The Effect of Short-Term Endurance Training on $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ Receptor mRNA Levels in Rat Lumbar Motoneurons

by

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Abstract

Serotonin receptor subtypes 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} are expressed in motoneurons and modulate motoneuron excitability. Serotonergic neurons, which increase their discharge with motor activity, make numerous contacts with motoneurons; however, little is known about the adaptability of motoneuron serotonin receptor expression in response to exercise. The purpose of this study was to determine the effect of a 7-day treadmill exercise protocol on 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptor mRNA levels in rat lumbar motoneurons. Lumbar motoneurons of exercised and sedentary animals were collected via laser capture microdissection. RNA was isolated from these samples and real-time reverse transcription polymerase chain reactions were performed to determine differences in receptor mRNA levels between exercised and sedentary animals. It appears that 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptor mRNA levels are unaltered following 7 days of treadmill exercise; however, future research must be done to determine if an exercise effect exists when motoneurons are differentiated by type.

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Abbreviations

5-HT – serotonin

5-HT_{1A}R – serotonin receptor subtype 1A

5-HT_{1B}R – serotonin receptor subtype 1B

5-HT_2R – serotonin receptor family 2 subtype

5-HT_{2A}R – serotonin receptor subtype 2A

5-HT_{2B}R – serotonin receptor subtype 2B

5-HT_{2C}R – serotonin receptor subtype 2C

5-HTR – serotonin receptor

BDNF – brain-derived neurotrophic factor

BSA – bovine serum albumin

Ca^{2+} – calcium

cDNA – complementary DNA

Cl^- – chloride

C_q – quantification cycle

DNase – deoxyribonuclease

GABA – γ-aminobutyric acid

GLUT4 – glucose transporter type 4

K^+ – potassium

L3 – segments 3 of the lumbar spinal cord

L5 – segments 5 of the lumbar spinal cord

L6/S1 – lumbar spinal cord segment 6/sacral sacral spinal cord segment 1

LCM – laser capture microdissection

mRNA – messenger ribonucleic acid

Na^+ – sodium

neurotrophin-3 – NT-3

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PEN – polyethylene naphthalate

PGC-1α – peroxisome proliferator activated receptor co-activator 1α

PTFE – polytetrafluoroethylene (Teflon)

qPCR – real-time (quantitative) polymerase chain reaction

qRT-PCR – real-time (quantitative) reverse transcription polymerase chain reaction

RNase – ribonuclease

RQ – relative quantification

RT-PCR – reverse transcription polymerase chain reaction

SDHA – succinate dehydrogenase complex subunit A

TASK – TWIK-related acid-sensitive potassium (K^+) channel

TBS – tris-buffered saline

TBST – tris-buffered saline and Tween 20

TWIK – tandem pore domain weak inward rectifying potassium (K^+) channel

TE – tris-ethylenediaminetetraacetic acid (EDTA)

Tris-HCl – tris-hydrochloric acid

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Introduction

Serotonin (5-HT) mediates a wide range of processes in the body, both centrally and peripherally; these include smooth muscle tone, platelet aggregation, sleep, appetite, sensory perception, mood, thermoregulation, anxiety, pain, learning, memory, aggression, sexual behaviour, endocrine function and motor function (for reviews see Hannon & Hoyer, 2008; Hoyer & Schoeffter, 1991; Kroeze, Kristiansen & Roth, 2002; Millan, Marin, Bockaert & Mannoury la Cour, 2008; Nichols & Nichols, 2008; Peroutka, 1988a; Roth, Berry, Kroeze, Willins & Kristiansen, 1998; Zifa & Fillon, 1992). The diverse actions of 5-HT are accomplished through numerous 5-HT receptor (5-HTR) subtypes. Though it had previously been suggested that there are 15 (Rekling et al., 2000; Roth et al., 1998) or 16 (Peroutka, 1995) distinct mammalian 5-HTR subtypes, the majority favours 14 subtypes organized into 7 different classes (or families) based on homology (Andrade, 1998; Barnes & Sharp, 1999; Hoyer, Hannon & Martin, 2002; Kroeze et al., 2002; Millan et al., 2008; Nichols & Nichols, 2008). Most 5-HTRs are G-protein coupled receptors, including subtypes 1A (5-HT_{1A}R), 2A (5-HT_{2A}R, previously known as the 5-HT_2 receptor) and 2C (5-HT_{2C}R, previously known as the 5-HT_{1C} receptor) (Barnes & Sharp, 1999; Hannon & Hoyer, 2008; Hoyer et al., 2002; Kroeze et al., 2002; Nichols & Nichols, 2008; Roth et al., 1998).

Serotonergic neurons originate in the raphe nuclei and parapyramidal region of the medulla and project to various parts of the central nervous system, including spinal motoneurons, the neurons that activate skeletal muscle (Alvarez et al., 1998; Ballion, Branchereau, Chapron & Viala, 2002; Liu & Jordan, 2005; Pilowsky, de Castro, Llewellyn-Smith & Voss, 1990). Serotonergic terminals appose both motoneuron soma

and dendrites, with the majority of synapses found on both proximal and distal dendrites (Alvarez et al., 1998; Pilowsky et al., 1990). 5-HT is known to alter motoneuron excitability and plays a role in modulating locomotion, perhaps through its actions on motoneurons (for reviews, see Rekling et al., 2000; Schmidt & Jordan, 2000). As each motoneuron makes more than 1500 contacts with serotonergic boutons, it is evident that 5-HT provides important synaptic input to motoneurons (Alvarez et al., 1998). In the case of endurance exercise, where motoneurons are repeatedly activated, 5-HT may represent an important means of regulating motoneuron excitability to ensure optimal motor output. However, there is limited information as to the adaptability of the serotonergic system to increased physical activity, and more specifically, the adaptability of serotonergic receptors in motoneurons to increased physical activity.

Review of Literature

Distribution of 5-HT Receptors in the Ventral Spinal Cord

5-HT$_{1A}$ Receptor

Many studies have examined the distribution of 5-HT$_{1A}$Rs in the ventral spinal cord, however, the findings of these studies have been largely conflicting. Early autoradiographic experiments showed virtually none or no binding of various radiographic ligands ([^{125}I]BH-8-MeO-N-PAT, [^{3}H]5-HT, [^{3}H]8OH-DPAT) to 5-HT$_{1A}$Rs in the ventral horn of human, rat and cat spinal cord at cervical, thoracic, lumbar and sacral levels (Giroux, Rossignol & Reader, 1999; Gozlan et al., 1988; Laporte, Fattaccini, Lombard, Chauveau & Hamon, 1995; Laporte et al., 1996; Marlier, Teilhac, Cerruti & Privat, 1991; Pazos & Palacios, 1985b; Thor, Nickolaus & Helke, 1993). There also

appears to be no labelling of $5\text{-}HT_{1A}R$ mRNA in rat ventral spinal cord (Pompeiano, Palacios & Mengod, 1992). However, binding of $[^3H]$5-HT and $[^3H]$8OH-DPAT to $5\text{-}HT_{1A}Rs$ was observed in the ventral horn of rat lumbar spinal cord, with Fischette and colleagues (1987), finding binding density of $[^3H]$5-HT to $5\text{-}HT_{1A}Rs$ to be nearly the same for dorsal and ventral horns in the lumbar-sacral segment (Croul, Radzievsky, Sverstiuk & Murray, 1998). Immunohistochemistry revealed labelling of $5\text{-}HT_{1A}R$ protein in the ventral horn of rat cervical and lumbar spinal cord (Kheck, Gannon & Azmitia, 1995; Kia et al., 1996; Otoshi et al., 2009). Kheck et al. (1995), found $5\text{-}HT_{1A}R$ protein was primarily on the axon hillock of motoneurons, with some additional immunoreactivity present on soma, but not dendrites, in cervical spinal cord. Otoshi et al. (2009), utilized two antibodies directed against different regions of the $5\text{-}HT_{1A}R$, and observed plasma membrane labelling of the axon hillock, soma and proximal dendrites of motoneurons in the lumbar spinal cord. Although these findings appear to be contradictory, Kheck and colleagues (1995), argue that electrophysiological studies confirm the presence of $5\text{-}HT_{1A}Rs$ on motoneurons and that the different approaches used to identify the distribution of these receptors in the spinal cord, each with varying sensitivity, account for the disparity of results.

$5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ Receptors

In contrast to $5\text{-}HT_{1A}Rs$, the presence of $5\text{-}HT_{2A}Rs$ and $5\text{-}HT_{2C}Rs$ in the ventral spinal cord is well documented. Several autoradiographic studies have found dense binding of $[^3H]$Ketanserin and $[^{125}I]$DOI to $5\text{-}HT_{2A}Rs$ and $5\text{-}HT_{2C}Rs$ found in the ventrolateral rat spinal cord at cervical, thoracic, lumbar and sacral levels (Croul et al., 1998; Fischette et al., 1987; Marlier et al., 1991; Thor et al., 1993). Other studies,

however, found limited binding of 5-HT_{2A}Rs and 5-HT_{2C}Rs in the ventral horn of human (cervical, thoracic and lumbar levels) and rat spinal cord with [^{3}H]Ketanserin and [^{3}H]Mesulergine (Laporte et al., 1996; Pazos, Cortes & Palacios, 1985; Pazos, Probst & Palacios, 1987a; Pazos, Probst & Palacios, 1987b). 5-HT_{2A}R mRNA and 5-HT_{2C}R mRNA has also been located in the ventral horn of mouse and rat spinal cord at cervical, thoracic, lumbar and sacral levels (Basura, Zhou, Walker & Goshgarian, 2001; Fonseca, Ni, Dunning & Miledi, 2001; Pompeiano et al., 1994; Ung et al., 2008). Both mRNAs are strongly expressed in Rexed Lamina IX (the motoneuron-containing region of the spinal cord), however, it appears that 5-HT_{2A}R mRNA and 5-HT_{2C}R mRNA are not co-localized within this area, perhaps indicating different roles for these populations of motoneurons (Fonseca et al., 2001). Immunohistochemistry has also revealed intense labelling of 5-HT_{2A}R protein in the ventral horn of rat spinal cord at cervical, thoracic, lumbar and sacral levels (Cornea-Hébert, Riad, Wu, Singh & Descarries, 1999; Doly et al., 2004; Fuller et al., 2005; Kong, Wienecke, Hultborn & Zhang, 2010; Lee, Johnson & Wrathall, 2007; Maeshima et al., 1998). This immunoreactivity was localized to motoneuron soma, proximal dendrites and cytoplasm within Lamina IX (Cornea-Hébert et al., 1999; Doly et al., 2004; Kong et al., 2010; Lee et al., 2007; Maeshima et al., 1998). Additionally, 5-HT_{2A}R protein labelling was more pronounced on rat soleus motoneurons, particularly the soma and proximal dendrites of these motoneurons, as compared to extensor digitorum longus motoneurons, where labelling was strictly dendritic (Vult von Steyern & Lomo, 2005). Interestingly, the soleus and extensor digitorum longus represent slow extensor and fast flexor muscles, respectively, perhaps indicating that function, firing rate or total daily activity levels may influence expression

of 5-HT_{2A}Rs (Vult von Steyern & Lomo, 2005). Motoneuron soma and proximal dendrites were also robustly labelled with TH8 antibody, which recognizes 5-HTR subtype 1B (5-HT_{1B}R), 5-HT_{2A}R and 5-HT_{2C}R protein (Ridet, Tamir & Privat, 1994). Although the exact location of 5-HT_{2C}Rs on spinal neurons, including motoneurons, is unknown, they are presumed to be found on the soma and dendrites of these structures (Leysen, 2004).

