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Hindlimb Stepping in Response to Treadmill Speed in Neonatal Spinal-Transected Rats

by:

Aimee L. Bozeman

A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in the Department of Psychology

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Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Aimee Lynn Bozeman find it satisfactory and recommend that it be accepted.

_____

Michele R. Brumley, Major Advisor

_____

Erin Rasmussen, Committee Member

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Jason Pilarski, Graduate Faculty Representative

March 8, 2016

Michele Brumley, PhD
MS 8112
Psychology Department
Pocatello, ID 83209

RE: Your application dated 3/1/2016 regarding study number 742: Spinal regulation of neonatal rat behavior

Dear Dr. Brumley:

Your request for approval of the new protocol listed above was reviewed at the 3/8/2016, meeting of the Idaho State University IACUC. This is to confirm that your protocol was approved. Your protocol number is 742.

You are free to proceed with your study as described in your protocol effective immediately. The study is subject to annual review on or before 3/8/2017, unless closed before that date.

Please note that any changes to the protocol as approved must be immediately reported and approved. Contact me (208-282-2179; fax 208-282-4723; email: anmlcare@isu.edu) if you have any questions or require further information.

Sincerely,

Tom Bailey
IACUC Coordinator

Acknowledgments

First, I would like to thank my advisor, *Michele Brumley*, for the energy she has invested into my growth as a scientist. I am a better scientist, writer, and person because of her. Through all of this, she was the person I could go to with any question or concern. Without her, none of this would be possible. Thank you for taking a chance on a girl from Georgia. Here's to the PhD!

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Finally, I would like to thank my family who has been there throughout the entire process, from Georgia to Idaho, I am eternally grateful.

Dedication

To my parents – thank you for supporting me no matter what, for loving me no matter the distance, and for always being available for phone calls no matter the time difference. I love y'all.

To my sister, Kasey – thanks for showing me how to run a t-test for my 10th grade science fair project. I didn't use them for this project, but you get the point.

To all my friends who were there for every tear, smile, laugh, and frantic phone call – I'm sorry you had to witness that.

To the rats who make everything possible – thank you.

And finally, to every little girl who has ever been told she can't be a scientist – *they're wrong.*

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Hindlimb Stepping in Response to Treadmill Speed in Neonatal Spinal Rats

Thesis Abstract -- Idaho State University (2021)

This study examined hindlimb stepping on different treadmill belt speeds in rats with an early spinal cord transection. Rats received a low-thoracic spinal transection or sham operation on postnatal day 1 (P1). On P5, subjects were tested on a treadmill for 30-min following an injection of quipazine (a selective serotonin agonist). Spinal-transected rats showed significantly more alternating hindlimb steps and total hindlimb movements, consistent with hindlimb supersensitivity to serotonin following spinal injury. Spinal-transected and sham rats differently adapted their intralimb coordination (i.e., hindlimb step cycle duration, step height, and step length) in response to different treadmill speeds. Overall, findings from this study show that the developing spinal cord is responsive to sensory feedback from treadmill stepping but suggest that further experience with weight-bearing locomotion may be needed to express more adult-like adaptations to differing belt speeds.

Keywords: spinal cord, development, alternating stepping, plasticity

Chapter I: Introduction

Behavioral and neurobiological studies have demonstrated that the neural mechanisms supporting locomotion begin developing before birth, and continue developing during the early postnatal period, in rats. Further, the control of interlimb coordination during locomotion in mammals lies within the spinal cord (Frigon, 2017). Sensory stimulation and pharmacological treatment can evoke patterns of interlimb coordination (i.e. alternating or synchronous) and influence the quantity and quality of movement in fetal and newborn rats. For example, the type of substrate (floor) that pharmacologically-treated perinatal rats are tested on influences type of locomotor-like stepping, as well as its frequency and duration (Brumley, Roberto, & Strain, 2012). Sensory stimulation and drug administration can be used to evoke and modulate alternating stepping in rats that have a spinal cord transection, as well. Often times, researchers use sensory stimulation and pharmacological treatment in conjunction with one another to examine their effects on locomotor behavior, as their effects together are stronger than either alone (Gerasimenko et al., 2007; Brumley, Guertin, & Taccola, 2017). The current study used pharmacological treatment (quipazine) and sensory stimulation (treadmill) to induce alternating stepping in spinal-transected rat pups to examine the ability of the isolated spinal cord to adapt to sensory conditions. This experiment is a follow-up to a study by Bozeman et al. (in preparation) that showed that intact neonatal rats are capable of modulating their stepping behavior in response to a moving treadmill belt. However, in the current study, we added in surgery condition as an additional variable, in order to examine the behavioral capabilities of the developing, isolated spinal cord.

The ability to recover significantly more function following a spinal cord injury (SCI) during early development than in adulthood was first experimentally described by Stelzner,

Ershler, and Weber (1974) and named the “survival of function” effect. In subsequent years, other researchers termed this display of physiological and behavioral plasticity following neural injury in developing animals the “infant lesion effect” (Bregman & Goldberger, 1983). More recently, researchers have conducted both *in vitro* and *in vivo* experiments to better understand the restoration of physiological mechanisms and behavioral responses following SCI in early, developmental periods (Hayes, Chang, & Hochman, 2008; Tillakarante, et al. 2010; Yuan et al., 2013). Research has shown that sensory stimulation and feedback during the neonatal period is important for motor behavior development and might act to promote enhanced recovery of function following neural injury or behavioral deficit (Dewolf et al., 2021; Swann, Kauer, Allmond, & Brumley, 2017). Treadmill training, as a form of sensory stimulation, is often used in studies examining sensory effects on stepping and as an intervention/therapy following SCI (Heng & de Leon, 2009; Fouad et al., 2000). Thus, the motivation behind the current experiment was to use a treadmill stepping paradigm to further understand behavioral recovery and the behavioral potential of the isolated spinal cord following an early SCI.

Stepping in Neonatal Rats

Although rats begin to regularly exhibit quadrupedal walking approximately two weeks after birth (Altman & Sudarshan, 1975; Swann & Brumley, 2019), research has shown that fetal rats possess the necessary neural circuitry to express an alternating pattern of interlimb coordination (Bekoff & Lau, 1980; Brumley & Robinson, 2005). However, largely due to immature postural control, expression of locomotor behavior is limited during the early postnatal period (Gramsbergen, 1998) and often needs to be induced via sensory stimulation or pharmacological treatment (e.g. Mendez-Gallardo & Robinson, 2014; Swann et al., 2016). Because this study will be examining the modulation of stepping behavior in spinal-transected

and intact neonatal rats, stepping will be induced via pharmacological stimulation (i.e. quipazine) in order to activate spinal locomotor circuitry. Additionally, subjects will be secured to a bar above the treadmill to reduce postural demands.

Evoking Alternating Stepping in Spinal Rats

Pharmacological treatment is a reliable form of stimulation used to evoke alternating stepping in spinal-transected rats. Specifically, serotonin (5-HT) receptor agonists are often used to activate motor circuits within the spinal cord (McEwen, Van Hartesveldt, & Stehouwer, 1997; Feraboli-Lohnherr, Barthe, & Orsal, 1999; Sławińska, Majczyński, Dai, & Jordan, 2012; Sławińska, Miazga, & Jordan, 2014). Since serotonin pathways are implicated in initiating and regulating stepping, as well as its rhythm and coordination, serotonergic drugs are often used to induce stepping in spinal rats in many experimental paradigms. Using serotonergic drugs to activate these neural systems allows researchers to examine the extent of locomotor behavior that is possible following injury, as these drugs also can promote recovery of locomotor function following SCI (McEwen, Van Hartesveldt, & Stehouwer, 1997; Feraboli-Lohnherr, Barthe, & Orsal, 1999).

The selective serotonin (5-HT2_A) agonist quipazine has been used to induce alternating stepping in fetal (Brumley & Robinson, 2005), neonatal (McEwen, Van Hartesveldt, & Stehouwer, 1997; Strain et al., 2014), and adult rats (Antri et al. 2005) that have received a spinal cord transection, as well as intact neonatal rats (Strain & Brumley, 2014; Swann et al., 2016). In an experiment using quipazine, Brumley and Robinson (2005) showed that alternating stepping behavior in complete spinal (transected at T5-T6) and sham rat fetuses was significantly increased after intraperitoneal administration of quipazine. Additionally, that experiment showed that spinal fetuses exhibited significantly more hindlimb steps than sham fetuses. It has been

suggested that serotonergic agonists, such as quipazine, play a role in hindlimb motor behavior of rats by acting at the level of spinal networks isolated by transection surgery (Brumley & Robinson, 2005). Recently, Swann and colleagues reported that intrathecal administration of quipazine (applied in the thoracolumbar area) evokes hindlimb steps in intact rats, further suggesting direct activation of locomotor networks by quipazine (Swann, Viall, & Brumley, 2021). Since previous research suggests the reliability of quipazine to induce alternating stepping behavior in intact and spinal neonatal rats (e.g. Strain et al., 2014), we use it here in combination with sensory stimulation for this experiment.

As previously mentioned, researchers often use a combination of methods to induce stepping in spinal rats. A combination of serotonin or quipazine injections and tail-pinching aids in inducing motor behaviors, such as hindquarter elevation and hindlimb flexion, in adult rats with a complete transection at thoracic-level (T8-T9; Feraboli-Lohnherr, Barthe, & Orsal, 1999). Although observable stepping behavior was not induced by injections of serotonin or quipazine alone, the injections did produce spontaneous motor rhythms (measured by electromyography, or EMG) that showed obvious alternation between right-left flexor hindlimb bursts, as well as flexor-extensor alternation. Drug treatment and mechanical stimulation produced the most robust effect on stepping behavior, and treatment with serotonin agonists increased the length of stepping sequences obtained by tail-pinch. Longer rhythmic locomotion durations co-occurred with improvement of locomotion parameters described as interlimb coordination, cycling velocity, and flexor-extensor burst amplitude (Feraboli-Lohnherr, Barthe, & Orsal, 1999). These results suggest that an additive effect of drug and sensory stimulation (serotonin + tail pinch) is a dependable method for evoking stepping in adult spinal rats (Feraboli-Lohnherr, Barthe, & Orsal, 1999).

The transected/isolated spinal cord is sensitive to the combined effects of sensory stimulation and pharmacological treatment in newborn rats as well. In a study by Strain and colleagues (2014), rats underwent a low-thoracic spinal or sham surgery on postnatal day one (P1) and were behaviorally tested on P10 in the prone position with limbs hanging freely. Following an injection of quipazine, range-of-motion (ROM) restriction was imposed on half of the subjects. For ROM restriction, a Plexiglas plate was placed beneath the limbs at 50% fully-extended limb length for 15 minutes. Following the restriction period, the Plexiglas was removed. Interlimb and intralimb coordination was scored and analyzed. Spinal P10 rats showed significantly more alternating hindlimb steps than sham rats. Additionally, spinal and sham subjects that received ROM restriction showed changes in intralimb coordination during the restriction period; however, only spinal subjects maintained the intralimb changes post-ROM restriction. This shows that spinal subjects showed intralimb adaptations to ROM-restriction. Interestingly, only spinal subjects showed persistent effects of ROM restriction in the hindlimbs suggesting that the immature isolated spinal cord is capable of supporting long-term behavioral changes following sensory perturbation (i.e. ROM restriction) (Strain et al., 2014).

Different pharmacological substances can be used to evoke stepping behavior in neonatal spinal rats. L-DOPA and quipazine independently induce stepping in intact and spinal-transected neonatal rats subjected to the air-stepping paradigm (McEwen, Van Hardesvelt, & Stehouwer, 1997). However, L-DOPA evokes forelimb (not hindlimb) alternating stepping in spinal and sham subjects, whereas quipazine evokes hindlimb alternating stepping in spinal and sham subjects. Because quipazine is effective at inducing alternating stepping behavior in the hindlimbs following spinal cord transection, we used quipazine to induce hindlimb stepping in the current experiment.

Sensory stimuli are an important aspect to the evocation, modulation, and continuation of locomotor behavior. Locomotor-like stepping has been shown to be evoked by cutaneous stimuli in neonatal rats (Noreel et al., 2003; Swann, Kauer, Allmond, & Brumley, 2017). For example, some researchers utilize a tail pinch (brief mechanical pressure applied to the base of the tail) to administer sensory stimulation. Specifically, the stimulation provided by the tail pinch acts on sacrocaudal afferent fibers. Activation of these afferents creates a neural signal that is then carried to the spinal cord. From there, the neural signal is transmitted to motor efferent fibers, which can activate flexor and extensor muscles (Lev-Tov, Etlin, & Blivis, 2010; Cherniak, Etlin, Strauss, Anglister, & Lev-Tov, 2014). The result is rhythmic motor activity in spinal and intact rats (Norreel et al., 2003; Swann et al., 2017).

In previous research, low-thoracic spinal-transected P1 rats that were treated with quipazine and then administered a tail pinch showed similar frequencies of hindlimb movements and alternating hindlimb steps to that of intact rats. The amplitude of hindlimb steps was also evaluated, and rats treated with quipazine showed significantly higher percentages of high-amplitude steps than saline-treated rats. However, sham pups exhibited higher percentages of high-amplitude steps than spinal rats (Swann et al., 2017). This suggests that while stepping behavior can be induced in rats with a SCI, it may not be of the same quality as pre-injury stepping. Differences in high-amplitude stepping observed between spinal and intact rats helps researchers better understand the therapeutic implications for humans with SCI. For example, in individuals with SCI, restoration of some motor function is possible through many therapeutic interventions. However, motor function may never be restored to pre-injury quality. Through evocation methods, such as treadmill training therapy, people with SCI or neurological disorders can improve locomotor function.

