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**GENE EXPRESSION ANALYSIS OF NEUROTROPHINS, TRK  
RECEPTORS, AND ASSOCIATED REGULATORY MOLECULES  
AFTER CONTUSIVE SPINAL CORD INJURY**

By

Matthew Tyler Hougland

B.S. University of Louisville 2007

M.S. University of Louisville 2010

A Dissertation

Submitted to the Faculty of the  
School of Medicine of the University of Louisville  
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for the Degree of

Doctor of Philosophy

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University of Louisville  
Louisville, Kentucky

August 2013



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## **ABSTRACT**

# **GENE EXPRESSION ANALYSIS OF NEUROTROPHINS, TRK RECEPTORS, AND ASSOCIATED REGULATORY MOLECULES AFTER CONTUSIVE SPINAL CORD INJURY**

Matthew Tyler Houglund

August 12, 2013

Traumatic spinal cord injury (SCI) results in changes to the anatomical, neurochemical, and physiological properties of cells in the central and peripheral nervous system. Neurotrophins, acting by binding to their cognate Trk receptors on target cell membranes, contribute to modulation of anatomical, neurochemical, and physiological properties of neurons in sensorimotor circuits in both the intact and injured spinal cord. Neurotrophin signaling is associated with many post-SCI changes including maladaptive plasticity leading to pain and autonomic dysreflexia, but also therapeutic approaches such as training-induced locomotor improvement. Here we characterize expression of mRNA for neurotrophins and Trk receptors in lumbar dorsal root ganglia (DRG) and spinal cord after two different severities of mid-thoracic injury and at 6 and 12 weeks post-SCI. There was complex regulation that differed with tissue, injury severity, and survival time, including reversals of regulation between 6 and 12 weeks, and the data suggest that natural regulation of neurotrophins in the spinal cord may continue for months after birth.

Our assessments determined that a coordination of gene expression emerged at the 12 week post-SCI time point and bioinformatic analyses address possible mechanisms. Additionally, we sought to determine if the regulatory patterns we observed were perhaps due to an inflammatory molecule that mediated the coordinated expression pattern that we observed at 12 weeks post injury, and identified the chemokine CCL2 as a potential candidate gene.

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## **CHAPTER 1**

### **GENERAL INTRODUCTION**

#### **1.1 Spinal cord injury**

Estimates of the global occurrence of spinal cord injuries (SCI) are in the range of 100000 to 300000 new cases reported each year (Barbeau H 1999). Many factors contribute to the spontaneous recovery (or lack thereof) observed in patients with SCI as a result of changes in the injured spinal cord, with many functional aspects of daily life being negatively affected. In particular, locomotion is among the most common and debilitating of these. Limited independent mobility is associated with lower life satisfaction and quality of life post-SCI, and enhancing sensory and locomotor capability of patients with SCI has a dramatically positive effect (Putzke JD 2002).

Traumatic injury to the spinal cord results in a variety of changes not only to neurons within the spinal cord but throughout the entire sensorimotor circuitry. Sensory neurons of the dorsal root ganglia (DRG) rapidly undergo long-lasting changes in their electrophysiological properties and growth capacity (Bedi SS 2010; Bedi SS 2012; Walters 2012). Locomotor circuitry in the spinal cord caudal to an injury site undergoes plasticity at the cellular, synaptic, and connectivity levels in an activity dependent manner after injury in humans and experimental models (Edgerton VR 2004; Rossignol S 2006; Petruska JC 2007). One strategy to restore function after spinal cord injury (SCI) is physical therapy and/or locomotor rehabilitation training (Wernig A 1995).

## **1.2 Locomotor rehabilitation training and activity dependent plasticity**

Factors that determine the functional outcome after training include both the severity and location of the injury. Based on these factors, some patients with incomplete SCI can improve walking ability after rehabilitation training (Wernig A 1995). A similar degree of improvement has not been shown in motor complete injuries, but several weeks of treadmill training can increase the load bearing capacity of clinically complete patients (Dietz V 1995). These changes can partially be attributed to a reorganization of sensorimotor circuits. For example, (Grasso R 2004) showed that in ASIA A/B patients (complete motor loss) and ASIA C/D patients (incomplete motor loss) with body weight supported treadmill training (BWST), levels of activation of motor neuron pools show drastic spatiotemporal changes when compared with controls (healthy subjects) (Figure 1), though foot placement was roughly equal between all groups. These and other studies (Beres-Jones JA 2004, Ivanenko YP 2003, for review see Dietz V 2002) highlight the ability of the injured human spinal cord to produce rhythmic patterns that are modulated with training.

A degree of locomotor capability can also be restored after training in animal models. After transection, cats trained to perform bipedal stepping of the hindlimbs improve on the basis of two behavioral criteria. Trained cats step at speeds six times faster and can produce 30-50 more successful steps during an evaluation session than non-trained cats. (Figure 2) During the swing phase, electromyography (EMG) burst amplitude increased in the tibialis anterior (TA, an ankle flexor) and decreased in the vastus lateralis (VL, a knee extensor) and medial gastrocnemius (MG, an ankle extensor),

similar to what is seen in uninjured cats. This finding is consistent with evidence in humans that shows after treadmill training, EMG amplitude in the MG is increased during the stance phase, and improper co-activation of the TA during the stance phase is decreased (Dietz V 1995). The differences seen in EMG activity were also reflected by differences in kinematics; vertical displacement of the ankle was higher in the step-trained cats, at the end of stance the hindlimbs extended further, and the swing phase projected further forward at placement in the trained versus untrained cats (de Leon RD 1998). When stand training is provided to transected adult cats for 12 weeks, the load bearing capacity of the hindlimbs increased. At the end of the 12 week training period, cats that received stand training were able to maintain full weight-bearing hindlimb extension for an average 545 s longer than cats that were untrained. Longer periods of standing are indicative of an enhanced ability to activate hindlimb extensors and thus maintain load-bearing extension at the knee and ankle joints. To test whether the characteristics of stepping were retained when the task is no longer practiced, EMG amplitude and kinematic assessments were done 6 and 12 weeks after the cessation of training. After 6 weeks, the hindlimb circuitry retained the characteristic patterns that were seen at the cessation of training, however by 12 weeks these acquired characteristics were lost. Despite this lack of task specific retention, when the animals were re-trained after the 12 week period, they acquired the ability to stand four times faster than with initial training (De Leon RD 1999).

Further evidence for reorganization of the sensorimotor circuitry after injury comes from rat models. In adult rats after contusion injury, treadmill training leads to restoration of kinematic stepping parameters seen in the normal state (Heng C 2009).

Treadmill training can not only compensate for the decrease elicited by a neonatal transection (assessments done in adult rats), but results in larger than normal EPSPs (a measure of the synaptic input onto a motoneuron), and reverses the SCI induced increase in AHP depth (part of what determines firing frequency of a neuron based on a given input current) (Petruska JC 2007). In the same model, step training enhanced the locomotor capability of rats, and this enhancement was not due to regrowth of either ascending or descending axons across the site of the lesion (Tillakaratne NJ 2010).

In the isolated lumbar spinal cord, reorganization of sensorimotor circuits can essentially occur in either the afferent neurons (DRG), interneurons in the spinal cord, or the motor neurons themselves (as mentioned above). Monosynaptic connections are made onto interneurons that project both ipsilaterally and contralaterally from group Ib and II afferents (Jankowska E 2009), and are important for the pattern generating characteristics of sensorimotor circuitry. These interneurons reside within laminae V-VIII and can be glutamatergic, glycinergic, or GABA/glycinergic (Bannatyne BA 2009). Following injection of the glycinergic inhibitor strychnine, transected adult cats were trained to either step or stand for twelve weeks, and stepping ability was then assessed after thirty minutes. The cats trained to step showed no difference in stepping ability before or after administration of strychnine, however the cats that were trained to stand were not initially able to step, but after injection were able to regain full weight-bearing stepping. The group of cats that were trained to step for the first twelve week period, were then re-trained to stand for twelve weeks and likewise the cats that were initially trained to stand were then re-trained to step for twelve weeks. Following this additional twelve weeks of training, the cats re-trained to stand (that were first trained to step)

showed similar results to the initial twelve week stand trained group in that they were not able to step initially but regained the ability after administration of strychnine. Similarly, the cats that were re-trained to step for twelve weeks lost their responsiveness to strychnine with results analogous to the step trained group after the first twelve weeks (de Leon RD 1999). Collectively, these studies demonstrate the potential for sensorimotor circuits in the lumbosacral spinal cord to develop and retain physiological information in an activity dependant manner after training.

### **1.3 Neurotrophins, Trk receptors and SCI**

The neurotrophins Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), and Neurotrophin 3 (NT3) are secreted growth factors that were first characterized for their important role in the survival of subpopulations of sensory neurons and in formation of spinal cord sensorimotor circuits during development (Barbacid M 1995; Lindsay RM 1996; Huang EJ 2001). In addition to these essential roles in establishing the physiological patterns of developing neural circuitry, neurotrophins are implicated as having a role in activity dependent changes associated with restoration of function after spinal cord injury.

When uninjured rats are trained for five days and sacrificed at different time points after training, BDNF levels increase two hours post training in the spinal cord and at all time points in the soleus muscle. NT3 shows a similar expression pattern to BDNF, with elevated levels two and six hours after training in the cord and at all time points in soleus muscle (Gomez-Pinilla, 2001). NT3 and its high affinity receptor TrkC were further analyzed at three and seven days after voluntary wheel running. NT3



mRNA and protein levels in the lumbar spinal cord were elevated both three and seven days after voluntary exercise. In the soleus muscle, NT3 mRNA and protein levels were elevated at all points. TrkC mRNA was elevated in the cord significantly at both time points, and protein after three days (Ying Z 2003). These studies suggest a similar pattern of regulation for the neurotrophins in the uninjured cord, but also provide a basis for how their regulation changes after injury.

Using spinal isolation (SI), which eliminates all supraspinal and afferent input to lumbar spinal cord, both NT3 and BDNF were down-regulated in the lumbar region bereft of input. In the same study, when botulinum toxin was injected unilaterally into the muscle, eliminating efferent output to the ipsilateral muscle but leaving the sensory input intact, NT3 expression was increased in the ipsilateral hemicord compared to the contralateral hemicord, while BDNF was decreased (Gómez-Pinilla F 2004). In the lumbar cord after hemisection, rats were placed in either a trained group or sedentary group and their levels of neurotrophins were analyzed at several time points. BDNF levels of trained rats decreased after injury and were restored after training, while NT3 stayed the same as controls after injury and with training expression levels were significantly elevated after 28 days (Ying Z 2005).

Rats with a moderate mid-thoracic contusion subjected to three different types of exercise (treadmill, stand, and swim) show altered expression levels of the neurotrophins BDNF and NT3 in both the spinal cord and soleus muscle (ankle extensor). As seen with hemisection, BDNF expression in the cord decreased after injury, but all forms of exercise restored expression to levels seen in controls. In the soleus muscle, only the group with treadmill training returned to pre-injury levels. Levels of NT3 expression

were elevated in both the cord and soleus muscle with all the exercise regimes (Figure 3) (Hutchinson KJ 2004). Delivery of exogenous BDNF and NT3 to the transected spinal cord improves recovery of hindlimb function (Blits B 2003)(in rats) and results in a level of function similar to that seen in animals receiving locomotor training after spinal transection (Figure 4) (Boyce VS 2007)(in cats). In light of the demonstrated and suggested roles in modulating sensorimotor physiology, characterizing the endogenous regulation of neurotrophins and their receptors after injury is particularly relevant.

#### **1.4 Neurotrophins and Physiology**

Neurotrophins have key roles in modulating the anatomical, neurochemical, and physiological properties of cells in the central and peripheral nervous system. The effects of neurotrophins on responses to stimuli in both the intact and injured nervous system have been extensively investigated and studies have demonstrated an important role in modulation of sensorimotor physiology (Huang EJ 2001; Huang EJ 2003; Reichardt LF 2006; Skaper SD 2008; Skaper SD 2012). The neurotrophins have therefore become a frequent target for manipulation after injury.

Physiological and behavioral studies have established NGF as a nociceptive agonist. NGF administration to neonatal, juvenile, and adult rats over the course of 9 weeks results in a decrease in both mechanical and thermal withdraw latency, indicated hypersensitivity to both types of stimuli (Lewin GR et al. 1993). These findings are also extended to the single cell level, evident by the finding that nociceptive DRG neurons responsive to capsaicin are sensitized upon application of NGF (Shu and Mendell 2001).

After subcutaneous injection of BDNF and NT3 into neonatal rats, an enhancement in the response of motoneurons to increasing intensities of dorsal root stimulation after BDNF was seen in the polysynaptic component, whereas no change is observed in the monosynaptic component. In contrast, after NT3 injection, the monosynaptic component showed the greatest increase, with an increase in the polysynaptic component also observed to a lesser degree. Additionally, four treatments were administered; BDNF, TrkB-IgG (blocks BDNF signaling), NT3, and TrkC-IgG (blocks NT3 signaling). TrkB-IgG and NT3 both increased the average amplitude of the EPSP, while TrkC-IgG and BDNF had the opposite effect (Figure 5) (Seebach BS 1999). These results indicate that the levels of these neurotrophins have potentially different effects following postnatal injection, in that NT3 potentiates the monosynaptic component of the EPSP and to a lesser degree the polysynaptic component, while BDNF enhances the exclusively the polysynaptic component.

These results are also consistent with the notion that TrkB activation has an effect on plasticity through facilitation of GABAergic transmission (i.e. interneuron) (Pezet S 2002), TrkB ligands have a suppressive effect on mechanosensory plasticity in the deafferented spinal cord (Ramer LM 2007), and have been shown to hypersensitize neurons of the superficial dorsal horn receiving high threshold primary afferent input (Garraway SM 2003), highlighting the broad physiological role of TrkB ligands in the function of the nervous system.

When the distal stump of the MG nerve in axotomized cats is placed in NT3, they exhibit Ia EPSPs 2.5 times normal, and 5 times larger than the axotomized that are left untreated. Conduction velocity of group I afferents in treated animals recovered to

normal values (Munson JB 1997). Removal of NT3 four to five weeks later results in a subsequent decline in both EPSP and conduction velocity within one week to values observed in the axotomized group, while NT4/5 (a TrkB ligand) causes a limited improvement in conduction velocity with no effect seen in the EPSP (Mendell LM 1999). This is also the case for chronic application of NT3, as fibroblast implants into the lumbosacral cord enhanced the EPSP from both central and segmental inputs (Arvanian VL 2003). Additionally, cultured dorsal root ganglion cells allowed to grow in BDNF and NT3 conditioned media from AAV infected HEK cells exhibit robust neurite outgrowth (Blits B 2003). Taken together, these studies suggest that neurotrophins can support both anatomical and physiological forms of plasticity

### **1.5 Neurotrophins/Trks expression in sensorimotor circuits after injury**

Neurotrophins influence cellular processes by binding to membrane-bound receptors which transduce the extracellular signal into intracellular effect – their high affinity tyrosine kinase receptors. In general, NGF binds TrkA, BDNF binds TrkB, and NT3 binds TrkC (Barbacid M 1995; Patapoutian A 2001; Huang EJ 2003), although cross-talk is recognized and there is a low-affinity pan-neurotrophin receptor p75, which we do not consider here. To determine the role of neurotrophins in any process or condition one must examine not only the neurotrophins, but also the receptors.

Prior characterizations of changes in neurotrophins and Trk receptors in lumbar neural circuitry have been instrumental in elucidating the complex regulation of these important molecules after injury (Table 1). However, these have largely focused on time

points of less than 6 weeks (Hayashi M 2000; Liebl, Huang et al. 2001; Liebl DJ 2001; Nakamura M 2001; Widenfalk J 2001; Qiao and Vizzard 2002; Qiao L 2002; Gulino R 2004; Zvarova, Murray et al. 2004; Qiao and Vizzard 2005; Qiao LY 2005; Qian DX 2006; Li XL 2007; Hajebrahimi, Mowla et al. 2008; Hajebrahimi Z 2008; Qian, Zhang et al. 2011; Qian DX 2011; Keeler BE 2012). Although valuable for elucidating the role of neurotrophin signaling in the first 6 weeks after SCI, these data are of uncertain value for relating to longer-term post-SCI function. Given the many demonstrations of continued changing conditions after SCI (Beattie, Hermann et al. 2002; Profyris, Cheema et al. 2004; Ung, Lapointe et al. 2008; Beck, Nguyen et al. 2010), it is important to recognize that the temporal character of experiments has a significant influence on the outcome.

## **1.6 Inflammation and SCI**

Another important aspect of SCI that affects cellular processes and influences how the nervous system responds to injury is inflammation. In humans after SCI, neutrophils, macrophages, and lymphocytes enter the cord through hemorrhage or extravasation, inducing a robust microglial response (Fleming, Norenberg et al. 2006). This cellular infiltration acutely after traumatic injury results in an upregulation of inflammatory factors in endogenous cells of the nervous system (neurons and microglia) such as IL1beta, IL6, and TNFalpha (Yang, Blumbergs et al. 2004). In rats, early transient increases in IL1beta, IL6 and TNFalpha are injury severity dependent, and as is the case in humans, is a result of upregulation of expression in neurons and microglia, not blood borne leukocytes (Yang, Jones et al. 2005). The acute early release of these cytokines leads to induction of their mRNA, as mice lacking the receptors for TNFalpha

and IL1 exhibit a diminished level of expression of these molecules (Pan, Ni et al. 2002). However, this expression pattern is transient, and returns quickly to levels seen in controls (Streit, Semple-Rowland et al. 1998).

In addition to these cytokines, many others have been identified as being upregulated after SCI in rodents such as IL-6, IL-1 $\alpha$ , IL-1 $\beta$ , IL-13, CCL2, MIP1 $\alpha$ , RANTES, and TNF $\alpha$  (Stammers, Liu et al. 2012). This finding can be extended to humans in the case of IL-6, IL-8, and CCL2, and has even been suggested as being a relevant biomarker for injury severity and subsequent recovery, as they were able to better predict the outcome of motor recovery 6 months after injury than the ASIA score (Kwon, Stammers et al. 2010). CCL2 has been shown to be upregulated in many models of peripheral nerve injury, and has a role in the development of pain after peripheral nerve injury (Tanaka, Minami et al. 2004; Jeon, Lee et al. 2008; Bhangoo, Ripsch et al. 2009; Jeon, Lee et al. 2009; Foster, Jung et al. 2011). Similar to IL1beta, induction of CCL2 expression can be accomplished through the actions of TNFalpha (Jeon, Sung et al. 2011). TNFalpha has also been shown to lead to hyperalgesia in models of peripheral inflammation, through regulation of both NGF (Woolf, Allchorne et al. 1997) and BDNF-TrkB (Lin, Ro et al. 2011). Thus, in studies investigating SCI and neurotrophin regulation, examination of cytokines and other markers of inflammation could prove to be instrumental in uncovering novel interactions that govern expression of plasticity related molecules.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2. Materials and methods

All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville, Louisville, KY. Experimental animals were 7 week old female Sprague–Dawley rats (Taconic Labs, Hudson, New York). Animals were housed in pairs throughout the course of our experiments.

##### 2.1 Surgical spinal cord injury

Rats ( $n = 47$ ) were anesthetized with 50mg/kg sodium pentobarbital (Sigma, St Louis, MO). Once sedated, Lacquer Lube was applied to the eyes to prevent drying. After skin incision, laminectomy was performed at vertebral level T9, to expose the T10 spinal cord. Contusion injuries were produced using the New York University (NYU) Impactor. Either “Moderate” or “Moderately severe” injuries were produced by releasing a 10g, 2mm rod from 12.5 mm or 25 mm height, respectively, onto the exposed dura mater of the spinal cord. These will subsequently be referred to as 12.5gcm and 25 gcm injuries. Immediately after producing the contusion the wound was closed in layers and the skin incisions were stapled. Rats received fluids (10cc 0.9% saline subcutaneously), and antibiotic treatment (0.1cc Gentamicin (50mg/mL) intramuscularly, and Bacitracin was topically applied on the incision site). Animals were housed overnight in a recovery

room with a heating pad under their cage, and were taken to the animal facilities in the morning.