5-HT Receptors and Glia

It should be noted that glial cells, which are also located in Lamina IX, express 5-HTR protein (Cornea-Hébert et al., 1999; Kheck et al., 1995; Kia et al., 1996; Maeshima et al., 1998; Maxishima et al., 2001; Ridet et al., 1994).

5-HT Pharmacology

As mentioned previously, 5-HT modulates motor function, having both hyperpolarizing and depolarizing actions on motoneurons (for reviews, see Rekling et al., 2000; Schmidt & Jordan, 2000). Activation of 5-HT_{1A}Rs has inhibitory and excitatory effects on motoneurons (Cotel, Berg, Smith & Perrier, 2009; Holohean, Hackman & Davidoff, 1990; Perrier, Alaburda & Hounsgaard, 2003; Perrier & Cotel, 2008; Takahashi & Berger, 1990; Wang & Dun, 1990). 5-HT_{1A}Rs are preferentially coupled to G-protein subunit G_i, and utilize negative coupling to adenylyl cyclase as a second messenger, resulting in membrane hyperpolarization through increased potassium (K^+) conductance and decreased neuronal firing (see Andrade, 1998; Bobker & Williams, 1990; Hamon et al., 1990; Hoyer & Schoeffter, 1991; Hoyer et al., 2002; Kroeze et al., 2002; Millan et al., 2008; Nichols & Nichols, 2008; Peroutka, 1988b; Peroutka, Schmidt, Sleight & Harrington, 1990; Zifa & Fillion, 1992). 5-HT_{1A}R signal transduction may

also inhibit calcium (Ca^{2+}) conductance (Andrade, 1998). Recently, it has been suggested that motoneuron hyperpolarization mediated by 5-HT_{1A}Rs at the axon initial segment, is due to inhibition of a sodium (Na^+) current (Cotel et al., 2009). In contrast, 5-HT_{1A}R mediated motoneuron depolarization (due to activation of somatodendritic 5-HT_{1A}Rs) occurs via inhibition of a TASK-1-like K^+ leak current and may be linked to an alternative transduction system (Andrade, 1998; Hamon et al., 1990; Hoyer & Schoeffter, 1991; Millan et al, 2008; Perrier et al., 2003; Perrier & Cotel, 2008; Zifa & Fillion, 1992). Activation of 5-HT_{1A}Rs has been shown to depress the 5-HT_{2A}R/5-HT_{2C}R facilitated spinal monosynaptic mass reflex, perhaps indicating a functional link between 5-HT_{1A}Rs and 5-HT receptor family 2 subtypes (5-HT_2Rs) in determining motoneuron excitability (Gajendiran, 2008). This antagonistic relationship has been observed in the modulation of locomotor activity in rats, with interactions likely occurring at the receptor or second messenger level (Gajendiran, 2008; Krebs-Thomson & Geyer, 1998).

Activation of 5-HT_2Rs has an excitatory effect on motoneurons (Hasegawa & Ono, 1996; Holohean et al., 1990; Roberts, Davies, Girdlestone & Foster, 1988; Wang & Dun, 1990). 5-HT_{2A}Rs and 5-HT_{2C}Rs are preferentially coupled to G-protein subunit G_q, with their activation triggering second messenger phospholipase C, increased Ca^{2+} signalling and neuronal depolarization (and therefore increased neuronal firing) through reduced K^+ conductance (see Barnes & Sharp, 1999; Bobker & Williams, 1990; Chojnacka-Wojcik, 1995; Frazer, Maayani & Wolfe, 1990; Hannon & Hoyer, 2008; Hartig, 1989; Hoyer et al., 1994; Hoyer et al., 2002; Kroeze et al., 2002; Leysen, 2004; Millan et al., 2008; Nichols & Nichols, 2008; Peroutka, 1988b; Peroutka et al., 1990; Shih, Chen & Gallaher, 1994; Zifa & Fillion, 1992). As well, 5-HT_{2A}Rs and 5-HT_{2C}Rs

are thought to increase chloride (Cl^-) conductance (Bobker & Williams, 1990; Hoyer & Schoeffter, 1991; Peroutka, 1988b). 5-HT_2Rs also modulate both the Na^+ and Ca^{2+} channel components of persistent inward currents in motoneurons and accordingly, facilitate long-lasting motoneuron excitability, with 5-HT_{2C}Rs believed to play a key role in this long-lasting excitability (Gajendiran, 2008; Harvey, Li, Li & Bennett, 2006; Perrier & Hounsgaard, 2003; Perrier & Cotel, 2008). Additionally, 5-HT_{2A}Rs contribute to the initiation of locomotion and may do so via direct action on motoneurons (Liu & Jordan, 2005; Madriaga, McPhee, Chersa, Christie & Whelan, 2004).

Although the transduction systems of 5-HTRs have been studied in a variety of neuronal cell types, for the most part it appears that there is conservation of receptor/G protein/ion channel coupling in the central nervous system and therefore, we can expect similar responses in motoneurons (Andrade, 1998). However, motoneuron responses to 5-HT, like other neurons, are dependent on the cellular location of 5-HTRs and the variety 5-HTR subtypes expressed by the cell (Andrade, 1998). Motoneuron responses to 5-HT are also likely affected by the state of an organism, such as its chronic physical activity levels, as G-protein coupled receptors are thought to adjust neuron electrophysiological properties (and therefore excitability) to ensure that any functional demands imposed on the cell are met (Andrade, 1998; Hille, 1994).

It is important to note that these studies utilize a variety of 5-HTR agonists and antagonists to examine the contribution of each receptor subtype to mediating motoneuron excitability. These drugs are often used to activate or block one (or more) 5-HTR subtypes in order to determine the role of each subtype. However, many of these drugs are not specific to one 5-HTR and although one subtype may have a greater affinity

for the drug, it may also activate or block other subtypes, potentially confounding any findings (International Union of Basic and Clinical Pharmacology, 2010). Therefore, the effects observed may, in fact, be mediated by multiple 5-HTRs or a different 5-HTR subtype than concluded. As well, the specificity of 5-HTR agonists and antagonists may be an issue in the autoradiography studies discussed previously.

5-HT and Exercise

Discharge of serotonergic neurons, including those innervating the spinal cord, is increased with motor output (for reviews, see Jacobs & Fornal, 1997; Jacobs, Martin-Cora & Fornal, 2002). Jordan and colleagues (2010), found that c-Fos labelling of yellow fluorescent protein-marked serotonergic neurons in the parapyramidal region of the brainstem is increased following 1 hour of treadmill exercise. During exercise, the activity of serotonergic neurons within the dorsal raphe nucleus appears to be intensity-dependent, as c-Fos expression was increased following a 30 minute session of low-speed treadmill running (15 m/minute), but not high-speed treadmill running (25 m/minute) (Otsuka, Kubota, Ichikawa & Kita, 2009). Additionally, brain 5-HT synthesis and metabolism are known to increase with exercise (for reviews, see Chaouloff, 1989; Chaouloff, 1997), but it should be noted that increased 5-HT metabolism does not necessarily indicate increased 5-HT release as 5-HT may be broken down prior to release (see Chaouloff, 1993). Despite this evidence, release of 5-HT from serotonergic terminals in the ventral lumbar spinal cord during intense treadmill exercise is unaltered compared to resting conditions (Gerin, Legrand & Privat, 1994). However, the high intensity of treadmill exercise and sampling location (Rexed Laminae VII and VIII as opposed to Lamina IX) may explain these findings.

In addition to modulating motoneuron activity, 5-HT has been suggested to play a role in the development of central fatigue – particularly with regards to the "brain component" of central fatigue (for reviews, see Blomstrand, 2001; Davis & Bailey, 1997; Meeusen, Watson, Hasegawa, Roelands & Piacentini, 2006; Newsholme & Bloomstrand, 2006). In terms of central fatigue at the motoneuron level, it has been proposed that activation of 5-HT_{1A}Rs at the axon initial segment by excess 5-HT released at serotonergic synapses inhibits a Na^+ current, thus preventing generation of action potentials and serving as a protective mechanism to avoid motoneuron hyperexcitability (Cotel et al., 2009; Perrier & Cotel, 2008).

Motoneurons and Exercise

Motoneurons alter their electrophysiological properties in response to varying levels of physical activity, becoming more excitable with increased neuromuscular activity (for reviews, see Gardiner, Dai & Heckman, 2006; Gardiner, 2006). Although these shifts in motoneuron properties are independent of serotonergic modulation, altered expression of 5-HTRs could provide an additional means of increasing or decreasing motoneuron excitability in response to chronic alterations in activity (Barnes & Sharp, 1999; Gardiner et al., 2006; Gardiner, 2006). While no published studies have examined the effect of exercise on 5-HTR mRNA or protein expression in the spinal cord, exercise does alter gene expression in the spinal cord and motoneurons. The neuromuscular junction adapts to increased activity levels, with alterations to protein expression and structure observed at the motor endplate (Ferraiuolo et al., 2009; Gharakhanlou, Chadan & Gardiner, 1999; for reviews see Panenic & Gardiner, 1998 & Wilson & Deschenes, 2005). Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-

4 and their receptors, are upregulated in the lumbar spinal cord following increased neuromuscular activity (Al-Majeed, Brushart & Gordon, 2000; Gomez-Pinilla, Ying, Opazo, Roy & Edgerton, 2001; Gomez-Pinilla, Ying, Roy, Molteni & Edgerton, 2002; Skup et al., 2000; Skup et al., 2002; Ying, Roy, Edgerton & Gomez-Pinilla, 2003). Expression of these neurotrophins is thought to promote neuroplasticity, perhaps mediating exercise-induced motoneuron adaptations (Gomez-Pinilla et al., 2001). Altered expression of mRNAs coding for K^+ and Ca^{2+} channels, glutamate receptor 3, Ca^{2+} ATPase, and a splicing factor affecting glutamate receptor subunit zeta-1 transcription, was observed in mouse spinal cord following exercise training (Hashimoto et al., 2009; Ferraiuolo et al., 2009).

In contrast to increased neuromuscular activity, microarray analysis revealed differential expression of several ion channels (including various Ca^{2+}, Na^+, Cl^- and K^+ channels) and neurotransmitter receptors (including glutamate, acetylcholine, γ-aminobutyric acid (GABA), adrenergic, glycine and dopamine receptors) in rat sacral motoneurons following spinalization (Wienecke, Westerdahl, Hultborn, Kiehn & Ryge, 2010). Interestingly, Wienecke and colleagues (2010), also observed a downregulation of 5-HTR subtype 2B (5-HT_{2B}R) – a member of the 5-HT_2 receptor family along with the 5-HT_{2A}R and 5-HT_{2C}R – 21 days after spinalization. These receptors increase motoneuron excitability and appear to play a minor role in generating locomotor-like activity (Gunther, Maroteaux & Schwarzacher, 2006; Holohean & Hackman, 2004; Ung et al., 2008). As well, $GABA_A$ receptor subunit γ2 immunoreactivity was increased in tibialis anterior motoneurons and decreased in soleus motoneurons following spinal cord transection (Khristy et al., 2009). However, after step training, $GABA_A$ receptor subunit

$\gamma 2$ protein labelling was restored near control levels in both tibialis anterior and soleus motoneurons in transected rats (Khristy et al., 2009). These observations, particularly altered expression of ion channels with increases or decreases (in the case of spinal lesion) in activity, demonstrate the plasticity of the spinal cord and motoneurons and seem to corroborate the changes in motoneuron electrophysiological properties observed with varying levels of physical activity.

