[Frontiers in Bioscience 17, 2158-2180, June 1, 2012]

Enabling techniques for *in vitro* studies on mammalian spinal locomotor mechanisms

Shawn Hochman[1,2], Elizabeth A. Gozal[1], Heather B. Hayes[3], JoAnna T. Anderson[2], Stephen P. DeWeerth[2], Young-Hui Chang[4]

[1]Department of Physiology, Emory University School of Medicine, Atlanta, Georgia, [2]Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia, [3]Center for Rehabilitation Medicine, Emory University School of Medicine, Atlanta, Georgia, [4]School of Applied Physiology, Georgia Institute of Technology, Atlanta, Georgia

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1. ABSTRACT

The neonatal rodent spinal cord maintained *in vitro* is a powerful model system to understand the central properties of spinal circuits generating mammalian locomotion. We describe three enabling approaches that incorporate afferent input and attached hindlimbs. (i) Sacral dorsal column stimulation recruits and strengthens ongoing locomotor-like activity, and implementation of a closed positive-feedback paradigm is shown to support its stimulation as an untapped therapeutic site for locomotor modulation. (ii) The spinal cord hindlimbs-restrained preparation allows suction electrode electromyographic recordings from many muscles. Inducible complex motor patterns resemble natural locomotion, and insights into circuit organization are demonstrated during spontaneous motor burst 'deletions', or following sensory stimuli such as tail and paw pinch. (iii) The spinal cord hindlimbs-pendant preparation produces unrestrained hindlimb stepping. It incorporates mechanical limb perturbations, kinematic analyses, ground reaction force monitoring, and the use of treadmills to study spinal circuit operation with movement-related patterns of sensory feedback while providing for stable whole-cell recordings from spinal neurons. Such techniques promise to provide important additional insights into locomotor circuit organization.

2. INTRODUCTION

Locomotion is defined as the act of moving from one place to another, and sufficient complexity in neural circuit operations is needed to allow for the range of locomotor behaviors observed. Limbed vertebrates must coordinate limb and trunk musculature to achieve smooth movements that integrate with postural and cardiorespiratory activity. The circuitry required for generating mammalian (and lower vertebrate) locomotor activity resides in the spinal cord (1), and many reviews consider its organization (e.g. (2-9)). Locomotor central pattern generators (CPGs) consist of sets of interconnected neurons that can generate rhythmical output patterns in the absence of phasic input. A fundamental CPG output for limbed locomotion comprises a 'half-center' alternation between flexors and extensors in a single limb and left-right alternation in flexors and extensors between limbs. It is thought that the CPG may be composed of multiple units, such as series of unit burst oscillators which can be flexibly coupled to produce different behaviors (2, 10) or a multi-level hierarchical structure with one level responsible for setting the timing (rhythm generator) and the pattern (pattern generators) (6). Several schematics have been drawn to explain experimental findings (e.g. (2, 11-16) including recent computational models by Rybak, McCrea

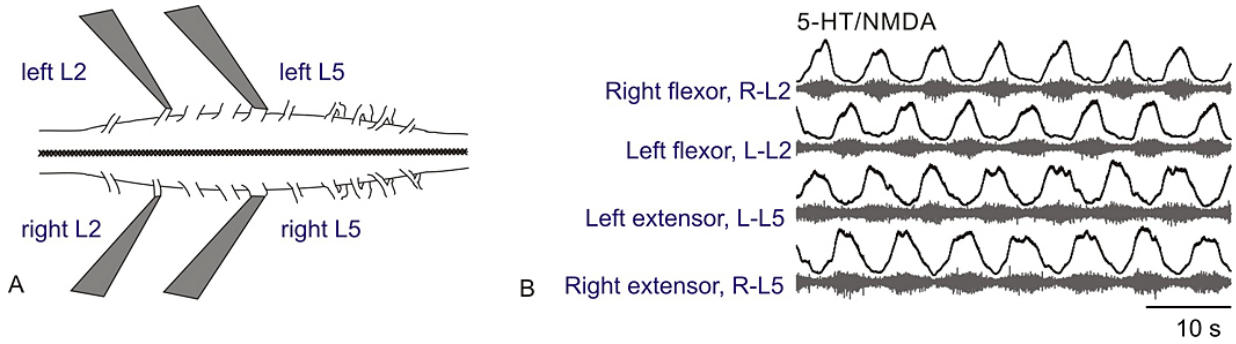

Figure 1. Experimental setup. (a) For *in vitro* studies on locomotor-like motor rhythms, suction electrodes are placed on lumbar L2 and L5 ventral roots bilaterally to monitor population motoneuron flexor and extensor activity. Drugs are applied to the bath. (b) Example of locomotor-like activity. Locomotor-like activity (LLA) is recruited with the addition of various neurochemicals with the combination of NMDA and 5-HT being the most common and illustrated here. Activity recorded from L2 ventral roots typically report flexor motoneuron activity while L5 ventral roots predominantly reflect extensor motoneuron activity. Rectified and low-pass filtered (top record) and raw records are displayed for each root recording.

and colleagues based on hypothesized half-center dynamics of a single cat hindlimb during locomotion (6, 17-19). Below, we explore the *in vitro* neonatal rodent (rat and mouse) spinal cord preparation as a model system to study properties of mammalian locomotion and the underlying circuitry. We provide a MATLAB-based code to analyze important measures of locomotor circuit operation. We then focus on experimental approaches that emphasize the contribution of sensory feedback and the importance of keeping hindlimbs attached.

2.1. The neonatal rodent spinal cord maintained *in vitro* for studies on locomotion

In mouse and rat, locomotor CPGs are present at birth and the spinal cord can be isolated from the brain and maintained *in vitro* (20). When activated by neurochemicals or electrical stimulation, the isolated spinal cord preparation produces a motor pattern consistent with locomotion that can be measured by using suction electrodes on ventral motor nerve roots or recordings from hindlimb muscles (21). While the full locomotor repertoire does not mature until a few weeks postnatal in rat (22), when the hindlimbs are left intact, the isolated spinal cord can produce limb kinematics and muscle activity patterns that are remarkably comparable to those seen in the adult (23-27). The greatest similarity to the adult requires specific afferent feedback, without which the pattern may more closely reflect a neonatal air-stepping pattern (23). One obvious difference between the adult and *in vitro* neonatal cord preparation, however, is in frequency, with those in the neonate being considerably slower than for adult locomotion *in vivo* (28).

Locomotion studies in the isolated *in vitro* spinal cord (taken at room temperature) afford distinct methodological advantages. Due to its small size, passive diffusion of oxygen in the CO2/bicarbonate buffered artificial cerebrospinal fluid (aCSF) maintains viability for many hours (21), though it is important to consider tissue depth-dependent gradients in pH, P02, and drug concentration (29-31). The extracellular medium can be controlled, and transport of drugs into the cord is not limited by the blood brain barrier, vastly expanding drug choice. The isolated cord also allows easy access for manipulation through global or restricted pharmacology, stimulation, and lesioning. Further, the surgery and electrode placement is relatively simple, and the absence of mechanical cardiorespiratory perturbations allows for stable whole-cell patch clamp recordings (16, 32). Defining the circuitry and function of CPGs in mammals has been greatly aided with the incorporation of complementary genetic approaches to this *in vitro* model. This includes expression-based neuron identification, conditional neuronal knockout/silencing of molecularly distinct cell populations, and optogenetics (3, 4, 7, 33-35).

While numerous bath-applied neuroactive substances can generate a patterned motor output consistent with locomotion, a combination of serotonin (5-HT) and N-methyl-D-aspartate (NMDA) is widely used to generate stable and long-lasting activity patterns consistent with a locomotor rhythm. The motor output recorded from lumbar L2 and L5/L6 ventral roots usually corresponds to activity in flexors and extensors, respectively, (36, 37) with left/right and ipsilateral alternation of bursts between these predominant flexor and extensor-reporting roots (Figure 1)(38, 39). When using the isolated spinal cord preparation the bursting pattern from ventral root neurograms is thought to provide a signature for activation of the locomotor circuitry and is commonly referred to as locomotor-like activity (LLA).

Normally, CPG circuit operation is initiated and controlled by activity in descending systems and/or primary sensory afferents. The intact *in vitro* brainstem-spinal cord preparation permits locomotor circuit activation via descending bulbospinal pathways (40-42). These pathways are clearly of great importance to understanding locomotor circuit operation. Recent *in vitro* studies have incorporated selective stimulation of descending pathways (33, 41), including activity-dependent imaging of recruited spinal neurons (43, 44) that promise to hasten our understanding of bulbospinal circuit function in locomotion.

Afferent activity-based recruitment of spinal locomotor circuits has also been demonstrated in the *in vitro* rodent spinal cord (35, 37, 45-52). However, activity-dependent afferent fatigue, difficulty in afferent fiber modality identification, and the lack of studies on movement-dependent sensory feedback on the operation of locomotor circuits make the study of afferents in the *in vitro* neonatal cord comparatively recalcitrant to detailed characterization (described further in Section 2 below).

Overall, while locomotor studies prior to weight-bearing may represent an inherent weakness of the neonatal *in vitro* model system for some studies (however, see (53)), the circuitry already expresses a mature motor pattern. This, in concert with discussed advantages, has catapulted the system into prominence in studies of mammalian locomotor circuits. Below we highlight enabling approaches and techniques that provide additional opportunities for experimentation in the *in vitro* model system.

2.2. Provision of a MATLAB-based code to analyze important measures of locomotion

To aid in quantifying and comparing rhythmic locomotor patterns, we developed a custom MATLAB® Graphical User Interface (GUI) called SpinalMOD (Spinal Motor Output Detector) for the analysis of LLA (Figure 2A & B). SpinalMOD determines the onset and offset of the bursts to calculate statistics on several variables including cycle period, frequency, burst duration, duty cycle, and phase (Figure 2C). SpinalMOD is a simple yet rather comprehensive single-screen GUI designed for ease of operation. SpinalMOD can open files collected with pCLAMP acquisition software, or files collected elsewhere and exported to appropriate Microsoft Excel formats. Four channels of data can be imported and compared at one time. Once opened, the raw data is rectified and processed through a low-pass filter. The burst threshold is calculated, and a series of IF-THEN logic statements are used for burst detection. This program is used for many of the subsequent analyses, and can be freely downloaded as-is from our website http://userwww.service.emory.edu/~shochm2/main_menu.html).

