MECHANISMS UNDERLYING SPONTANEOUS FUNCTIONAL SENSORY CHANGES FOLLOWING SPINAL DEAFFERENTATION

by

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ABSTRACT

Dorsal root injury (DRI) induces both pain and anatomical changes in pain-processing systems in the deafferented dorsal horn. Brain-derived neurotrophic factor (BDNF) is also upregulated in the spinal cord following DRI and is thought to participate in nociceptive transmission and stimulation of axon outgrowth. Transection of the C7 and C8 dorsal roots (C7/8 DRI) generates cold pain in the ipsilateral forepaw which peaks at 10 days, and resolves within three weeks after injury. In this thesis I sought to investigate the influence of BDNF on cold pain behaviour following C7/8 DRI.

Increased BDNF expression by Ox-42-positive microglia was observed up to 20 days post-C7/8 DRI. To determine the acute facilitatory effects of this increased BDNF on cold pain behaviour, intrathecal boli of TrkB-Fc ‘receptor bodies’ were administered following DRI and resulted in reduced peak response duration to peripheral cooling stimuli. However, long-term BDNF sequestration with continuous TrkB-Fc infusion failed to reduce cold pain and prevented the spontaneous recovery normally seen after three weeks. Prolonged BDNF sequestration also prevented the increase in serotonergic and GABAergic terminal densities which normally follows C7/8 DRI, and simultaneously stimulated sprouting of nociceptive primary afferents. These results dually implicate endogenous BDNF in modulating synaptic transmission and governing the plasticity of inhibitory circuitry in the deafferented spinal cord to, indirectly, promote the recovery from cold pain that develops following C7/8 DRI.

We also investigated the effects of exogenous BDNF treatment in DRI-induced cold pain and hypothesized that at high concentrations, BDNF would have a preventative effect. Surprisingly, exogenous BDNF treatment also impeded cold pain recovery, which
was not attributable to sprouting of nociceptive terminals. Exogenous BDNF did, however, increase the density of the inhibitory adrenergic/noradrenergic, dopaminergic and GABAergic terminals, whereas DRI-induced serotonergic sprouting was unaffected. BDNF also down-regulated spinal K\(^+\)/Cl\(^-\) co-transporter-2 (KCC2) expression, possibly resulting in conversion of GABAergic signaling (itself probably amplified subsequent to sprouting) from inhibitory to excitatory. These results illustrate the differential and exquisite levels of control which BDNF exerts over distinct populations of spinally-projecting axons, and the effects of exogenous BDNF on DRI-induced axonal plasticity and cold pain.
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<tbody>
<tr>
<td>Adv</td>
<td>Adenoviral vectors</td>
</tr>
<tr>
<td>AMCA</td>
<td>Aminomethylcoumarin amide</td>
</tr>
<tr>
<td>AMPA</td>
<td>Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BPI</td>
<td>Brachial plexus injury</td>
</tr>
<tr>
<td>CCC</td>
<td>Chloride-cation co-transporter</td>
</tr>
<tr>
<td>CCI</td>
<td>Chronic constriction injuries</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
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<td>CGRP</td>
<td>Calcitonin-gene related peptide</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Contra</td>
<td>Contralateral</td>
</tr>
<tr>
<td>CST</td>
<td>Corticospinal tract</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin B subunit</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCV</td>
<td>Dense core vesicles</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DREZ</td>
<td>Dorsal root entry zone</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
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<td>DRI</td>
<td>Dorsal root injury</td>
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<tr>
<td>DJβH</td>
<td>Dopamine-beta-hydroxylase</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory post-synaptic current</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;R</td>
<td>Gamma-aminobutyric acid A receptor</td>
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<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>i.m.</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>i.t.</td>
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<tr>
<td>IB4</td>
<td>Isolectin Bandeiraea simplicifolia 4</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
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<tr>
<td>Ipsi</td>
<td>Ipsilateral</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun kinase</td>
</tr>
<tr>
<td>KCC2</td>
<td>Potassium (K&lt;sup&gt;+&lt;/sup&gt;)/chloride (Cl&lt;sup&gt;-&lt;/sup&gt;) co-transporter 2</td>
</tr>
<tr>
<td>LOF</td>
<td>Loss-of-function</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeats</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>NDS</td>
<td>Normal donkey serum</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor-kappa-beta</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NgR</td>
<td>Nogo receptor</td>
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<td>NK-1</td>
<td>Neurokinin-1</td>
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<td>NKCC1</td>
<td>Sodium (Na⁺)/potassium (K⁺)/chloride (Cl⁻) co-transporter 1</td>
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<td>NMDA</td>
<td>N-methyl D-aspartate</td>
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<td>NPY</td>
<td>Neuropeptide tyrosine (Y)</td>
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<td>NR1</td>
<td>NMDA receptor subunit 1</td>
</tr>
<tr>
<td>NR2</td>
<td>NMDA receptor subunit 2</td>
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<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NT-4/5</td>
<td>Neurotrophin-4/5</td>
</tr>
<tr>
<td>OMgp</td>
<td>Oligodendrocyte myelin glycoprotein</td>
</tr>
<tr>
<td>P2X₃</td>
<td>Purinergic receptor 2X₃</td>
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<td>P2X₄</td>
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<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt;</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PAD</td>
<td>Primary afferent depolarization</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaductal grey</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PC1</td>
<td>Prohormone convertase 1</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PF</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC-γ1</td>
<td>Phospholipase C-gamma-1</td>
</tr>
<tr>
<td>PNI</td>
<td>Peripheral nerve injury</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>RSA</td>
<td>Rat serum albumin</td>
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<tr>
<td>RST</td>
<td>Rubrospinal tract</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral ventromedial medulla</td>
</tr>
<tr>
<td>s.c.</td>
<td>Sub-cutaneous (s.c.)</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean (SEM)</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin transporter (SERT)</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homologue 2-containing protein</td>
</tr>
<tr>
<td>SNL</td>
<td>Spinal nerve ligation (SNL)</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>Trk</td>
<td>Tropomyosin-related kinase</td>
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<tr>
<td>TrkB.FL</td>
<td>Full length TrkB receptor</td>
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<td>TrkB.T1</td>
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<td>TrkB.T4</td>
<td>Truncated TrkB variant 4</td>
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<tr>
<td>VGAT</td>
<td>Vesicular GABA transporter</td>
</tr>
<tr>
<td>VGLUT1</td>
<td>Vesicular glutamate transporter 1</td>
</tr>
<tr>
<td><strong>VGLUT2</strong></td>
<td>Vesicular glutamate transporter 1</td>
</tr>
<tr>
<td><strong>WDR</strong></td>
<td>Wide dynamic range</td>
</tr>
</tbody>
</table>
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“No duty is more urgent than that of giving thanks” – Unknown.

My sincere gratitude is extended to my supervisor, my mentor and my colleague, Dr. Matt Ramer. His conspicuous breadth of knowledge and technical expertise, as well as his zealous quests for that which is novel never cease to amaze me. It was an honour and a pleasure to have learned from him and to have been part of the Ramer Lab. Many thanks to the following co-authors of the manuscripts: Leanne Ramer, without whose seminal work I would have no Masters degree – I thank her for being a wonderful example of an intelligent and inexhaustible woman in science; Tim Kaan, for giving me hope to do more; and Dr. Lowell McPhail, for remaining the ever pragmatist. I wish to thank my labmates, my friends, who have collectively contributed to the maintenance of my sanity: Angela Scott – whom I thank for not only being a role model and confidante, but for more importantly being a true inspiration; Andrew Gaudet, the constant voice of reason and positivity – for always encouraging me to learn and improve; Jessica Inskip, whose creative and adventurous influences have never allowed me accept mediocrity; and Jacquelyn Cragg – for riding shotgun through crazy behavioural experiments and for her ever keenness thereafter. I would also like to thank the following ICORD trainees, past and present: Dr. Victoria Claydon, Dr. Ward Plunet, Byron Ramsey, Emily Lipinski, Jeremy Toma, Clarie Lam, Joe Sparling, Cody Mann, Jae Lee, Dr. Femke Streijger, Peggy Assinck and last but not least, Jason Plemel – whose unflagging support, encouragement, and critical insight have given me the confidence to persevere, I could not have done this without him. Finally, I must thank the members of my supervisory and examining committees: Dr. Bill Milsom, Dr. Shernaz Bamji, Dr. Tim O’Connor and
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DEDICATIONS

To my family

Mom, Dad and Tim

I hope I have made you proud
CO-AUTHORSHIP STATEMENT

The work presented in Chapters 2, 3 and 4 could not have been prepared and successfully completed without the contributions of the members of Dr. Matt Ramer’s laboratory at the International Collaboration of Repair Discoveries (ICORD), UBC.

Chapter 2 contributions: My role was in conducting the short- and long-term behavioural experiments with the IgG-, TrkB-Fc- and TrkC-Fc-treated animals, the immunohistological analyses of serotonergic axon density and the characterization of neurotrophin expression with the gracious aid of Leanne M. Ramer, Dr. Lowell T. McPhail, Timothy K.Y. Kaan, Jae H.T. Lee and Lucy P. Hwi. All other behavioural experiments (untreated and K252a-treated animals) and immunohistological analyses of VGLUT1 and CTB terminal densities were done by Leanne M. Ramer, Timothy K.Y Kaan and Lucy P. Hwi. The electrophysiological work was performed by Dr. Jaimie F. Borisoff and James W. Saunders. Experimental procedures were done under the guidance of Dr. Matt S. Ramer. Manuscript preparation was also conducted by myself, Leanne M. Ramer, Dr. Lowell T. McPhail with the supervision of Dr. Matt S. Ramer.

Chapter 3 contributions: I conducted the behavioural and immunohistological experiments and data analyses with the courteous help of Leanne M. Ramer, Timothy K.Y. Kaan under the supervision of Dr. Matt S. Ramer. I, Leanne M. Ramer and Dr. Lowell T. McPhail were directly involved in the manuscript preparation with the guidance of Dr. Matt S. Ramer.

Chapter 4 contributions: This study was conducted and manuscript was prepared by myself under the supervision of Dr. Matt S. Ramer. Dmitri Krassioukov-Enns provided some technical support with behavioural and imaging procedures.
CHAPTER 1

*Introduction: the sum of all parts*
1.1. Introduction

Deafferentation, or loss of sensory input to the central nervous system (CNS), is a common consequence of injuries at the level of the spinal cord. Clinical cases of brachial plexus injuries (BPIs), involving crushing, transecting and/or complete avulsion of the cervical sensory and motor spinal roots (brachial plexus), often result in monoplagia and loss of sensation. Spinal cord deafferentation also, inexplicably, leads to severe intractable pain in the majority of patients and can be the greatest impediment to their rehabilitation (Parry, 1980; Bruxelle et al., 1988; Frampton, 1996). Unfortunately the mechanisms that underlie such neuropathic pain have yet to be elucidated, attributing to the inadequacy of current analgesic treatments (Berman et al., 1998; Olson et al., 2007; Birch, 2009).

A relevant animal model of BPIs is the dorsal rhizotomy or dorsal root injury (DRI), which involves selective injury of the sensory spinal roots. This model has the distinct experimental advantage over complete brachial plexus avulsion in that it is sensory-specific: permitting the examination of sensory deficits that may arise following BPIs, independent of possible motor deficits. DRI in animals has also been demonstrated to result in the development of pain. For instance, single lumbar dorsal rhizotomy reportedly elicits touch or mechanically-evoked pain (Colburn et al., 1999; Li et al., 2000; Eschenfelder et al., 2000). More recently, it has also been found that rats with transection injuries of the 7th and 8th cervical dorsal roots (C7/8 DRI) develop profound and highly reproducible hypersensitivity to innocuous cold stimulation that becomes apparent by five days following the injury and spontaneously recovers 20 days later (Ramer et al., 2004).
The spontaneous development of, and recovery from sensory deficits following limited dorsal root lesions has been previously reported with other modalities, such as somatosensory postural reflexes in cats (Goldberger, 1988a, b), and voluntary hand movement in macaque monkeys (Darian-Smith and Ciferri, 2005, 2006). Since DRI directly damages sensory neuron processes, much of the observed changes in sensation have been commonly attributed to the re-wiring or plasticity of the spared spinal systems (Pubols and Goldberger, 1980; Darian-Smith and Brown, 2000; Darian-Smith, 2004). These anatomical and functional rearrangements subsequent to neurological damage, manifest into detrimental (e.g. development of pain) or beneficial (e.g. recovery from pain) consequences (Gomez-Pinilla et al., 2002, 2004; Ying et al., 2005; Galtrey et al., 2007; Deumens et al., 2008; Maier et al., 2009; Casals-Diaz et al., 2009). The work presented in this thesis is, therefore, focused on understanding the mechanisms governing plasticity of the intact or uninjured neurons in the spinal cord, and their potential to contribute to or compensate for deficits from the injury.

The first study (Chapter 2) investigates possible mechanisms underlying the anatomical and behavioural changes incurred following C7/8 DRI, with specific consideration into the role of the endogenous neurotrophin brain-derived neurotrophic factor (BDNF). Upregulation of endogenous spinal BDNF has also been implicated in the potentiation of neuropathic pain (Coull et al., 2005; Miletic and Miletic, 2008). Therefore, the experiments conducted in Chapter 3 evaluate the contribution of endogenous BDNF to neuroplastic events regulating cold pain following C7/8 DRI. Exogenous application of BDNF, on the other hand, has also been implicated in plasticity-mediated recovery of both motor (Zhou and Shine, 2003; Grider et al., 2005)
and respiratory (Baker-Herman et al., 2004) deficits following nervous system injury. In view of this, Chapter 4 examines the consequences of the exogenous BDNF treatment following C7/8 DRI to determine whether the exogenous neurotrophin regimen can enhance the plastic events harnessed by its endogenous counterpart. The present introductory chapter starts with an overview of the neuronal populations, intrinsic and extrinsic, influencing nociceptive processing in the spinal dorsal horn. Examples of spinal neuroplasticity following spinal deafferentation are subsequently described, and finally details of the molecular candidates underscoring such plastic events are discussed.

1.2 Organization of the spinal dorsal horn

The dorsal grey matter of the spinal cord, or the dorsal horn, serves as the first site of integration for a variety of peripheral and central neurons involved in sensory neurotransmission. Based on cytoarchitectural studies, the spinal grey matter is organized into ten segmented layers, for which the six most superficial segments form the bilateral dorsal horns (Fig. 1.1; Rexed, 1952). Lamina I, referred to as the marginal layer, is the most superficial; the substantia gelatinosa (SG) is represented by lamina II, which is further divided into lamina II outer (IIo) and lamina II inner (IIi); the nucleus proprius is comprised of laminae III and IV; and the most ventral layers of the dorsal horn are laminae V and VI (Rexed, 1952). Collectively, laminae I and II are recognized as the superficial laminae, a major site for pain or nociceptive propagation and processing. As such, the superficial laminae receive and harbour components from four major neuronal types including the central axon terminals of primary sensory neurons (Szentagotai, 1964; Light, 1988), descending modulatory axons from bulbospinal neurons (Westlund
and Coulter, 1980; Ruda et al., 1982; Millan, 2002), projection neurons whose axons ascend contralaterally to the brain and intrinsic interneurons with spinally-localized neuritic processes (Craig, 1995; Millan, 1999).

1.2.1 Peripheral primary sensory neurons

The unique morphology of primary sensory neurons facilitates the transduction of peripheral sensory information to the internal environment. Their somata are situated within posterior or dorsal root ganglions (DRGs) located in the invertebral foramen of the peripheral nervous system (PNS) (Devor, 1999). Symmetrical pairs of DRGs are attached to discrete spinal roots of collective vertebral segments of the spinal cord (Crosby and Lauer, 1967). Morphologically, primary sensory or DRG neurons are considered pseudo-unipolar as they extend a single axon bifurcated into two branches from their cell bodies: one process (the distal branch) projects to various peripheral targets, whereas the other (the primary afferent) travels via the dorsal roots and crosses the CNS-PNS interface, known as the dorsal root entry zone (DREZ), to enter the spinal dorsal horn (Fraher & Sheehan, 1987).

Archetypical classification of DRG neurons, based on cell size, state of myelination and receptive field modality, has commonly divided them into three groups: C, Aδ and Aβ neurons (Millan, 1999). Primary afferents of C and Aδ neurons are of particular interest with regards to nociceptive neurotransmission (Millan, 1999). Small-calibre, unmyelinated C fibres are generally slow conducting and respond to noxious mechanical, chemical and/or thermal stimuli (Bessou and Perl, 1969; Millan, 1999). Interestingly, some C fibres are solely responsive to one type of peripheral stimulus,
whereas others known as polymodal nociceptors respond to multiple stimuli (Bessou and Perl, 1969). The fibres of Aδ neurons are medium-sized in diameters and lightly myelinated, thus conduct at intermediate velocities and also respond to a wide range of innocuous to noxious thermal and mechanical stimuli (Burgess and Perl, 1967; Campbell et al., 1979; Light and Perl, 1979). In contrast, Aβ fibres do not normally respond to noxious stimuli, conducting primarily innocuous mechanical and proprioceptive information, but for the interest of comparison these afferents are the largest in diameter and heavily myelinated (Woolf, 1987; Koerber et al., 1995; Millan, 1999).

Primary afferent terminals in the rodent dorsal horn are also typically identified immunohistochemically by their expression of various neurotransmitters, neuropeptides and/or receptors. Nociceptive fibres (C and Aδ) in particular, are subdivided into two major groups: the first, contain neuropeptides such calcitonin-gene related peptide (CGRP), substance P and galanin, and express the neurotrophin receptor tropomyosin-related kinase A (TrkA) (Zhang et al., 1993; Averill et al., 1995; Molliver et al., 1995; Bennett et al., 1996; Lawson et al., 1997); whereas afferents of the second group do not contain any neuropeptides, but exhibit binding sites for the plant lectin IB4 and express the purinergic receptor P2X₃ and the receptor tyrosine kinase RET (Averill et al., 1995; Molliver et al., 1997; Bennett et al., 1998; Guo et al., 1999). Although it remains unclear as to whether these afferent phenotypes are functionally distinct, spatially, they terminate in discrete laminae with axons containing peptides (peptidergic fibres) in Rexed’s laminae I and IIo, and non-peptidergic axons terminating primarily in lamina III (Guo et al., 1999; Michael and Priestley, 1999).
1.2.2 Descending monoaminergic axons

Analagous to its afferent input, the dorsal horn also receives a variety of supraspinal monoaminergic input. Of note are axons of the serotonergic, dopaminergic, and noradrenergic/adrenergic systems (Millan, 2002). Serotonergic projections originate within the area of the rostral ventromedial medulla (RVM), specifically in the nucleus raphe magnus, while dopaminergic axons project from neurons in the hypothalamus and substantia nigra pars compacta and noradrenergic axons from the locus coeruleus (Millan, 2002). Axon terminals of these monoaminergic phenotypes are extensively distributed in the rat dorsal horn. Serotonergic varicosities, for example, are identified by their expression of the serotonin transporter (SERT) (Blakely et al., 1991; Qian et al., 1995; Zhou et al., 1998) and are present as a dense plexus in laminae I-IIo (MacDermid et al., 2004; Ramer et al., 2004; Ramer et al., 2007). Antibodies directed against the enzyme tyrosine hydroxylase (TH) identifies noradrenergic/adrenergic and dopaminergic cells, while the dopamine-β-hydroxylase enzyme is expressed exclusively by noradrenergic/adrenergic axons (Armstrong et al., 1981; Verney et al., 1982). Interestingly, the pattern of immunoreactivity of both of these enzymes has revealed a similar uniform expression throughout the spinal cord, except for a gap in lamina III (MacDermid et al., 2004; Ramer et al., 2004).

Melzack and Wall’s “Gate Control Theory of Pain” (1965) brought light to the integrative modulation of messages propagated by nociceptive primary afferents to their targeted projection neurons, prior to their onward transfer to the brain. Descending monoaminergic projections provide an intriguing modulatory influence as they are both inhibitory and facilitatory to noxious transmission (Millan, 1999, 2002). For instance,
Monoaminergic axons may synapse onto pre-synaptic nociceptive terminals to negatively regulate the release of neurotransmitters/modulators, such as glutamate and substance P, inhibiting noxious excitation of their projection neuron targets (Millan, 1999, 2002). Conversely, supraspinal projections may directly or indirectly (activating excitatory interneurons) excite the post-synaptic cell, facilitating pain transmission (Millan, 1999). Although the anatomical substrates subserving these opposing synaptic processes are common, it is likely that the type of modulation will be determined by which sub-type of receptors are activated and the site of input, pre-synaptic versus post-synaptic (Lopez-Garcia and King, 1996; Millan et al., 1996; Peng et al., 1996; Millan, 2002).

1.2.3 Spinal nociceptive projection neurons

The intrinsic neuronal elements of the dorsal horn involved in pain are the second-order nociceptive projection neurons and the spinal interneurons. Cell bodies of the former have been primarily localized to lamina I (Nakaya et al., 1994) and deeper laminae III and IV (Todd, 2002) by the expression of neurokinin-1 (NK-1), the receptor for the neuropeptide substance P. Considering that the release of substance P (and glutamate) from C and Aδ fibres occurs predominantly in lamina I (Lawson et al., 1997), the deeper projection neurons were found to dorsally extend their dendrites to the superficial laminae, most likely to facilitate the receipt of afferent input (Naim et al., 1997; Todd et al., 2002). There is also a population of projection neurons located in laminae IV-V that respond to innocuous and noxious stimuli, known as the ‘wide dynamic range’ (WDR) neurons (Mense, 1993; Ness and Gebhart, 1990; Gebhart, 1995). While most dorsal horn neurons in these deeper laminae receive input from
mechanoreceptive Aβ fibres, WDR neurons may be monosynaptically (directly) or polysynaptically (via interneurons) activated by C, Aδ and Aβ afferents (Light and Kavookjian, 1988; De Koninck et al., 1992; Todd et al., 1994). Collectively, axons of these nociceptive projection neurons cross the spinal cord midline and travel rostrally as the contralateral spinothalamic tract (Craig, 1995; Yu et al., 1999).

1.2.4 Resident inhibitory and excitatory interneurons

Relative to the number of nociceptive projection neurons, spinal interneurons, form the majority of dorsal horn neurons in laminae I-III (Szentagothai, 1964). Golgi staining has revealed interneuronal neurites to be fairly short, arborizing proximal to their cell bodies often within the same laminae (Sheibel and Scheibel, 1968; Beal and Cooper, 1978; Lima and Coimbra, 1986). Dorsal horn interneurons are also functionally segregated into inhibitory γ-aminobutyric acid (GABA) or glycinergic interneurons, and excitatory glutamatergic interneurons (McLaughlin et al., 1975; Barber et al., 1978; Campistron et al., 1986; Ottersen and Storm-Mathisen, 1987; van den Pol and Gorcs, 1988; Todd and Sullivan, 1990). With regard to the inhibitory interneurons, the glycinergetic phenotypes are typically in laminae III-IV, whereas GABAergic interneurons are in abundance throughout the dorsal horn (Todd and Sullivan, 1990). Thus, local inhibition of nociception involves both GABAergic and glycinergic neurons projecting pre- and post-synaptically on nociceptive afferent terminals and projection neurons, respectively (Todd, 1990; Powell and Todd, 1992). Interestingly, immunocytochemical (of the neurotransmitters themselves) and physiological data have also provided evidence for the co-localization and co-release of GABA in glycinergetic interneurons (van den Pol...
On the other hand, identification of excitatory glutamatergic interneurons has been more tedious because the detection of glutamate alone does not differentiate them from primary afferent terminals (Todd and Spike, 1993). Messenger RNA (mRNA) for the vesicular glutamate transporter 2 (VGLUT2), however, has been observed in dorsal horn neurons (Kullander et al., 2003) and may serve as a sufficient marker for excitatory interneurons since expression of VGLUT2 protein was located in CGRP-negative axons in the superficial laminae (Todd et al., 2003). Spinal interneurons have alternatively been classified based on their neuropeptide content. Neuropeptide Y (NPY) or galanin, for example, are detected exclusively in GABAergic interneurons (Rowan et al., 1993; Naim et al., 1997; Polgar et al., 1999). Whereas somatostatin and enkephalin were found in presumptive excitatory interneurons, as they were non-GABAergic VGLUT2-immunoreactive cells (Todd et al., 2003). As mentioned, excitatory interneurons also provide polysynaptic excitation for other dorsal horn neurons (Light and Kavookjian, 1988; De Koninck et al., 1992; Todd et al., 1994). Therefore, together with inhibitory interneurons, these cells provide dynamic control over dorsal horn processing.