While the effect of exercise on 5-HTR mRNA expression has not been examined in the spinal cord, it has been studied in other central nervous system regions. 5-HT_{1A} autoreceptor mRNA was increased in rat dorsal raphe nuclei and median raphe nuclei after 6 weeks of voluntary wheel running (Greenwood et al., 2003; Greenwood et al., 2005). Conversely, 5-HT_{1B} autoreceptor mRNA was decreased in rat cerebellum with intense treadmill training, in rat frontal cortex with both moderate and intense treadmill training and in rat dorsal raphe nuclei after voluntary wheel running (Chennaoui et al., 2001; Greenwood et al., 2005). These observations appear to indicate an exercise-induced shift toward decreased brain 5-HT neurotransmission in certain brain regions as 5-HT_{1A} autoreceptors inhibit discharge of serotonergic neurons, while 5-HT_{1B} autoreceptors stimulate serotonergic neurons (Chennaoui et al., 2001; Greenwood et al., 2003; Greenwood et al., 2005). Additionally, Loughridge and colleagues (2009), have also observed altered $5\text{-HT}_{2C}\text{R}$ mRNA expression in select brain regions after 6 weeks of voluntary wheel running.

Finally, preliminary data suggests that there is no change in $5\text{-HT}_{2A}\text{R}$ protein expression in lumbar and sacral motoneurons after 2 months of intense treadmill exercise (Wienecke, Ploug & Zhang, 2009). However, this study examined motoneurons at the

fifth and sixth lumbar levels and second sacral level, even though the majority of hindlimb musculature is innervated with motoneurons rostral to the fifth lumbar level of the spinal cord (Nicolopoulos-Stournaras & Iles, 1983). It is possible that changes in 5-$HT_{2A}R$ expression may be observed rostral to the fifth lumbar level as these pools of motoneurons correspond to the musculature more vigorously activated with treadmill running. As well, changes in 5-$HT_{2A}R$ mRNA levels may be observed in motoneurons at the levels studied, but these changes may not be translated, therefore not affecting 5-$HT_{2A}R$ protein expression.

Spinal Cord Lesion and Expression of 5-HT Receptors

Various studies have examined the effect of spinal cord lesion on the expression of 5-HTRs in the spinal cord. Giroux et al. (1999), found no change in [^{3}H]8OH-DPAT binding to 5-$HT_{1A}Rs$ in the ventral horn of cat spinal cord at cervical, thoracic and lumbar levels at any time point – both short term and long term – following spinal cord transection. In contrast, labelling of 5-$HT_{1A}R$ protein on motoneuron soma and proximal dendrites in rat lumbar spinal cord was increased 8 weeks after spinal cord transection and spinal cord isolation (Otoshi et al., 2009). This increase was observed in all of Lamina IX in spinal cord-transected animals and in the lateral region of Lamina IX in spinal cord-isolated animals and was more robust in spinal cord-transected as compared to spinal cord-isolated animals (Otoshi et al., 2009). 5-$HT_{1A}R$ protein distribution was also increased along the axon hillock of motoneurons in spinal cord-transected animals, but not spinal cord-isolated animals (Otoshi et al., 2009). These findings suggest that sensory input (which is left intact in spinal cord transection, but not spinal cord isolation) plays a key role in determining the expression of 5-$HT_{1A}R$ protein in the lumbar spinal

cord and is likely more important than ligand availability to the expression of these receptors (Otoshi et al., 2009). Labelling of 5-HT_{2A}R mRNA was not significantly increased in the medial or lateral ventral horn of mouse lumbar spinal cord at any time point from 3 hours to 28 days after spinal cord transection (Ung et al., 2008). 5-HT_{2A}R immunoreactivity in the ventral horn was, however, increased 2 months after spinal cord transection in rat sacral spinal cord, 4 weeks after contusive spinal cord injury in rat lumbar spinal cord and 2 weeks after cervical hemisection in rat cervical spinal cord (ipsilateral to the lesion) (Fuller et al., 2005; Kong et al., 2010; Lee et al., 2007). Spinal cord transection produced the greatest increase in 5-HT_{2A}R protein, prompting Kong and colleagues (2010), to suggest that complete absence of serotonergic projections to motoneurons (as is the case with spinal cord transection, but not contusive spinal cord injury or cervical hemisection as these are incomplete lesions) results in a large compensatory upregulation of 5-HT_{2A}Rs. Nonetheless, these studies did not examine the role of afferent input in determining the expression of 5-HT_{2A}Rs following spinal cord lesion and like 5-HT_{1A}Rs, their expression may be influenced by a reduction or elimination of 5-HT availability and sensory input.

Statement of Problem

To determine the effect of a 7-day treadmill exercise protocol on mRNA levels of 5-HT_{1A}Rs, 5-HT_{2A}Rs and 5-HT_{2C}Rs in rat lumbar motoneurons.

Rationale

While it is well documented that motoneurons adapt to changes in neuromuscular activity, the effect of exercise on the expression 5-HTRs in motoneurons remains to be elucidated. The purpose of this study was to examine the influence of exercise on 5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2C}R expression in lumbar motoneurons. Developing a greater understanding of how physical activity alters serotonergic modulation of motoneurons may allow for design of more effective exercise/rehabilitation programs for a variety of neurological conditions, including spinal cord injury.

Hypotheses

1. 5-HT_{1A} receptor mRNA levels in lumbar motoneurons will be decreased in the treadmill exercise rats compared to the sedentary control rats after the 7-day treadmill exercise protocol.

This is hypothesized because of the inhibitory actions of 5-HT_{1A}Rs on motoneurons, their antagonistic relationship with 5-HT_2Rs, and their proposed role in central fatigue. A downregulation of 5-HT_{1A}Rs would be a beneficial adaptation for exercise-trained motoneurons as it may shift the 5-HT_{1A}R/5-HT_2R relationship in favour of 5-HT_2Rs and prolong time to fatigue while exercising.

2. 5-HT_{2A} receptor mRNA levels in lumbar motoneurons will be increased in the treadmill exercise rats compared to the sedentary control rats after the 7-day treadmill exercise protocol.

This is hypothesized because of the excitatory actions 5-HT_{2A}Rs on motoneurons and their role in long-lasting motoneuron excitability and the initiation of locomotion. An upregulation of 5-HT_{2A}Rs would be a beneficial adaptation for exercise-trained motoneurons as locomotion may be initiated more easily at the onset of exercise and long-lasting motoneuron excitability is required for maintaining posture, especially for lumbar motoneurons.

3. 5-HT_{2C} receptor mRNA levels in lumbar motoneurons will be increased in the treadmill exercise rats compared to the sedentary control rats after the 7-day treadmill exercise protocol.

This is hypothesized because of the excitatory actions 5-HT_{2C}Rs on motoneurons and their role in long-lasting motoneuron excitability. An increase in 5-HT_{2C}Rs would be a beneficial adaptation for exercise-trained motoneurons as long-lasting motoneuron excitability is required for maintaining posture, especially for lumbar motoneurons.

Methods

Animals

Twelve female Sprague-Dawley rats were obtained from the University of Manitoba at approximately 8 weeks of age. Animals were be assigned to two groups: sedentary control (n=6) and treadmill exercise (n=6). Both groups were housed in standard plastic cages (two animals per cage) in a temperature controlled room (23°C) with a 12-hour light/dark cycle. Food and water were provided ad libitum. Animals were allowed to adjust to their surroundings for approximately 1 week prior to the start of the experimental protocol. Experimental procedures were approved by the University of Manitoba (Bannatyne Campus) Animal Care Committee and conducted in accordance with University of Manitoba and Canadian Council on Animal Care policies.

To determine the appropriate sample size for each group, a power calculation, based on our pilot data (see Appendix 1), was performed as follows:

$$n = 2\ (\ PI * \sigma\ /\ \mu_1 - \mu_2\ )^2$$

$$= 2\ (\ 2.80 * 0.65\ )^2$$

$$= 6.62$$

where

PI is the Power Index (based on α (two-tailed): 0.05, and β (one-tailed): 0.20) and

$\sigma / \mu_1 - \mu_2$ is the inverse of effect size as estimated from our pilot data.

Based on this calculation, we would require 7 animals per group; however, as we will be extracting individual lumbar motoneurons (a homogeneous tissue sample) in the present study, our findings should be less variable than our pilot data (which sampled Lamina IX,

a heterogeneous tissue sample). Based on this assumption, we selected a sample size of 6 animals per group.

Exercise Training Protocol

Treadmill training was performed with an Exer-3/6 Treadmill (Columbus Instruments) with two clear dividers in place (rat configuration). A towel was placed over the treadmill cover to create a dark running environment for the animals. Following the 1 week adjustment period, animals in the treadmill exercise group were acclimated to treadmill running for 3 days immediately prior to the 7-day treadmill exercise protocol. In order to acclimatize the animals to treadmill running, loud tapping noises (conditioned stimulus) coupled with brief pulses of foot shocks (unconditioned stimulus) were employed during the 3 day period whenever animals ceased running and rested on the shock grid. During the subsequent 7-day treadmill exercise protocol, only loud tapping noises were used to prompt animals to continue if they stopped running. The exercise protocol was modified from a 5-day treadmill exercise protocol previously used in the Gardiner Laboratory and can be found in the table below. Animals in the sedentary control group were brought into the exercise room and handled similarly to the treadmill exercise group, but were not placed on the treadmill.

Although the one-week training period is brief compared to many protocols used to investigate nervous system adaptations to physical activity (for example, Beaumont & Gardiner, 2002; Beaumont & Gardiner, 2003; Chennaoui et al., 2001; Wienecke, Ploug & Zhang, 2009), several studies have utilized shorter exercise periods, ranging from 3 to 7 days, and observed changes in gene expression (Gomez-Pinilla et al., 2001; Gomez-Pinilla et al., 2002; Greenwood et al., 2005; Molteni, Zheng, Ying, Gomez-Pinilla &

Twiss, 2004; Ying et al., 2003). In fact, 5-$HT_{1B}R$ mRNA levels in rat ventral dorsal raphe nuclei were reduced after 3 days of voluntary wheel running (Greenwood et al., 2005).

7-Day Treadmill Exercise Protocol			
Day	Speed (m/min.)	Incline (°)	Duration (min.)
Acclimation 1	10.85	0	10
Acclimation 2	17.30	0	15
Acclimation 3	22.08	0	15
1	15.61	0	15
2	15.61	5	15
3	17.30	5	20
4	19.40	10	20
5	20.65	10	30
6	22.08	10	30
7	22.86	10	30

Table 1. Overview of 7-Day Treadmill Exercise Protocol

Tissue Removal

Animals were sacrificed with a combination of isofluorane anaesthesia and decapitation, alternating between sedentary control and treadmill exercise animals. Treadmill-exercised animals were sacrificed approximately 4 hours after the final training session. Following decapitation, a tissue block containing segments 3 (L3) to 5 (L5) of the lumbar spinal cord was immediately removed, placed in a cryomold, covered in Tissue-Tech O.C.T. embedding compound (Gene Research Lab) and fresh-frozen in melting isopentane on liquid nitrogen; these segments were selected as motoneurons innervating both upper- and lower-hindlimb musculature are found in L3 to L5 and these muscles are vigorously activated with treadmill running (Nicolopoulos-Stournaras & Iles, 1983). The L3-L5 tissue block was then wrapped in aluminium foil and stored at -80°C for future use. Prior to sectioning, it was placed in a cryostat and allowed to warm to approximately -20°C. The L3-L5 tissue block was cross-sectioned at 10 μm and slide-

mounted on polytetrafluoroethylene (PTFE) -coated glass slides. Prepared slides were either used immediately or stored at -80°C for up to 3 days. Soleus and plantaris muscles were dissected bilaterally on ice-cold saline, blotted dry, weighed, placed in cryogenic vials and stored at -80°C for future use.

