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Locomotor Stepping and Serotonin 2A Receptor Plasticity in the Intact and Isolated Spinal Cord

of the Rat During Early Postnatal Development

Sierra Kauer

A dissertation

submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy in the Department of Psychology

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Committee Approval

To the Graduate Faculty,

The members of the committee appointed to examine the dissertation of Sierra Kauer find it satisfactory and recommend that it be accepted.

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Animal Welfare Research Committee Approval Page

September 4, 2012

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

RE: Your application dated 9/1/2012 regarding study number 699: Modulation of Action Patterns in Developing Rats

Dear Dr. Brumley:

Thank you for your response to requests from a prior review of your application for the new study listed above.

This is to confirm that your application is now fully approved. The protocol is approved through 9/4/2015 with an annual review at 9/4/2013.

You are granted permission to conduct your study as most recently described effective immediately. The study is subject to continuing review on or before 9/4/2013, unless closed before that date.

Please note that any changes to the study as approved must be promptly reported and approved. Some changes may be approved by expedited review; others require full board review. Contact Patricia Hunter (208-282-2179; fax 208-282-4529;
email: anmlcare@isu.edu) if you have any questions or require further information.

Sincerely,

Curt Anderson, PhD
IACUC Chair

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List of Abbreviations

5-HT: 5-hydroxytriptamine

5-HT_1: 5-hydroxytriptamine 1 receptors

5-HT_{1A}: 5-hydroxytriptamine 1A receptors

5-HT_{1B}: 5-hydroxytriptamine 1B receptors

5-HT_2: 5-hydroxytriptamine 2 receptors

5-HT_{2A}: 5-hydroxytriptamine 2A receptors

5-HT_{2C}: 5-hydroxytriptamine 2C receptors

BBB: Basso, Beatttie, and Bresnahan motor scale

BDNF: brain derived neurotropic factor

BSA: bovine serum albumin

°C: degrees Celsius

C2: cervical spinal level 2

C4: cervical spinal level 4

$[CL^-]i$: chloride ions

CO_2: carbon dioxide

CPGs: central pattern generators

E11: embryonic day 11: eleven days before birth

E12: embryonic day 12: ten days before birth

E13: embryonic day 13: nine days before birth

E14: embryonic day 14: eight days before birth

E15: embryonic day 15: seven days before birth

E16: embryonic day 16: six days before birth

E17: embryonic day 17: five days before birth

E19: embryonic day 19: three days before birth

E20: embryonic day 20: two days before birth

E21: embryonic day 21: day before birth

E22: embryonic day 22: day of birth

G: grams

Ga: gauge

GABA: *gamma*-Aminobutyric acid

Hrs: hours

IP: intraperitoneal

KCC2: potassium chloride co-transporters

Kg: kilogram

LER: leg extension response

L2: lumbar spinal level 2

L3: lumbar spinal level 3

L5: lumbar spinal level 5

L6: lumbar spinal level 6

$_{L}$-DOPA: dopamine precursor

NMA*: N*-methyl-D, L-aspartate

NMDA: *N*-methyl-D-aspartate receptor agonist

Mg: milligram

Min: minute

Ml: milliliter

mRNA: messenger ribonucleic acid

Mm: millimeter

NGS: normal goat serum

P0: postnatal day 0: day of birth

P1: postnatal day 1: 24 hours after birth

P2: postnatal day 2: 2 days after birth

P3: postnatal day 3: 3 days after birth

P4: postnatal day 4: 4 days after birth

P5: postnatal day 5: 5 days after birth

P6: postnatal day 6: 6 days after birth

P7: postnatal day 7: 7 days after birth

P8: postnatal day 8: 8 days after birth

P10: postnatal day 10: 10 days after birth

P12: postnatal day 12:12 days after birth

P14: postnatal day 14:14 days after birth

P15: postnatal day 15:15 days after birth

P17: postnatal day 17:17 days after birth

P20: postnatal day 20: 20 days after birth

P21: postnatal day 21:21 days after birth

PBS: phosphate buffered saline

PFA: paraformaldehyde

PIC: persistent inward current

ROM: range of motion

S: seconds

S4: sacral spinal level 4

SCI: spinal cord injury

SEM: standard error of the mean

T8: thoracic spinal level 8

T9: thoracic spinal level 9

T10: thoracic spinal level 10

Tx: treatment

µl: microliter

µm: micrometer

Vol: volume

Wt: weight

ABSTRACT

Quipazine (a 5-HT_{2A} receptor agonist) has been shown to activate locomotor-like air-stepping behavior in perinatal rats given a spinal cord transection. Previous research has shown that there are differences in hindlimb activity (hindlimb supersensitivity) in response to quipazine between transected and sham rat pups. We sought to determine whether hindlimb supersensitivity was influenced by time of surgery and/or age of testing. In the Experiment 1, we examined the developmental differences in 5-HT_{2A} activated stepping between different ages of surgery (P1 and P5) and different ages of testing (P5 and P10). We found that transected subjects exhibited hindlimb supersensitivity (increased total hindlimb movements and alternated hindlimb steps) in response to quipazine, and that hindlimb supersensitivity increased with recovery time from surgery (P1-P10 compared to P1-P5 and P5-P10). Furthermore, we found that age of subjects influenced percentage of alternated hindlimb steps where P10s were more coordinated than that of P5s. However, while transection treatment increased hindlimb activity compared to sham treatment, it did not increase coordination for transected subjects compared to shams tested on P10. Previous research has shown that there is upregulation of 5-HT_{2A} receptors in the spinal cord following spinal injury. We hypothesized that this was one explanation for observed hindlimb supersensitivity to quipazine. Thus, Experiment 2 used immunohistochemistry to examine 5-HT_{2A} receptor immunoreactivity and hypothesized an increase in area signal percentage as well as particle count. Results showed that transect subjects showed a significant increase in 5-HT_{2A} receptor area signal percentage and particle count compared to shams. However, when examining differences between different surgery and sacrifice ages, no significant

differences were found Thus, hindlimb supersensitivity in response to quipazine is partially associated with upregulation of 5-HT_{2A} receptors, but is the result of complex interactions of many biological factors. Understanding the biological and behavioral alterations that occur following spinal cord transection can help us in developing therapies for those with spinal cord injury and neurological disorders.

Locomotor Stepping and Serotonin 2A Receptor Plasticity in the Intact and Isolated Spinal Cord of the Rat During Early Postnatal Development

Spinal cord injury (SCI) results from physical trauma (e.g. car accidents) and disrupts connections between the brain and various levels of the spinal cord, depending on the site of the lesion (Brodwin, Siu, Howard, & Brodwin, 2009). Spina bifida is a neural tube defect where the backbone and membranes covering the spinal cord fail to close, often damaging the spinal cord and nerves. For individuals with SCI or spina bifida, the disruption of these connections prevents or interfere with sensory processing of information received from peripheral stimulation below the lesion, as well as voluntary and involuntary movements. Since spinal cord neurons are not known to regenerate, individuals with SCI or spina bifida may suffer from debilitating lifelong symptoms. However, rehabilitation and treatments for these individuals can improve quality of life through functional recovery (Field-Fote, 2009). Although cells do not regenerate, existing connections are plastic in that they are activity-and sensory-dependent (Parker, 2000), thus providing opportunity for activity-dependent recovery.

Current animal models using a SCI paradigm have demonstrated recovery of motor functioning through stimulating 5-HT_2 receptors (Antri, Mouffle, Orsal, & Barthe, 2003; Antri, Orsal, & Barthe, 2002; Sławińska, Majczyński, Dai, & Jordan, 2012) as well as through step training (Cha et al., 2007; Tillakarante et al., 2010). Furthermore, the plasticity of the spinal cord has been demonstrated by operant and classical conditioning of the hindlimbs of rats following complete SCI (Grau, Barstow, & Joynes, 1998; Joynes, Janjua, & Grau, 2004; Patterson & Grau, 2001). However, most of the existing literature using a SCI paradigm is on adult animal models. Although the nervous system is plastic

throughout the lifespan to some degree, it is more so during early development. This has important implications even for adults with SCI, since understanding how these systems develop initially can increase our knowledge of how to repair them when they are damaged. Specifically, by increasing our understanding of the plasticity of immature locomotor circuitry we can better develop treatments for spinal injured individuals and infants with neurological problems (i.e. spina bifida). To further our understanding of the plasticity of immature locomotor circuitry, the current study examined the development of locomotor stepping and serotonin receptor plasticity in the intact and isolated spinal cord of developing rats.

In Experiment 1 locomotor behavior was compared between postnatal day 5 (P5) and P10 rat pups that received a low thoracic (T8-T10) spinal cord transection or sham surgery. The spinal surgery took place on P1 (for pups tested on P5 or P10) or P5 (for pups tested on P10), so that developmental differences could be examined between pups that had received the transection at different ages. Previous research indicates that neonatal rats receiving a spinal cord transection show the greatest recovery when the transection occurs prior to P12 (Weber & Stelzner, 1977). However, motor functioning is limited prior to P12 in the rat and it is difficult to examine the full repertoire of motor behavior without the aid of pharmaceuticals. Therefore, the current study examined developmental differences in locomotor behavior by activating the 5-HT_{2A} receptor system via drug treatment. Previously quipazine, a 5-HT_{2A} receptor agonist, has been shown to induce locomotor behavior in spinal transected rats, including newborns (Antri et al, 2002; Brumley & Robinson, 2005; McEwen, Van Hartesveldt, & Stehouwer, 1997; Sławińska et al., 2012; Strain, Kauer, Kao, & Brumley, 2014). Thus, hindlimb locomotor

behavior was examined following administration of quipazine (3.0 mg/kg), or saline (vehicle control) using an air-stepping paradigm. Developmental differences were compared between P5 and P10 pups that had been injected with quipazine or saline, as well as pups that had received a sham or spinal cord transection on P1 or P5.

Experiment 2 used immunohistochemistry to examine the density of 5-HT_{2A} receptors in the lumbar spinal cord of P5 and P10 rat pups that had received a sham or spinal cord transection on P1 or P5. Previous research has found that low spinal transected (T9/T10) rat pups show a significant increase in alternated stepping following quipazine treatment, when compared to sham pups (Strain et al., 2014). This could possibly be due to lack of suprispinal inhibition or upregulation of 5-HT receptors in the isolated spinal cord. Research examining 5-HT receptors in adult rats that have received a sacral spinal cord transection have found upregulation of 5-HT_{2A} receptors in the spinal cord (Kong, Weinecke, Hultborn, & Zhang, 2010; Kong, Weinecke, Chen Hultborn, & Zhang, 2011). If this is the case with rat pups transected at T9/T0 as well, it could be contributing to increased sensitivity of the serotonin system, and therefore increases in observed locomotor activity (Strain et al., 2014). Thus, Experiment 2 used confocal microscopy to inspect the density of 5-HT_{2A} receptors in the lumbar spinal cord (L3-L5) of sham and spinal pups to determine if there is receptor upregulation that therefore may be contributing to any possible observed differences in locomotor behavior seen in Experiment 1. Together, the relationship between 5-HT activated locomotor activity and 5-HT receptor density was examined in the presence or absence of supraspinal inputs. By doing so, we are able further our understanding of the plasticity of mechanisms

underlying motor development that occur without supraspinal input which may occur following SCI.

MOTOR DEVELOPMENT IN RATS

Motor behavior encompasses a broad range of actions in animals and is initiated and maintained by an intricate integration of peripheral and central mechanisms. Motor behavior includes simple actions such as twitches and reflexes, as well more complex coordinated actions such as locomotion. Species can either be precocial or altricial in their locomotor behavior. Precocial species are those that are more mature in their locomotor development at birth (e.g. horses and chickens), while altricial species are those that are relatively more immature in their locomotor behavior at birth (e.g. humans and rats) (Muir, 2000). While it may seem like altricial species are at a disadvantage at birth (since they are dependent on a caregiver for their survival), motor development is experience-expectant, thus enabling altricial young to be able to adapt to their individual environments. Even early experience in the fetal period can influence both motor development and the neurological mechanisms of motor development.

Motor Development in Fetal Rats

Spontaneous motor activity is commonly observed in fetuses. In humans the observation of this activity is limited to ultrasound technology. However, with rats researchers have found ways to directly observe and manipulate fetal motor activity. One method of observation is to induce spinal anesthesia in the dam allowing the uterus to be exteriorized in a saline bath (Smotherman & Robinson, 1986). Fetuses are removed from the uterus while still in the transparent amniotic sac (with umbilical circulation and

umbilical attachment) and suspended in a saline bath, which allows observation. In rats prior to embryonic day 16 (E16) movements are limited, however on E17 and thereafter fetuses exhibit a variety of limb, mouth, and body movements. Using this method, researchers have examined the influence of environmental stimuli on motor capability (Robinson, 2015; Robinson, Kleven, & Brumley, 2008; Robinson 2005; Robinson & Smotherman, 1991; Smotherman & Robinson, 1987). One such example can be seen in the facial wiping response of rat fetuses. Facial wiping is elicited by an intraoral infusion of lemon solution after the fetus is externalized from the uterus and has the embryonic sacs removed, starting at E20 (Smotherman & Robinson, 1987). However, if the fetus is left within the amniotic sac on E19 they will exhibit a facial wiping response at that earlier age (Robinson & Smotherman, 1991). The reason for this is that E19 rats have less stability of the head than E20s, which prevents coordination with the forepaws, which is necessary for facial wiping. However, with assistance from the amniotic sac, E19s are able to stabilize their head while engaging in forelimb responses. This study provides evidence for the importance of sensory feedback in the development of motor behavior in the fetus. Another example of the influence of sensory feedback on motor development can be observed with limb training.

Rat fetuses have been shown to have the capacity to respond to kinesthetic and proprioceptive feedback, thereby altering their spontaneous motor activity (Robinson et al., 2008; Robinson 2005). Using a yoke-training paradigm, researchers have examined the ability of rat fetuses to adapt their interlimb coordination to a constraint. This paradigm uses a thread looped through polyethylene tubing attached to the hindlimbs of the fetus. During independent spontaneous limb movements one limb will pull the

homologous limb with it, resulting in a passive movement. Results of these studies show following removal of the yoke, E19, E20, and E21 fetuses show an increase in conjugate limb movements (e.g. limb movements initiated at the same time and following parallel trajectories) (Robinson et al., 2008). Additionally, E20 rats that have received a spinal cord transection show an increase in conjugate limb movements after yoke training, providing evidence that this learning is spinally mediated (Robinson, 2015). Furthermore, when the yoke is reapplied to the fetus, there appears to be retention of yoke motor learning since yoked subjects show conjugate limb movements more quickly after exposure to the yoke a second time, even if they have been transected (Robinson, 2015; Robinson, 2005). These studies show that spontaneous movements can be adapted to environmental stimuli, and are most likely providing the foundation for motor development after birth.

Motor Development in Postnatal Rats

As previously mentioned, rats are immature in their motor functioning at birth. Prior to 2 weeks of age rat pups exhibit pivoting using their forelimbs as “paddles” to push themselves in a circular pattern for locomotion (Altman & Sudarshan, 1975). Rats follow a rostral-caudal pattern of development (similar to humans) where by P8 they are able to raise their head and use their limbs to crawl (without supporting their trunk). Quadrupedal walking supported primarily by the forelimbs is displayed by P12, and by P20 full maturity of locomotion is reached. It is important to note however, that crawling or walking prior to P12 can be observed on occasion with presentation of a stimulus, such as an odor (Jamon & Clarac, 1998; Mendez-Gallardo & Robinson, 2014). Furthermore, locomotor and postural behaviors can be promoted as early as P1 by using the

serotonergic agonist quipazine (Swann, Kempe, Van Orden, & Brumley, 2016). Motor development can also be influenced by altering the perinatal environment. Researchers have examined the effects of an extra day of postnatal experience on the development of motor behaviors (Roberto & Brumley, 2014). E22 is the typical day of birth in rats, therefore subjects were delivered via caesarian section one day before term (E21), and then cross-fostered. Motor functioning of prematurely delivered pups was then compared to that of normal, vaginally delivered pups. Pups that were delivered prematurely and received one extra day of postnatal experience showed differences in motor behaviors such as an increase in bilateral facial wiping response, more mature righting response, and increases in the bilateral leg extension response (Roberto & Brumley, 2014). This study provides evidence that exposure to environmental factors such as the mother, nest, and littermates are a large factor in the development of motor behaviors.

Rat pups experience a wide variety of stimulation in the nest. Their littermates and mother provide them with thermal, tactile, and olfactory stimulation. They also experience vestibular and kinesthetic sensations. This stimulation allows the pup to adapt its motor behavior in response to its own experiences, making it more suitable to its individual environment. For example, newborn rats will alter their motor activity in response to sensory feedback. As previously mentioned, pups injected with quipazine show alternated air-stepping, when attached to a horizontal bar (Brumley et al., 2012; Strain & Brumley, 2014). However, if different substrates are placed underneath them after administration of quipazine, they will avoid contact with a stiff substrate when compared to an elastic substrate (Brumley et al., 2012). Thus, the pup's motor activity is

influenced by factors such as cutaneous and proprioceptive feedback provided by the environment.

Neurological Mechanisms of Motor Development in Rats

Ascending and descending pathways of motor functioning start developing prenatally (de Boer-van Huizen & ten Donkelaar, 1999; Lakke, 1997; reviewed in Vinay et al., 2005). However, differentiation of motor pathways is incomplete in the newborn rat, contributing to the immaturity of motor behavior. While spinal central pattern generators (CPGs) have been found to be functional at birth, rat pups are not very adept at controlling and coordinating movements due to the immaturity of supraspinal inputs, muscle control, and postural control (Vinay et al., 2005). Rapid development of the central nervous system occurs during the first two postnatal weeks of life for the rat, which allows for more mature control of the neurobiological mechanisms of motor behavior.

Descending Pathways

Descending motor pathways of the central nervous system travel from the brain to the spinal cord. These pathways allow for control of motor movements. In the developing rat fetus there are already brainstem fibers that innervate the cervical spinal cord at E14 (de Boer-van Huizen & ten Donkelaar, 1999; Lakke, 1997; Smith, 1983; reviewed in Vinay et al., 2005; Ziskind-Conhaim, Seebach, & Gao, 1993). These cells originate in the medullary and pontine reticular formation, the interstitial nucleus of the medial longitudinal fasciculus, as well as the lateral vestibular nucleus. The function of the medullary and pontine reticular formation in motor behavior is related to postural control

and allows for adjustments for postural stabilization in movements (Purves et al., 2012). The interstitial nucleus of the medial longitudinal fasciculus assists in coordinating conjugate eye movements and projects to the lateral vestibular nucleus, which assists in signaling abnormal posture. These axon projections have been reported to reach the cervical spinal cord as early as E12 (de Boer-van Huizen & ten Donkelaar, 1999). At approximately E14 they reach the thoracic level, and the low thoracic and lumbar levels by E15 (de Boer-van Huizen & ten Donkelaar, 1999; Lakke, 1997; reviewed in Vinay et al., 2005). While some of the vestibulospinal tract and reticular formation begin developing prenatally, the corticospinal tract begins developing postnatally.