1.3 Neural plasticity in the dorsal horn: consequences of spinal deafferentation

A hallmark of the spinal sensory system is its adaptive reorganization or plasticity in response to an injury. In general, anatomical damage (e.g. axonal injury) from lesions to the CNS leads to the permanent disruption of neuronal circuits (Maier and Schwab, 2006). Most CNS injuries are, however, considered incomplete (Bunge et al., 1993;
Bunge et al., 1997; Guest et al., 2005) with some neurons remaining intact and rearranging in a compensatory manner to promote the functional restitution of those interrupted circuits (Bregman et al., 1997; Raineteau and Schwab, 2001; Ding et al., 2005; Courtine et al., 2008). Similarly, DRI specifically disconnects primary afferents from their original targets in the dorsal horn, but also triggers a concomitant plastic response from neurons spared by the injury (Liu et al., 1958; McNeill et al., 1990, 1991; Polistina et al., 1990; Wang et al., 1991; Zhang et al., 1993; Sengelaub et al., 1997; Kinkead et al., 1998; Belyantseva et al., 1999; Darian-Smith, 2004; Ramer et al., 2004, 2007; MacDermid et al., 2004; Scott et al., 2005). Considering that the term ‘plasticity’ indistinctly describes the advent of change in the nervous system, anatomical and functional plasticity in the dorsal horn following DRI will be considered here.

1.3.1 DRI-induced anatomical plasticity

Since axon degeneration occurs to afferents directly affected from DRI, any observed anatomical plasticity or sprouting of axon terminals in the dorsal horn may be attributed to those spared by the lesion (Scott et al., 2006). Accordingly, partial spinal deafferentation (of a limited number of dorsal roots) is well-recognized for promoting intraspinal sprouting of intact adjacent afferent terminals (Liu et al., 1958; McNeill et al., 1990, 1991; Polistina et al., 1990; Belyantseva et al., 1999; Darian-Smith, 2004) and uninjured descending monoaminergic axons (Polistina et al., 1990; Wang et al., 1991; Zhang et al., 1993; Kinkead et al., 1998; Ramer et al., 2004, 2007; MacDermid et al., 2004; Scott et al., 2005). This rhizotomy-induced sprouting has often been considered a regulated process, rather than a randomized event (Wang et al., 1991). First, the
plasticity of different axon populations (primary afferents versus serotonergic axons) has been found in discrete, non-overlapping regions of the dorsal horn (Polistina et al., 1990). There has also been suggestion of hierarchical axon plasticity, whereby some axonal phenotypes exert marked plastic responses over others (Polistina et al., 1990; Wang et al., 1991). For example with monoaminergic axons, serotonergic sprouting has been readily observed following DRI (Polistina et al., Wang et al., 1991; Zhang et al., 1993; Ramer et al., 2004), whereas dopaminergic axon plasticity has been coaxed by more extensive rhizotomies (Mitchell et al., 2000; Ramer et al., 2004), and adrenergic and noradrenergic axons are supposedly not stimulated by DRI alone (Wang et al., 1991a, b; MacDermid et al., 2004).

1.3.2 Functional consequences of axonal sprouting

That DRI-induced axonal plasticity occurs in a regulated manner suggests that specific functional consequences are also likely to ensue (Scott et al., 2006). Perhaps the most convincing evidence for this is the reactivation of dorsal horn neurons deafferented from the initial DRI (Basbaum and Wall, 1976; Pubols and Goldberger, 1980; Darian-Smith and Brown, 2000). Early electron microscopy studies verified the degeneration of injured primary afferents (Knyihar-Csillik et al., 1982; Ralston and Ralston, 1982; Coimbra et al., 1984; Franson and Ronnevi, 1984) and subsequent loss of presynaptic terminals in superficial laminae by three days post-DRI (Zhang et al., 1993). In contrast, animals that survived to chronic timepoints (3-10 weeks post-DRI) exhibited a restoration in presynaptic terminal numbers comparable to intact controls, which was attributed to sprouting of uninjured axons from spared roots (Zhang et al., 1993). This
adaptive reorganization of intact sensory fibres re-wiring to deafferented second-order neurons has been suggested to contribute to the functional restoration of peripheral cutaneous or mechanical sensation (Basbaum and Wall, 1976; Darian-Smith and Brown, 2000) and recovery of postural somatosensory reflexes (Goldberger and Murray, 1974).

Because DRI has also been suggested to contribute to pain-related behaviour (Colburn et al., 1999; Li et al., 2000; Eschenfelder et al., 2000; Ramer et al., 2004) re-wired input to nociceptive dorsal horn neurons may also promote aberrant nociceptive transmission. Normal afferent input from the 5th lumbar (L5) dorsal root has been suggested to directly suppress pre-synaptic input from adjacent dorsal roots (Wall, 1995; Eschenfelder et al., 2000). Therefore subsequent to L5 DRI, unmasking of these intact adjacent afferents may lead to the acute reactivation of post-synaptic L5 nociceptive projection neurons and mechanical hypersensitivity, sustained by their collateral sprouting over time (Eschenfelder et al., 2000). Considering that supraspinal monoaminergic projections are thought to participate in nociceptive modulation (Milan, 2002), a delayed onset of deafferentation-induced pain (>5 days post-DRI) may alternatively result from increased descending input to deafferented dorsal horn neurons concomitant to serotonergic axon sprouting (Ramer et al., 2004).

1.3.3. Molecular candidates regulating spinal neuroplasticity

Unfortunately the molecular mediators and/or intracellular mechanisms governing the injury-induced plasticity remain somewhat unclear. It has been demonstrated that antagonism of the inhibitory myelin-derived proteins, such as NogoA, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (MacDermid et
al., 2004), genetic deletion of a functional myelin co-receptor (Scott et al., 2005) or inhibition of the convergent RhoA pathway (Ramer et al., 2004) enhances rhizotomy-induced sprouting. Known for inhibiting axon growth in the injured adult CNS, by binding the Nogo receptor (NgR) and downstream activation of the small GTPase RhoA (Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002), myelin-derived inhibitors have been suggested to limit the extent of serotonergic and TH-positive axon plasticity after DRI (MacDermid et al., 2004; Ramer et al., 2004; Scott et al., 2005). However, since DRI-induced growth is still prevalent in spite of these inhibitory molecules (Ramer et al., 2004; MacDermid et al., 2004) this suggests a dominant presence of local growth-promoting influences in the deafferented region. As it may be in the developing CNS, the best endogenous candidates for stimulating local neuronal growth in the deafferented dorsal horn are the neurotrophic factor family of neurotrophins.

1.4 The neurotrophin family of neurotrophic factors

Neurotrophic factors are proteins characterized by their capacity to support the growth and survival of distinct subpopulations of neurons. In line with the neurotrophic factor hypothesis, developing neurons compete for limited target-derived neurotrophic factors, which are responsible for maintaining neuronal phenotype in adulthood (Davies et al., 1996). Of these factors, the neurotrophin family has been the most extensively examined, especially with regards to influences on sensory neurons (McMahon et al., 1995; Verge et al., 1995; Munson et al., 1997). Nerve growth factor (NGF) was the first to be discovered in a search for unknown neuronal survival factors (Cohen et al., 1954, Cohen and Levi-Montalcini, 1956). Subsequent to this, brain-derived neurotrophic factor
(BDNF) was identified as the second member of this protein family (Barde et al., 1982). Isolated from pig brain, BDNF was responsible for the survival of dorsal root ganglion (DRG) neurons unresponsive to NGF (Barde et al., 1982). In total, there are four secreted mammalian neurotrophins: NGF, BDNF, neurotrophin-3 (NT-3) (Hohn et al., 1990; Maisonpierre et al., 1990) and neurotrophin-4/5 (NT-4/5) (Hallbook et al., 1991; Ip et al., 1992), which have been collectively found to regulate proliferation, survival, neurite growth and guidance, synapse formation and plasticity throughout the CNS and PNS (Poo, 2001; Reichardt, 2006; Chao et al., 2006).

1.4.1 Origins and structure of the neurotrophins

The mature dimeric neurotrophin forms arise from direct cleavage of their pro-isoforms, the proneurotrophins. The 30-35 kDa proneurotrophin sequences contain dibasic amino acid motifs recognized by endoproteases such as furin and prohormone convertase 1 (PC1) (Seidah et al., 1996a, b; Mowla et al., 1999). Thus upon enzymatic cleavage, the 12-14 kDa biologically active (mature) neurotrophin product is separated from the N-terminal pro-domain (Seidah et al., 1996a, b; Chao and Bothwell, 2002; Binder and Scharfman, 2004). There is also evidence that proneurotrophins exhibit biological activity, independent from that mediated by cleaved neurotrophins, by binding the tumor necrosis superfamily member p75 neurotrophin receptor (p75NTR) and sortilin, a type I transmembrane protein that acts as a receptor for neurotensin (Mazella et al., 1998; Lee et al., 2001; Nykjaer et al., 2004; Teng et al., 2005; Kenchappa et al., 2006). However, it remains unclear as to whether some of these proforms, such as proBDNF,
exist in substantive quantities (to exert such roles) throughout the adult CNS (Matsumoto et al., 2008; Yang et al., 2009; Barker, 2009).

1.4.2 Neurotrophin signaling via the Trk and p75NTR receptors

Mature neurotrophin function is primarily mediated by the tropomyosin related kinase (Trk) family of tyrosine kinase receptors (Barbacid, 1994). NGF binds with high-affinity to TrkA (Kaplan et al., 1991; Klein et al., 1991), the TrkB receptor binds both BDNF and NT-4/5 (Klein et al., 1991; Soppet et al., 1991; Berkemeier et al., 1991; Klein et al., 1992), and NT-3 not only binds TrkC with high-affinity (Lamballe et al., 1991), but is also a heterologous low-affinity ligand for TrkA (Cordon-Cardo et al., 1991) and TrkB (Klein et al., 1991; Soppet et al., 1991). The p75NTR has also been suggested to coordinate with individual Trk receptors to enhance the responsiveness and binding specificity of Trks with their cognate neurotrophin ligands (Hempstead et al., 1991; Lee et al., 1994; Clary and Reichardt, 1994; Bibel et al., 1999; Brennan et al., 1999). Interestingly, recent three-dimensional characterization of the receptor structures has stirred some debate over the mechanism of this functional interaction, contesting as to whether p75NTR and Trks directly interact (He and Garcia, 2004; Wehrman et al., 2007; Gong et al., 2008).

Each Trk receptor spans the membrane once, with an extracellular domain consisting of two cysteine clusters flanking tandem leucine-rich repeats (LRR) followed by two immunoglobulin-like motifs, the second one serving as the binding domain for neurotrophin homodimers (Urfer et al., 1998; Ultsch et al., 1999; Wehrman et al., 2007). The cytoplasmic domain contains a tyrosine kinase sequence and upon neurotrophin
binding Trk receptors form homodimers, triggering kinase activation and auto-
phosphorylation of the cytoplasmic tyrosine residues (Jing et al., 1992; Patapoutian and
Reichardt, 2001). Once phosphorylated, these residues serve as docking sites for specific
cytoplasmic adaptors and enzymes that are critical intermediates for the activation of
specific intracellular signaling cascades (Inagaki et al., 1995; Barbacid, 1995;
Patapoutian and Reichardt, 2001).

Downstream signaling cascades activated upon Trk phosphorylation include the
Ras GTPase protein (GAP), phosphoinositide 3-kinase (PI3-kinase) and phospholipase C-
γ1 (PLC-γ1) pathways (Patapoutian and Reichardt, 2001). Ras stimulation of the
downstream effector mitogen-activated protein kinase or extracellular signal-regulated
kinase (MAPK/ERK) cascade, via the protein kinase Raf, has been demonstrated to
promote the transcription of pro-survival and pro-differentiation genes (Bonni et al.,
1999; Mazzoni et al., 1999). Trk signaling via PI3-kinase, incidentally another Ras target
(Rodriguez-Viciana et al., 1994), activates the serine/threonine kinase Akt and has been
considered the most critical pathway in neuronal survival (Soltoff et al.,
1992; Obermeier et al., 1993; Dudek et al., 1997; Crowder and Freeman, 1998; Vaillant et al., 1999).
Finally, phosphorylation of PLC-γ1 generates inositol triphosphate (IP3) and
diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP2) and results in
the release of Ca^{2+} stores and activation of Ca^{2+}- and DAG-dependent protein kinases
(e.g. protein kinase C; PKC) required for activation of MAPK/ERK and neurite
outgrowth (Ohmichi et al., 1991; Corbit et al., 1999). The mature neurotrophins have
also been found to bind, albeit with low-affinity, to the p75NTR and activate the nuclear
factor-κβ (NF-κβ) and Jun kinase (JNK) pathways to initiate responses such as neuronal
apoptosis (Rabizadeh et al., 1993; Frade et al., 1996; Bamji et al., 1998; Aloyz et al., 1998; Sedel et al., 1999; Miller and Kaplan, 2001).

1.4.3 Neurotrophins as candidate regulators of spinal neuroplasticity

While neurotrophins exert predominant regulatory roles over cell fate and survival during developmental stages, in adulthood they progress into maintenance of anatomical and synaptic plasticity. In fact certain injurious manipulations, such as cervical DRI, have resulted in aberrant expression of neurotrophic factors in the adult spinal cord concomitant to such plastic events. Studies conducted by Gordon Mitchell’s group (Johnson et al., 2000) were amongst the first to reason that, similar to their neurotrophic influence in supraspinal areas (Mamounas et al., 1995, 2000), endogenous neurotrophins may also govern spinal neuroplasticity following DRI. For instance, dorsal rhizotomy results in the sprouting of descending serotonergic terminals adjacent to spinal phrenic motor nuclei (Kinkead et al., 1998). This spinal plasticity of serotonergic inputs has been suggested to contribute to synaptic plasticity of the spinal respiratory (phrenic) system (Zhang et al., 1993; Kinkead et al., 1998). Although the mechanism(s) manifesting such plasticity remained somewhat elusive, one week after bilateral transection of the C3-C6 dorsal roots, Johnson et al. (2000) found specific do novo expression of the growth promoting proteins BDNF and NT-3 in the ipsilateral ventral spinal cord.

Rhizotomy to a more limited number of cervical dorsal roots has also been demonstrated to promote serotonergic sprouting within corresponding dorsal spinal cord segments (MacDermid et al., 2000; Ramer et al., 2004). Work done from Matt Ramer’s laboratory (Ramer et al., 2004) has also shown that unilateral C7/8 DRI specifically
promoted the transient development of cold allodynia and hyperalgesia (cold pain) in response to acetone application to the palmar forepaw. That serotonergic sprouting in the dorsal horn increased coincident to cold pain progression suggested its participation to the pain-related behaviour (Millan, 2002; Ramer et al., 2004). Unlike in the ventral horn (Johnson et al., 2000), however, it was not determined whether local growth promoting factors were also present in the deafferented dorsal horn. Nevertheless, since serotonergic projections originating from the raphe nucleus specifically express the TrkB and TrkC neurotrophin receptors (King et al., 1999), BDNF and NT-3 have been suggested as the most likely candidates stimulating spinal neuroplasticity of this spared descending system (Johnson et al., 2000; Ramer et al., 2004).

1.5 BDNF in the adult sensory nervous system

BDNF is the most widely synthesized and released neurotrophin in the adult mammalian CNS (Binder and Scharfman, 2004). Both human and rat BDNF genes contain five exons (I-V), with exon V specifically encoding the mature BDNF form (Metsis et al., 1993; Timmusk et al., 1993). Neuronal expression of the mature BDNF protein in adult CNS regions include the somatosensory cortex, hippocampus, thalamus, brainstem nuclei such as the raphe magnus nucleus and locus coeruleus, and spinal cord (Ernfors et al., 1990; Wetmore et al., 1990; Conner et al., 1997; Yan et al., 1997; Kohara et al., 2001). Peptidergic C and Aδ sensory neurons also synthesize BDNF in the DRG and anterogradely transport it to their primary afferent terminals in the spinal cord (Wetmore and Olson, 1995; Altar et al., 1997; Michael et al., 1997; Kerr et al., 1999; Kohara et al., 2001; Luo et al., 2001; Zhou and Rush, 1993). For example in the dorsal
horns, terminals containing CGRP and substance P are also BDNF-positive (Michael et al., 1997; Salio et al., 2007). This capacity for anterograde BDNF transport (Zhou and Rush, 1996; Alter et al., 1997) was intriguing given that retrograde neurotrophin transport was classically described by the neurotrophic factor hypothesis (Davies et al., 1996).

However, support for anterograde transport of the neurotrophin came from ultrastructural and biochemical studies localizing BDNF in dense core vesicles (DCVs) at the terminals of central and peripheral neurons (Michael et al., 1997; Fawcett et al., 1997; Smith et al., 1997; Luo et al., 2001; Salio et al., 2007).

1.5.1 TrkB expression in the adult sensory nervous system

As mentioned, BDNF exerts its physiological effects in the adult CNS through its high-affinity TrkB receptor (Barbacid, 1994). Due to alternative mRNA splicing, TrkB in rodents exists in four major forms: the dominant catalytic full length TrkB (TrkB.FL) receptor, and the three truncated TrkB variants TrkB.T1, TrkB.T2, TrkB.T4 (Klein et al., 1990; Middlemas et al., 1991; Forooghian et al., 2001; Stoilov et al., 2002). The truncated TrkB receptors are homologous to TrkB.FL in their extracellular and transmembrane regions, yet lack a significant kinase domain due to their truncated cytoplasmic sequences (e.g. 23 and 21 residues in length for TrkB.T1 and TrkB.T2, respectively) (Klein et al., 1990; Middlemas et al., 1991). TrkB.T4 also lacks a tyrosine kinase sequence, but its intracellular domain is much longer than the other truncated isoforms as it has both a Shc binding region (Stoilov et al., 2002) and an internalization sequence (Forooghian et al., 2001).
TrkB mRNA and protein expression is ubiquitous throughout the adult CNS (Merighi et al., 2008). Markedly, TrkB.FL expression has been detected in many supraspinal regions involved in pain processing including the cortex, thalamus and periauductal grey (PAG), as well as throughout the brainstem in areas such as red nucleus, raphe magnus and locus coeruleus (Alter et al., 1994; Tetzlaff et al., 1994; Pitts and Miller, 1995; Fryer et al., 1996; Kobayashi et al., 1997; Yan et al., 1997; King et al., 1999; Miller and Pitts, 2000; Madhav et al., 2001; Yamuy et al., 2005). A small proportion of mechanoreceptive Aß neurons also express TrkB.FL in their cell bodies (McMahon et al., 1994; Wright and Snider, 1995), although whether it is detected on primary afferent terminals has been somewhat contentious (Bradbury et al., 1998; Salio et al., 2005). Regardless the majority of TrkB.FL expression in the dorsal horn has been attributed to intrinsic projection neurons and interneurons (Zhou et al., 1993; Mannion et al., 1999; Widenfalk et al., 2001; Slack et al., 2005; Bardoni et al., 2007; Carrasco et al., 2007).

Neuronal (Beck et al., 1993; Armanini et al., 1995; Bracken and Turrigiano, 2009) and non-neuronal expression (Klein et al., 1990; Frisen et al., 1993; Fryer et al., 1997) has been described for the truncated TrkB isoforms. In the spinal cord, for example, truncated TrkB expression was detected in both the grey and white matter (Liebl et al., 2001). Despite their incapacity for tyrosine kinase activity, the truncated receptors have been shown to negatively regulate BDNF-TrkB.FL interactions by physically hindering and binding to TrkB.FL (Eide et al., 1996) or sequestering BDNF (Fryer et al., 1997). TrkB.T1 and TrkB.T2 have also independently demonstrated intracellular signaling capability, kinetically distinct from that of TrkB.FL-mediated
signaling (Baxter et al., 1997). TrkB.T4 mRNA has been specifically located in the brain (Stoilov et al., 2002) and recently, regulation of its expression in the visual cortex was found to be experience-dependent, suggesting its possible contribution to cortical plasticity (Forooghian et al., 2001; Bracken and Turrigiano, 2009).

1.5.2 Injury-induced changes in BDNF and TrkB expression

That physiological expression of BDNF and TrkB is predominant along areas of the pain neuroaxis suggests their participation in nociceptive processing. In fact, many pathological pain conditions in animals result in the dysregulated expression of the neurotrophin and its high-affinity receptor (Pezet and McMahon, 2006). Those occurring in primary sensory neurons and the dorsal horn of the spinal cord will be considered here. Intraplantar injection of several pro-inflammatory substances, such as NGF (Michael et al., 1997), carageenan (Kerr et al., 1999) or complete Freund’s adjuvant (CFA; Cho et al., 1997a, b), increased basal BDNF mRNA and protein in small- and medium-diameter TrkA-postive DRG neurons. With the latter two inflammatory mediators, the changes in BDNF levels have also been suggested to occur in an NGF-dependent manner (Kerr et al., 1999; Thompson et al., 1999; Cho et al., 1997a, b). Injury to DRG neuron processes, such as those produce by neuropathic pain paradigms, has similarly resulted in extensive alterations of BDNF expression (Ernsfors et al., 1993; Cho et al., 1998; Zhang et al., 2000; Zhou et al., 2000; Ha et al., 2001). For example, axotomy of lumbar peripheral nerves decreased BDNF mRNA expression in small diameter DRG neurons, yet prompted do novo expression in the medium- and large-calibre counterparts (Michael et al., 1999). The Noguchi group, however, demonstrated that following partial peripheral
nerve injury (PNI) intact small-diameter axons remaining in the injured sciatic nerve actually increase their somal BDNF expression (Fukuoka et al., 2001). Chronic constriction injuries (CCI) of the sciatic nerve, which additionally elicit an inflammatory response, have been shown to further increase BDNF in small nociceptive DRG neurons (Obata et al., 2003; Zhao et al., 2003).

Despite these reports of modified BDNF levels in the DRG, conditional genetic deletion of the neurotrophin from small- and medium-diameter primary sensory neurons has suggested that nociceptor-derived BDNF does not contribute to neuropathic pain behaviour (Zhao et al., 2006). Alternatively, resident microglial cells in the spinal cord have become a novel source of injury-upregulated BDNF (Tsuda et al., 2003; Coull et al., 2005; Ulmann et al., 2008; Trang et al., 2009). As the innate immune cells of the spinal parenchyma, microglia remain in a fairly dormant state under normal circumstances (Inoue et al., 2005). Following PNI, adenosine triphosphate (ATP) activation of the microglial purinergic P2X4 receptor (Tsuda et al., 2003; Ulmann et al., 2008) triggers the glial cells to secrete BDNF into the superficial dorsal horn (Coull et al., 2005; Trang et al., 2009).

Injury-induced changes in TrkB expression, on the other hand, have been relatively less reported (Pezet and McMahon, 2006). One study examining both TrkB.FL and truncated TrkB expression following peripheral inflammation demonstrated that intraplantar CFA injection had no apparent effect on TrkB.FL levels, yet increased truncated TrkB mRNA expression in the DRG (Lee et al., 1999). In the dorsal horn, TrkB.FL mRNA and protein were elevated subsequent to intraplantar CFA injection (Mannion et al., 1999). Concomitantly, increased truncated TrkB protein was also
observed after CFA injection and, notably, more pronounced compared to TrkB.FL levels (Mannion et al., 1999). In contrast, reports of direct (non-inflammatory) injury-induced changes in spinal TrkB protein expression have been sparse. Partial spinal nerve ligation in mice was found to increase TrkB protein levels on non-specific plasma membranes in the ipsilateral spinal cord (Narita et al., 2000; Yajima et al., 2002). Whereas transcriptional TrkB changes have also been observed in the DRG after peripheral axotomy or dorsal rhizotomy, but without an apparent effect on TrkB mRNA (Ernfors et al., 1993).

1.5.3 Regulation of BDNF release from nociceptive primary afferents

Nociceptor-release of BDNF in the superficial dorsal horn transiently activates/phosphorylates TrkB receptors on nociceptive projection neurons (Michael et al., 1997; Luo et al., 2001; Lever et al., 2001, 2003; Walker et al., 2001). Since BDNF and neuropeptides are co-stored in individual DCVs, neurotrophin release has been associated with mechanisms similarly governing neuropeptide secretion (Balkowiec and Katz, 2000; Lever et al., 2001; Brigadski et al., 2005). Of those, activity has revealed to be a versatile regulator of BDNF release (Balkowiec and Katz, 2000; Lever et al., 2001; Walker et al., 2001). For example, high-frequency dorsal root stimulation (C-fibre stimulation) elicited BDNF and substance P co-release (Lever et al., 2001). Application of the noxious stimulant capsaicin has also been found to evoke similar bursting behaviour and co-release from small-diameter primary afferents (Lever et al., 2002). An increased and more diffuse release of nociceptor-derived BDNF has also been coupled with its aberrant synthesis subsequent to peripheral inflammation (Lever et al., 2001;
Walker et al., 2001). However noxious stimulation in general, may not necessarily serve as a wide effector seeing as other similar stimuli lead to substance P-release alone (Lever et al., 2001). Accordingly, this difference in stimulus-evoked release may be attributed to variable release rates of the heterogeneous DCV content (Berg et al., 2000; Brigadski et al., 2005).

