Lumbar spinal cord excitability: flexors vs. extensors, sensitivity to quipazine; effects of activity following spinal transection; and expression of post-synaptic serotonin receptors

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

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Winnipeg

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Abstract

Serotonin (5-HT) is a well-known modulator of spinal cord excitability and motor output. In the spinal cord, the actions of 5-HT are primarily mediated by the 5-HT_{1A}R, 5-HT_2Rs and the 5-HT_7R. Following a spinal cord transection, which results in a loss of supraspinal input, 5-HT agonists such as quipazine are used to provide excitation to the spinal cord to facilitate locomotor recovery. This is characterized by rhythmic alteration of left and right hindlimbs and ipsilateral flexor and extensor muscles. However, whether 5-HT has a global effect on spinal cord excitability or is confined to a specific motor group (i.e. flexors or extensors) is currently unknown. Furthermore, quipazine is used in conjunction with activity based interventions to enhance recovery following a spinal cord injury. However, the influence of limb activity on the responsiveness of the injured spinal cord to quipazine has not been examined. Lastly, the recovery of locomotion is at least in part thought to occur through an up-regulation of 5-HT receptors, although this has not been investigated in lumbar spinal cord.

Chapter 2 examines whether quipazine had a differential effect on flexor and extensor motor output assessed by recording flexor and extensor reflexes, motoneurons and Ia extracellular field potentials pre- and post-quipazine. It was determined that following an acute spinal transection, quipazine induced a larger flexor monosynaptic reflex (MSR) compared to the extensor MSR due to pre-synaptic but not motoneuron modulation.

Chapter 3 examines the influence of a chronic spinal transection with and without passive cycling on the hindlimb flexor and extensor MSR, both pre- and post-quipazine. It was found that three months post STx, the extensor but not flexor MSR demonstrated a hyperexcitable response, which was attenuated with passive cycling. Further, three

months of passive cycling extensor MSR response to quipazine was similar to that seen in the control intact group.

Chapter 4 examined 5-HT receptor expression in flexor and extensor motoneurons three months post spinalization with or without passive cycling. Following a chronic STx, the 5-HT_{1A}R and 5-HT_{2C}R are down regulated, whereas the 5-HT_{2A}R is up-regulated. Passive cycling further enhanced the 5-HT_{2A}R expression as well as up-regulated the 5-HT_7R in extensor but not flexor motoneurons.

Chapter 5 discusses the results and significance of these findings in detail.

Acknowledgements

I would like to first, thank my Ph.D supervisor and mentor Dr. Phillip Gardiner. Between my Masters and PhD we have been together for seven terrific years. You have always been positive and supportive and allowed me to pursue my curiosities. You have given me guidance, mentorship and taught me what it takes to be a positive role model for future students and scientists. Most importantly, you have given me the knowledge, skill set and passion to pursue a career in sciences. Thank you!

I would also like to thank my PhD committee – Dr. Larry Jordan, Dr. Brian (or Brain) Schmidt and Dr. Dean Kriellaars. When my committee was initially formed, I was a little intimidated and scared as there was such a wealth of knowledge and experience among the group. However, I quickly learned that all three of you were more than willing to share that knowledge and experience whenever it was needed. All three of you have taught me to think outside the box and critically assess the literature as well as my own results. Thank you!

I would also like to thank members of Dr. Gardiner's lab. Dr. Chris MacDonell, we have worked together for a long time now. It has been great times and sometimes frustrating (hurray electrophysiology) times. It has been great getting to know you as a researcher as well as a friend. I would like to thank Kalan Gardiner, Shannon Deschamps and Patricia Sheppard for your patience, guidance and support in teaching me animal care and support as well as various lab techniques. I would also like to thank Jason Au, the rat care specialist, for two great years. Your help and training of the rats were invaluable and I appreciate the work so much. Lastly, I would like to thank the honorary lab member Dr.

Kris Cowley. You have been very supportive during my time at the SCRC and have always allowed me to rant or bounce ideas to you and have sent me on the correct direction to find the answers. Thank you all!!

Thank you to the Manitoba Health Research Council and Canadian Institutes for Health Research for providing me with student scholarships. I would also like to thank the financial supporters for this research; Canadian Institutes for Health Research, Canada Research Chairs program and the Manitoba Paraplegic Foundation.

Dedication

I dedicate this thesis to my parents and Ashley. To my parents Paul and Colleen. You have provided me tremendous unconditional support from day one of my academic journey. Whether it was something small or something big both, you two have gone out of your way without a second thought to ensure the best for me. I gratefully appreciate every sacrifice you have made on my behalf and without a doubt I would not have achieved this without you.

To Ashley, we meet half-way through my program and all I can say is I wish I met you earlier. You have provided me tremendous support and a steady calming voice that I needed. I look forward to beginning the next chapter of our lives together in Halifax!

List of abbreviations

5-HT	serotonin
5-HTR	serotonin receptor
AHP	afterhyperpolarization
AHP_{amp}	afterhyperpolarization amplitude
AHP_{decay}	afterhyperpolarization ½ decay time
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium
CPG	central pattern generator
DCC	discontinuous current clamp
DSCT	dorsal spinocerebellar tract
EDL	extensor digitorium longus
ENG	electroneurogram
EPSP	excitatory post synaptic potential
FDD	frequency dependent depression
f-I	frequency-current
GABA	gamma amino butyric acid
GDNF	glial cell derived neurotrophic factor
GIRK	g-protein gated inward rectifying potassium current
GPCR	g-protein coupled receptor
i.p.	intraperitoneal
IR	input resistance
K^+	potassium
Mn	motoneuron
MSR	monosynaptic reflex
mV	millivolt

nA	nanoampere
Na	sodium
PIC	persistent inward current
PPR	parapyramidal region
RMP	resting membrane potential
s.c.	sub-cutaneous
SCI	spinal cord injury
STx	spinal transection
T	threshold
μV	microvolt
Vth	voltage threshold

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CHAPTER 1: GENERAL INTRODUCTION

Spinal cord excitability depends on various factors, including supraspinal input, monoamines, neurotransmitters and afferent feedback. The monosynaptic reflex (MSR), due to its simplicity of eliciting and recording, is often used to measure spinal cord excitability and indirectly the excitability of the motoneuron in mammals from mice to humans. The MSR is a simple spinal reflex circuit involving one synapse between the Ia afferents and the homonymous alpha-motoneurons. Eliciting the MSR or the analogous h-reflex allows for the monitoring of change in spinal cord excitability following an intervention such as the use of a drug or after periods of activity or inactivity, all of which influence the level of excitation of the spinal cord.

The use of MSR to measure the excitability of two distinct motor groups underlies a component of this thesis. This thesis examines the influence that the serotonergic agonist quipazine (5-HT_2R agonist) has on flexor (peroneal nerve) and extensor (tibial nerve) MSR excitability in activity dependent states. The last project of this thesis will aim to provide context in how alterations in the spinal cord excitability can occur by examining serotonin (5-HT) receptor gene expression in identified flexor and extensor motoneurons following a chronic spinal transection with and without passive cycling. The examination of the MSR both pre-and post- quipazine and 5-HT receptor gene expression was chosen for the following reasons and will be discussed further: 1) serotonin is a well-known modulator of locomotion, with the serotonergic agonist quipazine routinely used to excite the spinal cord following a spinal cord injury (SCI), 2) indirect evidence suggests flexor and extensor motoneurons respond differently to

serotonin, 3) both the MSR and motoneurons demonstrate a hyperexcitability to serotonin and serotonergic agonists following a SCI, 4) serotonin receptor expression is altered following a SCI, and 5) serotonin receptor expression is altered with increases in endurance activity.

Serotonin modulates motor output

The origin of descending 5-HT fibres to the spinal cord

The sources of serotonin for the spinal cord originate almost exclusively from supraspinal centres (Carlsson *et al.*, 1963;Carlsson *et al.*, 1963;Carlsson *et al.*, 1964). Specifically, serotonin originates from the brain stem in three distinct regions – raphe pallidus, raphe obscuris and the raphe magnus (Dahlstroem & Fuxe, 1964;Dahlstroem & Fuxe, 1965) and also from a portion of 5-HT neurons located in the parapyramidal region (PPR) of the mid-medulla (Liu & Jordan, 2005). In the rat, the remaining source of 5-HT may come from intraspinal neurons, located dorsal to the central canal between segments thoracic 3 and coccyx 1 (Newton & Hamill, 1988;Newton *et al.*, 1986). Although an intraspinal source of 5-HT exists, they are thought to play a role in the sympathetic system (Newton *et al.*, 1986) while their role, if any in locomotion remains unclear. Following a spinal transection only 2-15% of 5-HT content remains below the lesion in the rat and cat (Carlsson *et al.*, 1963;Clineschmidt *et al.*, 1971;Hadjiconstantinou *et al.*, 1984) and may number between 3-9 neurons in the rat spinal cord (Newton & Hamill, 1988).

The three raphe nuclei innervate distinct regions within the spinal cord. 5-HT neurons originating from the raphe magnus predominately project to the dorsal horn via the dorsal funiculus (Schmidt & Jordan, 2000). Serotonergic contacts within the dorsal

horn are found on dorsal spinocerebellar tract (DSCT) cells and group II muscle afferent interneurons, with DSCT cell innervation being diffuse and group II muscle afferent interneuron innervation being in close apposition (Jankowska *et al.*, 1995;Jankowska *et al.*, 1997;Maxwell & Jankowska, 1996). Serotonergic neurons located within the raphe obscuris and raphe pallidus predominately project to the intermediate grey matter and lamina IX (motoneuron level) via the ventrolateral and ventral funiculi respectively (Schmidt & Jordan, 2000). Whereas in the dorsal horn, descending 5-HT innervation tends to be diffuse, innervation of motoneurons seems to be specific with close apposition between serotonergic fibres and motoneuron dendrites and cell bodies accounting for more than 1500 dendritic and 52 somatic contacts per motoneuron (Alvarez *et al.*, 1998). Serotonin cells originating in the PPR appear to synapse on central pattern generator (CPG) neurons as well as motoneurons and are involved with generation of locomotion (Liu & Jordan, 2005).

Serotonin Receptors in the spinal cord

The actions of 5-HT are mediated by various receptors and their respective subtypes. Prior to the understanding of the various receptors in the spinal cord, bath application of 5-HT on the spinal cord or electrical stimulation of the 5-HT raphe nuclei would often result in conflicting reports, in which 5-HT could cause either an increase (Barasi & Roberts, 1974;Wang & Dun, 1990;Clineschmidt & Anderson, 1970;Hounsgaard *et al.*, 1988a) or decrease in spinal cord excitability (Wang & Dun, 1990;Clineschmidt & Anderson, 1970;Phillis *et al.*, 1968;Anderson EG & Shibuya T, 1966). With the use of selective 5-HT receptor agonists and antagonists the mechanisms by which serotonin acts on the spinal cord have become better understood. There are

seven known 5-HT receptor families (5-HT_{1-7}) with 14 distinct receptor subtypes (Hoyer *et al.*, 1994). Excluding the 5-HT_3R which is a ligand gated ion channel, 5-HT receptors modulate neuron excitability via G-protein coupled receptors (GPCR) (Hoyer *et al.*, 1994;Hoyer *et al.*, 2002). Of importance in modulating spinal cord excitability are the 5-HT_1Rs, 5-HT_2Rs and the 5-HT_7R which will be discussed.

5-HT_1 Receptors

The 5-HT_1 receptor family consists of five subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}) which when activated couples with the g-protein subunit Gi to inhibit adenyl cyclase and ultimately decrease cyclicAMP activity and Ca^{2+} conductance (Landry *et al.*, 2006b;Hoyer *et al.*, 2002). As well, the βγ dimer subunit of the GPCR activates the GIRK (g-protein-gated inward rectifying K^+ channel) which leads to hyperpolarization of the cell (Landry *et al.*, 2006b). In the spinal cord, the 5-HT_1 receptor subtypes are found almost exclusively in the dorsal horn (Marlier *et al.*, 1991), except for the 5-HT_{1A} receptor which is also found on the motoneuron (Otoshi *et al.*, 2009;Kheck *et al.*, 1995;Cotel *et al.*, 2013). Autoradiographic ligand binding has demonstrated that the 5-HT_{1A} receptor is located primarily in lamina I & II of the dorsal horn and demonstrates a rostro/caudal gradient of expression, with the highest expression located in the lumbar and sacral segments (Marlier *et al.*, 1991). On the motoneuron, the 5-HT_{1A} receptor is located at the axon initial segment (Otoshi *et al.*, 2009;Kheck *et al.*, 1995;Cotel *et al.*, 2013) while others also suggest the 5-HT_{1A} receptor is also located on the soma and proximal dendrites (Otoshi *et al.*, 2009). Excluding the 5-HT_{1A} receptor, the remaining 5-HT_1 receptors are thought to have a role in nociception and inhibition of sensory transmission to the motoneuron (Millan, 2002;Yoshimura & Furue, 2006). For example,

agonists for the 5-$HT_{1B/D}$ receptors have been shown to reduce spasticity in non-injured and spinal cord injured humans (D'Amico *et al.*, 2013a) by reducing sensory transmission of low threshold afferents to the motoneuron (Murray *et al.*, 2011b).

In general, through the use of agonists and antagonists, it is established that activation of the 5-HT_{1A} receptor results in a depressed monosynaptic reflex (Hasegawa & Ono, 1996;Gajendiran, 2007;Nagano *et al.*, 1988;Hedo *et al.*, 2002) and decreased motoneuron excitability demonstrated by hyperpolarization of the resting membrane potential (Talley *et al.*, 1997;Wang & Dun, 1990) and decreased input resistance (Wang & Dun, 1990), likely due to activation of the GIRK channel. Recently it has been demonstrated that the 5-HT_{1A} receptor may be responsible for central fatigue during prolonged activation (Cotel *et al.*, 2013). When the agonist 8-OH-DPAT (5-$HT_{1A/7}$R agonist) was applied to the axon initial segment, the motoneuron ceased to generate action potentials. However, when 8-OH-DPAT was applied to other regions of the soma or dendrites, no effect on the motoneuron was seen. It was suggested that as no pre-synaptic 5-HT terminals oppose the axon initial segment, that spillover from prolonged 5-HT release on the dendrites and soma eventually activates the 5-HT_{1A} receptor and terminates output by inhibiting Na^+ conductance (Cotel *et al.*, 2013).

Although a general consensus exists for an inhibitory effect from 5-HT_{1A} receptor activation, contradictory reports have also shown that the 5-HT_{1A} receptor depolarizes motoneurons in the neo-natal rat, by activation of the non-specific cation current (I_h) and inhibition of the K+ leak channel (Ziskind-Conhaim *et al.*, 1993;Takahashi & Berger, 1990). Furthermore, it has been shown that activation of the 5-HT_{1A} receptor facilitated recovery of locomotion in the spinalized rat (comparable to agonists of the 5-HT_2

receptor; (Antri *et al.*, 2003;Landry *et al.*, 2006a) and the general 5-HT_1 receptor antagonist propranolol, inhibited 5-HT induced locomotion in the *in-vitro* rat preparation (Cazalets *et al.*, 1992). However, these contradictory reports on motoneuron properties can partially be explained by a potential age dependent response of the spinal cord to 5-HT (Wang & Dun, 1990;Ziskind-Conhaim *et al.*, 1993). For example, activation of the 5-HT_{1A}R in the neonate, depolarizes the motoneuron, whereas in the juvenile rat, 5-HT_{1A}R activation hyperpolarizes the motoneuron (Ziskind-Conhaim et a., 1993).

5-HT_2 Receptors

The 5-HT_2 receptor family consists of three subtypes (5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}) which share a 46-50% overall sequence identity (Hoyer *et al.*, 2002) and are located in the spinal cord of rat, cat, monkey and humans (Helton *et al.*, 1994). 5-HT_2 receptors when activated, bind with the Gq subunit to increase phospholipase c activity and ultimately increase hydrolysis of inositol phosphates, elevate cytosolic [Ca^{2+}] and reduce K^+ conductance (Hoyer *et al.*, 2002;Landry *et al.*, 2006b).

Initial studies, prior to the identification of the receptor subtypes, demonstrated that the 5-HT_2 receptor is primarily found in grey matter, with a higher concentration in the ventral horn, specifically in lamina IX. The 5-HT_2R distribution demonstrated a rostro/caudal gradient with the highest expression seen in the mid and low thoracic levels (Marlier *et al.*, 1991). In situ hybridization studies have demonstrated that the 5-HT_{2A} receptor is located exclusively in lamina IX and absent in all other laminae of the spinal cord (Fonseca *et al.*, 2001). However, it should be noted, a study using immunocytochemistry, demonstrated that the 5-HT_{2A} receptor is also widely expressed in the grey matter, with a high expression in laminae I and II, primarily on postsynaptic cell

bodies (Doly *et al.*, 2004). The 5-HT_{2C} receptor is widely distributed in the grey mater except for lamina II and equally distributed throughout the spinal cord (Fonseca *et al.*, 2001). The location of the 5-HT_{2B} receptor has not been fully investigated, although recently using immunolabeling, the 5-HT_{2B} receptor was identified on the motoneuron and thought to regulate the calcium persistent inward current (Ca PIC) along with the 5-HT_{2C} receptor (Murray *et al.*, 2011a).

Various agonists and antagonists have demonstrated that activation of both the 5-$HT_{2A/2C}$ receptors results in an increase in amplitude of the MSR and spinal reflexes (Miller *et al.*, 1996;Machacek *et al.*, 2001;Shay *et al.*, 2005;Hasegawa & Ono, 1996;Gajendiran, 2008). Activation of 5-HT_2 receptors on the motoneuron has demonstrated an increase in input resistance (Wang & Dun, 1990;Elliott & Wallis, 1992;Hsiao *et al.*, 1997), depolarization of the resting membrane potential (VanderMaelen & Aghajanian, 1980;White & Fung, 1989;Wang & Dun, 1990;Elliott & Wallis, 1992;Hsiao *et al.*, 1997;Harvey *et al.*, 2006), hyperpolarization of the voltage threshold (Fedirchuk & Dai, 2004) and both a decrease (White & Fung, 1989) and increase in the AHP amplitude (Zhang, 1991). Activation of the 5-HT_2 receptors also enhances motoneuron excitability by activation of both the sodium (Na) and calcium PICs (Harvey *et al.*, 2006;Perrier & Hounsgaard, 2003) where the 5-HT_{2B} and $_{2C}$ receptors mediate the CaPIC (Murray *et al.*, 2011a) and the 5-HT_{2A} receptor likely mediates the NaPIC (Harvey *et al.*, 2006).

Facilitation of locomotion in the intact and spinalized animal is partially linked to the activation of 5-HT_2 receptor family (Schmidt & Jordan, 2000). Serotonin induced locomotion in the *in-vitro* neonatal rat and mouse preparations can be blocked by

antagonists for the 5-HT_{2A} and 5-HT_{2C} receptors (Cazalets *et al.*, 1992;Bracci *et al.*, 1998;MacLean *et al.*, 1998;Madriaga *et al.*, 2004). Conversely, in the spinalized preparation, agonists of the 5-HT_2 receptors facilitated hindlimb recovery, characterized by increased EMG amplitudes, frequency, coordination and weight support (Antri *et al.*, 2002;Barbeau & Rossignol, 1990;Kim *et al.*, 2001). As well, quipazine, a general 5-HT_2 receptor agonist is commonly used alone or in combination with activity based interventions or grafting of serotonergic neurons to facilitate locomotor recovery following a spinal transection (Feraboli-Lohnherr *et al.*, 1999;Fong *et al.*, 2005;Gerasimenko *et al.*, 2007;Ichiyama *et al.*, 2008;Slawinska *et al.*, 2012;Ung *et al.*, 2008;Slawinska *et al.*, 2013;Kim *et al.*, 2001;Musienko *et al.*, 2011).

Constitutive activity of 5-HT_2 Receptors

In the last decade, the concept of constitutive activity of the 5-HT_2 receptor on the motoneuron has been studied and linked to spasticity and recovery of locomotion following a spinal transection (Fouad *et al.*, 2010;Murray *et al.*, 2010;D'Amico *et al.*, 2013b). Constitutivly active receptors spontaneously couple to their respective G protein (Gq for 5-HT_2 receptors) and initiate intracellular signaling independent of whether a ligand is bound to its receptor (Seifert & Wenzel-Seifert, 2002). Inverse agonists, which bind to the receptor, uncouple the g protein from the respective receptor and terminate intracellular signaling (Seifert & Wenzel-Seifert, 2002). Specific inverse agonists for the 5-HT_{2C} receptor have implicated that constitutive activity in both rats and humans underlie spasticity due to larger PIC activation. When inverse agonists are administered in both rats and humans, spasticity and long lasting reflexes are abolished (Murray *et al.*, 2010;Murray *et al.*, 2011a;D'Amico *et al.*, 2013b). Similar in the spinalized rat, after

locomotion has been induced via 5-HT agonists, administration of a $5\text{-}HT_2$ and a $5\text{-}HT_{2C}$ receptor inverse agonist abolished locomotion, implicating a role of $5\text{-}HT_2$ receptor constitutive activity in locomotor recovery (Fouad *et al.*, 2010;Murray *et al.*, 2010).

