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Multilevel Analysis of Locomotion in Immature Preparations Suggests Innovative Strategies to Reactivate Stepping after Spinal Cord Injury

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Abstract

Locomotion is one of the most complex motor behaviors. Locomotor patterns change during early life, reflecting development of numerous peripheral and hierarchically organized central structures. Among them, the spinal cord is of particular interest since it houses the central pattern generator (CPG) for locomotion. This main command center is capable of eliciting and coordinating complex series of rhythmic neural signals sent to motoneurons and to corresponding target-muscles for basic locomotor activity. For a long-time, the CPG has been considered a black box. In recent years, complementary insights from in vitro and in vivo animal models have contributed significantly to a better understanding of its constituents, properties and ways to recover locomotion after a spinal cord injury (SCI). This review discusses key findings made by comparing the results of in vitro isolated spinal cord preparations and spinal-transected in vivo models from neonatal animals. Pharmacological, electrical, and sensory stimulation approaches largely used to further understand CPG function may also soon become therapeutic tools for potent CPG reactivation and locomotor movement induction in persons with SCI or developmental neuromuscular disorder.

Basic structure of locomotion: biomechanical basis and neural organization

Walking is a relatively stereotyped motor behavior that allows terrestrial movement of limbed animals. In these organisms, locomotion consists of the rhythmic reiteration of a basic motor scheme, the gait, which is characterized by the alternated activation of pairs of appendages and, within each limb, by the transition between the phase in which the base of support remains in contact with the ground (stance) and when it swings forwards (swing) [1]. The different number of limbs between bipeds and quadrupeds influences complexity of gait patterns. There is a large repertoire of locomotor gaits expressed by quadrupeds, mainly in relation to the speed of locomotion [2]. However, the most common type of locomotion is characterized by the double alternation between hind- and fore-limbs, and between ipsilateral limbs [3], particularly during to low-to-moderate speeds of locomotion.

Although different tetrapod species may exhibit different gaits as adults (i.e., walking, trotting, bounding, etc.), a pattern of interlimb coordination characteristic of walking (alternating homologous limbs) is shown by newborns of many species, including kittens, rabbits, rats, jerboas, gerbils, jirds, kangaroo rats, dormice, and voles [4-11]. Similarities in locomotor coordination may be due to similarities in body size and morphologies (short limbs, wide stance), and relative immaturity of the CNS (central nervous system), PNS (peripheral nervous system), and skeletal system. Specialization in locomotor patterns subsequently emerges in animals experiencing geometric and allometric growth and continued development of neural and motor systems. Even human infants, which can show a variety of crawling patterns (i.e., hands-and-knees, hands-and-feet, creeping, scooting, and mixes of these patterns), predominately exhibit an alternating interlimb pattern during crawling [12] that is kinematically similar to non-human primates and other mammals [13]. Furthermore, the development of bipedal walking locomotion in humans shows many parallels—e.g., gradual reduction of step cycle duration and variability, hyperflexion of leg joints, training effects—as with other animals [14].

In bipedal organisms, locomotion faces a further challenge. Notably, each step continuously pushes the center of mass forward and this requires a series of sophisticated systems of postural control to recover balance in response to this continuous instability [15]. Nevertheless, the imbalance following each step seems to obey to a functional significance. Indeed, the forward propulsion of the body to recover the center of mass becomes the necessary consequence to maintain the equilibrium, which is then compromised once again at the end of each step and at the beginning of the following one [16].

This rhythmic nature of locomotion requires a phasic activation of osteo-articular and muscular actuators in the periphery. As a matter of fact, muscles are recruited only in distinct phases to alternate the two limbs. Furthermore, flexor and extensor muscles around the joints of each limb are sequentially activated to allow swing and stance. Phasic muscular activation generates metabolic advantages with respect to a postural massive tonic contraction. Indeed, albeit the effort of moving the body in space, the energetic consumption during gait is quite similar to static posture [17]. The energetic cost of locomotion is concomitantly reduced also by passive elastic structures (e.g. tendons, ligaments and muscular components) that temporarily store the propulsive energy lost at one stage of the stride and return it in the following phase of gait [18]. From a kinetic point of view, limbed animals typically use gaits that are energetically favorable for body propulsion [19]. Note that further details about energetic considerations and locomotion in spinal cord-injured persons may be found in this issue in the paper by Nash and colleagues.

The most economical locomotor pattern is selected by continuously processing sensory input, including proprioceptive afferents that provide information about body mechanics [20; 21]. Nevertheless, continuous fluctuations from the nominal preferred gait naturally occur during normal walking, regardless of the increase in energy expenditure [22]. Step-by-step variability also plays an important role in optimizing locomotor performance, as it represents a robust control system that promptly adjusts the pattern in response to environmental perturbations [23]. This same logic of efficiency of movement and dynamic sensorimotor integration governs the systems responsible for generating and organizing rhythmic interlimb coordination during locomotion.

A remarkable network of spinal interneurons, mainly localized in the upper lumbar segments of the spinal cord, is responsible for producing the fundamental neural commands underlying basic locomotion [24-26]. Once activated, this network, named central pattern generator (CPG), sustains itself rather automatically [27]. This hierarchical arrangement thus reduces the need for constant supraspinal modulation of the locomotor rhythm. Supraspinal mechanisms may then be mostly limited to planning, triggering, and terminating locomotion [28; 29], modulating movement and posture in response to visual and auditory stimuli [30], and allocating neural resources toward the control of vital and cognitive functions. Although the locomotor CPG represents only a small portion of the spinal cord, a wider network of propriospinal neurons reverberates its rhythmic pattern along the whole axis [31], to integrate other rhythmic tasks, such as respiration or movement of upper limbs [32; 33].

During the embryonic stage of prenatal development, intrinsic rhythmogenicity of spinal networks and basic elements of the locomotor pattern (i.e. the double alternation among the two sides of the cord) are already expressed [34]. Shortly before and after birth the spinal CPG is tuned by descending fibers [35]. Hence, activity-dependent mechanisms of plasticity mediate the processing of afferent inputs and their regulation of the locomotor pattern [36]. This is especially apparent in immature animals that are undergoing continual development of muscle-skeletal actuators, overall body growth, and physiological systems. Even the earliest attempts to perform locomotion reveal a dynamic interplay between form and function. For example, rabbits [11] and some rodents [8] show quadrupedal walking during the early postnatal period, before the development of elongated hindlimbs and other forms of locomotion such as bounding and ricochetal locomotion. This suggests that although locomotor mechanisms are structurally in place and capable of functioning, that locomotor behavior is assembled in a dynamic fashion, and is dependent upon multiple factors that are necessary to physically support and move the animal's body.

Maximal efficiency in the hierarchical organization and sensorimotor integration of the neuromotor system is, in turn, supported to some extent by the redundancy of structures, which renders locomotion more resistant to occasional failures [37] and reduces vulnerability in response to peripheral or central lesions. Indeed, synergies in the activation of multiple muscles [38; 39] allow the alternated activation of limbs, even in the presence of localized muscular deficits [40; 41]. At the same time, the intrinsic variability in recruiting CPG interneurons [42] physiologically drives plastic rearrangements and neuronal compensation mechanisms, enabling gait to be expressed even after discrete neurological lesions [43]. Together, task efficiency and redundancy of structure sustain the function of the neuromotor system, even if the two principles are in contrast with one another because the maintenance of supernumerary replacing elements in case of damage requires a more consuming structure. Despite the redundancy, the compromise between these two elements reached by the neuromotor system still exposes locomotion to serious functional deficits in cases of severe impairments.

Although problems with peripheral actuators (such as osteoarticular, muscle or peripheral nerve lesions) may be allayed to some degree by using prostheses or orthoses, to permit locomotion, on the other hand, central damage is currently incurable, and may lead to paralysis. In this situation, the study of the neuronal bases of locomotion, including cellular substitution or reconfiguration of residual spinal circuits, can support targeted interventions to exploit spinal mechanisms of self-repair and plasticity. Such a multi-level understanding of CPG functions and supraspinal-peripheral system contributions to its

modulation is likely to yield the development of a multidisciplinary approach for functional recovery in persons with a spinal cord injury (SCI). Although locomotion in neurologically intact individuals involves continuous integration of neural networks throughout the CNS, including cortical, sub-cortical, cerebellar, and spinal areas, we chose to focus primarily in this review on spinal mechanisms, CPG properties, and clinically relevant research tools.