Treadmill Training in Spinal Rats

Treadmill training is often used as a form of sensory stimulation in behavioral studies utilizing spinal rats and other species, and has been shown to have a positive impact on the restoration of locomotor behavior following SCI (Smith and Knikou, 2016; Tillakaratne et al., 2010; Bigbee et al., 2007; Cha et al., 2007). The moving treadmill belt evokes and modifies expression of alternating stepping behavior in intact, P1 rats treated with quipazine (Bozeman et al., in preparation). In another study, rats received a mid-thoracic spinal transection on P5 and were assessed on locomotor behaviors, such as stepping, from the day of weaning (P26) to 12 weeks of age (Tillakarante, et al. 2010). In order to examine the effects of step training on recovery of locomotor function, subjects were assigned to receive motor training on a treadmill or no training. Through the use of treadmill training, rats that received a spinal cord transection significantly improved hindlimb stepping compared to spinal rats that did not receive treadmill training (Tillakarante et al., 2010). Additionally, following re-transection of the cord, rats that received treadmill training showed no significant difference in kinematic analysis of hindlimb stepping. These results suggest that employing motor training techniques, such as treadmill training, improves the quantity and quality of locomotor behavior in animals following injury.

Work completed by Cha and colleagues (2007) examined how the amount of activity imposed on the hindlimbs of spinal rats affected locomotor behavior. Rats received a mid-thoracic spinal cord transection on P5 and were assessed using robotic technology to determine the baseline ability of weight-supported hindlimb stepping on a treadmill on P21. Subjects were then divided into two experimental groups: one in which subjects were trained to perform 100 steps per training session and one in which subjects were trained to perform 1,000 steps per training session. A robotic device was used to train the subjects to step bipedally, and a body

weight support (BWS) arm controlled the amount of load on the hindlimbs. The BWS allowed researchers to control the force that was imposed on the hindlimbs, predicted as a percentage of each subject's body weight. Training began on P23 and continued for four weeks, five days a week. The results showed that the training did not affect the quantity of hindlimb weight-bearing steps, but further kinematic analysis revealed that the quality of stepping behavior (i.e. step area and step cycle duration) significantly differed between the two training groups. Specifically, the group that was trained to perform 1,000 steps per training session had significantly larger step areas at the end of the four-week training session compared to subjects in the 100 steps per training session group. Additionally, rats that were trained in the 1,000 steps per session had longer step cycle durations than rats in the 100 steps per session group, at the end of the four weeks. Similar to recent work discussed below (Bozeman et al., in preparation), the number of hindlimb steps performed was influenced by treadmill speed. The frequency of steps elicited by rats in both training groups was significantly higher on fast, compared to slow, treadmill belt speeds. Again, these results suggest that treadmill training changes the quality of steps following SCI, and that sensory feedback (i.e. a moving treadmill belt) is an important aspect in modulating alternating stepping behavior.

Many experiments using spinal cats have examined the role of treadmill training on recovery of locomotion following injury. For example, Martinez, Delivet-Mongrain, & Rossignol (2012) examined the recovery of alternating hindlimb stepping following a partial spinal cord lesion (i.e. incomplete SCI) in adult cats. Subjects either received a partial lesion at T10-T11 on the left side of the spinal cord or remained intact, and then were assigned to either receive treadmill training or not. Training consisted of 30-minute sessions every day on a treadmill for three weeks. During the training phase (one-week post-operation), a Plexiglas sheet

was placed beneath the forelimbs, so subjects were only trained on the hindlimbs. The next phase (two-weeks post-operation) consisted of training subjects to walk with all four limbs using perineal stimulation and body support when needed. By the final phase (three-weeks post-operation), subjects were able to walk with all four limbs without the need for perineal stimulation and little to no body support. Behavioral analyses showed that cats with a partial SCI that were trained using a treadmill showed no significant difference in hindlimb step cycle duration compared to trained, intact cats. Additionally, cats that received treadmill training showed longer hindlimb step cycle durations compared to subjects that did not receive training. There was a significant difference in left hindlimb stance duration between spinal and intact subjects that were untrained, but this effect was not present in spinal and intact subjects that received treadmill training. These findings suggest that changes in intralimb coordination that occur with treadmill training are capable of being supported by the injured spinal cord, and again illustrate the utility of the treadmill training paradigm for helping to restore locomotor function.

In a previous study in our lab, we investigated whether or not newborn rats would step on a treadmill. P1 rats were secured over a treadmill and were tested on a moving or non-moving treadmill belt (Brumley, Kauer, & Swann, 2015). Results showed that the moving treadmill did not effectively induce stepping behavior, and that rats tested on the non-moving belt showed a higher frequency of steps compared to rats tested on the moving belt. It is important to note that the frequency of stepping behavior of rat pups in both treadmill belt conditions (moving and non-moving) were very low (Brumley et al., 2015). More recently, a follow-up experiment was conducted to determine if newborn rats treated with quipazine (known to induce alternating stepping, as previously discussed) would alter stepping behavior in response to changes in treadmill belt speed (Bozeman et al., in preparation). The frequency of alternating forelimb

stepping was highest for pups tested on the fast treadmill belt speed. Additionally, subjects tested on the fast treadmill belt had shorter forelimb step cycle durations. They also showed no difference in swing phase duration when compared to rats tested on the non-moving (control), slow, and medium treadmill belt speeds. Thus, the difference in total step cycle duration was evident in the stance phase only: rat pups in the fast treadmill speed condition showed significantly shorter durations of forelimb stance compared to pups in all other belt speed groups. Interestingly, the change in stance phase duration that occurred in neonatal rats on the faster treadmill belt is characteristic of adult stepping (Pearson, 2008) and has also been observed in human infants (Thelen, Ulrich, & Niles, 1987). These results demonstrate that neonates have the ability to modulate locomotor stepping in response to a moving treadmill belt. Whether or not the immature spinal cord alone can support treadmill-induced adaptations in neonates is the subject of the current study.

Developmental Plasticity of the Spinal Cord

Although the spinal cord exhibits neural plasticity in adulthood, it displays greater plasticity during early development. For example, neonatal rats, compared to weanlings and adults, show more recovery of motor function following a complete spinal cord transection (Weber & Stelzner, 1977). Differences in hindlimb step frequency, duration of alternating stepping bouts, and increased quality of steps are evident in newborn rats when compared to adults (Weber & Stelzner, 1977; Cummings & Stelzner, 1988; Vinay et al., 2002). A spinal cord transection in adult rats typically leaves the animal more or less paralyzed, but newborn rats express spontaneous limb movements and develop more functional behavior caudal to the lesion site following a spinal transection surgery (Robinson & Smotherman, 1990; Robinson et al., 2000; Yuan et al., 2013). Even when pharmacological administration and sensory stimulation are

used, adult spinal rats do not display as much locomotor behavior or the same quality of hindlimb stepping. These differences in recovery of function are due to increased synaptogenesis, and decreased denervation and spinal shock within the immature spinal cord, rather than regrowth of nerve fibers across the lesion site (Tillakaratne et al., 2010; Yuan et al., 2013).

Age plays a crucial role in motor acquisition and maintenance following injury. Research has shown that newborn rats that receive a mid-thoracic spinal cord transection shortly after birth exhibit the same developmental changes in motor neuron morphology that is expected during normal development (in the absence of a lesion). However, rats that received the same transection as weanlings or adults showed regressive changes in motor neuron morphology (Cummings & Stelzner, 1988). Some of the changes include: soma surface changes (irregular number of somatic spines), irregular varicose swellings in primary dendrites, and the expanse of the dendritic tree is significantly smaller.

Recovery of hindlimb locomotor function in neonatal rats following SCI has been shown to be due to adaptation in the spinal circuitry caudal to the lesion, rather than the regeneration of axons across the injury site. Yuan and colleagues (2013) examined recovery of locomotor function and regrowth of spinal tracts following a transection in rats on P1, P7, P14, and in adulthood. Rats underwent a spinal transection surgery at thoracic-level 10 (T10) at one of the four ages, and locomotor function was assessed two months following surgery using the BBB (Basso, Beattie, & Bresnahan, 1995) open-field locomotor scale. Compared to rats that received a spinal cord transection at P1 or P7, subjects that received the transection later in life (P14 and adulthood) exhibited plantar placement of their hindpaws, but no weight-bearing support. Rats that received the transection earlier in development (P1 and P7 ages) scored between a range of 8

and 10 on the BBB and were able to move their hindlimbs (to an extent) and stepped with and without weight support in adulthood. The P14 and adult age surgery group rats scored between a range of 0 and 1 and exhibited almost complete paralysis and did not move their hindlimbs. Although the P1 and P7 surgery groups exhibited some functional recovery, they did not exhibit locomotion patterns comparable to intact rats (i.e. consistent plantar stepping, trunk stability, and coordinated gaits). Retrograde labeling with Fluorogold (FG) was used to highlight spinal pathways implicated in locomotion. The brain and spinal cord of each subject was dissected, and neurofilament immunohistochemistry was performed to examine the secondary damage around the lesion site. Rats that received a spinal transection at any of the four ages showed no signs of regeneration of axons across the lesion site. Yuan and colleagues (2013) also performed a re-transection surgery to determine the extent that rats transected on P1 exhibited motor learning. The BBB scores of rats transected on P1 and re-transected two months after the first surgery ranged from 8 – 10, with no significant difference to animals that did not receive the re-transection procedure. This suggests that the locomotor abilities of rats transected on P1 are not attributable to supraspinal input. Rather, this provides evidence that the isolated neural circuitry located caudal to the lesion site (T10) is capable of supporting some degree of locomotion following a neonatal spinal transection.

Experiments using neonatal spinal rats have increased our understanding about motor behavior and function following injury. McEwen and Stehouwer (2001) tested rat pups that received a spinal cord compression (90% or 95% compression groups) or sham injury four days after birth (P4) in one of two age conditions: one day post-operation (POD 1; P5) or eleven days post-operation (POD 11; P15). For behavioral testing, the trunk of the pup was secured, and the pup was suspended to allow free movement of the limbs (i.e. air-stepping paradigm). Subjects

were then administered L-DOPA and motor behavior was recorded. Results showed that POD 1 sham rats primarily stepped with all four limbs, POD 1 rats that received a 90% compression injury exhibited alternating stepping behavior in heterolateral limbs that was uncoordinated, and POD 1 rats that received a 95% compression injury showed mostly alternating forelimb stepping. POD 11 sham rats showed similar locomotor behaviors to POD 1 sham rats, in addition to some extension of the forelimbs in combination with alternating stepping in the hindlimbs. POD 11 rats with a 90% compression injury showed the highest frequency of stepping behavior in all four limbs, and POD 11 rats with a 95% compression injury showed similar locomotor activity to POD 1 rats with 95% compression injury. These results highlight the importance of type/degree of spinal injury, time since injury, and development with respect to locomotion in neonatal rats.

Work by Bradley and Smith (1988) with spinal kittens demonstrated developmental plasticity of the isolated spinal cord and its ability to produce stepping on a moving treadmill belt. Kittens underwent a low-thoracic spinal transection on P1 or P14 and were tested on a moving treadmill belt for two months. During the early phases of testing, exteroceptive stimuli (i.e., a brief, sustained tail pinch) was needed to elicit alternating stepping bouts on the treadmill for both age groups. These bouts were also characterized as brief (less than 5 step cycles) and using the hip joint to initiate movement, while the knee and ankle remained passive. The was no EMG activity in the knee extensor, ankle flexor, or ankle extensor corroborating behavioral observations of these steps in the early phase of injury for both P1 and P14 spinal subjects. However, by 3-4 days post-operation, most kittens in both surgical groups were able to take 5 or more sequential alternating steps on the treadmill. By post-operative day 6, all subjects were taking 5 or more sequential steps with light tail and/or perineal stimulation. EMG activity confirmed activation of the knee extensor, ankle flexor, and ankle extensor in all animals by

post-operative day 6. All kittens required postural assistance during treadmill stepping, especially during the stance (weight-bearing) phase of the step cycle. By the second week, all subjects showed some improvement, but minimal weight-support during the stance phase of the step cycle. Researchers had to assist kittens during the swing phase since paws often did not clear the treadmill belt, as well as prevent interlimb contact during locomotion. However, by the end of the second post-operative month (and late developmental period for kittens), P1 spinalized kittens intermittently showed full weight-bearing steps during the stance phase. Only half of the P14 spinalized kittens showed full weight-bearing during the stance phase. All kittens showed partial weight-bearing by the end of the two-month experiment. Further, vigorous kicking and hyperflexion was often observed after stimulation to the tail and trunk regions, and hyperflexion of the digits prevented full plantar steps during the stance phase. This study suggests that not only is the timing of injury crucial to recovery, but that the development of locomotion may play a role in step cycle duration and limb kinematics. This work showed that alternating step quality is not only affected by a spinal transection, but that sensory stimulation can also induce certain patterns of motor behavior in the absence of supraspinal input.