Assessment of mRNA expression in SCI animals was compared to control animals.

These consisted of naïve rats (2 per time point group) and rats receiving laminectomy only (3 rats per time point group), for a total of 5 controls per time point. There were 4 additional laminectomy-only control rats included with the animals used for the 6 week post-SCI DRG assessment. All surgical procedures (except for the spinal cord injury), were as described above for the laminectomy-only control rats.

## **2.2 Injury Characterization**

### **2.2.1. Behavior**

Experiments were performed on rats separated into groups based on injury severity, survival time, and the tissue to be analyzed for mRNA expression. Rats were familiarized with the testing procedures and personnel by handling for 1 week before injury. Pre-surgical behavioral assessments were done to ensure no pre-existing conditions were present that would subsequently affect our locomotor outcome measures. Seventeen rats received 12.5g-cm NYU (moderate) and sixteen rats received 25 g-cm NYU (moderately severe) injuries. Hindlimb locomotor function was assessed with the Basso, Beattie, and Bresnahan Locomotor Rating Scale (BBB) (Basso DM 1996). BBB testing was carried out prior to injury and 7, 14, 21, 28, 35, 42 for the six week spinal cord group, and 7, 14, 21, 28, 35, 42, 49, 56, 63, 72, 79, and 84 days post injury for the twelve week spinal cord (SC) groups, twelve week dorsal root ganglia (DRG) group, and



at 7, 14, 28, and 42 days post injury for the six week DRG group. For testing, rats were placed in an open field (a plastic tank that was 105 cm in diameter with 30 cm high walls) for 4 min. BBB testing was done after animal care in the morning. Hindlimb movement and locomotion were scored simultaneously by two observers who were blind to the treatment groups. We include the BBB measures as a means to characterize the injuries with commonly used assessments so that the mRNA measures can be placed in context.

### **2.2.2. Histology**

At the end of the testing period, rats were anesthetized with sodium pentobarbital and euthanized via transcardial perfusion with 30% RNA Later (Qiagen) in 0.1M Phosphate Buffered Saline (PBS). An approximately 10 mm long block of spinal cord containing the injury epicenter was removed from each animal and immersed in 4% paraformaldehyde. After 1 week cords were immersed in PBS containing 30% sucrose for cryoprotection until further processing. For sectioning, tissue was embedded in TissueTek® (VWR) and frozen. The blocks were cut 50µm thick in the transverse plane on a cryostat and were sampled every 250µm. A series of sections spanning the rostrocaudal extent of the lesion was stained with eriochrome cyanine (EC) to assess amounts of spared myelin as described (Rabchevsky AG 2007) . Light microscopy was used to determine spared white matter. Images were captured using a SPOT digital camera (Diagnostic Instruments) mounted on a Zeiss Axioskop. From these, the area of spared tissue was manually designated (Intuos drawing tablet; Wacom (Otone, Japan)). Areas of white matter sparing were calculated using the ImageJ program and expressed as a proportion of control (defined as group-mean of the smallest white matter area from an analogous

section of spinal cord from all control animals). For each injured animal, the spinal cord injury epicenter was defined quantitatively as the section containing the least amount of intact tissue. Percent white matter sparing is reported as mean ( $\pm$  standard deviation). As with the BBB, we include the WMS measures as a means to characterize the injuries with commonly used assessments so that the mRNA measures can be placed in context.

### **2.3. mRNA expression**

#### **2.3.1. Isolation and cDNA conversion**

Animals were euthanized after final behavioral assessments and exsanguinated by transcardial perfusion using 30% RNA later (Qiagen) in Phosphate Buffered Saline (PBS). Lumbar spinal cords (L4/5) and Dorsal Root Ganglia (DRG) were removed and immersed in 100% RNA later and stored at  $-20^{\circ}\text{C}$  until further processing. Spinal cords were homogenized on ice in 1mL Trizol and RNA was isolated using Trizol/chloroform extraction method. Briefly, homogenate was transferred to a 1.5mL tube and spun at 12000g for 10 min at  $2^{\circ}\text{C}$ . The supernatant was transferred to a new tube and 200uL chloroform added. This mixture was spun for 15 min at  $2^{\circ}\text{C}$  to separate into aqueous and organic phases. The aqueous phase was transferred to a new tube and alcohol precipitation was performed with 100% isopropanol, then 70% ethanol. After removal and drying of excess ethanol, the pellet was resuspended in 30uL nuclease free  $\text{H}_2\text{O}$ , solubilized in 600uL Buffer RLT with beta-Mercaptoethanol (BME) and processed through RNeasy MiniKit (Qiagen) per manufacturers protocol. DRGs were homogenized

directly in Buffer RLT + BME and processed through RNeasy MiniKit. RNA was analyzed by Nanodrop (ThermoScientific, Waltham, MA, USA) to obtain concentration and 500ng of RNA from each sample was reverse transcribed into cDNA using Quanta Biosciences qScript cDNA SuperMix. All RNA was converted to cDNA using the same lot of reverse transcriptase. Performing the reverse-transcription for all samples with the same reagents is a methodological procedure meant to reduce the cross-sample variability which in turn can enhance the reliability of statistical assessments.

### **2.3.2. qRT-PCR**

mRNA expression levels were quantified by qRT-PCR on Corbett Research 6000 (Qiagen) using FastStart Universal SYBR Green Master Mix(Roche). Duplicate reactions were run for each sample for both the gene of interest and the normalizer (Beta-3 Tubulin – demonstrated as a suitable normalizer gene for SCI work (Strube C 2008). Relative expression levels were calculated as  $\Delta\Delta CT$  of gene of interest vs. normalizer. Primer sequences for the genes analyzed are provided in Table 2, along with their relationship to the known gene structure and transcript species.

### **2.4. Statistics**

Statistical analyses were performed using SPSS (IBM, North Castle, NY, USA) or SigmaPlot/SigmaStat (Systat Software, San Jose, CA, USA). A Student's t-test was performed to determine if expression levels differed between control groups. In cases

where gene expression did not differ between control groups the six and twelve week control groups were combined and the expression values for the experimental groups are reported as a fold change of the unified control group. One-way analysis of variance (ANOVA) was performed on these values with post-hoc Tukey's test for all pairwise comparisons. All groups with  $p < 0.05$  difference are reported as significant. Pearson Product Moment was calculated to determine the relationships between the expression levels of the different transcripts, and to determine the relationships between BBB/WMS vs. expression levels. Differences between BBB scores were assessed using a mixed model repeated measures ANOVA with a post-hoc Bonferroni t-test.

## CHAPTER 3

### EXPRESSION OF NEUROTROPHINS AND TRK RECEPTORS IN SENSORIMOTOR CIRCUITS AFTER DIFFERING CONTUSION SEVERITIES

#### 3.1 Introduction

The impact of SCI varies depending on the location of the injury itself and the spatial relation of the investigated tissue to the SCI. The relative composition of types of tissues innervated changes throughout the course of the neuraxis as does the specific function of local circuitry. For example, in rat, the spinal components of bladder control are focussed on the T13/L1 and L6/S1 segments, colon function is focused in L6/S1, and the locomotor central pattern generator appears focussed in (though not limited to) the L1/2 segments, spinal sympathetic circuitry regulating outflow exists roughly from T1-L2, and spinal parasympathetic circuitry exists in the sacral-caudal spinal cord. Thus it follows that the effect on spared function and/or recovery is influenced by the level of the injury (Magnuson, Trinder et al. 1999; Magnuson, Lovett et al. 2005; Garcia-Alias, Valero-Cabre et al. 2006), but this also extends to less direct functions (Campagnolo, Bartlett et al. 2000; Lucin, Sanders et al. 2007). It is also very important to consider that both neural and non-neural tissues remote from the SCI can be affected (Collazos-Castro, Soto et al. 2005; Massey, Hubscher et al. 2006; Gris, Hamilton et al. 2008).

Sensory input to the spinal cord plays a role in establishing recovery and regulating spinal function after alteration of descending inputs. For example, urinary

bladder function after SCI is highly reliant on afferent input and plasticity of sensory components (Tai, Roppolo et al. 2006; de Groat and Yoshimura 2009), and SCI affects the trk receptor profile of neurons in DRG segments innervating bladder differently than for DRG innervating hindlimb (Qiao and Vizzard 2002; Qiao and Vizzard 2005), a finding that extends to spinal trk receptors as well (Zvarova, Murray et al. 2004). Additionally, the type and amount of sensory input can influence spontaneous recovery after SCI (Grau, Washburn et al. 2004; Ollivier-Lanvin, Keeler et al. 2010; Caudle, Brown et al. 2011; Ferguson, Huie et al. 2012; Ferguson, Huie et al. 2012; Grau, Huie et al. 2012) and also influence the effectiveness of physical therapy (Bouyer and Rossignol 1998; Bouyer and Rossignol 2003; Edgerton VR 2004; Gomez-Pinilla, Ying et al. 2004; Edgerton, Courtine et al. 2008; Frigon and Rossignol 2009; Ollivier-Lanvin, Keeler et al. 2010), all of which may involve neurotrophin signaling (Gomez-Pinilla, Ying et al. 2004; Hutchinson, Gomez-Pinilla et al. 2004; Boyce, Tumolo et al. 2007; de Leon 2007; Côté MP 2011; Boyce, Park et al. 2012). Further, autonomic dysreflexia (AD), a maladaptive condition frequently observed in patients with cervical or high thoracic SCI, is often triggered by nociceptive sensory input (Maiorov, Fehlings et al. 1998; Krassioukov and Fehlings 1999; Garstang and Miller-Smith 2007), and sprouting of central terminals of nociceptive neurons, thought to be NGF-dependent, is a proposed mechanism that contributes to AD (Weaver, Cassam et al. 1997; Krenz, Meakin et al. 1999; Marsh, Wong et al. 2002; Cameron, Smith et al. 2006; Ackery, Norenberg et al. 2007).

Hence, it is important to examine not only the spinal cord, but also the sensory neurons that provide signals to the spinal cord, and to consider that the effects of SCI on the afferent neurons may differ in relation to the SCI, and/or to the tissues they innervate

(Qiao and Vizzard 2002; Zvarova, Murray et al. 2004; Bedi, Yang et al. 2010; Bedi, Lago et al. 2012; Keeler BE 2012). With these considerations in mind, it is important to consider that the spatial parameters of experiments, with regard to both the level of SCI and how the SCI affects the tissue investigated, can significantly influence the outcome. Injury severity, or more specifically the location and degree of damage to the SC, is another key factor that determines the functional capabilities of the spinal cord caudal to a SCI. The literature is replete with examples of this when reports are considered together, (Rossignol and Frigon 2011), however far fewer single studies examine multiple injury severities (Magnuson, Lovett et al. 2005; Smith, Burke et al. 2006), despite the fact that the degree of injury severity also significantly influences the outcome.

Thus, we initially sought to characterize natural regulation of neurotrophin and trk receptor genes in tissues and conditions that were most applicable to experimental studies of long-term function and recovery after SCI and to conditions most often represented in animal models and human studies. We characterized the transcriptional response of neurotrophins and their cognate Trk receptors to spinal cord contusion temporally (6 and 12 weeks post-injury), spatially (in lumbar spinal cord and DRG), and relative to injury severity (12.5 g-cm and 25g-cm NYU contusions).

## **3.2 Results**

### **3.2.1. Injury Characterization**

To assess the degree of injury severity, we characterized spinal cord injuries based on two parameters; behavior as measured by BBB, and the amount of spared white

matter at the epicenter after staining with eriochrome cyanin (Rabchevsky AG 2007). BBB scores were significantly greater in the 12.5 g-cm injury groups than the 25 g-cm groups beginning at week 5 (Fig 6a). These differences in behavior were reflected in the amount of spared white matter, as the 25 g-cm groups had 8.5% ( $\pm 1.8\%$ ) and the 12.5 g-cm groups had 13.9% ( $\pm 3.6\%$ ) spared white matter at the epicenter. In accord with prior literature (Basso DM 1996; Schucht P 2002; Magnuson DS 2005), a significant correlation ( $r=0.88$ ,  $p<0.001$ ) was observed between white matter sparing at epicenter and BBB scores (Fig 6b). BBB scores of the 12.5 g-cm group showed a high degree of variability and continued to increase between 6 and 12 weeks instead of reaching a plateau. Within this group, 2 animals had BBB scores consistent with the range observed in previous literature (Basso DM 1996; Agrawal G 2010) (12 and 13) and 4 animals that had higher BBB scores than expected for this injury severity (mean 17.9) at 12 weeks post injury. We considered that these results may be due to both greater amount of spared white matter (SWM) and/or asymmetry of the lesion (Fig 6c, 6d). Indeed, of the 4 animals whose BBB scores continued to increase, all had a greater amount of spared white matter (mean 16.1% for 4 animals with higher BBB scores, 10.1% for 2 animals with lower BBB scores), and all had asymmetrical injuries (arbitrarily defined as more than 4% greater SWM on one side versus the other). Animals with the lower BBB scores in the 12.5 g-cm group did not represent statistical outliers (Grubbs outlier test). Separate statistical analyses of gene expression were performed with the exclusion of the 2 animals whose BBB scores did not continue to increase and the results generally did not differ from those found when all 6 animals were considered together. The lone exception was the results for expression of one Trk receptor in the spinal cord, which is noted



below. We thus consider all 6 animals together in the group in all subsequent figures and analyses of mRNA expression.

### **3.2.2. Expression of Trk receptors in the DRG**

One purpose of this study was to determine whether these different contusion severities result in a differential transcriptional response of neurotrophins and their Trk receptors in lumbar sensorimotor circuits. Hence, we sought to determine the expression level of Trk receptors in the DRG 6 and 12 weeks after our two severities of contusion injury. Expression of TrkA, TrkB, and TrkC each differed significantly between the 6 and 12 week groups, with the magnitude and direction of difference depending on receptor type and injury severity. Expression of TrkA mRNA in DRG from the 12 week group at both injury severities was significantly greater than that in DRG from the corresponding 6 week group. Expression of TrkA in DRG from the 12 week group that received 12.5g-cm injury was also elevated relative to the control groups. We also observed a difference in TrkA expression between injury severities at the 12 week time point. Similar to TrkA, expression of TrkC mRNA in DRG from the 12 week group was greater than that in DRG from the corresponding 6 week group at both injury severities, but the difference only reached significance in the 12.5 g-cm animals. Unlike the findings for TrkA, we detected no significant difference in TrkC expression between DRG from the 12.5 g-cm group and from the 25 g-cm group at the 12 week time point. Expression of mRNA for TrkB in DRG at 12 weeks after 25 g-cm injury was significantly lower than in DRG from

both the 6 week SCI and control groups. No significant difference in TrkB expression was observed between injury severities at 6 or 12 week time points in the 12.5 g-cm injury group (Fig. 7).

### **3.2.3. Expression of Neurotrophins in the DRG**

As with TrkA, NGF mRNA expression in DRG from the 12.5g-cm injury severity group was significantly greater in the 12-week group than in both the 6 week and control groups. However, no significant changes in NGF expression were observed between survival-time groups in the 25 g-cm injury severity group. As with TrkB, BDNF expression in the 12 week 25 g-cm group was significantly less than in the 6 week 25 g-cm group, but did not differ from the control group (Fig. 8). No other differences were observed in BDNF expression levels. There was a large increase in the mean expression of NT3 in DRG from the 12 week, 12.5 g-cm injury group, however due to high variance no significant differences were observed from 6 to 12 weeks.

### **3.2.4. Expression of Trk receptors in the spinal cord**

Expression levels of mRNA for neurotrophin receptors TrkA, TrkB, and TrkC were assessed from samples of lumbar spinal cord (L4/5). In the groups that received a 12.5 g-cm injury, the level of TrkA in spinal cord from the 12 week group was

significantly greater than that from the 6 week group, whereas there was no significant difference between the two post-SCI times in the 25g-cm injury group. Like TrkA, the level of TrkC in spinal cord from the 12 week 12.5g-cm group was significantly greater than that from the 6 week group, with no significant difference between the two post-SCI times in the 25g-cm injury group. No significant changes in TrkB expression levels were detected between any groups (Fig. 9).

### **3.2.5. Expression of neurotrophins in the spinal cord**

The results for neurotrophins in the spinal cord are displayed differently from the data regarding expression levels of neurotrophins and Trk receptors in the DRG, and Trk receptors in the spinal cord. In the aforementioned assessments, the expression of neurotrophins and trks did not differ between the 6 week and 12 week control animals. Thus, those data were analyzed and presented relative to the mean and variation of a single unified control group. This allowed us to simultaneously assess the effect of both injury severity and survival time on gene expression. For the neurotrophin genes in spinal cord, however, expression differed significantly between the 6 week and 12 week control groups (Figure 5A). We first analyzed these expression data exactly as was done for the other tissues – comparing each injury severity and survival time to the mean and variation of a single unified control group – but for the sake of clarity we have presented the data from the individual animals in each group. Caution must be taken when considering the expression data for the experimental groups in this analysis (Figure 10A) because of the

use of a unified control group – i.e., these data were generated exactly as were the other expression values, but are relative to a unified control group that, in this case, is not a suitable control group. We found decreases between our 6 and 12 week control groups in expression levels of NGF, BDNF, and NT3 in the spinal cord in the absence of spinal cord injury. It is worth noting that our quality control measures were repeated for these samples, but the assessments remained the same. In ruling out technical issues and variability due to the necessity of using animals from different litters, a single factor appears to account for the altered expression levels in the control groups, that being age.

Because the gene expression differed between the 6 week and 12 week control groups, we cannot incorporate the temporal characteristic of the experimental design in our assessment of neurotrophin expression in spinal cord. We are limited to analyzing the effect of injury severity on gene expression within each separate survival time group, where the data from experimental groups is expressed relative to the time-matched control group only (Figure 10B). Considered in this way, spinal cord injury itself did not significantly influence expression of any neurotrophin at any time considered, with the exception of NT3 at 12 weeks post-SCI. At this time, NT3 was elevated relative to the time-matched control group, with no effect of injury severity.

### **3.2.6. Relationship of transcriptional assessments to functional and anatomical assessments**

Our experimental design was intended to embrace the variability that exists with models of contusive SCI in that we also examined whether a statistical correlation existed

between expression levels of each transcript and BBB or white matter sparing on an animal by animal basis. We observed no statistically significant correlation between the expression levels of the transcripts and BBB score or white matter sparing.

### **3.3 Discussion**

Spinal cord injury leads to many changes that affect both the central and peripheral nervous system, indeed the entire organism, with residual functional capacity largely dependent on the location and severity of the injury. Many approaches have been used in efforts to re-establish function, including (but not limited to) enhancement of regeneration across the injury site (Bregman BS 2002; Moon L 2005; Sharma H 2012; Smith GM 2012) and plasticity of intact circuits below the level of the lesion (Edgerton VR 2004; Boulenguez P 2009; Rossignol S 2011). One means for achieving plasticity of intact circuits is through activity-dependent reorganization of inputs (Edgerton VR 2004)). This phenomenon has been described in both animal (Edgerton VR 2008) and human (Harkema SJ 2008) studies of spinal cord injury. Neurotrophins have been associated as playing a key role in induction of such changes (Hutchinson KJ 2004; Boyce VS 2007; Côté MP 2011; Boyce VS 2012). However, activity-dependent changes in locomotion often manifest at times later than those examined in studies of post-SCI expression of neurotrophins and trk receptors (De Leon RD 1998; De Leon RD 1999); (Table 3). Indeed, the dynamic period of spontaneous locomotor recovery generally lasts for approximately 6 weeks after SCI, a time well beyond the majority of previous studies (Table 3). In addition to a putative role in locomotor function, neurotrophin signaling has

also been implicated in pathologic outcomes of plasticity such as post-SCI pain and autonomic dysreflexia (Brown and Weaver 2012). The role of neurotrophin signaling has primarily been examined in terms of initiation of these conditions acutely after SCI in animal models (Krenz, Meakin et al. 1999; Marsh, Wong et al. 2002; Cameron, Smith et al. 2006), as opposed to later-phase initiation or maintenance. The regulation we have demonstrated at extended time points may provide new rationale for examining the role of neurotrophin signaling in later stages of these conditions.