3. AFFERENT ACTIVITY-BASED RECRUITMENT, MODULATION AND FEEDBACK CONTROL OF LOCOMOTION

3.1. Introduction

Locomotor activity is regulated by ongoing afferent feedback recruited during the behavior (54). How afferent input can alter LLA in the *in vitro* neonatal cord preparation, and how afferent input properties differ from the adult remain an important but under-investigated area. Repetitive stimulation of afferents in sacral or lumbar dorsal roots, or the cauda equina is capable of recruiting LLA in the isolated spinal cord (27, 35, 37, 45-49, 51, 52). In comparison to neurochemical induction of LLA, which can be maintained for epochs of over an hour (55, 56), or to brainstem electrical stimulation-induced locomotion that can last for many minutes (40) afferent electrical stimulation-based recruitment of LLA is typically short-lived. Studies on sensory stimulation-based recruitment of LLA utilize trains of low frequency stimuli (generally less than 5 Hz) to generate an activity that builds up, stabilizes, and then fatigues within about one minute (35, 37, 49, 57, 58). LLA cannot be reinstated by stimulation of the same afferents for over a minute due to a required recovery from fatigue, presumably due to transmitter depletion in afferent terminals in the neonate (48, 59).

The dorsal column is a white matter tract predominantly composed of primary afferents carrying touch, vibration and proprioceptive information as well as information from visceral afferents (60, 61). It issues collaterals as it ascends to influence and possibly recruit, coordinate, and control locomotion. In the adult rat dorsal column, most axons are fast-conducting myelinated Aβ fibers; however, almost one-third of the axons appear to be unmyelinated primary afferents (62). While sacral caudal afferent stimulation is a useful strategy for electrically evoked control of motor circuitry, as a white matter tract, stimulation of the sDC may provide a unique therapeutic opportunity when incorporated into the use of conformable multi-electrode arrays for surface stimulation (63). In support of this proposition, previous studies involving epidural stimulation in humans, cats, and rats implanted wire electrodes on the dorsal surface to activate locomotion, and based on their positioning, these are likely also activating dorsal column pathways (64-67).

3.2. General methods

For the studies in this section, the spinal cords of postnatal day (P) 0-3 rats were isolated and maintained in a static solution of oxygenated aCSF with the dorsal aspect of the cord facing upward. Glass suction electrodes were applied to ventral lumbar roots (L2s and L5s) for recording. For stimulation, glass suction electrodes were applied to a sacral dorsal root, or to the dorsal surface midline region (with ~30 μm tip diameter) of the sacral dorsal column (sDC) between segments S2 and S4. Rhythmic activity was induced with trains of stimuli between 1 and 2 Hz.

3.3. Effects of sacral dorsal column stimulation on activated locomotor-like activity

Figure 3A&B show that electrical stimulation of the sDC recruits afferents that activate the locomotor CPG with properties comparable to that observed following stimulation of sacral dorsal roots (e.g. (37, 47)). Figure 3B and subsequent panels are shown following stimulus artifact removal (a feature provided in SpinalMOD). Note that recruitment of alternating L2 ventral root activity does not necessarily couple to comparable activity in L5 ventral roots and so may not always recruit a full locomotor pattern. The observed fatigue can be overcome by stimulating alternate afferent fiber populations using multiple stimulation sites, as shown previously with switching stimulation to contralateral dorsal roots (57). Examples of the effects of recruiting additional populations of afferents to reinstate a motor rhythm after activity-dependent fatigue, either in dorsal roots or dorsal column, are shown in Figure 3C&D, respectively. These results demonstrate the possibility of extending the duration of

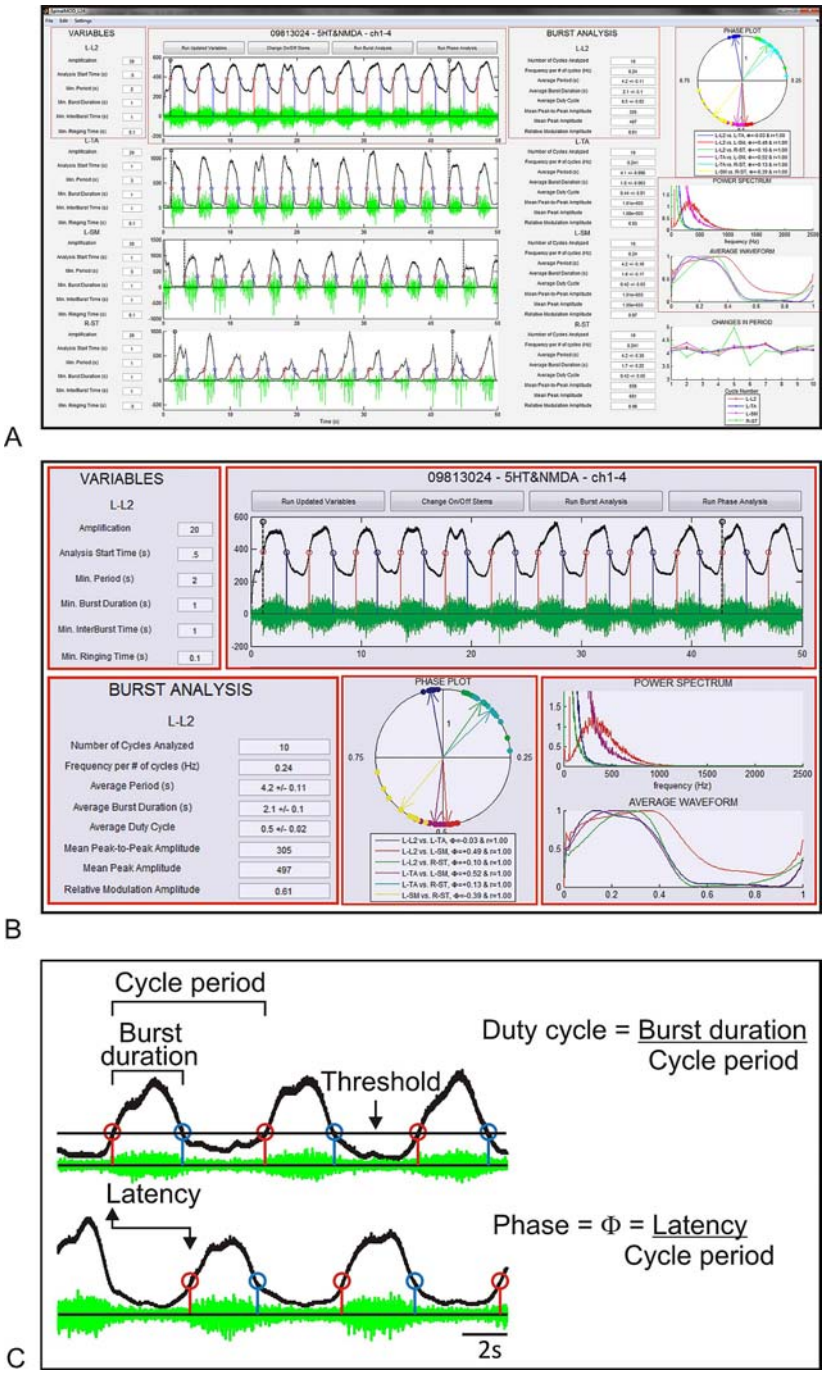

Figure 2. Commonly measured variables to describe locomotion analyzed with a custom Matlab-based GUI. SpinalMOD is designed to analyze ventral root, dorsal root, or muscle bursting for up to four channels. (a) Screenshot of the GUI with boxed regions enlarged in panel (b). Input variables are shown to the left and readily changed by the user by clicking in the box. The raw (green) and filtered (black) waveforms are graphed in the middle. Following burst detection, burst onset (red) and offset (blue) are denoted by vertical stems. "Run Updated Variables" allows the user to adjust the input variables and rerun the detection algorithm to correct misplaced burst markers. The stems can be also manually changed with "Change On/Off Stem Marks." Once burst detection is acceptable, burst analysis is run by selecting "Run Burst Analysis." The user then identifies the first burst to analyze and provides the number of bursts to be analyzed. Dashed black stems mark the beginning and end of the analyzed period. Once analyzed, the values calculated are shown in tabular and graphical form the Burst Analysis section to the right of the GUI. A phase plot figure as well as three of six other graphs, (average waveform, period, peak height, duty cycle, burst duration, and power spectrum), can be chosen for display. Finally, tabulated value and figures can be exported to Excel for subsequent use. (c). Blow-up of example raw and filtered waveform data shows commonly measured variables used to describe locomotion. For more information and to download SpinalMOD freely go to http://userwww.service.emory.edu/~shochm2/main_menu.html.

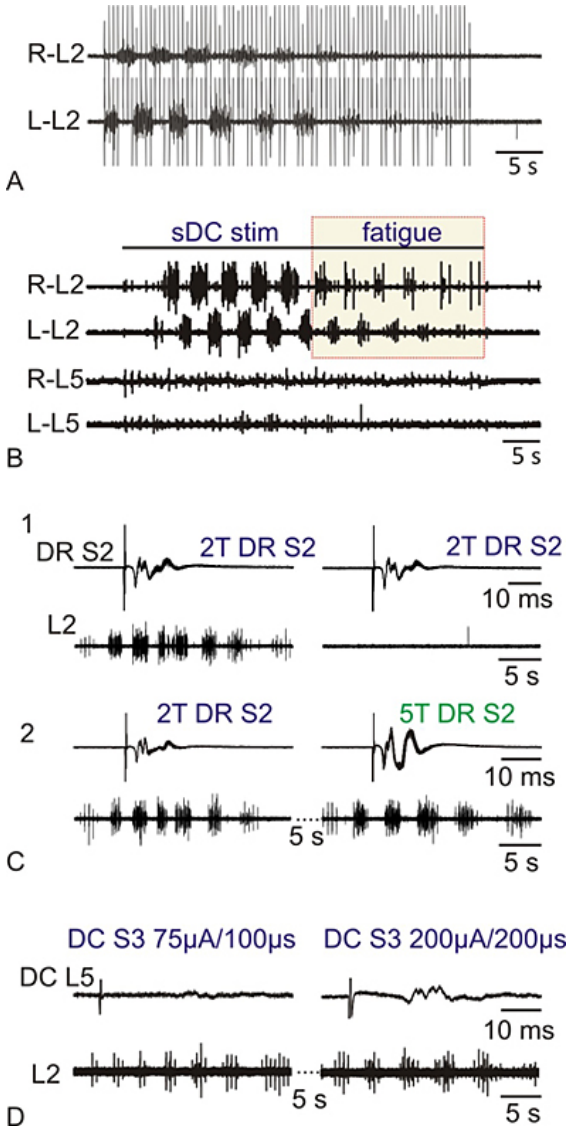

Figure 3. Afferent activity-dependent recruitment of rhythmic motor activity. (a) Sacral dorsal column (sDC) stimulation evoked rhythmic alternation in lumbar (L2) ventral roots shown in the presence of stimulation artifacts. Notice how the rhythm slowly fatigues even as stimuli are continually applied. (b) Continuous stimulation of the sDC recruits a rhythmic alternating pattern of motor activity with alternation between left and right L2 ventral roots. The recruited activity clearly beings to fatigue within 30s of its onset (2 Hz). Fatigability can last several minutes. (c and d) Stimulation intensity dependent recovery of fatigability in fiber pathways recruited in either sacral dorsal roots (sDR) or sDC. Top panels show fiber volleys recorded in the same dorsal root as stimulated (c) or in the same dorsal column tract as stimulated (d). Bottom rows show activity on a longer time scale in ventral roots. (c1) Stimuli were delivered to the sacral dorsal root (2 times threshold for afferent volley detection (2T) at 2 Hz) until motor activity fatigued. After a brief pause (less than five seconds), the stimulus train was repeated without recruitment of rhythmic L2 ventral root motor output. (c2) Fatigue of rhythmic activity at 2T can be quickly reinstated by recruiting a previously unstimulated fiber population at 5T to rescue the rhythm. (d) Similarly, when stimuli were delivered to the sacral dorsal column to fatigue the pattern (2 Hz) and subsequently delivered at higher intensity, rhythmic output was reinstated.

afferent stimulation-evoked LLA by interleaving stimulation at different sites.

