The Effects of Spinal Cord Injury on Expression of Pain-Relevant Genes in the Colon

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The Effects of Spinal Cord Injury on Expression of Pain-Relevant Genes in the Colon

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Abstract

Spinal cord injury (SCI) results in varying degrees of paralysis, loss of normal sensory function and, oftentimes, chronic pain originating below the level of injury. Post-SCI pain tends to be resistant to traditional analgesic therapies and has been shown to impair recovery of function, making it a significant healthcare concern. Approximately one third of SCI patients report abdominal pain as a primary and severe symptom with marked similarity to other functional gastrointestinal pain disorders (e.g. irritable bowel). The etiology of this intense pain phenotype is unclear given that visceral organs are left uninjured in SCI. Here we examine changes in expression of pain-relevant genes in the colon following SCI both over time and between two different specific injury parameters. Mice underwent spinal contusion injury resulting in complete paralysis below the level of the forelimbs (two injury severity conditions), were then sacrificed 1 or 7 days following SCI, and alterations in pain-relevant gene expression were determined using quantitative RT-PCR. Additionally, the presence of inflammation at 1 day post-SCI was performed using hematoxylin and eosin staining. The data indicate a pattern of both time- and injury-dependent differential gene expression within the colon following SCI including Gfra1-3, Trkb, Trkc, Asic1, Asic2, and Calca. Further, pilot hematoxylin and eosin studies suggest that differences in expression in the first 24 hours post-injury may be inflammation independent. Differentially expressed genes in the periphery may offer insight into novel therapeutic targets for prevention and treatment of abdominal pain following SCI.
Contributions

In the present study I completed the following: colon processing/RNA extraction, reverse transcription, and RT-PCR on all targets for all tissue collected after my start date in the lab in January 2017, re-runs for any samples with unusable RT-PCR values from before my start date in the lab, all RT-PCR for targets P2y1, P2x3, Trkc, Piezo2, and Calca on all samples used in the study (only the other targets had been run on samples processed before my arrival), RT-PCR data calculations, all statistical analysis, cryostat sectioning for the hematoxylin and eosin staining, the hematoxylin and eosin staining itself, and microscope images of the sections.

I also completed the following not in relation to or included in the present study: attempted piloting of immunocytochemistry and immunohistochemistry on colon sections, whole-mount colon immunohistochemistry, GFRα2, TRPV1, and P2X3 Western blot piloting, protein processing, cell culture as a favor to those who asked, tissue collection, single-cell RNA extraction, single-cell reverse transcription, and single-cell RT-PCR, and some bladder RNA processing, reverse transcription, and RT-PCR.
Introduction

Background

Spinal cord injury (SCI), a debilitating and life-altering injury, affects as many as 195 per million people worldwide, with the United States among one of the top affected developed countries at ~40 cases per million people (Massetti & Stein, 2017). SCI is more common in men with a nearly four-fold increased prevalence between men and women (Massetti & Stein, 2017). Additionally, the average age of SCI occurrence is around the age of 40 (Massetti & Stein, 2017). Although a rare type of injury, SCI has as an unusually high financial burden with the annual cost in the United States estimated at ~$9.7 billion per year, therefore demonstrating the complex medical needs of this patient population (French et al., 2007; Wyndaele & Wyndaele, 2006).

Patients experience varying degrees of paralysis in both voluntary and involuntary muscle groups depending on location and severity of injury: tetraplegia or paraplegia (Maynard et al., 1997). The most severe paralysis classification is tetraplegia, which is injury to the cervical region of the spinal cord with impairment of arms, trunk, legs, and pelvic organs (Maynard et al., 1997). Paraplegia, on the other hand, refers to injury at any of the other levels of the spinal cord (thoracic, lumbar, or sacral) that results in varying degrees of trunk, leg, and pelvic organ impairment, depending on the specific level of injury, and no arm impairment (Maynard et al., 1997). Further, depending on the degree of spinal cord transection, both tetraplegia and paraplegia can either be complete or incomplete (Maynard et al., 1997). If sensory or motor function is found anywhere below level of injury, including the sacral region, then the injury is considered incomplete (Maynard et al., 1997). Complete injuries, as the classification name
suggests, result in a complete absence of sensory and motor function below level of injury, particularly in the sacral region (Maynard et al., 1997).

The dual innervation of the somatic nervous system (SNS) and autonomic nervous system (ANS) is essential for proper organ function (Alexander et al., 2009). In SCI, these two pathways are often disrupted, with increasing severity as the level of injury gets higher, leading to many commonly experienced side effects that require close medical monitoring (Alexander et al., 2009). For example, in the cardiovascular and respiratory systems, SCI patients can have difficulty controlling blood pressure and heart rate, and develop shortness of breath, an inability to clear their airways by coughing, and/or heightened bronchial responses (Alexander et al., 2009). Patients also may experience problems with temperature regulation as the innervation of their sweat glands can be affected by SCI too (Alexander et al., 2009). Lastly, patients often lose control of their visceral organs including the bladder, bowel, and sexual organs (Alexander et al., 2009). While the aforementioned SCI related side effects are thought of as loss-of-function, one major symptom exists that would be considered gain-of-function: pain.

Pain, defined as “the unpleasant sensory and emotional experience associated with actual or potential tissue damage” (International Association for the Study of Pain) can be one of the most troublesome symptoms to treat in this patient population. Although SCI patients cannot feel their lower extremities, they experience pain that can severely decrease quality of life as well as deter recovery of function (Siddall et al., 2003). Additionally, once pain transitions from an acute to a chronic condition, it becomes very difficult to treat with traditional analgesics (Siddall et al., 2003).

Not all pain experienced by SCI patients is the same. Researchers have divided SCI-induced pain into two main categories: nociceptive pain and neuropathic pain (Finnerup &
Neuropathic pain is defined as “pain caused by lesion or disease of the somatosensory nervous system” (Finnerup & Baastrup, 2012; Jensen at al., 2011). This type of pain has been further classified into at-level and below-level pain and is characterized by both spontaneous and stimulus-evoked pain. Patients with neuropathic pain typically describe it as “burning,” “tingling,” “pins and needles,” and “shooting” (Finnerup & Baastrup, 2012). Alternately, nociceptive pain originates specifically from alterations to the activity of primary afferent neurons (International Association for the Study of Pain). Two of the main types of nociceptive pain seen in SCI patients are musculoskeletal pain and visceral pain (Finnerup & Baastrup, 2012). Much of what we know about post-SCI pain comes from studies on musculoskeletal pain. This type of “achy” pain can be caused by overuse of muscles like the upper body to compensate for a lack of mobility in lower extremities, poor posture, and muscle spasms (Finnerup & Baastrup, 2012).