Unlike plasticity that occurs in the mature nervous system, developmental plasticity emphasizes changes in neural networks (i.e. synaptic pruning, changes in neuron morphology, and synaptic connections) as a consequence of developmental processes and interactions with the environment during sensitive periods of development. Changes in the mechanisms supporting sensorimotor behavior, such as motor neuron morphology, as well as spinal sensory processing, undergo quick modifications during the perinatal period. During this period, the spinal cord increases in size, complexity, synaptic properties and organization, and the firing rate of locomotor-related neurons change (Gramsbergen, 1998; Vinay et al., 2002). Changes in sensory

processing within spinal networks also occur. Growth of afferent neuron terminals and their integration into the dorsal horn of the spinal cord, alteration of cutaneous and reflex thresholds, properties of receptive fields, and neurotransmitter regulation of sensorimotor circuits occur during this period (Baccei & Fitzgerald, 2004; Fitzgerald & Jennings, 1999). Thus, the robust changes that happen during early development are thought to be contributing mechanisms to the infant lesion effect. As previously mentioned, this phenomenon is characterized by an infant's ability to recover significantly more motor function following SCI than adults with the same injury.

By utilizing information provided from research on developmental plasticity of the spinal cord, researchers can inform practitioners and therapists about more promising or appropriate interventions for children with SCI or defect (i.e. spina bifida) and disorders and diseases affecting motor function (i.e. Down syndrome). Approximately 1,455 new cases of pediatric SCI are diagnosed in U.S. hospitals each year (American Spinal Injury Association, 2018) and 1,500 new cases of spina bifida occur in United States pediatric populations every year (Centers for Disease Control and Prevention, 2017). In order for practitioners to create better, more effective interventions for children with SCI or spina bifida, researchers should investigate experimental paradigms of SCI in neonatal animals. Most SCI research uses adult animals. However, neonatal animals exhibit significantly more recovery of function following injury (Murray et al., 2010), and therefore learning how to exploit the developmental plasticity of the spinal cord in pediatric injury/disease is therefore of paramount importance (Dewolf et al., 2021)**.**

Neural Mechanisms of Stepping Behavior

The necessary neural circuitry for producing alternating stepping is found within the spinal cord (Hayes, Chang, & Hochman, 2008; Petruska, Ichiyama, Jindrich, Crown, Tansey,

Roy, Edgerton, & Mendell, 2007; Lev-Tov, Etlin, & Blivis, 2010), and is made up of spinal interneurons which communicate incoming sensory signals to exiting motoneurons. Some conceptualizations of this circuitry model include motoneurons (efferents) and sensory neurons (afferents) in their definition, but not all (Guertin, 2009). However, most researchers agree that the spinal interneurons are necessary in describing the circuitry needed for locomotion. In both intact and spinal animals, the central pattern generators (CPGs) for interlimb coordination of forelimbs and hindlimbs are in the cervical and lumbar spinal cord, respectively. The CPG for locomotion is sensitive to sensory feedback even in the absence of supraspinal input (Hayes, Chang, & Hochman, 2008; Lev-Tov, Etlin, & Blivis, 2010). Sensory input allows for locomotor patterns to adapt to environmental demands, such as changes in the timing of step phases and transitions between stance and swing phases. Hindlimb locomotor rhythms are maximally produced from the thoracic and lumbar regions of the spinal cord (Cowley & Schmidt, 1997; Lev-Tov, Etlin, & Blivis, 2010). Both *in vivo* and *in vitro* experiments have elucidated the effects of lesions, pharmacological substances, and sensory stimulation on spinal locomotor circuitry. Understanding the neural circuitry supporting hindlimb alternating stepping is important in evaluating the behavioral adaptations observed in many treadmill studies.

Most of our understanding of the locomotor CPG and its cellular, neurophysiological, and response to pharmacological agents has come from *in vitro* research using the neonatal, rodent spinal cord. There are a few advantages to using a neonatal spinal cord preparation compared to an adult (fully developed) cord (Clarac et al., 2004). Although the neonatal cord is significantly smaller in size, the anatomy of structures, nuclei, and pathways remains somewhat comparable to the adult. The size of the cord (dependent on age) also allows for easy perfusion in the preparation. Further, dissection of the spinal cord is much simpler in neonates due to less

calcification of vertebrae around the cord. Thus, the time from euthanasia to experimentation is much shorter compared to using adult models. Another hallmark of the neonatal spinal cord is the ability to keep the cord alive longer in the *in vitro* set-up, since it is significantly less myelinated. Adult preparations require much more sophisticated systems of circulation in the bath due to myelination, but the neonatal cord can be kept alive for hours in an adequate bath system. The contact between neurons and modes of stimulation (i.e., drug or electrical) is direct, as opposed to an adult spinal cord which can be blocked by myelin and meningeal barriers. For example, in adult preparations, levodopa (L-DOPA) must be used since dopamine cannot cross the blood-brain barrier. In contrast, dopamine can be used in the neonatal preparation, eliminating the need for precursors to certain neuroactive substances. Since the neonatal cord can be used to study ion channels and ionic manipulations, past work has provided insight into the switch of gamma-Aminobutyric acid (GABA) and glycine from having excitatory to inhibitory effects during the perinatal period, which is important for the development of the alternating activity pattern recorded using *in vitro* preparations (Nakayama, Nishimaru, & Kudo, 2002).

In vitro studies of the isolated spinal cord often record electrical activity from the ventral roots, hindlimb nerves, or motoneurons. Many experiments investigating the spinal CPG supporting locomotion use pharmacological stimulation to activate rhythmic activity in the *in vitro* isolated spinal cord. The alternating rhythmic bursts of activity recorded from the isolated spinal cord are known as fictive locomotion. Fictive locomotion is characterized by both inter- and intra-rhythm activity: rising and falling patterns within a recording from a single side of the cord and left-right alternating rhythms between both sides of the cord. These neural rhythms occur in the absence of motor behavior and supraspinal input, but can be altered or disrupted via changes in sensory stimulation (i.e., electrical stimulation), pharmacological agents, and lesions.

Work from Cazalets and colleagues (1995) used a bath application of NMDA to induce fictive locomotion in the lumbar cord of newborn rats, but later experiments showed that adding serotonin (5-hydroxytryptamine; 5-HT) refines the left-right and flexor-extensor activity (Pearlstein et al., 2005). For their experiments, Pearlstein and colleagues removed the brainstem, spinal cord, and lumbar ventral roots from P0-P4 rats, and the preparations were perfused with a 95%O2/5%CO2 saline solution. All preparations received an application of NMDA to elicit fictive locomotion, and recordings were performed from the ventral roots 10-min after drug application. Then, serotonergic drugs were added to the bath solution: 5-HT, DOI (a 5-HT2_A/2_C agonist), SB269970 (a 5-HT_7 antagonist), or ketansarin (a 5-HT_2 antagonist). Results showed that NMDA application evoked an alternating fictive locomotion pattern from the L3 and L5 ventral roots, but the addition of 5-HT significantly increased the cycle period. However, the addition of 5-HT had no effect on burst pattern amplitude. Both left-right alternation and L3/L5 alternation (the classic fictive locomotion pattern) was significantly greater when using NMDA + 5-HT compared to NMDA alone. Further, the addition of DOI induced an increase in left-right and L3/L5 alternation, but it was not statistically significant. This shows that while 5-HT receptor activation can be used to refine fictive locomotion produced by NMDA, the amount of change is receptor-specific. Since increasing the NMDA concentrations alone also produced increased alternating activity, it was important to test the effects of endogenous 5-HT in conjunction with the NMDA activity pattern. Subjects pre-exposed to PCPA (a 5-HT synthesis inhibitor) produced disorganized and irregular patterns at all lumbar levels with burst overlaps. In preparations not pre-treated with PCPA, the addition of the 5-HT antagonists (SB269970 and ketanserin) induced a disorganized pattern significantly different from NMDA controls but did

not eliminate the rhythm. The use of PCPA and 5-HT antagonists in this context revealed that 5-HT is important in fine-tuning locomotor activity in the spinal cord.

Kjaerulff and Kiehn (1996) showed that the vital components of the hindlimb locomotor CPG are likely to be located medially within the spinal cord, rather than laterally, using the *in vitro* neonatal rat spinal cord preparation. The lower thoracic and complete lumbar sections of the spinal cord were dissected from euthanized rats on P0-P2, and ventral root recordings were made following bath application of NMDA and 5-HT after a series of lesions. Their work showed that pathways along the entire rostrocaudal axis in the caudal thoracic-lumbar cord are implicated in left-right alternating rhythms in neonatal rats.

Stimulation of sacrocaudal afferents can also produce left-right alternating rhythms in both the lumbar and sacral regions of the spinal cord (Lev-Tov, Delvolve, & Kremer, 2000). Neonatal rat cords (P3-P8) were isolated from T6 and below, with or without the intact tail. Activity was recorded from the ventral roots in the L2, S2, and S3 regions of the spinal cord. Following a brief tail-pinch at the base of the tail, alternating left-right and alternating flexor-extensor rhythms were recorded from both the L2 and S2 regions. This activity was not significantly different than activity produced by direct electrical stimulation of S4 dorsal roots in control preparations (no tail). Even after a midsagittal lesion of the thoracolumbar cord, the sacral rhythms were not perturbed, with no significant difference in rhythm onset. This study showed that the CPG supporting hindlimb locomotion is sensitive to sensory perturbations, such as a tail pinch or electrostimulation, and the pathways and mechanisms involved in sensory activation of rhythmic locomotor activity are located within spinal cord. Evidence that locomotor CPGs can be activated via sensory stimulation in the absence of the brain or descending control

shows the relative independence of the spinal circuitry, as well as suggests the importance of sensory feedback in inducing and modulating *in vivo* locomotion.

In a different preparation, Hayes, Chang, and Hochman (2008) studied the *in vitro* isolated spinal cord, pelvis, and hindlimbs of P1-P4 neonatal rats. The dissected portion of the subject (spinal cord and hindlimbs) was placed in an oxygenated, artificial cerebrospinal fluid (aCSF) bath solution chamber. Then, the preparation was secured over a treadmill belt (hindpaws made plantar contact with the belt) in the chamber to allow sufficient perfusion of the cord and hindquarters. To induce alternating stepping, NMDA and 5-HT was added to the bath. Results from this study showed that the neonatal *in vitro* spinal cord-hindlimb attached preparation produced similar kinematic data to that of adult *in vivo* preparations from previous studies. For example, when the speed of the treadmill belt speed was increased (i.e. change in sensory feedback), stride frequency significantly increased. Although behavioral experiments typically use *in vivo* preparations of the whole animal, this study showed that an *in vitro* preparation of the neonatal rat spinal cord can produce adaptations to sensory feedback, as well as mimic the locomotor behavior of adults. The results also show the importance of the spinal circuitry in evoking and modulating locomotion-like stepping and lend basis to the current study.

Chapter II: Current Study

The current study examined the behavioral potential of the developing spinal cord and its ability to respond to environmental input. On P1, rats received a complete low-thoracic spinal cord transection or sham operation. On P5, subjects were behaviorally tested on a treadmill, and hindlimb stepping was later scored and analyzed. The treadmill speed was set to a slow, medium, fast, or non-moving (control) speed. During treadmill testing, there was a 5-minute baseline period. After baseline, subjects were injected with quipazine, which induced alternating stepping, and behavioral recording continued for 30 minutes. Following testing, rats were euthanized and preserved in formalin for later dissection.

By eliminating supraspinal input to the lower spinal cord, we were able to examine the capabilities of the isolated spinal cord *in vivo*. Previously, research from our lab has shown that intact neonatal rats are capable of modulating their stepping behavior in response to a moving treadmill belt, when stepping is induced with quipazine (Bozeman et al., in preparation). However, the current experiment expanded on the previous study by using spinal-transected subjects which mimicked the "infant lesion effect" in an experimental paradigm. Further, this experiment dovetails with previous treadmill studies conducted in spinal injured adult animals and *in vitro* work previously discussed. Overall, this project provides insights into the role of the spinal cord in stepping, as well as its neuroplasticity during development and following injury.

Behavior Hypotheses: Effects of Treadmill Speed on Hindlimb Step Quantity

It was hypothesized that rat pups that received a complete spinal cord transection would show hindlimb stepping adaptations to different treadmill belt speeds. Previous research has shown that neonatal rats that have received a spinal cord transection are capable of altering motor behavior in response to changes in sensory stimuli (Strain et al., 2014; Swann, Kauer,

Allmond, & Brumley, 2017). Other research shows that intact neonatal rats treated with quipazine are capable of modulating their stepping behavior in response to differing treadmill belt speeds (Bozeman et al., in preparation). Specifically, we expected to see more stepping on faster treadmill belt speeds, which aligns with results from previous research (Cha et al., 2007; Bozeman et al., in preparation). For example, we expected that rat pups tested on a fast belt speed would express higher frequencies of hindlimb stepping compared to that of rats tested on medium, slow, or non-moving (control) belt speeds. Additionally, we expected to observe more hindlimb steps in spinal subjects compared to shams since previous research has shown that spinal-transected perinatal rats demonstrate hindlimb supersensitivity to quipazine (Brumley & Robinson, 2005; Strain et al., 2014). Furthermore, we expected that spinal subjects would show a higher a percentage of alternating steps across the test session compared to shams, meaning that more of their limb movements would be involved with stepping compared to other kinds of limb movements.

Kinematic Hypotheses: Effects of Treadmill Speed on Hindlimb Step Quality

We expected that spinal subjects would show adaptations to different treadmill speeds due to the isolated spinal cord's ability to respond to sensory feedback (i.e., treadmill stimulation). Thus, it was hypothesized that both spinal and sham subjects would show differences in step quality (i.e., kinematics) across the four treadmill belt speed groups, but show no difference as a function of surgery condition. Specifically, we expected to observe shorter step cycle durations on faster treadmill belt speeds, consistent with previous research (Leblond et al., 2003; Bozeman et al., in preparation). We also expected the changes in step cycle duration to result from changes in duration of the stance phase rather than changes in duration of the swing phase for both spinal and sham subjects. Additionally, we expected to see no difference in step

area between spinal and sham subjects, since the isolated spinal cord should be capable of maintaining step quality similarly to intact animals.

