Serotonin receptor and KCC2 gene expression in lumbar flexor and extensor motoneurons

post-transection with and without passive cycling

Jeremy W. Chopek¹, Patricia C. Sheppard¹, Kalan Gardiner¹, Phillip F. Gardiner¹,²

¹Spinal Cord Research Centre, Department of Physiology and, ²Faculty of Kinesiology and Recreation Management, University of Manitoba, Winnipeg, Manitoba, Canada

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Corresponding author:

P.F. Gardiner, Spinal Cord Research Centre, Dept. of Physiology, University of Manitoba, 436-745 Bannatyne Ave., Winnipeg, Manitoba, Canada, R3E 0J9

Tel: 204-474-8770  Fax: 204-261-4802

Email: gardine2@cc.umanitoba.ca

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ABSTRACT

Sacrocaudal 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. Also, 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 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, 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$_{7}$R and KCC2 expression. Our results demonstrate that passive cycling influences serotonin receptor and KCC2 gene expression and that extensor motoneurons compared to flexor motoneurons may be more plastic to activity based interventions post-transection.
KEYWORDS

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

INTRODUCTION

Gene expression is altered in sacrocaudal 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 that cause spasticity and the pharmacological methods to alleviate spasticity (D'Amico et al., 2014). However, the examination of gene expression in lumbar, as opposed to sacrocaudal 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 mRNA expression of mGluR1 and the potassium chloride co-transporter, KCC2 were also examined. mGluR1 modulates motoneuron excitability by enhancing glutamatergic input on the motoneuron and facilitatating plateau potentials (Delgado-Lezama & Hounsgaard, 1999), whereas KCC2 maintains chloride homeostasis; which is necessary for GABA to have an inhibitory or hyperpolarizing effect on the neuron. (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 that 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 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 was used. This allowed 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.
Further, 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 T9 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 antibiotic Baytril (s.c. injection 0.5 mg·kg$^{-1}$) twice daily for a week period and the analgesic Buprenex (buprenorphine, s.c. injection 0.05 mg·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 hindlimbs. 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 stored 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 manufacturer’s 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 H₂O, 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 weight 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**

Prior to sacrifice, the rats were weighed, with no difference in body mass observed

between spinal transection groups, whereas, 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) and 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 ± 0.08g) weights, similar
to that seen in the control group. Transection did not result in loss of mass in either flexor

muscle.

**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 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<sub>2A</sub>R gene expression is up-regulated post transection

Similar to previous reports in sacral motoneurons, 5-HT<sub>2A</sub>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<sub>2A</sub>R gene expression in the spinal transected group.

5-HT<sub>2C</sub>R, 5-HT<sub>1A</sub>R and mGluR1 gene expression is down-regulated post transection

The effect of spinal transection was similar between extensor and flexor motoneurons. 5-HT<sub>2C</sub>R gene expression decreased 35% and 42% in extensor and flexor motoneurons respectively (P < 0.03). 5-HT<sub>1A</sub>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<sub>7</sub>R or KCC2.

KCC2 and 5-HT<sub>7</sub>R gene expression is not altered post transection

Contrary to previous studies demonstrating a down-regulation of KCC2 following a spinal transection (Ziemlinska <i>et al.</i>, 2014; Boulenguez <i>et al.</i>, 2010; Bos <i>et al.</i>, 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<sub>7</sub>R expression in either extensor or flexor motoneurons.
Daily passive hindlimb cycling influences extensor motoneuron mRNA expression

Three months of passive cycling increased 5-HT$_7$R, 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 enhancement of 86%, which was significantly greater than that of the flexor motoneurons and of the spinal transection group (P < 0.03). Passive cycling increased 5-HT$_7$R 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 lumbar 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$_7$R 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 sacrocaudal 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). However, to properly assess and develop therapeutic interventions aimed at restoring locomotion or reducing hindlimb spasticity, an understanding of lumbar extensor and flexor motoneuron gene expression is necessary. To that extent, our findings will be discussed in context with the known literature on serotonin receptor regulation in sacrocaudal 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 sacrocaudal segments, the 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 mechanism of muscle spasms and necessary for locomotor recovery (Murray et al., 2010; Murray et al., 2011). Although the total amount of 5-HT$_{2C}$R mRNA was unchanged in these studies (Murray et al., 2010; Murray et al., 2011), Ren and colleagues have shown that 5-HT$_{2C}$R protein in sacrocaudal 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. (2010), 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 points 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 with 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$_{2A}$ 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, the 5-HT$_{1A}$R is also found on the soma and proximal dendrites of the motoneuron and may 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
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 a down-regulation of KCC2 following a spinal transection is linked to spasticity (see Boulenguez et al., 2010), KCC2 gene expression in lumbar extensor and flexor motoneurons was 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 days 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 growth inhibitor; Ghiani et al., 2007) and up-regulates 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). Therefore, it may be that our activity paradigm only activates extensor muscles thereby preferentially influencing the extensor MSR. However, 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., 2011). Passive cycling seems to fine-tune this paradigm by up-regulating the 5-HT$_{2A}$R and KCC2, which have a role in motoneuron excitability and attenuating 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 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, demonstrates 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 available 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 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.
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DISCLOSURES

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


Chopek JW, Macdonell CW, Gardiner K, & Gardiner P (2014). Daily passive cycling attenuates the hyperexcitability and restores the responsiveness of the extensor monosynaptic reflex to quipazine in the chronic spinal transected rat. *J Neurotrauma*.


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_{7}R 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.
## Table 1. Genes examined in study

<table>
<thead>
<tr>
<th>RefSeqID</th>
<th>Gene Symbol</th>
<th>Protein Symbol</th>
<th>Description</th>
<th>qPCR assay ID</th>
<th>Amplicon length(bp)</th>
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<tr>
<td>NM_130428.1</td>
<td>SDHA</td>
<td>SDHA</td>
<td>Succinate dehydrogenase complex, subunit A, flavoprotein</td>
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<td>NM_012585.1</td>
<td>HTR1A</td>
<td>5-HT 1A</td>
<td>5-HT receptor 1A</td>
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<td>NM_017254.1</td>
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<td>NM_012765.3</td>
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<td>mGluR1</td>
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<td>NM_134363.1</td>
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<td>Kcc2</td>
<td>Solute carrier family 12 potassium-chloride transporter member 5</td>
<td>Rn0059264_m1</td>
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## Table 2. Relative gene expression values of control spine intact rats

<table>
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<tr>
<th>Gene</th>
<th>Extensor Mns</th>
<th>Flexor Mns</th>
<th></th>
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<tbody>
<tr>
<td>5-HT1A</td>
<td>0.18 ± 0.09</td>
<td>0.10 ± 0.06</td>
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</tr>
<tr>
<td>5-HT2A</td>
<td>5.95 ± 0.90</td>
<td>5.79 ± 0.39</td>
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<tr>
<td>5-HT2C</td>
<td>0.29 ± 0.10</td>
<td>0.42 ± 0.08</td>
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<tr>
<td>5-HT7</td>
<td>1.45 ± 0.23</td>
<td>1.32 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>mGluR1</td>
<td>1.90 ± 0.54</td>
<td>0.86 ± 0.35</td>
<td>*</td>
</tr>
<tr>
<td>KCC2</td>
<td>1.13 ± 0.19</td>
<td>1.23 ± 0.13</td>
<td></td>
</tr>
</tbody>
</table>

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).