Researchers theorize that both peripheral and central sensitization occur following injury, which causes the transition from localized acute pain, to chronic widespread pain (Graven-Nielsen & Arendt-Nielsen, 2010). The International Association for the Study of Pain (IASP) defines peripheral sensitization as “increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields.” This type of sensitization is due to inflammatory mediators and neurotrophic factors during injury or inflammation that in turn activate G-coupled receptors and tyrosine kinase receptors (Woolf & Salter, 2000). When these receptors are activated, tyrosine kinases in the cell can phosphorylate sodium channels in the neuron therefore affecting the behavior of the channel by making it easier for the channel to be activated and propagate action potentials (Woolf & Salter, 2000). The definition of central sensitization is “increased responsiveness of nociceptive neurons in the
central nervous system to their normal or subthreshold afferent input” (IASP). In a non-injured setting, nociceptors composed of Aδ and C-fibers have their terminals in peripheral tissues (Graven-Nielsen & Arendt-Nielsen, 2002). These terminals can be activated by ligands for any of the sensory-related receptors found on them (Woolf & Salter, 2000). When sufficiently activated, the nociceptors transduce sensory information into action potentials that travel to the central terminals in the spinal cord on the dorsal horn (Woolf & Salter, 2000). Once there, excitatory glutamate is released and binds to AMPA and kainate ligand-gated ion channels and their signals get modulated by spinal cord inhibitory neurons (Woolf & Salter, 2000). In pathological injury or inflammatory states, there is an increase of nociceptor signal frequency that causes temporal summation and depolarization of additional NMDA receptors and voltage-gated calcium channels, resulting in action potential discharge windup (Woolf & Salter, 2000). These increases in nociceptor discharge frequency or intensity lead to sensations of pain experienced by patients (Koltzenburg, 2000). The mechanisms researched for musculoskeletal pain can apply to other types of pain, as well.

Visceral pain refers to pain originating in the abdominal organs and approximately 14-38% of all SCI patients report abdominal pain as a primary and severe symptom (Ebert, 2012; Finnerup et al., 2008). Described by patients as “cramping and tightening,” “tender,” and “shooting,” this specific type of pain begins at later time points following the initial injury and is accompanied by a host of symptoms similar to those of functional gastrointestinal disorders like Irritable Bowel Syndrome (IBS) (Finnerup & Baastrup, 2012). For example, patients report constipation, abdominal distension, incontinence, diarrhea, pain worse after eating, and pain relieved by bowel movements (Ebert, 2012). The etiology of this intense pain phenotype is unknown considering that visceral organs are left without damage following SCI (Finnerup &
Most of the research on post-SCI visceral pain have been clinical studies focusing on symptomology. Additionally, literature using animal models of SCI tend to explore methods for regaining motor function and, in the pain literature specifically, examining musculoskeletal pain. Currently, there is a lack of precise, mechanistic studies for understanding SCI visceral pain and which genes and proteins may be playing a role in its induction and maintenance. What we can take from the other available literature is that molecular changes seem to happen immediately following SCI. Therefore, despite visceral pain being a late onset symptom, there may be immediately targetable mechanisms that arise during the early phase following injury. If we can identify the early gene(s) involved in the eventual development of chronic visceral pain, it may be possible to develop novel precision pain treatment strategies to prevent the eventual onset of this phenotype. This type of early intervention is more plausible in SCI than in other injury types of injuries as patients seek medical attention immediately at the time of injury offering a unique window of opportunity to intervene before the phenotype has even been established.

**Targets of Interest**

The targets we chose to examine are all important for sensory processing. Further, they have been implicated, more specifically, in peripheral pain processing and are mechanistically tied to the development of pathological pain states responsible for hypersensitivity.

**GDNF family receptors.** The GDNF family receptors are well known for their role in the development of both central and peripheral neurons (Airaksinen & Saarma, 2002; Baloh et al., 2000). After development, the signaling between these receptors and their respective ligands (GFRα1-GDNF, GFRα2-Neurturin, GFRα3-Artemin) is still essential as it helps maintain the survival of neurons (Airaksinen & Saarma, 2002). In the peripheral nervous system specifically,
and why they are of interest to us in the present study, the GDNF family receptors are involved in controlling sensory neuron cell body size, the targets the neurons innervate, and pain processing (Airaksinen & Saarma, 2002). As a result, they are interesting potential targets for treating pain disorders (Airaksinen & Saarma, 2002).

**Tyrosine receptor kinases.** Similar to the GDNF family receptors, signaling between the tyrosine receptor kinase family and their neurotrophic ligands (NGF-TrkA, BDNF-TrkB, NT-3-TrkC) is important for neural development, adult sensory neuron survival and regeneration, and nociceptor function (Khan & Smith, 2015; Jankowski & Koerber, 2010; Shu et al., 1999; Lindsay, 1996). Mutations of TrkA in humans has been linked to the congenital insensitivity to pain while manipulations of TrkB and TrkC have both been associated with alterations in pain development and persistence (Indo, 2001; Wang et al., 2009; Jankowski & Koerber, 2010).

**Transient receptor potential channels.** Both transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1) have been implicated in pain transmission. TRPV1, most well-known for its role as the capsaicin receptor, is highly associated with visceral pain pathologies like IBS as increased TRPV1-immunoreactive nerve fibers have been found in colon biopsies from IBS patients (Akbar et al., 2008). TRPA1 seems to be more important in inflammatory models of pain, such as formalin, as blocking it can attenuate inflammation-associated behavioral and physiological activity (McNamara et al., 2007).

**Acid sensing ion channels.** The acid sensing ion channels (ASICs) are known for their sensitivity in detecting pH changes, however they also seem to be important players for the transduction of mechanical stimuli in the viscera, as well as both contributing to and modulating
pain responsiveness after inflammatory events (Lingueglia, 2007; Staniland & McMahon, 2012; Mamet et al., 2002).

**Purinergic receptors.** The purinergic receptors P2X3 (ATP receptor) and P2Y1 (ADP receptor) are also important for peripheral pain processing (Hockley et al., 2016). P2X3 has been shown to be a contributor to both chronic inflammatory pain states as well as colon mechanical hypersensitivity in IBS (North, 2004; Shinoda et al., 2009). Similarly, P2Y1 activation via ADP has been highlighted as a mechanism for human and mouse visceral pain pathologies (Hockley et al., 2016).

**Piezo-type mechanosensitive ion channel component 2.** Piezo2 has emerged as a main sensitive mechanical transducer, mostly researched in skin (Yang et al., 2016). When examined for a role in visceral mechanotransduction, researchers found that Piezo2 knockdown in the dorsal root ganglion (DRG) decreased visceral responses to stimuli, suggesting it could be important (Yang et al., 2016).

**Calcitonin-related polypeptide alpha.** *Calca*, the gene that encodes calcitonin gene related peptide (CGRP), has been associated with migraines and inflammatory pain (Ho et al., 2010; Hernanz et al., 2003; Donnerer et al., 1992). Further, CGRP is released by peptidergic nociceptors in response to noxious heat and inflammatory mediators (Kessler et al., 1999). If inflammation is present in the colon following SCI, it is possible CGRP could be involved in post-SCI visceral pain.

**Statement of Objective**

Due to the marked similarities between post-SCI visceral pain and gastrointestinal disease symptomology, the purpose of this study was to determine potential alterations in colon-
specific gene expression in the development of colon (i.e. visceral) pain following SCI. We evaluated gene expression changes over the first week following injury and also compared across different injuries to examine 1) the dynamic pattern of altered gene expression immediately post-SCI and 2) whether this pattern differs with injury. Toward this goal we used a mouse model of SCI to quantitatively analyze differential gene expression in the colon. We identified time- and injury-specific changes in colon-specific gene expression of several targets including Gfra1-3, Trkb, Trkc, Asic1, Asic2, and Calca. This study is a first step in identifying potential therapeutic targets to increase the specificity of pain treatment in patients.