Initiation and modulation of locomotion: considerations for recovery of locomotor activity following SCI

In individuals with a complete SCI, several sensory inputs can still access and modulate the output of the spinal locomotor CPG, including afferent feedback from muscle proprioceptors, joint receptors, and cutaneous afferents. Sensory stimulation and activity-dependent feedback has been shown to facilitate locomotion for those with an incomplete SCI (e.g., [44-47]). See also the paper from Pearcey and colleagues in this issue, for further details on cutaneous contribution to locomotion. However afferent input alone is not likely to lead to a functional recovery of locomotor ability in humans with complete SCI [48]. Therefore, it is critical to consider modulatory effects on spinal networks in combination (sensory, electrical, and pharmacological approaches) for the development of therapeutic techniques, as combined methodologies may ultimately achieve greater functional outcomes through synergistic actions. Here we review the sensory afferent control, electrical initiation, and pharmacological neuromodulation of locomotor activity in spinal preparations, which together represents a promising avenue for examining the function and plasticity of spinal locomotor networks.

Sensory afferent control of locomotion

Early experiments by Graham Brown [49; 50] were critical in determining that the spinal cord contains the necessary elements to produce basic, phasic locomotor activity produced by the limbs, devoid of sensory inputs. However, since the time of these crucial studies that discovered the independence of central mechanisms from external stimuli, it has become widely recognized that sensory afferent stimulation plays an important role in modulating spinal locomotor networks and plays a key role in the recovery of locomotion for individuals with SCI [51; 52]. Sensory stimulation not only facilitates the expression of locomotion, but it permits adaptation of locomotion to the environment, regulates reflex activity, promotes transitions between different phases of the locomotor cycle, and helps to induce plasticity in the injured spinal cord.

Proprioceptive stimulation from muscle and Golgi tendon organ receptors play an important role in regulating reflex activity in the spinal cord and during locomotion. Because central excitability is typically decreased or impaired following neural damage, understanding how reflexes may alter activity and plasticity in spinal locomotor networks is essential. It is well established that hip joint afferents activate appropriate patterns of leg muscle activity during walking and are important for initiating the transition from stance to swing. This has been shown to be the case for spinal cats [53-55], human infants [56], and adult humans with SCI [57; 58]. Additionally, in spinal cats, activation of group Ia and group Ib afferents from ankle extensors entrains the locomotor rhythm, prolongs extensors bursts, and inhibits flexor activity [59; 60], such that a reduction in extensor muscle Ib activity promotes the transition from stance to swing during locomotion [61]. Stimulation of ankle group Ia afferents and cutaneous nerve stimulation (delivered to the nerve innervating the plantar foot) also prolongs extensor activity. This suggests that both Ib and Ia afferent activity continually shape amplitude and timing of the locomotor step cycle [60]. Proprioceptive feedback from the hindlimbs is also thought to be important for regulating interlimb coordination and locomotor speed adaptations, as on a treadmill, for intact as well as spinal animals [62-65].

Strong cutaneous stimulation, delivered to the perineum [65-67] or to the tail (tail-pinch; [68]), can induce some locomotor stepping in spinal animals. In fact, recently it was shown that perineal stimulation alone was sufficient to induce stepping movements on a treadmill in spinal rats, thus permitting treadmill training to occur [67]. Modulation of locomotor behavior, such as altered foot contact and limb activity, occurs following mechanical stimulation of the skin on the back [69], section of cutaneous nerves innervating the foot [70], and electrical stimulation delivered to the foot dorsum [71]. Such studies indicate that cutaneous stimulation likely alters excitability of spinal circuits for locomotion and weight-supported posture, and are important for inducing plasticity following SCI (see [72] for review). In adult humans with chronic incomplete SCI, excitation of plantar cutaneous afferents modulated walking in a phase-dependent manner, suggesting interactions among locomotor mechanisms, peripheral afferents, and segmental reflex circuits [73].

Given that the spinal cord is capable of sensory-induced functional plasticity, activity-dependent mechanisms in the spinal cord are often exploited to try and rehabilitate locomotor function. For example, operant conditioning of the H-reflex modifies spinal reflex pathways in various animals [74-76], as well as improves locomotion in incomplete spinal rats [77] and chronic incomplete spinal humans [46]. Cycle training has been shown to

normalize spinal reflex excitability in spinal adult rats [78], as well as determine gait (an alternating or synchronous pattern) following several days of anti- or in-phase cycle training in young spinal rabbits [11]. More commonly, daily treadmill training is used to help restore locomotion in animals with SCI [62; 63; 65; 79].

It is likely that both proprioceptive and cutaneous afferents are involved in cycle and treadmill training effects. However, the necessary and sufficient mechanisms promoting activity-dependent functional plasticity in the spinal cord remain largely elusive. Possible neural mechanisms involve plastic changes (i.e, neural reconfigurations, receptor and transporter up- and down-regulation, axonal sprouting, long-term potentiation or depression, presynaptic modulation) occurring at the level of locomotor CPG, interneurons downstream from the locomotor CPG, or motoneurons. Regardless of the exact mechanisms [80], it is clear that the isolated or damaged spinal cord is capable of dynamic, sensorimotor integration that is dependent upon both endogenous and exogenous factors [81], and that understanding these mechanisms provides important opportunities for facilitating recovery and limiting further damage.

In fact, sensory afferent stimulation and use-dependent plasticity is a hallmark of physical therapy treatments. For decades now, stepping on a treadmill or use of gait orthoses has helped to restore gait in individuals with SCI (e.g., [82; 83]). For those with incomplete SCI, locomotor training improves many aspects of locomotion, including: interlimb coordination, endurance, walking speed, and limb kinematics (for review see [48]). However outcomes are typically better for individuals with an incomplete rather than complete SCI, indicating that supraspinal structures likely play a role in recovery of function for incomplete lesions. Although locomotor training typically has not resulted in recovery of walking locomotion in complete SCI individuals [57; 84], a case report of a 33-year-old man with complete SCI showed some over-ground walking function following task-specific practice coupled with robotic locomotor training as part of an intensive physical therapy program [85]. The authors asserted that intensive physical therapy and locomotor training together was likely more effective than locomotor training alone, and that training intensity, frequency, and task-specificity are likely important factors for improving motor outcomes.

Another promising application of afferent stimulation and use-dependent plasticity in promoting locomotor function can be seen with partial body-weight supported treadmill training (BWSTT) in infants that have developmental neuromuscular disorders. Parents of infants with Down Syndrome were provided small treadmills for the home and engaged their babies in treadmill-induced stepping practice 5 days a week, between the ages of 8-

10 months, in addition to traditional physical therapy. Although infants with Down Syndrome often start walking at 2 years of age (which is about one year later compared to typically developing infants), infants that received treadmill training learned to walk independently significantly earlier compared to infants that received physical therapy alone [86; 87]. Infants also showed improvement in other motor milestones, such as pulling to stand [86]. Similar early intervention strategies using BWSTT are currently being examined in infants that have myelomeningocele (MMC) [88]. MMC is the most severe form of spinal bifida in which the developing spine and neural tube do not close properly during prenatal development. This typically results in a small part of the lower spinal cord and meninges (forming a sac) protruding from the back of the individual, accompanied by severe motor and sensory deficits including bladder dysfunction and paralysis below the level of spinal damage, which is usually at the lumbar or sacral level. Infants with MMC start walking around 2.5-5 years of age [89], if they are able to walk at all. After 6 months of BWSTT as described above for infants with Down Syndrome, MMC infants showed earlier mean onset ages for motor items on the Bayley Scales of Infant Development, and higher bone mineral content in the legs compared to MMC infants who did not receive treadmill training [88]. Furthermore, enhancing sensory feedback via increasing overall friction on the treadmill belt increased the step rate on the treadmill for infants with MMC [90], suggesting that synergistic approaches may be more effective at triggering locomotor plasticity in the injured, developing spinal cord.

