The rat spinal cord following traumatic injury: An anatomical and behavioural study examining NADPH-d and fos

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This thesis is submitted in fulfilment
of the degree of
Doctor of Philosophy

Declaration

The scientific work contained within this thesis is entirely my own except where due reference is made within the text. The interpretations of the data presented here, both original and those of other workers, are also my own, as are any errors made. All animal experiments were approved by the Institutional Animal Ethics Committee of Royal North Shore Hospital, and animals were cared for according to the guidelines of the NHMRC, "Australian code of practice for the care and use of animals for scientific purposes".

http://www.nhmrc.gov.au/research/awc/code/cop.pdf

The work contained within this thesis has been presented in part as indicated below:

Allbutt HN, Siddall PJ & Keay KA. Projections to the Ventroposterior Nucleus of the Thalamus from distinct columns of the midbrain periaqueductal gray of the rat. *Proceedings of the Australian Neuroscience Society*. (1999) 10:178

Allbutt HN, Siddall PJ & Keay KA. Contusive spinal cord injury dramatically alters NADPH-diaphorase expression in
The the spinal cord: correlation with c-fos expression. *Proceedings of the Australian Neuroscience Society*. (2000) 11:107

Allbutt HN, Siddall PJ & Keay KA. Spinal cord injury alters activity in primary and secondary somatosensory cortices in rat. *Restorative Neurology and Neuroscience*. (2000) 16(3-4):231-232

Allbutt HN, Siddall PJ & Keay KA. Changes in laminae processing of non-noxious stimuli following contusion spinal cord injury in the rat. *Proceedings of the Australian Neuroscience Society*. (2004) 15:88

Haydn Nathanial Allbutt
March 2004

Acknowledgements

A PhD thesis, like life, has its ups and downs, and usually takes turns that you don't expect. This one was no exception and much like the bus trip between Sydney and Melbourne, seemed to take forever. However, with the help of a lot of very wonderful people I got through and have created something that due to its extreme thickness will likely collect dust marvellously on the shelves of the Burkitt Ford Library for the next hundred years. More importantly however was the creation of something that didn't exist before. It is this more than anything else that has attracted me to science. Creating knowledge and following in the footsteps of the best thinkers of our species such as Galileo Galilei, Leonardo da Vinci, Andreas Vesalius, Isaac Newton, Joseph Priestly, Robert Hooke, Gregor Mendel, Albert Einstein and Stephen Hawking, is at the same time a very exciting and also a very humbling experience. These scientists and countless like them have discovered and explained to humanity how the universe, the world around us and even our own bodies work, and have allowed the creation of technologies that not so long ago would have appeared as magic.

As a work of science I would like humbly acknowledge the scientists who have come before me, without whom I would not have been able to complete this work. Their thoughts, interpretations, patient experiments and at times great wisdom, has been the basis upon which my work is based.

Of course over the years that I have worked there have been many who have been equally important on a personal level without whom I could never have done what I have done. My friends and family have put up with, both, me in person, and increasingly, my absence, and despite sometimes not seeing me for years at a time they have always been there for me. While a PhD is a very lonely road it is those that walk the journey along side you that make it both memorable and possible.

Special thanks go to my various lab mates from over the years, Danielle Butler, Vanessa Mitchell, Annette Zawaliski, Angelo Jamamine for their support and laughs. As the ship from Red Dwarf was often heard to say. You've got to laugh. To Geoff Drew, even though he finished before me…and published before me…and got a post doc before me……

Alright on second thoughts he doesn't get any thanks, he's got enough already. ;-) Just gagin'.

To Michelle Gerke for her endless enthusiasm, wonderful conversations, editing prowess and just plain awesomeness, I thank her and will always think fondly of this PhD for having had her around.

Profound thanks go to Michael Cousins and the Department of Anaesthesia and Pain management for supporting me and my thesis financially at various points though out my degree. Without this sort of support producing a thesis just wouldn't be possible.

Most of all I would like to thank Kevin and Phil for believing in me despite my average grades and giving me this chance to become a scientist. I thank them for the skills they have given me and for all their help and advice. Particularly I thank them for putting up with all my wild ideas. Whenever I got discouraged or stuck they always had an encouraging word to say and some invaluable advice. Any science I do in the future would not have been possible without their help. I am privileged and deeply honoured to have had the opportunity to have learnt from them.

Lastly, not only my thanks but also my love must go to Gin. While she has only joined me for the last part of this journey, it was for the most important part. In her I have discovered the one that makes me whole and I thank her for supporting me and believing in me, even in my bleakest writing up moments.

We humans have discovered how to manipulate individual atoms, how to look inside a living human being, and have found explanations that go some way towards describing the massively powerful forces that created the universe. While this thesis and the work it describes represents only the tiniest part of the sum of human knowledge, I offer it now to the world in the hope that those that are yet to come may find it in some small way useful.

Abstract

The general aim of this current work was to examine spinal cord injury (SCI), and in particular to examine the pathology of injury as it relates to changes in sensory transmission. Due to the limited possibilities for experimentation in humans, a range of animal models of SCI have been developed and are reviewed here. The weight drop SCI model is the most similar to the clinical presentation of SCI in humans and has been widely used in the rat. It was selected for the series of experiments reported in this thesis.

Many of the functional deficits produced by SCI result from a cascade of biochemical events set into motion by the injury. Included amongst these is the activation of the enzyme nitric oxide synthase which produces the gaseous neuromodulator, nitric oxide (NO). NO is amongst the most widely distributed and widely utilised molecule in virtually all living organisms, and it is an important signalling molecule in the nervous system. One of the major functions performed by NO appears to relate to sensory transmission, and thus alterations in sensory transmission observed as a result of SCI may involve alterations to NO synthesis. One of the principal aims of this thesis was to examine the effect of SCI on the NO producing cells of the spinal cord and to consider what any changes in NO synthesis may suggest in regards to sensation. NO producing cells were examined using NADPH diaphorase (NADPH-d) histochemistry. As the symptoms of SCI such as motor loss and changes in sensory processing are functional changes, it was also useful to examine changes in neuronal function as a result of SCI. Widespread neuronal function was examined via immunohistochemical detection of the gene product of the immediate early gene, *c-fos*.

It is not known how extensive the biochemical changes resulting from SCI may be, thus another of the aims of the present thesis was to examine the effects of SCI on NO synthesis not only at the level of injury, but also distant to the injury. Findings of the present thesis indicated that traumatic SCI resulted in a decrease in the number of NADPH-d positive cells from the superficial dorsal horn (SDH) of the spinal cord, while the number of these cells are increased in the ventral horn. These changes were restricted to spinal segments adjacent to

the injury. Fos expression was also altered by injury and was found to decrease. The most profound changes were found to occur in lamina III, although the other laminae also demonstrated similar changes. Changes in fos expression however were notably more widespread than those for NADPH-d and were not restricted to the level of the injury, occurring at all levels of the spinal cord examined. It was interpreted that alterations in NO synthesis appear to be modulated by the local injury-induced environment while fos expression may be altered by widespread changes to the global level of activity within the central nervous system.

Having observed that the number of NADPH-d positive cells of the SDH is reduced following injury, it was of interest to determine whether these cells were in fact killed, or whether they were still present but with reduced NADPH-d activity. Cell counts suggested that the NADPH-d positive cells, which were likely to represent a population of inhibitory interneurons, were not killed following injury, but rather are disrupted such that their normal biochemistry is altered.

Since these cells were likely to be inhibitory and were located in laminae involved in sensory transmission, the question arose how disruption of these cells may relate to the neuropathic pain observed to develop following SCI. Thus both NADPH-d and fos expression were again examined, but this time in conjunction with the sensory function of the rats. Sensory thresholds to pain-like behaviour were determined prior to and after injury using Von Frey filaments. Rats that demonstrated a decrease in sensory threshold of at least two Von Frey filament gradations (>70%) were classed as allodynic, while those with a less than a 70% decrease in threshold were classed as non-allodynic. A subpopulation of each of the groups of rats (uninjured, non-allodynic and allodynic) underwent a somatic stimulation paradigm. It was found that stimulation resulted in an increase in the number of NO producing cells but only in the allodynic group of animals. Since this group of animals by definition would perceive this stimulation as noxious, it is likely that the noxious nature of the stimulation resulted in the increased number of NO producing cells observed. This effect occurred only in segments adjacent to the injury. When fos expression was examined in the uninjured animals it was noted that somatic stimulation resulted in a decrease in fos expression, almost exclusively in lamina III. Following injury, there was no change in fos expression in lamina III observed. Instead the only change observed was an increase in fos expression in the deep dorsal horn (DDH, lamina IV and V). This occurred most profoundly

in the allodynic group. These results suggested that SCI may lead to misprocessing of sensory signals such that non-noxious somatic stimuli are processed in the DDH rather than lamina III following SCI. It is proposed here that this change in laminae processing may be responsible for the perception of pain towards a non-noxious stimulus, and that the reported injury-induced loss of NO producing inhibitory interneurons in the SDH may be responsible for this alteration in sensory processing following SCI.

Sensation is also processed by a number of supraspinal structures and a number of these have been implicated in the development of neuropathic pain states. The effects of SCI on neuronal activity as well as NO synthesis were examined in the periaqueductal grey region of the mid brain (PAG). SCI was shown to result in reduced neuronal activity in the PAG. This reduction in activity did not follow the somatotopy of the lateral column of the PAG (lPAG). It was suggested the reduced activity may not be solely caused by reduced spinal input as a result of SCI. Reduced neuronal activity in the PAG may indicate reduced PAG function, which includes descending modulation of spinal sensory transmission. Injury was not found to alter NADPH-d expression in the PAG.

The effect of traumatic lumbar SCI on the parietal (sensorimotor) cortex of the rat was also examined, as loss of inputs following SCI have been shown to result in a profound reorganisation of the cortex. Results indicated that SCI results in a virtual cessation of neuronal activity in areas 1 and 2 of the parietal cortex, likely as a result of lost afferent drive. Theories of cortical plasticity suggest that while the primary inputs via the lumbar spinal cord may be lost following SCI, other less dominants input will remain and become more dominant. It has been proposed previously that cortical reorganisation involves a rapid reorganisation of the entire sensory system. It was interpreted that a similar process may explain the system-wide reduction in neuronal activity observed in the present series of studies.

Table Of Contents

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

References

Appendix

List of Figures in this thesis

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Appendix

List of Tables in this thesis

Chapter 3

Chapter 5

Chapter 1

Introduction

According to the national Australian Spinal Cord Injury Register (ASCIR), which is the national body that records all spinal cord injuries presenting at the six specialist spinal units in this country, each year approximately 255 people suffer spinal cord injury (SCI) (Cripps and O'Connor, 1998; O'Connor, 2000; O'Connor, 2001; O'Connor, 2003; O'Connor and Cripps, 1997; O'Connor and Cripps, 1998). While this number represents an incidence of only 0.0014% of the population, at the present time, the functional deficits that result from SCI are largely untreatable. As such the condition is persistent, resulting in an increasing number of spinally injured patients over time. At the end of the 2000/2001 financial year there were almost 11,000 registered SCI patients in Australia (O'Connor, 2003). It is estimated that the cost of the long term care for SCI patients ranges from about $600,000 for a paraplegic to more than $4 million for a ventilator dependent tetraplegic (O'Connor and Cripps, 1998). In the US these costs are greatly magnified with up to 11,000 spinal injuries occurring each year and the annual cost of treatment recently estimated to be $US 4 billion (Fehlings and Tator, 1999).

The problem of SCI is not just an economic one however. Added to this is the personal cost to the patient. A case of spinal cord injury is defined as “the occurrence of an acute, traumatic lesion of neural elements in the spinal canal resulting in temporary or permanent sensory deficit, motor deficit or bladder/bowel dysfunction” (O'Connor, 2003). Bladder and bowel dysfunction is just one manifestation of a loss of autonomic function which is also a major consequence of SCI (Kaplan et al., 1991; Lynch et al., 2001). Thus apart from the financial cost, a spinally injured patient must confront a loss of sensation, movement, respiratory control, sexual function, temperature and blood pressure regulation, the possible development of neurological conditions such as neuropathic pain or autonomic dysreflexia and the loss of bladder and bowel function (Burns et al., 2001; Eltorai et al., 1997; Erickson, 1980; Finnerup et al., 2003; King et al., 1994; Nockels, 2001; Roth et al., 1997; Sawka et al., 1989; Siddall et al., 1999a; Teasell et al., 2000).

In order to increase the quality of life for the spinally injured, as well as reducing the ever increasing burden on the health system, research must be done in order to discover methods of returning function to an injured nervous system. To this end a number of animal models have been developed that allow detailed study of many of the mechanisms that underlie the functional deficits of SCI. Initially such models involved the use of non-human primates, and larger animals such as dogs. In recent times, however, both ethical and economic considerations have lead to an increased use of rodents in the laboratory. While these, mostly rat and mouse models, may not be directly related to humans owing to very different morphologies and physiologies, they do allow experimental manipulation not available in humans. In addition, because of their ready availability, the experimental scale used by researchers may be very much greater. Lastly a major benefit of rodent models of injury is that due to their widespread use, there is now a large body of data available to which experimental results may be compared.

As one of the principal deficits of SCI is alteration of sensation a brief review will be made here of rat CNS circuitry with special emphasis on the sensory pathways. This will be followed by a section briefly outlining the major models of SCI currently in use to determine which might be the most appropriate for a study into the mechanisms of functional deficit following SCI.

1.1 Sensation and Sensory Processing

Sensory information is transmitted to the CNS from the periphery via three principal neuronal fibre types. These are designated Aβ, Aδ and C. Aβ fibres have a large diameter of 10-15μm and are heavily myelinated, conducting impulses at 30-70m/s. They transmit principally discriminative touch and proprioception. Aδ fibres on the other hand are thinner, having a diameter of 2-5μm. They are lightly myelinated, have a conduction velocity of 10-30m/s and transmit information from hair follicles as well as free nerve endings for some temperature and pain sensation. The last fibre type is the narrowest type, having a diameter of less than 1.5μm. These fibres are unmyelinated and are therefore the slowest conducting of the sensory fibres, transmitting at 0.5-2.0m/s (for example see Macefield et al., 1989; Martin and Jessell, 1991).

These fibres pass into the dorsal horn of the spinal grey matter via the dorsal root of the spinal nerve, however here the common pathway ends. The different fibre types, transmitting their different sensory modalities, then take a range of paths to centres in the CNS where sensory information is processed and appropriate reactions determined. The first point of synapse for many of the fibres is the spinal cord. It has been observed that sensory fibres enter the dorsal horn with a particular mediolateral distribution. The larger, myelinated fibres synapse more medially and to deeper laminae, while the smaller unmyelinated fibres pass laterally and tend to synapse in more superficial laminae (Light and Perl, 1979).

1.1.1 Spinal Synapses of Sensory Afferents

Having reached the dorsal horn, tracing studies have indicated that the larger Aβ fibres tend to synapse in lamina III-V of the dorsal horn (Fitzgerald et al., 1994; LaMotte et al., 1991; Maslany et al., 1992; Shortland et al., 1989; Woolf, 1987), while the smaller diameter fibre types synapse most prominently in laminae I and II (Light and Perl, 1979; Nagy and Hunt, 1983; Sugiura et al., 1986; Sugiura et al., 1989; Sugiura et al., 1993). In these laminae, Aδ have been suggested as being most prominent in lamina I while C fibres predominate in lamina II, however a strong projection of Aδ to outer lamina II (IIo) of the dorsal horn has been noted (Nagy and Hunt, 1983). In addition cutaneous unmyelinated afferents have also been found to project strongly to lamina II (Sugiura et al., 1989; Sugiura et al., 1993). This indicates that both lamina I and II receive a strong projection of nociceptive fibre types and are likely involved in the processing of these modalities.

Thinly myelinated Aδ fibres have also been found to project to lamina V and X where they take nociceptive information from muscles and viscera (Ciriello and Calaresu, 1983; Molander and Grant, 1987; Neuhuber et al., 1986; Wang et al., 1999)

1.1.2 Supraspinal Sensory Pathways

After entering the spinal grey matter sensory signals reach higher levels of the neuraxis via a number of pathways. The principal pathways include the dorsal column pathway, the spinoreticular tract, spinomesencephalic tract and spinothalamic tract. Sensation is transmitted by both second order neurons from the spinal laminae discussed above, and also from axon collaterals of the primary afferents themselves. The direct projections travel via the dorsal columns and consist mainly of large diameter fibres concerned with the

transmission of non-noxious stimuli such as light discriminative touch, vibration and proprioception. There is also some evidence to suggest that up to 23% of direct primary afferent axons in the dorsal columns are unmyelinated (Chung et al., 1987; Horch et al., 1976; Langford and Coggeshall, 1981). These collaterals of primary afferents constitute the direct dorsal column pathway. In addition to this, second order neurons from the spinal grey matter also transmit sensation via the dorsal columns. This appears to be largely nociception from the viscera and originates mostly in lamina IV and lamina X (Al-Chaer et al., 1996; de Pommery et al., 1984; Wang et al., 1999; Willis et al., 1999). The destination of the dorsal column pathways are the dorsal column nuclei in the medulla. Spinal projection neurons originating below approximately T6 project to the gracile nucleus, while those above T6 project mostly to the cuneate nucleus (Giesler et al., 1984).

In addition to the dorsal column pathways, sensation is also transmitted to supraspinal structures via other pathways. The reticular nuclei of the medulla and pons for instance receive a strong projection from the spinal cord, arising from laminae V, VII, VIII and X, via the spinoreticular tract (Chaouch et al., 1983; Van Bockstaele et al., 1989; Wang et al., 1999). These neurons project innocuous proprioception and also nociception to the reticular nuclei (Menetrey et al., 1984a; Ness et al., 1998; Villanueva et al., 1989), which are thought to be an important site for pain modulation (Janss and Gebhart, 1988).

Also in the medulla is the nucleus of the solitary tract which is thought to receive sensation from the viscera (Hubscher and Berkley, 1995; Torvik, 1956). Spinal neurons projecting to this nucleus arise from lamina I, V and X (Guan et al., 1998; Menetrey and Basbaum, 1987; Wang et al., 1999).

Another supraspinal area that receives sensory input from the spinal cord is the mid brain via the spinomesencephalic tract. Three main regions in the midbrain receive a projection from the spinal cord, these are the superior colliculus, the midbrain periaqueductal grey (PAG) and, as already mentioned the midbrain reticular formation (Yezierski and Mendez, 1991). The spinal neurons that project to the midbrain are located largely in the cervical and sacral spinal cord, particularly lamina X (Keay et al., 1997; Wang et al., 1999), and are involved in the reception and localisation of incoming nociception or in descending inhibition (Willis et al., 2001; Willis and Westlund, 1997). Spinal projections to the midbrain travel via the ventrolateral funiculus (Bernard et al., 1995; Bianchi et al., 1990).

The principal forebrain structure involved in sensory processing and integration is the thalamus. Spinal cells that project to the thalamus form the spinothalamic tract (STT), which passes to the diencephalon via the ventral or ventrolateral funiculus (Giesler et al., 1981). The STT is the principal pathway for nociception and temperature information (Willis and Westlund, 1997) and originates in lamina IV-VI of the spinal cord (Granum, 1986; Kemplay and Webster, 1986; Kobayashi, 1998). About 90% of these cells cross to the contralateral side of the cord with in a few segments of their origin (Granum, 1986; Kemplay and Webster, 1986), terminating finally in the ventroposteriorlateral nucleus (Peschanski et al., 1983), the intralaminar nucleui (Ma et al., 1987) and the posterior complex of the thalamus (Dado et al., 1994a; Ledoux et al., 1987). STT cells respond to a range of modalities with 50% responding to both noxious and non-noxious stimuli (wide dynamic range neurons; WDR), 44% responding to only noxious stimuli (high threshold), while 6% respond largely to non-noxious stimuli (Dado et al., 1994b). There are few STT cells in the lumbar spinal cord of the rat, however those that are present respond mostly to non-noxious stimuli (Dado et al., 1994b; Menetrey et al., 1984b). STT neurons in the sacral spinal cord on the other hand respond to noxious stimuli, particularly from the pelvic viscera (Katter et al., 1996).

In addition to direct spinal projections voa the STT, about 8% of spinoreticular neurons also send collaterals to the thalamus (Kevetter and Willis, 1983). These neurons are activated by noxious stimuli (Villanueva et al., 1989) and are thought to form and important link between the spinal cord and the medial thalamus (Villanueva et al., 1998).

1.1.3 Descending Sensory Projections

Ascending projections from the spinal cord to higher levels of the neuraxis form one level of sensory processing. The stimuli that these projections transmit however are also able to activate descending projections that modify the afferent inputs to the sensory system. These modifications in input, inturn, affect the afferent drive that the system receives.

The highest level of the neuraxis to send projections to the spinal cord is the cortex. Axons from these cells form the corticospinal tract and originate in both motor regions of the cortex and forelimb and hindlimb areas of the primary sensory cortex. In these cortical areas, spinally projecting neurons are restricted to layer 5b (Miller, 1987). Corticospinal neurons

are also found in the secondary sensory cortex (Par 2) as well as in the posterior parietal cortex (Par 1; Miller, 1987). The corticospinal tract runs in the contralateral dorsal columns of the spinal cord of the rat after having crossed the midline at the pyramidal decussation in the medulla (Armand, 1982). There is also a small proportion of axons which do not cross and travel to the spinal cord in the ventral white matter (Brosamle and Schwab, 1997). The sensory part of the corticospinal tract projects to all laminae of the spinal cord however it has stronger projections to lamina III-VII, with fewer terminations in lamina I and II and the ventral horn (Casale et al., 1988; Liang et al., 1991). In addition to the spinal cord, corticospinal neurons also send collaterals to the midbrain (Catsman-Berrevoets and Kuypers, 1981; Killackey et al., 1989).

Other projections which may be involved in modulating sensation include those from the hypothalamus, which projects to lamina I of the spinal cord (Cechetto and Saper, 1988; Palkovits, 1999), and the zona inserta of the thalamus, which projects to lamina IV and V and lamina X of the cervical and lumbar enlargements (Schwanzel-Fukuda et al., 1984; Watanabe and Kawana, 1982).

The rubrospinal tract from the red nucleus in the midbrain also projects to the spinal cord, terminating in laminae V-VII. Here it synapses with both excitatory and inhibitory interneurons (Antal et al., 1992). The PAG (Masson et al., 1991), midbrain reticular formation (Satoh, 1979; Veazey and Severin, 1980a; Veazey and Severin, 1980b; Waldron and Gwyn, 1969) dorsal raphae nucleus (Bowker et al., 1981; Kazakov et al., 1993; Skagerberg and Bjorklund, 1985) and the parabrachial nucleus of the pons (Blomqvist et al., 1994), also project to the spinal cord. So to do the noradrinergic neurons from the Locus Coeruleus (Jones, 1991), all of which are believed to be involved in the modulation of pain (Bowker et al., 1982; Mason, 1999; Skagerberg and Bjorklund, 1985; Villanueva et al., 1996; Wei et al., 1999).

Lastly there are propriospinal projections along the rostrocaudal extent of the spinal cord, particularly to the cervical spinal cord which is involved in sensory modulation. Axons projecting to the cervical spinal cord from more caudal levels are mostly from laminae III-V (Baker and Giesler, 1984; Giesler et al., 1988), though there are propriospinal projections connecting all levels of the spinal cord. These propriospinal connections have been implicated in the control of nociception (Sandkuhler et al., 1993).

It has been noted that sensation occurs under heavy inhibitory control. This has been found to involve a network of GABAergic and glycinergic interneurons located in the spinal cord (Sorkin and Puig, 1996). These are particularly prevalent in lamina I-III (Campistron et al., 1986; Fuji et al., 1985; Hunt et al., 1981; Magoul et al., 1987; Todd and McKenzie, 1989), with up to half of the cells in lamina III having been observed to be GABA positive for instance (Todd and Sullivan, 1990). While GABAergic interneurons may have a particular prevalence in the superficial laminae, they generally have a wide distribution in the spinal cord and are located in all laminae except IX (Barber et al., 1982; Carlton and Hayes, 1990).

While these pathways have been found to modulated sensation in general, nociception in particular is heavily modulated by a vast complex of descending modulatory systems each of which is activated, and itself modulated, by incoming primary afferent drive (Basbaum and Fields, 1978; Besson and Chaouch, 1987; Willis and Westlund, 1997).

Collectively these data indicate that sensation, far from simply arriving at a given nucleus and causing a response, is instead processed by a highly complex network of neurons, across a range of neuronal structures throughout the entire neuraxis. It is not only the input from each of the structures involved that is important but also how the over all tone of the system is regulated. As this has a great deal to do with the primary afferent drive and the interconnectivity of the network, any disruption of this system is likely to alter sensory processing, resulting in the neuropathies observed clinically.

In order to examine the mechanisms involved in sensory dysfunction following injury, a number of animal models of spinal cord injury have been developed. Each use different techniques to injure the cord and therefore result in differing pathologies. In order to examine which may be appropriate to the needs of the present thesis these models will be reviewed here.

1.2 Experimental models of spinal cord injury

The models of SCI available fall into three main categories, those that examine the physical disruption of traumatic injury, those that examine ischemic injury and those that examine biochemical excitotoxicity. Within these three groups the various mechanisms thought to

underlie the pathologies and loss of function associated with SCI can be studied. Animal modelling of different aspects of nervous system injury allows the study of particular mechanisms of injury without confounding or multiple factors. This may allow the study of the contribution of these factors to the functional or physiological deficits observed.

1.2.1 Physical injury models

The majority of SCI cases occur due to accident involving physical trauma to the spinal cord (O'Connor, 2003). In order to study the aetiology of this form of injury, and/or possible therapeutic treatments, a number of models in which the spinal cord is injured physically have been developed. These include the transection models of injury in which the spinal cord is surgically cut, the contusion models in which the spinal cord is struck by a weight and the compression models in which the spinal cord is crushed under a constant weight. Each have their own benefits and drawbacks, and the major features of each will be discussed here.

Transection

Transection was first utilised experimentally in the late 1840's by Charles Edouard Brown-Séquard, first as part of his doctoral thesis using frogs, birds and a number of mammal species, then in a series of papers published from 1849-1852 describing the syndrome that would later bare his name (Aminoff, 1996; Haas, 1998; Tattersall and Turner, 2000).

Since that time the transection model of SCI, particularly the hemisection has been used as a means of producing an injury that was technically easy to perform and did not need elaborate or specialized equipment (Christensen et al., 1996). In addition, due to its simplicity, the injury itself was highly reproducible. The hemisection, in which only half of the spinal cord is lesioned, had the added benefit that the integrity of one side of the spinal cord is maintained. This meant that the animal still retained a degree of function allowing behavioural testing to be readily performed. The plantar test, in which noxious heat is directed at the plantar surface of the foot, is an example of one such test. The plantar test requires that the animal is able to move its foot away from the heat source, a movement that the hemisection model allows as only one side is paralysed (Christensen et al., 1996; Kim et al., 2003). This may not be possible with other forms of injury as they tend to be bilateral. Also specific white matter tracts may be cut using the hemisection technique, while sparing the grey matter. This enables the axotomy of functional columns to be

performed, allowing their role in, for instance, motor control or sensation to be examined (e.g. Back et al., 2003; Huang et al., 1999; Sun et al., 2001).

In more recent times the transection and hemisection models have become popular particularly in relation to spinal cord regeneration research. This model is useful in producing a complete axotomy of fibres of passage and therefore may sever the connections of a particular tract more fully than other models. It may also be used to resection regenerated axons to observe functional or behavioural effects (Basso et al., 1996; for review see also Bregman et al., 2002; Shumsky et al., 2003).

The major draw back of the transection models of injury is that the injury is very clean, that is, there is minimal accessory tissue damage apart from the axotomy, unlike injuries observed clinically. For this reason the models may not reflect the clinical presentation of SCI. In order to better examine the aetiology of full traumatic injury a series of contusion models were developed with which this form of injury may be studied.

Contusion injury

The contusion model followed on from the transection models of SCI and was the next to be developed. In its initial form, it involved dropping a weight a known distance onto the exposed spinal cord. This weight drop model of traumatic SCI was developed by Allen in 1911, as a means of examining how much injury the spinal cord could sustain and still permit functional recovery (Allen, 1911). The instrument he designed consisted of a long metal frame which allowed a weight of 30g to be dropped onto the exposed spinal cord of a dog. This instrument had the advantage of being able to vary the height from which the weight was dropped, which inturn varied the force with which the cord was struck and therefore the severity of the injury.

Since this first use of the model, the original Allen weight drop protocol has been modified by many groups for use in different animals and to allow, amongst other factors, a more accurate strike of the cord. Freeman and Wright, for instance, later modified Allen's original technique by, instead of dropping a weight to directly impact upon the cord, placing a lucite "impounder" which was concave at one end, to sit upon the exposed surface of the cord (Freeman and Wright, 1953). A weight, which in this case was a glass rod filled with mercury, was then dropped a known distance, delivering a calculable force to the surface of

the cord via the impounder. Experiments using this modified weight drop method found a similar effect to Allen's original method. Using dogs it was found that similar impact forces in both versions of this model had similar effects, despite the indirect application of the force in the modified method (Freeman and Wright, 1953). Due to the convex shape of the surface of the cord it was thought that this concave impounder would more evenly distribute the force of the striking weight to the cord.

While the weight drop technique has gone on to become one of the most commonly used methods for producing experimental SCI, it is also reported to be the most variable of the methods available, in terms of the pathophysiology produced (Khan and Griebel, 1983; Khan et al., 1985; Koenig and Dohrmann, 1977; see also Tator and Deecke, 1973). Possible reasons for this are that small movements of the animal or the apparatus can vary the force and the location of the injury. Also, the individual technique of different groups using the model can vary; as does the apparatus they use (Khan and Griebel, 1983).

Other factors which can affect the injury resulting from a weight drop is the size and weight of the animals and the region of the cord, and therefore the qualities of the tissue (ie surface area, density, amount of cerebrospinal fluid), struck. While many of these factors may be controllable in larger animals, the variability introduced by them may be magnified in smaller animals such as the rat (Khan and Griebel, 1983).

In addition to this, it has been found that the force measurement used for the weight drop technique could lead to inaccuracies. The unit of measurement used in this model is the product of the weight (in grams) and the distance it was dropped (in centimetres) to yield a unit of gram-centimetres (g.cm). This has led to variations in injury however, since different combinations of weights and height can lead to the same product in g.cm, but the actual force applied to the cord will be different. For instance the force applied to the cord with a drop of 40g over 10cm is 100 times greater than the force applied by a 5g weight over 80 cm, even though they are both 400g.cm weight drops (Dohrmann and Panjabi, 1976; Tator and Deecke, 1973).

It was found that lesion volume is linearly related to the impulse (product of weight and velocity - g m/s) of the falling weight (Dohrmann and Panjabi, 1976). It was also suggested

that whether the spinal cord is struck over bone or an intervertebral disk may also affect the injury produced (Dohrmann et al., 1978).

A last disadvantage associated with this model is thought to be that it causes trauma largely to the posterior cord, whereas in humans cord trauma tends to involve anterior compression and burst fractures (Tator, 1983). To overcome this and other difficulties, such as the variability of functional deficit produced by the model and variability in the pathophysiology produced in the cord, the 1980's saw a range of modifications to Allen's weight drop models (Anderson, 1982; Ford, 1983).

Dogs, cats and primates were used for much of this early experimentation but towards more recent times, scientific, economic and ethical considerations have lead to the development of similar protocols in the laboratory rodent, particularly the rat. It was not until 1985 that the weight drop model was used extensively in rats but since then it has been found to provide an inexpensive, easily obtainable subject for SCI research (Wrathall et al., 1985). Also despite the drawbacks it remains the most similar injury model to the majority of clinical cases and is amongst the easiest to perform.

In order to address the problem of variability in placement of the wound and therefore the pathophysiology produced by the weight drop model, a last model of physical injury was developed. This was the compression model of injury.

Compression

The compression model of injury, like the weight drop models also delivered a force to the spinal cord, however this force was delivered over a longer duration of time resulting in a spinal cord compression. The first of the compression models of SCI to be developed was the balloon catheter model, developed by Tarlov and colleagues in 1953 (Tarlov et al., 1953). This model aimed to simulate both acute compression of the spinal cord, such as through vertebral fracture, and also long term, slowly developing compression, such as from the development of a spinal tumour (Matin et al., 1992).

The balloon catheter injury model is considered a closed injury model as the cord is injured within the confines of the vertebral canal. It is believed to mimic the closed nature of human

spinal cord injury, in which the majority of cases, the vertebrae are fractured and compress the spinal cord (Matin et al., 1992).

The balloon compression technique appears to produce damage by two mechanisms. Firstly, (in the rat) low volume (0.1cc) compression over a short duration results in reduced motor function, which recovered over 3-5 weeks back to control levels. A longer duration compression, however, at the same balloon volume resulted in permanent loss of function. A short duration compression at a high volume (0.2cc), on the other hand, also resulted in permanent functional deficit (Khan and Griebel, 1983). These results, together with examination of the tissue, suggest that low volume compression most likely injures the cord through ischemia while short duration, high volume compression leads to mechanical disruption of the cord.

A major benefit of the balloon compression technique is the minimal amount of soft tissue and bone which needs to be removed in order to perform the procedure. In addition, the duration of compression may be accurately controlled, unlike the instantaneous delivery of force involved in the weight drop technique, although the precise location of the balloon catheter in the epidural space was reported to be difficult (Khan and Griebel, 1983). Additionally the catheter model appears to have a steep dose-response curve. That is to say, the difference in the amount of compression which may be applied and still allow functional recovery, is quite close to the amount of compression required before no functional recovery is possible. This may be a problem if attempting to examine sub-maximal injury with this method (Khan and Griebel, 1983).

Another characteristic of the technique is that unlike tramatic injury the balloon catheter compression model reduced gliotic scar formation. This was of consequence as it is thought that the formation of a gliotic scar is a major obstacle to the regeneration of damaged axons. The functional recovery exhibited by this model, which does not include scar formation may therefore be different from traumatic injury (Matin et al., 1992).

Martin and Bloedel used Tarlov's original balloon catheter in cats while Khan and Griebel adapted it to rats (Khan and Griebel, 1983; Martin and Bloedel, 1973). The latter group compared this extradural catheter compression injury model with other models of SCI and

found that, because of the steep dose-response curve, it was difficult to produce sub maximal injuries.

Since this was the major drawback with Tarlov's balloon model, two main modifications were made on the technique in order to produce a sub-maximal injury. This was of importance in that the compression models of injury were developed to allow not only the study of the pathophysiology of the injury process, but also to examine possible therapeutics for SCI. In order to do this it was important to develop a model in which the return of function may be possible, in order to examine the efficacy of any treatment studied (Eidelberg et al., 1976).

The first of these modifications was developed in 1973 by Eidelberg, and was an adaptation of Allen's weight drop technique (Eidelberg, 1973). Similar to the impounder used in Freeman and Wright's modified weight drop (Freeman and Wright, 1953), the Eidelburg compression model made use of a "pressor element" (a 3mm diameter convex metallic head on a shaft) which was held in place against the spinal cord by a Teflon guide. The spinal cord was exposed by a laminectomy, the dura was left intact, and the vertebral column held rigid by clamping the spinous processes of the vertebrae caudal and rostral to the laminectomy. The desired force could then be applied to the cord by loading the pressor element with circular weights (Eidelberg et al., 1976; Eidelberg, 1973).

The second modification of the compression model of SCI was adapted from the intravertebral balloon catheter model but instead, utilised an inflatable cuff that could be placed around the spinal cord. This technique was developed using the larger spinal cord of the monkey. As well as applying an easily controllable and quantifiable amount of force to the cord, this model was reported to be more reproducible that the weight drop model that was being used extensively at the time (Tator and Deecke, 1973).

As with the other compression models, the cuff method was designed to more closely simulate one of the more common forms of SCI in humans, which is compression of the spinal cord following fracture of the vertebrae (Tator and Deecke, 1973). The inflatable cuff method of SCI also had some variation in the degree of severity and the functional deficit which results, but this variation was reported to be overcome by sufficiently high numbers of animals.

Due to the size of the inflatable cuff however this technique was only suitable for use in large animals. To adapt it for use in small lab animals Rivlin and Tator made use of a modified aneurysm clip in order to compress the cord (Rivlin and Tator, 1978). This model has the advantage that it is highly reproducible in terms of the pathophysiology produced (Khan and Griebel, 1983), but also that the actual force applied to the cord is easily quantifiable (Dolan and Tator, 1979) unlike for other models including the weight drop model and balloon catheter model (Dohrmann and Panjabi, 1976; Dohrmann et al., 1978). On the other hand since the aneurysm clip is applied extradurally to the spinal cord, one of the drawbacks of the technique is that a complete laminectomy must be performed to allow the anterior blade of the clip to pass beneath the cord with minimal displacement of the cord dorsally. Another disadvantage is that it was found that the experimenter must be well practiced in the technique to minimise unintentional damage to the cord (Khan and Griebel, 1983).

These then are the principal physical injury models that have been reported. One of the secondary pathologies that develops as a result of traumatic injury, and which was partially modelled by spinal cord compressions, is ischemia. This likely results from micro occlusion of blood vessels and develops soon after injury (Balentine, 1978; Goodman et al., 1979). Ischemia is thought to play a large part in the tissue degeneration that is observed to occur secondary to the initial physical trauma (Balentine, 1978; Goodman et al., 1979; Griffiths et al., 1978; Means et al., 1978; Osterholm, 1974; Sandler and Tator, 1976; Wagner et al., 1978). In addition, ischemia itself is a primary cause of neural damage as in the case of stroke. For these reasons, following the development of the models of physical SCI, a number of models have been also been developed that model the ischemic component of neural injury. These will be discussed in the following section.

1.2.2 Chemical Injury Models

Ischemia

The first model of ischemic injury was developed in 1982 by Zivin and colleagues and was produced by ligating the abdominal aorta distal to the left renal artery in rabbits (Zivin et al., 1982). This model was later refined further when Watson and Prado and colleagues (Prado et al., 1987; Watson et al., 1986) developed a model of spinal cord ischaemia which allowed the area of ischaemia to be restricted solely to the spinal cord through the use of a photo-

chemically induced blood clot. In this model the spinal cord was irradiated with a laser following systemic injection of the dye rose Bengal resulting in the formation of free oxygen atoms. These oxygen atoms were then believed to react with unsaturated fatty acids and other susceptible molecules of the endothelial cell membrane resulting in platelet aggregation and microvascular occlusion (Watson et al., 1986).

This model of SCI was developed in order to create a minimally invasive, highly reproducible model of injury (Watson et al., 1986), which was thought to represent the ischaemic component of traumatic SCI (Hao et al., 1991). It also enabled a more thorough study of this component of the injury process (Prado et al., 1987; Watson et al., 1986).

The photochemical ischaemia model, unlike the compression model (Martin and Bloedel, 1973; Tator and Deecke, 1973), or weight drop (Tator and Deecke, 1973) models, is highly reproducible and allows for the production of a graded injury series by varying the time of laser irradiation. The area of intact tissue following this form of ischemic injury was significantly correlated with the duration of irradiation. It has the added advantage of not requiring a laminectomy as other models do, reducing collateral damage to the cord (Prado et al., 1987).

Excitotoxicity

The last major model of SCI also examines a component of the secondary consequences of traumatic injury. It has been observed that excitatory amino acids (EAAs) are released following traumatic SCI (Liu et al., 1991; Liu et al., 1997; McAdoo et al., 1999; Meldrum, 1985; Panter and Faden, 1992; Panter et al., 1990; Rothman, 1984; Rothman and Olney, 1986; Simpson et al., 1990), which result in excitotoxic cell death (Choi et al., 1987; Globus et al., 1988; Liu et al., 1991; Liu et al., 1999a). Thus a major mechanism of injury following trauma appears to be excitotoxicity. Models have been developed to allow examination of the contribution of this form of neuronal injury to the injury-induced functional deficits.

Examination of the contribution of excitatory amino acids to neuronal injury began in the late 1950's when Lucas and Newhouse first found that injections of exogenous glutamate is neurotoxic to neurons of the retina in neonatal mice (Lucas and Newhouse, 1957). Twenty years later, Olney and colleagues found that oral glutamate could cause neuronal toxicity at regions of the brain which are devoid of blood brain barrier, and also that analogues of

glutamate had a neurotoxic activity which seemed to parallel their excitatory activity (Olney et al., 1971). These observations lead them to put forward the suggestion that excess excitatory agents may lead to the death of neurons, an activity which came to be known as excitotoxicity. At about the same time, glutamate excitotoxicity was observed and defined ultrastructurally in infant mouse hypothalami with the electron microscope (Olney, 1971), and later was also observed to occur in young primate brains (Olney et al., 1972).

Excitotoxicity itself has been studied in a large number of spinal injury models. However, it was not used specifically as a model of SCI until the early 1990's. Yezierski and colleagues used the model to examine the effects of excitotoxicity on neuronal degeneration and cavitation of the white matter of the spinal cord (Yezierski and Park, 1993; Yezierski et al., 1993). In this model, small quantities of quisqualic acid were microinjected into the spinal cord leading to morphological changes within the cord which are similar to those from traumatic and ischemic models of injury. This suggested that the activation of glutamate receptors may be responsible, at least in part, for the morphological changes resulting from these types of injury (Park et al., 2003; Plunkett et al., 2001; Rokkas et al., 1994; Yezierski et al., 1998; Yezierski et al., 1993). This observation was further reinforced by later work which showed injections of quisqualic acid into the grey matter of the spinal cord resulted in neuronal loss, cavitation, formation of an astrocytic scar and prominent inflammation (Yezierski et al., 1998). In addition, it was found that all animals following quisqualic acid injections developed some form of hypersensitivity to mechanical or thermal stimuli, despite the lack of any physical trauma to the cord (Yezierski et al., 1998). These results demonstrate that in addition to causing morphological changes, the EAA released following traumatic injury may also be involved with the onset of chronic pain, one of the common consequences of traumatic, physical injury (Yezierski et al., 1993).

Due to the lack of other confounding forms of tissue injury, the EAA model has largely been used to examine the contribution of secondary EAA release to the development of neuropathic pain. This in turn allows study into highly specific antagonists against these mechanisms that will hopefully alleviate the development of such pain following traumatic or ischemic SCI (Abraham et al., 2000; Abraham et al., 2001; Brewer and Yezierski, 1998; Plunkett et al., 2001; Yezierski et al., 1998). Another advantage of the EAA model is that it is a highly localised method of performing a lesion with in the CNS. The contusion, compression and ischemic models by their nature affect the entire diameter of the cord.

While the hemisection model affects a reduced volume of cord, it is difficult to restrict to specific levels within the cord without damaging all structures in a line from the surface. The EAA model on the other hand allows for highly selective lesioning. When injected into the dorsal horn of the spinal cord for instance, the superficial dorsal horn may be spared while only the deeper laminae are affected (Yezierski et al., 1998). This has the benefit of allowing study of the contribution of specific structures to the function of the spinal cord, and therefore to the deficits produced by injury to these structures. This may not be possible with other models.

