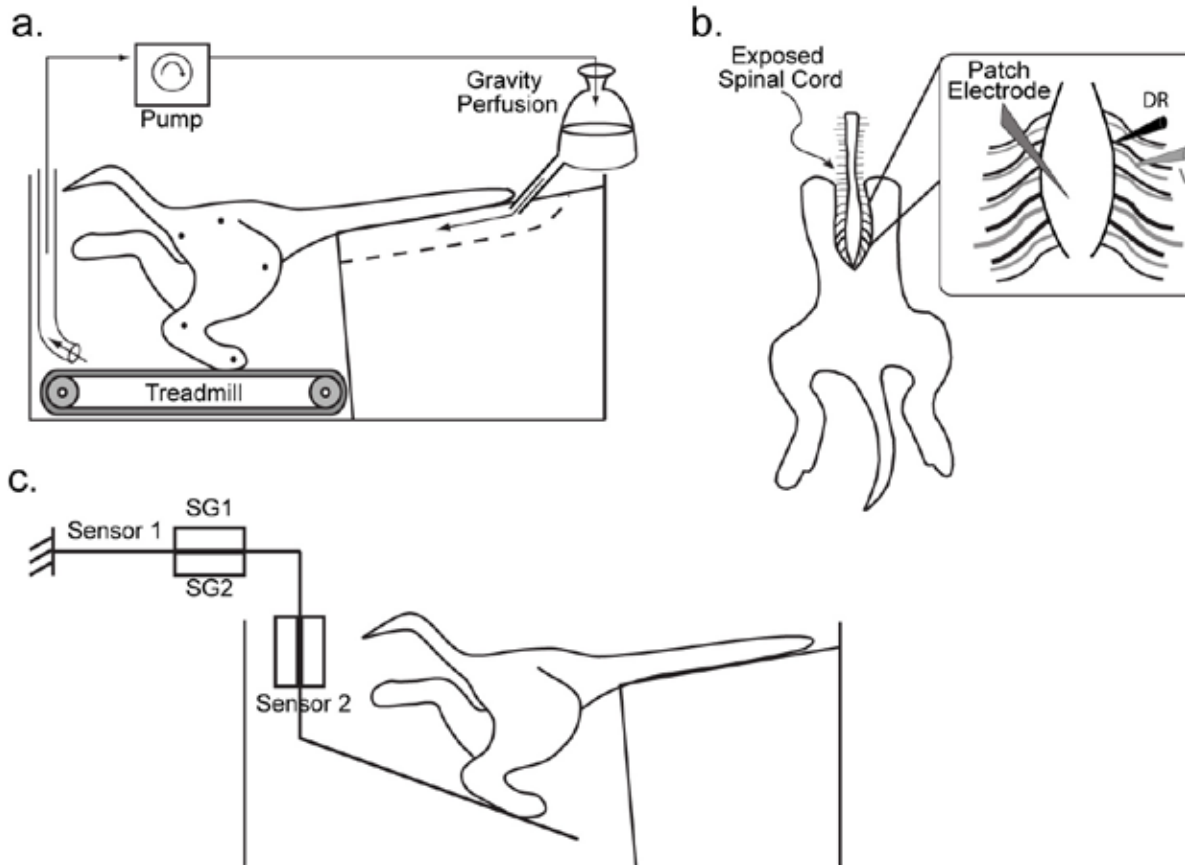
stimulation. The components of the circuitry can then be dissected using a broad array of techniques, including immunohistochemistry, pharmacology, electrophysiology, and transgenic approaches.<sup>6,7</sup> In the absence of the blood-brain barrier, drugs can be applied in known concentrations and the extracellular environment easily controlled. Further, the ability to mechanically stabilize the spinal cord allows for stable intracellular recordings which are difficult in vivo<sup>8</sup> and unprecedented during unrestrained hindlimb movement. Although these advantages make the in vitro spinal cord a powerful model for investigating spinal circuitry, the isolated spinal cord lacks key features required for translating neural mechanisms to rehabilitation applications. First, the isolated cord lacks sensory feedback from limb movement, which is known