Methods

Animals

34 female C57BL/6 mice were used that were either ordered directly from Jackson Laboratories or bred in-house from Jackson Laboratories founder strains. We decided to use females because females are more likely to develop pain and they weigh less which makes them less likely to develop scoliosis or other postural changes that could affect behavioral outcomes (Wiesenfeld-Hallin, 2005). Naïve animals were kept in on-site animal facilities and injured animals were moved to an SCI-specific on-site animal room following any surgical procedures. All animals were kept on a regular dark/light schedule and given food and water ad libitum. Injured animals and shams were given nutrient supplements in addition to regular food and water. Injured and sham animals were also given injections of 2cc saline once a day for the first four days following surgery (including day of surgery) and their bladders were expressed twice daily. All animals maintained proper bowel function. Animal health was closely monitored and, although not experienced by any of the animals used for tissue in this study, if any animals were to show signs of excess pain/distress or poor overall health (weight loss greater than 15%,
ruffling/hunching), they would have been euthanized. All experiments were in conducted in accordance with protocols approved by the UCONN Health Institutional Animal Care and Use Committee. All animal facilities at UConn Health are fully accredited by the AAALAC-international since June 1977. The experimental cohorts of animals used in the study were as follows: naïve; 1 day post-SCI sham; 7 days post-SCI sham; 1 day post-SCI of 65kD, 1 second dwell time; 7 days post-SCI of 65kD, 1 second dwell time; 1 day post-SCI of 70kD, 10 second dwell time; and 7 days post-SCI, 10 second dwell time.

**Spinal Cord Injury Model**

Mice were initially anesthetized using isoflurane flowed through to a plexiglass anesthesia induction chamber via an anesthesia machine set to 2.5% flow rate concentration. Once unconscious, mice were secured to an oxygen mask flowing both oxygen and 1.5% flow rate concentration of isoflurane for maintenance of anesthetic level during surgery. Each mouse’s back fur was then shaved and the skin sterilized using iodine and chlorhexidine. A small portion of the spinal cord around T10/T11 was exposed via delamination and cleared of bone debris. The mouse was then secured underneath the piston of an Infinite Horizons impactor (Precision Systems and Instrumentation; Fairfax Station, VA) and injury parameters were programed to deliver a specific severity of contusion injury (either 70kD impact, 10 seconds dwell time OR 65kD impact, 1 second dwell time). Two injuries were performed see if different injury severities may lead to different patterns of expression. The 70kD, 10 second dwell time injury leads to a much greater injury, closer to a transection, with little to no recovery of function over time (i.e. bladder and motor) and are more likely to have severe complications earlier following injury resulting in death or euthanasia. The 65kD, 1 second dwell time injury is still severe, but is more likely to be followed by some recovery of function (at later timepoints than those used in this
study) and the mice have better overall health with increased survival rates. After injury
induction, the incision was sutured and animals received a 5mg/kg injection of gentamicin
(Henry Schein; Melville, NY) and allowed to come out of anesthesia under close supervision.
Mice were given gentamicin injections once daily for the following two days after surgery, as
well. With both impact severities, the injury resulted in complete paralysis below the level of the
forelimbs. The mice were sacrificed either 1 day or 7 days after SCI. Sham control surgery
consisted of spinal cord exposure via delamination and tissue collection at 1 day and 7 days post-
surgery.

**RNA Extraction and Complementary DNA Preparation**

Animals were perfused with cold saline under anesthesia via transcardial perfusion until
the exudate ran clear from the atrium. The distal ~1.5 cm of colorectum was collected avoiding
the inclusion of the anus (i.e. somatic tissues). Samples were put immediately on dry ice and
then stored at -80°C for processing in batches. The colon was homogenized in 350µL of a
mixture of Buffer RLT and 10µL/1mL β-mercaptoethanol until no individual tissue pieces were
visible and the resulting tissue homogenate was used for collection of total ribonucleic acid
(RNA) (RNeasy MiniKit; Qiagen; Hilden, Germany). Final RNA concentrations and RNA
quality were determined using a Biospectrometer (Eppendorf; Hamburg, Germany). A 260/280
value of at least 2.00 was deemed as usable quality of RNA. Total RNA with a starting
concentration of 500ng per sample was made into complementary deoxyribonucleic acid
(cDNA) using the iScript cDNA Synthesis Kit according to the manufacturer’s instructions (Bio-
Rad; Hercules, CA).
Quantitative RT-PCR

Quantitative RT-PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad; Hercules, CA) with custom primers for each candidate gene (IDT; Coralville, IA). Primers were created for the following targets: GDNF family receptor alpha 1-3 (Gfra1, Gfra2, Gfra3), tyrosine receptor kinase a-c (Trka, Trkb, Trkc), transient receptor potential vanilloid 1 (Trpv1), transient receptor potential ankyrin 1 (Trpa1), acid sensing ion channels 1-3 (Asic1, Asic2, Asic3), purinergic receptor P2y 1 (P2y1), purinergic receptor P2x 3 (P2x3), piezo-type mechanosensitive ion channel component 2 (Piezo2), calcitonin-related polypeptide alpha (Calc-a), and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (Table 1). The primers were all determined to be 100% efficient (defined as 95% or better amplification between PCR cycles at specific concentrations of template/sample and primer used) using efficiency analysis (Pfaffl, 2001). Melt curve analyses were performed to confirm single product amplification, followed by PCR product analysis via gel electrophoresis to confirm that a single product was produced. All primers were used at their optimal concentration to maintain efficiency consistency between targets. Each sample was run in duplicate using 500ng of cDNA per well. The temperature cycle parameters (StepOne Plus Real-Time PCR System; Applied Biosystems; Foster City, CA) and were as follows: 30 seconds at 95°C followed by 40 cycles of 15 seconds at 95°C and then 60 seconds at 60°C. Following completion of the cycles, the PCR machine produced cycle threshold values (Ct values) that denote the number of amplification cycles needed for the amount of target product to reach a detectable threshold. Therefore, the higher the Ct value, the more cycles it took to detect the product, meaning it was lower in abundance in the sample. Gapdh was used as a housekeeping gene for every sample and each sample’s respective Gapdh average Ct value was subtracted from the average target gene Ct
value in order to calculate the $\Delta C_t$. All subjects for all conditions were then normalized to the average of the naïve control condition to calculate $\Delta \Delta C_t (\Delta C_{t\text{Subject}} - \Delta C_{t\text{NaiveMean}})$. $\Delta \Delta C_t$ values were then converted into fold difference in expression using the $2^{-\Delta \Delta C_t}$ method to examine the target gene expression differences between conditions.

**Hematoxylin and Eosin Staining**

We used a hematoxylin and eosin staining as a histological assessment to determine whether SCI may cause inflammation in the colon that could be related to alterations in gene expression early following injury. Hematoxylin and eosin stain is a common method for examining colonic inflammation as it can detect the presence of infiltrating immune cells and wall thickening (Wirtz et al., 2007). One naïve and one 1 day post-SCI (70kD, 10 second dwell time) animals were first fixed using 4% paraformaldehyde, then their colons were dissected and post-fixed for 24 hours in 4% paraformaldehyde, 4% sucrose before subsequent embedding in OCT. Colons were cross-sectioned at a thickness of 5 micrometers using a cryostat and placed on charged microscope slides for hematoxylin and eosin staining (National Diagnostics; Atlanta, Georgia).

**Statistical Analysis**

One-way Analysis of Variance (ANOVA) were used to determine significant differences in gene expression between both naïve, 1 day post-SCI, and 7 days post-SCI time points for both injury conditions (70kD, 10 second dwell time and 65kD, 1 second dwell time), as well as naïve, 1 day sham, and 7 day sham. Planned comparison tests were used to determine an effect of injury level on gene expression at both 1 day and 7 days post-SCI. A result was considered significant
if the $p$ value was less than or equal to 0.05. Post-hoc analysis was conducted on all significant ANOVA using Tukey’s honestly significant difference test (HSD).