The post-exercise sacrifice timeline was selected as multiple studies documented the greatest changes in mRNA levels (in a variety of tissue types) between 2 and 6 hours after exercise (Jemiolo & Trappe, 2004; Kawai et al., 2007; Nedergaard, Vissing, Overgaard, Kjaer & Schjerling, 2007). Additionally, expression of c-Fos, c-Jun, and zif/268 (immediate-early genes and transcription factors), peroxisome proliferator activated receptor co-activator 1α (PGC-1α, a transcription factor implicated in mitochondrial biogenesis) and BDNF (a potential signal for altered gene expression in motoneurons) are increased at varying times from 30 minutes to 2 hours after exercise (Cullinan, Herman, Battaglia, Akil & Watson, 1994; Gardiner, 2006; Gomez-Pinilla et al., 2001; Pilegaard, Saltin & Neufer, 2003; Timofeeva, Huang, & Richard, 2003); an upregulation of these genes may provide a signal for changes in gene expression and if so, would likely precede any exercise-induced changes in transcription. Therefore, based on this evidence, treadmill-exercised animals were sacrificed approximately 4 hours after the final training session.

Laser Capture Microdissection

Prepared slides were immersed in acetone (pre-chilled to -20°C) for 2 minutes and then fixed and stained according to the LCM Staining Protocol (LCM Staining Kit, Ambion). Briefly, tissue was fixed with decreasing concentrations of ethanol, stained with cresyl violet, washed with increasing concentrations of ethanol, cleared with xylene

and dried with a vacuum desiccator – all at room temperature. Fixed and stained slides were not removed from xylene or dried until just prior to laser capture microdissection. Motoneurons were dissected individually, from the ventral horn of lumbar spinal cord sections, with the Arcturus PixCell II (Molecular Devices) laser capture microdissection system. Once slides were removed from the vacuum desiccator, motoneurons were collected for up to 30 minutes, after which the slide was discarded. After a collecting cap was filled with motoneurons, the adhesive of a sticky note was placed on the collecting surface of the cap to remove any pieces of tissue adhered to the thermofilm by static electricity, preventing contamination of the sample. The collecting cap was then placed on a 200 µl PCR-clean microcentrifuge tube pre-filled with 100 µl of lysis solution (RNAqueous-Micro Kit, Ambion), inverted and incubated at 42°C for 30 minutes. Approximately 700 motoneurons were collected from each animal.

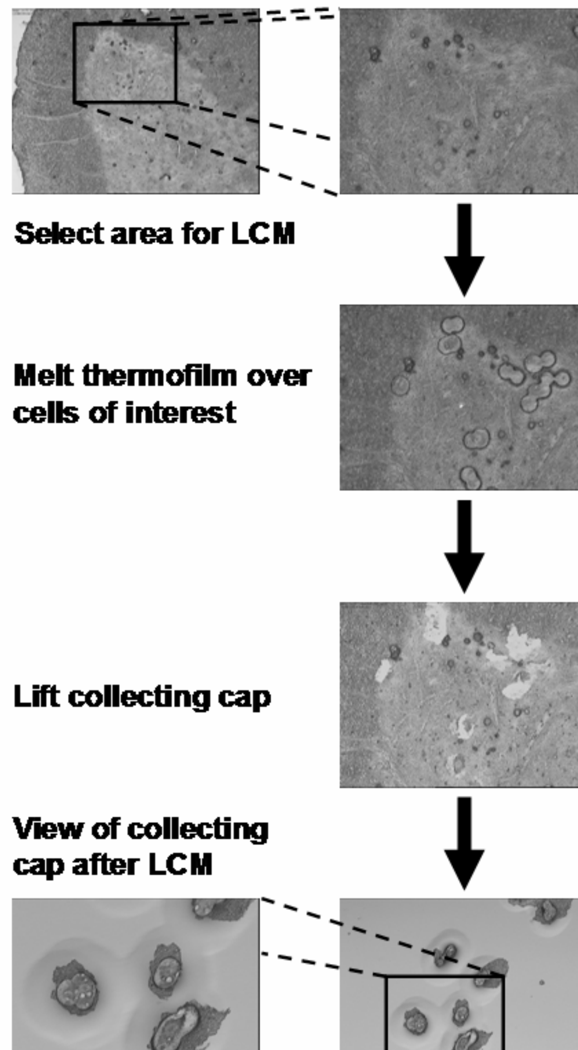

Figure 1. Overview of laser capture microdissection

Although the goal was to collect strictly alpha motoneurons, we cannot definitively state that was the case. Due to the limitations of the PixCell II LCM system (see limitations section below), we were unable to label or size motoneurons – therefore, "presumed alpha motoneurons" were collected based on their size (which was assessed visually) and location. From this point forward, when referring to the results of this study, "presumed alpha motoneurons" will simply be termed as "motoneurons".

RNA Isolation

RNA was isolated from LCM samples with the RNAqueous-Micro Kit (Ambion), using the protocol for LCM. Briefly, RNA from the LCM samples was trapped on a silica filter, with only large RNA species being eluted and recovered from the filter (20 µl of elution solution was used to pass RNA through the filter). The optional deoxyribonuclease (DNase) I Treatment and DNase Inactivation steps in this protocol were performed to remove any trace amounts of genomic DNA prior to amplification. The RNA concentration, protein contamination and RNA integrity of each sample was assessed with a 2100 Bioanalyzer, 2100 Bioanalyzer RNA Pico 6000 kit and 2100 Expert Software (Agilent Technologies).

Real-Time Reverse Transcription Polymerase Chain Reactions

Reverse transcription was performed with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). The volume of RNA used for each reaction varied by sample – 14 µl of the least concentrated sample was used, with the volume of other samples being adjusted to ensure the same quantity of RNA (pg) was utilized in each reaction. Nuclease-free water was added to RNA volumes less than 14 µl to a total volume of 14 µl. A Master Cycler Gradient thermal cycler (Eppendorf) was used to incubate reverse transcription

reactions. Due to the limited quantity of complementary DNA (cDNA) produced from samples, synthesized cDNA was preamplified with the TaqMan PreAmp Master Mix Kit (Applied Biosystems), prior to real-time polymerase chain reactions (qPCR). The 14 preamplification cycle protocol was followed, with pooled and diluted TaqMan Gene Expression Assays (Applied Biosystems) for 5-$HT_{1A}R$, 5-$HT_{2A}R$, 5-$HT_{2C}R$ and succinate dehydrogenase complex subunit A (SDHA) being used. The Master Cycler Gradient was also used to incubate preamplification reactions. qPCR reactions were set-up (in triplicate) in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems) as follows: 1.25 µl TaqMan Gene Expression Assay, 6.25 µl nuclease-free water, 12.5 µl TaqMan Gene Expression Master Mix (Applied Biosystems) and 5 µl preamplified cDNA. 5-$HT_{1A}R$, 5-$HT_{2A}R$, 5-$HT_{2C}R$ (genes of interest) and SDHA (reference gene) Gene Expression Assays were used. Pooled whole lumbar spinal cord cDNA was loaded on all plates to act as a calibrator sample, allowing data comparison between multiple plates. Additionally, a water lane was loaded on all plates to ensure contamination of plates or reagents was not present. Reactions were run in the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) for 40 cycles under standard settings.

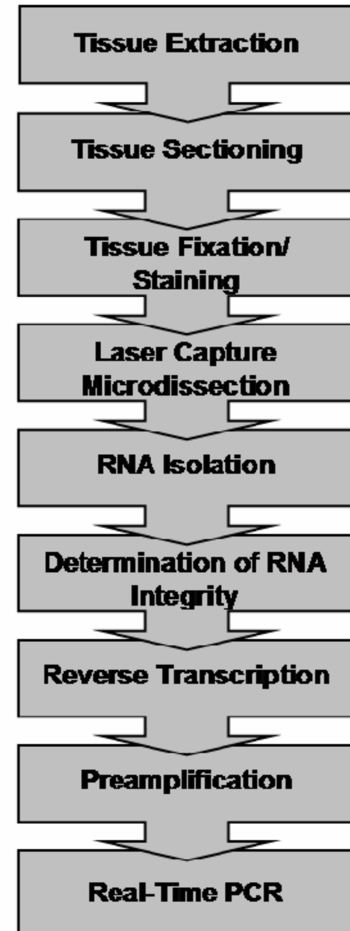

Figure 2. Overview of real-time reverse transcription polymerase chain reaction experiments

Verification of Training Adaptations

To confirm that the 7-day treadmill exercise protocol provided an adequate stimulus for training adaptations, soleus and plantaris glucose transporter type 4 (GLUT4) content was examined. These muscles have been previously selected to study biochemical adaptations to exercise training as they represent two distinct muscle phenotypes (predominantly slow- and fast-twitch, respectively) and respond to treadmill training (see Betik, Thomas, Wright, Riel & Hepple, 2009). Additionally, human skeletal muscle GLUT4 protein has been shown to increase after two bouts of intermittent exercise and therefore provides a good indicator of the training status of these animals (Green et al., 2008). Right soleus and plantaris muscles were removed from storage at -80°C and ground with a mortar and pestle over liquid nitrogen. Approximately 5 to 6 mg of ground muscle was homogenized on ice in a radioimmunoprecipitation buffer with a PowerGen 125 homgenizer (Fisher Scientific). Samples were centrifuged at 13 000 revolutions per minute for 30 minutes at 4°C and the supernatant, representing total protein, was collected. The concentration of each sample was determined with the Bio-Rad Protein Assay (Bio-Rad) and Ultrospec 2100 Pro UV/Visible spectrophotometer (Biochrom) set to 595 nm. Fifty μg of protein from each sample was loaded in a 15-well 1 mm thick 10% polyacrylamide gel and run for 45 to 60 minutes at 200 V in the Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad). Protein was transferred to a nitrocellulose membrane for 60 minutes at 100 V in the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Following protein transfer, the membrane was blocked in tris-buffered saline and Tween 20 (TBST)/5% milk for 1 hour and incubated in rabbit GLUT4 (H-61) antibody (Santa Cruz) (diluted 1:500 in TBST/5% milk) overnight at 4°C. The

membrane was then washed in TBST, incubated in goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz) (diluted 1:2500 in TBST/5% milk) for 1 hour at room temperature, washed in TBST and incubated in SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 minute. Chemiluminescent detection of GLUT4 antibody binding was performed with a Fluor-S MultiImager (Bio-Rad) and exposed images were analyzed with Quantity One Basic software version 4.6.5 (Bio-Rad). To ensure equal protein loading across lanes, the membrane was stripped in Restore Western Blot Stripping Buffer (Thermo Scientific) for 20 minutes, washed in TBST, reblocked in TBST/5% milk for 1 hour and incubated in mouse β-actin antibody (Sigma) (diluted 1:7500 in TBST/5% milk) for 2 hours at room temperature. The membrane was then washed in TBST, incubated in goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz) (diluted 1:2500 in TBST/5% milk) for 1 hour at room temperature, washed in TBST and incubated in SuperSignal West Pico Chemiluminescent Substrate for 1 minute. Exposed images of β-actin antibody binding from the Fluor-S MultiImager were analyzed with Quantity One Basic software. The membrane was stored in tris-buffered saline (TBS) at 4°C for future use.