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**Figure 1.** In vitro spinal cord-hindlimb preparation (SCHP). (A) Sagittal view of the SCHP with the hindlimbs stepping on a custom-designed treadmill. The treadmill is composed a polyurethane belt mounted around plastic rollers and metal shafts. The shafts are secured through the walls of the perfusion chamber. The treadmill is then front roller-driven by a small, speed-adjustable DC motor. The preparation is maintained within the perfusion chamber by circulating continuously oxygenated artificial cerebrospinal fluid (aCSF) with a peristaltic pump and gravity-fed perfusion system. Finally, joint centers are marked for sagittal plane video capture and kinematic analyses. Adapted and used with permission from Hayes HB, Chang Y-H, Hochman S. An in vitro spinal cord-hindlimb preparation for studying behaviorally relevant rat locomotor function. *J Neurophysiol.* 2009;101:1114-1122. (B) Overhead view of the SCHP showing suction electrodes recording from ventral roots (VR) and dorsal roots (DR) along with a patch clamp electrode inserted into the cord to record intracellularly from single neurons. (C) Force platform system with sensors for recording fore-aft (Sensor 1) and vertical (Sensor 2) ground reaction forces. Each sensor is composed of 2 opposing strain gauges (SG), whose output is then fed through Wheatstone bridge circuitry and DC amplifiers.

to strongly modulate motor output<sup>9,10</sup> and is key to retraining the injured cord.<sup>11-13</sup> In addition, without the limbs intact, the behavioral impact of pharmacological, electrical, and neural manipulations is very difficult to quantify. Although in vivo models offer both intact sensory feedback and the ability to quantify behavior, the limited neural accessibility of intact preparations hinders the ability to directly monitor underlying neural mechanisms.

Recently, we developed an in vitro spinal cord-hindlimb preparation (SCHP) in the neonatal rat

that combines the neural accessibility of classic in vitro preparations with intact sensory feedback from hindlimb movement and behavioral observability.<sup>14</sup> The SCHP is composed of a fully-exposed neonatal rat spinal cord maintained in artificial cerebrospinal fluid (aCSF) with hindlimbs intact and hanging pendant to step on a custom-designed treadmill or instrumented force platforms (Figure 1). Hindlimb stepping is activated by bath application of N-methyl D-aspartate (NMDA), serotonin (5HT), and/or dopamine (DA). As is common in human clinical research, we

can then monitor muscle activation patterns with electromyography (EMG), hindlimb kinematics, and ground reaction forces and apply mechanical perturbations to the limbs. However, unlike in clinical research, we can directly monitor neural activity at the single neuron level with intracellular recordings, motor output at the ventral roots (VR), and sensory input at the dorsal roots (DR), allowing us to relate neural mechanisms to behavioral outcomes and to quantify how mechanical manipulations like those used in rehabilitation influence neural activity.

Here we discuss several investigations undertaken in the SCHP on the use of mech-anosensory manipulations to modulate, reinforce, and initiate spinal locomotion in the absence of descending brain input. We also demonstrate the ability to monitor individual neuron activity using intracellular recordings during unrestrained hindlimb movement and mechanical perturbations. These initial studies demonstrate the ability to use an in vitro rat preparation to ask questions similar to those asked in the clinic with the future promise of investigating the underlying neural mechanisms. Some of these results have been presented previously.<sup>14</sup>