In summary, primary afferent-evoked LLA fatigues quickly in the *in vitro* neonatal preparation. Strategies for interleaving recruitment of afferent fiber subpopulations or recruitment by natural afferent stimuli with more asynchronous recruitment may help considerably (23, 68). Further systematic exploration of these methods is needed especially as continuous afferent activation with epidural stimulation can recruit the hindlimb locomotor CPG after chronic spinal injury (66, 69-71).

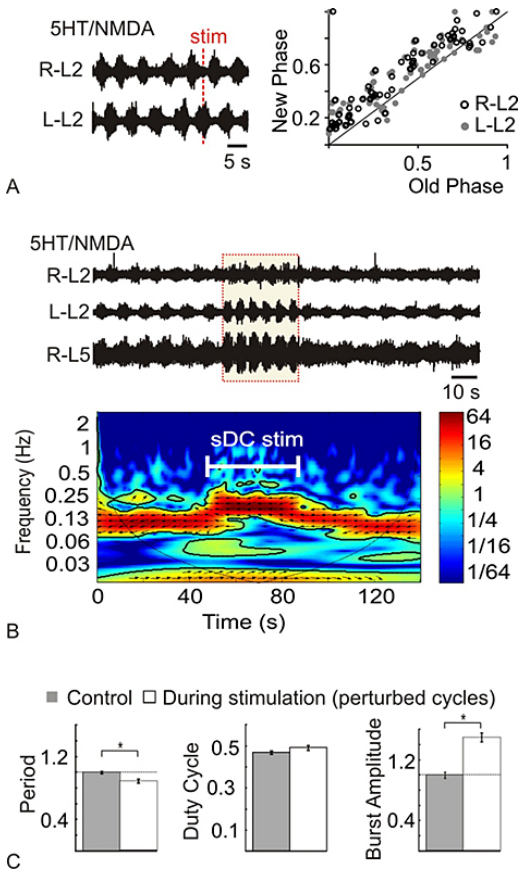

Figure 4. (a) Single pulses of sDC stimulation during 5HT/NMDA induced locomotor-like activity abruptly altered the bursting ventral root's activity. When analyzed for phase changes, these pulses result in shifts in the expected phase. These shifts are generally phase advances in both ventral roots regardless of the phase at which the stimuli occur as shown at right. To calculate the phase of the CPG, a "zero phase marker" (ZPM) was defined as the onset of bursting. At the time of stimulation, the "old phase", φ_{old}, was calculated as:

$$\varphi_{old} = \frac{t_{stim} - t_{ZPM}^{N}}{\bar{T}}$$

where t_{stim} is the time of stimulation, t_{ZPM}^{N} is the time of the most recent burst onset, and $\bar{T}$ is the mean cycle length measured during unperturbed bursting. After the stimulus occurs, the "new phase", φ_{new}, was calculated as:

$$\varphi_{new} = 1 - \frac{t_{ZPM}^{N+1} - t_{stim}}{\bar{T}}$$

where t_{ZPM}^{N+1} is the time of the next burst onset after the stimulation. Each stimulus pulse, therefore, generates a pair of "old" and "new" phases (φ_{old}, φ_{new}), which is plotted as a phase response curve. If the stimulus has no effect on the ongoing rhythm, then the old phase will equal the new phase. (b) Stimulus trains (2 Hz) significantly changed the cycle period and burst amplitude of the 5HT/NMDA locomotor-like activity. Wavelet analysis visualization of changes in frequency and phase over time for this data is provided below the raw data. Note that during stimulation there is a shift in the power of the low frequency component at locomotor frequency to a larger value that then returns to its original values after termination of stimulation. (c) Combined results from analysis of three experiments as in (b) are shown in (c). Data is displayed as mean ± S.E.M. Asterisks indicate significance with $p<0.05$.

3.4. Effects of sacral dorsal column stimulation during ongoing neurochemical locomotion

Previous studies have demonstrated that afferent activation can reset or entrain locomotor rhythms (39, 72-76). Resetting occurs when a stimulus alters the length of the ongoing flexor or extensor burst resulting in a phase delay or advancement of future bursts which occur at the same frequency as before (76). Entrainment occurs when the frequency of the CPG locomotion is altered by and follows the frequency of an external stimulus (15, 39, 75).

In comparison to in vivo studies predominantly undertaken in cat, where intensity of electrical stimuli correlates well with recruitment of afferent fiber modalities (e.g. (77, 78)), it

is much more difficult to target specific afferent fiber populations in the neonatal rodent using electrical stimulation (e.g. (79)) at least in part due to incomplete myelination (80). Nonetheless, activation of muscle afferents during 5-HT/NMDA-induced rhythms also produces resetting in the neonate (73, 81) and stimulation of lumbar dorsal roots may entrain locomotion (39). Last, locomotor frequency can be modulated by thermal stimuli applied selectively to the tail (82) or hindlimb (68) with frequency reversibly increasing and decreasing as a function of temperature deviations from physiological skin temperature (68).

Here we show that single pulse stimuli delivered to the sDC alter LLA, but do not cause phase resetting of the rhythmic pattern (Figure 4A). To analyze effects, changes in phase were calculated and displayed as a phase response curve (83) (see Figure 4 legend). In such plots, phase advances lie above the equality diagonal, and phase delays lie below. In three experiments, we found that the vast majority (87%) of stimulus pulses resulted in a phase advance in both right and left ventral roots (Figure 4A). This demonstrates that a single afferent stimulus acts on the rhythm generator to support a brief locomotor frequency increase. In comparison, short trains of pulses (500 μA/200 μs, 1-2 Hz) delivered to the sDC during ongoing 5-HT/NMDA LLA increased both burst amplitude and frequency (n=3/3; Figure 4B&C). The rhythm did not entrain to the stimulus frequency, and there was no change in duty cycle, suggesting the trains of stimuli provide additional excitatory drive to the neurochemically-activated locomotor CPG. Overall, these data demonstrate that sDC afferent stimuli facilitate locomotion. This is of potential importance to the development of future neuroprosthetic devices, such as spinal cord stimulators in closed-loop feedback strategies as considered below.

As shown in Figure 4B, the stimulation-induced increase in locomotor frequency coincides with a corresponding shift in predominant wavelet coherence. Wavelet spectral analysis is advantageous over traditional Fourier analyses for visualizing coherent frequency components and phase relationships for biological signals since many biological signals are nonstationary, and wavelet analysis methods do not assume stationarity. Moreover, its sensitivity facilitates detection and comparison of signals weakened by lesioning or pharmacological means (84, 85).

3.5. Afferent stimulation in closed-loop feedback strategies for locomotor control

The importance of afferent activity in the control of locomotor output is well established, and can be used to control locomotion after injury. For example, Edgerton and colleagues demonstrated that continuous epidural afferent stimulation recruits locomotor activity in the chronic spinal rat (66, 69-71). In cases of incomplete injury, a strategic output-based control of afferent stimulation may be preferred in order to augment but not overwhelm compromised locomotion. Below we discuss and explore close-loop feedback as a control strategy. Closed-loop feedback control strategies enable the system to autonomously operate and alter the inputs by comparing the output to a desired outcome. This methodology utilizes information regarding the input-output relationships of different system parameters to develop algorithms for feedback control. For example, in the cat, Mushahwar and colleagues used intraspinal microstimulation to show that basic proportional-derivative controllers mimic the phasic nature of neural signals and could produce locomotor stepping rhythms using a surprisingly small number of stimulating electrodes (86). Additionally, Vogelstein and colleagues developed a closed-loop control scheme to regulate directionality of simulated lamprey swimming using electrical stimulation of a spinal hemicord (87).

There are two basic forms of feedback control. Negative feedback works to maintain an equilibrium set point by altering the feedback to negate the error in the output. Positive feedback loops augment small perturbations to the system. If left unchecked, positive feedback systems are unstable, requiring an external variable or counter-signal to break the loop and limit the positive feedback. In the case of locomotion, the circuitry itself may intrinsically limit positive feedback controls due to neural fatigability, in their afferent inputs or in the CPG itself.

Using the isolated neonatal rat spinal cord preparation, we developed a simple positive feedback system based on detecting locomotor bursts in real-time. With sufficient levels of 5-HT/NMDA to induce a subthreshold erratic bursting pattern, a thresholding closed-loop simulation protocol was used to ascertain the impact of sDC stimulation on the ongoing locomotor pattern. The rectified, low-pass filtered signal from a single, flexor-related L2 ventral root was used. A burst was identified as beginning once the filtered trace rose above a specified threshold. At the onset of a detected burst, a stimulus pulse was delivered to the sDC. The stimuli served as positive reinforcement, presumably by providing additional excitatory input to the locomotor CPG. Prior to instituting the closed-loop feedback system, the phase relationships between the recorded ventral roots were irregular with a rather variable cycle period. After the closed-loop was initiated, cycle period variability was reduced to provide a more stable rhythm (n=3). An example of the effects of closed-loop stimulation is shown in Figure 5. While theoretically closed-loop positive feedback stimuli should become unstable, as demonstrated above, if afferent transmission fatigues or is limited by presynaptic inhibitory mechanisms, such mechanisms could limit the duration of productive afferent stimulation-induced actions. Because fatigue-based negative feedback may lessen with development, alternatively, a stopping external signal may be programmed into the closed-loop feedback to control for any tendency towards instability by inhibiting the rhythm or by simply ceasing the positive-feedback stimulation temporarily.

We conclude that afferent stimulation may be incorporated in closed-loop feedback strategies for locomotor control, and the *in vitro* model provides for a highly accessible high-throughput system to explore such strategies (63).