Corticospinal tract fibers are part of the medullary pyramids and terminate in the brainstem or spinal cord and are involved in voluntary movement (Purves et al., 2012). In the rat the corticospinal tract originates with layer V cortical neurons where they decussate (cross) in the medulla, and the axons then terminate in the dorsal horn of the spinal cord (Leslie, 1986). In the developing rat the decussating pyramidal axons of the corticospinal tract reach the cervical spinal cord by the day of birth (P0) (Gribnau, de Kort, Dederen, & Nieuwenhuys, 1986). By P2 they reach the mid-thoracic level, and by P5 they reach the lumbar level. Axonal growth is followed by axonal loss (pruning) in the developing corticospinal tract (Purves et al., 2009). The red nucleus of the tegmentum in the midbrain projects via the rubrospinal tract and terminates in the ventrolateral region of the dorsal horn (Purves et al., 2012, pg. 395; Leslie, 1986, pg. 320). This tract works with projections from the motor cortex to control forelimbs (Leslie, 1986). The rubrospinal tract in developing rats has been found to be present at birth (Shieh, Leong, & Wong, 1983). Although many axonal projections are developed or developing early,

myelination of the axons does not take place until about P10 (Gorgels, de Kort, Van Aanholt, & Nieuwenhuys, 1989).

The development of 5-HT systems in particular have important relevance to the proposed study, since the serotonergic agonist quipazine is used to induce motor behavior in neonatal rats. The the raphe nuclei contain high amounts of 5-HT neurons (Leslie, 1986). In the rat, the raphe nuclei neurons project to the spinal cord in the ventral and dorsolateral funiculi and form synapses onto motoneurons. 5-HT receptor expression in the lower spinal cord precedes the arrival of descending 5-HT projections (Vinay et al., 2000). 5-HT projections from the raphe nuclei have been observed as early as E16 in the lumbar spinal cord and continue to advance until E20-E21 (Lakke, 1997; reviewed in Vinay et al., 2005; Ziskind-Conhaim et al., 1993). At birth, 5-HT projections are present throughout the spinal cord (although they are less dense than in adults) and follow a rostral-caudal/ventral-dorsal pattern of development (Bregman, 1987). By P14 the full adult pattern and density of 5-HT fibers is reached in the cervical spinal cord, and by P21 the full adult pattern and density of 5-HT fibers is reached in the thoracic and lumbar cord.

Ascending Pathways

Ascending pathways of the central nervous system are those that travel from the spinal cord to the brain. These pathways largely convey sensory information. Dorsal column pathways are located in the middle to posterior spinal cord and make up the white matter (Purves et al., 2012). First-order dorsal column pathway neurons are the ascending collaterals of primary afferents and have long axonal processes and contain cell bodies in the dorsal root ganglion (Leslie, 1986; Purves et al., 2012). The fasciculus

gracilis of the dorsal column conveys information about the lower limbs, while the fasciculus cuneatus conveys information about the upper limbs, trunk, and neck (Purves et al., 2012). Second-order neurons of the dorsal column pathway convey somatosensory information to the thalamus. In the fetal rat, dorsal root ganglion are present between E11 and E15 and are bipolar (which is typical of immature ganglion cells), sensory axons in the periphery are present at about E14, and dorsal cutaneous nerve branches have formed (Smith, 1983). By P17 there is a clear distinction between the fasciculus gracilis and fasciculus cuneatus (Purves et al., 2009). Other ascending pathways that start developing prenatally in the rat are the spinothalamic pathway and spinocerebellar pathways.

The spinothalamic pathways originate in the dorsal horn and ascend to the thalamus (Leslie, 1985). These pathways convey information from receptors about pain and temperature. In fetal rats these pathways develop from E13 to E15 (Beal & Bice, 1994). Clarke's nucleus (i.e. dorsal nucleus) are neurons that form the dorsal spinocerebellar tract extending to the cerebellum and are contacted by the lumbar and thoracic afferents (Leslie, 1986). This tract is involved in conveying proprioceptive information for the lower body. Clarke's neurons are present between E13 and E15 in the rat (Purves et al., 2009). Spinothalamic and spinocerebellar tracts in the lumbar spinal cord are distinct and have their own patterns of neurogenesis between E13 and E15 (Beal & Bice, 1994).

MOTOR BEHAVIOR IN SPINAL CORD INJURY MODELS

SCI paradigms not only allow researchers to study potential therapies for individuals with spinal injuries, but enable basic research in understanding neurological mechanisms of motor behavior in isolated areas of the spinal cord. When examining

research using animal models of SCI it is important to distinguish between early developing models and adult models. There is a great deal of difference between the functional recovery of young and old animals in their recovery following SCI (Petruska et al., 2007). This may be because the developing nervous system is more plastic than the adult nervous system, allowing for a more flexible response to post-injury training. This functional recovery in neonates is not due to the re-growth of axons across the transection site per se, but is attributed to alterations in the lumbosacral neural circuitry (Tillakaratne et al., 2010). In order to examine the plasticity of motor behavior following SCI, researchers employ a variety of methods including using neonatal SCI models, motor training, and drug treatments.

Perinatal Rat Motor Functioning Following Spinal Cord Transection

Pharmacological methods are commonly used to study the role of 5-HT in the development of locomotion in the rat pup, including those that have received a spinal cord transection (Brumley et al., 2012; Brumley & Robinson, 2005; Cazalets, Squalli-Houssaini, & Clarac, 1992; Kjaerulff & Kiehn, 1996; Strain et al., 2014). Stimulation of 5-HT receptors has been shown to induce an alternated stepping pattern in the hindlimbs of animals *in vivo (*Brumley et al., 2012; Brumley & Robinson, 2005; Strain et al., 2014; Ung et al. 2008). Likewise, *in vitro* studies of the isolated spinal cord have shown that 5-HT induces fictive motor bursts alternating between both sides of the lumbar spinal cord, as well as between flexors and extensors (Cazalets et al., 1992; Kjaerulff & Kiehn, 1996; Pearlstein, Mabrouk, Pflieger, & Vinay, 2005). The 5-HT rhythmic activity is different from dopaminergic-activated activity in that it is fast and regular, while the dopamine pattern is slow and irregular (Kjaerulff & Kiehn, 1996). This distinction between

different types of locomotor rhythms is important for the current study, since the serotonergic agonist quipazine will be used to specifically induce (and evaluate) an alternated stepping pattern. Furthermore, it is important to note that sensory feedback can influence motor behavior in spinal rat pups, even in the absence of supraspinal input (Robinson, 2015; Strain et al., 2014).

Effects of 5-HT Stimulation on Motor Behavior in the Spinal Perinatal Rat

As previously mentioned, in the rat supraspinal mechanisms of motor control begin developing prenatally. This includes the raphe nuclei that project into the lumbar spinal cord and is comprised of serotonin-containing neurons (Lakke, 1997; reviewed in Vinay et al., 2005; Ziskind-Conhaim et al., 1993). Although many studies have looked at the presence of 5-HT tracts in the spinal cord, few have examined the influence of these systems on behavior. However, one study has examined the 5-HT system and its influence on fetal locomotor behavior in spinal rats using the exteriorized uterus preparation discussed earlier (Brumley & Robinson, 2005). In this study E20 rats were treated with a mid-thoracic spinal transection or sham surgery while maintained in a saline bath. They were then IP injected with varying doses of quipazine (5-HT_{2A} agonist), CGS-12066A (5-HT_{1B} agonist), α-methylserotonin (5-HT_2 agonist), or saline and observed for a 10-min period. The different subtypes of 5-HT receptor agonists were found to induce different motor behaviors for spinal and sham fetuses. For example, quipazine induced more alternated stepping in spinal fetuses than shams, while CGS-12066A caused a significant increase in hindlimb steps in shams, but not spinal pups. Additionally, α-methylserotonin did not produce stepping in shams or spinal pups. This study provides evidence for the role of different 5-HT receptor subtypes in producing

different types of motor behaviors, as well as the presence of 5-HT mechanisms of locomotor behavior during prenatal development. Additional research has examined the effects of quipazine on locomotor stepping in the postnatal developing rat.

Spinal cord transection models that combine pharmacological treatments allow researchers to examine the effects of various neurotransmitter systems on motor behavior by isolating sections of the spinal cord. This helps inform which neurotransmitter systems influence various limb movements (i.e. forelimb vs hindlimbs). In developmental models these methods enable researchers to examine when and where these systems are likely developing. For example, one study examined the influence of both L-DOPA and quipazine on motor behavior in spinal and sham rat pups (McEwen et al., 1997). Rat pups received a mid-thoracic spinal cord transection or sham surgery on P4. On P5 pups were injected with L-DOPA (dopamine precursor) and quipazine, L-DOPA only, quipazine only, or vehicle control and evaluated for a 20-min test period while suspended in a harness from a horizontal bar. Pups that received vehicle control were relatively inactive during the testing period, regardless of surgery condition. Sham pups that received either L-DOPA or quipazine exhibited increased air-stepping with all four limbs. Spinal pups that received L-DOPA showed an increase in forelimb stepping only, while spinal pups injected with quipazine showed an increase in hindlimb stepping only. Additionally, hindlimb stepping was faster in pups that had received both L-DOPA and quipazine when compared with pups that had received only one of the two drugs. The results of this study suggest that dopamine and serotonin systems differentially influence locomotor behavior. Furthermore, the combination of the two appears to have an additive effect that contributes to a quadrupedal pattern of locomotion.

Effects of Sensory Feedback on Motor Behavior in Spinal Rat Pups

Apart from examining the effects of 5-HT system activation on motor behavior, quipazine can be a useful tool to induce motor behavior in order to examine the role of sensory feedback on the development of caudal mechanisms of motor behavior. In one study, quipazine was used to examine the influence of a perturbation on limb adaptations following a spinal cord transection (Strain et al., 2014). Rat pups underwent a low-thoracic spinal surgery or sham procedure on P1, then were injected with quipazine on P10 with a range of motion (ROM) restriction (i.e. a Plexiglas plate) or no restriction. Pups injected with quipazine adapted their stepping behavior in response (i.e. less hindlimb stepping) to the ROM restriction regardless of surgery condition. However, spinal pups that received ROM restriction showed a persistent response in that they maintain a flexed position following the removal of the Plexiglas. This research provides evidence for the plasticity of the spinal cord and its ability to adapt to environmental constraints in the absence of supraspinal inputs. This is important for research that examines recovery of function after SCI, since it shows that motor behavior is responsive to aspects of physical therapy that take mechanisms of sensory feedback into consideration.

Adult Rat Motor Functioning Following Spinal Cord Transection

The literature on adult rat motor functioning following a spinal cord transection is more extensive than that on developmental models. This is possibly since in humans SCI is more common in adults than it is in infants. Adult SCI models attempt to investigate the neurological and behavioral mechanisms of recovery following spinal trauma. The adult spinal cord mechanisms may not be as plastic as the mechanisms in the developing

spinal cord, however this system is far from fixed. Research with SCI models in adult rats shows that recovery of motor function can be achieved with the use of pharmacological treatments and motor training (Antri, et al, 2003; Antri et al., 2002; Sławińska, Majczyński, Dai, & Jordan, 2012; Tillakaratne et al., 2010). This recovery of function is not due to regrowth of axons across the lesion site, but often appears to be due to the plasticity of existing connections (Leszyńska, Majczyński, Wilczyński, Sławińska, & Cabaj, 2015). However, it is important to note that this is not a full recovery of function, since locomotor impairments persist.

Pharmacological Effects on Motor Functioning in Spinal Adult Rats

Pharmacological treatments in SCI models commonly examine the effects of neurotransmitter activation on recovery of motor function. 5-HT agonists in particular are used to activate the underlying neurological mechanisms mediating motor behavior. This activation can improve motor behavior (Antri et al., 2003; Antri et al., 2002; Sławińska et al., 2012). For example, in one study adult female rats were given a complete low-thoracic spinal cord transection or sham surgery and given chronic quipazine (or saline) administration via implanted osmotic pumps at the lumbar level (Antri, Orsal, & Barthe, 2002). Locomotor recovery was assessed via treadmill activity at various times during the month after the surgery. Spinal animals that had chronic administration of quipazine showed improved locomotor recovery (e.g. could support their own weight and walk) assessed by the Basso, Beatttie, and Bresnahan (BBB) motor scale compared to saline-treated subjects following a tail pinch. Similarly, chronic administration of the 5-HT_{1A} receptor agonist 8-OHDPAT increased locomotor recovery of treadmill stepping, even though 5-HT_1 receptor agonists have been shown to block fictive locomotion *in vitro*

(Cazalets et al., 1992). It is important to note as well, that evaluation of motor recovery was evaluated on a treadmill at multiple time points during a 1-month period. Thus, in addition to drug treatment rats received small time periods of motor training on treadmills. This could have contributed to their recovery since treadmill training has been shown to facilitate recovery after spinal cord transection (Cha et al., 2007). Additionally, posture has been shown to affect motor behavior in spinal rats.

Remarkably, if spinal adult rats are given a tail pinch while on a treadmill, being held in an upright posture (in contrast to horizontal posture) improves plantar stepping (Sławińska, Majczyński, Dai, & Jordan, 2012). This effect appears to be mediated by cutaneous and proprioceptive feedback, since lidocaine (anesthesia) administration to the paw impeded plantar stepping. Quipazine or 8-OHDPAT administration was not found to facilitate motor functioning for subjects held in an upright posture (and in fact impeded it for quipazine subjects). However, both were found to increase plantar walking (with body weight support) in response to a tail pinch while in the horizontal posture. Taken together, findings from this study indicate that locomotor stepping in rats occurs as a complex integration of multiple systems. These systems work in conjunction with one another to produce different motor behaviors. This is especially important for SCI research models since motor recovery following injury appears to work more efficiently in response to a combination of multiple therapies (e.g. pharmaceuticals + motor training) (Brumley, Guertin, & Taccola, 2017).

Learning in Spinal Adult Rats

The spinal cord has the capacity to learn responses to sensory stimuli, even without supraspinal input. This can be seen with classical and operant conditioning of

spinally mediated behaviors. A conditioning paradigm can be used to induce an antinociceptive response in rats that have been given an upper thoracic spinal transection (Patterson & Grau, 2001). The antinociceptive response is a tail flick in response to an intense shock. This antinociceptive response can be differentially conditioned in spinal rats. For example, if the tail shock is paired with stimulation of one of the limbs, both spinal and sham subject's exhibit increased antinociceptive responses (longer tail flick latencies) to stimulation of the hindlimb alone. Additionally, if spinal rats are given a shock to one hindlimb when it is extended they will exhibit longer flexions of that hindlimb compared to controls (Grau et al., 1998). However, use of an NDMA receptor antagonist eliminates this learned flexion response, which suggests that central plasticity is necessary for this learning response (Joynes et al., 2004). The occurrence of habituation and learning that takes place in SCI animal models indicates that the mechanisms supporting associative and non-associative learning are present within the rat spinal cord.

5-HT UPREGULATION IN SPINAL CORD INJURY MODELS

Immunohistochemistry use in SCI research is useful for investigating some biological changes that can occur following injury. This review will focus mainly on the regulation of 5-HT receptors in the rat spinal cord following transection, since examination of the density and distribution of 5-HT_{2A} receptors in the neonatal lumbar spinal cord was conducted in the current study. Cells produce receptors; however receptors have a life cycle and can be reprocessed by the cell (Sherwood, 2004). This reduces the amount of that cell's receptors thereby decreasing sensitivity of responsiveness to ligands. This process is called downregulation. Conversely, if a cell is

receiving a weak signal it can respond by producing more receptors, which can increase sensitivity to ligands. This is referred to as upregulation. Upregulation of receptors in the spinal cord following injury has been observed in rats by using microscopy techniques (Kong et al., 2010; Kong et al., 2011). Receptor upregulation may be due to the absence of presynaptic communication, as a consequence of the abolishment of many neurotransmitter-releasing cells from the transection surgery.

There is little immunohistochemistry research that has been done in examining the regulation of receptors in the SCI neonatal rat model. However, there is research that has examined density and distribution of 5-HT_{2A} receptors in the spinal cord of adult rats. 5-HT_{2A} receptors have been implicated as being important in locomotor recovery following SCI, and 5-HT_{2A} antagonists have been found to impede recovery (Saruhashi, Hukuda, & Maeda, 1991). In one study, sacral (S2) spinal cord transections or sham surgeries were performed on adult rats and S4 segments were observed over a 2-month period (Kong et al., 2010). Immunohistochemistry (ABC peroxidase and immunofluorescence) analyses revealed that the density of 5-HT_{2A} receptors was greater for spinal rats when compared with shams. An additional study corroborated these results and showed that 5-HT_{2A} receptor upregulation occurred in the ventral and dorsal horns of the sacral (S4) spinal cord (Kong et al, 2011). Also, a time course for upregulation was analyzed where upregulation was present the day after the transection and continued until the 4^{th} week, where it plateaued. Similar results have been found for 5-HT_{2C} receptors in the sacral spinal cord following transection (Ren et al., 2013). Lee et al. (2007) found a moderate increase in 5-HT_{2A} receptors in the lumbar (L5-L6) region following T8 contusion (i.e. not complete injury). Upregulation of 5-HT_{1A} receptors (concentrated in

the dorsal horn) has also been observed in the lumbar (L3-L5) sections of spinal transected rats (Otoshi et al., 2009). Furthermore, the upregulation of 5-HT receptors following SCI is not confined to the sacral and lumbar segments, but has been observed in the cervical spinal cord segments as well.

5-HT receptors in the cervical spinal cord are necessary for phrenic motor output. Cervical injury damages the activation of the phrenic nerve inhibiting respiratory responses. In one study, rats were given a cervical (C2) hemisection or sham operation (Fuller et al., 2005). Expression of C4 5-HT_{2A} receptors was then analyzed using immunostaining techniques. 5-HT_{2A} receptors were found to be concentrated in the ventral spinal cord, regardless of surgery condition, but was denser in the surgery condition compared to shams. This upregulation is thought to be one of the neurological processes that is mediating the spontaneous respiratory recovery that is observed in the 2 months following SCI. Furthermore, rats that have had a complete thoracic spinal cord transection show an increase in 5-HT_{2A} receptor gene expression on the lumbar flexor and extensor motoneurons (Chopek, Sheppard, Gardiner, & Gardiner, 2015). This expression is further increased on the extensor motoneurons following 3 months of daily passive cycling. Additionally, an increase of 5-HT fibers and terminals in the lumbar segment has been found to be correlated with locomotor recovery following a low-thoracic chronic spinal cord transection (Saruhashi, Young, & Perkins, 1996). Taken together, these studies provide evidence that examining 5-HT receptors in relation to locomotor activity is informative in SCI models and can further our understanding of the mechanisms that influence recovery following spinal cord trauma. However, these processes are not well understood. More research is needed to better understand these

processes, particularly as these relate to behavioral motor function and plasticity. Research examining any changes in amounts of 5-HT receptors in the spinal cord of neonates is particularly lacking, but could be greatly beneficial to studies of basic developmental processes, adult and infant SCI models, and developmental neurological disorders.