#### **Limb Loading and Posture Establish Task-Appropriate Kinematics and Muscle Activity**

One common strategy in spinal cord injury rehabilitation is to use the sensory cues typically experienced by stepping limbs to re-establish functional rhythmic muscle activation patterns and limb movements. One of the most important cues for establishing such patterns is rhythmic limb loading.<sup>15-17</sup> Limb loading has been shown to strongly modulate muscle activation amplitude and timing in both cats and humans.<sup>18-22</sup> Using the SCHP, we tested the effect of limb loading and posture on motor output generated by the isolated neonatal rodent spinal cord in the absence of descending brain input. We compared the kinematic and muscle activation patterns in a ventral-up stepping posture (no ground contact) and a dorsal-up stepping posture (ground contact and limb loading).<sup>14</sup> Representative kinematics and EMG patterns are shown in **Figure 2**. Several significant changes were observed. First, the knee joint trajectory underwent a near reversal, extending during stance in the unloaded ventral-up condition but flexing during stance in the loaded dorsal-up condition (**Figure 2A**). As a result, joint coordination across the 3 joints differed significantly. All 3 joints were largely in-phase in the ventral-up condition, undergoing mass extension followed by mass flexion, while the knee was out-of-phase with the hip and ankle in the dorsal-up condition. The dorsal-up patterns more closely

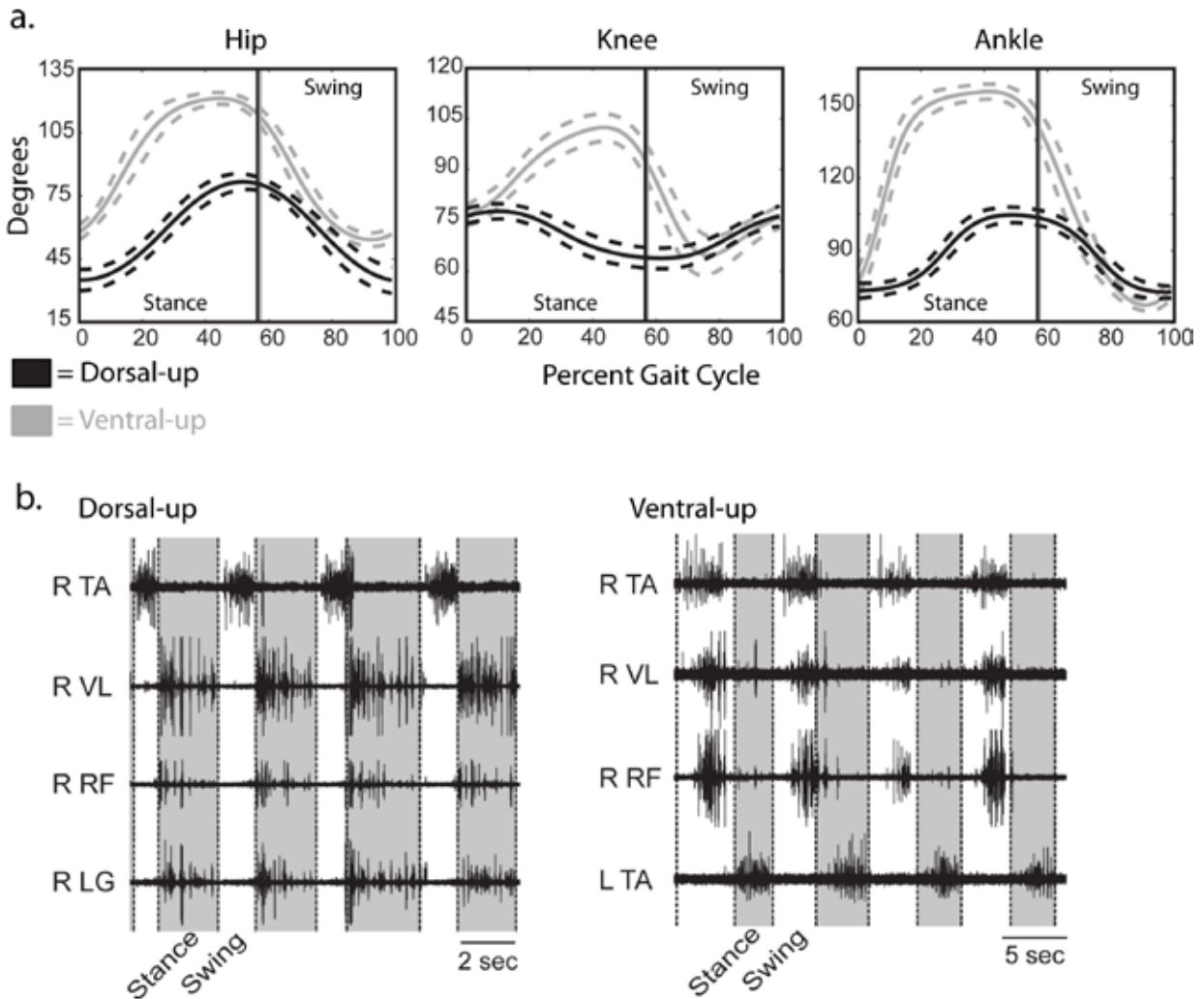
resembled those typically observed in the intact rat,<sup>14,23</sup> suggesting that sensory cues related to ground contact and loading are important for establishing stepping patterns similar to the intact rat.

