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Altered transcription of glutamatergic and glycinergic receptors in spinal cord dorsal horn following spinal cord transection is minimally affected by passive exercise of the hindlimbs

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ABSRACT

Gene expression is altered following a spinal transection (STx) in both motor and sensory systems. Exercise has been shown to influence gene expression in both systems post-STx. Gene expression alterations have also been shown in the dorsal root ganglia and nociceptive laminae of the spinal cord following either an incomplete spinal cord injury (SCI) or a contusive SCI. However, the effect of STx and exercise on gene expression in spinal cord laminae I-III has not fully been examined. Therefore, the purpose of this study was to determine if gene expression in laminae I-III is altered following STx and determine if superimposed passive exercise of the hindlimbs would influence gene expression post-STx in laminae I-III. Laser capture microdissection was used to selectively harvest laminae I-III of lumbar spinal cord sections and quantitative RT-PCR was used to examine relative expression of 23 selected genes in samples collected from control, STx and STx plus exercise rats. We demonstrate that post-STx, gene expression for metabotropic glutamate receptors 1, 5 and 8 were up-regulated, whereas ionotropic glutamatergic receptor (Glur2) and glycinergic subunit GLRA1 expression was down-regulated. Daily exercise attenuated the down-regulation of Glur2 gene expression in laminae I-III. Our results demonstrate that in a STx model, gene expression is altered in laminae I-III and that although passive exercise influences gene expression in both the motor and sensory systems, it had a minimal effect on gene expression in laminae I-III post-STx.

INTRODUCTION

Following spinal cord injury (SCI), gene alteration in the nociceptive, proprioceptive, motor and sensory systems has been shown (Nesic *et al.*, 2005; Ryge *et al.*, 2010; Wienecke *et al.*, 2010; Keeler *et al.*, 2012; Navarrett *et al.*, 2012; Chopek *et al.*, 2015). The consequences of altered gene expression include spasticity due to alteration in motoneuron excitability (Murray *et al.*, 2011) and persistent chronic neuropathic pain, which is present in up 40% of individuals with a SCI (Nesic *et al.*, 2005; Mehta *et al.*, 2013). As well, altered gene expression and the subsequent development of neuropathic pain is present following axotomy or nerve injury (Waxman *et al.*, 1994; Woolf & Mannion, 1999; Costigan *et al.*, 2002; Yang *et al.*, 2004)

In these models, in which varying degrees of ascending and descending fibres remain intact (Novakovic *et al.*, 1999; Woolf & Mannion, 1999; Tsuzuki *et al.*, 2001; Michael *et al.*, 2015), gene expression has been quantified by homogenizing the entire spinal cord or the dorsal horn, whereas the perception of pain is mediated in the superficial lamina (I to III) of the dorsal horn (Huang *et al.*, 2016). As such, an examination of mRNA expression specific to the laminae associated with nociceptive signaling is an appropriate next step. In addition, using a complete spinal transection (STx) model eliminates all descending input controlling for the influence of intact fibers that remain after incomplete spinal cord injury and to assess gene expression alteration that is solely from spinal or peripheral influence.

A second part of this investigation was to examine the potential influence "passive exercise" has on gene expression in laminae I-III following a STx. Exercise in the form of daily passive cycling has been demonstrated to influence gene expression in

the motoneuron, dorsal root ganglia and lamina VII following STx (Keeler *et al.*, 2012; Chopek *et al.*, 2015). Also, post-STx, daily passive cycling exercise has been shown to attenuate the change in motoneuron biophysical properties (Beaumont *et al.*, 2004), normalize spinal reflexes (Cote *et al.*, 2014; Chopek *et al.*, 2015), and in the contusive SCI model, reduce allodynia and aberrant afferent sprouting (Detloff *et al.*, 2014). In addition, exercise is one of the only non-pharmacological approaches to attenuate neuropathic pain in incomplete spinal cord injury and peripheral nerve injuries (Dobson *et al.*, 2014). Therefore, the influence of exercise was examined post STx on gene expression in lamina I-III.

Previously we have used laser capture microdissection (LCM) in combination with qRT-PCR to isolate specific motoneuron types and examine gene expression following a complete spinal transection (STx; Chopek et al. 2015). In this current investigation we used LCM and qRT-PCR to isolate the superficial laminae I-III of the lumbar spinal cord and examine gene expression between three groups: 1) control, 2) three months following a complete STx with no exercise, and 3) complete STx that received daily exercise for three months. We compared the expression of 21 genes that express products both pre-synaptically and post-synaptically, in addition to genes that express products destined exclusive to pre-synaptic terminals or post-synaptic neurons.