CURRENT STUDY

The purpose of the current study was to examine locomotor stepping and serotonin receptor plasticity in intact and spinal developing rats. In Experiment 1 quipazine-induced stepping was observed in P5 and P10 rat pups that had a low-thoracic spinal transection or sham surgery on P1 or P5. In Experiment 2, immunohistochemistry was used to examine the density of 5-HT_{2A} receptors in the lumbar spinal cord to determine if upregulation occurred in spinal vs. sham rat pups. Furthermore, density of 5-HT_{2A} receptors in the lumbar spinal cord of spinal subjects was compared between the different surgery age conditions to determine if upregulation increased over time.

Although some studies have examined the effects of quipazine on stepping in neonatal and adult rats, to our knowledge this is the first to examine developmental changes in locomotor activity in spinal neonatal rats between time of surgery and time of testing. Thus, we were able to examine whether coordinated stepping is altered as a function of age and/or time since injury. Additionally, to our knowledge there are no studies examining the density of 5-HT receptors in the neonatal lumbar spinal cord following a low-thoracic transection. We chose the subtype 5-HT_{2A} receptors because (1) upregulation of this subtype has been observed in adult rats following SCI (Fuller et al., 2005; Kong et al., & Zhang, 2011; Kong et al., 2010; Lee et al., 2007), and (2) this

receptor subtype is associated with recovery of function following SCI in rats (Saruhashi et al., 1991). We also chose to focus on expression in lamina IX and the ventral funiculi of ventral horn of the lumbar region because 5-HT_2 receptors are primarily found in the ventral horn and on motoneurons (Marlier, Teilhac, Cerruti, & Privat, 1991). Because neonatal transected rats show increased recovery of locomotor functioning following SCI when compared to adult transected rats (Yuan, Su, Chiu, Wu, & Lin, 2013; Weber & Stelzner, 1977), we were interested in determining if upregulation of 5-HT_{2A} receptors occurs along with this response. Furthermore, we had preliminary data examining alternated stepping in 11 P5s and 6 P10s that underwent spinal transection on P1. Our preliminary data showed that there was an increase in sensitivity (i.e. increased alternated stepping) to quipazine for spinal pups when compared to shams. Immunohistochemistry confirmed that this was associated with upregulation of 5-HT_{2A} receptors. While 5-HT_{2A} receptor upregulation has been observed in the sacral segments of the spinal cord following complete SCI, this project focused on immunohistocological data for the lumbar segments. This is also a novel aspect of the proposed study, as 5-HT_{2A} receptor upregulation has yet to be observed (to our knowledge) in the complete transected lumbar spinal cord. Another justification for examining the lumbar segments in this study is that fictive locomotion of alternating patterns of flexors and extensors are observed in the lumbar segments (Cazalets, Squalli-Houssaini, & Clarac, 1992; Kiehn & Kjaerulff, 1996; Pearlstein et al., 2005) and Experiment 1 will examine alternated stepping. Thus, observations of locomotor behavior can be complemented with a neurological mechanism of locomotion.

EXPERIMENT 1: LOCOMOTOR STEPPING

For Experiment 1, the first hypothesis was that there would be developmental differences in quipazine-induced hindlimb alternated stepping (analyzed as a percentage of alternated steps as a function of overall hindlimb movements) between P5 and P10 rats, regardless of surgery or drug condition. The justification for this hypothesis was that for preliminary data we had observed an increase in quipazine-induced alternated stepping for P10s compared to P5s. The second hypothesis of Experiment 1 was that there would be a significant increase in the frequency of hindlimb alternated steps for spinal pups compared to shams. As previously mentioned, the justification for this was that our preliminary data showed that individual spinal pups showed an increase in alternated stepping when compared to shams. This is also why we chose total hindlimb movements, alternated hindlimb steps, and percentage of alternated hindlimb steps as our dependent variables. In past research, we have found that P1s treated with quipazine show increased alternated hindlimb steps compared to P10s, but that P10s show a higher percentage of alternated steps (Strain & Brumley, 2014). Thus, we wanted to parse apart hindlimb activity and coordination. We also hypothesized that frequency of stepping between ages would be influenced by time of surgery for spinal pups, due to possible upregulation of 5-HT receptors in the lumbar spinal cord. The logic behind this was that if surgery is taking place earlier (on P1) and upregulation is present following surgery and then continues, as it does in adult rats (Kong et al., 2011), then P10 subjects that have had the transection surgery earlier (on P1) will have an increase in sensitivity to quipazine when compared to P10 subjects that have had a transection on P5. Furthermore, P10s will have had more recovery time and have more sensory feedback

prior to testing than P5s. Following this logic, P10s that have had the transection surgery on P1 would show an increased frequency of alternated stepping, compared to P5s that have had a transection on P1.

The justification for the choice of ages for surgery and testing is based on past research using rat pups. First, Strain & Brumley (2014) showed that quipazine produces a robust stepping response in P1 and P10 rat pups. However, there appears to be an age effect of quipazine, where it wears off sooner for P10 pups and frequency of alternated stepping slows over the testing period. Thus, we determined that P10 would be the maximum age of testing. We also used comparable methods to studies performing a spinal transection on P1 (Strain et al., 2014), P2 and P3 (Kao et al., 2011), P4 (McEwen et al., 1997), and P5 (Ichiyama et al, 2011). These studies used hypothermia to anesthetize pups, however as pups increase in age hypothermia is less effective as an anesthetic. Thus, we chose P5 as the maximum surgery age, to be able to use consistent methods across developmental age.

Methods

Subjects

Subjects were 72 male Sprague-Dawley rat pups: 24 tested at P5, and 48 tested at P10. Adult animals were obtained from the animal facility at Idaho State University (ISU) and bred in the Brumley Laboratory. Pregnant females were housed in pairs until 2 days before they gave birth, after which they were housed individually. Animals were kept in a temperature-controlled room on a 12 hr light: 12 hr dark cycle and provided ad libitum food and water. All animals were cared for and used in accordance with

guidelines established by the National Institutes of Health, and by the Institutional Animal Care and Use Committee at ISU.

Experimental Design

A total of 6 subjects were tested in each of 12 groups. Previous research using similar methods have also used a sample size of 6 (Strain et al., 2014). Group characteristics are outlined in Table 1. Subjects received a complete low thoracic spinal cord transection surgery or sham spinal surgery on P1 or P5. Pups within the same litter received the same type of surgery in order to control for maternal behavior. Litters were culled to 6 pups on P1. Subjects receiving a spinal cord surgery on P1 were tested on P5 or P10, and treated with quipazine or saline. Subjects receiving a spinal cord surgery on P5 were tested on P10, and treated with quipazine or saline. Subjects within the same group were selected from different litters to control for litter effects. Only males were used to control for possible sex differences.

Characteristics of Groups in Experiment 1

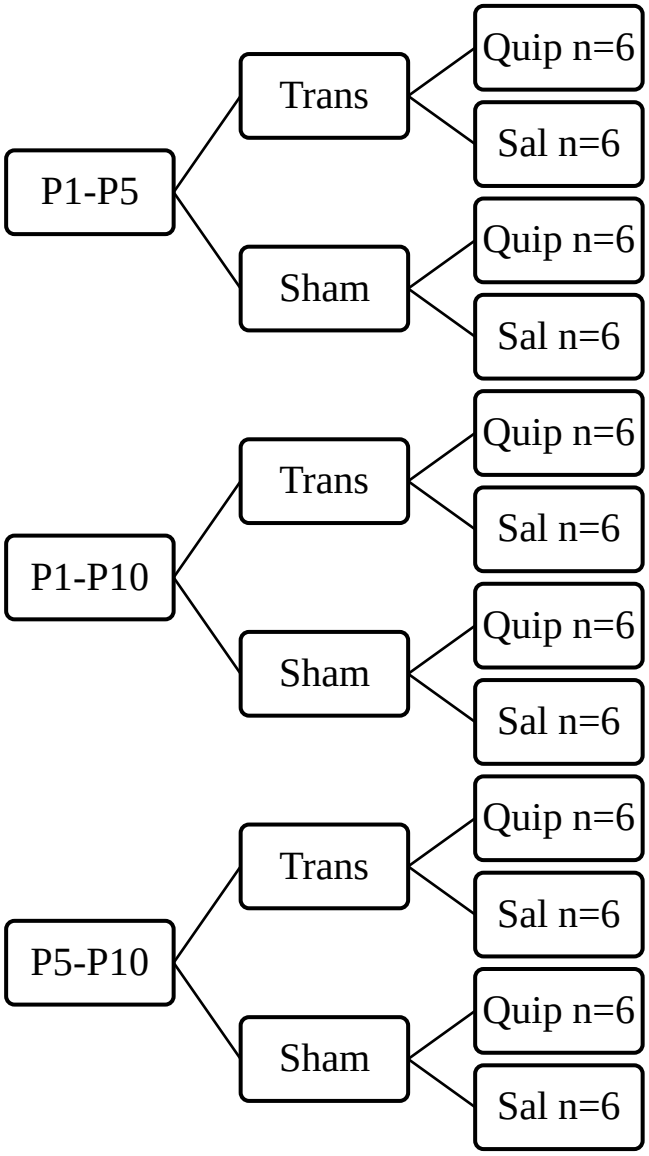


Figure 1. Flowchart of Experiment 1 design. Subjects were categorized into three groups based on surgery age and age of test surgery conditions (i.e. P1-P5, P1-P10, and P5-P10). Within those three groups subjects were assigned to transection or sham surgery. Within the surgery conditions subjects were injected with quipazine or saline treatment during testing. A total of 12 groups was used in the design with n=6.

Spinal Surgery

Spinal cord transection or sham surgeries were performed on P1 or P5, and followed the methods of Strain et al. (2014). Subjects were removed from their home cage dam, manually voided with a soft paintbrush prior to surgery, and were only selected if they had recently fed (evident by the presence of a milk band) and appeared healthy (i.e. pink in color, typical in size and activity, and no apparent damage). Subjects were then anesthetized via hypothermia. After the subject was anesthetized (evident by shallow breathing in the lower abdomen) the back was prepared with betadine. An incision was made from the mid-back to the shoulders. The fat pad was rolled up, exposing the artery that crosses over the vertebral thoracic level 6 (T6). This was used as a landmark to count the vertebrae in order to find vertebra T10. At T10, a partial laminectomy was performed to expose the spinal cord. Irridectomy scissors were inserted into the midline of the spinal cord at one spinal level between T8-T10 for the subjects in the transection group. The scissors were opened and closed mediolaterally, cutting the cord, then moved laterally on both sides of the cord to ensure all fibers were cut (Kao et al., 2011). A collagen matrix was then injected into the transection site. The cut muscle lateral to the cord was sutured to cover the transection site. The back-skin incision was then sutured. Surgery for sham subjects identical except the spinal cord was not cut and no collagen was injected.

Subjects were given a subcutaneous hip injection of both 0.9% (wt/vol) surgical saline and Buprenex SQ (0.1 ml of 0.04 mg/kg solution) following surgery. Subjects were then placed in a plastic container with their littermates in an infant incubator to recover. Subjects were returned to the dam after recovery from anesthesia (evidenced by pink

coloring and increased motor activity). They remained with the dam until the day of testing on P5 or P10 in an opaque cage. Pups from the same litter received the same type of spinal surgery in order to ensure the dam would take care of them equally. Pups were checked daily for possible infections or complications resulting from the surgery. Saline injections were given as needed to assist with hydration and weight gain.

Behavioral Testing

Behavioral testing happened on P5 or P10. Subjects were healthy and had fed recently, as evidenced by a milk band across the abdomen. Subjects had to meet a minimum weight cut-off of three standard deviations from the average body weight for a P5 or P10 rat pup (i.e. 8.46 g for P5s and 15.46 g for P10s) to be included in the study. Subjects showing infection or illness (total 3) were euthanized. On the day of testing, subjects were transferred from their home cage into an incubator with a temperature of 30°- 33°C (33° at P5; 30° at P10). They were manually voided using a small paintbrush, to standardize the time since last bladder expression. Subjects were placed in a small plastic dish with 2 littermates to allow acclimation to incubator conditions for 30 minutes. To begin testing, subjects were placed in a fitted jacket that supports the neck and abdomen, but leaves the limbs free for movement. Subjects were fitted into a jacket appropriately sized for their age group. They were attached in a prone position to a vinyl covered horizontal bar, to allow the limbs to hang pendently.

After a 5-minute baseline, subjects were given an IP injection of quipazine (3.0 mg/kg) or saline, with volume of injection based on weight (i.e. 25 µl for every 5 grams of body weight). Subjects were then recorded from a ventral camera angle for a 30-

minute test (post-injection) period onto DVD for later playback and scoring. Subjects were euthanized via CO_2 immediately following behavioral testing.

Behavioral Scoring

Behavior was scored using JWatcher, which is a real-time event-recording program that categorizes behavior and the time it is expressed (± 0.01s). The scorer was blind to pup group association. A 90% inter-rater reliability rate with a standardized scoring video file was required before scoring pup behavior. Scoring of stepping behavior was done during DVD playback of the ventral camera view of the baseline and test session. All hindlimb movements, (including alternated stepping) were scored (Brumley et al., 2012). Alternated stepping is defined as the sequential extension and flexion in one limb followed by the sequential extension and flexion in the homologous limb.

Histology

After testing all subjects were euthanized and preserved in 10% (wt/vol) formalin (Brumley & Robinson, 2005). Subjects were later examined under low magnification to verify complete spinal cord separation between the spinal levels of T8 and T10 for spinal transected pups, or a fully intact spinal cord for sham pups. Pups not meeting these criteria (total of 12) were excluded from the study.

Data Analysis

Analyses examined group differences in frequencies of hindlimb alternated steps and total hindlimb movements (the combined sum of all hindlimb movements). Alternated steps also were analyzed as a percentage of total hindlimb movements. Hindlimb stepping was analyzed during the 35-minute session (5-minute baseline and 30-minute post-injection period). Dependent variables were frequency of total hindlimb movements, alternated steps, and percentage of alternated steps calculated as a function

of overall hindlimb movements. Each variable was summarized into 5-minute time bins. Independent variables were surgery condition (spinal or sham), drug (quipazine or saline), age of surgery (P1 or P5), and age of testing (P5 or P10). Data were analyzed usinga 3-way ANOVA with repeated measures with surgery condition, drug condition, age of surgery, and age of testing as between subjects variables and time as the within subjects (repeated measure) variable. Post hoc analyses were performed using Tukey's HSD. Data was analyzed using StatPlus statistical software. A significance level of 5% was adopted for all tests.

EXPERIMENT 1 RESULTS

Effect of Age of Testing on Hindlimb Behavior

Analyses examined group differences between time of testing age conditions for subjects that received spinal cord transection or sham surgery on P1 and were tested on P5 (P1-P5) or P10 (P1-P10). Omnibus analyses were first done for each movement category (total hindlimb movements, alternated hindlimb steps, and percentage of alternated steps) using a repeated measures ANOVA (TestAge*Drug*Surgery*Time), with age of test, drug, and surgery as between subjects variables and time as the within subjects (repeated measures) variable. All significant main effects and interactions are reported. Non-significant effects were $p > 0.05$. Follow-up tests were conducted as needed.

Total Hindlimb Movements

For frequency of total hindlimb movements for subjects that underwent surgery on P1, test age approached significance [$F(1, 40) = 4.04$, $p = 0.051$], and there were main

effects of drug [$F(1, 40) = 26.6$, $p < 0.001$], surgery [$F(1, 40) = 9.96$, $p = 0.003$], and time [$F(6, 240) = 14.36$, $p < 0.001$]. There were 2-way interactions between test age and drug [$F(1, 40) = 7.32$, $p = 0.01$], drug and surgery condition [$F(1, 40) = 9.53$, $p = 0.004$], drug and time [$F(6, 240) = 11.92$, $p < 0.001$], and surgery condition and time [$F(6, 240) = 11.92$, $p < 0.001$]. Also, there were 3-way interactions between test age, drug, and time [$F(6, 240) = 2.66$, $p = 0.02$], drug, surgery, and time [$F(6, 240) = 7.11$, $p < 0.001$]. Follow-up analyses for test age showed that while subjects tested on P10 showed more hindlimb movements (M=90.12, SD = 113.3) than subjects tested on P5 (M = 52.22, SD = 71.73), this was not significant [$F(1, 47) = 1.92$, $p = 0.173$]. Follow-up analyses for the main effects revealed that quipazine-treated subjects showed significantly more hindlimb movements than saline-treated subjects, spinal subjects showed significantly more hindlimb movements than shams, and that hindlimb movements significantly increased after baseline (T5-T30). Figure 2A and 2B shows the number of total hindlimb movements for subjects that underwent surgery on P1 and were tested on P5 and P10, respectively.

Follow-up analyses of the 2-way interaction between test age and drug revealed that P10 quipazine-treated subjects showed significantly more hindlimb movements than test age P10 saline-treated subjects, P5 quipazine-treated subjects, and P5 saline-treated subjects. Follow-up analyses for the 2-way interaction between drug and surgery revealed that for quipazine-treated subjects, spinal subjects showed significantly more hindlimb movements than sham subjects. Follow-up analyses for the 2-way interaction of drug and time showed that hindlimb movements increased significantly for quipazine-treated subjects compared to saline-treated subjects after baseline (T5-T30). For the interaction

of surgery and time, sham subjects showed significantly more hindlimb movements than spinal subjects during baseline, but spinal subjects showed significantly more hindlimb movements towards the end of the test session (T25-T30).

To interpret the 3-way interaction between test age, drug, and time, drug and time were examined within each of the test ages. For P5, quipazine-treated subjects showed a significant increase in total hindlimb movements compared to saline-treated subjects at the end of the session (T30). For test age P10, quipazine-treated subjects showed significantly more hindlimb movements for every time point following the baseline (T5-T30). When test age and time were examined within the two drug conditions, no significant differences were found in hindlimb movements between the two test ages for any of the time points (all *p*s > 0.05). However for saline-treated subjects, test-age P5 subjects showed more hindlimb movements (M = 29.41, SD = 30.96) compared to P10 subjects at T25 of the test session test (M = 11.25, SD = 5.53), but this trend only approached significance [$F(1, 23) = 4.0$, $p = 0.058$].

For quipazine-treated subjects, P10 subjects showed more hindlimb movements (M = 227.67, SD = 192.1) compared to P5 subjects (M = 94.08, SD = 133.94) at T25 of the test session, but this trend only approached significance [$F(1, 23) = 3.91$, $p = 0.06$].