$5\text{-}HT_7$ Receptors

The $5\text{-}HT_7$ receptor binds with a Gs subunit which in turn leads to increases in cAMP activation and ultimately an increase in intracellular $[Ca^{2+}]$ and conductance (Hoyer *et al.*, 2002). Initial studies demonstrated that the $5\text{-}HT_7$ receptor is primarily found in the dorsal horn with the highest density in lamina I & II, suggesting a role in pain transmission (Doly *et al.*, 2005;Meuser *et al.*, 2002). More recently, the $5\text{-}HT_7$ receptor has been identified in the dorsal horn as well as lamina VII in the ventral horn, often co-localized with $5\text{-}HT_{2A}$ and $5\text{-}HT_{1A}$ receptors and restricted to locomotor interneurons (Noga *et al.*, 2009).

It is believed that the $5\text{-}HT_7$ receptor does not directly modulate the motoneuron as the receptor has not been identified on the motoneuron, but rather it appears the $5\text{-}HT_7$ receptors modulates motor output by modulating neurons that comprise the CPG (Noga *et al.*, 2009;Slawinska *et al.*, 2013;Liu & Jordan, 2005). Activation of the $5\text{-}HT_7$ receptor with 8-OH-DPAT results in locomotion in the *in-vivo* spinalized mouse preparation (Landry *et al.*, 2006a) and the adult spinalized rat (Slawinska *et al.*, 2012;Slawinska *et al.*, 2013). Furthermore, antagonists of the $5\text{-}HT_7$ receptor abolishe 5-HT induced locomotion in the *in-vitro* mouse and rat preparation (Landry *et al.*, 2006a;Madriaga *et al.*, 2004;Pearlstein *et al.*, 2005;Liu *et al.*, 2009). Locomotion induced by electrical or chemical stimulation is abolished when $5\text{-}HT_7$ receptor antagonists are applied to the rostral (level of CPG) but not caudal portion of the lumbar spinal cord (Liu & Jordan,

2005). Lastly, using a double knock-out mouse model of the 5-HT_7 receptor (5-$HT7^{-/-}$), it has been suggested that during 5-HT induce locomotion, the 5-HT_7 receptor is necessary for coordinated left and right hindlimb alternation (Liu *et al.*, 2009).

Modulating flexor and extensor motor output

Motoneurons can be divided into subtypes based on their metabolic and physiological profiles (Burke, 1967;Burke *et al.*, 1973). This has resulted in the classification of slow and fast motoneurons which differ in their biophysical properties and their ability to generate force and resist fatigue (Burke, 1967). For example fast and slow motoneurons have distinct differences in the size and duration of their AHP (Zengel *et al.*, 1985;Gardiner, 1993;Gardiner, 1993;Gardiner, 1993) . Also, slow motoneurons compared to fast motoneurons can generate plateau potentials more readily (Lee & Heckman, 1998b;Lee & Heckman, 2000;Lee & Heckman, 1998a;Heckman *et al.*, 2008). While a basic understanding of fast and slow motoneurons has been established, understanding differences between flexor and extensor motoneurons has not extensively been studied. The little evidence that does exist (Hounsgaard *et al.*, 1988a;Cotel *et al.*, 2009), would suggest that a fundamental difference exists between identified flexor and extensor motoneurons in their ability to activate persistent inwards currents, which will be discussed below and provides the rationale for project#1.

Project #1 "Examination of flexor and extensor motor output in response to quipazine in the intact and acute spinalized adult decerebrate rat"

Rationale for Project #1

Flexor and extensor motoneurons and their ability to generate PICs

Persistent inward currents have been extensively studied since they were first identified in motoneurons by Schwindt and Crill (Schwindt & Crill, 1977). Persistent inward currents defined as depolarizing currents generated by non- or slow-inactivating voltage gate Na^{+} and Ca^{2+} ion channels (Li & Bennett, 2003;Lee & Heckman, 1999) are essential for sustained firing in the absence of synaptic input to the motoneuron. When outward K^{+} currents are blocked, and the membrane potential is depolarized above activation threshold (~ -45mV to -55mV) PICs can mediate a plateau potential (action potential is blocked (Heckman *et al.*, 2003). A persistent inward current can be initiated by a short depolarizing current that produces a sustained depolarization or self-sustained firing (action potential is not blocked) that remains for long periods. A subsequent brief hyperpolarizing pulse or inhibitory input to the motoneuron restores the cell to the quiescent state. This is referred to as “bistable behaviour” in which excitatory and inhibitory inputs can toggle the cell between the depolarized and quiescent states (Heckman *et al.*, 2003).

In the 1980’s Hultborn and colleagues studied the role of monoamines in generating plateau potentials. First, it was demonstrated that plateau potentials could be generated in the adult decerebrate preparation likely due to decerebration resulting in a tonic activity of brainstem (serotonergic) neurons, which projected to the spinal cord (Crone *et al.*, 1988). Subsequent studies in the cat demonstrated that a spinal transection eliminated the bistability of motoneurons, which could be restored with the administration of 5-HT or L-DOPA (Conway *et al.*, 1988;Hounsgaard *et al.*, 1988a). During this time it was suggested that extensor motoneurons had a greater ability to generate plateau potentials compared to flexor motoneurons (Hounsgaard *et al.*, 1988a). Recently, in the

in-vitro neonatal rat preparation, this discrepancy was confirmed. The vast majority of extensor motoneurons (70%) studied demonstrated self-sustained firing, whereas none of the flexor motoneurons demonstrated self-sustained firing (Cotel *et al.*, 2009). These two studies demonstrated a potential fundamental difference in modulating extensor and flexor motor output.

Differences in flexor and extensor motor output to 5-HT and 5-HT agonists

The notion that extensor and flexor motoneurons differ in their ability to generate plateau potentials or self-sustained firing was a large part of the rationale for project #1. It is established that 5-HT and 5-HT agonists modulate both the NaPIC and CaPIC likely through activation of the 5-HT_{2A} and 5-$HT_{2B/2C}$ receptors respectively (Murray *et al.*, 2011a). The study by Hounsgaard et al. (1988), which demonstrated extensor motoneurons had a greater ability to generate plateau potentials also demonstrated that following application of 5-HT, the discrepancy of extensor motoneurons compared to flexor motoneurons in generating plateau potentials was even greater.

A study by Vult von Steyern and Lomo (2005) examined the distribution of the 5-HT_{2A} receptors on the soleus (extensor) and EDL (flexor) motor pools. Using immunological staining, a greater intensity and percentage of staining for the 5-HT_{2A} receptor was found on soleus motoneurons compared to EDL motoneurons. Furthermore, 5-HT_{2A} receptors on the soleus were found both on the dendrites and soma of the motoneuron, whereas 5-HT_{2A} receptors were only found on the soma of EDL motoneurons. However, as the authors pointed out, the purpose of the study was to

examine physiological fast (EDL) and slow (soleus) motor pools not flexor and extensor motor pools.

Lastly, quipazine is routinely used to facilitate stepping following a spinal cord injury. A study by Edgerton's group has suggested that following a spinal transection in the rat, quipazine may act on two distinct pathways to modulate flexor and extensor output (Gerasimenko *et al.*, 2007). Using a transfourier analysis of EMG activity, the author's suggested that quipazine may act on flexors via a monosynaptic pathway whereas quipazine acted on extensors via a polysynaptic pathway. Taken together, these studies suggest that serotonin or serotonergic agonists such as quipazine may have a differential effect on flexor and extensor motoneurons.

Hypothesis #1: Quipazine will enhance extensor motor output defined as increased monosynaptic reflex amplitude and motoneuron excitability to a larger extent than that demonstrated in the flexor monosynaptic reflex and flexor motoneurons in both the intact and acute spinalized preparation.

The effect of chronic spinal transection on spinal cord excitability

Following the initial period of spinal shock, when the spinal cord is characterized by hypo-reflexive responses and lack of muscle tone (Ditunno *et al.*, 2004), the spinal cord undergoes remodeling that results in supersensitivtiy to exogenously applied monoaminergic such as the serotonin precursor 5-HTP (Barbeau & Bedard, 1981). This "de-innervation supersensitivtiy" on the motoneuron is at least in part thought to be the result of up-regulation of serotonin receptors (Barbeau & Bedard, 1981;Wienecke *et al.*, 2010) and increase in constitutive activity of serotonin receptors (Murray *et al.*, 2010).

Regulation of 5-HT Receptors following a spinal transection

The understanding of gene regulation following a spinal transection is relatively new, but in the last several years research has begun to examine the re-distribution or re-organization of receptors post-spinal transection. Global gene expression studies in isolated sacral motoneurons using laser capture microdissection have examined the time course of gene clusters following a sacral spinal transection up to 60 days post injury (Wienecke *et al.*, 2010;Ryge *et al.*, 2010). It was demonstrated that initial or early alteration in gene expression was associated with genes that mediate immunological and inflammatory responses, whereas late alterations in gene expression were associated with genes that regulate neuron excitability (Wienecke *et al.*, 2010;Wienecke *et al.*, 2010;Ryge *et al.*, 2010). In general, during the late response (60 days post injury), neuromodulator pathways (serotonergic, dopaminergic, and adrenergic) are up-regulated, while inhibitory neurotransmitter pathways such as the GABAergic and glycinergic pathways are down-regulated, likely responsible for the motoneuron hyperexcitability seen post transection (Ryge *et al.*, 2010;Wienecke *et al.*, 2010).

While initial examination in global gene expression has been undertaken, a more thorough understanding of serotonin receptors post transection is established. In particular, the time course of altered 5-HT_{2A} receptor gene expression has recently been investigated using immunohistochemistry (Kong *et al.*, 2010;Kong *et al.*, 2011). These studies demonstrated that 1 day post spinal transection, the 5-HT_{2A} receptor is up-regulated 3.5-fold, increasing to 5-fold at day 28 and persisting until day 60 in sacral motoneurons. However, there was no correlation associated between the development of tail spasticity and the up-regulation of the 5-HT_{2A} receptor, suggesting that the 5-HT_{2A}

receptor was not responsible for mediating tail spasticity. 5-HT_{2A} receptor mRNA has also been shown to be up-regulated in the lumbar ventral horn following a spinal transection in rats. Specifically, 5-HT_{2A} receptor mRNA was up-regulated 3 hours post transection in the lateral intermediate zone, persisting for 14 days then eventually returning to control levels (Ung *et al.*, 2008). However, the alteration in 5-HT_{2A} receptor in lumbar motoneurons has not been examined.

Similar to the 5-HT_{2A} receptor, the time course and alteration of 5-HT_{2C} receptor expression post spinalization has been extensively examined. However, a lack of consistency in 5-HT_{2C} receptor expression post injury has been demonstrated. An initial study conducted by Bennett's group demonstrated the 5-HT_{2C} receptor undergoes mRNA editing following a sacral transection to produce a 5-HT_{2C} receptor that has a higher level of constitutive activity (see earlier discussion), although the total amount of 5-HT_{2C} receptor mRNA in the sacral ventral horn was unchanged (Murray *et al.*, 2010). A similar study was conducted, however a thoracic spinal transection was used and lumbar mRNA levels were examined (Navarrett *et al.*, 2012). It was demonstrated that the 5-HT_{2C} receptor did not undergo editing nor did the total amount of 5-HT_{2C} receptor mRNA alter, however an up-regulation of 5-HT_{2A} receptor mRNA was detected below the lesion (Navarrett *et al.*, 2012). Whereas these two studies examined 5-HT_{2C} receptor mRNA in the whole cord below the lesion, a recent study has examined 5-HT_{2C} receptor protein expression on sacral motoneurons post sacral transection using immunohistochemistry (Ren *et al.*, 2013). The 5-HT_{2C} receptor was found to significantly increase in the ventral horn at day 28 post transection and on sacral motoneurons at day 45, increasing 1.8 and

1.6 fold respectively. Further, the increase in 5-HT_{2C} receptor expression correlated with the onset of tail spasticity in these rats (Ren *et al.*, 2013).

From these studies it is obvious that serotonin receptors are differentially regulated following a spinal transection and partly contribute to the increased excitability of the motoneuron and spasticity. However, serotonin receptor gene expression in isolated lumbar motoneurons has not been examined. Further, studies have not examined whether the demonstrated super-sensitivity of the motoneuron (Harvey *et al.*, 2006) is found in flexor and extensor motoneurons. This lack of information provides rationale for projects #2 and #3 and will be discussed below. The rationale for examining passive cycling for projects #2 and #3 will be discussed in a subsequent section.

Project #2 "Daily passive cycling attenuates the hyperexcitability and restores the responsiveness of the extensor monosynaptic reflex to quipazine in the chronic spinal transected rat"

Project #3 "Serotonin receptor and KCC2 gene expression in lumbar flexor and extensor motoneurons post-transection with and without passive cycling"

Rationale for Projects #2

It is well established that activation of serotonin receptors is necessary for motoneuron excitability and locomotor recovery following a spinal transection. In particular, serotonergic agonists acting on the 5-HT_{2A} receptor (Ung *et al.*, 2008) and 5-HT_{2C} receptor (Fouad *et al.*, 2010;Murray *et al.*, 2010) have been found to facilitate locomotor recovery as well as modulate motoneuron excitability (Miller *et al.*, 1996;Murray *et al.*, 2011a;Harvey *et al.*, 2006) post-spinal transection. However,

whether or not serotonergic agonists such as quipazine act differently on flexor and extensor motoneurons in the chronic state of a spinal transection has not been investigated. Results from my project #1 demonstrated that in the intact rat, no difference between the response of the flexor and extensor MSR to quipazine existed (Chopek *et al.*, 2013). However, following an acute spinal transection, both the flexor and extensor MSR demonstrate a dramatic increase in amplitude in response to quipazine, with a significantly greater response seen in the flexor MSR compared to the extensor MSR (5.7-fold vs 3.6-fold increase respectively, (Chopek *et al.*, 2013). Whether a difference exists between the extensor and flexor MSR to quipazine following a chronic spinal transection is unknown.

Recently it has been demonstrated, in the chronic spinal transection rat, that quipazine has a defined role in facilitating extension, demonstrated by an increase in stance duration and an increase in duration and amplitude of medial gastrocnemius EMG bursts (Musienko *et al.*, 2011). Also in the cat, it has been demonstrated that spinal reflexes undergo remodeling from the acute to chronic state of a spinal cord transection. In the acute state, the triceps surae stretch-reflex did not elicit EMG activity of the extensor muscles (lateral gastrocnemius or soleus) but rather generated EMG activity from the antagonist flexor muscles (tibialis anterior, sartorius and semitendinosus). One month later in the chronic state the triceps surae stretch-reflex elicited EMG activity of both the extensor and flexor muscles, producing greater extensor torque (Frigon *et al.*, 2011). These studies suggest there is a differential transformation of flexor and extensor reflexes between the acute and chronic states.

Hypothesis #2.1 The extensor monosynaptic reflex amplitude, elicited by afferent stimulation will be larger in the three month spinal transected group compared to the control group. No difference in flexor monosynaptic reflex amplitude will be seen between the three month transected group compared to the control group.

Hypothesis #2.2 Three months post spinal transection, the extensor monosynaptic reflex will demonstrate a larger increase in amplitude in response to quipazine compared to the flexor monosynaptic reflex.

Rationale for project #3

Although numerous studies have examined serotonin gene or protein expression following a spinal transection (Kong *et al.*, 2010;Navarrett *et al.*, 2012;Ung *et al.*, 2008;Otoshi *et al.*, 2009;Murray *et al.*, 2010;Kong *et al.*, 2011;Ryge *et al.*, 2010;Wienecke *et al.*, 2010;Ren *et al.*, 2013), to date gene expression specifically in lumbar motoneurons post spinalization has not been examined. As pharmacological agents targeting serotonin receptor activation to promote locomotor recovery (Gerasimenko *et al.*, 2007;Barbeau & Rossignol, 1990;Barbeau & Rossignol, 1991;Antri *et al.*, 2002) or reduce spasticity (D'Amico *et al.*, 2013b;Murray *et al.*, 2011b;Murray *et al.*, 2011a) are used, a better understanding of the regulation of serotonin receptors post-spinalization in lumbar motoneurons is needed. Further, in the control spinal cord intact state, potential differences in serotonin receptor expression between flexor and extensor motoneurons has not been examined. A previous study has demonstrated, using immunohistochemistry, that soleus motoneurons demonstrated a higher level of 5-HT_{2A}R staining compared to EDL motoneurons (Vult & Lomo, 2005). If interventions such as

activity are to be used to promote recovery (Gerasimenko *et al.*, 2007;Ichiyama *et al.*, 2011;Fong *et al.*, 2005;Barbeau & Rossignol, 1987), an understanding of serotonin receptor expression in flexor and extensor motoneurons needs to be examined to appreciate any potential impact of an intervention post spinal cord injury.

Hypothesis #3.1 In the intact control animal, extensor motoneurons will have higher levels of 5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2C}R gene expression compared to flexor motoneurons.

Hypothesis #3.2 Three months post-transection, 5-HT_{2A}R and 5-HT_{2C}R expression will be up-regulated in both extensor and flexor motoneurons, whereas 5-HT_{1A}R expression will be down-regulated.

Hypothesis #3.3 Three months post transection, 5-HT_{2A}R and 5-HT_{2C}R expression will be up-regulated to a greater extent in extensor motoneurons compared to flexor motoneurons.

Influence of activity on the spinal cord following a spinal cord injury

Activities such as treadmill training or passive cycling are used alone or in combination with serotonergic agonists to preserve the spinal circuitry and promote functional recovery following a complete spinal cord transection. In the late 1980s, it was demonstrated that cats regained the ability to step following a complete thoracic spinal transection and that with daily treadmill training, the ability to step and the kinematics of stepping was superior (Barbeau & Rossignol, 1987;de Leon *et al.*, 1998;de Leon *et al.*, 1999;Lovely *et al.*, 1986;Lovely *et al.*, 1990) . This was subsequently demonstrated in rat (Cha *et al.*, 2007;Timoszyk *et al.*, 2005) and mice (Cai *et al.*, 2006;Fong *et al.*, 2005). It

is hypothesized that the sensory input received during locomotion activates the proper spinal circuitry and may prune or eliminate the aberrant connections that occur following a spinal transection (Fong *et al.*, 2009). However, the exact mechanism by which treadmill training facilitates the recovery of stepping is unknown.

Treadmill training in conjunction with pharmacological agents appears to have an additive effect. In the cat, clonidine, an α2 noradrenergic agonist reduces the recovery time in which the cat can regain stepping, prolong the cycle duration and increase the burst duration and amplitude of flexor and extensor EMG (Giroux *et al.*, 1998;Chau *et al.*, 1998)**,** however a direct comparison between treadmill training and the use of clonidine alone were not made. In the complete spinal transected rat, the use of quipazine and epidural stimulation promoted coordinated stepping, longer and larger EMG burst amplitudes in treadmill trained rats compared to non-treadmill trained rats (Gerasimenko *et al.*, 2007) . Lastly, in complete spinal transected mice, quipazine in combination with robotic step training further enhanced the number of steps performed and the consistency of stepping compared to robotic step training alone (Fong *et al.*, 2005)**.** Despite the understanding of exercise and pharmacological agents on locomotor recovery following a spinal transection, information on the influence exercise has on pharmacological agents is lacking (i.e. efficacy) as well as the influence of exercise on extensor and flexor motor output. This provides the rationale for using passive cycling to study the influence activity has on the extensor and flexor MSR pre- and post- quipazine for Project #2: "Daily passive cycling attenuates the hyperexcitability and restores the responsiveness of the extensor monosynaptic reflex to quipazine in the chronic spinal transected rat"

Rationale to study activity in project #2

Similar to treadmill training post spinalization, passive cycling has been shown to be beneficial in preserving the neuromuscular system below the lesion. Passive cycling for thirty to sixty minutes a day, five days a week for one to three months preserved the muscle mass of the hindlimbs (Houle *et al.*, 1999;Murphy *et al.*, 1999;Peterson *et al.*, 2000), maintained the frequency dependent depression of the monosynaptic reflex (Skinner *et al.*, 1996;Reese *et al.*, 2006), attenuated spasticity (Garrison *et al.*, 2011) preserved type Ia and II afferents on the motoneuron (Ollivier-Lanvin *et al.*, 2010), maintained the firing frequency of motoneurons (Beaumont *et al.*, 2004) as well as up-regulated the neurotrophic factors BDNF and GDNF in motoneurons (Keeler *et al.*, 2012). This allowed us to use passive cycling to examine the influence of activity post-spinalization on both the extensor and flexor MSR as well as on the responsiveness of the extensor and flexor MSR to an acute application of quipazine.

There is evidence that extensor spinal circuitry may be influenced to a larger extent than the flexor spinal circuitry following a spinal cord injury as well as to exercise post-transection. Following a spinal transection, extensor muscles atrophy earlier and to a larger extent compared to flexor muscles (West *et al.*, 1986;Roy & Acosta, Jr., 1986). As well, treadmill training post-transection increased the number of cholinergic contacts apposing extensor but not flexor motoneurons (Skup *et al.*, 2012).