There were also significant changes in muscle activation patterns.<sup>14</sup> As seen in **Figure 2B**, ankle flexors (tibialis anterior [TA]) and knee extensors (vastus lateralis [VL]) were nearly out-of-phase in the dorsal-up condition ( $0.375 \pm 0.057$ ;  $n=4$ ) but nearly in-phase in ventral-up ( $0.0961 \pm 0.101$ ;  $n=5$ ). This change was statistically significant ( $P = .002$ ). Additionally, the relative duty cycles of flexors and extensors changed significantly between the 2 conditions. During dorsal-up stepping, the duty cycle of both recorded extensors, vastus lateralis (VL;  $0.52 \pm 0.064$ ) and lateral gastrocnemius (LG;  $0.56 \pm 0.073$ ), was significantly longer than the duty cycle of TA ( $0.37 \pm 0.098$ ) (VL vs TA:  $P < .01$ ,  $n=4$ ; LG vs TA:  $P < .05$ ,  $n=5$ ). In contrast, during ventral-up stepping, the duty cycle of TA ( $0.47 \pm 0.094$ ) was significantly longer than that of VL ( $0.34 \pm 0.12$ ;  $P < .01$ ,  $n=5$ ). When comparing a single muscle between conditions, the duty cycle of VL was significantly longer during dorsal-up stepping compared to ventral-up stepping ( $P < .05$ ), whereas TA tended to longer during ventral-up stepping ( $P = .06$ ). As observed for the kinematic trajectories, the muscle activation pattern exhibited in the dorsal-up condition is typical of intact animals experiencing rhythmic limb loading and ground contact,<sup>23,24</sup> while flexor-dominated patterns are usually only seen during fictive locomotion where all sensory cues are absent.<sup>25</sup>

In sum, the sensory cues associated with ground contact and limb loading, such as muscle stretch and tension, seem to be vitally important for establishing functional motor outputs. Even in the absence of descending input, removing or adding ground contact limb loading and changing limb orientation profoundly affected joint coordination and muscle activation, showing the importance of loading-related sensory cues in sculpting spinal motor output. Our findings affirm previous work in the cat showing that ground contact loading, and the consequent Golgi tendon group Ib afferent activity, are vital for ankle extensor activation.<sup>20</sup> Additionally, in humans, inhibition of Ib reflexes<sup>26</sup> and muscle activation patterns and joint kinematics in reduced gravity<sup>22</sup> all depend on phasic limb loading (for earlier review, see ref. 27).