**Results**

**Differential Gene Expression**

Visceral pain induced by SCI is not a symptom reported by patients until later following injury. However, we assessed differences in pain-relevant gene expression at two time points across the first week post-SCI to evaluate changes early on that may reflect pathological processes that could contribute to subsequent visceral pain. If we can identify and target these early changing genes, we could prevent chronic visceral pain before it even starts. We also examined two different injury levels following SCI in order to determine injury-specific alterations in gene expression. We detected both time and SCI condition significant differences for multiple targets.

**GDNF family receptors.** We examined expression differences in a group of glial-derived neurotrophic factor family receptors, $Gfra1$-3 (Figure 1). For the severe injury condition (70kD, 10 second dwell time), we found a significant difference in expression between naïve, 1 day post-SCI, and 7 days post-SCI for $Gfra2$ ($F(2,10) = 5.502, p = 0.024$), but not $Gfra1$ or $Gfra3$ ($F(2,10) = 3.661, p = 0.064$; $F(2,10) = 4.059, p = 0.051$, respectively). Tukey’s HSD post-hoc analysis demonstrated elevated $Gfra2$ expression at 7 days post-SCI compared to 1 day post-SCI ($p = 0.020$). For the moderate injury condition (65kD, 1 second dwell time) we found the opposite in that both $Gfra1$ and $Gfra3$ had significant differences in expression ($F(2,10) = 6.460, p = 0.016$; $F(2,10) = 6.112, p = 0.018$, respectively), but $Gfra2$ did not ($F(2,10) = 2.050, p = 0.179$). Tukey’s HSD post-hoc analysis determined that compared to naïve mice, both 1 day and
7 days post-SCI animals had reduced Gfra1 expression (\(p = 0.026; \ p = 0.019\), respectively), but only 1 day post-SCI animals showed a decrease in Gfra3 (\(p = 0.016\)). Similarly, sham surgery also demonstrated significant differences in both Gfra1 and Gfra3 expression (\(F(2,11) = 11.431, \ p = 0.002\); \(F(2,11) = 11.028, \ p = 0.002\), respectively). According to Tukey’s HSD post-hoc, 1 day sham and 7 day sham animals both had decreases in Gfra1 expression (\(p = 0.003\) for both) and Gfra3 expression (\(p = 0.003; \ p = 0.005\), respectively) compared to naïve.

When assessing effect of injury condition (70kD, 10 second dwell time vs. 65kD, 1 second dwell time) on Gfra1-3 expression at 1 day post-SCI, we found no significant differences (\(F(1,7) = 0.659, \ p = 0.444\); \(F(1,7) = 2.212, \ p = 0.181\); \(F(1,7) = 1.476, \ p = 0.264\), respectively) (Figure 1). However, there was a significant effect of injury condition on expression of both Gfra1 and Gfra3 (\(F(1,9) = 5.546, \ p = 0.043\); \(F(1,9) = 5.664, \ p = 0.041\), respectively) at 7 days post-SCI, but not Gfra2 (\(F(1,9) = 2.015, \ p = 0.189\)). There was more Gfra1 and Gfra3 expression in the moderate injury condition (M = 0.31, SD = 0.09; M = 0.49, SD = 0.31, respectively) than the severe injury condition (M = 0.18, SD = 0.09; M = 0.19, SD = 0.04).

**Tyrosine receptor kinases.** We also examined differential expression within the family of tyrosine receptor kinases, Trka-c (Figure 2). There was a significant difference of Trkc expression in the severe injury condition (\(F(2,10) = 11.581, \ p = 0.002\)), but not of Trka or Trkb (\(F(2,10) = 0.915, \ p = 0.432\); \(F(2,10) = 0.297, \ p = 0.749\), respectively). Tukey’s HSD post-hoc analysis showed that, compared to naïve, 1 day post-SCI and 7 day post-SCI animals both had decreased expression of Trkc (\(p = 0.005; \ p = 0.003\), respectively). Moderate injury condition did not demonstrate any significant differences in expression of Trka, Trkb, or Trkc (\(F(2,10) = 1.713, \ p = 0.229\); \(F(2,10) = 3.870, \ p = 0.057\); \(F(2,10) = 0.273, \ p = 0.767\), respectively) and
neither did sham ($F(2,11) = 0.453, p = 0.647; F(2,11) = 0.627, p = 0.552; F(2,11) = 2.623, p = 0.117$, respectively).

In the assessment of injury condition, at day 1 post-SCI, there were no significant differences in expression of Trka-c ($F(1,7) = 0.050, p = 0.830; F(1,7) = 3.260, p = 0.114; F(1,7) = 1.868, p = 0.214$, respectively) (Figure 2). At 7 days post-SCI, there was no significant effect of injury level on Trka or Trkc expression ($F(1,9) = 3.626, p = 0.089; F(1,9) = 3.825, p = 0.082$, respectively), but there was on Trkb expression ($F(1,9) = 6.395, p = 0.032$). Trkb expression was higher in the moderate injury condition ($M = 45.46, SD = 40.29$) than the severe injury condition ($M = 3.81, SD = 5.75$).

**Transient receptor potential channels.** We also included transient receptor potential channels, Trpv1 and Trpa1, in our investigative panel (Figure 3). Severe injury condition demonstrated no significant changes in expression for either Trpv1 or Trpa1 ($F(2,10) = 1.256, p = 0.326; F(2,10) = 0.562, p = 0.587$, respectively). Moderate injury condition also did not have any significant changes in Trpv1 or Trpa1 expression ($F(2,10) = 1.633, p = 0.243; F(2,10) = 1.246, p = 0.329$, respectively). Similarly, shams had no significant changes in expression of Trpv1 or Trpa1 either ($F(2,11) = 0.255, p = 0.780; F(2,11) = 2.761, p = 0.107$, respectively).

There was no effect of injury condition on expression of Trpv1 or Trpa1 at 1 day post-SCI ($F(1,7) = 0.263, p = 0.624; F(1,7) = 0.302, p = 0.600$, respectively) or at 7 days post-SCI ($F(1,9) = 3.255, p = 0.105; F(1,9) = 3.733, p = 0.085$, respectively) (Figure 3).

**Acid sensing ion channels.** When examining the acid sensing ion channels, Asic1-3, we found no significant changes in expression for the severe injury condition ($F(2,10) = 1.233, p = 0.332; F(2,10) = 0.716, p = 0.512; F(2,9) = 1.089, p = 0.377$, respectively) (Figure 4). Moderate
injury condition did not have any significant changes in expression of Asic1-3 either ($F(2,10) = 3.140, p = 0.087$; $F(2,10) = 1.125, p = 0.363$; $F(2,9) = 1.152, p = 0.359$, respectively). For sham treatment, Asic1 and Asic3 had no significant changes in expression ($F(2,11) = 0.135, p = 0.875$; $F(2,10) = 0.452, p = 0.649$, respectively), however, Asic2 expression was significantly altered ($F(2,11) = 12.241, p = 0.002$). Tukey’s HSD post-hoc analysis determined that, compared to naïve, 1 day sham and 7 day sham both had decreased levels of Asic2 ($p = 0.001$; $p = 0.016$, respectively).

There was no effect of injury condition on expression of Asic1-3 at 1 day post SCI ($F(1,7) = 1.106, p = 0.328$; $F(1,7) = 0.790, p = 0.404$; $F(1,7) = 0.644, p = 0.449$, respectively) (Figure 4). However, it did significantly affect expression of Asic1 at 7 days post-SCI ($F(1,9) = 14.186, p = 0.004$), but not Asic2 or Asic3 ($F(1,9) = 0.743, p = 0.411$; $F(1,9) = 1.896, p = 0.202$). Asic1 expression was greater at the moderate injury level ($M = 2.47$, $SD = 1.16$) than the severe injury level ($M = 0.65$, $SD = 0.28$).