4. METHODOLOGIES TO STUDY CIRCUIT OPERATION WITH INTACT HINDLIMBS: THE *IN VITRO* SPINAL CORD HINDLIMBS-RESTRAINED PREPARATION

4.1. Introduction

While ventral root recordings offer a simple way to monitor spinal motor output, they do not identify nor report recruitment of individual muscles. Individual lumbar ventral roots contain axons projecting to flexors and extensors (88, 89), axial musculature (90), and sympathetic preganglionic neurons that exit from thoracic and upper lumbar segments (91). Thus, one cannot a priori assume that observed rhythmical activity in these roots reliably

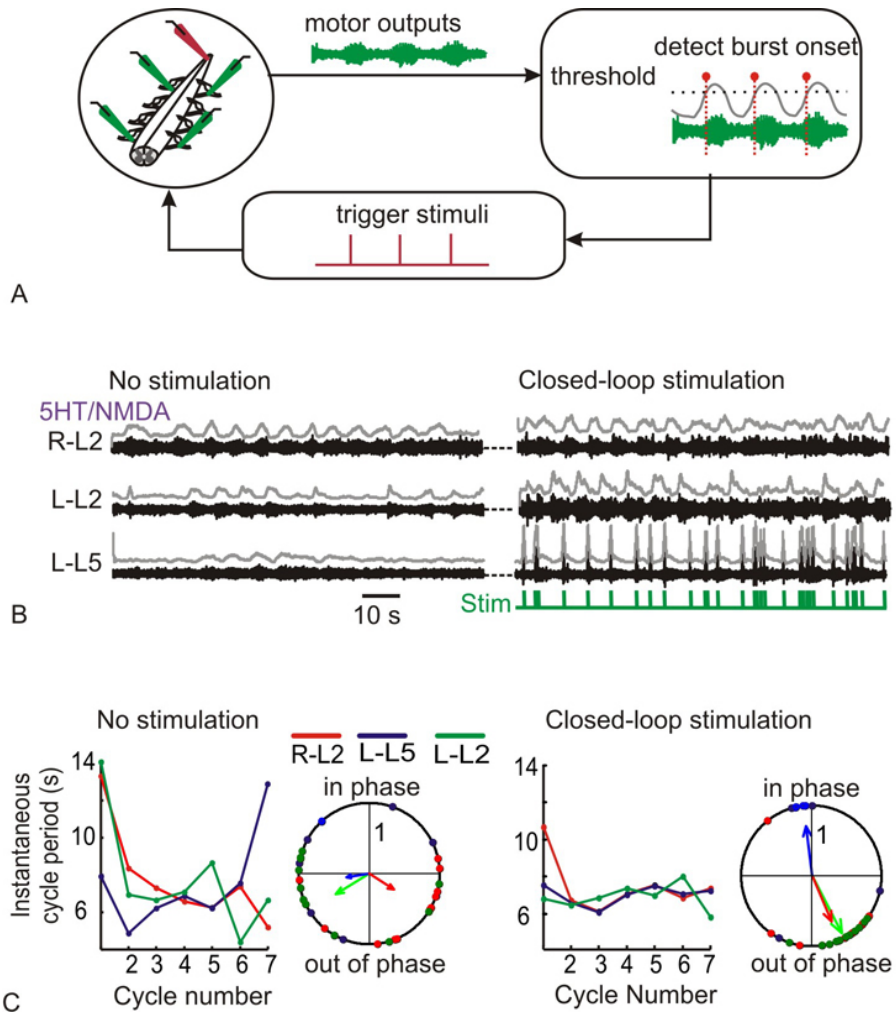

Figure 5. (a) Real-time burst detection feedback for triggering stimuli to alter ongoing 5HT/NMDA locomotion. (b) Irregular locomotor patterns with inconsistent instantaneous ventral root phase relationships. (c) Closed-loop stimulation reinforced the motor pattern, and phase relationships between roots were more consistent. Phase plots compare indicated root pairs.

represent the activity of individual flexors and extensors (92), particularly after experimental interventions that could alter actions on efferents not associated with hindlimb movement. For example, sympathetic preganglionic axons also exit the L2 ventral root, and may also express rhythmical patterns of activity (93, 94). An additional limitation of ventral root recordings is that important information on motor coordination is lost, including sequential activation of muscles, and distinctive patterns that reflect activation of different locomotor programs (e.g. swim vs. step) as can be observed with different bath-applied neuromodulators or following afferent electrical stimulation (25, 27). Finally, without the hindlimbs and their innervation intact, the influences of distinguishable sensory inputs on circuit function are limited. Demonstrated advantages of the SCH-R for studies on locomotion are shown below.

4.2. General methods

The *in vitro* spinal cord hindlimbs-restrained preparation (SCH-R) permits EMG recordings with suction electrodes attached to hindlimb muscles. In order to accomplish this, the spinal cord and attached hindlimbs are immobilized ventral side upward with insect pins affixed to Sylgard-lined chamber bottom (Dow Corning). Lower thoracic and the lumbar ventral roots innervating the hindlimbs remain intact, and the vertebral column surrounding the cervical and most of the thoracic spinal cord is removed to allow for sufficient cord superfusion. Thoracic and lumbar dorsal roots were cut in experiments on neurochemically-evoked locomotion, but remained intact for studies on afferent feedback. Independent micromanipulators with attached glass suction electrodes are used. We typically record from lumbar L2 ventral root en passant and 7 hindlimb muscles (Figure 6A) (see also (27)). Example recordings provided here were acquired from the following muscles: tibialis anterior (TA; ankle flexor), medial gastrocnemius (MG; ankle extensor), semitendinosus (St; knee flexor / hip extensor), semimembranosus (Sm; knee flexor / hip extensor), vastus medialis (VM; knee extensor), rectus femoris (RF; knee extensor / hip flexor), and adductor magnus (AM; hip

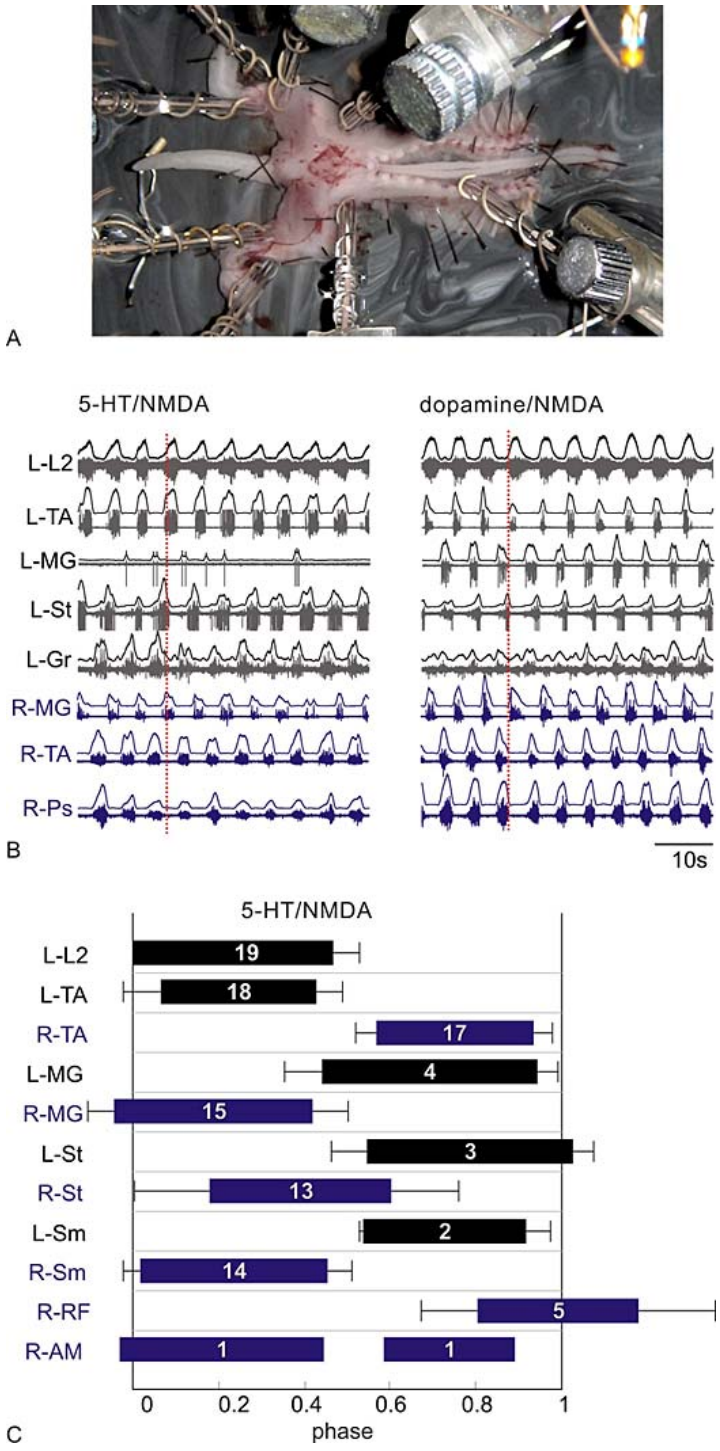

Figure 6. Multiple suction EMG electrode recordings in the *in vitro* neonatal rodent cord provide information regarding the locomotor pattern. (a) The isolated neonatal rat spinal cord with attached hindlimbs maintained *in vitro*. For studies on the output of muscle coordination, glass suction electrodes are placed on the left lumbar L2 ventral root and typically 7 muscles of the hindlimb. Drugs are applied to the bath. (b) EMG recording of LLA comparing motor patterns recruited in an animal in the presence of 5-HT and NMDA (left) or dopamine and NMDA (right). (c) Phase diagram summarizing activity periods observed during 5-HT/NMDA LLA. All phases are referenced to the onset of the L-L2 ventral root. Bars represent the average burst duration of muscles normalized to cycle duration. Burst onset is on the left and burst termination on the right. The number of rats used to obtain the diagrams is given in the middle. One difference observed from the earlier reports using 5-HT (Kiehn and Kjaerulff 1996) or 5-HT and NMDA (Klein *et al.* 2010) is that AM can also be observed during the flexor phase.

adductor), gracilis (Gr; hip flexor / hip adductor), iliopsoas (Ip; hip flexor), and paraspinal (Ps; movement of vertebrae). The left lumbar L2 ventral root (L-L2VR) was always recorded: (i) to enable comparison of the ventral root activity profile obtained in the isolated spinal cord preparation, and (ii) to provide a common reliable comparison between experiments where muscle EMG recruitment was variable (see also (27)).

4.3. Recording muscle EMG recruitment patterns during locomotion: complex phasing and expression of spontaneous burst deletions

Bath-applied 5-HT with NMDA produced EMG locomotor phase relations consistent with those seen previously for 5-HT by Kiehn and Kjaerulff, or 5-HT with NMDA by Tresch and colleagues (25, 27) (Figure 6B, left). While there was inter-animal variability in muscle recruitment, TA and MG were active during the flexor and extensor phases, respectively. The phase diagram in Figure 6C compares these phase relations and those of other recorded muscles in the sample population. Under these conditions Sm and St, both knee flexor and hip extensors, were usually both active during the extensor phase, while RF, a knee extensor and hip flexor was active during the flexor phase. Locomotor rhythms generated with bath-applied dopamine were previously reported to be slower, more variable, and require much higher doses than 5-HT (25). Interestingly, though flexor/extensor coordination patterns were preserved, they observed clear phase differences in recruitment patterns of bifunctional muscles, supporting a neuromodulator-dependent recruitment of different locomotor programs. Here, when applied with NMDA, 5-HT or dopamine generated locomotor rhythms that occurred at comparable frequencies and with muscle recruitment patterns (Figure 6B, right; n=4). Though preliminary, these findings highlight the contribution of co-applied NMDA to locomotor pattern expression.