#### **Stepping-like Assistance Can Reinforce or Initiate Neurochemically Induced Spinal Locomotion**

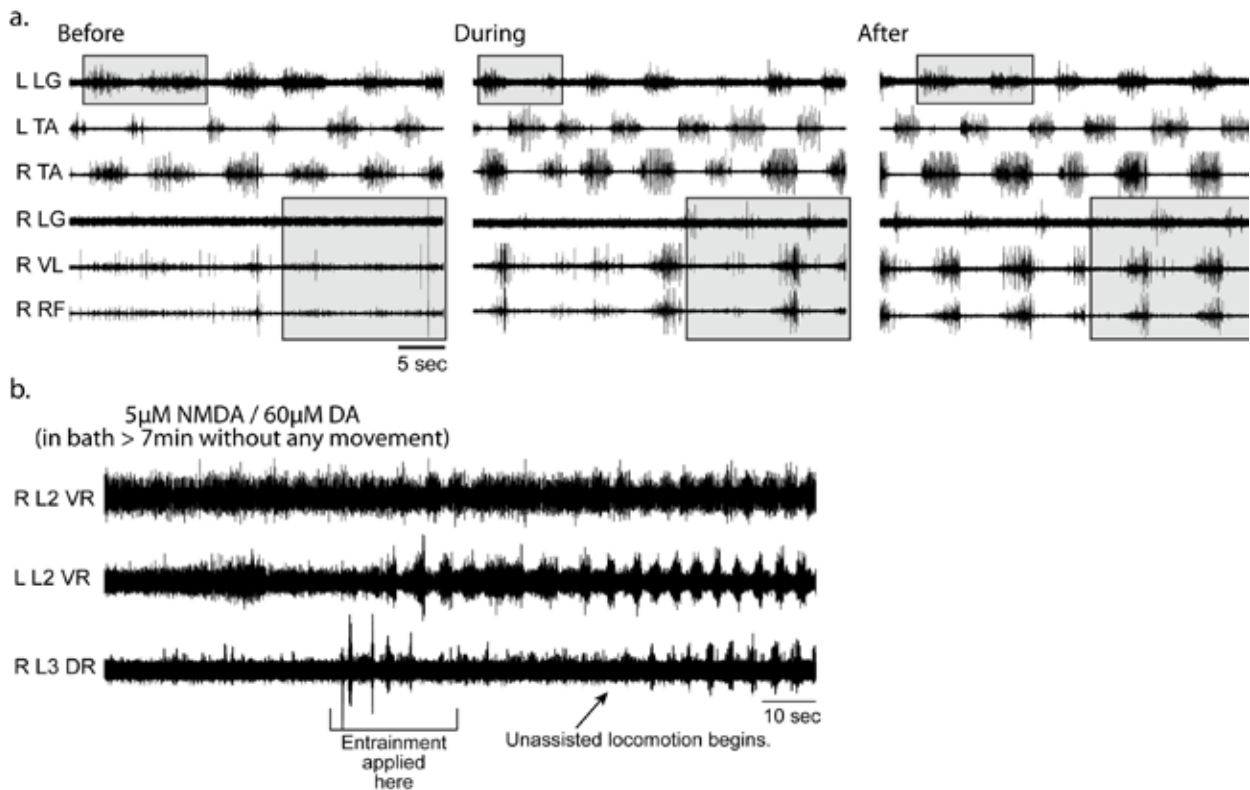
We also considered whether assisting stepping-like movements could reinforce weak locomotor patterns. As



**Figure 2.** Kinematics and EMG activity during dorsal-up treadmill stepping and ventral-up stepping. (A) Kinematic joint angle trajectories from dorsal-up (black) and ventral-up (gray) stepping. Solid trajectories represent average (17 cycles) time normalized joint angle trajectories from the hip, knee, and ankle plotted across the gait cycle. The dashed lines represent the average trajectory  $\pm 1$  SD. Increasing angles indicate joint extension and decreasing angles flexion. 0% gait cycle indicates stance onset (anterior extreme position). Vertical lines indicate swing onset (posterior extreme position). (B) Muscle activation patterns from 4 cycles of dorsal-up (left) and ventral-up (right) stepping. Recorded muscles include the right tibialis anterior (R TA), vastus lateralis (R VL), rectus femoris (R RF), lateral gastrocnemius (R LG), and left tibialis anterior (L TA). Gray shaded regions represent stance phase. Adapted, with permission, from Hayes HB, Chang Y-H, Hochman S. An in vitro spinal cord-hindlimb preparation for studying behaviorally relevant rat locomotor function. *J Neurophysiol.* 2009;101:1114-1122.