To interpret the second 3-way interaction between drug, surgery, and time, surgery and time were examined within the two drug conditions. For quipazine-treated subjects, those treated with a spinal transection showed a significant increase in total hindlimb movements compared to shams during the second half of the test session (T20-T30). Next drug and time were examined within the two surgery conditions. For shams, quipazine-treated subjects showed a significant in hindlimb movements compared to

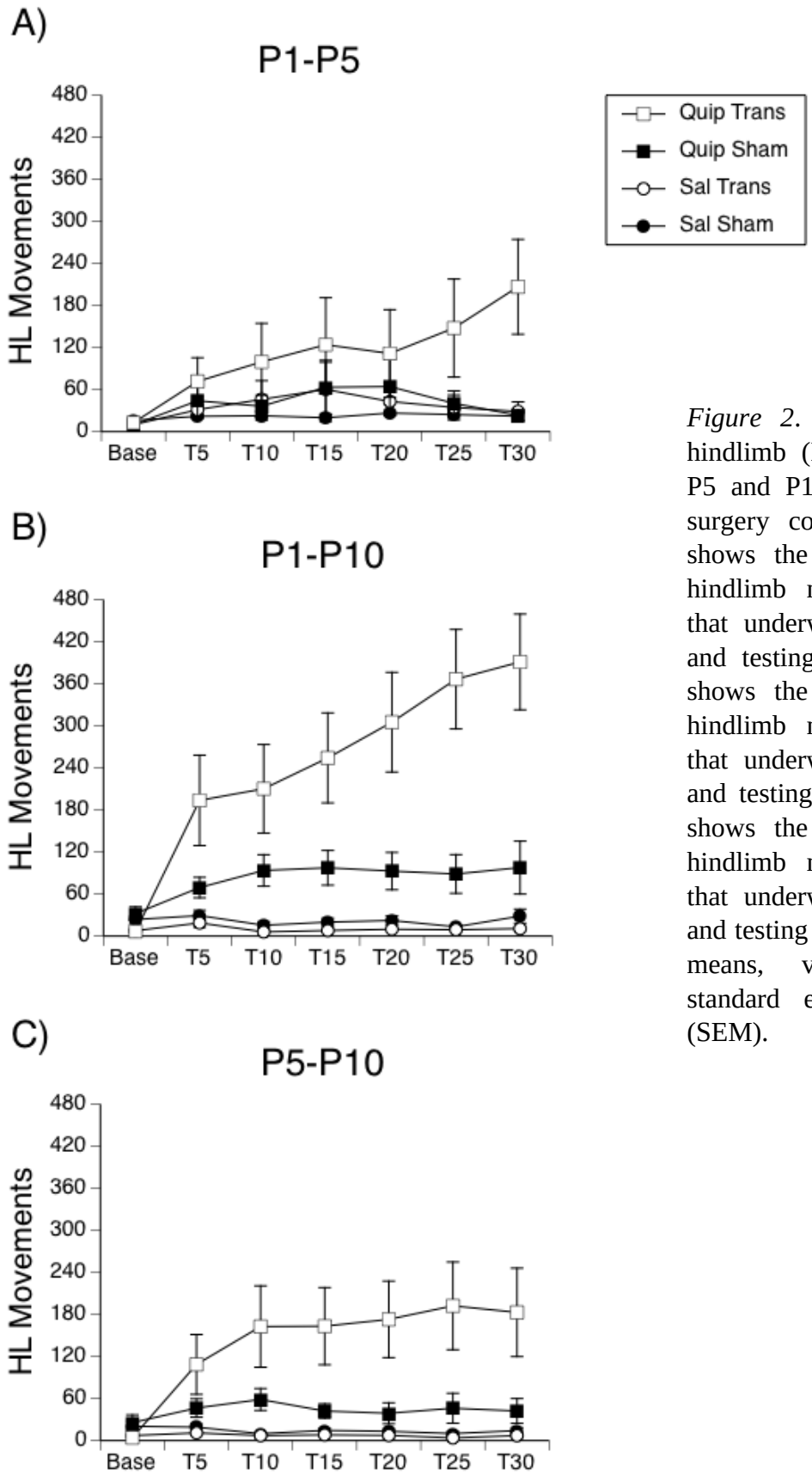


Figure 2. Frequency of total hindlimb (HL) movements for P5 and P10 rats by drug and surgery condition. Graph (A) shows the frequency of total hindlimb movements for rats that underwent surgery on P1 and testing on P5. Graph (B) shows the frequency of total hindlimb movements for rats that underwent surgery on P1 and testing on P10. Graph (C) shows the frequency of total hindlimb movements for rats that underwent surgery on P5 and testing on P10. Points show means, vertical lines are standard error of the mean (SEM).

saline-treated subjects at T15-T25 of the test session. For spinal subjects, those treated with quipazine showed significantly more hindlimb movements compared to those treated with saline for all time points following the baseline (T5-T30).

Alternated Hindlimb Steps

For frequency of alternated hindlimb steps for subjects that underwent surgery on P1, test age approached significance [$F(1, 40) = 3.75$, $p = 0.06$], and there were main effects of drug [$F(1, 40) = 26.7$, $p < 0.001$], surgery [$F(1, 40) = 7.54$, $p = 0.009$], and time [$F(6, 240) = 10.71$, $p < 0.001$]. There were 2-way interactions between test age and drug [$F(1, 40) = 4.74$, $p = 0.04$], drug and surgery condition [$F(1, 40) = 7.34$, $p = 0.01$], drug and time [$F(6, 240) = 9.63$, $p < 0.001$], and surgery condition and time [$F(6, 240) = 4.01$, $p < 0.001$]. Also, there was a 3-way interaction between drug, surgery, and time [$F(6, 240) = 7.11$, $p < 0.001$]. Follow-up analyses for test age showed that while subjects tested on P10 showed more alternated steps (M=55.79, SD = 85.04) than subjects tested on P5 (M = 26.89, SD = 55.62), this trend was not significant [$F(1, 47) = 1.94$, $p = 0.17$], Follow-up analyses for the main effects revealed that quipazine-treated subjects showed significantly more alternated steps than saline-treated subjects, spinal subjects showed significantly more alternated steps than shams, and alternated steps significantly increased after baseline (T5-T30).

Follow-up analyses of the 2-way interaction between test age and drug revealed that for test age P10s subjects, quipazine-treated subjects showed significantly more alternated steps compared to saline-treated subjects, and that among quipazine-treated subjects, test age P10s showed significantly more alternated steps than test age P5s. Figure 3A and 3B shows the number of alternated hindlimb steps for subjects that

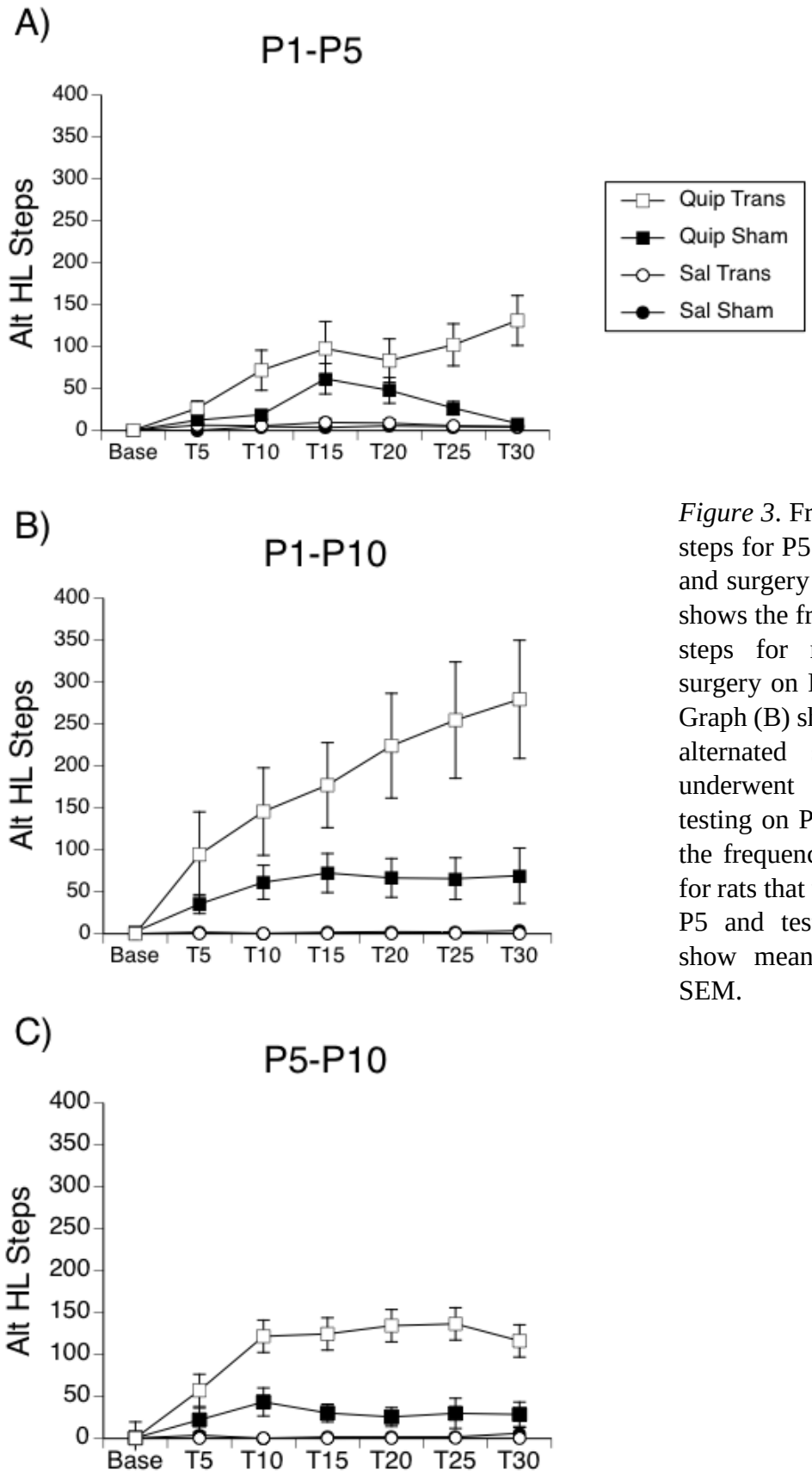


Figure 3. Frequency of alternated steps for P5 and P10 rats by drug and surgery condition. Graph (A) shows the frequency of alternated steps for rats that underwent surgery on P1 and testing on P5. Graph (B) shows the frequency of alternated steps for rats that underwent surgery on P1 and testing on P10. Graph (C) shows the frequency of alternated steps for rats that underwent surgery on P5 and testing on P10. Points show means, vertical lines are SEM.

underwent surgery on P1 and were tested on P5 or P10. Follow-up analyses for the 2-way interaction between drug and surgery revealed that for quipazine-treated subjects, those treated with a spinal cord transection showed significantly more alternated steps than shams. Follow-up analyses for the 2-way interaction of drug and time showed that alternated steps increased significantly for quipazine-treated subjects compared to saline-treated subjects after baseline (T5-T30). For the interaction of surgery and time, spinal subjects showed significantly more alternated steps towards the end of the test session (T25-T30). To interpret the 3-way interaction between drug, surgery, and time, surgery and time were examined within the two drug conditions. Follow-up analyses revealed that there were no differences in alternated steps between surgery conditions for quipazine or saline-treated subjects for any of the time points (all *p*s > 0.05). Drug and time were examined within the two surgery conditions. For shams, those treated with quipazine significantly increased alternated hindlimb steps compared to those treated with saline between T5-T25 of the test session. For spinal subjects, those treated with quipazine showed significantly more alternated steps than those treated with saline across all time points following the baseline (T5-T30).

Percent Alternated Hindlimb Steps

For percent alternated hindlimb steps for subjects that underwent surgery on P1, there were main effects of age of test age [$F(1, 40) = 74.45$, $p = 0.05$], drug [$F(1, 40) = 27.94$, $p < 0.001$] and time [$F(6, 240) = 29.16$, $p < 0.001$]. There were 2-way interactions between test age and drug [$F(1, 40) = 8.74$, $p = 0.01$] and drug and time [$F(6, 240) = 19.62$, $p < 0.001$]. Follow-up analyses for the main effect of test age showed that test age P10 subjects showed a significantly higher percent alternated steps compared to test age

P5s. Figure 4A and 4B shows the percent alternated steps for subjects that underwent surgery on P1 and were tested on P5 or P10. Follow-up analyses for the main effects of drug and time revealed that quipazine-treated subjects showed a significantly higher percent alternated steps compared to saline-treated subjects and that percent alternated steps increased significantly after baseline, respectively. Follow-up analyses for the 2-way interaction between test age and drug showed test age P10 subjects treated with quipazine showed a significantly higher percent alternated steps compared P5 subjects treated with quipazine. Quipazine increased percent alternated steps for spinal test age P5 subjects compared to sham subjects and saline-treated subjects. Follow-up analyses for the interaction of drug and time revealed that quipazine-treated subjects significantly increased in their percent alternated steps compared to saline-treated subjects for all time points following baseline (T5-T30).

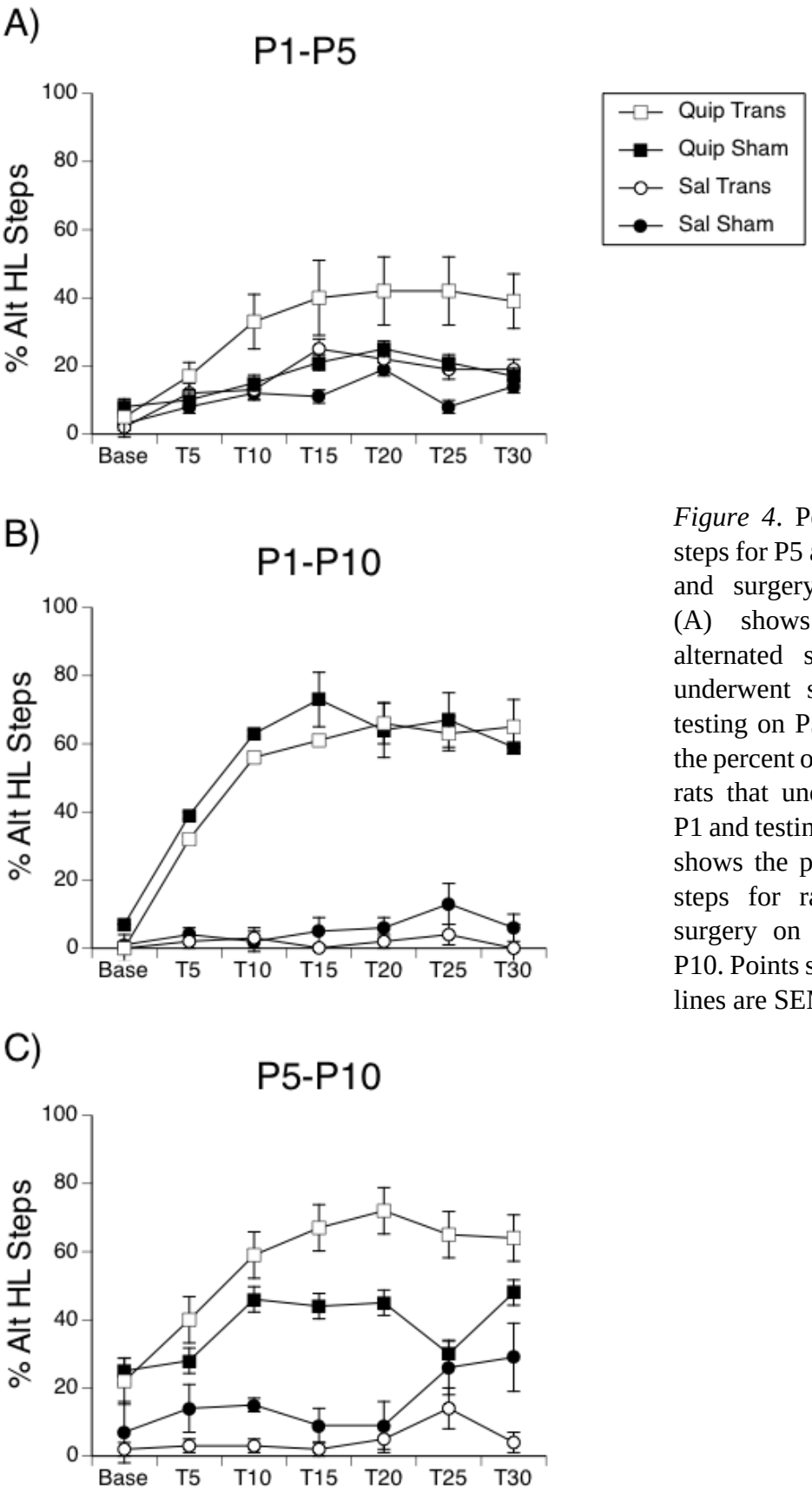


Figure 4. Percent of alternated steps for P5 and P10 rats by drug and surgery condition. Graph (A) shows the percent of alternated steps for rats that underwent surgery on P1 and testing on P5. Graph (B) shows the percent of alternated steps for rats that underwent surgery on P1 and testing on P10. Graph (C) shows the percent of alternated steps for rats that underwent surgery on P5 and testing on P10. Points show means, vertical lines are SEM.

Effect of Age of Surgery on Hindlimb Behavior

Analyses examined group differences of hindlimb behavior between subjects that received spinal cord transection or sham on P1 (P1-P10) or P5 (P5-P10) and were tested on P10. Analyses examined group differences in frequencies of total hindlimb movements and alternated steps. Alternated steps were analyzed as a percentage of total movements as described previously.

Total Hindlimb Movements

For frequency of total hindlimb movements for subjects that underwent testing on P10 there were main effects of surgery age [$F(1, 40) = 4.73$, $p = 0.04$], drug [$F(1, 40) = 41.64$, $p < 0.001$], surgery condition [$F(1, 40) = 11.82$, $p = 0.001$], and time [$F(6, 240) = 16.16$, $p < 0.001$]. There were 2-way interactions between surgery age and drug that approached significance [$F(1, 40) = 3.59$, $p = 0.066$], drug and surgery condition [$F(1, 40) = 15.74$, $p < 0.001$], drug and time [$F(6, 240) = 25.8$, $p < 0.001$], and surgery condition and time [$F(6, 240) = 15.84$, $p < 0.001$]. Also, there were 3-way interactions between surgery age, drug, and time [$F(6, 240) = 4.13$, $p = 0.05$], drug, surgery, and time [$F(6, 240) = 15.12$, $p < 0.001$]. Follow-up analyses for the main effect of surgery age revealed that while P1s showed a higher frequency of total hindlimb movements (M = 90.12, SD = 113.3) than P5s (M = 51.47, SD = 77.82), this trend was not significant [$F(1, 47) = 1.89$, $p = 0.18$]. Follow-up analyses for the main effects revealed that quipazine-treated subjects showed significantly more hindlimb movements than saline-treated subjects, that spinal subjects showed significantly more hindlimb movements than sham subjects, and that hindlimb movements increased significantly after baseline.

Follow-up analyses for the 2-way interaction between surgery age and drug revealed that for quipazine-treated subjects, P1s showed significantly more hindlimb movements when compared to P5s, and that for both surgery ages quipazine-treated subjects showed significantly more hindlimb movements compared to saline-treated subjects. Follow-up analyses for the 2-way interaction between drug and surgery condition revealed that spinal subjects treated with quipazine showed significantly more hindlimb movements than sham subjects treated with quipazine and spinal subjects treated with saline. Follow-up analyses for the 2-way interaction between drug and time showed that hindlimb movements significantly increased for quipazine-treated subjects for all time points following baseline (T5-T30). Follow-up analyses for the 2-way interaction of surgery condition and time showed that sham subjects had significantly more hindlimb movements than spinal subjects during baseline, but that spinal subjects had significantly more hindlimb movements during T15-T30 of the test session.