Spontaneous absences of bursting in specific motor pools, often referred to as deletions, provide clues to the underlying modular organization of CPGs in vertebrate limb pattern generation. For mammalian hindlimb locomotion, McCrea and colleagues examined spontaneous burst deletions observed in motor pools from one hindlimb of the decerebrate cat during electrical stimulation of the mesencephalic locomotor region (MLR). These deletions are of two forms; non-resetting and resetting. Non-resetting deletions are identified when the reappearing activity emerges without phase shift in the locomotor cycle. In contrast, resetting deletions undergo a shift in the phase of the re-emerging rhythm relative to the rhythm that preceded it. The presence of resetting and non-resetting deletions provided strong evidence of a minimally two-layered CPG organization with rhythm-generating and subordinate pattern-generating circuits to control ipsilateral patterns of locomotor activity (6, 18, 19, 95). While compelling, these studies are nevertheless limited to the experimental model system utilized. Studies incorporating bilateral hindlimb and forelimb EMG or ENG recordings, will undoubtedly provide more complete insight into the organization of locomotor CPGs (24, 96). In comparison, Stein and colleagues undertook an analysis of deletions and the modular structure of the turtle rostral scratch CPG and observed an organization consistent with independent 'unit burst generators' as originally hypothesized by Grillner (2, 97).

Deletions are also observed during LLA in the isolated *in vitro* neonatal spinal cord of mouse (Ron Harris-Warrick, personal communication) and rat (below). Comparison of expressed deletions to the adult decerebrate cat may identify both conserved deletions and interesting differences in deletions during the uncoupling of modular elements of the activated locomotor circuit. Experimental approach, species, and developmental stage could all conceivably alter deletion expression patterns. To our knowledge locomotor studies examining spontaneous motor pool or muscle activity deletions in the *in vitro* hindlimbs-attached preparations have yet to be utilized. Here, with bilateral EMG recordings, many spontaneous deletions are observed during neurochemically-induced locomotion. For example, Figure 7A shows a non-resetting deletion in the left ankle flexor TA during the flexion phase and this occurs without obvious changes in activity in its strict antagonist MG, or in right flexor and extensors. These results are consistent with independent flexor and extensor pattern generators across a joint, an observation supporting Grillner's unit burst generator hypothesis (2). As loss of left TA activity is not associated with a loss in left L2 VR activity, rhythmic activity in ipsilateral flexors can be independently controlled. Figure 7B shows spontaneous coincident deletions of activity in left TA and right MG with corresponding complementary actions in the opposite limb: prolonged activity in right TA and left MG (shaded bars). Such deletions support the existence of a bilateral pattern generator that couples flexor activity in one limb with extensor activity in the opposite limb. This is consistent with a deletion in the population of descending contralateral interneurons identified by Butt and Kiehn (2003) that couple ipsilateral flexion with contralateral extension (98). Spontaneous resetting deletions are also seen in this preparation as shown in Figure 7C. Loss of the right TA corresponds with continued right extensor MG and Sm EMG activity with a subsequent maintained phase shift in timing. While the data presented here is preliminary, deletions during neurochemical locomotion were observed in most experiments, and the variations remain to be fully analyzed.

Overall, the much greater ease of interneuronal recordings, and flexibility for manipulation of motor output in the *in vitro* preparation, provides a high-yield platform to dissect the organization of locomotor pattern-generating circuits. When integrated with the guidance of genetic manipulations that now include optogenetics and high-throughput connectivity mapping (33, 99), a rather complete dissection of mammalian locomotor operation seems tantalizingly within reach. Yet, it is important to acknowledge evidence of at least two, and possibly three, distinct interneuronal circuits that generate locomotion in the phylogenetically older limbless vertebrates (100, 101). It is reasonable to assume these circuits are evolutionarily

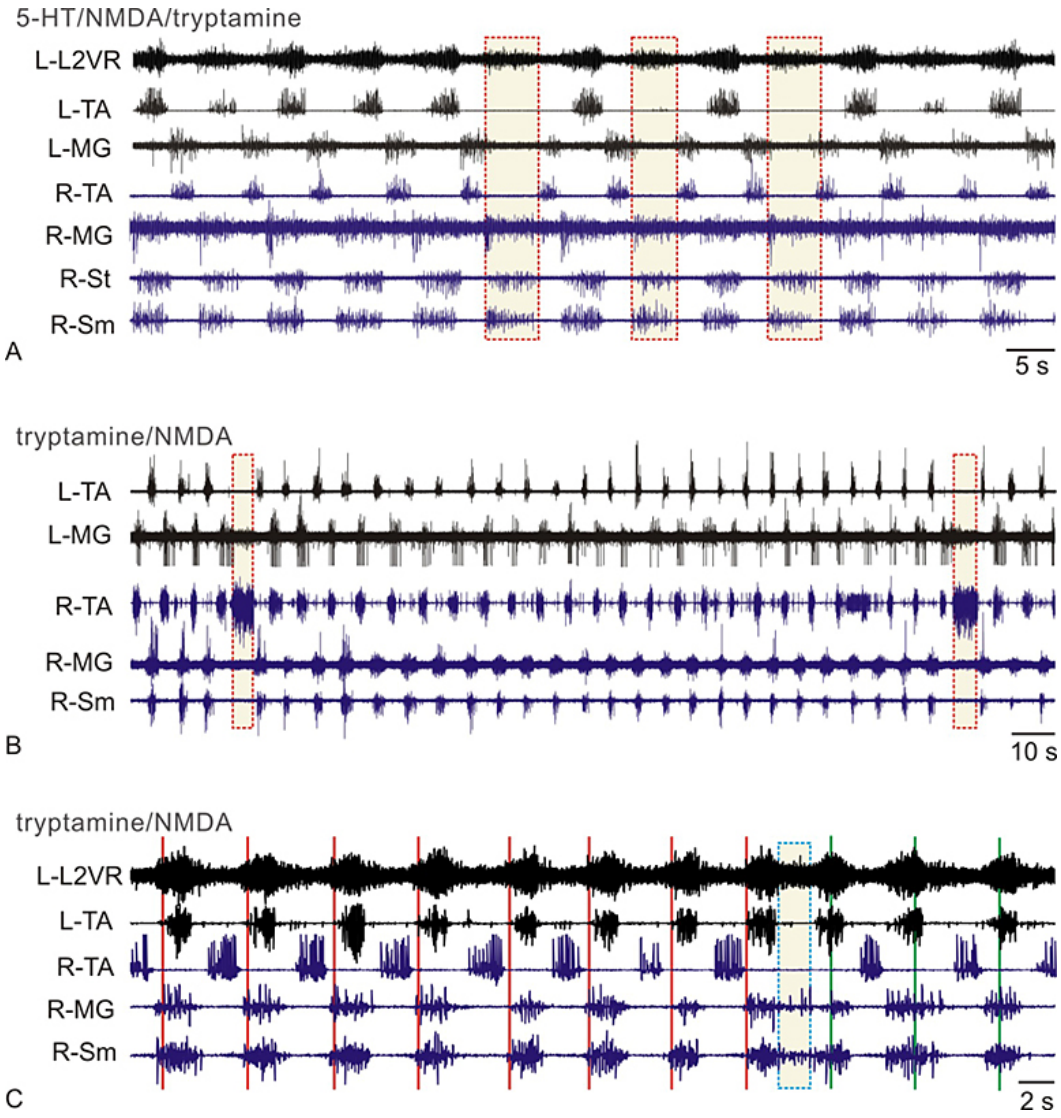

Figure 7. Multiple EMG recordings provide insight into the organization of central patterns generators based on spontaneous intermittent loss in muscle recruitment (deletions). (a) The left TA flexor phase burst is absent at expected time (shaded bars) but there is otherwise no obvious alteration in EMG activity in extensor phase or flexor phase activity in other channels recorded bilaterally. Note the loss of left TA activity is associated with only a reduction in left L2 ventral root amplitude. These results are consistent with several flexor modules, as well as an independence from extensor module. (b) Spontaneous coincident deletions of activity in left TA and right MG associate with corresponding complementary actions in opposite limb (prolonged activity in right TA and left MG (shaded bars). Such deletions support a bilateral pattern generator of mutual inhibitory interactions between flexors and extensors. (c) Evidence of a spontaneous resetting deletion. Red lines mark approximate same locomotor phase (onset of left TA activity), whose average duration is used to predict subsequent burst onsets (green lines). Note that after spontaneous deletion of the right TA and corresponding continued right extensor activity, subsequent locomotor activity has undergone a phase shift in timing consistent with a resetting of the locomotor rhythm.

conserved in limbed vertebrates including mammals. If true, studies undertaken during differing behavioral drives and/or afferent input patterns, particularly when combined with the silencing of distinct neuronal populations (3, 4) may uncover additional independent mammalian locomotor CPG circuitries, with differing modular organizational structures. The SCH-R and spinal cord hindlimbs-pendant preparation (SCH-P; see section 5 below) provide powerful model systems for such analyses.

4.4. Afferent activity can perturb ongoing locomotion: Activation of nociceptive flexion reflexes

Nociceptors and other higher-threshold afferents that converge onto interneuronal populations to produce ipsilateral flexion reflexes can be classified as flexor-reflex afferents (FRA) (72, 102-105). This includes the longer-latency FRA pathways unmasked by L-dopa in the spinal cat that generate spinal stepping (13, 106). Flexor reflex afferents can reset fictive locomotion to flexion in the cat

