Serotonin receptor and KCC2 gene expression in lumbar flexor and extensor motoneurons posttransection with and without passive cycling

Jeremy W. Chopek, Patricia C. Sheppard, Kalan Gardiner, and Phillip F. Gardiner

Sacrocaudal motoneuron gene expression is altered following a spinal transection. Of interest here is the regulation of serotonin (5-HT) receptors (R), glutamate receptor, metabotropic 1 (mGluR1), and potassium-chloride cotransporter (KCC2), which mediate motoneuron excitability, locomotor recovery, and spasticity posttransection. The examination of these genes in lumbar motoneurons posttransection 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 postinjury, a further examination of neuromuscular activity on gene expression posttransection is warranted. The purpose of this study was to examine motoneuronal gene expression of 5-HT receptors, KCC2, and mGluR1 at 3 mo 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 quantitative RT-PCR analysis. We demonstrate that postranssection 5-HT₁₅R, 5-HT₂₃R, and mGluR1 expression was downregulated, whereas the 5-HT₂₃αR was upregulated. 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-HT₁₅R expression and increased 5-HT₂₃R and KCC2 expression. Our results demonstrate that passive cycling influences serotonin receptor and KCC2 gene expression and that extensor motoneurons compared with flexor motoneurons may be more plastic to activity-based interventions posttransection.

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

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, postspinal 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 (R) on the alpha motoneuron, given the fundamental role 5-HT has in recovery of motoneuronal excitability and locomotion postinjury (Ung et al. 2008; Schmidt and Jordan 2000). Further mRNA expression of glutamate receptor, metabotropic 1 (mGluR1) and potassium-chloride cotransporter (KCC2) was also examined. mGluR1 modulates motoneuron excitability by enhancing glutamatergic input on the motoneuron and facilitating plateau potentials (Delgado-Lezama and 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; Boulguenez et al. 2010).

Following a spinal cord transection, the 5-HT₂₃αR is upregulated in sacral motoneurons (Kong et al. 2010, 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-HT₂ receptors and enhance spinal cord excitability and promote locomotor recovery posttransection 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 posttransection has not been reported.

Recently, we demonstrated that at 3 mo following a complete spinal cord transection the extensor but not flexor mono-synaptic reflex (MSR) demonstrated a fivefold increase in amplitude, an effect that was attenuated with a program of daily passive cycling. Furthermore, it was demonstrated that the extensor MSR of passively cycled rats responded to quipazine whereas the MSR of noncycled 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 upregulation 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 the 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 mo 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 quantitative (q)RT-PCR analysis. We demonstrate that following a spinal transection, the 5-HT2AR is upregulated and the 5-HT1AR, 5-HT2C-R, and mGluR1 are downregulated in both flexor and extensor motoneurons. Furthermore, passive cycling altered gene expression in extensor but not flexor motoneurons, resulting in a further enhancement of 5-HT2AR expression and upregulation of 5-HT-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 and 300 g 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 12:12-h 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 and postoperative care. The surgical techniques and postoperative 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 ~2 mm. 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. Postsurgery, the rats were given the antibiotic Baytril (subcutaneous injection 0.5 mg/kg) twice daily for a week period and the analgesic Buprenex (buprenorphine, subcutaneous injection 0.05 mg/kg) twice daily for the first 2 days. A subcutaneous injection of 5 ml of saline was also given immediately postsurgery to aide in rehydration. Manual bladder expression was performed three times daily until the voiding reflex was reestablished.

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 3 mo (n = 7); and 3) a spinal transected group that received passive cycling for 3 mo (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 1 wk 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 1 h daily for a 3-mo period at a rate of 30–50 rpm. It was found that the number of revolutions per minute needed to be adjusted for each rat daily as certain speeds produced spasticity.