often observed in humans with spinal cord injuries,<sup>3,15,28</sup> muscle activation patterns produced by the isolated spinal cord can be weak in amplitude and irregular in frequency, and some muscles remain silent. In 5 SCHPs with initially weak muscle activation patterns, we facilitated swing by moving the right hindlimb forward with a glass probe on the plantar surface of the paw at swing onset. These assistive movements resulted

in a more robust locomotor pattern.<sup>14</sup> An example is shown in **Figure 3A**. Prior to assistance, the muscle activation patterns were irregular in frequency and LG, VL, and RF remained largely silent or tonic. With swing assistance, these extensors began to show more regular bursting. Even when assistance was terminated, the stronger pattern persisted at a more regular frequency, with each muscle being cyclically recruited



**Figure 3.** Stepping-like assistance can reinforce weak locomotion or initiate locomotion. (A) Reinforce weak locomotion (adapted from ref. 14). Muscle activation patterns from right (R) or left (T) lateral gastrocnemius (LG), tibialis anterior (TA), vastus lateralis (VL), and rectus femoris (RF) are shown before, during, and after application of swing assistance. Each panel shows 40 s of activity. Gray boxes highlight changes in frequency and muscle bursting. Adapted and used with permission from Hayes HB, Chang Y-H, Hochman S. An in vitro spinal cord-hindlimb preparation for studying behaviorally relevant rat locomotor function. *J Neurophysiol.* 2009;101:1114-1122. (B) Initiate locomotion. Root activity from the right lumbar ventral root (R L2 VR), left lumbar ventral root (L L2 VR), and right lumbar dorsal root (R L3 DR) are shown before, during, and after assistive stepping-like movements. The entrainment period (bracket) indicates the period in which the limbs were cyclically moved through stepping-like patterns. Before entrainment, the subthreshold concentration of neurochemicals failed to initiate rhythmic locomotor bursting. Stepping-like movements initiated rhythmic locomotor bursting in all roots, which persisted after the entrainment period.

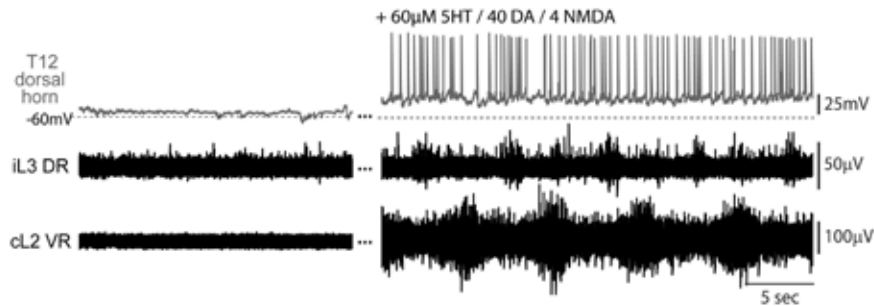
into rhythmic bursting. Similarly, when neurochemical application failed to elicit hindlimb stepping, repeatedly moving the limbs through alternating, stepping-like movements initiated locomotion (Figure 3B; n=6 bouts in n=4 animals). In sum, sensory feedback from behaviorally relevant movements not only can modulate ongoing locomotion, but also can be used to facilitate activation of the spinal locomotor circuitry. Thus, rehabilitation techniques that provide rhythmic sensory cues by moving the limbs through stepping-like movements, such as the Lokomat (Hocoma AG, Volketswil, Switzerland) and physical therapist-assisted body weight-supported treadmill training, can be used to recruit muscles that remain silent during voluntary activity and enhance activation of residual

spinal circuitry. Recent studies on persons with both incomplete and complete spinal cord injuries affirm this assertion.<sup>3,15,29</sup> In future studies, the SCHP could be used to test which assistive techniques are most effective and to identify the responsible sensory pathways and motor targets using direct neural recordings as well as reflex testing.