Electrostimulation facilitates locomotion

In humans, spinal locomotor circuits can be directly activated, even in the absence of any voluntary control, by relatively nonspecific stimuli such as direct electrical non-patterned stimulation of the lumbar cord [91], continuous vibration of the quadriceps and hamstring muscle groups [92], tonic electrical stimulation of the peroneal or sural nerves [93], transdermal spinal cord stimulation (see companion paper from Minassian and colleague) or electromagnetic stimulation at the level of the lumbosacral spinal cord [94]. The automatic stepping movements generated by these approaches suggests that the CPG can function independently from brain control and thus opens the door to new paradigms for the recovery of posture and locomotion in individuals with severe SCI.

Among these methods, epidural stimulation dorsally applied over lumbosacral segments promotes reproducible locomotor patterns that can be recorded from adult spinal rats in vivo [95-97]. In humans, epidural stimulation is a minimally invasive technique that has been used for several years to alleviate spasticity and pain. Clinical use

confirmed that epidural stimulation of the thoraco-lumbar spinal cord enables bursts of electromyographic activity in lower limb muscles and few step-like alternating flexion and extension movements after complete SCI [91]. More recently, epidural stimulation associated with intense training reactivated motor functions in persons with a chronic spinal lesion [98; 99]. In these cases, electrical stimulation was not able to automatically trigger locomotion *per se*, but facilitated locomotor-like patterns evoked by afferent stimuli and reactivated voluntary commands, but only during protocol delivery [98; 99]. Likewise, transcutaneous electrical stimulation generated similar results in five SCI subjects [100]. Overall, electrical stimulation of the spinal cord enabled all of the nine subjects tested with complete paralysis to voluntarily move their lower limbs. Therefore, it now represents one of the most promising strategies to restore locomotor function following SCI.

However, the potential of electrical stimulation has not been fully disclosed yet. In fact, while research on both animals and humans has assessed the best parameters of intensity, frequency and location for stimulation, there has not been a full exploration of stimulating patterns and their motor consequences. For example, only trains of square pulses have been used [101], without varying the wave shape of single pulses. Another issue that requires further study is the combined use of electrical stimulation with pharmacology, in order to find agents more specifically targeted to enhancing locomotor CPG function. Indeed, some experimental pharmacological interventions have already been associated with potential recovery of locomotion in individuals with SCI [102]. More recently, results of a phase I/IIa trial with a first oral CPG activator called Spinalon^{TM} has provided evidence of safety as well as promising preliminary efficacy data (induced rhythmic EMG activity in both legs) in 45 complete SCI persons (paper from Radhakrishna and colleagues, this issue; for corresponding preclinical results in mice, see [103]). It is thus straightforward to consider the adoption of complementary and synergistic strategies as a logical direction for translating some basic biological concepts into clinical settings.

The possibility of identifying a methodology for reactivating human spinal locomotor mechanisms after SCI does not imply that spinal injured persons could easily and safely just get up and walk voluntarily. An essential component of successful over ground locomotion are the neural mechanisms for maintaining posture and recovering stability after an occasional imbalance, which are severely compromised following a spinal lesion, both in animals [104] and in humans [105]. Nevertheless, epidural electrical stimulation significantly improved posture and recovery after a loss of balance, when applied to the lumbar segments of spinal animals [106; 107] as well as to spinal cord injured persons [98;

99]. These findings suggest that electrical stimulation may therefore be a promising component to a rehabilitation strategy for recovering walking.

Pharmacological modulation of locomotion

In this section we briefly highlight findings on in vitro and in vivo animal models regarding some of the main neurotransmitters and neuromodulators that are known to stimulate and modulate synaptic spinal locomotor function. These chemical signals principally influence locomotor CPG functioning by altering motoneuron and CPG interneuron electrical properties, altering synaptic responses between motoneurons and CPG interneurons, or both. It is important to note that the effect of neuromodulators on locomotor network activity occasionally differs among species. For a more comprehensive review of pharmacological neuromodulation of locomotor networks, see Miles and Sillar [108] or Guertin [109].

The rhythmic activity produced within the spinal locomotor CPG is mainly produced by glutamate-mediated excitation and GABA- and glycine-mediated inhibition between spinal interneurons. Both ionotropic [110; 111] and metabotropic glutamate receptors [112] modulate aspects of CPG activity such as excitability, speed, and rhythmicity. Inhibitory neurotransmission regulates the left-right alternating pattern, and the speed and stability of the locomotor rhythm [113]. Renshaw cells, Ia inhibitory neurons, inhibitory commissural neurons, and several other classes of inhibitory neurons are involved [113-115].

Monoaminergic systems also play a key role in activating and modulating spinal locomotor networks. Activation of 5-HT receptors induces locomotor activity in the isolated rodent spinal cord in vitro (e.g., [116; 117]) and in spinal rodents in vivo (e.g., [118-121]). Depending on the receptor class that is activated, 5-HT receptor activation in some cases also increases the frequency and amplitude of locomotor bursts, increases the regularity of stepping, and can decrease stepping (reviewed in [108]). Activation of dopamine receptors stimulates locomotor activity in spinal rodents in vivo [122], but in the isolated spinal cord in vitro the rhythm is slower than that which is induced by 5-HT [123]. Stimulation of noradrenergic receptors induces locomotor activity in spinal cats (e.g., [124; 125]) and modulates network activity, such as increasing tonic spinal activity and locomotor bursts, in the isolated spinal cord of the neonatal rat in vitro [126; 127]. Additional modulators of spinal locomotor circuits are acetylcholine [128; 129] various neuropeptides [130; 131], and trace amines [132].

In animals with SCI, the availability of some neuromodulators to influence spinal circuits changes drastically. Acutely after SCI, glutamate and aspartate levels increase to

>400% in the spinal cord and this contributes to tissue injury [133]; their levels, and levels of GABA and glycine decrease thereafter [134]. Thus following SCI, the balance between excitation and inhibition in the spinal cord is disrupted [81]. Furthermore, the brain is the main source of monoaminergic-containing cells in the CNS, including 5-HT [135]. Following SCI, these substances no longer can be released from supraspinal projections caudal to the site of injury. Part of the consequence then is the up-regulation of 5-HT and noradrenergic receptors caudal to the lesion [136-139]. Hence, levels of endogenous neuromodulators, and likewise receptor levels, after a SCI change in relation to the time of injury, and may thereby influence responses to both drugs and sensory or electrical stimulation.

Pharmacological modulation is one way to help induce plasticity in the injured spinal cord, though combination efforts may be more fruitful than drug treatments alone. For instance, spinal adult rats treated with subthreshold doses of serotonergic agonists, provided electric epidural stimulation, and step-trained improved their hindlimb stepping coordination and muscle activation patterns within one week following SCI [97]. Comparable results without electrical stimulation may be obtained using higher doses of synergistic combinations with 5-HT agonists and NA/DA agonists or precursors in spinal-transected mice and turtles [103; 140]. As with other combinatorial approaches, the potent CPG-activating effects of suprathreshold doses of proper drug cocktails (e.g., Spinalon^{TM}) can further improve overtime with repeated training (drug administration 3-5 times/week) [141; 142]. These findings suggest that sensory afferent feedback from step training interacts with electrical and/or pharmacological activation of spinal networks to induce neuronal plasticity changes following SCI. CPG activation through locomotor training increases the percentage of active motoneurons in the spinal cord [143]. In turn, these results suggest that afferent feedback may act on enhanced motoneuron excitability, induced by serotonergic receptors and electrical stimulation. Serotonergic stimulation also has been shown to influence spinal reflex pathways and to presynaptically influence segmental afferent projections (reviewed in [144]). Although using multiple, concurrent treatments in humans with SCI may not be the most desired approach to reinstating locomotor behavior, experimental paradigms such as those using rodent or cat models are explicating many important principles of reawakening spinal locomotor networks that are important to understand in approaching this challenge in humans. In fact, recent work with humans indicates that coupling stimulation with training promotes more adaptive plasticity and improves motor performance, and suggests that augmenting training with stimulation helps to better activate spinal circuitry [145]. Understanding and treating the

pharmacological bases of this plasticity should help to further facilitate improvements in function.