Retrograde motoneuron labeling and tissue extraction. One week before death, 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 0.1 M PBS, each muscle) or 7% dextran tetramethylrhodamine 10,000 MW (fluororuby, 18 µl in saline, each muscle) with a Hamilton syringe. The muscle group injected was alternated between each tracer to ensure an 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 death (24 h 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 7 days. Slides were immersed in prechilled acetone (−20°C) for 1 min, followed by a series of alcohol washes (75, 50, 50,75, 90, and 100%) and air dried for 2 min. The lumbar enlargements were then scanned and photographed using a 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 (Fig. 1). To limit RNA degradation, samples...
were collected for no longer than 60 min 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 min and stored at −8°C. Lysates from the same animal were pooled before 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). An RNA integrity number of 6.5 or greater was accepted for analysis. Total numbers of motoneuron sections were comparable between groups and muscles (~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. 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 succinate dehydrogenase complex, subunit A (SDHA) mRNA levels and were expressed as a percent relative quantitation (%RQ) of control spinal cord intact rats. All reactions were performed in triplicate, and the coefficient of variation was <5% for each triplicate.

**Hindlimb muscle dissection.** After death 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 RQ values calculated with the 7500 Software version 2.0 (Applied Biosystems) using the 2−ΔΔCq method (Livak and 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 were 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 5% 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-m posttransection, all three extensor muscles demonstrated a 24% 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 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-HT2AR (5.95 ± 0.90 and 5.79 ± 0.39, extensors and flexors, respectively), whereas the lowest RQ value was the 5-HT2CR (0.18 ± 0.09 and 0.10 ± 0.06, extensors and flexors, respectively).

**5-HT2AR gene expression is upregulated posttransection.** Similar to previous reports in sacral motoneurons, 5-HT2AR gene expression was upregulated following spinal transection (Fig. 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-HT2AR gene expression in the spinal transected group.

**5-HT3R, 5-HT1AR, and mGlur1 gene expression is downregulated posttransection.** The effect of spinal transection was similar between extensor and flexor motoneurons. 5-HT3R gene expression decreased 35 and 42% in extensor and flexor motoneurons respectively (P < 0.03). 5-HT1AR gene expression was downregulated 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-HT7R or KCC2.

### Table 1. Genes examined in study

<table>
<thead>
<tr>
<th>RefSeqID</th>
<th>Gene Symbol</th>
<th>Protein Symbol</th>
<th>Description</th>
<th>Quantitative PCR Assay ID</th>
<th>Amplicon Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_130428.1</td>
<td>SDHA</td>
<td>SDHA</td>
<td>Succinate dehydrogenase complex, subunit A, flavoprotein</td>
<td>Rn00590475_m1</td>
<td>59</td>
</tr>
<tr>
<td>NM_012585.1</td>
<td>HTR1A</td>
<td>5-HT 1A</td>
<td>5-HT receptor 1A</td>
<td>Rn00561409_s1</td>
<td>75</td>
</tr>
<tr>
<td>NM_017254.1</td>
<td>HTR2A</td>
<td>5-HT 2A</td>
<td>5-HT receptor 2A</td>
<td>Rn00562748_m1</td>
<td>100</td>
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<td>NM_012765.3</td>
<td>HTR2C</td>
<td>5-HT 2C</td>
<td>5-HT receptor 2C</td>
<td>Rn00568473_m1</td>
<td>71</td>
</tr>
<tr>
<td>NM_022938.2</td>
<td>HTR7</td>
<td>5-HT 7</td>
<td>5-HT receptor 7</td>
<td>Rn00576048_m1</td>
<td>85</td>
</tr>
<tr>
<td>NM_000114330</td>
<td>GMR1</td>
<td>mGlur1</td>
<td>Glutamate receptor, metabotropic 1</td>
<td>Rn00566625_m1</td>
<td>83</td>
</tr>
<tr>
<td>NM_134363.1</td>
<td>Slc12a5</td>
<td>Kcc2</td>
<td>Solute carrier family 12 potassium-chloride transporter member 5</td>
<td>Rn0059264_m1</td>
<td>79</td>
</tr>
</tbody>
</table>