Hypothesis #2.3 Three months of passive cycling will produce no change in the extensor and flexor monosynaptic reflex amplitude compared to the control intact group. No differences will be seen between the extensor and flexor MSR amplitude.

Hypothesis #2.4 Following three months of passive cycling, both the extensor and flexor monosynaptic reflex amplitudes will increase in response to quipazine, similar to that seen in the control group.

Activity and gene expression following a spinal transection

Rationale to examine activity in project #3

There has been little investigation on whether or not activity influences serotonin receptor gene expression on the motoneuron post spinal transection. This information would be vital and a potential explanation as to why activity promotes recovery and maintenance of the spinal cord circuitry. It is known that the neuromuscular system demonstrates activity-related plasticity in both the intact and spinal transected animal demonstrated by the modulation of motoneuron properties (Beaumont & Gardiner, 2002;Beaumont & Gardiner, 2003;Beaumont *et al.*, 2004;Cormery *et al.*, 2000;Cormery *et al.*, 2005) and gene expression in motoneurons (Keeler *et al.*, 2012;Woodrow *et al.*, 2013) and the lumbar spinal cord . Three weeks of voluntary wheel running in mice resulted in an increase in gene expression for neuronal health and signalling, synaptic reorganization and the adaptation of the neuromuscular junction (Ferraiuolo et al., 2009;Perreau et al., 2005). Neurotrophic factors also respond to exercise. Following one week of endurance training in the spine intact rat, BDNF, NT-3, TrkB, TrkC, synapsin I, Gap-43 and CREB are up-regulated in the lumbar spinal cord (Gomez-Pinilla et al., 2001;Gomez-Pinilla et al., 2002;Ying et al., 2003) with a similar enhancement found in laser-captured motoneurons following one month of passive cycling in the spinal transected rat (Keeler et al., 2012). Recently, in the intact rat following 16 weeks of

wheel running, the 5-$HT_{1A}R$, which inhibits motoneuron firing was down-regulated by 80% in laser captured motoneurons (Woodrow *et al.*, 2013)**.** These studies demonstrate that activity alters gene expression in the lumbar spinal cord as well as in the motoneuron and provides the rationale to examine the influence passive cycling has on serotonin receptor gene expression in flexor and extensor motoneurons in Project #3 "Serotonin receptor and KCC2 gene expression in lumbar flexor and extensor motoneurons post-transection with and without passive cycling."

Hypothesis #3.4 In the chronic spinalized rat, following three months of passive cycling, serotonin receptor gene expression in extensor motoneurons will have a similar expression to that of the control intact extensor motoneurons.

Hypthesis #3.5 Three months of passive cycling will not alter the gene expression in flexor motoneurons, having a similar expression to that seen in the spinal transected no cycling group.

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CHAPTER 2: REMOVAL OF SUPRASPINAL INPUT REVEALS A DIFFERENCE IN THE FLEXOR AND EXTENSOR MONOSYNAPTIC REFLEX RESPONSE TO QUIPAZINE INDEPENDENT OF MOTONEURON EXCITATION

Running title: quipazine and flexor and extensor motor output

Keywords: motoneuron, monosynaptic reflex, serotonin, motor output

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ABSTRACT

The purpose of this study was to determine if quipazine, a serotonergic agonist, differentially modulates flexor and extensor motor output. This was achieved by examining the monosynaptic reflex (MSR) of the tibial (extensor) and peroneal (flexor) nerves, by determining the basic and rhythmic properties of extensor and flexor motoneurons, and by recording extracellular Ia field potentials of the tibial and peroneal nerves in the *in vivo* adult decerebrate rat in both spinal intact and acute spinalized preparations. In the spinal intact preparation, the tibial and peroneal MSR amplitude significantly increased compared to baseline in response to quipazine, with no difference between nerves ($p < 0.05$). In the spinalized preparation, the MSR was significantly increased in both the tibial and peroneal nerves in response to quipazine with the latter increasing more than the former (5.7x vs. 3.6x, $p < 0.05$). Intracellular motoneuron experiments demonstrated that rheobase decreased, while input resistance, AHP amplitude and the firing rate at a given current injection increased in motoneurons following quipazine administration with no differences between extensor and flexor motoneurons. Both the tibial and peroneal nerve extracellular Ia field potentials increased with the peroneal demonstrating a significantly greater increase (7% vs. 38%, $p < 0.05$) following quipazine. It is concluded that in the spinal intact preparation, quipazine does not have a differential effect on flexor or extensor motor output. However, in the acute spinalized preparation, quipazine preferentially affects the flexor MSR compared to the extensor MSR, likely due to the removal of a descending tonic inhibition on flexor Ia afferents.

INTRODUCTION

The neuromodulating effects of serotonin (5-HT) on motor output are well established (Schmidt and Jordan 2000). Of interest, the 5-HT_1 and 5-HT_2 receptor families are important for modulating motor output through inhibition and excitation of interneurons, afferent feedback and motoneuron discharge. Activation of the 5-HT_2Rs results in motoneuron depolarization, increased input resistance and discharge rate (Wang and Dun 1990; Elliott and Wallis 1992; Harvey et al. 2006; White and Fung 1989), whereas activation of the 5HT_{1A}R causes hyperpolarization of the resting membrane potential and decreased input resistance (Wang and Dun 1990). It has been shown that when 5-HT or 5-HT_2R agonists are applied on the dendrites of the motoneuron, plateau potentials are facilitated and when 5-HT or 5-HT_{1A}R agonists are applied at the perisomatic region inhibition of motoneuron output occurs, leading to the notion that 5-HT_2Rs are located on the dendrites and 5-HT_{1A}Rs are located on the perisomatic region (see Perrier and Cotel 2008). As well, the effects of 5-HT on the monosynaptic reflex (MSR) are well documented. Similar to the motoneuron, activation of the 5-HT_2R facilitates the MSR (Miller et al. 1996; Machacek et al. 2001; Shay et al. 2005; Hasegawa and Ono 1996; Gajendiran 2008) and activation of the 5-HT_{1A}R inhibits the MSR (Hasegawa and Ono 1996; Gajendiran 2007; Nagano et al. 1988; Hedo et al. 2002).

5-HT is of interest because indirect evidence suggests that this neuromodulator may have a different effect on flexor and extensor motoneurons, in particular with regards to the ability of 5-HT to induce plateau potentials. Plateau potentials are mediated by both the sodium and calcium persistent inward currents (NaPIC and CaPIC) and both of which have been shown to be directly modulated by the 5-HT_2R on the motoneuron

(Harvey et al. 2006; Perrier and Hounsgaard 2003; Murray et al. 2011). It was suggested by Hounsgaard et al. (1988) that in the intact spinal cord, the ability to generate plateau potentials may favour extensor motoneurons compared to flexor motoneurons. Recently, in the *in vitro* neonate rat preparation, Cotel et al. (2009) showed that extensor motoneurons, but not flexor motoneurons, were able to generate self-sustained firing. The difference between extensor and flexor motoneurons to generate PICs may be due to differences in the 5-HT_2R activation or distribution as immunohistological staining for the 5-HT_2R has shown a greater staining in the soleus motoneuron pool compared to that of the extensor digitorium longus (EDL) (Vult and Lomo 2005).

If the ability to generate PICs is greater in extensor motoneurons, and it is known that the 5-HT_2R activates both the CaPICs and NaPICs, then one could suggest that extensor and flexor motoneurons are potentially under differential modulation from 5-HT. Following an acute spinalization, the ability to generate PICs is lost and 5-HT has been shown to restore or generate PICs (Hounsgaard et al. 1988). The purpose of this study was to investigate if the MSR of the tibial and peroneal nerves, as well as, the rhythmic and basic properties of identified flexor and extensor motoneurons respond differently to an intraperitoneal (i.p.) injection of the 5-HT_2R agonist quipazine, in the adult decerebrate rat. This was investigated in both spinal intact and acute spinalized preparations.

METHODS

Animal care

All animal treatment, surgical and experimental procedures were in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of Manitoba Animal Ethics Committee.

Adult female Sprague-Dawley rats weighing 275-350g obtained from the University of Manitoba were used for all experiments described. The rats were housed in groups of three in plastic cages situated in an environmentally controlled room maintained at 23°C with a 12h-12h light-dark cycle. The rats had unlimited access to water and rat chow throughout the experiment period.

General surgical preparation

Surgical anaesthesia was induced in 5% isofluorane mixed with 100% oxygen and maintained at 2-2.5% isofluorane mixed with 100% oxygen once a tracheotomy was performed. A tracheal tube was inserted to ventilate (Harvard Apparatus, St. Laurent, QC, Canada) the rat on pure oxygen at a tidal volume of 2 ml with a ventilation rate of approximately 48-60 strokes per min. Expired carbon dioxide levels were measured (Capstar 100 CO_2 analyzer; CWE Inc. Androme, PA, USA) and maintained at 3-4%. Once the tracheal tube was inserted the following procedures were performed. A catheter was inserted into the carotid artery to monitor mean arterial pressure (Pressure Monitor BP-1; World Precision Instruments, Saratosa, FL, USA), which was maintained between 80-100 mmHg. As well, a constant infusion of a dextrose/saline solution delivered at 0.9 ml per hour was administered through the carotid catheter. The left hindlimb tibial and peroneal nerves were dissected away from the musculature and isolated for mounting of electrodes, and the back musculature was dissected free from the vertebrae. The rat was

then moved to a stereotaxic frame where the head, thoracic and lumbar vertebrae and left foot were immobilized. A T12 to L3 laminectomy was performed to expose the lumbar spinal segments L2-L5. The dura mater was incised, the large dorsal roots (L3 and L4) were cut and, depending on the experiment protocol, were either mounted for stimulation (MSR) or brushed to the side of the spinal cord (motoneuron intracellular recording). Back and left hindlimb pools were then prepared, and were filled with mineral oil. The rectal temperature of the rat was monitored and maintained at 37°C using a Homeothermic Blanket Control Unit and UV lamp (Harvard Apparatus). A craniotomy was performed followed by a precollicular decerebration. Once the decerebration was complete, the animal was removed from anaesthetic and pancuronium bromide (0.2 ml) was given intravenously every hour to ensure no movement. For the intracellular motoneuron experiments a pneumothorax was performed to limit respiratory related movements.

Acute spinalization

In half of our MSR and intracellular motoneuron recording experiments an acute complete spinal transection was performed to eliminate descending input to the spinal cord. The transection was performed by dissecting away the back musculature and performing a laminectomy at either C4 (MSR experiments) or T8 (intracellular experiments). The spinal cord was then transected with fine forceps and suction, the 2 mm incision was then packed with gelfoam. The differences in the location of the spinal transections were due to difficulty in providing a stable preparation for intracellular recording with a C4 spinal transection (i.e. increased movement, large blood pressure increases).

Monosynaptic reflex recordings

Eight female Sprague-Dawley rats were used for the MSR experiments in the acute spinalized preparation and four rats were used for the MSR experiments with the spinal cord intact. The MSR was elicited by stimulation of the L4/L5 dorsal roots and the electroneurogram (ENG) activity of the tibial (extensor) and peroneal (flexor) nerves were recorded with bipolar Ag-Cl hook electrodes. The ENG signal was collected with custom software (capture, SCRC) differentially amplified (10000x; band-pass filtered 50 - 1000 Hz), digitized at 10 kHz (12-bit A/D), and stored for offline processing. ENG activity was recorded at threshold (T), 1.25T, 1.5T, 2T, 3T. Threshold for the MSR was defined as the smallest current producing a detectable extracellular compound action potential volley at the cord dorsum recording electrode. The dorsal roots were stimulated at 4 Hz (0.1 ms duration). A minimum of 85 responses were collected and averaged at each time point. A minimum of four baseline averages were taken five minutes apart to ensure a reliable baseline measure. After the baseline measurements were collected, an intraperitoneal (I.P.) injection of quipazine in saline (15 mg kg^{-1}) was administered and the MSR was recorded every five minutes for a two-hour duration in four experiments. Since it was noted that the largest MSR increase occurred between five and twenty minutes, the MSR response was recorded every five minutes for 60 minutes in the remaining experiments.

For analysis, the ENG recordings were full-wave rectified and averaged (minimum 85 responses per stimulation). The area under the MSR response was calculated at 1 ms, 1.5 ms, 2 ms and 2.5 ms following the initiation of the ENG response.

The area under the MSR response was then normalized to the average baseline response for each nerve.

Intracellular recordings

Thin-walled 1.0 mm glass microelectrodes (World Precision Instruments, USA) were pulled to an impedance of approximately 10 MΩ (Kopf Vertical Pipette Puller, David Kopf Instruments, Tujunga, CA, USA) and filled with 2 M potassium citrate. The electrode was advanced through the spinal cord at 6-10 μm steps using the Burleigh inchworm microdrive system (Burleigh Instruments Inc., USA). Both the tibial and peroneal nerves were stimulated with bipolar silver electrodes at a frequency of 2 Hz, while the microelectrode was advanced through the spinal cord, and field potentials identified as originating from either extensor motoneurons or flexor motoneurons were continuously monitored. A membrane potential change ≥ 55 mV in the spinal transected and ≥ 60 mV in the spinal intact rats, accompanied by an antidromic action potential spike height of ≥ 55 or 60 mV respectively were used to determine successful impalement of a motoneuron. Once the motoneuron was identified and the resting membrane potential stabilized, basic and rhythmic properties were recorded using an intracellular amplifier system (Axoclamp 2B, Axon Instruments, Molecular Devices, USA). Only motoneurons, in which both the baseline and post-drug values were recorded in the same motoneuron, were used for analysis.

Measurements of motoneuron properties

Basic Properties

Rheobase, defined as the minimum current required to elicit an action potential 50% of the time, was recorded in response to a 50 ms intracellular depolarizing pulse in discontinuous current clamp (DCC) mode (5-10 kHz). Resting membrane potential (RMP) and voltage threshold (Vth), defined as the membrane potential at which depolarization increased at ≥10V S^{-1} (Power et al. 2010), was also determined from these recordings. Input resistance (IR) was determined from an averaged membrane response to approximately 60, 1 nA hyperpolarizing pulses each lasting 150 ms. Afterhyperpolarization amplitude (AHP_{amp}) and afterhyperpolarization ½ decay time (AHP_{decay}) were measured from an average of approximately 40 orthodromic spikes evoked by 0.5 ms supramximal intracellular current pulses recorded in bridge mode.

Rhythmic Properties

Following successful recording of the basic motoneuron properties, the motoneuron was subjected to a slow triangular current ramp (5-second rise, 5-second decay) in DCC mode to determine the active firing properties of the motoneuron. The peak current amplitude of the ramp for each cell was determined by trial and error such that the cell would begin firing within 1-2 seconds of the peak of the ramp, and this amplitude was maintained for the quipazine trial. From the triangular current injection, the following measurements were obtained: 1/ slope of frequency-current (F-I) relationship (Hz nA^{-1}); 2/ instantaneous firing frequency at recruitment and derecruitment; 3/ maximum firing frequency achieved on ramp current injection; and 4/ the number of spikes achieved on ramp current injection. After completion of a successful intracellular recording, the microelectrode was backed out of the motoneuron

and the extracellular voltage was recorded to correct membrane voltages recorded intracellularly.

Ia extracellular field potential recordings in spinalized rats

It was noted following the intracellular motoneuron experiments that no difference existed between flexor and extensor motoneurons in response to quipazine (see results). Therefore, in several experiments (n = 7) extracellular field potentials were recorded pre- and post- quipazine injection to determine if presynaptic modulation affected the response of the MSR to quipazine. Recording extracellular field potentials allowed for comparison of pre- and post- quipazine values of both the peroneal and tibial nerve in the same experiment. In the Ia extracellular field potential experiments a low resistance microelectrode (~ 1-2MΩ) was advanced through the spinal cord in 6 μm steps until a location was found with the maximum tibial and peroneal antidromic potentials (minimum depth of 700 μms to ensure microelectrode was in the ventral-lateral horn).

Once the optimal location was determined, stimulation of the tibial and peroneal nerves was set at 1.25T to ensure only the Ia extracellular potentials were recorded via the micro electrode. An average of 60 stimulus responses from each nerve were recorded and analyzed. The negative deflection of both the tibial and peroneal nerve were compared pre- and post-quipazine injection for a twenty-minute period. Recordings at 1.5T, 2T and 3T were also made to ensure saturation of the field potential had not occurred at 1.25T.

Statistics

The data was subjected to a two-way analysis of variance with repeated measures (RM ANOVA) analysis, with the factors of motoneuron type (flexor vs. extensor) and drug (pre vs. post quipazine) and time being a repeated measure unless otherwise stated. A Tukey's *post hoc* analysis was used when a significant interaction was found. Significance was determined at an alpha level of $p < 0.05$. Data are presented as mean (standard deviation) throughout the text.

RESULTS

In total, eight acute spinalized and four spinal intact rats were used for MSR recordings and seven acute spinalized rats were used for the Ia extracellular field potential experiments. A total of 15 motoneurons were recorded from the spinal intact rat (seven extensors, eight flexors) and eight motoneurons (four extensors, four flexors) in the acute spinalized rat. For the intracellular experiments, only motoneurons in which both pre-quipazine and stable post-quipazine recordings were made were selected for analysis. Furthermore, to ensure that a stable intracellular penetration occurred during the recording phase, only motoneurons that fired rhythmically and had a stable RMP (+/- 5 mV) were used for analysis.

The effect of quipazine on the MSR

Figure 1 demonstrates the average response obtained from the eight acute spinalized rats (Figure 1A) and a representative individual response to quipazine (Figure 1B). Data represents the area under the response collected at 1.5 ms at 2T. The MSR responses at the various stimulus strengths and time epochs described in the methods

section produced similar results as described and presented in Figure 1 (peroneal MSR response was significantly larger than the tibial MSR response). The results are normalized to their own baseline value. The coefficient of variation of the baseline values was 16% (range: 1-22%) for the tibial nerve and 19% (range: 9-27%) for the peroneal nerve. A two-way RM ANOVA indicated a significant drug response and a significant motoneuron type effect ($p < 0.05$). Post hoc analysis revealed that the area under the peroneal (flexor) MSR response was increased significantly larger compared to the area under the tibial (extensor) MSR following quipazine. No interaction of time was found. The average increase in the area under the MSR following quipazine injection in the tibial and peroneal nerve was 3.6- and 5.7- fold respectively. Although large variability exists in the responsiveness of the MSR to quipazine, it is important to note that in all eight acute spinalized rats from which the MSR was recorded, the increase in the peroneal MSR was always greater than that of the tibial MSR.

To determine the influence of descending inputs, the MSR of spine intact rats was also evaluated. In these four experiments, the MSR was recorded similarly to the spinalized animals. In the spinal intact animals, the average increase was 1.8- and 2.0-fold for the tibial and peroneal MSR respectively (data not shown). A significant drug effect was found ($p < 0.05$) with no difference between nerves. Therefore, the difference in the increase in the tibial and peroneal MSR following quipazine requires the removal of a descending tonic influence on the peroneal reflex pathway.

In two MSR experiments (acute spinalization), after collecting the MSR response to quipazine, ketanserin (0.45 mg kg^{-1}) was administered to determine if the results seen were likely due to the activation of the 5-HT_{2A}R (data not shown). Following the

injection of ketanserin both the tibial and peroneal MSR amplitudes reached steady state at 50% of their control pre-quipazine baseline values, indicating that the increase in the tibial and peroneal MSR following quipazine is likely due to the activation of the 5-$HT_{2A}R$.

The effect of quipazine on flexor and extensor motoneuron properties

In the spinal intact preparation, a two-way RM ANOVA revealed a significant main effect of drug (enhanced excitability of motoneurons) for several properties but no significant difference for type (flexors vs. extensors). Motoneuron basic and F-I relationship properties are summarized in Table 1. The basic and rhythmic firing properties of flexor and extensor motoneurons were similar at baseline and demonstrated a similar responsiveness to quipazine.

Overall, motoneuron excitability increased after quipazine injection. For both flexor and extensor motoneurons, rheobase decreased by approximately 30% following quipazine injection. Average IR at baseline for flexor and extensor motoneurons was 1.9 ± 0.9 and 2.1 ± 1.0 MΩ respectively. Following quipazine injection, input resistance increased 60% (3.1 ± 1.9 MΩ) for flexor motoneurons and 20% (2.5 ± 0.8 MΩ) for extensor motoneurons ($p < 0.05$). The AHP_{amp} increased for both flexor (0.9 ± 0.4 mv to 1.4 ± 0.7 mV) and extensor (2.0 ± 1.0 mV to 2.8 ± 2.1 mV) motoneurons ($p < 0.05$). Quipazine did not have an effect on $AHP_{decay,}$ RMP or Vth.