1.6 BDNF is a central neuromodulatory of pain

To date, there is considerable acceptance of a neuromodulatory role for BDNF in spinal nociceptive transmission (Pezet and McMahon, 2006; Merighi et al., 2008). Neurotransmitter-like activation of BDNF was first demonstrated in areas such as the hippocampus, cerebellum and cortex (Kafitz et al., 1999). Subsequently, multiple groups have demonstrated that BDNF released within the dorsal horn modulates excitatory nociceptive neurotransmission (Kerr et al., 1999; Arvanian and Mendell, 2001; Garraway et al., 2003; Pezet et al., 2002a, b; Matayoshi et al., 2005). Binding of BDNF to TrkB.FL, for example, has been found to potentiate N-methyl D-aspartate (NMDA) glutamate receptor activation on nociceptive projection neurons (Kerr et al., 1999; Guo et al., 2002; South et al., 2003; Brenner et al., 2004). The ionotropic NMDA receptor is a heterotetramer composed of two NR1 and two NR2 subunits (Paoletti and Neyton, 2007). Phosphorylation of residues on either subunit potentiates receptor activation and is considered to be critical for inducing the pain phenomenon of central sensitization (Sucher et al., 1996; Levine et al., 1998; South et al., 2003). Described as the activity-dependent hyperexcitability of spinal nociceptive projection neurons, central sensitization is thought to ensue after brief stints of nociceptive afferent input (Woolf, 1983). As such,
exogenous BDNF applied to an *in vitro* spinal cord preparation has been suggested to contribute to central sensitization by rapid phosphorylation of NMDA receptors on projection neurons (Kerr et al., 1999; Thompson et al., 1999). Furthermore, depressed nociceptive output was measured in BDNF-deficient mice (Heppenstall and Lewin, 2001), as well as following sequestration of endogenously released BDNF (Kerr et al., 1999).

Two distinct signaling cascades involved in BDNF-induced NMDA receptor phosphorylation are the MAPK/ERK and PLC/protein kinase C (PKC) pathways (Pezet et al., 2002a, b; Garraway et al., 2003; Slack et al., 2004). Activation of both pathways has been observed not only subsequent to neurotrophin-Trk interaction (Ohmichi et al., 1991; Obermeier et al., 1993; Bonni et al., 1999; Mazzoni et al., 1999; Corbit et al., 1999), but also upon nociceptive stimulation (Ji et al., 1999, 2002; Zhuang et al., 2005; Shi et al., 2008). As such, *in vitro* or *in vivo* administration of BDNF to the spinal cord resulted in ERK phosphorylation and activation in the superficial dorsal horn (Pezet et al., 2002), whereas subsequent MAPK kinase (MEK) inhibitor treatment diminished NR1 subunit phosphorylation (Slack et al., 2004). Similarly, NMDA receptor-facilitated excitatory post-synaptic currents (EPSCs) and NR1 phosphorylation in lamina II neurons were prevented with PLC (Garraway et al., 2003; Slack et al., 2004) and PKC inhibitors (Slack et al., 2004) in the presence of BDNF.

**1.6.1 Intracellular consequences of BDNF-TrkB signaling in post-synaptic neurons**

In addition to receptor phosphorylation, activation of the MAPK/ERK pathway by BDNF has been suggested to exert transcriptional control in the dorsal horn. ERK
phosphorylation is readily observed following BDNF administration to the spinal cord (Pezet et al., 2002; Kawasaki et al., 2004), especially in neurons of the spinothalamic tract (Slack et al., 2005). Correspondingly, the synthesis of the neuropeptides and enzymes involved in pain modulation, such as NPY and glutamic acid decarboxylase (GAD), has been suggested to increase in spinal neurons upon ERK activation (Kim et al., 2000). Consequent downstream expression of noxious-specific immediate early genes has also been observed (Kerr et al., 1999; Ji et al., 1999, 2002; Kim et al., 2000; Jongen et al., 2005; Zhaung et al., 2005). Noxious stimulation of peripheral nerves specifically results in immediate early gene expression, such as of c-fos, in the superficial dorsal horn (Hunt et al., 1987). Likewise, BDNF administered into the intrathecal space in rats incites spinal expression of c-fos, c-jun and krox 24 (Kerr et al., 1999; Kim et al., 2000; Jongen et al., 2005).

1.6.2 Microglial-derived BDNF in spinal pain processing

Modulation of neuronal anion reversal potential has alternatively emerged as another post-synaptic factor modulated by BDNF (Cordero-Erausquin et al., 2005; Coull et al., 2005). BDNF, released from activated microglia, has been suggested to bind TrkB receptors on nociceptive projection neurons to increase neuronal intracellular chloride levels $[\text{Cl}^-]_i$, likely as a result of reduced post-synaptic potassium-chloride co-transporter 2 (KCC2) levels (Coull et al., 2005; Miletic and Miletic, 2008; Trang et al., 2009). KCC2 protein is ubiquitously expressed throughout the adult CNS, and is thought to be the major regulatory chloride-cation co-transporter (CCC) in anion reversal potential maintenance (Lu et al., 1999; Kanaka et al., 2001; Li et al., 2002). Therefore, such an
alteration has been suggested to not only disrupt fast chloride-dependent GABAergic inhibition, but concomitantly heighten the excitability of lamina I nociceptive projection neurons (Coull et al., 2005).

1.6.3 Behavioural evidence supporting a pro-nociceptive role for BDNF

Some of the most visible indices for BDNF contributing to nociceptive signaling have been gathered from behavioural studies coupling loss-of-function (LOF) in vivo approaches (Pezet and McMahon, 2006). For instance, reduced pain-related behaviour has been observed in conditional BDNF knockout mice (Zhao et al., 2006) and in rats treated with BDNF-sequestering fusion proteins (Kerr et al., 1999; Thompson et al., 1999; Mannion et al., 1999) after peripheral inflammation. Similarly, intrathecal infusion of a function blocking TrkB antibody (anti-TrkB) or TrkB-Fc has been found to reverse the mechanical allodynia and thermal hyperalgesia from PNI (Coull et al., 2005) and sciatic nerve ligation (Yajima et al., 2005), respectively. Although it has been suggested that nociceptor-derived BDNF is not necessary for the induction of neuropathic pain (Zhao et al., 2006), the latter pronociceptive examples for the neurotrophin can be reconciled from the identification of spinal microglia as a neuropathic source of BDNF (Coull et al., 2005; Trang et al., 2009). Recently, direct pharmacological inhibition and genetic deletion of the TrkB receptor itself (Wang et al., 2009) has also suggested that the full-length isoform is required for the induction and maintenance of thermal and mechanical neuropathic, but not inflammatory, pain. Therefore regardless of where it is derived, endogenous BDNF signaling via its TrkB.FL receptor has been suggested to exert a potent influence on nociceptive interpretation.
1.7 NT-3 and TrkC in the adult sensory nervous system

As the third member of the neurotrophin family to be discovered, the rat NT-3 amino acid structure shares approximately 50% homology to those of NGF and BDNF (Maisonpierre et al., 1990; Hohn et al., 1990). During perinatal development is when NT-3 has been found to be highly expressed (Maisonpierre et al., 1990; Ernfors et al., 1991; Friedman et al., 1991), particularly in neurons of the ventral spinal cord, DRG, cortex and hippocampus (Maisonpierre et al., 1990; Schecterson and Bothwell, 1992; Miranda et al., 1993; Elkabes et al., 1994). In contrast, NT-3 expression is at its most minimal in adulthood (Maisonpierre et al., 1990; Ernfors et al., 1991; Friedman et al., 1991), with a pattern of expression in the brain fairly similar to that of BDNF (Hofer et al., 1990; Maisonpierre et al., 1990). With respect to neuronal elements of the spinal sensory system, NT-3 expression has also been localized within the DRG, specifically in large-diameter mechanoreceptive neurons (Zhou and Rush, 1993).

The trkC gene for the high-affinity NT-3 receptor encodes four distinct TrkC isoforms (Lamballe et al., 1993; Tsoufas et al., 1993; Valenzuela et al., 1993). The primary signaling isoform, TrkC K1 (also referred to as TrkC) is structurally comparable to both TrkA and TrkB (Lamballe et al., 1991). Whereas the TrkC K14, TrkC K25, TrkC K39 isoforms contain additional amino acids in the putative autophosphorylation site, rendering them incapable of normal TrkC catalytic activity (Tsoufas et al., 1993; Valenzuela et al., 1993). TrkC expression has been demonstrated in various neurons of the adult cortex, hippocampus, spinal cord and sensory and sympathetic ganglia (Rodriguez-Tebar et al., 1992; Dechant et al., 1993; Altar et al., 1994). Also similar to
the expression of its ligand, TrkC is predominantly expressed by large-diameter primary sensory neurons (Carroll et al., 1992).

### 1.7.1 Injury-induced changes in NT-3 and TrkC expression

Discrete alterations in NT-3 and TrkC expression levels have also been reported in the DRG, sensory neuron processes and their corresponding peripheral and central targets following various peripheral pain states (Funakoshi et al., 1993; Watanabe et al., 2000; Zhou et al., 1999; Wang et al., 2008). Sciatic nerve transection was demonstrated to elicit a small and transient decrease in NT-3 mRNA in the distal part of the nerve, whereas TrkC mRNA was increased in surrounding Schwann cells (Funakoshi et al., 1993). NT-3 protein expression has alternatively been suggested to increase in glial cells surrounding DRG neurons after PNI, as well as in the inflamed skin of the hindpaw following intraplantar CFA injection (Zhou et al., 1999; Watanabe et al., 2000). More recently, selective ganglionectomy of the DRGs surrounding the L6 DRG was found to promote an acute increase in NT-3 immunoreactivity in lamina II (Wang et al., 2008). Such changes in spinal NT-3 expression were associated with an increase in NT-3 mRNA and protein in small- and medium-diameter neurons, and corresponding decrease in large-diameter neurons of the spared L6 DRG (Wang et al., 2008). Therefore, this shift in NT-3 expression to superficial laminae may then facilitate an injury-induced shift in function for the neurotrophin in pain processing.
1.7.2 NT-3 in spinal pain processing

Relative to the expansive contributions of endogenous BDNF to spinal nociceptive processing, endogenous NT-3 has been suggested to exert a more restricted influence on mechanosensory processing (Mendell et al., 2001; Pezet and McMahon, 2006). For example, NT-3 has been suggested to restore the normal conduction properties of mechanosensory Aβ afferents, as well as reactivate their post-synaptic motorneuron targets after axotomy (Munson et al., 1997a, b). There have been some reports, however, of its direct (Chaudhry et al., 2000) and indirect (Zhou et al., 1999; Deng et al., 2000) pro-nociceptive roles in animal models of pain. Direct intraplantar delivery of NT-3 was found to evoke moderate, localized pain (Chaudhry et al., 2000). NT-3 expression has also been found to increase in satellite cells that surround neurons in injured DRGs following PNI (Zhou et al., 1999). Some models of neuropathic pain, including PNI and CCI, have been demonstrated to trigger sympathetic axon sprouting into injured DRGs resulting in the formation of pericellular sympathetic baskets around large-diameter neurons (Ramer and Bisby, 1997, 1998a, b). Administration of neutralizing antibodies directed against NT-3 has been shown to reduce this neuropathic sympathetic basket formation (Zhou et al., 1999; Deng et al., 2000) and partially attenuate accompanying mechanical hyperalgesia (Zhou et al., 2000). Activity of small-diameter nociceptive afferents, on the other hand, does not appear to be directly influenced by NT-3, unlike with NGF or BDNF (Shu and Mendell, 1999).
1.8 Experimental objectives

Although development of pain and local neuroplasticity have been found to result from deafferentation of the cervical spinal cord (Johnson et al., 2000; Ramer et al., 2004), the mechanisms that underlie these sequelae have yet to be fully elucidated. For this thesis, my overall aim was to determine whether mechanisms underlying the plasticity of spared spinal systems contribute to spontaneous functional sensory changes that occur following cervical DRI. Therefore the experimental C7/8 DRI model was utilized in the proceeding studies to test the following hypotheses:

1. Neurotrophins are upregulated in the dorsal horn after C7/8 DRI and contribute to local synaptic and morphological plasticity
2. Endogenous BDNF governs axonal plasticity to regulate cold pain following C7/8 DRI
3. Exogenous BDNF treatment will lessen the severity of, or accelerate the recovery from C7/8 DRI-induced cold pain by stimulating recovery-promoting neuroplasticity

It is important to note that while the data in Chapter 2 are primarily concerned with describing deficits in mechanosensation, any neurochemical and anatomical alterations within the dorsal horn can be expected to have consequences on nociception. Accordingly, the histological characterization of injury-upregulated neurotrophins (Figure 2.2), the behavioural testing for cold hypersensitivity (Figure 2.5b,d) and the characterization and quantification of the descending serotonergic projections (Figure
2.7) should be specifically considered in the context of the overall aim and hypotheses of this thesis.
Figure 1.1 Schematic overview of the Rexed laminae in the cervical dorsal horn.

The top left image is a cross-section of the cervical region of the spinal cord, approximately within the C7 segment. The left dorsal horn of the grey matter has been highlighted in the boxed region, and the image to the bottom right is a magnified view of this. The spinal grey matter is divided into ten Rexed laminae and the first six laminae (I-VI) comprise the dorsal horn, which is represented here. The first two laminae, I and II (the latter subdivided into IIo and Ii) are referred to as the superficial laminae and serve as the major site of nociceptive integration. In contrast, deeper laminae III-VI are primarily concerned with processing mechanosensory and proprioceptive information.
Figure 1.2 Diagram of the major neuronal phenotypes present in the cervical dorsal horn. Sensory information from the periphery first travels via specific dorsal root ganglia (DRG) neurons: C and Aδ-neurons transmit pain and temperature information and project primarily to nociceptive projection neurons (in pink) in the superficial laminae, whereas Aβ-neurons transmit mechanosensory and proprioceptive information and project to either deeper laminae or ipsilateral medullary nuclei (e.g. cuneate nucleus). In the dorsal horn, sensory information can be modulated by descending monoaminergic projections (in black) from various bulbospinal nuclei, including the rostral ventromedial medulla (5-HT neurons), locus coeruleus (noradrenergic axons), substantia nigra and hypothalamus (dopaminergic axons), as well as by local spinal interneurons (in purple). Processed nociceptive information is ultimately propagated to the brain by the dorsal horn projection neurons whose axons cross the spinal cord midline and travel rostrally to discrete targets in the thalamus.
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CHAPTER 2

*Endogenous TrkB ligands suppress functional mechanosensory plasticity in the deafferented spinal cord*¹

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2.1 Introduction

It is widely-recognized that spontaneous recovery of function following spinal cord injury (SCI) depends on synaptic and/or axonal plasticity of spared neurons. While mechanisms remain largely undefined, such plasticity is evident in limited functional recovery following incomplete SCI (Murray and Goldberger, 1974; Goldberger and Murray, 1974; Kaegi et al., 2002). Dorsal root injury (DRI), which disconnects sensory axons from the spinal cord, is a useful model in which to investigate injury-induced structural and functional changes in spared spinal axons: it is sensory-specific, precisely titratable in the sense that the extent of deafferentation can be controlled by varying the number of roots cut, and like incomplete SCI, some recovery occurs when lesions are limited. For example, postural reflexes partially return in the rhizotomized cat (Goldberger and Murray, 1974), and restricted DRI leads to a transient loss of manual dexterity in monkeys (Darian-Smith and Ciferri, 2005). Such recovery implicates improvements in mechanosensation.

DRI also results in the upregulation of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) in spinal grey matter (Johnson et al., 2000), where they have been implicated in enhancing synaptic plasticity in respiratory circuitry (Kinkead et al., 1998; Baker-Herman et al., 2004). Several convergent lines of evidence suggest that BDNF and/or NT-3 may also mediate spontaneous mechanosensory recovery following DRI, and that this may occur through their modulatory effects on synaptic transmission and/or via promoting morphological plasticity (sprouting) of relevant axonal populations.
For example, both BDNF and NT-3 are known to facilitate glutamatergic synaptic efficacy between primary afferent axons and second-order neurons in the spinal cord (Kerr et al., 1999; Ji et al., 2003; Arvanian et al., 2003). In addition, the normally inhibitory effect of gamma-aminobutyric acid (GABA) on spinal projection neurons becomes excitatory in the presence of elevated BDNF (Coull et al., 2005), increasing excitability and enhancing sensory transmission. BDNF and NT-3 also play essential roles in establishing and maintaining mechanosensory circuitry (Airaksinen et al., 1996; Carroll et al., 1998), and NT-3 promotes functional mechanosensory axon regeneration into and within the spinal cord following both SCI and DRI (Bradbury et al., 1999; Ramer et al., 2000; Ramer et al., 2002). Given that mechanosensory recovery and spinal primary afferent sprouting have been shown to occur following dorsal rhizotomy (Darian-Smith, 2004), and that mechanosensory axons express TrkB and TrkC but not TrkA (McMahon et al., 1994), it is also important to ask whether DRI-induced expression of these neurotrophins effects sprouting of their terminal arbors.

Here we establish a model of mechanosensory dysfunction following partial forepaw deafferentation in the rat. In this injury model, mechanosensory behaviour partially recovers spontaneously within 10 days of injury, in the absence of spinal primary afferent sprouting. However, mechanosensation does not recover to preoperative levels, and mechanosensory deficits in the deafferented forepaw persist for months following injury. We hypothesized that spinally-upregulated neurotrophins might underpin the early partial functional restitution. To our surprise, sequestering
endogenous BDNF/NT-4, but not NT-3, resulted in more complete behavioral recovery and stimulated sprouting of mechanosensory primary afferent axons in the spinal cord.

2.2 Materials and methods

Surgery and animal care

All procedures conformed to the Canadian Council on Animal Care guidelines on the use of experimental animals, and were approved by the University of British Columbia animal care committee. Adult male Sprague-Dawley rats (UBC Animal Care Facility) were housed in groups of three to five, on a 12-hour light/12-hour dark cycle, and had ad libitum access to standard rodent chow and water. Following pre-operative behavioral testing (see below) rats were anesthetized with an intraperitoneal injection of ketamine HCl/medetomidine HCl (75 mg/kg and 0.5 mg/kg, respectively). Following laminectomy and durotomy, the exposed 7th and 8th cervical dorsal roots were transected with microscissors or left intact (sham).

Intrathecal infusions: For continuous intrathecal infusions, osmotic minipumps (Alzet, Cupertino, CA, models 2001 and 2002) and intrathecal cannulae (0.64 mm O.D., 0.3 mm I.D.) were filled with a solution of either phosphate-buffered saline (PBS), whole human IgG (Sigma-Aldrich Canada Ltd, Oakville, ON) in PBS, TrkB-Fc (Sigma) in PBS, TrkC-Fc (R&D Systems Inc., Minneapolis, MN) in PBS, 10% dimethyl sulfoxide (DMSO) in PBS, or the pan-Trk antagonist K252a in 10% DMSO in PBS. Cannulae were inserted into the intrathecal space via the atlanto-occipital membrane such that the tip rested above the C6 spinal cord. Minipumps were inserted into a subcutaneous
pocket just below the scapulae. Based on previous experiments demonstrating efficacy of these and similar drugs in vivo (Kerr et al., 1999; Sung et al., 2003; Yajima et al., 2005; McPhail et al., 2006), IgG and Trk-Fc chimeras were delivered at a rate of 3 µg/day for ten or twenty days; K252a was delivered at a rate of 2 µg/day. In the twenty-day animals the 2002 (two week) model pump was exchanged for a 2001 (one week) model pump on day 15. Individuals carrying out the surgeries coded the animals for blind behavioral analysis. At the end of the behavioural study, rats were re-anesthetized and their ipsilateral median nerve was exposed in the upper forelimb. Using a pulled glass micropipette fitted to a Hamilton syringe, the nerve was injected with 0.5 µl of a 1% solution of the B fragment of cholera toxin (CTB, Cedarlane, Ltd., Hornby, ON) to transganglionically label the spinal terminal fields of myelinated primary afferent axons.

For bolus infusions of IgG or TrkB-Fc, saline-filled intrathecal cannulae were inserted into ketamine/medetomidine-anesthetized rats on the seventh post-operative day. These were externalized over the skull and sealed with cyanoacrylate gel. On days nine and ten, rats received either a bolus injection of IgG or TrkB-Fc (0.2 mg/ml, 10 µl injections followed by 10µl saline flush). Beginning thirty minutes following infusions and continuing for no more than one hour, rats underwent blind behavioral assessment (see below).

Mechanosensory Testing

All behavioral experiments were carried out by observers who were blind with respect to treatment. Rats underwent training for habituation to the testing environment
on two occasions prior to preoperative behavioral testing. Low-threshold cutaneous mechanosensation across the palmar surface of the forepaw was assessed using an adhesive removal test (Thallmair et al., 1998; Bradbury et al., 2002; Starkey et al., 2005; Onifer et al., 2005; Moreno-Flores et al., 2006). Circular stickers (6.4 mm in diameter) were applied to the palm of either forepaw and the rat was placed in a cage for observation. The time required for the rat to sense the sticker, indicated by a brisk paw shake and/or bringing the paw to the mouth, was measured for each paw to a maximum time of 150 seconds. This maximum was imposed in order to distinguish sticker sensation in the palm from accidental sticker discovery during grooming, behavior which typically begins following 2-3 minutes of test cage exploration. Trials in which the sticker was discovered during grooming before the 150 second maximum were excluded and repeated. In order to characterize the injury-induced mechanosensory deficit, rats that received sham injury or C7/8 DRI only were tested over 90 postoperative days. To examine the effects of neurotrophin signaling antagonism, rats that received C7/8 DRI plus IgG, Trk-Fc, K252a, or K252a vehicle (10% DMSO in PBS) were tested over 20 postoperative days. In all cases, daily test scores represent averages of two trials, and trials were separated by at least one hour. Between trials, rats underwent a mock trial, in which the experimenter picked up the rat and applied pressure to the forepaws to mimic sticker application.

The Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy) was used to test for mechanical allodynia. Rats were positioned in a raised cage with a wire mesh floor over the stimulator unit. The filament was applied to the center of the palmar
surface of the forepaw, and upward force was increased from 1 to 50 grams over 7 seconds. Force at withdrawal was recorded for both forepaws.

Cold sensitivity was examined by measuring the duration of response to acetone (10 µl) applied to the palmar forepaw, as we have done previously (Ramer et al., 2004). Rats were placed in a raised cage with a wire mesh bottom, and acetone was applied from below. A response was defined as withdrawal, biting, licking or shaking of the paw. A brief withdrawal without further attention to the paw was assigned a score of 1s. Response duration was recorded for both forepaws.

**Electrophysiology**

In a separate group of rats that did not undergo behavioral testing, we recorded dorsal horn activity using a 16-electrode matrix (2x8) microarray inserted into the superficial dorsal horn between the 7<sup>th</sup> and 8<sup>th</sup> cervical segments (Borisoff et al., 2006). Neural activity in urethane-anesthetized (1.5 g/kg, intraperitoneal), paralyzed (gallamine triethiodide, 60 mg/kg, intra-arterial) and ventilated rats was amplified and recorded using a multi-channel *Pentusa* neurophysiology workstation (Tucker-Davis Technologies, Alachua, FL). Sensory receptive fields were electrically-stimulated in left forepaw digits using steel pin electrodes (1 ms pulse width, 4 mA current amplitude, 0.5 Hz). Post-synaptic responses from 100 trials were recorded from each electrode in the array and stored for off-line analysis. At the end of the experiment rats were killed with an intra-arterial injection of chloral hydrate (1 g/kg).
We have described in detail a quantitative cluster analysis-based method to characterize electrophysiological activity in the dorsal horn arising from electrical stimulation of individual digits (Borisoff et al., 2006). This method is based on the discrimination accuracy of specific digit stimulation sites, solely determined from an automatic analysis of recorded neural activity in the spinal cord. Briefly, signal features based on spike rate data were calculated for each stimulation event. The features consisted of the number of threshold-detected fast (Aβ fibre-evoked) spikes in each of 12 consecutive bins of 4 ms in length, calculated for each of the 16 channels. Thus 192 features were generated for each event from the all but 2.5 ms of the first 48 ms of recorded data following stimulation onset. Data from the very first 2.5 ms post-stimulus were not included in the analysis to eliminate the influence of stimulus artifact.