To interpret the 3-way interaction between surgery age, drug, and surgery condition, drug and surgery condition were examined within each surgery age. Figure 2B and 2C shows the number of total hindlimb movements for subjects that underwent surgery on P1 and P5 and were tested on P10. For quipazine-treated surgery age P1s, those treated with spinal cord transection showed significantly more hindlimb movements than quipazine-treated shams. For spinal surgery age P1s, those treated with quipazine showed significantly more hindlimb movements than those treated with saline. For quipazine-treated surgery age P5s, those treated with spinal cord transection showed significantly more hindlimb movements than shams. For spinal P5s, those treated with quipazine showed significantly more hindlimb movements than those treated with saline.

Additionally, sham P1s that were treated with saline showed significantly more hindlimb movements compared to spinal P1s that were treated with saline. For quipazine-treated surgery age P1s, those that were treated with spinal cord transection showed significantly more hindlimb movements than quipazine-treated surgery age P5 spinal subjects.

To interpret the second 3-way interaction between surgery age, drug, and time, drug and time were examined within the two surgery age conditions. For both surgery age P1s and P5s quipazine-treated subjects showed significantly more hindlimb movements for all time points following baseline (T5-T30).

To interpret the third 3-way interaction between drug, surgery condition, and time, surgery condition and time were examined within the two drug conditions. Shams treated with saline showed significantly more hindlimb movements than spinal subjects treated with saline at baseline, and between T10-T30 of the test session. For quipazine-treated subjects, shams showed significantly more hindlimb movements during baseline compared to those treated with spinal cord transection. However, quipazine-treatment increased hindlimb movements for those treated with spinal cord transection compared to shams for every time point following baseline (T5-T30). When drug and time were examined within the two surgery conditions, for both shams and spinals, quipazine-treated subjects significantly increased hindlimb movements compared to saline-treated subjects for every time point following baseline (T5-T30).

Alternated Hindlimb Steps

For frequency of alternated hindlimb steps for subjects that underwent testing on P10 there were main effects of drug [$F(1, 40) = 36.42$, $p < 0.001$], surgery [$F(1, 40) =$

10.6, $p = 0.002$], and time [$F(6, 240) = 12.96$, $p < 0.001$]. There were 2-way interactions between drug and surgery condition [$F(1, 40) = 11.42$, $p = 0.002$], drug and time [$F(6, 240) = 12.49$, $p < 0.001$], and surgery condition and time [$F(6, 240) = 4.87$, $p < 0.001$]. Also, there was a 3-way interaction between surgery age, drug, and time that approached significance [$F(6, 240) = 2.01$, $p = 0.065$] and a 3-way interaction between drug, surgery, and time [$F(6, 240) = 5.15$, $p < 0.001$]. Figure 3B and 3C shows the number of total alternated steps for subjects that underwent surgery on P1 and P5 and were tested on P10. Follow-up analyses for the main effects revealed that quipazine-treated subjects showed significantly more alternated steps than saline-treated subjects, spinal subjects showed significantly more alternated steps than shams, and that alternated steps significantly increased for all time points following baseline (T5-T30).

Follow-up analyses for the 2-way interaction between drug and surgery condition revealed that spinal subjects treated with quipazine showed significantly more alternated steps than sham subjects treated with quipazine and spinal subjects treated with saline. Follow-up analyses for the 2-way interaction between drug and time showed that quipazine-treated subjects significantly increased in alternated stepping compared to saline-treated subjects for every time point following baseline (T5-T30). Follow-up analyses for the 2-way interaction of surgery and time showed that spinal subjects had a significant increase in alternated stepping compared to shams at T20-T30 of the test session.

To examine the 3-way interaction between surgery age, drug, and time, drug and time were examined within each of the two surgery ages. Quipazine-treatment significantly increased alternated steps for P1s and P5s for all time points following

baseline (T5-T30). When examining surgery age and time within the two drug conditions for quipazine-treated surgery age P1s showed significantly more alternated steps than surgery age P5s at T25-T30 of the test session.

To examine the second 3-way interaction between drug, surgery condition, and time, surgery and time were examined within the two drug conditions. For quipazine-treated subjects those treated with spinal cord transection showed more alternated steps compared to shams for all time points following baseline (T5-T30). When examining drug and time within the two surgery conditions, quipazine-treatment significantly increased alternated steps compared to saline-treatment subjects for every time point following baseline for both sham and spinal subjects.

Percent Alternated Hindlimb Steps

For percent alternated steps for subjects that underwent testing on P10 there were main effects of drug [$F(1, 40) = 138.54$, $p < 0.001$] and time [$F(6, 240) = 26.16$, $p < 0.001$]. There was a 2-way interaction between drug and time [$F(6, 240) = 20.35$, $p < 0.001$]. Follow-up analyses for the main effects revealed that quipazine-treated subjects showed a significantly higher percent of alternated steps and that percent alternated steps increased significantly after baseline. The 2-way interaction between drug and time revealed that for quipazine-treated subjects, percent alternated steps increased significantly for all time points following baseline (T5-T30) when compared to saline-treated subjects. Figure 4B and 4C shows the percent alternated steps for subjects that underwent surgery on P1 and P5 and were tested on P10.

Effects of Equal Recovery Time Since Surgery on Hindlimb Behavior

Analyses examined group differences between subjects that received spinal cord transection or sham on P1 or P5 and were tested on P5 or P10, but had the same amount of recovery time since surgery (i.e. P1-P5 and P5-P10). Hindimb stepping analyses examined group differences infrequencies of total hindlimb movements and alternated hindlimb steps. Alternated steps were analyzed as a percentage of total movements as previously described. Surgery age and test age were both represented by test age (since these represent the same group variable.

Total Hindlimb Movements

For frequency of total hindlimb movements for subjects that had the same recovery time between surgery and testing there were main effects of drug [$F(1, 40) = 12.11$, $p < 0.001$], surgery condition [$F(1, 40) = 5.76$, $p = 0.02$], and time [$F(6, 240) = 9.8$, $p < 0.001$]. There were 2-way interactions between drug and surgery condition [$F(1, 40) = 4.85$, $p = 0.03$], drug and time [$F(6, 240) = 7.5$, $p < 0.001$], and surgery condition and time [$F(6, 240) = 6.29$, $p < 0.001$]. Also, there was a 3-way interaction between drug, surgery condition, and time [$F(6, 240) = 7.11$, $p < 0.001$]. Figure 2A and 2C shows the number of total hindlimb movements for subjects that underwent surgery on P1 or P5 and were tested on P5 or P10. Follow-up analyses for the main effects revealed that quipazine-treated subjects showed significantly more hindlimb movements than saline-treated subjects, that spinal subjects showed significantly more hindlimb movements than sham subjects, and that hindlimb movements increased significantly after baseline.

Follow-up analyses for the 2-way interaction between drug and surgery condition revealed that among quipazine-treated subjects, spinals showed significantly more hindlimb movements than shams. Additionally, for spinal subjects, quipazine-treatment significantly increased hindlimb movements compared to saline-treatment. For the 2-way interaction of drug and time, quipazine-treated subjects showed an increase in hindlimb steps compared to saline-treated subjects for all time points following baseline (T5-T30). Follow-up analyses for the 2-way interaction between surgery condition and time revealed that shams showed significantly more hindlimb movements than spinals during baseline, but that spinals showed significantly more hindlimb movements towards the end of the test session (T25-T30).

To examine the 3-way interaction between drug, surgery condition, and time, surgery condition and time were examined within the two drug conditions. Follow-up analyses for saline-treated subjects revealed that shams showed significantly more hindlimb movements during baseline than spinal subjects. For those treated with quipazine, spinal subjects significantly increased in their hindlimb movements compared to shams towards the end of the test session (T25-T30). When drug and time were examined within the two surgery conditions for shams, quipazine-treated subjects showed significantly more hindlimb movements than saline-treated subjects at T10 of the test session. For spinal subjects, those treated with quipazine showed a significant increase in hindlimb movements compared to those treated with saline for all time points following baseline (T5-T30). Figure 2A and 2C shows the number of total hindlimb movements for subjects that underwent surgery on P1 or P5 and were tested on P5 or P10.

Alternated Hindlimb Steps

For frequency of alternated hindlimb steps for subjects that had the same recovery time between surgery and testing there were main effects of drug [$F(1, 40) = 14.41$, $p = 0.001$], surgery condition [$F(1, 40) = 4.2$, $p = 0.05$], and time [$F(6, 240) = 9.51$, $p < 0.001$]. There were 2-way interactions between drug and surgery condition [$F(1, 40) = 4.11$, $p = 0.05$], drug and time [$F(6, 240) = 12.94$, $p = 0.001$], and surgery condition and time [$F(6, 240) = 6.85$, $p = 0.01$]. Also, there was a 3-way interaction between drug, surgery, and time [$F(6, 240) = 7.47$, $p = 0.01$]. Figure 3A and 3C shows the number of alternated hindlimb steps for subjects that underwent surgery on P1 or P5 and were tested on P5 or P10. Follow-up analyses for the main effects revealed that quipazine-treated subjects showed significantly more alternated steps than saline-treated subjects, that spinal subjects showed significantly more alternated steps than sham subjects, and that alternated steps increased significantly after baseline.

Follow-up analyses for the 2-way interaction of drug and surgery condition revealed that for quipazine-treated subjects, those with a spinal cord transection showed significantly more alternated steps than shams. For shams, quipazine-treated subjects showed significantly more alternated steps than saline-treated subjects. For spinal subjects, those treated with quipazine showed significantly more alternated steps than Those treated with saline. Follow-up analyses for the 2-way interaction between drug and time showed that quipazine-treated subjects significantly increased in alternated hindlimb steps compared to saline-treated subjects for all time points following baseline (T5-T30). Follow-up analyses for the main effect of surgery showed that spinal subjects showed a

significant increase in alternated hindlimb steps towards the end of the test session (T25-T30).

To examine the 3-way interaction between drug, surgery condition, and time, surgery condition and time were examined within the two drug conditions. Follow-up analyses for quipazine-treated subjects, showed that those treated with spinal cord transection significantly increase in alternated hindlimb steps compared to shams between T20-T30 of the test session. When drug and time were examined within the two surgery conditions for shams, those treated with quipazine significantly increased in their alternated hindlimb steps compared to those treated with saline between T5-T25 of the test session. For spinal subjects, those treated with quipazine significantly increased in their alternated steps compared to those treated with saline between T10-T30 of the test session.

Percent Alternated Hindlimb Steps

For percent alternated steps for subjects that had the same recovery time between surgery and testing there were main effects of test age [$F(1, 40) = 5.23$, $p = 0.03$], drug [$F(1, 40) = 42.03$, $p < 0.001$], and time [$F(6, 240) = 14.03$, $p < 0.001$]. There were 2-way interactions between test age and drug [$F(1, 40) = 4.64$, $p = 0.04$], and between drug and time [$F(6, 240) = 8.18$, $p < 0.001$]. Figure 4A and 4C shows the percent alternated steps for subjects that underwent surgery on P1 or P5 and were tested on P5 or P10. Follow-up analyses for the main effect of test age revealed no significant differences between test age P5s and test age P10s. Follow-up analyses for the main effects of drug and time revealed that quipazine-treated subjects showed a significantly higher percent alternated steps compared to saline-treated subjects and that percent alternated steps increased

significantly after baseline. Follow-up analyses for the 2-way interaction between test age and drug revealed that among quipazine-treated subjects, test age P10s showed significantly higher percent alternated steps compared to test age P5s. Follow-up analyses for the interaction of drug and time revealed that quipazine-treated subjects significantly increased in their percent alternated steps compared to saline-treated subjects for all time points following baseline (T5-T30).

Summary

Quipazine treatment increased total hindlimb movements, alternated hindlimb steps, and percent alternated steps for all surgery age and test age groups (P1-P5, P1-P10, and P5-P10). Furthermore, spinal quipazine-treated subjects showed an increase in total movements and alternated steps compared to sham quipazine-treated subjects. Following treatment with quipazine, P1-P10 (Tx-Test Age) subjects showed more total hindlimb movements, alternated steps, and percent alternated steps, when compared to P1-P5 subjects. Spinal P1-P10 subjects showed an increase in hindlimb movements and alternated stepping compared to P5-P10 subjects. Quipazine treatment increased percent alternated steps for spinal P1-P5 subjects compared to quipazine-treated sham subjects, but for P1-P10 and P5-P10 subjects there were no differences between quipazine-treated spinal subjects and quipazine-treated sham subjects. However, quipazine-treated P1-P10 and P5-P10 subjects showed higher percent alternated steps compared to quipazine-treated P1-P5 subjects.

Discussion

Quipazine-Induced Hindlimb Supersensitivity in Spinal Subjects

The hypothesis that there would be more hindlimb steps following quipazine administration for spinal subjects compared to shams was supported. This is consistent with our preliminary data and previous research indicating "hindlimb supersensitivity" following SCI in rat pups (Norreel et al., 2003; Strain et al., 2014). For example, Norreel et al. (2003) found that mechanical stimulation of the tail (tail pinch) increased hindlimb activity of P1-P7 rats transected on P0 when compared to sham controls. Furthermore, application of the *N*-methyl-D-aspartate (NMDA) receptor agonist *N*-methyl-D, L-aspartate (NMA) to the isolated *in vitro* spinal cord of P2-P3 rats that underwent surgery on P0 showed that spinal transected subjects had a shorter latency to express fictive locomotion (alternating ventral root motor bursts) compared to shams. Thus, it appears that there is increased excitability of the CPGs controlling motor responses when the spinal cord is isolated from the brain. One possible mechanism for this is there are alterations in the availability of neuromodulators following SCI (reviewed in Brumley et al., 2017).

Glutamate is necessary for mediating excitatory activation of CPGs while GABA and glycine are necessary for inhibitory neurotransmission (Beato, Bracci, & Nistri, 1997; Brumley et al., 2017; Nishimaru & Kakizaki, 2009; Taplar & Kiehn, 2010). The balance of excitatory and inhibitory neurotransmission is important for regulating aspects of locomotion such as speed, rhythmicity, and the left-right alternating pattern (Nistri, Ostroumov, Sharifulina, & Taccola, 2006). However, following SCI, glutamate increases while GABA and glycine decrease (Demediuk, Daly, & Faden, 1989). Thus, the balance

between excitation and inhibition is disrupted. This disruption may explain an observed switch in the *in vitro* spinal cord from a left-right alternating motor burst pattern in P1-P3 spinal transected pups to a more synchronous pattern in P6-P7 transected pups (Norreel et al., 2003). However, 5-HT_2 administration restores the left-right alternating pattern (Norreel et al., 2003).

Hindlimb supersensitivity appears to be particulary responsive to 5-HT administration. This has been observed in excitability changes of motor neurons from spinal adult rats (Harvey et al., 2006). Administration of a 5-HT_2 agonist in spinal rats has been shown to produce a depolarization of the resting membrane potential, an increase input resistance, and larger persistent inward currents (PICs). Thus, in spinal adult rats, motor neurons become easier to excite via 5-HT_2 administration. As previously mentioned, one of the possible mechanisms of hindlimb supersensitivity is also upregulation of receptors following injury (Fuller et al., 2005; Kong et al., 2011; Kong et al., 2010; Lee, Johnson, & Wrathall, 2007). The upregulation of 5-HT receptors, along with a decrease in GABA and glycine (e.g. less inhibition) is very likely contributing to the observed excitability of motoneurons (Harvey et al., 2006). Specifically, this study examined the effects of the 5-HT_{2A} agonist quipazine or saline on hindlimb stepping in spinal neonatal rats. Spinal subjects did indeed exhibit hindlimb supersensitivity, but only in response to 5-HT_{2A} administration supporting the role of 5-HT_{2A} receptors in excitation of CPGs controlling motor behavior during the early postnatal period. Furthermore, in the current study we found that hindlimb supersensitivity to 5-HT_{2A} administration during early development in the rat is influenced by time of injury and the amount of time that had passed since surgery.

Time of Surgery and Age of Testing Effects on Hindlimb Supersensitivity in Spinal Subjects

The hypothesis that P10 rats that underwent spinal cord transection surgery on P1 would show an increase in quipazine-induced alternated stepping compared to P1-P5s and P5-P10s was supported. This supports our preliminary data where spinal P10s showed increased quipazine-induced alternated stepping compared to spinal P5s. To determine whether our observations were due to pup age, lack of supraspinal inhibition, or amount of recovery time from the spinal surgery, we examined quipazine-induced alternated stepping between pups of the same age (P10) but that had undergone spinal transection at a different age (P1 or P5). The results of the current study show that the increased alternated stepping in P10s we observed was not due to age or supraspinal inhibition alone, but can also be attributed to having a longer time in between surgery and testing (i.e. 9 days vs 4). One possible explanation for this is that as time since surgery increases, the alterations of neuromodulators also increase.

As previously mentioned, Norreel et al. (2003) found disruptions from a left-right alternating motor burst pattern *in vitro* in P6-P7s following P0 spinal surgery, but not in P1-P3s. In conjunction with the results of the current study, one possible explanation for this is that as time since injury increases, glutamate increases while GABA and glycine continue to decrease, which may contribute to an increased excitatory response to 5-HT administration. It is important to note however that surgeries in the Norreel et al. (2003) study were performed at the same age, so the results may be due to developmental age at testing.

Upregulation of 5-HT_{2A} receptors in the caudal spinal cord also may explain the increased hindlimb supersensitivity to quipazine of subjects that had a longer recovery time since surgery. Kong et al. (2011) found that in sacral spinal transected adult rats, upregulation of 5-HT_{2A} receptors caudal to the transection site occurred 1-day following injury and continued for 28 days thereafter. Thus increased supersensitivity to quipazine for P1-P10s when compared to P1-P5s and P1-P10s may in part be due to an increase in upregulation of 5-HT_{2A} receptors. This issue is investigated in Experiment 2 of the current study.

Another possible explanation for increased hindlimb motor behavior in P1-P10s is that there was increased amount of recovery time in the nest environment and as a result increased motor experience following injury. Research shows that motor training following SCI improves functional recovery in immature and adult rats (Cha et al., 2007; Petruska et al., 2007; Tillakaratne et al., 2010). While we did not give our subjects additional motor experience per se, pups were maintained within the nest environment along with their mothers and littermates, providing a great deal of sensory stimulation. Maternal care has been shown to influence biological development in rats, such as changes in expression of brain-derived neurotropic factor (BDNF) mRNA in the hippocampus, which is associated with increased plasticity (Liu et al., 2000), and development of the spinal-mediated leg extension response in postnatal rats (Kauer et al., 2016). P1-P10 subjects had the most recovery time, thus more time for plastic changes to occur following injury. However, very little is known about how BDNF mRNA expression is altered in the neonatal rat spinal cord following injury, or if there are differences in maternal care for spinal vs. sham pups. Future research into plasticity of

the neonatal spinal cord should consider how spinal transection at varying ages influences mechanisms related to neural plasticity (i.e., BDNF mRNA expression) and how maternal care is influenced by pup surgery condition.