Examining spinal mechanisms of locomotion from different levels of analysis in immature preparations: novel strategies for activating locomotor stepping following SCI

Numerous experimental paradigms have been developed to study locomotion. Here we focus on the isolated spinal cord in vitro, and behavioral analysis in vivo, discussing recent insights provided by our laboratories using electrostimulation, pharmacological, and sensory feedback manipulations. Our research illustrates how an integrative approach to the study of locomotor mechanisms in immature animals reveals important dynamic interactions among levels of analysis, and strengths and limitations of specific experimental approaches. Together this work has important implications for neurorehabilitation strategies for SCI, including opening new avenues for combinatorial approaches.

Selective electrostimulation of dorsal roots triggers locomotor patterns in the isolated spinal cord

Electrostimulation through a bipolar hook electrode selectively applied to dorsal roots (DRs) cut distally from the spinal cord has been shown to evoke bouts of locomotion in spinal cats ([146]; see also Lev Tov and colleagues, this issue). A similar outcome was observed more consistently on in vitro spinal cords isolated from neonatal rats. In these preparations, electrical stimulation with stereotyped trains of square impulses triggered brief episodes of electrical oscillations, alternating between flexor and extensor motor pools on both sides of the cord (fictive locomotion rhythm, FL; [25]), when selectively delivered through tight fitting electrodes to either DRs [147] or sacrocaudal afferents [148]. In addition, activation of multiple DRs with staggered pulses [149; 150] effectively generated FL, indicating a multi-segmental convergence of afferent inputs on neuronal circuits during electrical spinal cord stimulation, as also reported in both in vivo animals [151] and in humans [100; 152].

However, still to be defined are both the neurophysiological mechanisms of electrical stimulation for triggering locomotor activity and the involved spinal wiring. Supposedly, the origin of FL episodes in response to DR stimulation may relate to the cumulative depolarization of distinct post-synaptic sites able to vary extracellular ionic concentrations [153] and facilitate the release of neurotransmitters that selectively activate network

elements crucial for generating the locomotor pattern. Indeed, selective stimulation of a subpopulation of spinal neurons is sufficient to trigger the locomotor pattern [154]. Modeling studies also have demonstrated that it is possible to effectively activate the CPG through even a few afferent projections [155]. Functional projections from the periphery to the CPG have been identified in both Ia afferents from muscle spindles and, mostly, in Ib afferents from Golgi tendon organs [156].

A peculiarity of locomotor episodes evoked by electrical stimulation in the spinal cord in vitro is that they spontaneously decay, regardless of continuous delivery of trains, and only can be transiently rescued by varying either intensity or stimulation site. The cause of this failure is not related to impairment of action potential invasion toward afferent terminals, nor to changes in the passive properties of the motoneuron membrane [157]. On the other hand, at the presynaptic level, stimulation with trains of impulses decreases glutamate release [157], even though this effect does not seem to be linked to the disappearance of locomotor cycles [147]. Rather, progressive deterioration of FL episodes and pattern ceasing during continuous DR electrostimulation can be caused, at the post-synaptic level, by the membrane shunt determined by the depolarization that derives from increased potassium concentrations [153] and by the release of inhibitory neurotransmitters eventually reducing FL oscillations [158; 159]. High frequency stimulation may also involve receptor desensitization, since recovery (e.g. for glutamate receptors) can require up to hundreds of milliseconds [160], and depend on the quantity of the receptor agonist and the composition of the receptor subunit [161].

Overall, the spontaneous cessation of the pattern induced by afferent stimulation may be a property of the functional organization of the spinal locomotor circuit, which attributes a triggering role to afferents, with intrinsic self-limiting properties. Indeed, volleys in afferent fibers induce presynaptic inhibition on their own terminals, thus stopping excitation [162], in a manner dependent upon the frequency of incoming input [163]. Moreover, spinal interneurons that are rhythmically active during locomotor activity are modulated by the ongoing phasic rhythm and might filter sensory input out of phase with their oscillation frequency, thus stopping the pattern shortly after its onset [164]. Cellular properties, and peculiar channel expression patterns shown by crucial classes of dorsal interneurons, also may be involved in sensory motor integration and gating [165].

However, in vitro studies allow induction of FL through different experimental modalities to optimally trace the dynamics of CPG recruitment [116; 166]. A comparison between electrically- and pharmacologically-induced FL patterns indicates that neurochemicals added to the perfusion bath generate FL with a much slower onset, but

that once established remains stable for many hours. Moreover, unlike electrical stimulation, it is possible to finely modulate frequency of pharmacologically-induced FL by titrating concentrations of pharmacological agents [147]. This might imply that modulation of the locomotor pattern requires involvement of a more widespread region of the spinal cord rather than the few segments activated by electrical stimulation of a single DR [167].

Albeit variations in frequency of stereotyped trains of pulses within a relatively broad range (1-25 Hz) does not affect number (nor periodicity) of locomotor cycles [147], stimulation with trains of distinct pairs of frequencies, even simultaneously delivered to different DRs, activates longer episodes of FL [150]. This suggests that, rather than the selection of a specific frequency, optimal DR electrostimulation to evoke FL must provide a minimum level of input range variability. Several studies suggest that critical levels of variability in CPG input are required to engage neural control mechanisms, even in a highly repetitive motor task. For example, lack of variation in step trajectories interferes with the normal cycle progression that the networks execute, which can result in an inability to learn or improve the performance of motor tasks [42; 168; 169].

Innovative protocols of electrostimulation exploit the intrinsic rhythmogenic potential of spinal circuits

Locomotor-like activity in the in vitro spinal cord (Fig. 1 A) has been optimally evoked by stimulating one DR or the cauda equina with intrinsically variable asynchronous (i.e. noisy) patterns, obtained by sampling biosignals corresponding to rhythmic motor patterns in vitro or in vivo, from either a ventral root (VR, Fig. 1 B), a muscle, or a single motoneuron [131; 150; 170; 173; 174]. The clear advantage of this approach relies on stimulation strength, which, unlike canonical electrostimulation, is much lower than the minimum one required to induce a reflex response (i.e., sub-threshold). Moreover, when compared to the classic protocols of electrical stimulation [147], noisy biosignals induce locomotor-like oscillations of longer duration and with a greater number of cycles [170; 173], although the pattern still does not last throughout the protocol. The reason behind the improved efficiency of the protocols that use noisy biosignals is still under investigation. A possible explanation could rely on the presence of an intrinsic variability in amplitude and frequency of noisy protocols that accommodates the variability required by the locomotor network, mimicking the volley of physiological input that reach the spinal cord during locomotion [175]. Noise-derived high variability of the stimulus per se is not sufficient to elicit FL, as a phasic component in the lower frequencies seems also to be required, as demonstrated by the inefficacy of stimulation using either the sole Gaussian

noise (Fig. 1 C) or biosignals sampled during tonic muscle activation [174]. At the same time, FL could not even be induced by noise-free phasic input such as pure sinusoids (Fig. 1 D), or artificial noisy waveforms, software-designed by adding to a pure sinusoid either the spontaneous baseline activity at rest [170] or Gaussian noise (Fig. 1 E). These results indicate that input able to optimally trigger the CPG must contain both the low frequency component of rhythmic motor tasks and the high frequency spectral density of motor-related biosignals. As a result, effectiveness of noisy waveforms might be linked to the relative contribution of such distinct stimulus frequencies particularly efficient in activating frequency-dependent CPG elements [150]. Moreover, variability in the amplitude of the stimulating patterns might play a crucial role reminiscent of the control over sacral network output, using amplitude-modulated signals delivered to the peripheral nerve [171; 172]. The possibility to deliver these protocols at subthreshold intensity makes them an elective tool to exploit the intrinsic rhythmogenic potential of spinal circuits.