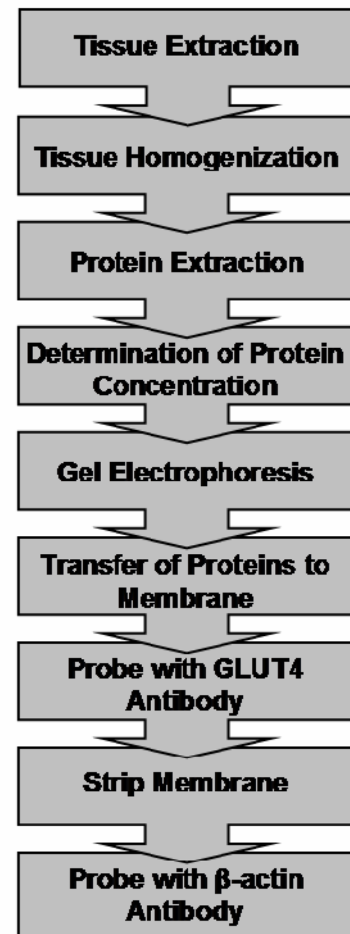

Figure 3. Overview of experiment to verify training adaptations

Data Analysis

The results of qPCR experiments were expressed in relative quantification (RQ) values. These values were calculated by 7500 Software version 2.0 (Applied Biosystems) and represent "the quantity of target, normalized to an endogenous control and relative to a reference sample" (Applied Biosystems, 2008, p. A-7), with 5-$HT_{1A}R$, 5-$HT_{2A}R$ and 5-$HT_{2C}R$ being the targets, SDHA being the endogenous control and preamplified pooled whole lumbar spinal cord cDNA being the reference sample. It was determined as follows:

$$2^{-\Delta\Delta C_q}$$

where

$$\Delta\Delta C_q = (\overline{\text{target } C_q} - \overline{\text{endogenous control } C_q}) - (\overline{\text{target } C_{q\text{ reference sample}}} - \overline{\text{endogenous control } C_{q\text{ reference sample}}})$$

and C_q is quantification cycle of a qPCR sample (Livak & Schmittgen, 2001). Preamplified pooled whole lumbar spinal cord cDNA served as the calibrator tissue for all plates, allowing comparison of data from multiple qPCR plates. Two-tailed independent Student's t-tests were used to compare RQ values between sedentary control and treadmill exercise groups for each gene of interest. The p-value was set at <0.05 and an approximate false discovery rate adjustment was used as multiple comparisons were made; we examined an additional 21 genes separate from those included in this project and therefore, required a less conservative adjustment to our p-value as we were analyzing data (from the same samples) for 24 genes total. The Approximate False Discovery Rate Adjustment (Hassard, 2010) with $p<0.05$ was calculated as follows:

$$\frac{0.05*(t+1)}{2t}$$

where t is the number of tests conducted.

Results

5-HT Receptor mRNA Levels

Figures 5, 6 and 7 illustrate the results of the qPCR experiments examining 5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2C}R mRNA levels in sedentary control and treadmill exercise groups. No significant differences in mRNA levels between groups were found for any of the 5-HTRs examined. (5-HT_{1A}R: p=0.520, 5-HT_{2A}R: p=0.303 and 5-HT_{2C}R: p=0.081)

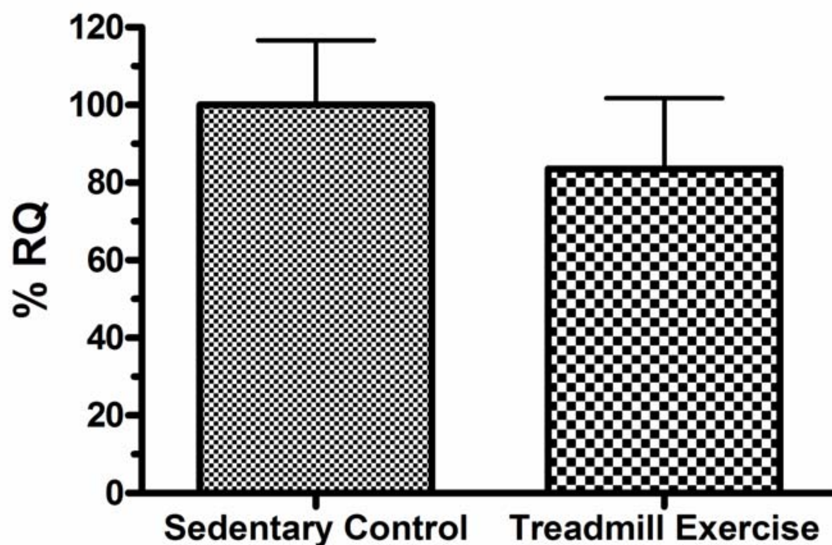

Figure 4. Effect of exercise on 5-HT_{1A}R mRNA levels in lumbar motoneurons
Lumbar motoneuron (L3-L5) mRNA levels presented in percent relative quantification (% RQ). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.026$

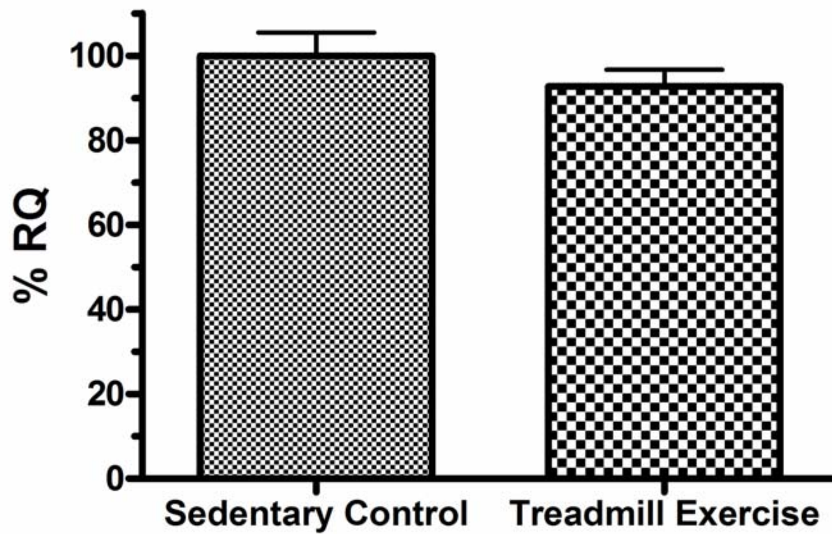

Figure 5. Effect of exercise on 5-HT_{2A}R mRNA levels in lumbar motoneurons
Lumbar motoneuron (L3-L5) mRNA levels presented in percent relative quantification (% RQ). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.026$

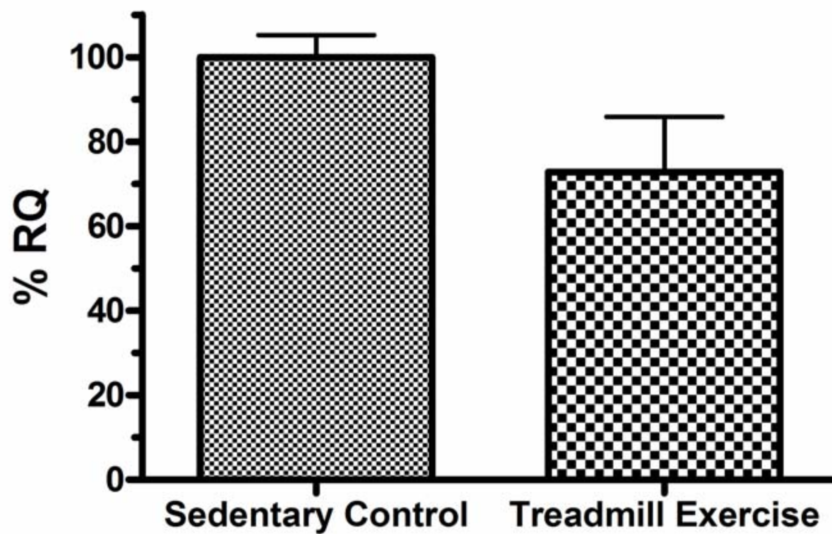

Figure 6. Effect of exercise on 5-HT_{2C}R mRNA levels in lumbar motoneurons
Lumbar motoneuron (L3-L5) mRNA levels presented in percent relative quantification (% RQ). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.026$

GLUT4

Figures 8 and 9 illustrate the results of immunoblot experiments examining soleus muscle and plantaris muscle total protein GLUT4 content in sedentary control and treadmill exercise groups. No significant differences in GLUT4 content between groups were found for either soleus or plantaris muscles. (soleus: p=0.116 and plantaris: p=0.097)

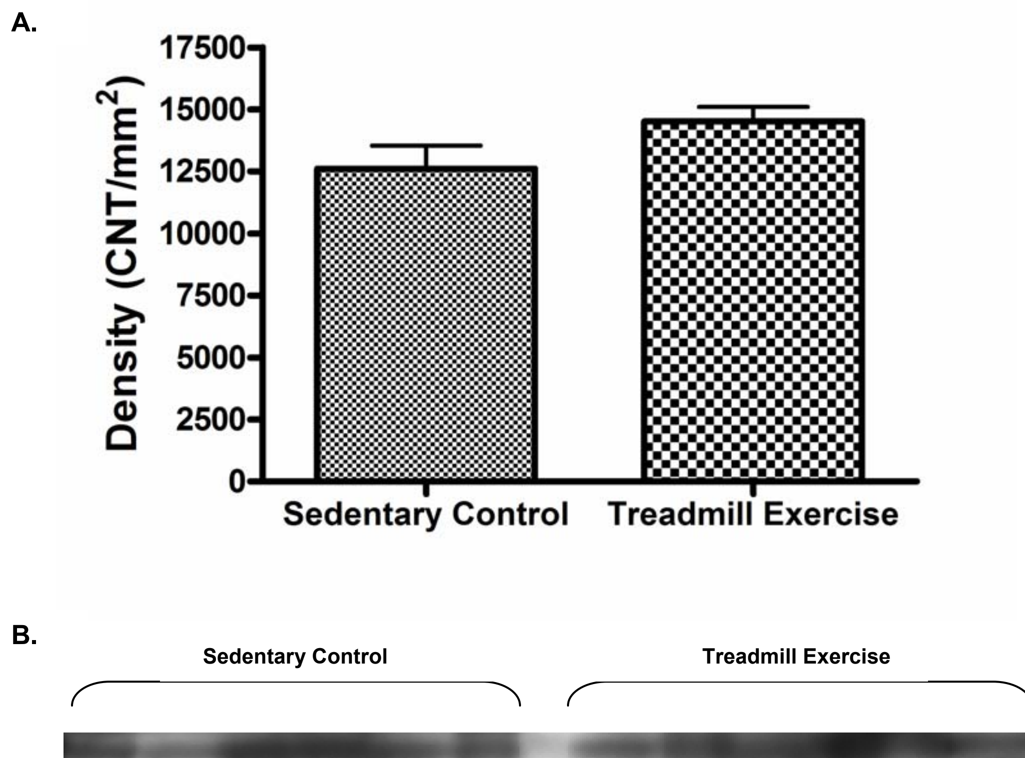

Figure 7. Effect of exercise on GLUT4 content in soleus muscle
A. Soleus GLUT4 protein content presented in protein density (CNT/mm^2). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.05$
B. Immunoblot showing GLUT4 content in soleus muscle total protein for Sedentary Control and Treadmill Exercise groups.