Neurotrophins exert modulatory effects on cellular physiology through activation of their cognate Trk receptors (Lindsay RM 1996; Patapoutian A 2001; Huang EJ 2003). In the DRG, expression of neurotrophin receptors is restricted to specific populations of cells. Generally, TrkA is expressed in neurons with small soma size, TrkB in neurons with intermediate size, and TrkC in neurons with large soma size; populations of TrkA and TrkC expressing neurons remain largely separate, whereas TrkB is co-expressed in overlapping populations of TrkA and TrkC positive cells (Mu X 1993; McMahon SB 1994; Wright DE 1995; McMahon SB 1996; Phillips HS 1996). Trk receptors are not ubiquitous, however, as there is a large subpopulation of small diameter DRG neurons which do not express any of the Trk receptors or the low-affinity neurotrophin receptor p75 in the adult (McMahon SB 1994; Molliver DC 1997; Bennett DL 1998). In the mammalian spinal cord, TrkA is expressed in second order nociceptors of the dorsal horn, TrkB has a broad pattern of expression which overlaps with both TrkA and TrkC expression, and TrkC is expressed in neurons of the intermediate and ventral horn (Duberley RM 1997; Curtis R 1998; Schober A 1999; Copray S 2000; Liebl DJ 2001); also available from: <http://mousespinal.brain-map.org>.

As long as 6 weeks after spinal cord transection injury, the number of cells expressing TrkA and TrkB protein in L1 and L6/S1 DRG (containing bladder afferents) increases over controls, though the numbers of cells expressing these genes does not significantly change in L4/5 DRG (Qiao L 2002; Qiao LY 2005). Our analysis of Trk expression, which was also performed in L4/5 DRG, found no significant change in the *trkA* or *trkB* mRNA levels for either severity of contusion injury at 6 weeks post injury, in agreement with the prior work. In intact sensory and sympathetic ganglia of the adult rat, NGF and NT3 (as well as TrkA, full length TrkB, and TrkC), localize exclusively to neurons; BDNF and the truncated isoform of TrkB are expressed more extensively, however, localizing to neuronal cells and some glial and satellite cells (Wetmore C 1995). These observations are consistent with the notion that full length Trk expression predominantly occurs in neurons, though since the latter study was performed with intact animals, we cannot exclude the possibility that our injuries potentially resulted in ectopic expression in other cell types. Indeed, there are numerous reports of *trk* receptor expression by non-neuronal cells. In particular Schwann cells can express *trks*, as can cancer cells (Funakoshi, Frisen et al. 1993; Tacconelli, Farina et al. 2005; Hess, Scott et al. 2007; Jin, Lee et al. 2011). Further, neurotrophins are often expressed in non-neuronal cells, most notably by cells outside the nervous system where they influence both developmental and adult processes (Lewin 1996; Petruska and Mendell 2004).

Previous assessments of changes in neurotrophin/Trk receptor expression levels after spinal cord injury have typically focused at time points of less than 6 weeks. BDNF expression increases up to two weeks after injury in the spinal cord after thoracic transection and crush injury (Hayashi M 2000; Li XL 2007), though both increases and

decreases in expression have been reported after hemisection during a similar time period post injury (Gulino R 2004; Qian DX 2006). Expression levels of NGF and NT3 in the cord increase for up to 3 weeks after spinal cord injury (Hayashi M 2000; Li XL 2007). In another study, NGF and BDNF transcripts were found to increase up to 4 days following injury in the adult cord, however, by 2 weeks post injury all neurotrophins were expressed at levels similar to that of control (Nakamura M 2001; Widenfalk J 2001), suggesting expression decreases after an early increase, though these studies used different injury models. Trk mRNA expression is downregulated acutely in the spinal cord at and around the injury site after contusion (Liebl DJ 2001; Hajebrahimi Z 2008), however by 6 weeks expression levels are not different from control (Liebl DJ 2001). However, after spinal cord transection TrkC has been shown to increase after two weeks (Qian DX 2011). Similarly, in a recent study assessing mRNA and protein changes after transection at 10 and 31 days post injury, whole spinal cord TrkB mRNA was elevated at 10 days post injury, and whole spinal cord NT3 and TrkB protein was elevated at 31 days post injury, with expression differences also observed depending on the location within the parenchyma of the spinal cord (Keeler BE 2012). Table 1 summarizes the findings of recent experiments to facilitate comparison of these results.

We found TrkA expression increases in both the DRG and spinal cord of animals after contusion in a manner that was dependent on injury severity. This finding is of particular interest with regards to the functions of NGF and TrkA. NGF plays a well-defined role in sensitization of nociceptive afferent neurons (Shu X 1999; Shu X 2001; Galoyan SM 2003; Zhu W 2004). Nociceptive DRG neurons undergo changes after SCI, including development of spontaneous activity (Bedi SS 2010) and an enhanced intrinsic



growth promoting state (Bedi SS 2012). Such changes in anatomical and physiological properties of nociceptors may contribute to development of conditions such as autonomic dysreflexia (Marsh DR 2002)). TrkA antagonists prevent the sensitization (thermal and mechanical hyperalgesia) normally induced by partial nerve injury (Ma WY 2010), and antagonism of TrkA signaling has been effective for controlling human pain (Mantyh PW 2011). Hence, elevation in the levels of TrkA and NGF in response to contusive injury could play a role in some of the maladaptive processes after incomplete SCI.

TrkB activation has also been implicated in hypersensitivity to nociceptive input and sensitization of nociceptors (Kerr BJ 1999; Shu XQ 1999; Garraway SM 2003). However, after either SC transection or contusion injury, BDNF induced facilitation of afferent responses in lamina II of the dorsal horn is significantly reduced (Garraway SM 2005; Garraway SM 2007). Our results could suggest a mechanism for those physiological observations. In addition to TrkB expression in populations of second order nociceptive neurons (Schober A 1999), it is expressed robustly throughout the interneuronal circuitry, and also co-expressed along with NT3 in motoneurons (Buck CR 2000), a finding corroborated in humans (Josephson A 2001). Notably, BDNF administration to the injured spinal cord can improve locomotor outcomes, however because of its influence on nociceptive circuitry its therapeutic utility may be limited (Boyce VS 2012).

In DRG, TrkC is present on medium to large diameter muscle spindle afferents that make monosynaptic connections with motoneurons and cutaneous low threshold mechanoreceptors (Klein R 1994; Oakley RA 1997; Josephson A 2001) in the intermediate and ventral horns of the spinal cord. NT3, likely acting via TrkC, can exert

a modulatory effect on sensorimotor circuits in both intact (Petruska JC 2010) and injured preparations (Mendell LM 2001; Arvanian VL 2003; Arvanian VL 2006; Arvanian VL 2006; García-Álías G 2011; Schnell L 2011). Locomotor training after spinal cord injury is associated with increased expression levels of TrkB and TrkC agonists in rats (Hutchinson KJ 2004; Côté MP 2011). In addition, co-administration of both BDNF and NT3 to the injury site has been shown to improve hindlimb locomotion after transection in both rats (Blits B 2003) and cats (Boyce VS 2007). Taken together, these findings suggest a potential role for Trk activation in modulation of lumbar sensorimotor circuitry in both intact and injured animals.

## CHAPTER 4

### NEUROTROPHINS AND AGING IN THE SPINAL CORD

#### 4.1 Introduction

The apparent age-related regulation of NGF, BDNF, and NT3 in non-injured spinal cord was unexpected and we made significant efforts to identify possible technical and sampling issues. While those factors that often account for variability did not satisfactorily account for the expression patterns we observed, the single factor of age did appear to fully account for the differences. Expression of the neurotrophins has been examined in the context of embryonic and postnatal development and in aging (e.g., (Timmusk, Belluardo et al. 1994; Nosrat 1998; Bergman, Ulfhake et al. 2000). However, to the best of our knowledge, there has been no systematic assessment of the regulation of the neurotrophins at such late postnatal times, leading us to postulate that perhaps there was a previously undocumented age related decrease in neurotrophin expression that persisted throughout the lifespan of the adult rat.

Much of the literature concerning neurotrophins and aging comes from assessments of expression levels in the forebrain. In the cortex and hippocampus, the mature form of NGF levels decrease relative to age in naïve animals, while BDNF levels remain unchanged (Perovic, Tesic et al. 2012). However, other studies that sampled multiple areas throughout the forebrain and also the sciatic nerve found no significant difference in levels of NGF, BDNF, or NT3. These changes appear to be region specific however, as increases in the levels of BDNF in the hippocampus, as well as increases in

levels of NT3 in the cerebral cortex have been documented (Katoh-Semba, Semba et al. 1998). Thus, we sought to determine whether the changes we observed from 6 to 12 weeks in the spinal cord were due to regional specificity within the spinal cord and whether these changes persisted throughout the lifespan of the adult rat

## **4.2 Results**

### **4.2.1 Expression of NGF, BDNF, and NT3 at 3, 6, 9, 12, and 15 months after birth in the uninjured rat spinal cord**

Unlike the decreases that we observed for all neurotrophins from 3 to 6 months in the adult rat, these changes did not persist throughout the course of adulthood based on further analysis at 9, 12, and 15 months after birth (NGF Fig 11, BDNF Fig 12, NT3 Fig 13). The only significant change in the levels for any of the neurotrophins was an increase in the levels of NT3 at 9 months after birth, above the levels of 3 and 6 month uninjured rats (Fig 13).

## **4.3 Discussion**

Though the observation that the trend of decreasing expression did not persist through the lifespan of the adult rat was somewhat surprising, there are several factors that provide rationale for the explain differences we observed. The animals we used at our 9 and 15 month time points had been used for the purpose of breeding and were housed at a different location. This could have resulted in several differences between these animals and the animals used for the 3, 6, and 12 month after birth groups. One was simply that the 9 and 15 month groups had been pregnant. During pregnancy, uterine

receptivity to implantation is dependent on levels of progesterone (Bazer, Burghardt et al. 2008). Progesterone plays a role in neuroprotection via regulation of BDNF (Su, Cunningham et al. 2012; Singh and Su 2013). Progesterone has also been shown to maintain levels of NGF (Tometten, Blois et al. 2005), thus a putative link between neurotrophins and hormones regulated during pregnancy is plausible. Another difference between the groups of rats were the environments in which they were placed. Additionally, environmental enrichment in the adult rodent nervous system has been shown to protect the brain from age-related dysfunction, which can be accomplished through induction of NGF (Mohammed, Henriksson et al. 1993) and BDNF (Cirulli, Berry et al. 2010) expression, and could also play a role in regulation of neurotrophin levels. Though these mechanisms might certainly be involved, the observation that no 12 and 15 month after birth groups differed in their expression levels (12 month animals were conditioned in a similar environment to the 3 and 6 month groups, and did not give birth) raises questions as to the validity of this argument.

## CHAPTER 5

### COORDINATED EXPRESSION OF NEUROTROPHINS AND TRK RECEPTORS IN DRG AT 12 WEEKS POST INJURY

#### 5.1 Introduction

12 weeks after injury there were analogous increases in the NGF and NT3 from the expression levels we observed at our 6 weeks time points. Since all assessments were obtained from samples that were derived from the same animals, we sought to determine whether these increases extended concomitantly within each sample. Thus, we next calculated the correlation coefficient between all neurotrophins(i.e. NGFvNT3, NGFvBDNF, BDNFvNT3), their Trk receptors(i.e. TrkAvTrkC, TrkAvTrkB, TrkBvTrkC), and between each neurotrophin ligand and its cognate receptor(NGFvTrkA, BDNFvTrkB, NT3vTrkC). Such interactions that regulate expression levels of different neurotrophins have been reported in different cellular and animal models previously (Leingärtner A 1994; Canossa, Griesbeck et al. 1997; Gratto and Verge 2003; Mallei, Rabin et al. 2004; Kuo, Groves et al. 2007), however none have investigated whether such patterns emerge between all neurotrophins and Trk receptors at more chronic time points after contusive SCI.

## **5.2 Results**

To further characterize the relationship between the neurotrophins and their receptors in lumbar DRG and spinal cord, we analyzed the expression levels of neurotrophins and Trk receptors relative to each other, and without respect for injury severity. In the spinal cord, the only significant relationship was that of TrkB and TrkC in the control and 6 week groups. No relationship was found between any other expression levels at any time points in the spinal cord (Table 2). In the DRG, there was a relationship between NGF and NT3 in all groups. In the 6 week groups the only other significant correlation observed was between BDNF and TrkB. After 12 weeks there was a significant correlation in the expression levels of all neurotrophins in the DRG, a relationship that existed for the Trk receptors as well (Table 3). Additionally, a significant correlation was observed between expression levels of neurotrophins and their cognate Trk receptors at 12 week time points (Table 3). This coordinated expression pattern occurred in all animals independent of injury severity (Figures 14 & 15). The reliability of this statistical assessment is enhanced by our performing the reverse-transcription for all samples with the same reagents, a procedure which reduces the cross-sample variability.

## **5.3 Discussion**

12 weeks post injury a coordinated expression pattern emerged when comparing all neurotrophins and Trk receptors to one another. This pattern was independent of injury severity, and was also present between the neurotrophins and their cognate Trk receptors in the DRG (Tables 1 and 2, fig's. 5 and 6), a relationship that was not evident at

6 weeks post-SCI, nor for expression levels in the SC. Although there are reports of smaller groups of neurotrophins and/or Trks being regulated in a coordinated fashion (Widenfalk, Olson et al. 1999), to our knowledge this degree of coordination has not been reported. One obvious possibility is a feedback/feedforward relationship between some/all of these genes, and these sorts of relationship do exist (Michael, Averill et al. 1997; Wyatt, Middleton et al. 1999; Gibbons and Bailey 2005).

Neurotrophin dependent neurotrophin expression has been demonstrated in vitro in NIH3T3 and PC12 cells (Canossa M 1997; Mallei A 2004), hippocampal neurons (Canossa M 1997), and cerebellar granule neurons (Leingärtner A 1994). In vivo, intrathecal administration of NT3 to intact adult animals for one week results in reduced expression of TrkA protein in the DRG, but has no effect on levels of TrkC (Gratto KA 2003). After unilateral axotomy, sub-cutaneous administration of exogenous NT3 similarly causes a decrease in TrkA on the side contralateral to the injury. This contrasts to the increase in TrkA expression seen on the side ipsilateral to the injury; the effect of NT3 on expression levels of TrkB and TrkC however is not affected by injury in this paradigm, as levels of these transcripts show increased expression up to 4 weeks post axotomy in both ipsi- and contralateral DRG (Kuo LT 2007). Such coordinated expression patterns could result from changes at the epigenetic level or from potentially from interactions between the different transcription factors associated with expression of specific transcripts.

In (Houglund et al. 2012), bioinformatic analyses were performed in an attempt to elucidate potential underlying factors involving miRNA and common transcription factors that may underlie the expression patterns that were observed between



neurotrophins/Trks. Though no common miRNA targets were revealed through a TargetScan analysis of 3'-UTRs, this was likely due to the search criteria inherent in the bioinformatic platform that was used. TargetScan only retrieves targets that have been experimentally confirmed, thus the absence of a finding likely reflects a lack of preceding investigations into the subject rather than simply a negative finding. Likewise, in searching for common transcription factors that regulate expression of these genes, no TF was retrieved that involved regulation of TrkC. This was due to stringent filtering processes that relegated all returned queries to those validated as being expressed in the nervous system (Houglund, Harrison et al. 2012). Despite the exclusion of TrkC as a result of this filtering process, TrkC has several well established transcription factors that play a role in determining its expression in the nervous system, notably; Runx3 (Levanon, Bettoun et al. 2002), Runx1, Brn3a, (Zou, Li et al. 2012) and REST (Nakatani, Ueno et al. 2005).

During development, Runx1 and Runx3 transcription factors play essential roles in cell fate determination of nociceptive (Chen CL 2006) and proprioceptive (Inoue K 2002) neurons, respectively. Much attention regarding transcriptional regulation of neurotrophin expression in the mature nervous system has been given to BDNF, due to its role in activity dependent mechanisms during long-term potentiation (LTP). Such investigations have revealed several important transcriptional regulators including; cyclic AMP response element binding protein (CREB), calcium-responsive transcription factor (CaRF), and methyl CpG-binding protein 2 (MeCP2) (Tao X 1998; Tao X 2002; Chen WG 2003; Reichardt LF 2006). Such findings may facilitate future efforts to determine

the mechanisms regulating the expression of the neurotrophins and Trk receptors in the injured adult spinal cord and sensory ganglia.

Despite the absence of TrkC from the analysis, (Houglund et al 2012) identified 4 TFs that potentially interacted with the entire set of remaining genes. The majority of literature regarding these TFs and their involvement in regulation of neurotrophins and Trks is in the context of development or cancer. Accordingly, the authors could not identify any studies examining Pax3, NeuroD, or MafB in spinal cord or DRG in the context of SCI. Maf has been studied in relation to neurodegeneration (Kobayashi, Tsukide et al. 2011) and in stress (Machiya, Shibata et al. 2007). Pax3 was studied in relation to nerve injury, where it was found to not be regulated (though this does not imply it not being active) (Vogelaar, Hoekman et al. 2004).

There are studies examining CREB in spinal cord (Canossa, Griesbeck et al. 1997; Copray and Kernell 2000; Crown, Ye et al. 2006; Cote, Azzam et al. 2011) or DRG (Qiao and Vizzard 2005) in the context of SCI, with the latter study examining the TrkA, TrkB, and CREB, though not in direct relation to each other. Interestingly, the expression of activated CREB in the DRG changed over the course of the first 6 weeks after SCI, with the levels at 6 weeks being significantly greater than controls, though not in the DRG we examined here. After a conditioning lesion to the peripheral nerve, enhanced regeneration of the central branches can be observed and is dependent on elevation of cAMP within the DRG. (Neumann, Bradke et al. 2002; Yang and Yang 2012) Other studies demonstrate induction of CREB in injured/stressed neurons and also in neurons post-synaptic to stressed sensory neurons (Ji and Rupp 1997; Bedogni, Pani et al. 2003; Choi, Kim et al. 2003; He, Csiszar et al. 2003; Zhu, Lau et al. 2004), while others

demonstrate CREB regulating multiple NTs (Bender, Lauterborn et al. 2001), in at least one case by interacting with cytokines (Otten, Marz et al. 2000).

Our data was derived from homogenized tissue, thus we cannot make conclusions about apparent coordinated expression on the basis of individual cells, though we can draw from a number of sources to make inferences about what may potentially be happening.

1) There is some evidence that at 6 weeks after SCI trk receptors are expressed almost exclusively in DRG neurons, similar to before the SCI (Qiao and Vizzard 2002; Qiao and Vizzard 2005). However, it must be noted that there is a wealth of evidence that suggest expression of NTs and Trks in non-neuronal cells (e.g., (Funakoshi, Frisen et al. 1993; Elkabes, Peng et al. 1998; Nemoto, Fukamachi et al. 1998; Noga, Englmann et al. 2002; Hess, Scott et al. 2007), although much of this is in the context of cancer (e.g., (Tacconelli, Farina et al. 2005; Howe, Cochrane et al. 2011; Jin, Lee et al. 2011). Studies which identify the cell types expressing the NTs or Trks are necessary as it is possible that at least a portion of the tissue-level regulation could be due to invading cells. Certainly the complement of immune cells in the spinal cord is affected by injury, even in segments spatially remote from the injury (e.g., (Popovich, Wei et al. 1997). Immune cells invade the DRG after nerve injury (e.g., (Nguyen, O'Barr et al. 2007; Vega-Avelaira, Geranton et al. 2009; Kim and Moalem-Taylor 2011), but this possibility has not been examined in DRG at any time after SCI. However, evidence suggests that the immune cells and their functions throughout the body may be affected by SCI (e.g.,

(Popovich, Stuckman et al. 2001), and some express Trk receptors and/or neurotrophins (e.g., (Noga, Englmann et al. 2002; Nassenstein, Kerzel et al. 2004; Tabakman, Lecht et al. 2004).