Chapter III: Methods and Materials

Subjects

Subjects were 48 male offspring of Sprague-Dawley rats that were mated at ISU. Pregnant females were housed in pairs until a few days before birth, at which point they were housed individually. On P1 (~24 hours after birth), pups received a low thoracic spinal cord transection (T8-T10) or sham operation. Following surgery, litters were culled to 6-8 pups, and then returned to the homecage with the dam until the day of testing. Pups were tested on P5. While housed, animals received food and water ad libitum and were kept on a 12-h light:dark cycle. Animals were maintained in compliance with guidelines for animal care and use established by the National Institutes of Health, Institutes of Laboratory Animal Resources, and Institutional Animal Care and Use Committee at Idaho State University.

Experimental Design

In the current study, a total of 48 male rat pups underwent surgery on P1 and were tested on a treadmill at P5. Half of the subjects received a spinal cord transection (n=24), and the other half received a sham operation (n=24). On P5, all subjects received an injection of quipazine and were divided into four treadmill belt speed groups: slow, medium, fast, and non-moving (control). There was a total of six subjects per treadmill speed group and surgery condition, as illustrated in Figure 1. In order to avoid litter effects, no more than one pup per litter was assigned to each group. Additionally, only males were tested to avoid sex effects.

Immediately prior to testing on P5, pups were manually voided via perineum stimulation, weighed, and placed in an infant incubator (35º C and 40% humidity) for an acclimation period (30 minutes). After acclimation to incubator conditions, subjects were gently secured in the prone posture to a horizontal bar with a rubber surface, using hair tape to secure the head and

upper-torso, but allowing the pelvis and hindlimbs to move freely. The bar was positioned just above a miniature treadmill, until subjects' hind paws could make full plantar contact with the treadmill belt. Security to the bar ensured the subject remained in the center of the treadmill without slipping or moving off (Bozeman et al., in preparation). A small ruler was attached to the horizontal bar for calibration during later kinematic analyses. Another bar held a Plexiglas plate which was placed under the forelimbs of the subject, leaving only the hindlimbs touching the treadmill belt. Figure 2 shows an illustration of a rat pup during the test period and a timeline for the experiment. Pups were recorded from a sagittal view with both hindlimbs in clear view for a 5-minute baseline and 30-minute test session on the treadmill. For the baseline, the treadmill belt was moving or non-moving (per belt speed group) and was meant to provide an acclimation to test conditions (i.e., treadmill and Plexiglas) for the subject. It was turned off during drug (quipazine) administration and turned back on once the pup was placed back on the treadmill belt for the 30-minute test session.

Surgical Techniques and Post-Operative Care

Neonatal rat subjects underwent spinal surgery on P1. All subjects had a milk band present across the abdomen suggesting that they had recently fed. Immediately before surgery, subjects were manually voided and then anesthetized via hypothermia. The spinal transection technique used was the same as Strain and colleagues (Strain et al., 2014) and occurred under aseptic conditions. A partial laminectomy exposed the thoracic spinal cord from T8 to T10. Using iridectomy scissors, the cord was severed completely via a single cut between the levels of T8 and T10, and a collagen matrix was injected into the site for subjects that received a spinal cord transection. Back muscles and skin were then sutured. Subjects that received a sham

operation underwent all procedures listed above, except the physical spinal cord transection was not performed and no collagen matrix was injected.

After surgery, all subjects were given a 50 µl subcutaneous injection of Buprenex (0.1 ml of 0.04 mg/kg solution) and 0.9% (wt/vol) saline. Subjects were placed in an infant incubator maintained at 35º C alongside littermates and bedding from the home cage. Once subjects fully recovered from anesthesia, they were returned to the dam in their home cage. Subjects were checked regularly until P5 to ensure they were properly gaining weight and sutures were intact. Until the day of testing (P5), subjects remained with the dam. Litters were not mixed (i.e., all pups received spinal transection or sham operation) to ensure the dam would equally care for her pups. From P2-P5, subjects were checked daily to ensure there were no surgical complications or infections, and subcutaneous injections of saline were given if needed to help with proper weight gain and hydration.

Quipazine Administration

On P5, after being placed on the bar and suspended above the treadmill for a 5-min baseline, subjects were given a 75 µl intraperitoneal injection of quipazine maleate (3.0 mg/kg) using a 30-guage needle to induce alternating stepping. The drug was obtained from Sigma-Aldrich (St. Louis, MO). Quipazine is a selective 5-HT_2 receptor agonist that is often used to induce alternating stepping in neonatal rats (Strain & Brumley, 2014). At this dose, neonatal rats exhibit alternating air-stepping behavior, patterns of locomotion, and posture for longer than 30 minutes (Swann et al., 2016).

Treadmill

The miniature treadmill used for behavioral testing was custom-made, with a belt that was 9.5 cm long by 4.5 cm wide. A latex dental dam was used for the belt surface. The treadmill

was connected to a computer via USB and controlled by a custom-made software program. Treadmill speeds were determined by using belt speeds from a previous experiment conducted in our lab (Bozeman et al., in preparation). Because the previous experiment tested rats on P1, the treadmill belt speed rate needed to be increased in the current study to accommodate subjects' increased abilities in motor function. The fast belt speed from the previous experiment was used as the medium belt speed in the current experiment. This speed was then increased by 50% to create the fast belt speed and decreased by 50% to create the slow belt speed for the current experiment. Treadmill belt speeds were as follows: slow speed (1.6 cm/s), medium speed (3.2 cm/s), fast speed (4.8 cm/s), and non-moving (control) speed. After securing the pup to the bar, the treadmill was turned on for a 5-minute baseline. Following baseline, the treadmill was turned off, and subjects received an injection of quipazine. The treadmill was then turned back on for the 30-minute test session (see Figure 2B).

Euthanasia and Histology

Immediately following testing, subjects were euthanized via carbon dioxide inhalation. Next, subjects had the incision site on their back reopened and were placed in formalin (10% wt/vol) for later dissection. Dissections revealed that all spinal cord transections were complete between the T8-T10 levels.

Behavioral Scoring and Kinematic Analyses

All test sessions were recorded onto DVD. Scoring of stepping behavior during the 30-min post-injection period occurred during DVD playback using the software program JWatcher (Version 1.0), which records the time of entry (± 0.01 s). One person (ALB) completed the scoring, after achieving an intra- and inter-reliability rate (when compared to a standard file) of ≥ 95%. All videos and DVDs were labeled with the experiment name, subject's identification (ID),

and date of testing. No information was included about the surgery condition or treadmill belt speed to ensure blind scoring. Only hindlimb activity was scored and analyzed because we were interested in comparing treadmill stepping supported by the isolated (below lesion level) versus intact spinal cord. The following hindlimb behaviors were scored or calculated: alternating step frequency, total hindlimb movements (including unilateral steps and toe drags), percentage of alternating steps, step cycle duration, stance phase duration, swing phase duration, and step area.

Both hindlimbs were observed for scoring alternating step frequency and hindlimb movements. An alternating step was scored when a bilateral alternating pattern of flexion and extension of the hindlimbs occurred (Strain & Brumley, 2014). Unilateral stepping was scored when either of the hindlimbs showed a pattern of flexion and extension without the other hindlimb alternating in cycle. Toe drags were scored when the toes passively dragged along the treadmill belt. The percent of alternating steps during the test session was calculated by dividing the alternating step frequency by total hindlimb movements, multiplied by two (for the two hindlimbs), and then converted to a percentage.

For kinematic analyses, alternating step frequency was divided into six 5-min time bins, and then the first two to five consecutive alternating steps per time bin were selected for further limb kinematic analyses (Swann et al., 2016; Bozeman et al., in preparation). If there were not enough consecutive alternating steps per time bin, kinematic analyses were not performed for that subject at that time bin. Using the two to five consecutive steps identified from the initial scoring pass, step cycle duration, stance phase duration, swing phase duration, and step area was measured or calculated for the left hindlimb (Bozeman et al., in preparation). These measurements were made manually using frame-by-frame analysis of the consecutive steps. Swing duration began when the hind paw lifted from the treadmill belt and ended just before the

paw made contact again with the belt. Stance duration began when the hind paw made contact with the treadmill belt and ended before the paw lifted from the belt to begin the swing phase. Step cycle duration was the combined times of swing phase and step phase durations. Step area was calculated by determining the highest and lowest point the limb reached (height) and the farthest forward and back point the limb reached (length) and then multiplying those two values (Cha et al., 2007). A small ruler on the bar was used for calibration.

Statistical Analyses

A repeated-measures ANOVA was used for behavioral analyses. Surgery condition and treadmill belt speed were between-subjects variables, with time (six 5-min time bins) as the within-subjects variable. Alternating step frequency, total hindlimb movements, and percent of alternating steps were the dependent variables. For kinematic analyses, a mixed repeated measures model was used since some subjects did not show alternating steps in some time bins (unequal n sizes). Step area (step length and step height), stance phase duration, swing phase duration, and step cycle duration were the dependent variables in these analyses. Fisher's Least Significance Difference test was used for all post-hoc analyses. The significance level was $p \leq 0.05$. All statistical analyses were completed in IBM SPSS Version 27. Graphs show group means ± SEM.

Chapter 4: Results

Behavior Results: Step Quantity

Hindlimb Alternating Stepping. There was no three-way interaction of surgery condition, belt speed, and time, $F(15, 200) = .655$, $p = .826$. However, we found a two-way interaction of time and surgery condition $F(5, 200) = 9.99$, $p < .001$, as shown in Figure 3. Spinal subjects showed significantly more alternating hindlimb steps across each time bin of the test session compared to sham subjects. There was a main effect of time, $F(5, 200) = 6.62$, $p < .001$, and a main effect of surgery condition, $F(1, 40) = 16.24$, $p < 001$. Spinal subjects showed significantly more alternating steps than sham subjects.

Percentage of Hindlimb Alternating Steps. In order to calculate percent hindlimb steps, the number of hindlimb movements was analyzed first. There was no three-way interaction of time, surgery condition, and treadmill belt speed, $F(5, 200) = .77$, $p = .711$. There was a significant interaction between time and surgery condition, $F(5, 200) = 7.94$, $p < .001$ (Figure 4), but no interaction between time and belt speed condition, $F(15, 200) = .46$, $p = .96$. There was a main effect of surgery. Spinal subjects showed significantly more hindlimb movements across the test session at each time bin (alternating steps, unilateral steps, and drags) compared to sham subjects, $F(1, 40) = 20.69$, $p < .001$. There was a significant main effect of time, $F(5, 200) = 5.2$, $p < .001$, but there was no significant main effect of treadmill belt speed, $F(3, 40) = .2.16$, $p = .11$

For percent hindlimb steps, there was a significant three-way interaction of time, surgery condition, and belt speed condition ($F(15, 200) = 4.07$, $p < .001$), a significant two-way interaction of surgery condition and time ($F(5, 200) = 6.48$, $p < .001$), and a significant main effect of time ($F(5, 200) = 4.77$, $p < .001$). There was not a main effect of surgery condition ($F(1, 40) = 1.15$, $p = .29$) or treadmill belt speed ($F(3, 40) = .57$, $p = .638$). To analyze the three-

way interaction, we examined the effect of treadmill belt speed across time for each surgery condition separately (see Figure 5A and 5B). For spinal subjects, there were no differences in the percentage of alternating hindlimb steps among belt speeds or across the test session. As can be seen in Figure 5A, spinal subjects in the medium speed condition showed the highest percentage of steps across the test period, but this was not significant. However, for sham subjects there was a significant effect of time and belt speed. During the second time bin, sham subjects on the control speed had significantly lower percentages of alternating steps compared to the fast speed ($p = .03$). Shams showed a more consistent higher percentage of steps across the test session on the faster belt (Figure 5B), but again this was not significant compared to other speeds. Because there was only one significant effect for time and belt speed when split by surgery condition, we also show here the percentage of hindlimb steps for surgery condition collapsed across speed. As can be seen in Figure 5C, spinal subjects showed a significantly higher percentage of hindlimb steps across the test session compared to shams.

Kinematic Results: Step Quality

Hindlimb Step Cycle Duration. For step cycle duration (stance + swing phase durations), there was a significant interaction effect of surgery condition and belt speed, $F(3, 174.3) = 5.32$, $p = .002$ (note: non-integer degrees of freedom are due to the type of analysis used). Spinal subjects showed a significantly longer step cycle duration on the control (n=6) non-moving treadmill belt compared to the slow (n=6), medium (n=6), and fast (n=5) moving belt speeds, as can be seen in Figure 6A. In a bit of a reversal, sham subjects showed a significantly shorter step cycle duration on the control (n=6) belt compared to both medium (n=6) and fast (n=6) belt speeds, as shown in Figure 6B. There were no other significant interaction or main effects for step cycle duration.

Hindlimb Stance Phase Duration. As shown in Figure 7, there was a significant interaction effect of surgery condition and time, $F(5, 58.78) = 2.61$ $p = .034$. Spinal subjects showed no significant differences in stance phase duration across the test session. Sham subjects showed a significantly shorter stance phase duration during the first-time bin compared to the fifth and sixth time bins. There were no significant main effects of surgery, belt speed, or time for stance phase duration.