**RESULTS**

**Daily passive cycling attenuates extensor muscle mass loss.** Before death, the rats were weighed, with no difference in body mass observed between spinal transection groups, whereas, 3 mo posttransection, all three extensor muscles demonstrated a significant decrease in muscle weight (Fig. 2). The gastrocnemius decreased by 24% (2.6 ± 0.4 vs. 3.4 ± 0.4 g), and the soleus and plantaris muscle weights decreased by 16% (0.26 ± 0.05 vs. 0.31 ± 0.04 g) and 17% (0.55 ± 0.06 vs. 0.66 ± 0.09 g), respectively. Daily passive cycling attenuated the loss in muscle mass, preserving the gastrocnemius (3.01 ± 0.39 g), soleus (0.31 ± 0.06 g), and plantaris (0.62 ± 0.08 g) weights, similar to that seen in the control group. Transection did not result in loss of mass in either flexor muscle.

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**KCC2 and 5-HT7-R gene expression is not altered posttranssection.** Contrary to previous studies demonstrating a downregulation of KCC2 following a spinal transection (Ziemlinska et al. 2014; Boulenguez et al. 2010; Bos et al. 2013), we demonstrated no change in KCC2 gene expression 3 mo posttransection in either extensor or flexor motoneurons. Although there was a 20% decrease in KCC2 expression in flexor motoneurons posttransection, which is similar to the decrease reported in the above studies, this was not significant. Spinal transection did not result in alteration in 5-HT7R expression in either extensor or flexor motoneurons.

**Daily passive hindlimb cycling influences extensor motoneuron mRNA expression.** Three months of passive cycling increased 5-HT7R and KCC2 gene expression and further enhanced 5-HT2AR gene expression in extensor but not flexor motoneurons compared with spinal transection alone. The increase in 5-HT7R 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-HT7-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-HT2CR, 5-HT1AR, and mGluR1 gene expression associated with spinal transection in both extensor and flexor motoneurons.

### DISCUSSION

This article is the first to examine mRNA expression in two distinct lumbar motoneuron types following a complete thoracic spinal transection and the influence 3 mo of passive cycling would have on gene expression. It was demonstrated that 3 mo postspinal transection 5-HT2AR expression is upregulated, whereas 5-HT2CR, 5-HT1AR, and mGluR1 expression is downregulated. Passive cycling influenced extensor but not flexor motoneurons of spinal transected rats, resulting in an upregulation of 5-HT7-R and KCC2 gene expression, a further enhancement in the 5-HT2AR spinal transection associated upregulation and the preservation of extensor muscle mass.

**Gene expression postspinal 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. 2010, 2011; Murray et al. 2010; Navarret 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 extensor and flexor motoneurons.

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

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

Data are presented as means ± SD. Relative expression values (relative quantification), using SDHA as the housekeeping gene, of each gene in hindlimb extensor and flexor motoneurons (Mns) of control spine intact rats.

*Significant difference between extensor and flexor Mns (*P* < 0.03).
in light of recent findings of KCC2 expression on lumbar motoneurons postthoracic 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, 2011). Although the total amount of 5-HT_{2C}R mRNA was unchanged in these studies (Murray et al. 2010, 2011), Ren et al. (2013) have shown that 5-HT_{2C}R protein in sacrocaudal motoneurons is upregulated 45 days postsacral transection and was associated with the development of tail spasticity. Similar to Murray et al. (2010), Navarrett et al. (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 postinjury, as well as tissue used (whole cord vs. motoneuron). Furthermore, 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 downregulation in 5-HT_{2C}R expression, we demonstrated a robust upregulation of the 5-HT_{2A}R in both flexor and extensor motoneurons, suggesting that the 5-HT_{2A}R may enhance lumbar motoneuron excitability posttranssection. 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. Furthermore, behavioral and electrophysiological studies have linked the 5-HT_{2A}R with mediating lumbar spinal cord excitability postspinal 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) demonstrated that the 5-HT_{2A}R mediates lumbar motoneuron excitability when locomotion is generated by stimulation of the parapyramidal region in the in vitro rat preparation. The use of selective serotonin agonists and antagonists for the 5-HT_{2A}R and 5-HT_{2C}R will need to be used to confirm which receptor subtype mediates lumbar motoneuron excitability posttranssection.