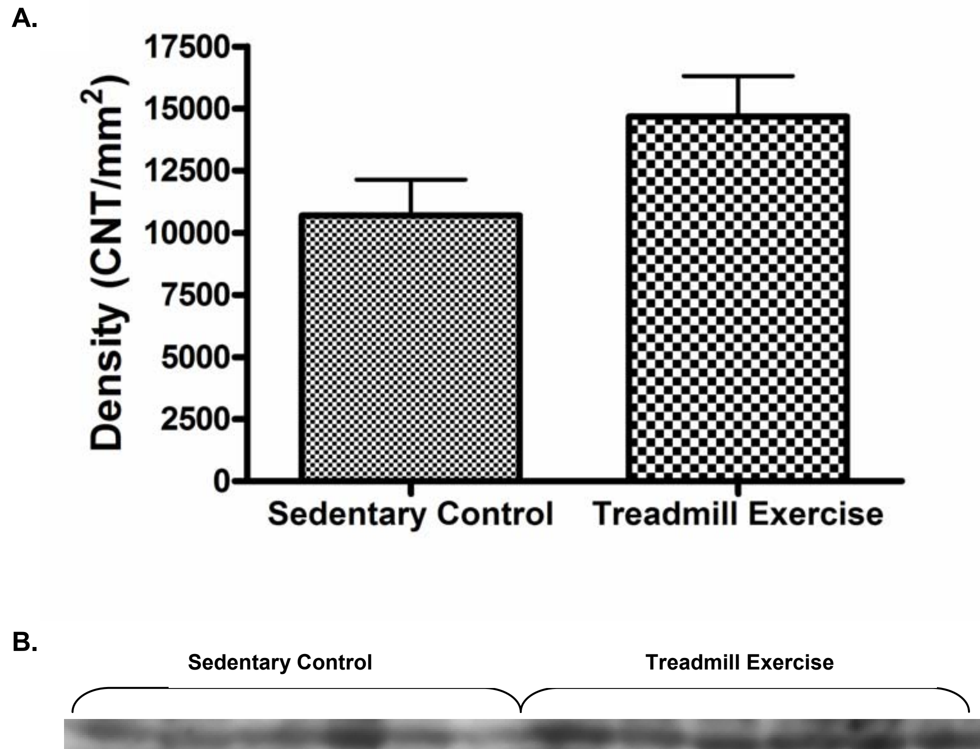

Figure 8. Effect of exercise on GLUT4 content in plantaris muscle
A. Plantaris GLUT4 protein content presented in protein density (CNT/mm^2). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.05$
B. Immunoblot showing GLUT4 content in plantaris muscle total protein for Sedentary Control and Treadmill Exercise groups.

Discussion

Training Stimulus and Sacrifice Timeline

Was the training stimulus sufficient to elicit changes in muscle gene expression?

The treadmill exercise protocol utilized in this study was not sufficient to elicit changes in soleus muscle or plantaris muscle total protein GLUT4 content. Although exercise training has been consistently shown to increase skeletal muscle GLUT4 content (see Holmes & Dohm, 2004), it has also been demonstrated that these increases occur several hours after an exercise session (Kuo, Browning & Ivy, 1999; Ren, Semenkovich, Gulve, Gao & Holloszy, 1994). It seems likely that our 4 hour post-exercise sacrifice time was too soon after exercise training to allow GLUT4 protein to be upregulated in the soleus and plantaris and therefore, we observed no significant differences between sedentary control and treadmill exercise groups.

How does the current protocol compare to other training protocols?

Multiple studies have examined the effect of short-term exercise training on mRNA expression in the lumbar spinal cord. Gomez-Pinilla and colleagues (2001), utilized a similar protocol to the present study, treadmill training rats for either 1 or 5 days at 27 m/minute with a 3% incline for 30 minutes each session. Although not identical, both protocols vigorously activate hindlimb musculature and likely require 80% or more of the maximal oxygen uptake of the animals (interpolated from Bedford, Tipton, Wilson, Opplinger & Gisolfi, 1979). Despite its short duration, the Gomez-Pinilla protocol was sufficient to elicit changes in BDNF and NT-3 mRNA levels in the lumbar spinal cord after both 1 and 5 days of treadmill training (Gomez-Pinilla et al., 2001).

Similar studies have also employed voluntary wheel training to examine changes in spinal cord gene expression (Gomez-Pinilla et al., 2002; Ying et al., 2003). Increased BDNF, NT-3, growth associated protein 43, synapsin I, TrkB and TrkC mRNA levels in the lumbar spinal cord were observed following either 3 and/or 7 days of voluntary wheel running (Gomez-Pinilla et al., 2002; Ying et al., 2003). Additionally, a positive relationship between running volume and mRNA levels was observed for both BDNF and Synapsin I (Gomez-Pinilla et al., 2002). While both short-term training paradigms have been shown to elicit changes in lumbar spinal cord mRNA levels, each training mode provides a different stimulus for adaptation; voluntary wheel training emphasizes exercise volume and treadmill training emphasizes exercise intensity. Although it is unclear whether utilizing a voluntary wheel training protocol would have affected the outcome of this study, some motoneuron physiological properties are known to be more sensitive to one training mode over the other (Gardiner et al., 2006). It is possible however, that neither protocol is appropriate for inducing changes in motoneuron 5-HTR mRNA levels as 5-HT_{1A}R mRNA levels in peripheral blood cells were only altered after exhaustive exercise, but not aerobic exercise (Kawai et al., 2007).

Was the sacrifice time after exercise appropriate?

As discussed previously, the post-exercise sacrifice timeline was selected as multiple studies found optimal changes in mRNA levels between 2 and 6 hours after exercise (Jemiolo & Trappe, 2004; Kawai et al., 2007; Nedergaard, Vissing, Overgaard, Kjaer & Schjerling, 2007). However, as these studies did not examine motoneurons (or any kind of nervous tissue), 4 hours post-exercise may not be the optimal sacrifice time to observe robust changes in motoneuron mRNA levels. The timeline selected may also not

be suitable to observe changes in 5-HTR mRNA levels as 5-HT_{1A}R mRNA levels in peripheral blood cells were altered 24 hours post-exercise, but not 4 hours post-exercise (Kawai et al., 2007).

Although it seems likely that potential signals for altered gene expression in motoneurons are "turned on" at approximately 30 minutes to 2 hours after exercise (Cullinan et al., 1994; Gardiner, 2006; Gomez-Pinilla et al., 2001; Pilegaard et al., 2003; Timofeeva et al., 2003), it is possible that by waiting until 4 hours post-exercise, we missed the downstream effects of the signals – either collecting samples before or after the peak of altered transcription. It is apparent though, that the RNA integrity of our samples remained largely intact for the duration of the experiment and therefore, did not obscure our findings. Evidently, a time course study is needed to determine the optimal time after exercise at which to examine changes in 5-HTR mRNA levels in motoneurons. The variability of the animals studied should also be considered as it is likely that the ideal post-exercise sacrifice time will not be uniform for all animals.

Agreement With Previous Studies

How can we account for differences between thesis and pilot data?

Although the findings of this study do not completely agree with our pilot data – where Lamina IX 5-HT_{2C}R mRNA levels were higher in exercised animals – there are several possible explanations for these differences. Firstly, the spinal cord segments that were sampled vary between the current study and pilot study; pilot experiments sampled tissue from lumbar segment 6/sacral segment 1 (L6/S1), representing different pools of motoneurons than presently examined (L3-L5). It is possible that different types of motoneurons express a discrete pattern of neurotransmitter receptors/ion channels

specialized to their physiological role and that motoneurons in L6/S1 rely on altered expression of 5-HT_{2C}Rs to modify their excitability, while motoneurons in L3-L5 do not.

It is also possible that the increased 5-HT_{2C}R mRNA levels observed after exercise training (in the pilot study) may be attributed to glia rather than motoneurons. As discussed previously, glial cells also express 5-HTRs and because we sampled Lamina IX in the pilot study (as opposed to individual motoneurons) the changes that we observed in this study may be largely due to glia. In fact, the BDNF immunoreactivity in Lamina IX astrocytes was more intense following 5 days of treadmill exercise, demonstrating the capacity of glia to alter their gene expression in response to exercise training (Gomez-Pinilla et al., 2001).

Finally, questionable tissue quality and RNA integrity, and unsuitable equipment for measuring RNA concentrations in small samples reduced the amount of control at various stages of the pilot experiment. As this aspect of real-time reverse transcription polymerase chain reaction (qRT-PCR) experiments is critically important, it is possible that the pilot data are completely invalid and the increased 5-HT_{2C}R mRNA levels observed in the exercised animals are not genuine.

How do these results compare to similar studies?

While no previous studies have examined the effect of exercise training on 5-HTR mRNA levels in the spinal cord, several studies have investigated how increased physical activity alters 5-HTR mRNA expression in various brain regions. 5-HT_{1A}R mRNA expression was increased in dorsal and median raphe nuclei after 6 weeks of voluntary wheel running, but not after 3 days or 3 weeks of training (Greenwood et al., 2005). On the other hand, 5-HT_{1B}R mRNA expression was decreased in the dorsal raphe nuclei after

3 days of voluntary wheel running and remained reduced after both 3 and 6 weeks of training (Greenwood et al., 2005). This suggests that 5-HTRs respond to exercise at varying time points, with some subtypes, such as 5-HT_{1A}Rs, requiring a longer period of time to respond to exercise training than others. Additionally, 5-HT_{2C}R mRNA expression was altered in multiple brain regions after 6 weeks of voluntary wheel running (Loughridge et al., 2009). Considering these findings, it is possible that our training protocol was not long enough to observe changes in 5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2C}R mRNA levels in motoneurons.

Chennaoui and colleagues (2001), observed decreased 5-HT_{1B}R mRNA expression after 7 weeks of treadmill training in select brain regions; no changes were found in the striatum or hippocampus, but 5-HT_{1B}R mRNA expression was reduced in the cerebellum (with intense treadmill training) and frontal cortex (with moderate and intense treadmill training) (Chennaoui et al., 2001). These results suggest that endurance exercise may alter 5-HTR expression differently in distinct components of the central nervous system, with expression of some 5-HTRs being more stable in certain areas (Chennaoui et al., 2001). Additionally, 5-HT_{1B}Rs in the substantia nigra were desensitized after both 5 and 7 weeks of treadmill exercise (Chennaoui et al., 2000; Seguin, Liscia, Guezennec & Fillion, 1998); similar levels of desensitization were observed in moderately and intensely trained rats after 5 weeks of training, but greater desensitization was observed in intensely trained rats (compared to moderately trained rats) after 7 weeks of training (Chennaoui et al., 2000; Seguin et al., 1998). These observations may indicate that endurance exercise modifies serotonergic neuromodulation in multiple ways – first, by altering 5-HTR expression at the

transcriptional level and second, by altering 5-HTR sensitivity at the post-transcriptional level (Chennaoui et al., 2001). As expression of 5-HTR mRNA and protein (Wienecke, Ploug & Zhang, 2009) in motoneurons appears to be unchanged with exercise, modified 5-HTR sensitivity could potentially provide an alternative mechanism for altered motoneuron excitability in the absence of changes in 5-HTR expression. It should be noted however, that Wienecke and colleagues (2009), utilized quantitative immunohistochemistry and did not differentiate between motoneuron types (see below) in their study, limiting the conclusions that can be made from this work. Therefore, changes in 5-HTR protein expression may still be a viable mechanism for altering serotonergic modulation of motoneurons in response to exercise training.