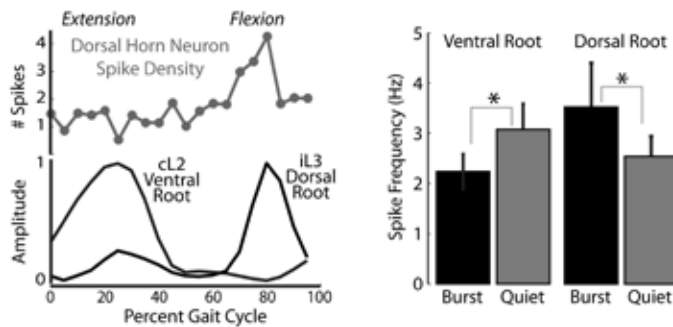
#### Monitoring Cellular Responses to Peripheral Perturbations Using Intracellular Recordings

One of the greatest advantages of the SCHP is the ability to mechanically stabilize the spinal cord while retaining sensory feedback and intact limb movement. The stability of the spinal cord allows for technically

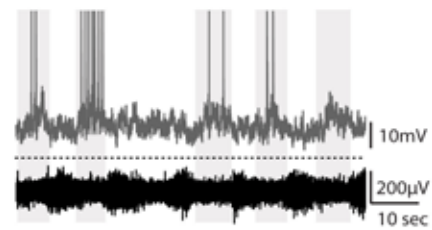
**a. Quiescent at rest & rhythmically modulated during locomotion**



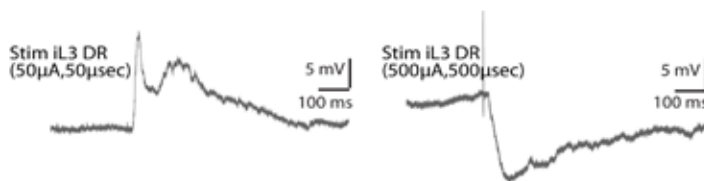
**b. Max firing during flexion & in-phase with L3 DR**



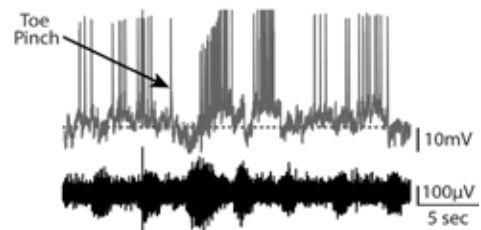
**c. Hyperpolarization reveals drive potentials**



**d. Excited by low threshold input & inhibited by high threshold input**



**e. Inhibited by toe pinch (natural high threshold)**



**Figure 4.** Intracellular patch clamp recordings during unrestrained hindlimb locomotion. Activity recorded from a T12 dorsal horn interneuron (dark gray) is shown during several conditions relative to ipsilateral (i) and contralateral (c) ventral root (VR) and dorsal root (DR) activity (black). Note that L2 VR activity generally corresponds to ipsilateral flexion phase.<sup>35</sup> (A) Resting membrane potential for this neuron was  $-60\text{mV}$ . The neuron was quiescent at rest, but rhythmically spiked during neurochemically induced locomotion. (B) Mean spike density (no. of spikes/bin) and rectified integrated root activity were averaged across 47 cycles at 5% time bins. Spike density was maximum during the flexion phase, out-of-phase with cL2 VR activity, and in-phase with iL3 DR activity. Mean spike frequency was significantly higher during cL2 VR quiescence and iL3 DR bursting ( $P < .05$ ). (C) Slightly hyperpolarizing the membrane potential highlighted the underlying rhythmic drive potentials. (D) The neuron was excited by low-threshold stimulation ( $50\mu\text{A}$ ,  $50\mu\text{s}$ ), but inhibited by high-threshold stimulation ( $500\mu\text{A}$ ,  $500\mu\text{s}$ ). (E) Natural high-threshold noxious stimuli, such as toe pinch, also inhibited neuron firing. Inhibition was followed by rebound spiking.