Hindlimb Swing Phase Duration. There was a significant interaction of surgery and belt speed, $F(3, 164.16) = 11.21$, $p < .001$. Spinal subjects showed significantly longer swing phase durations on the control non-moving belt compared to the slow, medium, and fast moving belt speeds (see Figure 8A). Sham subjects showed significantly longer swing phase durations on the control belt speed compared to slow and medium speeds. Furthermore, shams showed significantly longer swing durations on the fast treadmill belt speed compared to the control speed (see Figure 8B).

Additionally, there was a significant main effect of belt speed, $F(3, 164.16) = 3.65$, $p = .014$, but not a main effect of surgery or time. Subjects in the control belt speed condition had significantly longer swing phase durations compared to the slow and medium speeds. The difference between the control and fast speeds approached significance.

Hindlimb Step Area. For step area, there were no significant interaction effects. However, there was a significant main effect of treadmill belt speed, $F(5, 185.3) = 3.27$, $p = .022$. As can be seen in Figure 9, subjects tested on the control non-moving belt had significantly larger step areas compared to subjects in the slow and fast belt speeds. There was no main effect of surgery or time.

Because step area is a factor of both step length and step height, we decided to examine each of these separately as well. For step length, there were no significant interaction effects. However, there was a significant main effect of surgery, $F(1, 186.5) = 17.7$, $p < .001$. Spinal subjects showed significantly longer step lengths than sham subjects, as can be seen in Figure 10A. Additionally, there was a main effect of time, $F(5, 66.7) = 2.55$, $p = .036$. Subjects had significantly shorter length steps in the first 5-min time bin compared to the third, fourth, fifth, and sixth time bins (see Figure 10B). There was no main effect of treadmill belt speed condition on step length.

For step height, there was no three-way interaction of surgery condition, belt speed, and time, $F(15, 69.54) = .39$, $p = .977$. There was a significant two-way interaction of surgery and speed condition, $F(3, 164.57) = 6.1$, $p = .001$, as can be seen in Figure 11. On the fast belt speed, spinal subjects showed significantly shorter step heights compared to sham subjects, $F(1, 57) = 10.78$, $p = .002$ (Figure 11A). Spinal subjects also showed significantly shorter step heights on the fast belt speed compared to the control and medium belt speeds, and significantly shorter step heights on the slow belt speed compared to the medium belt speed (Figure 11B). Sham subjects showed significantly shorter step heights in the slow condition compared to the control and fast speed conditions (Figure 11C). Additionally, there was a significant main effect of belt speed, $F(3, 164.57) = 5.07$, $p = .002$ (see Figure 11D). Subjects' steps were significantly higher in the control condition compared to the slow and fast speed conditions. Subjects' steps were significantly shorter in the slow speed condition compared to the medium speed condition. There was no main effect of surgery or time on step height.

Chapter 5: Discussion

Experimental treadmill training paradigms are most often used as a manipulation (i.e., activity-based training before other behavioral testing) or to examine if animals can step following injury, rather than to assess real-time changes in stepping behavior. Some studies have employed different treadmill belt speeds to help induce stepping in conjunction with other stimuli (i.e., mechanical, epidural, or tactile) in adult animals (LeBlond et al., 2003; Cha et al., 2007). However, this is the first study to examine hindlimb stepping on different belt speeds in the immature rat. Findings from the current experiment indicate that both spinal-transected and sham rats treated with a 5-HT_{2A} receptor agonist (quipazine) demonstrated alternating hindlimb stepping on the treadmill. Both spinal and sham subjects showed changes in step quality, or limb kinematics, that was sometimes belt speed-dependent. Taken together, our results indicate that the developing spinal cord can produce and maintain interlimb coordination (step quantity) and alter intralimb coordination (step quality) in the absence of supraspinal control on a moving treadmill belt.

Hindlimb Stepping

We found that spinal-transected P5 rats showed significantly more hindlimb alternating steps compared to sham rats, and that spinal rats had higher percentages of alternating steps across the test session compared to shams. However, there were no consistent differences in the frequency or percentage of alternating steps across the treadmill belt speed groups for both spinal and sham subjects. It is well known that quipazine administration produces alternating hindlimb stepping in neonatal and adult animals, as well as intact and spinal injured animals (Fong et al., 2005; McEwen, Van Hartesveldt, & Stehouwer, 1997). Thus, it is likely that the results on hindlimb stepping that are reported in the current study are due to the robust effects of quipazine,

particularly in spinal-transected subjects. Furthermore, it may be the case that because quipazine induces alternating stepping so well, the effects of the drug overpowered sensory input from the treadmill such that the differing belt speeds did not provide strong enough stimulation to affect interlimb stepping.

Spinal animals, including immature rats, exhibit hindlimb supersensitivity to quipazine, meaning that the drug usually produces much stronger effects (i.e., more alternating stepping) in spinal animals compared to intact animals (e.g., Fong et al., 2005; Strain et al., 2014). Following a spinal cord transection, serotonin receptors (specifically 5-HT_{2A} and 5-HT_{2C}) are upregulated in motoneurons of the spinal cord by approximately threefold (Ganzer et al., 2018; Fabbiana et al., 2018; Kong et al., 2010). This receptor upregulation is apparent within 24 hr following spinal transection (Kong et al., 2011), and is thought to be responsible for the robust behavioral response following administration of quipazine in spinal animals compared to shams. Given the upregulation of serotonin receptors that likely occurred in spinal-transected rats in the current study, and the reported effect of quipazine in spinal neonatal rats (Strain et al., 2014), it is possible that quipazine may have maximally activated alternating hindlimb stepping. In doing so, it may have masked any sensory input (the moving treadmill belt) effects on interlimb coordination, or it may be possible that subjects were trying to maintain a certain level of motor activity regardless of sensory input.

In a previous study by Strain and colleagues (Strain et al., 2014), P10 rats with an early spinal transection showed significantly more alternating hindlimb steps compared to shams when administered quipazine, similar to the results of the current study. Additionally, spinal subjects in the range-of-motion (ROM) restriction condition (a form of sensory stimulation) showed lower frequencies of alternating stepping compared to spinal subjects that did not receive ROM

restriction, but these effects were not persistent. However, sensory stimulation (ROM restriction) effects were most pronounced when examining intralimb coordination, similar to the results of the current study. Here, rather than the frequency of alternating hindlimb stepping being affected by treadmill belt speed, the effect of sensory input was illuminated in the kinematic results of the current experiment, but at an even younger age than reported by Strain and colleagues (2014). Taken together, this suggests that serotonin system activation was important to induce and maintain interlimb coordination in the current study, while sensory input appeared to more so influence intralimb coordination.

Intralimb Coordination during Hindlimb Stepping

In the current experiment, spinal P5 rats showed a significant decrease in hindlimb step cycle duration as the treadmill belt speed increased, consistent with forelimb stepping in Bozeman et al. (in preparation) and hindlimb stepping in the intact adult mouse (Leblond et al., 2003). This means that on faster treadmill belts, the hindlimbs of spinal-transected rats were making stepping motions more quickly. On the other hand, sham animals showed a significant increase in step cycle duration as the treadmill belt increased. This finding is novel and non-intuitive (i.e., slower steps on a faster treadmill belt). Further examination of the kinematic aspects of the stepping motion helps to reveal more clearly how the limbs were adapting to the treadmill speeds across experimental conditions.

Previous research in intact adult mice, adult and developing kittens, and humans has shown that step cycle durations change as a function of stance phase duration (i.e., shorter step cycles are due to shorter stance phases, with no changes in swing phase) when increasing treadmill belt speed (LeBlond et al., 2003; Yang et al., 2005; Bradley & Smith, 1988). The stance phase of the step cycle is when the foot is on the ground, and thus typically what is seen at

faster walking speeds is a reduction in time that the foot is in contact with the ground in the stance phase. However, this was not demonstrated in spinal adult mice (Leblond et al., 2003). Interestingly, in the current study, the change in step cycle duration was found to result from changes in the swing phase of the step cycle (when the foot is in the air swinging forward before making ground contact), not the stance phase as originally hypothesized and shown in some other studies (Leblond, et al., 2003; Yang et al., 2005; Bozeman et al., in preparation). At faster treadmill belt speeds, spinal P5 rats showed significantly shorter swing phase durations, while sham rats had significantly longer swing phase durations. Additionally, stance phase durations did not differ significantly for spinal subjects across the four belt speed conditions, but there were differences in stance phase durations for shams across the test session (but no difference dependent on belt speed).

Regardless of surgery condition, all subjects showed smaller step areas as the treadmill belt speed increased, except for the medium belt speed condition. Thus, the effect was not perfectly linear. It is possible that the medium belt speed is the "average" hindlimb stepping speed for P5 pups. This means the slow speed is slower than average for hindlimb stepping at this age, resulting in pups having larger step areas compared to the medium, or average, speed. Furthermore, the fast speed is faster than average, so pups showed smaller step areas on this speed. This may also be why step height was non-significant between the control and medium belt speeds. Step height did not differ between the medium and control conditions for all subjects, regardless of surgery condition. If the "normal" speed of alternating hindlimb stepping is approximately the speed of the medium belt, then there should be no difference between the non-moving belt condition (as seen in some kinematic data), as seen in step area measures.

These findings are novel and have interesting implications for motor development and recovery following an early neonatal spinal transection. At P5, rats have little experience using their hindlimbs for locomotion or weight-bearing stepping (Altman & Sudarshan, 1975; Swann & Brumley, 2019; Theodossiou et al., 2019). Between P1 and P5, locomotion in rats mainly involves the forelimbs, such as pivoting and crawling (Altman & Sudarshan, 1975; Swann & Brumley, 2019). Therefore, placing the hindpaws of P5 rats on a moving treadmill belt may have been the first time they attempted to engage in stepping with the hindlimbs only. This leads to the suggestion then that perhaps experience with weight-bearing movement may be critical for animals to learn how to alter aspects of the step cycle in a mature fashion. Although stepping can be produced at an early age in rats (Bekoff & Lau, 1980; Brumley & Robinson, 2005), it may be necessary to experience actual weight bearing on the limbs for animals to distinguish between weight-bearing (stance) and non-weight bearing (swing) phases of the step cycle. This interpretation is consistent with previous research in spinal kittens (Bradley & Smith, 1988) that showed that once postural support and hindlimb weight-bearing was achieved, improvements in alternating stepping on the treadmill occurred. Additionally, this is consistent with previous work in adult, spinal rats showing that altering weight-support while on a treadmill affects the frequency of weight-bearing steps produced (de Leon, See, & Chow, 2011). Further, a study with rats spinalized as neonates showed that later recovery of hindlimb stepping was significantly improved when treadmill training was enhanced with robotic loading of the hindlimbs during the stance phase of the step cycle, further suggesting the importance of weight-bearing on the hindlimbs in facilitating behavioral recovery of locomotion (See & de Leon, 2013).

For step area, both spinal and sham animals showed smaller step areas on the slow and fast treadmill belt speeds compared to the control speed. The step areas on the medium speed did

not differ from the control speed. There was no difference in step area between spinal and sham rats, suggesting that spinal animals have similar step quality (intralimb coordination) compared to sham (intact) animals. To better understand how step area changed as a function of surgery condition and treadmill belt speed, we evaluated step length and step height separately.

We found that spinal subjects showed significantly longer steps compared to shams regardless of treadmill belt speed, which is consistent with previous work in adult mice (Leblond et al., 2003). Moreover, spinal subjects in the current experiment showed an increase in step length as the test session progressed. Given that the time-dependent changes in step length are consistent with the time course of quipazine effects on hindlimb stepping in neonatal rats (Swann et al., 2016), and that belt speed did not affect step length, it may be the case that the longer hindlimb steps in spinal subjects were due to the strong effect of quipazine in spinal animals, and therefore not variations in sensory stimulation.

Both surgery condition and treadmill belt speed influenced hindlimb step height. Spinal rats showed significantly shorter step heights compared to shams on the fast treadmill belt speed, showed the tallest steps on the control and medium belt speeds, and showed the shortest steps on the slow and fast speeds. Conversely, sham rats showed the tallest steps on the control and fast speeds, and the shortest steps on the medium and slow belt speeds. Previous research showed a linear pattern of step height in spinal, neonatal kittens: as the belt speed increased, step heights decreased (Bradley & Smith, 1988). While spinal subjects in the present study showed shorter step heights on the fast belt speed, this effect was not perfectly linear. Again, this may be due to P5 rats having little experience with hindlimb weight-bearing activity (Theodossiou et al., 2019), and thus little experience clearing obstacles with the hindlimbs during locomotion.

Although both spinal and sham rats demonstrated the ability to regulate hindlimb step length and height, our results suggest that spinal subjects show a different quality of steps (Swann et al., 2017). While the steps may look different compared to sham controls, spinal subjects have seemingly adapted their alternating stepping by using different strategies (i.e., changes in swing phase and step length). This suggests that while a spinal cord transection causes the quality of steps to look atypical, it does not rule out the ability of the developing spinal cord to adapt in other ways following injury to produce rhythmic motor coordination in the presence of pharmacological and sensory stimulation.