**Purinergic receptors.** The purinergic receptors P2y1 and P2x3 demonstrated no significant changes with severe injury condition ($F(2,10) = 0.880, p = 0.445$; $F(2,10) = 1.179, p = 0.347$, respectively) (Figure 5). Similarly, moderate injury condition did not display any significant changes in P2y1 or P2x3 expression ($F(2,10) = 0.638, p = 0.548$; $F(2,10) = 0.854, p = 0.455$ respectively). Sham treatment also did not have any significant changes in P2y1 or P2x3 gene expression ($F(2,11) = 0.238, p = 0.792$; $F(2,11) = 0.641, p = 0.545$, respectively).

There was no effect of injury condition on expression of P2y1 or P2x3 at 1 day post-SCI ($F(1,7) = 1.018, p = 0.347$; $F(1,7) = 3.266, p = 0.114$, respectively) or at 7 days post-SCI ($F(1,9) = 0.036, p = 0.854$; $F(1,9) = 0.112, p = 0.746$, respectively) (Figure 5).
Piezo-type mechanosensitive ion channel component 2. We also assessed *Piezo2* (Figure 6). Severe injury condition, moderate injury condition, and sham treatment all had no significant changes in *Piezo2* expression ($F(2,10) = 1.662, p = 0.238$; $F(2,10) = 0.676, p = 0.530$; $F(2,11) = 2.084, p = 0.171$, respectively).

There was no effect of injury condition on expression of *Piezo2* at either 1 day post-SCI ($F(1,7) = 0.818, p = 0.396$) or 7 days post-SCI ($F(1,9) = 0.274, p = 0.614$) (Figure 6).

**Calcitonin-related polypeptide alpha.** Lastly, we examined changes in *Calca* expression (Figure 6). Severe injury condition, moderate injury condition, and sham treatment all had no significant changes in *Calca* expression ($F(2,10) = 1.784, p = 0.218$; $F(2,10) = 0.638, p = 0.548$; $F(2,11) = 0.772, p = 0.485$, respectively).

There was no effect of injury condition on *Calca* expression at 1 day post-SCI ($F(1,7) = 2.040, p = 0.196$), but there was a significant effect at 7 days post-SCI ($F(1,9) = 6.832, p = 0.028$) (Figure 6). *Calca* expression was greater at the moderate injury condition (M = 4.95, SD = 4.31) compared to the severe injury condition (M = 0.40, SD = 0.18).

**Hematoxylin and Eosin Staining**

When assessing for the presence of inflammation using hematoxylin and eosin stain on one naïve and one 1 day post-SCI colon tissue sections, we determined that there was no overt sign of inflammation due to no overt immune cell infiltration, no thickening of organ walls, and no loss of goblet cells (Wirtz et al., 2007) (Figure 7). The sections from each condition look nearly identical to each other, suggesting a lack of physical change in the colon at that early of a timepoint following SCI. The sample size is much smaller than it should be, but if it the trend holds true then this would suggest that any changes in target expression, especially in the 1 day
post-SCI condition, may be inflammation independent. More specifically, even though we are seeing differences in gene expression, it is not mimicked by morphological changes in the colon within the first 24 hours after injury. More subjects and more time-points needed to be assessed to make any solid conclusions.

Discussion

We successfully analyzed a set of differentially expressed pain-related genes following SCI. The pattern of differentially expressed genes differed both as a result of injury condition and over time following SCI. In the severe injury condition, \textit{Gfra2} increased in expression at 7 days post-SCI compared to 1 day post-SCI while \textit{Trkc} had sustained decreased expression following SCI. In the moderate injury and sham conditions, we detected decreases in both and \textit{Gfra1} and \textit{Gfra3}, but decreased \textit{Asic2} expression only in the sham condition. Additionally, there were effects of specific injury condition on expression of several genes at 7 days post-SCI including \textit{Gfra1}, \textit{Gfra3}, \textit{Trkb}, \textit{Asic1}, and \textit{Calc\alpha} with moderate injury condition demonstrating a pattern of higher expression compared to severe injury condition. These findings are important first steps in identifying potential injury- and time-sensitive therapeutic targets for better precision medicine in treating post-SCI visceral pain.

We detected a significant increase in \textit{Gfra2} expression at 7 days following SCI in the 70kD, 10 second dwell time condition. Signaling between the GDNF family receptors and their respective ligands (GFR\alpha1-GDNF, GFR\alpha2-Neurturin, GFR\alpha3-Artemin) are essential for neurodevelopment in the enteric, autonomic, and sensory systems (Balogh et al., 2000). Aside from their promotion of neural survival and proliferation, the GDNF family receptors have also emerged as important players in the realm of pain research. Previous findings indicate that these receptors are transcriptionally regulated by their respective ligand and, therefore, a feedback loop
exists that helps to ensure a change in the neurotrophic factor ligands corresponds to a change in neurotrophic response (Airaksinen & Saarma, 2002). Therefore, it is likely that in the context of the GDNF family receptors, their differences in transcription would be mimicked by differences in translation. In the context of other pain-associated pathologies, such as human pancreatic cancer, increased neural GFRα2 is associated with increased severity of pain in patients (Wang et al., 2014). Further, in models of inflammatory pain, GFRα2 knockout mice had a deficient inflammatory pain response, but normal responses to innocuous stimuli, suggesting an important role for GFRα2 in pathological pain responses (Lindfors et al., 2006). Based on these previous studies, we would predict that the increased Gfra2 expression found in the colon could contribute to an increased visceral pain phenotype if translated into protein. This would mean that the colon would become more sensitive to the effects of neurotrophic factors, neurturin specifically.

In the moderate injury condition (65kD, 1 second dwell time), there were significant decreases of both Gfra1 and Gfra3 early following injury with Gfra1 maintaining low expression at the 7 day mark, but Gfra3 recovering so that it was no longer significantly different from naïve. Similarly, sham animals also demonstrated decreased expression of both of these transcripts. Antisense oligodeoxynucleotides have been used against Gfra1 mRNA causing an inhibition of induced muscle hyperalgesia (Alvarez et al., 2012). If this result was true for other pain models, it may suggest that decreased Gfra1 may be analgesic in our SCI model. In a cutaneous inflammatory model, known to induce hyperalgesia, researchers reported increases in Gfra3 mRNA, suggesting that Gfra3 is likely important for the development of inflammation induced hyperalgesia (Malin et al., 2006). If more Gfra3 is correlated to increased pain, then it’s possible that decreased Gfra3 seen in our SCI model would be analgesic similar to Gfra1. Next steps in following up on these findings would be to make sure the differences in mRNA
translated to differences in protein and to examine the amount of each ligand present in the colon following SCI to see if they match the expression of their respective receptors. Decreases in the receptors could mean decreased colon sensitivity to the influence of their neurotrophic factor ligands. Protein levels for the receptors and ligands could be assessed with Western blots or enzyme-linked immunosorbent assays (ELISAs) making sure to assess both injury conditions and the same time points used in the present study.