2) There are certainly studies which examine the chronic post-SCI condition, but we could not identify any that could provide data relevant to these specific considerations (i.e., they examined other readouts).

Almost irrespective of the outcome of the above considerations, there is still another consideration that can be brought to bear. Although there are papers describing co-expression of some of these genes in single cells where common genetic/molecular regulation could possibly be at play, it is highly unlikely that all the coordinated expression is accounted for by single cells. Even in the condition where expression is limited to neurons, and even to the same population of neurons that expressed these genes in the intact system (i.e., differences in expression would be based on volume regulation in any given cell and not on recruitment/de-recruitment of cell populations), what is the likelihood that this degree and scope of coordinated expression could occur across different cell types independently? It seems unlikely that each of the genes considered would change in a single cell type independent of its regulation in any other cell type, and still display such relatively similar expression levels. However, because there is little-to-no cellular expression data here or in the literature from which to extrapolate the identity of the cells expressing these genes (i.e., immunocytochemical or in situ hybridization assessment of DRG 12 weeks post-SCI), we must acknowledge that this is possible.

There is, however, virtually no reason to expect that individual cells would express all of

the “coordinated” genes and thus have the mechanism of coordinated regulation exist fully inside of those given single cells. Therefore, at least some of the coordination must arise *across* cells which express one or more of the “coordinated” genes, though whether this is due to the protein products of the genes themselves or an outside factor is unclear.

Additionally, there might also be a shared biological process(es) or response(es) being executed in various different cells – a process that has similar outcomes in terms of gene regulation but arrives there through actions of different specific molecular entities. For “simplicity”, let us consider that only the neurons of the DRG are involved. Even this cell population is not homogeneous in function, form, or sensitivity. Each of the Trk receptors is expressed largely in separate subpopulations. Given the dissimilarities of their regulatory sequences, they may each be directly regulated by distinct factors. However, conditions may arise that induce the non-homogeneous neuronal types, regardless of the specific trk they express (and thus which specific factors will act on the DNA and/or mRNA), to coordinately regulate the expression of their trk receptor. It is possible that the regulation of those specific factors may be under a control mechanism that is itself shared across the different neuron types. Our analysis would not detect this. As an example, consider cellular stress or injury. Numerous authors have reported on the regulation of Trks and neurotrophins in response to nerve injury, and the change in expression over time (Ernfors, Rosario et al. 1993; Sebert and Shooter 1993; Krekoski, Parhad et al. 1996; Yamamoto, Sobue et al. 1996; Bergman, Fundin et al. 1999; Lee, Zhuo et al. 2001; Kuo, Groves et al. 2007), and many aspects of our data agree with reported regulation after nerve injury or neuronal stress. Intriguingly, there was another

report of “coordinated regulation” associated with DRG neurons and glia in conditions of injury and/or stress (Cameron, Vansant et al. 2003).

Presently it is unclear whether SCI induces any long-term injury or stress on DRG neurons. Certainly the central axons of some DRG neurons are damaged in SCI, particularly those terminating in the affected cord, or with long axons ascending through the dorsal columns (Huang, Robson et al. 2006). However, effects of injury to central axons differs from those of injury to peripheral axons (Stam, MacGillavry et al. 2007), and the long-term effects on expression of neurotrophins and trk receptors has not been examined. Injury to central axons is not the only possible source of stress to sensory neurons, however. The inflammatory condition of the spinal cord and continued spread of damage may induce injury or stress in sensory neurons at times remote from the acute SCI, and at locations remote from the lesion (Popovich, Wei et al. 1997; Popovich 2000; Bao, Chen et al. 2004; Fleming, Norenberg et al. 2006; Gris, Hamilton et al. 2008; Kwon, Stammers et al. 2010; Bao, Brown et al. 2011; Lubieniecka, Streijger et al. 2011; Ng, Stammers et al. 2011; Stammers, Liu et al. 2012). There is a systemic inflammatory condition (Fleming, Norenberg et al. 2006; Gris, Hamilton et al. 2008; Bao, Omana et al. 2012) that has unknown effects on these neurons. Additionally, one must consider the effects of SCI on peripheral tissues innervated by sensory and motor neurons. The inflammation and altered activity/mobility/use state can impact these tissues (Edwards-Beckett and King 1996; Lynch, Anthony et al. 2000; Gris, Hamilton et al. 2008) with uncertain consequences for the innervating neurons. The increased expression of galanin, a neuropeptide induced in DRG neurons by stress/injury (Suarez, Guntinas-Lichius et al. 2006), in the DRG innervating bladder and bowel (but not other DRG) after SCI

(Zvarova, Murray et al. 2004) suggests that the histopathology secondary to SCI may stress the sensory neurons innervating those tissues. Tissue damage has been shown to induce stress/injury responses in sensory neurons (Ivanavicius, Ball et al. 2007; Hill, Harrison et al. 2010; Thakur, Rahman et al. 2012), and has been shown to affect regulation of multiple neurotrophins in the injured tissue (Vizzard 2000).

## CHAPTER 6

### EXPRESSION OF INFLAMMATORY MARKERS AFTER SCI

#### 6.1 Introduction

Cytokines such as IL-6 have been shown to induce expression of NGF, NT4/5, and NT3 from cultured astrocytes (Otten, Marz et al. 2000). In rodents after injury, IL-6, IL-1 $\alpha$ , IL-1 $\beta$ , IL-13, CCL2, MIP1 $\alpha$ , RANTES, and TNF $\alpha$  are elevated acutely in the spinal cord (Stammers, Liu et al. 2012). In humans, protein levels measured from CSF of injured patients indicate that assessment of some of the same molecules can serve as a biomarker for injury severity, including IL-6, IL-8, CCL2, tau, S100beta, and glial fibrillary acidic protein (Kwon, Stammers et al. 2010). Based on the data presented in the previous chapters, we deduced several scenarios that could be at play. It is possible that neurotrophins alone could be regulating their own expression and that of Trk receptors. We also considered, based on the data mentioned above, that a common factor or mechanism might be influencing expression, such as infiltration of immune cells expressing inflammatory cytokines, or simply cytokines endogenously expressed by cell types resident within the nervous system.

To investigate whether a cytokine/receptor or other inflammatory molecule is responsible for the expression patterns observed among the set of neurotrophins/Trks, we limited our targets to a specific (and partially overlapping) subset of factors that we felt represented a broad range of molecules involved in these processes. These included: IL6, LIF, and CNTF, a related set of ligands that bind the common GP130 co-receptor(a



molecule which was also included), the cytokine TNF $\alpha$ , iNOS, a protein that is induced to form Nitric Oxide as an immune defense mechanism, CD11b, an integrin present on the surface of leukocytes that mediates immune cell adhesion, Interferon gamma and the chemokine CCL2(aka MCP1).

## **6.2 Results**

### **6.2.1 Gene targets without appreciable expression levels**

Several of the genes we chose to investigate were not expressed to detectable levels in the DRG. All reactions were run in duplicate, and the criteria for detectable gene expression was when *more than one* of the samples(1 set of duplicates) within *more than one* of the 6 groups(6wk control, both 6wk injury groups, 12wk control, and both 12wk injury groups) failed to reach the Ct threshold for the gene of interest. The genes included in this list are: IL6, IL10, iNOS, and Interferon gamma (data not shown).

### **6.2.2 Gene targets whose controls were unchanged from 6 to 12 weeks**

Genes that were expressed at appreciable levels were divided into two groups depending on whether or not expression of their control groups changed significantly from 6 to 12 weeks post surgery. There were four genes that had no change in the control groups from 6 to 12 weeks; CD11b, LIF(Fig 16), TNF $\alpha$  in DRG and SC(Fig 17), and gp130. Of these four genes, only gp130 showed any significant changes. For gp130, in the 12.5gcm 12 weeks post injury, expression levels were elevated significantly above the control group, as well as both of the 25 gcm injury groups. In animals that received a 25

gcm injury, expression levels were reduced significantly from 6 to 12 weeks post injury.(Fig 18)

### **6.2.3 Gene targets whose controls changed from 6 to 12 weeks**

Genes that had changes in the expression of controls from 6 to 12 weeks were CNTF and CCL2. For CNTF, all 12 week groups showed a significantly increase relative to the 6 wk control and 6 wk 12.5 gcm groups. However, since controls increased from 6 to 12 weeks, it also helps to assess levels relative to time matched control. If this is done, the 6 wk 25gcm group is significantly above the 6 wk control group, while all other relative expression levels are not significant. (Figure 19) For CCL2, control expression levels decreased from 6 to 12 weeks, but in contrast the injury groups were substantially increased. (Fig 20)

Note: Figure 21 and 22 for discussion purposes

## **6.3 Discussion**

Interleukins are cytokines that exhibit a diverse array of effects across different cell types and tissues (Brocker, Thompson et al. 2010). Interleukins can exert both pro- and anti-inflammatory effects, and serve to activate and modulate differentiation of immune cells in response to inflammation (Commins, Borish et al. 2010). IL6 belongs to a family of cytokines that have a broad range of effects stemming from their various roles in inflammation, immune response, and cell survival and differentiation (Jazayeri, Carroll et al. 2010), and can be further characterized based on whether or not they are

biologically active as monomers or heterodimers. Those of the monomeric subdivision bind through the common co-receptor subunit gp130 (also known as IL6ST) (Boulay, O'Shea et al. 2003; Heinrich, Behrmann et al. 2003), which also includes (among others) LIF and CNTF. LIF signals through gp130 in combination with LIF receptor (LIFR) (Plun-Favreau, Perret et al. 2003), while CNTF signals through a heterotrimeric complex that includes both gp130 and LIFR as well as the CNTF receptor (CNTFR) (Stahl and Yancopoulos 1994).

Expression of IL6 can be stimulated through signaling complexes of other cytokines such as TNF $\alpha$ , IL1, and LIF, as well as in an autocrine fashion. This occurs through binding of nuclear factor IL6 (NF-IL6) to an IL1-responsive element within the IL6 promoter (Akira, Isshiki et al. 1992), and also activates transcription through phosphorylation of STAT3 tyrosine kinase (Akira 1997). In contrast to IL6, IL10 is biologically active as a homodimer and acts through the JAK/STAT pathway to suppress expression of pro-inflammatory cytokines, and is thus an important anti-inflammatory molecule (Murray 2007). These cytokines, along with the evolutionary related Interleukins in both of their family, are expressed by white blood cells in response to infection and injury (Dinarello and Mier 1986). In addition, CD11b is an integrin expressed on the cell surface of white blood cells that mediates adhesion and migration of leukocytes (Solovjov, Pluskota et al. 2005). They are important mediators of the immune response and are highly expressed after insult to the body, thus due to the lack of expression (or change in expression in the case of CD11b) we observed, it is unlikely that the infiltration of white blood cells that express these molecules into the DRG can explain the patterns of expression that were observed in our previous studies. Despite our data

suggesting that the IL6 cytokines that are biologically active as monomers and TNF $\alpha$  do not appear to be responsible for the expression pattern of neurotrophins 12 weeks post SCI, we did not investigate the IL6 cytokines biologically active as heterodimers, nor did we investigate other Interleukins or cytokines. Since these molecules comprise expansive families of molecules that mediate immune response in a wide variety of ways, our analysis is by no means exhaustive.

IL6, LIF, and CNTF have differing effects on the survival of cultured newborn rat DRG neurons. Specifically, both CNTF and LIF are able to promote survival of these neurons (Simon, Thier et al. 1995), and enhances neurite outgrowth in both large diameter and small diameter cells of the DRG, while IL6 does not (Sango, Yanagisawa et al. 2008). This finding however, is likely due to the IL6 receptor not being sufficiently expressed at this developmental stage, as supplying both the IL6 ligand and soluble receptor does support survival (Thier, Marz et al. 1999). In vivo, CNTF is expressed by Schwann cells, and after peripheral nerve injury is released to promote axonal regeneration and neuron survival. However, when primary DRGs are explanted and cultured, neurons begin to also express CNTF, which is localized to both the soma and dendrites (Sango, Yanagisawa et al. 2007). This could be due either to disruption of cell-cell contact, or potentially a response to injury by the explanted neurons. We observed that CNTF levels were elevated above that of controls 6 weeks post injury in a moderately severe (25 gcm) contusion. However, though the expression levels of both the 12 week control and injury groups are elevated above controls in the 6 week post injury groups, CNTF levels returned to that of controls by 12 weeks post injury *relative to the time matched control*. Thus, the increase in CNTF seen at 6 weeks does not persist,

and the further increase in CNTF expression above 6 week levels is likely only a function of aging, a finding that is supported in previous literature (Nakamura and Bregman 2001).

Our investigations of inflammatory markers that potentially influence expression of the neurotrophins and their Trk receptors targeted three groups of molecules that serve separate, yet overlapping functions: cytokines, interferons, and chemokines. Cytokines mediate growth, differentiation, and activation during an immune response. This contrasts with chemokines, which serve to direct immune cells to sites of inflammation via chemotaxis; and inteferons, which directs cellular responses to viral infections (Commins, Borish et al. 2010). Interferon gamma was the only interferon that was assessed, due to its important role in cell-mediated immunity (Farrar and Schreiber 1993), and had undetectable levels of expression. The only chemokine (White, Sun et al. 2005) assessed was Chemokine ligand 2(CCL2), also known as Monocyte Chemotactic Protein(MCP1). Expression of CCL2 was essentially the same as control at our 6 week time point, but at 12 weeks post injury had risen to approximately 8 times the level of the control group in both injury severities. CCL2 leads to hyperexcitability of DRG neurons both in culture and in vivo (White, Sun et al. 2005). Additionally, many models of neuropathic pain induce potent expression of CCL2, (Tanaka, Minami et al. 2004; Jeon, Lee et al. 2008; Bhangoo, Ripsch et al. 2009; Jeon, Lee et al. 2009; Foster, Jung et al. 2011) and knockout mice lacking its receptor CCR2 (Abbadie, Lindia et al. 2003), or pharmacological blockade (Serrano, Pare et al. 2010) impairs the development of neuropathic pain. This hyperalgesic response is due to upregulation of the current density

and expression of TRPV1 (Kao, Li et al. 2012) and Nav1.8 (Belkouch, Dansereau et al. 2011).

In the rodent and human spinal cord, CCL2, along with several other inflammatory cytokines, can be used as a correlative biomarker for injury severity (Kwon, Stammers et al. 2010; Stammers, Liu et al. 2012). We observed that CCL2 was upregulated in our 25 gcm group at 12 weeks post-injury. Though the discrepancy between our findings and that of the previous studies is unclear, there are a number of parameters that were different between the experiments. In the previous rodent studies, the time points assessed only extended to 24 hours post injury. Though a contusion injury was also used for the purposes of their studies, the injury was performed using the OSU impactor rather than the NYU device. As shown earlier, not only can the type of contusion effect the levels of expression but even the severity within the same type of injury model. These previous also assessed protein levels, whereas only mRNA was examined for the purposes of our study. In terms of the human study, protein levels were also examined, though from the CSF and the blood serum, and the time period was up to 72 hours post injury. These vastly different time points and methods of injury very likely contributed to the differences observed between the findings of these previous studies and our own.

## **CHAPTER 7**

### **GENERAL DISCUSSION**

Through our characterization of neurotrophins and their Trk receptors we found that from 6 to 12 weeks post-contusion there were differences in the mRNA expression levels of these molecules, and this difference was dependent on the severity of the contusion. Based on previous data that use different models of spinal cord injury and assess neurotrophin/Trk levels at various times (Table 1), the observation that there is an injury severity dependent difference in expression of individual transcripts is not surprising. This highlights the importance of characterizing any experiment in terms of injury model, time point, and injury severity. However, it nevertheless elicits the question of why the difference exists? One explanation is that there are different levels of activity below the level of the injury. It is feasible to think that differing levels of descending input (based on which fibers are spared) lead to concomitant changes to the motoneurons basal levels of excitability, perhaps through remaining monoaminergic synapses (Heckman, Lee et al. 2003; Anelli, Sanelli et al. 2007; Murray, Stephens et al. 2011). This could in turn result in tonic levels of muscle activity and subsequent levels of afferent activity that are affected relative to the amount of spared descending input.

Another possibility is that the nervous system is reacting to the injury by regulating certain plasticity-inducing factors. Operating under this notion, there are several scenarios that one could use to explain these observations, 1) such factors could be regulated in a manner meant to achieve the degree of homeostatic plasticity (aka

“normalcy”) that was present in the circuitry before the injury 2) such factors could be regulated based on the *capacity* for the circuitry to achieve a certain degree of homeostatic plasticity (that is, based on severity of the injury and how well the animal(s) recovered). If the former is the case, then one would expect that the further the animal is from how it functioned before the injury (i.e. more severely injured), the more highly a plasticity inducing molecule would have to be expressed to achieve the level of pre-injury function. If the latter is the case, then one would expect essentially that the higher the capacity for functional recovery (i.e. less severely injured), the higher the level of expression of these plasticity-inducing factors.

It is important to note, that the “factors” being referred to could either be the neurotrophins/Trks themselves and/or some other molecule that regulates expression of all the neurotrophins/Trks. If it is a single neurotrophin, the prime candidate would be NT3, as it is the only neurotrophin that has been shown to be able to bind to and activate all three Trk receptors (Davies, Minichiello et al. 1995). If it is all the neurotrophins, then it would likely be acting through the pan-neurotrophin receptor p75 (Dechant, Rodriguez-Tebar et al. 1994). There is also the possibility that it is not the neurotrophins acting through Trks or p75, but through a separate signaling mechanism that acts on all subclasses of neurons in the DRG that express neurotrophins/Trks and accordingly coordinates levels of expression, such as an inflammatory mediator. Indeed, inflammatory molecules such as TNF $\alpha$  and IL6 have been suggested to be associated with NGF (Woolf, Allchorne et al. 1997) and BDNF (Lin, Ro et al. 2011) mediated hyper excitability of DRG neurons in models of neuropathic pain. The observation that CCL2 is drastically upregulated at 12 weeks is particularly interesting, given that CCL2 can effect



DRG neurons of all sizes (i.e. large, medium, small) (White, Sun et al. 2005), which theoretically encompasses all subpopulations of neurons that express Trk receptors in the DRG. Finally, these putative interactions could be occurring synergistically, such that an external factor initiates a particular expression pattern which is then overtaken by neurotrophin/Trk mediated neurotrophin/Trk expression in a feed-forward manner, or vice versa.

Upon further analysis, by calculating the correlations between expression levels of the different neurotrophins and Trks in the context of each individual animal, we were able to uncover what appears to be a coordinated pattern of expression that exists among the neurotrophins and Trk receptors in the DRG (but not spinal cord) at 12 weeks (but not at 6 weeks) post injury. This pattern was *independent* of injury severity (i.e. expression values were correlated between all molecules regardless of whether the injury was 12.5gcm or 25 gcm). Furthermore, we investigated expression of cytokines as potential elements involved in the changes we observed, and identified elements that were regulated in either a time(i.e. CCL2) or injury severity dependent (i.e. gp130) fashion. Based on these assessments, future investigations into the cause(s) of initiation of these mechanisms will be better informed as to what might or might not be a useful starting point.

The finding that at 12 weeks there was a coordinated expression pattern in the DRG that was independent of injury-severity while certain transcripts increase (i.e. TrkA) from 6 to 12 weeks, but other transcripts decrease (i.e.TrkB), warrants further clarification. Why is this happening? More specifically, what is causing a different direction of change in one versus the other if they are eventually being regulated in an

analogous manner in the different constituent subpopulations of neurons? One possibility is that each set of neurotrophin/Trk receptor has an initial response to loss of descending input/alteration of afferent input acutely (i.e. TrkB increases, TrkA decreases), from which it is still “recovering” at 6 weeks, that by 12 weeks has been overtaken by a differing response. Such a response could be activity- or factor-dependent (or both) in such a way that modulates their expression in a manner that dictates when expression of one of these molecules (neurotrophin, Trk) is relatively low/high in the DRG of a certain animal, that the other molecules of their family (neurotrophin, Trk) is relatively low/high as well. It is also possible that both are active at the same time, with a more robust response that masks the effect of the other until the initial robust response becomes inactive and thus the other becomes more apparent.