Treadmill Stepping in Neonatal, Spinal Rats

In the current experiment, we set out to examine the developing, isolated spinal cord *in vivo* and better understand its ability to produce alternating stepping adaptations on a moving treadmill belt. In summary, we showed that following administration of quipazine, neonatal spinal rats can step on a treadmill (a form of interlimb coordination) and adapt their step quality (intralimb coordination) differently than intact and adult animals (Bradley & Smith, 1988; LeBlond et al., 2003; Bozeman et al., in preparation). Even at such a young age, P5 rats can produce more mature patterns of locomotion, as well as alter the quality of their steps in response to sensory stimulation. However, given previous research on the development of locomotion in rats, it is possible that experience with weight-bearing locomotion is important in how rat pups (both intact and spinal) respond to a moving treadmill belt and change their stepping behavior. The results from this experiment expand the current understanding of the developing spinal cord's ability to produce and adapt alternating stepping in the absence of supraspinal input.

Limitations and Future Directions

The current study has a few limitations. As previously mentioned, experience with weight-bearing locomotion may be important for initiating stepping on a treadmill and adapting to different belt speeds. Some forms of weight-bearing hindimb locomotion are established in rats by postnatal day 10 without the use of serotonergic drugs (Swann & Brumley, 2019; Theodossiou et al., 2019), and pilot data from our lab has shown that P10 spinal rats can step on a moving treadmill belt without additional stimulation. Therefore, it is possible that testing spinal rats at a later age without administering quipazine would be a better test for evaluating sensory effects on stepping using a treadmill paradigm. Future directions could explore the parameters of treadmill stepping in developing rats without the use of quipazine by testing subjects at an age when some weight-bearing locomotion has been established.

Because neonatal rats often need stimulation to evoke stepping, we used a 3.0 mg/kg dose of quipazine for our experiment. This dose produces robust effects on alternating hindlimb stepping, which may have masked sensory effects on interlimb coordination. Previous research in our lab has examined the effects of different doses of quipazine (1.0 mg/kg, 3.0 mg/kg, and 10.0 mg/kg) on hindlimb alternating stepping in developing rats (Swann et al., 2016). While 10.0 mg/kg and 3.0 mg/kg doses produced significantly more hindlimb alternating steps compared to the 1.0 mg/kg dose, this was done in an air-stepping paradigm. It is possible that using a dose of 1.0 mg/kg quipazine would be sufficient for producing alternating stepping on a treadmill without overshadowing sensory input from the moving belt, thus resulting in changes in interlimb coordination caused by changes in treadmill belt speed. Thus, future experiments could utilize this lower dose to perhaps better examine the effects of different treadmill belt speeds during early development.

Chapter 6: Conclusions

Alternating stepping in spinal animals has been shown to rely on communication within isolated spinal circuitry, as well as integration of sensory input from the periphery (Frigon, 2017). Previous work has shown that adult spinal animals (i.e., rats and cats) can show weight-supported alternating stepping on treadmills and following treadmill training (Cha et al., 2007; Shurrager & Dykman, 1951), highlighting the isolated spinal cord's ability to maintain alternating interlimb coordination despite having no input from descending tracts. Work in neonatal kittens has demonstrated plasticity of the developing spinal cord, as well as the ability of the immature nervous system to produce alternating stepping in the absence of supraspinal input (Bradley & Smith, 1988). However, previous experiments have not examined how the immature, developing spinal cord supports stepping adaptations on a treadmill.

Instead of examining activity-dependent exercise on locomotor outcomes, here we were interested in the quantity (interlimb coordination) and quality (intralimb coordination) of alternating steps on a treadmill belt during a single session. To our knowledge, this is the first experiment examining alternating hindlimb stepping on different belt speeds in neonatal animals. Although spinal subjects do not regain full behavioral function following a complete spinal cord transection, we showed that developing rats have the necessary circuitry in place for producing and maintaining alternating stepping on a treadmill and can alter their step quality to different treadmill belt speeds. However, development of weight-bearing locomotion may be a critical component in how step quality is altered on a moving treadmill belt. The current study showed that the developing isolated spinal cord *in vivo* can respond to changes in sensory stimulation in the absence of supraspinal input. Our results suggest that the plasticity of the developing spinal

cord contributes to recovery of stepping function and sensory responsiveness following an early spinal cord injury.

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Appendix
Figures and Tables

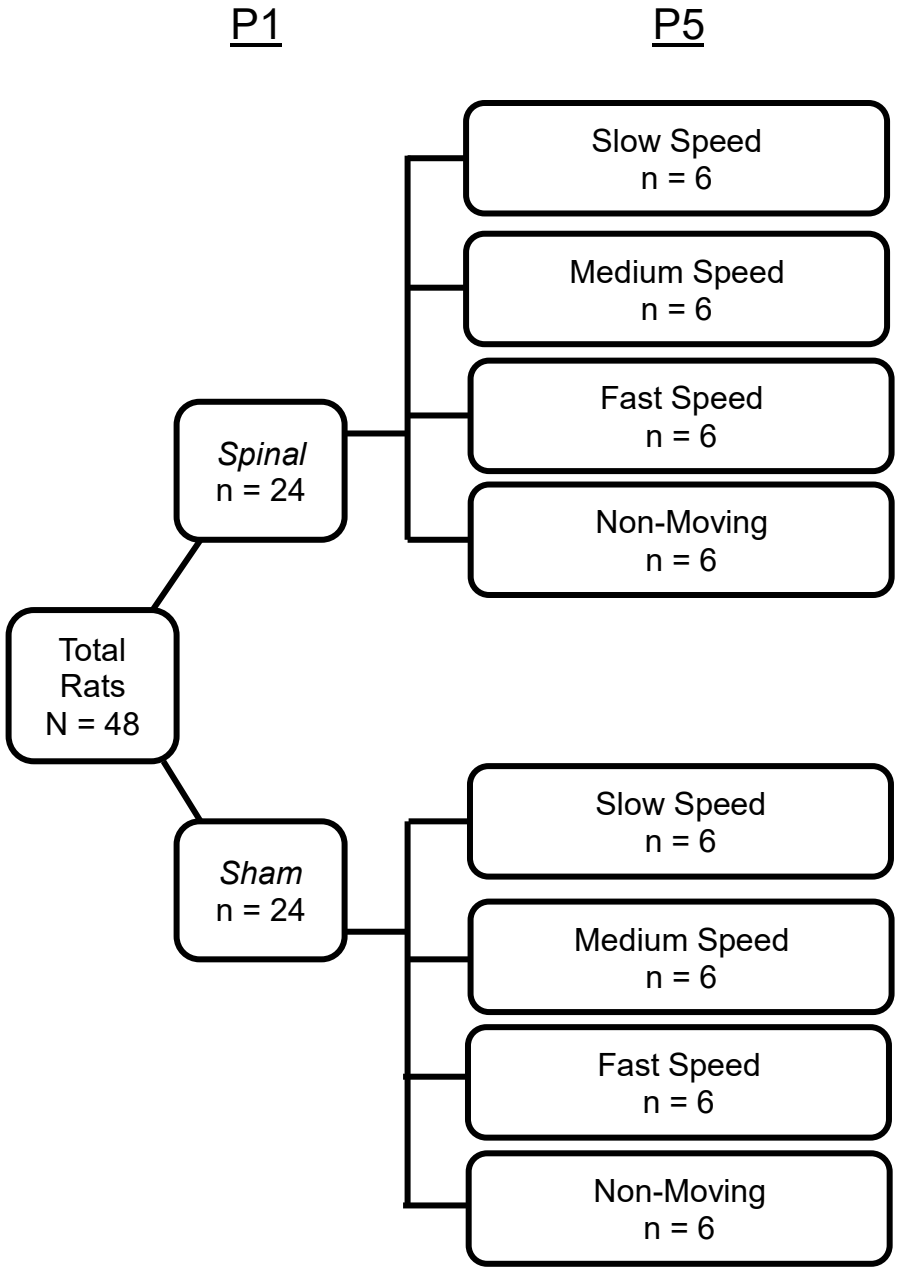


Figure 1. Experimental design. On P1, rats received a low-thoracic spinal cord transection or sham operation. On P5, subjects were tested on one of four treadmill belt speeds: slow (1.6 cm/s), medium (3.2 cm/s), fast (4.8 cm/s), or non-moving. Following testing, subjects were euthanized and preserved for later dissection.

A)

B)

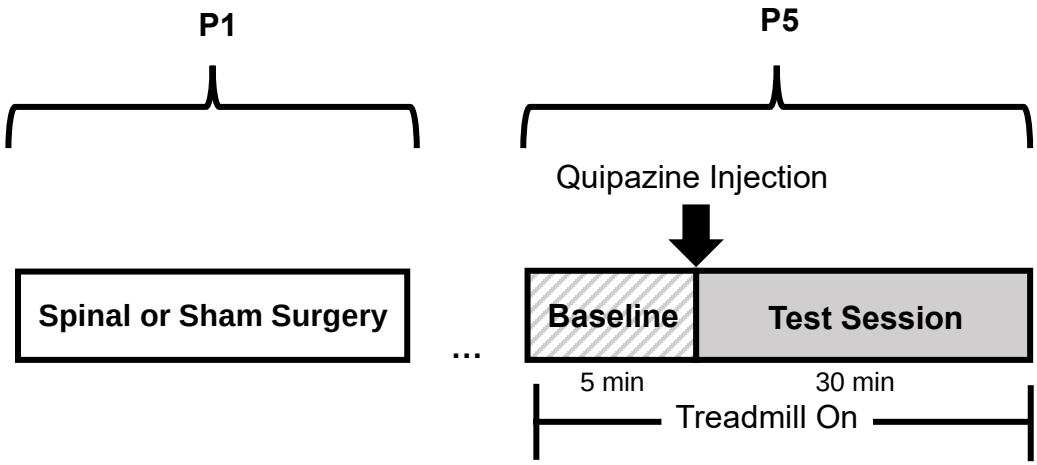


Figure 2. A) Picture depicting how subjects were secured above the treadmill. Note that the forelimbs were placed on a Plexiglas plate, and only the hindlimbs were on the treadmill belt. B) Experimental timeline. Following surgery on P1, subjects were tested on the treadmill on P5. There was a 5-min baseline, followed by an injection of quipazine, and behavior was recorded for an additional 30-min on the treadmill.

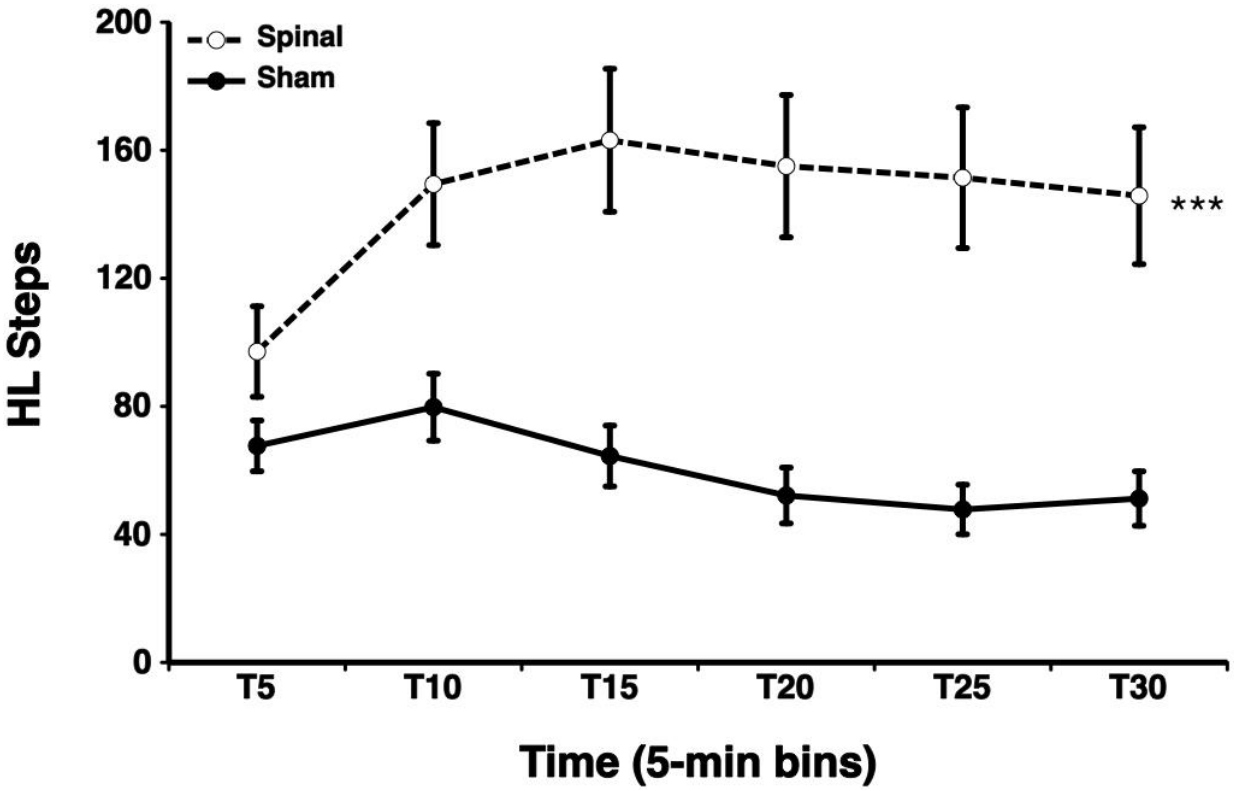


Figure 3. Frequency of alternating hindlimb steps across surgery condition and time, during the 30-min treadmill test period following quipazine administration. Spinal subjects showed significantly more hindlimb movements compared to shams. Note: *** show significance at $p < .001$.