A classification scheme using a k-means-derived codebook, calculated from the training feature sets was used. The feature set dimensionality was first reduced to the top three principal components using principal component analysis (PCA). The PCA transform matrix was calculated during codebook generation and later used during classification of the test features. During codebook generation, each class of the three dimensional training features was reduced to \( k = 2 \) codebook vectors using k-means clustering. During testing, one-Nearest Neighbors classification was used to discriminate test features, using the previously calculated two-vector classes as a codebook.
Tissue processing and image analysis

At the end of the behavioral experiments, animals were killed with an overdose of chloral hydrate (1 g/kg intraperitoneally, Sigma) and perfused with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Cords and cannulae were inspected for damage and/or blockage, and those animals in which evidence for either was overt were removed from all behavioral and anatomical analyses. Final sample sizes for each group are presented in Table 1. The cervical spinal cords (segments C6-T1) were harvested, post-fixed overnight, cryoprotected in 20% sucrose in 0.1 M PB, frozen over liquid nitrogen and sectioned on a cryostat at 16 µm. Sections were thaw-mounted onto glass slides and stored at -80°C. Slides were blocked in a 10% solution of normal donkey serum in PBS plus Triton-X-100 (0.1%) for 20 minutes, followed by primary antibody solutions overnight (see Table 2). Following three 15-minute washes in PBS, secondary antibodies raised in donkey and conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA), Alexa 488 (Molecular Probes Inc., Eugene, OR) or AMCA (Jackson) were applied (1:200) for two hours. Coverslipped slides were inspected using a Zeiss Axioplan II microscope (Zeiss, Jena, Germany), and digital images were captured using Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada) via a digital camera (QImaging, Burnaby, British Columbia, Canada). Exposure, gain and offset settings were set to optimize signal-to-noise ratios for each antigen.

Quantitative analyses of terminal density in the dorsal horn were performed by measuring terminal density as a function of depth in the dorsal horn, as we have done previously (Ramer et al., 2001; MacDermid et al., 2004; Ramer et al., 2004; Scott et al.,
Densitometric analyses of VGLUT1- and CTB-positive terminals were performed within the consistent deafferentation gap apparent in C7 following C7/8 DRI. Images of five randomly-selected sections from the C7 spinal segment were captured for each animal listed in Table 1. To measure density, images were first passed through a Laplacian omnidirectional edge-detection filter (SigmaScan Pro v. 5.0), which optimizes the signal-to-noise ratio and corrects for small variations in background staining across images. Terminal profiles in the filtered images were selected with a threshold overlay, to give all immunopositive pixels equal weight irrespective of their brightness in the original image. The density of terminals was measured from the overlay in laminae II or III/IV of the deafferentation gap. To control for variability in tracing efficacy, the density of CTB-positive terminals within the gap was normalized to that of laterally-adjacent spared terminals, directly beneath the dorsal horn apex (arrow in Figure 1C). Behavioral and densitometric results are presented as means ± standard errors of the mean.

We also quantified the size-distribution of VGLUT1-positive terminals in deafferented regions of the dorsal horn. To do so, images were captured at high power (63X, oil immersion), and the diameter of all structures distinguishable as individual boutons was measured in each field using SigmaScan Pro v. 5.0. Since the distribution of profiles can lead to overestimation of the proportion of small diameter boutons (due to end-cuts of larger-diameter ones), recursive translation (Rose and Rohrlich, 1988) was used to convert the distribution of bouton profiles to the distribution of whole boutons.
**Statistics**

A list of sample sizes for all experiments is presented in Table 1. All quantitative behavioural, histological and electrophysiological data are expressed as means ± standard errors of the means. For all statistical outcomes, significance was set at $p < 0.05$.

**Behavior:** For the sticker, dynamic plantar anaesthesiometer and acetone tests, differences between ipsilateral and contralateral latencies were normally distributed, and compared using Student’s t-test. For the sticker test, mean latency difference scores (ipsilateral minus contralateral) were averaged over plateau phases (days 10-20), and compared using a one-way ANOVA followed by Holm-Sidak test for pairwise differences. To detect differences between IgG and TrkB-Fc bolus infusions in the same animals, we used a paired t-test.

**Electrophysiology:** The single trial digit-discrimination accuracy for each animal was calculated using five separate sequential (80% training and 20% test) feature sets and averaging the results (five-fold cross-validation). A parametric one-way ANOVA was used to detect differences in discrimination accuracy.

**Histology:** Terminal densities were subjected to one-way ANOVAs followed by Holm-Sidak post hoc tests. Because DRI can result in contralateral effects (Ramer et al., 2004), ipsilateral densities following rhizotomy were compared to those in uninjured animals. Densities were all normally-distributed. Statistical differences in size distributions of VGLUT1-positive terminals were detected using the Kolmogorov-Smirnov goodness-of-fit test.
2.3 Results

**Partial mechanosensory recovery after C7/8 DRI**

We first characterized changes in low-threshold mechanosensation following unilateral transection of the 7\textsuperscript{th} and 8\textsuperscript{th} cervical dorsal roots (C7/8 DRI). In experimental and sham-operated rats, we measured the time to attend to a sticker applied to the palm (Fig. 2.1a) (Bradbury et al., 2002). Normally, rats sensed the sticker within 11 ± 7s of its application. Two days following C7/8 DRI, response latencies increased significantly ($p < 0.001$), to 128 ± 20s. By two weeks post-injury, detection latency had decreased to 56±19s, but was still significantly different from the contralateral side ($p = 0.026$), and remained so thereafter. Response latencies were unaffected in sham-operated rats, assessed concurrently in a blinded fashion. These results demonstrate a rapid and spontaneous, but incomplete return of mechanosensory function following C7/8 DRI.

It is conceivable that increased detection rate may have been the result of supraspinal learning: i.e. an association between handling/deposition into the test environment (a potential conditioning stimulus) and the presence of a sticker (the test stimulus). Such associative learning was ruled out through mock behavioral trials in which the rats were handled and deposited into test cages in exactly the same way, but did not have stickers applied. In these mock trials rats did not attend to their forepaws as though stickers were present.
An electrophysiological correlate of behavioral recovery

Although partial spontaneous behavioral recovery implied emergent connectivity between decentralized dermatomes and spinal neurons, and although mock behavioural trials (without stickers) did not elicit responses, we wished to further verify that more rapid attention to the sticker was not purely a result of associative learning (Onifer et al., 2005). If primary afferent-driven activity at the level of the spinal cord did not return in parallel with behavioral recovery, this may indicate that cues other than the sticker elicit faster response times in behavioral experiments. We therefore tracked recovery electrophysiologically in the spinal cord (Borisoff et al., 2006) (Fig 2.1b). Using an electrode microarray inserted into the dorsal horn, we sought to discriminate specific locations of peripheral electrical digit stimulation from post-synaptic neural activity. Evoked activity from each of four digits was subjected to machine learning classification using principal component analysis and k-means clustering (Borisoff et al., 2006). Such classification generated identifiable clusters in three-dimensional feature spaces and reliably discriminated between the digits stimulated in intact animals. Acute C7/8 DRI significantly reduced the probability of digit discrimination ($p < 0.001$). However, ten days following C7/8 DRI, discernable sites of digit stimulation re-emerged, and classification accuracy was restored to that of the uninjured state. Together with mock sticker trials, these data indicate that more rapid sticker detection does not occur through associative learning, but is mediated by changes in the spinal cord. They also show that even though changes in connectivity occur at the level of the dorsal horn to such an extent
that digit discrimination probability is fully restored, they are insufficient to completely restore normal behavior.

**C7/8 DRI produces a stable deafferentation gap in the dorsal horn**

We next investigated changes in primary afferent innervation of the dorsal horn. Two markers were used: vesicular glutamate transporter 1 (VGLUT1), and the axon tracer CTB (cholera toxin B), both of which label mechanosensory afferents in the rat dorsal horn (Todd et al., 2003; Alvarez et al., 2004; Persson et al., 2006) (Fig. 2.1c). VGLUT1 is a particularly useful marker since it only labels terminal boutons, and not pre-synaptic growth cones – we have found that CTB-labeled axotomized C7 and C8 axons halted at the dorsal root entry zone do not contain VGLUT1 (data not shown). C7/8 DRI produced a consistent deafferentation gap in laminae III-IV of the C7 dorsal horn.

Densitometric analyses of VGLUT1- and CTB-positive terminals were performed within the deafferentation gap, by measuring terminal density as a function of depth in the dorsal horn (Ramer et al., 2001; MacDermid et al., 2004; Ramer et al., 2004; Scott et al., 2005). Following C7/8 DRI, the density of both VGLUT1 and CTB-labeled terminals was decreased, but remained unchanged for at least twenty days postoperatively. VGLUT1 immunohistochemistry was not carried out at the three-day timepoint because of the confounding influence of slow clearance of degenerating primary afferents (George and Griffin, 1994). Spontaneous partial recovery therefore occurs in the absence of spinal mechanosensory axon sprouting.
C7/8 DRI induces BDNF and NT-3 expression in the dorsal horn

Because BDNF and NT-3 are upregulated in the spinal ventral horn following DRI (Johnson et al., 2000), we asked whether the same was true in the dorsal horn (Fig. 2.2). In uninjured animals, BDNF was restricted to superficial primary afferent terminals (Fig. 2.2a). C7/8 DRI induced BDNF expression in Ox-42-positive microglia/macrophages throughout the ipsilateral dorsal grey and white matter (Fig. 2.2e). Although the NT-3 antibody used efficiently labeled cerebellar Purkinje cells and their processes (Fig. 2.2f, inset), NT-3 immunoreactivity was undetectable in uninjured spinal cord (Fig. 2.2f). However, C7/8 DRI induced NT-3 expression in the ipsilateral grey and white matter (Fig. 2.2g-i). This was localized to blood vessel-associated astrocytic cell bodies and their processes (Fig. 2.2h, j). Both neurotrophins were upregulated by three days post-injury, and persisted for at least 20 days following DRI. While nerve growth factor (NGF) and neurotrophin-4 (NT-4) immunoreactivities were obvious in positive control tissues (injured dorsal roots and cerebellar Purkinje cells, respectively), they were undetectable in uninjured or deafferented cords at all timepoints (Fig. 2.3).

Continuous TrkB-Fc treatment improves mechanosensory recovery

To test the hypothesis that upregulated neurotrophins mediate partial recovery, we administered Trk-Fc "receptor bodies" intrathecally to sequester spinal BDNF/NT-4 and NT-3 (Cabelli et al., 1997; Seebach et al., 1999; Chan et al., 2001). An antibody specific for the human Fc moiety was used to assess spinal Trk-Fc penetration (Fig. 2.4a). Prominent staining was evident in dorsal white matter, and more so in dorsolateral white
and gray matter, as we have demonstrated previously with a similar fusion protein (MacDermid et al., 2004). Behaviorally, IgG treatment resulted in a pattern of behavior similar to untreated rats in which mechanosensory ability reached a plateau that remained significantly different from the contralateral side (Fig. 2.4b). Surprisingly, intrathecal TrkC-Fc was without effect, and TrkB-Fc treatment actually enhanced recovery: ipsilateral and contralateral detection latencies were statistically equivalent (p = 0.08) by the 6th postoperative day and remained so thereafter. To directly determine whether the extent of recovery differed between groups, we compared response latency differences (ipsilateral minus contralateral response times) over the plateau phase (averaged data from days 10–20). There was a significant difference between IgG-treated (41 ± 9 s) and TrkB-Fc treated (8 ± 3 s; p = 0.016) rats, but not between IgG-treated and TrkC-Fc-treated rats (46 ± 16 s; p = 0.711).

Possible mechanical allodynia after C7/8 DRI was examined using the Dynamic Plantar Aesthesiometer. These experiments were done to determine whether more rapid sticker detection was related to evolution of mechanical allodynia [i.e., did any pain (potentially associated with the presence of the sticker) motivate more rapid detection? When increasing punctate force was applied to the center of the palmar forepaw, no differences in force at withdrawal were detected between forepaws in IgG-treated rats and TrkB-Fc-treated rats (Fig. 2.4c). In rats that received TrkC-Fc, withdrawal thresholds were increased significantly at 5 d (p = 0.005), 10 d (p = 0.003), and 15 d (p = 0.02) after DRI, possibly as a result of decreased synaptic efficacy between deep-terminating mechanosensory axons and motoneurons (Arvanian et al., 2003). These results indicate that mechanical allodynia did not incite more rapid sticker withdrawal in TrkB-Fc-treated
rats. Because of the unexpected improvement in mechanosensation afforded by TrkB-Fc treatment, we did not pursue electrophysiological assessment further: even in untreated rats, digit discrimination probability recovered to close to 100% by 10 d after DRI, rendering additional electrophysiological experiments superfluous.

**TrkB-Fc treatment enhances mechanosensory recovery independent of effects on synaptic transmission**

Of the two TrkB ligands, only BDNF has acute effects on neurotransmission in the spinal cord. Unlike BDNF, NT-4 has no effect on activity-dependent synaptic plasticity or neuropathic pain (Heppenstall and Lewin, 2001; Yajima et al., 2002). To determine whether potential neuromodulatory effects of BDNF contributed to mechanosensory recovery, we gave rats bolus intrathecal injections of either IgG or TrkB-Fc (2 µg of protein) on days 9 and 10 after DRI. We chose this time point based on the statistically complete recovery in rats treated continuously with TrkB-Fc. Mechanosensory deficits were unaffected by acute BDNF antagonism (Fig. 2.5c), indicating that recovery was not attributable to acute effects of BDNF on synaptic transmission. To verify efficacy of bolus TrkB-Fc infusions, we measured rhizotomy-induced cold hypersensitivity in the same animals (Ramer et al., 2004). We found that a 2 µg intrathecal bolus of TrkB-Fc reduced ipsilateral response durations to forepaw acetone stimulation from 9.2 ± 1.1 to 5.8 ± 0.8 s ($p = 0.03$) (Fig. 2.5d). Contralateral responses were unaffected. These results indicate a neuromodulatory role for endogenous spinal BDNF after DRI that contributes to cold pain and provide additional support for an
absence of the acute synaptic involvement of BDNF in mediating improved mechanosensation.

**TrkB-Fc treatment induces spinal mechanosensory axon sprouting**

Could the unexpected improvement conferred by TrkB-Fc treatment be mediated by mechanosensory axon sprouting? VGLUT1-positive terminal density in the deafferentation gap was significantly increased by TrkB-Fc (Fig. 2.6a, c, d) at both 10 d ($p = 0.008$) and 20 d ($p = 0.01$) after C7/8 DRI, correlating with behavioral improvement. Because it is also expressed in corticospinal axons (Persson et al., 2006), we measured VGLUT1 density within lamina II, where corticospinal but not myelinated sensory axons terminate. No increases in density occurred in lamina II in any treatment group. TrkB-Fc treatment, but not IgG or TrkC-Fc-treatment, also increased CTB-positive terminal density in laminas III–IV (Fig. 2.6b, e) at 10 d ($p = 0.02$) and 20 d ($p < 0.001$) after DRI; CTB-positive terminals were absent from lamina II.

Because VGLUT1-containing mechanosensory terminals are larger than corticospinal boutons (Valtschanoff et al., 1993), we quantified the size distribution of VGLUT1-positive puncta in the deafferentation gap of 20 d IgG-treated ($n = 1111$ puncta from five rats) and TrkB-Fc-treated ($n = 1270$ puncta from five rats) animals. This was performed to determine whether the increase in density could have been attributable to corticospinal axons. Terminals were significantly larger ($p < 0.05$) in TrkB-Fc-treated rats, indicating that mechanosensory axon terminals were present in higher proportions in TrkB-Fc-treated rats than IgG-treated controls (Fig. 2.6f). Larger terminals were also CTB filled (Fig. 2.6g, arrowheads). Thus, endogenous TrkB ligands suppress
mechanosensory axon sprouting after DRI. These data also validate VGLUT1 as a reliable marker of mechanosensory axons.

**C7/8 DRI induces TrkB-dependent serotonergic sprouting in the dorsal horn**

To determine whether endogenous DRI-induced neurotrophins had a generalized suppressive effect on axonal plasticity, we analyzed changes in spinal serotonergic axon density. Serotonergic sprouting is a well recognized consequence of spinal deafferentation (Polistina et al., 1990; Wang et al., 1991; Zhang et al., 1993; Kinkead et al., 1998; MacDermid et al., 2004; Ramer et al., 2004; Scott et al., 2005), although the underlying mechanism has remained unknown. Because descending serotonergic axons express TrkB and TrkC (King et al., 1999), we asked whether the responsible stimulus was BDNF/NT-4 and/or NT-3. Serotonergic axon density had increased significantly in outer lamina II (IIo; \( p = 0.004 \)), inner lamina II (IIi; \( p = 0.01 \)), and laminae III/IV (\( p < 0.001 \)) by 20 d after DRI in rats treated with IgG (Fig. 2.7a–c). The same was true for TrkC-Fc-treated rats (IIo, \( p < 0.001 \); IIi, \( p = 0.005 \); III/IV, \( p < 0.001 \)). TrkB-Fc treatment completely prevented serotonergic sprouting, illustrating that for some axonal populations, BDNF/NT-4 does indeed enhance plasticity. These results support the notion of hierarchical plasticity in the spinal cord after spinal deafferentation (Polistina et al., 1990): processes that favor sprouting of some axonal populations (serotonergic) also suppress sprouting in others (mechanosensory primary afferent).
**TrkB antagonism promotes functional plasticity independent of NT-3**

We next asked what role spinally upregulated NT-3/TrkC signaling might play in functional recovery and plasticity in the absence of BDNF/NT-4/TrkB signaling. We continuously administered the pan-Trk antagonist K252a (Knusel and Hefti, 1992) intrathecally in animals with C7/8 DRI, reasoning that if NT-3 was responsible for functional improvements and enhanced plasticity in the absence of TrkB signaling, additionally blocking TrkC signaling would abolish both effects. Like TrkB-Fc, intrathecal K252a significantly improved the behavioral performance of rhizotomized animals (Fig. 2.8a): ipsilateral and contralateral detection latencies were equivalent by the 8th postoperative day ($p = 0.08$) and remained so thereafter. Plateau latency difference scores were significantly higher in vehicle-treated rats than in K252a-treated rats ($56 \pm 16$ s vs $18 \pm 6$ s; $p = 0.04$). K252a treatment also significantly increased the density of VGLUT1-positive terminals in the deafferentation gap in laminas III–IV (Fig. 2.8b, c) ($p < 0.001$). Elevated NT-3 therefore does not contribute to enhanced recovery and plasticity when TrkB is antagonized.

**2.4 Discussion**

We set about this work to test the hypothesis that spinally upregulated BDNF and/or NT-3 mediate partial spontaneous mechanosensory recovery after limited spinal deafferentation, a rationale supported not only by the known neuromodulatory effects of these neurotrophins (particularly those of BDNF) (Kerr et al., 1999; Arvanian et al., 2003; Ji et al., 2003; Baker-Herman et al., 2004; Coull et al., 2005; Vaynman and Gomez-Pinilla, 2005), but also by their roles in mechanosensory development (Airaksinen et al.,
1996; Carroll et al., 1998) and regeneration (Bradbury et al., 1999; Ramer et al., 2000, 2002). We find that spontaneous recovery during the early postoperative period occurs in the absence of primary afferent sprouting and is, in fact, neurotrophin independent: recovery was identical between Trk antagonizing treatments and their respective controls. The more surprising outcome is that beyond the spontaneous recovery period, endogenous TrkB ligands have a suppressive effect on mechanosensory plasticity: TrkB antagonism results in complete recovery of mechanosensation. Acute neuromodulatory effects of BDNF do not underpin recovery because bolus injections of TrkB-Fc were without effect. Finally, we show that, in addition to promoting behavioral recovery, TrkB antagonism stimulates mechanosensory axon sprouting in the deafferented spinal cord. Endogenous BDNF/NT-4 do not have a general, plasticity-limiting influence on spinal axons, because TrkB-Fc treatment prevented rhizotomy-induced sprouting of serotonergic axons in the dorsal horn.

**Potential mechanisms of spontaneous recovery after DRI**

The reactivation of dorsal horn neurons by electrical stimulation of decentralized digits not only provides an electrophysiological correlate of behavioral improvement but also illustrates the spinal nature of spontaneous recovery. It is conceivable, for example, that faster response times could have been caused solely by learning-associated changes at supraspinal loci and may not have manifested as increased discrimination probability at the level of the spinal cord. However, the reappearance of primary afferent-driven activity occurred in the absence of training (a prerequisite for associative learning). The same holds for experiments in which cats underwent similar electrophysiological studies.
in the absence of behavioral training (Basbaum and Wall, 1976). These findings argue very strongly against supraspinal associative learning as underlying neurophysiological recovery at the level of the spinal cord.

The present results implicate a recovery mechanism operating locally, within the partially deafferented cord. Synaptic unmasking and/or strengthening is the most plausible mechanism. A physiological process that may underlie partial recovery is the GABAergic presynaptic control [via primary afferent depolarization (PAD)] of impulse transmission along branches of individual mechanosensory axons (Wall, 1995): although some collaterals of myelinated axons fail to propagate action potentials in intact animals, this conduction block is reversed with time after rhizotomy of caudal segments. Successful conduction emerges by 5–7 d after DRI, correlating remarkably well with the partial behavioral recovery reported here.

In the rat, many C6 and T1 sensory neurons also innervate C7 and C8 dermatomes (Ramer et al., 2004). Therefore, the most likely mechanism underlying spontaneous recovery involves the (neurotrophin-independent) unmasking and/or strengthening of synapses between C6/T1 primary afferents and postsynaptic targets. Although others have suggested that this functional connectivity emerges subsequent to deafferentation-induced sprouting (Sengelaub et al., 1997; Darian-Smith and Brown, 2000; Darian-Smith, 2004), such axonal plasticity occurs too slowly to account for rapid partial spontaneous recovery after C7/8 DRI.
TrkB ligands arrest full spontaneous mechanosensory recovery independent of synaptic neuromodulation

BDNF (but not NT-4) has been shown to both enhance and suppress synaptic transmission in the dorsal horn (Pezet and McMahon, 2006). Our original hypothesis was based in part on the previously demonstrated GABAergic suppression of impulse transmission along branches of mechanosensory axons (Wall and McMahon, 1994; Wall, 1995) and on the known effects of BDNF on this type of inhibitory control. Because exogenous BDNF enhances GABA release in the spinal cord (Pezet et al., 2002), one might have expected DRI-induced BDNF to augment GABA-mediated PAD. Strengthened PAD would lead to successful action potential propagation in previously blocked Aβ fiber collaterals and activation of second-order neurons, a mechanism that has been proposed to underlie increased activation of pain-responsive second-order neurons in nerve injury and inflammation (Willis, 1999). BDNF is also known to render postsynaptic GABAergic inhibition of nociceptive projection neurons excitatory (Coull et al., 2005). The potential applicability of this mechanism to mechanosensory second-order neurons, or to wide dynamic-range neurons, had initially led us to predict that the effects of BDNF on GABAergic transmission participated in partial spontaneous recovery.

On the other hand, BDNF has been shown to inhibit glutamatergic signaling in the nucleus of the solitary tract (Balkowiec et al., 2000) and in lateral motor nuclei in the spinal cord (Seebach et al., 1999; Arvanian and Mendell, 2001). It might therefore have been argued that endogenous BDNF prevents complete recovery by inhibiting glutamatergic transmission in the dorsal horn. However, because recovery occurred at its
usual rate over the first several days after DRI even in the presence of TrkB-Fc (and K252a), we conclude that it is attributable neither to the effects of BDNF on GABAergic transmission nor on glutamatergic transmission during this period. Furthermore, acute treatment with a bolus injection of TrkB-Fc had no effect on mechanosensory ability during the plateau phase after DRI, despite significantly reducing cold pain.

In neonatal rats, chronic TrkB-Fc treatment has been shown to strengthen the connection between Ia afferents and motoneurons, evident as an increase in the size of EPSPs (Seebach et al., 1999). It might therefore be argued that TrkB-Fc treatment results in larger EPSPs in dorsal horn neurons subsequent to peripheral stimulation of cutaneous mechanosensory axons. Although this is an interesting question, its relevance to behavioral recovery becomes questionable when we consider that because of sprouting and synapse formation (the latter implied by the increased density of VGLUT1-positive terminals), more dorsal horn neurons are being driven by the same number of dorsal root axons. That is, the behavioral improvement that occurs with TrkB-Fc treatment may simply be the result of the larger population of neurons activated by C6/T1 primary afferents.

**How does TrkB-Fc treatment promote primary afferent sprouting?**

It is important to define putative direct and indirect processes by which BDNF (and possibly NT-4) suppresses plasticity. Evidence for a direct mechanism comes from work on dissociated adult sensory neurons (Gavazzi et al., 1999) where addition of BDNF had a marked inhibitory effect on spontaneous neurite outgrowth from large-diameter cells. Such inhibition may involve the p75 neurotrophin receptor, which in DRG neurons
is coexpressed with Trk receptors (Wright and Snider, 1995): activating p75 with BDNF in TrkB-negative cells inhibits outgrowth mediated by NGF/TrkA signaling (MacPhee and Barker, 1997; Kimpinski et al., 1999; Kohn et al., 1999), suggesting that in the present experiments, spinal upregulated BDNF (or NT-4) may have been similarly interfering with NT-3/TrkC signaling. However, TrkB-Fc and K252a data were remarkably similar, indicating that whether or not p75 is involved, improved function and anatomical changes occurring in TrkB-Fc-treated animals were NT-3 independent. In combination with the absence of TrkA from low-threshold mechanosensory axons (McMahon et al., 1994; Wright and Snider, 1995), and no evidence for NGF expression in the spinal cord or upregulation after DRI, the K252a data also argue against a role for NGF-TrkA signaling in mechanosensory improvement.