1.3 Summary of models of SCI

Taken together then, it appears that due to the intractable nature of SCI and the current lack of effective treatment, there is a need to develop methods by which some function may be returned following SCI. A number of studies have been performed, and continue to be performed, seeking to develop therapies for the dysfunction that results from SCI. Underlying these, however, is an incomplete understanding of the mechanisms that are set into motion following injury resulting in the functional deficits observed. Only a limited number of study types may be performed in humans for ethical and legal reasons and so animal models must be used in order to gain a greater understanding of the pathophysiology of SCI. One of the purposes for discussing these animal models of SCI here is to determine which may be suitable to use in the series of experiments described in this thesis.

Despite the reported variability in pathophysiology of the weight drop model, this model is most similar to the clinical presentation of SCI in the human. It is also the only model (including the few variations on the technique) of full traumatic injury. Each of the other types of SCI were designed to examine specific characteristics of traumatic injury and thus do not allow study of the full range of mechanisms that may play a role in a traumatic injury. In addition to this, the weight drop model is one of the most widely used models of SCI, particularly in the laboratory rodent thus providing a large basis of comparison for the results of such studies. The method is not technically difficult to carry out, and while a laminectomy must be performed, it is not as extensive as that for the inflatable cuff or aneurysm clip models of compression therefore minimising the risk of accessory damage to the spinal cord. Thus the weight drop model of traumatic SCI was selected for use in the present series of experiments. Lastly, because of the extensive use of this model in the

laboratory rat, as well as the low cost and ready availability of this animal, the rat was chosen as the animal appropriate for the experiments to be reported here.

In order to understand the results of experiments examining the mechanisms underlying the functional deficits of SCI it is important to briefly examine some of the pathological processes set into motion by this injury. These processes are often referred to as the secondary mechanisms of injury since they occur secondary to, or as a result of, the initial physical trauma. It is the combined effect of the primary injury and the secondary degeneration that results in the pathology and loss of function observed following SCI. As suggested by Allen (Allen, 1911) in his work on traumatic SCI, there is little that can be done after the fact to reduce the physical disruption to spinal tissue following injury. Instead it may be more practical to focus on ways of reducing or preventing the secondary degenerative consequences as a means of reducing injury severity. While it is beyond the scope of the current chapter to examine all mechanisms of secondary injury, the following section will outline some of the principal ones. The intention of the following sections is to, a) illustrate the extensive biochemical changes that can occur as a result of traumatic injury, b) to indicate what effects these changes may have on the tissue and the nervous system, and most importantly c) to indicate which mechanisms may warrant further study.

1.3.1 Secondary changes of SCI

Traumatic SCI results in a large series of biochemical changes to be set into motion. These range from the very rapid changes in lipid break down and phospholipase activation which occur within minutes of injury (Faden et al., 1987; Panter and Faden, 1992; Saunders et al., 1987), to changes of enzyme levels (Benton et al., 2000) and cytotoxic oedema which are still occurring days after injury (Willard-Mack et al., 1996). Included between these extremes are events such as the release of toxic amounts of EAAs (Liu et al., 1991; Liu et al., 1997; McAdoo et al., 1999; McIntosh, 1994; Meldrum, 1985; Panter and Faden, 1992; Panter et al., 1990; Rothman, 1984; Rothman and Olney, 1986; Simpson et al., 1990), and release of lipid metabolites such as arachidonic acid, thromboxane and peptidoleukotrienes which may be involved in the formation of microocclusions of the spinal capillaries following SCI (Faden, 1987; Hall and Braughler, 1986; Panter and Faden, 1992). Free radical release is also reported to increase following SCI resulting in further damage to lipid membranes and cellular proteins (Hall and Braughler, 1986; Hall and Braughler, 1993)

Downstream from these initial events is uncontrolled ion influx (Chan et al., 1979; Choi et al., 1988; Janssen and Hansebout, 1989; Koh et al., 1990; Rothman, 1984), activation of enzymes including proteases, lipases and endonucleases (Choi, 1988; Choi, 1992; Choi et al., 1987; Eimerl and Schramm, 1994; Faden, 1987; Regan and Choi, 1994; Siesjö, 1988), decoupling of the electron transport chain and reduced energy production (Anderson et al., 1980; Walker et al., 1977; Walker et al., 1979), and increased gene transcription of genes such as the immediate early genes (Dragunow et al., 1990; Yakovlev and Faden, 1994), TNF-α (Yakovlev and Faden, 1994), and preprodynorphin (Faden, 1987).

From the above then, it is clear that injury sets into motion a large number biochemical changes, many of which result in damage to neural tissue. A review of the literature finds that a great deal of evidence points towards the earlier changes as being largely responsible for the pathology and loss of function as a result of injury. The initial changes in blood flow for instance can reduce the perfusion of spinal tissue thereby exacerbating any initial traumatic injury with ischemia. Perhaps more importantly though, the increases in EAAs, that occur within the first few minutes of injury, have been found to induce cell death through a large variety of mechanisms. These have been examined to varying degrees and there is mounting evidence to implicate EAA induced excitotoxicity as a major contributor to spinal degeneration as a result of traumatic SCI.

In order to discuss the mechanisms of EAAs in SCI pathology, and in particular to determine a candidate molecule for further study, the role of EAAs following SCI will be outlined in more detail here.

As mentioned above, traumatic injury or ischemia to the spinal cord, results in the rapid release and increase in extracellular concentration of excitatory amino acids (EAAs) such as glutamate and aspartate (Liu et al., 1991; Liu et al., 1997; McAdoo et al., 1999; McIntosh, 1994; Meldrum, 1985; Panter and Faden, 1992; Panter et al., 1990; Rothman, 1984; Rothman and Olney, 1986; Simpson et al., 1990). Furthermore, these are released in sufficient concentration to be toxic to neurons (Choi et al., 1987; Globus et al., 1988; Liu et al., 1991; Liu et al., 1999a).

The degree of release of EAAs such as glutamate and aspartate is related to the degree of trauma, with the concentration increasing more, and for a greater length of time, in more

severe trauma. Although interestingly, the mode of tissue injury, eg physical trauma, ischemia or hypoglycaemia, does not appear to affect the injury-induced changes (Panter et al., 1990; Simon et al., 1984; Yezierski et al., 1998).

The toxicity of glutamate appears to be mediated largely by the NMDA receptor since inhibiting the NMDA receptor has been found to increase the number of surviving neurons after both the application of exogenous glutamate and also that released following injury (Choi et al., 1988; Freese et al., 1990; Hartley and Choi, 1989; Rothman and Olney, 1987; Vornov et al., 1995). Excitotoxicity can also be mimicked by NMDA receptor (NMDAr) agonists and this death can also be blocked by NMDAr antagonists (Freese et al., 1990; Simon et al., 1984). Non-NMDA EAA receptors have also implicated in mediating glutamate toxicity (Chen et al., 1995; Wrathall et al., 1997).

So given that the glutamate released as a result of injury appears to be toxic to neurons, the question becomes how does glutamate stimulation lead to cell death, and therefore, how might this relate to the functional deficits observed following SCI? The toxicity observed is brought about by two principal mechanisms. The first is an immediate influx of sodium and chloride ions resulting in cellular swelling and lysis, the second is more delayed and is induced by a derangement of intracellular Ca^{2+} homeostasis.

1.4 EAA-induced toxicity

1.4.1 Neuronal swelling and lysis

One of the first activities of glutamate is to activate both NMDA and non-NMDA receptors (kainite and AMPA receptors), allowing entry of Na^+, K^+, Cl^- and Ca^{2+} (Collingridge and Bliss, 1987; MacDermott and Dale, 1987; Rothman and Olney, 1987). The salt influx draws water into the cells resulting in swelling, loss of energy reserves and in severe cases cell lysis (Chan et al., 1979; Choi et al., 1988; Janssen and Hansebout, 1989; Koh et al., 1990; Rothman, 1984). Inhibition of excitatory neurotransmission with kynurenate on the other hand is found to reduce the swelling in a model of ischemic injury (Simon et al., 1986) while glutamate treatment of neurons cultured in a medium without Na^+ prevents swelling (Rothman and Olney, 1987). This indicates that the activation of EAA receptors appears to be the mechanism by which the swelling occurs and that this depends on the presence of Na^+.

Swelling is not the sole cause of death following activation of EAA receptors, however. In addition to this there appears to be a second more delayed mechanism which is mediated by Ca^{2+}.

1.4.2 Calcium-induced delayed cell death

Cultured neurons treated with glutamate that include Ca^{2+} in the extracellular medium, will swell and degenerate over the next 24 hours. However, if Ca^{2+} is excluded from the medium, brief treatment with glutamate results in cell swelling, but cells do not degenerate. Treating cells in a medium with no Na^{+} but with Ca^{2+} results in no swelling but cells go on to degenerate in a delayed fashion. This indicates that while in extreme cases NMDAr-induced cell swelling may lead to cell lysis and death, Ca^{2+} in the extracellular fluid also plays a major role in NMDAr-induced excitotoxicity (Rothman and Olney, 1987). Treatment of the spinal cord with a concentration of glutamate within the range released following traumatic injury, resulted in the death of 75% of neurons by 24 hours after administration. However, when examined immediately after a 1hour perfusion, no neuronal loss was detectable (Liu et al., 1999a). This result illustrates the delayed nature of the cell death. Lastly, inhibiting NMDAr not only decreases the degree of cell death but also has the effect of decreasing intracellular Ca^{2+} when compared to cells without an antagonist (Simon et al., 1984), indicating that the death may indeed caused by Ca^{2+} dependent mechanisms.

This delayed toxicity then, appears to be mediated by increases in intracellular Ca^{2+}, and appears to be a major mechanism in the sequelae and functional deficits that occur as a result of SCI. In order to treat the symptoms of SCI, more must be known about the mechanisms by which the physical injury may result in tissue damage. There are a number of mechanisms by which Ca^{2+} influx may result in cellular damage and death. These include mitochondrial dysfunction, induction of free radicals and reactive oxygen species, failure of Na^{+}-K^{+} ATPase, DNA fragmentation and the activation of Ca^{2+}-dependent enzymes. These important mechanisms are likely to be the underlying causes of glutamate toxicity and thus the secondary degeneration resulting from SCI. These mechanisms will be examined briefly here in order to gain an understanding of the major processes set into motion by SCI and to determine which mechanisms may warrant further study.

1.4.3 Mitochondrial dysfunction

Following glutamate stimulus of NMDA receptors, intracellular Ca^{2+} is increased and is buffered to a large degree by sequestration into mitochondria. Ca^{2+} is sequestered by diffusing down the proton electrochemical gradient across the mitochondrial membrane, which inturn depolarises the mitochondria. The reduction of the electrochemical gradient reduces ATP synthesis at a time when the energy-dependent, cell membrane-Ca^{2+} pumps are working maximally to restore Ca^{2+} concentrations. In addition to this, the reduction of the mitochondrial electrochemical gradient leads to the opening of the permeability transition pore of the inner membrane of the mitochondria and the further collapse of the proton electrochemical gradient. The combined result is a decrease in ATP production and, if severe enough, cell death (Khodorov et al., 1996; Peng et al., 1998; Schinder et al., 1996). Earlier studies also report a decrease in the high energy molecule phosphocreatine following traumatic brain injury, which they also suggest indicates a disruption to mitochondrial function (Vink et al., 1987). Disruption to mitochondrial ion homeostasis is associated with mitochondrial swelling due to disruption of ion gradients, and this is followed later by neuronal degeneration (Wingrave et al., 2003), likely due to loss of mitochondrial function. Lastly, blocking non-NMDA glutamate receptors following contusion SCI was found to improve mitochondrial function, in addition to reducing production of reactive oxygen species (ROS) and also the products of lipid peroxidation (Mu et al., 2002). Results such as these indicate that the disruption of mitochondrial ion concentration and therefore ATP synthesis may be an important mechanism in post injury neuronal degeneration.

1.4.4 Induction of free radicals and reactive oxygen species

A second mechanism by which Ca^{2+} is believed to lead to neuronal toxicity subsequent to injury is through the formation of free radicals. This may occur through two principal Ca^{2+} dependent mechanisms; the uncoupling of the electron transport chain, associated with mitochondrial dysfunction and the generation of free radicals (Frantseva et al., 2001; Paschen, 2000; Schinder et al., 1996) or through the activation of enzymes such as nitric oxide synthase {NOS; \Pou, 1992 #685;Klatt, 1993 #684;Culcasi, 1994 #683}. NOS is activated by increased intracellular Ca^{2+} and produces the non-charged, gasseous free radical, nitric oxide, one of the most widespread and ubiquitous signalling molecules in, not only the nervous system, but the entire body (Bredt and Snyder, 1990; Bredt and Snyder, 1992; Forstermann, 1994; Stuehr et al., 1991). Nitric oxide is involved in both the function

of the normal CNS, and the pathology that occurs as a result of injury, thus is an important molecule in the subsequent effects of SCI.

Once formed, free radicals including nitric oxide (NO), superoxide and the reactive oxygen species peroxynitrite, lead to lipid peroxidation and the break down of lipid membranes (Beckman et al., 1990; Chan et al., 1985; Dawson and Dawson, 1996; Demediuk et al., 1985; Janssen and Hansebout, 1989). This in turn has been associated with neuronal degeneration likely through mitochondrial dysfunction, again leading to cellular energy depletion (Fiskum, 1985), induction of apoptosis (Virag et al., 2003; Wei et al., 2000), or the oxidation of membrane lipids or cellular proteins including enzymes and ion channels (Chan et al., 1985; Demediuk et al., 1985; Janssen and Hansebout, 1989; Virag et al., 2003).

1.4.5 Failure of Na^+-K^+ ATPase

A third mechanism by which Ca^{2+} is believed to lead to neuronal toxicity subsequent to injury is the failure of the Na^+/K^+ ion pump, Na^+-K^+ ATPase, as a result of loss of lipid membrane integrity. The Na^+-K^+ ATPase pump is a membrane bound protein that is responsible for actively transporting Na^+ out of, and K^+ back into a neuron after it has depolarised. This reduced Na^+-K^+ ATPase activity is then thought to result in increased intracellular Na^+ and failure of the neuron to repolarise, which exacerbates the detrimental effects of SCI (Agrawal and Fehlings, 1996).

Lipid membranes are degraded following injury as a result of free radical lipid peroxidation (White et al., 1985), in addition to the activation of calcium dependent phospholipases (Demediuk et al., 1985). The activity of the membrane bound Na^+-K^+ ATPase requires an intact membrane, thus injury is likely to reduce its level of activity. It has been found following traumatic spinal cord injury that there is a reduction in spinal Na^+-K^+ ATPase activity (Faden et al., 1987; Kurihara, 1985). Treatment of isolated membrane Na^+-K^+ ATPase with superoxide radicals caused an irreversible inactivation of the enzyme that could be prevented by addition of superoxide dismutase, an enzyme that inactivates the superoxide radical (Hexum and Fried, 1979). This reduction in Na^+-K^+ ATPase activity was found to be likely due to free radical lipid peroxidation and membrane integrity as apposed to alterations in the ATPase molecule itself (Jamme et al., 1995).

In addition to the reduction of existing Na^+-K^+ ATPase activity, the mRNA for this molecule also appears to be reduced following injury. In situ hybridisation analysis following spinal transection shows a reduction in the mRNA for one of the subunits of this enzyme in large and medium neurons of the ventral horn. Furthermore the treatment with glucocorticoids which have been shown to be helpful in the restoration of function after spinal injury was found to increase the expression of Na^+-K^+ ATPase mRNA following transection (Gonzalez et al., 1996).

1.4.6 DNA fragmentation

Another mechanism that results in neuronal death following SCI is DNA fragmentation. In addition to DNA damage from free radical formation (Floyd and Carney, 1992), injury induced Ca^{2+} influx also appears to result in DNA degradation. In this case, the degradation appears to be due to Ca^{2+}-dependent activation of endonucleases leading to DNA fragmentation (Joseph et al., 1993). Such DNA fragmentation is a morphological characteristic of cells undergoing apoptosis, as apposed to the injury induced necrosis associated with the excitotoxic cascade. Endonuclease-mediated DNA cleavage is thought to play a part, amongst other mechanisms, in the induction of cellular apoptosis (Krystosek, 1999).

Evidence for this mechanism of Ca^{2+}-mediated cell death lies in studies that indicate that hypoxia resulted in increased Ca^{2+} in the nucleus, hence the activation of endonucleases and apoptotic mechanisms (Delivoria-Papadopoulos et al., 2003). Also, increasing extracellular glutamate results in neuronal degeneration, which was found to be partially prevented by the inhibition of Ca^{2+}-dependent endonucleases (Rothstein and Kuncl, 1995). Inhibiting endonucleases also prevented apoptosis following in vitro treatment with increased calcium (Michel et al., 1994), growth factor withdrawal (Batistatou and Greene, 1993), and the cell death following hypoxia and NMDA treatment in vivo (Roberts-Lewis et al., 1993).

1.4.7 Other mechanisms

In addition to the activation of endonucleases a number of other similar mechanisms are also thought to be involved in Ca^{2+}-induced cell death. These include activation of the Ca^{2+}-dependent protease calpain, and the lipase, phospholipase A_2. Neurons contain high levels of calpain which is a proteolytic enzyme. It is activated by increased calcium levels, leading

to excessive proteolysis (Melloni and Pontremoli, 1989). This can include the breakdown of cytoskeletal proteins (Kupina et al., 2003) followed by fragmentation of dendritic processes and subsequent neuronal degeneration (Neumar et al., 2001). Calpain activity may also result in mitochondrial damage again leading to neuronal death (Wingrave et al., 2003). Ca^{2+} activation of Phospholipase A_2 on the other hand leads to the degeneration of membrane lipids of both the plasma membrane and those of organelles. This results in membrane rigidity and loss of permeability. It is thought that this Ca^{2+}-induced activation of phospholipases may work in concert with free-radical induced lipid peroxidation to affect cell viability via this mechanism (Farooqui and Horrocks, 1998).

Collectively these results indicate that Ca^{2+} mediates glutamate neurotoxicity through a number of mechanisms, and that these mechanisms often overlap and reinforce one another. The end result of this is that glutamate activation of NMDA and non-NMDA receptors results in a cascade of cellular events which leads to the disruption of cellular processes and increased levels of cellular damage. It is via these mechanisms that cellular and tissue degeneration secondary to the initial physical trauma are thought to occur.

As stated earlier there is little that can be done to ameliorate the effects of physical trauma to the spinal cord, after the fact. Rather the greatest hope for improving the functional outcome of SCI lies in understanding and manipulating the processes that follow on from this initial injury. These include the biochemical changes just discussed. By understanding how these biochemical changes lead to altered function within the CNS, methods may be developed by which some of the functional deficit of SCI may be returned.

While the loss of cellular energy production or membrane integrity may be deleterious to a tissue, the results of this are reasonably well defined. Less is known about how SCI may affect the expression of molecules important to the normal functioning of the CNS. One molecule that is involved in both the normal functioning of the CNS and the pathology of SCI is NO.

As mentioned earlier, NO is one of the most widespread signalling molecules in the body. It is involved in the regulation of function in the cardiovascular system, the immune system, the gastrointestinal system, urogenital system, the nervous system, skeletal muscle and cardiac muscle (Bredt and Snyder, 1992; Choi, 1993; Eu et al., 1999; Furchgott and

Vanhoutte, 1989; Hibbs et al., 1987; Janero, 2001; Kingwell, 2000; Marletta et al., 1988; Moncada et al., 1988b; Snyder, 1992). In the CNS NO is also involved in the regulation of a number of neuronal systems in the normal animal including, cell signalling, neuromodulation, brain development, response to stress, hormone release, memory and learning, sensory transmission and sexual behaviour (Esplugues, 2002; Keay and Bandler, 2001; Luo and Cizkova, 2000; Riedel, 2000; Shibuki and Okada, 1991). Because of this any changes in the amount of NO produced following SCI may result in changes in the normal functioning of these systems. Of particular relevance to the present discussion also, is the involvement of NO in a number of the deleterious effects of over-stimulation of glutamate receptors following CNS injury (Bredt and Snyder, 1992; Dawson et al., 1993; Dawson et al., 1991b).

It has been reported that while only 1-2% of neurons in the cerebral cortex synthesise NO, virtually all neurons are contacted by NOS positive fibres (Bredt and Snyder, 1992; Esplugues, 2002). In the spinal cord too NOS positive neurons are distributed throughout the entire rostrocaudal extent of the cord in addition to the dorsal root ganglia and the brain stem (Aimar et al., 1998; Nakamura, 1997; Onstott et al., 1993; Rodella et al., 1998; Trudrung et al., 2000; Vincent and Kimura, 1992).

It appears then, that study of NO may provide useful information concerning the effect of traumatic injury on the CNS. This is suggested by its ubiquity as a signalling molecule, its wide distribution throughout the CNS, the role it plays in a number of systems in the normal CNS and the role it appears to play in mediating a number of the injury-induced effects of glutamate. The following section will examine in more detail the role that NO may play in the CNS and will outline the benefit in studying this molecule in relation to neural function following SCI.

1.5 What is NO?

The role of NO as a biological agent was first discovered in 1977 by Ferid Murad and colleagues during their research into the activity of the enzyme, guanylate cyclase. When examining agents found to alter guanylate cyclase activity, Maurad found that nitroprusside, nitroglycerin and sodium azide all lead to an increase in cGMP production, and were associated with a relaxation of smooth muscle cells (Katsuki et al., 1977a; Katsuki et al., 1977b). While, at the time, it was not known why these compounds were able to activate

guanylate cyclase, Maurad suggested that they may do so through the formation of NO since he had found that NO was also able to increase the activity of guanylate cyclase (Arnold et al., 1977; Katsuki et al., 1977a). Soon after, it was discovered that this was indeed the case, and also that NO was the agent responsible for vasodilation in response to acetylcholine and was released from endothelial cells (Feelisch et al., 1994; Furchgott and Vanhoutte, 1989; Furchgott and Zawadzki, 1980; Hutchinson et al., 1987; Ignarro, 1989; Moncada et al., 1988a; Moncada et al., 1988b; Palmer et al., 1987). Since that time, NO has come under increasing attention, in particular due to the multiple roles it plays in the CNS.

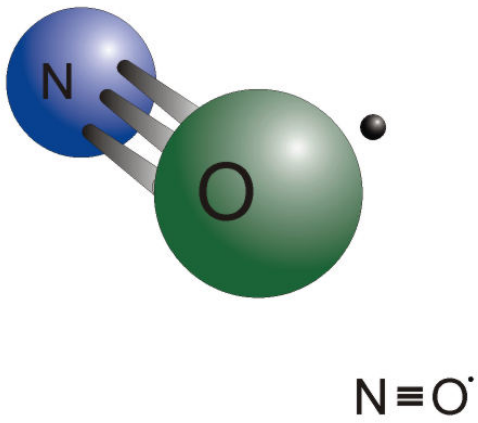


Figure 1.1 Molecular diagram of nitric oxide. Nitric oxide is a non-charged free radical, with the free electron associated with the oxygen atom. The nitrogen atom is covalently bonded to the oxygen atom via a triple bond.

Nitric oxide (Figure 1.1) is a gaseous free radical molecule and as such can not be stored within synaptic vesicles, as are the traditional neurotransmitters. Similarly it is not degraded by enzymatic break down, rather, NO activity is terminated upon reacting with target substrates (Arnold et al., 1977; Brann et al., 1997; Callsen-Cencic et al., 1999; Dawson and Snyder, 1994; Gally et al., 1990; Philippides et al., 2000). Thus the activity, and the regulation of NO is governed at the level of synthesis, being produced on demand, with the activity of the synthetic enzyme altered by a number of mechanisms. These include substrate limitation, Ca^{2+} sequestration, enzyme phosphorylation, NO autoregulation and a small peptide called protein inhibitor of NOS (Alagarsamy et al., 1994; Alderton et al., 2001; Bereta et al., 1994; Bredt et al., 1992; Egberongbe et al., 1994; Jaffrey and Snyder, 1996; Kiedrowski et al., 1992; Nathan and Xie, 1994).

NO is produced through the enzyme catalysed reaction converting L-arginine to citrulline. During this reaction L-arginine is reduced by electrons taken from the cofactor NADPH and the guanyl amino group of L-arginine is combined with oxygen to produce NO and citrulline

(Bredt and Snyder, 1990; Bredt and Snyder, 1992; Forstermann, 1994; Stuehr et al., 1991). See Figure 1.2 below for an illustration of this reaction.

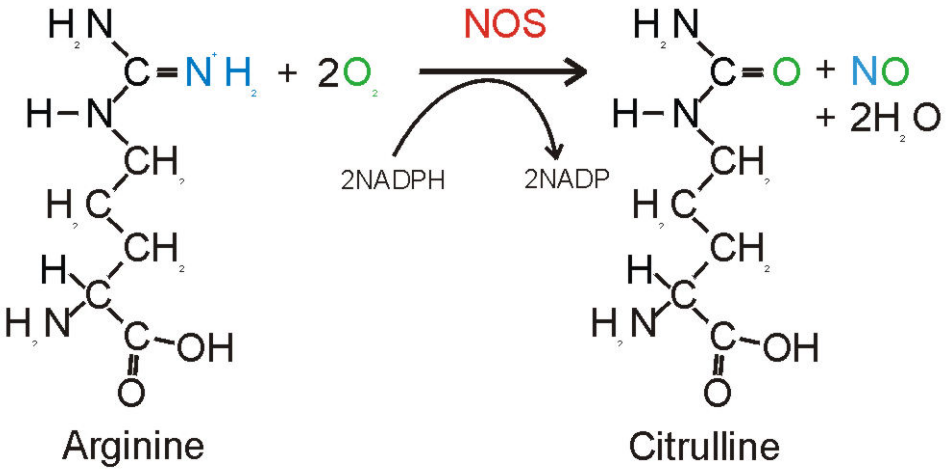


Figure 1.2 Reaction catalysed by NOS, combining the guanyl amino group of L-arginine with oxygen to produce NO and citrulline. NADPH is utilised as a cofactor supplying the electrons for the reaction.

Due to the labile nature of NO, it has been found to diffuse for up to 300μm in all directions from the point of synthesis in the CNS, potentially acting upon any cell within that radius (Gally et al., 1990; Kelm et al., 1988; Kim et al., 1993; Lancaster, 1997; Philippides et al., 2000). For this reason NO has been described as a volume transmitter (Callsen-Cencic et al., 1999; Philippides et al., 2000).

There is little general agreement in the literature as to the particular action of NO in the CNS. This confusion revolves around the difficulty in determining exactly what it is acting upon. NO does not bind to a cell surface receptor as do conventional transmitters, rather it is able to diffuse freely across membranes where one of its mechanisms of action is to upregulate the activity of the enzyme guanylate cyclase (GC, Bredt and Snyder, 1990; Bredt and Snyder, 1992; Gally et al., 1990).

Taken together these findings suggest that the action of NO appears to depend not so much on whether a target cell is in contact with a cell able to synthesise NO, but rather its distance from that cell. Also important is whether or not the putative target cell bares molecules capable of reacting to the presence of NO. Since NO is present in the normal

CNS, disruption to this may result in functional changes related to the role of NO in the CNS.

The following sections will examine the likelihood of this by firstly, reviewing briefly the synthetic enzymes that produce NO then outlining the principal functions of NO in the normal CNS. This will be followed by an examination of insults to the CNS that may affect NO synthesis, before, lastly, discussing the functional deficits that would therefore be expected to arise following SCI.

1.5.1 The Enzymes

While NO as a biological signalling molecule was first discovered in endothelial cells (Furchgott and Zawadzki, 1980; Moncada et al., 1988a; Palmer et al., 1988; Palmer et al., 1987), the chemical reaction by which it is produced from L-arginine was first elucidated in macrophages (Marletta et al., 1988) and later confirmed in other tissue (Moncada et al., 1989; Palmer and Moncada, 1989).

Later the synthetic enzyme for NO was purified from rat brain where it was found to require a cofactor, calmodulin, for its synthetic activity (Bredt and Snyder, 1990). Since that time two other isoforms have been discovered, baring 51-57% homology between them in the human. The three isoforms of NOS then are; neuronal nitric oxide synthase or nNOS which was the first purified and is the major NOS isoform present in neural tissue (also called NOS type I, NOS-I, NOS-1, brain NOS [bNOS] or neural constitutive NOS [neural cNOS]). The second type appears to be inducible in a number of cell types, particularly macrophages and neutrophils and is thus called inducible NOS or iNOS (also called NOS type II, NOS-II or NOS-2). The last isoform is the form expressed largely by endothelial cells and so is called endothelial NOS or eNOS (also called NOS type III, NOS-III, NOS-3 or endothelial cNOS). Both nNOS and eNOS are constitutively expressed. These three isoforms are the products of different genes, have different regulators, different levels of activity and are localised in different cellular compartments (Alderton et al., 2001; Bredt et al., 1991b; Forstermann, 1994; Murphy et al., 1993; Nathan and Xie, 1994)

All NOSs are dimers in their active form and contain binding sites for various co-factors essential to their synthetic activity including, haem, tetrahydrobiopterin (BH_4), NADPH and FAD and FMN, as well as the active site for binding of the substrate L-arginine (for review

see (for review see Alderton et al., 2001; Marletta, 1993). As illustrated in Figure 1.3, there is a binding site for calmodulin between FMN and BH_4, the binding of which allows the catalytic activity of the enzyme to take place. This occurs through the transfer of electrons between FAD and FMN. Without calmodulin binding this transfer of electrons would occur only very slowly (Matsuda and Iyanagi, 1999). Because of this, both eNOS and nNOS remain inactive until intracellular Ca^{2+} rises high enough to stimulate the binding of calmodulin to NOS resulting in the synthesis of NO for several minutes (Alagarsamy et al., 1994; Kiedrowski et al., 1992; Malinski and Taha, 1992). iNOS on the other hand was found to synthesise NO in an apparently Ca^{2+}- independent fashion (Bredt and Snyder, 1992).

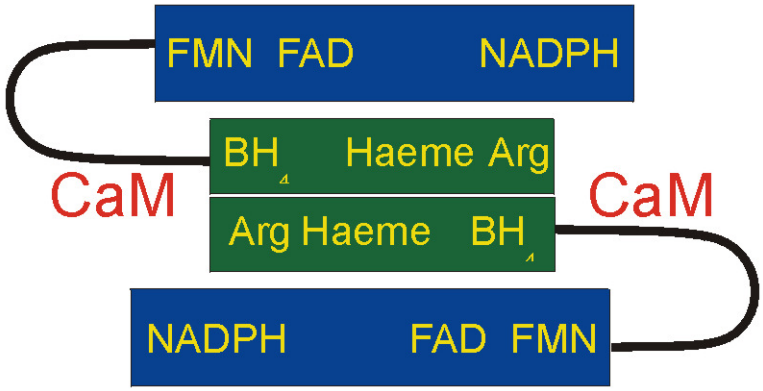


Figure 1.3 Schematic diagram of NOS dimer showing binding sites for NADPH, FAD, FMN, tetrahydrobiopterin (BH_4), haeme and arginine. Also shown is the binding site for calmodulin (CaM), located between the flavin molecules and BH_4.

However it is now known that the calcium dependence of nNOS and eNOS is due to an auto regulatory loop between the FMN and FAD groups resulting in an instability in the binding of calmodulin at resting levels of intracellular Ca^{2+}. iNOS which does not have this loop is able to bind calmodulin, activating the synthetic activity of the NOS, even at resting levels of Ca^{2+}, reducing the dependence of iNOS on calmodulin (Alderton et al., 2001; Cho et al., 1992). Due to the relative sensitivities to Ca^{2+} then, the two constitutive forms of NOS remain expressed within the cells and are modulated largely by Ca^{2+} influx, such as via EAA-mediated NMDA receptor activation (Alagarsamy et al., 1994; Kiedrowski et al., 1992). iNOS on the other hand due to its reduced dependence on Ca^{2+} concentrations is instead regulated at the level of gene transcription, being newly expressed following appropriate cell stimulation, such as by various cytokines (Bereta et al., 1994; Egberongbe et al., 1994; Koprowski et al., 1993; Lowenstein et al., 1993; Lyons et al., 1992; Minc-Golomb et al., 1994; Vodovotz et al., 1994; Wallace and Bisland, 1994).

Lastly once activated nNOS and eNOS synthesise only a small amount of NO (picomoles) for a short period of time (Hibbs et al., 1988; O'Dell et al., 1994), while iNOS generates large amounts of NO (nanomoles) for as long as 5 days (Vodovotz et al., 1994).

1.5.2 What does NO do in the CNS?

One of the most important observations of the present discussion is that the role played by NO in the normal CNS appears to be related to its anatomical localization. In other words there is evidence to suggest that cells synthesizing NO are involved in the normal functioning of the neural systems in which they are located. For instance, NO synthesizing cells have been observed in both the hippocampus and the cerebellum (Bredt et al., 1991a; Bredt and Snyder, 1992) and NO has been implicated in the processes of memory and learning (Esplugues, 2002). Many studies have been performed supporting and confirming this suggestion (e.g. Chapman et al., 1992; Izumi et al., 1992; Noda et al., 1997; Shibuki and Okada, 1991; Yamada et al., 1995). Secondly, NO synthesising neurons have also been located in the hypothalamus and appear to modulate the release of pituitary hormones (Brann et al., 1997; Riedel, 2000). Thirdly, the dorsolateral column of the periaqueductal gray matter of the mid brain (PAG) is characterised by a dense plexus of NO synthesising cells (Onstott et al., 1993; Rodella et al., 1998; Vincent and Kimura, 1992). Since this structure has been reported to mediate the defensive behaviours of an animal to a range of threats (Bandler et al., 1991b; Bandler and Shipley, 1994; Keay and Bandler, 2001), it is likely that NO may be involved in mediating a portion of this defensive response. There is evidence to suggest that this is the case. Inhibiting NOS in the dorsal PAG (d-PAG) results in a reduction in anxiety-like behaviour (de Oliveira and Guimaraes, 1999; Guimaraes et al., 1991; Guimaraes et al., 1994; Matheus et al., 1994). In addition, injection of NO donors into the dl-PAG resulted in a directed defensive flight reaction (de Oliveira et al., 2000b) which is similar to the response provoked by injection of NMDAr agonists into (Bandler and Carrive, 1988), or electrical stimulation of, the same area (Shaikh and Siegel, 1990).

Lastly high densities of NOS and reduced nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) have been found in the spinal cord. The superficial dorsal horn in particular contains the highest number of labelled cells in the cord (Aimar et al., 1998; Bredt et al., 1991a; Nakamura, 1997; Trudrung et al., 2000). Since these laminae primarily receive incoming nociception via Aδ- and C fibres (Baba et al., 1994; Keay and Bandler, 2002), it is thought that NO may be involved in mediating nociception. Certainly the

superficial dorsal horn has been proposed to form a part of an endogenous pain control mechanism that regulates the transmission of nociception to higher brain centres (Basbaum and Fields, 1978; Basbaum and Fields, 1984; Melzack and Wall, 1965), and the involvement of NO in nociception has recently been reviewed (Luo and Cizkova, 2000).

From this then, the functional role NO plays in the normal CNS appears to be directly related to its anatomical location within the nervous system. Any insult to the CNS that may alter NO synthesis may therefore be expected to result in physiological, and perhaps functional, changes within the particular system where the alteration occurred. The next section of this introduction will therefore address the insults to the CNS that have been examined to date, that appear to result in an alteration in NO synthesis. This will then be followed by a brief discussion on what the functional upshot of these alterations may therefore be.

1.5.3 *Factors that affect NO expression in the CNS*

There are a number of insults, both peripheral and central that have been found to alter the expression of NO in the CNS. These include spinal root avulsion and ligation, peripheral nerve transection, sciatic nerve ligation, peripheral inflammation, central ischemia and physical injury to the CNS.

One of the more commonly used models to study neuronal injury is that of spinal root avulsion which has been found to increase NO synthesis in, amongst other cells, the motoneurons of the ventral horn (He et al., 2000; Wu, 1993; Wu and Li, 1993; Wu et al., 1994). Spinal root ligation can also induce NOS expression in motor neurons and decrease the number of labelled cells in the SDH (Orendácová et al., 2000). These indicate that various treatments of the spinal nerve roots may alter CNS expression of NOS. Similar results have also been found to occur following ligation of the sciatic nerve. This procedure resulted in an increase in the number of NOS positive cells and fibres in the DRG and in the deep dorsal horn (laminae III-V), as well as a decrease in the number of NO labelling in Lam I and II (Cizkova et al., 2002; Goff et al., 1998). This decrease was observed for up to 28 days after ligation, reaching a maximum at 7 days (Goff et al., 1998).

While sciatic nerve transection is more often used as an injury model than spinal root avulsion, unlike avulsion, this model of nerve injury does not appear to result in NOS upregulation in the spinal motor neurons of the adult animal (Clowry, 1993; Fiallos-Estrada et al., 1993; Yu, 1994). It is thought that this effect may be due to the presence of Schwann cells on the remaining portion of the proximal length of the nerve (Wu, 1993). Sciatic nerve transection however does lead to a long lasting increase in NOS expression in the cells of the dorsal root ganglia (DRG), which project into the dorsal horn of the spinal cord and may therefore affect the amount of NO released there (Fiallos-Estrada et al., 1993; Verge et al., 1992).

Peripheral inflammation too has been shown to alter NOS expression centrally, resulting in increased expression of neuronal NOS in the dorsal horn after formalin injection into the hind paw (Herdegn et al., 1994; Lam et al., 1996), or postoperative abdominal inflammation (Dolan et al., 2003). As detailed above, the expression of iNOS, the isoform found principally in macrophages and glial cells, can also be induced in neurons following peripheral inflammation (Heneka and Feinstein, 2001; Petrov et al., 2000; Wu et al., 1998).

Focal cerebral ischemia has been found to induce an increase in expression of endothelial NOS (eNOS) in cerebral blood vessels (Veltkamp et al., 2002), as well as nNOS and iNOS in neurons of the cortex (Holtz et al., 2001). nNOS and iNOS expression was also increased in motor neurons of the spinal cord following transient occlusion of the abdominal aorta (Zhou et al., 1999). Nitrate production is increased in the hippocampus and the striatum after carotid artery occlusion indicating an increased production of NO due to reduction of perfusion (Adachi et al., 2000; Calapai et al., 2000), as is the proportion of nNOS positive cells immunoreactive for citrulline following ischemia (Eliasson et al., 1999). In addition to demonstrating that ischemia is capable of inducing new expression of nNOS and iNOS in neurons, it is also capable of inducing normally quiescent nNOS to begin synthesising NO.

Lastly, physical injury of the CNS tissue itself also appears to result in the alteration of NOS expression. The acceleration model of brain injury for instance has been shown to increase expression of iNOS in endothelial cells, macrophage-like cells and neurons (Petrov et al., 2000), while transection of the pituitary stalk results in an increase in intensity, and number of cells labelled with NADPH-d in the supraoptic nucleus and the paraventricular

hypothalamic nucleus (Lumme et al., 1997). Cortical stab wounds and cortical freeze lesion resulted in increased iNOS expression in macrophages and astrocytes around the wound (Grzybicki et al., 1998; Wallace and Bisland, 1994), while macrophages but not astrocytes appeared to express iNOS in response to an impounder weight drop spinal cord injury (Satake et al., 2000). nNOS is also up regulated by traumatic injury to the cortex (Rao et al., 1999) or stab wound to the hippocampus, in both neurons and astrocytes, while iNOS was upregulated in microglia (Stojkovic et al., 1998).

Spinal hemisection or full spinal transection results in an increase in the number of neurons expressing nNOS in the nucleus dorsalis as well as the red nucleus (Wu et al., 1994; Xu et al., 2000), while both weight drop SCI or static weight compression result in an increase in eNOS and nNOS activity in the spinal cord surrounding the injury (Diaz-Ruiz et al., 2002; Nakahara et al., 2002). Aneurism clip compression on the other hand results in a decrease in nNOS activity 15 minutes after compression (Miscusi, 2002).

Collectively, these studies indicate that there are a number of insults to both the peripheral and the central nervous systems that are capable of altering NO synthesis. Together with the previous section this indicates that the function of the neural networks so affected may also be changed. Little study has been done to examine the extent of such functional deficits, particularly in relation to deficits that have been brought about through injury-induced changes to NO synthesis. Previous studies in this regard have been restricted almost entirely to the effect of NO-changes on tissue pathology following injury, or only to changes in sensation as a result of injury. Few studies have examined the functional consequences of injury-induced changes in NO synthesis.

In order to examine the effect of NO synthesis following SCI, however, a method of easily and accurately detecting NO synthesising cells is required. One of the most widely used methods utilises the biochemistry of NO synthesis to label cells containing the synthetic enzyme NOS. This process is known as NADPH diaphorase (NADPH-d) histochemistry and will be discussed briefly here to determine the validity of using this marker for the detection of NO synthesising cells.

1.5.4 NOS and NADPH-d

As mentioned earlier, during the synthesis of NO, electrons are taken from the cofactor NADPH and are used to reduce L-arginine to citrulline and NO. As part of this process the electrons are first transferred from NADPH to the NOS enzyme. The presence of calmodulin then allows the electrons to then be transferred to the active site for the catalytic reaction that produces NO. However the electrons may also be passed to alternate electron acceptors such as nitroblue tetrazolium, leading to the reduction of these molecules (Bredt et al., 1991a; Bredt and Snyder, 1990). The reduction of soluble nitroblue tetrazolium results in the formation of an insoluble blue reaction product which can then be observed histologically, marking the cells that have produced it. These cells are said to contain NADPH-diaphorase (NADPH-d) activity. Since NOS is capable of transferring electrons from NADPH to nitroblue tetrazolium, this reaction has been found to be a useful and convenient one for the localisation of NOS protein intracellularly (Bredt et al., 1991a; Bredt et al., 1991b; Dawson et al., 1991a).

It must remembered however that this reaction does not necessarily mark the NOS enzyme itself, therefore any cell containing NADPH-d activity may or may not be NOS positive. Although, while less research has been done on either eNOS or iNOS, NADPH-d has been found to co-localise reliably with nNOS in neuronal tissue (Bredt et al., 1991a; Dawson et al., 1991b; Hope et al., 1991).

Experiments were performed in which nNOS was cloned into cultured kidney cells, which themselves did not express either NOS or NADPH-d activity. Following transfection of nNOS DNA, the kidney cells were found to express both NOS and NADPH-d activity in an amount proportional to the amount of DNA transfected (Dawson et al., 1991a). These results indicate that NOS possesses NADPH-d activity.

Examination of brain tissue shows that despite only 1-2% of cells containing NOS in the cerebral cortex, all of these cells demonstrated NADPH-d activity. So too did the NOS reactive cells and fibres found in the cerebellum, olfactory bulb, caudate-putamen, hippocampus, hypothalamus, various brainstem centres, the colliculi, periaqueductal gray and substantia gelainosa of the spinal cord (Bredt et al., 1991a; Dawson et al., 1991a; Vincent and Kimura, 1992). These co-localisation studies support the suggestion that NADPH-d reliably labels neurons positive for NOS.