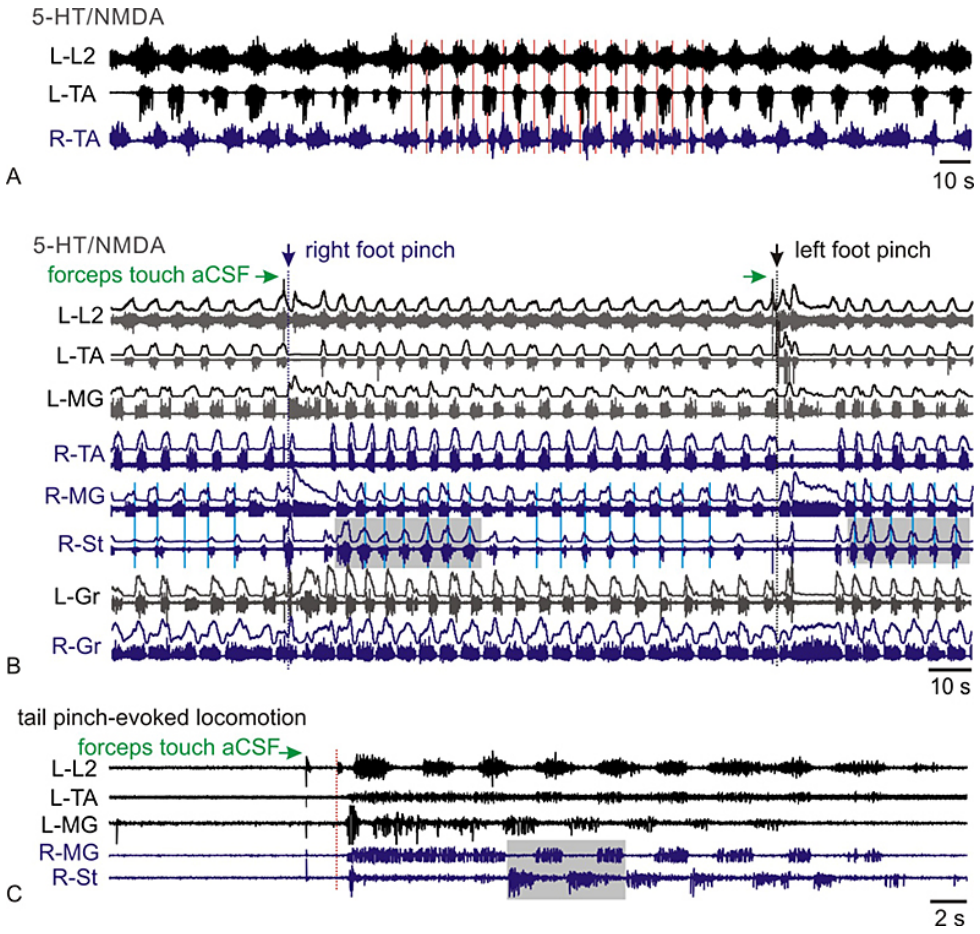

Figure 8. Sensory stimuli modify locomotor pattern expression. (a) High intensity lumbar dorsal root stimulation (1 mA, 1 ms) is able to increase and entrain frequency at half the simulation frequency (0.2 Hz at red bar) with signs of 1:1 entrainment at the end of the stimulus train. (b) Pinch evoked increase in locomotor frequency. Fast initial right pinch or left pinch-evoked ipsilateral right or left flexion reflexes, respectively, but this is difficult to discern on the slow time scale displayed. After initial response, both pinch events result in a suppression of ankle flexor muscle actions bilaterally with coincident bilateral strong recruitment of extensors. This event precedes a subsequent frequency increase that couples to greatly facilitated activity in the knee flexor, St, which now includes temporal overlap with ankle extensor activity (blue timing lines in gray shaded boxes). (c) Tail pinch-evoked action on locomotor circuits. Tail pinch (at red bar) initiates several cycles of LLA (top series) that is faster than neurochemically-evoked LLA (bottom series).

(107), and high threshold sensory stimuli in the neonate also reset to flexion (73). Studies in the *in vitro* neonatal spinal cord have demonstrated nociceptor activation of LLA (82) as well as facilitation of ongoing LLA as measured from ventral root recordings (68, 79). Ventral root recordings of LLA obviously limit interpretations of afferent-evoked alterations in LLA and motor coordination. In contrast, the response signature recorded from many muscles can provide a more complete assessment of afferent-evoked changes – from those that span multiple joints, that includes actions on bi-functional muscles, adductors and abductors, and even in the associated axial musculature (e.g. paraspinal muscle in Figure 6B).

We provide example actions of high threshold afferent electrical stimulation as well as mechanonociceptor pinch-evoked actions on EMG activity recorded bilaterally from several hindlimb muscles. High intensity lumbar dorsal root stimulation increased locomotor frequency and locomotion entrained to the stimulus frequency of 0.2 Hz (Figure 8A). Observations based on multiple EMG recordings identify recruitment patterns clearly more complex than observable from ventral roots. EMG recordings from many individual muscles during hindlimb pinch provided an interesting and complex sequence of motor coordination transitions, as shown in Figure 8B. Left or right hindlimb pinch produced an initial ipsilateral activation of flexors (flexion reflex) followed by a longer-lasting bilateral suppression of ankle flexors (TA) with concomitant strong recruitment of ankle extensors (MG). Note that the hip flexor/adductor Gr had more differentiated actions. Thereafter, a frequency increase emerged, lasting several cycles. This increase included a phase shift in patterning as St switched from flexor-related to extensor related activity, and with greatly facilitated motor recruitment. Figure 8C shows that tail pinch initiated

several bouts of locomotion in the absence of ongoing neurochemically-induced locomotion. This included several cycles of continuous bilateral tonic EMG activity. While the behavioral relevance of the aforementioned examples require additional study and interpretation, it is clear that hindlimb-attached preparations greatly extend the power of the *in vitro* studies of spinal locomotor circuits.

In summary, the SCH-R allows for easy placement of suction EMG electrodes that can be maneuvered for EMG recordings from multiple muscles bilaterally. Combined with the capacity for studying locomotor modifiability with various neurochemicals, both electrical and natural afferent stimulation, and expression of spontaneous deletions, it is clear that this approach will enable considerable further insight into locomotor mechanisms.

5. METHODOLOGIES TO STUDY CIRCUIT OPERATION WITH INTACT HINDLIMBS: UNRESTRAINED HINDLIMB LOCOMOTION

5.1. Introduction

While aspects of sensory feedback can be studied as described above, these studies cannot recreate the complex interactions between sensory modalities produced by natural limb movement, nor determine the behavioral impact of pharmacological, electrical, or mechanical manipulations on hindlimb kinematics. To overcome these limitations, we developed a novel *in vitro* spinal cord hindlimbs-pendant preparation (SCH-P) in the neonatal rat that combines the neural accessibility of the classic *in vitro* preparations with the natural patterns of sensory feedback and behavioral observability of more intact preparations (23). The SCH-P is composed of the nearly isolated, fully exposed neonatal rat spinal cord oriented dorsal-up and maintained in continuously oxygenated aCSF. The hindlimbs, as well as the corresponding dorsal and ventral lumbosacral roots, are left intact, with the limbs hanging pendant for stepping (Figure 9). Hindlimb stepping can then be activated by bath application of 5-HT or dopamine with or without NMDA. Sensory-modulated behavior can then be characterized using sagittal plane kinematics, EMG recordings of muscle activity, ventral and dorsal root recordings, and intracellular recordings. Thus, the SCH-P allows us to relate behavior to underlying neural substrates and to quantify the effect of pharmacological, electrical, or mechanical manipulations on neural function and behavior in ways not typically possible *in vitro*. Here we review the technologies developed for use with the SCH-P, demonstrate the use of the SCH-P to study sensory modulation and reinforcement of spinally-generated locomotion, and report the first intracellular recordings undertaken during unrestrained hindlimb locomotion.

5.2. General methods

Because dorsal-up hindlimb stepping had not been previously undertaken *in vitro*, several enabling technologies were needed to establish an environment amenable to hindlimb stepping and to enable extensive mechanical characterizations. First, we developed a custom perfusion chamber and treadmill system to facilitate stepping in the aqueous solution and at the slow speeds characteristics of *in vitro* preparations (23) (Figure 9A&B). The spinal cord was mounted dorsal-up on a Sylgard step with a small canal under the ventral surface to direct the incoming aCSF for sufficient ventral perfusion and drug diffusion. The treadmill belt was constructed from a 30mm wide by 130mm long polyurethane belt (McMaster-Carr) and mounted around two metal shafts with plastic rollers (Tamiya Inc). The shafts passed through holes in the perfusion chamber walls that were subsequently sealed with epoxy or petroleum jelly. The treadmill was front-driven by one of two radio-controlled car motors, a brushed DC motor (Tamiya Inc, GM7) or a brushless DC motor with electronic speed controller (Novak, Goat Brushless Crawler System). The back rollers were passively turned by friction with the belt. Treadmill speed could be adjusted between 2-12 mm/s to match the slow step frequency of *in vitro* stepping. Altering treadmill speed could also entrain SCH-P stepping frequency across a narrow range of speeds (23). The treadmill allowed us to monitor stepping more similar to that of the intact rat, rather than stepping without ground interaction or having to overcome high friction interactions with the perfusion chamber floor.

Ground reaction forces are commonly monitored in the intact animal to quantify the strength of limb-environment interactions and joint torques (e.g.(108-110)). Typical force platform systems are unsuitable for an aqueous environment and are insensitive to the small forces exerted by neonatal limbs *in vitro*. Thus, we developed miniaturized 2D force platforms specialized for the SCH-P and capable of measuring both vertical and fore-aft forces of each hindlimb (Figure 9C&D). The basics of design were similar to previous models (111), but specialized for the *in vitro* SCH-P using force plates composed of an acrylic-based photopolymer (Objet 3D printer) and highly sensitive strain gauges (Omega Engineering) to transduce the smaller forces. An example of the monitored forces is shown in Figure 9E. Measuring ground reaction forces allows us to relate muscle activity, ventral root activity, or single neuron activity to the forces exerted on the environment. We can also consider how neural activity and circuit function is modulated by the forces experienced by the limbs and their mechanical interactions. Finally, the force platforms can be readily repositioned to alter ground height, slope, or even temporarily eliminate ground interaction.

5.3. Studies on sensory modulation and reinforcement in the spinal cord-hindlimbs pendant preparation

Studies in the intact animal indicate that sensory cues related to limb posture and limb loading play significant roles in determining the spatiotemporal features of motor output (112, 113). Sensory feedback regulates both timing and amplitude of muscle activation, particularly the timing of flexor activation (114, 115) and the amplitude of extensor activation (116, 117). Initial studies in the SCH-P confirmed the importance of these sensory cues in establishing intact rat-like motor patterns *in vitro* (23). To understand the influence of limb posture and limb loading in the SCH-P, we compared joint kinematics and muscle activation patterns produced during unloaded