TrkB activation is more likely to indirectly suppress mechanosensory axon sprouting by selectively enhancing plasticity in other spinally projecting systems. Both BDNF and NT-4, whether delivered via osmotic minipumps or genetically modified cell grafts, can induce growth of serotonergic fibers in the injured spinal cord (Bregman et al., 1997; Tobias et al., 2003; Blesch et al., 2004). TrkB-expressing descending serotonergic fibers (Bradbury et al., 1998; King et al., 1999) sprout earlier and more profusely after DRI than primary afferent and descending noradrenergic axons (Zhang et al., 1993), and the present data show for the first time that removing the influence of endogenous TrkB ligands prevents rhizotomy-induced serotonergic sprouting. Although exogenous or virally overexpressed NT-3 has also been shown to promote serotonergic axon growth after CNS lesions (Bregman et al., 1997; Grider et al., 2005), the present results demonstrate that endogenous NT-3 is not necessary for DRI-induced sprouting.
It is now clear that endogenous TrkB ligands have an important negative impact on mechanosensory plasticity after DRI. Although the relative contribution of NT-4 to these results has yet to be formally defined, it is likely to be minor because its mRNA is expressed at very low levels compared with other neurotrophins (Timmusk et al., 1993; Ibanez, 1996; Widenfalk et al., 2001) and its protein is undetectable in the intact and deafferented cord (data not shown). Because optimizing synaptic and axonal plasticity of CNS-projecting axons remains a necessary objective in the search for a viable therapy for neural trauma (Scott et al., 2006) clarifying the positive and negative effects of injury-induced molecules such as BDNF represents a vital intermediate step.
### Table 2.1 Sample sizes

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<sup>1</sup> Thirteen intact animals underwent terminal electrophysiological experiments. Of these, six also received acute C7/C8 rhizotomies.  
<sup>2</sup> Animals with 3-day, 10-day and 20-day C7/8 DRI used to map spinal deafferentation gap and assess mechanosensory sprouting therein.  
<sup>3</sup>Tissue processed from behaviourally-tested animals.  
<sup>4</sup>Tissue from the 20-day group used as control material in assessing Trk-Fc and IgG penetration.
Table 2.2 Primary antibodies used

<table>
<thead>
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<th>Host</th>
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<th>Dilution</th>
<th>Source</th>
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Figure 2.1 Behavioral and physiological deficits after C7/8 DRI partially recover in the absence of mechanosensory axon sprouting. (a) Time to detect a sticker on the palm in sham-operated and C7/8 DRI rats. In rhizotomized animals, all postoperative ipsilateral (ipsi)–contralateral (contra) latencies differed significantly. (b) Electrophysiological assessment of functional deficits and recovery. Left, Experimental setup and a sample trace from a single channel of the electrode array. Right, Feature space plots in various experimental conditions (Borisoff et al., 2006). Acute C7/8 rhizotomy decreased the discrimination of digit simulation clusters. Ten days after C7/8 DRI, all clusters were distinct, and the probability of correctly classifying evoked responses as being from separate digits was restored. Principal component (PC) axes are indicated. Asterisk indicates significant reduction in probability of correct digit classification. (c) C7/8 DRI produces a prominent deafferentation gap in VGLUT1- and CTB-positive terminals in the C7 dorsal horn (box); density did not change therein for 20 d after injury. The arrow points to spared C6/T1 axons. Scale bar = 100 µm.
**Figure 2.2** DRI induces BDNF and NT-3 expression in the dorsal horn.

(a) In the intact spinal cord, BDNF immunoreactivity was restricted to the terminals of primary afferent axons. Higher-power images from the same sections are shown on the right (boxes in lower-power images indicate their approximate origins). (b-d) BDNF was upregulated in glial cells by 3 d after C7/8 DRI and remained elevated for at least 20 d. *e*, Ox-42 immunohistochemistry revealed BDNF expression by microglia in early (arrowhead) and intermediate (arrow) stages of reactivity. (f-h) NT-3 immunoreactivity was intense in cerebellar Purkinje cells (inset), absent from the uninjured dorsal horn, but present in blood vessel-associated (asterisk) cellular processes as early as 3 d after DRI (g, h arrows). (h, i) DRI-induced NT-3 expression persisted for at least 20 d. (j) NT-3 was expressed in glial fibrillary acidic protein (GFAP)-positive astrocytes around rat endothelial cell antigen 1 (RECA-1)-positive vasculature (a white-matter astrocyte from the degenerating dorsal columns is shown). Scale bars: a = 100 µm; e, i = 25 µm.
**Figure 2.3** Neurotrophin-4 and nerve growth factor immunoreactivities are not detectable in the uninjured dorsal horn and are not upregulated by C7/8 DRI. (a) NT-4 immunoreactivity was absent from the intact dorsal horn despite robust signal in cerebellar Purkinje cells (inset), a known source of NT-4 (Friedman et al., 1998, Neuroscience, 84:101-14). (b) NT-4 immunohistochemistry in the deafferented cord. NGF was also absent from the intact and deafferented spinal cord but obvious in Schwann cells in the injured dorsal root (c, d). Scale bars: in a = 50 µm; in b = 100 µm.
**Figure 2.4** Sequestration of injury-induced BDNF promotes complete mechanosensory recovery after C7/8 DRI. (a) Fc immunohistochemistry demonstrating spinal penetration of intrathecally delivered proteins. Scale bar, 100 µm. (b) Only continuous TrkB-Fc treatment improved mechanosensation. (c) Mechanical allodynia did not emerge in IgG- or TrkB-Fc-treated rats, and withdrawal thresholds were increased with TrkC-Fc treatment. Asterisks in b and c indicate significant ipsilateral (ipsi)–contralateral (contra) differences.
**Figure 2.5** Acute BDNF antagonism does not affect mechanosensation 10 d after rhizotomy. (a) Time course of mechanosensory recovery over the first 10 d after DRI. (b) Time course of forepaw cold-pain development (increased response duration to an acetone squirt) in the same animals. Arrows indicate days on which intrathecal boli of IgG or TrkB-Fc were administered. (c) Intrathecal boli of TrkB-Fc did not alter mechanosensory response latencies 30–90 min after administration. (d) Acute BDNF antagonism reduced response durations to acetone in the same animals. Asterisks in a and b indicate significant ipsilateral (ipsi)–contralateral (contra) differences. Asterisks in c and d indicate significant differences between IgG and TrkB-Fc treatments.
**Figure 2.6** TrkB-Fc promotes mechanosensory axon sprouting after C7/8 DRI.

(a)VGLUT1-positive and (b) CTB-positive terminal density in superficial (II) and deep (III–IV) spinal laminas in uninjured animals. DRI reduced density in deeper laminas, which was partially reversed by intrathecal TrkB-Fc. Asterisks indicate significant differences between IgG- and Trk-Fc-treated rats. (c) Low-power micrographs of VGLUT1 immunoreactivity (lamina II indicated). Higher-power micrographs of VGLUT1 (d) and CTB (e) staining from regions similar to that indicated in c (arrow indicates spared C6/T1 axons). (f) The size distribution of VGLUT1-positive terminals was significantly right-shifted in TrkB-Fc-treated rats. (g) Large VGLUT1-positive terminals were also CTB filled (arrowheads). Clusters of small VGLUT1-positive terminals are indicated by arrows. Scale bars: in c = 100 μm; in g = 20 μm.
**Figure 2.7** Endogenous TrkB ligands stimulate serotonergic sprouting after C7/8 DRI.

(a) Serotonergic axon density measurements from superficial (IIo and IIi) and deeper (III/IV) spinal laminae: TrkB-Fc treatment prevented DRI-induced sprouting. Asterisks indicate significant differences from intact rats. (b) Low-power micrographs of serotonin transporter (SERT)-expressing axons in the spinal dorsal horn. (c) Higher-power micrographs of SERT immunohistochemistry from regions similar to that indicated in (b). Scale bar = 100 µm.
**Figure 2.8** K252a improves mechanosensation and enhances mechanosensory sprouting after C7/8 DRI. (a) Ipsilateral-contralateral latencies remained significantly different in vehicle-treated rats; differences disappeared by 8 d after DRI in K252a-treated rats. Asterisks indicate significant ipsilateral (ipsi)–contralateral (contra) differences. (b) VGLUT1 density (pooled 10 and 20 d animals) was elevated after K252a treatment. Asterisks indicate significant difference from deafferented control rats. (c) VGLUT1 immunohistochemistry. Scale bar = 100 µm.
a  

![Graph showing response latency over test days for Vehicle and K252a treatments.](image)

b  

![Bar graph showing VGLUT1 density across different groups.](image)

c  

![Images comparing Vehicle and K252a treatments.](image)
2.5 Bibliography


neurons from loss and atrophy, and provides limited regeneration. Exp Neurol 184: 97-113.


CHAPTER 3

Spinal brain-derived neurotrophic factor governs neuroplasticity and recovery from cold-hypersensitivity following dorsal rhizotomy

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3.1 Introduction

While brain-derived neurotrophic factor (BDNF) was initially isolated based on its ability to promote neuronal survival and neurite outgrowth (Barde et al., 1982), it is now widely recognized as a modulator of synaptic transmission. For example, BDNF facilitates excitatory glutamatergic transmission at the level of the spinal cord dorsal horn, illustrating its role in the modulation of nociception (Mannion et al., 1999; Thompson et al., 1999; Pezet et al., 2002; Malcangio and Lessmann, 2003; Pezet and McMahon, 2006). Synthesized in a sub-population of nociceptive DRG neurons, BDNF is anterogradely transported to the central terminals in the dorsal horn (Zhou and Rush, 1996; Michael et al., 1997; Luo et al., 2001), where it contributes to enhanced pain-related behaviour in animal models of peripheral inflammation (Kerr et al., 1999; Thompson et al., 1999; Zhao et al., 2006).

Although DRG neurons upregulate BDNF or express it de novo following peripheral nerve injury (Cho et al., 1997; Cho et al., 1998; Zhou et al., 1999; Fukuoka et al., 2001; Ha et al., 2001; Obata et al., 2004; Obata et al., 2006), genetic elimination of BDNF from nociceptive DRG neurons has no effect on the development of neuropathic pain (Zhao et al., 2006). Activated spinal microglia, however, which contribute to neuropathic pain following peripheral nerve injury (Tsuda et al., 2003; Inoue et al., 2003; Jin et al., 2003; Kempermann and Neumann, 2003; Tsuda et al., 2003; Tsuda et al., 2004), also upregulate BDNF (Coull et al., 2005). Microglial-derived BDNF interacts with its high affinity tropomyosin-related receptor kinase B (TrkB) to disrupt the inhibitory GABAergic control of nociceptive projection neurons and facilitate excitatory noxious transmission (Coull et al., 2005).
We have previously characterized a rat model of spinal deafferentation pain, in which C7 and C8 dorsal root injury (C7/8 DRI) induces cold pain that emerges by 5 days, peaks at approximately ten days, and resolves by 20 days post-injury (Ramer et al., 2004). Others have also described rhizotomy-induced pain (Colburn et al., 1999; Eschenfelder et al., 2000; Li et al., 2000). Interestingly, C7/8 DRI also elicits upregulation of BDNF by activated microglia (Ramer et al., 2007), but not by intact DRG neurons (Obata et al., 2006). Acute BDNF antagonism via a single intrathecal bolus of TrkB-Fc partially reduced peak pain responses (Ramer et al., 2007), indicating that BDNF contributes to cold pain in this model through enhancing nociceptive transmission.

In addition to its effects on synaptic function, endogenous BDNF may influence structural plasticity in nociceptive circuitry. We have previously shown that the serotonergic sprouting which follows DRI (Polistina et al., 1990; Wang et al., 1991; Zhang et al., 1993; Kinkead et al., 1998; MacDermid et al., 2004; Ramer et al., 2004; Scott et al., 2005) is dependent upon endogenous BDNF (Ramer et al., 2007). On the other hand, continuous TrkB-Fc treatment after C7/8 DRI elicited intraspinal sprouting of mechanosensory primary afferents (Ramer et al., 2007), presumably due to increased availability of synaptic space normally taken over by sprouting serotonergic and other BDNF-sensitive populations of spinal axons.

Here we ask whether small-caliber primary afferents, like their large-caliber counterparts, sprout in response to BDNF antagonism. We also investigate plasticity of intrinsic GABAergic neurons, which, like serotonergic neurons, are BDNF-sensitive (Pezet et al., 2002; Bardoni et al., 2007; Carrasco et al., 2007). Finally, we examine the effects of continuous BDNF antagonism on cold pain behaviour following C7/8 DRI.
We intrathecally administered TrkB-Fc from the time of C7/8 DRI until 20 days post-injury. TrkB-Fc treatment induced sprouting of nociceptive primary afferents and prevented a DRI-induced increase in density of GABAergic terminals. These findings were correlated with a prolonged cold hypersensitivity in TrkB-Fc-treated animals. The results illustrate a dual role for BDNF in pain which follows spinal deafferentation: it not only potentiates nociceptive transmission synaptically, but also governs plasticity of nociceptive circuitry which may underlie the resolution of DRI-induced cold pain.

3.2 Materials and methods

Surgery

All surgical procedures were conducted according to the guidelines of the Canadian Council for Animal Care and the University of British Columbia Animal Care Committee. Male Sprague Dawley rats (200-250g) were anesthetized with ketamine hydrochloride (75 mg/kg, Bimeda-MTC, Cambridge, ON) and medetomidine hydrochloride (0.5 mg/kg, Novartis, Mississauga, ON), by intra-peritoneal (i.p.) injection. For C7/8 dorsal root injury (C7/8 DRI), the left C7 and C8 dorsal roots were exposed via hemilaminectomy and durotomy, and transected with microscissors. Anesthesia was reversed with an intramuscular (i.m.) injection of atipamezole hydrochloride (5 mg/ml, Novartis, Mississauga, ON). The twenty-five animals (n = 25) included in this study were the same as those used in the behavioural and histological experiments in Chapter 3.
**Drug Treatment**

Rats had cannulae (0.64 mm outer diameter, 0.3 mm inner diameter) inserted through the atlanto-occipital membrane as far as C6 for intrathecal (i.t.) drug delivery. Cannulae were connected to an osmotic minipump (#2001, 2002, Alzet, Cupertino, CA) inserted into a subcutaneous pocket just caudal to the scapulae and filled with whole human IgG (n = 13; Sigma-Aldrich Canada, Oakville, ON) in phosphate-buffered saline (PBS), or human TrkB-Fc (n = 12; Sigma, St. Louis, MO) in PBS. These were delivered at a dose of 3 µg per day. Fourteen-day osmotic minipumps (2002) were implanted at the time of C7/8 DRI. Rats that survived for 20 days were re-anesthetized after behavioural testing on day 15, and two-week pumps were replaced with seven-day osmotic minipumps (2001) which delivered IgG or TrkB-Fc at the same dose. Chronic intrathecal delivery is also likely to reach the DRG. However, TrkB is expressed by only a few DRG neurons (typically non-nociceptive) (McMahon et al., 1994; Mu et al., 1993), and BDNF is not upregulated in intact ganglia following transection of neighbouring roots (Obata et al., 2006).

**Behaviour**

Behavioural testing was done blind and conducted by an investigator other than the surgeon. On at least two occasions, naïve rats were handled and habituated to the behavioural setup prior to preoperative testing. Testing was performed on two pre-operative days (days -2 and -1), and on days 2, 5, 10, 15 and 20 post-C7/8 DRI. Also before each trial, rats were placed in the behavioural apparatus for at least 5 minutes to
allow further habituation. Daily test scores for each test were averaged from three trials separated by 1 hour, for both forepaws.

A modified acetone test (Choi et al., 1994) for the palmar forepaw was used to assess cold pain, as done previously (Ramer et al., 2004; Ramer et al., 2007). Briefly, animals were individually placed in clear cages on a raised wire mesh bottom, and the duration of response to acetone applied to the palmar surface was measured. A micropipette was used to squirt 10 µl of acetone from below the wire mesh bottom. Cold pain responses were defined as a shaking, withdrawal, biting or licking of the tested forepaw. Responses were recorded for both ipsilateral and contralateral forepaws. Behavioural scoring ranged from a minimum of 1s (a brief withdrawal without excessive attention to the forepaw), to a maximum of 20s. A prolonged withdrawal without accompanying biting, licking or shaking was assigned a score of 3s, even if the paw remained elevated beyond this time.

Thermal allodynia was also tested using the plantar (in this case palmar) radiant heat test (Ugo Basile, Camerio, Italy). Animals were again placed in clear cages, this time on a glass floor. A moveable infrared light source below the glass was then positioned under the center of the forepaw. Withdrawal latency from stimulus onset was recorded for both forepaws. For all behavioural tests, averaged daily scores from each treatment group were plotted using graphing software (Sigma Plot 2001; SPSS, Chicago, IL), where error bars indicate standard error of the mean (SEM).
**Immunohistochemistry**

An overdose of chloral hydrate (1 g/kg) by i.p. injection was used to kill all rats, and once breathing ceased they were transcardially perfused with PBS, and 4% paraformaldehyde (PF). Animals in which cannulae were blocked or had penetrated the spinal cord were excluded from anatomical and behavioural analyses. Five additional unoperated (uninjured group; n = 5) rats were killed for immunohistochemical analysis. Spinal cord segments C6-T1 were removed and immediately postfixed in 4% PF for 12 hours, then cryoprotected in 20% sucrose in 0.1 M phosphate buffer (PB) for at least 24 hours. After, tissue was frozen over liquid nitrogen and cut with a cryostat into 16 µm-thick transverse sections. To prevent non-specific binding, sections were incubated with 10% normal donkey serum (NDS) with 0.2% Triton X-100 in PBS for 30 minutes. For the isoelectin B4 (IB4) immunohistochemistry, lectin from *Bandeiraea simplicifolia* (1:400; Sigma-Aldrich Canada, Ltd., Oakville, ON) was applied overnight prior to primary antibody incubation.

Rabbit anti-calcitonin gene-related peptide (CGRP) (1:4000; Sigma-Aldrich Canada Ltd., Oakville, ON), goat anti-IB4 (1:2000; Vector Laboratories, Burlingame, CA), guinea-pig anti-vesicular glutamate transporter 1 (VGLUT1) (1:4000; Chemicon International Inc., Temecula, CA), anti-growth associated protein-43 (GAP-43) (1:4000; Chemicon International Inc., Temecula, CA), rabbit anti-vesicular GABA transporter (VGAT) (1:500; Synaptic Systems, Goettingen, Germany), and rabbit anti-neuropeptide Y (NPY) (1:1000; Peninsula Laboratories Inc., San Carlos, CA) primary antibodies were applied overnight at room temperature. Secondary antibodies raised in donkey and conjugated to Cy3 or aminomethylcoumarin amide (AMCA) (Jackson ImmunoResearch,
West Grove, PA) or Alexa 488 (Molecular Probes Inc., Eugene, OR) were applied at 1:200 for 2 hours at room temperature.

**Image Analysis**

Spinal cord sections were examined using an Axioplan II microscope (Zeiss, Jena, Germany), and digital images were taken with a digital camera (QImaging, Burnaby, BC) and Northern Eclipse software (Version 6.0; Exmpix Imaging, Mississauga, ON). SigmaScan Pro software (SPSS, Chicago, IL) was used to measure fibre density in the dorsal horn. In order to optimize the signal-to-noise ratio and correct for variations in background fluorescence, threshold overlays of dorsal horn images were generated from images processed with a Laplacian omni-directional edge detection filter. From all threshold overlays, immunopositive terminal density was measured as a function of depth in the dorsal horn, as we have done previously (MacDermid et al., 2004; Ramer et al., 2004; Scott et al., 2005; Ramer et al., 2007). All measurements were taken from five transverse sections per animal (C7) and terminal density was measured from the overlays within the deafferentation gap for primary afferent markers, and throughout the mediolateral extent of laminae II to III/IV of the dorsal horn for all other histological markers. Average axonal density measurements from each treatment group were plotted as bar graphs using graphing software (Sigma Plot 2001; SPSS), where error bars indicate standard error of the mean (SEM).
Statistics

All quantitative histological and behavioural data are expressed as means ± SEMs. In all instances significance was taken as $p < 0.05$. Statistical differences in terminal densities were detected by one-way ANOVA, followed by Holm-Sidak post-hoc tests where appropriate, between IgG- and TrkB-Fc treated groups. DRI can result in contralateral effects (Ramer et al., 2004), thus ipsilateral densities after injury were compared with uninjured animals. For the acetone and radiant heat behavioural tests, differences between ipsilateral (ipsi) and contralateral (contra) were normally distributed and compared using a one-way repeated measures ANOVA for both IgG- and TrkB-Fc treated groups.

3.3 Results

C7/8 DRI created a consistent deafferentation gap in the C7 dorsal horn.

Since primary afferents do not regenerate back into the spinal cord, dorsal rhizotomy results in a persistent loss of afferent input to the dorsal horn. We have previously shown that neurons in the C6-T1 dorsal root ganglia contribute to forepaw innervation (Ramer et al., 2004). Therefore, we examined the corresponding spinal segments to identify the region of the spinal cord that incurs the most dramatic loss of primary afferent input as a consequence of C7/8 DRI. The deafferentation zone was delineated immunohistochemically in five rats that received C7/8 DRI only, using antibodies against VGLUT1 to label large-diameter primary afferents (Persson et al., 2006; Alvarez et al., 2004; Ramer et al., 2007) and antibodies against CGRP and IB4 to label peptidergic and non-peptidergic nociceptors, respectively (Snider and McMahon,
At 10 days after C7/8 DRI, spinal deafferentation was apparent in the ipsilateral dorsal horn between C6 and C8 (Fig. 3.1). However, the deafferentation gap was most pronounced and consistent in the C7 dorsal horn (Fig. 3.1; area bounded by hash marks). We chose this region to examine the effects of C7/8 DRI and BDNF sequestration on plasticity of spinal axons involved in pain processing (Figs. 3.2-3.7).

**BDNF sequestration promoted the sprouting of nociceptive primary afferents following C7/8 DRI**

We have previously shown that following C7/8 DRI, spinally upregulated TrkB ligands such as BDNF suppress the plasticity of large diameter mechanosensory primary afferents (Ramer et al., 2007). We therefore asked whether DRI-induced BDNF would similarly suppress the plasticity of another population of DRG neurons, nociceptive primary afferents.

Peptidergic (CGRP-expressing) nociceptive primary afferents are depleted in the ipsilateral dorsal horn after dorsal rhizotomy and sprouting does not occur in the early post-operative weeks (Traub et al., 1989; Piehl et al., 1992; MacDermid et al., 2004; Scott et al., 2005; Hannila and Kawaja, 2005). As expected, C7/8 DRI reduced CGRP-positive fibre density in lamina II outer (IIo) of the C7 dorsal horn and produced a distinct gap in the termination pattern of the afferent terminals (Figs. 3.1, 3.2). To determine whether endogenous BDNF prevents peptidergic axon sprouting, animals received continuous intrathecal (i.t.) infusions of either BDNF sequestering TrkB-Fc or IgG control (IgG; both at 3 µg/day) (Cabelli et al., 1997; Seebach et al., 1999; Chan et al.,
In IgG-treated rats, the density of CGRP-expressing axons in the deafferentated area was significantly lower than in the uninjured rats (Fig. 3.2). TrkB-Fc treatment, however, resulted in a significant reappearance of CGRP-positive axons in the deafferentation gap at 10 days, and to a greater extent at 20 days post-DRI (Fig. 3.2a, c-e).

Although less is known regarding the response of non-peptidergic primary afferents to acute rhizotomy, chronic rhizotomy studies indicate that IB4-binding afferents have a smaller capacity for terminal sprouting than their peptidergic counterparts (Belyantseva and Lewin, 1999). C7/8 DRI dramatically reduced IB4-binding axon density in lamina II inner (Ili) of the C7 dorsal horn (Fig. 3.3). In IgG- and TrkB-Fc treated rats, the density of IB4-binding axons was significantly reduced compared to the uninjured rats at both 10 and 20 days after C7/8 DRI (Fig. 3.3c-e). At the later timepoint some IB4-binding axons occupied this denervated territory in TrkB-Fc treated rats, but not in IgG-treated rats (Fig. 3.3c-e). These results indicate that endogenous BDNF suppresses the plasticity of both peptidergic and non-peptidergic nociceptive primary afferent terminals after C7/8 DRI.