Despite this however a number of studies have reported that, while NADPH-d may co-localise with both eNOS and nNOS in the brain, there are cells that demonstrate NADPH-d activity but not NOS positivity, probably representing another enzyme using NADPH as a co-factor for electron transfer (Hope et al., 1991; Wehby and Frank, 1999). Following hind paw inflammation too it was found that despite NADPH-d being co-localised with NOS containing neurons, NADPH-d activity was occasionally disassociated with NOS immunoreactivity (Traub et al., 1994). The same has also been found in the periphery with both the liver and the adrenal cortex demonstrating NADPH-d activity but not NOS positivity (Bredt et al., 1991a). As with similar studies finding a dissociation in the brain, it was suggested that this peripheral dissociation may reflect other non-NOS enzymes utilising NADPH-d as a cofactor for the transfer of electrons, thereby demonstrating NADPH-d activity. This suggestion is supported by chromatographic studies attempting to purify NADPH-d activity. These studies found that NADPH-d activity separated as a number of protein bands that were separate from NOS immunoreactivity. NOS bands however all showed NADPH-d activity (Dawson et al., 1991a; Hope et al., 1991).

Lastly it has been observed that NADPH-d appears to be more sensitive than NOS immunoreactivity particularly in respect to smaller and less strongly labelled neurons (Herdegn et al., 1994). Failure to find co-localisation between NOS and NADPH-d may on occasion be related to the sensitivity of the antibody used for NOS compared with the sensitivity of the NADPH-d reaction. In addition it has been found that the NADPH-d reaction is influenced by the fixation of the tissue (Nakamura, 1997). All nNOS neurons exhibit NADPH-d activity, however this activity is altered by the level and type of fixation and so interpretation, particularly of earlier results, must be made with this in mind (Stanarius et al., 1997).

Taken together these findings indicate that as suggested earlier NOS is able to transfer electrons from NADPH to either arginine, or to another electron acceptor such as nitroblue tetrazolium. Therefore NOS containing neurons always demonstrate NADPH-d activity. Other enzymes however such as DT diaphorase and NADPH dehydrogenase in neurons, or possibly oxidative enzymes involved in steroid production in the adrenal cortex, may also demonstrate this activity (Bredt et al., 1991a; Hope et al., 1991; Wehby and Frank, 1999). These result in instances where NADPH-d activity does not co-localise with NOS positivity.

It is suggested however that despite these minor differences that, in the CNS, virtually all NADPH-d positive neurons represent NOS containing cells (Bredt et al., 1991a; Dawson et al., 1991a; Hope et al., 1991; Saxon and Beitz, 1996; Vincent and Kimura, 1992).

It would appear then that with the exception of very rare instances, NADPH-d histochemistry offers an accurate and highly sensitive marker for NO synthesising cells. Care must be taken with fixation, but examination of labelling in conjunction with previously reported literature is likely to provide an accurate measure of the number of NO synthesising cells following SCI. In order to examine the effect of SCI on the distribution of NADPH-d positive neurons in the spinal cord, a brief review of the distribution of these cells in the normal animal will be given here.

1.5.5 Distribution of NADPH-d positive cells in the Normal CNS

Even before NADPH-d activity was reported to coincide with NOS immunoreactivity, it had been reported that a number of cell populations in the spinal cord demonstrated this enzymatic activity (Mizukawa et al., 1989; Scherer-Singler et al., 1983; Thomas and Pearse, 1964). Descriptions of labelled cells have indicated that NADPH-d histochemistry allows the demonstration of very detailed neuronal morphology, of a quality similar to that achieved by Golgi staining (Valtschanoff et al., 1992a).

When the distribution of these cells is examined, studies reveal that the neuronal somata stained by this process are found most prominently in laminae I and II and lamina X of the entire spinal cord, and also the intermediolateral cell column (IML) of thoracic levels. In addition to these, a few neurons are found sparsely distributed in laminae III-V and the ventral horn.

The morphology of NADPH-d positive cells in laminae I and II, were small bipolar or fusiform cells (~6-10μm), and were elongated along the rostrocaudal dimension with poorly branching dendrites (Aimar et al., 1998; Bredt et al., 1991a; Nakamura, 1997; Spike et al., 1993; Tang et al., 1995; Trudrung et al., 2000; Valtschanoff et al., 1992a). The dedritic trees of lamina II NADPH-d positive cells was in the rostrocaudal axis (Aimar et al., 1998). Under electron microscopy the nitroblue tetrazolium reaction product can be clearly seen filling the cytoplasm of the somata of positive cells and their processes (Aimar et al., 1998). In addition to labelled somata well defined NADPH-d positive fibres can be found

throughout the spinal cord. The plexuses of greater density may be found in lamina I and II outer, the IML of the thoracic cord and also the sacral parasympathetic plexus of the sacral cord (Aimar et al., 1998; Laing et al., 1994; Spike et al., 1993; Valtschanoff et al., 1992a). Most of these run rostrocaudally within the laminae of origin (Aimar et al., 1998; Bredt et al., 1991a; Nakamura, 1997; Spike et al., 1993; Tang et al., 1995; Trudrung et al., 2000; Valtschanoff et al., 1992a).

In addition to cells in lamina I and II, there was also a distinct population of fusiform neurons along the border of laminae IIi and III. The dendrites of these neurons occasionally extended short distances up into lamina IIi as well as ventrally for up to 100μm, while axons ran rostrocaudally, generally within the same laminae, for up to several hundred micrometres (Valtschanoff et al., 1992a; Valtschanoff et al., 1992b).

Lamina X demonstrated a loosely packed but prominently labelled population of neurons. Neurons were a mixture of multi- and bipolar cells with long dendrites. These dendrites extended throughout much of the spinal grey matter, and were sometimes observed to enter the lateral funiculus. Many dendrites also crossed the midline and travelled in the contralateral grey matter, while axons were observed to travel rostrocaudally (Nakamura, 1997; Spike et al., 1993; Tang et al., 1995; Valtschanoff et al., 1992a).

Lastly a prominent population of NADPH-d positive cells were present in the intermediolateral cell column (IML), which was characterised by a very densely packed column of NADPH-d positive, multipolar neurons. No unlabelled cells have been found in the IML (Valtschanoff et al., 1992a). Neurons were medium sized and triangular to multipolar in shape (Anderson, 1992; Nakamura, 1997; Tang et al., 1995). The neurons form regularly spaced clusters along the rostrocaudal axis of the of the thoracic cord. The dendrites of these cells were long and generally organised into bundles. Bundles of processes ran largely in three directions, towards the central canal, laterally through the white matter, and in a rostrocaudal direction connecting other clusters of neurons that make up the IML (Valtschanoff et al., 1992a).

Only a few neurons were seen, sparsely distributed in the deeper dorsal horn laminae (III-VI). These neurons were larger than those in more superficial laminae and were multipolar to pyramidal in shape. Their processes were long and radiating, extending for up to

1000μm rostrocaudally and also dorsoventrally down towards lamina X. Axons ran either ventrally or into the dorsal funiculus. In addition there were a few smaller bipolar neurons, largely in sections taken from the cervical or lumbar cord (Aimar et al., 1998; Nakamura, 1997; Spike et al., 1993; Tang et al., 1995; Valtschanoff et al., 1992a). Electron microscopy in these deeper laminae indicate that NADPH-d positive processes were largely dendrites which were sparsely scattered through out the laminae (Aimar et al., 1998).

In the ventral laminae there were only a few scattered bipolar neurons. The dendrites of these cells radiated in the horizontal plane from the medial to lateral borders of the ventral horn and were reported to resemble Renshaw cells. A few axons could also be seen to exit with the ventral roots. (Spike et al., 1993; Valtschanoff et al., 1992a)

This pattern of labelling is identical to that shown with NOS immunoreactivity (Dun et al., 1993; Laing et al., 1994; Saito et al., 1994).

Given then that NO appears to play a functional role in the CNS, related in particular to the neural networks in which it is located, it would be of interest to examine how changes in NO synthesis may be altered by various pathological states. As detailed above there are a number of insults which have been shown to alter NO synthesis. Due to the functional nature of NO in the CNS it is likely that any changes in the synthesis of this molecule would alter the function of the system of which it forms a part. The following section will examine the major pathological effects mediated by NO reported in the literature.

1.5.6 Effect of altered NO expression

Tissue pathology

As indicated above, nervous system injury may result in altered NO synthesis. One process mediated by this alteration appears to be, at least in part, the tissue degeneration that results from injury. While the precise mechanism is not known, manipulating NO synthesis following injury has been found to alter the tissue degeneration that resulted from injury. Inhibiting NOS with L-NAME for instance resulted in a reduction in both cerebral and striatal infarct volumes following middle cerebral artery (MCA) occlusion (Buisson et al., 1992; Nagafuji et al., 1992; Nowicki et al., 1991). The intravenous administration of nNOS specific inhibitor ARL 17477 was also found to reduce infarct volume following MCA occlusion (Zhang et al., 1996).

In the spinal cord, spinal hemisection was found to result in spongiosis, demyelination and oedema. Topical application of anti-NOS antibodies was found to reduce the sponginess, oedema and to reduce NOS expression. Pre-absorbed antiserum had no effect on NOS expression (Sharma et al., 1996). Similar findings were reported upon static compression of the spinal cord. The resulting cavitation was reduced with administration of NOS inhibitor L-NMMA, as was the injury-induced increase in firing latency (Suzuki et al., 2001). This last result indicates a functional change to these neurons as well. Intraocular injection of non-specific NOS inhibitors NOLA or L-NAME increases retinal ganglion cell survival after axotomy (Koeberle and Ball, 1999).

These results indicate that injury-induced changes to NO synthesis may indeed result in at least some of the tissue pathology observed. In addition to the gross morphological changes discussed above, the other principal effect of injury-induced alterations in NO synthesis studied is a change in sensory processing, particularly nociception.

Sensory effects

As discussed earlier, NO has been reported to be involved in sensory transmission in the normal animal (Basbaum and Fields, 1978; Basbaum and Fields, 1984; Luo and Cizkova, 2000; Melzack and Wall, 1965). Thus the changes in NO synthesis following injury observed in areas such as the SDH of the spinal cord may indicate a resulting change in function (Goff et al., 1998; Orendácová et al., 2000).

In support of this suggestion are studies examining the effect of NO on the neuropathic pain that results from nervous system injury. Loose ligation of the sciatic nerve, for instance, results in a thermal hyperalgesia. This condition is reversed by L-NAME, Nω-nitro-L-arginine, or methylene blue (guanylate cyclase inhibitor), but not the inactive isomer D-NAME or saline, indicating that the hyperalgesia produced depends on NO synthesis and GC activation (Meller et al., 1992; Salter et al., 1996; Yamamoto and Shimoyama, 1995). Hyperalgesia was blocked by L-NAME both at the level of the spinal cord, but also at higher supraspinal centres responsible for processing nociception (Salter et al., 1996). The increase in DH neuron firing rate produced by formalin-induced inflammation of the hind paw was also found to be inhibited by L-NAME (Haley et al., 1992).

Intradermal injection of capsaicin has been reported to result in the sensitisation of spinothalamic tract neurons, and that this sensitisation may be due to a decrease in descending inhibitory tone from supraspinal centres (Lin et al., 1999a). Blocking NO synthesis with microdialysis of nNOS specific inhibitor, 7-NI into the dorsal horn, reduced the sensitisation induced by capsaicin injection (Lin et al., 1999b). Also, both L-NAME and 7-NI were found to dose dependently reduce the allodynia induced by spinal cord ischemia. This reduction in allodynia by blocking NOS was reversed by treatment with L-arginine, the substrate used in the synthesis of NO (Hao and Xu, 1996). Lastly, behavioural experiments looking at the attenuation of autotomy behaviour, which is believed to be an indicator of neuropathic pain, also found that L-NAME in addition to NMDA receptor antagonists reduced autotomy behaviour in response to brachial plexus transection (Wong et al., 1998).

Taken together these results provide strong evidence that the changes in NO expression as a result of, for example, loose ligation of the sciatic nerve, formalin-induced inflammation, intradermal injection of capsaicin or peripheral nerve axotomy, may result in central changes in sensory processing. These changes may in turn be responsible for the development of chronic neuropathic pain following injury. Further study is needed to elucidate the underlying mechanisms that may be responsible for this process however.

Lastly, one of the few other functional effects examined in relation to injury-induced NO changes are the motor deficits. While the injury-induced expression of NO in motoneurons of the spinal cord have been interpreted as responsible for decreased motor control, there is some evidence to suggest that alterations in NO may *follow* changes in motor function.

Motor effects

Spinal root avulsion, for instance, has been found to result in NOS expression and subsequent degeneration of motoneurons (MN). Some of this degeneration was attenuated with the general NOS inhibitor nitroarginine (Wu and Li, 1993), suggesting that NO may be responsible for loss of motor function. In addition, the motor neurons of the wobbler strain of mouse have also been found to express NOS. The motoneurons of this strain of mouse degenerate due to a lack of the potent antioxidant enzyme superoxide dismutase (SOD). This again indicated that NO may be responsible for the degeneration of the motor neurons observed in this animal model.

In a study examining the hind limb immobilisation of the guinea pig, however, it was found that simply immobilising the limb resulted in increased NO synthesis in motor neurons (as indicated by increased NADPH-d expression). This increase in NO expression did not result in tissue damage, and NADPH-d expression was reversible upon release of the limb (He et al., 1997). This result indicated that NO synthesis in MN may be as a result of loss of function, rather than NO synthesis being the cause of the loss of function. It was speculated by the authors of the latter study that NO may be produced in MN as a protective mechanisms against possible neuronal degeneration following damage.

While little more has been done to establish directly the degree to which alterations in NO synthesis in MN may be responsible for motor dysfunction, these results indicate that it may be a possibility.

1.6 Fos

As discussed above, a number of insults to the nervous system appear to result in changes to NO expression within the CNS, and it is likely that these changes may result in alterations in neuronal function. While a number of studies have reported experimental evidence for the involvement of NO in the pathology of nervous system injury, little has been done to examine the effect of traumatic contusion of the spinal cord on this molecule. This form of SCI is the most common clinically, particularly as a result of motor vehicle or sporting accidents (Cripps and O'Connor, 1998; O'Connor and Cripps, 1998). Therefore a better understanding of the processes that underlie morbidity as a result of this form of injury may serve to assist further research aimed at preventing them.

As the anatomical evidence discussed here suggests, NO may be involved in the functional changes observed following injury. A means of examining neuronal activity would therefore be useful in determining how injury-induced changes in NOS expression may be related to changes in neuronal activity following SCI.

Two principal methods for examining activity at the neuronal level are electrophysiology and also immunohistochemistry. Electrophysiology involves the measurement of electrical activity of individual or groups of neurons, while immunohistochemistry involves labelling neurons for markers of neuronal activity. The latter allowing visualisation of the neurons of interest. The advantages of electrophysiology are that measurements can be made from

live cells, with a very high temporal resolution. This means that very rapid responses can be recorded. Also because cells are alive they can be observed continuously over time, and can be treated with various substances to probe and test the function observed. The disadvantages however include that many tissue preparations for electrophysiology require the use of anaesthetics and paralysis or decerebration, particularly to record at the single cell level. Also, they can generally only record from a limited number of cells at once and the electrodes are invasive.

Measuring neuronal activity immunohistochemically on the other hand has the drawbacks that the technique requires dead, and for best results, fixed tissue, and therefore cannot provide information on neuronal activity over time. Also, because of this, the technique can only provide limited information about the fluxes of ions or other dynamic cellular changes. Despite these however, using an anatomical marker for activated neurons allows examination of the entire CNS in an awake unanaesthetised animal and also allows examination of the associations between those neurons and other cell markers and their anatomical locations. Lastly it has the further advantage that it is not as technically difficult to perform and requires less specialised equipment.

In order to examine the relationship between NO and changes in neuronal function that occur as a result of SCI, the present study will therefore utilise an immunohistochemical measure of neuronal activity. This will allow easy examination of the distribution of NO producing cells, both in the normal animal and in the injured animal, and also allow them to be compared to where in the CNS neuronal activity had been altered. One of the most widely used molecules for examining neuronal activity is fos protein.

1.6.1 What is Fos?

c-fos is a member of a family of genes known as the immediate early onset genes (IEG) which are activated and involved in regulating the expression of late-onset genes and are therefore thought to act as a stimulus-transcription coupling mechanism (Morgan and Curran, 1991). IEG are defined as genes that are capable of being expressed in the presence of protein synthesis inhibitors, and thus require no protein synthesis for their own transcription (Guzowski, 2002). Activation of a range of receptors and in particular the NMDA receptor, has been found to activate IEG including the c-fos gene within minutes of receptor stimulation, likely as a result of Ca^{2+} influx (Chapman et al., 1995b; Greenberg et

al., 1985; Greenberg et al., 1992; Halazonetis et al., 1988; Roche et al., 1996; Szekely et al., 1989).

The c-fos gene codes for a nuclear protein that forms a dimer with another IEG gene product, jun protein, via a leucine zipper motif. The dimer then binds to the promoter region of late-onset genes that bare the activator protein 1 recognition site (Halazonetis et al., 1988; Herdegen et al., 1991; Ruggiero et al., 1997; Ryseck and Bravo, 1991). These include proteins and peptides, such as the opioid peptides (Naranjo et al., 1991; Rauscher et al., 1988), enkephalin (Comb et al., 1988; Hunter et al., 1995; Won et al., 1998b; Ziolkowska et al., 1998), tyrosine hydroxylase (Gizang-Ginsberg and Ziff, 1990), nerve growth factor (Hengerer et al., 1990), heme oxygenase (Alam and Den, 1992) and dynorphin (Hermanson et al., 1998; Hunter et al., 1995; Naranjo et al., 1991; Ziolkowska et al., 1998). Thus it has been suggested that Fos may act as a 3rd messenger to activate target genes involved with long term adaptive change in response to extracellular stimuli (Morgan and Curran, 1989; Morgan and Curran, 1991; Szekely et al., 1989).

1.6.2 The time course of Fos expression

One of the benefits of using Fos as a marker for neuronal activation is that it is expressed very rapidly after cellular stimulation. While the precise time until peak expression depends on the stimulus and mode of application, c-fos gene transcription has been reported to begin within minutes (Greenberg et al., 1985; Greenberg et al., 1992; Szekely et al., 1989), while mRNA levels peak by 30-45 minutes and return to control levels by as little as 2 hours (Delander et al., 1997a; Gu et al., 1997; Jacobs et al., 1994; Yakovlev and Faden, 1994). Fos protein expression on the other hand follows the mRNA expression and reaches a maximum by about 2-4.5 hours after the stimulus returning to baseline generally by 24 hours (Bullitt et al., 1992; Gu et al., 1997; Luo et al., 1998) (Herdegn et al., 1994; Hunt et al., 1987; Williams et al., 1990).

1.6.3 The relationship between NO synthesis and Fos expression

Of particular relevance to the present series of studies is the suggestion that Fos expression may be related to NO synthesis. In vitro studies have demonstrated that NO can promote expression of fos via activation of the cyclic GMP pathway in PC12 cells (Haby et al., 1994), while other studies have described an anatomical relationship between the two molecules. Following peritoneal injection of acetic acid, for instance, NADPH-d positive

processes in the parabrachial complex, the hypothalamus and the cortex were observed to be adjacent to or apposed to fos labelled neurons (Rodella et al., 1999). Similarly, NADPH-d positive cells of the deep dorsal horn of the spinal cord appear to be associated with fos labelled cells. In this region NADPH-d positive neurons appear to contact up to 15 immediate early gene (IEG) labelled cells per section. Since both NADPH-d fibres and IEG labelled neurons are relatively rare in the DDH, this relationship suggests that NO may be involved in IEG induction in these cells. No such relationship was observed in the superficial dorsal horn however (Herdegn et al., 1994). In other studies nitroglycerin has been shown to induce fos in the paraventricular and supraoptic nuclei of the hypothalamus, sites where the induced fos was related to NOS containing neurons or processes (Amir et al., 1997; Tassorelli and Joseph, 1995).

While this physical proximity between NOS- and fos positive cells suggests NO may induce fos expression directly, at least some of this anatomical correlation may be related to the role of NO on descending or spinal inhibitory systems. As reviewed in the previous chapter, NO is involved in the modulation of sensory transmission including the transmission of nociception (Luo and Cizkova, 2000; Riedel and Neeck, 2001) as well as through activation of spinal- and supraspinal inhibitory mechanisms (Pineda et al., 1996; Prast and Philippu, 2001; Tsuruoka et al., 2003). Thus NO may indirectly affect neuronal activity and therefore fos expression through these excitatory or inhibitory mechanisms. Indeed stimulating animals with systemic nitroglycerine results in increased fos expression in regions of the brain stem involved with regulating descending inhibitory tone such as the locus coeruleus, nucleus tractus solitarius (Tassorelli and Joseph, 1995). Combined studies have also found NO to be involved in both nociceptive transmission and descending inhibition, and that inhibition of NO synthesis may have opposite effects on fos expression depending on the mechanism studied (Gao and Qiao, 1998).

While generally speaking NO has been reported to be involved in the increase in fos expression as a result of noxious stimulation (e.g. Chapman et al., 1995a; Lee et al., 1992; Roche et al., 1996; Wu et al., 2000), the effect of NO on Fos expression appears to relate to the cell types examined. NO synthesis for instance has been reported to inhibit fos expression in the hippocampus (Won et al., 1998a).

Lastly, studies into the molecular mechanisms of fos expression indicate that NMDA receptor activation may induce Fos through calmodulin-dependent phosphorylation by CREB (Dampney and McAllen, 1988), though NO may also induce CREB which inturn induces Fos (Ohki et al., 1995) perhaps though potentiating Ca^{2+} influx (Peunova and Enikolopov, 1993). Though other research has found that the NOS gene, at least in humans, contains CREB and FOS binding sites (Hall et al., 1994) suggesting that Fos may play a role in the induction of NO.

Thus there appears to be an anatomical relationship between NO and fos in the CNS. This may be related to a direct action by NO of fos expression or may involve the influence of NO on sensory transmission and therefore neural activity indirectly. In addition it is noted that due to the molecular mechanisms of fos induction via CREB, fos expression may be stimulated via the same initial mechanisms which can lead to the synthesis of NO. These results indicate that there is good reason to expect that NO synthesis may be involved in fos expression, or at least in the mechanisms that in turn relate to fos expression. Thus if as suggested fos is reliable marker for neuronal activity, alterations in NO synthesis as a result of injury may in turn be related to alterations in neural activity.

1.6.4 Justification for using fos as a marker for neuronal activity

Related to its function as a possible regulator of long term cellular adaptation through the promotion of late-onset gene transcription (Morgan and Curran, 1989; Morgan and Curran, 1991; Szekely et al., 1989), fos has most often been used as a histological marker for neuronal activation. In particular since it was first shown that fos could be induced in the spinal cord in response to the noxious application of both radiant heat or mustard oil (Hunt et al., 1987), fos has been extensively used in the study of nociception as well as an increasing number of other studies.

In these studies very little fos is observed in control animals (Bullitt, 1990; Ruggiero et al., 1997), however fos may be induced by a wide range of stimuli. These include, spinal cord transection (Ruggiero et al., 1997), loose constrictions of the cauda equina (Orendácová et al., 2000), sciatic nerve chronic constriction injury (CCI) (Delander et al., 1997a; Huang and Simpson, 1999b; Orendácová et al., 2000; Presley et al., 1990), sciatic nerve transection (Gu et al., 1997; Molander et al., 1992; Molander et al., 1998), sciatic nerve crush (Molander et al., 1998), noxious mechanical stimulation (Bullitt, 1991; Clement et al., 1996;

Lima et al., 1993; Menetrey et al., 1989), neuropathic pain such as allodynia (Siddall et al., 1999b), irritation of the lower urinary (Birder and de Groat, 1992), itch (Yao et al., 1992), nerve growth factor (Curran and Morgan, 1985; Milbrandt, 1986), acetylcholine (Greenberg et al., 1986), intraplantar bee venom (Luo et al., 1998), capsaicin (Wu et al., 2000), Freund's adjuvant (Woolf et al., 1994), or formalin (Elliott et al., 1995; Hagihira et al., 1997; Lima et al., 1993; Presley et al., 1990; Todd et al., 1994; Williams et al., 1990), intramuscular formalin or carrageenan (Clement et al., 2000), immersion of the hind foot into a >50°C hot water bath (Huang and Simpson, 1999a; Williams et al., 1990), ip acetic acid (Rodella et al., 1999), exposure to radiant heat >58°C (Lima et al., 1993) intravenous injection of 5-HT (Clement et al., 2000), or spinal injection of quisqualic acid (Abraham and Brewer, 2001).

Since most of these stimuli are nociceptive it is thought that fos may be a marker for cells involved in nociceptive transmission. Indeed following a nociceptive stimulus the neurons that are induced to express fos are localised in regions of the neuraxis related to the transmission and processing of nociception. Including laminae I and II (Bullitt, 1991; Clement et al., 2000; Hunt et al., 1987; Luo et al., 1998; Orendácová et al., 2000; Presley et al., 1990; Ruggiero et al., 1997; Woolf et al., 1994), and lam IV and V of the spinal cord (Bullitt, 1991; Clement et al., 2000; Hunt et al., 1987; Luo et al., 1998; Presley et al., 1990; Ruggiero et al., 1997; Wu et al., 2000), the upper cervical spinal cord (Clement et al., 2000; Keay et al., 1997), the PAG (Keay and Bandler, 1993; Keay et al., 2002), the locus coeruleus (Baulmann et al., 2000; Tsuruoka et al., 2003), ventrolateral medulla (Bullitt, 1990) and thalamus (Bullitt, 1989; Bullitt, 1990).

In addition fos expression in the spinal cord appears to be dependant upon activity of small-diameter, high threshold primary afferents (Hunt et al., 1987; Lynn and Shakhanbeh, 1988). Also many treatments which modify pain related behaviours are also found to modify the fos expression induced by these treatments for instance nociception induced fos expression is reduced by analgesic drugs such as morphine (Gogas et al., 1991; Jasmin et al., 1994b; Presley et al., 1990) or drugs which interfere with nociceptive transmission, eg noradrenaline (Jones, 1992) or antagonists to NMDA receptors (Chapman et al., 1995b; Elliott et al., 1995). Lastly fos labelling in animals with allodynia (hypersensitivity to non-noxious stimuli) following contusive spinal cord injury is significantly increased in lam I and II following a non-noxious stimulus (Siddall et al., 1999b). All of these observations again suggest that fos expression may be particularly induced by noxious stimuli. The benefits

and drawbacks of using Fos as a marker for studying nociception has been reviewed previously (Harris, 1998).

There is evidence to suggest however that it may not be nociception specifically that induces the expression of fos. Activation of NMDA receptors for instance has been reported as a possible mechanism behind the expression of fos (Cole et al., 1989; Elliott et al., 1995; Jacobs et al., 1994; Mitsikostas et al., 1998; Szekely et al., 1989; Zhang et al., 1998), likely through the influx of Ca^{2+} (Bading et al., 1993; Griffiths et al., 1998; Morgan and Curran, 1986; Szekely et al., 1989). The cells from many of the areas in which fos is induced have been found to express glutamate terminals and EAA receptors (Battaglia and Rustioni, 1988; Jansen et al., 1990; Menetrey et al., 1989; Tavares et al., 1993). In addition fos can be induced in neurons following a number of non-noxious stimuli such as walking (Jasmin et al., 1994a), non-noxious somatic stimulation (Hunt et al., 1987), swim stress (Bellchambers et al., 1998), disruption of sleep (Grassi-Zucconi et al., 1993), strong odours (Guthrie et al., 1993; Jin et al., 1996), restraint (Chowdhury et al., 2000), novel environment (Handa et al., 1993; Wirtshafter et al., 1998), tactile exploration (Staiger et al., 2002), sexual behaviour (Curtis et al., 2003), predation (Comoli et al., 2003), airpuff startle (Palmer and Printz, 1999) and the separation stress of pups from their mother (van Oers et al., 1998).

Anatomically non-noxious stimulation induces expression of fos in laminae of the spinal cord responsible for transmitting non-noxious stimuli, including lamina II inner, lamina III and lamina IV (Hunt et al., 1987; Jasmin et al., 1994a; Sheng and Greenberg, 1990). A number of studies have also been done that indicate that fos may be induced in systems that are involved in the regulation of synaptic transmission, as apposed to simply being induced by direct stimulation of nociceptive neurons. Administration of fos anti-sense for instance was found to increase pain behaviour in response to noxious stimuli (Hunter et al., 1995), indicating fos expression was required for anti-nociception. In support of this is the observation that formalin injection has also been found to up regulate fos expression in GABAergic neurons (Todd et al., 1994). Fos is also able to activate endogenous opioid systems (Hunter et al., 1995; Morgan and Curran, 1991; Naranjo et al., 1991; Noguchi et al., 1991; Sheng and Greenberg, 1990; Todd et al., 1994), and is activated in descending inhibitory systems following nociceptive or stressful stimuli (Bellchambers et al., 1998; Pinto et al., 2003; Sandkühler, 1996). Lastly activation of antinociceptive structures in the medulla results in fos expression in some spinal neurons (Bett and Sandkuhler, 1995),

indicating that supraspinal structures are also able to induce fos in other parts of the CNS, and not necessarily as a result of incoming nociception.

Taken together these results indicate that fos expression does not occur simply due to the direct peripheral stimulation at the primary or secondary synapse, since activation of descending projections also appear to activate fos expression. This indicates that modulation of neural activity, whether by incoming peripheral stimuli or through the activation of modulatory circuits within the CNS its self is more likely to be responsible for fos expression. The ability of non-noxious stimuli to induce fos expression indicates that fos expression is not solely indicative of nociception despite the extensive use of fos in these types of studies. Though stimuli that induce the expression of fos generally appear to be stimuli that are stressful to the animal, or in other words that stimulate levels of activity in neurons that are above their basal level of activity. Hence the preponderance of studies indicating that nociception is capable of inducing fos. The laminae specificity discussed above for either nociceptive or non-noxious stimuli further indicate that only neurons that are receiving a stimulus that is capable of activating them above basal levels is likely to induce fos. Neurons in the SDH, for instance respond to noxious stimulation, thus foot immersion in a water bath below 46°C does not induce fos expression. Temperatures greater than this however have been found to result in an increase (Abbadie et al., 1994).

As a result of evidence such as that presented here, fos has become well established as a valuable marker for activated neurons (Abraham and Brewer, 2001; Bullitt, 1990; Chan et al., 1993; Hochstenbach and Ciriello, 1996; Hughes and Dragunow, 1995; Hunt et al., 1987; Morgan and Curran, 1986; Morgan and Curran, 1991; Munglani and Hunt, 1995; Parkes et al., 1993; Sagar et al., 1988; Sheng and Greenberg, 1990).

As with any technique there are a number of limitations with using fos as a marker for activated neurons. These include the observation that not all neurons express fos when activated eg DRG cells or those of particular nuclei of the thalamus; or that fos is an image of a particular instant in time (at the point of animal sacrifice) and results must be interpreted with this in mind; or that it can not necessarily be excluded that a neuron that does not express fos has not been activated (Bullitt, 1990; Harris, 1998). So long as these are kept in mind, it appears likely that fos may be a reliable, reproducible and well reported tool to determine the level of neuronal activity.

1.7 Summary

Spinal cord injury results in functional deficits that are both expensive to treat and debilitating to the patient. There are however few successful therapies for this form of injury due to the low regenerative capacity of mammalian neural tissue. For this reason research must be performed to better understand the mechanisms of injury that lead to loss of function. In this way perhaps more effective treatments may be developed.

As the range of experimentation possible is severely limited in humans for ethical and legal reasons, a number of animal models of SCI have been developed. A range of models were discussed in this chapter and it was determined that the weight drop model of SCI would be an appropriate model for the present thesis. It is most similar to the injury presented in the clinical setting and allows study of the full range of pathologies associated with this type of injury.

It was observed that contusive SCI resulted in a physical disruption to the spinal cord, followed by a series of biochemical processes that are set into motion by the initial injury. These so called secondary processes result in greater functional deficit than the initial injury. As little can be done to ameliorate the initial physical disruption of the spinal tissue after the fact, it was suggested that study of the secondary processes may offer some insight into potential therapies. Principal amongst the secondary mechanisms of injury is the release of toxic amounts of excitatory amino acids from the intracellular compartment. These inturn activate both NMDA and non-NMDA type glutamate receptors. Examination of this process of neuronal toxicity indicate that two principal mechanisms are at work, an initial neuronal swelling, and in severe cases lysis, mediated by ion influx. In addition there is a more delayed mechanism of cell death mediated by influx of Ca^{2+}. Increased intracellular Ca^{2+} resulted in cell death by a number of mechanisms and included amongst these was an increase in the synthesis of an atypical signalling molecule, NO.

Examination of nitric oxide indicates that it is amongst the most ubiquitous signalling molecules in the body and, in particular, in the nervous system. It is involved in a range of functions in the normal CNS including memory and learning, release of pituitary hormones, modulation of behaviour, autonomic reflexes and sensory transmission. It was observed that the functions in which NO is involved are related to the anatomical localisation of cells

that synthesise this molecule within the nervous system. Thus the anatomical localisation of any injury-induced alteration in the number of these cells may reflect an alteration in the function of the system of which they form a part. It was found that a range of insults resulted in alterations to NO synthesis, although peripheral nerve lesions were most often studied. Little work has been done on the effect of traumatic spinal cord injury on synthesis of NO in the CNS. Thus it was determined that examination of the anatomical localisation of NO, both before and after contusive SCI, may give some indication as to the possible role of this molecule in the loss of function that occurs as a result of injury.

1.8 Aims of thesis

As discussed above then, it is likely that alterations in NO synthesis as a result of SCI may result in functional changes with in the CNS. For this reason it is one of the principal aims of this thesis is to examine the effects of SCI on NO synthesis in the spinal cord. This will be done at spinal levels both adjacent to and distant from the site of injury to determine how related such changes may be to the local environment produced by the injury.

As it is of interest to determine whether any alteration in NO synthesis may be related to functional changes within the CNS a second aim of this thesis will be to examine the effects of SCI on fos expression. The expression of fos protein has been well reported as a reliable marker of neuronal activity and so will be used to examine the effect of SCI on neuronal activity. This will be done at spinal levels both adjacent to and distant from the site of injury to also determine the relationship between neural activity and the local injury induced environment.

As one of the major consequences of SCI is the abnormal processing of sensation and the development of neuropathic pain, the relationship between nitric oxide expression, neuronal activity and the neuropathic pain that develops as a result of spinal cord injury will also be examined.

While SCI may certainly affect the local tissue of the spinal cord, alterations in function may also be related to changes in the CNS at supraspinal levels. A fourth major aim of this thesis will, therefore, be to examine the effects of SCI on NOS expression and neuronal activity at supraspinal structures. This will seek to determine SCI may affect not only the spinal cord but also the whole CNS.

Lastly this thesis will aim to consider how such changes to nervous system NO synthesis and neuronal activity may be related to the functional changes commonly observed following SCI, particularly in relation to sensation.

Chapter 2

NADPH-d and fos expression in the spinal cord after contusive lumbar spinal cord injury

As discussed previously, NO has been implicated in the modulation of a large number of biological processes including the synaptic efficacy through long term potentiation and depression, memory, learning, brain development, opioid tolerance and withdrawal, respiratory pattern formation, sexual behaviour and sympathetic outflow (Esplugues, 2002), modulation of gene expression (Dawson and Dawson, 1998), inhibition of the NMDA receptor (Prast and Philippu, 2001), Ca^{2+} homeostasis (Lincoln and Cornwell, 1991; Lincoln et al., 1994), modulation of stress related behaviours (de Oliveira and Guimaraes, 1999; Guimaraes et al., 1991; Guimaraes et al., 1994; Matheus et al., 1994) and the transmission of nociception (Luo and Cizkova, 2000; Riedel and Neeck, 2001).

As such, any nervous system insult which results in the alteration of NO synthesis may potentially affect a number of these systems. As discussed extensively in Chapter 1, the anatomical localisation of NO synthesising cells is likely to give an indication of the systems that would be affected by changes to this molecule following SCI. For example, there is a high density of NOS labelled cells in the SDH, an area of the spinal cord that receives largely nociceptive sensory impulses (Aimar et al., 1998; Baba et al., 1994; Bredt et al., 1991a; Keay and Bandler, 2002; Nakamura, 1997; Trudrung et al., 2000; Yaksh and Hammond, 1982). As discussed in the introduction to this thesis, it has also been shown functionally that NO is involved in the transmission of nociception. If expression of NO is altered by injury then, it is possible then that sensory transmission would also be altered.

Little work has been done on the effect of traumatic spinal cord injury on NO synthesis, and what has been done has been restricted to spinal segments immediately adjacent to the injury (for example Chatzipanteli et al., 2002; Diaz-Ruiz et al., 2002; Liu et al., 2000; Nakahara et al., 2002; Trudrung et al., 2000). In addition, since NO is involved in a range of functions within the CNS, examination of neural activity in conjunction to changes in the

levels of NO synthesis would provide valuable information concerning the effect of injury on those neural systems.

One of the objectives of the work described in this thesis as a whole will therefore be to investigate whether the sensorimotor deficits that arise following SCI may be as a result of changes in NO synthesis in the spinal cord. The present chapter will discuss changes in the anatomical distribution of NADPH-d activity in spinally injured animals and compare this to uninjured animals to determine the effect of injury on this distribution. This will be done using histochemical detection of NADPH-d which is accepted as a useful method of detecting nitric oxide synthase (NOS) containing neurons (Bredt et al., 1991a; Dawson et al., 1991a; Hope et al., 2001).

Importantly, this will be done not only at spinal segments adjacent to, but also at segments distant from the injury. This will be done in order to determine whether any changes in NADPH-d activity observed were due to the local injury-induced environment. Another alternative would be that any changes observed may be due to a modification to the function of the CNS, and therefore independent of the distance from the injury site. This will be investigated. The sensory consequences of any alteration discovered will be discussed in chapter 4.

NO was monitored at one week following a contusive injury, a time point that appeared to be important in the development of more chronic conditions (Xu et al., 1992), as well as being representative of ongoing pathological processes in the cord. In addition there are a number of studies examining both peripheral and central nervous system injures at more acute time points (e.g. Chapman et al., 1995a; Delander et al., 1997b; Gonzalez et al., 2001; Gu et al., 1997; Hengerer et al., 1990; Yamazaki et al., 2001). However there is evidence to suggest that the pathology of SCI may continue past these earlier time points (e.g. Abraham et al., 2000; Blight, 1991; Castro-Lopes et al., 1993; Soblosky et al., 1996; Trudrung et al., 2000; Xu et al., 1992). Thus examining the time point of seven days would provide additional data concerning the effect of contusive SCI on NADPH-d activity and fos expression not currently available. In the same animals, immunohistochemical detection of fos protein, the gene product of the immediate early gene *c-fos*, was utilised as an anatomical marker for neuronal activation. This technique has been reported previously

(Morgan and Curran, 1989; Morgan and Curran, 1991) and was discussed extensively in chapter 1 of the present thesis.

These experiments allowed the examination of altered levels of neuronal activity following SCI, both at the level of the contusive injury, and also at spinal segments distant to the injury. They also allowed us to determine any association between changes in the patterns of neuronal activity and changes in nitric oxide synthesis. This was the first study to examine patterns of neuronal activity together with NADPH-d expression at the chronic time point of seven days for a traumatic model of SCI. It was also the first study to examine the changes in these molecules over an extended number of spinal levels.

2.1 Methods

2.1.1 Experimental groups

A total of 19 female Wistar rats (200-300g) were used. Of these, 14 were subjected to contusive SCI and 5 served as uninjured controls. All experiments were conducted following approval of the study protocol by the institutional animal care and ethics committee, and observed the animal welfare guidelines of the National Health and Medical Research Council of Australia and the International Association for the Study of Pain (Zimmermann, 1983).

2.1.2 Spinal cord contusion

Rats were initially anaesthetised with 4% halothane in 100% O_2 in an airtight box, and subsequently maintained using a face mask and 2% halothane. A laminectomy was performed at the T13 vertebra (~L3-4 spinal level) and a brass guide tube was then lowered over the exposed spinal cord. A 10g stainless steel weight (tip 2mm in diameter and protruding 3mm below the end of the guide tube) was then dropped onto the dorsum of the cord from either a 3 or 5cm height. The tube was left in place for 10 seconds before being removed. Once haemostatic, the incision was then closed in layers and the level of halothane gradually reduced. Each rat was allowed to breathe oxygen enriched air until head movement was regained. Amoxycillin (150mg/kg; s.c.; Norbrook Laboratories) was administered as antibiotic and, once awake, buprenorphine (0.3mg/kg; s.c.; Reckitt and Colman) administered as an analgesic. The rats were then returned to their home cage and monitored closely over the next 48 hours for any signs of distress.

2.1.3 Perfusion

One week after the SCI, and approximately two hours following behavioural testing, each of the rats was deeply anaesthetised with pentobarbitone sodium (120mg/kg i.p., Boehringer Ingelheim) and given an intracardiac injection of heparin (250 IU in 250µl) and sodium nitrite (50%: 250µl). The rat was perfused intracardially with 0.9% saline (250ml) followed by cold (4°C) 4% paraformaldehyde (pH 7.6; 500ml). The brain and spinal cord were then removed and postfixed at 4°C overnight in the same fixative and cryoprotected in 30% sucrose for a minimum of 24 hours.

2.1.4 Histology

The spinal cord was divided into three blocks: i) a block termed "cervical" was taken from the mid-cervical enlargement (5mm); ii) a block termed “rostral” was taken from rostral to the site of injury (10mm) and iii) a block termed “caudal” was taken from caudal to the site of injury (10mm) (see Figure 2.1 for details). The rostral and caudal tissue blocks were determined as being 10mm rostral and caudal from the visible epicentre of the contusion.

The point of contusion was visible as a darkened, bruised indentation of the tissue, with some degree of scarring often apparent around the entire circumference of the cord. The spinal cords of uninjured animals were matched segmentally to ensure that the same spinal segments were examined between injured and uninjured groups. This was done by blocking the spinal cord into the same regions as was done for the injured cords, despite the absence of injury site in the uninjured tissue.

A one in three series of 40µm coronal sections from each of these blocks was cut together on a freezing microtome. Free-floating sections from each animal were then processed to detect the presence of NADPH-d. The sections were washed twice in 0.1M phosphate buffer (PB, pH 7.4) for 10min, before being incubated for 2.5-3 hours at 37°C in a solution containing 0.3% triton X-100, 15mM malic acid, 0.5mM β-NADPH and 0.2mM nitroblue tetrazolium (Sigma). Sections from both injured and uninjured animals were reacted together to ensure that any observed differences in staining were due to differences in the experimental group rather than differences in tissue processing. Sections were then transferred into 0.1M PB to stop the reaction.