The coordinated expression pattern can also be understood as a predictive factor that, given a set of data, one could deduce which animal expressed a certain level of a neurotrophin/Trk based on the expression level of another neurotrophin/Trk. For example, view the range of all injured animals at 12 weeks post injury and their TrkA expression values as lying on a spectrum from low to high. When compared with the highest value from NGF, BDNF, NT3, TrkB, and TrkC, the highest expression in 4 of 5 of these molecules are from the same animal as that for TrkA. Though it might be useful for one to view the coordinated expression pattern in this way to aid conceptualization, it is important to note that the predictive power of any given point is directly related to the correlation coefficient between two sets of molecules, and since none of the coefficients were 1, this is not an absolute predictor (as demonstrated above).

Due to the finding that control levels of certain genes changed between 6 and 12 weeks, plots were constructed for display purposes and to identify any robust trend that might have been occurring that our statistical analyses failed to elucidate. These analyses failed to uncover any novel findings, but served to demonstrate that there was a higher degree of variability in neurotrophin expression than Trk receptor expression (Fig 21 and 22). Subsequent power analysis was performed post-hoc to determine the ideal group size based on the average means and variability for all neurotrophins and Trk receptor transcripts assessed. These analyses revealed that had we used an n of 12 for all groups, we would perhaps have also found changes between groups relative to both injury severity and survival time in the following analyses: TrkC in the DRG, NT3 in the DRG, TrkB in the DRG, TrkA in the spinal cord, and TrkA in the DRG.

Several other factors that could have influenced the results we observed warrant further consideration. For instance, as the animals that were used in this experiment were all females, it is important to note that the potential role of the estrous cycle was not investigated. (Baker and Hagg 2005) found that after spinal cord contusion, the stage of the estrous cycle did not affect sensory axon degeneration, though neurotrophin levels were not examined. Investigations of the role of estrous cycle on neurotrophin/Trk receptor levels have been performed primarily in the hippocampus. In one study, it was shown that TrkA fluctuates by as much as 16-fold during proestrous in reactive astrocytes. This effect was not observed in rats that were ovariectomized, however with reintroduction of estrogen, TrkA increased 12-fold (McCarthy, Barker-Gibb et al. 2002). This increase in expression of TrkA is also observed in medial septum neurons, and for levels of BDNF in the hippocampus, but not NGF (Gibbs 1998). Mutated BDNF can lead

to increased anxiety behavior, impaired memory, and increased expression of BDNF and its receptor TrkB in the hippocampal formation, and also alters the fluctuation of protein expression observed across the estrous cycle (Spencer, Waters et al. 2010). Additionally, in the hippocampus of mice, TrkB is phosphorylated during high-estradiol states (i.e. proestrus in females) than lower estradiol states (estrus and diestrus in females and males) (Spencer-Segal, Waters et al. 2011), an increase that also is accompanied by a period of greater hippocampal excitability (Scharfman, Mercurio et al. 2003). These studies highlight 1) that different stages of the estrous cycle can lead to differential changes in gene expression of neurotrophins 2) that different stages of the estrous cycle can alter BDNF levels, which leads to changes both to the activity of individual neurons and behavior of the animal. Despite these documented interactions, the exact contributions of hormone levels and stages of the estrous cycle to our data are unclear.

Another consideration is activity levels of the animals before sacrifice. Specifically, changes in the activity level could alter the induction of immediate early genes, such as c-Fos. c-Fos has been used as a marker for many cellular processes, including activity (Jasmin, Gogas et al. 1994), hormonal activation (Li, Hand et al. 1997), as well as neurotrophin binding (Boyce, Park et al. 2012). Spinal cord injury can also induce expression of immediate early genes. After a spinal cord crush injury, levels of immediate early genes, cytokines, and neurotrophins show a sequential expression pattern that differs with time. c-fos and c-Jun reach peak levels an hour after injury, persisting for up to 6 hours; followed by cytokines TNF $\alpha$  and IL6 which reach their peak expression levels around 3 hours, persisting up to 12 hours; and then the neurotrophins NT3 and BDNF begin to be upregulated at 12 hours (Hayashi, Ueyama et al. 2000). Relating this

to activity period after sacrifice, animals that were sacrificed earlier in the day (close to the night, their period of highest activity), would be more likely to have increased c-Fos (for example) levels relative to those sacrificed at later times in the day. From the study above it appears that this induction of immediate early genes does not begin to affect neurotrophin levels until around 9 hours after these genes reach their peak, thus neurotrophin levels would likely be higher later in the day, assuming a direct link between activity, immediate early gene expression, and neurotrophin expression. The fact that the animals were sacrificed in a sequential manner throughout the day (from approximately 10am to 5 pm) and that we still found consistently elevated expression levels in some groups, it seems unlikely that these factors played a significant role in influencing the data. Nevertheless, it certainly would have been useful to have some measure of activity, and similar future studies should attempt to address these concerns.

Though the manipulation of expression of the neurotrophins and/or Trk receptors might seem beneficial when viewed alone for purposes of cell survival, or alteration of excitability of a certain population of cells, these studies also highlight an idea that has not been explored thus far with regard to neurotrophins. Though neurotrophins/Trk receptors have been largely been investigated under that assumption that they have discrete functions on discrete populations of cells, it is also possible that manipulation of any one component of their signaling or expression system could alter the others in a way that could be maladaptive. Take for instance the effects of NT3, which has established functions as potentiating the excitability of proprioceptive neurons in both the DRG and spinal cord (Klein, Silos-Santiago et al. 1994). If one were to attempt to overexpress NT3 as a means of enhancing the excitability of this system, it could inadvertently lead to

overexpression of NGF as well (Mendell, Albers et al. 1999), which has documented effects of leading to hyperexcitability of nociceptive neurons (though NT3 can have anti-nociceptive properties (Shu XQ 1999) as well, thus may serve to mitigate this effect to an extent). Conversely, reducing expression of NGF to ameliorate pain could lead to unwanted effects on proprioceptive circuits. Accordingly, investigations that involve neurotrophins/Trk receptors should take into account the effect that manipulation of one component could have on all others, both through expression analysis and functionally. Furthermore, as side effects are a common reason for cessation of many therapies, this is also important with regard to clinical studies that involve neurotrophins.

Taken together, these findings point to what appears to be a set of overlapping interactions that ultimately produce a coordinated transcriptional network. After a contusive injury to the thoracic spinal cord, a degree of descending input to the lumbar cord is lost based on the exact nature of the injury. These inputs are heterogeneous in nature in terms of neurotransmitter content of the spared tracts, and the location in the lumbar cord onto which the tracts synapse. Despite this heterogeneity, the resulting effect of this loss of descending input is loss of function of the output of lumbar circuits (i.e. lower motoneurons), and loss of molecular interactions between synapses of the descending tracts and the axons/dendrites/synapses present in the lumbar cord. Likely at play in the acute setting of the injury is the release of inflammatory/stress molecules from the spared descending synapses that is due to their interaction with the still dynamic molecular setting of the injury site. After time has passed and the cord has “adjusted” to the different environment and dynamic events have ceased, it appears based on our work that an external factor begins to regulate the expression of the neurotrophins and their

cognate Trk receptors in a “coordinated” manner. Based on our results, CCL2 seems to be a potential contributor to such a process, as it is dramatically upregulated at time points when we see this phenomenon of coordinated expression (Fig 20) and has been previously shown to effect large, medium, and small diameter cells of the DRG, which represent all different subclasses of neurons on which Trk receptors are present (White, Sun et al. 2005).

Though this is one mechanism that may be present, a separate mechanism, perhaps one that is activity dependent also appears to regulate the levels of the neurotrophins and the Trk receptors. Such an activity dependent mechanism could be based on a level of activity that is determined by injury severity, i.e. motor neuron pools with greater levels of spared descending input lead to more motor output which leads to a greater level of basal activity in the DRG. Summarily, both activity dependent and independent mechanisms could be at play simultaneously to achieve a spectrum of expression levels. Alternatively, differing levels of motoneuron output could lead to release of a common factor/molecule in the periphery that is *motoneuron* activity dependent but not dependent on levels of afferent activity. Whether this process is beneficial or maladaptive to the entire system depends on a variety of factors, including (but not limited to); the relative contributions of each trophic factor to the overall activity of the system, how much the expression level of one factor influences the other, and the severity of the injury and what types of information are relays to more rostral levels of the nervous system. These findings have an impact not only as novel discoveries, but could also have implications for the utility of different therapeutic approaches used to help patients with spinal cord injury. Ultimately, only further investigation into the

precise mechanisms that govern such processes will determine their impact on future scientific research.



Figure 1

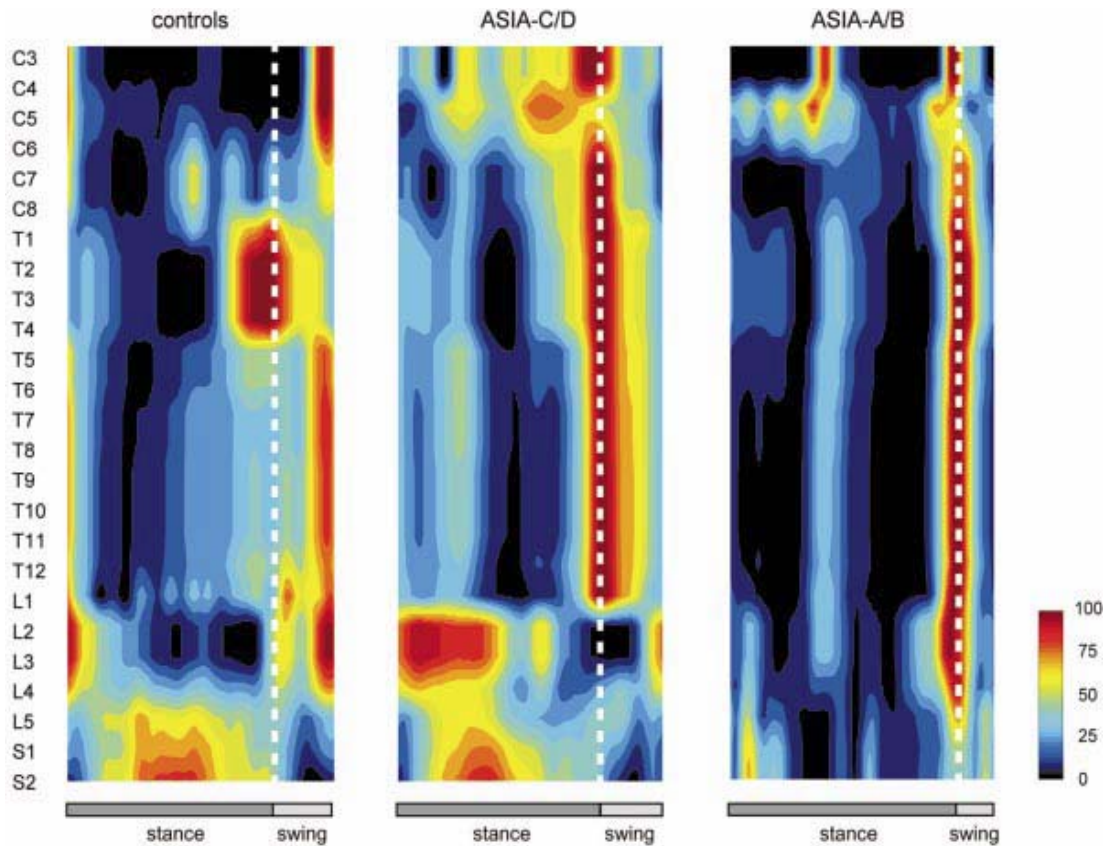


Figure 1: In ASIA A/B patients (complete motor loss) and ASIA C/D patients (incomplete motor loss) with body weight supported treadmill training (BWST), levels of activation of motor neuron pools show drastic spatiotemporal changes when compared with controls (healthy subjects)

Figure 2

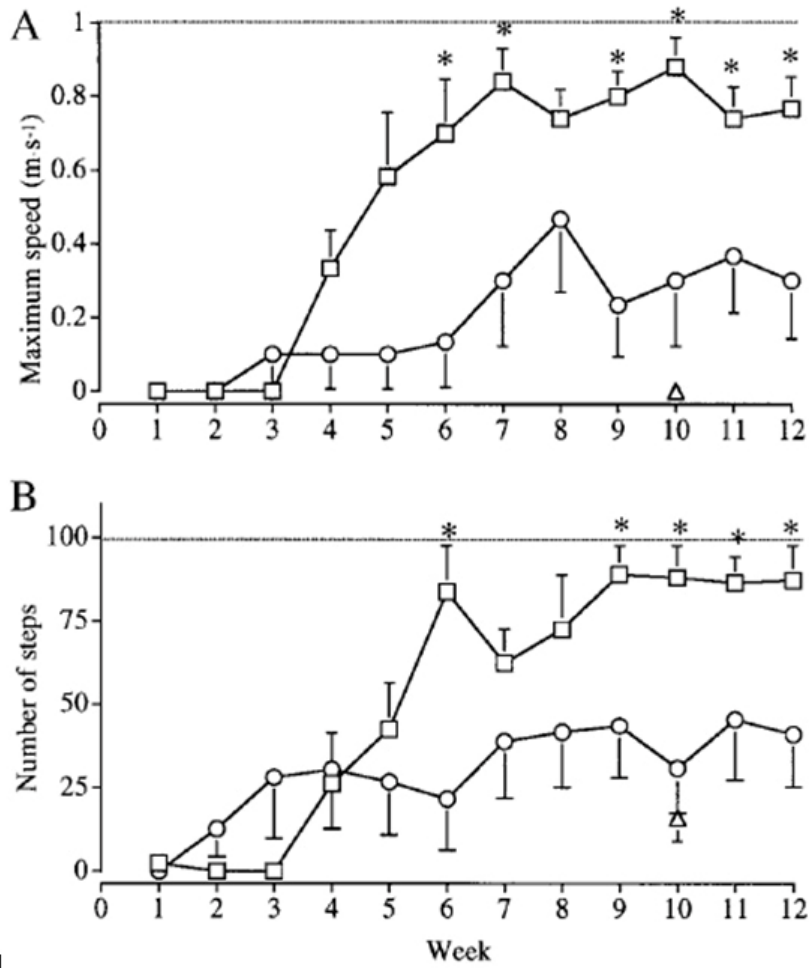


Figure 2: Treadmill training improves both maximum speed of stepping and the number of successful steps that are able to be performed after thoracic transection in the cat.

Figure 3

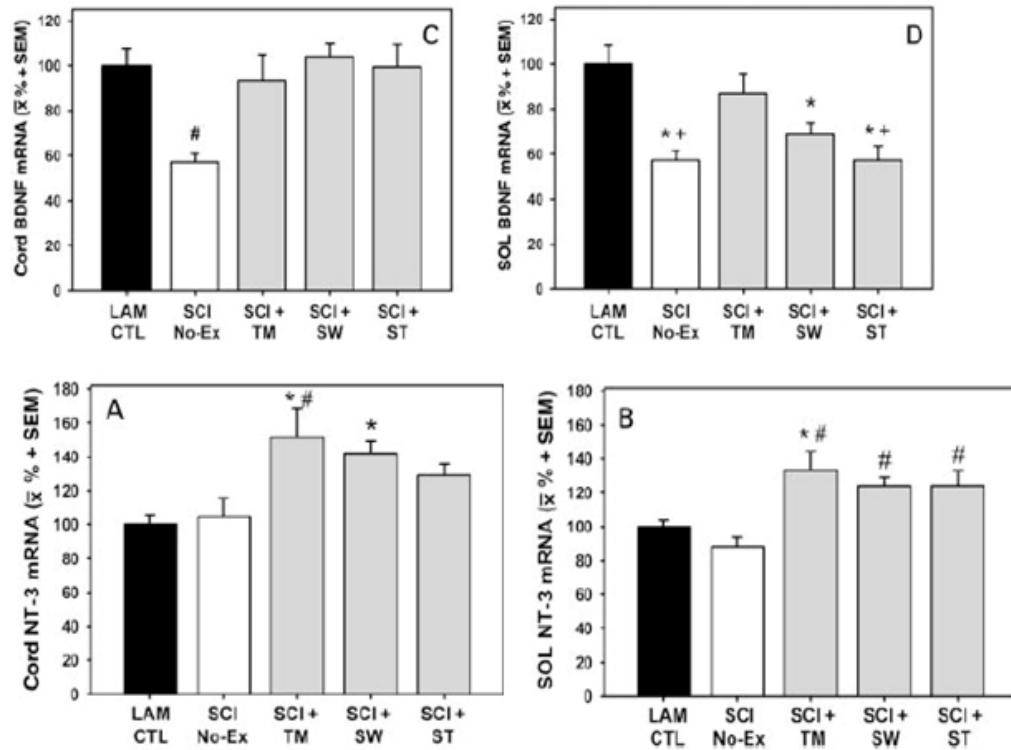


Figure 3: Contusive spinal cord injury results in decreased expression of BDNF, but does not change NT3 expression. Exercise after contusive spinal cord injury results in levels of BDNF and NT3 expression that are elevated relative to what is observed after contusive spinal cord injury.

Figure 4

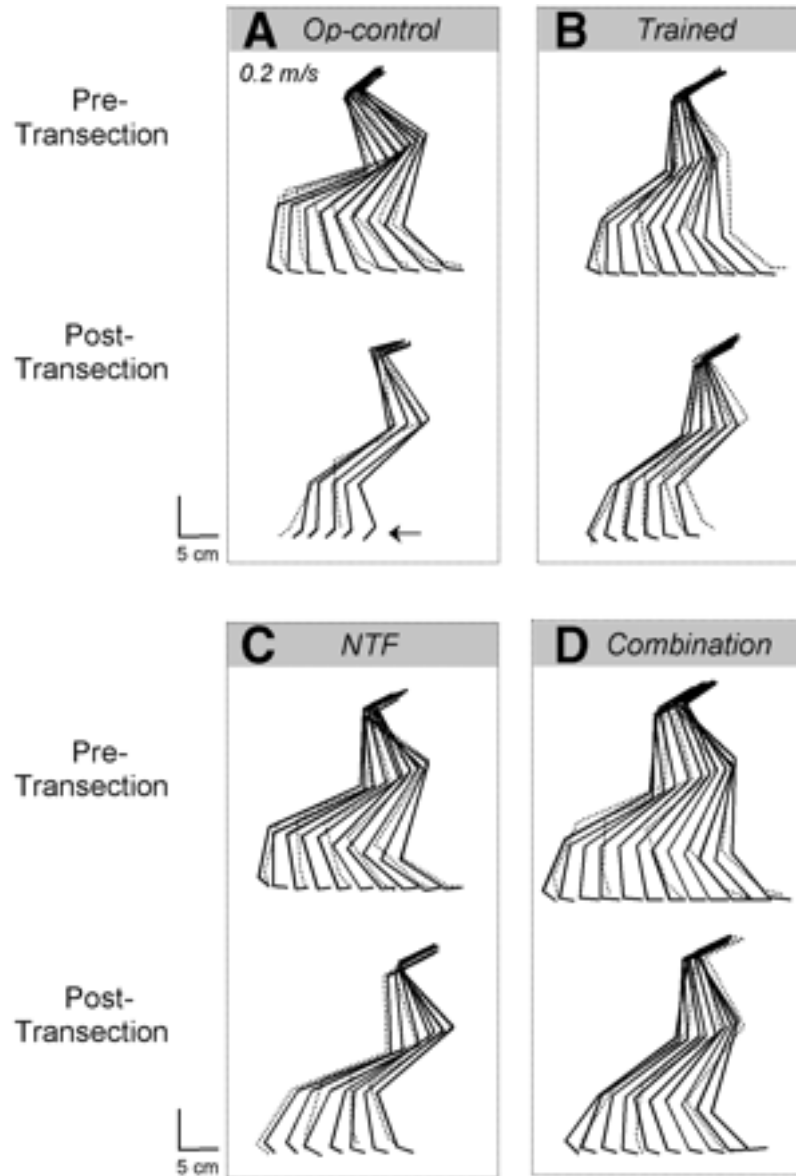


Figure 4: Neurotrophic factor treatment(NTF) after thoracic transection of the cat spinal cord produces the same effect as treadmill training(trained) after transection.