**Endogenous BDNF promotes the overall plasticity of intraspinal axons after C7/8 DRI**

Differential responsiveness to endogenous factors and/or competition for synaptic space (Polistina et al., 1990) may regulate the varied sprouting responses of pain-processing axon populations after spinal deafferentation. Because chronic TrkB-Fc
treatment not only promotes primary afferent plasticity (current findings) (Ramer et al., 2007), but also suppresses spinally-projecting serotonergic axon sprouting (Ramer et al., 2007), we sought to determine whether BDNF has an overall plasticity-promoting or suppressing effect on spinally-projecting axons. Growth associated protein-43 (GAP-43) is expressed in axons which have an increased propensity for growth (Schwab, 1996). We therefore examined GAP-43 expression in the dorsal horn of IgG- and TrkB-Fc treated rats following C7/8 DRI.

Rhizotomy induced a significant increase in GAP-43-positive axon density in lamina IIo of IgG-treated rats at 10 and 20 days post-DRI (Fig. 3.4a, c; daggers). GAP-43 expression in TrkB-Fc treated rats did not change compared to uninjured animals (Fig. 3.4a). GAP-43-positive fibre density was significantly lower in TrkB-Fc-treated rats than in IgG-treated rats in lamina IIi and III-IV at 10 days, and in all laminae 20 days after injury (Fig. 3.4a, d, e; asterisks).

C7/8 DRI stimulated BDNF-dependent increases in the density of GABAergic terminals.

Spinal GABAergic interneurons are also BDNF-sensitive (Bardoni et al., 2007; Carrasco et al., 2007) implicating TrkB expression. The inhibitory role of the GABAergic system in nociceptive processing has been well-studied (Enna and McCarson, 2006). Following spinal deafferentation, both GAD67 and GABA content are increased in the superficial dorsal horn (Dumoulin et al., 1996). Because DRI-induced serotonergic sprouting (Polistina et al., 1990; Wang et al., 1991; Ramer et al., 2004; MacDermid et al., 2004) is mediated by endogenous BDNF (Ramer et al., 2007), we
were prompted to ask what the effects of endogenous BDNF were on the plasticity of GABAergic interneurons following C7/8 DRI.

The vesicular GABA transporter (VGAT) is highly concentrated in the terminals of all GABAergic axons (Chaudhry et al., 1998), and is thus a reliable marker for analyzing GABAergic synaptic density. VGAT-positive terminal density was compared between groups in laminae IIo, IIIi, and III-IV. In uninjured animals, punctate VGAT labeling was apparent throughout the dorsal horn, although it was most dense in superficial laminae (Fig. 3.5b). In IgG-treated controls, C7/8 DRI resulted in a significant increase in VGAT-positive interneuron density in lamina II by 10 days, which persisted until at least 20 days post-injury (Fig. 3.5a, c, e; daggers). In contrast, VGAT density in TrkB-Fc treated rats was significantly lower than in IgG-treated animals in lamina IIIi and III-IV at 10 days (Fig. 3.5a, asterisks), and in all examined laminae at 20 days post-DRI (Fig. 3.5d, e; asterisks).

A subset of GABAergic interneurons, with axons containing neuropeptide Y (NPY), has been implicated in disynaptic transmission between nociceptive peptidergic primary afferents and the thalamus (Rowan et al., 1993; Naim et al., 1997; Polgar et al., 1999), and is thus likely to have a modulatory role in spinal pain processing. Although little is known about rhizotomy-induced effects on NPY-containing interneurons in vivo, BDNF has been shown to increase NPY expression in spinal neurons (Siuciak et al., 1995; Kim et al., 2000). Therefore we also examined the influence of both C7/8 DRI and BDNF sequestration on NPY-expressing neurites in the C7 dorsal horn.

In all groups, NPY-positive processes were most dense in the superficial laminae (Fig. 3.6b-e). In IgG-treated rats, C7/8 DRI stimulated an increase in NPY-positive
process density, which was elevated above uninjured levels in lamina IIi and III-IV at 10 days (Fig. 3.6a-c, e; daggers), but only in lamina III-IV at 20 days post-DRI (Fig. 3.6a, daggers). In TrkB-Fc-treated rats, the density of NPY-positive neurites in the dorsal horn did not change relative to the uninjured rats at either 10 or 20 days after rhizotomy (Fig 3.6a, b, d, e). NPY-positive neurite density was significantly lower in TrkB-Fc-treated rats than in IgG-treated rats in all examined laminae at 10 days after injury (Fig. 3.6a, asterisks). At 20 days post-injury, this effect of BDNF sequestration was only significant in deeper laminae of the dorsal horn (Fig. 3.6a, asterisks).

Continuous BDNF sequestration prevented recovery from cold pain following C7/8 DRI

We have previously characterized cold pain that develops following rat C7/8 DRI (Ramer et al., 2004). In untreated rats, hypersensitivity to cooling is apparent by 5 days after C7/8 DRI and resolves within three weeks (Ramer et al., 2004). We also found that acute BDNF sequestration (a single 2 µg i.t. bolus of TrkB-Fc) reduced the duration of peak cold pain responses to forepaw stimulation with acetone (Ramer et al., 2007). This indicated that BDNF was exerting a neuromodulatory role contributing to cold pain generated by C7/8 DRI. Given the anatomical plasticity of nociceptive circuitry (current findings) and previous acute involvement in synaptic transmission with TrkB-Fc treatment (Ramer et al., 2007), we hypothesized that continuous BDNF sequestration would also affect the long term pain behaviour after C7/8 DRI.

Cold pain was assessed by measuring the duration of withdrawal, biting, licking or paw shaking after a squirt (10 µl) of acetone onto the palmar surface (Choi et al., 1994;
Ramer et al., 2004; Ramer et al., 2007) (Fig. 3.7). C7/8 DRI-induced cold pain has both allodynic and hyperalgesic components, since a non-noxious cooling stimulus (acetone application) elicits nocifensive responses (allodynia), and becomes more exaggerated with time after injury (hyperalgesia). Thus the inclusive term “cold pain” is used to describe the sensitivity to cold stimulation that develops in this injury model.

Acetone stimulation of the uninjured or normally-innervated (contralateral) forepaw evoked a brief withdrawal with little licking or shaking, and no biting of the forepaw and/or limb (Fig. 3.7a, b). In IgG-treated controls, acetone applied to the injured or partially-denervated (ipsilateral) paw 5-10 days after C7/8 DRI elicited a significantly prolonged elevation of the forepaw (Fig. 3.7a), and was generally accompanied by vigorous licking and/or shaking of the forepaw. Biting of the paw and of the upper forelimb and vocalization occurred occasionally. None of the rats exhibited signs of autotomy or self-mutilation of the affected forepaw or limb (i.e. nocifensive responses did not draw blood and occurred only after acetone application). This cold-induced pain resolved by 20 days post-DRI, similar to untreated rats (Ramer et al., 2004). Ten days after C7/8 DRI there were no significant differences in response durations between IgG and TrkB-Fc treated rats (Fig 3.7a, b). At 20 days post-injury the ipsilateral response duration in TrkB-Fc treated rats was still significantly elevated. These results demonstrate that continuous BDNF sequestration prevented the resolution of cold pain.

Previous experiments demonstrated that continuous BDNF sequestration has no effect on the responses to punctate mechanical stimulation (Ramer et al., 2007). To determine whether heat sensitivity after spinal deafferentation was also BDNF-independent, we assessed thermal (heat) allodynia in the same IgG- and TrkB-Fc treated
rats. The plantar test (Hargreaves et al., 1988) was used on the palmar surface to test for thermal allodynia (Fig. 3.7c, d). The latency to withdrawal did not decrease in the partially-denervated forepaws in either group. At five days post-DRI, IgG-treated animals exhibited an increased latency to withdrawal in the ipsilateral forepaw (Fig. 3.7c), but this effect was small and transient. These responses to radiant heat are similar to our previous observations in untreated rats (Ramer et al., 2004).

3.4 Discussion

Here we have taken advantage of the C7/8 DRI model to demonstrate the consequences of endogenous BDNF on the plasticity of pain circuitry in the dorsal horn and on deafferentation-induced cold pain. We show that although endogenous BDNF suppresses nociceptive primary afferent sprouting, on the whole it has a plasticity-enhancing influence on spinal axons. As is the case for serotonergic axons (Ramer et al., 2007) endogenous BDNF is necessary for the increase in density of spinal GABAergic terminals following DRI. We also find that chronic TrkB-Fc treatment prevents recovery from deafferentation-induced cold pain, illustrating that BDNF contributes to its resolution.

Primary afferent plasticity is suppressed by endogenous BDNF

Interestingly, the majority of nociceptive DRG neurons are TrkB-negative (McMahon et al., 1994) but their responsiveness to other neurotrophins is well-documented: peptidergic nociceptors express TrkA, conferring responsiveness to nerve growth factor (NGF), while non-peptidergic nociceptors express receptor components for
glial cell line-derived neurotrophic factor (GDNF) (Snider and McMahon, 1998). Despite the absence of TrkB from small caliber primary afferent axons, our current findings implicate endogenous BDNF in suppressing both peptidergic and non-peptidergic nociceptor sprouting in the first three weeks following C7/8 DRI. These results are consistent with BDNF-mediated suppression of plasticity of mechanosensory axons (Ramer et al., 2007), the majority of which are also TrkB-negative (Mu et al., 1993; McMahon et al., 1994; Wright and Snider, 1995).

The plasticity-restricting influence of endogenous BDNF may reflect either a direct or indirect effect on nociceptive primary afferents. Based on the distribution of neurotrophin receptors in DRG neurons and the changes in neurotrophin expression in the deafferented spinal cord (with BDNF being more abundant than NT-3, NGF or GDNF (Johnson et al., 2000; Ramer et al., 2007), we favour a scenario in which nociceptor sprouting is inhibited indirectly by BDNF-sensitive axons. That is, under conditions of elevated BDNF expression, TrkB-expressing descending serotonergic axons (King et al., 1999; Loudes et al., 1999; Madhav et al., 2001) and local interneurons (Bardoni et al., 2007; Carrasco et al., 2007), have a competitive advantage over TrkB-negative axons when it comes to occupying vacated post synaptic membrane.

Direct inhibitory effects of BDNF on TrkB-negative sensory neurons cannot be ruled out, however. Previous work has shown that BDNF added to cultured adult sensory neurons inhibited both NGF-evoked neurite outgrowth from peptidergic nociceptors and neurotrophin-independent neurite outgrowth from non-peptidergic nociceptors (Kimpinski et al., 1997, 1999; Gavazzi et al., 1999). In the case of peptidergic axons, BDNF can compete with NGF for p75 and subsequently decrease NGF-TrkA binding
(Barker and Shooter, 1994). The importance of such a mechanism in vivo is questionable, however, given the lack of NGF in the deafferented spinal cord (Ramer et al., 2007). The negative influence of BDNF on IB4-binding fiber growth must occur via some unknown p75-independent mechanism, since these neurons do not express the receptor (Wright and Snider, 1995).

**BDNF-dependent plasticity of GABAergic neurons following DRI**

While the reappearance of nociceptive axons in the TrkB-Fc-treated deafferentation gap is indicative of primary afferent sprouting (as opposed to changes in phenotype of the few remaining axons), less can be said about changes in the density of GABAergic terminals which normally follows DRI. It has been convincingly demonstrated in quantitative EM studies that synapse replacement occurs relatively quickly following dorsal rhizotomy (by 10 days) in the cat (Murray, 1986) and rat (Zhang et al., 1993). While the increase in density of VGAT-containing terminals in Lamina II demonstrated here may well contribute to the previously-reported reappearance of synapses, it should also be noted that there is an increase in the number of neurons expressing GABA-synthesizing enzymes following rhizotomy (Dumoulin et al., 1996). This change in GABAergic interneuron number could also underlie the increase in VGAT-positive terminal density in the spinal cord. Interestingly, in hippocampal granule cells, glutamatergic neurons can be induced to take on a GABAergic phenotype by TrkB activation (Gomez-Lira et al., 2005), and it is tempting to speculate that rhizotomy might trigger an increase in the number of GABAergic neurons that is also BDNF-dependent. While the present results show that the increase in density of NPY-containing processes
(also known to be GABAergic) (Rowan et al., 1993; Naim et al., 1997; Polgar et al., 1999) is indeed dependent on endogenous BDNF, we do not yet know whether it is a reflection of increased sprouting or BDNF-mediated upregulation, as NPY is upregulated by BDNF in spinal cord neurons \textit{in vitro} (Kim et al., 2000) and \textit{in vivo} (Siuciak et al., 1995). Ultimately, it is likely that both sprouting and phenotypic alterations in interneurons account for the increased density of GABAergic terminals in the deafferented spinal cord.

**A dual role for BDNF in DRI-induced cold pain**

While the relationship between anatomical and behavioural outcomes of C7/8 DRI remains enigmatic, both are clearly BDNF-dependent. Complicating the issue is that there are both fast (synaptic) and slow (sprouting and phenotypic changes) components to BDNF’s effects. The fast component is evident in the effect of a single bolus of TrkB-Fc into the intrathecal space at 10 days post-DRI when cold pain is maximal (Ramer et al., 2007). Acute BDNF antagonism significantly reduces the duration of response to cooling, an effect illustrating a pro-nociceptive role for BDNF. The slow component is apparent in chronic TrkB-Fc treated animals, in which cold pain fails to resolve by 20 days post-injury, demonstrating that in the longer term, endogenous BDNF is anti-nociceptive. The absence of any apparent effect of TrkB-Fc on behaviour at 10 days post-DRI in the present experiment is probably due to elimination of both BDNF’s fast (pro-nociceptive) and slow (anti-nociceptive) components.

The development of BDNF-mediated cold pain following C7/8 DRI likely involves N-methyl-D-aspartate (NMDA) receptor activation. Many synapses between
primary afferent and second-order neurons are of the “silent” type: they express NMDA receptors but no α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Li and Zhuo, 1998). Central sensitization, a process dependent on NMDA receptor activation and phosphorylation (Ji et al., 2003; South et al., 2003), is enhanced by BDNF in isolated neonatal spinal cords (Kerr et al., 1999), and is markedly reduced in BDNF-deficient transgenic mice (Heppenstall and Lewin, 2001). TrkB-Fc treatment in adult ex vivo spinal cord preparations also reduces NMDA receptor phosphorylation following high-intensity stimulation of the attached roots (Slack et al., 2004). Silent glutamatergic synapses may also be activated heterosynaptically by 5-HT, which recruits AMPA receptors to otherwise purely NMDA synapses (Li and Zhuo, 1998; Li et al., 1999; Wang and Zhuo, 2002). This may be particularly germane to pain following DRI, since the density of serotonergic axons increases in relevant spinal laminae (Polistina et al., 1990; Wang et al., 1991; Zhang et al., 1993; Kinkead et al., 1998; Ramer et al., 2004; MacDermid et al., 2004; Scott et al., 2005; Ramer et al., 2007).

That the resolution of cold pain occurs concomitant with an increase in density of GABAergic terminals suggests a causal relationship. GABA activates GABAA receptors on post-synaptic cells, resulting in chloride efflux and hyperpolarization. Spinal NPY, acting through the Y1 receptor, is also generally considered to be antinociceptive (Naveilhan et al., 2001; Mahinda and Taylor, 2004; Miyakawa et al., 2005), and the increase in density of VGAT- and NPY-positive terminals after DRI might be an adaptive response of GABAergic interneurons to initiate recovery from cold pain. BDNF is also known to increase inhibitory GABAergic synaptogenesis in the cortex (Palizvan et al.,
Recently, however, it has been demonstrated that BDNF derived from activated microglia changes the postsynaptic response to GABA from hyperpolarizing to depolarizing, and results in hyperalgesia (Coull et al., 2005). This effect is probably attributable to a BDNF-mediated reduction in the expression of the K⁺-Cl⁻ cotransporter (KCC2), and an associated change in postsynaptic neurons’ ability to extrude chloride (Wardle and Poo, 2003; Rivera et al., 2004). It is therefore possible that KCC2 downregulation induced by endogenous BDNF may contribute to the development of cold pain following C7/8 DRI. Arguing against such a mechanism, however, is the fact that the resolution of cold pain occurs at a time when spinal BDNF is still elevated. Future experiments will be necessary to determine the role of the chloride gradient and KCC2 function in the DRI model.

Conclusions

The present results emphasize the multifunctionality of BDNF, and that manipulating BDNF-TrkB signaling for the purposes of altering its neuromodulatory effects must be undertaken with the understanding that it may also alter spinal circuitry via its effects on axonal plasticity. Dissecting mechanisms underlying both processes may lead to new therapies targeting BDNF-mediated pain, and to treatments exploiting BDNF’s neurotrophic effects in promoting functional recovery following nervous system injury.
**Figure 3.1** C7/8 DRI created a consistent deafferentation gap in the C7 dorsal horn. Photomicrographs of spinal cord sections from C6, C7, C8 and T1 segments ipsilateral and contralateral to C7/8 DRI. This representative animal was chosen from five rats that received C7/8 DRI only. Immunohistochemical characterization of large-diameter mechanosensory primary afferents (expressing vesicular glutamate transporter 1; VGLUT1) and small-diameter peptidergic and non-peptidergic nociceptive primary afferents (expressing calcitonin gene-related peptide [CGRP] and binding isolectin Bandeiraea simplicifolia [IB4], respectively) revealed a deafferentation gap which, while apparent in C6-C8, was most consistent in C7 (area bounded by hash marks). Scale bar = 200 μm.
**Figure 3.2** BDNF sequestration promoted the sprouting of peptidergic nociceptive primary afferents following C7/8 DRI. (a) CGRP-positive axon density in the C7 dorsal horn. C7/8 DRI resulted in decreased CGRP-fibre density in lamina IIo (b, c). CGRP-positive axon density was increased with TrkB-Fc treatment 10 days, and to a greater extent 20 days post-DRI (a, d). (e) Higher power images of axons from regions similar to those indicated by the boxed region in b. In a, asterisks indicate significant differences between TrkB-Fc and IgG-treated controls; daggers indicate significant differences between uninjured and IgG-treated controls. All rhizotomized groups (n = 5 for each group) were significantly different from uninjured animals (n = 5). Scale bar = 100 µm.
Figure 3.3 BDNF sequestration increased the sprouting of non-peptidergic nociceptive primary afferents following C7/8 DRI. (a) IB4-binding axon density in the C7 dorsal horn. In IgG-treated animals, the density of IB4-binding axons was reduced in lamina IIi after C7/8 DRI (b, c). (d) TrkB-Fc treatment resulted in an increase in IB4-binding axon density 20 days following C7/8 DRI. (e) Higher power images of axons from regions similar to those indicated by the boxed region in b. In a, asterisks indicate significant differences between TrkB-Fc and IgG-treated controls; daggers indicate significant differences between uninjured and IgG-treated controls. All rhizotomized groups (n = 5 for each group) were significantly different from uninjured animals (n = 5). Scale bar = 100 µm.
Figure 3.4 Endogenous BDNF promotes the overall plasticity of intraspinal axons after C7/8 DRI. (a) GAP-43-positive axon density in the C7 dorsal horn. (b) Axons expressing GAP-43 were apparent in superficial laminae of the uninjured dorsal horn. DRI stimulated an increase in GAP-43 expression in lamina IIo (a, c). (D) BDNF sequestration prevented this DRI-mediated increase, and GAP-43-positive fibre density was similar to intact levels (a, b). (e) Higher power images of GAP-43-positive axon density from regions similar to those indicated by the boxed region in b. In a, asterisks indicate significance differences between TrkB-Fc (n = 5 for each timepoint) and IgG-treated controls (n = 5 for each timepoint); daggers indicate significant differences between uninjured (n = 5) and IgG-treated rhizotomized controls. Scale bar = 100 μm.
**Figure 3.5** C7/8 DRI stimulated BDNF-dependent increases in the density of VGAT-expressing GABAergic interneurons. (a) VGAT-positive interneuron density in the superficial dorsal horn. (b) In the intact spinal cord, punctate VGAT immunoreactivity was evident throughout the dorsal horn. C7/8 DRI elicited an increase in VGAT-positive interneuron density in superficial laminae (IIo and IIIi) (c). (d) BDNF sequestration attenuated this increase 20 days post-DRI. (e) Higher power images of VGAT-positive interneuron density from regions similar to those indicated by the boxed region in b. In a, asterisks indicate significant differences between TrkB-Fc (n = 5 for each timepoint) and IgG-treated controls (n = 5 for each timepoint); daggers indicate significant differences between uninjured (n = 5) and IgG-treated rhizotomized controls. Scale bar = 100 μm.
Figure 3.6 C7/8 DRI increased the density of NPY-expressing neurites in a BDNF-dependent manner. (a) NPY-positive neurite density in the superficial dorsal horn. NPY-positive fibre density increased in laminae IIi and III-IV, 10 days after rhizotomy (b, c). (D) TrkB-Fc treatment prevented this increase in NPY-positive neurite density which followed C7/8 DRI. (e) Higher power images of NPY-positive fibre density from regions similar to those indicated by the boxed region in b. In a, asterisks indicate significant differences between TrkB-Fc (n = 5 for each timepoint) and IgG-treated controls (n = 5 for each timepoint); daggers indicate significant differences between uninjured (n = 5) and IgG-treated rhizotomized controls. Scale bar = 100 µm.
Figure 3.7 Continuous BDNF sequestration prevented recovery from cold pain following C7/8 DRI. (a) The duration of response to a cold stimulus increased in IgG-treated rats by five days post-DRI, and returned to preoperative levels within 15 days (20 days post-C7/8 DRI). (b) TrkB-Fc treatment prevented normal recovery from cold pain. (c) The latency to withdraw from an infrared beam (with the exception at five days post-rhizotomy in IgG-treated controls in which there was a small but significant ipsilateral increase in withdrawal threshold), changed little after C7/8 DRI and/or TrkB-Fc treatment (d). Asterisks indicate significant differences between ipsilateral (ipsi, white) and contralateral (contra; black) responses.
### 3.5 Bibliography


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CHAPTER 4

Exogenous brain-derived neurotrophic factor alters nociceptive circuitry, reduces potassium-chloride co-transporter expression and facilitates cold pain following dorsal root injury.\(^3\)

\(^3\) A version of this chapter has been submitted for publication. Soril, L.J.J. and Ramer, M.S. (2009) Exogenous brain-derived neurotrophic factor alters nociceptive circuitry, reduces potassium-chloride co-transporter expression and facilitates cold pain following dorsal root injury.
4.1 Introduction

Neurotrophins are pivotal regulators of pain. At the level of the spinal cord, pathological neurotrophin-mediated modifications to nociceptive circuitry may involve the strengthening of existing synapses (Ji et al., 2003), the unmasking of silent synapses (Li and Zhou, 1998), and the establishment of new connections subsequent to axonal sprouting (Romero et al., 2000). One neurotrophin in particular, brain-derived neurotrophic factor (BDNF), is commonly accredited for such modifications (Pezet and McMahon, 2006) through its interaction with the tropomyosin-related kinase B (TrkB) receptor (Kaplan and Stephens, 1994; Kaplan and Miller, 1997). For instance, in the presence of BDNF specific pain-modulating systems, such as descending monoaminergic axons and intrinsic inhibitory interneurons, undergo terminal sprouting and increase neurotransmitter release in the spinal dorsal horn (Pezet et al., 2002; Ramer et al., 2007; Hayashida et al., 2008; Soril et al., 2008).

While there have been suggestions of a pro-nociceptive role for elevated spinal BDNF levels in models of neuropathic pain (Kerr et al., 1999; Thompson et al., 1999; Zhou et al., 2000; Yajima et al., 2002, 2005; Coull et al., 2005), other reports of high-dose exogenous BDNF treatment have claimed analgesic or anti-nociceptive effects (Pezet and McMahon, 2006). For example, direct administration (Siuciak et al., 1995; Cirulli et al., 2000), cellular graft delivery (Cejas et al., 2000), and viral-vector expression (Eaton et al., 2002) of BDNF have all been reported to alleviate painful neuropathic behaviour in rats and mice.

Spinal deafferentation by cervical dorsal root injury (DRI) results in the spinal upregulation of BDNF (Johnson et al., 2000; Ramer et al., 2007). Interestingly, DRI of
the C7 and C8 dorsal roots (C7/8 DRI) in the rat reproducibly evokes the development of both cold hyperalgesia and allodynia (cold pain) that resolves spontaneously within three weeks of injury (Ramer et al., 2004). Recently we determined that this recovery from cold pain is regulated by microglia-derived BDNF (Soril et al., 2008): animals treated continuously with the BDNF sequestering fusion protein TrkB-Fc did not recover from DRI-induced cold pain. Furthermore, the axonal plasticity associated with this behavioural recovery was prevented (Soril et al., 2008).