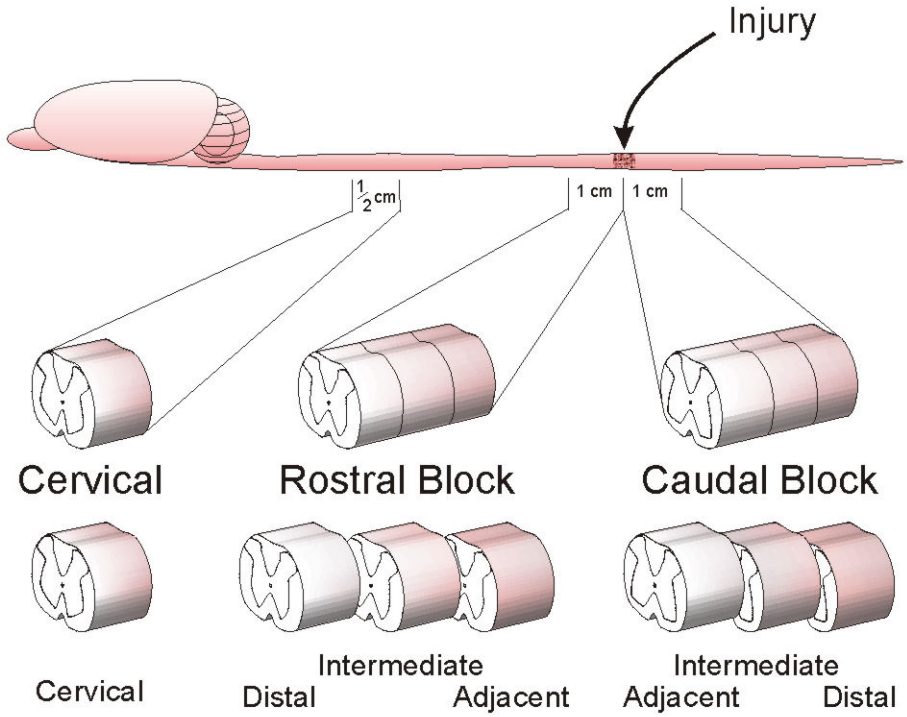


Figure 2.1 Diagram illustrating the segments of the spinal cord examined in the present study. Three blocks of tissue were cut from the spinal cord, a 5mm block from the mid cervical enlargement, a 10mm block from the visible centre of the injury rostrally, and a 10mm block taken from the visible centre of the injury caudally. Five random sections from the cervical tissue block and from each third of the rostral and caudal blocks (from the third adjacent to the injury, the third intermediate to the injury, and the third most distal to the injury) were then examined for both NADPH-diaphorase activity and c-fos immunoreactivity.

A one in three series of 40µm coronal sections from each of these blocks was cut together on a freezing microtome. Free-floating sections from each animal were then processed to detect the presence of NADPH-d. The sections were washed twice in 0.1M phosphate buffer (PB, pH 7.4) for 10min, before being incubated for 2.5-3 hours at 37°C in a solution containing 0.3% triton X-100, 15mM malic acid, 0.5mM β-NADPH and 0.2mM nitroblue tetrazolium (Sigma). Sections from both injured and uninjured animals were reacted together to ensure that any observed differences in staining were due to differences in the experimental group rather than differences in tissue processing. Sections were then transferred into 0.1M PB to stop the reaction.

2.1.5 Immunohistochemistry

Following visualisation of NADPH-d, sections were rinsed twice, for 10 minutes, in PB then incubated in 20% normal horse serum in PB for 20 minutes. Sections were then incubated in a polyclonal rabbit anti-Fos (Santa Cruz Biotechnology) at 1:2,500-5,000 dilution for 2-3

days at 4°C. The sections were then washed twice in PB for 10 minutes, placed into biotinylated donkey anti-rabbit IgG (Amersham; 1:500) and incubated for two hours at room temperature. Sections were again washed twice in PB for 10 minutes and then placed into Extr-avidin peroxidase (Sigma) at 1:1000 for 2.5 hours at room temperature. Following two PB rinses, the bound peroxidase was then visualised using Diaminobenzidine (DAB) as the chromogen and using the glucose oxidase method of peroxide liberation. Briefly, the sections were incubated in a 0.05% DAB solution containing 0.0056% ammonium chloride and 0.28% D-glucose for 20 minutes. Glucose oxidase was then added to the solution (final concentration 1μL/mL) and the sections incubated until the desired level of staining had been reached. The reaction was terminated by rinsing in several changes of PB, when the positively stained nuclei could be clearly seen under microscopic visualisation at X400 magnification. Sections were then mounted onto glass slides with 0.5% gelatine solution and left to air dry at room temperature before being dehydrated through serial alcohols to histolene and coverslipped with DPX.

2.1.6 Plotting

Sections were observed under the light microscope and five randomly selected sections from each spinal cord division (Figure 2.1), in each rat were plotted using a camera-lucida attachment. The numbers of: i) NADPH-d labelled; ii) fos-positive; and iii) double labelled cells (ie., containing both NADPH-d and Fos-like immunoreactivity) were counted on both sides of the spinal cord, on each section of each animal. Positive cellular labelling was determined by direct visual inspection using light microscopy to determine whether labelling fulfilled specific criteria. A cell was determined as being positive for NADPH-d if it was of a distinct neuronal morphology and intensely labelled. Length of time for the diaphorase reaction was chosen to produce intense labelling in cells which contained the enzyme. A few cells which were also of a neuronal morphology were found to be very lightly NADPH-d positive, but these were distinct from the intensely labelled cells and most likely represented non-specific diaphorase activity. These were not counted as positive.

The chromogenic reaction utilised for fos labelling was allowed to proceed to an extent which very lightly labelled the background cytoarchitecture. This together with the diaphorase activity in the same section, removed the need for cellular counterstaining as the cytoarchitecture of the tissue was clearly visible, allowing the spinal laminae to be easily delineated. As with the diaphorase histochemistry, the length of time the fos

immunohistochemistry was allowed to progress resulted in dark, intensely labelled nuclei which were well above background intensity. Those that were not, were not counted. It was not possible to determine cellular morphology from fos labelling, but all clear nuclear profiles were counted within the confines of the grey matter.

It is important to note that in the series of studies reported in this thesis that the experimenter was not blinded to the injury status of the animals. In both the spinal cord and the cortex the status of the animal was immediately obvious from the pattern of labelling of either NADPH-d activity or fos immunoreactivity. The morphology of the spinal cord was also drastically altered following SCI thus enabling immediate recognition of injury status. Change in labelling was less obvious in the midbrain. To ensure unbiased and accurate cell counts, positive cell labelling was decided on the following criteria which was used in all groups. NADPH-d labelled cells must have demonstrated strong cytoplasmic labelling of the soma. Cells, depending on their plane of orientation, also generally showed strong labelling in the first few major divisions of their processes. While these would often pass out of the plane of section, strong labelling of fine processes was also observed. Low intensity labelling of larger multipolar cells sometimes observed in the ventral horn were not counted.

Positive fos immunoreactivity was determined by a strong orange-brown reaction product in the nuclei of cells. It was not possible to determine the neuronal/non-neuronal nature of the cell examined in this manner thus all positive cells were counted. The majority of cells were clearly immuno positive in regards to fos, however lightly stained cells which tended towards background were not counted.

2.1.7 Statistical Analysis

The mean number of labelled cells at each spinal cord level and within each spinal cord lamina were then compared between SCI and uninjured control groups. Significance was determined using the “G-Test”, which examines for difference between two populations (Sokal and Rohlf, 1995), with a significance level of 0.05.

2.2 Results

2.2.1 Tissue Morphology

Contusive SCI resulted in a destruction of the spinal tissue that was visible from the surface of the cord as a small, darkened, bruised area that occasionally spread up to the diameter

of the cord. When the tissue was cut into 40μm sections and mounted onto glass slides, the injury site would typically be observed as a profound loss of cytoarchitecture, disruption of tissue integrity and multiple microcysts and cavitations (e.g. Figure 2.2). If the sections were followed rostral to the injury, the grey matter regains integrity first, although the tissue still appears quite disrupted, and the blood vessels demonstrate strong NADPH-d labelling. Sections rostral to the injury are characterised by a large central cavity in the white matter between the dorsal horns, which extends roughly six spinal segments to approximately T11 (e.g. Figure 2.3). The ventral and lateral white matter tracts appear intact by approximately 4 spinal segments from the injury site. In contrast, caudal to the injury, rather than a well defined cavity in the dorsal white matter, a series of microcysts can be observed in the ventral white matter, and occasionally in the grey matter of the ventral horn (Figure 2.4). The injury-induced changes do not appear to spread as extensively along the cord caudally as they do rostrally (Figure 2.5).

2.2.2 NADPH-d Labelling in Uninjured Control Rats

Injury level

In spinal cord sections taken from control uninjured animals, at the level corresponding to the injury level in the experimental group, laminae I and II contained NADPH-d labelled neurons. Sections taken from the rostral tissue block (~Th13-L4) contained a mean of 14.6 ± 1.89 (SEM) cells per 40μm section in the distal segment, 15.2 ± 1.99 cells in the intermediate segment and 19.3 ± 2.61 cells in the segment adjacent to the injury site. Sections taken from the caudal tissue block (~L5-S2) contained a mean of 25.4 ± 1.57 labelled cells per section in the segment adjacent to the injury, 26.6 ± 3.24 cells in the intermediate segment and 17.7 ± 2.84 cells per section in the segment distal most to the injury (Figure 2.6).

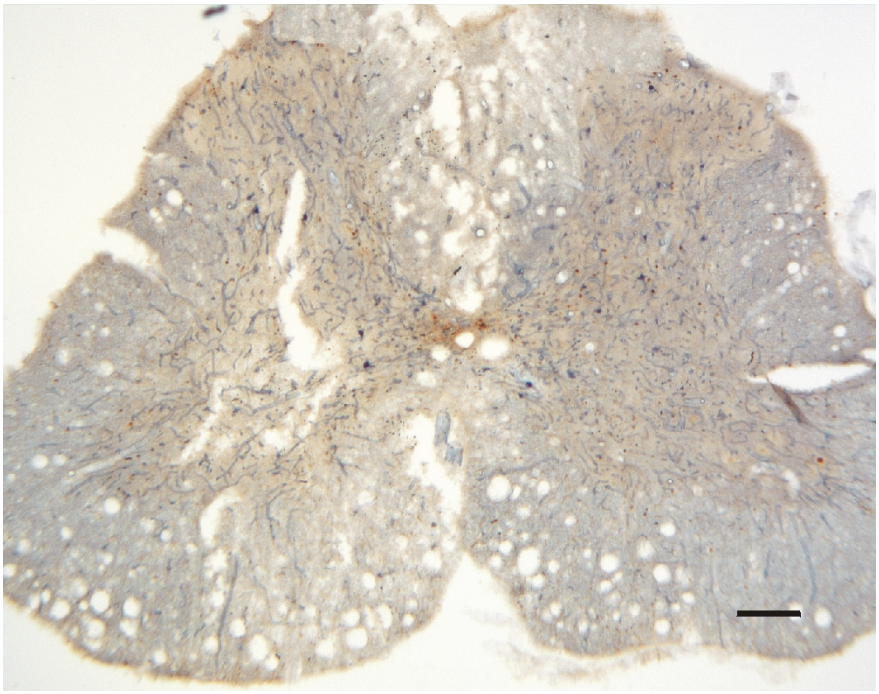


Figure 2.2 Coronal section through the spinal cord at approximately L3/L4, taken from a rat that had undergone a contusive weight drop spinal cord injury. This picture was taken slightly rostral to the epicentre of the injury as section integrity is minimal at the actual injury site. Immediately adjacent to the injury site, at the level shown, the morphology of the grey matter is lost and there is prominent cavitation of the white matter. Scale bar = 200μm.

Labelled cells in these laminae had an homogenous, bipolar morphology, being small (10-15μm in diameter) and oval in shape with a low cytoplasmic to nuclear volume. They were only rarely seen to project any distance dorsally or ventrally in the section (Figures 2.7A,B). There were few labelled cells in laminae III, IV or V (range 1-6 cells per lamina per section). The cells in lamina III shared similar morphology to those of laminae I and II (Figure 2.7C), whereas the cells that were observed in laminae IV and V were either multipolar with short processes in the coronal plane, or occasionally bipolar with dendritic processes extending ventromedially across both laminae IV and V (Figure 2.7D).

In the ventral horn, the number of cells per section ranged from 5.8 ± 0.88 (SEM) cells per 40 μm section in the distal segment of the rostral tissue block to 9.2 ± 0.62 cells per section in the adjacent segment. Sections from the caudal tissue block were found to range from 6.0 ± 0.40 cells per section in the adjacent segment to 4.6 ± 0.88 cells per section in the distal segment. These neurons were a mixture of bipolar and multipolar cells, whose soma were

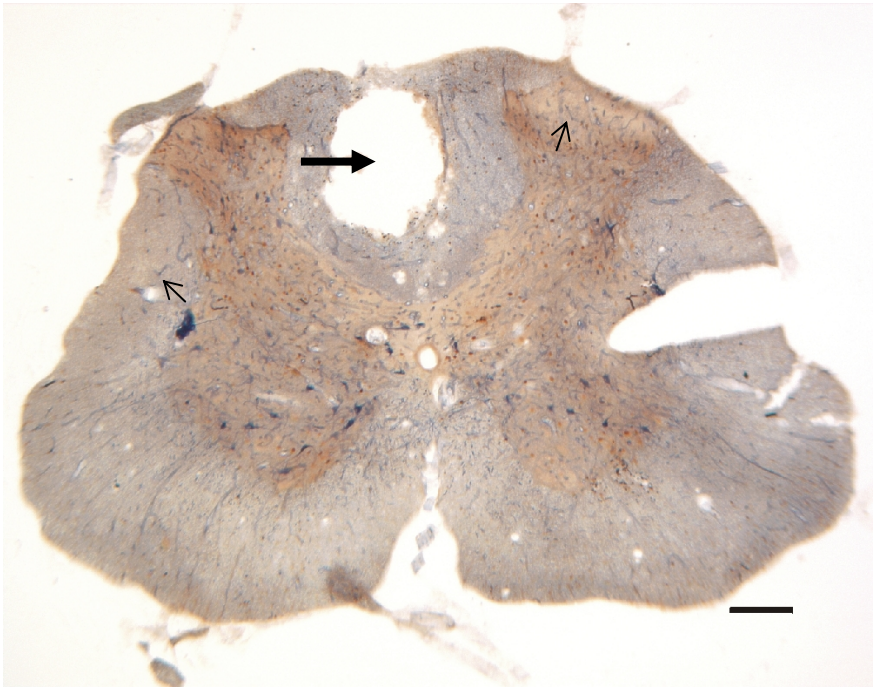


Figure 2.3 A section taken from rostral to the injury at approximately T13. Outline of spinal grey matter is more defined than closer to the injury site. Tissue still appears disrupted however. Large dorsal cavitation is prominent in the white matter between the dorsal horns (closed arrow), while some smaller microcysts are present in the ventral white matter (arrow heads). Blood vessels also appear labelled with NADPH-d reaction product (open arrows). Scale bar = 200μm.

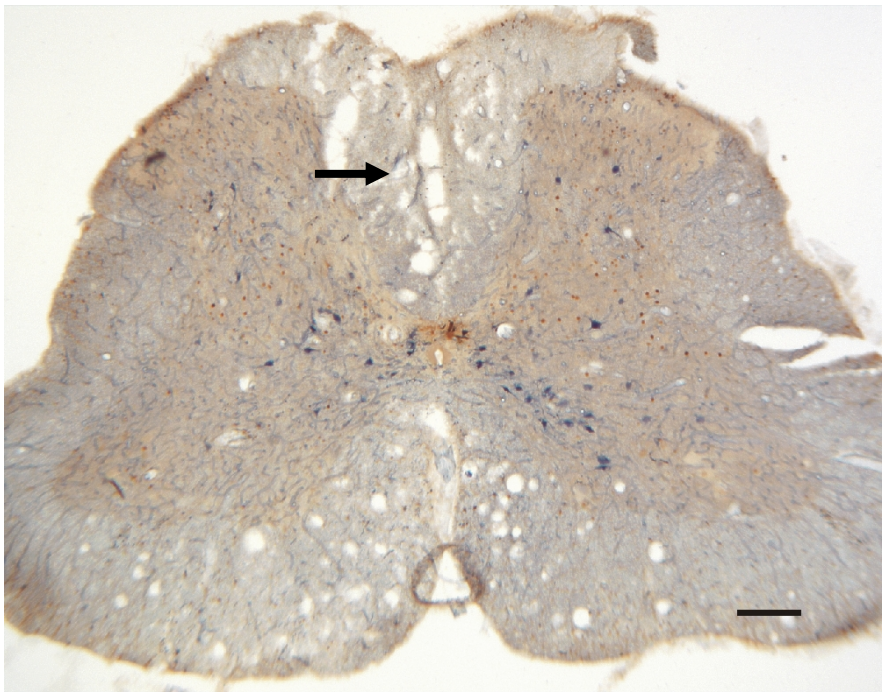
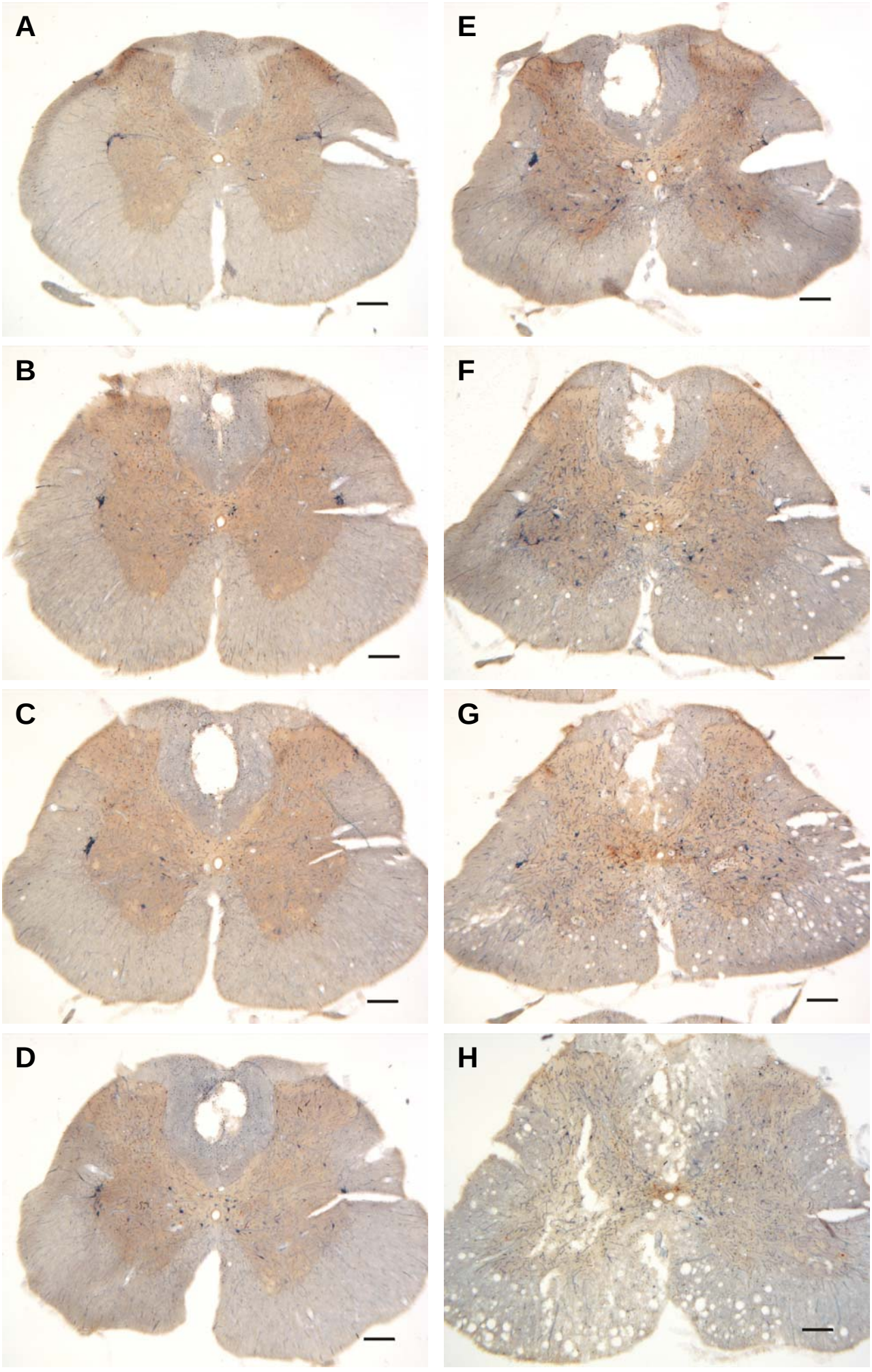


Figure 2.4 Coronal section taken from caudal to the injury at approximately L5. The cavitation of the dorsal white matter is less pronounced (closed arrow) and there is a greater degree of microcyst formation in the ventral white matter (arrow heads). Scale bar = 200μm.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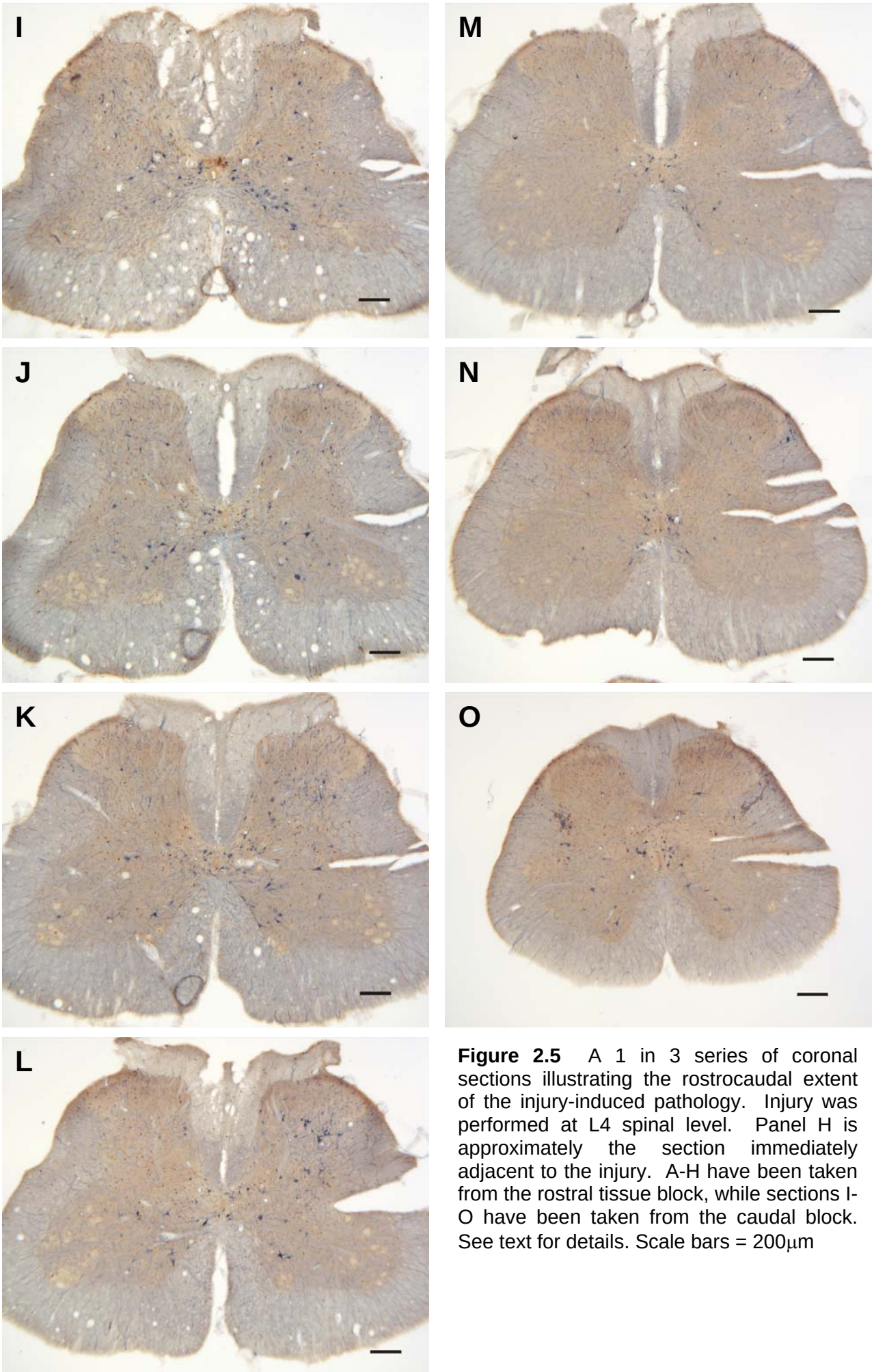
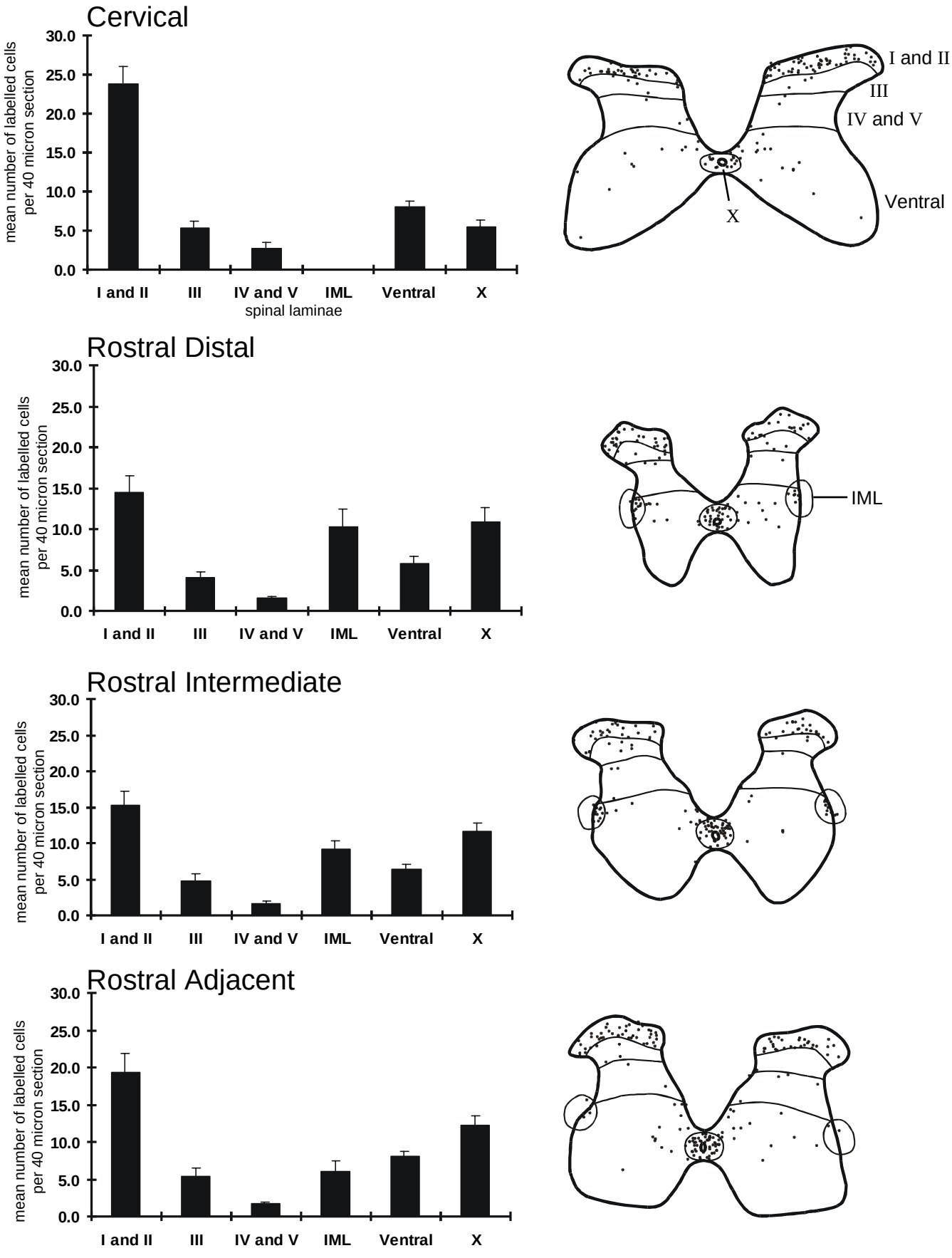


Figure 2.5 A 1 in 3 series of coronal sections illustrating the rostrocaudal extent of the injury-induced pathology. Injury was performed at L4 spinal level. Panel H is approximately the section immediately adjacent to the injury. A-H have been taken from the rostral tissue block, while sections I-O have been taken from the caudal block. See text for details. Scale bars = 200μm


Cervical
mean number of labelled cells per 40 micron section
30.0
25.0
20.0
15.0
10.0
5.0
0.0
I and II
III
IV and V
IML
Ventral
X
spinal laminae
I and II
III
IV and V
Ventral
X
Rostral Distal
mean number of labelled cells per 40 micron section
IML
Rostral Intermediate
mean number of labelled cells per 40 micron section
Rostral Adjacent
mean number of labelled cells per 40 micron section

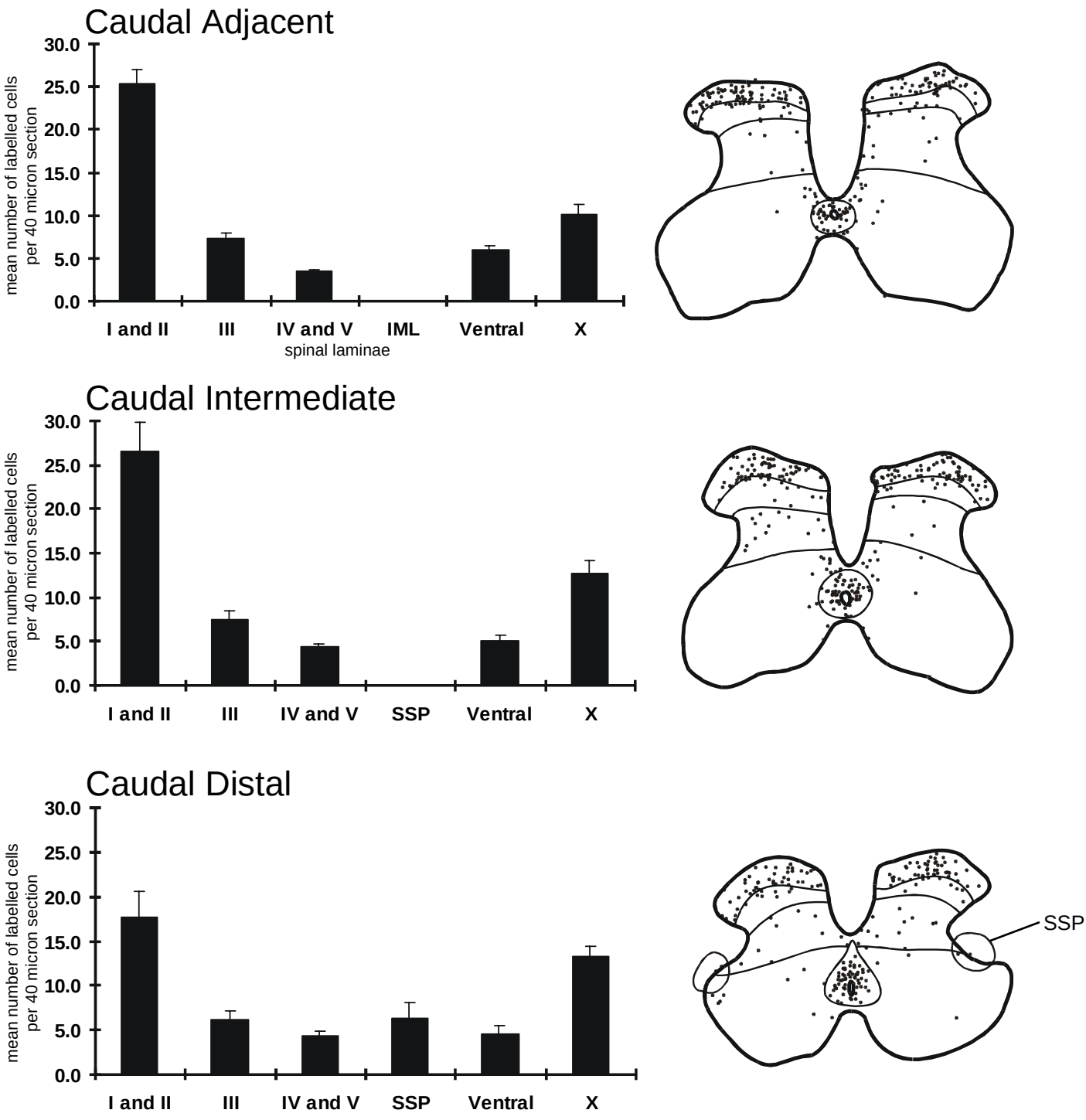


Figure 2.6 Left panel: Histograms showing the mean number (±SEM) of NADPH-d positive cells per 40µm section (y-axis) in laminar subdivisions of the grey matter of the spinal cord of normal, uninjured rats (Rexd's laminae I and II; III; IV and V; intermediolateral cell column [IML] or, sacral sympathetic plexus [SSP]; ventral horn [laminae VII, VIII, and IX] and lamina X). Right panel: Camera lucida reconstructions of the locations of NADPH-d positive cells derived from five 40µm sections of the spinal cord, at the same rostrocaudal level illustrated in the adjacent histogram.

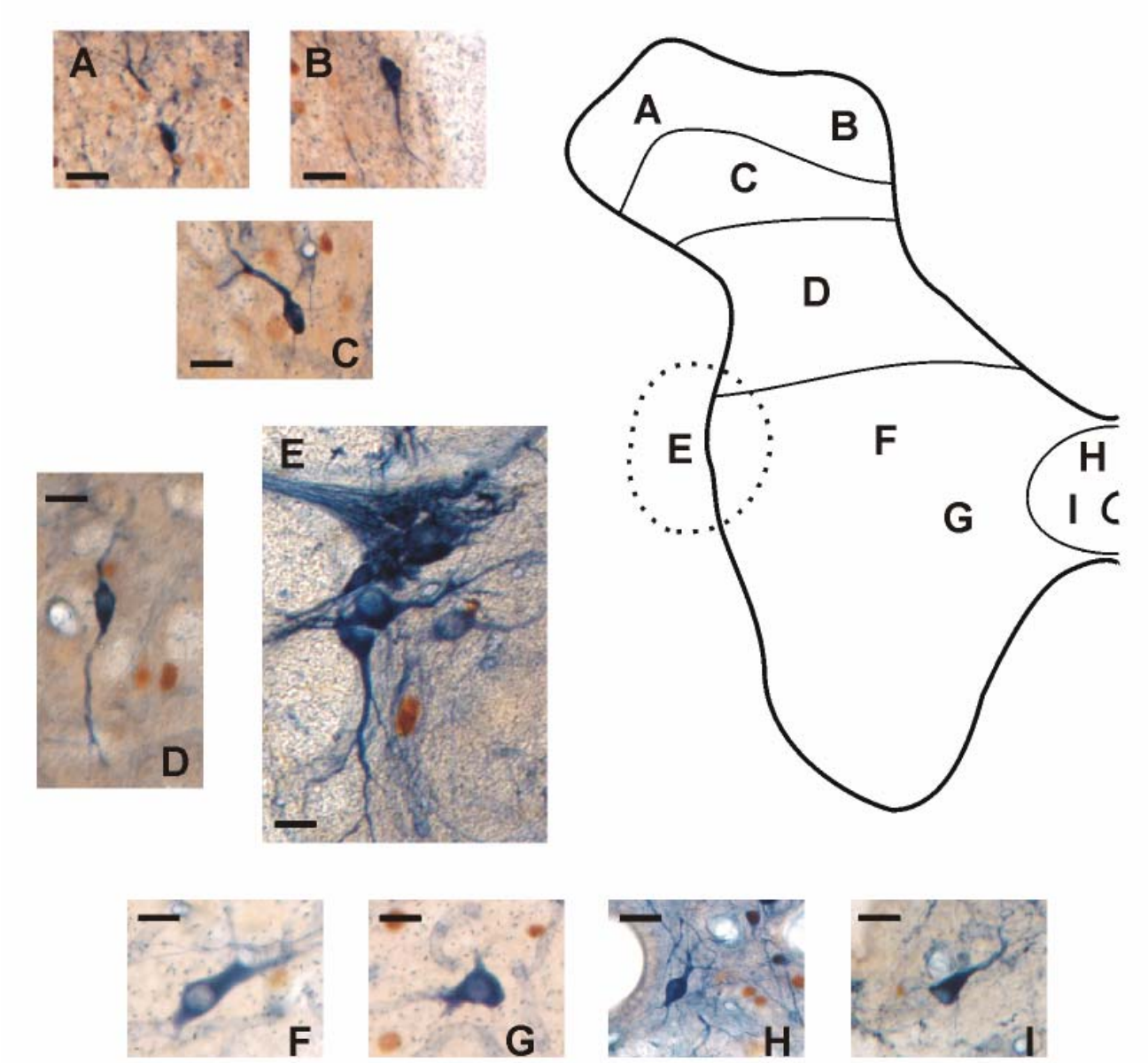


Figure 2.7 Photomicrographs illustrating the typical morphologies of NADPH-d positive neurons in laminar subdivisions of the grey matter of the spinal cord of normal "uninjured" rats. A and B: show islet cells from laminae I and II; C shows a bipolar, fusiform cell from lamina III; D shows a bipolar with dendritic processes extending ventromedially across both laminae IV and V. E: shows neurons of the intermediolateral cell column; F and G: show multipolar neurons of the ventral horn; H and I: show multipolar cells surrounding the central canal within lamina X. See text for further details. The scale bars indicate 20μm.

approximately 20μm in diameter (Figures 2.7F,G). Larger multipolar cells (approximately 40-50μm diameter) which were much less darkly stained were also occasionally visible.

Also distinctive at the boundary of the dorsal and ventral horns is the intermediolateral cell column (IML), which appears as a closely packed collection of 6-10 intensely stained

neurons on the lateral edge of the grey matter (Figure 2.7E). IML cells were often seen to project across lamina VII into lamina X. Fibres were also observed running ventrally along the border of the white and grey matter of the ventral horn, before turning to exit the spinal cord with fibres of the ventral ramus. Lastly, a cluster of NADPH-d stained neurons were found in lamina X. Usually around 10 medium sized (20-25µm diameter) multipolar cells surrounded the central canal. The dendritic processes of these cells were often seen to envelop other cells of this lamina (Figure 2.7 H,I). It was notable that the large motoneurons of the ventral horn were never seen to contain NADPH-d.

Mid cervical enlargement

In sections taken from the C6-7 spinal segments, the distribution and numbers of NADPH-d labelled cells in laminae I and II was similar to that seen at the site of injury, (mean 23.8 ± 2.33 cells per section). Similarly, only 1-5 labelled cells were found in laminae III, IV or V at this level. The cells in laminae III, IV and V shared the morphologies of cells in these laminae described in the adjacent segments (Figures 2.7C,D). Ventral horn laminae at this level contained on average 8.1 ± 0.70 labelled cells per section and lamina X of these sections contained a mean of 5.5 ± 0.77 labelled cells per section. The size and morphology of these cells were identical to that described above.

2.2.3 NADPH-d Labelling in Spinal Cord Injured Rats

Injury level

Weight drops were performed from both 3 and 5 cm. There was no significant difference in the number of either NADPH-d activity or fos expression between these groups thus data was pooled for the studies reported in this thesis.

Following spinal cord injury, both the numbers of NADPH-d labelled cells and the density of neuropil labelling within laminae I and II of the dorsal horn decreased dramatically in the tissue segments adjacent to the injury in both the rostral and caudal tissue blocks. Thus, at the injury site, the number of NADPH-d labelled cells decreased from 19.3 ± 2.61 SEM to 4.6 ± 1.24 cells per section, a 76% decrease (“G-test”; $p<0.005$). Such a decrease however, was not observed within the distal segment of the rostral tissue block of the injured animals, where the numbers of NADPH-d labelled cells did not change from control levels. In contrast, significant decreases in the number of NADPH-d labelled cells in laminae I and II were observed throughout the entire caudal tissue block. In the adjacent segment of

the caudal tissue block, there was a 62% decrease from 25.4 ± 1.57 cells per section to 9.7 ± 3.81 ($p<0.01$), in the intermediate segment there was a 49% decrease from 26.6 ± 3.24 cells per section to 13.6 ± 4.78 ($p<0.05$), while in the distal segment of the caudal tissue block there was a 58% decrease from 17.7 ± 2.84 cells per section to 7.4 ± 2.96 ($p<0.05$; see Figure 2.8).