Pharmacological synergism of electrically-induced locomotor patterns

In spinal animals, superior locomotor performances so far have been found with suprathreshold doses of specific drug cocktails or with subthreshold doses of 5-HT agonists combined with electrical stimulation of the spinal cord [103; 141; 142; 176-179]. This suggests that innovative neurorehabilitation strategies to improve sensorimotor functions following neuromotor disorders could combine pharmacotherapy, training and electrical stimulation. In neonatal rat isolated spinal cords, FL was activated by the association of neurochemicals at low doses and noisy protocols at weak intensity (but not conventional trains of rectangular pulses), both unable to generate a locomotor pattern on their own. Moreover, this combination modulated cycle frequency and increased duration of FL episodes beyond the limits of electrical stimulation alone, even if delivered at optimal intensity [173]. However, these effects were not seen in the presence of a generic increase in the overall neuronal excitability of the spinal cord mediated by a shift in extracellular ionic concentrations [173], indicating that locomotor circuits, once optimally triggered by low intensity noisy patterns, can be modulated by a likewise selective (low concentration) pharmacological stimulation.

In this regard, it has been recently demonstrated that even nanomolar concentrations of the neuropeptide oxytocin, which alone is unable to elicit FL, can synergize with weak noisy stimulating protocols to elicit locomotor network activation [131]. These findings suggest that combining low doses of oxytocin with direct sub-threshold electrical stimulation helps to exploit the automatic locomotor capacities of isolated spinal circuits.

This perspective is even more interesting, in light of the ongoing clinical trials targeting safety of oxytocin for spinal cord dysfunction (http://clinicaltrials.gov).

Strengths and limitations of the in vitro approach

Newborn rat spinal cord networks are organized in a very similar way as adult networks [180], but the former ones allow advantages in terms of easier surgical isolation of the spinal cord, technical access to multiple electrophysiological recordings and electrical stimulations, as well as a longer in vitro availability compared to older tissue [181]. In addition, spinal cord isolation reduces the basic modulatory tone [182], in turn increasing consistency of motor output. As a result, it is possible to unveil even the slightest modulatory effects that could only be barely identified in vivo even using a very high number of repetitions. In general, however, the in vitro approach also allows recordings of the motor output with a pure neuronal origin, thus excluding any influence from the activation of either compensatory muscle contractions or modulators of peripheral circulation. Moreover, the clear distinction between input from DRs and motor output from VRs makes the isolated spinal cord an elective model for assessing the recruitment of locomotor networks by afferent electrical pulses. As a result, we can carefully determine the efficacy of the different protocols of stimulation, by quantifying the number of FL oscillations or by assessing the minimum duration of stimulation required to induce an episode of FL. For example, the most selective protocols available in vitro are efficient even when delivered for periods as short as 500 ms [150].

Nevertheless, the in vitro model does have a few limitations. For example, it does not allow a full analysis of motor control in terms of fine-tuning abilities, such as kinematic analysis, which is available with in vivo animal preparations. Furthermore, using in vitro preparations, we cannot identify the neuronal output that corresponds with maintenance of standing posture nor to the different coordination among muscle groups, considering the complexity of the motor behavior displayed by the behaving animal [3]. Thus for example, this does not permit confirmation of whether distinct protocols of electrical stimulation can generate different motor behaviors in vivo. For all these reasons, in order to propose innovative strategies to reactivate stepping after spinal cord damage, and to consider pediatric incidence of SCI [183], it would be profitable to adopt a multilevel analysis of locomotion in immature preparations. An extremely useful research approach could thus consider the serial application of the same experimental treatments to the same animals in each setup, to integrate initial kinematic assessments of real behavior and electrophysiological recordings of spinal network activity, after spinal cord isolation.

Stimulation of stepping behavior in vivo

To confirm the function of spinal circuits in vivo, behavioral paradigms in animals have been developed. The in vivo complement to the isolated spinal cord in vitro comes in the form of air-stepping. During air-stepping, animals typically are provided body-weight support by being held in a sling, with limbs unobstructed so they can move in the air (Fig. 2). Using the air-stepping paradigm, the function of locomotor circuits may be examined in immature and SCI animals that may not have the postural control or muscle strength for independent walking. To evoke air-stepping, pharmacological, sensory, or electrical stimulation is often used.

For example, when newborn rats are suspended in a sling, air-stepping may be evoked by treatment with the dopamine precursor $_{L}$-DOPA [184] or the 5-HT_{2A} receptor agonist quipazine [185]. Both $_{L}$-DOPA-induced and quipazine-induced air-stepping produce alternating limb kinematic patterns consistent with walking locomotion [186; 187]. A mid- or low-thoracic spinal transection eliminates $_{L}$-DOPA-induced hindlimb stepping [188], however it does not eliminate quipazine-induced stepping [120; 121; 188; 189], suggesting that 5-HT receptors in the spinal cord engage spinal locomotor networks. Pharmacological stimulation of air-stepping has led to better understanding of the development [120; 190; 191], mechanisms [188; 192], function [187], and sensory modulation [121; 185] of locomotor circuits in vivo, including for animals with SCI [119; 189; 193; 194].

Sensory stimulation such as tail-pinch [68] and olfactory stimulation (bedding material; [195]), and electrical stimulation delivered by epidural [196] or intraspinal methods [197], also stimulates air-stepping. Air-stepping is not a phenomenon limited to rodents, as it has been reported in cats [198], dogs [199], monkeys [197], and human infants [201] and adults [202].

There are several advantages for using the air-stepping paradigm to examine locomotion. First, air-stepping occurs in a living, animal body that is equipped with a complex anatomy and physiology for supporting behavior. Thus compared to in vitro models, it is more behaviorally relevant. Second, because of this complex physiology, it allows examination of interactions among factors that may influence ongoing locomotor behavior, such as neurotransmitter receptor stimulation and movement-produced sensory feedback. Third, it permits investigation of locomotor activity without the need for balance control and body-weight support via reduction of external resistance. This is useful for studying developing animals that have immature postural systems and weak muscles, and

humans and animals that have weakened or damaged sensorimotor systems such as with SCI. Fourth, and related to the reduction of external resistance, it allows for study of the integrity of locomotor mechanisms separate from postural mechanisms. This separation may be useful to understand in some situations where balance and posture problems may interfere with phasic limb patterning.

However, the air-stepping paradigm alone will not reveal all mechanisms involved with locomotion. Techniques at additional levels of analysis, and use of other paradigms such as the isolated spinal cord in vitro, are necessary to more precisely identify cellular properties, molecular signaling cascades, and genetic regulation of spinal locomotor networks. Further, while air-stepping resembles locomotor behavior in terms of alternating limb activity, it is still not actual locomotion. True locomotion involves integration among sensory, motor and cognitive systems and movement of the body center of mass through space. Thus air-stepping is a rather contrived experimental situation that is quite removed from the complex, dynamic interactions experienced by walking individuals. Therefore it is necessary to combine findings from behavioral experiments using the air-stepping paradigm with more reductionist, as well as more sophisticated, paradigms and preparations to more accurately depict the control and regulation of locomotion. This kind of multilevel analysis of locomotion is necessary for approaching the myriad factors that are necessary for addressing SCI.

Synergistic effects of pharmacological and sensory stimulation on locomotor behavior in developing rats in vivo

Recent research has focused on the development and regulation of locomotor behavior in the developing nervous system, using the in vivo perinatal rat as a model system. Understanding how such factors promote development and shaping of locomotor mechanisms during ontogeny has implications for facilitating recovery of function following SCI or developmental neuromuscular disorders, particularly as we now recognize that these mechanisms are activity-dependent [88].

In rats, the neural mechanisms controlling locomotion begin developing during the prenatal period [120; 203; 204], with much continued development occurring during the early postnatal period [4; 205]. During this early time in development, the spinal cord exhibits remarkable plasticity. For instance, following a spinal cord transection, immature rats recover significantly more motor function compared to older animals, mainly due to increased synaptogenesis and decreased denervation and spinal shock [206-209]. Thus

by studying locomotor function in spinal cord transected immature rats, the function of the isolated spinal cord in vivo may be evaluated at the height of spinal plasticity.