Age of Testing Effects on Stepping Coordination During Early Postnatal Development

The hypothesis that P1-P10 and P5-P10 quipazine-treated subjects showed increased coordination (i.e. percent alternated steps) when compared to P1-P5 subjects was supported. This is consistent with our preliminary data and with previous research in our lab showing that P1s are less coordinated compared to P10s (Strain et al., 2014). Thus, the left-right stepping alternation appears to become more coordinated during early postnatal development with age (Norreel et al., 2003).

Spinal P1-P5s showed a higher percentage of alternated hindlimb steps compared to sham P1-P5s. However, it is interesting to note that while spinal P1-P10s and P5-P10s had a higher *frequency* of alternated hindlimb steps compared to shams, the *percentage* of alternated steps was equal. This is because spinal P1-P10s and P5-P10s had a higher frequency of total hindlimb movements compared to shams. Previously in our lab we have shown that while quipazine-treated P1s showed increased frequency of alternated hindlimb steps compared to P10s, P10s showed a higher percentage of alternated steps, which shows that they are becoming more coordinated during development (Strain & Brumley, 2014). In the current study, rats received a spinal cord transection on P1 or P5. During this time, rats do not yet have corticospinal tract innervation of the lumbar spinal cord (Gribnau, et al., 1986; Lakke, 1997). Thus, what we can determine from the current study is that there is an increase in activity of spinal transected P10s compared to shams, but not coordination. As previously mentioned, excitation of CPGs increases following

SCI, but CPG activity becomes irregular (i.e. right-left alterations are out of phase) after P6 in spinal transected rats (Norreel et al., 2003). However, 5-HT administration restores this coordination. Thus, quipazine not only increased motor activity, but it may have restored coordination in spinal P10s equal to that of shams. This was not seen in P1-P5s since shams are already less coordinated and show a lower percentage of alternated steps at this age.

EXPERIMENT 2: 5-HT$_{2A}$ IMMUNOHISTOCHEMISTRY

The first hypothesis for Experiment 2 was that there would be an increase in the density (i.e. upregulation) of 5-HT$_{2A}$ receptors in the ventral spinal cord of spinal subjects when compared to shams. The justification for this hypothesis was based on past research that has observed upregulation of 5-HT$_{2A}$ receptors in the ventral horn of the sacral segments of adult animals following complete spinal cord transection (Fuller et al., 2005; Kong et al., 2011; Kong et al., 2010; Lee et al, 2007). While 5-HT$_{2A}$ receptors are present in the superficial dorsal horn (DH), the intermediate zone, and around the central canal, we chose to image the ventral horn motoneuron pool (lamina IX) and the ventral funiculi since this is where 5-HT$_{2A}$ receptors are most dense and are more likely to be associated with motor functioning (Kong et al., 2011). Additionally, we expected to find differences in density based on time of surgery and age of testing. We hypothesized that there would be an increase of 5-HT$_{2A}$ receptors for P10 subjects when compared with P5s (justification for this is the same for Experiment 1). And finally, it was hypothesized that there would be an increase of 5-HT$_{2A}$ receptors for P10s transected on P1 when compared to P10s transected on P5, owing to the increased amount of time since the spinal cord transection.

Methods

Subjects

Subjects were 36 male Sprague-Dawley rat pups that received a spinal cord transection surgery or sham surgery on P1 or P5 and were sacrificed on P5 or P10. Subjects were euthanized via decapitation and L3-L5 spinal cords were extracted and immediately placed in 4% paraformaldehyde (PFA) for 24-48 hrs. All other information is the same as Experiment 1.

Experimental Design

A total of 6 subject spinal cords were used for immunohistochemistry in each of the 6 groups. Group characteristics are outlined in Table 2. Subjects received a complete low thoracic spinal cord transection surgery or sham surgery on P1 or P5. Pups within the same litter received the same type of surgery to control for maternal behavior. Spinal surgery procedures are described in Experiment 1. At age of sacrifice, subjects were euthanized via decapitation. Lumbar sections between L3 and L5 were dissected out and prepared on slides. Four images per subject were imaged from each cord to reduce subjectivity. Two planes of the ventral horn were imaged per slice; both the left and right lamina IX and ventral funiculi. $5HT_{2A}$ receptor positive signal percentage and particle count in the lumbar sections of the spinal cord was compared between surgery (sham or spinal) groups, age of surgery (P1 or P5) groups, and age of testing (P5 or P10) groups. Subjects within the same group were selected from different litters to control for litter effects. Only males were used to control for possible sex differences.

Characteristics of Groups in Experiment 2

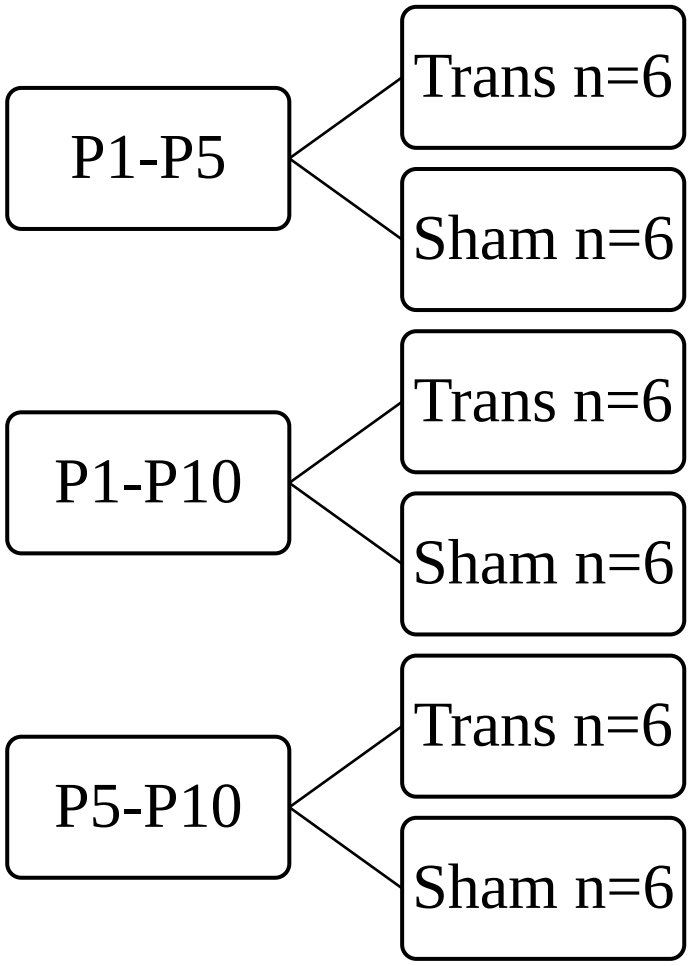


Figure 5. Flowchart of Experiment 2 design. Subjects were categorized into three groups based on surgery age and age of test surgery conditions (i.e. P1-P5, P1-P10, and P5-P10). Within those three groups subjects were assigned to transection or sham surgery. A total of 6 groups was used in the design with n=6.

Immunohistochemistry

Following tissue fixation in PFA, samples were placed in 30% sucrose solution overnight in 4˚C. Samples were mounted in OCT embedding compound, and frozen at -80 °C. Five sections per tissue sample were cut into 20 µm thick transverse tissue sections between L3 and L5, using a Leica cryostat. Slides were washed 3x for 5 min in phosphate buffered saline (PBS) to remove the OCT. A blocking/permeabilizing buffer, 10% normal goat serum (NGS) and 0.25% Triton X-100 was administered to samples for 1 hr. A primary Abcam rabbit 5-HT_{2A} antibody (1:300) was mixed in a 5% NGS, 2% bovine serum albumen (BSA), 0.03% Triton X-100 solution and added to the sample and left overnight at 4˚C. Slides were washed 3x for 10-min. A goat, anti-rabbit Alexa Fluor 488 secondary antibody (1:700; Life Technologies), and Hoechst's blue nuclear counterstain (1:1000; Pierce Biotechnology) mixed in the same solution as the primary, was administered to the slides for 2 hrs at room temperature. The secondary antibody and Hoescht's was washed with PBS, 3x for 10 min and then mounted with a coverslip. Specificity of this antibody was performed with primary and secondary controls, as well as tissue from a *Xenopus laevis* adult. These images were negative for signal.

Data Analysis

An Olympus FV1000 confocal laser-scanning microscope was used to collect a series of Z-stacked images of transverse spinal cord sections with a 40X objective (N.A 0.9). Two contiguous planes were imaged per slice, each image within a field of view that was 512 µm X 512 µm. Image stacks with equal section thickness were taken and averaged for quantitative analysis. Quantitative analysis of 5-HT_{2A} density was performed using NIH Image J software. 5-HT_{2A} density is defined by total positive signal

percentage (percentage of positive signal/total pixel area) as well as by the particle count analysis (e.g. count of individual segmented particles above 0.03 µm). Threshold levels (total pixel area) are set by case with reference to the background level of the negative control (i.e. secondary antibody) sections. Only pixels above threshold were counted as positive 5-HT_{2A} signal labeling. Data were analyzed using multi-way ANOVA tests with surgery condition, age of surgery, and age of sacrifice as independent variables and 5-HT_{2A} signal percentage and particle count as dependent variables. Subjects were identified with a number produced from a random number generator to ensure experimenter blindness to group association during data collection and analyses.

EXPERIMENT 2 RESULTS

5-HT_{2A} Receptor Density

Analyses examined group differences for images from subjects that underwent the sham procedure or spinal transection surgery on P1 and P5 and were sacrificed on P5 and P10. Analyses examined group differences for percent positive signal and particle count. Omnibus analyses were first done for percent positive signal and particle count using a multi-way ANOVA with surgery age, sacrifice age, and surgery condition as the independent variables. All significant main effects and interactions are reported. Non-significant effects were $p > 0.05$. Follow-up tests were conducted as needed.

Percent Positive Signal

For percent positive signal, there was a main effect of surgery condition [$F(1, 35) = 6.03$, $p < 0.02$], surgery age [$F(1, 35) = 5.4$, $p < 0.03$], and sacrifice age [$F(1, 35) = 5.81$, $p < 0.02$]. Follow-up analyses revealed that images from spinal subjects had a higher percent positive signal when compared to shams. Further analyses revealed that

while P1-P10s had a higher percent positive signal compared to P1-P5s and P5-P10s, this was not significant [$F(1, 23) = 3.57$, $p = 0.07$], [$F(1, 23) = 3.93$, $p = 0.06$] respectively. Figure 6A shows the percent positive signal for spinal subjects vs. shams.

Particle Count

For particle count there was a main effect of surgery condition [$F(1, 35) = 6.93$, $p < 0.01$]. Follow-up analysis showed that the images from spinal subjects had a significantly higher particle count compared to shams. Figure 6B shows the particle count for spinal subjects vs. shams.

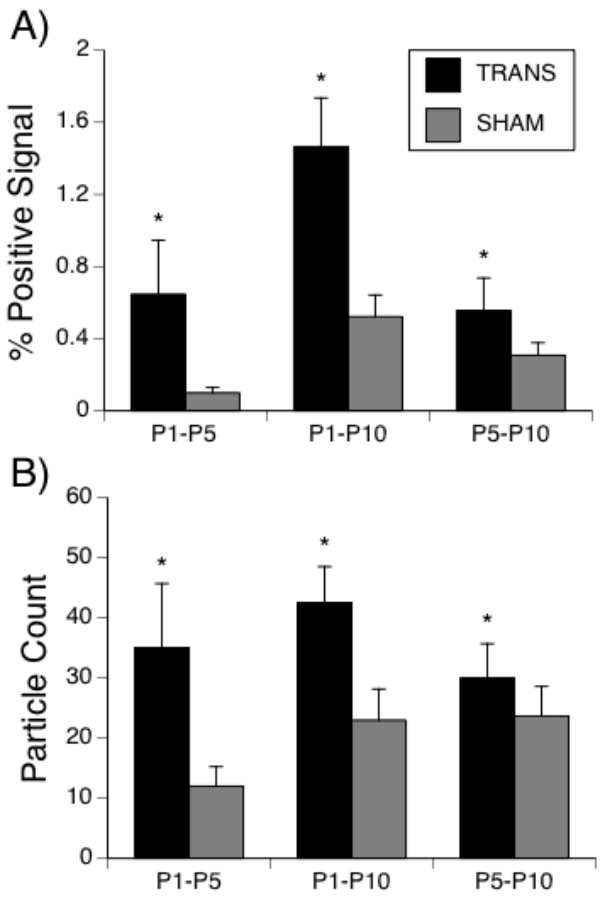
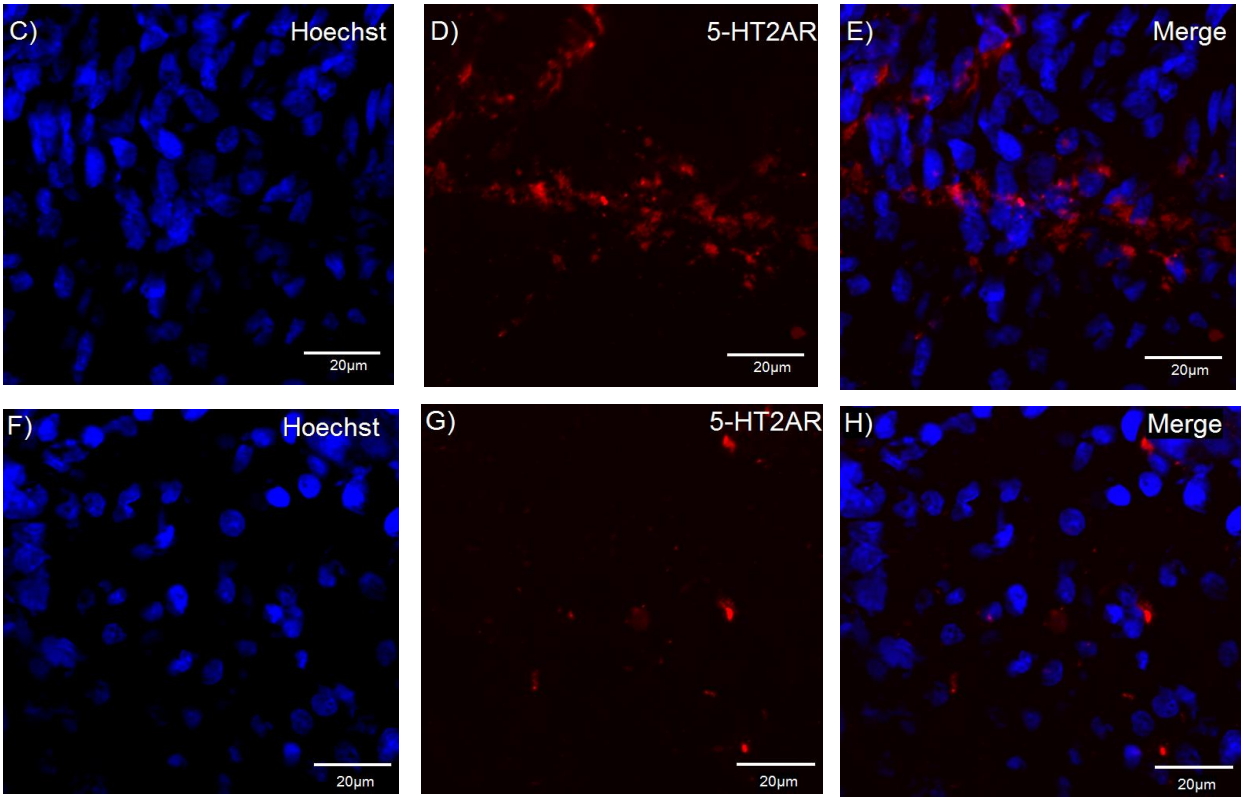


Figure 6. Density of 5-HT_{2A} receptor immunoreactivity in the L3-L5 lamina IX and ventral funiculi of the rat spinal cord between subjects that underwent spinal or sham surgery on P1 or P5 and were sacrificed on P5 or P10. Graph (A) shows the percent of positive 5-HT_{2A} signal. Graph (B) shows the 5-HT_{2A} individual particle count. vertical lines are SEM. (C-H) Representative images for Hoechst stain and 5-HT_{2A} positive signal for (C-E) p1-P5 spinal subject (F-H) or P1-P10 sham. Scale bar = 20 µm. 5-HT_{2A} signal is pseudo-colored red.



Summary

Spinal subjects showed a significantly higher percent positive signal and particle count compared to sham subjects. No significant differences were found between age of surgery and age of sacrifice groups.

Discussion

Upregulation of 5-HT$_{2A}$ Receptors

The hypothesis that spinal subjects would show upregulation of 5-HT$_{2A}$ receptors in the ventral lamina IX (motoneuron pool) and ventral funiculi between L3-L5 compared to sham subjects was supported. This is consistent with previous research that also reported upregulation of 5-HT receptors following SCI (Fuller et al., 2005; Kong et al., 2011; Kong et al., 2010; Lee et al., 2007). Immunoreactivity was observed in lamina IX, ventral funiculi, dorsal horn, and intermediate zone, which is consistent with previous reports of the distribution of 5-HT$_{2A}$ receptors in rat spinal cord sections. This supports the specificity of our primary antibody to 5-HT$_{2A}$ receptors.

It is interesting to note that these results contrast somewhat with studies looking at 5-HT$_{2A}$ mRNA levels in the spinal cord following injury. Basura et al. (2001) found no upregulation of 5-HT$_{2A}$ receptor mRNA levels in the cervical spinal cord following a hemisection. Ung et al. (2008) found upregulation of 5-HT$_{2A}$ receptor mRNA levels in the intermediate zone of L1-L3 of mice for 2 weeks following spinal cord transection, but no upregulation in the ventral horn. The results of the Basura et al. study may reflect the differences that can occur in upregulation due to severity or type of injury. Hemisection and contusion models may not produce upregulation of mRNA and receptors to the same degree as complete spinal cord transections. In the Ung et al. study, species differences

could be attributed to different receptor distribution patterns between the mouse and rat. However, the correlation between mRNA expression and protein expression is poor (Maier, Güell, & Serranano, 2009). Thus, it is possible that the mechanisms guiding upregulation of mRNA and protein expression are different. Time from surgery, location, and experience all may be critical factors in upregulation of genes vs. proteins. For example, Chopek et al. (2015) found 5-HT_{2A} receptor gene upregulation in the flexor and extensor muscle motoneurons 3 months following complete spinal cord transection. Upregulation was also increased in rats that had undergone daily passive cycling. Thus, future research should look at the different mechanisms guiding upregulation of mRNA and protein expression since the expression of both is likely to be influenced by epigenetic processes.

What is particularly interesting about the results of our immunohistochemistry is that we did not find support for our hypothesis that upregulation would be increased in P1-P10s compared to P1-P5s and P5-P10s. We based our hypothesis on the research of Kong et al. which established a time course for 5-HT_{2A} receptor upregulation. In the Kong et al. study they found 5-HT_{2A} receptor upregulation in spinal transected rats compared to shams a day after the surgery and that these values increased for 28 days after. We did find that the mean percentage of signal area and mean particle count 5-HT_{2A} receptor expression was increased in P1-P10s compared to P1-P5s and P5-P10s (see Figure 4A and 4B), but that this was not statistically significant. However, the differences in our results and that of Kong et al. could be attributed to our analyses and experimental design. Kong et al. (2011) found that 5-HT_{2A} receptor expression for spinal transected subjects increased over time compared to shams, but unlike in our study, they

did not make a comparison between different groups of spinal transected subjects. They also found differences in upregulation up to 28 days after transection, whereas the current study only went out to 9 days maximum post-transection. To our knowledge, we are the first to make comparisons of 5-HT_{2A} receptor upregulation between subjects that have undergone spinal transection surgery at different time points, and we did not find this to be statistically significant.