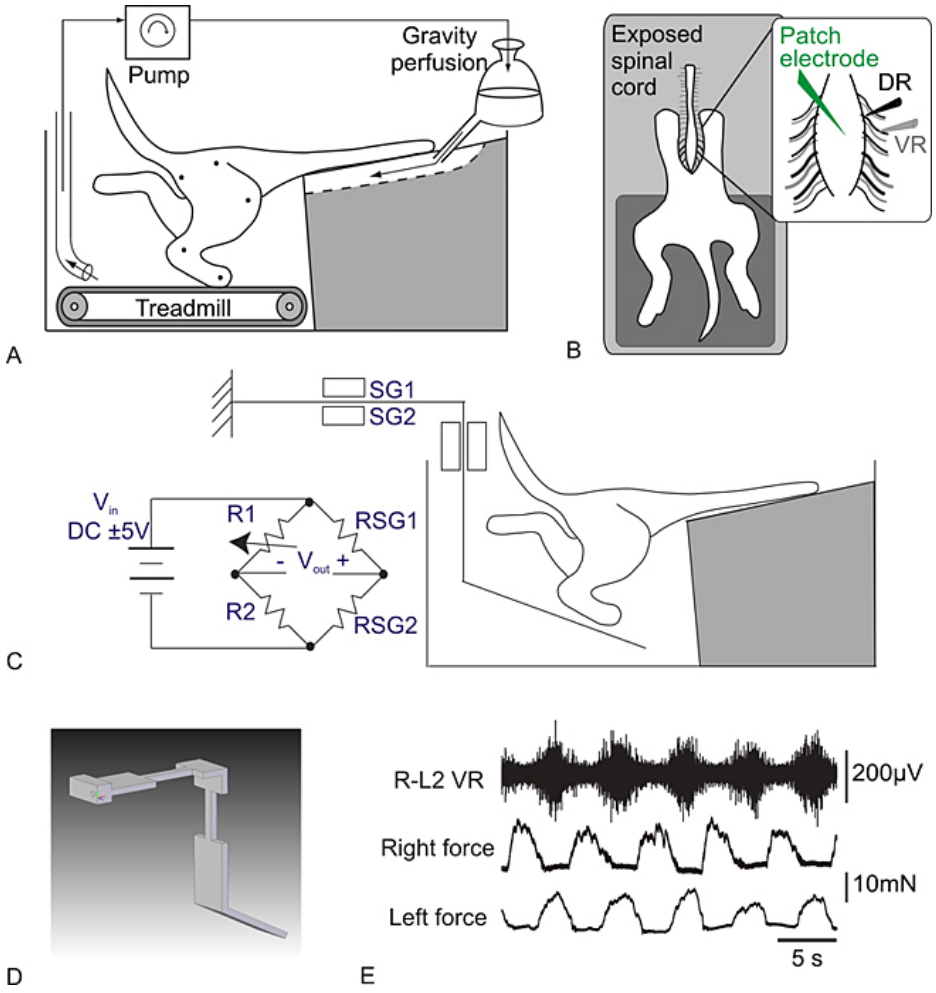

Figure 9. *In vitro* spinal cord-hindlimb preparation (SCH-P) experimental methods. (a) Sagittal view of the SCH-P oriented dorsal-up with hindlimbs hanging pendant to walk on a custom-built treadmill. The spinal cord and limbs are maintained in continuously oxygenated aCSF circulated by a peristaltic pump and gravity-fed perfusion system. aCSF was directed through a canal under the ventral surface to facilitate cord perfusion and drug diffusion to the ventral motor circuitry. The joint centers of the right hindlimb are marked for sagittal plane video collection and kinematic analyses. (b) Overhead view of the SCH-P. The inlay shows the setup for extracellular recordings at the ventral and dorsal roots (VR and DR) and intracellular patch clamp recordings in the exposed spinal cord. (c) 2D force platform to monitor forces exerted by a single hindlimb. Sagittal view of SCH-P-force platform interaction and accompanying Wheatstone bridge circuitry. Strain produced by strain gauges SG1 and SG2 of Sensor 1 and fed into the Wheatstone bridge circuit. Strain sensed by Sensor 2 is fed into a separate but identical Wheatstone bridge circuit. Output voltages are amplified by a DC amplifier and then converted to vertical and fore-aft forces offline. (d) Schematic2D force platform as shown in C with the SCH-P. The force platforms were calibrated by applying n known weights. The influence matrix (I) was then calculated according to: (V2xn) = (I2x2) · (L2xn) such that (I2x2) = (V2xn) · (L2xn)-1 where (V) is the voltage output in response to the known applied loads in μV, (L) is the known loads in mN, (I) is the influence matrix describing the relationship, and n is the number of known weights. After calibration, vertical and fore-aft forces could be computed from the recorded voltage responses according to: (F2xn) = (I2x2)-1 · (V2xn) where (I2x2)-1 = { (V2xn) · (F2xn)-1}-1 where (F) is the force matrix that includes both vertical and fore-aft forces computed from the conversion matrix (I)-1 . (e) Samples recordings of right and left hindlimb vertical ground reaction forces measured during locomotion and displayed relative to the right L2 ventral root activity (R-L2 VR).

ventral-up stepping to those produced during loaded dorsal-up treadmill stepping. Representative patterns are show in Figure 10. In the absence of ground contact, the hip, knee, and ankle joint trajectories were all in-phase, going through mass extension during stance and mass flexion during swing. In contrast, in the dorsal-up posture with ground contract, the knee trajectory was nearly reversed, such that the knee flexed during stance. The dorsal-up pattern much more closely resembled patterns typically observed in the intact rat (23, 118).

The muscle activation patterns also differed significantly between the ventral-up and dorsal-up conditions (23). For example, the phasing between flexors

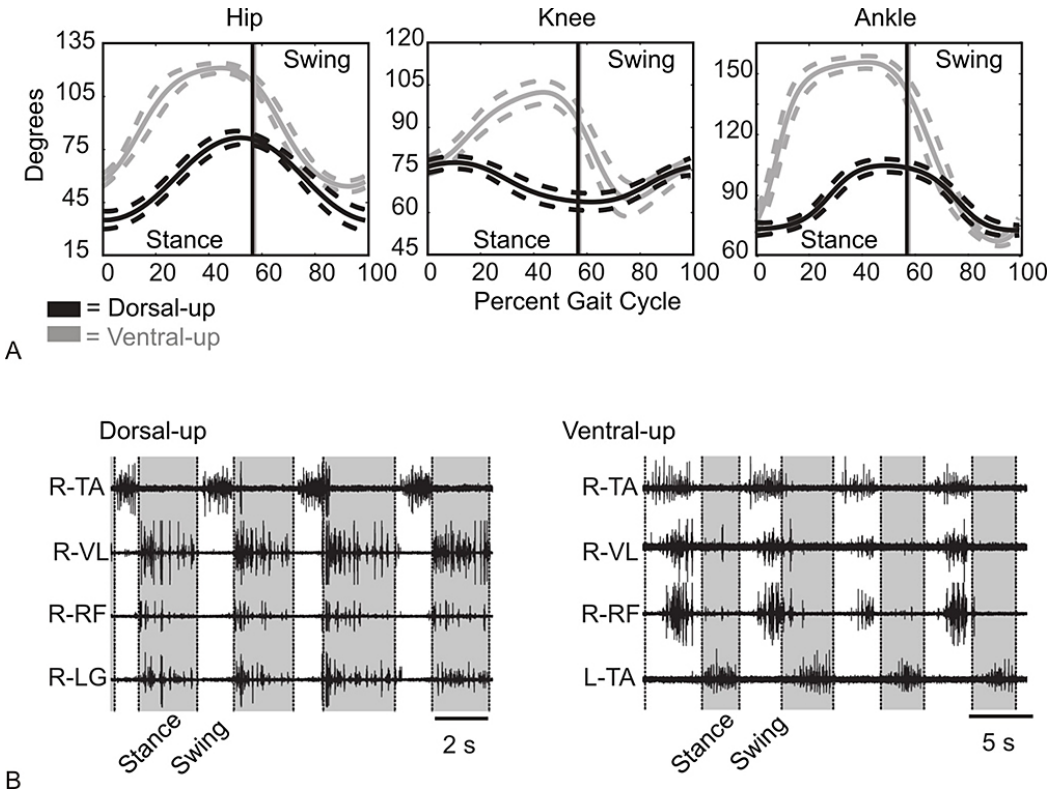

Figure 10. Kinematics and EMG activity during dorsal-up treadmill stepping and ventral-up stepping (adapted from Hayes *et al* 2009). (a) Kinematic joint angle trajectories from dorsal-up (black) and ventral-up (gray) stepping. Joint angle trajectories at the hip, knee, and ankle were time normalized and averaged across 17 cycles and plotted across the gait cycle. Average trajectories (17 cycles) are shown as mean (solid) plus or minus standard deviation (dashed). 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 four cycles of dorsal-up (left) and ventral-up (right) stepping are shown for 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 boxes represent stance phase.

and extensors differed significantly between the two conditions. In addition, extensors exhibited longer duty cycles than flexors during dorsal-up stepping, whereas flexor duty cycles tended to exceed extensor duty cycles during ventral-up stepping. The extensor-dominated patterns exhibited in the dorsal-up posture is characteristic of rat stepping, particularly at slower speeds like those seen *in vitro*, while flexor-dominated patterns are often seen during fictive locomotion in which muscle have been paralyzed and no sensory feedback is present.

Together these results suggest that ground contact and limb posture are important for establishing intact rat-like limb movements because they establish the appropriate sensory cues for locomotion, such as stretch and loading. Such cues have been previously shown to enhance extensor activation for body-weight support (116, 119). Loading and ground contact have also been shown to affect reflex patterns, joint kinematics, and muscle activation in humans (120, 121) Without these sensory cues, the isolated spinal circuitry functions without the vital influences of sensory feedback that is known to alter both its properties and final output, preventing us from studying this circuitry in a behaviorally-relevant context. By providing appropriate sensory cues, the SCH-P allows us to harness the many advantages of *in vitro* preparations, such as neural and pharmacological access, without reducing behavioral relevance.

Most importantly though, retaining sensory feedback from ongoing limb movement creates a more robust model for studying locomotion *in vitro* by reinforcing or even initiating spinally-generated locomotion. For example, cyclical sensory cues provided by applying repetitive swing assistance on the plantar surface of the hindlimb paw reinforced initially weak and irregular locomotion, resulting in a more regular frequency and reliable bursting in previously inactive muscles. This more robust pattern persisted after termination of the assistance (Figure 11A, (23)). Similarly, when drugs failed to initiate locomotion, alternating cyclical assistance on both limbs could actually initiate strong locomotion for the first time that persisted after bilateral assistance was terminated (Figure 11B). Thus, retaining the hindlimbs and sensory feedback can not only establish a behaviorally-relevant context, but can also be used to reinforce and