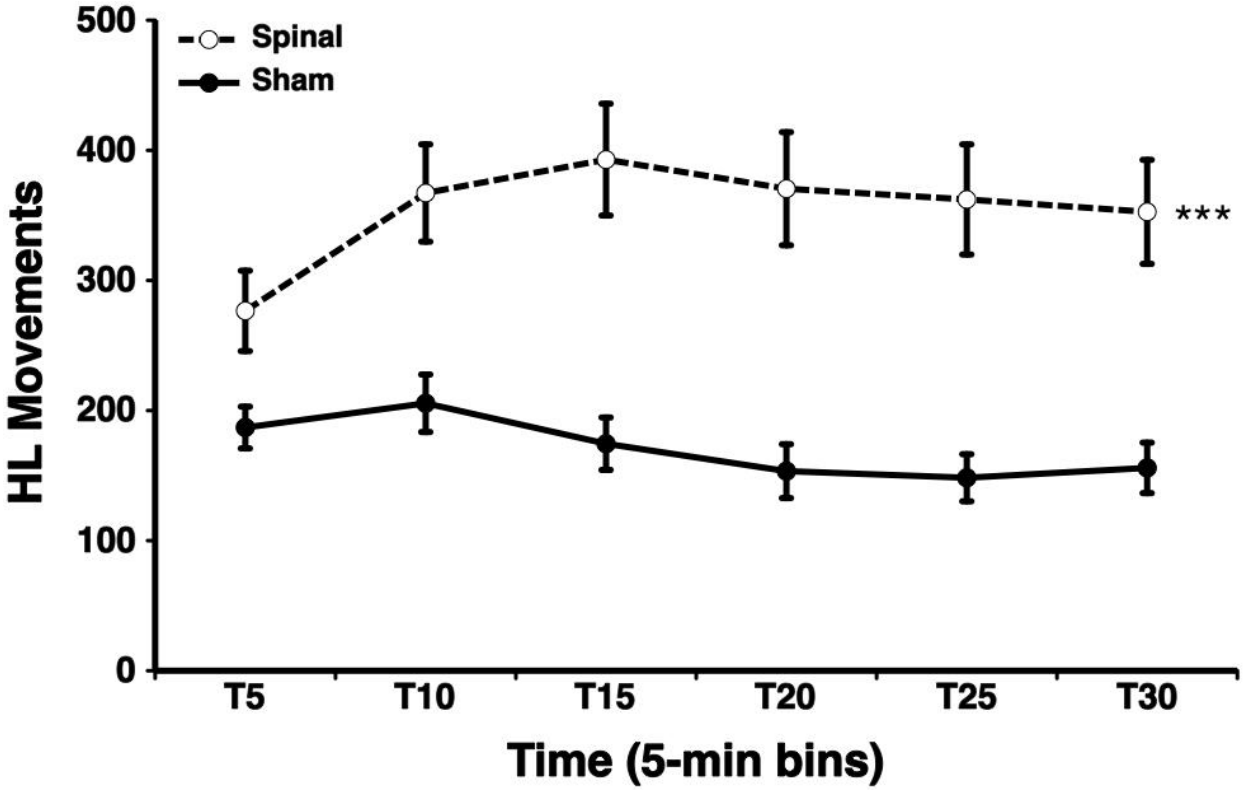
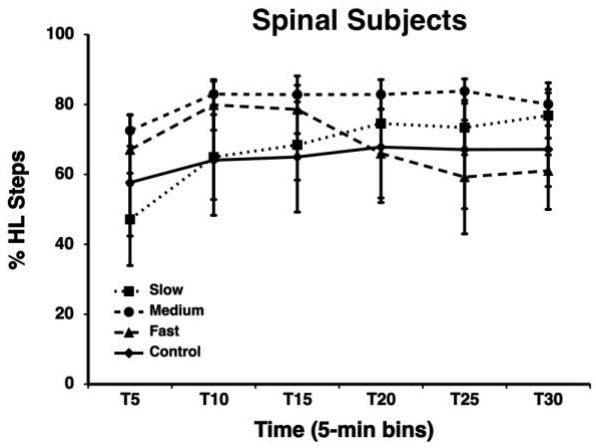
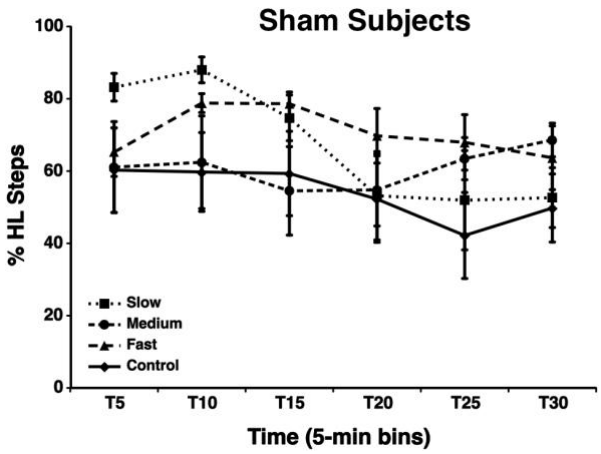


Figure 4. Total hindlimb movements across surgery condition and time. Spinal subjects showed significantly more hindlimb movements compared to shams. Note: *** show significance at $p < .001$.

A)



B)



C)

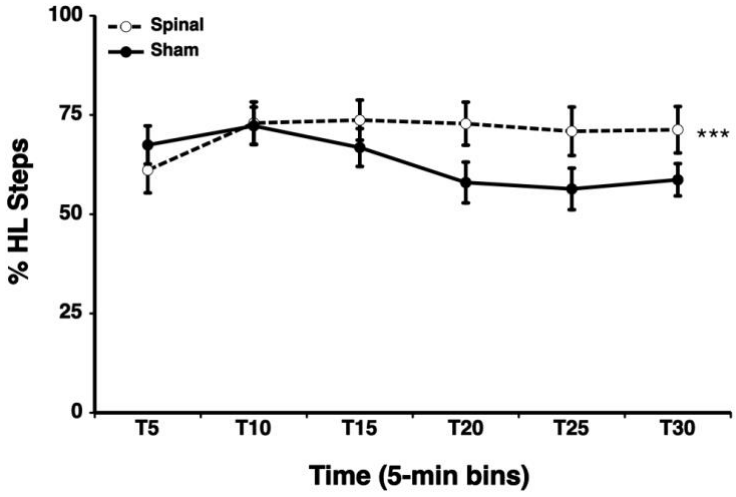
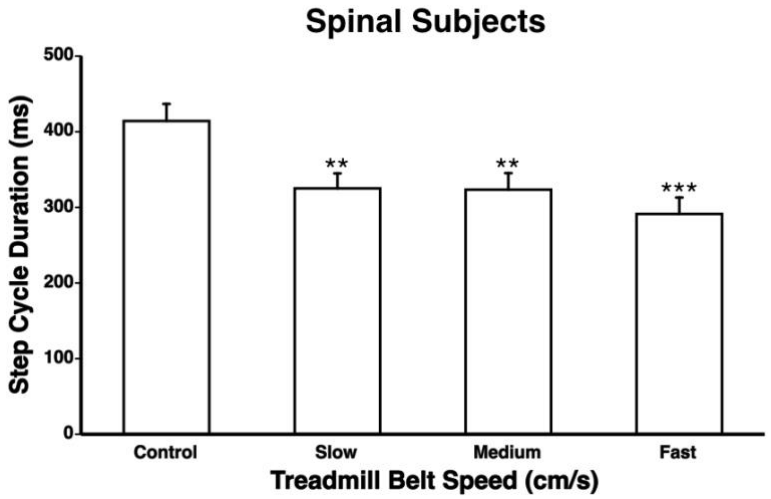


Figure 5. A) Percentage of hindlimb alternating steps for spinal subjects, across time and by treadmill belt speed condition. B) Percentage of steps for sham subjects, across time and by treadmill belt speed condition. C) Percentage of steps across surgery condition and time. Spinal subjects showed a significantly higher percent of alternating steps compared to shams. Note: *** show significance at $p < .001$.

A)



B)

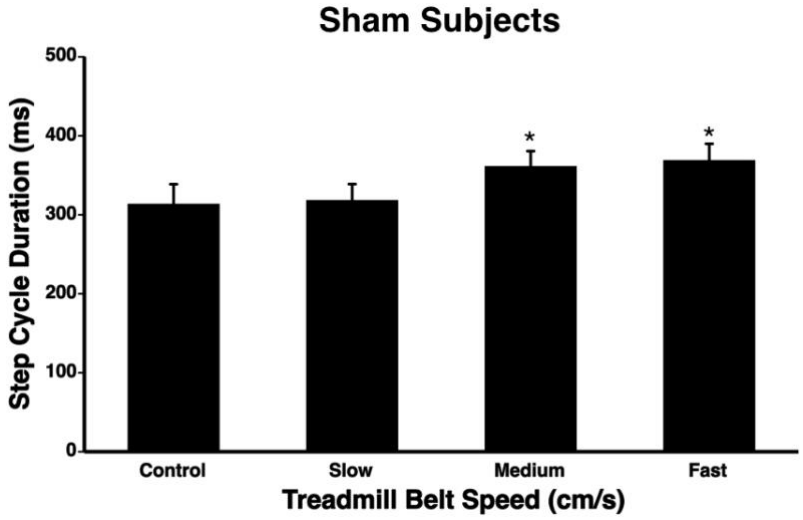


Figure 6. A) Step cycle duration for spinal subjects across treadmill belt speed conditions. Step cycle durations were significantly longer on the control belt speed compared to slow, medium, and fast belt speeds. B) Step cycle for sham subjects across treadmill belt speeds. Step cycle durations were significantly shorter on the control belt speed compared to medium and fast belt speeds. Note: * show significance at $p < .05$, ** show significance at $p < .01$, and *** show significance at $p < .001$.

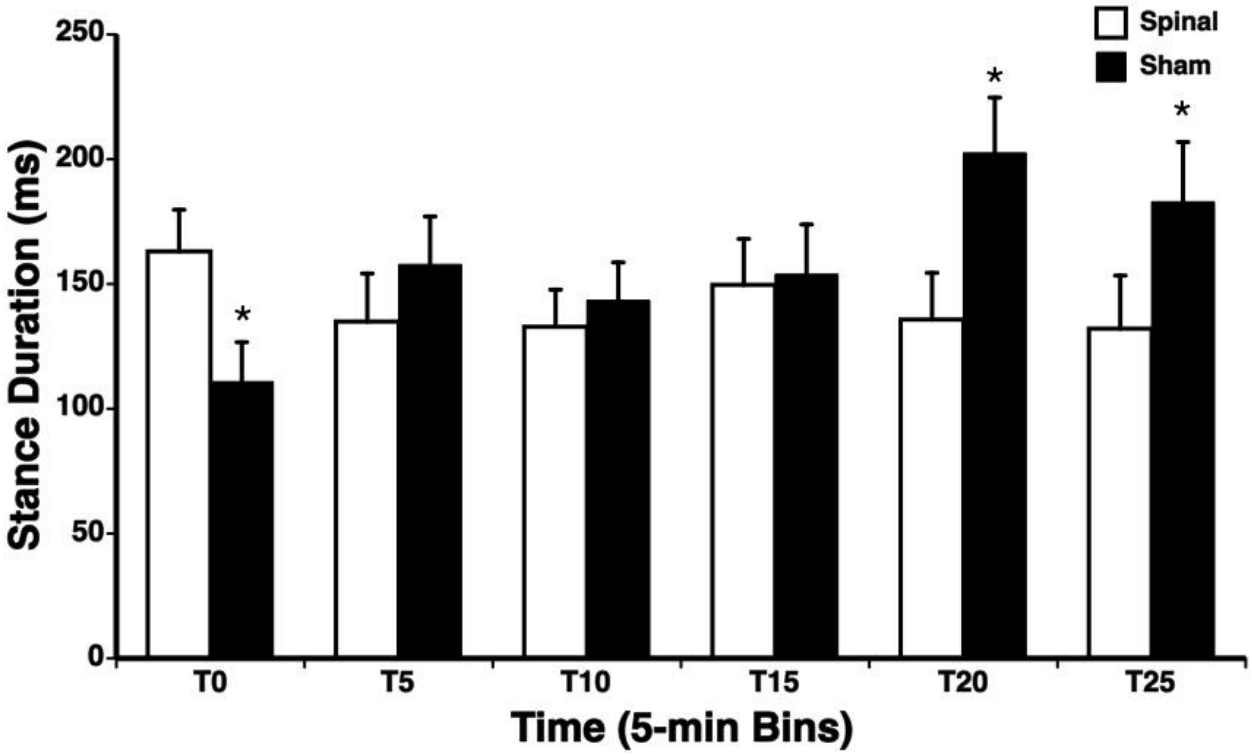
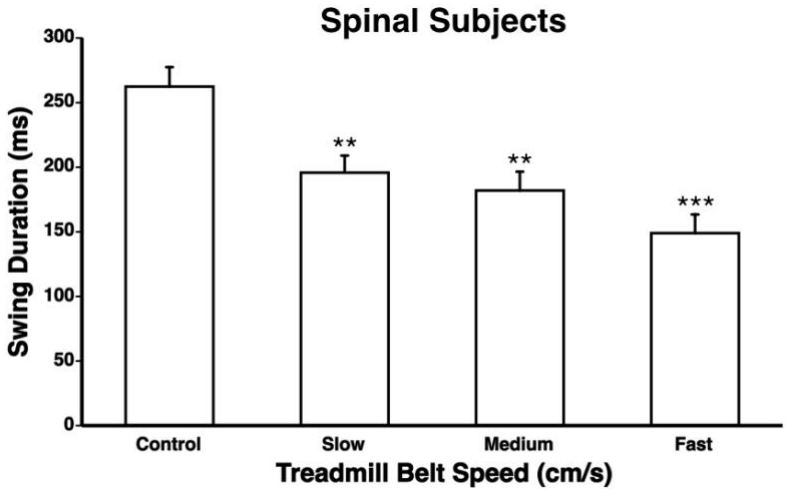


Figure 7. Stance phase duration by surgery across time. Sham subjects showed significantly shorter stance durations during the first time bin compared to the fifth and sixth time bins. Note: * show significance at $p < .05$.