We show that despite previously demonstrated pronounced gene expression changes in the motoneurons and the sensory system post SCI, changes in gene expression in laminae I-III are modest. In addition, daily passive exercise of the hindlimbs had a minimal effect on the altered gene 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 grams 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 hour light-dark cycle. The rats had unlimited access to water and chow throughout the experimental period. Following the STx, rats were individually caged for ease of monitoring.

Spinal transection procedure and post-operative care

The surgical procedure and post-operative care were previously described in detail (Chopek *et al.*, 2014). Briefly, rats were initially anesthetized at 5% isoflurane and then maintained at 2-3% isoflurane mixed with 100% oxygen throughout the procedure. A laminectomy was performed at T8 followed by a small incision in the dura mater. The spinal cord segment at T9 was then completely transected with microdissection scissors and gentle aspiration to ensure a complete transection of approximately 2mm. Gelfoam was inserted into the gap and the musculature was sutured (Ethicon 4-0) and the skin was closed with vet bond. Post-surgery, the rats were given the antibiotic Baytril (s.c. injection 0.5 mg/kg) twice daily for a week period and the analgesic Buprenex (s.c. injection 0.05 mg/kg) twice daily for the first two days.

Experimental groups

Harvested dorsal laminae I-III were collected from the following three groups: 1) a control spine intact group (CON, N=6); 2) a spinalized group that did not receive any intervention for three months (STx, N=5); and 3) a spinalized group treated with daily passive exercise for three months (STx-Ex, N=5).

Exercise protocol

Daily exercise (passive cycling) as previously described (Skinner *et al.*, 1996; Chopek *et al.*, 2014) commenced 1 week following spinal transection. The exercise regimen consisted of the rat hindlimbs manipulated through a full range of motion with left and right alternation and rhythmic extensor and flexor muscle stretch via motorized pedals. The exercise protocol consisted of 1-hour sessions daily at a rate of 30-50 revolutions per minute for a three-month duration.

Tissue extraction, laser capture microdissection and qRT-PCR

In an initial group of animals (n=5), gene expression profiles in laminae I-III, lamina VII, lamina VIII and lamina IX were compared using LCM. This allowed us to 1) validate the use of LCM to dissect various lamina by comparing our gene expression results to previous studies that have examined differential expression in spinal laminae, and 2) determine which genes have a higher level of expression in laminae I-III in the healthy control rats compared to other spinal cord laminae.

For the rats in the STx plus exercise study, at the 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, immersed in Tissue-Tec O.C.T. embedding compound (Gene Research Lab), fresh-frozen in isopentane and stored at -80 °C for future use. Twelve-μm cross-sections of the lumbar enlargement were cut on a cryostat and mounted on polytetrafluorethylene-coated glass slides. Slides were then immersed in prechilled acetone (-20°C) for 1 minute, stained with cresyl violet, followed by a series of alcohol washes (75, 50, 50, 75, 90 and 100%) and air dried for 2 minutes. Transverse lumbar enlargement sections for each rat were then scanned and photographed on a Zeiss microscope. In order to identify laminae I-III a superimposed template of Rexed laminae was used in combination with visually identifying the border between laminae III and IV based on neuronal size and density from the cresyl violet staining. Lamina I-III was then dissected using the PALM laser microdissection and capture system and collected in a PALM microfuge tube with an adhesive cap. 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 MicroKit; Ambion), and stored upside down for 20 min at 42°C to aid tissue digestion. The tubes were then vortexed and centrifuged at 10062 g for 1 minute and stored at -80°C. Total RNA was isolated from the LCM samples with the RNAqueous MicroKit (Ambion). Total RNA concentration and integrity were determined with the RNA Pico 6000 Kit and the Agilent 2100 Bioanalyzer (Agilent

Technologies). Samples with a RNA integrity number of 6.5 or greater were accepted for analysis.

Reverse transcription was performed on equal amounts of sample RNA, with the Superscript Vilo cDNA Synthesis Kit (Invitrogen). Synthesized cDNA was preamplified with the TaqMan PreAmp Master Mix Kit (Applied Biosystems) for 14 preamplification cycles. Preamplified cDNA was diluted to 1ml final volume with TE buffer. qPCRs were set up with 12.5 µl TaqMan Gene Expression Master Mix (Applied Biosystems), 6.25 µl nucleases free H_20, 1.25 µL TaqMan Gene Expression Assays and 5µL preamplified cDNA per reaction. Table 1 contains a complete list of GEAs used. Reactions were run with the ABI 7500 Fast Real-Time PCR system (Applied Biosystems) for 40 cycles. Levels of mRNA were normalized to succinate dehydrogenase complex subunit A (SDHA) mRNA levels and were expressed as a percent relative quantification (%RQ) of control spine cord intact rats. All reactions were performed in triplicate and the coefficient of variation was <5% for each triplicate.