Motoneuron Types and Intensity of Involvement in Exercise

Are we losing the effect of exercise by pooling all motoneurons?

Although it is unclear if expression of 5-HTRs varies by motoneuron type (for example, flexor motoneurons versus extensor motoneurons, fast motoneurons versus slow motoneurons, etc.), there appears to be some evidence suggesting that this may be the case. As mentioned previously, Fonseca and colleagues (2001) found little to no co-localization of 5-HT$_{2A}$R mRNA and 5-HT$_{2C}$R mRNA within Lamina IX at all spinal cord levels, perhaps indicating that there are two functionally distinct populations of motoneurons within this region, with each population requiring different modulation by 5-HT for optimal motor output. Basura et al. (2001), observed expression of 5-HT$_{2A}$R mRNA and 5-HT$_{2C}$R mRNA in cervical motoneurons, however only 5-HT$_{2A}$R mRNA was expressed above background levels in phrenic motoneurons. It is unclear if 5-HT$_{2A}$R mRNA and 5-HT$_{2C}$R mRNA were co-localized on cervical motoneurons, but these

findings suggest that 5-HTR expression is not consistent for all motoneurons and that function is an important determinant of 5-HTR expression in motoneurons. Vult von Steyern & Lomo (2005), reported a greater percentage of soleus motoneurons expressing 5-HT_{2A}R protein as compared to extensor digitorum longus motoneurons. As these muscles represent slow extensor and fast flexor muscles, respectively, it seems logical that function, firing rate and/or total daily activity levels may influence expression of 5-HTRs (Vult von Steyern & Lomo, 2005). Finally, Slawinska (2010) observed rostral-caudal gradients for both 5-HT_{2A}R protein and 5-HT_{2C}R protein in the lumbar spinal cord; 5-HT_{2A}R protein expression was greater rostrally, while 5-HT_{2C}R protein expression was greater caudally (Slawinska, 2010). As the populations of motoneurons found at each lumbar segment are different (Nicolopoulos-Stournaras & Iles, 1983), these findings appear to lend themselves to the idea that 5-HTR expression may not be uniform for all motoneurons.

While function, firing rate and/or total daily activity level appear to be critical to the expression of 5-HTRs in motoneurons, sensory input also seems to be an important factor (Otoshi et al., 2009). As these characteristics vary by motoneuron type, it would seem reasonable to assume that the expression of 5-HTRs in motoneurons is not consistent across all types. Although an effort was made to keep samples as consistent as possible from animal to animal (for example, sampling from approximately the same lumbar segments, sampling from approximately the same number of sections, collecting approximately the same number of motoneurons for each animal, etc.) it was impossible to collect identical samples from each animal – especially because we did not use any labelling to ensure we collected a specific type or types of motoneurons. As well, the

inherent variability between animals makes it extremely difficult to collect similar samples; the number of motoneurons innervating specific muscles, the distribution of motoneuron firing frequencies, the segments in which certain motoneurons are found and the training response of motoneurons would likely be somewhat variable from animal to animal. These variables are perhaps sufficient enough to affect the results of this study such that we observed no change in 5-HT_{1A}R, 5-HT_{2A}R or 5-HT_{2C}R mRNA levels. Additionally, by pooling all motoneurons in this experiment, without regard for type, it is possible that we negated any significant exercise effects.

Other Adaptations

What else could account for changes in motoneuron excitability with exercise training?

In addition to 5-HTRs, motoneurons are known to express a variety of ion channels and neurotransmitter receptors (Alilain & Goshgarian, 2008; Charles et al., 2001; Day, Campeau, Watson, Jr. & Akil, 1997; Dedek et al., 2001; Frazao, Nogueira & Wassle, 2007; Doly, Fischer, Brisorgueil, Verge & Conrath, 2005; Khristy et al., 2009; Gabriel, Abdallah, Yost, Winegar & Kindler, 2002; Ma et al., 2006; Matus-Leibovitch et al., 1996; Muennich & Fyffe, 2003; Petri et al., 2005; Reyes et al., 2000; Stocker & Pedarzani, 2000; Tolle, Berthele, Zieglgansberger, Seeburg & Wisden, 1993; Veh et al., 1995; Welton, Stewart, Kerr & Maxwell, 1999; Zhang et al., 2006). In a parallel study, we examined an additional 21 genes in the same tissue samples collected for this project. As chronic physical activity is known to increase motoneuron excitability, the goal of this parallel study was to gain a greater understanding of the changes in ion channel subunit and/or neurotransmitter receptor gene expression that may contribute to these

adaptations. Potassium channel subunit $K_v1.1$ (↓ 21.49% ± 3.40%, $p < 0.026$), sodium channel subunits $Na_v\beta1$ (↓ 17.62% ± 4.47%, $p < 0.026$) and $Na_v\beta4$ (↓ 11.49% ± 2.85%, $p < 0.026$), and GABA receptor subtype $GABA_{B1}$ (↓ 14.59% ± 3.78%, $p < 0.026$) mRNA levels were significantly lower in the lumbar motoneurons of treadmill-exercised animals compared to sedentary controls. These findings suggest that exercise training may enhance motoneuron excitability through a decreased potassium conductance (both directly and indirectly) and altered voltage-gated sodium channel kinetics (Aman et al., 2009; Charles et al., 2001; Gardiner et al., 2006; Isom, De Jongh & Catterall, 1994). For a complete list of the genes examined and results, see Appendices 2 and 3.

Limitations

The greatest limitation of this study was the Arcturus PixCell II LCM system; specifically, the system did not allow for definitive identification of alpha motoneurons. As the PixCell II is not capable of measuring the diameter of cells, presumed alpha motoneurons were collected based on their size (which was assessed visually) and location. Although it would have been possible to use a screen-mounted ruler scaled to the microscope magnification to measure motoneuron diameters, this would have been impractical due to the strict 30 minute collection time limit and large number of alpha motoneurons to be collected from each animal. Additionally, we were unable to fluorescently label motoneurons as the PixCell II is not equipped for LCM from still images. Therefore, we would have been forced to expose slides to fluorescence multiple times, destroying tissue and likely jeopardizing RNA integrity. Due to these restrictions, we cannot definitively state that the tissue samples collected were alpha motoneurons or even motoneurons, but based on the size of cells collected and their location, we suggest

that our samples represent "presumed alpha motoneurons". Despite these caveats, the Arcturus PixCell II LCM system is far superior to the Leica AS LMD LCM system, which was used for the pilot component of this project. Although the AS LMD allows fluorescent labelling and sizing of motoneurons, it is not possible to remove individual motoneurons without removing substantial amounts of surrounding tissue or threatening RNA integrity due to the radiation emitted by the ultraviolet laser. While being able to definitively identify alpha motoneurons would have been ideal, poor RNA integrity and large quantities of excess tissue relative to the alpha motoneurons collected would have confounded our results and been more detrimental to this study.

Also absent from this study was the determination of 5-HTR expression in distinct types of motoneurons. While it is unclear whether the expression of 5-HTRs varies by type of motoneuron (for example, flexor motoneurons versus extensor motoneurons, fast motoneurons versus slow motoneurons, etc.), we may have failed to observe any significant differences in 5-HTR mRNA levels between exercised and sedentary animals because all motoneurons collected, regardless of type (although no differentiation was made), were pooled together. Conducting a study examining distinct populations of motoneurons, while possible, would present many challenges: first, an LCM system capable of fluorescence and collecting individual motoneurons with minimal tissue damage would be necessary; second, tissue samples may be too small or require pooling from multiple animals as only certain alpha motoneurons (not all alpha motoneurons from L3-L5, for example) would be collected, effectively limiting the number of motoneurons available for sampling. Despite these issues, automated LCM systems and

optimized qPCR techniques would likely allow a study like this to be performed in the future.

Additionally, no measure of 5-HTR protein expression or sensitivity was conducted as part of this study. Although it appears that exercise does not affect 5-HTR expression in motoneurons at the transcriptional level (at least, in a short-term treadmill training regime), it remains unclear as to whether exercise alters motoneuron 5-HTR regulation at some post-transcriptional level.

Finally, our study lacked sufficient statistical power to detect any real differences (if present) in lumbar motoneuron 5-HT_{2C}R mRNA levels between sedentary control and treadmill exercise groups ($p=0.081$). Based on data from the present study, the appropriate sample size for each group would be as follows:

$$n = 2 (PI * \sigma / \mu_1 - \mu_2)^2$$

$$= 2 (2.80 * 0.82)^2$$

$$= 10.54$$

where

PI is the Power Index (based on α (two-tailed): 0.05, and β (one-tailed): 0.20) and

$\sigma / \mu_1 - \mu_2$ is the inverse of effect size as determined from the present study.

Based on this calculation, we would require 11 animals per group to detect any real differences between groups.

Conclusions

Although this is the first study to examine the effect of exercise training on 5-HTR mRNA levels in motoneurons, no significant differences between treadmill exercise and sedentary control groups were found. While it remains unclear if the expression of 5-HTR mRNAs is altered by exercise, there are several variables that may have contributed to the findings of this study: training program length, training mode, post-exercise sacrifice time, lack of differentiation of motoneurons, regulation of 5-HTRs post-transcriptionally and altered expression of other genes affecting motoneuron excitability. Of these considerations, the lack of differentiation of motoneurons seems to be the most critical to answering this question; by separating different types of motoneurons we will likely get a better sense of whether 5-HTR mRNAs are altered by exercise.

Future Research

Moving forward, there are several additional studies that could be conducted to complement this work. As this project examined changes in motoneuron 5-HTR mRNA levels in response to short-term exercise training, it would be logical to see the effect of a long-term (and more intense) exercise protocol on motoneuron 5-HTR mRNA levels. This would represent the chronic and more stable response of motoneurons to exercise and would provide greater insight into the response of 5-HTRs to increased physical activity. It would also be interesting to determine if the sensitivity of 5-HTRs in the spinal cord is altered by exercise as it is unknown at what level (if at all) exercise regulates these receptors.

As well, this project investigated changes in 5-HTR mRNA levels in lumbar motoneurons without regard for motoneuron type (for example, flexor motoneurons versus extensor motoneurons, fast motoneurons versus slow motoneurons, etc.). It is unknown if the expression of 5-HTR subtypes is varied amongst different types of motoneurons and therefore, should be studied. If a difference in the expression of 5-HTR subtypes between motoneuron types does exist, this work could be extended to examine how this relationship is affected by exercise training, in both the short-term and long-term. Examining the co-expression 5-HT_{2A}R and 5-HT_{2C}R mRNA in the spinal cord with double-label in situ hybridization would also complement this work, as it would determine if these mRNAs are not co-localized in Lamina IX (as suggested by Fonseca et al., 2001) and may demonstrate that there are functionally discrete (based on 5-HTR subtype expression) populations of motoneurons within Lamina IX.