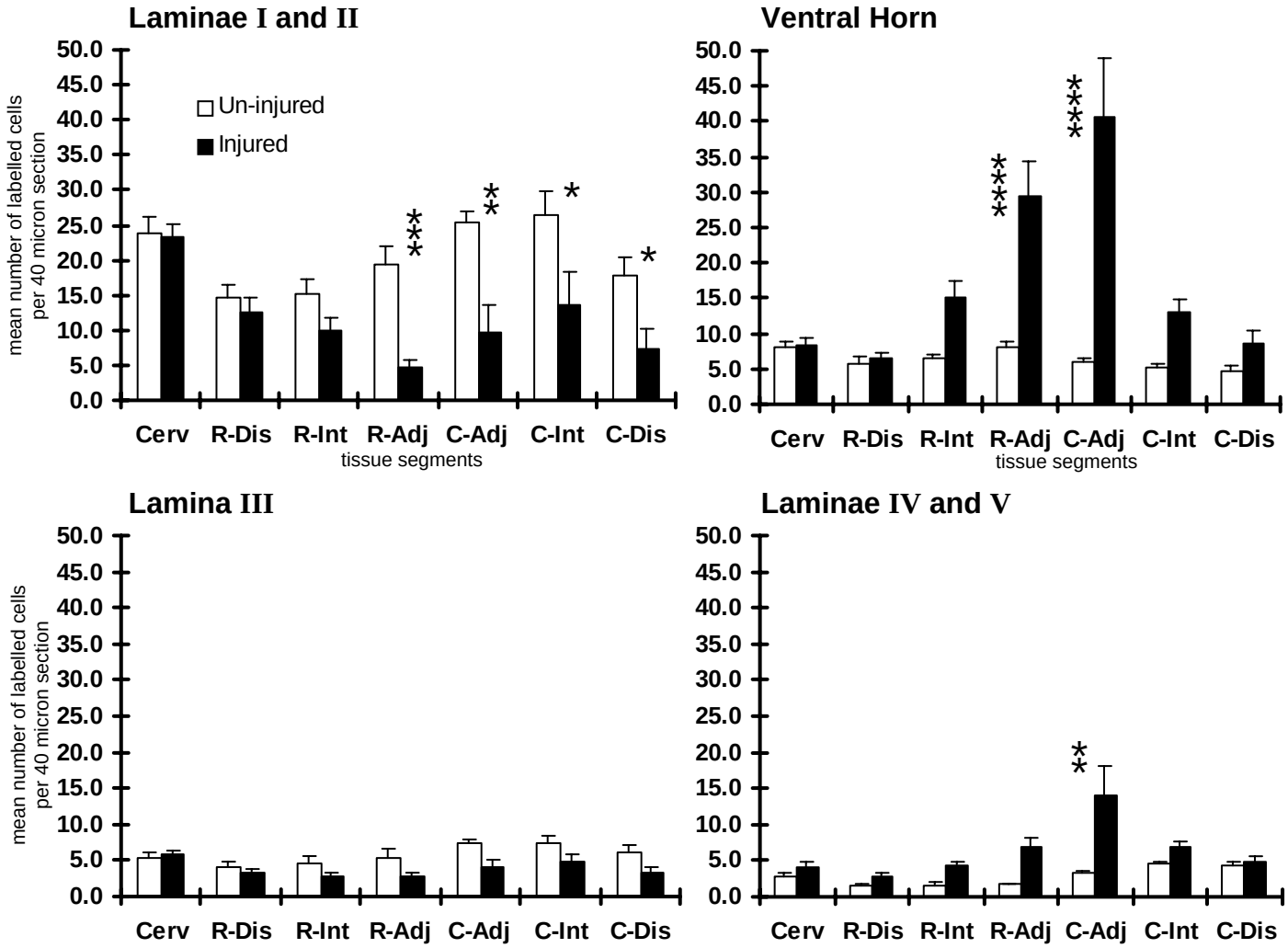


Figure 2.8 Histograms comparing the rostrocaudal distributions (mean ±SEM) of NADPH-d positive cells within specific spinal cord laminar subdivisions in normal, uninjured versus injured rats. Y-axis shows the mean number of NADPH-d labelled cells per 40μm section. *P<0.05; **P<0.01; ***P<0.005; ****P<0.001

In contrast to the decreases seen in laminae I and II, contusion injury evoked *increases* in the numbers of NADPH-d labelled cells in laminae IV and V (the deep dorsal horn: DDH) and in laminae VII, VIII and IX of the ventral horn grey matter.

In the segments adjacent to the injury of both the rostral and caudal tissue block, there was a notable increase in the numbers of NADPH-d labelled cells in laminae IV and V. This

increase was only significant however in the adjacent segment of the caudal tissue block in which a 4.1 fold increase in the number of labelled cells was observed following injury (ie, 3.4 ± 0.23 SEM cells per section increased to 14.0 ± 4.07 SEM cells per section, $p<0.01$).

There were few NADPH-d labelled cells in the ventral horn prior to injury, an observation which remained unchanged in sections from the distal segment of the rostral tissue block after injury. However, closer to the site of injury in the adjacent segment of the rostral tissue block there was a 3.6 fold increase in the number of ventral horn cells in which NADPH-d was detected (change from a mean of 8.2 ± 0.62 SEM cells per section, to 29.5 ± 4.90 SEM cells per section, $p<0.001$). An even larger increase (6.8 fold) was detected in the adjacent segment of the caudal tissue block, changing from 6.0 ± 0.40 SEM with a mean of 40.6 ± 8.21 SEM cells per section ($p<0.001$). These increases were due to labelling in both the neurons which shared the morphology of the small densely stained cells of the ventral horn observed before injury (described above) and large motoneurons expressing NADPH-d (Figures 2.9 & 2.10).

Mid cervical enlargement

There were no changes in either the number or distribution of NADPH-d labelled cells in the mid-cervical enlargement of injured compared with uninjured control rats.

2.2.4 Fos-like Immunoreactivity in Uninjured Control Rats

In uninjured control rats, fos-like immunoreactive (IR) nuclei were easily detected under the light microscope, since the amber coloured, DAB reaction product was distinct from the background labelling (for example, Figure 2.13). Fos-like IR cells were found in all spinal cord laminae at each of the levels investigated with the exception of the intermediolateral cell column (IML). At each of the spinal levels investigated, the largest number of fos-like IR cells were found in lamina III and the ventral horn. The nuclei labelled in laminae I, II and III were small and round (5-10µm diameter), whereas the nuclei of other laminae tended toward an ovoid shape (>10µm diameter). The fos-like IR cells appeared to be randomly distributed with no medio-lateral or sub-laminar distribution patterns. Larger numbers of fos-like IR cells were found in spinal cord sections of greater surface area, ie, there were higher numbers of fos-like IR neurons in sections taken from the mid-cervical enlargement compared to lower thoracic segments (Figure 2.11).

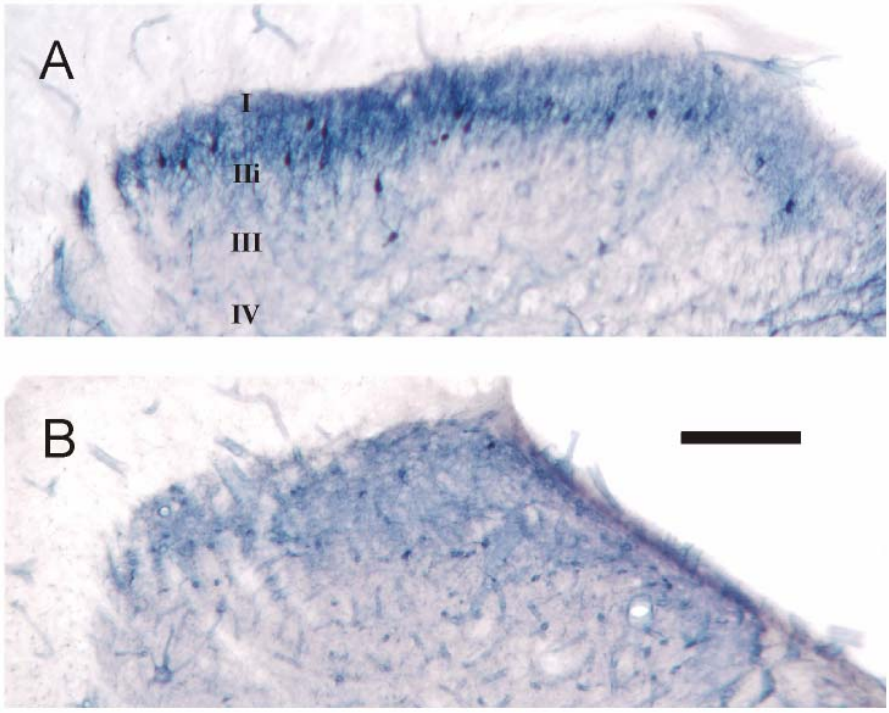


Figure 2.9 Photomicrographs of the superficial dorsal horn of a normal uninjured (A), and injured (B) animal. Note in the uninjured animal (A), that the neuropil of laminae I and II show strong labelling of both somal (mainly fusiform cells) and non-somal elements, in a distinct band over the entire width of the laminae. In the injured animals (B), the intensity of the labelling is decreased and most of the somal labelling is gone. The approximate lamina divisions are labelled in roman numerals in panel A while the scale bar indicates 150µm.

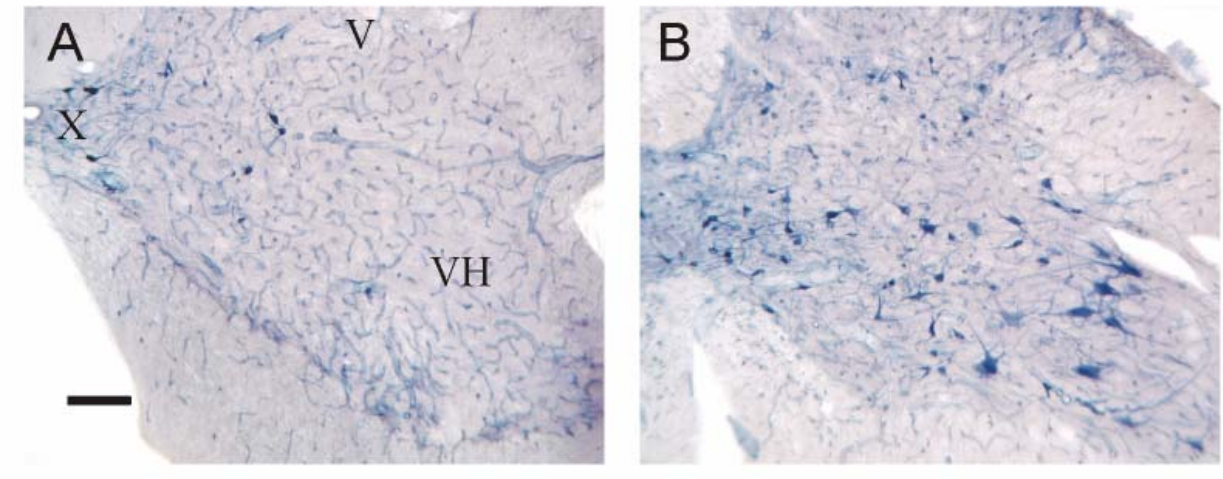


Figure 2.10 Photomicrographs of the ventral horn of a normal "uninjured" (A) and injured (B) animal. Note in the injured animal (B) the large number of NADPH-d labelled cells within the entire mediolateral aspect of the ventral horn, this contrasts greatly with the absence of NADPH-d containing neurons in uninjured animals (A). X denotes lamina X, V denotes lamina V and VH denotes the ventral horn. The scale bar indicates 200µm.

2.2.5 Fos-like Immunoreactivity in Spinal Cord Injured Rats

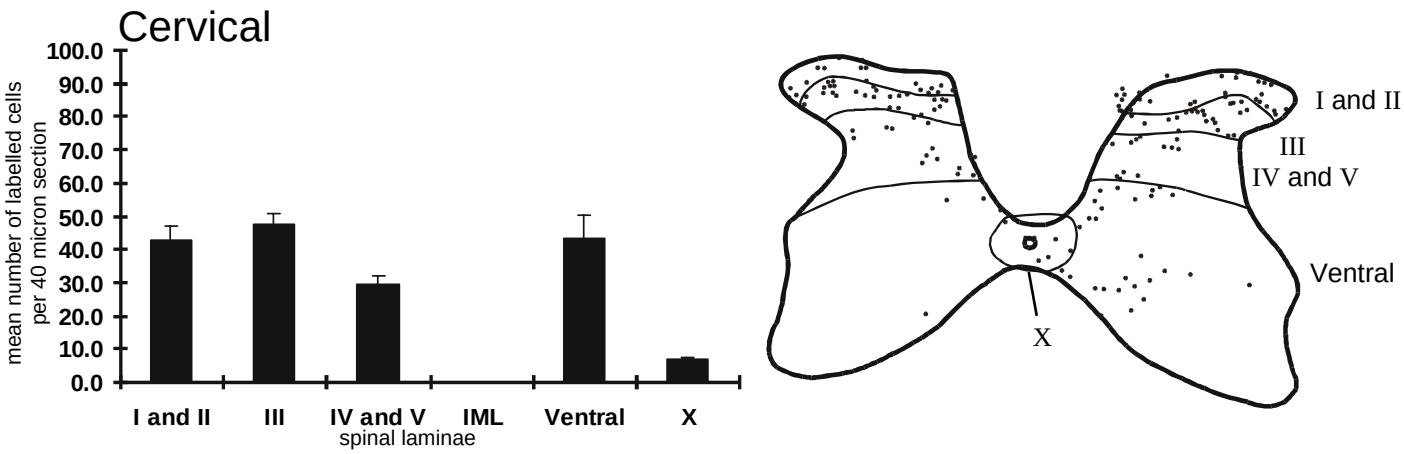
Following spinal cord injury, there was an overall decrease in the number of fos-like IR cells in the spinal cord segments examined. This reduction was greatest in lamina III both rostral and caudal to the injury but also at segments distant to the site of injury (ie, cervical enlargement). The reduction in fos expression in lamina III ranged from a decrease of 52% in the intermediate segment of the rostral tissue block (falling from 48.5 ± 5.17 SEM cells per section to 23.1 ± 3.37 SEM cells per section, $p<0.005$) to 80% in the intermediate segment of the caudal tissue block (falling from 76.3 ± 7.44 SEM cells per section to 15.6 ± 2.42 SEM cells per section, $p<0.001$). The largest reductions in fos expression were seen in the adjacent and intermediate segments of the caudal tissue block (Figure 2.12).

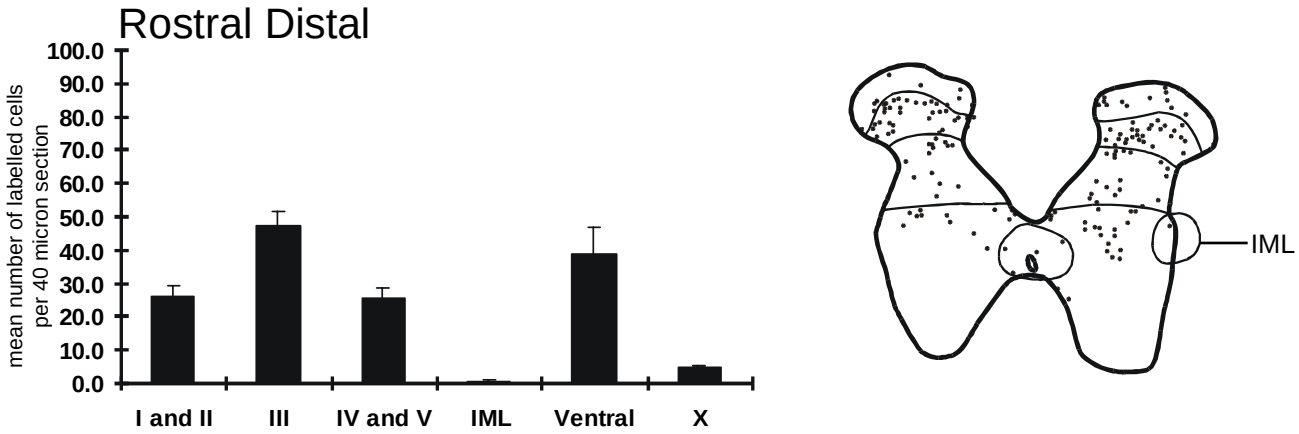
Significant decreases in fos expression were also seen in laminae I & II following injury, however these were restricted to sections taken from the caudal tissue block ("adjacent" -46%, $p<0.01$; "intermediate" -48%, $p<0.005$; and "distal" -48%, $p<0.01$) as well as in the mid-cervical enlargement (-57%, $p<0.005$). In contrast, *c-fos* expression was decreased in the ventral horn, in sections taken from the rostral tissue block ("distal" –42%, $p<0.025$; and "adjacent" -47%, $p<0.025$).

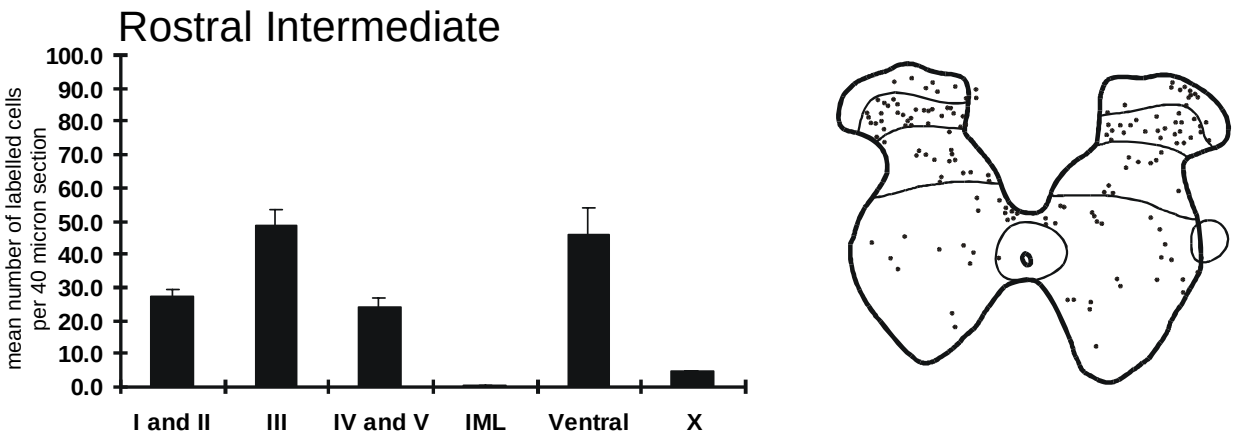
In addition, a smaller reduction (-32%, $p<0.025$) was observed in the caudal tissue block adjacent to the injury site (Figure 2.12). The only region of the spinal cord in which a significant increase in fos-expression was detected was the sacral parasympathetic nucleus (SPN). An increase from a mean of 7 ± 1.6 SEM to 17 ± 5.17 SEM fos-like IR cells was revealed following injury (2.4 fold increase, $p<0.05$; Figure 2.12 and 2.13).

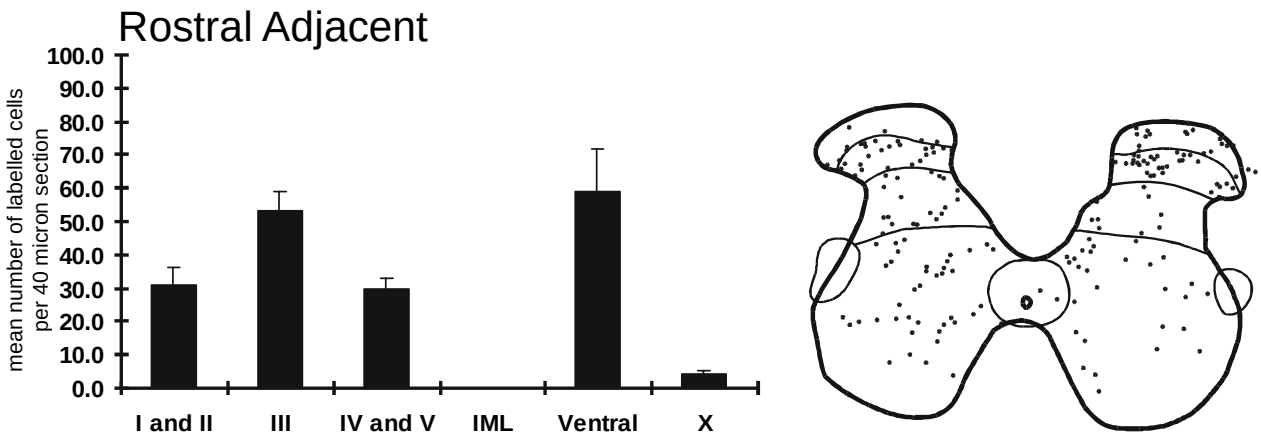
2.2.6 Double Labelling (NADPH-d and fos-IR) in Uninjured Control Rats

In uninjured control animals, double-labelled (NADPH-d/fos-like IR) cells were found in greatest numbers in laminae I & II. Here the number of double labelled cells ranged from 3.1 ± 0.33 cells per section in the distal segment of the rostral tissue block to 8.9 ± 1.8 cells per section in the intermediate section of the caudal tissue block. These neurons were predominantly of a single morphology, and most often found within the inner division of


Cervical
mean number of labelled cells per 40 micron section
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
I and II
III
IV and V
IML
Ventral
X
spinal laminae
I and II
III
IV and V
Ventral
X


Rostral Distal
mean number of labelled cells per 40 micron section
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
I and II
III
IV and V
IML
Ventral
X
IML


Rostral Intermediate
mean number of labelled cells per 40 micron section
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
I and II
III
IV and V
IML
Ventral
X


Rostral Adjacent
mean number of labelled cells per 40 micron section
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
I and II
III
IV and V
IML
Ventral
X

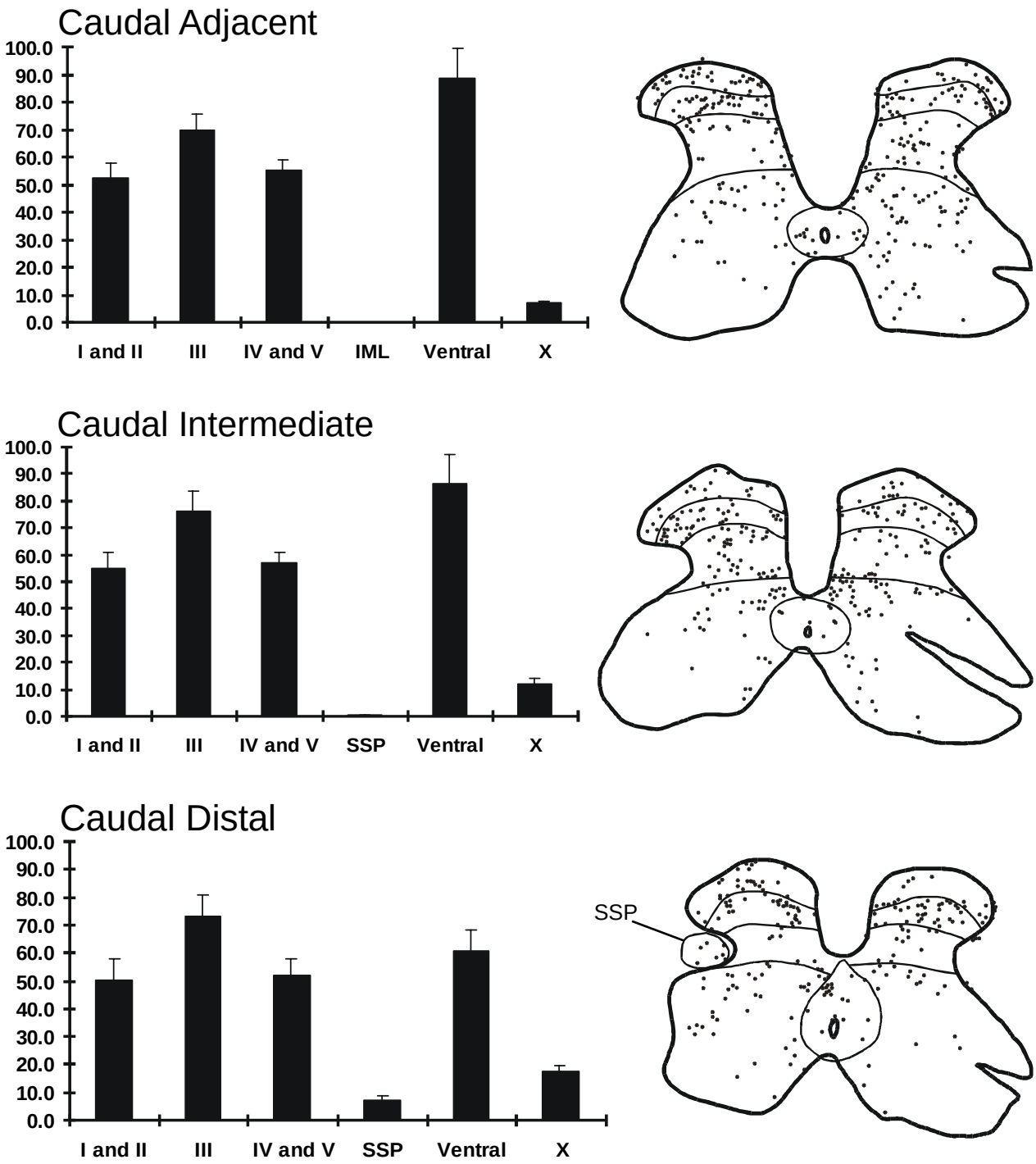


Figure 2.11 Left panel: Histograms showing the mean number (±SEM) of Fos-like IR cells per 40µm section (y-axis) in laminar subdivisions of the grey matter of the spinal cord of normal "uninjured" rats (Rexed's laminae I and II; III; IV and V; intermediolateral cell column [IML] or, sacral parasympathetic plexus [SSP]; Ventral Horn [laminae VII, VIII, and IX] and lamina X). Right panel: Camera lucida reconstructions of the locations of Fos-like IR cells derived from five 40µm sections of the spinal cord, at the same rostrocaudal level illustrated in the adjacent histogram.

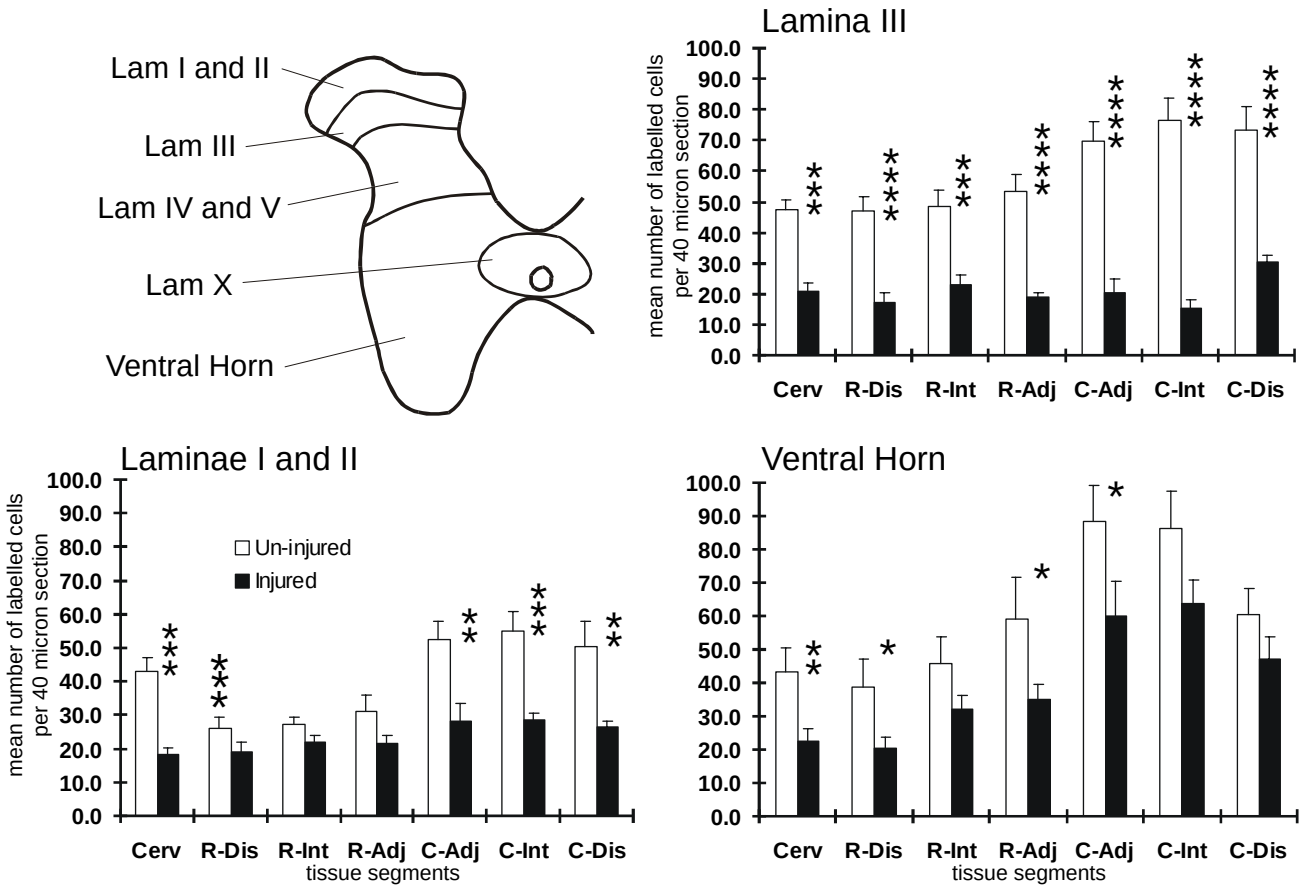


Figure 2.12 Histograms comparing the rostrocaudal distributions (mean ±SEM) of Fos-like IR cells within specific spinal cord laminar subdivisions in normal "uninjured" versus injured rats. Cerv, cervical segment; R-Dis, rostral tissue block distal segment; R-Int, rostral tissue block intermediate segment; R-Adj, rostral tissue block adjacent segment; C-Adj, caudal tissue block adjacent segment; C-Int, caudal tissue block intermediate segment; and C-Dis caudal tissue block distal segment. Y axis shows the mean number of c-fos labelled sections per 40μm section. *P<0.05; **P<0.01; ***P<0.005; ****P<0.001

lamina II. The cells were bipolar, spindle shaped, with the low cytoplasmic to nuclear volume ratio, typical of all NADPH-d containing neurons of the superficial dorsal horn. In the plane of sectioning used in this study, the double-labelled cells were usually dorso-ventrally oriented, occasionally displaying short processes which projected dorsally into the outer portion of lamina II and/or ventrally into lamina III. Very few double-labelled cells were noted in other spinal cord laminae of the segments observed (ie, $\leq$1 cell per lamina, per section).

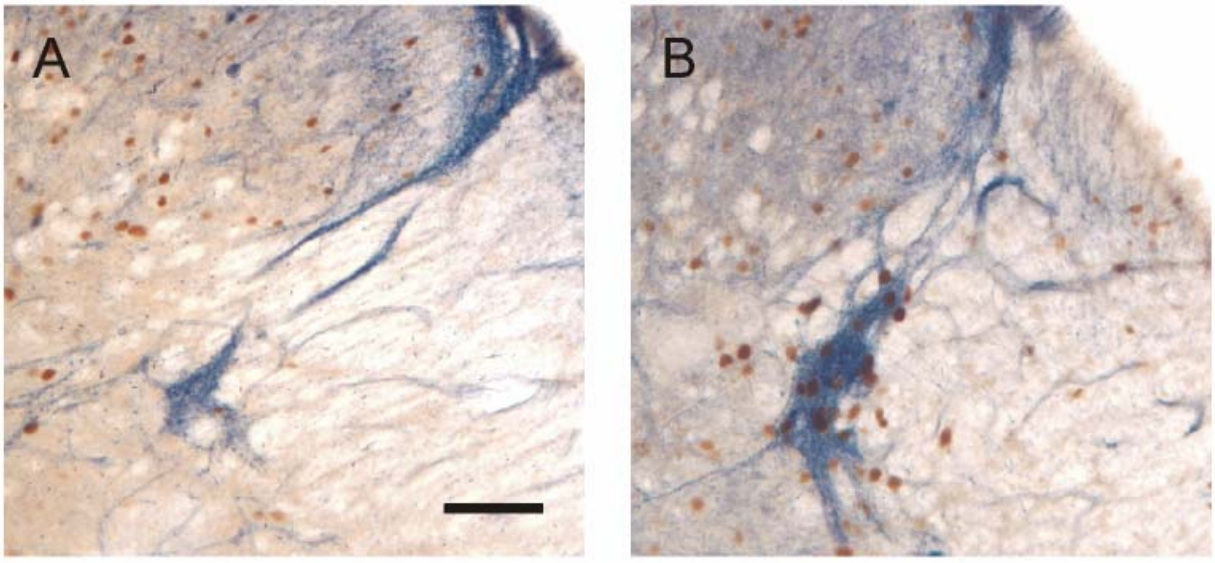


Figure 2.13 Photomicrographs of sacral parasympathetic nucleus in (A) "uninjured" control animal and (B) injured animal showing increased Fos expression with in the nucleus following spinal cord injury. Fibres of the nucleus are labelled blue with a strong NADPH-d reaction product. The scale bar indicates 75μm.

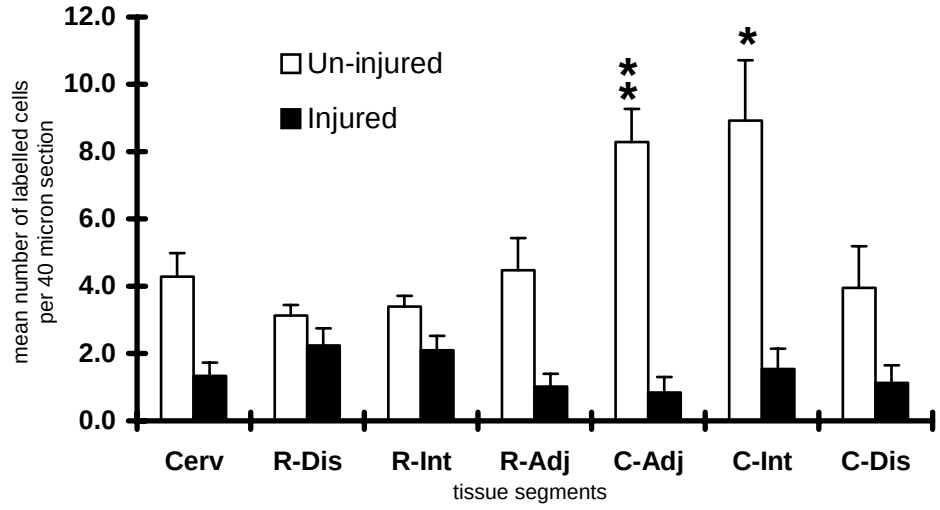


Figure 2.14 Histogram comparing the rostrocaudal distributions (mean ±SEM) of double labelled, NADPH-d positive and Fos-like IR cells within laminae I and II of the spinal cord in normal "uninjured" versus injured rats. Y axis shows the mean number of double labelled cells per 40μm section. Cells counted at sections taken from the cervical tissue block (Cerv), and the distant, intermediate and adjacent segments from the rostral tissue block (R-Dis, R-Int, R-Adj), as well as those from the caudal tissue block (C-Adj, C-Int, C-Dis). *P<0.025; **P<0.01

2.2.7 Double Labelling (NADPH-d and fos-IR) in Spinal Cord Injured Rats

Following injury, the number of double-labelled cells in laminae I & II were decreased, reaching statistical significance in the adjacent and intermediate segments of the caudal tissue block, where numbers are decreased from 8-9 double-labelled cells per section to 1-2 cells per section ("adjacent" -90%, $p<0.01$ and "intermediate" -82%, $p<0.025$; Figure 2.14, see Figure 2.15 for examples of double labelled cells).

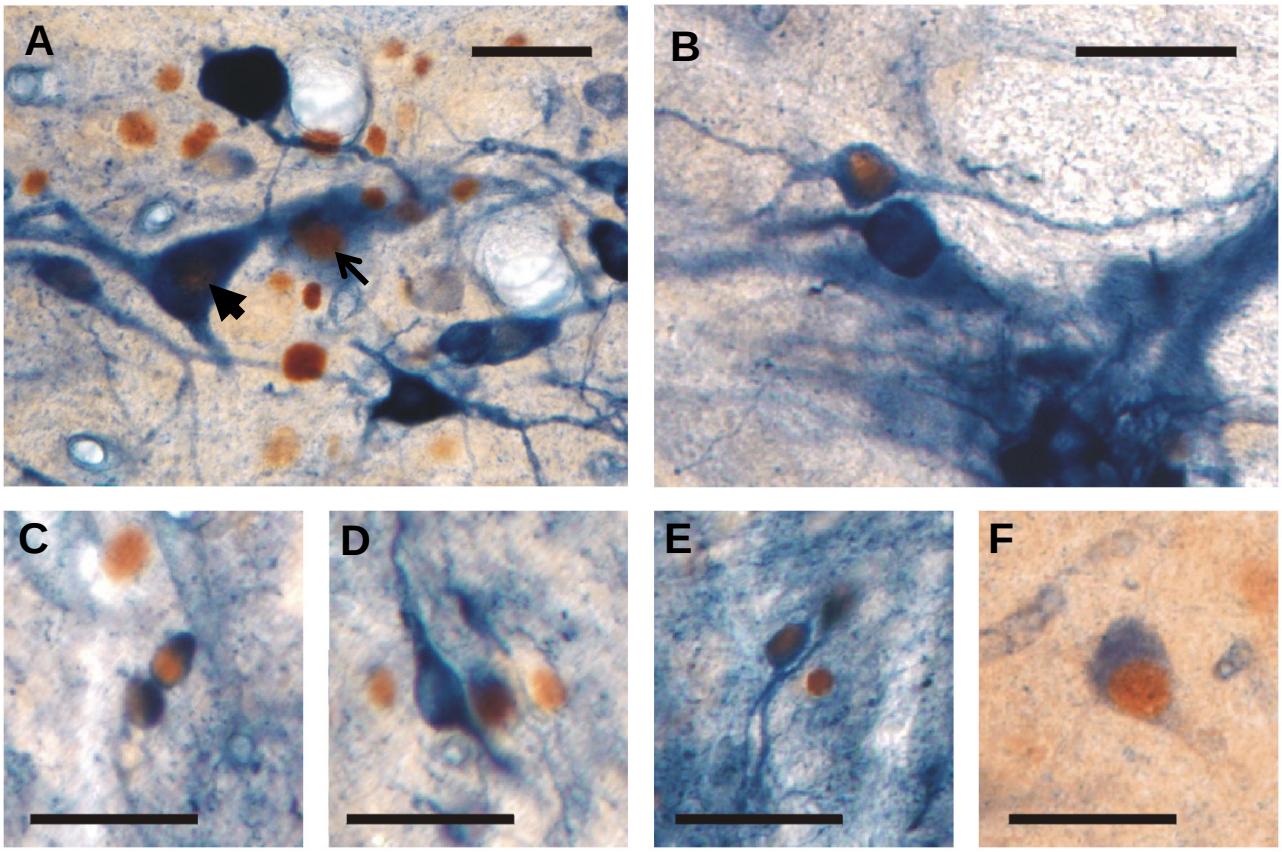


Figure 2.15 Micrographs showing typical examples of NADPH-d/fos double labelled cells. NADPH-d labelling appears as blue reaction product, orange brown nuclei are labelled against fos protein. A, shows cells in lamina X just dorsal to the central canal. Two double labelled cells are shown, one with lighter cytoplasmic NADPH-d labelling (open arrow) and another in which the labelling is more intense. NB fos reaction product in this cell is more difficult to see in the micrograph, cell is readily apparent under the microscope despite dense NADPH-d reaction product. B, shows cells from intermediolateral cell group (IML). Panels C-E show small fusiform cells from lamina II. The cells in panel C and E are from superficial lamina IIi. Panel D shows an unlabelled border cell from the junction between laminae IIi and III to demonstrate the readily apparent, unlabelled nucleus. Panel F shows a ventral horn cell from lateral lamina VII. Panels C-F are shown at the same magnification. Scale bar represents 70μm.

2.3 Discussion

It was the aim of the present experiment to examine the distribution of NADPH-d positive cells within the spinal cord in both normal, uninjured animals, as well as in animals that had undergone a traumatic SCI. It was thought that the anatomical localisation of NO synthesising cells in normal animals may indicate neural systems that may be affected by alterations in NO synthesis following injury. In addition to this, fos expression was also examined in the same sections to determine how neuronal activity may be related to changes in NADPH-d activity or visa versa. Lastly, sections were examined from spinal levels both adjacent to the injury (up to 1cm rostral and caudal to ~L3-4) as well as distant to the injury (mid cervical enlargement). This enabled the dependence of any changes observed to be correlated to the local injury-induced environment. This will be discussed further later in this discussion.

The principal finding of this study was that traumatic SCI results in a significant decrease in the number of NADPH-d positive cells in the SDH of the spinal cord. Conversely, the number of NADPH-d positive cells in the ventral horn was found to be significantly increased following injury. These changes were found to be dependent on the local injury induced environment since no changes were observed in sections distant from the injury. When fos expression was examined however, it was found that the number of fos positive neurons was significantly decreased following traumatic SCI. This change was most profound in lamina III. Furthermore the changes in fos expression was independent of the proximity to the injury site, since sections taken from the mid cervical enlargement were equally affected by the injury as were sections taken from adjacent to the injury site. These findings give an indication as to the cellular effects that traumatic SCI have towards these molecules at seven days after injury.

Clinically, most spinal cord injuries are associated with contusion. Contusive models of SCI have been used for many years in the study of SCI-related sensory, motor and regenerative processes (see for example Allen, 1911; Freeman and Wright, 1953). The contusive SCI model used in the present study is an aetiologically valid model because it includes most of the components associated clinically with SCI in humans, including tissue damage, physical trauma and both sensory and motor changes (Siddall et al., 1997; for review see also Wrathall, 1992). This study reports, for the first time, a detailed description of the changes in both NADPH-d expression (an indicator of nitric oxide synthase activity) and fos-like IR

(an indicator of neuronal activity), seven days after a contusive SCI. This is at a time during which more chronic conditions appear to develop (Xu et al., 1992), and at which both sensory and motor changes are manifest (Siddall et al., 1995). Furthermore, it has been shown that following the development of this chronic condition, inhibitors of nitric oxide synthesis are able to reverse the allodynia observed (Hao et al., 1994a), suggesting that alterations to NO synthesis may have important behavioural ramifications.

2.3.1 Technical Considerations

NADPH-d histochemistry is a well established technique that is widely used as a marker of NOS activity and thus NO production (Bredt et al., 1991a; Dawson et al., 1991a; Hope et al., 2001; Laing et al., 1994; Wu et al., 1994). Thus the present data likely reflect changes in nitric oxide production following SCI. The significance of the expression of fos protein has been discussed in many other publications. It is clear that immediate early genes, such as *c-fos*, are induced by membrane depolarisation and increased intracellular calcium levels (Morgan and Curran, 1986; Sheng and Greenberg, 1990), and there is general agreement that increases in *c-fos* expression are a consequence of increased neuronal activity (Anton et al., 1991; Dragunow and Faull, 1989; Hunt et al., 1987; Sagar et al., 1988; Strassman and Vos, 1993).

It should be noted that in the present study there was a relatively high basal level of fos protein expression that was probably related to the handling and behavioural testing the animals underwent as part of their normal upkeep. These occurred up to and including the morning of sacrifice. It has been shown previously that stressful or novel environments enhance neuronal fos expression, as does light restraint (Chowdhury et al., 2000; Cullinan et al., 1995; Handa et al., 1993; Otake et al., 2002; Staiger et al., 2002; Wirtshafter et al., 1998). This was validated in the present series of studies and reported in chapters 5 and 6 of this thesis in which an unhandled group of rats was compared with uninjured rats that were behaviourally tested. While behavioural data only formed a part of the study reported in chapter 4 of this thesis, all rats were monitored behaviourally.

2.3.2 Spinal Cord Morphology after Injury

Cavitation following SCI is a well reported phenomenon and has been found to occur both rostral and caudal to the injury (for example Gorio et al., 2002; Kao and Chang, 1977; Schwab and Bartholdi, 1996; Takami et al., 2002). This is in agreement with the findings of

the present study, which also indicate that spinal morphology is disrupted both rostral and caudal to the injury.

The pattern of degeneration rostral to the injury following a single contusive SCI involves a large central cavity in the white matter between the dorsal horns. At its widest point the cavity takes up over 50% of the white matter tracts, although generally spares the dorsal columns. Caudal to the injury the pattern is slightly different, appearing as a series of microcysts, generally within the ventral white matter and occasionally the grey matter of the ventral horns.