For example, in a series of studies, how newborn rats adapt their stepping behavior to a range of motion (ROM) restriction manipulation was examined. In these studies alternating air-stepping behavior was induced with the 5-HT_{2A} receptor agonist quipazine (3.0 mg/kg), and ROM restriction was imposed by placing a Plexiglas plate beneath the limbs of the rats at a distance of 50% of limb length when the limbs were fully extended. Intact postnatal day 1 (P1; 24 hr after birth) and P10 rats adapted their stepping behavior to the ROM restriction, such that they accommodated the ROM restriction task by altering intralimb coordination to apparently preserve the alternating pattern of interlimb coordination [191]. Specifically subjects made larger hindlimb step cycle excursions moving their limbs more towards the front and back of the body, rather than directly underneath the body. When subjects were administered a low-thoracic spinal cord transection on P1, such that hindlimb locomotor networks were now isolated from the rest of the CNS, hindlimb stepping behavior on P10 was abundant and intralimb adaptations to the ROM restriction also were made in these spinal subjects [121]. In fact, hindlimb stepping in spinal subjects (~450 bilateral hindlimb steps per 5 min bin) occurred approximately three times as much compared to intact subjects. This may be due in part to an up-regulation of 5-HT receptors in the caudal spinal cord following a spinal cord transection [137-139]. But in spinal subjects that received ROM restriction, frequencies of hindlimb stepping decreased to intact levels of stepping (~150 bilateral hindlimb steps per 5 min bin) during, but not after, ROM restriction (Fig. 3 A). Hence the cutaneous and proprioceptive stimulation provided by ROM restriction may have acted to specifically reduce stepping behavior or, alterations in intralimb coordination may have compromised the ability to maintain such high levels of alternating interlimb coordination in the isolated spinal cord. Intralimb adaptations to ROM restriction were much more drastic in spinal compared to sham subjects [121]. Together, these studies are suggestive of strong synergistic actions between pharmacological stimulation and sensory afferent feedback in permitting locomotor adaptations to environmental perturbations in the isolated spinal cord in vivo. To establish if 5-HT_{2A} receptor up-regulation is a mechanism of hindlimb behavioral supersensitivity producing these effects, specifically in the area of the hindlimb locomotor CPG, an investigation is underway which is examining hindlimb stepping parameters and 5-HT_{2A} receptor density in the lumbar cord, in relation to age at spinal cord transection.

Additionally, because it is becoming clear that sensory and pharmacological stimulation may often have synergistic effects on spinal function, recently the effect of

quipazine on sensory responsiveness in acute spinal transected rats was examined (unpublished data by Swann, Kauer, Allmond & Brumley). Response to tail pinch was recorded in newborn rats that were prepared by mid-thoracic spinal transection and pretreated with quipazine, and compared to controls. All subjects showed an immediate and robust motor response to tail pinch that consisted mainly of hindlimb steps (Fig. 3 B). In shams, both quipazine-treated and saline-treated subjects showed persistent effects of the tail pinch. However in spinal animals it was only quipazine-treated subjects that showed persistent effects, while saline-treated subjects did not. This study suggests that serotonergic stimulation in spinal subjects helps to recover sensory responsiveness to sham levels. However, it is important to note that quality of movement was different in spinal and sham subjects: spinal subjects including those treated with quipazine showed a higher percentage of low amplitude and smaller excursion hindlimb steps in response to tail pinch, whereas sham subjects showed a high percentage of high amplitude and large excursion steps. Thus serotonergic stimulation may help to restore excitation in the spinal cord, but not necessarily the amplitude and kinematics of leg movements.

Examination of non-neural factors in the regulation of locomotor function in spinal injured rats also has been investigated in the immature rat model in vivo. In this study, rats were treated with a thoracic hemisection on P3 and injected into the lesion site with human placental pericytes (unpublished data by Mayo, Kauer, Brumley and Bearden). Pericytes are cells of the microvascular wall that have been shown to stimulate angiogenesis in vitro [210; 211], promote functional recovery in ischemic heart repair [212], muscle regeneration following injury [213], and regulate blood-brain barrier permeability [214]. On P10, spinal injured subjects were examined for locomotor function. Pericyte treatment significantly improved hindlimb locomotor function and increased neurofilament density in both male and female rats. Additionally, placental pericytes were found in the tissue of all subjects, and migrated both rostral and caudal from the site of injury. Vessel density increased only in males. These results indicate that vascular changes within the spinal cord play a role in locomotor recovery from SCI in rats, and suggest some possible sex differences in vascular organization, function, or timing of repair in spinal tissue (unpublished data by Mayo, Kauer, Brumley and Bearden). Thus pericytes may be useful as a therapeutic cell treatment following SCI, perhaps limiting vascular dysfunction and/or playing a role in supporting neuronal reconfigurations. Intriguingly, assays with endothelial cells or spinal cord tissue culture showed faster wound healing and greater vascular density when pericytes were stimulated with $CoCl_2$ (to activate hypoxia-inducible pathways known for stimulating capillary growth) in vitro [211]. However when examined in spinal tissue in vivo

(described above), naïve pericytes, but not pericytes stimulated with $CoCl_2$, promoted better recovery of locomotor function in SCI subjects (unpublished data by Mayo, Kauer, Brumley and Bearden). Thus results at one level of analysis may not necessarily be predicative of results at another level of analysis (i.e., cellular/in vitro ≠ behavioral/in vivo; under exact conditions), though each approach can reveal important insights to inform a different level of analysis (i.e., increasing angiogenic activity in vitro and improving locomotor function in vivo; under modified conditions).

While the spinal in vivo neonatal rat preparation is more directly relevant to SCI, the intact neonatal rat offers important insights into general issues of neurobehavioral development and plasticity as well. For example, it has been shown that locomotor behavior in intact newborn rats is modulated by the substrate that the animal is stepping on [188], ROM restriction [191], treadmill speed [215], posture [216], and testing environment [187]. Thus even before the onset of independent walking and maturation of neural pathways (e.g., corticospinal tract development, myelination), it is clear that locomotor mechanisms demonstrate plasticity and are responsive to the environment. This principle is evident in developing humans as well [88]. Understanding how the development of locomotion typically occurs at multiple levels of analysis and factors that go into the shaping of locomotor circuits is crucial for developing therapies of locomotor recovery for infants and children that experience motor dysfunction due to pediatric SCI, stroke, or congenital disorders (e.g., neural tube defects such as spina bifida). For example, basic research has yielded insights of clinical significance, such as early identification and empirically-based treatments of motor dysfunction to optimize neurobehavioral outcomes in children [217]. Implementation of activity-based treatments for infants with Down syndrome and MMC were discussed earlier in this review. To further our understanding of these disorders, mechanisms affected, and treatment options, experimental paradigms with animals such as the in vivo perinatal rat is crucial as it permits testing at earlier ages, cellular and systems manipulations, and evaluation of possible treatments.

Conclusion/Perspective

The spinal locomotor system is complex and, undoubtedly, still incompletely understood. From the seminal work of Sherrington and Graham Brown a century ago, which suggested the existence of this ‘black box’ for locomotion in the spinal cord, up to the pivotal insights since the 1980s about cellular and pharmacological properties of the CPG gained with the development of different in vitro isolated spinal cord preparations

(e.g, isolated spinal cords from lampreys, tadpoles, turtles, rats and mice both wild-type and genetically-engineered), significant advances have been made. As challenging as it is, carefully comparing data from in vitro and in vivo approaches has already begun to yield the development of promising combinatorial approaches that remain to be clinically tested. If one day, some of these CPG-activating approaches get approval by regulatory authorities, they may not cure SCI, but, combined with proper training, they may lead to significant benefits on health, as holistic approaches designed to prevent or reverse metabolic diseases, cardiovascular problems and other chronic illnesses associated generally with SCI-related physical inactivity.

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Author Disclosure Statement

MRB declares having no conflict of interest.

PAG declares having no conflict of interest.