Combination of training and neurotrophic factor treatment results in similar kinematic parameters as seen before transection.

Figure 5

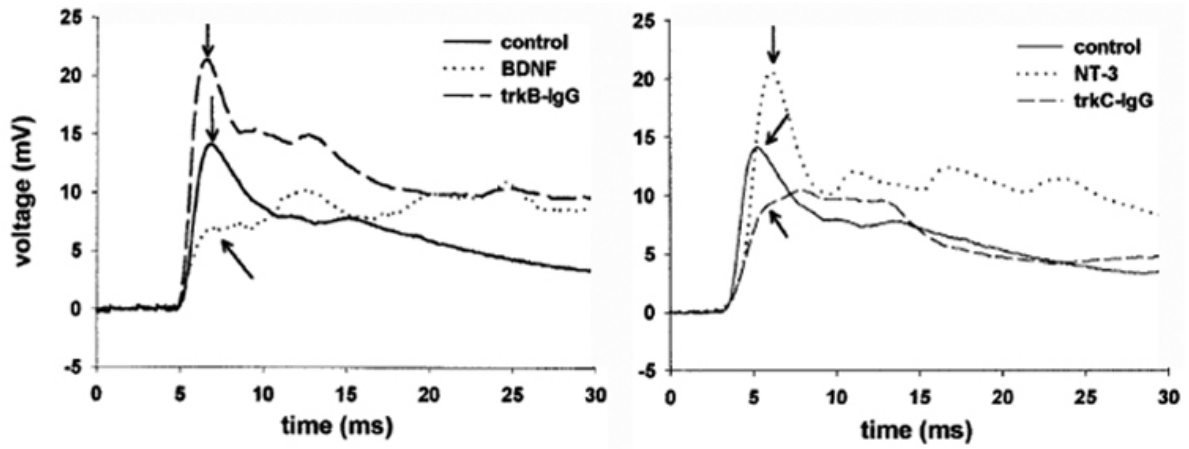


Figure 5: BDNF decreases the amplitude of the EPSP in motoneurons after afferent stimulation. NT3 increases the amplitude of the EPSP in motoneurons after afferent stimulation, demonstrating a difference in the effects of the two Trk receptor ligands.

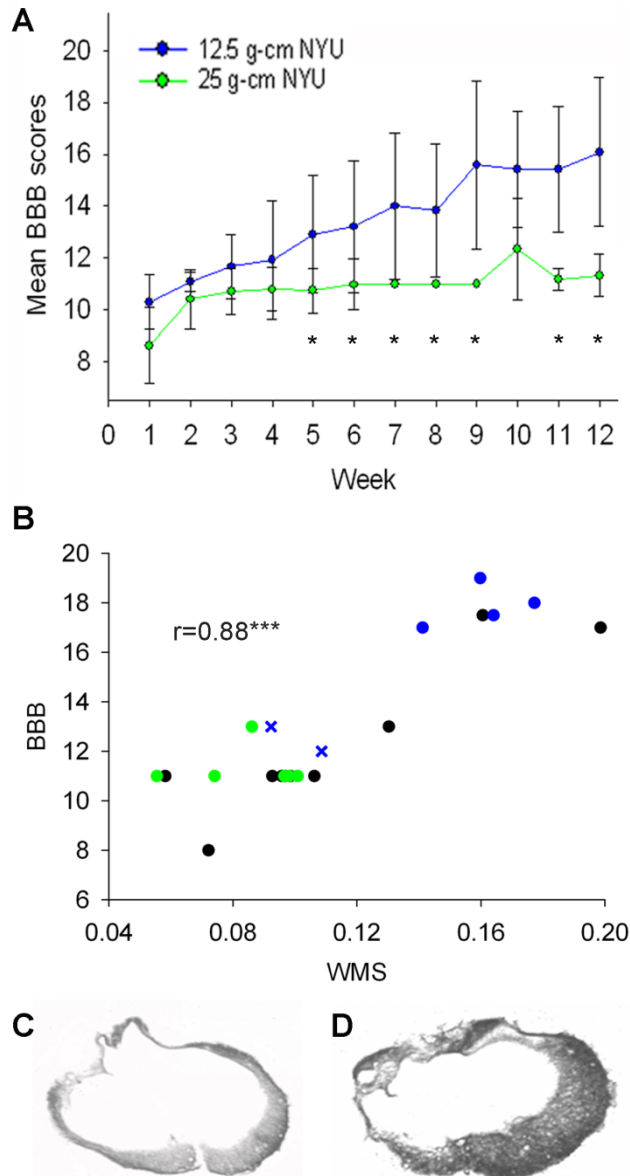
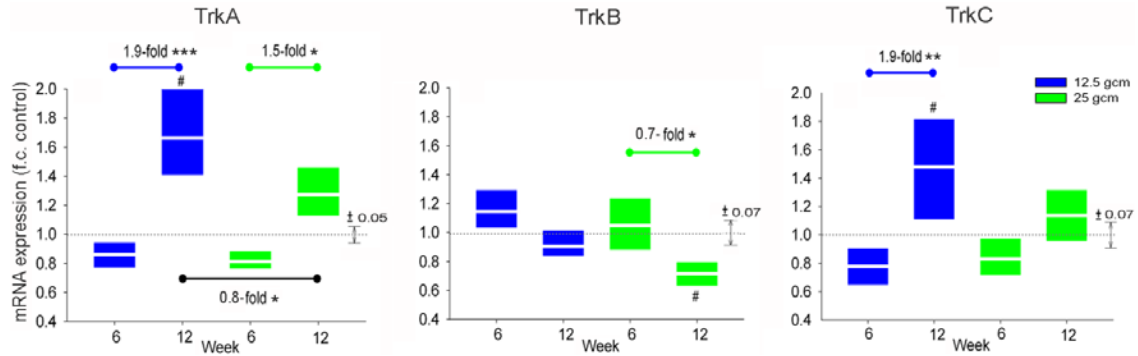


Figure 6. Injuries were characterized using BBB scores to assess hindlimb locomotor function and white matter sparing (WMS) at epicenter using EC stain. (A) BBB scores of groups that received 12.5 g-cm (blue) or 25 g-cm (green) NYU injuries. Starting at week 5, a significant difference was observed between the two injury severities. Both groups were significantly different ( $p < 0.05$ ) from controls (not shown) at all time points. (B) White matter sparing at epicenter (x-axis) plotted versus BBB score. Black dots represent 6 week values. Green dots represent 12 week, 25 g-cm injured animals. Blue x represent the two animals from the 12 week, 12.5

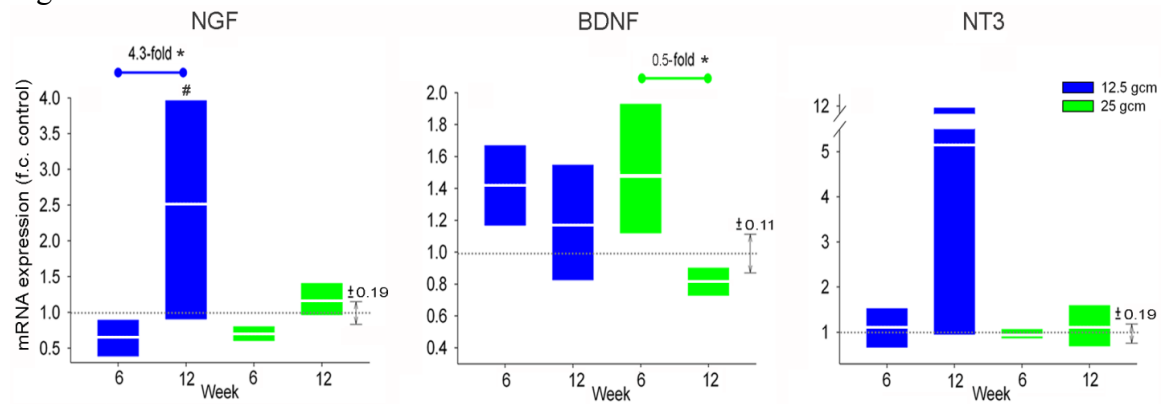
g-cm group with the lowest BBB scores within the group. Blue dots represent the four animals from the 12 week, 12.5 g-cm group with the highest BBB scores within the group. (C) Image taken from a 12.5 g-cm contused animal showing a laterally-symmetrical injury pattern at the epicenter. Note the difference from (D), which was taken from an animal that also received a 12.5 g-cm spinal cord contusion but which yielded an asymmetrical injury at epicenter. \* $p < 0.05$ , \*\*\* $p < 0.001$

Figure 7.



mRNA expression of Trk receptors is altered in L4/5 DRG at 6 and 12 weeks after receiving either 12.5 g-cm (blue) or 25 g-cm (green) NYU contusion injury to spinal cord at vertebral level T9. Fold change (F.C.) is reported as change of 12 week relative to 6 week time points in all figures. Black bar on TrkA reports fold-change (f.c.) of 25 g-cm at 12 weeks relative to 12.5 g-cm at 12 weeks. X axis denotes weeks post injury. White lines in box-plots indicate group mean. Dotted gray lines indicate expression level of controls (normalized to 1), with  $\pm$  s.e.m. indicated by the vertical arrows at right end of the control line. # $p < 0.05$  vs. control, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

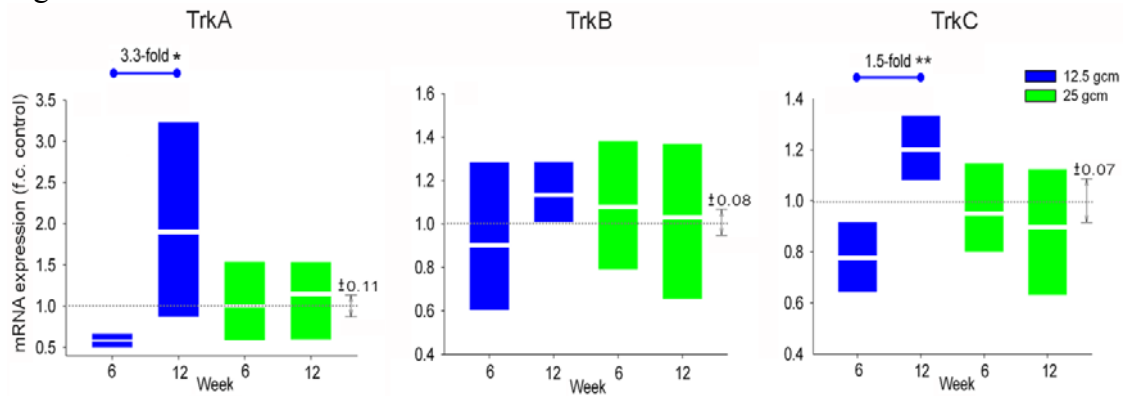
Figure 8



mRNA expression of Neurotrophins is altered in L4/5 DRG at 6 and 12 weeks after receiving either 12.5 g-cm (blue) or 25 g-cm (green) NYU contusion injury to spinal cord at vertebral level T9. Fold-change (f.c.) is reported as change of 12 week relative to 6 week time points in all figures. X axis denotes weeks post injury. White lines in box-plots indicate group mean. Dotted gray lines indicate expression level of controls (normalized to 1), with  $\pm$  s.e.m. indicated by the vertical arrows at right end of the control line. # $p < 0.05$  from control. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

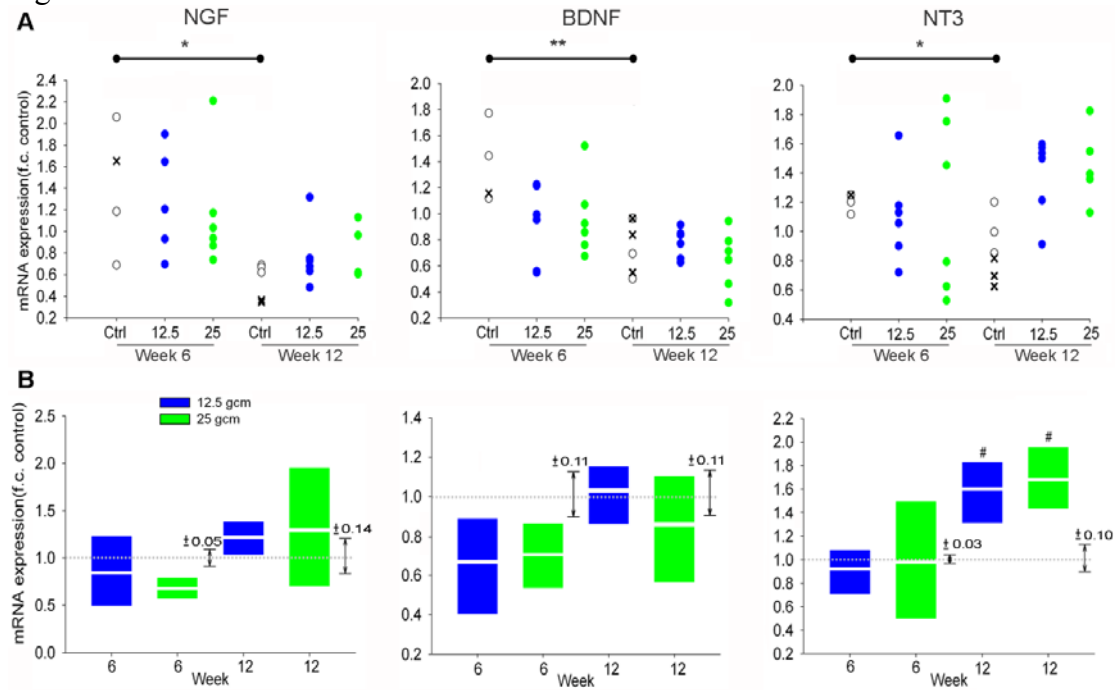


Figure 9.



mRNA expression of Trk receptors is altered in L4/5 spinal cord at 6 and 12 weeks after receiving either 12.5 g-cm (blue) or 25 g-cm (green) NYU contusion injury to spinal cord at vertebral level T9. Fold-change (f.c.) is reported as change of 12 week relative to 6 week time points in all figures. X axis denotes weeks post injury. White lines in box-plots indicate group mean. Dotted gray lines indicate expression level of controls (normalized to 1), with  $\pm$  s.e.m. indicated by the vertical arrows at right end of the control line. # $p < 0.05$  from control. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

Figure 10.



A) Scatterplots of Neurotrophin mRNA expression in L4/5 SC at 6 and 12 weeks after receiving either 12.5 g-cm (blue) or 25 g-cm (green) NYU contusion injury to spinal cord at vertebral level T9, relative to the unified control group as was done for the other data. x's represent mRNA expression of age-matched naive animals. Open circles represent expression of age-matched laminectomy control animals. \* $p < 0.05$ , \*\* $p < 0.01$

B) mRNA expression of Neurotrophins in L4/5 SC at 6 and 12 weeks after receiving either 12.5 g-cm (blue) or 25 g-cm (green) NYU contusion injury to spinal cord at vertebral level T9, relative to their respective age matched control groups. X axis denotes weeks post injury. White lines in box-plots indicate group mean. Dotted gray lines indicate expression level of controls (normalized to 1), with  $\pm$  s.e.m. indicated by the vertical arrows to the right of each time-point pair. # $p < 0.05$  from control.

Figure 11: NGF expression as a function of age in the L4/5 spinal cord of naïve rats

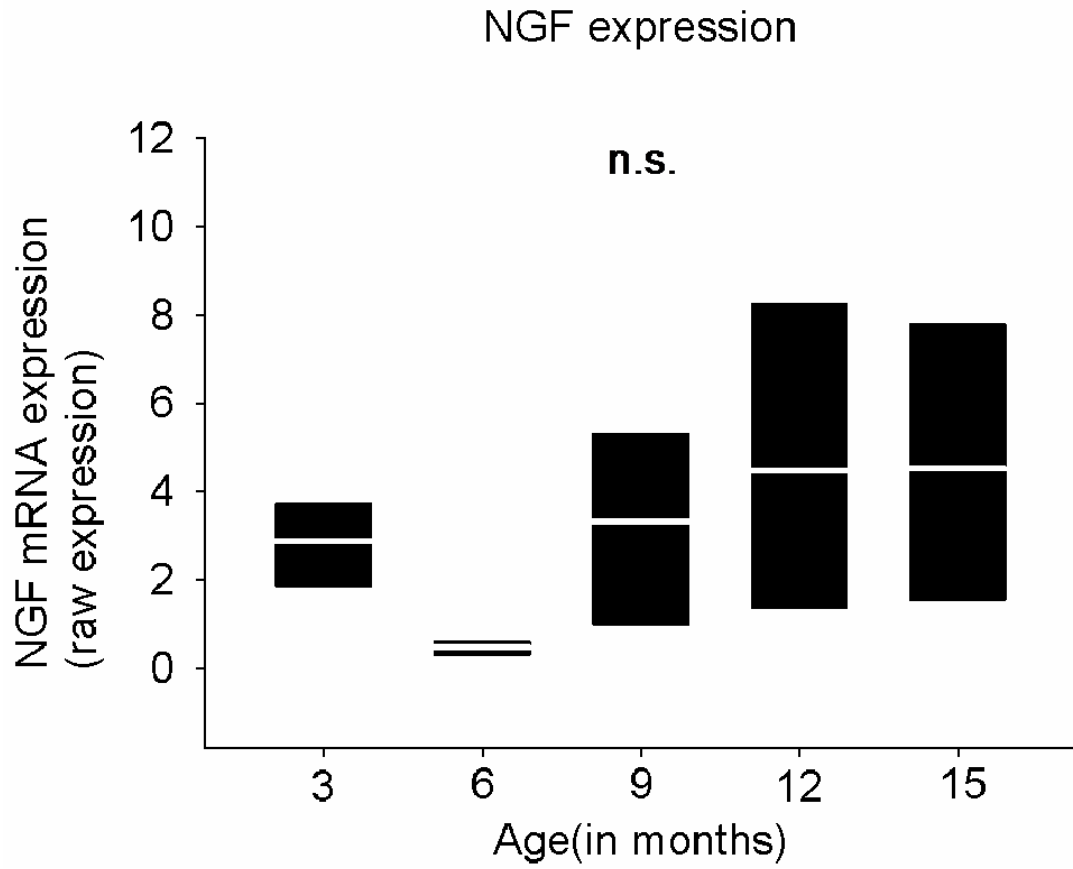


Figure 11: No age-related change in mRNA expression of NGF.