Although this study focused on the effect of exercise training on lumbar motoneuron 5-HTR mRNA levels, future studies should examine how increased physical activity (both short- and long-term) affects other genes that alter motoneuron excitability. As discussed previously, motoneurons adjust their electrophysiological properties in response to increased motor activity, with the changes in gene expression that lead to these physiological adaptations remaining unclear. By broadening the scope of the study and exploring several genes related to motoneuron excitability, we can gain greater understanding of the mechanisms by which motoneurons adapt to exercise. Additionally, by examining a variety of genes after both short- and long-term exercise programs, we can learn which genes respond more rapidly to exercise and which require a greater training stimulus. These studies should also be expanded to include both treadmill-

exercised and voluntary wheel-exercised animals to obtain a better understanding of which genes are affected by training intensity and which are affected by training volume.

Finally, lumbar motoneuron protein expression (for 5-HTRs and other genes) could also be examined in acutely and chronically trained animals to provide a more complete picture of how exercise training affects motoneuron gene expression. By examining both mRNA and protein, we can gain insight into the level at which each gene is regulated in response to a training stimulus.

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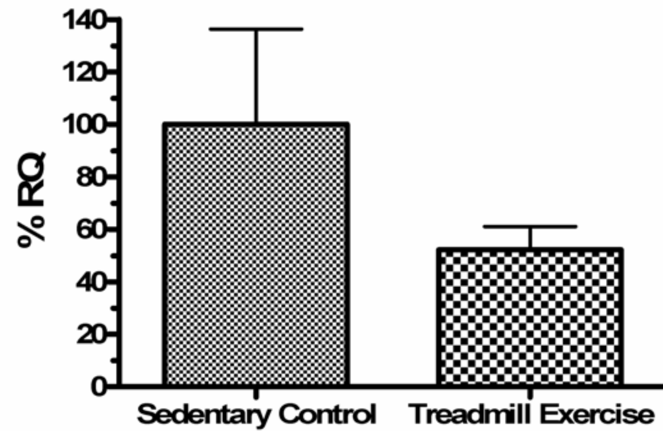
Appendix 1 – Results of Pilot Study

A.



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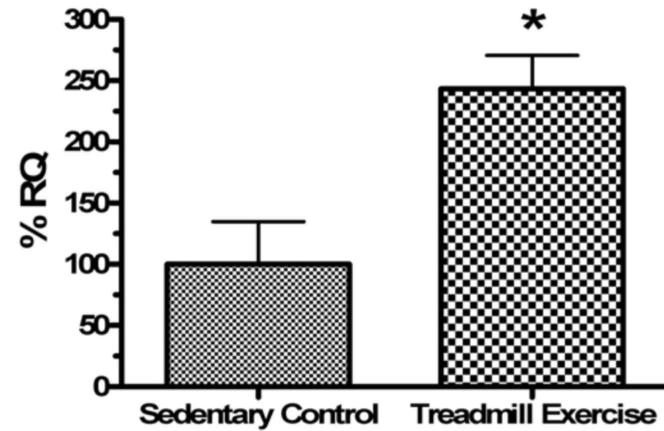
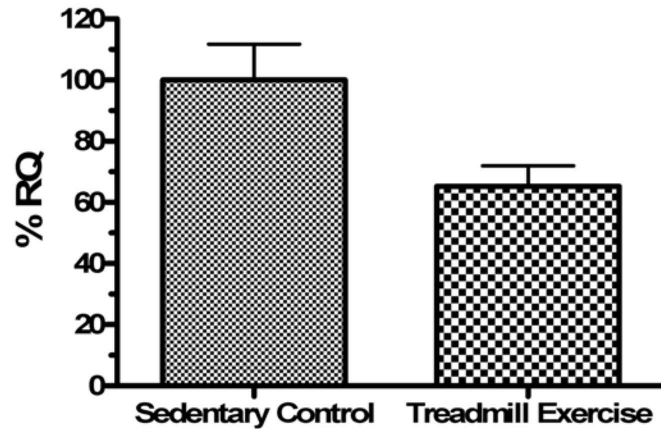


Effect of exercise on 5-HTR mRNA levels in Lamina IX (L6/S1)
A. Lamina IX (L6/S1) 5-HT_{2A}R mRNA levels presented in percent relative quantification (%RQ). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.025$
B. Lamina IX (L6/S1) 5-HT_{2C}R mRNA levels presented in percent relative quantification (%RQ). A significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.025$

A.



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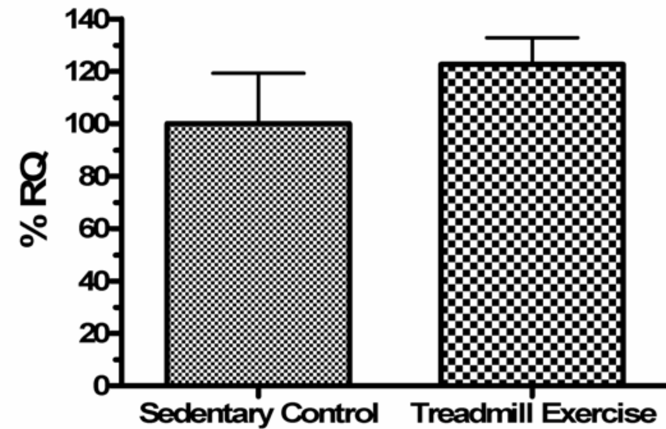
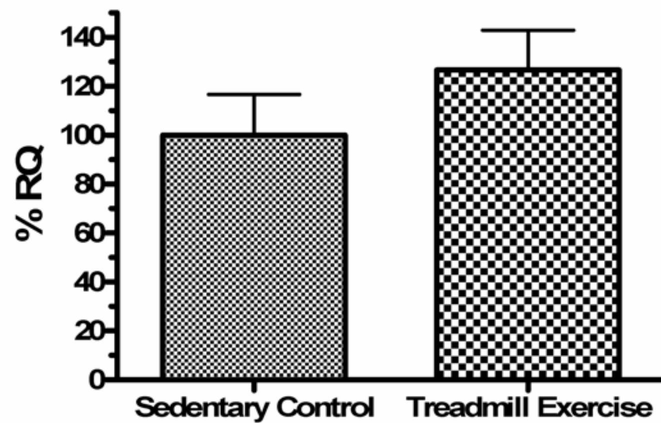


C.

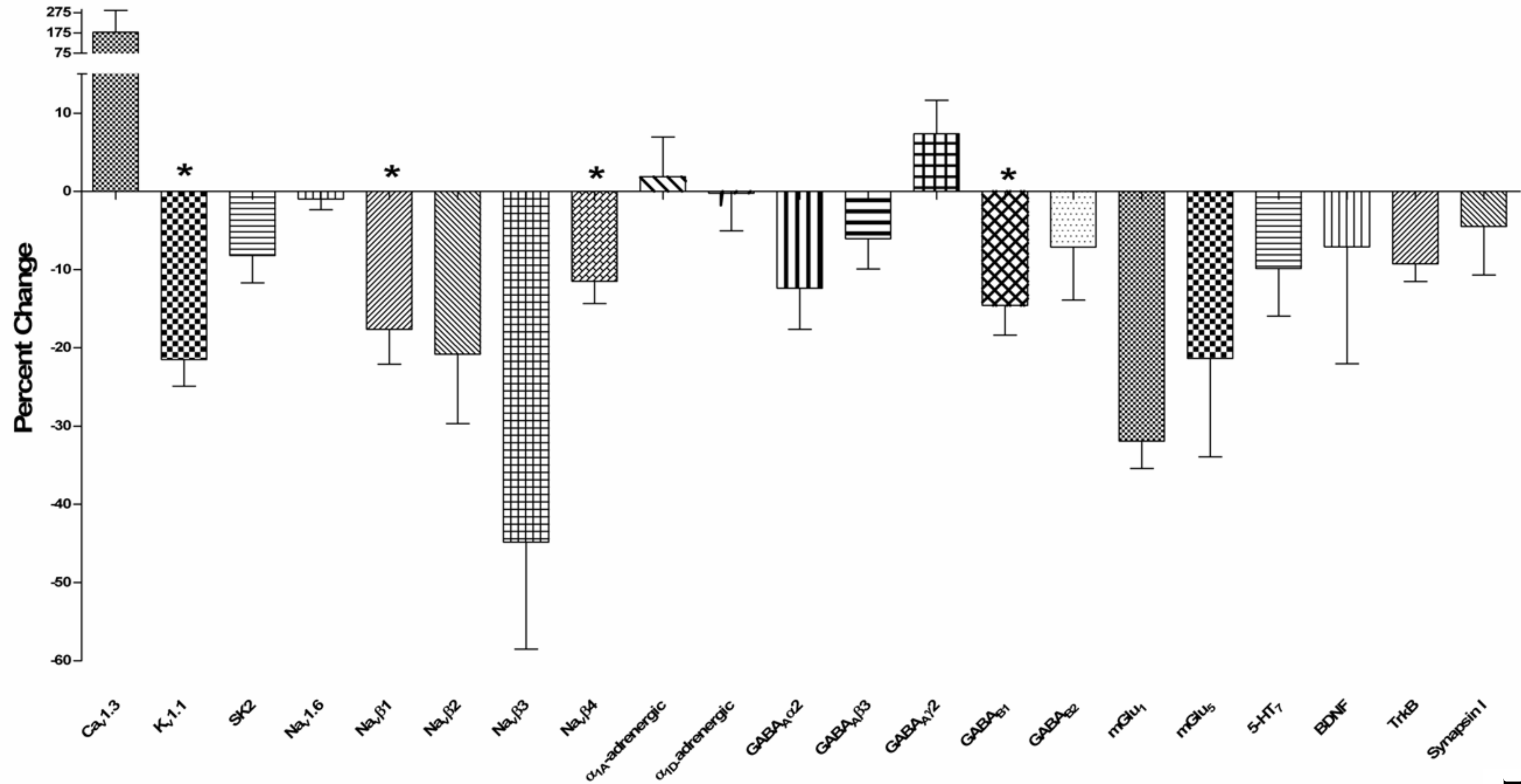


Effect of exercise on 5-HTR mRNA levels in Lamina IX (L3/L4)
A. Lamina IX (L3/L4) $5\text{-}HT_{1A}R$ mRNA levels presented in percent relative quantification (%RQ). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.017$
B. Lamina IX (L3/L4) $5\text{-}HT_{2A}R$ mRNA levels presented in percent relative quantification (%RQ). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. $p<0.017$
C. Lamina IX (L3/L4) $5\text{-}HT_{2C}R$ mRNA levels presented in percent relative quantification (%RQ). No significant difference between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups was observed. Error bars represent standard error. Error bars represent standard error. $p<0.017$

Appendix 2 – List of Additional Genes Examined

	Category	Gene
1	Ion Channel Subunits	Cav1.3
2		Kv1.1
3		SK2
4		Nav1.6
5		Navβ1
6		Navβ2
7		Navβ3
8		Navβ4
9	Adrenergic Receptors	α-1A
10		α-1D
11	GABA Receptor Subunits	GABAAα2
12		GABAAβ3
13		GABAAγ2
14		GABAB1
15		GABAB2
16	Metabotropic Glutamate Receptors	mGluR1
17		mGluR5
18	Serotonin Receptors	5-HT7R
19	Other	BDNF
20		TrkB receptor
21		Synapsin I

Appendix 3 – Results of Parallel Study



Effect of exercise on select ion channel subunit/neurotransmitter receptor mRNA levels in lumbar motoneurons
Lumbar motoneuron (L3-L5) mRNA levels of treadmill-exercised animals presented in percent change relative to sedentary controls. Significant differences between Sedentary Control (n=6) and Treadmill Exercise (n=6) groups were observed for $K_v1.1$, $Na_v\beta1$, $Na_v\beta4$ and $GABA_{B1}$. Error bars represent standard error. $p<0.026$