These differing patterns of cavitation are thought to derive from alternate mechanisms and are likely to occur as a result of the secondary consequences of injury. In particular, the initial physical trauma of the injury results in endothelial damage as a result of the pressure wave produced at impact (Balentine, 1978; Goodman et al., 1979; Griffiths et al., 1978; Osterholm, 1974). This then leads to a cascade of events that result in ischaemic damage to the cord as a result of occlusion of the microvasculature (Balentine, 1978; Goodman et al., 1979; Griffiths et al., 1978; Means et al., 1978; Osterholm, 1974; Sandler and Tator, 1976; Wagner et al., 1978). The early work by Allen also suggests that oedema and haemorrhage constrained by the meninges results in increased pressure and damage of the neural tissue (Allen, 1911).

It is these changes that lead to the cavitation observed following traumatic SCI. Interestingly it has been reported that the initial necrosis as a result of injury begins at a point of the grey matter just dorsal to the central canal, where the maximum pressure due to oedema occurs (Balentine, 1978; Ducker et al., 1971; Faden et al., 1981a; Faden et al., 1981b; Lewin et al., 1974; Osterholm, 1974; Wagner et al., 1978; Yakovlev and Faden, 1995). Thus it is likely that the central dorsal cavitation that forms rostral to the injury is likely due to the increased pressure resulting from injury-induced oedema constrained by the meninges, in addition to vascular changes.

Why the pattern of degeneration caudal to the injury should take the form of a series of microcysts restricted largely to the ventral spinal cord is not known. However Guizar-Sahagun and colleagues (Guizar-Sahagun et al., 1998) do report that after both spinal transection and contusion microcysts have formed by about 7 days after injury and are still

present up to 90 days later. The authors found that the formation of microcysts is immediately preceded by the swelling of axons to produce structures descried as axon- or terminal clubs (Kao and Chang, 1977). These terminal clubs were then found to express NADPH-d activity immediately before collapsing and degenerating, leaving a microcyst. Microcysts were found to form for up to 5mm from the injury site which is similar to the distance observed in the present study. Similar results have also been reported elsewhere (Basso et al., 1996).

The laminar, or rostro-caudal distribution of the microcysts however is not mentioned in the Guizar-Sahagun study, therefore it is not clear as to why they should be distributed in largely the ventral aspect of the cord, caudal to the injury in the present study. However, it has been observed previously that, following traumatic SCI, the fibres of the ventral cord demonstrate an increase in NADPH-d activity caudal to the injury, while ventral fibres rostral to the injury have been found to decrease levels of NADPH-d expression (Lukacova et al., 2000). Guizar-Sahagun and colleagues found that axons were induced to express NADPH-d activity immediately prior to degeneration (Guizar-Sahagun et al., 1998), thus these caudal axon fibres may degenerate preferentially resulting in the microcysts observed. Furthermore, it has been suggested that NO may be involved in axonal degeneration in the neurodegenerative disorder multiple sclerosis (Smith and Lassmann, 2002), and also, inhibiting NO synthesis has been found to reduce axonal degeneration in ganglion cells of the retina following axotomy (Koeberle and Ball, 1999).

While the numbers of studies into this process are limited, it seems possible then that the dorsal cavitation rostral to the injury and the ventral microcysts caudal to the injury may reflect these differing mechanisms of degeneration following traumatic injury.

2.3.3 Injury Evoked Decreases NADPH-d in the Superficial Dorsal Horn

As described above, contusive SCI reduced neuronal NADPH-d expression in laminae I and II of the superficial dorsal horn, and increased its expression in the ventral horn (laminae VIII and IX), most notably in motoneurons. These changes were observed at seven days following injury and were strongest adjacent to the site of injury. The expression of NADPH-d in the mid-cervical segments remained unchanged, resembling that of control animals.

Other studies have also examined NO synthesis following various forms of SCI, although often at more acute time points. Five hours following a spinal cord hemisection, for instance, increased numbers of NOS-containing neurons have been reported in laminae I and II, both caudal and rostral to the injury (Sharma et al., 1996). In a microdialysis study also, there was found to be an increase in NO synthesis immediately after injury and this occurred as two waves of NO production. Following static weight compression of the spinal cord, an initial wave occurred immediately upon compression, and decreased by 24 hours. The second wave of NO production occurred after 24 hours and lasted until approximately 3 days after injury. It is thought that the first wave may be due to constitutively expressed nNOS activity while the second wave may be a product of iNOS, requiring protein expression (Nakahara et al., 2002). In the present study, on the other hand, rather than increased numbers of NADPH-d positive cells a decrease in NADPH-d was observed in these laminae. This occurred at 7 days following injury. The decreased NADPH-d expression reported here then appears to be related to the length of time following injury.

Increased NO production in the early post-injury phase (i.e. at 5 hours) is thought to be related to inflammatory processes resulting from the injury, as peripheral application of inflammatory mediators have been found to evoke increased NADPH-d activity in the SDH (Dolan et al., 2003; Goff et al., 1998; Herdegn et al., 1994; Lam et al., 1996; Orendácová et al., 2000). Taken together, it appears that there is a biphasic response in NO production in the SDH following injuries of the spinal cord likely comprising of an immediate increase in the number of cells producing NO (i.e., increased NOS immunoreactivity) followed (at least one week later), by a decrease in NO production (i.e., decreased NADPH-d labelling).

This suggestion of a longer term reduction in NO production in the SDH, is supported by the data from Mense's group (Trudrung et al., 2000) who report that six weeks following a complete spinal cord transection the number of NADPH-d labelled cells in the SDH had decreased. In addition, they also found that the number of NADPH-d labelled neurons was more markedly reduced caudal to the injury site, which is in agreement with the present study. It is interesting to note that a decrease in the number of NOS labelled neurons in the SDH has also been reported 7-14 days following nerve injury to both the sciatic nerve (Goff et al., 1998) and the cauda equina (Orendácová et al., 2000).

Whether the decrease in nitric oxide synthesis observed plays a role in the expression of longer term sensory and motor changes is not clear. A high density of NADPH-d positive cells has been found to be located in the SDH, both in the present study and others (Aimar et al., 1998; Bredt et al., 1991a; Nakamura, 1997; Trudrung et al., 2000). These laminae are thought to be responsible largely for the transmission of nociception (Baba et al., 1994; Keay and Bandler, 2002; Yaksh and Hammond, 1982). NO has also been reported to be involved in sensory transmission, particularly nociception (Luo and Cizkova, 2000; Riedel and Neeck, 2001). Thus, a decrease in NADPH-d positive cells, and therefore NO, in the SDH might be expected to alter sensory function, particularly in relation to nociception.

Chronic constriction of the L5/L6 spinal nerves has been found to result in the development of neuropathic pain which is fully expressed by one week following injury and remained significantly different for at least 20 weeks (Choi et al., 1994). This indicates that at approximately the time that the present study noted a decrease in NO synthesising cells in the SDH following SCI, neuropathic pain is found to develop in another model. However, in this model of nerve injury, nitric oxide inhibitors were shown to *decrease* mechanical and cold allodynia, in addition to on-going cold induced pain. Other studies too, have found that inhibition of NO synthesis attenuates the neuropathic pain that develops as a result of SCI and peripheral nerve injury (Hao and Xu, 1996; Meller et al., 1992; Salter et al., 1996; Wong et al., 1998; Yamamoto and Shimoyama, 1995). This is interesting in that, at this time point, spinal cord and peripheral nerve injury had already reduced the number of NO producing cells. Some mechanism must therefore exist whereby further reduction in NO synthesis attenuates the neuropathic pain that develops as a result of injury. This conclusion requires additional consideration and will be addressed further in Chapter 4. The functional deficit that may result from altered NO synthesis in the SDH may be, at least partly, explained by an examination of the identity of the cells that are lost following SCI.

2.3.4 Which cells are "lost"?

The present study provides no evidence as to whether the reduction in NADPH-d positive cells occurs as a result of cell death or simply a downregulation of the proteins expressed. The functional consequences of NADPH-d reductions in the SDH however, can be considered.

As discussed above, a decrease in the number of NADPH-d positive cells appears to occur at approximately the same time that neuropathic pain develops following injury (Hao and Xu, 1996; Meller et al., 1992; Salter et al., 1996; Wong et al., 1998; Yamamoto and Shimoyama, 1995). The question becomes then, how might a decrease in the number of NADPH-d producing cells in the SDH be responsible for the development of this neuropathic pain?

The SDH receives largely nociceptive sensation transmitted by Aδ- and C fibres (Baba et al., 1994; Keay and Bandler, 2002; Yaksh and Hammond, 1982). Amongst the cells that receive these inputs are the NADPH-d containing neurons of the SDH. It has been reported that these NADPH-d containing cells virtually all contain γ-aminobutyric acid (GABA) (Gobel, 1978; Spike et al., 1993; Valtschanoff et al., 1992b). Using anti-GABA antibodies it was found that the highest concentration of GABA +ve cell bodies were in the SDH (Magoul et al., 1987). Furthermore, the morphology of the majority of NADPH-d expressing cells in the SDH of the present study were identical to those described by Valtschanoff and colleagues (Valtschanoff et al., 1992b) as gelatinosa, or border cells. These also correspond to the islet and spiny cells, described in the Golgi studies of Beal *et al*. (1989). The border cells and the islet cells of the latter two studies are the major NO containing neurons of lamina II (Aimar et al., 1998) and in addition, they are a thoroughly investigated population of inhibitory neurons (Barber et al., 1982; Gobel, 1975; Gobel, 1978; Todd and McKenzie, 1989; Valtschanoff et al., 1992b). Most GABAergic terminals in the substantia gelatinosa of the rat spinal cord are thought to be the axon terminals of neurons from the same laminae, indicating that these GABAergic cells are likely to be interneurons (Barber et al., 1978; Bernardi et al., 1995).

Thus, the majority of NADPH-d expressing cells revealed in the SDH of the present study are highly likely to be inhibitory interneurons, suggesting that following contusive SCI, inhibitory interneurons either die or stop synthesising NO, one week following injury. It has also been shown that blocking NO synthesis in the spinal cord results in an increase in the background “activity” of SDH neurons without alterations in sensory-evoked responses (Hoheisel et al., 1995). In addition, topical administration of L-arginine, a NO precursor, to the spinal cord significantly reduces SDH neuronal responses to electrical stimulation of Aβ and C fibres (Haley et al., 1992). It is possible, therefore, that the decreased number, or altered biochemistry, of the inhibitory cells of the SDH observed in the present study

following SCI may be responsible for changes in sensation observed in other studies. Reduced numbers or altered function of inhibitory cells may result in alterations in background activity of neurons within these laminae. These changes one week following contusive SCI may underlie the enhanced sensory processing and reduced somatosensory thresholds which are often observed following injuries to the cord (Bowsher, 1996; Christensen et al., 1996; Siddall et al., 1995; Xu et al., 1992; Yezierski et al., 1998).

2.3.5 Injury Evoked Increases in NADPH-d in the Ventral Horn

Seven days following SCI, many cells of the ventral horn, including motoneurons begin to express NADPH-d, suggesting NO synthesis is triggered in this region as a result of injury. A number of reports describe injury induced synthesis of NO in motoneurons. These include ventral root avulsion (Wu, 1993; Wu et al., 1994), ligation of the cauda equina (Orendácová et al., 2000) and transection of cranial nerves (Yu, 1994). These manipulations all affect the peripherally directed fibres of motoneurons, thus axonal damage appears to increase NO production in these cells. On the other hand it has been reported that sciatic nerve transections do not effect NADPH-d expression in adult rats (Brecht et al., 1997; Clowry, 1993; Fiallos-Estrada et al., 1993; Yu, 1994) unless the transection is associated with a subsequent nerve ligation (Carr et al., 1998). It would appear then that motoneurons only synthesise NO if their function is disrupted. This includes the regeneration of their axons, a characteristic ability of peripheral nerves. If the damaged axons of motoneurons are able to regenerate normally then NO does not appear to be synthesised by this cell type. Motoneuronal integrity is critically dependent on the presence of target derived neurotrophic factors, including those produced by the Schwann cells of their peripheral axons. Thus, it is possible that transected, but not ligated axons are able to generate the trophic support for axonal regeneration and/or motoneuronal survival, whereas avulsed ventral roots (Wu, 1993; Wu et al., 1994), ligated cauda equinae (Orendácová et al., 2000), and transected cranial nerves (Yu, 1994) are not.

In addition to physical injury, it has been shown that hind limb immobilisation also results in increased NADPH-d activity in ventral horn motoneurons. This increase is reversed by re-mobilisation of the joint (He et al., 1997), indicating the functional dependence of motoneuron NO synthesis.

Taken together, the data from these studies suggests that expression of NADPH-d in motoneurons is related largely to loss of function, whether that be due to loss of trophic support or physical restraint. In the present study, therefore, the increase in NADPH-d in motoneurons adjacent to the level of injury may be associated with either (i) disruption of motoneuronal integrity as a result of injury to the cord, or (ii) dramatically altered activity due to damaged descending tracts to the motoneurons themselves due to the SCI.

2.3.6 Injury Evoked Decreases in fos expression

When fos expression was examined in the same spinal cord sections as those for NADPH-d activity, the number of fos expressing cells was found to be reduced in animals following traumatic SCI. The reduction in fos positive cells was observed in laminae I, II and III and in the ventral horn (laminae VII, VIII and IX), both rostral and caudal to the level of injury. While this occurred at spinal segments adjacent to the injury, surprisingly, this pattern was seen also in segments distant to the injury taken from the mid-cervical enlargement. Increased fos expression was seen only in the sacral parasympathetic nucleus lying caudal to the injury site.

In the present study, uninjured control rats showed moderate levels of fos expression in the spinal cord. These levels of fos expression are most likely due to the handling and behavioural testing animals underwent prior to sacrifice as part of their normal care and maintenance. It has been established previously that a two-hour post-stimulus window is the most effective for revealing maximal fos expression following a stimulus (Siddall et al., 1999b). Thus, in the present study, basal levels of fos expression in the spinal cord were slightly higher than controls in other studies of spinal cord function (Bullitt, 1990; Ruggiero et al., 1997; Woolf et al., 1994).

Seven days following SCI, the levels of spinal fos expression were significantly reduced, both at the site of injury and at the level of the mid-cervical enlargement. This is the first report of reduced fos expression in the spinal cord of SCI rats one week following injury. Previous studies have focussed on the acute phase (3-24 hours) of either spinal cord or peripheral nerve injury. This phase is characterised by a rapid increase in fos expression in the spinal cord at the level of the injury, or at the level of primary afferent terminations, reaching maximal levels within three hours and returning to baseline within 24 hours (Delander et al., 1997a; Gu et al., 1997; Luo et al., 1998; Ruggiero et al., 1997; Wu et al.,

2000; Yakovlev and Faden, 1994). Continued peripheral stimulation results in elevated levels of fos expression at the level of primary afferent input for up to 10 days following a peripheral nerve injury (Kajander et al., 1996). At seven days post-injury, we report decreases in fos expression. This effect extends into spinal regions distant from the site of injury, where NADPH-d activity did not change, as well as at spinal segments adjacent to the injury where NADPH-d activity did change. This suggests that changes in fos expression are i) not directly related to NADPH-d activity and ii) may be related to more global changes in spinal cord neural function following SCI.

One possible explanation for the reduction in fos expression observed following SCI may be due to reduced neural activity as a result of spinal shock. Spinal shock is a period following SCI when neuronal activity is reduced such that spinal reflexes are lost and is characterised by flaccid paralysis. It is thought that spinal shock may be due to the sudden loss of input from supraspinal centres following injury. In humans spinal shock has been reported to last anything from a couple of days to a couple of weeks depending on the severity of the injury (Hiersemenzel et al., 2000).

Following this stage of flaccid paralysis, a period occurs when spinal reflexes begin to return. This leads to muscle spasticity, in which tendon reflexes become hyper-responsive, increased muscle tone and involuntary muscular contractions (Hiersemenzel et al., 2000).

Few studies on spinal shock have been performed in rats, however the condition has been reported in passing in a number of experiments, particularly those in relation to loss of autonomic function as a result of SCI. These studies indicate that in the rat, as in the human, there is a loss in spinal reflexes following spinal injury resulting in hypotension, hypothermia and hypoventilation as well as loss of ano-urethral reflexes and colonic motility (Holaday and Faden, 1980; Holmes et al., 1998; Meshkinpour et al., 1985). Unlike the human however, loss of reflexes occurs over a much shorter timeframe in rats, appearing to resolve within 24 hours (Holmes et al., 1998; Meshkinpour et al., 1985; Schouenborg et al., 1992; Shaker et al., 2003).

Indeed studies examining return of locomotor activity following contusive SCI report either a return of function, or begin exercise training, as quickly as the first or second day after injury

(Basso et al., 1995; Hutchinson et al., 2001; Van Meeteren et al., 2003). These illustrate the rapid recovery of the rat from spinal shock.

The present study was performed at 7 days following injury which is well after these earlier studies indicated a return of function. Rats in the present series of studies likewise demonstrated a return of locomotor function within 24 hours of injury, though the hind limbs of many rats displayed muscular rigidity which generally resolved over the next 2-4 days. By 7 days following injury there was a significant improvement in motor score (Figure 4.2, Chapter 4) demonstrating that spinal shock had resolved in these animals over the time course examined. Also, as the upper limbs appear unaffected by injury from approximately 12hours onwards, it is unlikely that cervical spinal cord was affected by spinal shock at 7 days. However, this part of the cord demonstrates the same decrease in Fos expression observed in spinal segments adjacent to the injury. Thus it is unlikely that the reduced levels of fos expression observed in the present study was due to spinal shock-induced loss of neuronal activity.

Another possible explanation for the reduced levels of neural activity (fos expression) was that spinally injured rats may have been less physical active than the uninjured group. Certainly this may have been true within the first couple of days following injury, however by 7 days a significant degree of locomotor activity had returned. The hind limbs of injured animals remained less functional than those of the uninjured group (See Chapter 4, Figure 4.2) but the rats appeared otherwise active and alert. Also simple locomotion does not induce increased fos expression, rather, in addition to noxious stimuli, fos is induced by prolonged or novel stressors such as swim stress, disruption of sleep, strong odours, restraint or a novel environment (Bellchambers et al., 1998; Chowdhury et al., 2000; Grassi-Zucconi et al., 1993; Guthrie et al., 1993; Handa et al., 1993; Jin et al., 1996; Wirtshafter et al., 1998). Therefore it is unlikely that the slightly increased levels of locomotion alone, in the uninjured group of animals, would have resulted in significantly greater levels of fos in this group. Lastly, in Chapter 5 and Chapter 6 a third group of naïve, uninjured animals was compared to the uninjured and injured group of behaviourally tested animals. This group of animals was not handled in any way before sacrifice and therefore represented the basal level of fos expression in the naïve animal. While the spinal cord was not examined in this group of animals, in both the PAG and the parietal cortex, the levels of fos expression was less in the naïve group of animals than that of the injured group. This was despite the naïve

group of animals having fully functional limbs and therefore, presumably, greater levels of spontaneous locomotion. For these reasons it is unlikely that reduced levels of physical activity in the injured animals would have resulted in the decreased fos expression reported here.

One way in which neural activity of the spinal cord can be altered is via supraspinal mechanisms. It has been shown for example that neural activity (fos expression) in the SDH depends on the activity of descending inhibitory pathways which travel in the dorsolateral fasciculus, (an area which remained intact following the contusive injury performed in the present experiments) and that these inhibitory pathways can be activated by both noxious stimuli and tissue injury (Bhandari et al., 1999; Harris et al., 1995; Jasmin et al., 1994b; Liu et al., 1999b; Sandkühler, 1996; Zhang et al., 1993; Zhang et al., 1994b). It is possible, therefore, that at seven days post-injury, the levels of activity in the SDH of the spinal cord are reduced by increased activity in these supraspinal descending inhibitory circuits, an hypothesis which awaits experimental verification. In addition, the reduction in fos expression in ventral horn neurons may reflect reduced supraspinal and local segmental motor outflow, which is observable as a persistent reduction in function of the hind limbs following SCI.

2.3.7 Injury Evoked Increases in fos expression in the Sacral Parasympathetic Nucleus

The only region in which there was increased c-fos expression, and thus a presumed increase in neuronal activity following SCI, was the sacral parasympathetic nucleus (SPN). Neurons of the SPN are responsible for both afferent sensory reception from-, and motor efferent control to the bladder. This afferent and efferent drive is mostly parasympathetic, originating from S2-S3 in rats (Arsdalen and Wein, 1991; Sutherst et al., 1990). The increases in neural activity in the SPN may underlie the profound changes in both bladder and colon function observed following spinal cord injuries in humans (Gutierrez et al., 1993; Yalla and Fam, 1991).

Increased stretching of the bladder wall with filling results in a greatly increased afferent discharge, which reaches the spinal cord through spinal nerves to the S2-S4 sacral segments, as well as the sympathetic hypogastric nerve to T10 - L2 spinal segments (Sutherst et al., 1990). The sacral fibres convey conscious touch, and pain from the bladder

and its mucosa, and also proprioceptive information such as a sense of fullness. The thoracolumbar afferents are active only when the bladder is very full (Sutherst et al., 1990). SPN neurons exhibit an extensive axon collateral system which projects to many laminae of the spinal cord including lamina X, the dorsal commissure and the lateral dorsal horn. It is thought that these neurons may therefore be involved in the spinal integration of reflex bladder function including inhibition and modulation of sphincter reflexes (de Groat, 1993).

In the present experiment, the most likely explanation for the observed increased c-fos expression in the SPN is that spinal shock associated with SCI resulted in loss of bladder reflex activity. This activity is reported to return within 6-8 weeks following injury in humans (Yalla and Fam, 1991). The loss of this reflex results in acute urinary retention and bladder distension. Experimentally, bladder distension has been shown to increase c-fos expression in the SPN (Birder and de Groat, 1992; Vizzard, 2001). The bladders of the animals in the present experiments were not manually evacuated, although a degree of bladder function was observed to return within the seven day experimental period. The increase in c-fos observed here in the SPN is therefore likely due to injury-induced bladder distension.

2.3.8 Reduction in Double Labelled Cells

Lastly, it was noted that the number of NADPH-d/fos double labelled cells was reduced in lamina I and II, in particular in the adjacent and intermediate segments of the caudal tissue block. It was also noted that these laminae demonstrated significant decreases in both NADPH-d activity as well as fos activity. It is perhaps not surprising then, that the number of NADPH-d/fos double labelled cells is also decreased accordingly.

2.4 Conclusions

While previous studies have examined the effect of various forms of neuronal injury on NO synthesis in the spinal cord, the present study was the first to examine both the dorsal and ventral horns over such a large number of spinal levels. In addition, this was the fist study to examine the SDH at 7 days following injury and to note the inhibitory nature of the NADPH-d cells that were “lost” following SCI. Lastly this is the first time that the effect of SCI on NO synthesis was compared to the effect of SCI fos expression, also over such a large spinal distribution, allowing the observation of the differing effect of SCI on these two molecules.

It was the aim of this experiment to determine the effect of traumatic SCI on the number of NO synthesising cells within the spinal cord of the rat at seven days following injury. This was done utilising NADPH-d histochemistry. The effect of the same injury on neuronal activity was also examined utilising fos immunohistochemistry. It was the findings of this study that the number of NO synthesising cells was reduced in the SDH, one week following injury. The NADPH-d positive cells observed in the present study bore the same morphology as GABA positive inhibitory interneurons, reported previously. Thus it appears that traumatic SCI results in a decrease in the number or activity of inhibitory interneurons from the SDH at seven days following injury. Furthermore these laminae from which they are lost are involved in receiving and processing nociception. Loss of inhibitory cells in these laminae is therefore likely to result in changes in sensory processing. This possibility will be examined further in chapter 4 of this thesis.

It must be noted that while the number of NADPH-d positive cells in the present study was found to be reduced in the SDH, it was not possible with the methodology used here to determine whether the cells were killed or whether the level of NADPH-d activity was simply reduced. To attempt to provide further details of this potential “loss” of cells, the effect of traumatic SCI on the total number of neurons in the SDH will be addressed in the following chapter of this thesis.

As well as changes in the number of NADPH-d positive cells in the SDH following SCI it was also found that traumatic injury resulted in an increase in the number of NO synthesising cells in the ventral horn. This was interpreted to occur as a result of either a disruption of the cellular integrity of the motoneurons as a result of injury directly, or altered activity due to a reduced motor output stemming from damaged descending motor pathways.

Lastly, the effects of traumatic SCI on neuronal activity was also examined in the same sections and was found to be reduced as a result of traumatic SCI. It was not known why neuronal activity should be reduced at seven days following injury, especially considering the possibility that the inhibitory cells of the SDH were lost following injury. Loss in inhibition generally leads to an increased level of activity. However it was noted that the reduction in neuronal activity was reduced even at spinal levels distant from the injury. This indicated

that the changes in activity were not related to biochemical changes at the site of the injury but instead may have reflected more global changes to the activity levels of the CNS. One suggested possibility was that supraspinal structures may be involved in either mediating this global change, or may indicate other possibilities. These hypotheses will be examined further in chapters 5 and 6 of this thesis.

Chapter 3

Effects of traumatic spinal cord injury on the total number of neurons in the superficial dorsal horn of the rat: a study utilising NeuN

As discussed in the previous chapter the number of NO synthesising cells in the SDH was found to decrease at seven days following traumatic SCI. The method used to determine this utilised NADPH-d histochemistry. While the number of NADPH-d positive cells was reduced it was not possible to determine whether this was due to a decrease in the number of cells, or whether the expression of NADPH-d activity was simply reduced. In order to address this question the present experiment sought to determine the effect of traumatic SCI on the total number of neurons in the SDH both before and following traumatic SCI.

As the NADPH-d labelled cells in the previous chapter bore the morphology of neurons, a marker for neuronal cells was utilised in order to determine whether the total number of neurons in the SDH was altered. The marker selected for this purpose was the neuronal nuclear marker (NeuN), a protein that is expressed exclusively in the nuclei of neurons, and has been increasingly used as a marker for neuronal cells throughout the nervous system (Mullen et al., 1992). Not all neurons in the CNS however have been found to express NeuN. A number of regions in the brain, in addition to the sympathetic chain ganglia, have been identified as containing neurons that are negative for NeuN immunoreactivity (Harkany et al., 2002; Mullen et al., 1992; Wolf et al., 1996). The present study however is restricted to the spinal cord and it has been reported that NeuN appears to be expressed in all neurons of the spinal cord (Todd et al., 1998).

In order to further characterise the effect of traumatic SCI then, it is the aim of the present study to determine whether there is an overall decrease in the number of neurons in the SDH following traumatic SCI. If a decrease is found, the reduced number of inhibitory, NO producing cells reported in the SDH in the previous chapter may be interpreted as a loss of

cells as apposed to a functional down regulation in NO synthetic activity. This may have a bearing on the function of these cells and the potential for therapeutic intervention.

3.1 Methods

3.1.1 Animals and surgery

A total of 11 female Wistar rats (200-300g) were used in the present study. Of these, five were subjected to contusive SCI and six served as uninjured controls. Details of the injury performed were described in the previous chapter, with the exception that all animals received a weight drop 3cm in height for the present experiment. Sacrifice and perfusion of the animals was likewise performed as described previously.

3.1.2 Histology

Briefly, the spinal cord was divided into three blocks of tissue taken from the mid cervical enlargement (5mm); rostral to the injury at approximately L4 (10mm) and caudal to the injury (10mm). The blocks taken from rostral and caudal to the injury were taken from the visible epicentre of the contusion (Figure 3.1).

A one in five series of 20µm coronal sections from each of these blocks was cut on a freezing microtome. Sections underwent NADPH-diaphorase (NADPH-d) histochemistry to enable clear visualisation of the superficial dorsal horn which is heavily positive for NADPH-d reaction product. The sections were washed twice in 0.1M phosphate buffer (PB, pH 7.4) for 10min, before being incubated for 2.5-3 hours at 37°C in a solution containing 0.3% triton X-100, 15mM malic acid, 0.5mM β-NADPH and 0.2mM nitroblue tetrazolium (Sigma). Sections were then transferred into 0.1M PB to stop the reaction.

3.1.3 Immunohistochemistry - NeuN

Following visualisation of NADPH-d, sections were rinsed twice for 10 minutes in PB, then incubated in 20% normal horse serum in PB for 20 minutes. Sections were then incubated in primary antibody diluted in phosphate buffered horse serum (PBH; 0.1% BSA, 2% normal

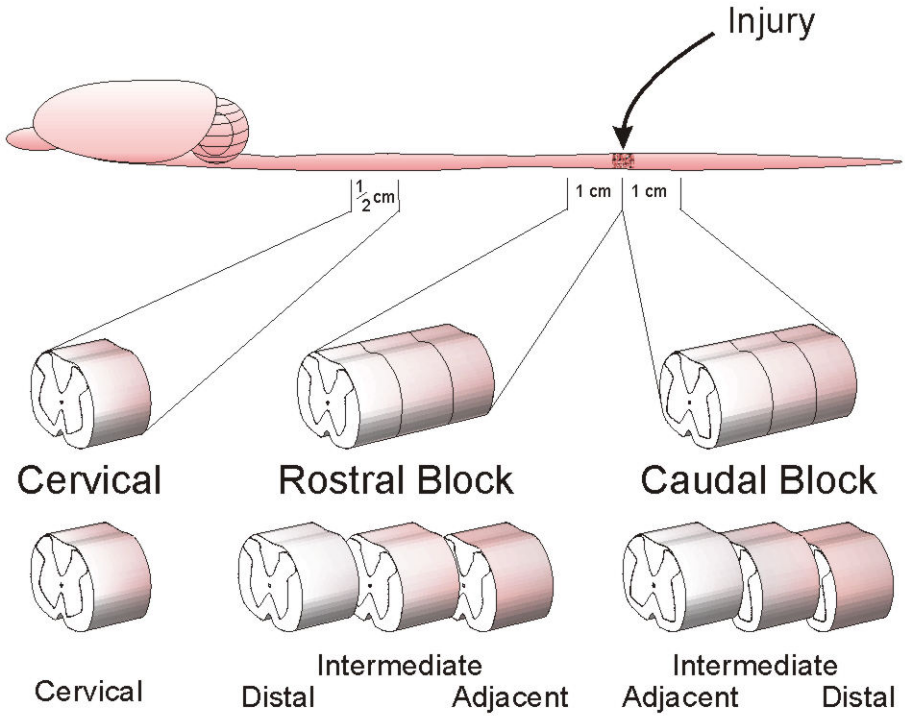


Figure 3.1 Diagram illustrating the segments of the spinal cord examined in the present study. The spinal segments examined were the same as those examined in chapter 2. These were the three principal blocks of tissue taken from the mid cervical enlargement (5mm), from the visible centre of the injury rostrally (10mm), and from the visible centre of the injury caudally (10mm). Five random sections were then examined from the cervical tissue block and from each third of the rostral and caudal blocks (from the third adjacent to the injury, the third intermediate to the injury, and the third most distal to the injury), processed for NeuN immunoreactivity.

horse serum, 0.2% Triton X-100 in 0.1M PB), for 2-3 days at 4°C. Primary antibody was a monoclonal mouse anti-NeuN (Chemicon) antibody used at 1:2000. The sections were then washed twice in PB for 10 minutes, placed into biotinylated goat anti-mouse IgG (Amersham; 1:500) and incubated for two hours at room temperature. Sections were then washed twice in PB for 10 minutes and then placed into Extr-avidin peroxidase (Sigma) at 1:1000 for 2.5 hours at room temperature.

Following two PB rinses, the bound peroxidase was then visualised using diaminobenzidine as the chromogen and using the glucose oxidase method of peroxide liberation (see Clement et al., 1996 for details, also descibed in Chapter 6). The reaction was terminated by rinsing in several changes of PB, when the positively stained nuclei could be clearly seen under microscopic visualisation at 10x magnification. Sections were then mounted onto glass slides with 0.5% gelatine solution and left to air dry at room temperature before being dehydrated through serial alcohols to histolene and cover slipped with DPX.

3.1.4 Plotting and Statistical Analysis

Coverslipped sections were examined under the light microscope and five random sections were plotted from the cervical tissue block and also from each third of both the rostral- and caudal tissue block. The rostral most level plotted was equivalent to the low thoracic spinal level, the mid rostral level was upper lumbar, while the level adjacent to the injury in the rostral tissue block was the equivalent of upper-mid lumbar. The level of the caudal tissue block adjacent to the injury was the equivalent of mid-low lumbar level, the mid-caudal block of low lumbar upper sacral, while the caudal most block was the equivalent to mid sacral spinal level (Figure 3.1).

Sections were plotted using a camera lucida attachment. The numbers of NeuN-positive cells were then counted bilaterally in the SDH of the sections selected in both injured and uninjured animals. The mean number of labelled cells bilaterally, within each of the spinal levels, were then compared. Significance was determined using an ANOVA, at a 0.05 level of significance.

3.2 Results

A total of 195,079 neurons were counted in the superficial dorsal horns (SDH; laminae I and II) of 385, twenty micron sections taken from 11 rats. Thirty-five sections were taken from each rat used in the study. The six uninjured rats demonstrated a mean of 18,527 (±786.4 SEM) neurons per rat in the SDH of the sections counted, while five spinally injured rats demonstrated a mean of 16,783 (±644.3 SEM) neurons per rat in the same sections. As indicated in Figure 3.1, the spinal cord was blocked into three regions, a 5mm block taken from the mid cervical enlargement, a 10mm block taken rostrally from the visible epicentre of L3-4 injury, and a 10mm block taken form the injury epicentre, caudally. The tissue blocks were then sectioned at 20μm as described above and stained for NeuN immunoreactivity. Once stained, five random sections were counted from the cervical enlargement, and from each third of the rostral and caudal tissue blocks such that sections were examined from a total of seven spinal levels. The adjacent, intermediate and distal spinal segments of the rostral and caudal tissue blocks represent the spinal cord over 10mm both rostral and caudal to the contusion injury, while the block taken from the mid

cervical enlargement represent sections distant to, and therefore not likely to be directly influenced by, the injury.

3.2.1 NeuN positive cells in the SDH of the uninjured animal

The SDH was found to vary in area according to spinal level examined. The number of NeuN positive neurons in the SDH was related to the relative area of the SDH, ie the bigger the laminae, the more neurons they contained. The NeuN labelling in the SDH was restricted to the nucleus, whereas the larger neurons of deeper laminae demonstrated cytoplasmic labelling also. The profiles in the SDH were all small (5-10μm) rounded nuclei and demonstrated no particular distribution throughout the lamina, i.e. in cords, lamella etc (Figures 3.2 - 3.4).

3.2.2 NeuN positive cells in the SDH following SCI

Following a L3-4 (spinal level) injury the number of NeuN positive cells in the SDH tended towards a decrease across the vertebral levels studied. An ANOVA was then performed however there was no significant difference in the total number of neurons in the SDH between the uninjured and injured animals. These data are summarised in Table 3.1 shown in Figure 3.5.

3.3 Discussion

It was the aim of this experiment to determine whether the total number of neurons in the SDH of the rat is altered following a L3-4 spinal contusion. To this end the total number of neurons labelled with NeuN immunoreactivity were counted in the SDH of six uninjured and five spinally injured rats. The neurons were counted in five random sections taken from seven spinal levels both adjacent to and distant from the injury. When the counts were examined using an ANOVA, there was no significant difference between uninjured and injured animals found.

Table 3.1 Mean number of NeuN labelled cells per 20μm section from both uninjured and injured groups of rats. Sections were examined at seven spinal levels, the mid cervical enlargement (mid cervical), each third of the rostral tissue block (rostral distal, rostral middle and rostral adjacent) and each third of the caudal tissue block (caudal adjacent, caudal middle, and caudal distal). Rostral and caudal tissue blocks extend from approximately T11-S3.

Spinal Level	Uninjured (mean number of labelled cells per 20 μm section)	Injured (mean number of labelled cells per 20 μm section)
mid cervical	523.9 ± 33.07 (SEM)	480.3 ± 23.82 (SEM)
rostral distal	410.3 ± 29.17	412.1 ± 21.23
rostral middle	472.6 ± 23.89	404.9 ± 16.12
rostral adjacent	531.4 ± 62.73	422.7 ± 23.24
caudal adjacent	572.4 ± 63.73	544.6 ± 48.43
caudal middle	613.7 ± 25.99	573.0 ± 48.79
caudal distal	580.4 ± 25.18	519.0 ± 35.44

3.3.1 Technical considerations

The present study utilised NeuN as a marker to determine whether neurons were lost following traumatic SCI. NeuN is an expressed protein, albeit a constitutively expressed protein, thus it is possible that a perturbation of the cell may reduce expression of the protein without necessarily a loss of the cell. Since the structure or function of NeuN has not yet been elucidated, it is not possible to estimate how likely it is that this may occur. However, decreases in NeuN immunoreactivity were attributed to a loss of neurons following middle cerebral artery occlusion (Kokubo et al., 2002). In addition, NeuN labelling is reduced with the loss of neurons in the hippocampus at 24 hours and 1 week after induction of epileptic seizures (Poirier et al., 2000); activated microglia mediated neurotoxicity in culture (Zujovic et al., 2000), axotomy of neurons of the entorhinal cortex (Peterson et al., 1996), fluid percussion of the temporal cortex (Sato et al., 2001) and NMDA excitotoxicity of the basal forebrain in vivo (Harkany et al., 2002). Over a longer time course of 1-4 months too, experimentally induced glaucoma resulted in a reduction of NeuN labelling in the lateral geniculate nucleus, and this was also interpreted as representing a loss of neurons (Wang et al., 2000).

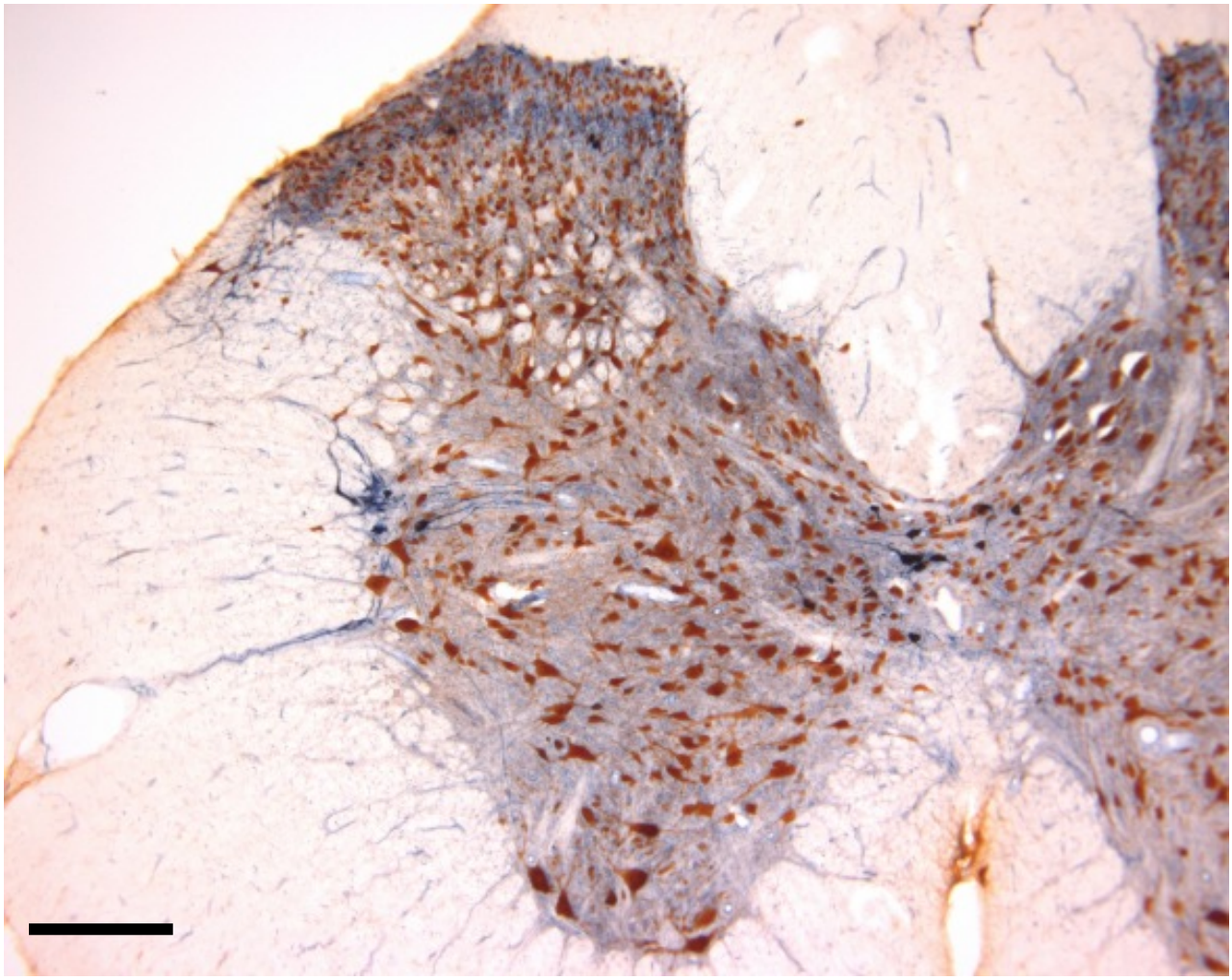


Figure 3.2 Low power micrograph of a 20μm coronal section taken from the spinal cord of an uninjured animal. Section has been processed for NADPH-d histochemistry (blue reaction product), and NeuN immunohistochemistry (brown reaction product). Scale bar represents 200μm.

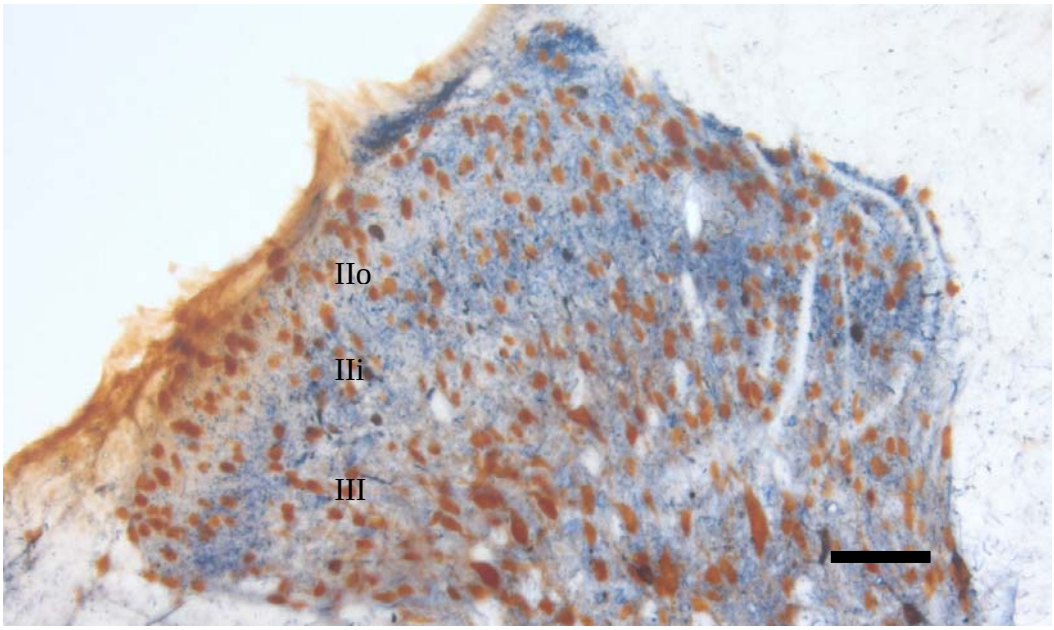


Figure 3.3 Micrograph of a 20μm coronal section showing the SDH of an uninjured animal. Section has been processed for NADPH-d histochemistry (blue reaction product), and NeuN immunohistochemistry (brown reaction product). The dense NADPH-d positive neuropil of inner lamina II (IIi) is clearly visible just under the less intensely stained outer lamina II (IIo). Lamina III likewise demonstrates a less intense NADPH-d positive neuropil and is visible as a thin band situated over the lateral reticulated portion of lamina IV (not shown). The NeuN labelling is restricted to the nucleus of the small neurons of the SDH. Scale bar represents 60μm.