GENERAL DISCUSSION

The purpose of the current study was to examine the impact of a low thoracic spinal cord transection on quipazine-induced locomotor activity and 5-HT_{2A} receptor levels in the spinal cord of the developing rat. We found that thoracic spinal cord transection causes hindlimb supersensitivity in response to quipazine administration. While this may be partially mediated by lack of supraspinal inhibition, there are likely to be other biological alterations that occur in response to spinal cord transection. Indeed, we did find that there was upregulation of 5-HT_{2A} receptor immunoreactivity in the lumbar spinal cord in response to thoracic spinal transection. Thus, there is likely to be a relationship between 5-HT_{2A} receptor upregulation and hindlimb supersensitivity during the early postnatal period in the rat.

Furthermore, we found that the amount of time between surgery and testing significantly influenced hindlimb supersensitivity to quipazine since spinal P1-P10s showed more hindlimb movements and alternated steps compared to P1-P5s and P5-P10s. This supported our original hypothesis that hindlimb supersensitivity would increase with the amount of recovery time between surgery and testing. However, the justification for this hypothesis was that there would be an even greater increase in

upregulation of 5-HT_{2A} receptor levels in L3-L5 of the ventral spinal cord of P1-P10 spinal subjects based on previous research (Kong et al. 2011), which was not supported by our data.

There are several possible explanations for the discrepancies in our behavioral data and immunohistochemistry data (and that of previous research). One possible explanation is that while 5-HT_{2A} upregulation is contributing to hindlimb supersensitivity following spinal cord transection, it is not the only factor. As previously mentioned, the balance between excitation and inhibition of CPGs is disrupted due to increases in glutamate and decreases in GABA and glycine (Beato, Bracci, & Nistri, 1997; Brumley et al., 2017; Nishimaru & Kakizaki, 2009; Taplar & Kiehn, 2010). It is very likely that this contributed hindlimb supersensitivity via dysregulation of chloride homeostasis (Gackière & Vinay, 2014). In the intact spinal cord, GABA and glycine activation leads to chloride entry causing membrane hyperpolarization (i.e. inhibition). This is because potassium chloride co-transporters (KCC2) maintain intracellular chloride ions ($[CL^-]_i$) at low levels by extruding them from the cell (Blaesse, Airaksinen, Rivera, & Kaila, 2009; Chamma, Chevy, Poncer, & Lévi, 2012; reviewed in Gackière & Vinay, 2014). Following SCI, KCC2 is downregulated in lumbar motoneurons (Boulenguez et al., 2010). This alters $[CL^-]_i$ levels causing a depolarizing shift (i.e. less inhibition). This leads to increased spasticity (Boulenguez et al., 2010) and disorganization of the left-right alternating pattern (Norreel et al, 2003). Interestingly, 5-HT_{2A} activation has been shown to upregulate the function of KCC2 following SCI, thereby increasing synaptic inhibition and reducing spasticity in adult rats (Bos et al., 2013). During early postnatal development glycine receptors are upregulated while GABA receptors are downregulated

(reviewed in Gackière & Vinay, 2014; Sadloud et al, 2010). KCC2 is upregulated leading to the switch of inhibitory postsynaptic potentials from depolarizing to hyperpolarizing (Jean-Xavier et al., 2006). This switch (along with the downregulation of GABA) is prevented via neonatal spinal cord transection. Chronic administration of 5-HT_{2A} restores the hyperpolarizing shift (Bos et al., 2013). However, in the current study we did not use chronic administration of quipazine and may not have restored the shift to hyperpolarization. Thus, it is possible that the hindlimb supersensitivity to quipazine administration we observed in the current study is due to the fact that we prevented the switch from depolarization to hyperpolarization via spinal cord transection.

Another possible explanation is that quipazine does not have an affinity for 5-HT_{2A} receptors alone, but to other 5-HT_2 receptor subtypes as well. While quipazine-induced locomotor activity has been shown to be blocked by 5-HT_{2A} antagonists (Ung et al., 2008), quipazine also has been characterized as a 5-$HT_{2A/2C}$ agonist (Landry & Guertin, 2004). 5-HT_{2C} upregulation has been observed in the rat spinal cord (Ren et al., 2013) and has been shown to be important for motor recovery and functioning following SCI in rats (Murray et al, 2010). Furthermore, 5-HT_{2C} receptor upregulation has been found to be correlated with behavior (i.e. spasticity) (Ren et al., 2013), while 5-HT_{2A} receptors was not (Kong et al., 2011). Therefore, if quipazine is a partial agonist for ligands other than 5-HT_{2A}, and those receptors are also upregulated after transection, then hindlimb supersensitivity to quipazine may increase for subjects having a longer recovery time between surgery and testing, while not necessarily having significantly more upregulated 5-HT_{2A} receptors. Future research should examine the role of 5-HT_{2C} receptors in quipazine-induced stepping and upregulation following SCI.

As previously mentioned, unlike Kong et al. (2011), we did not find that a longer recovery time between spinal transection surgery and sacrifice increased 5-HT_{2A} receptor upregulation. One possible explanation for this is differences in methodology. We performed a spinal cord transection at one spinal level between T8-T10 and examined 5-HT_{2A} receptors in L3-L5 spinal segments, while Kong et al. (2011) performed an S2 spinal cord transection and examined 5-HT_{2A} receptors at S4. L3-L5 is further from the transection site than in the Kong et al. study, and while upregulation was observed, it may be occuring at a different rate than that of segments closer to the injury. The established time course for 5-HT_{2A} receptor levels in the Kong et al. study showed that upregulation occurred one day post transection, and increased thereafter. If segments caudal to the injury site are affected with respect to how close they are to the injury site, it may take more time for upregulation to occur in segments further from the injury. However, it is important to note that Ren et al. (2013) did not report upregulation of 5-HT_{2C} receptors at S4 following S2 transection until day 14, which may reflect 5-HT_2 receptor subtypes. Future research should examine differences in the time-course of upregulation of receptors in relation to the injury site.

Another possible explanation for the differences in upregulation of 5-HT_{2A} receptors in our spinal cord transected subjects compared to the Kong et al. study could be developmental. One of the novel aspects of the current study was that we were looking at developmental differences of quipazine-induced motor behavior and 5-HT_{2A} receptor levels in developing spinal rat pups. To our knowledge, this is the first study to examine this issue. There are considerable differences between the locomotor recovery of neonatal rats and adult rats following SCI (Weber & Stelzner, 1977; Yuan et al., 2013). Neonatal

spinal transected rats show significantly more recovery if the transection occurs prior to P15, whereby recovery starts to decline with age. Research shows that improved recovery in eary transected rats is not due to regrowth across the lesion site, but to plasticity of the lumbosacral circuitry (Tillakaratne et al. 2010). It is because of this that we had originally expected to see higher levels of 5-HT_{2A} receptors in spinal P1-P10s compared to P1-P5s and P5-P10s. While there was an observed increase, this was not significant. Thus it may be that receptor upregulation is occuring at a slower rate during early development compared to adulthood. One possible reason for this may be that during the early postnatal period supraspinal connections are not fully formed and dendritic bundles onto motoneurons have not fully formed (Westerga & Gramsbergen, 1997). Thus, there may already be less 5-HT_{2A} receptor activation in neonatal rats, leading to more pronounced upregulation in adults. Therefore it is possible that if we had looked at later ages following the P1 transection that we would have observed significant differences in upregulation compared to younger pups.

Limitations

As with any study, the current study has limitations. First, the spinal cords extracted from spinal and sham pups used in the immunohistochemistry are not from the same subjects used in Experiment 1. This was due to improper storage of subjects in formalin for Experiment 1 causing degradation of tissue and thus non-specific signaling during confocal microscopy. It was also because of this that we were not able to perform a meaningful correlation between 5-HT_{2A} receptor density and behavior. However, the benefit of using separate subjects for Experiment 1 and 2 is that there is not an additional variable of drug condition that could cause unknown alterations in receptor upregulation.

Additionally, 5-HT_{2A} receptor expression has not been found to be correlated with behavior until 20 days following spinal cord transection (Kong et al., 2011).

Another limitation is that for the immunohistochemistry we had originally planned to use 10 images per subject to reduce subjectivity. Due to a lack of signal in some slices (primarily sham P1-P5s), we were unable to obtain these images for analyses. The lack of signal could be due to methodology, or because the 5-HT_{2A} receptors were not present in those slices. We also did not test out different concentrations of primary and secondary antibodies between spinal and sham subjects. Due to alterations in biochemistry following SCI, antigen binding to epitopes can be reduced (OSU Spinal Cord Injury Training Program, 2017). Thus, future research using immunohistochemistry to examine differences in SCI vs. sham animal models should test different concentrations before imaging and analysis. We also recognize that the signal we obtained using our immunohistological methods does not represent individual receptors. Due to the high densities of receptor expression high performance liquid chromatography (HPLC) would be needed to examine individual receptor counts.

Finally, this study did not employ immunohistochemistry of ChAT to identify motoneurons and determine if 5-HT_{2A} receptor immunoreactivity is colocalized on motoneurons. Qualitative observations in the current study were that 5-HT_{2A} receptor immunoreactivity was primarily in the ventral funiculi, which could represent developmental differences since more ventral lateral projections have been found in spinal neonatal rats compared to adults (Petruska et al., 2007). Research has shown that 5-HT_{2A} receptor and ChAT immunoreactivity are colocalized (overlap) in adult rats (Kong et al. 2010). Future research should examine colocalization of 5-HT_{2A} receptor and

ChAT immunoreactivity to determine if there are differences in distribution of 5-HT_{2A} receptors on the motoneurons of neonates and adults.

Implications

The current study contributes to the growing body of literature examining locomotor and 5-HT receptor plasticity that occurs in response to SCI. However, most of the previous literature has used adult animal models and the current study looked at plasticity during early development since there are many anatomical differences between neonates and adults. Due to the vast amount of behavioral, biochemical, and anatomical reorganization the spinal cord goes through caudal to the lesion site, the injured spinal cord has often been referred to as a "new spinal cord" (Edgerton et al., 2001; Edgerton et al., 1997; Hodgson et al., 1994). But what does this mean for a spinal cord that is already new? This was the question we sought to answer with the current study.

Spontaneous partial recovery of motor functioning following SCI has been observed in both animal models (Ballermann & Fouad, 2006) and humans (Fawcett et al., 2007). For one, spontaneous recovery of phrenic motoneurons following cervical hemisection has been attributed to 5-HT_{2A} receptor upregulation (Fuller et al., 2005). Thus, the spinal cord is adaptive in response to injury and spontaneous locomotor recovery of hindlimbs may be in part due to upregulation of 5-HT_{2A} receptors. As previously mentioned, spontaneous locomotor recovery is greater in neonatal spinal transected rats compared to those transected after P15 (Weber & Stelzner, 1977; Yuan et al., 2013). While much of this recovery is often attributed to reorganization of CPGs (Tillakaratne et al. 2010), we have shown here that spinal cord receptor density changes as well, and therfore may influence recovery through regulation of activity levels.

We did not examine spontaneous locomotor recovery following SCI, but instead examined locomotor activity via quipazine administration. Neonatal rats do not exhibit the same repertoire of voluntary motor behavior as adults, due to less developed corticospinal tract innervation (Gribnau, et al., 1986; Lakke, 1997). Thus, we required the use of pharmaceuticals to activate motor behavior in neonatal rats. We specifically chose a 5-HT agonist (quipazine) since recovery of motor functioning in spinal transected adults has been found to be enhanced by $5HT_2$ receptor agonists (Antri et al, 2003, Antri et al., 2002; Sławińska et al., 2012), as well as through step training (Cha et al., 2007; Tillakarante et al., 2010). While some spontaneous recovery of locomotion can be observed following spinal cord transection, demonstrating the ability of the spinal cord to learn and adapt on its own, many of the supraspinal mechanisms controlling voluntary motor activity are lost, resulting in a reduction of use. Activation of CPGs via drug administration and motor training increase this use in the absence of supraspinal control, thereby influencing plasticity of existing connections and the ability of the spinal cord to recover some motor output. Therefore, by using quipazine to increase activity we can examine how a 5-HT receptor agonist influences motor output following SCI. Not much is currently known about how 5-HT agonists influence motor recovery in humans following SCI. The current study demonstrates the ability of a $5\text{-}HT_{2A}$ receptor agonist to activate motor output, which may facilitate recovery following SCI. Although this concept is not entirely new, our findings suggest the importance of timing in employing interventions for those with SCI due to the quickly occurring biological changes that follow post-injury, and many interventions do not occur for months following injury (Behrman & Harkema, 2000). Thus, the plasticity of the spinal cord following injury may

be more beneficially influenced by 5-HT_{2A} administration and physical therapy early on. Furthermore, many studies are currently exploring the role of stem cell transplants in recovery from SCI in adults (Lu, Jones, Snyder, & Tuszynski, 2003; McDonald et al., 1999; Teng et al., 2002), highlighting the importance of understanding how a "new" spinal cord organizes itself in the absence of supraspinal input.

Future Directions

The present study examined the influence of quipazine, a 5-HT_{2A} receptor agonist, on alternated hindlimb stepping following a spinal cord transection. However, further research is needed to establish the extent to which 5-HT_{2A} receptors are involved in this behavior. To elucidate the role of 5-HT_{2A} receptors in locomotor stepping, future research should examine the role of additional drug receptor agonists and antagonists in evoking alternated activity in the neonatal isolated and intact spinal cord. Quipazine-induced locomotor movements have been shown to be blocked by 5-HT_{2A} antagonists, but not 5-HT_{2B} and 5-HT_{2C} receptor antagonists, in spinal cord transected mice (Ung et al. 2008). However, it is possible that other receptor subtypes are involved as well. In addition to drug studies, the roles of different receptors may further be illuminated by using 5-HT receptor-specific knockout models. The site of receptor activation (spinal cord vs. periphery) also should be investigated to more precisely uncover the mechanisms by which 5-HT is involved in controlling and regulating motor behavior.

Future studies should also examine the role of sensory feedback in quipazine-induced stepping in spinal transected neonatal rat pups. As previously mentioned, step training has been shown to enhance locomotor recovery following spinal cord injury (Cha et al., 2007; Tillakarante et al., 2010). Additionally, spinal rat pups have been shown to

respond to tactile and proprioceptive sensory feedback (Strain et al., 2014) and quipazine-induced air-stepping in spinal transected P1s is increased via tail-pinch (Swann, Kauer, Allmond, & Brumley, 2017). Thus, future directions should examine the combined roles of quipazine-induced stepping and sensory feedback on motor movements following spinal cord transection. For example, quipazine and tail-pinch have been shown to recover frequency of hindlimb steps, but not quality of movements, where transected subjects show more low amplitude steps (i.e. more flexed limbs) compared to shams (Swann et al., 2017). Thus, it would be interesting to see if plantar surface contact via treadmill training recovers quality of quipazine-induced stepping.

Conclusions

In this study, we found that there is considerable locomotor and 5-HT_{2A} receptor plasticity following SCI in the neonatal rat. The use of a 5-HT agonist (quipazine) allowed us to examine hindlimb supersensitivity in response to complete spinal cord transection. Importantly, we found that this was not merely an effect of age or supraspinal inhibition, but that the amount of recovery time between surgery and behavioral testing was influential on hindlimb supersensitivity. One interesting aspect of our behavioral data is that quipazine increased total hindlimb activity and alternated hindlimb steps for spinal transected subjects compared to shams, but not the percentage of alternated steps. This highlights the importance of inhibitory mechanisms involved in coordinated locomotor development. To our knowledge, this is the first study to examine the effects of spinal cord transection on quipazine-induced hindlimb supersensitivity between time of surgery and age of testing conditions. We also found that this hindlimb supersensitivity was in part due to the upregulation of 5-HT_{2A} receptors in the lumbar spinal cord.

Additionally, we are the first to show upregulation of 5-HT_{2A} receptors occurs in the neonatal rat lumbar spinal cord following complete spinal cord transection. However, we had hypothesized that 5-HT_{2A} receptors would increase significantly over time, and that this was the cause of increased hindlimb supersensitivity in spinal P1-P10s compared to P1-P5s and P5-P10s. We found that this was not the case. While we have shown that hindlimb supersensitivity is due in part to upregulation of 5-HT_{2A} receptors, it is important to note that this is not the only cause. It is important to understand that behavior (including motor behavior) is the result of complex interactions of a myriad factors. We cannot simplify complex behavior by attributing it to one simple biological process (mainly because biological processes are not simple). Rather, we must look at all contributing biological and environmental influences to elucidate causes of behavior. This is especially important for developing treatments for those with diseases, disorders and injury. The important implications of the current research is one piece in solving the puzzle of restoring recovery of function following SCI (in both adults and neonates).

References

Altman, J., & Sudarshan, K. (1975). Postnatal development of locomotion in the laboratory rat. *Animal Behaviour, 23*(4), 896-920.

Antri, M., Mouffle, C., Orsal, D., & Barthe, J. (2003). 5-HT_{1A} receptors are involved in short- and long-term processes responsible for 5-HT-induced locomotor function in chronic spinal rat. *European Journal of Neuroscience, 18*(7), 1963-1972.

Antri, M., Orsal, D., Barthe, J., & Pierre, Â. (2002). Locomotor recovery in the chronic spinal rat : Effects of long-term treatment with a 5-HT_2 agonist. *European Journal of Neuroscience, 16*(3), 467-476.

Behrman, A.L., & Harkema, S.L. (2000) Locomotor training after human spinal cord injury: A series of case studies. *Physical Therapy, 80*(7), 688-700.

Ballermann, M., & Fouad, K. (2006). Spontaneous locomotor recovery in spinal cord injured rats in accompanied by anatomical plasticity of reticulospinal fibers. European Journal of Neuroscience, 23(8), 1988-1996.

Basura, G.J., Zhou, S.Y., Walker, P.D., Goshgarian, H.G. (2001). Distribution of serotonin 2A and 2C receptor mRNA expression in the cervical ventral horn and phrenic motoneurons following spinal cord hemisection. *Experimental Neurology, 169*(2), 255-263.

Beal, J.A., & Bice, T.N. (1994). Neurogenesis of spinothalamic and spinocerebellar tract neurons in the lumbar spinal cord of the rat. *Developmental Brain Research , 78*(1), 49-56.

Beato, M, Bracci, E., & Nistri, A. (1997). Contribution of NMDA and non-NMDA glutamate receptors to locomotor pattern generation in the neonatal rat spinal cord. *Proceedings of the Royal Society of London B: Biological Sciences, 264*(1383), 877-884.