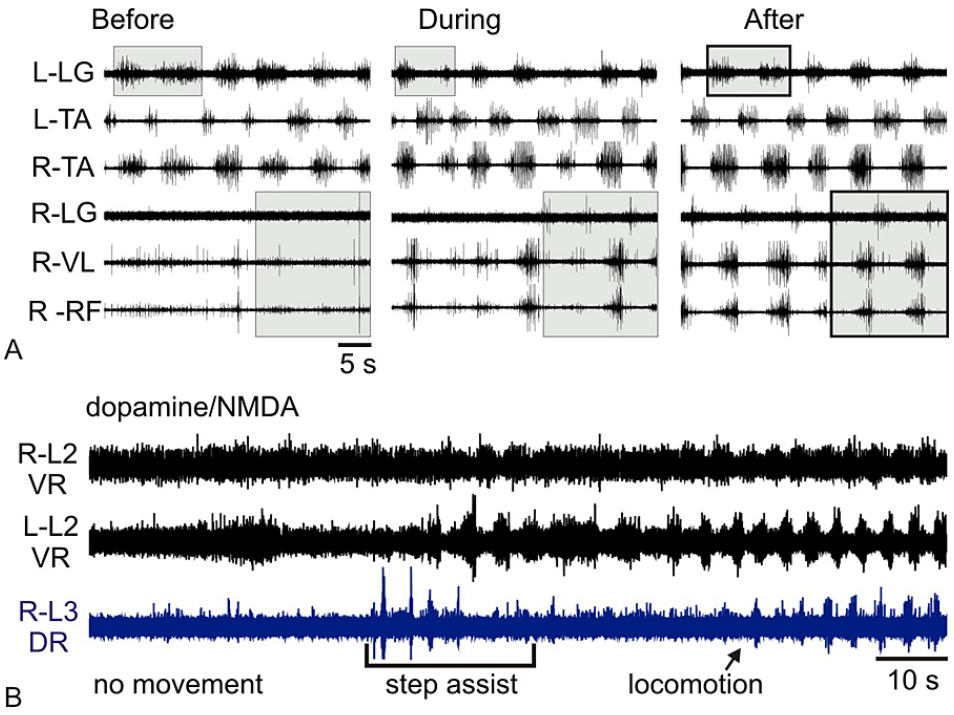

Figure 11. Unilateral swing assistance reinforced ongoing weak locomotion and bilateral stepping-like assistance initiated locomotion. (a) Application of swing assistance with a glass probe pushing on the right plantar paw surface reinforced otherwise weak locomotion (adapted from Hayes *et al* 2009). Forty seconds of muscle activation patterns from right (R-) or left (L-) lateral gastrocnemius (LG), tibialis anterior (TA), vastus lateralis (VL), and rectus femoris (RF) are shown before, during, and after application of swing assistance. Gray boxes emphasize changes in frequency and muscle bursting. (b) Bilateral cyclical stepping-like assistance initiated 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, blue) are shown before, during, and after assistive stepping-like movements. The bracket shows the period in which the limbs were cyclically moved through stepping-like patterns. Before entrainment, the sub-threshold concentration of neurochemicals failed to initiate rhythmic bursting in the ventral an dorsal roots. Stepping-like assistance initiated rhythmic locomotor bursting in all roots, which persisted after the assistance period.

facilitate CPG activation, making the SCH-P a powerful model for relating the spinal circuitry of locomotion to movement-related sensory feedback.

5.4. Intracellular recordings during unrestrained hindlimb stepping in the spinal cord-hindlimbs pendant preparation

One of the greatest advantages of the SCH-P is the potential for studying interneuronal activity and CPG function during natural, sensory-influenced behavior. Many have performed intracellular recordings in the isolated spinal cord without limbs attached or with neuromuscular blockage or deafferentation (e.g. (122, 123), for review see (124)). Although these studies provide insight into the neuronal contributions to rhythmogenesis, without movement-related sensory feedback, numerous inputs to these neurons are inactive. Because sensory inputs have profound effects on motor output, they undoubtedly influence the functioning of neurons including those comprising spinal locomotor circuitry.

Intracellular recordings performed during sensory-modulated, behaviorally-relevant locomotion provide additional insight into the functional synaptic and cellular properties of spinal interneuronal populations during locomotion. Wheatley and Stein made progress towards incorporating limb movement with intracellular recordings by developing the mudpuppy spinal cord-forelimb preparation (125), but no such model exists for mammals or for hindlimb locomotion. Further, impalement by the traditional sharp electrodes, as used in the mudpuppy, may induce current leaks that alter the passive membrane properties, while whole-cell patch recordings are thought to more accurately report these properties (126).

Using the dorsal-up SCH-P, we recently achieved whole-cell patch recordings from spinal interneurons during unrestrained locomotion. Stable recordings were easily achieved with adequate cord stabilization with insect pins. An example recording is shown in Figure 12. This T12 dorsal horn interneuron was recruited into spiking during locomotion and exhibited rhythmic spiking that increased with ipsilateral L3 dorsal root activity and reached maximum during ipsilateral flexion (Figure 12). Slightly hyperpolarizing the membrane with current injection revealed rhythmic depolarizing drive potentials (Figure 12B) similar to those previously observed in

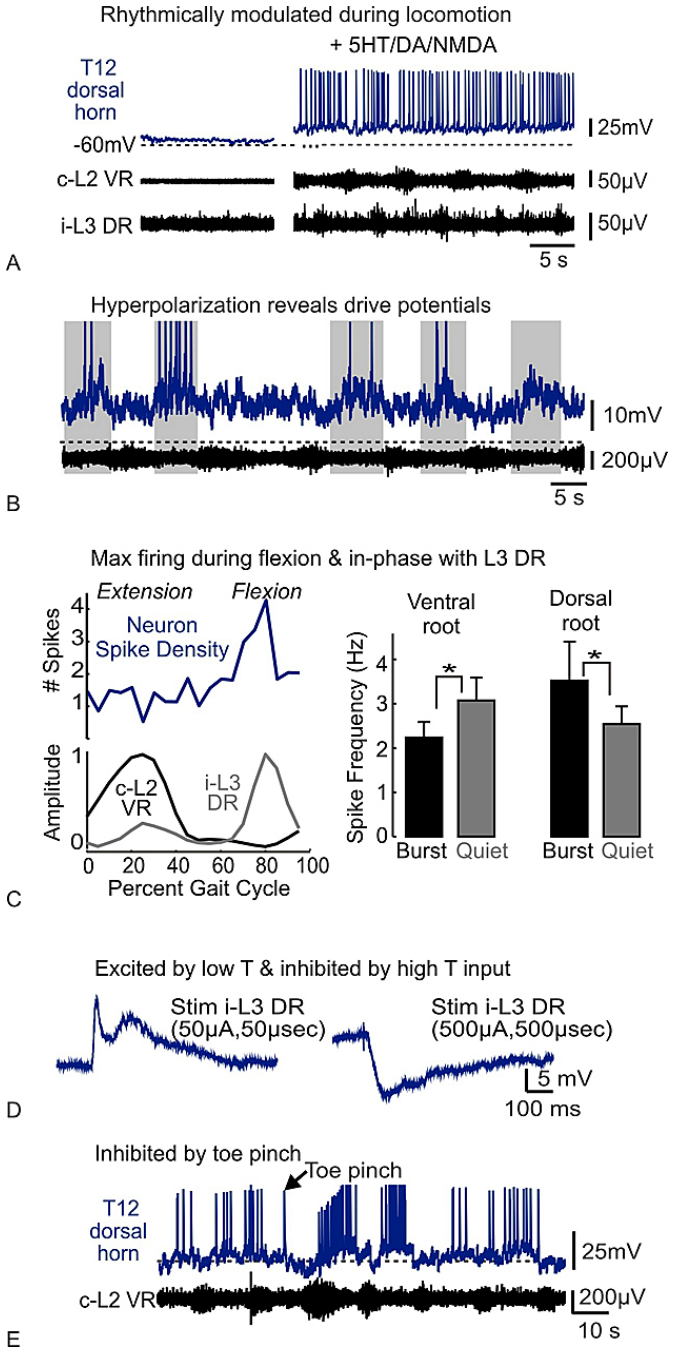

Figure 12. Intracellular patch clamp recordings during unrestrained hindlimb stepping. Activity from a T12 dorsal horn interneuron was recorded using whole cell patch clamp techniques. Interneuron activity (blue) is shown relative to ipsilateral (i-) and contralateral (c-) ventral root (VR) and dorsal root (DR) activity (black). As reported previously, the L2 VR activity corresponds to the flexion phase. (a) Resting membrane potential for this neuron was near -60mV. The neuron was quiescent at rest, but rhythmically spiked during neurochemically-induced locomotion. (b) Slightly hyperpolarizing the membrane potential revealed rhythmic drive potentials underlying the neuron's rhythmic spiking. (c) Left: Mean spike density (# spikes/bin) is shown relative to 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 c-L2 VR activity, and in-phase with i-L3 DR activity. Right: Mean spike frequency was significantly higher during cL2 VR quiescence and i-L3 DR bursting ($p < 0.05$). (d) Stimulation of the ipsilateral L3 dorsal root (i-L3 DR) showed that the neuron was excited by low-threshold stimulation (50µA, 50µsec), but inhibited by high-threshold stimulation (500µA, 500µsec). (e) Toe pinch showed that natural high-threshold noxious stimuli also inhibited the neuron's firing. Inhibition was followed by rebounding spiking.

motoneurons and interneurons (16, 32, 127). During locomotion, spike frequency for this interneuron was significantly higher during L3 dorsal root bursting and lower during contralateral L2 ventral root activity (i.e. out-of-phase with contralateral flexion, in-phase with ipsilateral flexion) (Figure 12C). In the dorsal horn, rhythmic drive potentials likely reflect afferent mediated synaptic actions from cyclic limb movements and may well contribute to locomotor drive potentials involved in the shaping of rhythmic motor output (122). This interneuron received strong, excitatory low-threshold afferent input, but was inhibited by high threshold inputs (Figure 12D). Toe pinch during locomotion evoked inhibition followed by rebound firing (Figure 12E), consistent with inhibition from high threshold stimulation.

Stable intracellular recordings and various imaging approaches (e.g. two–photon Ca2+ imaging) maintained through unrestrained hindlimb locomotion should further advance the understanding of spinal locomotor circuitry.

6. CONCLUSIONS

It has been known for over two decades that the isolated neonatal *in vitro* spinal cord offers distinct methodological advantages for the study of mammalian locomotor circuits. This includes easy access for pharmacology, stimulation, and lesioning, as well as stability for whole-cell patch clamp recordings. The more recent addition of complementary genetic approaches has provided for unprecedented identification and control of distinct neuronal populations implicated in locomotor rhythmogenesis. However, interpretations are limited when based solely on ventral root recordings that contain heterogeneity in motor and autonomic axons. Further integration of the *in vitro* model with afferent feedback and intact hindlimbs, particularly the SCH-P that can assess locomotor function with techniques previously restricted to *in vivo* studies, promise to provide important additional insights into locomotor circuit organization.

7. ACKNOWLEDGEMENTS

The authors are indebted to Dr. Patrick Whelan for providing helpful comments to improve the manuscript. The authors generously acknowledge the support of various funding agencies. S.H. - Christopher and Dana Reeve Foundation, Craig Neilsen Foundation, Paralyzed Veterans of America, National Science Foundation #0745164, NIH awards EB006179, NS 045248, NS40893, NS40440. E.G. - NIH NRSA. H.H. and J.A. - NSF IGERT DGE-0333411, and NSF GRFPs. S.D. – NIH EB006179. Y-H. C. - NIH AR054760.

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Key Words: Spinal cord, Locomotion, *In vitro*, Dorsal column, EMG, Central pattern generator, CPG, Rhythmic, Review, Afferent, Sensory, Review

Send correspondence to: Shawn Hochman**,** Department of Physiology, Emory University School of Medicine, Atlanta, Georgia, Tel: 404-712-3131, Fax: 404-727-2648, E-mail: shawn.hochman@emory.edu