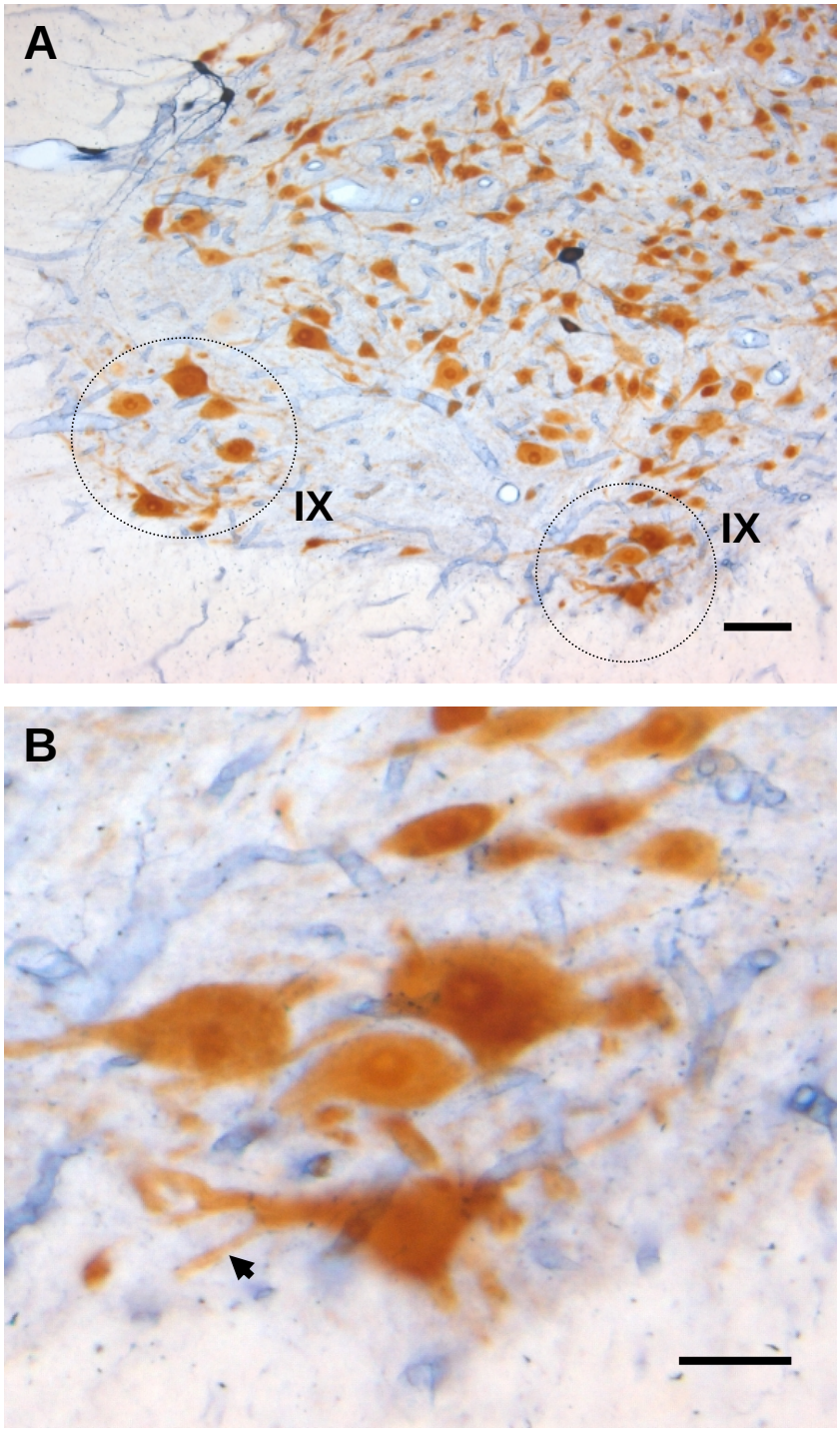


Figure 3.4 Micrographs of 20μm coronal sections showing the ventral horn of an uninjured animal. Section has been processed for NADPH-d histochemistry (blue reaction product), and NeuN immunohistochemistry (brown reaction product). A; Clusters of motor neurons comprising lamina IX are clearly visible (IX). B; High power micrograph of the lower lamina IX shown in panel A. NeuN reaction product is clearly visible in the nuclei but as in all larger neurons (greater than approximately 15μm in diameter) is also present in the cytoplasm though does not to label more than the second generation of neural processes (arrow head). Scale bar represents 70μm in panel A and 30μm in panel B.

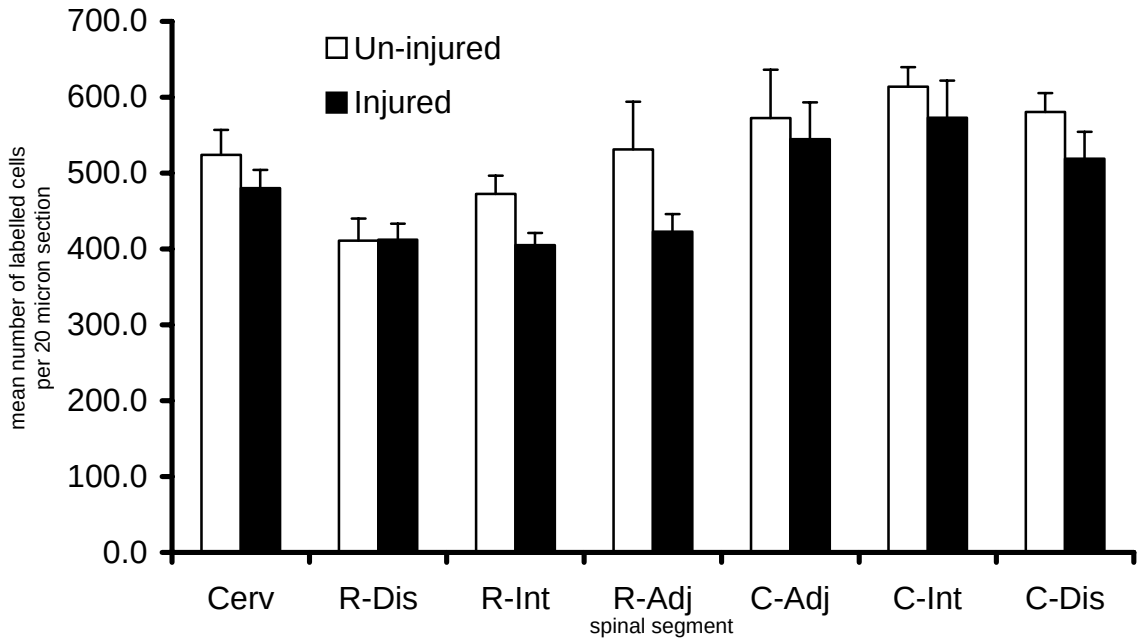


Figure 3.5 Histogram showing the mean number of NeuN labelled cells (plus SEM) per 20μm section in uninjured animals compared with animals receiving a traumatic lumbar spinal cord injury. Cerv, cervical; R-Dis, rostral distal spinal segment; R-Int, rostral intermediate segment; R-Adj, rostral segment adjacent to the injury; C-Adj, caudal segment adjacent to the injury; C-Int, caudal intermediate segment; C-Dis, Caudal distal segment. No significant difference was found between Uninjured and Injured animals.

In a study combining NeuN expression with cresyl violet staining of adjacent sections, it was found that the loss of labelled cells was concurrent with the area of tissue demonstrating degenerated pyknotic nuclei stained by cresyl violet (Andsberg et al., 1998). Lastly, the cytotoxic cell death induced by Kainic acid results in the apoptotic death of neurons five days after treatment. These are still found to be NeuN positive at the point of death (Tan et al., 2001). Other studies too have observed that cells undergoing apoptosis, and therefore irreversible cell death are still positive for NeuN, in both excitotoxic models (Neystat et al., 2001) and models of traumatic brain injury (Sanchez Mejia et al., 2001), These studies indicate that NeuN may be a reliable marker for neurons, even up to the point of death. Specifically at one week following administration of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine, the same time point used in the present study, another study found spinal cord neurons to be swollen, and TUNEL positive, while some appeared necrotic, and demonstrated granular NeuN labelling in the cytoplasm (Chera et al., 2002). This suggests that even one week following treatment sufficient to kill neurons NeuN is still found to be present within these cells, although labelling had become cytoplasmic possibly due to fragmentation of the nucleus associated with apoptosis. It should be noted too that NeuN

labelling in apoptotic cells is less intense than in normal cells suggesting that NeuN expression in cells is decreased quantitatively somewhat as cells die (Neystat et al., 2001) although notably, it is not absent.

So while there appear to be populations of neurons which do not label for NeuN (Andsberg et al., 1998; Harkany et al., 2002; Mullen et al., 1992), those that do label for this marker appear to be positive to the point of death. Thus NeuN appears to be a reliable marker for neurons and is likely to reflect the true number of neurons in the SDH following the contusion SCI utilised in the present study.

3.3.2 NeuN labelling in the SDH following SCI

As discussed in the previous chapter of this thesis, contusive SCI results in a profound decrease in the number of NADPH-d labelled cells in the SDH. Furthermore this decrease appeared to be dependent on local injury-induced changes. It should be noted however, that neuron counts were taken from spinal levels at which grey matter morphology was intact allowing examination of the SDH. Thus the results reported in this thesis indicate changes that occur at sections *adjacent* to the injury site, and will not represent the full level of neuronal loss that occurred at the epicentre of the injury.

The study reported here was performed to determine whether the decrease in NADPH-d labelling in sections adjacent to the injury, observed in the previous chapter, may be due to a decrease in cell numbers as a result of injury. While the possibility that NADPH-d positive neurons are selectively lost can not be eliminated, the results of the present study indicate that the total number of cells are not reduced in the SDH following contusive SCI. The reduction in the number of NADPH-d positive cells observed may therefore be due to an altered expression of NADPH-d activity following injury.

NADPH-d histochemistry has been utilised in the present study to indicate cells that are positive for the enzyme NOS and thus which synthesise NO. The NADPH-d activity of a NOS positive cell refers to the ability of that enzyme to transfer a pair of electrons from the carrier molecule, NADPH to a receiver molecule. In the process of NO synthesis that receiver molecule is L-arginine, however other molecules such as nitroblue tetrazolium may also receive the electrons from NADPH. This biochemistry is utilised in the histochemical

process to detect cells with NADPH-d activity (Bredt et al., 1991a; Bredt et al., 1991b; Dawson et al., 1991a; Hope et al., 1991).

A decrease in NADPH-d activity is likely to reflect a decreased ability of NOS to transfer electrons from NADPH-d, rather than an injury induced reduction in co-factors, since NADPH-d is supplied in the reaction solution. This suggests that NO synthesis will also be reduced as synthesis relies on this transfer of electrons (Bredt and Snyder, 1990; Bredt and Snyder, 1992; Forstermann, 1994; Stuehr et al., 1991).

Taken together then, while it appears that the NO producing cells of the SDH are not reduced in number following contusive injury, it is likely that injury does result in a reduced NO synthesis by these cells. Since the SDH of the normal rat is characterised by a dense population of NO synthesising cells (Aimar et al., 1998; Bredt et al., 1991a; Nakamura, 1997; Trudrung et al., 2000) it is likely that that normal function of the SDH is dependent on NO synthesis.

The SDH is involved in the reception and processing of nociception (Baba et al., 1994; Keay and Bandler, 2002; Yaksh and Hammond, 1982). This appears to involve NO, as the tonic release of NO in the normal SDH has been reported to inhibit the discharges of nociceptive dorsal horn neurons (Mense and Hoheisel, 2001). Also, microdialysis of 8-bromo-cGMP into the SDH resulted in a decreased neuronal response to pinch in the receptive neurons of the SDH. Background activity was also slightly reduced in these neurons (Lin et al., 1997). These studies indicate that, in the SDH, NO results in an inhibition of neural activity, particularly in cells transmitting nociception.

The previous chapter of this thesis indicated that the morphology of the NADPH-d positive cells in the SDH was the same as those described previously as border or islet cells (Beal et al., 1989; Valtschanoff et al., 1992b). Furthermore these cells have been described as the major NO synthesising cells of the SDH (Aimar et al., 1998) and are a population of inhibitory interneurons (Barber et al., 1982; Gobel, 1975; Todd and McKenzie, 1989; Valtschanoff et al., 1992b). Collectively these data suggest that these inhibitory neurons may inhibit incoming nociception or modulate the activity of dorsal horn neurons, at least in part, through the synthesis of NO. The present study however indicates that the ability of

these cells to synthesise NO is reduced following contusive SCI, therefore it is likely that the inhibitory activity of the SDH is also reduced following SCI.

It has been suggested previously that the neuropathic pain that develops as a result of SCI may be mediated by alterations in inhibitory drive within the spinal cord (Cui et al., 1996; Drew et al., 2001; Hao et al., 1991; e.g. Hao et al., 1992). This notion is also supported by studies indicating that alterations to GABA and glycinergic inhibitory neurotransmitter systems in the spinal cord may similarly lead to the development of neuropathic pain in the absence of injury (Hao et al., 1991; Loomis et al., 2001; Onaka et al., 1996; Reeve et al., 1998; Zhang et al., 2001).

3.4 Conclusions

The present study has found that there is no reduction in the total number of neurons in the SDH of the rat following contusive SCI. The reduction in the number of NADPH-d labelled cells in the SDH following injury, reported in the previous chapter, is likely due to a reduction in NADPH-d activity and not to a loss of cells. This reduction in NADPH-d activity is likely reflected in a reduced ability to synthesise NO which inturn is likely to reflect a reduced inhibitory drive in the SDH.

Since altered inhibitory drive to the spinal cord has been implicated in the development of neuropathic pain following SCI, it is possible that the changes observed in the present study may represent a mechanism by which such pain may develop. Certainly alterations in the normal inhibitory drive of the spinal cord are likely to result in functional changes in neuronal activity with in the cord. In order to test this hypothesis, the next chapter of this thesis will examine the effects of SCI on the levels of neural activity within the various laminae of the spinal cord. If the inhibitory drive of the spinal cord is changed as these previous two chapters have suggested, then it is likely that changes in neuronal activity will be observed within the spinal cord following injury.

Chapter 4

Changes in the laminar processing of non-noxious stimuli following contusive SCI in the rat

As discussed in the previous Chapter, the NO synthesising cells of the SDH of the rat appear to demonstrate an altered biochemistry following SCI such that their level of NADPH-d activity is much attenuated. This is likely to indicate a decreased ability to synthesise NO, which is dependent on the NADPH-d activity of the enzyme NOS to reduce L-arginine to citrulline and NO (Bredt and Snyder, 1990; Bredt and Snyder, 1992; Forstermann, 1994; Stuehr et al., 1991).

Since the inhibitory function of these neurons appears to, at least in part, depend on the NO they synthesise (discussed in Chapter 1), it was suggested that the injury-induced alterations to that synthetic activity would likely result in altered inhibitory drive within the spinal cord. It was hypothesised, as a result, that, if this was true, the neuronal activity of neurons within the dorsal horn of the spinal cord would be altered following SCI as a result of the altered inhibitory drive.

In support of this idea is the observation that the somatosensory system is one of the principal systems that remains affected long after the initial SCI. SCI has been found to result in the development of a number of neuropathic conditions characterised by the mis-transmission of sensory signals, including dysaesthesias, phantom pain, windup, hyperalgesia and allodynia (e.g. Beric et al., 1988; Christensen et al., 1996; Defrin et al., 2001; Donovan et al., 1982; Eide et al., 1995; Nikolajsen et al., 1996; Ralston et al., 2000; Stormer et al., 1997; Yezierski, 2000). The mechanisms underlying the development of these conditions is not yet known and is the subject of ongoing research.

The allodynia that develops as a result of nervous system injury is characterised by a reduction in sensory thresholds such that normally non-noxious stimuli elicit pain-like behaviours from the rat. It is thought that evoked pains such as allodynia may be caused,

in part, by alterations in neural circuitry either at the level of the spinal cord or at higher supraspinal levels (Christensen et al., 1996). In particular, these alterations are thought to involve changes in inhibitory tone and therefore sensory processing, resulting in the perceptual consequences of SCI; sensitisation and allodynia (Drew et al., 2001; Fukuoka et al., 1998; Lin et al., 1999a; Lin et al., 1999b; Malan et al., 2002; Wiesenfeld-Hallin et al., 1997).

Thus in order to determine the functional upshot of some of the anatomical findings described in the previous two Chapters, it is the aim of the present study to examine the effect of SCI on sensation. These changes in sensation will be correlated with changes in neuronal activity within the laminae of the spinal cord and will be discussed in terms of the alterations in inhibition likely to have occurred as a result of SCI.

In order to examine the effects of SCI on sensation, a somatic stimulation paradigm was used to demonstrate the active processing of a non-noxious, somatic stimulus within the spinal cord. This somatic stimulation technique will be described later. Lastly, to allow examination of widespread neuronal activity as a result of somatic stimulation, the immuno-detection of the immediate early gene *c-fos* was utilised as a marker.

4.1 Methods

4.1.1 Animals

A total of sixty-two 250-300g Wistar rats were used in the present study. Fifty-two were characterised behaviourally, while 33 were examined behaviourally and histologically. Animals were housed individually and were given free access to food and water.

4.1.2 Spinal cord injury

The SCI utilised in the present experiments was described in Chapter 2. The weight drop was performed from either 2, 3 or 5cm.

4.1.3 Behavioural testing

On 3-5 occasions for 8 days prior to the weight drop and for up to 8 days after, rats underwent behavioural testing to examine both their sensory (tactile) and motor function. To examine tactile nociceptive threshold, rats were held lightly and observed for reaction to application of a graded series of von Frey filaments. Beginning at an arbitrary minimal force,

filaments were pressed seven times against the animal to the point of bending at three locations bilaterally: upper flank, lower flank and thigh. The animals were observed closely during testing and the thresholds at which behaviours such as, attacking the filament, strong attempts to escape the filament, strong skin twitching and vocalisation occurred were noted. If the rat failed to react to the filament, the next filament of increasing strength was selected and the test repeated. When the rat reacted more than 50% of the time (4 out of 7 trials), the filament strength was noted as the tactile nociceptive threshold. For comparison, the force applied by the threshold von Frey filament was converted to grams then the mean force in grams calculated for each day of testing both before and after injury for all rats. The percentage change from pre-injury threshold was then calculated for each group of rats.

In addition to changes in tactile sensitivity, the motor function of rats was scored in the free field according to a modified Tarlov scale (Tarlov and Klinger, 1954): 0, No function; 1, Slight hip flexion, no dorsiflexion of feet; 2, Stronger hip flexion, slight dorsiflexion; 3, plantar placing, no weight bearing; 4, Weight bearing, no fine motor control; 5, Full function. Fine motor control was determined by the ability to place feet on a metal grid and to grip bars with the hind feet. Both non-injured and injured rats were tested up to and including the day of sacrifice.

4.1.4 Pre-perfusion somatic stimulation

Two hours prior to sacrifice, a subgroup of uninjured and injured animals were subjected to somatic stimulation in order to examine neural processing to a non-noxious stimulus within the spinal cord. A von Frey filament two gradations below the pre-injury nociceptive threshold was selected for each rat and was applied bilaterally to the flank seven times each side every two minutes for an hour. The rat was then left for an hour before being perfused intracardally.

4.1.5 Perfusion

One week following the SCI, approximately 3 hours after behavioural testing and 2 hours after somatic stimulation (if received), each of the rats was deeply anaesthetised with pentobarbitone sodium (120mg/kg i.p.; Boehringer Ingelheim) and perfused intracardally as described in Chapter 2.

4.1.6 NADPH-d histochemistry

A 10mm length of spinal cord was taken from the visible epicentre of the injury site rostrally (level matched in uninjured animals). The lesion was visible as a darkened, bruised indentation of the tissue, with some degree of scarring often apparent around the entire circumference of the cord. A one in three series of 40μm coronal sections was cut on a freezing microtome and collected free floating. Sections from each animal were then processed to detect the presence of NADPH-d activity as described in Chapter 2 (see Figure 4.1).

4.1.7 Fos immunohistochemistry

Following visualisation of NADPH-d, sections were rinsed twice, for 10 minutes, in PB then processed for fos protein immunohistochemistry as described in Chapter 2.

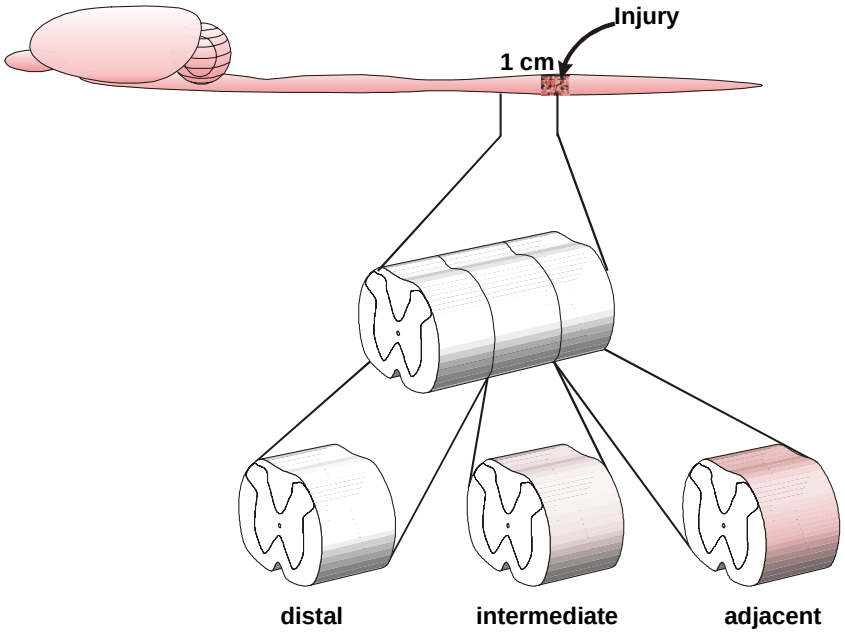


Figure 4.1 Diagram illustrating segments of tissue examined in the present study. A 1cm block of tissue was taken from the visible epicentre of the injury and sectioned free floating in a 1 in 3 series and mounted onto gelatinised glass slides. Five random sections from each of the three segments illustrated above were then plotted for both NADPH-d activity and fos immunohistochemistry.

4.1.8 Plotting

Once stained, sections were observed under the light microscope and five randomly selected sections from each third of the tissue block were plotted using a microscope with a

camera lucida attachment. Each third of the tissue plotted was described according to its relation to the injury site, with the segment most distant from the injury referred to as the "distal" segment, the middle segment referred to as the "intermediate" segment, while the segment nearest the injury was referred to as the "adjacent" segment (Figure 4.1).

The numbers of NADPH-d labelled, and fos immunoreactive cells were counted in each laminae bilaterally, for each of the sections counted. Positive cellular labelling was determined by direct visual inspection using light microscopy to determine whether labelling fulfilled specific criteria. A cell was determined as being positive for NADPH-d if it was of a distinct neuronal morphology and if intensely labelled. Length of time for the diaphorase reaction was chosen to produce intense labelling in cells which contained the enzyme. A few cells which were also of a neuronal morphology were found to be very lightly NADPH-d positive, but these were distinct from the intensely labelled cells and most likely represented non-specific diaphorase activity. These were not counted as positive.

The chromogenic reaction utilised for fos labelling was allowed to proceed to an extent which very lightly labelled the background cytoarchitecture. This, together with the diaphorase activity in the same section, removed the need for cellular counterstaining, as the cytoarchitecture of the tissue was clearly visible. As with the diaphorase histochemistry, the length of time the fos immunohistochemistry was allowed to progress, resulted in dark, intensely labelled nuclei which were well above back ground intensity. Faintly labelled nuclei were not counted. It was not possible to determine cellular morphology from fos labelling, but all clear nuclear profiles were counted within the confines of the grey matter.

The mean number of labelled cells at each spinal cord segment and within each spinal cord lamina was then compared between uninjured and spinally injured animals.

4.1.9 Statistical analysis

Motor score changes over the post injury survival time and changes in fos expression following somatic stimulation in the injured-non-allodynic group of animals was analysed ANOVA. A Fisher's Least Significant Differences post hoc test was used to determine the significance between the treated groups. The change in fos expression between the uninjured groups following stimulation was analysed using a Mann-Whitney U test. Significance was determined at $p<0.05$. There were insufficient animals in the allodynic,

non-stimulated (n=2) and allodynic stimulated group (n=3) to perform a statistical analysis. However their trends were commented upon.

4.2 Results

4.2.1 Effects of SCI on motor score

Uninjured animals were able to weight bear on all limbs, were qualitatively observed to perform acts of fine motor discrimination (eg grasp metal bars with hind feet, place feet accurately on metal bars) and were able to move with a co-ordinated quadrupedal gait. Animals with this level of motor function were assigned a motor score of 5. Following contusion injury to the lumbar spinal cord, a degree of motor function was lost in all animals. The day following injury, motor scores were dramatically reduced in rats receiving a 2cm, 3cm and 5cm weight drop SCI with an average motor score of 0.71 (± 0.29 SEM), 0.70 (± 0.42 SEM) and 0.33 (± 0.23 SEM) respectively. There was no significant difference in the motor score between these groups of animals. By seven days following injury, all groups demonstrated a significant improvement in motor function. Mean motors scores for the three groups were increased to 2.46 (± 0.37 SEM), 1.9 (± 0.55 SEM) and 1.67 (± 0.39 SEM) for the 2cm, 3cm and 5cm respectively (ANOVA; $p<0.0001$, with a Fisher's PLSD post hoc test indicating $p<0.0001$, $p=0.0428$, $p=0.0007$). As there was no difference in the motor score between animals with weight drops of different heights, the data from these animals was pooled for the purposes of histological examination on the basis of nociceptive thresholds (see Figure 4.2).

4.2.2 Effects of SCI on sensory function

Animals were tested for the nociceptive threshold required to elicit pain-like behaviours both before and after injury. Allodynic animals were defined as animals that underwent at least a 2 gradation drop in von Frey filament sensitivity following injury. This equated to at least a 70% decrease in nociceptive threshold.

Across all injured animals, the nociceptive threshold to the application of von Frey filaments was found to decrease from 13.83% to 83.94% of pre-injury levels. Of the 52 injured rats tested for nociceptive threshold, 15 demonstrated a decrease in nociceptive threshold of at least 70% of their pre-injury levels and were classified as allodynic (28.9% incidence). This decrease was found to develop by 4 days after injury and remained stable for at least the

remaining days that nociceptive thresholds were taken in the present study (up to 8 days after injury; see Figure 4.3).

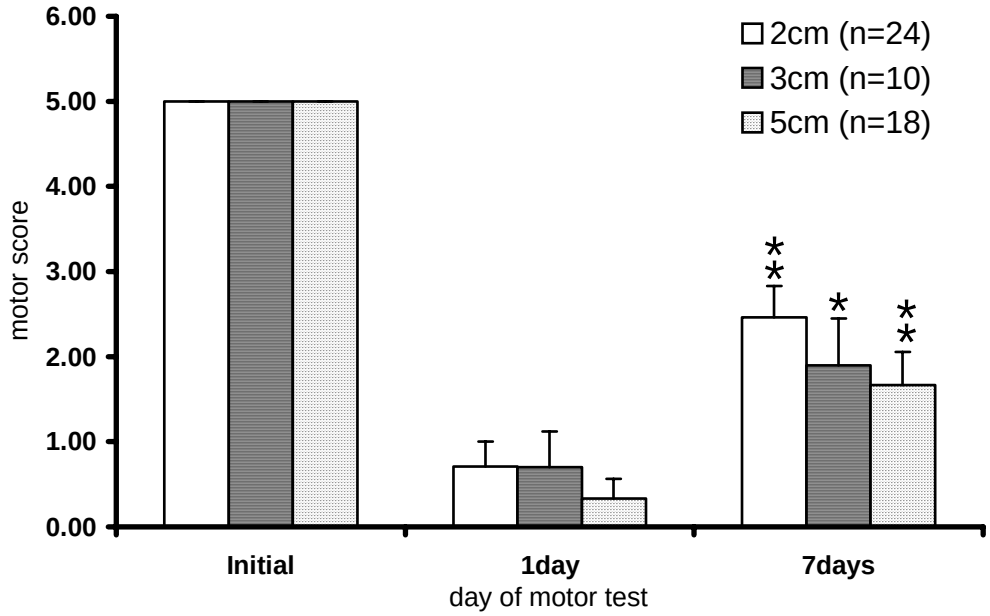


Figure 4.2 Histogram of motor score for rats grouped into the height of weight drop received. On the day after injury motor score dropped dramatically indicating a successful lesion. Following one week all groups demonstrated a significant increase in motor function (ANOVA; $p<0.0001$, with a Fisher's PLSD post hoc test indicating $p<0.0001$, $p=0.0428$, $p=0.0007$ for the 2cm, 3cm and 5cm group respectively). There were no significant differences in motor score between the three groups at either one day following injury or at 7 days. ** = $p<0.001$, * = $p<0.05$

4.2.3 Effects of somatic stimulation on fos in the normal animals

Of the 62 rats used in this study 33 were examined histologically. It should be noted that due to the low incidence of allodynia, a total of five allodynic rats only were obtained for this part of the study. Three of these underwent somatic stimulation while two remained unstimulated. Somatic stimulation was performed for 1 hour then the rats were left for another hour just prior to perfusion in a sub group of both uninjured and injured rats. This time frame has been previously reported as maximal for fos protein expression (Bullitt et al., 1992; Gu et al., 1997; Herdegn et al., 1994; Hunt et al., 1987; Luo et al., 1998; Williams et al., 1990).

In Chapter 2, it was reported that the most profound changes in fos expression, following SCI, occurred in laminae III of the spinal cord. The findings of the present study suggest that the effects of somatic stimulation in the uninjured animal also occurred most prominently there.

In laminae III, somatic stimulation caused the number of fos expressing cells to decrease by 48.94% in the rostral most "distal" segment, by 59.09% in the "intermediate" segment and 67.70% in the "adjacent" segment nearest the injury (Mann-Whitney U test; p=0.0283, p=0.0143, p=0.0143 respectively). While a similar trend towards stimulation-induced decrease is observed in other laminae, these did not reach significance (see Figure 4.4).

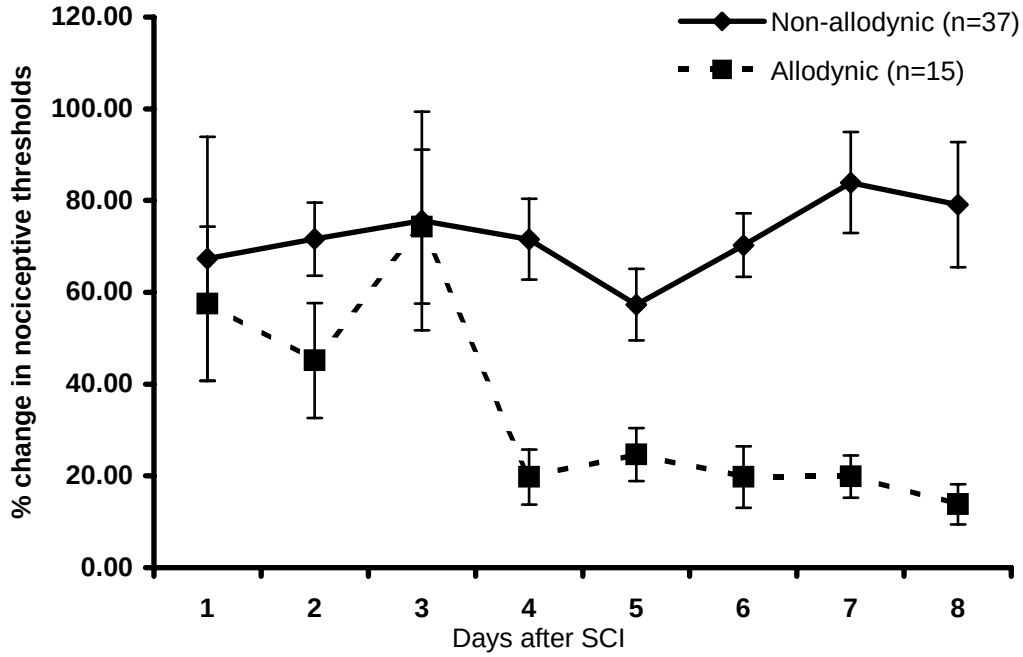


Figure 4.3 Graph of percentage change in the threshold of sensitivity to illicit pain-like behaviours measured by von Frey filaments. On 4-5 occasions over 8 days rats were tested at three locations, upper flank, lower flank and thigh. The filament was apposed to the skin of the animal to the bending point of the filament up to 7 times at each location. If rat demonstrated pain-like behaviours (attacking the filament, strong attempts to escape the filament, strong skin twitching and vocalisation) at least 50% of the time, this filament was taken as the nociceptive threshold. Filament strength (in Newtons) was then converted to grams and a mean taken across all rats tested for each time point. The mean sensitivities before weight drop were then averaged to determine a mean pre-weight drop sensitivity for uninjured rats. The mean sensitivity following injury at each time point was then converted to a percentage change from the pre-weight drop value and plotted. Allodynic rats demonstrate a drop in sensitivity of at least 70% which occurs at 4 days following injury and is maintained for the duration of the present experiment. The vertical axis is the percentage change from the pre-weight drop sensitivity, while the marks on the horizontal axis represent the days following injury. Error bars represent the standard errors of the mean.

4.2.4 Effects of somatic stimulation on fos in the injured animals

As already reported in Chapter 2, following SCI, the "basal" level of fos expressing cells in the uninjured animals is decreased following contusive SCI. This effect was also observed in the present study. When the effect of somatic stimulation was examined in injured animals, the only laminae in which a change was found to occur was the DDH. Here the number of fos positive cells tended towards an *increase* at all spinal segments examined in the allodynic animals. In the distal segment there was a 118.49% increase, while in the intermediate segment there was a 112.70% increase and at the adjacent segment there was a 127.14% increase. The only other change in the DDH occurred in the distal segment of the non-allodynic animals. Here, stimulation lead to a 114.34% increase in the number of fos positive cells (ANOVA; $p=0.0004$; with a Fisher's least difference of squares post hoc test to determine inter group differences, $p=0.0006$; see Figure 4.5). There were no changes associated with non-noxious somatic stimulation in any other laminae in the injured animals.

4.2.5 Effect of somatic stimulation on NO

As discussed in Chapter 2 and Chapter 3, the greatest density of NADPH-d cells in the spinal cord occurs in the SDH. More importantly these laminae are involved in sensory reception and are likely to be involved in the functional changes resulting from SCI. Thus, the results of somatic stimulation were examined in these laminae.

When the spinal cords of uninjured rats were examined for NADPH-d activity, somatic stimulation did not appear to affect the number of NADPH-d positive cells in these animals. The same too was observed in the spinal cords of the injured non-allodynic group of animals. However, when the allodynic animals were examined following somatic stimulation, a trend towards an *increase* in the number of NADPH-d labelled cells was observed in the SDH of the adjacent and intermediate tissue segments when compared to the non-allodynic injured animals (see Figure 4.6).

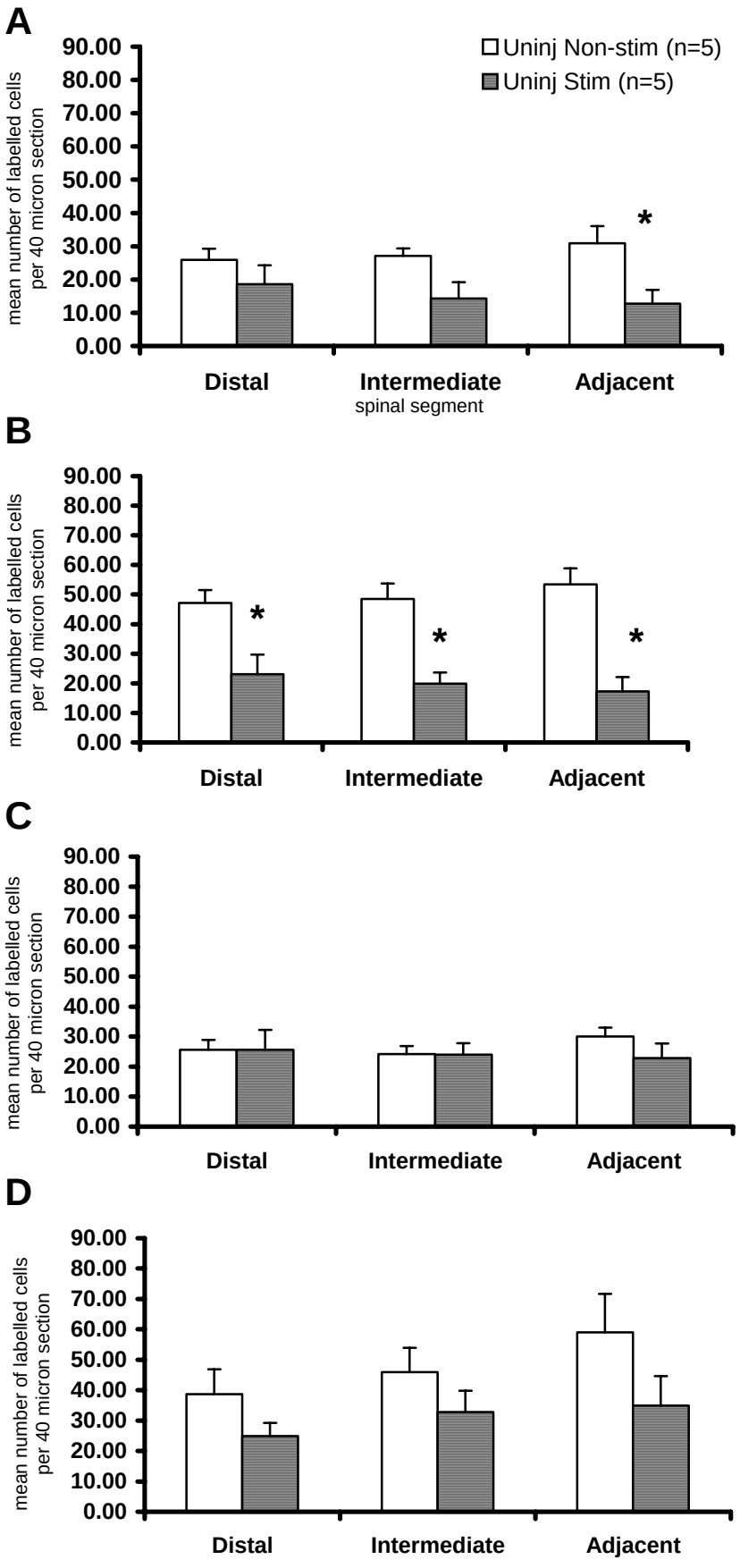


Figure 4.4 Histograms showing the effect of somatic stimulation on fos expression in laminae I and II (A), lamina III (B), laminae IV and V (C) and the ventral horn (D) of the normal animal. Spinal segments were taken from rostral to the injury. The mean number of fos positive cells per 40μm section was compared using a Mann-Whitney U test. The greatest stimulation-induced change was a decrease in lamina III across all the segments studies. Here the number of fos cells decreased following stimulation by 48.94% in the distal segment, by 59.09% in the intermediate segment and 67.70% in the adjacent segment (Mann-Whitney U test; p=0.0283, p=0.0143, p=0.0143 respectively). The vertical axis represents the mean number of fos positive cells per 40μm section. The horizontal axis represents the spinal segments examined. * indicate a significant change in the number of fos expressing cells relative to the non-stimulated animals.