Blaesse, P., Airaksinen, M., Rivera,C., & Kaila, K. (2009) Cation-cloride cotransporters and neuronal function. *Neuron, 61*(6), 820-838.

Bos, r., Sad laoud, K., Boulengues, P., Buttigieg, D., Labeuf, S., Brocard, C., …& Vinay, L.(2013). Activation of 5-HT2A reeceptors upregulates the function of the neuronal K-CL cotransporter KCC2. *Proceedings of the National Academy of Sciences, 110*(1), 348-353.

Boulenguez, P., Liabeuf, S., Bos, R., Bras, H., Jean-Xavier, C., Brocard, C., …& Marsala, M. (2010). Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. *Nature Medicine, 16*(3), 302-307.

Bregman, B.S. (1987). Development of serotonin immunoreactivity in the rat spinal cord and its plasticity after neonatal spinal cord lesions. *Developmental Brain Research, 34*(2), 245-263.

Brodwin, M.G., Siu, F.W., Howard, J., & Brodwin, E.R. (2009). *Medical, Psychosocial, and Vocational Aspects of Disability* (3rd ed.). Athens, GA: Elliot & Fitzpatrick, Inc.

Brumley, M.R., Guertin, P.A., Taccola, G. (2017). Multilevel analysis of locomotion in immature preparations suggests innovative strategies to reactivate stepping after spinal cord injury. *Current Pharmaceutical Design, 23*(12), 1764-177.

Brumley, M.R., Roberto, M.E., & Strain, M.M. (2012). Sensory feedback modulates quipazine-induced stepping behavior in the newborn rat. *Behavioural Brain Research*, *229*(1), 257–264.

Brumley, M.R., & Robinson, S.R. (2005). The serotonergic agonists quipazine, CGS-12066A, and α-Methylserotonin alter motor activity and induce hindlimb stepping in the intact and spinal rat fetus. *Behavioral Neuroscience, 119*(3), 821–833.

Cazalets, J.R., Squalli-Houssaini, Y., & Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in the neonatal rat. *The Journal of Physiology, 455*(1), 187-204.

Cha, J., Heng, C., Reinkensmeyer, D.J., Roy, R.R., Edgerton, V.R., & Leon, R.D. (2007). Locomotor ability in spinal rats is dependent on the amount of activity imposed on the hindlimbs during treadmill training. *Journal of Neurotrauma, 24*(6), 1000–1012.

Chamma, I., Chevy, Q., Poncer, J.C., & Lévi, S. (2012). Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission. *Frontiers in Cellular Neuroscience, 6*(5), 1-15.

Chopek, J.W., Sheppard, P.C., Gardiner, K., & Gardiner, P.F. (2015). Serotonin receptor and KCC2 gene expression in lumbar flexor and extensor motoneurons posttransection with and without cycling. *Journal of Neurophysiology, 113*(5), 1369-1376.

de Boer-van Huizen, R.T., & ten Donkelaar, H.J. (1999). Early development of descending supraspinal pathways: A tracing study in fixed and isolated rat embryos. *Anatomy & Embryology, 199*(6), 539–547.

Demediuk, P, Daly, M.P., & Faden, A.I. (1989). Effect of impact trauma on neurotransmitter and nonneurotransmitter amino acids in the rat spinal cord. *Journal of Neurochemistry, 52*(5), 1529-1536.

Edgerton, V.R., Leon, R.D., Harkema, S.J., Hodgson, J.A., London, N., Reinkensmeyer, D., …&Tobin, A. (2001). Retraining the injured spinal cord. *The Journal of Physiology, 533*(1), 15-22

Edgerton, V.R., Roy, R.R., DeLeon Niranjala Tilakaratne, R., & Hodgson, J.A. (1997). "Does motor learning occur in the spinal cord?". *The Neuroscientist, 3*(55) 287-294.

Fawcett, J.W., Curt, A., Steeves, J.D., Coleman, W.P., Tuszynski, M.H., Lammertse, D., … & Dobkin, B.H. (2007). Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP panel: Spontaneous recovery after spinal cord injury and statistical power needed for therapeudic clinical trials. *Spinal Cord, 45*(3), 190-205.

Field-Fote, E. (2009). *Spinal Cord Injury Rehabilitation*. Philadelphia, PA: FA Davis.

Fuller, D.D., Baker-Herman, T.L., Golder, F. J., Doperalski, N.J., Watters, J.J., & Mitchell, G. S. (2005). Cervical spinal cord injury upregulates ventral spinal 5-HT_{2A} receptors. *Journal of Neurotrauma, 22*(2), 203-213.

Gackière, F., Vinay, L. (2014). Seroternergic modulation of post-synaptic inhibition and locomotor alternating pattern in the spinal cord. *Frontiers in NeuralCircuits, 8*(102), 1-7.

Gribnau, A.A.M., de Kort, E.J.M., Dederen, P.J.W.C., & Nieuwenhuys, R. (1986). On

the development of the pyramidal tract in the rat II . An anterograde tracer study of the outgrowth of the corticospinal fibers. *Anatomy & Embryology, 175*(1), 101-110.

Gorgels, T.G.M.F., de Kort, E.J.M., Van Aanholt, H.T.H., & Nieuwenhuys, R. (1989). A quantitative analysis of the development of the pyramidal tract in the cervical spinal cord in the rat. *Anatomy & Embryology, 179*(4), 377–385.

Grau, J.W., Barstow., D.G., & Joynes, R.L. (1998). Instrumental learning within the spinal cord : I. Behavioral properties. *Behavioral Neurosciece, 112*(6), 1366-1386.

Harvey, P.J., Lui, X., Li, Y., & Bennett, D.J. (2006). $5HT_2$ receptor activation facilitates a persistent sodium current and repetitive firing in spinal motoneurons of rats with and without chronic spinal cord injury. *Journal of Neurophysiology, 96*(3), 1158-1170.

Hodgson, J.A., Roy, R.R., de Leon R., Dobkin, b., Edgerton, V.R. (1994). "Can the mamalian lumbar spinal cord learn a motor task?". *Medical Science Sports Exercise, 26*(12), 1491-1497.

Ichiyama, R.M., Broman, J., Roy, R.R., Zhong, H., Edgerton, V.R., & Havton, L. A. (2011). Locomotor training maintains normal inhibitory influence on both alpha-and gamma-motorneurons after neonatal spinal cord transection. *The Journal of Neuroscience, 31*(1), 26-33.

Jamon, M., & Clarac, F. (1998). Early walking in the neonatal rat: A kinematic study. *Behavioral Neuroscience, 112*(5), 1218-1228.

Jean-Xavier, C., Pfleiger, J.F., Liabeuf, S., & Vinay, L. (2006). Inhibitory post-synaptic potentials in lumbar motoneurons remain depolarizing after neonatal spinal cord

transection in the rat. *Journal of Neurophysiology, 96,* 2274-2281.

Joynes, R.L., Janjua, K., & Grau, J.W. (2004). Instrumental learning within the spinal cord: VI : The NMDA receptor antagonist, AP5, disrupts the acquisition and maintenance of an acquired flexion response. *Behavioural Brain Research, 154*(2), 431-438.

Kao, T., Shumsky, J.S., Knudsen, E.B., Murray, M., & Moxon, K. A. (2011). Functional role of exercise-induced cortical organization of sensorimotor cortex after spinal transection. *Journal of Neurophysiology, 106*(5), 2662-2674.

Kauer, S.D., Allmond, J.T. Belnap, S.C., & Brumley, M.R. (2016). Maternal behavior influences the development of a reflexive action pattern in the newborn rat. *Developmental Psychobiology, 58*(8), 1043-1054.

Kjaerulff, O., & Kiehn. O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord *in vitro*: A lesion study, *The Journal of Neuroscience, 16*(18), 5777-5794.

Kong, X.Y., Wienecke, J., Chen, M., Hultborn, H., & Zhang, M. (2011). The time course of serotonin 2A receptor expression after spinal transection of rats: An Immunohistochemical study. *Neuroscience, 177*, 114–126.

Kong, X.Y., Wienecke, J., Hultborn, H., & Zhang, M. (2010). Robust upregulation of serotonin 2A receptors after chronic spinal transection of rats : An immunohistochemical study. *Brain Research, 1320,* 60-68.

Lakke, E. (1997). The projections to the spinal cord of the rat during development: A

timetable of descent. *Advances in Anatomy, Embryology, and Cell Biology, 135,* 1-143.

Landry, E.S. & Guertin, P.A. (2004). Differential effects of 5-HT_1 and 5-HT_2 receptor agonists on hindlimb movements in paraplegic mice. *Psychopharmachology and Biological Psychiatry, 28*(6), 1053-1060.

Lee, J.K., Johnson, C.S., & Wrathall, J.R. (2007). Up-regulation of 5-HT_2 receptors is involved in the increased H-reflex amplitude after contusive spinal cord injury. *Experimental Neurology, 203*(2), 502-511.

Leslie, R. A. (1986). *The Rat Nervous System. Volume 2: Hindbrain and Spinal Cord: Edited by George Paxinos.* Kensington, Australia: Harcourt Brace Jovanovich, Publishers

Leszyńska, A.N., Majczyński, H., Wilczyński, G.M., Sławińska, U., & Cabaj, A.M. (2015). Thoracic hemisection in rats results in inititial recovery followed by a late decrement in locomotor movements, with changes in coordication correlated with serotonergic innervation of the ventral horn. *PloS One, 10*(11), e0143602.

Liu, D., Diorio, J., Francis, D.D., & Meany, M.J. (2000). Maternal care, hippocampal synaptogenesis, and cognitive development in rats. *Nature Neuroscience, 3*(8), 799-806.

Maier, T., Güell, M., & Serranano, L. (2009). Correlations of mRNA and protein in complex biological samples. *FEBS Letters, 583*(24), 3966-3973.

Marlier, L., Teilhac, J. R., Cerruti, C., & Privat, A. (1991). Autoradiographic mapping of

5-HT_1, 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors in the rat spinal cord. *Brain Research, 550*(1), 15-23.

McEwen, M.L., Van Hartesveldt C., Stehouwer, D.J. (1997). $_L$-DOPA and quipazine elicit air-stepping in neonatal rats with spinal cord transections. *Behavioral Neuroscience, 111*(4), 825-833.

Mendez-Gallardo, V., & Robinson, S.R. (2014). Odor-induced crawling locomotion in the newborn rat: Effects of amniotic fluid and milk. *Developmental Psychobiology, 56*(3), 327-339.

Muir, G. D. (2000). Early ontogeny of locomotor behaviour: A comparison between altricial and precocial animals. *Brain Research Bulletin, 53*(5), 719-726.

Murray, K.C., Nakae, A., Stephens, M.J., Rank, M., D'amico, J., Harvey, P.J., …& Heckman, C.J. (2010). Recovery of motoneuron and locomotor function after spinal cord injury depends on constitutive activity in 5-HT_{2C} receptors. *Nature Medicine, 16*(6), 694-700.

Nishimaru, H., & Kakizaki, M. (2009). The role of inhibitory neurotransmission in locomotor circuits of the developmin mammalian spinal cord. *Acta Physiologica, 197*(2), 83-97.

Nistri, A., Ostroumov, K., Sharifullina, E., & Taccola, G. (2006). Tunins and playing a motor rhythm:How metatropic glutamate receptors orchestrate generation of motor patterns in the mammalian central nervous system. *The Journal of Physiology, 572*(2), 323-334.

Norreel, J.C., Plieger, J.F., Pearlstein, Simeoni-Alias, J., Clarac, F., & Vinay, L. (2003). Reversible disorganization of the locommotor pattern after neonatal spinal cord transection in the rat. *Journal of neuroscience, 23*(5), 1924-1932.

Otoshi, C.K., Walwyn, W.M., Tillakaratne, N.J.K., Zhong, H., Roy, R., & Edgerton, V.R. (2009). Distribution and localization of 5-HT_{1A} receptors in the rat lumbar spinal cord after transection and deafferentiation. *Journal of Neurotrauma*, *26*(4), 575–584.

Parker, D. (2000). Spinal-Cord Plasticity. *Molecular Neurobiology, 22*(1), 55-80.

Patterson, M.M., & Grau, J.W. (2001). *Spinal Cord Plasticity: Alterations in Reflex Function*. Norwell, Massachusets: Kluwer Academic Publishers

Pearlstein, E., Mabrouk, F. Ben, Pflieger, J. F., & Vinay, L. (2005). Serotonin refines the locomotor-related alternations in the *in vitro* neonatal rat spinal cord. *European Journal of Neuroscience, 21*(5), 1338-1346.

Petruska, J.C., Ichiyama, R.M., Jindrich, D.L., Crown, E.D., Tansey, K.E., Roy, R.R., … Mendell, L.M. (2007). Changes in motoneuron properties and synaptic inputs related to step training after spinal cord transection in rats. *The Journal of Neuroscience, 27*(16), 4460–4471.

Purves, D., Augustine, G., Fitzpatrick, D., Hall, W., Lamantia, A S., & White, L. (2012) *Neuroscience* (5^{th} ed.). Sunderland, Massacheusetts: Somaier Associates.

Ren, L.Q., Wienecke, J., Chen, M., Møller, M., Hultborn, H., & Zhang, M. (2013). The time course of serotonin 2C receptor expression after spinal transection of rats: An immunohistochemical study. *Neuroscience, 236*, 31-46.

Roberto, M.E., & Brumley, M.R. (2014). Prematurely delivered rats show improved motor coordination during sensory-evoked motor responses compared to age-matched controls. *Physiology & Behavior, 130*, 75–84.

Robinson, S.R. (2015). Spinal mediation of motor learning and memory in the rat fetus. *Developmental Psychobiology, 57*(4), 421-434.

Robinson, S.R. (2005). Conjugate limb coordination after experience with an interlimb yoke : Evidence for motor learning in the rat fetus. *Developmental Psychobiology, 47*(4), 328-344.

Robinson, S.R., Kleven, G.A. & Brumley, M.R. (2008). Prenatal development of interlimb motor learning in the rat fetus. *Infancy*, *13*(3), 1–23.

Robinson, S.R., & Smotherman, W.P. (1991).The amniotic sac as scaffolding: Prenatal ontogeny of an action pattern. *Developmental Psychobiology*, *24*(7), 463-485.

Sadlaoud, K., Tazerart, S., Brocard, C., Jean-Xavier, C., Portalier, P., Brocard, F., …& Bras, H. (2010). Differential plasticity of the GABAnergic and glycinergic synaptic transmission to rat lumbarmotoneurons after spinal cord injury. *Journal of Neuroscience, 30*(9), 3358-3369.

Saruhashi, Y., Young, W., & Perkins, R. (1996). The recovery of 5-HT immunoreactivity in lumbosacral spinal cord and locomotor function after thoracic hemisection. *Experimental Neurology, 139*(2), 203-213.

Saruhashi, Y., Hukuda, S., & Maeda, T. (1991) Evidence for a neural source of acute accumulation of serotonin platelets in the injured spinal cord of rats. An

experimental study using dihydroxytryptamine treatment. *Journal of Neurotrauma, 8*(2), 121-128.

Sherwood, L. (2004). *Human Physiology: From Cells to Systems* (5th ed.). Belmont, California: Thompson Learning

Shieh, J.Y., Leong., S.K., & Wong, W.C. (1983). Origin of the rubrospinal tract in neonatal, developing and mature rats. *Journal of Comparative Neurology, 214*(1), 79-86.

Sławińska, U., Majczyński, H., Dai, Y., & Jordan, L.M. (2012). The upright posture improves plantar stepping and alters responses to serotonergic drugs in spinal rats. *The Journal of Physiology, 590*(7), 1721-1736.

Smith, C.L. (1983). The development and post-natal organisation of primary afferent projections to the rat thoracic spinal cord . *Journal of Comparative Neurology, 220*(1), 29-43.

Smotherman, W.P., & Robinson, S.R. (1987). Prenatal expression of species-typical action patterns in the rat fetus (*Rattus norvegicus*). *Journal of Comparative Psychology, 101*(2), 190-196.

Smotherman, W.P., & Robinson, S.R. (1986). Environmental determinants of behaviour in the rat fetus. *Animal Behaviour, 34*(6), 1859-1873.

Strain, M.M., & Brumley, M.R. (2014). Range of motion (ROM) restriction influences quipazine-induced stepping behavior in postnatal day one and day ten rats. *Behavioural Brain Research, 274*, 365-381.

Strain, M.M., Kauer, S. D., Kao, T., & Brumley, M.R. (2014). Inter- and intralimb adaptations to a sensory perturbation during activation of the serotonin system after a low spinal cord transection in neonatal rats. *Frontiers in Neural Circuits, 8*(80), 1-13.

Swann, H.E., Kauer, S.D., Allmond, J.T., & Brumley, M.R.. (2017). Stimulation of 5-HT2A receptors recovers sensory responsiveness in acute spinal neonatal rats. *Behavioral Neuroscience, 131*(1), 92.

Swann, H.E., Kempe, R.B., Van Orden, A.M., & Brumley, M.R. (2016). Serotonergic activation of locomotor behavior and posture in one-day old rats. *Behavioural Brain Research, 302*, 104-114.

Taplar, A.E., Kiehn, O. (2010). Glutamergic mechanisms for speed control and network operation in the locomotor CPG. *Frontiers in Neural Circuits, 4*, 1-14.

Tillakaratne, N.J.K., Guu, J.J., de Leon, R.D., Bigbee, A.J., London, N.J.L., Zhong, H., … Edgerton, V.R. (2010). Functional recovery of stepping in rats after complete neonatal spinal cord transection is not due to regrwoth across the lesion site. *Neuroscience 166*(1), 23-33.

Ung, R.V., Landry, E. S., Rouleau, P., Lapointe, N.P., Rouillard, C., & Guertin, P.A. (2008). Role of 5-HT_2 receptor subtypes in quipazine-induced hindlimb movements after a low-thoracic spinal cord transection. *European Journal of Neuroscience, 28*(11), 2231-2242

Vinay, L., Ben-Mabrouk, F., Brocard, F., Clarac, F., Jean-Xavier, C. (2005). Perinatal development of the motor systems involved in postural control. *Neural Plasticity*,

12(2), 131–140.

Vinay, L., Brocard, F., Pflieger, J. F., Simeoni-Alias, J., & Clarac, F. (2000). Perinatal development of lumbar motoneurons and their inputs in the rat. *Brain Research Bulletin, 53*(5), 635-647.

Weber, E.D., & Stelzner, D.J. (1977). Behavioral effects of spinal cord transection in the developing rat. *Brain Research, 125*(2), 241-255.

Westerga, J., Gramsbergen A. (1997). Structural changes of the soleus and tibialis anterior motoneuron pool during development in the rat. *Journal of Comparative Neurology, 319(*3), 406-416.

Yuan, Q., Su, H., Chiu, W., &, Lin, Z. (2013). Contrasting neuropathology and functional recovery after spinal cord injury in developing and adult rats. *Neuroscience Bulletin. 29*(4), 509-516.

Ziskind-Conhaim, L., Seebach, B. s., & Gao, B. X. (1993). Changes in serotonin-induced potential during spinal cord development. *Journal of Neurophysiology*, *69*(4), 1338-1349.