Statistical analysis. The mRNA results were expressed in 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 for comparison of data from multiple qPCR plates. Data were subjected to a one-way ANOVA to test for a main effect of group and Newman-Keuls analysis was used to test for difference between means. The *P* value was set at < 0.05. The following equation controlled the False Discovery Rate (Hassard & Becker, 1986), where t is the number of tests conducted:

$$Adjusted\ pvalue = pvalue \times \frac{(t+1)}{2t}$$

The adjusted *P* values used in this study were P < 0.028 for comparison of laminar gene expression and P < 0.025 for comparison of laminae I-III gene expression in the STx groups.

Results for the STx and STx-EX groups were expressed as a percent relative to the control spinal cord intact group.

RESULTS

mRNA levels for various receptors and ion channels isolated from laminae I-III, lamina VII, lamina VIII and lamina IX were compared to mRNA levels of whole lumbar spinal cord in a subset of control rats. In addition, gene expression of 23 genes associated with both pre- and post- synaptic receptors and ion channels in laminae I-III were compared in an uninjured control group, a chronic spinal transection group and a chronic spinal transection group that exercised for three months. Data is presented below as mean ± SD percent compared to the CON group (100%).

Gene expression distribution between different laminae in the adult spinal cord

Expression of twelve genes associated with neuronal excitability and synaptic plasticity from isolated laminae I-III, lamina VII, lamina VIII and lamina IX were compared to mRNA levels acquired from whole lumbar spinal cord. All twelves genes demonstrated differential laminar expression summarized in Table 1. Eight of the 12 genes had a higher level of expression in laminae I-III compared to lamina VII, lamina VIII, and lamina IX. Gene expression for 5-HT1A (8-fold), KCC2 (2-fold), mGluR1 (2

to 3- fold), mGluR5 (4.5 to 7-fold), mGluR8 (2 to 3-fold), GDNF (85-fold), TAC1 (20 to 40-fold) and STX1A (10-27-fold) in lamiae I-III was significantly higher compared to laminae VII, VIII and IX. In addition, GABRA2 (3- to 5-fold), 5-HT2A (4 to 6-fold) and TrkA (150-fold) had a higher level of expression in Lamina IX compared to laminae I-III, lamina VII, and lamina VIII. 5-HT2C demonstrated a lower level of expression in lamina IX compared to laminae III, VII and VIII. These results are similar to previous studies in which serotonin receptors (Marlier *et al.*, 1991), mGlu receptors (Berthele *et al.*, 1999), STX1A (Aguado *et al.*, 1999) and TAC1 (Mccarson & Krause, 1994) have demonstrated region-specific distribution in the spinal cord.

Effect of spinal transection and exercise on gene expression in laminae I-III.

Metabotropic glutamate receptors

Our initial results showed that mGluR1, mGluR5 and mGluR8 demonstrated a 2-7 fold higher level of expression in laminae I-III. In the STx experiment, a significant group effect was found for mGlur1 ($F_{2,13}$=5.9, P = 0.014), mGluR5 ($F_{2,13}$ = 12.4, P = 0.0001) and mGluR8 ($F_{2,13}$ = 15.2, P = 0.0005). mGluR1, mGluR5, mGluR8 were significantly higher by 22, 17 and 21% in the STx group (P = 0.04, 0.01 & 0.001 respectively, Figure 1) and 33, 23 and 18% in STx-Ex group (P = 0.01, 0.001 & 0.002) respectively compared to the CON group. No difference was seen between the STx and STx-Ex groups.

Ionotropic glutamate receptors

Gene expression for subunits of AMPA receptor (GluR2, GluR3) and the NMDA receptor (GRIN1) were examined. A significant group effect was found for GluR2 ($F_{2,13}$ = 5.1, P = 0.024). GluR2, which can undergo splicing to render the AMPA channel impermeable to calcium, was down-regulated 13% in the STx group compared to the control group (P = 0.045). Furthermore, exercise influenced GluR2 expression, preventing the down-regulation seen in the STx group (P = 0.019), with gene expression between the STx-EX and control group being similar. No effect of STx was seen for GluR3 or GRIN1 compared to the control group (Figure 1).