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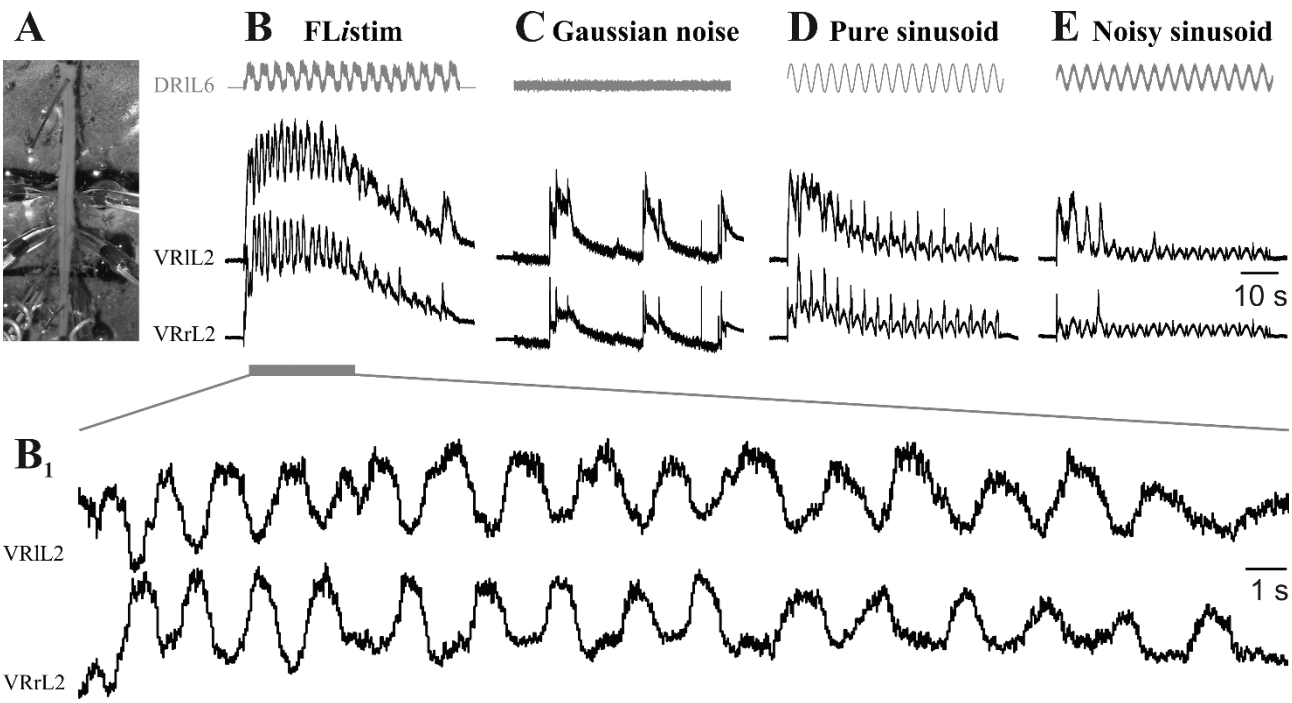


Figure 1. Innovative protocols of electrostimulation applied to a DR optimally trigger fictive locomotion patterns in the isolated spinal cord. **A**: The isolated spinal cord from a neonatal rat (one day post-natal) continuously perfused with physiological solution remains long-lastingly viable, allowing multiple recordings and stimulations through suction glass electrodes connected to ventral and dorsal roots, respectively. **B**: A 60 s trace sampled from VRrL5 during a stable FL induced by NMDA (5 µM) + 5HT (10 µM) is exported through off line analysis to a programmable electrical stimulator, to design the protocol named FL*i*stim (Fictive Locomotion *induced* stimulation). FL*i*stim is delivered (6 µA, 0.3 threshold, Th, defined as the minimum intensity required to induce a reflex response using a single square pulse) to the DRlL6 of the same isolated spinal cord, now perfused in physiological solution after extensive wash out from neurochemicals. In response to stimulation, a cumulative depolarization appears superimposed by an episode of fictive locomotion (FL) pattern, consisting of 15 oscillations fully alternated among the bilateral L2 VRs (see magnification on $\mathbf{B_1}$). After 30 seconds, traces repolarize to baseline, while FL cycles fade away despite continuous stimulation. **C**: Delivery of a trace of Gaussian noise artificially created through software failed to elicit FL, which is replaced by multiple synchronous bursts. **D**: A pure sinusoid of the same main frequency and amplitude of FL*i*stim induces a first cumulative depolarization that eventually ceases, while FL cycles are replaced by synchronous discharges time-locked with peaks of the stimulating waves. **E**: An artificial noisy waveform, constructed by adding the Gaussian noise to a pure sinusoid, does not induce any alternating cycles but only a cumulative depolarization with few synchronous cycles.

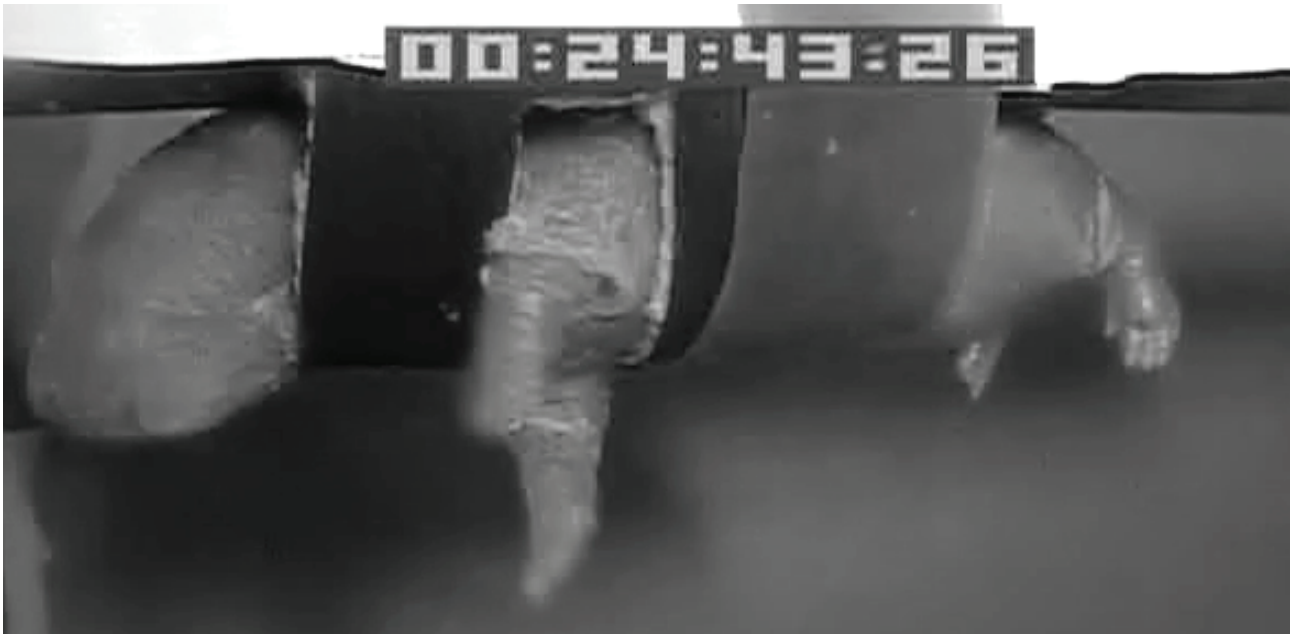
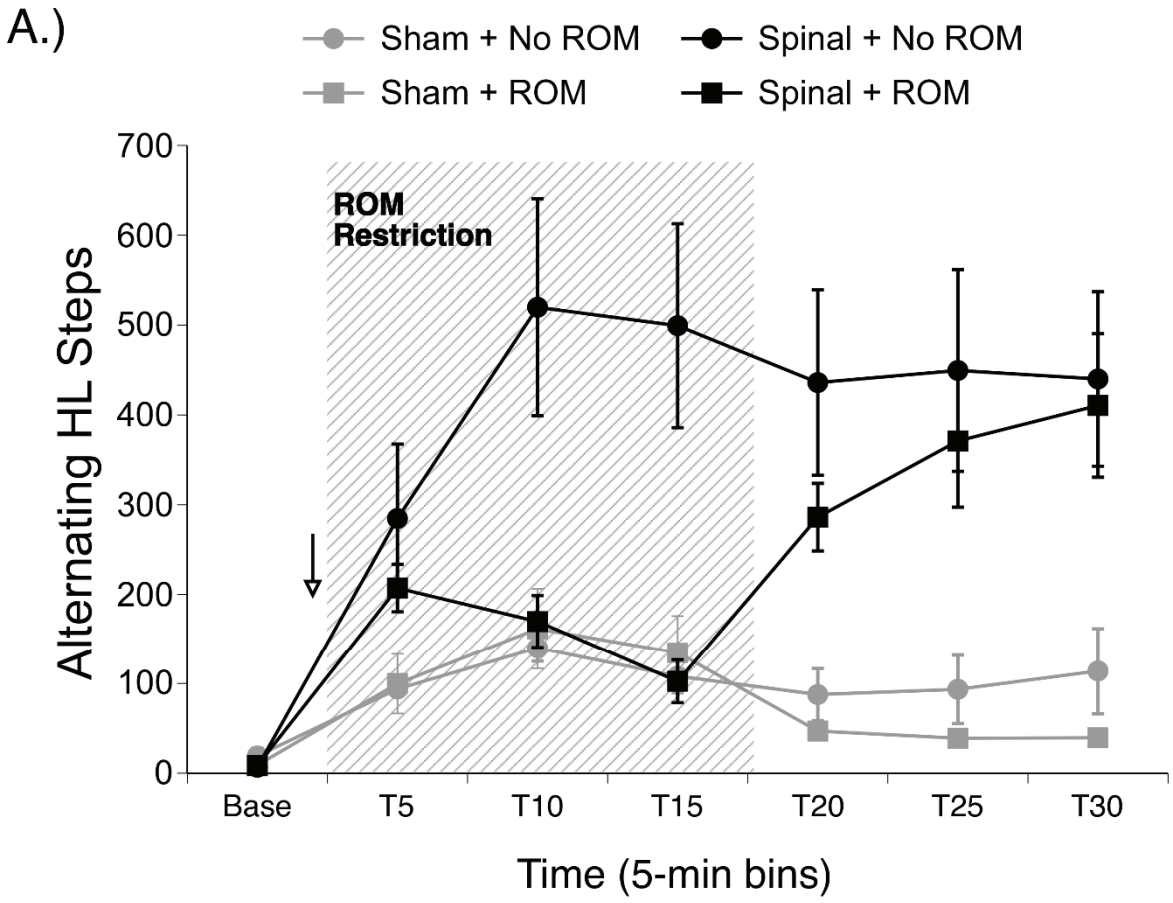