The increase in the excitability following quipazine injection was evident when examining motoneuron firing properties from a ramp current injection (see properties 7-12 in Table 1, Figure 2). Similar to the basic properties, no significant differences were

seen between flexor and extensor motoneurons pre- or post- quipazine injection. Current at spike recruitment for flexor motoneurons significantly decreased 28% from 10.1 to 7.3 nA and 45% from 8.5 to 4.8 nA for extensor motoneurons ($p < 0.05$). Current at spike derecruitment decreased from 10.4 to 7.8 nA (33%) and 8.2 to 5.0 nA (40%) for flexor and extensor motoneurons respectively ($p < 0.05$). The maximum ramp firing rate increased by 40% for flexor motoneurons and 30% for extensor motoneurons ($p < 0.05$). The slope of the F-I relationship following quipazine was shifted to the left for both flexor and extensor motoneurons with no changes in the slope.

The motoneurons recorded from the acutely spinalized group (Table 2) following quipazine (four flexors, four extensors) showed a similar enhancement of rhythmic firing as measured by current ramp injections to that was seen in the control spine intact group. Current at spike recruitment for flexor motoneurons significantly decreased 42% from 14.2 to 8.2 nA and 23% from 11.3 nA to 8.6 nA for extensor motoneurons ($p < 0.05$). Current at spike derecruitment decreased from 14.7 to 10.25 nA (30%) and 11.4 to 8.8 nA (23%) for flexor and extensor motoneurons respectively ($p < 0.05$). The F-I relationship was shifted to the left for both flexor and extensor motoneurons with no change in the slope.

The effect of quipazine on flexor and extensor Ia extracellular field potentials

In seven acute spinalized rats the extracellular Ia field potentials of both the tibial and peroneal nerves were measured pre- and post- quipazine (Figure 3). A two-tail student t-test revealed a significant difference in the increase of the Ia extracellular field

potential amplitudes following quipazine ($p < 0.05$). The average increase for the tibial and peroneal Ia extracellular field potentials was 7% and 38% respectively.

DISCUSSION

The most important finding of this study is that flexor and extensor motor output is differentially modulated by quipazine, a 5-HT_2R agonist in the acute spinal transected model. Furthermore, to our knowledge, this was the first time that a serotonergic agonist was used in an *in-vivo* decerebrate adult rat preparation in which both the flexor MSR and the extensor MSR were examined simultaneously. In doing so, it was shown that quipazine enhanced the flexor MSR to a greater extent than the extensor MSR. When the MSR, MN properties and Ia extracellular field potentials data are considered together, it is evident that the enhanced flexor MSR was due to presynaptic modulation and not due to differences in motoneuron modulation. The difference in presynaptic modulation is believed to result from the removal of a descending inhibition on the flexor Ia afferents as the difference was only seen in the spinal transected preparation.

Excitation of the monosynaptic reflex

In our initial studies, the primary goal was to determine if flexor and extensor output was preferentially modulated via 5-HT_2R activation. To achieve this, we recorded and compared the peroneal and tibial MSR pre- and post- quipazine injection. The peroneal nerve and tibial nerves were selected as they primarily innervate flexor and extensor muscles respectively. It was found that quipazine enhanced both the flexor and extensor MSR with a greater enhancement seen in the flexor MSR.

The finding that the MSR was increased when quipazine was administered is consistent with the literature, in which, 5-$HT_{2A/C}$ receptors are shown to be responsible for the enhancement of the MSR (Machacek et al. 2001; Shay et al. 2005; Hasegawa and Ono 1996; Gajendiran 2008), whereas inhibition of the MSR is mediated through the 5-HT_{1A}R (Hasegawa and Ono 1996; Gajendiran 2007; Nagano et al. 1988; Hedo et al. 2002). It was hypothesized that the extensor MSR would be preferentially modulated compared to flexor MSR. However, the opposite was seen in which the enhancement of the MSR was larger in the flexor compared to the extensor in the acute spinalized preparation. No differences between the flexor and extensor MSR were seen in the spinal intact animals, with quipazine having a minimal effect on the MSR amplitude as well.

To ensure the effects we saw were independent of the dose of quipazine, an experiment was performed in which 0.3mg kg^{-1} was given (versus 15 mg kg^{-1}). This dose is comparable to what is commonly used to facilitate stepping in the spinalized rat (0.2 - 0.5 mg kg^{-1}, Ichiyama et al. 2008). In this experiment, the flexor MSR increased in magnitude similar to that seen at the higher dose (5.9-fold increase), and the extensor MSR response was considerably smaller (1.5-fold increase).

Importance of descending inhibition on motor output

5-HT has been shown to increase the firing rate of primary and secondary ending of muscle spindles (Ellaway and Trott 1975). Our finding that the Ia extracellular field potentials increased following quipazine would also support this. However, since the difference in the MSR was restricted to the acute spinalized preparation, it is unlikely that

direct excitation of primary afferents is responsible for the difference in the increased MSR.

A more plausible explanation for the difference in the MSR responses following acute spinalization would be the removal of a descending inhibition, most likely the removal of descending inhibition on the flexor Ia terminals as previous literature has shown that flexor reflex afferents (Holmqvist and Lundberg 1961) and Ia afferents are under descending tonic inhibition (Quevedo et al. 1993). Our results support the idea that flexor and extensor Ia afferents are under different modulation from supraspinal sources and also that a difference in modulation exists between flexors and extensors.

Direct monosynaptic control of flexor and extensor motoneurons from supraspinal sources has been shown to exist (Grillner and Lund 1968; Lund and Pompeiano, 1968). Lund and Pompeiano (1968) have shown that stimulation of the ipsilateral Deiter's nucleus evoked excitatory post synaptic potentials (EPSPs) in extensor motoneurons and inhibitory post synaptic potentials in flexor motoneurons, while others have shown that the likely source for EPSPs in flexor motoneurons originates in the ipsilateral upper medullary or lower pontine reticular formation (Grillner and Lund 1968). Although it is unlikely that direct descending modulation of alpha motoneurons explains the difference in the MSR responses, these studies show evidence that the flexor and extensor pathway are under different modulation from supraspinal sources. Despite the lack of direct evidence, this conclusion is supported by the increased Ia extracellular field potentials in the spinal transected preparation, along with the disproportionate increase in the flexor Ia extracellular field potentials compared to extensor Ia extracellular field potentials after quipazine administration.

Enhanced excitability of motoneuron properties

The responses of motoneurons to quipazine are similar to what has been noted previously in the literature, but also differ slightly. The increased input resistance (Wang and Dun 1990; Elliott and Wallis 1992; Hsiao et al. 1997) and firing rate (White and Neuman 1983; White 1985) shown here are consistent with previous findings that the activation of 5-HT_2R enhances the overall excitability of the motoneuron (Harvey et al. 2006; Miller et al. 1996; Perrier and Hounsgaard 2003). However, we found no change in resting membrane potential (VanderMaelen and Aghajanian 1980; White and Fung 1989; Wang and Dun 1990; Elliott and Wallis 1992; Hsiao et al. 1997; Harvey et al. 2006) or voltage threshold (Fedirchuk and Dai 2004), and the AHPamp increased (White and Fung 1989).

There are several explanations that could account for these differences. First, a 5-HT_2R agonist was used rather than 5-HT. Harvey et al. (2006) noted that 5-HT administration caused depolarization and changes in membrane potential, whereas the specific 5-HT_2R agonist (DOI) did not change resting membrane potential or IR in the spinalized rat preparation. Second, it appears that age influences the effect of 5-HT on the motoneuron. For example, embryonic and neonatal rat motoneurons depolarize in response to 5-HT_{1A} and 5-HT_2R activation (Ziskind-Conhaim et al., 1993; Takahashi and Berger, 1989), whereas in the juvenile rat, the 5-HT_{1A}R and the 5-HT_2R activation results in hyperpolarization and depolarization of the motoneuron respectively (Talley et al. 1997).

This age-dependent effect is also highlighted by the differential effect 5-HT has on the AHPamp. In the neonatal rat, 5-HT_{1A}R activation results in the suppression of the AHPamp, whereas in the juvenile rat, the AHPamp is unaffected (Talley et al. 1997). As well, in the adult *in-vivo* cat preparation, 5-HT and the 5-HT_2R agonist DOI, increased the AHPamp (Zhang, 1991), which is consistent with our finding that quipazine increased the AHPamp in the adult in-vivo rat preparation.

Differences in flexor and extensor motoneurons

Contrary to what was hypothesized, no difference in the level of excitation or modulation of flexor and extensor motoneuron properties and their discharge rates occurred following quipazine injection in either the intact or acute spinalized preparation. Based on previous studies in which differences in the ability of extensor and flexor motoneurons to activate PICs in the adult *in vivo* cat (Hounsgaard et al. 1988) or self-sustained firing in the *in vitro* neonatal rat preparation (Cotel et al. 2009), it was unexpected to see no difference in modulation of the motoneuron type with quipazine, as 5-HT activates both the NaPIC (Harvey et al. 2006) and CaPIC (Perrier and Hounsgaard 2003) However, it has been recently shown that the 5-$HT_{2B/2C}$ receptors are responsible for mediating the CaPIC (Murray et al. 2011). As well, immunohistological staining for the 5-HT_{2A}R has shown that identified slow extensor motoneurons (soleus motoneurons) express a larger number of the 5-HT_{2A}R compared to fast flexor motoneurons (Vult and Lomo 2005). However, as the authors suggest, this may be due to differences between fast and slow motoneurons. As well, a previous study that examined spontaneous alpha and gamma motoneuron discharge rates in response to 5-HTP found no difference between extensor filaments (gastrocnemius) and flexor filaments (semitendinous)

(Myslinski and Anderson 1978). Our results seem to support this initial finding, which suggests that no differences between flexor and extensor motoneuron discharge rate and excitability exist in both the spinal intact and acute spinalized preparation.

CONCLUSIONS

This study concludes that, in the spinal intact state, quipazine does not have a differential effect on the MSR, due to a descending tonic inhibition on the flexor reflex pathway. In the acute spinal preparation, with the descending tonic inhibition removed, quipazine is found to have a preferential effect on the flexor MSR compared to the extensor MSR. The difference in excitation is believed to be the result of presynaptic modulation as evident by a consistently larger increase in the peroneal extracellular Ia field potential compared to the tibial Ia field potential, and by the lack of a differential effect of quipazine on the biophysical or rhythmic firing properties of the flexor and extensor motoneurons.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Larry Jordan and Dr. David McCrea for their assistance during this project.

GRANTS

This work was supported by grants to P.F.G. from the Canadian Institutes of Health Research, Canadian Research Chairs program, and the Manitoba Paraplegic Foundation. Funding support was provided to J.W.C from Manitoba Health Research Council and the Canadian Institutes of Health Research and to C.W.M. from the Manitoba Health Research Council.

DISCLOUSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHORS CONTRIBUTIONS

J.W.C. Conception and design of the experiments. Collection, analysis and interpretation of data. C.W.M. Collection, analysis and interpretation of data. K.E.P. Collection, analysis and interpretation of data. K.G. Collection, analysis and interpretation of data. P.F.G. Conception and design of the experiments. Collection, analysis and interpretation of data. All authors were involved in drafting article or revisiting it critically for important intellectual content, and all authors approved the final version.

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TABLES

Table 1. Passive and active motoneuron properties of decerebrate spine intact rats

Motoneuron Property	Flexor Mns Pre-quipazine	Post-quipazine	Extensor Mns Pre-quipazine	Post-quipazine	P value
RMP (mV)	-66.8 ± 5.5	-68.5 ± 3.4	-65.3 ± 2.2	-62.9 ± 2.3	
Vth (mV)	-47.5 ± 8.5	-48.4 ± 8.4	-44.5 ± 5.5	-44.3 ± 3.8	
Rheobase current (nA)	8.0 ± 4.3	5.6 ± 2.8	7.4 ± 3.4	5.5 ± 2.9	*
IR (MΩ)	1.9 ± 0.9	3.1 ± 1.9	2.1 ± 1.0	2.5 ± 0.8	*
AHP amplitude (mV)	0.9 ± 0.4	1.4 ± 0.7	2.0 ± 1.0	2.8 ± 2.1	*
AHP 1/2 decay time (ms)	17.9 ± 9.7	16.8 ± 9.8	17.8 ± 3.9	17.0 ± 3.6	
Current at spike recruitment (nA)	10.1 ± 5.8	7.3 ± 4.8	8.0 ± 4.7	4.4 ± 3.3	*
Current at spike derecruitment (nA)	10.4 ± 6.0	7.8 ± 5.6	7.5 ± 4.8	4.5 ± 3.6	*
Instantaneous frequency at spike recruitment (Hz)	25.4 ± 13.9	21.6 ± 4.1	21.5 ± 14.7	12.4 ± 5.4	*
Instantaneous frequency at spike derecruitment (Hz)	21.5 ± 7.4	17.9 ± 8.5	15.0 ± 7.6	11.6 ± 5.5	*
Max firing frequency on ramp current injection (Hz)	47.9 ± 16.7	67.9 ± 19.3	37.8 ± 22.7	49.3 ± 23.8	*
Number of spikes on ramp current injection (#)	52.3 ± 39.8	150.8 ± 57.3	45.0 ± 18.1	172.6 ± 72.4	*
Slope (Hz nA -1)	9.0 ± 4.5	8.4 ± 4.6	7.3 ± 2.1	5.3 ± 0.9	

Summary of basic and active motoneuron properties in the spinal intact preparation. Data are represented by mean and SD for each group. *Two-way ANOVA revealed a significant drug effect ($P < 0.05$). No significance of motoneuron type was found. RMP, resting membrane potential; Vth, voltage threshold; IR, input resistance; AHP, after-hyperpolarization. N = 7 flexor motoneurons, 8 extensor motoneurons.

Table 2. Rhythmic motoneuron properties of acute spinalized rats

Motoneuron Property	Flexor Mns Pre-quipazine	Post-quipazine	Extensor Mns Pre-quipazine	Post-quipazine	P value
Current at spike recruitment (nA)	14.2 ± 4.6	8.2 ± 2.4	11.3 ± 4.6	8.6 ± 4.3	*
Current at spike derecruitment (nA)	14.7 ± 5.0	10.25 ± 4.0	11.4 ± 5.0	8.8 ± 4.9	*
Instantaneous frequency at spike recruitment (Hz)	20.8 ± 3.0	23.0 ± 3.0	21.6 ± 9.9	27.3 ± 16.1	*
Instantaneous frequency at spike derecruitment (Hz)	18.7 ± 4.8	23.5 ± 9.4	20.0 ± 8.4	22.3 ± 7.6	*
Max firing frequency on ramp current injection (Hz)	35.8 ± 7.6	70.4 ± 12.7	40.9 ± 11.6	59.9 ± 20.5	*
Number of spikes on ramp current injection (#)	36.5 ± 21.3	178.0 ± 81.9	46.3 ± 34.6	152.3 ± 86.3	*
Slope (Hz nA -1)	4.5 ± 2.9	4.0 ± 0.9	6.0 ± 1.6	5.6 ± 2.1	

Summary of rhythmic motoneuron properties in the acute spinalized preparation. Data are represented by mean and SD for each group. *Two-way ANOVA revealed a significant drug effect ($P < 0.05$). No significance of motoneuron type was found.

N = 4 flexor motoneurons, 4 extensor motoneurons

FIGURES

Figure 1

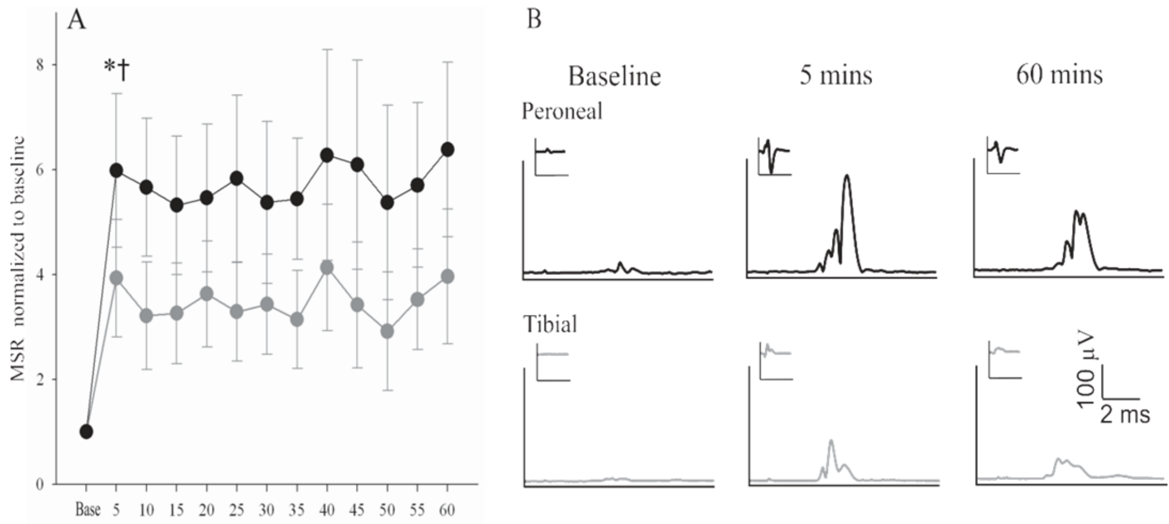

Figure 2

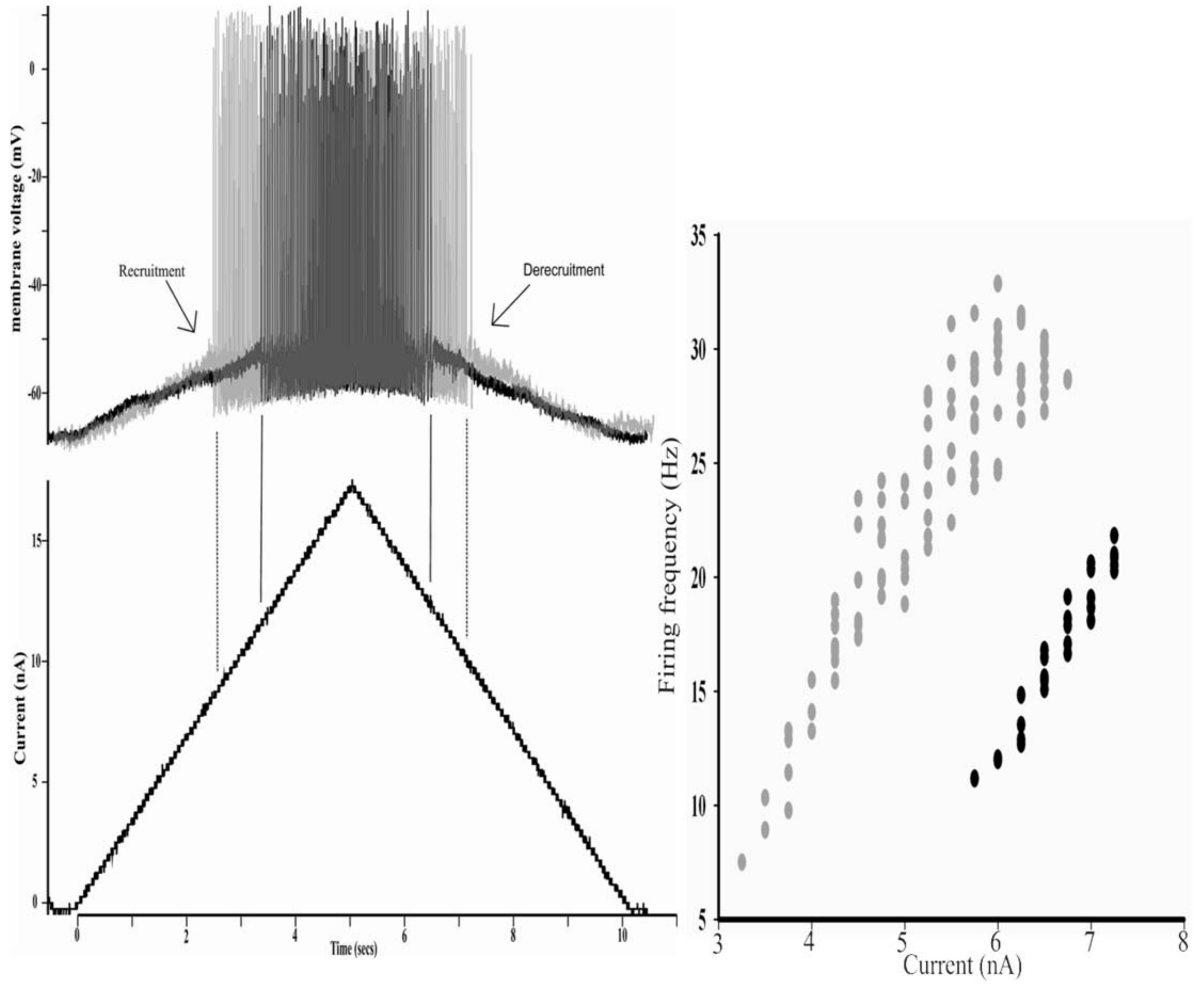

Figure 3

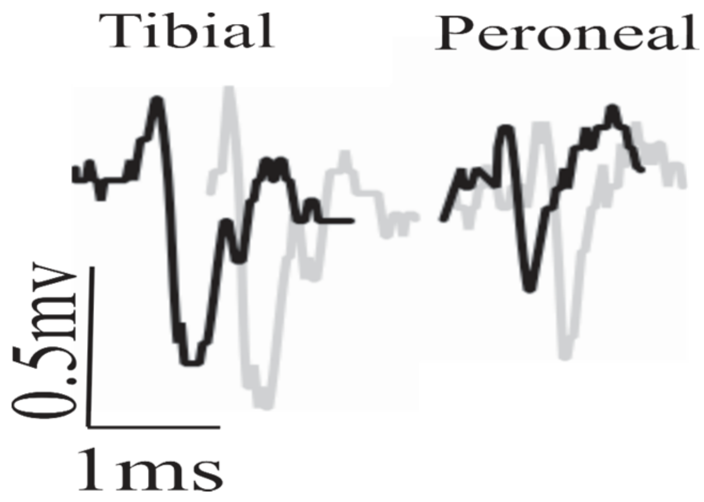

FIGURE LEGENDS

Figure 1. The effect of quipazine on the MSR in acute spinalized rats

A) Average MSR response of the tibial (grey) and peroneal (black) nerves following quipazine. Results are presented from the data collected at 2T and from the area under the response for 1.5 ms. * Significant increase in the MSR response for both the tibial and peroneal nerve was found compared to their respective baseline measurements. † Significant difference between the peroneal and tibial nerve (5.7 fold vs. 3.6 fold increase respectively, $p < 0.05$). Mean ± SD (n=8). **B)** Representative rectified ENG recordings from the tibial and peroneal nerves from one rat pre- and 5 and 60 minutes post quipazine. Inset is the raw ENG recordings. Responses shown are from 2x threshold.