Figure 12: BDNF expression as a function of age in L4/5 spinal cord of naïve rats

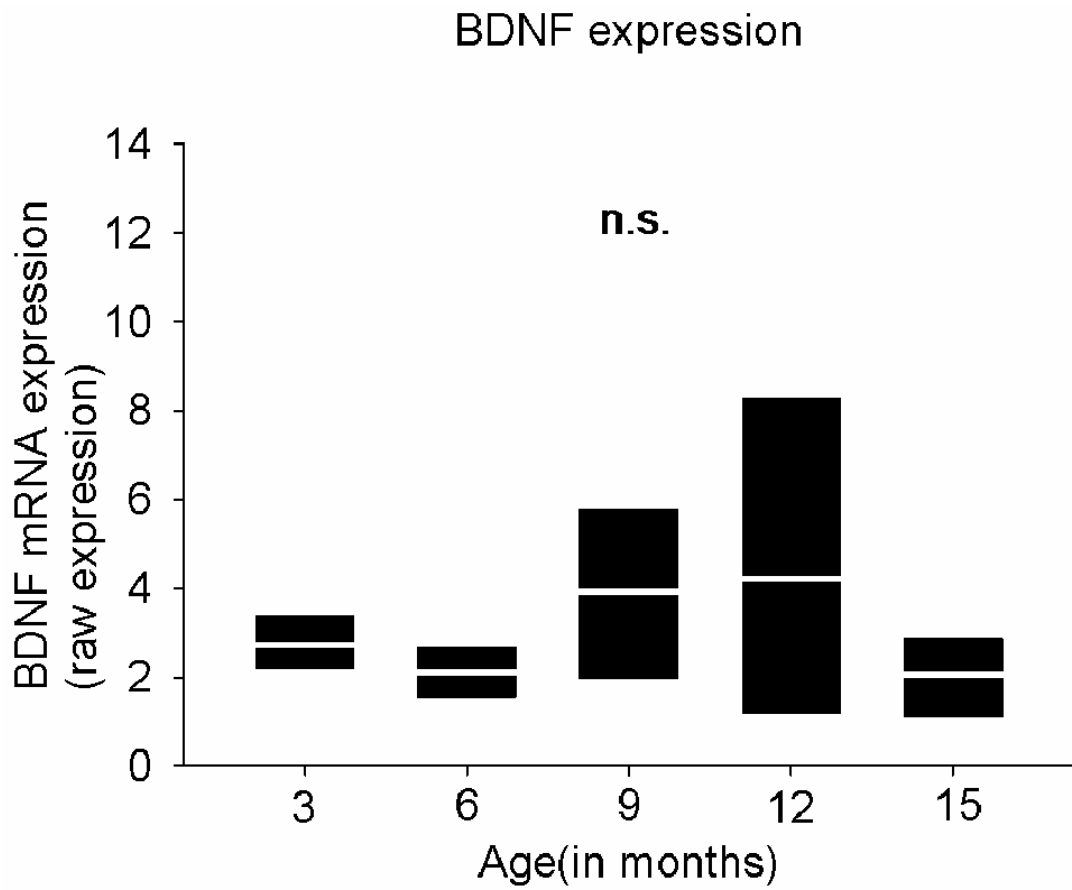


Figure 12: No change in age-related expression of BDNF.

Figure 13: NT3 expression as a function of age in L4/5 spinal cord of naïve rats

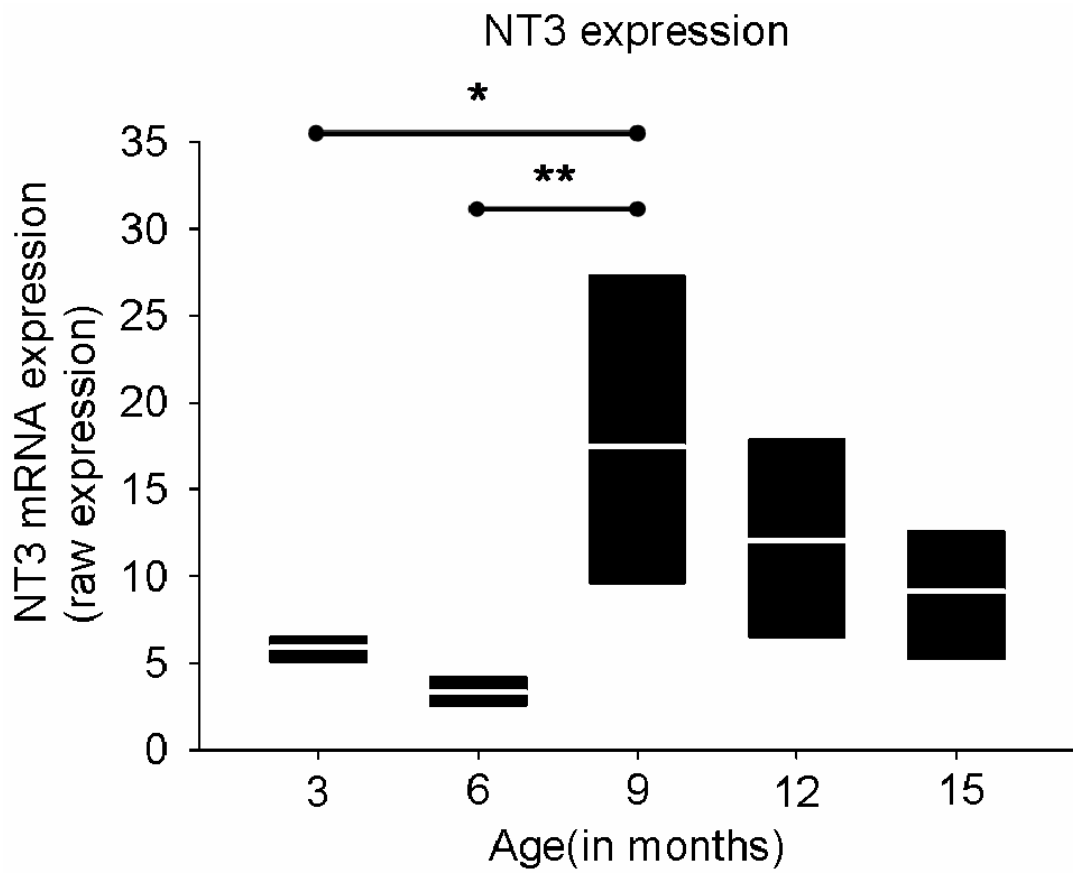


Figure 13: NT3 expression is elevated at 9 months post birth relative to both 3 and 6 months post birth. \* $p < 0.05$ . \*\* $p < 0.01$

Figure 14.

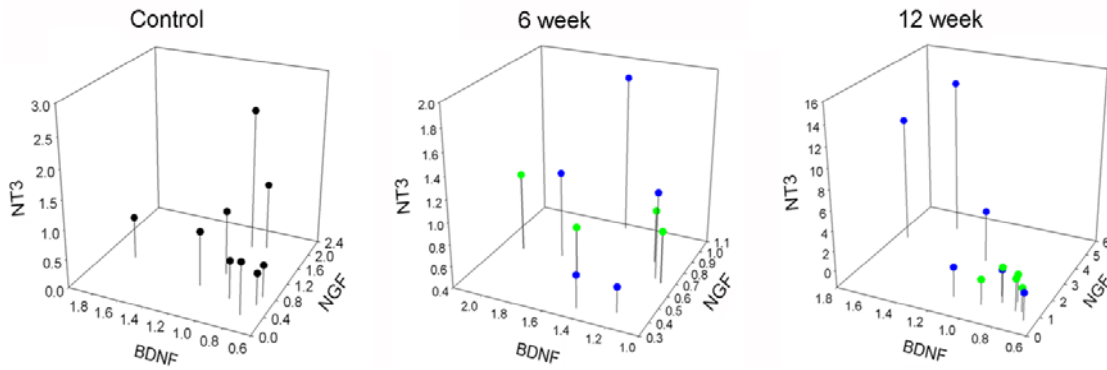


Figure 14: Correlated expression of neurotrophins in DRG emerges at chronic time points. Blue dots represent animals with 12.5 g-cm injuries. Green dots represent animals with 25 g-cm injuries. Values represent fold-change of the individual animals versus mean of control group.

Figure 15.

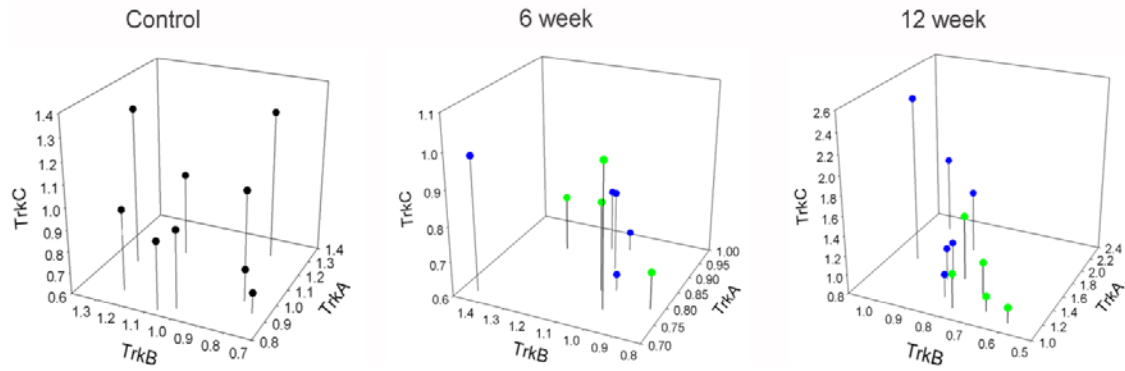


Figure 15: Correlated expression of Trk receptors in DRG emerges at chronic time points.

Values represent fold-change of the individual animals versus mean of control group.

Blue dots represent animals with 12.5 g-cm injuries. Green dots represent animals with 25 g-cm injuries.

Figure 16

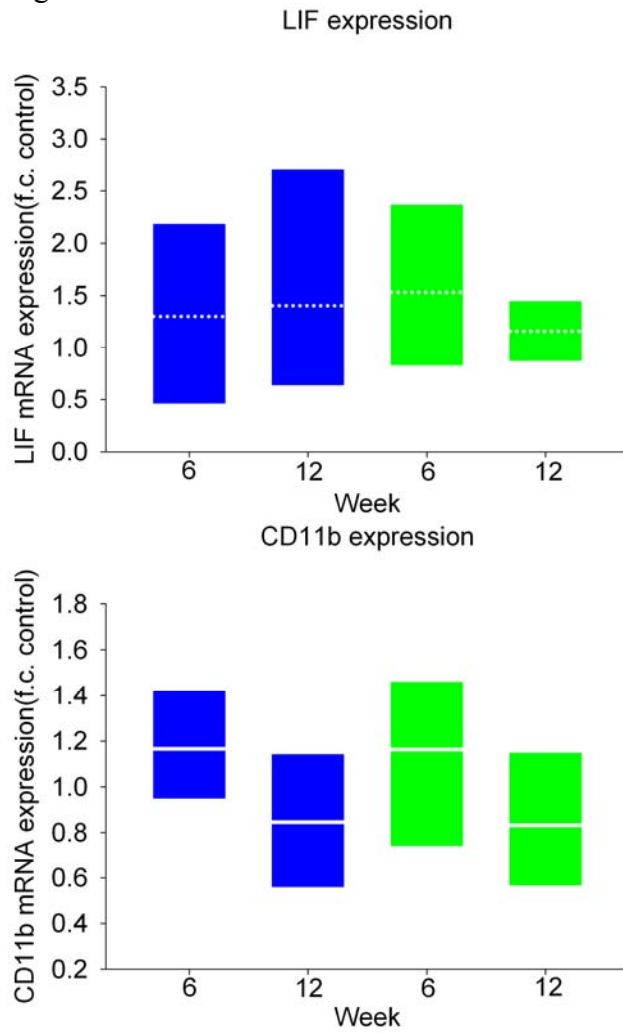


Figure 16: No change was observed between any injury severity or time point in the DRG for LIF or CD11b expression.



Figure 17

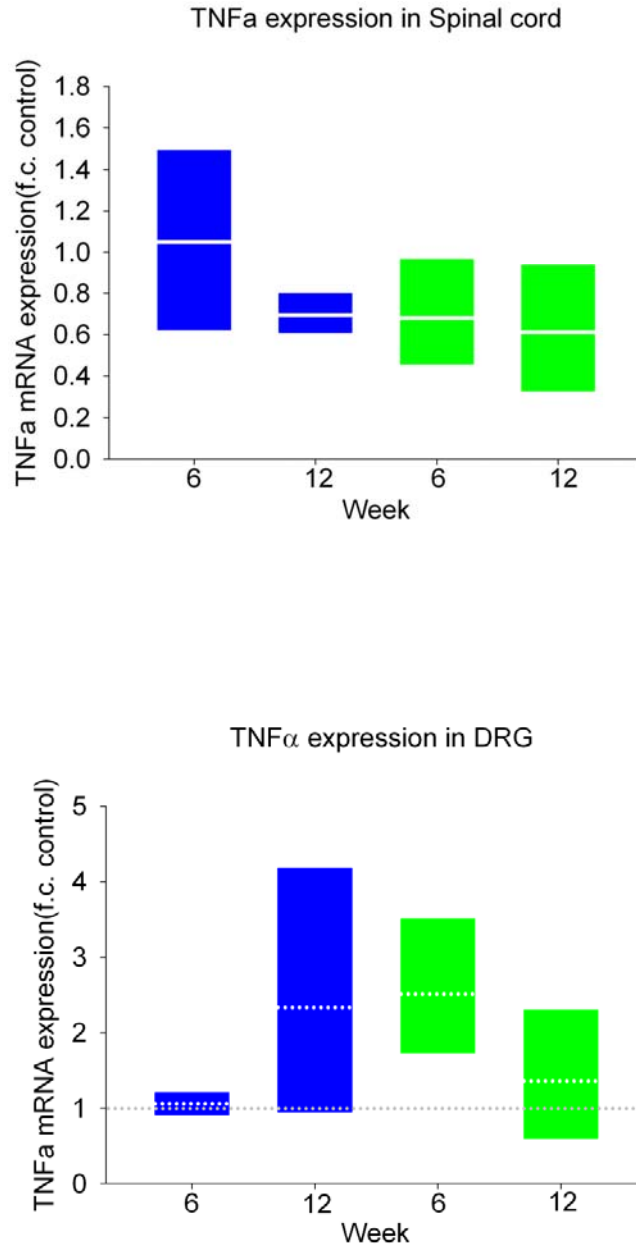


Figure 17: No change was observed between any injury severity or time point in the DRG or spinal cord in TNF $\alpha$  expression

Figure 18

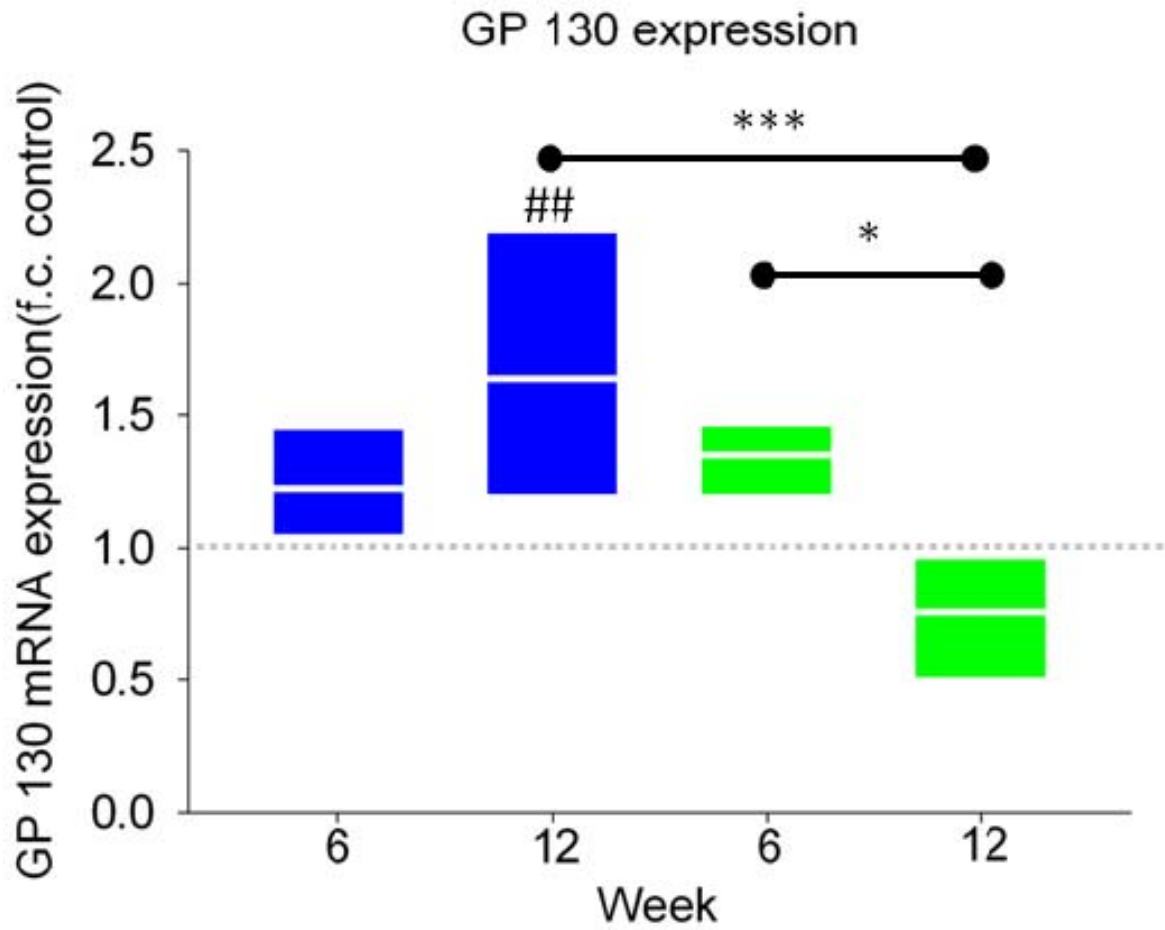


Figure 18: GP130 is decreased in 25 g-cm injury severity group at both time points relative to 12 weeks 12.5 g-cm injury severity group. \* $p < 0.05$ . \*\*\* $p < 0.001$ . ## $p < 0.01$  vs control group.

Figure 19

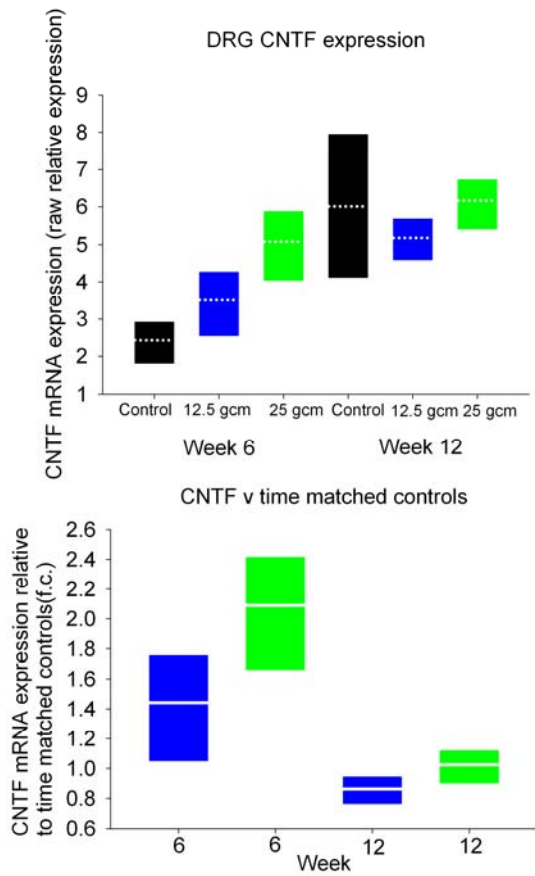


Figure 19: CNTF expression increases at 12 weeks in both injury severities. Control group at 12 weeks time point also increases(top). Relative to time matched control, CNTF expression is increased at 6 weeks in the 25 g-cm injury severity, no change relative to 12 week time matched control. Black bar represents mRNA expression of control group. Blue bar represents mRNA expression of 12.5g-cm injury severity group. Green bar indicates mRNA expression of 25 g-cm injury severity group.