A)



B)

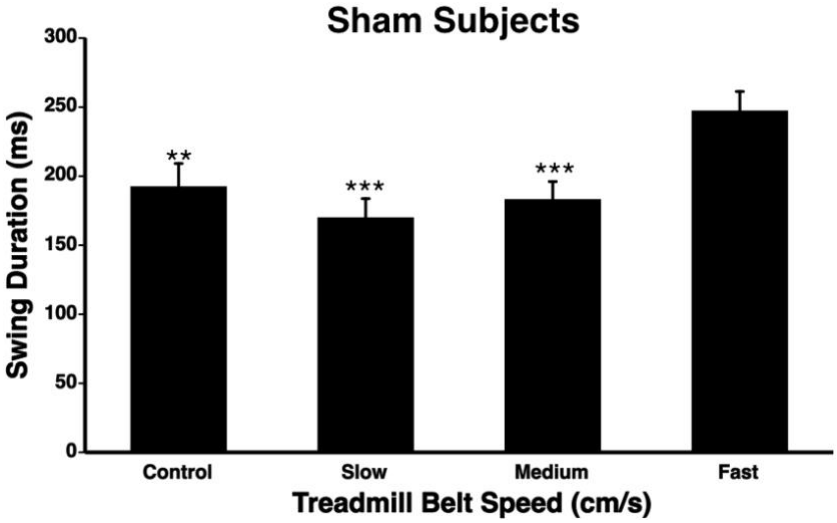


Figure 8. A) Swing phase durations were significantly longer on the control belt speed compared to slow, medium, and fast belt speeds for spinal subjects. B) Swing phase durations were significantly longer on the control belt speed compared to slow and medium speeds in sham subjects. Furthermore, shams showed significantly longer swing durations on the fast treadmill belt speed compared to the all other speeds. Note: ** show significance at $p < .01$, and *** show significance at $p < .001$.

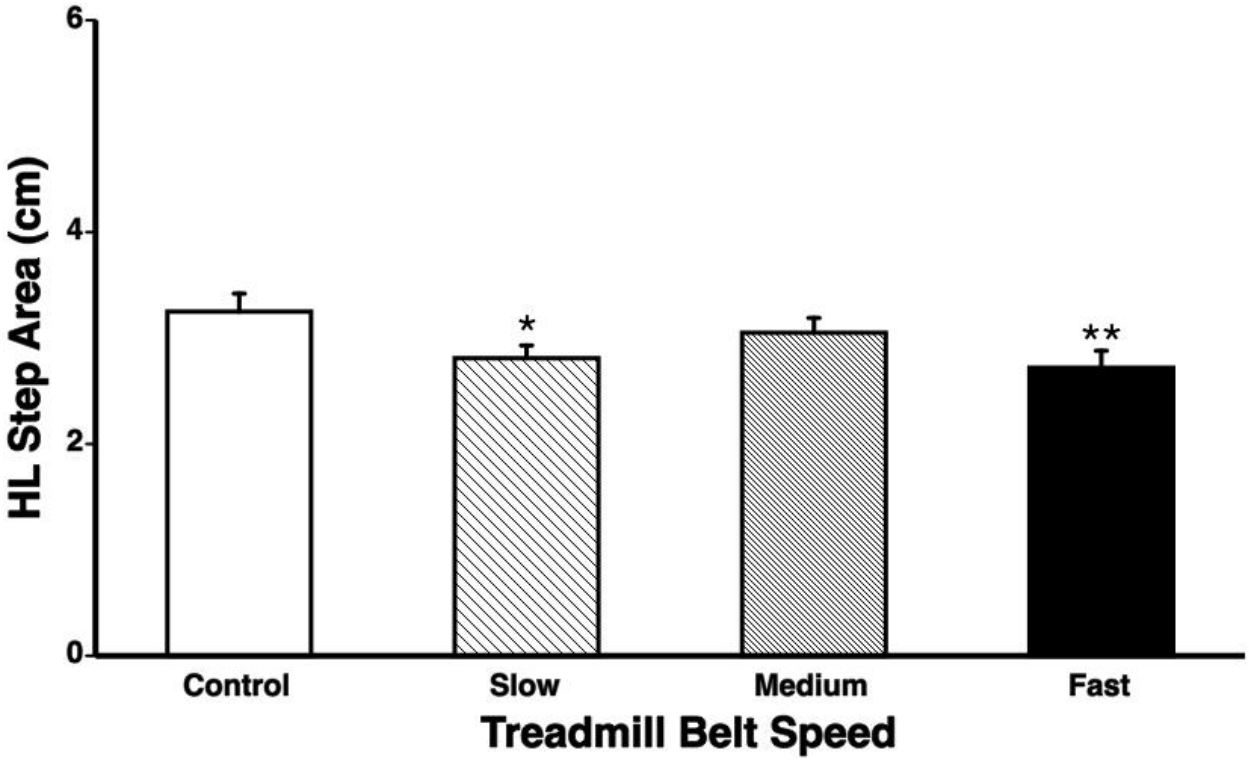
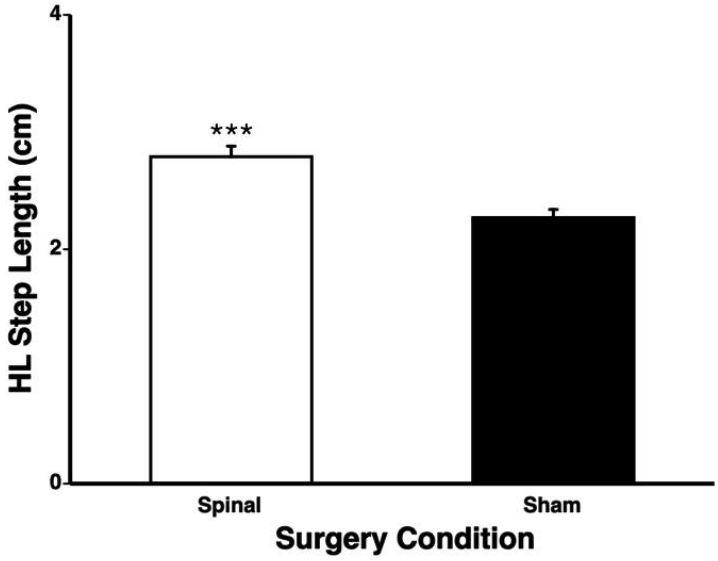


Figure 9. Step area for all subjects across treadmill belt speed conditions. Subjects tested on the control non-moving belt had significantly larger step areas compared to subjects in the slow and fast belt speed conditions. Note: * show significance at $p < .05$, and ** show significance at $p < .01$.

A)



B)

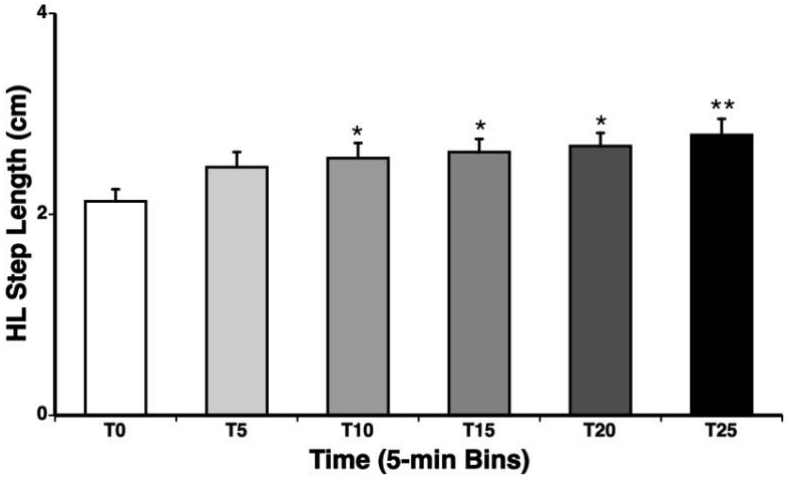


Figure 10. A) Hindlimb step length for both surgery conditions. Spinal subjects had significantly longer step lengths compared to sham subjects. B) Hindlimb step length for all subjects across time. Subjects had significantly shorter length steps in the first 5-min time bin compared to the third, fourth, fifth, and sixth time bins. Note: * show significance at $p < .05$, ** show significance at $p < .01$, and *** show significance at $p < .001$.

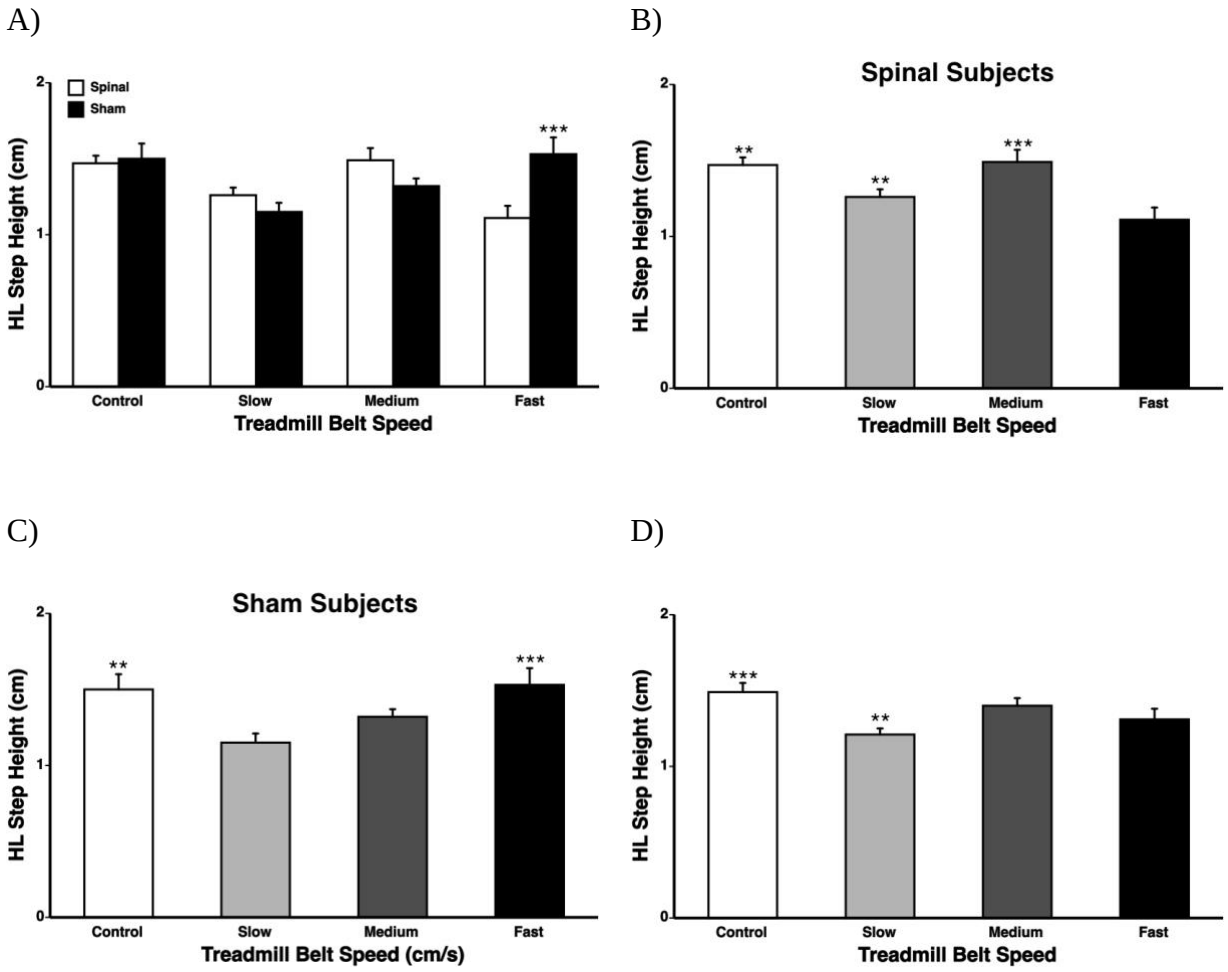


Figure 11. A) Step height across surgery and treadmill belt speed conditions. Spinal subjects had significantly shorter step heights in the fast speed condition than sham subjects. B) Step height for spinal subjects across speeds. Step height was significantly shorter on the fast treadmill belt speed condition compared to the non-moving and slow belt speed for spinal subjects. Step height was significantly lower in the slow speed condition compared to the medium speed condition for spinal subjects. C) Step height for sham subjects across speed. Sham subjects showed significantly shorter step heights in the slow compared to the control and fast speed conditions. D) Step height for all subjects across belt speed condition. Subjects' steps were significantly

higher in the non-moving condition compared to the slow and fast speed conditions. Subjects' steps were significantly lower in the slow compared to the medium speed condition. Note: ** show significance at $p < .01$, and *** show significance at $p < .001$.