Glycinergic and GABAergic receptors

A significant group effect was found for the Glycine receptor alpha subunit ($F_{2,13}$ =11.2, P = 0.001, GLR_{A1}). GLR_{A1} expression was down-regulated 19% in the STx group compared to the control group (P = 0.001). In the STx-EX group, the down regulation was 11% (P = 0.018), with no difference in the down-regulation when compared to the STx group. Expression of the subunits for the ionotropic GABA A receptor (GABRA2, GABRB3, GABRG2) and the metabotropic GABA B receptor (GABBR1, GABBR2) was not altered in the STx group compared to the control group (Figure 2).

Serotonin receptors

$5\text{-}HT_{1A}R$ activation is linked to pain (Avila-Rojas *et al.*, 2015) and in our initial study demonstrated region-specific distribution with an 8-fold higher expression in laminae I-III compared to laminae VII, VIII and IX. Despite this, no alteration in gene expression was seen for the 5-HTRs. $5\text{-}HT_{1A}R$, $5\text{-}HT_{2A}R$ and $5\text{-}HT_{2C}R$ expression in the

STx group was 106 ± 15%, 104 ± 12% and 102 ± 4% compared to the CON group, respectively. In the STx-EX group, 5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2C}R expression was 108 ± 11%, 111 ± 16% and 105 ± 15% respectively compared to the CON group.

Opioid Receptors

Gene expression for the opioid receptors delta (DOR), kappa (KOR) and mu (MOR) were examined given their role in modulating nociception in the spinal cord (Przewlocki & Przewlocka, 2005) and that the MOR is up-regulated in the brain and down-regulated in the spinal cord following a contusive SCI (Michael *et al.*, 2015). In our present study no difference in gene expression was seen between the control and STx groups for any of the opioid receptors in laminae I-III. DOR expression was 98 ± 21 % and 84 ± 20 % in the STx and STx-EX groups compared to the CON group. KOR and MOR expression was 113 ± 8% and 111 ± 8 % in the STx group and 120 ± 15% and 116 ± 18 % in the STx-EX group compared to the CON group.

STX1A, TAC1, KCC2 expression

STX1A, which is involved in presynaptic docking of neurotransmitter vesicles demonstrated a 10-fold higher level of expression in Lamina I-III. However, spinal transection did not influence STX1A expression. STX1A expression in the STx and STx-EX compared to the control group was 104 ± 10% and 103 ± 10% respectively. Similar, TAC1, which has a role in nociceptive processing, was expressed 20-fold higher in lamina I-III demonstrated no change in gene expression seen in the STx groups. TAC1 expression was 90 ± 7% and 98 ± 10% in the STx and STx-EX groups compared to the

CON group. Lastly, KCC2 which is expressed significantly higher in laminae I-III and is down-regulated in the motoneuron and linked to spasticity following STx (Boulenguez *et al.*, 2010a) demonstrated no alteration in expression in the STx groups. Compared to the CON group, KCC2 expression was 96 ± 7% and 104 ± 8% in the STx and STx-EX groups.

GDNF AND TRK A expression

GDNF expression demonstrated an 85-fold higher expression in lamina I-III compared to laminae VII, VII and IX. However no difference in GDNF gene expression between the control and STx groups was seen in laminae I-III. Compared to the CON group, GDNF expression was 113 ± 17% and 94 ± 37% in the STx and STx-Ex groups. TrkA demonstrated a trend between groups ($F_{2,13} = 4.2$, $P = 0.037$) and post-hoc analysis suggests that trkA tended to be up-regulated 29 ± 17% in the STx-EX group ($P = 0.038$) and 19 ± 21% in the STx group ($P = 0.008$) compared to the CON group.

DISCUSSION

The purpose of this study was two-fold. First, we sought to examine gene expression related to the modulation of sensory signaling in laminae I-III following a complete STx. Second, we wished to determine if exercise alters genes expression in laminae I-III following a complete STx. Similar to peripheral nerve injury or an incomplete SCI where extensive alterations are noted in laminae I-III, our results demonstrate that alteration in gene expression is present following a complete STx. Of the 23 genes examined associated with pre- and post-synaptic excitability and sensory

signaling in laminae I-III, five genes related to glutamatergic and glycinergic receptors demonstrated alteration in their expression compared to the control group. In addition, daily exercise had a minimal effect on gene expression, attenuating the down-regulation of GluR2.