Figure 20

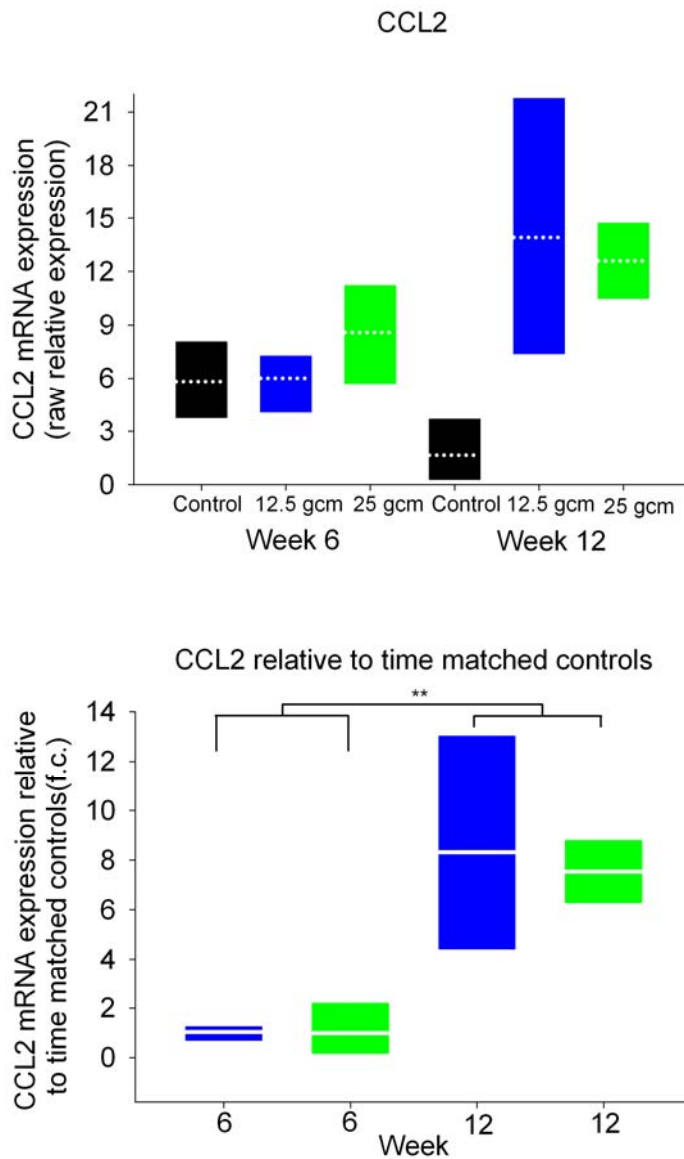


Figure 20: CCL2 expression increases at 12 weeks post contusive spinal cord injury.

Black bars represent mRNA expression of control group. Blue bars represent mRNA expression of 12.5 g-cm injury severity group. Green bars represent mRNA expression of 25 g-cm injury severity group. \*\*p<0.01

Figure 21

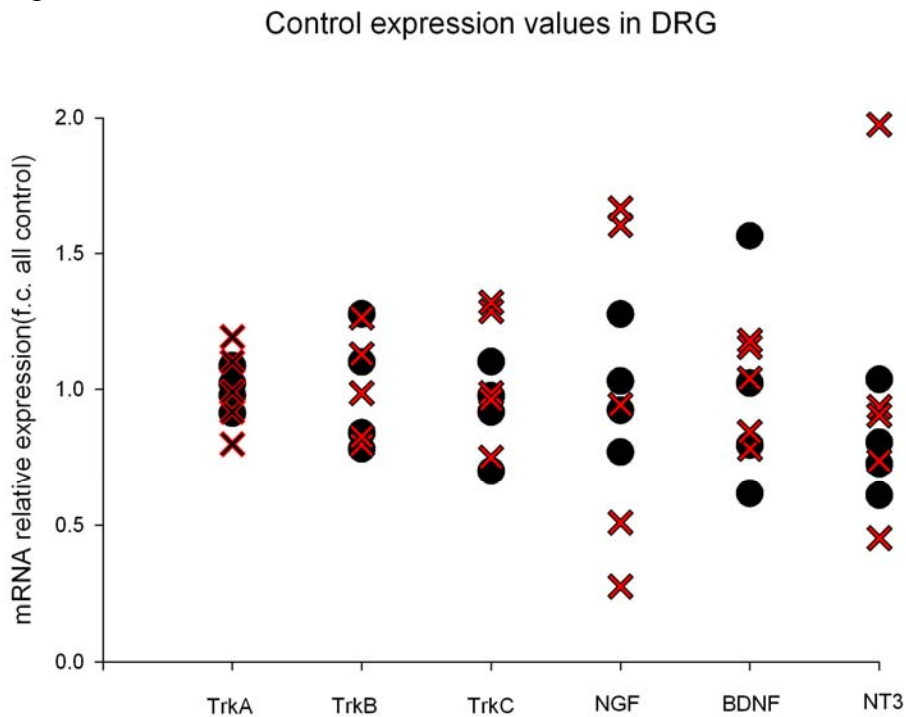


Figure 21. Control expression values of all groups assessed for mRNA levels of neurotrophins and Trk receptors in the DRG. An initial statistical analysis (t-test) was performed to determine if an age dependent change existed between our 6 and 12 week control groups. After performing this analysis we determined that no age related changes existed for our expression values in the DRG. Thus, all control relative expression values were combined, averaged, and then normalized to the combined average of all control values for each molecule labeled on the x-axis (denoted as f.c. all control on y-axis). Red X: 12 week control expression values. Black dot: 6 week control expression values.

Figure 22

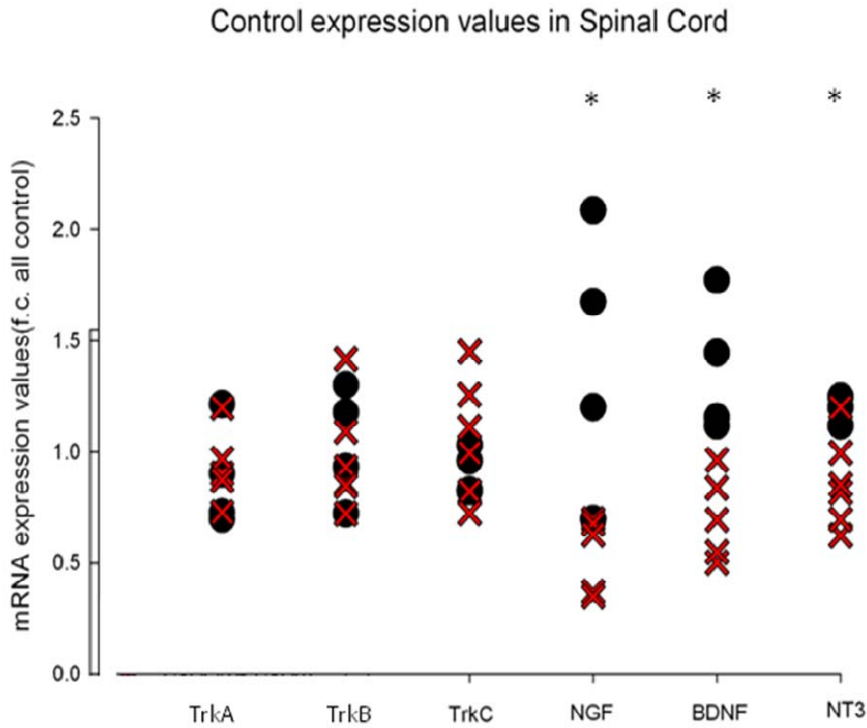


Figure 22. Control expression values of all groups assessed for mRNA levels of neurotrophins and Trk receptors in the spinal cord. An initial statistical analysis(t-test) was performed to determine if an age dependent change existed between our 6 and 12 week control groups. After performing this analysis we determined that no age related changes existed for our Trk receptor expression values in the spinal cord, but an apparent age related change in controls did exist for the neurotrophins in the spinal cord. All control relative expression values were combined, averaged, and then normalized to the combined average of all control values for each molecule labeled on the x-axis (denoted as f.c. all control on y-axis) Due to the difference observed for neurotrophin expression values in controls, further analysis was also performed relative to expression values of time matched controls at 6 and 12 week time points (Figure 10). Red X: 12 week control expression values. Black dot: 6 week control expression values. \*p<0.05

Table 1. Summary table of previous findings for NTs and Trk. (Houglund et al 2012)

PMID	Reference	Molecule(s)	Injury model	Injury site	Sampling site	Experimental methods	Post injury time course	Findings	Notes
10757326	Hayashi et al. (2000)	NGF, BDNF, NT3, TrkA, TrkB, TrkC	Spinal cord crush (60g, 1 s)	Under T10 vertebra	Five segments centered on epicenter	ISH	Six times; up to 3 days	Increase in BDNF and NT3, weaker increase for NGF; TrkA and TrkC not detected; TrkB detected in non-neurons and motoneurons, and increased in both with SCI	Functional status of animals was not assessed, but reference was given to Guth et al., 1994 (modal shows mild motor deficit after awakening from anesthesia with apparent full recovery at 72 hr; BDNF observed in non-neurons after SCI; qualitative data only; no statistics)
11161589	Liebl et al. (2001)	TrkA, TrkB, TrkC	12.5 gcm NYU contusion	Under T9, T10 vertebra	Entire SC	ISH	1 day	No difference in TrkA, TrkB, or TrkC expression rostral or caudal to injury	Absent Trk expression around injury site and reduced in penumbra, no statistics
11331375	Widenfalk et al. (2001)	NGF, BDNF, NT3, TrkA, TrkB, TrkC	25 gcm NYU contusion, transaction	Under T9 vertebra	Cross-sections taken from regions throughout length of spinal cord; injury epicenter and up to 1 cm caudal	ISH	Six times; up to 6 weeks	No change in TrkA, TrkB, TrkC; increase in NGF and BDNF up to 1 day, but no change vs. intact at 6 week; NT3 not detected in either intact or injured spinal cord	Functional status of animals with contusion was not assessed; reports on multiple injury types; statistical analysis uses optical density measures for ISH, and radioactivity for RPA
						RPA (NGF, BDNF; NT3)	6 weeks after contusion	No change in NGF and BDNF; NT3 not detected	ISH, and radioactivity for RPA
							1 day after transaction	Increase in NGF and BDNF; NT3 not detected	

(Continued)

Table 1(continued)

PMID	Reference	Molecule(s)	Injury model	Injury site	Sampling site	Experimental methods	Post injury time course	Findings	Notes
11358464	Nakamura and Bregman (2001)	NGF, BDNF, NT3, NT4	Lateral over hemisection	Under T6 vertebra	Entire SC	RPA	Five times; up to 2 weeks	Increase in NGF and BDNF up to 4 days, NT3 and NT4 not detected	Used whole SC mRNA, no assessment of injury or post-SCI function, expression data represented as % GAPDH
12115676	Qiao and Vizzard (2002)	TrkA, TrkB	Transection	Under T8-T10 vertebrae	L1-S1 DRG	IHC	5-6 weeks	Increase in # of TrkA and TrkB positive cells in L1, L6/S1, no change at L4/5	No assessment of post-SCI function, data expressed as # of Trk-IR positive cells
15193526	Gulino et al. (2004)	BDNF, NT4	Lateral hemisection	Under T9 vertebra	L4/5 SC	IHC	Four times; up to 2 weeks	Decrease in BDNF, NT4 starting at 30 min, lasting up to 2 weeks	Coronal sections; no assessment of post-SCI function; data expressed as relative optical density of IR positive cells in ipsilateral vs. contralateral hemisection cord
15235239	Zvarova et al. (2004)	NGF, BDNF	Transection	Under T7-T9 vertebrae	T7-S1	ELISA	< 1, 6 weeks	Increase in NGF T7-T8 (rostral), and T13-L1, L6-S1 (caudal) 6 weeks post injury; Increase in NGF T9-T10 (caudal), and T13-L1, L6-S1 (caudal) < 1 week post injury; increase in BDNF T7-T10, T13-L1, L6-S1 6 weeks post injury; Increase in BDNF T7-L1, L3-S1 < 1 week post injury	No assessment of post-SCI function; neurotrophin concentration expressed as proportion of total protein

(Continued)



Table 1(continued)

PMID	Reference	Molecule(s)	Injury model	Injury site	Sampling site	Experimental methods	Post injury time course	Findings	Notes
15611995	Qiao and Vizzard (2005)	TrkA, TrkB	Transsection	Under T8-T10 vertebrae	L1-S1 DRG	IHC	2 days and 2 weeks	Increase in # of TrkA and TrkB positive cells in L1, L6/S1, no change at L4/5	No assessment of post-SCI function; data expressed as # of Trk-IR positive cells
17055159	Qin et al. (2008)	NGF, BDNF, NT3	Lateral hemisection	Under T10 vertebra	Ventral horn caudal to T10 injury site	IHC	Three times; up to 3 weeks	Increase in # of BDNF, NT3, NGF positive cells in ventral horn	Characterized injuries by spared function (BBB locomotor score); data expressed as optical density of IR positive cells in hemisection cords relative to control cords
17159471	Li et al. (2007)	NGF, BDNF, NT3	Transsection	Under T9-T10 vertebra	Laminae IX, ~1.5cm caudal to injury site	IHC	Four times; up to 3 weeks	Increase in # of NGF IR cells and relative IR in laminae IX up to 3 weeks; # of NT3 IR cells and relative IR in laminae VIII and IX up to 3 weeks and laminae I-VII at 2 weeks; # of BDNF IR cells and relative IR in laminae IX up to 7 days	Characterized injuries by spared function (BBB locomotor score); data expressed as relative optical density of IR positive cells in hemisection v.s. control cords
18585435	Hajebrahimi et al. (2008)	NGF, BDNF, NT3, TrkA, TrkB, TrkC	25 gcm NYU contusion	Under T9-T10 vertebra	~1 cm block of SC	EthBr staining intensity	Eight times; up to 3 weeks	Decrease in NGF after 6h that increases until 3 week where greater than control; BDNF and NT3 decrease after 6h up to 3 weeks; TrkA, TrkB, TrkC decrease up to 3 weeks	B2m used as internal control; no assessment of injury or post-SCI function; data expressed as levels of mRNA relative to B2m

(Continued)

Table 1(continued)

PMID	Reference	Molecule(s)	Injury model	Injury site	Sampling site	Experimental methods	Post injury time course	Findings	Notes
21441969	Qian et al. (2011)	TrkC	Transsection/resection	Under T8-T9 vertebrae	Motor cortex; SC; adjacent to injury, rostral/caudal to injury	mRNA, protein	Four times; up to 2 weeks	Decrease at and around injury site from 1 to 7 days, then rapid increase until 14 days	mRNA more highly expressed at injury site than neighboring segments; protein shows same pattern; no assessment of post-SCI function
22244304	Keeler et al. (2012)	BDNF, NT4, NT3, TrkB, TrkC	Transsection	Under T9 vertebra	L4-L6 SC (laser-captured motoneurons, select laminae); L4-6 DRG (large neurons)	Laser-capture, qPCR, WB	up to 31 days	Increase in NT4 and TrkB mRNA at 10 days; NT4 mRNA at 31 days; increase in NT3, NT4, BDNF protein at 31 days; increases in NT4 at 10 days, TrkB at 31 days in motoneurons; no change in expression in intermediate gray matter or large DRG neurons	More robust increase in expression after exercise; no assessment of post-SCI function; data expressed as mRNA or protein relative to control
	Houglund et al. (this article)	NGF, BDNF, NT3, TrkA, TrkB, TrkC	12.5 and 25 gcm NYU contusion	Under T9 vertebra	L4/5 spinal cord and DRG	qPCR	6 and 12 weeks	Gene regulation differed by injury severity and by post-SCI time; correlated expression of genes at 12 weeks in DRG	Characterized injuries by spared function (BBB locomotor score) and by histology (white matter sparing); examined coregulation of genes

Abbreviations: n.c., no change; IHC, immunohistochemistry; ISH, in situ hybridization; WB, western blot; RPA, ribonuclease protection assay; EthBr, ethidium bromide; LCM, laser-capture microdissection; qPCR, quantitative polymerase chain reaction; SC, spinal cord; DRG, dorsal root ganglion.

Table 2. Correlations between expression of mRNA for trk receptors in spinal cord.

	Control		6 week		12 week	
	r-value	p-value	r-value	p-value	r-value	p-value
TrkA vs TrkB	0.47	0.2	0.25	0.46	0.19	0.56
TrkA vs TrkC	0.42	0.26	0.21	0.53	0.4	0.19
TrkB vs TrkC	0.75	<b>0.01</b>	0.76	<b>0.004</b>	0.44	0.16

Table 3. Correlations between expression of mRNA for neurotrophins, Trk receptors, and cognate pairs.

	Control		6 week		12 week	
	r-value	p-value	r-value	p-value	r-value	p-value
NGF vs BDNF	0.20	0.61	0.15	0.69	0.84	<b>0.001</b>
NGF vs NT3	0.72	<b>0.03</b>	0.87	<b>0.003</b>	0.92	<b>0.00006</b>
BDNF vs NT3	0.10	0.80	0.33	0.38	0.88	<b>0.0004</b>
TrkA vs TrkB	0.04	0.90	0.005	0.989	0.89	<b>0.0007</b>
TrkA vs TrkC	0.56	0.11	0.61	0.08	0.79	<b>0.006</b>
TrkB vs TrkC	0.40	0.28	0.38	0.31	0.78	<b>0.004</b>
NGF vs TrkA	0.65	0.06	0.55	0.13	0.88	<b>0.0004</b>
BDNF vs TrkB	0.45	0.22	0.69	<b>0.04</b>	0.77	<b>0.005</b>
NT3 vs TrkC	0.57	0.11	0.61	0.08	0.77	<b>0.006</b>

Table 4. Transcription factor binding sites for neurotrophin and trk receptor genes.

TF Binding-site name	HGNC symbol	TrkA	NGF	TrkB	BDNF	TrkC	NTF3
AhR	AHR	x		x	x		
AhR:Arnt					x		
AP-1	FOS; FOSB; JUN; JUND		x		x		
AP-2	TFAP2A	x	x	x	x		
AR	AR			x	x		
Arnt	ARNT			x			
ATF	ATF	x		x			
ATF2	ATF2	x	x	x	x		
ATF2:cJun			x				
Brn-2	POU3F2				x		
C/EBP	CEBPA,B,D,E,G,Z	x			x		
CAR	NR1H3	x					
c-Ets-2	ETS2		x				
c-Jun	JUN	x		x			
c-Myc:Max		x					
COUP-TF1	NR2F1		x		x		x
CREB	CREB1	x	x	x	x		x
CREB,ATF					x		
CREM	CREM	x		x			
DEC	BHLHE40	x	x		x		
E2A	TCF3			x			
Ebox	TCF3; MYOD1; MYOG			x			
ER-alpha	ESR1				x		x
Ets	ETS1,2; ETV 1,2,3,4,5,6,7		x		x		x
Foxj1	FOXJ1						x
FOXO1	FOXO1						x
GATA-3	GATA3				x		x
GR	NR3C1		x	x	x		
HES1	HES1			x			x
HOXA5	HOXA5		x				
HOXB8	HOXB8	x					
KROX	EGR1,2; ZNF22; ZBTB7B				x		
MAF	MAF		x	x	x		x
MAFB	MAFB	x	x	x	x		x
Max	MAX	x			x		x
MEF-2	MEF2A						x
MEF-2C	MEF2C				x		
Myc	MYC	x			x		
Neuro D	NEUROD1	x	x	x	x		x
NFAT1	NFATC2	x	x		x		x
NF-AT4	NFATC3	x	x		x		x
NF-kappaB	NFKB1		x				
NKX2B	NKX2-2		x				
NRSF	REST		x				
NIJRR1	NR4A2	x	x		x		
Oct-1	POU2F1		x		x		
Octamer	POU family of proteins		x		x		
Oct-x	STAT1		x				
p53	TP53						x
Pax-3	PAX3	x	x	x	x		x
Pax-6	PAX6						x
Pax-8	PAX8		x	x	x		
Pbx1	PBX1		x		x		x
POU6F1	POU2F1				x		
POUF2F1	POU6F1		x				
PPARgamma	PPARG			x			
PPARgamma:RXR-alpha				x			
PXR	NR1H2	x					
RXR-alpha	RXRA			x	x		
SF1	SF1						x
SMAD	MADH family of proteins				x		x
Smad3	SMAD3				x		x
Sox1	SOX1				x		x
Sox2	SOX2		x				
Sp1	SP1	x	x		x		x
SRF	SRF		x				
Sry	SRY		x		x		x
STAT	SOAT1		x		x		x
STAT1	STAT1		x				
STAT3	STAT3		x		x		
Tax	CNTN2			x			
Tax/CREB					x		x
Tbp	TBP			x	x		x
TCF-4	TCF4	x					
Tcfap2a	TFAP2A				x		
Tcfap2b	TFAP2B				x		
Tst-1	CCDC6		x				
USF	USF1				x		
USF2	USF2				x		
VDR	VDR	x					
VDR, CAR, PXR					x		x

Entries with transcription factors separated with a “.” have binding sites situated such that they act in a cooperative fashion, rather than independent from each other. Entries with transcription factors separated by a “,” share binding sites or have binding sites situated near each other such that the factors act in competition with each other.

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