We were also able to detect a consistent decrease of Trkc expression following SCI in the 70kD, 10 second dwell time condition. Signaling between the Trk family and their neurotrophic ligands (NGF-TrkA, BDNF-TrkB, NT-3-TrkC) is important for proper neural development and nociceptor function (Khan & Smith, 2015; Jankowski & Koerber, 2010; Shu et al., 1999). TrkC has specifically been shown to be expressed in both the enteric nervous system of the colon and sensory afferents (Lamballe et al., 1994; McMahon et al., 1994). Pain research involving TrkC and its ligand NT-3 have had conflicting results with some reporting anti-nociceptive effects in nerve injury and neuropathic pain models, but others showing pro-nociceptive effects in different conditions like colitis models (Jankowski & Koerber, 2010). In a model of skeletal pain, researchers demonstrated analgesic effects of a Trk inhibitor (Ghilardi et al., 2011). Although not TrkC specific, this finding could suggest that decreased Trkc in our SCI model would be anti-nociceptive. Future experiments should examine protein levels of TrkC and its ligand NT-3 following SCI at the same time points as this study using either Western blots or ELISAs.

Multiple significant differences in gene expression were seen in sham animals including reductions of aforementioned Gfral and Gfra3, but also Asic2, known to be involved in detecting changes in pH, visceral mechanotransduction, and modulating pain responsiveness following inflammation (Lingueglia, 2007; Staniland & McMahon, 2012). How could a sham
surgery with no actual spinal cord damage cause any changes in pain-related genes in the colon? It is possible that the act of removing bone and bleeding in that area triggers a cascade of events in the spinal cord that could cause changes to the nearby dorsal root ganglia or central terminals of the sensory afferents. If there are changes to colon-specific DRG, which happen to be right below the surgery site, it’s possible that transcriptional effects in the cell body could also be seen down in the nerve terminals within the colon. Skeletal biology studies have demonstrated a release of pro-inflammatory mediators, such as prostaglandins, following bone fracture and pinning (Dekel et al., 1981). As mentioned earlier, pro-inflammatory mediators can increase sensitivity of sensory neurons (Woolf & Salter, 2000). If this is true, then even in a sham injury condition, the sensory afferents could experience changes in pain-related gene expression that corresponds to the gene differences we found in the present study. To further investigate this hypothesis, immunohistochemistry could be performed on sections of sham condition spinal cords and DRG to assess for the presence of pro-inflammatory mediators. Further, a different sham model without any bone removal could be used to determine if this would prevent the pain-related gene expression changes we see in the current sham model.

We found significant effects of injury condition on expression of Gfra1, Gfra3, TrkB, Asic1, and Calca in the colon at the 7 day post-SCI time point. In all cases, expression was higher at the 65kD, 1 second dwell time injury than the 70kD, 10 second dwell time injury. If mimicked by more protein, there would be an increase sensitivity to neurotrophic factors, particularly GDNF, Artemin, and BDNF, as well as increased sensitivity to pH changes, peptides like CGRP, and visceral mechanotransduction compared to the higher injury (Khan & Smith, 2015; Jankowski & Koerber, 2010; Shu et al., 1999; Han et al., 2015; Lingueglia, 2007). This finding could suggest that moderate injury conditions yield larger pain burdens, perhaps due to
more neural tracts left intact with lesser injuries. However, although we did see these differences in gene alterations, we cannot presently say that one injury is more severe than the other. Much of the current literature on SCI is performed in rats or primates, not mice. In order to say there was a difference in severity, a lesion reconstruction would have to be performed for both conditions and compared.

While detecting gene expression changes in the colon following SCI is a good first step in identifying potential candidates for visceral pain involvement, there are several limitations to the present study. Firstly, the study is lacking any sort of assessment of a functional or behavioral outcome. We at least know that bowel function is maintained in SCI animals, however since we’re curious about a visceral pain phenotype, a good way to determine if it is present in our mouse SCI model would be to do colorectal distension (Christianson & Gebhart, 2007). This method measures the colon’s visceromotor response (VMR) via electromyograph (EMG) to specific pressures accomplished by inflation of a transanally inserted balloon. In models of other pathological abdominal pain phenotypes, animals typically demonstrate increased sensitivity to distension (Jones et al., 2007). Therefore, if SCI animals do have this phenotype, we would see increased VMR to distension pressures compared to naïve mice. We would at least test the two different injury levels and the time points used in this study, however it would be ideal to extend the time frame as far as 30 days or more as visceral pain can often develop at later times following injury (Finnerup & Baastrup, 2012).

An additional limitation is that we only used female mice in the present study. This means that our results may not generalize to males with SCI. Although not SCI specific, there are some differences in pain processing between males and females which may mean there could be differences in expression of pain-related genes in peripheral tissues (Berkley, 1997).
It would also be important to determine the specific source of the differences in expression. There are many cell types present in the colon including autonomic nervous system terminals, sensory afferents terminals, the enteric nervous system, circulating immune cells, endocrine cells, as well as the cell types that help make up the colon including epithelial cells, connective tissue, and muscle (Canadian Cancer Society; Buffa et al., 1978; Janig & Koltzenburg, 1991; Murray et al., 2004; Furness, 2008; Turner, 2009). It is possible that several of these could be contributing to changes in gene transcripts. By using immunohistochemistry to stain sections of the colon for our candidate genes and different cell type markers, I would be able to locate the most likely cell type causing the results we see. In situ hybridization could also be a useful tool to determine the specific cell type contributing to expression differences. For these experiments, we would have to collect colon from shams and both injury levels at the same time points used in the present study, with naïve mice serving as a control. This would provide us with a visual representation of expression differences in cell types within the colon between injuries and in time.

This study examined effects in the colon whole-tissue level, but not specifically how the colon sensory afferents may be changing due to SCI. There is a large possibility that the visceral pain phenotype seen in SCI patients is due to alterations in visceral afferent activity. Painful stimuli sensitive afferents, called nociceptors, transmit pain signals coming from the colon and other organs throughout the body (Basbaum et al., 2009). Nocicpetors are made up of two types of neurons: Aδ fibers which are myelinated and faster transmitting that are likely responsible for the initial sting of pain and C-fibers, which are small, unmyelinated, slow transmitting neurons responsible for poorly localized, later onset pain (Basbaum et al., 2009). C-fibers an also be divided into two subgroups, peptidergic and non-peptidergic, which are known to be mostly
polymodal (both heat and mechanically sensitive), but variation does exist (Basbaum et al., 2009). Peptidergic fibers release peptides in response to stimulation (i.e. CGRP, Substance P) and are TrkA expressing (Basbaum et al., 2009). Non-peptidergic fibers do not express peptides and are both isolectin B4 (IB4) and c-Ret, a receptor responsive to GDNF family ligands, expressing (Basbaum et al., 2009). Using retrograde labeling, researchers have determined that the distribution of these subclasses in the colon specifically is approximately 80% peptidergic (CGRP expressing) and 20% non-peptidergic (IB4 expressing) (Robinson et al., 2004). By taking advantage of retrograde dyes injected into the colon, we could investigate what is happening strictly at the sensory afferent level through either immunohistochemistry or single-cell quantitative RT-PCR. To use single-cell quantitative RT-PCR, we would inject retrograde dye into the colon specific for the non-peptidergic (IB4) and peptidergic (WGA) populations, collect and dissociate the appropriate DRGs (T12/T13-L1, L5/6-S1), use fluorescent microscopy and a micromanipulator with electrodes to visualize and pick-up the backlabelled cells of different sizes, extract their RNA, and create a panel similar to the one in this study to see if any pain-related transcripts are different following SCI. It would be important to begin with this exploratory method because the significantly different genes in the colon may not be the ones changing in the afferent cell body. This method would allow us to see what genes are differentially expressed on a nociceptor-type specific basis to see which type may be contributing more to the visceral pain phenotype following SCI.