In this current study, we investigated the effects of exogenous BDNF on C7/8 DRI-induced cold pain. The intraspinal plasticity of nociceptive primary afferents, local inhibitory interneurons and descending monoaminergic projections was also examined in order to determine whether exogenous BDNF would stimulate morphological changes beyond those of DRI alone. Given the effects of TrkB-Fc treatment, we initially hypothesized that a post-operative exogenous BDNF regimen would lessen the severity of, or accelerate the recovery from C7/8 DRI-induced cold pain. However, with the application of BDNF, DRI-induced cold pain unexpectedly failed to resolve, and we provide evidence that this was underpinned by BDNF-mediated plasticity of descending and intraspinal inhibitory systems combined with disinhibition of the deafferented dorsal horn.

4.2 Materials and methods

Surgery and drug treatment

Experimental procedures conformed to the Canadian Council for Animal Care and the University of British Columbia Animal Care Committee guidelines. Intra-
peritoneal (i.p.) injections of ketamine hydrochloride (75 mg/kg, Bimeda-MTC, Cambridge, ON) and medetomidine hydrochloride (0.5 mg/kg, Novartis, Mississauga, ON) were used to anaesthetize adult male Sprague Dawley rats (n = 24; 200-250g). C7/8 DRI was performed as done previously (Ramer et al., 2007; Soril et al., 2008), by transecting the exposed dorsal roots with small spring scissors. Dorsal muscle layers were then sutured with absorbable vicryl sutures (4-0; Ethicon, Piscataway, NJ) and intrathecal (i.t.) silastic cannulae (0.64 mm outer diameter, 0.3 mm inner diameter) were inserted through the atlanto-occipital membrane and advanced to C6. For i.t. drug infusion, cannulae were connected to fourteen-day osmotic minipumps (2002, Alzet, Cupertino, CA) inserted subcutaneously caudal to the scapulae and filled with rat serum albumin (RSA) (1 mg/ml; Sigma-Aldrich Canada, Oakville, ON) dissolved in sterile saline (n = 8), or recombinant human brain-derived neurotrophic factor (BDNF) (0.5 mg/ml; a gift from Dr. Wolfram Tetzlaff, UBC) in sterile saline (n = 8). Drugs were delivered at a dose of 6 µg and 12 µg per day for BDNF and RSA, respectively. Finally, rats were given 5 ml of lactated ringers sub-cutaneously (s.c.) and anaesthesia was reversed with an intramuscular (i.m.) injection of atipamezole hydrochloride (5 mg/ml, Novartis, Mississauga, ON).

**Behavioural testing**

All behavioural tests were conducted by blinded investigators. One week prior to pre-operative testing, animals were handled, numbered and acclimatized to the behavioural apparati. Behavioural tests were conducted on two pre-operative occasions (days -2 and -1), and every other day (starting at day 2) until 20 days post-C7/8 DRI.
Behavioural scores for each forepaw were averaged from three trials, where animals were given a least 1 hour in between trials.

Acetone stimulation of the palmar forepaw was used to assess cold sensitivity (Choi et al., 1994, Ramer et al., 2004; Ramer et al., 2007, Soril et al., 2008). Animals were placed in individual clear glass cages on an elevated wire mesh platform and 10 µl of acetone was squirted onto the palmar surface from below. The duration of response to the cold stimulus was measured in seconds, where withdrawal, shaking, biting or licking of the stimulated forepaw were considered cold pain responses. Response durations were recorded up to a maximum of 20s.

The radiant heat test (Ugo Basile, Camerio, Italy) was also used to assess thermal (heat) sensitivity. In similar glass cages, animals were positioned on top of a glass platform and an infrared light source below the glass was manoeuvred under the forepaws. The time in seconds for the animal to withdraw from the light sources (withdrawal latency) was recorded. Withdrawal latency was cut-off at a maximum of 20s to avoid stimulus evoked tissue damage. For both cold and radiant heat assessments, the averaged daily scores for BDNF- and vehicle-treated animals were plotted (Sigma Plot 2001; SPSS, Chicago, IL) and error bars indicate standard error of the mean (SEM).

**Tissue processing and immunohistochemistry**

Following the final behavioural testing day, animals were killed with an i.p. overdose of chloral hydrate (1 g/kg). Transcardial perfusion with PBS, and 4% paraformaldehyde (PF) was initiated upon cessation of breathing and loss of nociceptive reflexes. The C6-T1 spinal cord segments were removed with the corresponding dorsal
roots and dorsal root ganglia (DRG) attached and post-fixed in 4% PF for 12 hours. Finally, tissue was cryoprotected in 20% sucrose in 0.1 M phosphate buffer (PB) for a minimum of 24 hours. Animals whose spinal cords were damaged by the cannula were excluded from analyses. For the KCC2 immunohistochemistical analysis, tissue from 5 additional uninjured rats was similarly harvested and processed.

All immunohistochemical procedures were carried out at room temperature. First, tissue was snap frozen over liquid nitrogen, transversely sectioned with a cryostat (16 µm) and thaw-mounted onto glass slides. After two 10 min PBS washes, sections were blocked in 10% normal donkey serum (NDS) with 0.2% Triton X-100 in PBS for 30 minutes to prevent non-specific binding, and incubated overnight with the following primary antibodies: rabbit anti-calcitonin gene-related peptide (CGRP) (1:4000; Sigma-Aldrich Canada Ltd., Oakville, ON), rabbit anti-serotonin transporter (SERT) (1:1200; Immunostar, Hudson, WI), sheep anti-tyrosine hydroxylase (TH) (1:200; Chemicon, Temecula, CA), mouse anti-dopamine-β-hydroxylase (DβH) (1:1000; Chemicon), rabbit anti-neuropeptide Y (NPY) (1:1000; Peninsula Laboratories Inc., San Carlos, CA), and rabbit anti-K⁺/Cl⁻ cotransporter 2 (KCC2) (1:1000; Chemicon). Sections were washed three times (15 min) in PBS and secondary antibodies raised in donkey and conjugated to either Cy3 (Jackson ImmunoResearch, West Grove, PA) or Alexa 488 (Molecular Probes Inc., Eugene, OR) were applied (1:200) for two hours. Slides were then rinsed three final times with PBS (15 min) and coverslipped.
Image Analysis

To visualize spinal cord sections for densitometric analyses, an Axioplan II microscope (Zeiss, Jena, Germany) was used and images were captured with a digital camera (QImaging, Burnaby, BC) and Northern Eclipse computer software (Version 6.0; Empix Imaging, Mississauga, ON). Measurements of immunopositive terminal density were expressed as a function of depth in the dorsal horn, as done previously (MacDermid et al., 2004; Ramer et al., 2004; Scott et al., 2005; Ramer et al., 2007; Soril et al., 2008). Five randomly selected images were taken from the C7 dorsal horn of each animal (n = 5 per group) and processed through a Laplacian omnidirectional edge-detection filter (SigmaScan Pro version 5.0). The resulting images were thresholded, and densities of immunoreactive processes were measured across the mediolateral extent of laminae II to III/IV of the dorsal horn.

To identify contacts between NPY-positive somal profiles and dopaminergic and/or noradrenergic projections in the dorsal horn, a LSM 5 Pascal confocal microscope (Zeiss, Jena, Germany) was used. Two images per animal (n = 6 per treatment group) containing at least one NPY-positive dorsal horn neuron were taken. Images were captured as Z stacks of 15-30 images. From each image in a stack, all axo-neuritic (axons and dendrites) and axo-somatic contacts were counted. These were then expressed as the total number of contacts per mm$^3$. Terminal density and contact measurements from each treatment group were plotted as bar graphs using graphing software (Sigma Plot 2001; SPSS), and error bars indicate standard error of the mean (SEM).
Statistics

For behavioural data, one-way repeated measures ANOVA and Holm-Sidak post-hoc tests were used to detect statistical differences between ipsilateral and contralateral responses. Statistical differences in histological data were detected by one-way ANOVA, followed by Holm-Sidak post-hoc tests where appropriate, between uninjured (for contact analysis and KCC2-immunoreactivity), vehicle- and BDNF-treated groups. All data are expressed as means ± SEMs, and significance was taken as p < 0.05.

4.3 Results

Exogenous BDNF prevented the normal resolution of C7/8 DRI-induced cold pain

Considering that continuous sequestration of endogenous BDNF impeded the resolution of DRI-induced cold pain (Soril et al., 2008), we first asked whether i.t. treatment with exogenous BDNF would, conversely, facilitate cold pain recovery after C7/8 DRI. As expected, vehicle-treated animals exhibited a transient increase in response duration to innocuous acetone stimulation (10 µl) of the partially-denervated forepaw, compared to the uninjured side (Fig. 4.1a). The increase in response duration was significant at days 8 and 10 (p < 0.05 for both days) post-DRI, and recovery was again attained by 20d (Fig. 1a). Surprisingly, by 4d-post DRI, BDNF-treated animals displayed elevated response durations to acetone stimulation of the ipsilateral forepaw, which, but for one day (day 16), persisted throughout the 20 day test period (Fig. 4.1b). Thermal (heat) allodynia, as indicated by decreased withdrawal latency, did not develop in either vehicle- or BDNF-treated groups (Fig. 4.1c, d). There were some days when animals in both treatment groups exhibited elevated response thresholds in the ipsilateral
forepaw (Fig. 4.1c, d). These responses to radiant heat are similar to previous observations in untreated animals (Ramer et al., 2004; Soril et al., 2008). These results therefore demonstrate that exogenous BDNF treatment did not attenuate C7/8 DRI cold pain, but rather prolonged cold hypersensitivity throughout the entire post-operative period.

Plasticity of peptidergic nociceptive primary afferents did not underlie BDNF-mediated cold pain.

Sprouting of peptidergic nociceptive fibers containing calcitonin gene-related peptide (CGRP) has been suggested to contribute to the observed pain behaviours in several models of neuropathic pain (Ma and Bisby, 1998; Ma et al., 1999; Ondarza et al., 2003; Hoschouer et al., 2009). In our previous work, animals that displayed sustained cold pain responses beyond 20d-post DRI also exhibited intraspinal plasticity of CGRP-positive terminals after C7/8 DRI (Soril et al., 2008). This prompted us to examine whether the prolonged cold pain behaviour of BDNF-treated animals here was accompanied by sprouting of peptidergic nociceptors. By 20d post-C7/8 DRI there was reduced CGRP-positive fibre density in the medial superficial laminae of vehicle-treated animals (Fig. 4.2b), as previously described (Soril et al., 2008). In BDNF-treated animals (Fig. 4.2c), there was no change in the density of CGRP-positive fibers in the dorsal horn compared to vehicle-treated animals (Fig. 4.2a). Therefore, sprouting of CGRP-positive nociceptive afferents is unlikely to contribute to persistent cold pain in BDNF-treated animals.
Exogenous BDNF treatment differentially regulates the plasticity of descending monoaminergic inputs to the deafferented dorsal horn.

Descending modulation of sensory information is integral to both facilitation and inhibition of nociceptive processing (Millan, 2002), and changes to the extent of this modulation accompany injury. Serotonergic sprouting, for example, readily occurs following spinal deafferentation (Polistina et al., 1990; Wang et al., 1991; Zhang et al., 1993; Kinkead et al., 1998; MacDermid et al., 2004; Ramer et al., 2004; Scott et al., 2005; Ramer et al., 2007). Given this plasticity is stimulated by endogenous BDNF (Ramer et al., 2007), we sought to determine whether exogenous BDNF treatment would enhance DRI-induced serotonergic sprouting. The density of serotonin transporter (SERT)–positive fibres did not differ between vehicle- and BDNF-treatment groups (Fig. 4.3). Thus, although rhizotomy-induced serotonergic sprouting is BDNF-dependent, it is not enhanced with additional exogenous BDNF.

The density of dopaminergic and adrenergic/noradrenergic projections in the spinal cord, on the other hand, is only marginally affected by DRI (MacDermid et al., 2004; Ramer et al., 2004; Scott et al., 2005). We found that the density of tyrosine hydroxylase (TH)–positive axons increased only in response to more extensive dorsal rhizotomies (Ramer et al., 2004). Both axonal populations also express TrkB (King et al., 1999), and intraspinal BDNF injections given to rats after spinal nerve ligation was shown to stimulate sprouting of spinal noradrenergic profiles (Hayashida et al., 2008). These suggest that the greater the deafferentation and/or availability of trophic support, the greater the plastic effects. Twenty days following C7/8 DRI, BDNF-treated animals displayed a significant increase in TH-positive terminal density in superficial laminae
(Fig. 4.4c) compared to vehicle-treated controls (Fig. 4.4a, b). To discern whether either noradrenergic or dopaminergic axons contributed to the increase in TH-positive axon density, we also quantified the density of dopamine-β-hydroxylase (DβH)-positive fibers, which are exclusively noradrenergic. DβH expression was significantly elevated in BDNF-treated rats relative to vehicle controls (Fig. 4.4d-f), but the increase was not sufficient to account for the overall increase in TH-positive axon density. We can therefore conclude that both dopaminergic and noradrenergic fibre populations sprout in response to BDNF treatment in rhizotomized animals.

**BDNF treatment enhanced C7/8 DRI-induced plasticity of neuropeptide Y-positive terminals**

Local pain modulation is also dependent on fast chloride-dependent inhibition by GABAergic interneurons in the dorsal horn (Enna and McCarson, 2006). Antibodies directed towards neuropeptide Y (NPY) immunolabel a subset of spinal GABAergic interneurons (Rowan et al., 1993; Naim et al., 1997; Polgar et al., 1999) and the density of NPY-positive profiles is increased following C7/8 DRI, an effect that also appears to be BDNF-dependent (Soril et al., 2008). Twenty days post-DRI, vehicle-treated animals exhibited NPY-positive fibre density similar to previous observations (Soril et al., 2008), predominantly in laminae Iii and Ilo (Fig. 4.5b). With BDNF treatment, NPY-immunoreactivity was further increased throughout the superficial dorsal horn (Fig. 4.5c), by approximately twice that exhibited by vehicle-treated animals (Fig. 4.5a). These results demonstrated that unlike serotonergic sprouting, exogenous BDNF enhanced the DRI-induced increase in NPY-positive GABAergic process density.
Characterization of contacts between NPY-positive terminals and noradrenergic and/or dopaminergic projections in the dorsal horn

Dorsal horn interneurons also serve as sites for integrating descending monoaminergic input (Millan, 2002). Considering that exogenous BDNF increased the density of both TH- and NPY-positive profiles (Figs. 4.4 & 4.5), we wanted to determine whether and how these populations make contact in the dorsal horn. Confocal image stacks revealed that in the uninjured dorsal horn, TH-positive terminals were frequently found apposing NPY-positive cell bodies and neurites (axons and dendrites) (Fig. 4.6a-c). These two contact types were referred to as axo-somatic (Fig. 4.6b) and axo-neuritic (Fig. 4.6c), respectively. To determine whether C7/8 DRI and/or exogenous BDNF-treatment altered these contacts, the number of contact types in the C7 dorsal horn were compared amongst intact, vehicle- and BDNF-treated animals. The number of axo-neuritic contacts was equivalent between all groups (Fig. 4.6f). Twenty days after C7/8 DRI there was a significant increase in the total number of axo-somatic contacts in BDNF-treated animals compared to the vehicle-treated controls (Fig. 4.6g). The increase in the number of DβH-positive axons forming axo-neuritic contacts with NPY-positive processes (Fig. 6h) also nearly reached statistical significance ($p = 0.057$) (Fig. 4.6i). These results provide evidence that descending noradrenergic and dopaminergic inputs are in contact with NPY-positive interneuronal processes, and that BDNF-treatment increased the extent to which they form axo-somatic contacts.
Neuronal K⁺/Cl⁻ co-transporter 2 (KCC2) expression decreased following dorsal rhizotomy and BDNF-treatment

The lack of cold pain resolution in BDNF-treated animals was unexpected, given that the densities of descending (Fig. 4.4) and intrinsic (Fig. 4.5) inhibitory components of nociceptive circuitry were increased by the treatment. Therefore, we asked if exogenous BDNF was somehow converting normal inhibitory input into excitation to sustain cold pain. Recent implications for BDNF contributing to neuropathic pain have centered on decreases in neuronal K/Cl⁻ co-transporter 2 (KCC2) following both central and peripheral nerve injury and in peripheral neuropathies (Coull et al., 2005; Miletic and Miletic, 2008; Zhang et al., 2008; Jolivalt et al., 2008; Cramer et al., 2008). Fast GABAergic synaptic transmission is dependent on low intracellular chloride ([Cl⁻]ᵢ) of projection neurons maintained by KCC2 (Lu et al., 1999; Rivera et al., 1999; Hubner et al., 2001; Li et al., 2002; Payne et al., 2003). Reductions in KCC2 expression can accordingly convert chloride-dependent GABAergic inhibition into excitation.

To determine whether the same could occur following DRI, densitometric measurements of KCC2 immunoreactivity were made in the dorsal horn (Fig. 4.7). Prior to deafferentation, KCC2 expression was abundant throughout the dorsal grey matter (Fig. 4.7b), particularly in laminae III-IV (Fig. 4.7a, b). There was a marked reduction in KCC2 expression levels 20d-post DRI (Fig. 4.7c), although the difference was only significant in deeper laminae relative to intact controls (Fig. 4.7a). KCC2 is not expressed in DRG neurons (Price et al., 2006), in which [Cl⁻]ᵢ is high, therefore the decrease in the dorsal horn cannot be directly attributed to loss of primary afferent axons. The decrease in KCC2 was further pronounced with BDNF treatment and statistically
significant compared to the uninjured animals throughout all examined laminae (Fig. 4.7a, d). These findings indicate that the BDNF-mediated decrease in KCC2 levels may alter the intracellular chloride concentration of dorsal horn neurons and subsequently disrupt the normal inhibitory tone after C7/8 DRI.

4.4 Discussion

Our previous pharmacological interventions following C7/8 DRI indicated that increased endogenous BDNF levels in the spinal cord were necessary for the resolution of cold pain (Soril et al., 2008). The present study, however, demonstrated that the recovery-promoting plasticity that is stimulated by deafferentation alone (Soril et al., 2008) can be masked by events mediated by pharmacological doses of exogenous BDNF. Figure 4.8 summarizes our proposed model of these events. After C7/8 DRI exogenous BDNF treatment promotes the plasticity of descending adrenergic/noradrenergic and dopaminergic projections and those of intrinsic interneuronal processes. TH- (and DβH-) positive terminals are in contact with NPY-positive neurons in the dorsal horn, and increase in the extent to which they form axo-somatic contacts following BDNF treatment. At the same time, BDNF suppresses KCC2 protein expression in nociceptive projection neurons, raising resting \([Cl^-]_i\). Therefore, when GABA is released from inhibitory interneurons in superficial laminae and binds GABA\(_A\) receptors (GABA\(_A\)Rs) on projection neurons, neuronal Cl\(^-\) undergoes efflux rather than influx, ultimately depolarizing the neurons and resulting in propagation of nociceptive cold pain signals (Fig. 4.8).
**BDNF and the plasticity of monoaminergic axons**

Hierarchical plasticity of monoaminergic axons is a consequence of spinal deafferentation (Polistina et al., 1990; Wang et al., 1991; McDermid et al., 2004; Ramer et al., 2004). Previous work from our group has suggested that such differential plasticity is due to the availability of neurotrophins, particularly of BDNF, upregulated by rhizotomy (Johnson et al., 2000; Ramer et al., 2004; Ramer et al., 2007). We have found, for example, that more extensive rhizotomies (and, presumably greater BDNF upregulation) led to more extensive sprouting of both serotonergic and TH-expressing axonal populations (Ramer et al., 2004). Here we find that TH-positive, but not SERT-positive axon sprouting increases with exogenous BDNF. It is not the case that serotonergic sprouting is maximally-effected by endogenous BDNF after C7/8 DRI, since it increases with the number of roots involved. These results rather suggest that while endogenous BDNF is necessary, it is insufficient on its own to elicit sprouting of serotonergic axons. In contrast, BDNF is clearly sufficient for TH-positive axon sprouting, but endogenous BDNF is not present in effective concentrations following more restricted DRI.

It could be argued that in the case of C7/8 DRI, the lack of TH-positive terminal sprouting in the vehicle-treated group may be due to a competitive advantage of serotonergic fibres in binding available endogenous BDNF (Polistina et al., 1990). However, ablation of serotonergic axons with the toxin 5,7-dihydroxytriptamine (5,7-DHT) does not enhance TH- or DβH-positive axon sprouting after C7/8 DRI (M.S.R., unpublished observations). This suggests that even in the absence of competition, upregulation of endogenous BDNF remains insufficient to stimulate their plasticity.
Differential influences of myelin-derived growth inhibitors may also underlie the reticence of TH-positive axons to sprout. In rhizotomized animals, removing these influences via Rho kinase inhibition (Ramer et al., 2004), soluble Nogo fusion proteins (MacDermid et al., 2004), or in dysmyelinated mutant rats (Scott et al., 2005), has a proportionally larger effect on TH-positive axon sprouting than on serotonergic sprouting. It is thus the balance between growth-promoting and growth-suppressing signals which governs axon growth in response to injury.

Integration of inhibitory circuitry at the level of the dorsal horn

That the plasticity of descending (TH-positive axons) and intrinsic (NPY-positive terminals) components of nociceptive circuitry occurred concomitantly upon exogenous BDNF treatment, and that exogenous BDNF prevents behavioural recovery, makes it tempting to invoke a causal relationship. In terms of descending inhibition, adrenergic/noradrenergic and dopaminergic axons terminate directly onto nociceptive projection neurons and signal via α2A and D2 receptors, respectively (Millan, 2002). Additionally, these projections also directly activate resident inhibitory GABAergic interneurons through the adrenergic receptor α1A and the dopaminergic receptor D1 eliciting inhibitory primary afferent depolarization (PAD) or postsynaptic inhibition of projection neurons (Millan, 2002; Orii et al., 2002). While we cannot discount the advent of both mechanisms in our model of DRI, the latter may be favored in the BDNF treatment group due to the observed increase in axo-somatic contacts between TH- and NPY-positive terminals (Fig 4.6). NPY-containing axons of spinal GABAergic interneurons have also been shown to project onto neurokinin1- (NK1) immunoreactive
projection neurons in laminae I-IV (Polgar et al., 1999). We would therefore expect that the increase in density and connectivity of noradrenergic, dopaminergic and GABAergic inhibitory components in BDNF-treated animals would result in an overall increase in the inhibitory or antinociceptive influence on cold pain processing. However, the paradoxical sustained cold pain raises the possibility that exogenous BDNF may also be converting this inhibition to excitation or pronociceptive activity.

**BDNF-mediated changes in spinal KCC2 levels**

Developmental changes in neuronal \([\text{Cl}^-]_i\) are governed by the complimentary expression of both cation chloride co-transporters \(\text{Na}^+/\text{Cl}^-\) co-transporter 1 (NKCC1) and KCC2 (Payne et al., 1996; Gillen et al., 1996; Plotkin et al., 1997; Rivera et al., 1999; Lauf and Adragna, 2000; Li et al., 2002; Payne et al., 2003). Expression of the former is predominant throughout early postnatal development, and by harnessing the \(\text{Na}^+\) electrochemical gradient, NKCC1 facilitates a high \([\text{Cl}^-]_i\) during this time (Plotnick et al., 1997; Lu et al., 1999; Li et al., 2002). KCC2 expression, on the other hand, increases with maturation (Lu et al., 1999; Kanaka et al., 2001; Li et al., 2002) and couples \(\text{K}^+\) moving down its electrochemical gradient with \(\text{Cl}^-\) exit from the cell, subsequently converting the \([\text{Cl}^-]_i\) from high to low. Therefore GABA-mediated hyperpolarization of adult neurons, via the ionotropic \(\text{GABA}_A\)R, is converted to depolarization in the absence of KCC2 (Payne et al., 1996, 1997; Rivera et al., 1999).

Trauma-evoked decreases in the levels of KCC2 mRNA and protein have also been reported in several experimental models of epilepsy and neuropathic pain (Rivera et al., 2002; Jin et al., 2005; Coull et al., 2005; Wake et al., 2007; Miletic and Miletic, 2008;
Cramer et al., 2008). Under such pathophysiologi-
cal conditions, increases in endogenous BDNF are thought to mediate KCC2 downregulation and convert GABA_{A}R currents from hyperpolarizing to depolarizing (Rivera et al., 2002; Coull et al., 2005). Our current findings suggest that a similar mechanism may underlie sustained cold pain following DRI, since a decrease in KCC2 expression occurred in BDNF-treated animals (Fig. 4.7). Although DRI alone elicited a modest decrease in KCC2 expression (likely due to the endogenous upregulation of BDNF), this decrease was probably insufficient to confer dorsal horn disinhibition in the long run, as vehicle-treated animals recovered from cold pain (Fig. 4.1a).