Gene expression in laminae I-III post-spinal transection

To date, studies have focused on laminae I-III gene expression in models of spinal cord contusion, incomplete spinal cord injury or peripheral nerve injury with little focus on a complete STx model (Michael et al., 2015; Novakovic et al., 1999; Tsuzuki et al., 2001), as these models induce a high level of neuropathic pain. However, the use of a complete STx model allows one to determine if the maladaptive gene expression changes described in the previous injury models are present in the absence of spared descending supraspinal input and conscious awareness. In addition, the use of a complete STx model allowed us to make comparisons of passive cycling exercise on gene expression in laminae I-III with those changes noted in the sensory and motor systems following a complete STx.

Interestingly, our results demonstrate that in the absence of descending input, alterations in laminae I-III gene expression were still present. Of significant interest is the alteration of the metabotropic glutamate receptors, in which maladaptive mGluR expression has been linked to neuropathic pain, allodynia and hyperalgesia (Mills *et al.*, 2001; Kolber, 2015; Chiechio, 2016). Our results demonstrate that mGluR expression increased between 15-25% following a STx in laminae I-III. These results are similar to that seen in spinal contusive injury and hemisected models in which immunofluorescence

intensity increases in the superficial laminae for mGluR1 receptors (Mills *et al.*, 2001; Gwak & Hulsebosch, 2005) . However, they demonstrated no increase in mGluR5 expression, which may be due to the different injury models and methodology used between our studies. In addition, we also demonstrated an increase in mGluR8 expression. Previous studies have shown mGluR8 is expressed in the somas of nociceptive afferents in the dorsal root ganglia (Carlton & Hargett, 2007) , but expression within spinal cord laminae I-III has not been demonstrated (Valerio *et al.*, 1997). This warrants further investigation as mGluR8 mRNA was detected in the control group and also significantly increased in the STx groups.

Based on sequence, pharmacology and intracellular signaling pathways, mGluRs are classified into three groups. Specifically mGluR1 and mGluR5 belong to group 1 which are predominately post-synaptic and regulate neuronal excitability through an increase in phospholipase C and a decrease in K^+ channel conductance. mGluR8 belongs to group 3 receptors which are predominately presynaptic and regulate neurotransmitter release (Chiechio, 2016). Taken together, our results demonstrate that complete STx alters both pre- and post-synaptic excitability in the superficial laminae. This could suggest that neuropathic pain, allodynia and hyperalgesia, may be influenced by a change in the regulation of these genes, although our study did not include measurements of pain or changes in sensitivity to touch and temperature.

To our knowledge, this is the first study to examine ionotropic AMPA and NMDA glutamate receptor mRNA post STx in the dorsal horn. Ionotropic glutamate receptors are proposed to be involved in acute pain transmission and neuronal plasticity that underlies chronic pain and inflammation in neuropathic states (Dray *et al.*, 1994), in

addition to mediating the intensity and duration of nociceptive neuron activity (Dickenson *et al.*, 1997). Whereas AMPA receptors set the baseline activity of nociceptive neurons, NMDA receptors are linked to prolonged pain states through prolonged depolarization and increased neuronal activity termed "wind-up" (Dickerson et al., 1997). Of the three iontropic glutamate receptor genes we examined, a decrease in AMPA receptor subunit 2 (GluR2) expression post STx was noted. This is of interest as the GluR2 receptor is known to undergo splice variants which make the receptor impermeable to calcium and thus reducing its activity. Further investigation is needed to determine if the reduction in GluR2 involves splice variants and whether this is pre-synaptic or post-synaptic alteration in laminae I-III.

In addition to metabotropic and ionotropic glutamate receptors, GABAergic receptor expression has also been linked to pain (Roberts *et al.*, 1986; Sivilotti & Woolf, 1994; Latremoliere & Woolf, 2009) and central sensitization (Gjerstad *et al.*, 2001; Washburn *et al.*, 2007). In healthy controls, in which descending fibres are intact, central sensitization does not occur (Gjerstad *et al.*, 2001; Washburn *et al.*, 2007). Interestingly, our results demonstrated that gene expression for both ionotropic and metabotropic GABA receptors were not altered following a complete STx in which the descending fibres are completely removed.

Although no alteration in GABA receptor gene expression was found, we demonstrated that glycinergic receptor subunit alpha 1 (glra1) expression was down-regulated in the STx group. Glycinergic receptors are exclusively post-synaptic ligand-gated chloride channels that mediate fast-synaptic inhibition. Neuronal excitability is predominately controlled by the balance of excitatory ionotropic receptors and inhibitory

glycinergic receptors (Lynch, 2004). In addition, calcium influx via kainite receptor activation can reduce membrane bound glycine receptor expression to regulate the excitatory-inhibitory balance. Taken together, this could suggest that neuronal homeostasis is altered in laminae I-III following a STx, and that receptor regulation is a potential mechanism to maintain homeostasis post-injury.