Once candidate genes are established, immunohistochemistry could be used to determine whether the differences in mRNA are mimicked by differences in protein. To do this, I could use the same retrograde dyes used in single-cell, collect, fix, and section the appropriate DRGs, and co-stain for the identified candidate genes. To go a step further, I could see if there were any
differences in candidate expression between the two sensory inputs for the colon, the splanchnic nerve and the pelvic nerve, as past studies have demonstrated that they differ in level of mechanical sensitivity, chemical sensitivity, and receptor expression (Brierley et al., 2004; Brierley et al., 2005). In vitro colon-nerve preps could also be used to assess any differences in physiological activity of these afferents that could contribute to post-SCI visceral pain (Shinoda et al., 2009).

The present study is a first step in the process of identifying the source of the visceral pain following SCI. We demonstrated that a central injury like SCI can lead to peripheral effects in uninjured tissues. Even in assessing fifteen pain-related genes, we were able to detect time- and injury-dependent differences in expression. While there are many other targets to test and many next steps, the overarching goal is to one day create more specific and directed treatments for post-SCI pain in order to minimalize negative side effects from blanket-style drugs.
Table 1. Pain-related target genes, the forward and reverse primers used, and the PCR product size.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Forward and Reverse Primer Sequences (5’→3’)</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid sensing ion channel 1</td>
<td>Asic1</td>
<td>F: CAG-GCC-AGC-TCT-CCA-ATC-TC</td>
<td>100 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACG-TAC-ACA-GGG-TAC-CTG-CC</td>
<td></td>
</tr>
<tr>
<td>Acid sensing ion channel 2</td>
<td>Asic2</td>
<td>F: GCA-CCT-GTG-GAG-GAA-GTA-CG</td>
<td>111 bp</td>
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<tr>
<td></td>
<td></td>
<td>R: CCC-GCC-CCA-AA-AAT-AAT-CAG</td>
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<tr>
<td>Acid sensing ion channel 3</td>
<td>Asic3</td>
<td>F: GCA-ACA-CTC-TGC-TCC-AGG-AA</td>
<td>135 bp</td>
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<td></td>
<td></td>
<td>R: CGA-GGT-AA-AGG-TAC-GGT-GG</td>
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<tr>
<td>Calcitonin-related polypeptide alpha</td>
<td>Calc-α</td>
<td>F: TGA-CAG-CAT-GGT-TCT-GGC-TT</td>
<td>122 bp</td>
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<td>R: GTC-ACC-AGA-GCA-CAG-GAG-AG</td>
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<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>F: ATG-AAT-AGC-GCT-ACA-GCA-ACA-GG</td>
<td>105 bp</td>
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<td></td>
<td></td>
<td>R: CTC-CTG-CTC-AGT-GTC-CTT-GCT-GT</td>
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<td>GDNF family receptor alpha 1</td>
<td>Gfra1</td>
<td>F: GAC-TGC-GAA-TCC-AGC-CTA-GG</td>
<td>149 bp</td>
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<td></td>
<td></td>
<td>R: TCT-GCA-CTC-GTC-CTC-TGC-CTG</td>
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<td>GDNF family receptor alpha 2</td>
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<td>102 bp</td>
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<td>R: AGG-AGA-AGA-GAG-GGG-CAA</td>
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<tr>
<td>GDNF family receptor alpha 3</td>
<td>Gfra3</td>
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<td>117 bp</td>
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<td>Purinergic receptor P2X 3</td>
<td>P2x3</td>
<td>F: GGT-GCC-TAA-GCC-TCT-TCT-AG</td>
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<td>R: AGG-GAT-GGC-GCT-GAG-CTA-AC</td>
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<tr>
<td>Purinergic receptor P2Y 1</td>
<td>P2y1</td>
<td>F: GCC-AGG-ACA-CTA-ACC-CAT-CG</td>
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<td>R: AAC-TGA-AGG-CCC-ACA-AAC-CTC</td>
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<td>Piezo-type mechanosensitive ion channel component 2</td>
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<tr>
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<td>Trka</td>
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<td>138 bp</td>
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<td></td>
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<td>R: GAT-GCT-GGC-CAT-GAA-GCA-AG</td>
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<tr>
<td>Tyrosine receptor kinase B</td>
<td>Trkb</td>
<td>F: AAG-ATG-TCT-CCC-TGG-GCT-TC</td>
<td>77 bp</td>
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<td></td>
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<td>R: AAG-ATG-GTC-AGT-CAG-TC</td>
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<tr>
<td>Tyrosine receptor kinase C</td>
<td>Trkc</td>
<td>F: CTG-CTC-CCC-AGT-GTC-GTA-AG</td>
<td>149 bp</td>
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<td></td>
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<td>R: GGG-AGG-ATG-GAA-AAG-ATG-AGG-TC</td>
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<td>Transient receptor potential ankyrin 1</td>
<td>Trpa1</td>
<td>F: TGA-GCC-ACA-TGA-CAG-AAG-TCC</td>
<td>164 bp</td>
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<td></td>
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<td>R: CTA-AGC-AGC-GAG-GAC-GTG-TGC</td>
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<tr>
<td>Transient receptor potential vanilloid 1</td>
<td>Trpv1</td>
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<td>R: TCA-TCC-ACC-CTG-AAG-ATT-GC</td>
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**Figure 1. Changes in mRNA expression of the GDNF family receptors following SCI.**

**a.** For the severe injury condition (70kD, 10 second dwell time), we found a significant difference in expression between naïve, 1 day post-SCI, and 7 days post-SCI for Gfra2 ($F(2,10) = 5.502, p = 0.024$), but not Gfra1 or Gfra3 ($F(2,10) = 3.661, p = 0.064$; $F(2,10) = 4.059, p = 0.051$, respectively). Tukey’s HSD post-hoc analysis demonstrated elevated Gfra2 expression at 7 days post-SCI compared to 1 day post-SCI ($p = 0.020$).

**b.** Moderate injury condition (65kD, 1 second dwell) had significant differences in expression of both Gfra1 and Gfra3 ($F(2,10) = 6.460, p = 0.016$; $F(2,10) = 6.112, p = 0.018$, respectively), but not Gfra2 ($F(2,10) = 2.050, p = 0.179$). Tukey’s HSD post-hoc analysis determined that compared to naïve mice, both 1 day and 7 days post-SCI animals had decreases in Gfra1 expression ($p = 0.026$; $p = 0.019$, respectively), but only 1 day post-SCI animals showed a decrease for Gfra3 ($p = 0.016$). Sham surgery had significant changes in both Gfra1 and Gfra3 expression ($F(2,11) = 11.431, p = 0.002$; $F(2,11) = 11.028, p = 0.002$, respectively). Tukey’s HSD post-hoc showed that 1 day sham and 7 day sham animals both had decreases in Gfra1 expression ($p = 0.003$ for both) and Gfra3 expression ($p = 0.003$; $p = 0.005$, respectively) compared to naïve.