Our results would suggest that enhanced lumbar motoneuron excitability posttranssection is likely mediated by the upregulation of the 5-HT_{2A}R but also may in part be explained by the downregulation 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 (Cotel et al. 2013); thus a downregulation would likely lead to enhanced motoneuron excitability postsipinal 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. Furthermore, the 5-HT_{1A}R has also been shown to be unchanged (Giroux et al. 1999) or upregulated on lumbar motoneurons postsipinal transection (Otoshi et al. 2009). Thus it appears the 5-HT_{2A}R is likely mediating lumbar motoneuron excitability posttranssection.

Lastly, the mGluR1 is the main postsynaptic 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 downregulated 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 postsipinal transection.

In light of recent findings that demonstrate a downregulation 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 postspinal 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 Boullenguez et al. (2010) at 45 days posttransection (22% decrease in protein expression). As well, Ziemlinska et al. (2014) demonstrated that KCC2 downregulation was greater in L1-L3 segments compared with L3–L6 segments following a thoracic transection. Therefore, it is plausible that the downregulation of KCC2 expression posttransection 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 signaling, 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, downregulate the myelin-associated glycoprotein MAG (axon growth inhibitor; Ghiani et al. 2007), and upregulate BDNF, NT-3, TrkB, TrkC, synapsin I, Gap-43, and CREB in the lumbar spinal cord (Gomez-Pinilla et al. 2001, 2002; Ying et al. 2003; Macias et al. 2002, 2007) with a similar enhancement found in laser-captured motoneurons following 1 mo 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-HT7R and KCC2 expression and further enhances 5-HT2AR expression, exclusively in extensor motoneurons. The exact mechanism by which gene expression is upregulated 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 postspinal 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 antigravitational and thus affected to a greater extent than flexor muscles postinjury (West et al. 1986; Roy and Acosta 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 upregulating serotonin receptor and KCC2 mRNA.

Upregulation of 5-HT2AR 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 and 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 upregulating the 5-HT2AR and KCC2, which have a role in motoneuron excitability and attenuating spasticity, respectively (Schmidt and Jordan 2000; Bos et al. 2013; Cote et al. 2014).

Recently, it has been demonstrated that passive cycling posttransection resulted in the upregulation of KCC2 protein expression on lumbar motoneurons. This upregulation 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 mo postspinal transection the extensor but not flexor MSR was potentiated and this was attenuated with passive cycling. Thus our results, in combination with others, demonstrate that passive cycling attenuates spasticity (Cote et al. 2014) and MSR hyperexcitability (Chopek et al. 2014), likely through an upregulation of KCC2 expression, which is exclusive to extensor motoneurons.

5-HT7R expression is enhanced in extensor motoneurons. A novel finding was that passive cycling upregulated 5-HT7R gene expression in extensor motoneurons. Immunohistochemistry and immunocytochemistry studies have demonstrated that the 5-HT7R is present in the ventral horn and on motoneurons (Noga et al. 2009; Doly et al. 2005), although the role of the 5-HT7R on the motoneuron is poorly understood, likely due to the lack of available specific agonists for the 5-HT7R. It has, however, been demonstrated that the 5-HT7R reduces the medium afterhyperpolarization in presumed jaw-closing motoneurons (Inoue et al. 2002) and induces long-term motor facilitation in phrenic motoneurons (Hoffman and Mitchell 2011), demonstrating an excitatory effect on the motoneuron. The 5-HT7R has also been linked to locomotor generation in the in vitro rat preparation when stimulating the parapyramidal
region and both the 5-HT7R and 5-HT2AR are required for the induction of locomotion to occur (Liu and Jordan 2005), although it was believed that agonists for the 5-HT2AR acted on potential central pattern generator neurons whereas 5-HT2AR agonists acted directly on the motoneuron. Further studies are required to understand the role of the 5-HT2AR on the motoneuron to determine if an upregulation 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-HT2AR is upregulated, whereas the 5-HT7R, 5-HT1A, and mGluR1 are downregulated in both extensor and flexor motoneurons. With passive cycling, KCC2 and 5-HT7R expression is increased and 5-HT2AR 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 hyperexcitability 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 author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


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