Lastly, we did not see any alteration in KCC2 expression post STx, whereas others have seen a down-regulation of the membrane-bound to cytoplasmic ratio of KCC2 in whole cord homogenates below an acute STx. KCC2 has also been implicated in spasticity post-SCI, as a down-regulation of KCC2 alters the equilibrium of chloride, increasing the excitability of the cell (Boulenguez *et al.*, 2010b). In addition, we previously demonstrated a 23% decreases in KCC2 expression in flexor but not extensor motoneurons (Chopek *et al.*, 2015) and Cote et al., showed a 20% decrease in protein expression in motoneurons (Cote *et al.*, 2014; Chopek *et al.*, 2015). Taken together, these results would suggest that KCC2 likely mediates neuronal excitability in the ventral horn and that although it is linked to nociceptive sensitization in the acute STx model (Huang et al.,) this may not be achieved through alteration in KCC2 gene expression.

Influence of exercise on gene expression in laminae I-III

Exercise is effective in alleviating neuropathic pain in incomplete or contusive SCI models or after peripheral nerve injury, although the exact mechanisms are unknown (Dobson *et al.*, 2014). Following a contusive cervical spinal injury, exercise was shown to reduce neuropathic pain by reducing aberrant c-fibre sprouting by attenuating the increase in GDNF and atremin expression post injury (Detloff *et al.*, 2014). In addition, the benefits of passive cycling exercise have been well documented in multiple systems

post-STx. These changes include attenuating muscle atrophy (Houle *et al.*, 1999; Murphy *et al.*, 1999; Peterson *et al.*, 2000), normalizing spinal reflexes (Skinner *et al.*, 1996; Chopek *et al.*, 2014; Cote *et al.*, 2014), maintaining motoneuron biophysical properties (Beaumont *et al.*, 2004) and gene expression (Keeler *et al.*, 2012; Chopek *et al.*, 2015) and preserving proprioceptive transmission (Ollivier-Lanvin *et al.*, 2010). Passive cycling exercise in the current study influenced expression of only one of five genes that were altered following STx, which maintained the expression of GluR2. GluR2 is found at both pre and post-synaptic sites and maintains neuronal excitability, thus passive cycling may maintain neuronal homeostasis in laminae I-III neurons.

The minimal impact of passive cycling exercise on gene expression in laminae I-III is likely due to a combination of the following two reasons. First, training-specific alterations are seen below the lesion post STx (see Edgerton *et al.*, 2001a) and passive cycling which includes rhythmic sensory afferent and motoneuron activation likely excludes activations of nociceptive afferents. Second, we examined the impact of passive cycling exercise in the complete STx model whereas others have used a spinal contusion model. It is very likely that the remaining descending fibres impose alterations in the spinal cord which are not seen in the complete STx model and therefore exercise does not substantially influence gene expression.

Conclusion

We demonstrated that in the complete STx model, gene expression involving glutamatergic and glycinergic transmission at both pre- and post- synaptic sites in laminae I-III are altered. In addition, we demonstrate that passive cycling exercise can

influence gene expression in laminae I-III following a STx by maintaining expression of the AMPA receptor GluR2.

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COMPETING INTERESTS

The authors state no competing or conflicting interests.

AUTHOR CONTRIBUTIONS

J.C. performed experiments, data analysis and wrote the manuscript. C.W. performed data analysis and contributed to the manuscript. K.G. and P.S. performed the data collection. P.G. conceived the study and contributed to the manuscript.

DATA ACCESSIBILITY

Data files are available upon request to corresponding author.

ABBREVIATONS

5-HT: serotonin, DOR: delta opioid receptor, GABBR1: GABA B receptor subunit 1, GABBR2: GABA B receptor subunit 2, GABRA2: GABA A receptor alpha subunit 1, GABRB3: GABA A receptor beta subunit 3, GABRG2: GABA A receptor gamma subunit 2, GDNF: glial cell-derived neurotrophic factor, GLR_{A1}: glycine receptor alpha subunit 1, gluR: AMPA glutamate receptor, GRIN1: NMDA glutamate receptor subunit 1, KOR, kappa opioid receptor, LCM: laser capture microdissection, mGluR: metabotropic glutamate receptor, MOR: mu opioid receptor, SCI: spinal cord injury, STx: spinal transection, STX1A: syntaxin 1A, TAC1: tachykinin precursor 1, trkA: tyrosine kinase receptor A