Figure 2. Rhythmic firing of a motoneuron pre- and post- quipazine

A) An example of increased rhythmic firing of a motoneuron following quipazine. Current at recruitment and derecruitment decreased by 30% and maximum firing rate increased by 36%. **B)** A shift to the left in the F/I relationship was seen for all motoneurons, with no change in F/I slope. Black = pre quipazine, Grey = post quipazine.

Figure 3. The effect of quipazine on the tibial and peroneal Ia extracellular field potentials

A representative recording of the tibial and peroneal Ia extracellular field potentials pre (black) and post (grey) quipazine. The tibial and peroneal nerves were stimulated as a microelectrode was passed through the spinal cord (minimum depth of 700 μm) to determine the optimal location for recording the tibial and peroneal antidromic field potentials. In this example, the

tibial and peroneal nerves were stimulated at 1.25T and an average of 60 stimulus responses from each nerve were recorded. The negative deflection of both the tibial peroneal nerves was compared pre- and post- quipazine injection for a twenty-minute period. In this example, the tibial and peroneal Ia field potentials increased by 21% and 42% respectively. The traces have been separated (tibial and peroneal) and offset (pre and post) for ease of comparison.

CHAPTER 3: DAILY PASSIVE CYCLING ATTENUATES THE HYPEREXCITABILITY AND RESTORES THE RESPONSIVENESS OF THE EXTENSOR MONOSYNAPTIC REFLEX TO QUIPAZINE IN THE CHRONIC SPINAL TRANSECTED RAT

Running Title: Passive cycling and the MSR response to quipazine

Keywords: monosynaptic reflex, spinal transection, serotonin, exercise, motor output

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Chopek JW, MacDonell CW, Gardiner K, Gardiner PF (2014). Daily passive cycling attenuates the hyperexcitability and restores the responsiveness of the extensor monosynaptic reflex to quipazine in the chronic spinal transected rat. Journal of neurotrauma 31: 1083-7.

ABSTRACT

Activity based interventions such as locomotor training or passive cycling have a positive influence on the spinal circuitry and recovery following a spinal cord injury (SCI). The use of quipazine in combination with exercise training has demonstrated a greater functional recovery compared to exercise training alone. However, the influence of exercise or training on the responsiveness of the spinal cord to quipazine has not been examined following a chronic spinal transection. The purpose of this study was to characterize the flexor and extensor monosynaptic reflex (MSR) response pre- and post- quipazine in chronic complete spinal transection rats that either underwent daily passive cycling for three months or did not receive passive cycling. Following a chronic spinal transection, the extensor MSR demonstrated a hyperreflexive response (5-fold increase) to afferent stimuli and did not respond to quipazine injection. With daily passive cycling, the extensor MSR hyperexcitability was attenuated and the MSR amplitude increased 72% following quipazine injection ($P < 0.004$), which was comparable to the extensor MSR response (94%) in the control intact group. For both chronic spinal transection groups, the flexor MSR amplitudes were not altered following quipazine injection, whereas in the control intact group the flexor MSR amplitude increased 86% in response to quipazine ($P<0.004$). These results demonstrate that passive cycling attenuates the hyperreflexive response of the extensor MSR following a chronic SCI and restores the MSR response to quipazine.

INTRODUCTION

Following a spinal cord injury (SCI), intrinsic spinal cord circuitry undergoes remodelling, characterized by a period of spinal shock in which spinal reflexes are in a depressed state eventually transitioning to a chronic state characterized by exaggerated spinal reflexes and muscle spasticity. However, the idea that spinal reflexes are exclusively hyperreflexive in the chronic SCI state is misleading (Fong *et al.*, 2009). In fact, depending on the reflexes involved (monosynaptic or polysynaptic), the time post injury (one month vs. three month), and the muscle groups involved (flexor vs. extensor or fast vs. slow), spinal reflexes may be also be depressed. This discrepancy in hypo- vs. hyper-responsiveness of the spinal cord can be highlighted by the fact that both GABA and 5-HT receptors can be up-regulated or down-regulated following a chronic SCI (Edgerton *et al.*, 2001;Kong *et al.*, 2010).

During the remodelling of the spinal cord, activities such as locomotor training appear to maintain spinal circuitry for motor output (Fong *et al.*, 2009). With locomotor training, the step cycle, number of steps, and alternation of the left and right hindlimbs are improved. Similar to treadmill training, daily passive cycling has been shown to have a positive effect maintaining the spinal cord circuitry. Passive cycling, characterized by alternation of the flexor and extensor muscles and left and right hindlimbs, which demonstrate hindlimb EMG bursts similar to that seen during locomotion, maintains the frequency dependent depression (FDD) of the spinal reflexes (Houle *et al.*, 1999). FDD is typically seen in the spinal cord intact animal and is lost following a spinal transection (STx), due to the removal of descending inhibition and is believed to correlate with a loss of pre-synaptic inhibition and exaggerated spinal reflex activity (Skinner *et al.*, 1996;Reese *et al.*, 2006). Furthermore, daily passive cycling has been shown to maintain the resting membrane potential and firing properties of motoneurons (Beaumont *et al.*, 2004),

up-regulate the neurotrophic factors BDNF, GDNF (Dupont-Versteegden *et al.*, 2004) and attenuate hindlimb muscle atrophy (Houle *et al.*, 1999;Murphy *et al.*, 1999;Peterson *et al.*, 2000).

Despite the use of activity based interventions to facilitate locomotion and maintain the spinal cord circuitry following a chronic SCI, our understanding of the sites of action and mechanisms responsible for functional improvements is not complete. Furthermore, the influence of exercise or training on the responsiveness of the spinal cord following an acute exposure to pharmacological agents such as quipazine has not been examined following a spinal cord transection (STx). Quipazine (5-HT_2R agonist) enhances the excitability of the spinal cord and promotes locomotion (Ichiyama *et al.*, 2008), but the action of quipazine at the flexor and extensor motor output level is unknown after chronic STx. It has been suggested that following a spinal transection, quipazine may enhance flexor activity through a monosynaptic pathway, whereas quipazine enhances extensor activity through a polysynaptic pathway (Gerasimenko *et al.*, 2007). The mono- versus poly- synaptic pathway may also account for MSR activation differences between flexors and extensors demonstrated by our previous study (Chopek et al., 2013).

Following an acute STx, we have shown that both the flexor and extensor MSR demonstrate a large increase in amplitude, with a greater increase seen in the flexor MSR. The larger response of the flexor MSR, compared to the extensor MSR, was attributed to presynaptic modulation and not modulation of the motoneuron (Chopek *et al.*, 2013). However, in the chronic state of a STx, extensor muscles, compared to flexor muscles, atrophy earlier and to a larger magnitude (Roy *et al.*, 1999;West *et al.*, 1986;Roy & Acosta, Jr., 1986). Extensor motoneurons also respond better to locomotion training following a chronic STx compared to flexor motoneurons (Skup *et al.*, 2012). Given the potential differential effect of quipazine on

flexor and extensors, as well as the benefits of activity on spinal cord function after spinal cord transection, quipazine may have a differential effect on extensor and flexor motor output when comparing an acute and chronic STx. Therefore, the purpose of this study was to characterize the flexor and extensor MSR response pre- and post- quipazine in chronic STx rats that either received daily passive cycling for three months or did not receive passive cycling.

METHODS

Animal Care

All animal treatment, surgical and experimental procedures were in accordance with the guidelines of the Canadian Council of Animal Care and approved by the University of Manitoba Animal Ethics Committee.

Adult female Sprague-Dawley rats weighing between 250-300g obtained from the University of Manitoba were used for all experiments described. The rats were housed in groups of two in plastic cages situated in an environmentally controlled room maintained at 23°C with a 12h-12h light-dark cycle. The rats had unlimited access to water and rat chow throughout the experiment period. Following the spinal transection, rats were individually caged for ease of monitoring.

Spinal Transection Procedure

The rats were initially anaesthetized with 5% isoflurane mixed with 100% oxygen. During surgical preparation and the surgery, anaesthesia was maintained with 2-3% isoflurane, ensuring a surgical level of anaesthesia was achieved. All surgical procedures were performed

under aseptic conditions. The rat's heart rate, oxygen saturation and core body temperature were monitored throughout the procedure.

A small incision was made on the dorsal midline over the thoracic vertebrae. The musculature overlying the vertebrae from T6-T9 was separated from the spinal column. A laminectomy was performed at T8 followed by a small incision in the dura mater. The spinal cord was completely transected with microdissection scissors and gentle aspiration was applied ensuring a complete spinal transection of ~2mm. Gelfoam was packed into the gap and the surrounding fascia and musculature was sutured (4-0 Ethicon). The skin incision was closed with vet bond and secured with microstaples, which were removed one week post-operation.

Post-operative care

Following the STx, rats were given the antibiotic Baytril (s.c. injection 0.5 $mg \cdot kg^{-1}$) twice daily for a week period and the analgesic Buprenex (buprenorphine, s.c. injection 0.05 $mg \cdot kg^{-1}$) twice daily for the first two days. As well, immediately after the surgery, a sub-cutaneous injection of 5-10 ml of saline was given to aide in rehydration. Manual bladder expression was performed 3 times daily until the voiding reflex was re-established.

Experimental Groups

The MSR amplitude from flexor and extensor motor pools were recorded pre- and post-quipazine in the following three groups: 1) spinal cord intact control group (N = 6), 2) a spinal transected group (N = 6) that received a passive cycling intervention for three months and 3) a spinal cord transected group that did not receive any intervention for three months (N =7).

Daily Passive Cycling

Motorized pedals with a body hammock as described by Skinner et al., (Skinner *et al.*, 1996) were used for the passive cycling. Following one week of recovery from the spinal transection surgery, the rats in the daily passive cycling group began their cycling. The rat was positioned in a body supported hammock/sling that allowed for the hindlimbs to be passed through holes in the hammock and secured to motorized pedals. The feet were secured using paper tape (3M Micropore) and the height of the hammock was adjusted to an optimal position that allowed for full extension and flexion of the hindlimb. The motorized pedals also allowed for the rhythmic alteration of the left and right hindlimbs. Each rat underwent passive cycling for one hour daily for a three month period at a rate of 30-50 revolutions per minute (rpm). It was found that the number of rpm needed to be adjusted for each rat daily as certain speeds produced spasticity.

Surgery for MSR recordings

Three months post STx, the rats were prepared for MSR recordings similar to that previously described (Chopek *et al.*, 2013). Briefly, surgical anesthesia was induced at 5% isoflurane mixed with 100% oxygen and maintained at 2-3% isoflurane mixed with 100% oxygen once a tracheotomy was performed. The following surgical procedures were then performed: 1) insertion of a tracheal tube for ventilation (Harvard Apparatus, St. Laurent, QC, Canada), 2) insertion of a catheter into the left carotid artery for constant infusion of a saline/bicarbonate solution and to monitor mean arterial pressure (Pressure Monitor BP-1; World Precision Instruments, Sarasota, FL), 3) dissection of the tibial (predominately extensor nerve) and peroneal (predominately flexor nerve) nerves from the left hind limb musculature for

mounting on bi-polar AgCl electrodes, 4) dissection of the back and vertebral musculature, 5) laminectomy and exposure of the lumbar spinal cord and 6) craniotomy and precollicular decerebration. Following the decerebration, isoflurane was discontinued. The vertebral column and left foot were immobilized with clamps, and the open leg and back incisions were used to make a mineral oil bath around the spinal cord and tibial and peroneal nerves.

MSR recordings

The MSR was elicited by stimulation of the L4/L5 dorsal roots and the electroneurogram (ENG) activity of the tibial (extensor) and peroneal (flexor) nerves were recorded with bipolar AgCl electrodes. ENG activity was collected with custom software (capture, SCRC), differentially amplified (10, 000x; band pass filtered 50-1,000 Hz), digitized at 10 kHz (12-bit A/D), and stored for offline processing. The ENG responses were recorded at 1.25x threshold. Threshold was defined as the minimum current needed to produce a detectable extracellular compound action potential volley at the cord dorsum recording electrode. Dorsal roots were stimulated at 4 Hz with a 0.1 ms pulse width. A minimum of 50 frames were collected and averaged at each time point. A minimum of three separate baseline measurements were collected to ensure a reliable baseline response. Following collection of the baseline measurements, an intraperitoneal injection of quipazine in saline (15 $mg \cdot kg^{-1}$) was administered and the MSR was continuously recorded for a 20 minute period. For analysis, the ENG recordings were full-wave rectified and averaged. The area under the MSR response was calculated for 1ms following the initiation of the ENG response.

Statistics

Pair-wise t-tests were used to compare the MSR response between the flexor and extensor nerves and pre- and post- quipazine injection. A two-sample t-test was used to compare the effects of cycling. A modified Bonferoni's correction was used to correct for multiple comparisons. Significance was determined at $P < 0.004$ (12 t-tests). Data are presented as means ± SD. Data from intact spinal cord (control) rats as previously described in Chopek et al., (2013) were included for comparison of MSR response pre- and post-quipazine.

RESULTS

The mean extensor and flexor MSR responses, pre- and post-quipazine for each group are illustrated in Figure 1.

The extensor reflex is hyperexcitable following chronic spinalized rats

Following a chronic STx, the extensor MSR demonstrated a hyperreflexive response to afferent stimuli. The response of the MSR increased five-fold compared to the intact control group (320 ± 140 µV vs. 57 ± 23 µV, $P < 0.004$). However, the flexor MSR in the chronic STx group did not differ from the intact control group (60 ± 30 µV and 40 ± 23 µV, respectively).

Passive cycling attenuates extensor hyperexcitability in chronic spinalized rats

Rats that were passively cycled for three months show similar MSR responses to the control group. While chronic STx induced an increase in extensor MSR excitability, daily passive cycling normalized the response (68 ± 30 µV). In keeping with the results shown in the

non-cycling group, the flexor MSR response remained unchanged (46 ± 23 μV) and similar between the groups.

Passive cycling normalizes the response of the extensor MSR to quipazine in chronic spinalized rats

The typical response of an intact, properly functioning spinal cord to quipazine is an increase in MSR amplitude. As expected, the intact control group showed a 94% increase in the extensor MSR response (57 ± 24 μV to 112 ± 56 μV, $P < 0.004$) and a 86% in the flexor MSR response (40 ± 23 μV to 75 ± 43 μV, $P < 0.004$) after administering quipazine. Although no change in the flexor MSR response was seen in either of the chronic STx groups in response to quipazine, the extensor MSR demonstrated a difference. The extensor MSR of the daily passive cycling group increased 72% in response to quipazine (68 ± 30 μV to 117 ± 52 μV, $P < 0.004$), which was similar to the control group, while the extensor MSR of the non-cycling group showed no change in response to quipazine.

DISCUSSION

Combinational approaches involving pharmacological agents and training are used to facilitate locomotor recovery following a SCI. The purpose of this study was to investigate the effect daily passive cycling had on the MSR pre- and post-quipazine in two distinct motor pools following a chronic spinal transection. Daily passive cycling attenuated the hyperreflexive extensor MSR response to afferent stimuli and maintained the responsiveness to quipazine, similar to that demonstrated in the control group. Quipazine did not enhance the flexor MSR in the cycling group, nor did it alter the extensor or flexor MSR of the non-cycling group. These

results demonstrate the importance of an activity-based intervention in attenuating pathological hyperactivity of the extensor MSR to afferent excitation and excitation to the serotonergic agonist quipazine. The mechanisms responsible for these results remain to be elucidated, but it may be related to the postural (anti-gravity) role extensor muscles perform, which makes the extensor system more sensitive to changes in activity. For example, following a STx, others have shown that extensor muscles tend to atrophy earlier and to a greater extent than flexor muscles (West *et al.*, 1986;Roy & Acosta, Jr., 1986) while the findings within this manuscript suggest that the extensor muscle groups circuitry is preserved by exercise.

The effect of chronic spinalization on the MSR

This study in combination with our early work (Chopek *et al.*, 2013), demonstrated that spinal reflexes undergo remodeling and differ in responsiveness to quipazine from the transition of an acute STx to a chronic STx. Following a chronic STx, the extensor MSR demonstrates a hyperreflexive response (5x larger than control) following afferent stimulation. Evidence for functional reorganization has also been shown in the cat between acute and chronic STx. Following an acute STx, the triceps surae stretch-reflex fails to elicit EMG activity of the extensor muscles (lateral gastrocnemius or soleus) but rather generates EMG activity from the antagonist flexor muscles (tibialis anterior, sartorius and semitendinosus). One month post STx (chronic), the triceps surae stretch-reflex elicits EMG activity of extensor muscles (lateral gastrocnemius), and the flexor muscles (tibialis anterior, sartorius and semitendinosus) (Frigon *et al.*, 2011). The EMG activity change after one-month of having descending control eliminated supports the findings found herein, and the premise that re-organization of the spinal circuitry may lead to a hyper-reflexive extensor MSR following chronic STx.

The effect of daily passive cycling on the MSR

A number of studies reveal the benefits of daily passive cycling on the neuromuscular system following spinal cord injury. The benefits of daily passive cycling, which is characterized by alternating flexor and extensor EMG activity and left right alteration, (Houle *et al.*, 1999) include: 1) maintaining or restoring the resting membrane potential and firing rate of hindlimb motoneurons (Beaumont *et al.*, 2004), 2) preventing atrophy of the soleus (Houle *et al.*, 1999;Murphy *et al.*, 1999), tibialis anterior and plantaris muscles (Houle *et al.*, 1999;Murphy *et al.*, 1999;Peterson *et al.*, 2000) and 3) maintaining the FDD of the H-reflex (Skinner *et al.*, 1996;Reese *et al.*, 2006). Our findings expand on this literature by demonstrating that daily passive cycling preferentially maintains the response of the extensor MSR to afferent stimuli pre- and post-quipazine. It is thought that dorsiflexion produced during passive cycling provided a mechanism to produce a stretch-reflex or reflexive excitation that in turned influenced or maintained extensor spinal circuitry. In addition, this mode of exercise activates Group I and II afferent fibers, which are necessary for preserving the FDD of the MSR (Ollivier-Lanvin *et al.*, 2010). In a similar study, Skup et al., (2012) demonstrated daily treadmill training maintained acetylcholine contacts on extensor (soleus) motoneurons while failing to do so on flexor (tibialis anterior) motoneurons.

The effect of daily passive cycling on the responsiveness of the MSR to quipazine

This is the first study to demonstrate that previous activity modulates the responsiveness of the spinal reflex pathways to quipazine. This finding is important for two reasons. First, the 5-HT_{2C}R undergoes editing in response to environment changes (Werry *et al.*, 2008) . During periods of inactivity, such as a chronic spinal transection, the 5-HT_{2C}R mRNA undergoes

editing, producing a receptor that is less responsive to serotonin agonists (Murray *et al.*, 2010). Maintenance of afferent stimulation or activity of the motoneuron may explain why the cycling group responded to quipazine and why the non-cycling group did not. Second, understanding the influence of previous activity on the responsiveness of MSR to quipazine is also important as quipazine is often used to enhance or prime the spinal cord circuitry to facilitate functional recovery. This is demonstrated with the use of quipazine and treadmill training in spinalized rats (Feraboli-Lohnherr *et al.*, 1999;Antri *et al.*, 2002), cats (Barbeau & Rossignol, 1990;Barbeau & Rossignol, 1991), and robotic step trained mice (Fong *et al.*, 2005).