**Conclusions**

While the upregulation of endogenous BDNF promotes recovery from cold pain following DRI (Soril et al., 2008), exogenous BDNF prevents it (present results). These results emphasize the importance of dose-dependent effects when performing *in vivo* neurotrophin manipulations and highlight the diverse control that BDNF has on spinal nociceptive circuitry. We also reveal a novel mechanism of BDNF-mediated disinhibition of nociceptive cold pain transmission after cervical dorsal rhizotomy involving sprouting of descending monoaminergic and intrinsic interneurons, combined with downregulation of the KCC2 K^+ /Cl^− co-transporter. Future therapeutic interventions for spinal deafferentation pain must be considerate of the innate recovery-promoting plasticity of the spinal cord alone, as the benefit of endogenous reparative mechanisms can be obscured by exogenous neurotrophin manipulations.
**Figure 4.1** Exogenous BDNF prevented the normal resolution of C7/8 DRI-induced cold pain. Animals received either i.t. RSA (vehicle-treated) (a), or exogenous BDNF (b) following C7/8 DRI. Acetone stimulation of the ipsilateral forepaw elicited a transient increase in nocifensive response duration (s), which resolved after 20d post-DRI (a). BDNF-treatment prevented this resolution, prolonging cold pain throughout the behavioural testing period (b). (c, d) Thermal (heat) hypersensitivity did not appear to develop, and there were animals in both vehicle- and BDNF-treated groups that demonstrated increased thermal withdrawal latencies of the ipsilateral forepaw. Asterisks indicate significant differences between ipsilateral (ipsi, black) and contralateral (contra; white) responses; n = 8 for each treatment group.
Figure 4.2 Plasticity of peptidergic nociceptive primary afferents did not underlie cold pain in BDNF-treated animals. Proportional density of calcitonin gene-related peptide (CGRP)-positive primary afferent terminals was measured across laminae II-IV of the C7 dorsal horn, quantification shown in (a). (b) In vehicle-treated animals, CGRP-positive terminals are depleted from the medial superficial dorsal horn after C7/8 DRI. Exogenous BDNF treatment did not significantly alter the peptidergic nociceptor density (a, c). n = 5 for each treatment group. Scale bar = 100 µm.
Figure 4.3 Exogenous BDNF had no effect on DRI-induced plasticity of bulbospinal serotonergic terminals. (a) The density of trkB-expressing bulbospinal serotonergic axons in the C7 dorsal horn was measured by serotonin transporter (SERT)-immunoreactivity. Similar to previous observations, SERT-positive terminal density was most prominent in the superficial lamina IIo and deeper laminae III-IV in vehicle-treated animals after C7/8 DRI (b). This post-operative pattern of SERT-immunoreactivity was not affected by exogenous i.t. BDNF treatment (a, c). n = 5 for each treatment group. Scale bar = 100 µm.
Figure 4.4 Descending dopaminergic and noradrenergic projections sprout in response to exogenous BDNF treatment. Tyrosine hydroxylase (TH) labels both bulbospinal dopaminergic and noradrenergic projections, whereas dopamine-β-hydroxylase (DβH) selectivity labels noradrenergic (and adrenergic) projections to the dorsal horn. The density of (a) TH- and (d) DβH-immunoreactivity was measured in laminae II-IV of the C7 dorsal horn. TH-positive axon plasticity is not normally stimulated by C7/8 DRI alone (b), however with exogenous BDNF treatment, the density of TH-positive axons in lamina II was significantly increased relative to vehicle-treated controls (a, c). (e) DβH-immunoreactivity in the C7 dorsal horn of a vehicle-treated animal. (b, f) Exogenous BDNF significantly increased the density of DβH-positive terminals, compared to vehicle-treatment, in all examined laminae. In a, asterisks indicate significant differences between BDNF-treated (n = 5) and vehicle-treated controls (n = 5). Scale bar = 100 μm.
Figure 4.5 BDNF treatment enhanced C7/8 DRI-induced plasticity of neuropeptide Y-positive terminals. (a) Densitometric analysis of neuropeptide Y (NPY)-expression in the C7 dorsal horn. (b) Vehicle-treated animals exhibited an injury-induced increase in NPY-positive terminal density in the deafferented dorsal horn. Exogenous BDNF treatment potentiated this increase in NPY-expression almost two-fold in the superficial dorsal horn (a, c). In a, asterisks indicate significant differences between BDNF-treated (n = 5) and vehicle-treated controls (n = 5). Scale bar = 100 µm.
**Figure 4.6** Noradrenergic and dopaminergic axons contact NPY-positive processes in the deafferented dorsal horn. Spinal cord sections were double-labeled with antibodies directed against TH and NPY. (a) In naïve animals, there were several TH-positive axonal puncta (red) in contact with NPY-positive dendrites and axons (green) (axo-neuritic contacts; arrow heads) in the C7 dorsal horn. There were also some TH-positive axons in contact with NPY-positive cell bodies (axo-somatic contacts; arrows). (b, c) Higher power images of the contacts identified in a. Representative confocal slices demonstrating axo-neuritic (d), and axo-somatic (e) contacts in vehicle- and BDNF-treated animals, respectively. (f, g) From TH and NPY double-labeled sections, there was a significant increase in the number of axo-somatic contacts in the BDNF-treated group, compared the vehicle-treated controls. There was, however, no difference in the number of axo-neuritic contacts between all groups. There were also similar number of axo-neuritic contacts in sections labelled with antibodies against DβH and NPY (h). (i) The number of axo-somatic contacts with BDNF treatment appeared to be increased relative to the vehicle-treated group, but was in fact not significantly different ($p = 0.057$). In g, asterisks indicate significant differences between BDNF-treated ($n = 6$) and vehicle-treated controls ($n = 6$). Scale bars: in a, d, e = 10µm; in b = 5 µm; in c = 5um.
**Figure 4.7** Neuronal $K^+/{Cl}^-$ co-transporter 2 expression decreased following dorsal rhizotomy and BDNF-treatment. (a) Quantification of density measurements for $K^+/{Cl}$ cotransporter 2 (KCC2) expression in the deafferented C7 dorsal horn. KCC2 expression was present throughout the uninjured dorsal horn, with prominent reactivity in lamina I and deeper laminae (b). (c) Vehicle-treated animals demonstrated a significant reduction in KCC2 expression in deeper laminae (a, c). Exogenous BDNF-treatment, however, significantly decreased the density of KCC2-immunoreactivity throughout the dorsal horn (a, d). In a, crosses indicate significant differences between uninjured controls (n = 5) and rhizotomized animals (n = 5 in each treatment group). Scale bar = 100 µm.
Figure 4.8 Exogenous BDNF-mediated changes to spinal circuitry following C7/8 DRI. DRI leads to increases in the density of NPY-expressing GABAergic neurite density, an effect which is enhanced by exogenous BDNF. BDNF also promotes sprouting of TH and DβH-expressing descending axons and formation of axo-somatic contacts with GABAergic interneurons. It also decreases the expression of KCC2 in second-order nociceptive projection neurons, and the resulting increase in [Cl\(^{-}\)], renders GABAergic transmission excitatory. Thus, cold pain fails to resolve in BDNF-treated DRI animals.
4.5 Bibliography


CHAPTER 5

Discussion: the good, the bad and the ugly
5.1 Discussion

The results presented in this thesis have illustrated an intriguing “yin and yang” perspective for BDNF-TrkB signaling following spinal deafferentation. Remarkably, BDNF was found to exert both a pro- (Chapters 2 and 4) and an anti-nociceptive role in C7/8 DRI cold pain (Chapter 3). Overall it was my intent to determine whether mechanisms underlying intraspinal plasticity were contributing to the spontaneous sensory changes that occur following DRI. I found that the use of the C7/8 DRI model enabled a narrowed investigation of BDNF and its putative roles in 1) anatomical plasticity in the deafferented dorsal horn, as well as 2) cold pain behaviour.

As hypothesized from previous work (Johnson et al., 2000; Ramer et al., 2004), all three studies supported a role for BDNF in spinal neuroplasticity following deafferentation (Table 5.1). In Chapter 2, while both BDNF and NT-3 were found to increase in the ipsilateral dorsal horn, sequestration of BDNF with TrkB-Fc treatment specifically prevented the DRI-induced serotonergic sprouting. Because of this, TrkB-Fc treatment may have also indirectly promoted the plasticity of spared mechanosensory primary afferents. The experiments from Chapters 3 were a direct follow-up of those in Chapter 2, and they ultimately demonstrated a positive influence of endogenous BDNF on the plasticity of inhibitory GABAergic processes. As well, analogous to their large caliber counterparts, the plasticity of nociceptive primary afferents was found to be indirectly suppressed by the increased density of intrinsic inhibitory circuitry. Finally in Chapter 4, the gain of exogenous BDNF was found to potentiate the DRI-induced plasticity of the GABAergic processes, while coaxing descending dopaminergic and adrenergic/noradrenergic axon sprouting. However exogenous BDNF was also
demonstrated to, for the first time, decrease the expression of the Cl⁻ co-transporter KCC2 following partial spinal deafferentation.

Germane to BDNF and cold pain, these studies also demonstrated that 1) early endogenous increases of BDNF (<10 days post-DRI) are likely to contribute to cold pain via synaptic modulation in the dorsal horn (Fig. 5.1a; Chapter 2); whereas 2) over time (by 20 days post-DRI) endogenous BDNF was able to sufficiently stimulate the plasticity of relevant inhibitory circuitry (serotonergic and GABAergic terminals) promoting cold pain recovery (Fig. 5.1b; Chapter 3); yet 3) if large exogenous doses of BDNF are applied, the plasticity of inhibitory circuitry is not only enhanced (GABAergic and TH-positive terminals), but consequently masked by altered KCC2 levels and possible post-synaptic disinhibition manifesting into sustained cold pain (Fig. 4.8; Chapter 4).

The discrepancies between the behavioural outcomes from Chapters 3 and 4 were attributed to the differences in plasticity of spared axonal populations and KCC2 expression. Although these findings were somewhat surprising, they were likely a result of assuming that BDNF would exert the same generalized influence regardless of its source, dose and/or temporal presence. It is has since been evident that these factors were major determinates by which BDNF promoted differential anatomical and functional changes following C7/8 DRI. Also despite the convincing evidence for the neuromodulatory role of endogenous BDNF, there may be other early contributions from BDNF-independent mechanisms since single TrkB-Fc boli injections only partially attenuated peak cold pain at 10 days (Chapter 2). This suggestion is not foreign to other pain states; in fact, chronic pain from neuropathic and inflammatory insults has been
attributed to mechanisms such as protein tyrosine kinase Src phosphorylation of NMDA receptors, as well as BDNF-dependent GABAergic depolarization (Liu et al., 2008).

Despite the fact that many of these BDNF-induced responses are novel outcomes of DRI, they have been exemplified in other forms of neurological damage (Blesch and Tuszynski, 2002; Navarro et al., 2007; Fiumelli and Woodin, 2007).

5.2. The good: BDNF and recovery-promoting plasticity

Although considered fairly traumatic, similar to DRI, most spinal cord injuries (SCI) in patients are neurologically incomplete (Bunge et al., 1993; Bunge et al., 1997; Guest et al., 2005). In view of this, many therapeutic interventions used in experimental models of SCI have also focused on harnessing the plastic potential of spared neurons to promote functional recovery (Raineteau and Schwab, 2001; Ding et al., 2005; Maier and Schwab, 2006). One noteworthy strategy has involved the delivery of neurotrophic factors, such as BDNF, to stimulate the plasticity of intact spinal systems (Bregman et al., 1997; Mendell et al., 2001; Blesch and Tuszynski, 2002; Lu and Tuszynski, 2008).

5.2.1 BDNF contributes to neuroplasticity after SCI

BDNF responsiveness of injured neurons, as well as the potential for BDNF-mediated plasticity, has been exemplified from studies initially examining the effects of direct cell body treatment to injured spinally-projecting neurons (Giehl and Tetzlaff, 1996; Kobayashi et al., 1997; Kwon et al., 2002). Somata of motor axons descending to the spinal cord, such as those of the corticospinal tract (CST) and rubrospinal tract (RST), have been found to express both TrkB and TrkC receptors (Giehl and Tetzlaff, 1996;
Kobayashi et al., 1997). One week after axotomy, injury-induced atrophy of corticospinal neurons was prevented when BDNF was continuously delivered near the vicinity of their cell bodies (Giehl and Tetzlaff, 1996). Furthermore, BDNF delivery to the red nucleus was also demonstrated to counter the atrophy of axotomized rubrospinal neurons (RSN), as well as restore their expression of the regeneration-associated genes (RAG) GAP-43 and Tα1-tubulin (Kobayashi et al., 1997). Decreased RAG expression after injury (Skene, 1989; Tetzlaff et al., 1991, 1994) may hinder the propensity for axonal growth, since restored RAG expression with BDNF treatment promoted RST growth into spinally-implanted peripheral nerve graphs (Kobayashi et al., 1997; Kwon et al., 2002).

Subsequent variations of proximal (cell body) BDNF treatment have also demonstrated distal sprouting of CST axons after SCI (Zhou and Shine, 2003; Vavrek et al., 2006). For instance, adenoviral vectors (Adv) carrying the BDNF gene were delivered in the vicinity of the sensorimotor cortex in combination with Adv coding for NT-3 in the lumbar spinal cord (Zhou and Shine, 2003). Following CST lesion at the pyramidal level, this mode of proximal BDNF administration enhanced intraspinal CST sprouting (Zhou and Shine, 2003), which was beyond that observed with NT-3 Adv delivery alone (Zhou et al., 2003). Direct infusion of BDNF to the same cortical region after dorsal over-hemisection of the thoracic (T8) spinal cord also resulted in collateral sprouting of CST fibres within the cervical spinal cord, while local i.t. NT-3 administration did not promote similar plasticity (Vavrek et al., 2006). This BDNF-mediated plasticity of the CST may only occur in response to injury, as cell body
treatment with the neurotrophin in uninjured animals did not result in aberrant sprouting (Vavrek et al., 2006).

Endogenous alterations in spinal BDNF levels also support a role for the neurotrophin in injury-specific plasticity after SCI (Gomez-Pinilla, 2001, 2002, 2004). SCI to the thoracic spinal cord decreases endogenous BDNF expression below the level of injury (Gomez-Pinilla, 2004, Ying et al., 2005). Considering that voluntary exercise, in the form of wheel running, was found to elevate spinal BDNF (Gomez-Pinilla, 2001) and TrkB levels (Gomez-Pinilla, 2002) in naïve rats, a similar exercise regimen was proposed to also restore the neurotrophin levels in the injured spinal cord (Gomez-Pinilla, 2004, Ying et al., 2005). Accordingly, voluntary exercise in SCI rats normalized BDNF mRNA levels after 3 days, which continued to increase and exceeded that of intact controls by 1 month-post injury (Ying et al., 2005). More recently, this exercise-stimulated BDNF has been suggested to regulate spinal synaptic plasticity ameliorating locomotor recovery after SCI, most likely via the restoration of proteins necessary for synaptic activity (Ying et al., 2008).

5.2.2 BDNF contributes to neuroplasticity after PNI

By stimulating the plasticity of inhibitory circuitry at both functional and anatomical levels, BDNF has also been found to promote the recovery from PNI-induced pain (Pezet et al., 2002; Lever et al., 2003; Bardoni et al., 2007; Hayashida et al., 2008). As we have found endogenous and exogenous BDNF to alter the density of NPY-positive GABAergic processes in vivo (Chapters 2 and 3), topical application of BDNF to in vitro spinal cord preparations has been demonstrated to inhibit nociceptor release of substance
P, while concurrently evoking interneuron GABA release into the superficial dorsal horn (Pezet et al., 2002; Lever et al., 2003; Bardoni et al., 2007). Furthermore, these alterations to the intrinsic inhibitory system were postulated to be of significance to synaptic transmission in vivo, since an i.t. BDNF injection was found to act anti-nociceptively decreasing the thermal hyperalgesia after PNI (Lever et al., 2003).

On another note, many analgesics have also been developed as pharmacological mimetics of common inhibitory neurotransmitters (e.g. GABA, serotonin and noradrenaline) (Tanabe et al., 2005; Gwak et al., 2006). The mechanisms by which they promote pain relief have, however, extended beyond that of post-synaptic neurotransmitter receptor activation (Hayashida et al., 2007, 2008). The efficacy of the analgesic clonidine, for example, has been suggested to be dependent on intraspinal noradrenergic sprouting (Hayashida et al., 2008). Analogous to our findings following C7/8 DRI (Chapter 4), the plasticity of these inhibitory descending projections was found to be under the control of local increases in BDNF after spinal nerve ligation (SNL). Specifically, L5/6 SNL elicited a concomitant increase in primary afferent-derived BDNF and DβH-immunoreactivity in the adjacent dorsal horn (Hayashida et al., 2008). Such noradrenergic sprouting was prevented upon BDNF antagonism, via i.t. anti-BDNF delivery, suggesting a necessary role for the neurotrophin in recovery-promoting noradrenergic axon plasticity (Hayashida et al., 2008).

5.3 The bad: BDNF and pathological synaptic plasticity

Perhaps one of the most striking findings from the preceding data chapters is the possible conversion of normal inhibitory input to that of post-synaptic excitation and
facilitation of cold pain (Chapter 4). This dramatic switch in synaptic modality was initially characterized from aberrant GABAergic depolarization following various models of neuronal insult (van den Pol et al., 1996; Nabekura et al., 2002). Specifically after heating, replating, osmotic imbalance or transection injury to neurons in vitro, GABA release was found to promote their depolarization via the efflux of intracellular Cl\(^-\) (van den Pol et al., 1996). While GABAergic inhibition of mature CNS neurons is conventionally dependent on Cl\(^-\) influx through the ionotropic GABA\(_A\)R, this channel actually works to shift the membrane potential towards the Cl\(^-\) reversal potential (E\(_{Cl}^\text{R} \)) by facilitating either the outward or inward flux of the ion (Kaila, 1994). Considering that chloride-permeable membrane transporters and/or pumps largely determine the electrochemical gradient of Cl\(^-\) (Fiumelli and Woodin, 2007), it was hypothesized that mechanisms impairing the expression and/or function of these proteins may underlie GABAergic excitation (van den Pol et al., 1996).

### 5.3.1 BDNF regulates the plasticity of GABAergic transmission by modulating KCC2 expression in traumatized adult neurons

As the expression of the dominant Cl\(^-\) co-transporter evolves with CNS maturation, from that of the NKCC to the KCC co-transporter (Lu et al., 1999; Rivera et al., 1999; Yamada et al., 2004), the latter subtype has become of increasing interest to the pathophysiological changes in \(\left[\text{Cl}^\text{-}\right]_i\) of adult neurons (Rivera et al., 2002, 2004; Wardle et al., 2003; Woodin et al., 2003; Fiumelli et al., 2005; Coull et al., 2005). Of the four mammalian KCC isoforms (KCC1-4), KCC2 is the most predominantly expressed in postnatal and mature CNS neurons (Payne et al., 1996; Payne et al., 1997; Rivera et al.,
1999; Gulyas et al., 2001; Kanaka et al., 2001) and, as mentioned, is necessary for maintaining their nominal resting $[\text{Cl}^-]$. (Rivera et al., 1999; DeFazio et al., 2000; Kakazu et al., 2000). Therefore, neuronal $\text{Cl}^-$ extrusion via KCC2 has been tightly correlated with setting the reversal potential for chloride-dependent GABAergic responses ($E_{\text{GABA}}$) (Payne et al., 1996, 1997; Rivera et al., 1999).

A presumptive role for KCC2 in post-synaptic GABA-mediated inhibition was initially inferred from in vitro LOF experiments, wherein KCC2 mRNA knock-down in hippocampal slices was found to convert normal hyperpolarizing GABA$_A$R currents to depolarizing (Rivera et al., 1999). Additionally in vivo KCC2 disruption, not only resulted in a lack of post-synaptic inhibition leading to decreased muscle tone and motor ability (Hubner et al., 2001), but also the generation of seizure-like behaviour in KCC2-deficient mice (Vilen et al., 2001). Interestingly, it has been established that seizure-like or epileptiform activity in humans may be generated from the pathological depolarization of CA1 hippocampal neurons upon interneuronal release of GABA (Cohen et al., 2002). Reminiscent of the findings with injured neurons (van den Pol, et al., 1996; Nabekura et al., 2002), this spontaneous activity from depolarizing GABAergic currents may be regulated by pathological factor(s) that converge onto the modulation of KCC2 (Aguado et al., 2003).

BDNF expression in the adult hippocampus has also been described concomitant to seizure-like activity (Gall, 1993). Unlike its putative role in promoting pre-synaptic GABA release in the spinal cord (Pezet et al., 2002; Lever et al., 2003; Bardoni et al., 2007), BDNF was found to contribute to synaptic plasticity in the hippocampus by potentiating excitatory synaptic input (Kang and Schuman, 1995) and suppressing post-
synaptic GABA<sub>A</sub>R-mediated hyperpolarization (Tanaka et al., 1997; Frerking et al., 1998; Brunig et al., 2001). Subsequent to this, studies concerned specifically with the rat hippocampus demonstrated that KCC2 mRNA and protein levels, as well as neuronal Cl<sup>-</sup> extrusion capacity, were all negatively regulated by either exogenous BDNF treatment to organotypic cultures (Rivera et al., 2002), or endogenous BDNF upregulation following in vivo seizure paradigms (Rivera et al., 2002, 2004). Thus BDNF may be provoking the hyperexcitability and onset of seizure-like behaviour primarily via the conversion of GABA<sub>A</sub>R currents (Rivera et al., 2004). With the ubiquitous neuronal expression of KCC2, this coupled regulation of Cl<sup>-</sup> co-transport and GABA excitation is also likely to underlie the BDNF-mediated noxious transmission in the dorsal horn following PNI (Coull et al., 2005; Miletic and Miletic, 2008) and in the present work with exogenous BDNF after C7/8 DRI (Chapter 4).

5.4 The ugly: concluding remarks

Quite simply, the role of neurotrophins in the adult CNS is complicated. While most neurotrophins, including BDNF, have been regarded as positive regulators of neuronal phenotype and function, novel roles often drastically and complicatedly develop following traumatic insult. Particularly with regards to experimental models of neuropathic pain, the field of pain research has yet to reach an accordant understanding as to whether BDNF is beneficial or detrimental to various pain-related states. This dissonance is largely attributable to the site and type of injury, and the source and dose of BDNF under investigation. Therefore prior to any therapeutic intervention it is necessary to determine the specific cellular and molecular mechanisms that underscore individual
pathologies. Still, as the preceding manuscript chapters have illustrated, this feat is also not likely to be trivial. The work in this thesis did demonstrate a pivotal role for endogenous BDNF in mechanisms underlying the spontaneous development of, as well as recovery from DRI cold pain. These findings may not have elucidated a direct approach to alleviate pain from peripheral cold stimulation in patients with DRI (Bruxelle et al., 1988; Bonica, 1990), however, through future dose-response studies determining an optimal concentration/dose of exogenous BDNF to accelerate inhibitory circuitry plasticity, without significantly altering KCC2 levels, may serve as a long-term analgesic treatment for mitigating DRI-induced cold pain.
Table 5.1 Summary of histological data

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<th>Treatment</th>
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<th>SERT</th>
<th>CGRP</th>
<th>GAP-43</th>
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1 Changes (increase: + or decrease: -) represent average immunoreactivity 20d post-C7/8 DRI relative to uninjured controls.
2 Similar changes to CTB immunoreactivity.
3 Similar changes to IB4 immunoreactivity.
4 Similar changes to DβH immunoreactivity.
5 Not determined (n/d).
**Figure 5.1** A dual role for endogenous BDNF in DRI-induced cold pain.

(a) Microglial-derived BDNF is pro-nociceptive in the acute period after C7/8 DRI (<10d), likely exerting a fast (synaptic) effect within the dorsal horn contributing to the development of cold pain. Such an effect may be attributed to increased activation of NMDA receptors on nociceptive projection neurons, resulting in facilitation of noxious cold neurotransmission. This early neuromodulatory role for BDNF was evident from the partial attenuation of peak cold pain responses following a single i.t. bolus injection of TrkB-Fc. (b) Over time, sustained increases of endogenous BDNF in the deafferented dorsal horn may have slowly promoted sprouting and/or phenotypic changes to relevant inhibitory circuitry (serotonergic and GABAergic systems). The BDNF-mediated plasticity was apparent after 10d and may have, therefore, contributed to a delayed inhibition of noxious cold neurotransmission and spontaneous resolution of cold pain by 20d post-DRI. This slow anti-nociceptive role for endogenous BDNF was supported by the lack of cold pain recovery in animals that received continuous TrkB-Fc-treatment.
a Acute setting:

- PNS
- CNS
- DRG (spared)
- DRG (rhizot.)
- Microglia
- BDNF (endogenous)
- NMDA-R (?)
- SERT
- GABA
- NPY

b Chronic setting:

- PNS
- CNS
- DRG (spared)
- DRG (rhizot.)
- Microglia
- BDNF (endogenous)
- SERT
- GABA
- NPY
5.5 Bibliography