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TABLES

Table 1. Gene expression assays for qPCR

Gene Symbol	Protein	QPCR Assay ID	RefSeqID	Amplicon Length, bp
SDHA	SDHA	Rn00590475_m1	NM_130428.1	59
GRM1	mGluR1	Rn00566625_m1	NM_001114330	83
GRM5	mGluR5	Rn00566628_m1	NM_017012.1	112
GRM8	mGluR8	Rn00573505_m1	NM_022202.1	74
GRIA2	GluR2	Rn00568514_m1	NM_001083811.1	122
GRIA3	GluR3	Rn00583547_m1	NM_001112742.1	74
Grin1	NMDA1	Rn01436034_m1	NM_001270602.1	73
GLRA1	GLRA1	Rn00565582_m1	NM_013133.1	61
GABRA2	GABAAα2	Rn01413643_m1	NM_001135779.1	123
GABRB3	GABAAβ3	Rn00567029_m1	NM_017065.1	139
GABRG2	GABAAγ2	Rn00788325_m1	NM_183327.1	76
GABBR1	GABAB1	Rn00578911_m1	NM_031028.3	113
GABBR2	GABAB2	Rn00582550_m1	NM_031802.1	87
HTR1A	5-HT1A	Rn00561409_s1	NM_012585.1	75
HTR2A	5-HT2A	Rn00568473_m1	NM_017254.1	71
HTR2C	5-HT2C	Rn00562748_m1	NM_012765.3	100
OPRD1	DOR	Rn00561699_m1	NM_012617.1	70
OPRM1	MOR	Rn01430371_m1	NM_001038597.2	64
OPRK1	KOR	Rn01448892_m1	NM_017167.2	66
STX1A	Syntaxin-1A	Rn00587278_m1	NM_053788.2	70
TAC1	TKN1	Rn01500392_m1	NM_001124768.1	112
Slc12a5	Kcc2	Rn0059264_m1	NM_134363.1	79
GDNF	GDNF	Rn00569510_m1	NM_019139.1	122
NTRK1	TrkA	Rn00572130_m1	NM_021589.1	65

Table 2. Relative gene expression in spinal cord laminae

Gene	Laminae I-III	Lamina VII	Lamina VIII	Lamina IX	F value
5-HT1A	9.0 ± 1.2*	1.2 ± 0.4	1.2 ± 0.2	0.7 ± 0.2	189.6
5-HT2A	0.8 ± 0.1	0.5 ± 0.1	1.4 ± 0.5	5.9 ± 2.4$^{\Psi}$	22.4
5-HT2C	48.0 ± 0.2	45.9 ± 1.8	44.85 ± 5.1	29.3 ± 3.2^	15.5
KCC2	3041 ± 673*	1896 ± 417	1651 ± 268	1686 ± 184	11.8
mGluR1	142 ± 60.5*	56.2 ± 24.1	40.7 ± 14.1	31.7 ± 6.7	11.6
mGluR5	277 ± 54.5 *	61.9 ± 3.8	38.1 ± 7.0	32.3 ± 7.1	89.3
mGluR8	0.4 ± 0.01*	0.2 ± 0.01	0.1 ± 0.0 1	0.1 ± 0.02	65.8
GDNF	85.8 ± 8.8*	4.1 ± 4.7	1.4 ± 0.5	1.6 ± 1.4	339.1
TAC1	0.4 ± 0.1*	0.02 ± 0.01	0.01 ± 0.01	0.007 ± 0.005	81.5
STX1A	1.38 ± 0.1*	0.1 ± 0.08	0.09 ± 0.02	0.05 ± 0.02	318.8
TrkA	0.002 ± 0.00	0.007 ± 0.00	0.006 ± 0.00	0.3 ± 0.01 $^{\Psi}$	14.5
GABRA2	0.2 ± 0.02	0.1 ± 0.02	0.2 ± 0.08	0.7 ± 0.3 $^{\Psi}$	13.6

* significantly higher expression in Laminae I-III compared to Laminae VII, VIII and IX respectively (P<0.001)

Ψ significantly higher expression in Lamina IX compared to Laminae I-III, VII and VIII respectively (P<0.001)

^ significantly lower expression in Lamina IX compared to Laminae I-III, VII and VIII respectively (P<0.001)

FIGURE CAPTIONS

Figure 1 Glutamatergic receptor gene expression in laminae I-III of spinal rats. Relative expression of metabotropic and ionotropic glutamatergic receptors in the STx and STx-EX groups compared to the CON group. The metabotropic glutamatergic receptors 1, 5 and 8 were up-regulated, while the ionotropic GluR2 was down regulated in the STx-group. Exercise prevented the down-regulation of GluR2. * Significant difference from the CON group (P<0.02). Ψ Significant difference between the STx and STx-EX group (P<0.02). Data are presented as mean ± SD.