**d.** We found no significant differences in Gfra1-3 expression at 1 day post-SCI between the two different injury conditions ($F(1,7) = 0.659, p = 0.444$; $F(1,7) = 2.212, p = 0.181$; $F(1,7) = 1.476, p = 0.264$, respectively). There was a significant effect of injury condition on expression of both Gfra1 and Gfra3 ($F(1,9) = 5.546, p = 0.043$; $F(1,9) = 5.664, p = 0.041$, respectively), but not Gfra2 ($F(1,9) = 2.015, p = 0.189$). There was more Gfra1 and Gfra3 expression in the moderate injury condition (M = 0.31, SD = 0.09; M = 0.49, SD = 0.31, respectively) than the severe injury condition (M = 0.18, SD = 0.09; M = 0.19, SD = 0.04). (* means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$).
Figure 2. Changes in mRNA expression of the tyrosine kinase receptors following SCI. a) Severe injury condition had a significant alteration of TrkC expression ($F(2,10) = 11.581$, $p = 0.002$), but not of Trka or TrkB ($F(2,10) = 0.915$, $p = 0.432$; $F(2,10) = 0.297$, $p = 0.749$, respectively). Tukey’s HSD post-hoc analysis showed that, compared to naïve, 1 day post-SCI and 7 day post-SCI animals both had decreased expression of TrkC ($p = 0.005$; $p = 0.003$, respectively). b) Moderate injury condition did not demonstrate any significant changes in expression of Trka, Trkb, or TrkC ($F(2,10) = 1.713$, $p = 0.229$; $F(2,10) = 3.870$, $p = 0.057$; $F(2,10) = 0.273$, $p = 0.767$, respectively). c) Sham surgery did not demonstrate any significant changes in expression of Trka, Trkb, or TrkC ($F(2,11) = 0.453$, $p = 0.647$; $F(2,11) = 0.627$, $p = 0.552$; $F(2,11) = 2.623$, $p = 0.117$, respectively). d) At day 1 post-SCI, there were no significant differences in expression of TrkA-c ($F(1,7) = 0.050$, $p = 0.830$; $F(1,7) = 3.260$, $p = 0.114$; $F(1,7) = 1.868$, $p = 0.214$, respectively). e) At 7 days post-SCI, there was no significant effect of injury level on Trka or TrkC expression ($F(1,9) = 3.626$, $p = 0.089$; $F(1,9) = 3.825$, $p = 0.082$, respectively), but there was on Trkb expression ($F(1,9) = 6.395$, $p = 0.032$). Trkb expression was higher in the moderate injury condition ($M = 45.46$, $SD = 40.29$) than the severe injury condition ($M = 3.81$, $SD = 5.75$). (* means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$).
Figure 3. Changes in mRNA expression of the transient receptor potential channels following SCI. a) Severe injury condition demonstrated no significant changes in expression for either *Trpv1* or *Trpa1* ($F(2,10) = 1.256, p = 0.326$; $F(2,10) = 0.562, p = 0.326$, respectively). b) Moderate injury condition did not have any significant changes in *Trpv1* or *Trpa1* expression ($F(2,10) = 1.633, p = 0.243$; $F(2,10) = 1.246, p = 0.329$, respectively). c) Sham treatment had no significant changes in expression of *Trpv1* or *Trpa1* either ($F(2,11) = 0.255, p = 0.780$; $F(2,11) = 2.761, p = 0.107$, respectively). d) There was no effect of injury condition on expression of *Trpv1* or *Trpa1* at 1 day post-SCI ($F(1,7) = 0.263, p = 0.624$; $F(1,7) = 0.302, p = 0.600$, respectively). e) There was no effect of injury condition on expression of *Trpv1* or *Trpa1* at 7 days post-SCI ($F(1,9) = 3.255, p = 0.105$; $F(1,9) = 3.733, p = 0.085$, respectively).
Acid Sensing Ion Channels

**Figure 4.** Changes in mRNA expression of the acid sensing ion channels following SCI. 

- **a)** There were no significant changes in expression of *Asic1-3* in the severe injury condition ($F(2,10) = 1.233$, $p = 0.332$; $F(2,10) = 0.716$, $p = 0.512$; $F(2,9) = 1.089$, $p = 0.377$, respectively). 
- **b)** Moderate injury condition did not have any significant changes in expression of *Asic1-3* ($F(2,10) = 3.140$, $p = 0.087$; $F(2,10) = 1.125$, $p = 0.363$; $F(2,9) = 1.152$, $p = 0.359$, respectively). 
- **c)** Sham surgery had no significant changes in expression of *Asic1* and *Asic3* ($F(2,11) = 0.135$, $p = 0.875$; $F(2,10) = 0.452$, $p = 0.649$, respectively), however, *Asic2* expression was significantly altered ($F(2,11) = 12.241$, $p = 0.002$). Tukey’s HSD post-hoc analysis determined that, compared to naive, 1 day sham and 7 day sham both had decreased levels of *Asic2* ($p = 0.001$; $p = 0.016$, respectively). 
- **d)** There was no effect of injury condition on expression of *Asic1-3* at 1 day post SCI ($F(1,7) = 1.106$, $p = 0.328$; $F(1,7) = 0.790$, $p = 0.404$; $F(1,7) = 0.644$, $p = 0.449$, respectively). 
- **e)** Injury condition did significantly affect expression of *Asic1* at 7 days post-SCI ($F(1,9) = 14.186$, $p = 0.004$), but not *Asic2* or *Asic3* ($F(1,9) = 0.743$, $p = 0.411$; $F(1,9) = 1.896$, $p = 0.202$). *Asic1* expression was greater at the moderate injury level ($M = 2.47$, SD = 1.16) than the severe injury level ($M = 0.65$, SD = 0.28). (* means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$).
Figure 5. Changes in mRNA expression of the purinergic receptors following SCI. a) Severe injury condition did not have any significant changes in P2y1 or P2x3 (F(2,10) = 0.880, p = 0.445; F(2,10) = 1.179, p = 0.347, respectively). b) Moderate injury condition did not display any significant changes in P2y1 or P2x3 expression (F(2,10) = 0.638, p = 0.548, F(2,10) = 0.854, p = 0.455, respectively). c) Sham surgery also did not have any significant changes in P2y1 or P2x3 gene expression (F(2,11) = 0.238, p = 0.792, F(2,11) = 0.641, p = 0.545, respectively). d) There was no effect of injury condition on expression of P2y1 or P2x3 at 1 day post-SCI (F(1,7) = 1.018, p = 0.347; F(1,7) = 3.266, p = 0.114, respectively). e) There was no effect of injury condition on expression of P2y1 or P2x3 at 7 days post-SCI (F(1,9) = 0.036, p = 0.854; F(1,9) = 0.112, p = 0.746, respectively).
**Figure 6. Changes in mRNA expression of Piezo2 and Calc-α following SCI.**  

a) Severe injury condition had no significant changes in Piezo2 or Calc-α expression ($F(2,10) = 1.662, p = 0.238$; $F(2,10) = 1.784, p = 0.218$). b) Moderate injury condition did not display any significant changes in Piezo2 or Calc-α expression ($F(2,10) = 0.676, p = 0.530$; $F(2,10) = 0.638, p = 0.548$, respectively). c) Sham surgery also did not have any significant changes in Piezo2 or Calc-α gene expression ($F(2,11) = 2.084, p = 0.171$; $F(2,11) = 0.772, p = 0.485$, respectively). d) There was no effect of injury condition on expression of Piezo2 at 1 day post-SCI ($F(1,7) = 0.818, p = 0.396$) or Calc-α ($F(1,7) = 2.040, p = 0.196$). e) There was no effect of injury condition on expression of Piezo2 at 7 days post-SCI ($F(1,9) = 0.274, p = 0.614$), but there was of Calc-α expression ($F(1,9) = 6.832, p = 0.028$) (Figure 6). Calc-α expression was greater at the moderate injury condition ($M = 4.95, SD = 4.31$) compared to the severe injury condition ($M = 0.40, SD = 0.18$). (* means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$).
Figure 7. Lack of colon inflammation 1 day following SCI using hematoxylin and eosin stain. Cross sections of colon from naïve and greater injury severity 1 day post-SCI animals in order to assess for the presence of inflammation following SCI. There is no difference between the different conditions and, therefore, no inflammation early after SCI.
References


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