Figure 2. Air-stepping in the neonatal rat. Photograph of a 1-day-old rat showing alternating stepping behavior, following treatment with the 5-HT2A receptor agonist quipazine. The subject was secured to a horizontal bar, injected with quipazine, and recorded from a camera at a lateral angle. Behavioral testing occurred inside of an infant incubator that is temperature- and humidity-controlled.


A.)
Sham + No ROM
Spinal + No ROM
Sham + ROM
Spinal + ROM
ROM Restriction
Alternating HL Steps
700
600
500
400
300
200
100
0
Base
T5
T10
T15
T20
T25
T30
Time (5-min bins)

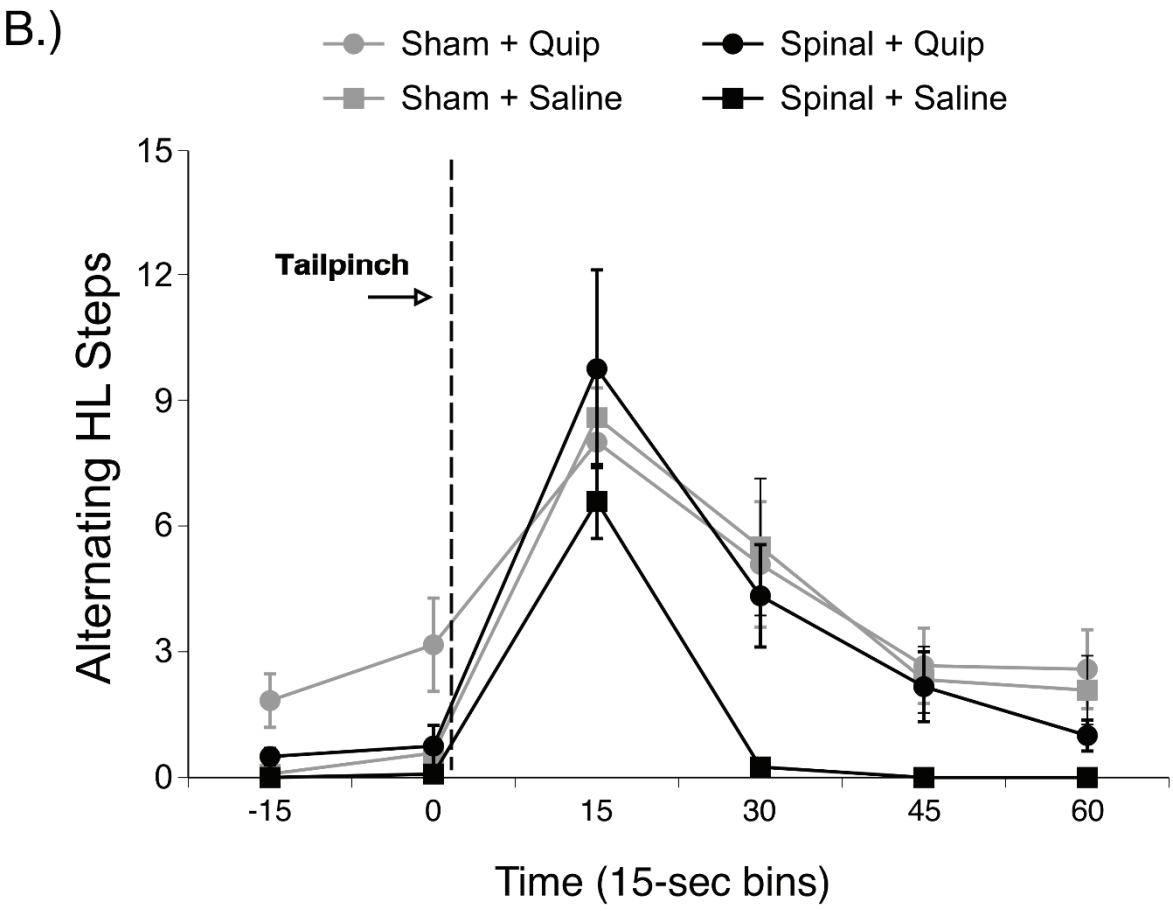

B.)
Sham + Quip
Spinal + Quip
Sham + Saline
Spinal + Saline
Tailpinch
Alternating HL Steps
15
12
9
6
3
0
-15
0
15
30
45
60
Time (15-sec bins)

Figure 3. Alternating hindlimb stepping in neonatal rats following pharmacological and sensory stimulation. **A**: Rats were given a low thoracic spinal transection or sham surgery on P1, and tested for quipazine-induced hindlimb stepping on P10. Following a 5-min baseline, half of the subjects experienced ROM restriction (shaded region), whereby a Plexiglas plate was placed beneath their limbs. They were also injected with 3.0 mg/kg quipazine (arrow) to induce stepping behavior. Note that spinal subjects showed significantly more hindlimb stepping across the test session, except for ROM-restricted subjects during the period of restriction (they fell to sham levels). **B**: Rats were prepared by acute mid-thoracic spinal transection and tested for sensory responsiveness to a tail pinch on P1. Ten minutes before tail pinch, subjects were pretreated with 3.0 mg/kg quipazine. Tail pinch (dashed line) was administered by gently squeezing forceps around the base of the tail. Response to tail pinch occurred immediately and persisted for about 1-min in sham subjects and spinal subjects pretreated with quipazine. Points show means; bars depict SEM.

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