Figure 2. GABAergic receptor gene expression in laminae I-III of spinal rats. Relative expression of metabotropic and ionotropic GABAergic receptors in the STx and STx-EX groups compared to the CON group. The ionotropic GLRA1 receptor was down-regulated in the STx-groups. * Significant difference compared to the CON group ($P<0.01$). Data are presented as mean ± SD.

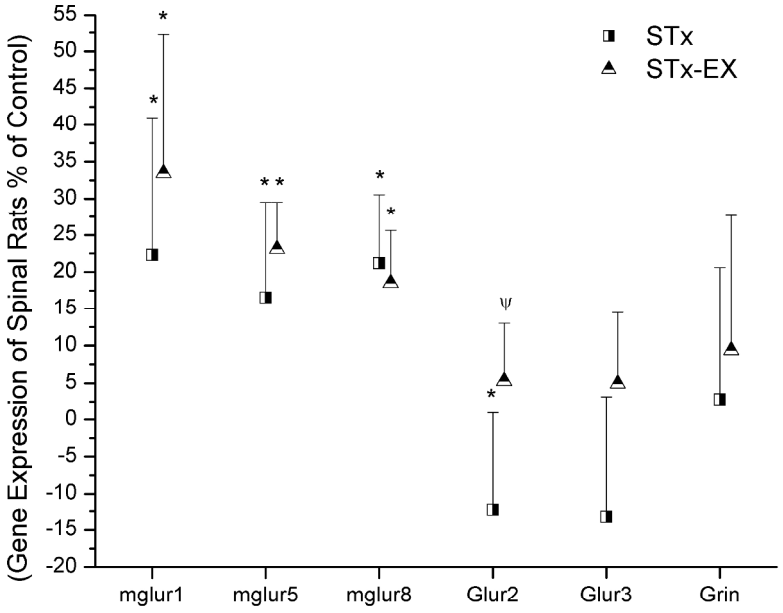

Figure 1 Glutamatergic receptor gene expression in laminae I-III of spinal rats. Relative expression of metabotropic and ionotropic glutamatergic receptors in the STx and STx-EX groups compared to the CON group. The metabotropic glutamatergic receptors 1, 5 and 8 were up-regulated, while the ionotropic GluR2 was down regulated in the STx-group. Exercise prevented the down-regulation of GluR2. * Significant difference from the CON group ($P<0.02$). Ψ Significant difference between the STx and STx-EX group ($P<0.02$). Data are presented as mean ± SD.

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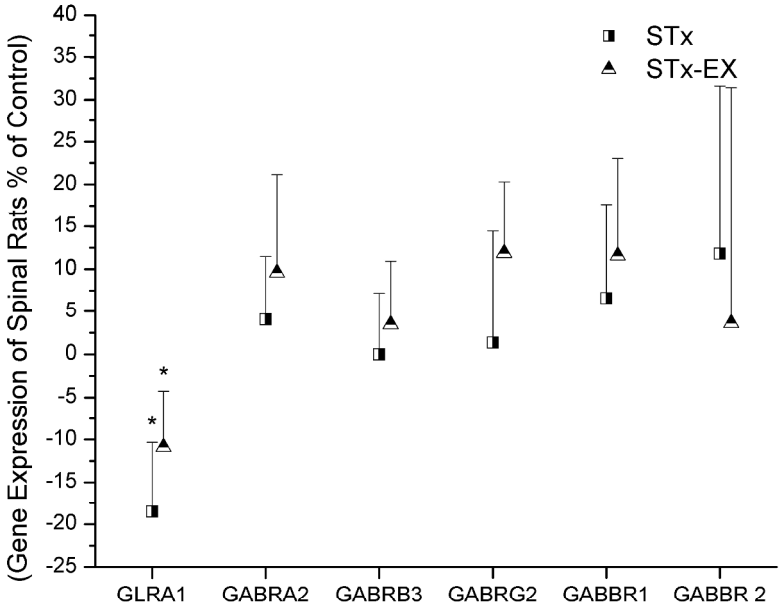

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