challenging intracellular patch clamp recordings, which are typically reserved for the isolated spinal cord or fictive locomotion in which limb movement is paralyzed (eg, refs. 30, 31; for review, see ref. 7). To demonstrate this capability, we recently performed whole-cell patch clamp recordings during unrestrained, nonfictive hindlimb locomotion. To the best of our

knowledge, these are the first such recordings during unrestrained hindlimb locomotion. Using blind patch methods similar to those reported previously,<sup>32,33</sup> we successfully recorded from 7 dorsal horn and 2 ventral horn thoracolumbar interneurons in 4 SCHPs. All recorded neurons were silent at rest, but 7 interneurons ( $n = 7/9$ ) showed increases in synaptic activity ( $n=4$ ) or

began spiking ( $n=3$ ) with locomotion. An example of a rhythmically spiking dorsal horn neuron in the T12 segment is shown in **Figure 4**. Spiking in this neuron increased with ipsilateral L3 dorsal root activity and was maximal during ipsilateral flexion. Spike frequency was significantly higher during L3 dorsal root bursting and significantly lower during contralateral L2 ventral root activity (ie out-of-phase with contralateral flexion and in-phase with ipsilateral flexion) ( $P < .05$ ; **Figure 4B**). Slightly hyperpolarizing the membrane with current injection revealed underlying rhythmic depolarizing drive potentials (**Figure 4C**) similar to those previously observed in motoneurons and interneurons.<sup>33,34</sup> Such rhythmic drive potentials in dorsal horn interneurons likely reflect excitatory and inhibitory sensory inputs from rhythmic limb movements. In terms of inputs, this interneuron received strong, excitatory low-threshold nonpain afferent input but was inhibited by high-threshold inputs (**Figure 4D**). Toe pinch during locomotion evoked inhibition followed by rebound firing (**Figure 4E**), consistent with the inhibition observed with high-threshold electrical stimulation.

These preliminary recordings confirm that stable intracellular recordings can be performed in the SCHP during unrestrained hindlimb locomotion. The interneurons remained healthy for extensive characterization, including afferent stimulation, mechanical perturbations, and descending tract stimulation (not shown). Even sensory inputs that evoked strong limb movement, such high-threshold dorsal root stimulation or pinch, did not disturb the intracellular recording. Intracellular recordings from spinal cord neurons during unrestrained hindlimb locomotion promise to provide detailed understanding of the operation of spinal locomotor circuitry during locomotion, including identification of the essential neuronal elements involved in rhythmogenesis, sensory integration, and motor coordination. The SCHP is unique in the ability to directly relate behavioral changes in limb function to cellular changes in individual neurons, as well as

the ability to apply mechanical, pharmacological, or electrical perturbations while simultaneously monitoring cellular and behavioral responses.

### Conclusions and Future Directions

Overall, the in vitro SCHP greatly expands our ability to study locomotor circuitry and the influence of sensory feedback over this circuitry in the absence of descending brain input and with increased acuity and manipulability. Here we demonstrated how the SCHP can be used to investigate sensory modulation of spinally generated locomotion, revealing its importance in sculpting motor output in behaviorally relevant ways. We also showed that sensory feedback from stepping-like assistance can both reinforce ongoing locomotion and even initiate locomotion in the presence of subthreshold neurochemical concentrations. Finally, we demonstrated the ability to perform stable intracellular recordings during hindlimb locomotion and characterize neuronal response to both electrical and mechanical perturbations. In sum, the SCHP allows for simultaneous behavioral and neuronal measurements, allowing us to relate behavioral outcomes to underlying neural mechanisms. This capacity makes the SCHP a very powerful model system for studying spinal circuitry and spinal control of limb movement.

Future studies in the SCHP promise to advance our understanding of neuro-rehabilitation therapies. For example, the SCHP can be used to apply drugs in known concentrations to test the role of neuromodulators in eliciting locomotion and even identify their site of action using split bath techniques. Intracellular interconnectivity studies can be used to dissect the locomotor and sensorimotor circuitry to identify the elements that must remain intact for specific locomotor functions. Finally, the SCHP also offers a tractable, high-throughput platform for testing neural interfaces, spinal cord stimulators, and other therapeutic agents.

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