Although it is plausible that 5-HT_{2C}R mRNA editing could explain why the cycling group responded to quipazine and the non-cycling group did not, we suggest that activity modulates the MSR through presynaptic mechanisms that alter the responsiveness of the MSR to quipazine for several reasons. First, after a chronic STx in rat, the motoneuron demonstrates a supersensitivity to the non-specific 5-HT_2R agonist DOI (Harvey *et al.*, 2006). Second, our previous work demonstrated that the differential modulation of the flexor and extensor MSR response to quipazine in the acute STx preparation was presynaptic and not postsynaptic. Third, primary and secondary afferent excitability is enhanced following serotonin application (Ellaway & Trott, 1975). Lastly afferents respond to activity. For example it has been demonstrated that passive cycling maintains the appropriate spinal circuitry necessary to elicit FDD of the MSR by maintaining group I and II afferent connections on the motoneuron (Ollivier-Lanvin *et al.*, 2010). Treadmill training in spinal cord hemisectioned mice was shown not to alter the intrinsic properties of spinal neurons but rather enhance the excitatory synaptic drive from dorsal column pathways(Flynn *et al.*, 2013). Finally, five weeks of treadmill training of STx rats demonstrated an increase of synaptophysin (marker of presynaptic terminals) around the motor nuclei in L3/L4

compared to STx control rats (Macias *et al.*, 2009). Although the site of action responsible for passive cycling maintaining the responsiveness of the MSR to quipazine is not known, taken together, the literature would suggest passive cycling induces a presynaptic mechanism responsible for maintaining the responsiveness of the MSR to quipazine.

CONCLUSION

Taken as a whole, the evidence suggests training alters presynaptic or afferent connections onto the motoneuron and maintains the MSR response to quipazine following a chronic spinal transection. It appears that daily passive cycling maintains the extensor MSR and responsiveness of the MSR to quipazine by preserving afferent connections on the motoneuron.

ACKNOWLEDGMENTS

The authors would like to thank Shannon Deschamps for her assistance with animal care and Jason Au for his assistance with animal care and passive cycling of the rats.

This work was supported by grants to P.F.G. from the Canadian Institutes of Health Research, Canadian Research Chairs program, and the Manitoba Paraplegic Foundation. Funding support was provided to J.W.C. from the Canadian Institutes of Health Research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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cord transection and its relation to expression of brain-derived neurotrophic factor and presynaptic markers. BMC. Neurosci. 10:144

FIGURES

Figure 1.

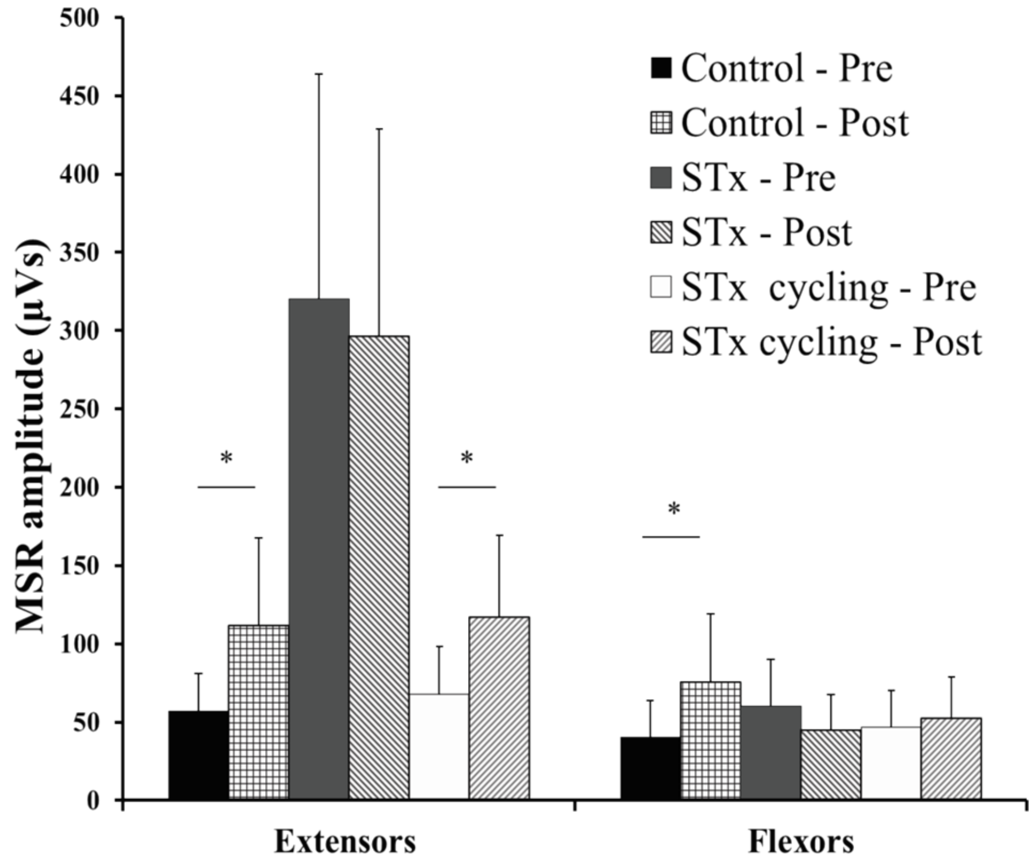

FIGURE LEGENDS

Figure 1. Daily passive cycling attenuates the extensor MSR hyperexcitability and maintains the responsiveness to quipazine.

Comparison of the averaged MSR pre- (solid colors) and post- (dashed lines) quipazine were made among the control, chronic STx non-cycling and cycling groups. Daily cycling maintained the MSR response (68 ± 30 μVs) within the control group values, compared to the hyperexcitable response found in the non-cycling group (320 ± 140 μV vs. 57 ± 23 μV, * $P < 0.004$). Flexor MSR amplitudes were similar among the groups. Quipazine increased the extensor and flexor MSR in the control group (94% and 86%, respectively; * $p < 0.004$) and the extensor MSR of the passive cycling group (72%, * $P < 0.004$) compared to pre-quipazine. Quipazine had no effect on the flexor MSR response of the cycling group or the chronic STx non-cycling group. Data are expressed as mean ± SD, with the MSR amplitude expressed in microvolts (μVs).

CHAPTER 4: SEROTONIN RECEPTOR AND KCC2 GENE EXPRESSION IN LUMBAR FLEXOR AND EXTENSOR MOTONEURONS POST-TRANSECTION WITH AND WITHOUT PASSIVE CYCLING

Running title: Lumbar extensor and flexor motoneuron gene expression

Keywords: Spinal transection, gene expression, serotonin receptors and KCC2, exercise, lumbar flexor and extensor motoneurons

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ABSTRACT

Sacral motoneuron gene expression is altered following a spinal transection. Of interest here is the regulation of serotonin (5-HT) receptors, mGluR1 and KCC2 which mediate motoneuron excitability, locomotor recovery and spasticity post-transection. The examination of these genes in lumbar motoneurons post-transection has not been studied which is necessary for developing potential pharmacological interventions aimed at restoring locomotion and or reducing spasticity. In addition, if activity is to be used to promote recovery or reduce spasticity post injury, a further examination of neuromuscular activity on gene expression post-transection is warranted. The purpose of this study was to examine motoneuronal gene expression of various 5-HT receptors, KCC2 and mGluR1 at three months following a complete thoracic spinal cord transection, with and without the inclusion of daily passive cycling. Physiological hindlimb extensor and flexor motoneurons were differentially identified with two retrograde fluorescent tracers, allowing for the identification and separate harvesting of extensor and flexor motoneurons with laser capture microdissection, and the subsequent examination of mRNA content using qRT-PCR analysis. We demonstrate that post-transection, 5-HT_{1A}R, 5-HT_{2C}R, and mGluR1 expression was down-regulated, whereas the 5-HT_{2A}R was up-regulated. These alterations in gene expression were observed in both flexor and extensor motoneurons similarly post-transection, whereas passive cycling influenced gene expression in extensor but not flexor motoneurons. Passive cycling in extensor motoneurons further enhanced 5-HT_{2A}R expression and increased 5-HT_7R and KCC2 expression. Our results demonstrate that daily passive cycling influences serotonin receptor and KCC2 gene expression and that extensor motoneurons compared to flexor motoneurons maybe more plastic to activity based interventions post transection.

INTRODUCTION

Gene expression is altered in sacral motoneurons following a sacral spinal cord injury (Wienecke *et al.*, 2010;Ryge *et al.*, 2010). The tail spinal transection model is important for studying spasticity (Bennett *et al.*, 1999) and has provided insight into the mechanisms and pharmacological methods to alleviate spasticity (D'Amico *et al.*, 2014). However the examination of gene expression in lumbar, as opposed to sacro-caudal motoneurons, post spinal transection is necessary for developing pharmacological interventions aimed at restoring locomotion or reducing limb spasticity and to date has not fully been investigated. Of interest is the regulation of the serotonin (5-HT) receptors on the alpha motoneuron, given the fundamental role 5-HT has in recovery of motoneuron excitability and locomotion post-injury (Ung *et al.*, 2008;Schmidt & Jordan, 2000). Further mGluR1 and KCC2 were also examined as mGluR1 also modulates motoneuron excitability by enhancing glutamatergic input on the motoneuron and facilitates plateau potentials (Delgado-Lezama & Hounsgaard, 1999) whereas KCC2 provides inhibitory input to the motoneuron by regulating chloride homeostasis (Payne *et al.*, 2003;Boulenguez *et al.*, 2010).

Following a spinal cord transection, the 5-HT_{2A}R is up-regulated in sacral motoneurons (Kong *et al.*, 2010;Kong *et al.*, 2011) and, in the lumbar cord, is necessary for the recovery of hindlimb locomotion induced by serotonergic agonists such as quipazine (Ung *et al.*, 2008). Following a thoracic spinal cord transection, quipazine is used to activate 5-HT_2 receptors and enhance spinal cord excitability and promote locomotor recovery post-transection alone or in combination with activity-based interventions such as treadmill training (Fong *et al.*, 2009). However, the influence of neuromuscular activity on expression of 5-HT receptor genes in motoneurons post-transection has not been reported.

Recently we demonstrated that at three months following a complete spinal cord transection, the extensor but not flexor monosynaptic reflex (MSR) demonstrated a 5-fold increase in amplitude, an effect which was attenuated with a program of daily passive cycling. Further, it was demonstrated that the extensor MSR of passively-cycled rats responded to quipazine whereas the MSR of non-cycled rats did not (Chopek *et al.*, 2014). Cote et al. (2014), have recently demonstrated that passive cycling following a spinal transection reduced muscle spasticity via an up-regulation of the potassium chloride-cotransporter 2 (KCC2) on the motoneuron. Whether passive cycling suppresses the development of extensor hyper-reflexia in the chronic spinalized rat was through alterations in serotonin receptor, mGlur1 or KCC2 gene expression in these motoneurons is unknown.

The purpose of this study was to examine gene expression of various 5-HT receptors at 3 months following complete thoracic spinal cord transection, with and without the inclusion of daily passive cycling. As we and others have shown that extensor but not flexor motoneurons respond differently to spinal transection and exercise (Chopek *et al.*, 2014;Skup *et al.*, 2012) a novel approach in which physiological hindlimb extensor and flexor motoneurons were differentially identified with two retrograde fluorescent tracers, allowing for the identification and harvesting of extensor and flexor lumbar motoneurons with laser capture microdissection, and the subsequent examination of mRNA content using qRT-PCR analysis. We demonstrate that following a spinal transection the 5-HT_{2A}R is up-regulated and the 5-HT_{1A}R, 5-HT_{2C}R and mGluR1 are down-regulated in both flexor and extensor motoneurons. Passive cycling altered gene expression in extensor but not flexor motoneurons, resulting in a further enhancement of 5-HT_{2A}R expression and up-regulation of 5-HT_{7}R and KCC2 expression.

METHODS

Animal Care

All animal treatment, surgical and experimental procedures were in accordance with the guidelines of the Canadian Council of Animal Care and approved by the University of Manitoba Animal Ethics Committee.

Adult female Sprague-Dawley rats weighing between 250-300g obtained from the University of Manitoba were used for all experiments described. The rats were housed in groups of two in plastic cages situated in an environmentally controlled room maintained at 23°C with a 12h-12h light-dark cycle. The rats had unlimited access to water and rat chow throughout the experiment period. Following the spinal transection, rats were individually caged for ease of monitoring.

Spinal Transection Procedure & Post-Operative Care

The surgical techniques and post-operative care procedures have been previously described in detail (Chopek *et al.*, 2014). Briefly, the rats were initially anesthetized with 5% isoflurane and maintained at 2-3% isoflurane mixed with 100% oxygen for the duration of the surgery. A laminectomy was performed at T8 followed by a small incision in the dura mater. The spinal cord at segment T10 was completely transected with microdissection scissors and gentle aspiration was applied, ensuring a complete spinal transection of ~2mm. Gel foam was packed into the gap and the surrounding fascia and musculature was sutured (4-0 Ethicon) while the skin was closed with vet bond. Post-surgery, the rats were given the antiobiotic Baytil (s.c. injection 0.5 $mg \cdot kg^{-1}$) twice daily for a week period and the analgesic Buprenex (buprenorphine, s.c. injection 0.05 $mg \cdot kg^{-1}$) twice daily for the first two days. A sub-cutaneous injection of 5 ml

of saline was also given immediately post-surgery to aide in rehydration. Manual bladder expression was performed 3 times daily until the voiding reflex was re-established.

Experimental Groups

Identified flexor and extensor motoneurons were collected from the following three groups: 1) control, spinal cord intact group that did not receive any intervention (N=7), 2) a spinal transected group that did not receive any intervention for three months (N=7) and, 3) a spinal transected group that received passive cycling for three months (N=6).

Daily Passive Cycling

Motorized pedals with a body hammock as described by Skinner et al. (1996) were used for the passive cycling. Following one week of recovery from the spinal transection surgery, the rats in the daily passive cycling group began their cycling. The rat was positioned in a body supported hammock/sling that allowed for the hindlimbs to be passed through holes in the hammock and secured to motorized pedals. The feet were secured using paper tape (3M Micropore) and the height of the hammock was adjusted to an optimal position that allowed for full extension and flexion of the hindlimb. The motorized pedals also allowed for the rhythmic alteration of the left and right hindlimbs. Each rat underwent passive cycling for one hour daily for a three month period at a rate of 30-50 revolutions per minute (rpm). It was found that the number of rpm needed to be adjusted for each rat daily as certain speeds produced spasticity.

Retrograde motoneuron labelling and tissue extraction

One week prior to sacrifice, physiological hindlimb flexor (extensor digitorum longus, tibialis anterior) and extensor (lateral gastrocnemius, soleus) muscles were injected with either 0.1 % cholera toxin subunit B Alexa 488 conjugate (10 µl in 0.1M PBS, each muscle) or 7%

dextran tetramethylrhodamine 10 000MW (fluororuby) (18 µl in saline, each muscle) with a Hamilton syringe. The muscle group injected was alternated between each tracer to ensure equal number of flexor and extensor muscles were injected with both dyes to prevent potential bias. As well, during each day of injections, one rat from each group was used to ensure consistency. At time of sacrifice (24 hours after last passive cycling session), the rat was deeply anesthetized with 5% isoflurane, followed by decapitation. The lumbar enlargement of the spinal cord was immediately removed, placed in a cryomold, covered in Tissue-Tec O.C.T. embedding compound (Gene Research Lab), fresh-frozen in isopentane and stored at -80 °C for future use.

Laser capture microdissection and qRT-PCR

Horizontal sections (11 µm) of the lumbar enlargement were cut on a cryostat and mounted on polytetrafluorethylene-coated glass slides. Slides were either used immediately or store at -80 °C for up to seven days. Slides were immersed in pre-chilled acetone (-20 °C) for one minute, followed by a series of alcohol washes (75%, 50%, 50%75%, 90%, 100%) and air dried for two minutes. The lumbar enlargements were then scanned and photographed using Zeiss filter set 38 for Alexa 488 and filter set 43 for fluororuby fluorescence on a Zeiss microscope to identify backfilled motoneurons. Individual motoneuron somas were dissected using the PALM laser microdissection and capture system and flexor and extensor motoneurons were collected in separate PALM microfuge tubes with adhesive caps (Figure 1). To limit RNA degradation, samples were collected for no longer than 60 minutes per slide. The collected material in the adhesive cap was treated with 20 µl of lysis buffer (RNAqueous Micro Kit, Ambion), inverted to wet the cap and stored upside down for 20 min at 42 °C to aid tissue digestion. The tubes were then vortexed and centrifuged at 10 000 RPM for 1 minute and stored

at -80 °C. Lysates from the same animal were pooled prior to RNA isolation (i.e., all flexor motoneurons pooled, all extensor motoneurons pooled). Total RNA was isolated from LCM samples with the RNAqueous Micro Kit (Ambion) according to manufacturer's recommendations. Total RNA concentration and integrity were determined with the RNA Pico 6000 Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies). A RNA integrity number of 6.5 or greater was accepted for analysis. Total numbers of motoneuron sections were comparable between groups and muscles (approximately 600-800 fragments) with comparable amounts of total RNA collected.

Reverse transcription was performed on equal amounts of sample RNA, with the SuperScript Vilo cDNA Synthesis Kit (Invitrogen) according to the manufacturers recommendations. Synthesized cDNA was preamplified with the TaqMan PreAmp Master Mix Kit (Applied Biosystems) for 14 preamplification cycles. Preamplified cDNA was diluted to 1 ml final volume with TE buffer. Quantitative polymerase chain reactions (qPCRs) were set-up with 12.5 μL of TaqMan Gene Expression Master Mix (Applied Biosystems), 6.25 μL nuclease free H_20, 1.25 μL TaqMan Gene Expression Assays (GEAs, see Table 1) and 5 μL preamplified cDNA per reaction. Reactions were run with the ABI 7500 Fast Real-Time PCR system (Applied Biosystems) for 40 cycles. Levels of mRNA expression were normalized to SDHA mRNA levels and were expressed as a %RQ (relative quantification) of control spinal cord intact rats. All reactions were performed in triplicate and the coefficient of variation was less than 5% for each triplicate.

Hindlimb muscle dissection

After sacrifice and removal of the spinal cord, muscles of both the left and right hindlimbs were dissected and the weights were recorded in grams. The following flexor muscles were dissected: 1) tibialis anterior and 2) extensor digitorum longus. As well the following extensor muscles were dissected: 1) gastrocnemius, 2) soleus and 3) plantaris.

Statistical analysis

The mRNA results were expressed in relative quantification (RQ) values calculated with the 7500 Software version 2.0 (Applied Biosystems) using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001). Preamplified pooled whole lumbar spinal cord cDNA served as the calibrator for all plates, allowing comparison of data from multiple qPCR plates. Data was subjected to a mixed-design ANOVA with group designation used for the between-subjects variable and flexor and extensor motoneuron %RQ as the within-subject variables. Fisher's least significant difference was used when a significant interaction was found. The p-value was set at < 0.05 and a False Discovery Rate adjustment was calculated (6 tests) for significance determined at ($P < 0.03$). Results for the spinal transection (STx) and STx – cycling group were expressed as percent relative to the control spinal cord intact group.

The muscle weights data was subjected to a one-way ANOVA to test for a main effect of group and a Newman-Kuels post hoc analysis was used to test for differences between means. Significance was set at $P < 0.05$.

RESULTS

Daily passive cycling attenuates extensor muscle mass loss

Three months post-transection, all three extensor muscles demonstrated a significant decrease in muscle weight (Figure 2). The gastrocnemius decreased by 24% (2.6 ± 0.4g vs 3.4 ± 0.4g) whereas the soleus and plantaris muscle weights decreased by 16% (0.26 ± 0.05g vs 0.31 ± 0.04g) and 17% (0.55 ± 0.06g vs 0.66 ± 0.09g) respectively. Daily passive cycling attenuated the loss in muscle mass, preserving the gastrocnemius (3.01 ± 0.39g), soleus (0.31 ± 0.06g) and plantaris (0.62 ± 0.08) weights, similar to that seen in the control group. Transection did not result in loss of mass in either flexor muscles.

Relative mRNA values of the control spine intact animals

Gene expression levels in control flexor and extensor motoneurons are presented in Table 2. The relative quantification (RQ) values for each gene, compared between extensor and flexor motoneurons, were not significantly different except for mGluR1. The RQ values for mGluR1 were 1.90 ± 0.54 and 0.86 ± 0.35 in extensor and flexor motoneurons respectively ($P<0.03$). The highest RQ value was for the 5-HT_{2A}R (5.95 ± 0.90 and 5.79 ± 0.39, extensors and flexors respectively) whereas the lowest RQ value was the 5-HT_{1A}R (0.18 ± 0.09 and 0.10 ± 0.06, extensors and flexors respectively).

5-HT_{2A}R gene expression is up-regulated post transection

Similar to previous reports on sacral motoneurons, 5-HT_{2A}R gene expression was up-regulated following spinal transection (Figure 3) in lumbar motoneurons. Both extensor and flexor motoneurons demonstrated a 62% and 55% increase respectively ($P < 0.03$). No

difference was seen between extensor and flexor 5-$HT_{2A}R$ gene expression in the spinal transected group.

5-$HT_{2C}R$, 5-$HT_{1A}R$ and mGluR1 gene expression is down-regulated post transection

The effect of spinal transection was similar between extensor and flexor motoneurons. 5-$HT_{2C}R$ gene expression decreased 35% and 42% in extensor and flexor motoneurons respectively ($P < 0.03$). 5-$HT_{1A}R$ gene expression was down-regulated 46% and 54% in extensor and flexor motoneurons ($P < 0.03$). Extensor and flexor motoneurons also demonstrated a 65% and 50% decrease in mGluR1 gene expression ($P < 0.05$). Spinal transection did not alter gene expression of the 5-HT_7R or KCC2.

KCC2 and 5-HT_7R gene expression is not altered post transection

Contrary to previous studies demonstrating a down-regulation of KCC2 following a spinal transection (Ziemlinska *et al.*, 2014;Boulenguez *et al.*, 2010;Bos *et al.*, 2013), we demonstrated no change in KCC2 gene expression three months post transection in either extensor or flexor motoneurons. Although there was a 20% decrease in KCC2 expression in flexor motoneurons post transection which is similar to the decrease reported in the above studies, this was not significant. Spinal transection did not result in alteration in 5-HT_7R expression in either extensor or flexor motoneurons.

Daily passive hindlimb cycling influences extensor motoneuron mRNA expression

Three months of passive cycling increased 5-HT_7R, and KCC2 gene expression and further enhanced 5-$HT_{2A}R$ gene expression in extensor but not flexor motoneurons when compared to spinal transection alone. Increase in 5-$HT_{2A}R$ gene expression in flexor

motoneurons was similar to that seen in the spinal transection group (53% increase, $P < 0.03$) whereas the extensor motoneurons of the passively cycled group demonstrated an 86% enhanced in expression which was significantly greater than that of the flexor motoneurons and of the spinal transection group ($P < 0.03$). Passive cycling following a spinal transection increased 5-HT_7R gene expression by 25% ($P < 0.03$) in extensor motoneurons, with no change seen in flexor motoneurons. KCC2 gene expression significantly increased in extensor motoneurons by 40% ($P < 0.03$) due to passive cycling, and was unchanged in flexor motoneurons. Passive cycling had no effect on the decrease in 5-$HT_{2C}R$, 5-$HT_{1A}R$ and mGluR1 gene expression associated with spinal transection in both extensor and flexor motoneurons.

DISCUSSION

This paper is the first to examine mRNA expression in two distinct motoneuron types following a complete thoracic spinal transection and the influence three months of passive cycling would have on gene expression. It was demonstrated that three months post spinal transection, 5-$HT_{2A}R$ expression is up-regulated, whereas 5-$HT_{2C}R$, 5-$HT_{1A}R$, and mGluR1 expression is down-regulated. Passive cycling influenced extensor but not flexor motoneurons of spinal transected rats, resulting in an up-regulation of 5-HT_7R and KCC2 gene expression, a further enhancement in the 5-$HT_{2A}R$ spinal transection associated up-regulation and the preservation of extensor muscle mass.

Gene expression post spinal transection

Previous studies have examined gene expression in sacral/caudal motoneurons that innervate the tail following a sacral cord lesion or in the whole lumbar cord following a thoracic lesion (Ryge *et al.*, 2010;Kong *et al.*, 2011;Kong *et al.*, 2010;Murray *et al.*, 2010;Navarrett *et al.*, 2012;Ung *et al.*, 2008;Wienecke *et al.*, 2010). It is vital that an understanding of lumbar motoneuronal gene expression is examined separately in extensor and flexor motoneurons following a complete transection to properly assess and develop therapeutic interventions aimed at restoring locomotion or hindlimb spasticity post injury. To that extent, our findings will be discussed in context with the known literature on serotonin receptor regulation in sacro-caudal tail motoneurons as well as in light of recent findings of KCC2 expression on lumbar motoneurons post thoracic transection.

Following a sacral spinal transection, in the sacro-caudal segments, 5-HT_{2C}R undergoes editing producing a 5-HT_{2C}R with high levels of constitutive activity, which has been proposed to be an underlying mechanisms of muscle spasms and necessary for locomotor recovery (Murray *et al.*, 2010;Murray *et al.*, 2011a). Although the total amount of 5-HT_{2C}R mRNA was unchanged in this study following a sacral transection (reference), Ren and colleagues have shown that 5-HT_{2C}R protein in sacral motoneurons is up-regulated 45 days post sacral transection and was associated with the development of tail spasticity (Ren *et al.*, 2013). Similar to Murray et al., Navarrett and colleagues (2012) found that total 5-HT_{2C}R mRNA was unchanged in whole lumbar cord homogenates following a complete thoracic spinal transection, whereas, we demonstrated a decrease in 5-HT_{2C}R expression in both lumbar flexor and extensor motoneurons. These differing results are likely due to different analyses used (protein vs.

mRNA), time point examined post injury as well as tissue used (whole cord vs motoneuron). Further, it is assumed in this adapted state the mRNA levels reflect protein levels, however this may not necessarily be the case.

Whereas we demonstrated a down-regulation in 5-HT_{2C}R expression, we demonstrated a robust up-regulation of the 5-HT_{2A}R in both flexor and extensor motoneurons, suggesting that the 5-HT_{2A}R may enhance lumbar motoneuron excitability post transection. This is supported by Jordan et al. (2010), who demonstrated that the 5-HT_{2A}R demonstrated a higher level of expression in the lumbar spinal cord whereas the 5-HT_{2C}R demonstrated a higher level of expression in the sacral cord. Further, behavioural and electrophysiological studies have linked the 5-HT_{2A}R to mediating lumbar spinal cord excitability post spinal transection. Ung et al. (2008) demonstrated that quipazine induced hindlimb locomotion was mediated by the 5-HT_{2A}R but not the 5-HT_{2C}R. Similar, Liu and Jordan (2005) have demonstrated that the 5-HT_{2A}R mediates lumbar motoneuron excitability when locomotion is generated by stimulation of the parapyramidal region in the in-vitro rat preparation. The use of selective serotonin agonists and antagonists for the 5-HT_{2A}R and 5-HT_{2C}R will need to be used to confirm which receptor subtype mediates lumbar motoneuron excitability post-transection

Our results would suggest that enhanced lumbar motoneuron excitability post-transection is likely mediated by the up-regulation of the 5-HT_{2A}R but also may in part be explained by the down-regulation of the 5-HT_{1A}R. The 5-HT_{1A}R, located on the axon hillock, inhibits action potential generation during periods of prolonged activity, thus a down-regulation would likely lead to enhanced motoneuron excitability post spinal transection. However, 5-HT_{1A}R is also found on the soma and proximal dendrites of the motoneuron and may also enhance excitability

by inhibiting a potassium leak channel. Further the 5-HT_{1A}R has also been shown to be unchanged (Giroux *et al.*, 1999) or up-regulated on lumbar motoneurons post spinal transection (Otoshi *et al.*, 2009). Thus, it appears the 5-HT_{2A}R is likely mediating lumbar motoneuron excitability post transection.

Lastly, the mGluR1 is the main post-synaptic mGluR in the ventral horn of the spinal cord (Valerio *et al.*, 1997). On the motoneuron, activation of the mGluR1 facilitates glutamatergic inputs and may also inhibit potassium channels and facilitate plateau potentials (Nistri *et al.*, 2006;Delgado-Lezama *et al.*, 1999). Consistent with the results by others (Alvarez *et al.*, 1997;Wienecke *et al.*, 2010), we demonstrated that mGluR1 expression is down-regulated following a spinal transection. Therefore, as the extensor MSR is potentiated following a spinal transection, mGluR1 gene expression is low in the motoneuron and passive cycling had no effect on expression, the mGluR1 likely does not contribute to the physiological responses seen post spinal transection.

In light of recent findings that demonstrate KCC2 mediates inhibitory control on motoneurons and is linked to spasticity following a spinal transection via a down-regulation in expression, KCC2 gene expression in lumbar extensor and flexor motoneurons were examined in our study. It is interesting that in our study we did not demonstrate a decrease in KCC2 expression post-spinal transection. However, flexor motoneurons demonstrated a 23% in expression which is similar to that seen in Cote et al (2014)at 28 days (-20% decrease in protein expression) and Boulenguez et al. (2010) at 45 day post transection (22% decrease in protein expression). As well, Ziemlinska et al.(2014) demonstrated that KCC2 down-regulation was greater in L1-L3 segments compared to L3-L6 segments following a thoracic transection.

Therefore, it is plausible that the down-regulation of KCC2 expression post-transection is exclusive to hindlimb flexor motoneurons. This warrants further investigation as in our current study the difference was not significant and likely due to the large variability in expression seen in the spinal transected group.

Passive cycling influences gene expression

This is the first study to demonstrate that an activity-based intervention such as passive cycling alters serotonin receptor expression in lumbar motoneurons following a spinal transection. The neuromuscular system demonstrates activity-related plasticity in both spine intact and spinal transected animals, demonstrated by the modulation of motoneuron properties and gene expression in lumbar motoneurons and spinal cord. Three weeks of voluntary wheel running in mice results in an increase in gene expression for cell signalling, ion channels, synaptic reorganization and growth and reinforcement of the neuromuscular junction (Ferraiuolo *et al.*, 2009;Perreau *et al.*, 2005). Neurotrophic factors also respond to exercise - endurance training in the spine intact rat, down-regulates the myelin associated glycoprotein MAG (axon gwoth inhibitor; Ghiani *et al.*, 2007) and upregulates BDNF, NT-3, TrkB, TrkC, synapsin I, Gap-43 and CREB in the lumbar spinal cord (Gomez-Pinilla *et al.*, 2001;Gomez-Pinilla *et al.*, 2002;Ying *et al.*, 2003;Macias *et al.*, 2002;Macias *et al.*, 2007) with a similar enhancement found in laser-captured motoneurons following one month of passive cycling in the spinal transected rat (Keeler *et al.*, 2012). Our results further the scope on activity related gene expression, demonstrating that passive cycling increases both 5-HT$_7$R and KCC2 expression and further enhances 5-HT$_{2A}$R expression - exclusively in extensor motoneurons. The exact mechanism by which gene expression is up-regulated is unknown but passive cycling has

previously been found to activate and preserve group I and II afferent connections on the motoneuron (Ollivier-Lanvin *et al.*, 2010), which would likely provide a level of daily afferent input on the motoneuron to increase gene expression.

Extensor but not flexor motoneurons respond to exercise

Our results are consistent with others that extensor motoneurons respond to neuromuscular activity following a spinal transection to a greater extent than flexor motoneurons (Chopek *et al.*, 2014;Skup *et al.*, 2012). We previously demonstrated that passive cycling attenuated the hyperexcitability of the extensor MSR and maintained the responsiveness of the extensor MSR to quipazine, whereas no effect was seen in the flexor MSR (Chopek *et al.*, 2014). Passive cycling results in rhythmic left and right alternation and flexor and extensor muscle length changes, with EMG confirming at least extensor activation; however, EMG activity was not monitored in flexor muscles (Houle *et al.*, 1999;Dupont-Versteegden *et al.*, 2004). It is possible that our activity paradigm only activates extensor muscle thereby preferentially influencing the extensor MSR, we consider this unlikely, as a similar result was found when treadmill training was used (Skup *et al.*, 2012) which activates both flexor and extensor muscles (Slawinska *et al.*, 2012). Treadmill training was shown to increase the number of cholinergic contacts on extensor motoneurons but not flexor motoneurons post spinal-transection (Skup *et al.*, 2012). Why extensor motoneurons respond to activity whereas flexor motoneurons do not is unknown, but is thought to be the result of extensor muscles being anti-gravitational and thus affected to a greater extent than flexor muscles post-injury (West *et al.*, 1986;Roy & Acosta, Jr., 1986). Similar to others, we demonstrated that following a spinal transection extensor muscle mass is lost and that with passive cycling, muscle mass is preserved (Houle *et al.*, 1999;Murphy

et al., 1999;Peterson *et al.*, 2000). Therefore, passive cycling appears to influence the extensor spinal circuitry to a greater extent than the flexor circuitry by preserving muscle mass, attenuating the pathological increase in the MSR, maintaining the responsiveness of the MSR to quipazine (Chopek *et al.*, 2014) and up-regulating serotonin receptor and KCC2 mRNA.

Up-regulation of 5-HT_{2A}R and KCC2 in passively cycled extensor motoneurons

Following a spinal transection, with the loss of excitatory descending monoaminergic input, the spinal cord compensates by increasing excitatory receptors on the motoneuron (Wienecke *et al.*, 2010), resulting in hyperexcitability (Li & Bennett, 2003). Unfortunately, this increase in excitability also leads to unwanted long lasting reflexes or spasticity (Murray *et al.*, 2011a). Passive cycling seems to fine-tune this paradigm by up-regulating both the excitatory 5-HT_{2A}R and the inhibitory KCC2 which have a role in motoneuron excitability and spasticity respectively (Schmidt & Jordan, 2000;Bos *et al.*, 2013;Cote *et al.*, 2014).

Recently it has been demonstrated that passive cycling post-transection resulted in the up-regulation of KCC2 protein expression on lumbar motoneurons. This up-regulation was associated with a decrease in spasticity and restoration of the frequency dependent depression of the h-reflex (Cote *et al.*, 2014). Our results expand on this novel finding by demonstrating that passive cycling positively influences KCC2 expression in extensor but not flexor motoneurons. Although we did not measure spasticity to correlate our findings, we previously have demonstrated that 3 months post spinal transection the extensor but not flexor MSR was potentiated and this was attenuated with passive cycling. Thus our results in combination with others demonstrate that passive cycling attenuates spasticity (Cote *et al.*, 2014) and MSR

hyperexcitability (Chopek *et al.*, 2014) likely through an up-regulation of KCC2 expression which is exclusive to extensor motoneurons.

5-HT$_7$R expression is enhanced in extensor motoneurons

A novel finding was that passive cycling up-regulated 5-HT$_7$R gene expression in extensor motoneurons. Immunohistochemistry and immunocytochemistry studies have demonstrated that the 5-HT$_7$R is present in the ventral horn and on motoneurons (Noga *et al.*, 2009;Doly *et al.*, 2005) although the role of the 5-HT$_7$R on the motoneuron is poorly understood, likely due to lack of availability of specific agonists for the 5-HT$_7$R. It has however, been demonstrated that the 5-HT$_7$R reduces the mAHP in presumed jaw-closing motoneurons (Inoue *et al.*, 2002) and induces long-term motor facilitation in phrenic motoneurons (Hoffman & Mitchell, 2011), demonstrating an excitatory effect on the motoneuron. The 5-HT$_7$R has also been linked to locomotor generation in the in-vitro rat preparation when stimulating the parapyramidal region and that both the 5-HT$_7$R and 5-HT$_{2A}$R are required for the induction of locomotion to occur (Liu & Jordan, 2005), although it was believed that agonists for the 5-HT$_7$R acted on potential CPG neurons whereas 5-HT$_{2A}$R agonists acted directly on the motoneuron. Further studies are required to understand the role of the 5-HT$_7$R on the motoneuron to determine if an up-regulation of the receptor leads to a measurable outcome.

CONCLUSION

This is the first study to examine serotonin gene expression in two distinct lumbar motoneuron pools following spinal transection with and without passive cycling. We

demonstrate that following a spinal transection, the 5-$HT_{2A}R$ is up-regulated, whereas the 5-$HT_{2C}R$, 5-$HT_{1A}R$ and mGluR1 are down regulated in both extensor and flexor motoneurons. With passive cycling KCC2 and 5-$HT_{7}R$ expression is increased and the 5-$HT_{2A}R$ expression is further enhanced in extensor but not flexor motoneurons. The increase in gene expression likely explains our previous results in which passive cycling attenuated the hyper-excitability of the extensor MSR and maintained the MSR response to quipazine. Finally, our results would suggest that extensor motoneurons may be more plastic to activity based interventions following a spinal cord injury.

ACKNOWLEDGEMENTS

The authors would like to thank Shannon Deschamps for her assistance with animal care, Jason Au for his assistance with animal care and passive cycling of the rats and Dr. Larry Jordan for his advice and valuable comments on the manuscript.

This work was supported by grants to P.F.G. from the Canadian Institutes of Health Research, Canadian Research Chairs program, and the Manitoba Paraplegic Foundation. Funding support was provided to J.W.C. from the Canadian Institutes of Health Research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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TABLES

Table 1. Genes examined in study

RefSeqID	Gene Symbol	Protein Symbol	Description	qPCR assay ID	Amplicon length(bp)
NM_130428.1	*SDHA*	SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein	Rn00590475_m1	59
NM_012585.1	*HTR1A*	5-HT 1A	5-HT receptor 1A	Rn00561409_s1	75
NM_017254.1	*HTR2A*	5-HT 2A	5-HT receptor 2A	Rn00568473_m1	71
NM_012765.3	*HTR2C*	5-HT 2C	5-HT receptor 2C	Rn00562748_m1	100
NM_022938.2	*HTR7*	5-HT 7	5-HT receptor 7	Rn00576048_m1	85
NM_001114330	*GRM1*	mGluR1	Glutamate receptor, metabotropic 1	Rn00566625_m1	83
NM_134363.1	*Slc12a5*	Kcc2	Solute carrier family 12 potassium-chloride transporter member 5	Rn0059264_m1	79

Table 2. Relative gene expression values of control spine intact rats

Gene	Extensor Mns	Flexor Mns	
5-HT$_{1A}$	0.18 ± 0.09	0.10 ± 0.06	
5-HT$_{2A}$	5.95 ± 0.90	5.79 ± 0.39	
5-HT$_{2C}$	0.29 ± 0.10	0.42 ± 0.08	
5-HT$_{7}$	1.45 ± 0.23	1.32 ± 0.18	
mGluR1	1.90 ± 0.54	0.86 ± 0.35	*
KCC2	1.13 ± 0.19	1.23 ± 0.13	

Relative expression values (RQ), using SDHA as the housekeeping gene, of each gene in hindlimb extensor and flexor motoneurons of control spine intact rats. Data are presented as means ± SD. * Significant difference between extensor and flexor mns (P < 0 .03).

FIGURES

Figure 1

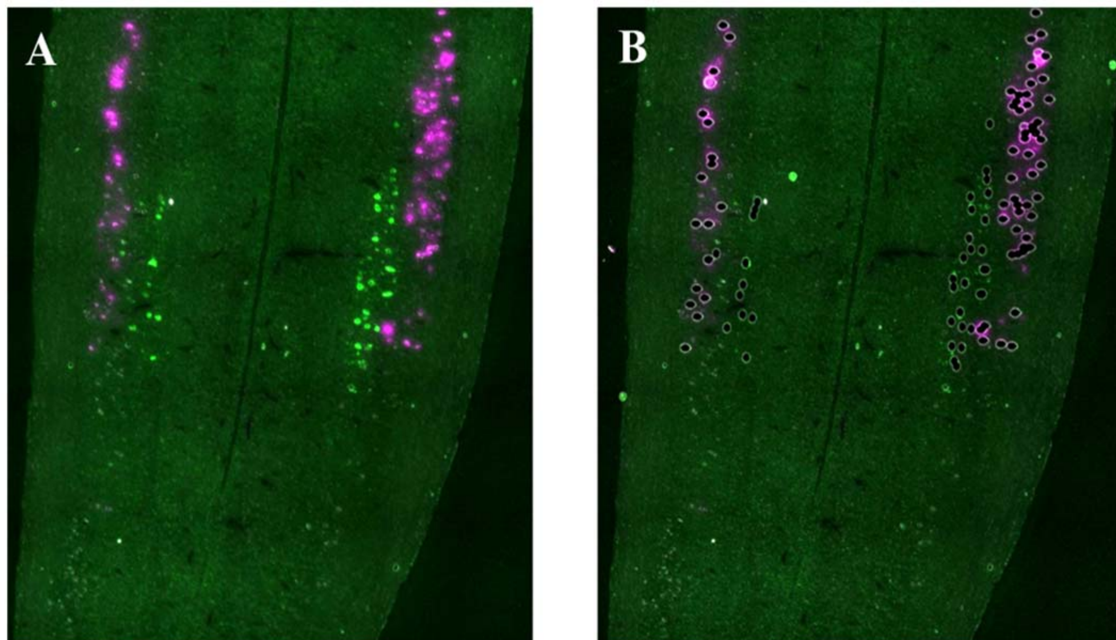

Figure 2

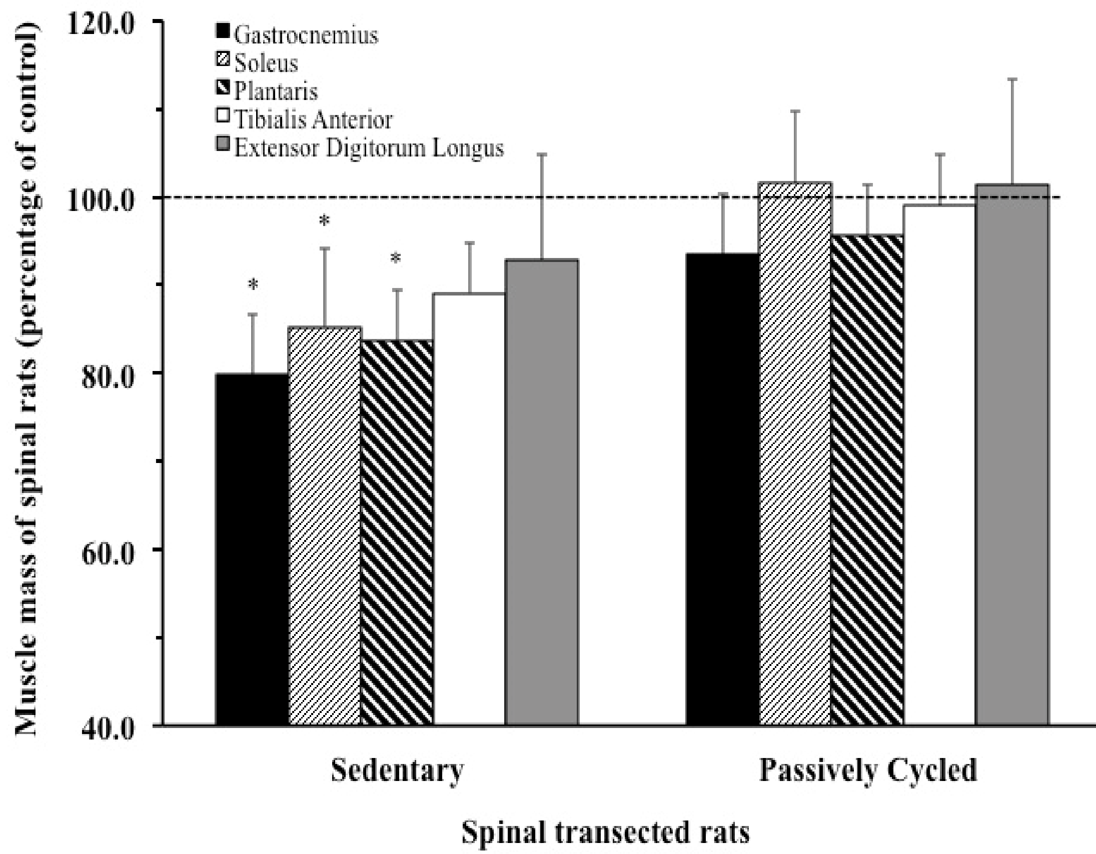

Figure 3

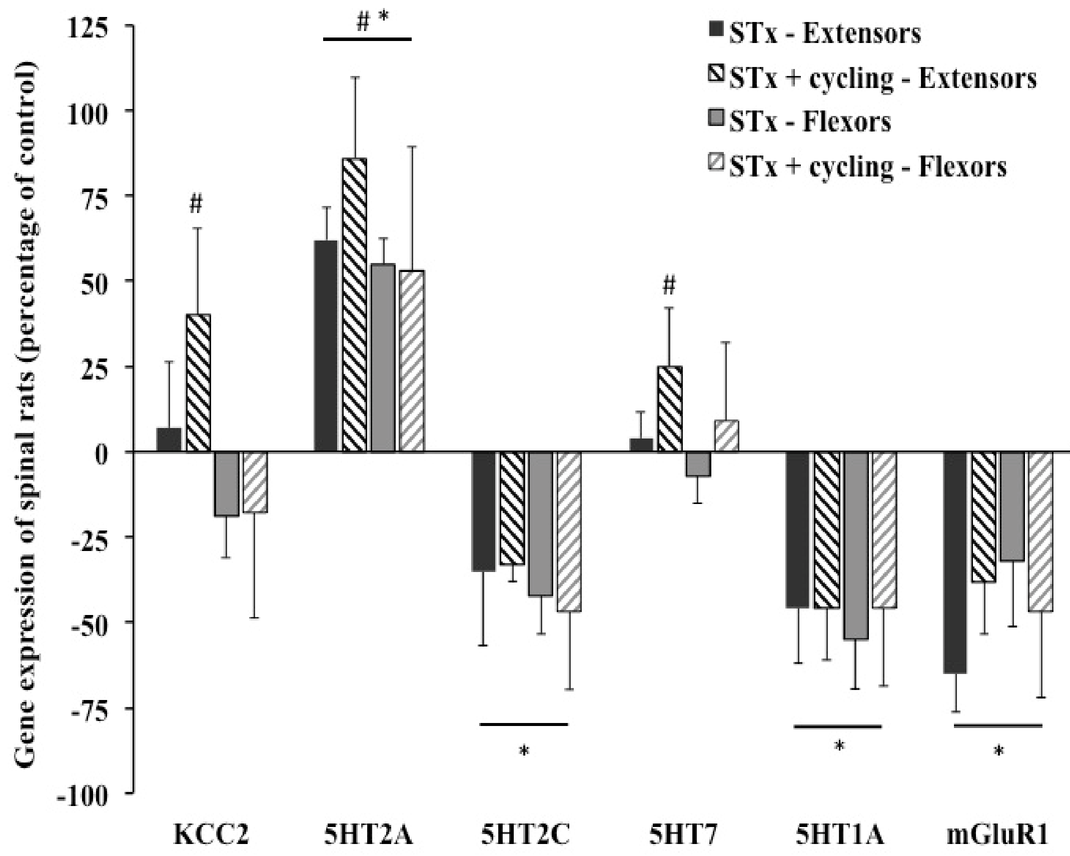

FIGURE LEGENDS

Figure 1. Laser capture microdissection of extensor and flexor motoneurons.

One week prior to sacrifice, hindlimb flexor (tibialis anterior & extensor digitorium longus) and extensor muscles (lateral gastrocnemius & soleus) were injected with either 0.1% cholera toxin subunit B Alexa 488 conjugate or 7% dextran tetramethylrhodamine 10000MW (fluororuby). Upon sacrifice, horizontal sections (11 µm) of the lumbar enlargement were mounted on polytetrafluorethlene-coated glass slides for laser capture microdissection. **A.** In this example, extensor motoneurons were injected with cholera toxin and fluoresce green whereas flexor motoneurons were injected with fluororuby and fluoresce pink. **B.** Scan of the lumbar segment, shown in A after extensor and flexor motoneurons were isolated separately in PALM microfuge tubes using the PALM laser microdissection and capture system.

Figure 2. Passive cycling attenuates extensor muscle mass loss post spinal transection.

Three months after a spinal transection, a significant decrease in extensor muscle mass of the gastrocnemius, soleus and plantaris was seen when compared to the respective muscle mass of control spine intact rats (* $P < 0.05$). Spinal transection was not associated with decrease mass of the hindlimb flexor muscles – extensor digitorium longus or tibialis anterior. Three months of daily passive cycling attenuated the loss of the extensor muscle mass, with muscle mass comparable to the control spine intact group. Bars represent means ± SE.

Figure 3. Gene expression in extensor and flexor motoneurons of spinal transected rats and spinal transected rats that underwent passive cycling.

Three months after a spinal transection, 5-HT_{2A}R expression was up-regulated in both flexor and extensor motoneurons, whereas 5-HT_{2C}R, 5-HT_{1A}R and mGluR1 expression were down-regulated. No differences in gene expression between flexor and extensor motoneurons post-transection were seen. Following three months of passive cycling, a further enhancement of 5-HT_{2A}R expression was seen in extensor motoneurons. Passive cycling also up-regulated 5-HT_7R and KCC2 expression in extensor but not flexor motoneurons. * Indicates a significant difference compared to the control spine intact group ($P < 0.03$). # Indicates a significant difference of the passive cycled extensor motoneurons compared to the passive cycled flexor motoneurons and extensor and flexor motoneurons of the spinal transected group ($P < 0.03$). Bars represent means ± SE.

CHAPTER 5: GENERAL DISCUSSION

This thesis comprises three studies in which two distinct motor pools were examined in activity dependent states, examining the influence passive cycling had on the responsiveness of motoneurons and the MSR to quipazine and on serotonin receptor gene expression. Six key highlights arose from these studies: 1) this was the first study in which intracellular motoneuron recordings of two distinct motoneuron pools were made pre- and post-drug in the adult *in-vivo* decerebrate rat spine intact and acutely spinalized preparation, 2) the preferential effect of quipazine on the flexor and extensor MSR differed from the control spine intact to the acute spinalized rat and lastly to the chronic spinalized rat 3) in the acute spinal preparation, the preferential effect of quipazine was presynaptic and not due to differences in motoneuron modulation, 4) in the chronic spinal preparation, passive cycling influenced the extensor MSR both pre-and post-quipazine, 5) this was the first study to examine serotonin receptor gene expression in extensor and flexor lumbar motoneurons following a spinal transection and 6) three months of passive cycling altered gene expression in extensor but not flexor lumbar motoneurons. These findings were discussed in detail in the respective manuscripts; therefore the remaining general discussion will be reserved to discussion of the inter-related findings, the limitations of the studies and the potential significance of these findings.

As the rhythmic alternation of left and right hindlimbs and flexor and extensor muscles underlie basic movements such as locomotion, an understanding of how serotonin modulated these motor outputs was essential. The first study was to determine whether or not extensor and flexor motoneurons are differentially influenced by the serotonergic agonist quipazine as previous literature has alluded to a potential difference (Hounsgaard *et al.*, 1988a;Cotel *et al.*,

2009;Vult & Lomo, 2005); however this had never been investigated. Our initial preparation allowed for us to simultaneously record the tibial (extensor) and peroneal (flexor) MSR elicited by L4/L5 dorsal root stimulation pre- and post-quipazine. Our findings demonstrated that in the intact control animal, no difference between the extensor and flexor MSR to quipazine existed, however in the acutely spinal transected rat, a significant increase in MSR amplitude was seen in both the flexor and extensor MSR with a greater increase in amplitude seen in the flexor MSR compared to the extensor MSR. From this and subsequent experiments (discussed in detail in chapter 2), it was suggested that in the spine intact animal, the flexor Ia afferents are under a stronger tonic inhibition compared to the extensor Ia afferents. As well, for the difference in the modulatory effect of quipazine to be apparent the removal of this descending inhibition was required. Future studies are essential to determine the precise mechanism or source of this difference in presynaptic inhibition on the flexor and extensor Ia afferents.

A second supportive explanation for a difference in the flexor and extensor MSR to quipazine post-spinal transection is that immediately following a spinal transection, which is thought to be a hypo-reflexive state (Ditunno *et al.*, 2004) is in fact also characterized by aberrant and exaggerated flexor reflexes (Fong *et al.*, 2009). It has been demonstrated in the acute spinalized cat, that the triceps surea stretch reflex elicited EMG activity of the flexor muscles but not extensor muscles (Frigon *et al.*, 2011). Furthermore, in the acute stage of a spinal transection, hindlimb kinematics and EMG recordings of the cat during treadmill locomotor training demonstrate a predominance of flexor activity and reduction in extensor activity (Belanger *et al.*, 1988). These results in addition to our own work, suggest that consideration of differences in extensor and flexor motor output need to be examined for future studies using pharmacological agents, as it may either increase excitability to produce unwanted

spasms or may not have an effect on motor output, depending on the muscle group targeted. This is highlighted by a recent study examining the effect of serotonergic drugs in humans with an incomplete spinal cord injury (Leech *et al.*, 2014). An acute dose of a selective serotonin reuptake inhibitor (SSRI, escitalopram) increased flexor EMG activity with no effect on extensor EMG activity whereas the serotonergic antagonist cyproheptadine decreased extensor EMG activity with no effect on flexor EMG activity. However, neither the SSRI nor the 5-HT antagonist resulted in improved locomotion, although only one dose of each drug was examined during locomotor testing (Leech *et al.*, 2014).

Following the initial finding the extensor and flexor MSR were differently modulated by quipazine in the acute spinalized state, we set to determine whether this was due to pre-synaptic or post-synaptic modulation. Based on previous findings in motoneurons (Hounsgaard *et al.*, 1988b), the motoneuron was examined pre- and post-quipazine application. This in itself was a feat as *in-vivo* intracellular motoneuron recording pre- and post-drug was very difficult to achieve. We presented data from 23 motoneurons (both spinal cord intact and acutely spinalized) successfully recorded pre- and post-quipazine. Our results demonstrated that quipazine did not preferentially modulate either flexor or extensor motoneurons. However several limitations to this study existed which should be addressed in future studies

First, our hypothesis was based on the premise that extensor and flexor motoneurons have differences in the ability to generate PICs (Hounsgaard *et al.*, 1988a;Cotel *et al.*, 2009). Our study attempted to examine this using the ePIC method which can be estimated by a slow triangular current injection and subtracting the current at de-recruitment to recruitment (Bennett *et al.*, 2001;Button *et al.*, 2006) . Unfortunately, due to the amount of quipazine injected in the

animal, immediate hyperexcitability of the motoneuron was achieved and the ePIC method was determined unreliable to determine the influence quipazine may have had on the underlying PIC. Future studies should be done using voltage clamp to record the PIC and determine if quipazine modulates flexor and extensor PICs differentially. Second, we gave a large bolus injection of quipazine in the intra-peritoneal region, therefore we cannot determine the exact mechanism or site in which quipazine is working, however we assumed the main site of action is the motoneuron as 5-HT2Rs are located predominately in the ventral horn and motoneuron (Marlier *et al.*, 1991;Fonseca *et al.*, 2001;Doly *et al.*, 2004).

A large injection of quipazine in the intra-peritoneal region was given for technical reasons. Recording intracellularly, the stability of the penetrating electrode can be compromised with large rises in blood pressure, which occurs when quipazine is injected. Therefore a large injection was given to reduce the time in which we had to maintain our position within the motoneuron while waiting for the quipazine induced excitability to occur. Several subsequent experiments were done with a dose of quipazine that is comparable to other studies that use quipazine to elicit stepping in the spinalized rat (Ichiyama *et al.*, 2008), which produced similar results seen when the large dose was given. Future studies should be done with dual electrodes to apply a microinjection of quipazine or various serotonergic agonists directly near the motoneuron to determine the site of action in which quipazine is acting or use the in-vitro rat preparation in which several specific agonists and antagonists can be directly applied. However, the *in-vitro* rat preparation has been shown to produce conflicting results to what we found, such as a lowering of voltage threshold (Fedirchuk & Dai, 2004) and resting membrane potential (Elliott & Wallis, 1992) and a decrease in AHPamp (White & Fung, 1989), likely owing to the fact that the *in-vitro* preparation generally uses an immature rat pup whereas our in-vivo

preparation uses an adult rat and that serotonin has known age dependent effects on motoneuron modulation (Ziskind-Conhaim *et al.*, 1993;Takahashi & Berger, 1990;Talley *et al.*, 1997;Schmidt & Jordan, 2000).

An important finding from the first study was that the differential effect quipazine had on the extensor and flexor monosynaptic reflex was pre-synaptic (Chopek *et al.*, 2013). This was confirmed with simultaneous Ia extracellular field potentials of the tibial and peroneal nerves. This allowed us to subsequently focus on the monosynaptic reflex for project 2 (Chopek *et al.*, 2014) instead of attempting to record intracellularly in three-month spinal transected rats. Ideally we would have recorded from motoneurons in addition to the MSR but given the technical challenges involved and the time and expenses involved with the animals, this was not pursued. Future studies should focus on recording intracellularly from motoneurons as it is plausible a difference in extensor and flexor motoneurons would be seen at three months post spinal transection. It has been demonstrated that extensor muscles atrophy earlier and to a larger extent than flexor muscles (West *et al.*, 1986;Roy & Acosta, Jr., 1986) and intracellular recordings of tibial motoneurons in the rat and cat demonstrate that chronic spinal transection alters the biophysical properties and rhythmic firing profiles of tibial motoneurons (Cope *et al.*, 1986;Hochman & McCrea, 1994;Beaumont *et al.*, 2004;Button *et al.*, 2008). However, our gene expression analysis did not demonstrate a difference in 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptor gene expression between extensor and flexor motoneurons, which may suggest that although motoneuron properties are altered following a chronic spinal transection, the properties that 5-HT receptor activation influences may not be differentially affected.

Another important concept of this thesis was to understand the role activity had on extensor and flexor motoneurons pre- and post-quipazine and whether or not any of the electrophysiological results could correlate with differences in serotonin receptor gene expression. As activity based intervention are used alone or in combination with serotonergic agonists (Fong *et al.*, 2009), it was important to determine if the effects of exercise or activity are global within the spinal cord or whether the effects are limited to a subpopulation of neurons. Previous studies have demonstrated that exercise (voluntary wheel running) alters the biophysical properties of slow but not fast motoneurons (Beaumont & Gardiner, 2002) and that following a spinal transection, extensor motoneurons respond to treadmill training by increasing the number of apposing cholinergic contacts whereas flexor motoneurons do not (Skup *et al.*, 2012), suggesting activity may have specific effects on specific motoneuron subtypes.

Our results demonstrated that three months of passive cycling preferentially affect the extensor MSR as well as altered serotonin receptor and KCC2 gene expression in extensor but not flexor motoneurons. Three months of passive cycling attenuated the hyperexcitability of the extensor MSR and maintained the responsiveness of the MSR to quipazine administration. However, it is uncertain whether passive cycling maintains the amplitude of the extensor MSR or that in the non-cycling group, the extensor MSR had already plateaued and quipazine did not produce a further increase in the extensor MSR amplitude. This ceiling effect and subsequent lack of a further increase in extensor EMG following an intervention has been suggested for spinal cord injury in humans as well other neurological disorders (Wirz *et al.*, 2006;Leech *et al.*, 2014;Little *et al.*, 1989). However, it is believed a ceiling effect did not occur as the flexor MSR of the non-cycling group did not respond to quipazine as well, which demonstrated no hyperexcitability pre-quipazine administration.

Whether or not a ceiling effect occurred, it is important to highlight that we demonstrated passive cycling attenuated the hyper-excitability of the extensor MSR. Unfortunately we did not measure spasticity pre- or post-passive cycling to determine if in fact passive cycling has a functional outcome on reducing spasticity. However, it has been previously demonstrated that passive cycling reduces spasticity following a complete spinal transection in the rat (Garrison *et al.*, 2011). We have also demonstrated that passive cycling up-regulated KCC2 gene expression in extensor motoneurons. KCC2 activation has recently been shown to be influenced by 5-$HT_{2A}R$ activity (Bos *et al.*, 2013) and linked to spasticity (Boulenguez *et al.*, 2010;Bos *et al.*, 2013). An increase in 5-$HT_{2A}R$ activity led to an up-regulation of KCC2 on the motoneuron membrane and reduced spasticity, whereas inhibition of 5-$HT_{2A}R$ activity resulted in a decrease in KCC2 expression on the motoneuron membrane and increased spasticity (Bos *et al.*, 2013). Similar previous studies have shown passive cycling up-regulates BDNF (Keeler *et al.*, 2012) in laser-captured motoneurons and that KCC2 expression is also regulated by BDNF expression (Boulenguez *et al.*, 2010). Studies using passive cycling alone (Rosche *et al.*, 1997;Kakebeeke *et al.*, 2005) or in combination with functional electrical stimulation (Krause *et al.*, 2008;Rayegani *et al.*, 2011) in spinal cord injured humans have also demonstrated a reduction in spasticity. Our results combined with others suggest that passive cycling can be used as a non-pharmacological intervention to reduce spasticity.

One of our limitations in the gene expression project was not examining a functional outcome such as locomotor function, weight-bearing, stepping or spasticity to potentially correlate our results. Future studies should address this concern, especially whether or not the increase in KCC2 gene expression in extensor motoneurons resulted in reduced spasticity. Also, an interesting future study would be to examine other various activities (i.e stand training,

treadmill training) on gene expression and functional outcome. Our results showed that extensor motoneurons and MSR were positively influenced by passive cycling which was similar to extensor motoneurons being positively influenced by treadmill training (Skup *et al.*, 2012). However in the complete spinal transected cat, medial gastrocnemius muscle mass loss was attenuated with stand training but not step training (Roy *et al.*, 1999), demonstrating that at least extensor muscles respond differently to the type of activity. As well, in future studies EMG should be examined prior to, during and after the activity to determine if both the flexor and extensor systems are in fact being activated. For our study, EMG was not recorded and therefore we cannot rule out that flexor muscles were not activated as previous studies have also only examined EMG activity in the soleus muscle to demonstrate that passive cycling activated muscles (Houle *et al.*, 1999;Dupont-Versteegden *et al.*, 2004).

Overall these three manuscripts demonstrate that the flexor and extensor monosynaptic reflex system undergoes remodeling from the acute to chronic stage of a spinal cord transection. This remodeling includes hyperexcitability of both the flexor and extensor MSR to quipazine, with a preferential effect on the flexor MSR in the acute stage, whereas in the chronic state the extensor MSR demonstrated hyperexcitability and neither the flexor nor extensor MSR demonstrated a response to quipazine. Lastly, we demonstrated that passive cycling preferentially modulated the extensor spinal circuitry demonstrated by the attenuation of the MSR amplitude, maintenance of the MSR response to quipazine, attenuation of extensor muscle mass loss and the alteration of serotonin receptor and KCC2 gene expression in extensor motoneurons.

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