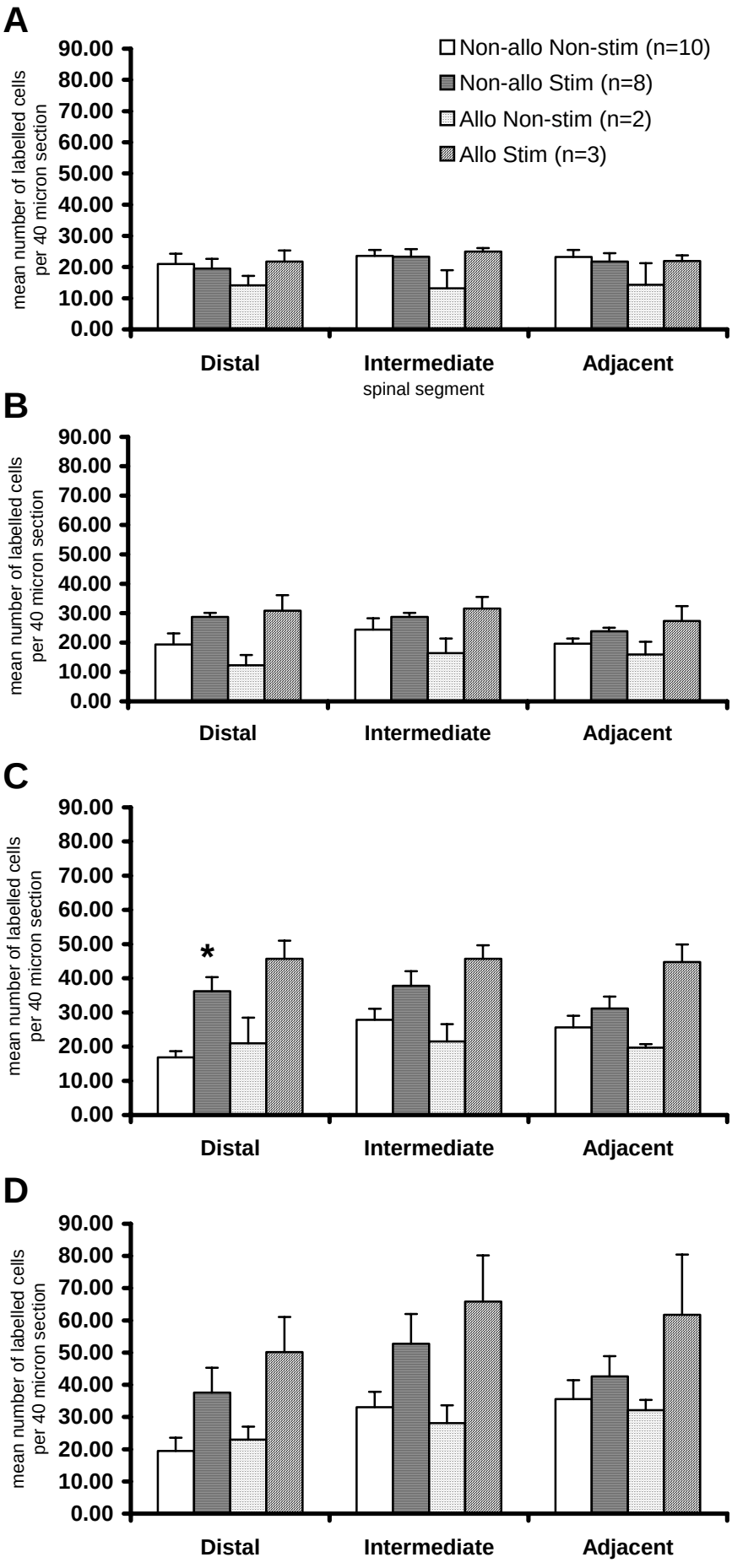


Figure 4.5 Histograms showing the effect of somatic stimulation on fos expression in laminae I and II (A), lamina III (B), laminae I and II (C) and the ventral horn (D) of the injured groups of animals. Changes in the number of fos expressing cells as a result of somatic stimulation largely occurred in the DDH following SCI. The greatest stimulation-induced change appeared to occur in the allodynic animals where somatic stimulation induced a notable increase in the number of fos positive cells relative to the allodynic, non-stimulated animals. This occurred in all spinal segments examined (C). In the distal segment the number of fos positive cells was increased by 118.49%, in the intermediate segment there was a 112.70% increase and in the adjacent segment there was a 127.14% increase. An increase was also observed in the DDH of non-allodynic animals as a result of somatic stimulation but only in the distal segment. Here, stimulation lead to a 114.34% increase in the number of fos positive cells (ANOVA; p=0.0004; with a Fisher's least difference of squares post hoc test to determine inter group differences, p=0.0006). The vertical axis represents the mean number of fos positive cells per 40μm section. The horizontal axis represents the spinal segments examined. * indicate a significant change in the number of fos expressing cells relative to the non-stimulated animals in the same group.

The non-allodynic non-stimulated group had a mean of 4.33 (±1.43 SEM) NADPH-d positive cells per 40μm section, while the stimulated non-allodynic group had a mean of 4.73 (±1.43 SEM). The stimulated allodynic group on the other hand had a mean of 12.70 (± 1.95 SEM) NADPH-d positive cells per 40μm section which was a 2.9 fold increase over the non-stimulated non-allodynic group and a 2.7 fold increase over the stimulated non-allodynic group. There was only a 1.7 and a 1.9 fold increase between the same groups in the intermediate spinal segment respectively (see Figure 4.6).

It was also noted that following injury there was a decrease in the number of NADPH-d labelled cells in the superficial dorsal horn (SDH; laminae I and II) of injured- when compared to uninjured animals, as was reported in Chapter 2.

4.3 Discussion

It was observed previously that contusive SCI results in a decrease in the NADPH-d activity of a population of inhibitory interneurons in the SDH of the rat. It is likely that this reflects a decreased NO synthesis by these cells. Additionally, it has also been reported that NO is involved in the tonic inhibitory drive of the superficial laminae (discussed in chapters 2 and 3). As a result it was hypothesised that contusive SCI would result in an altered neuronal activity in the dorsal horn due to the changes in inhibitory drive. Alterations in spinal inhibitory drive, whether from spinal or supraspinal sources, have been implicated in the development of neuropathic pain states such as allodynia (e.g. Drew et al., 2001; Fukuoka et al., 1998; Lin et al., 1999a; Lin et al., 1999b; Malan et al., 2002; Wiesenfeld-Hallin et al., 1997). Therefore, the aim of the present study was to examine the effect of contusive SCI on the level of neuronal activity within the spinal cord and to determine how that relates to any changes in sensory processing observed. In particular this was related to the development of neuropathic pain that likely develops as a result of changes in sensory processing.

The mechanisms by which neuropathic pain develop following SCI are not yet known, therefore, it is of interest to determine whether animals that develop neuropathic pain

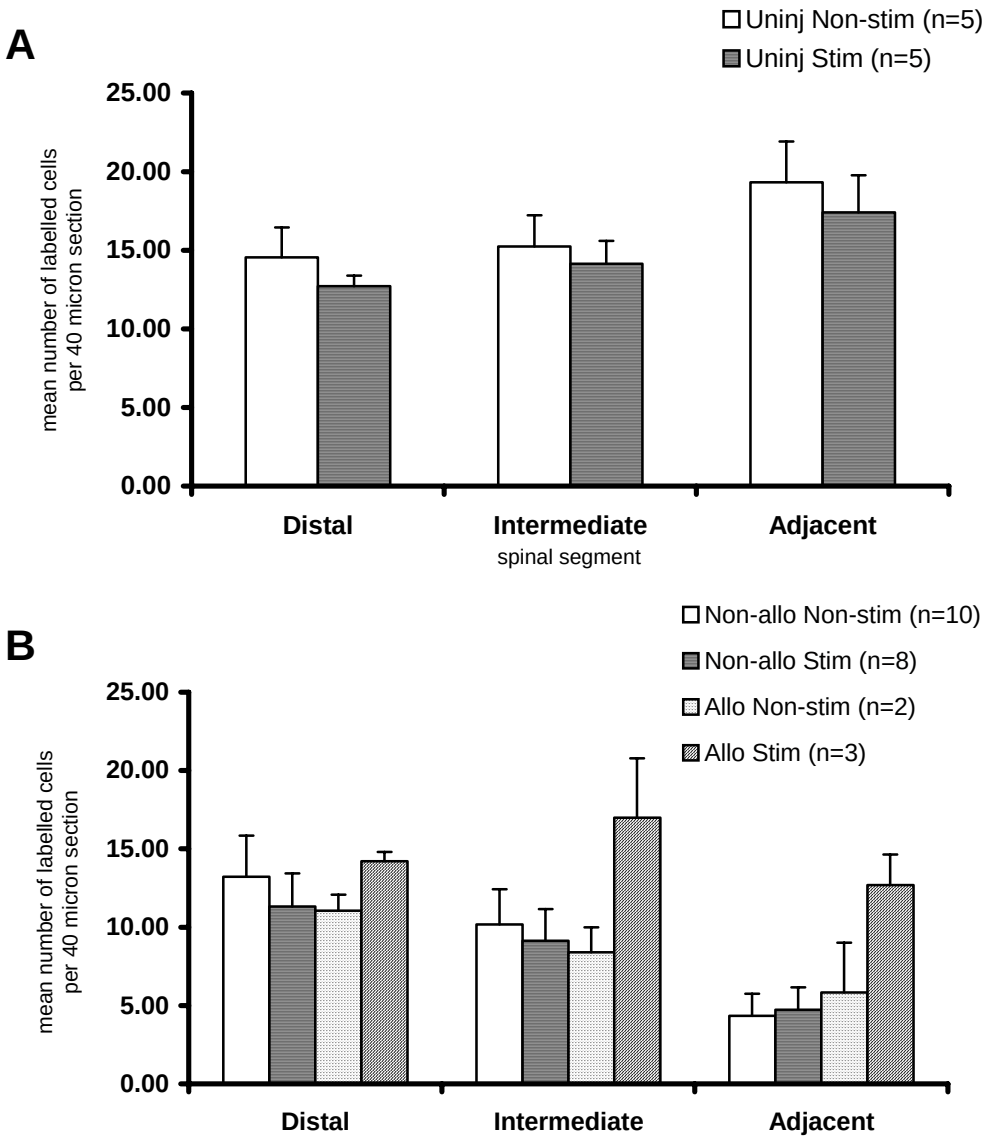


Figure 4.6 Histograms showing the effect of somatic stimulation at each of the spinal segments examined in both the uninjured- (A) and injured (B) animals. Spinal segments were taken from rostral to the injury. The number of NADPH-d positive cells per 40μm section in laminae I and II are shown for each of the groups. The most notable effect of somatic stimulation was to more than double the number of NADPH-d positive cells in the stimulated allodynic group of animals over the two non-allodynic groups in the caudal segment. Stimulation caused the number of NADPH-d positive cells to increase by 2.9 fold over the non-stimulated non-allodynic group and 2.7 fold over the stimulated non-allodynic group. While a similar sort of trend was noted in the intermediate spinal segment this change was not as great. The vertical axis is the mean number of NADPH-d positive cells per 40μm section. Error bars represent the standard errors of the mean.

demonstrate changes in neuronal function that are different to those without neuropathic pain. Any differences observed may give an indication as to the underlying mechanisms behind the development of such pain states.

4.3.1 Effect of somatic stimulation on fos in normal animals

In order to examine the active processing of a non-noxious stimulus within the spinal cord, animals were stimulated on the flank with a von Frey filament prior to sacrifice. This somatic stimulation protocol was adapted from that reported previously (Siddall et al., 1999b), except that the filament selected was two gradations below the filament that elicited pain like behaviours prior to surgery for each rat. Since the stimulating filament was below the threshold that elicited pain-like behaviours prior to surgery, the stimulation was likely to be perceived as non-noxious in the uninjured and injured non-allodynic rats, the equivalent of firm touch in humans.

Following the hour long stimulation protocol, rats were left for another hour in order to allow fos protein expression to become maximal (Hunt et al., 1987; Luo et al., 1998; Williams et al., 1990). The effect of this non-noxious somatic stimulus on neuronal activity in the spinal cord was then examined.

As with the effect of SCI on fos expression reported in chapter 2, the most profound changes in fos expression as a result of somatic stimulation also occurred in lamina III. In this lamina, of uninjured animals, somatic stimulation resulted in a significant *decrease* in neuronal activity across all spinal segments examined. The only other significant change in neuronal activity, in any other spinal laminae, occurred in the segment adjacent to the injury in the SDH (Figure 4.4). While it is not known why non-noxious stimuli would cause a decrease in neural activity almost exclusively in lamina III of the normal animal, it is likely that this result is related to the inputs received by this lamina.

It is thought that lamina III receives largely non-noxious cutaneous input (Presley et al., 1990), particularly from hair follicles (Mense, 1990; Todd et al., 1991), but also from low threshold, mechanoreceptors via Aβ and Aδ fibres (Maxwell, 1985; Powell and Todd, 1992; Rethelyi et al., 1982). These mechanoreceptors originate almost exclusively from the skin (Mense, 1990). Thus the cutaneous somatic stimulation given in the present study would be expected to be processed largely in lamina III.

It has been suggested that spinal sensory processing occurs under a strong inhibitory drive in the normal animal, involving GABAergic and glycinergic interneurons (Sorkin and Puig,

1996). Prominent localisation of GABA and glycine positive cells has been found to occur in lamina III (Campistron et al., 1986; Magoul et al., 1987; Todd and McKenzie, 1989), with approximately half of the cells of this lamina observed to be GABA positive (Todd and Sullivan, 1990). The GABAergic cells of lamina III were found to be of the sort that receive large diameter fibres from mechanoreceptors (Powell and Todd, 1992). Hair follicle afferents, with the conduction velocity of Aβ fibres, have also been observed to make synaptic contact with glycine immunoreactive dendrites (Todd et al., 1991) that lie mainly in lamina III. It was also observed that some of the larger fibres formed what appeared to be the central axon of the type II glomeruli previously described by Ribeiro-da-Silva and Coimbra (Powell and Todd, 1992; Ribeiro-da-Silva and Coimbra, 1982; Ribeiro-da-Silva and Coimbra, 1984). These are observed to occur exclusively in lamina IIi and III (Ribeiro-da-Silva and Coimbra, 1984; Todd, 1996).

The type II glomerulus is synaptic structure comprised of a central axon derived from a large diameter, myelinated, primary afferent forming a synapse with dendrites of the spinal cord. This is then contacted by a series of axon terminals derived from the spinal cord which make both presynaptic and postsynaptic connections. These spinal axon terminals have been reported to be almost entirely GABAergic (95%), while 88% have been found to be glycinergic. It is thought that these inhibitory inputs to the glomeruli are derived from local inhibitory interneurons (Todd, 1996). Because of this it is likely that primary afferents synapsing in lamina III are under inhibitory control from a series of local inhibitory neurons (Maxwell and Noble, 1987; Todd, 1990), and may themselves activate the inhibitory circuits located in this lamina (Todd, 1989; Wall, 1995).

Thus, taken together, it appears that the fibres likely to transmit the non-noxious stimulus used in the present study will be from cutaneous low threshold mechanoreceptors, which have been shown to synapse in lamina III. Certainly some of these fibres appear likely to synapse with, and be modulated by, a network of inhibitory cells that occur in this lamina. The reduction in neural activity observed in lamina III of uninjured rats then, is most likely due to the activation of inhibitory circuits by the non-noxious stimulation. Due to the behavioural testing used in the present study, the level of fos expression in the spinal cord appeared to be elevated above the basal level observed in naïve rats. This revealed alterations in neural activity as a result of the somatic stimulation which would not have

been apparent otherwise. As such this study offered an insight into the sensory processing that occurs at the level of the spinal cord in the normal rat.

4.3.2 Effect of somatic stimulation on fos in injured animals

Contusive SCI was found to result in a decrease in fos expression across the three spinal levels examined, and in all lamina of the spinal cord. This effect has been reported in Chapter 2. In addition, it was noted that somatic stimulation had a different effect on fos expression in injured animals than it did in the uninjured group. The decrease in fos expression induced by stimulation in lamina III of uninjured animals was abolished in injured animals. This was as a result of the injury-induced decrease in fos expression in this same lamina. Injury appears to reduce fos to the same levels in lamina III as did somatic stimulation in the uninjured animals. Somatic stimulation in the *injured* animals therefore resulted in no further reduction, possibly indicating a maximal level to the mechanism by which the reduction in fos occurs.

A further observation however is of interest. In the uninjured animals it was noted that somatic stimulation did not result in any change in the number of fos expressing cells in the DDH. Following injury, in the allodynic group of animals however, there tended towards an *increase* in the number of fos positive cells compared with their non-stimulated counterparts. The only other change as a result of somatic stimulation in the injured animals was a slight increase in the number of fos expressing cells in the non-allodynic group of rats in the distal tissue segment. This also occurred in the DDH (see Figure 4.5C).

These findings suggest that sensory processing of non-noxious somatic stimulation may be shifted from lamina III, in the uninjured animals, to deeper laminae following a contusive spinal cord injury. Furthermore this change in laminar processing appeared to occur preferentially in the allodynic animals. Certainly stimulation did not have any effect on the DDH in the uninjured animals. This mechanism may underlie the changes to sensation that result in the development of allodynia following contusive SCI.

In support of this suggestion is work by Drew and colleagues, in which the electrical activity of neurons of the deep dorsal horn was found to be increased only in allodynic animals as a result of low intensity electrical stimulation (Drew et al., 2001). This was interpreted as possibly being related to a loss of descending inhibition as a result of SCI.

Lamina IIi of the SDH as well as lamina III has a profuse network of inhibitory interneurons believed to be involved in regulating sensory processing in the spinal cord (Sorkin and Puig, 1996; Todd, 1989; Todd, 1990; Wall, 1995; Zhuo and Gebhart, 2002). It has been a well reported phenomenon of both peripheral injury (Castro-Lopes et al., 1993; Eaton et al., 1998; Ibuki et al., 1997; Moore et al., 2002) and central injury (Sharma and Sjoquist, 2002; Zhang et al., 1994a) that inhibitory cells are lost from the cord as a result of those injuries, and this loss is associated with the development of neuropathic pain (Eaton et al., 1998; Eide, 1998; Stiller et al., 1996; Wiesenfeld-Hallin et al., 1997).

Functionally, it is thought that disinhibition of spinal neurons may be involved in the sensitisation of dorsal horn neurons resulting in neuropathic pain (Traub, 1997; Zhuo and Gebhart, 2002). In support of this suggestion are studies in which the inhibitory GABA receptors are blocked resulting in a localised allodynia and thermal hyperalgesia similar to that produced by spinal nerve ligation (Hao et al., 1994b; Malan et al., 2002; Sorkin et al., 1998; Yaksh, 1989; Zhang et al., 2001). Intrathecal administration of the glycine receptor antagonist strychnine, in the normal rat, also results in the development of tactile allodynia (Beyer et al., 1985; Sherman and Loomis, 1994; Sorkin and Puig, 1996; Yaksh, 1989). This hyper-responsiveness induced by blockade of inhibitory receptors is characterised by an enhanced response to hair deflection, enlargement of the low threshold receptive fields, a reduced tactile sensitivity required to illicit the withdrawal reflex and an increase in afterdischarge in some cells (Sivilotti and Woolf, 1994; Sorkin and Puig, 1996; Sorkin et al., 1998). Findings such as these provide good evidence to suggest that any perturbation in spinal inhibitory activity may result in the development of neuropathic pain. Lastly, is has been observed that Aβ fibre stimulation is insufficient to elicit fos expression in the deep dorsal horn of the normal animal. Following an intraplantar injection of Freund's adjuvant, a stimulus well known to induce allodynia, Aβ fibre stimulation was found to increase fos expression in the DDH (lamina IV and V), similar to the results shown here.

Taken together these findings suggest that spinal cord injury, can indeed result in changes in spinal processing of afferent signals, and that these probably occur through a reduction in the normal inhibitory tone of the spinal cord. Thus the alteration in the laminar processing of non-noxious stimulation observed in the present study is likely due to these mechanisms. While almost all other studies examining the loss of spinal inhibition following injury make

the observation that the number of inhibitory cells are lost following injury, by examining neural activity the present study has determined what may be the functional upshot of this loss. That is that the spinal processing of somatic, non-noxious stimuli appears to change from the SDH to the DDH following SCI.

It is not known why increased amounts of low-threshold afferent input reaching the deeper laminae may result in the perception of pain, however it is reported that neurons of the DDH become sensitised and fire abnormally following injury (Lin et al., 1997; Simone, 1992; Treede et al., 1992). Neurons in these laminae receive both non-noxious and noxious peripheral input via Aβ and Aδ fibres (Brown and Culberson, 1981; Brown and Fuchs, 1975; Nagy and Hunt, 1983; Willis and Coggeshall, 1991). Thus the increased neural activity in these laminae following non-noxious stimulation in allodynic animals may be mis-perceived due to the usual information received by these laminae.

4.3.3 Effects of somatic stimulation on NO synthesis

In addition to neuronal activity, since NO synthesis appears to be involved in the processing of sensation in the normal animal, the number of NO synthesising neurons was also examined in the SDH in this study. The present results suggest that non-noxious somatic stimulation does not result in alteration in the number of NADPH-d positive cells in either uninjured or injured non-allodynic animals. When the allodynic group of animals were examined however, stimulation was found to lead to an *increase* in the number of cells expressing NADPH-d in the SDH when compared with non-allodynic animals. All animal groups were treated, handled and tested in the same way, thus the only difference between these groups is that this last group had allodynia.

For the present study, a rat was defined as allodynic if it demonstrated a decrease in pre-lesion nociceptive threshold of at least 70%. Due to the non-linear scale of von Frey filaments this corresponded to a decrease in two von Frey filament gradations. Thus since the stimulus used for all rats was two gradations below the pre-injury nociceptive threshold, and allodynic rats had demonstrated a decrease in threshold of *at least* two von Frey filament gradations, the somatic stimulation given to allodynic rats was likely to be perceived as noxious in this group of animals. The increase in the number of NADPH-d cells in the SDH in this group of rats was therefore likely to be due to the noxious stimulation.

In support of this suggestion are studies examining nitric oxide synthesis in relation to peripheral noxious events. These include injection of formalin, complete Freund's adjuvant, zymosan and carrageenan into the glabrous skin of the hind feet (Dreyer et al., 2003; Goff et al., 1998; Herdegn et al., 1994; Lam et al., 1996; Maihofner et al., 2000a; Maihofner et al., 2000b; Traub et al., 1994), ip acetic acid (Kovacs et al., 2001) or postoperative abdominal inflammation (Dolan et al., 2003). In these studies the number of cells expressing NOS or NADPH-d is also upregulated in the SDH following the noxious stimulation. In addition, studies using microdialysis and in vitro, spontaneous NO release, have found that there is an increased production of metabolites of NO synthesis in the SDH of rats with inflamed paws (Lin et al., 1999a; Lin et al., 1999b; Maihofner et al., 2000a; Wu et al., 1998).

Taken together these results suggest that a peripheral, noxious stimulus results in an increased number of NO synthesising cells in the SDH, which appear to be associated with increased synthesis of NO. The increased number of NADPH-d positive cells in the SDH of allodynic animals, observed in the present study, is therefore likely due to the noxious nature of the stimulus in this group of animals. Interestingly allodynic animals that do not receive somatic stimulation did *not* show an increased number of NADPH-d positive cells, although they displayed allodynia. That is increased NO is not required in order to maintain allodynia. This observation is at odds with what has been reported previously.

Previously it has been suggested that NO synthesis underlies the presence of allodynia since inhibiting NOS reduces the hypersensitivity that develops in various models of neuropathic or inflammatory pain (Kitto et al., 1992; Lin et al., 1999b; Malmberg and Yaksh, 1993; Meller et al., 1994; Milne et al., 2001; Moore et al., 1991; Wu et al., 2001; Yonehara et al., 2003).

This difference is likely due to the timing of NO synthesis. As discussed in chapter 2, the present study was performed at one week following SCI, as this was a time when more chronic conditions were found to be developing, and at which allodynia was well established. When NO synthesis is examined in peripheral nerve injury or inflammatory models soon after injury, NOS or NADPH-d was found to be *increased* in the SDH (Gonzalez et al., 2001; Mabuchi et al., 2003; Meller et al., 1994; Moore et al., 1991; Wu et al., 2001). Three days after central injury, an increase in NOS in the SDH was also observed (Diaz-Ruiz et al., 2002; Gonzalez et al., 2001; Marsala et al., 1997; Nakahara et

al., 2002; Sharma et al., 1996). The present results suggest that by seven days, while allodynia has been established and is still present, NO synthesising cells are not increased above uninjured levels. Similar results were also found in a study examining spinal root ligation. In this study, allodynia was found to develop but, without any other form of stimulation, at seven days there was no increase in NOS expressing cells in the SDH (Luo et al., 1999). Time course studies also indicate that any injury-induced increase in NOS expression is resolved by approximately 3 days (Diaz-Ruiz et al., 2002; Nakahara et al., 2002; Sharma et al., 1996).

Results of previous studies taken together with the present findings then, suggest that, while NO may not be involved in the maintenance of allodynia at seven days, it may be involved in the earlier development of the condition. Indeed as described above NOS appears to be upregulated in the SDH within the first 3 days following injury which corresponds to the time at which allodynia developed in the present study (see Figure 4.3). Other studies too have made a similar observation, finding that paw withdrawal latencies following nerve ligation, inflammation, spinal nerve transection or chronic constriction injury become maximal from approximately 3-5 days after injury (Goff et al., 1998; Luo et al., 1999; Mabuchi et al., 2003; Meller et al., 1992).

4.4 Conclusions

In summary the present study used non-noxious somatic stimulation to examine the effect of SCI on the processing of such stimuli. As a result, it was possible to observe injury-induced changes in the cord which would not otherwise have been apparent. At seven days following injury, for instance, somatic stimulation allowed neural activity in the uninjured and injured animals to be examined. Using fos immunohistochemistry, it was found that non-noxious stimulation resulted in alterations in neural activity in the uninjured animal. More importantly it was observed that following injury the spinal laminae in which non-noxious stimuli was processed was altered, particularly in allodynic animals. This suggests that changes in laminar processing of non-noxious stimuli, likely as a result of injury-induced alterations in inhibitory tone may be involved in the development of neuropathic pain as a result of spinal cord injury.

In addition to this, it was found that allodynic animals do not demonstrate increased numbers of NO producing cells, over their non-allodynic counterparts. However a non-

noxious somatic stimulation was found to result in an increased number of NO producing cells in the SDH. This indicates that while NO may not be involved in the maintenance of allodynia it is likely indicative of noxious spinal processing despite using a stimulus that in the same animals was perceived as non-noxious before injury. Thus somatic stimulation allowed these different functions of NO, in regards to sensory processing, to be elucidated.

Together with the previous two chapters these results indicate that contusive SCI results in a decreased level of NO synthesis in the SDH by seven days following injury. This decrease in NO synthesis appears to be involved in altered laminar processing for non-noxious stimuli, which may represent an underlying mechanism for the development of neuropathic pain conditions such as allodynia. An interesting finding, reported in chapter 3, was that, while the number of NO synthesising cells was reduced in the SDH following SCI, this reduction was not as a result of the death of the cells. Since the NO producing cells of the SDH appear to be a population of inhibitory interneurons this finding possibly represents a therapeutic avenue in the future. If the inhibitory cells are not dead then it may be possible for function to be returned. This is a possibility however that remains to be examined.

These previous chapters have examined the effect of SCI on cellular mechanisms within the spinal cord. It was found that these changes that occur may well contribute to the sensory dysfunction known to develop following SCI. It has been suggested, however, that supraspinal mechanisms may also be involved in the development of sensory dysfunction following SCI (Christensen et al., 1996; Drew et al., 2001; Gerke et al., 2003; Lin et al., 1999a; Lin et al., 1999b; Narita et al., 2003). Less is known, however about the effects of SCI on supraspinal structures. The following two chapters will therefore examine two supraspinal structures, the PAG and the sensorimotor cortex. Chapter 5 will examine the PAG which is involved in a number of functions including the behavioural and physiological responses to stressful situations and also modulating sensory transmission in the spinal cord. Chapter 6 will examine the sensorimotor cortex, which gives rise to the principal motor output from the brain. The aims of these chapters will be to a) examine the effect of spinal cord injury on supraspinal structures, distant to the injury, but also b) to consider how spinal cord injury may affect not only the spinal cord but the whole nervous system. The PAG and the sensorimotor cortex will therefore be considered as representative structures in the system.

Chapter 5

The effect of traumatic SCI on fos and NADPH-d expression in the PAG

A number of studies examining sensory dysfunction as a result of spinal cord injury or peripheral inflammation have suggested that such dysfunction may arise through changes in descending modulation of sensation from supraspinal structures (Christensen et al., 1996; Drew et al., 2001; Gerke et al., 2003; Lin et al., 1999a; Lin et al., 1999b; Narita et al., 2003). Certainly the stimulation of structures such as the nucleus raphae magnus in the medulla, locus coeruleus in the pons and the PAG in the midbrain have been found to modulate sensory processing at the spinal level (Jones, 1991; Jones and Gebhart, 1988; Li et al., 1998; Monhemius et al., 2001). Yet while it has been observed that spinal cord injury leads to alterations in sensation (e.g. Christensen et al., 1996; Eide et al., 1995; Stormer et al., 1997; Yezierski, 2000), little is known about the contribution supraspinal structures may make. The PAG has been implicated in mediating the development of sensory dysfunction including neuropathic pain as a result of peripheral nerve lesions (Monhemius et al., 2001; Narita et al., 2003; Pertovaara et al., 1997). However less is known about the role of the PAG on the sensory dysfunction that results from SCI. It is likely that any effect of SCI on the PAG would be represented as a change in activity in these neurons following injury. In order to better understand any changes observed a brief review of the function of the PAG will be given here.

The PAG is a region of grey matter surrounding the midbrain aqueduct as it passes from the third ventricle to the fourth. It consists of a dense collection of small to medium sized cells (5-20μm) with a range of morphologies including bipolar, pyramidal and multipolar. The somata and processes of these neurons runs in a rostrocaudal orientation and are arranged as a series of functionally, if not anatomically distinct columns (See Figure 5.1 for diagram of PAG columns, also for review see Bandler et al., 2000b; Bandler and Shipley, 1994; Keay and Bandler, 2004).

The over all function of the PAG is to mediate behavioural responses to stressful or threatening situations. These were first examined through the electrical stimulation of the PAG and resulted in displays of defensive behaviours (e.g. Morgan and Franklin, 1988; Sandner et al., 1987; Schmitt and Karli, 1980). The columnar arrangement of the PAG was later delineated further through the microinjection of excitatory neurotransmitters and the careful mapping of behavioural responses. It was noted that excitation of the ventrolateral column of the PAG (vlPAG) resulted in a decreased responsiveness of the animal to the environment including, quiescence and immobility, decreased vigilance, hyporeactivity, hypotension and bradycardia. Excitation of either the lPAG or dlPAG on the other hand resulted in more active defensive behaviours including, vocalisation, orienting towards and backing away from the stimulus (the experimenter), hypertension and tachycardia (Bandler and Carrive, 1988; Bandler and Depaulis, 1988; Depaulis et al., 1989; Depaulis et al., 1992; Depaulis et al., 1994; Krieger and Graeff, 1985; Yardley and Hilton, 1986; Zhang et al., 1990). It was also found that the lateral and dorsolateral columns demonstrated a rostrocaudal difference in response to stimulation. Stimulation of the rostral portion of the lPAG and dlPAG results in orientation towards the stimulation, where as stimulation of the caudal part of these columns results in a flight response. These responses are accompanied by appropriate changes in blood flow, to the face with rostral stimulation and to the limbs with caudal stimulation (Bandler et al., 2000a; Krieger and Graeff, 1985; Yardley and Hilton, 1986; Yardley and Hilton, 1987).

Related to the role of the PAG in mediating defensive behaviours to a threatening stimulus, the PAG has been found to play a role in the descending modulation of nociception. It has been found that different analgesic mechanisms may be elicited following stimulation of the various columns. Stimulation of either the lPAG or dlPAG results in a short term non-opioid mediated analgesia (Bandler et al., 1985; Depaulis et al., 1992; Krieger and Graeff, 1985; Lovick, 1992; Morgan et al., 1998), where as stimulation of the vlPAG results in an opioid-mediated analgesia which is longer lasting (Millan et al., 1986; Millan et al., 1987; Morgan et al., 1998; Nichols et al., 1989; Thorn et al., 1989). It is thought the non-opioid-mediated mechanisms of the lateral and dorsolateral columns may help to inhibit reflexes that may compete with defensive behaviours such as the withdrawal reflexes or the favouring of an injured limb. The longer acting opioid based mechanisms on the other hand are thought to assist in the induction of quiescence which may aid in recovery and healing.

These excitatory studies were supported functionally by noting that when the animal is exposed to escapable threatening stimulus such as exposure to a cat (Canteras and Goto, 1999) or radiant heat (Keay and Bandler, 1993) fos expression is increased in the lateral or dorsolateral PAG. Exposure to inescabable stimuli such as a clip in the back of the neck, intramuscular formalin or intravenous 5-HT, fos expression is increased in the vlPAG (Keay et al., 2001; Keay et al., 2000). This fos expression indicates activation of areas of the PAG that mediate appropriate behavioural responses to these stimuli (Keay and Bandler, 2002).

Lastly, the biochemistry of the PAG is relatively uniform however the dlPAG, but not the other columns, has been shown to be characterised by a dense plexus of nitric oxide synthesising neurons. This population of neurons define the column along the entire rostrocaudal extent of the structure (Guimaraes et al., 1994; Onstott et al., 1993; Traub et al., 1994). The nitric oxide produced here appears to be involved in the behavioural responses to anxiety as the injection of NO donors into this column resulted in flight responses similar to those seen by injection of excitatory neurotransmitters (de Oliveira et al., 2001; de Oliveira et al., 2000b). Inhibitors of nitric oxide on the other hand had an anxiolytic effect (de Oliveira et al., 2001; Guimaraes et al., 1994).

Taken together then, it appears that the PAG is highly involved in mediating various defensive behaviours to a range of stimuli both somatic and deep, as well as psychological. Anti-nociceptive mechanisms appear be involved in this function. It is not currently known to what degree alterations in function of supraspinal structures may be involved spinal changes. Though there have been implications that supraspinal structures may be involved in alterations in sensory processing following SCI (Christensen et al., 1996; Drew et al., 2001; Gerke et al., 2003; Lin et al., 1999a; Lin et al., 1999b; Narita et al., 2003).

Thus a principal aim of the present chapter was to examine the effect of traumatic spinal cord injury on neuronal activity in the PAG and to relate these cellular changes to changes in function mediated by the PAG. In order to perform these experiments the spinally injured rats underwent regular behavioural testing as part of their maintenance and upkeep. The uninjured controls to which they were compared were subjected to the same maintenance. To examine the effect of this handling and light restraint on fos expression in the PAG,

however, another group of controls were added. The "naïve", non-handled rats were compared to the behaviourally tested uninjured animals and any change in fos levels noted to examine the effect of behavioural testing on these PAG neurons.

Lastly, since the dlPAG is characterised by a dense plexus of nitric oxide synthesising neurons and this has been shown to be involved in mediating defensive behaviours, the effect of SCI on NO synthesis in the PAG was also examined.

5.1 Methods

5.1.1 Experimental groups

A total of 16 female Wistar rats (200-300g) were used in the present study. Of these, 6 were subjected to contusive SCI, 5 served as uninjured controls which underwent behavioural testing (uninjured), while another 5 served as uninjured controls which had undergone no behavioural testing (naïve). All experiments were conducted following approval of the study protocol by the institutional animal care and ethics committee, and observed the animal welfare guidelines of the National Health and Medical Research Council of Australia and the International Association for the Study of Pain (Zimmermann, 1983).

5.1.2 Spinal cord injury

Rats underwent a traumatic spinal cord injury under halothane anaesthesia, receiving a weight drop of 2, 3 or 5cm as described in Chapter 2. Sacrifice and perfusion were also performed as described in Chapter 2.

5.1.3 Behavioural testing

Rats undergoing spinal cord injury also underwent regular behavioural testing as part of their normal upkeep. Behavioural testing including both sensory and motor testing and was done as a means of monitoring the effect of the contusive SCI on these animals. The "uninjured" group of animals underwent the same testing, while the "naïve" group of animals were neither tested nor handled.

Behavioural testing was performed as described in Chapter 4.

5.1.4 Tissue processing

The brain was blocked prior to microtomy coronally through the level of the optic chiasm, and through the anterior third of the cerebellum. A one in three series of 40µm coronal sections was cut on a freezing microtome and collected free-floating. Sections from each animal were then processed to detect the presence of NADPH-d histochemically and fos protein immunohistochemically, as described in Chapter 2.

5.1.5 Plotting

Sections were observed under the light microscope and plotted in order from rostral to caudal PAG, using a camera-lucida attachment. The numbers of fos and NADPH-d-positive cells were plotted in slightly different fashions. While the experimenter was not blind to the animal groups, bias was minimised by using very specific criteria for determining whether a given cells was positively labelled. This was described in the "plotting" subsection of the methods of Chapter 2. Briefly fos immunoreactivity was determined by a strong orange-brown reaction product in the nuclei of cells. All strongly labelled cells were counted as it was not possible to determine cell type from this methodology. Cells demonstrating faint labelling, tending towards background were not counted.

All NADPH-d positive neurons in the PAG demonstrated strong labelling of the somata and processes. No partially or faintly labelled cells were observed. All positive soma were counted.

5.1.6 Fos

Fos cells were plotted in all columns of the PAG at 4x objective. As for the NADPH-d cells, a one in three series was plotted and level matched across the PAG. The generally 18 sections that represented the entire PAG were divided into 6 groups of 3 sections, in which all fos cells were counted bilaterally across all rats in each treatment group. The total number of cells per PAG level was determined and a mean number of cells per 40µm section calculated for each PAG level.

The mean number of labelled cells within each column was then compared between SCI, uninjured- and naïve uninjured controls, for both NADPH-d and fos.

In addition to the mean number of fos expressing cells per 40μm section at each of the six PAG levels studied, the mean number of cells per section was calculated for the entire column. The columns examined were the dorsal column, the dorsolateral column, the lateral column and the ventrolateral column, the boundaries of which have been described previously (Bandler et al., 1991a).

5.1.7 NADPH-d

NADPH-d cells were plotted in the dorsolateral column of the PAG bilaterally at 10x objective. Plots from each animal were then level matched to ensure the same level of the PAG was being compared across animals. The entire PAG was generally represented by eighteen 40μm sections in the 1:3 series. These 18 sections were then grouped into 6 groups of 3, consisting of the most rostral three sections of the PAG, followed by the next most rostral three sections and so on, until the last group which consisted of the caudal most three sections of the PAG. Each group of three sections represents approximately 280μm of PAG. The total number of cells at each level of the PAG was then summed across all of the animals in each group, then the mean number of cells per 40μm section determined for each of the six PAG levels examined.

Feret diameters of NADPH-d labelled cells were also taken at 6.16mm, 6.88mm and 7.60mm behind bregma in the uninjured animals. This involved measuring the longest and shortest diameter of the cell and taking the mean of these values. Five cells from each level from each rat studied was used for this as a measure of changes in neuronal population of the length of the PAG.

5.1.8 Statistical analysis

Significance was determined using a Mann-Whitney U test with significance determined at $p<0.05$.

5.2 Results

5.2.1 The effect of behavioural testing on fos expression in the PAG

Within the PAG of the naïve control animals few fos positive neurons were observed. Throughout the entire rostrocaudal extent of the dorsomedial column there were approximately 13 cells per 40μm section; in the dorsolateral column there were

approximately 7 cells per section; in the lateral column approximately 5 cells per section; while in the ventrolateral column there were approximately 3 cells per section.

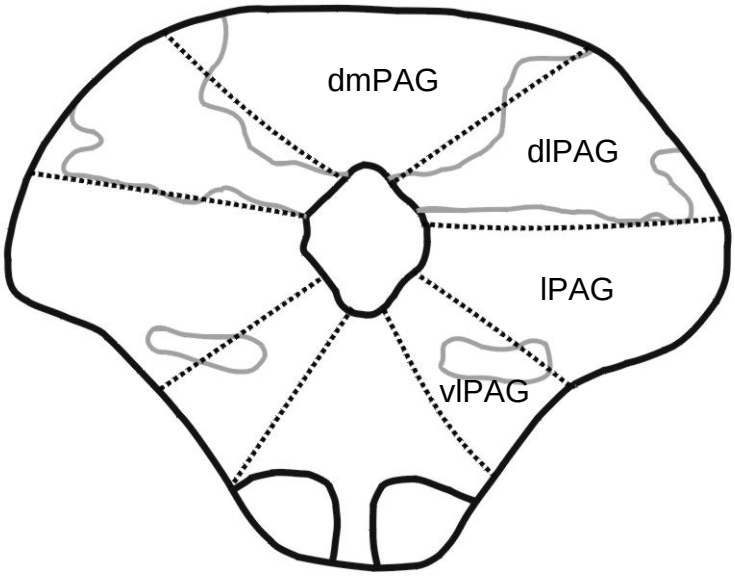


Figure 5.1 Diagram illustrating functional columns of the mid brain PAG as reviewed previously (e.g. Bandler et al., 1991a; Bandler et al., 1991b; Bandler and Shipley, 1994). Columns include the dorsomedial column (dmPAG), dorsolateral column (dlPAG), lateral column (lPAG) and ventrolateral column (vlPAG).

Each column was also divided into six levels to examine the rostrocaudal distribution of fos positive cells within the PAG. The dorsomedial column (dmPAG) for instance demonstrated 13 fos positive cells per 40μm section over the whole length of the column. When divided into six equal segments, the mean number of cells per level was found to be 33.4 (± 6.42 SEM) cells per section in the first, most rostral level of the PAG, 16.1 (± 3.48) cells per section in the second, 11.9 (± 3.32) cells per section in the third, 6.5 (± 1.78) cells in the fourth, 4.9 (± 1.87) cells in the fifth and 3.3 (± 1.43) cells per 40μm section in the sixth level of the PAG. This indicated that the greatest activity in this column then appears to occur rostrally, with very few active cells at caudal levels of the column. The remaining columns examined are listed in Table 5.1.

The act of lightly restraining the rats as part of their normal handling routine up until the day of sacrifice greatly increased the number of cells expressing fos in all columns of the PAG examined. Rats that were uninjured but were behaviourally tested showed increases in the number of fos expressing cells in the dmPAG ranging from a 2.6 fold increase at the most rostral level to a 14.5 fold increase at the fourth PAG level. The increase in fos shown in

Table 5.1 The mean number of fos expressing cells (± SEM) per 40μm section is shown at the six levels of the PAG examined in the dorsomedial, dorsolateral, lateral and ventrolateral columns in the naïve, uninjured rat. The mean number of cells per section (± SEM) for the entire column is also shown. The numbers in parentheses after the PAG level are the corresponding number of millimetres behind bregma of that level.

PAG level	dorsomedial cells/40μm section (±SEM)	dorsolateral cells/40μm section (±SEM)	lateral cells/40μm section (±SEM)	ventrolateral cells/40μm section (±SEM)
Entire column	11.8 (± 1.62)	13.4 (± 1.25)	10.2 (± 0.92)	5.6 (± 0.58)
1 (5.80-6.08)	33.4 (± 6.42)	17.2 (± 3.07)	5.6 (± 1.70)	3.8 (± 0.66)
2 (6.16-6.44)	16.1 (± 3.48)	20.2 (± 3.73)	6.1 (± 0.97)	4.9 (± 1.04)
3 (6.52-6.80)	11.9 (± 3.32)	14.9 (± 2.55)	12.1 (± 1.99)	5.6 (± 0.90)
4 (6.88-7.16)	6.5 (± 1.78)	20.4 (± 2.84)	16.3 (± 3.02)	8.1 (± 1.19)
5 (7.24-7.52)	4.9 (± 1.87)	8.4 (± 2.03)	9.1 (± 1.52)	6.4 (± 2.17)
6 (7.60-7.88)	3.3 (± 1.43)	6.0 (± 1.60)	10.3 (± 2.13)	3.3 (± 1.33)

the dorsolateral (dlPAG) ranged from a 6.7 fold increase at the second PAG level to a 13.8 fold increase at the fifth PAG level. The largest changes in fos expression occurred at selected levels of the lateral (lPAG) and ventrolateral (vlPAG) where the increase in fos expressing cells ranged from a 10.2 fold increase at the fourth PAG level to a 23.9 fold increase at the first PAG level of the lPAG and from a 7.8 fold increase at the first PAG level to a 33.6 fold increase at the sixth PAG level in the vlPAG (Figure 5.2).

5.2.2 The effect of SCI of fos expression in the PAG

Animals that were spinally injured were handled and/or behaviourally tested on 3-5 days during the week following SCI as part of their upkeep and maintenance. The uninjured control group to which they were compared were also handled in a similar way to enable comparison. Thus, it was expected that the difference in fos expression within these two groups be due to the spinal cord injury that had been performed on the injured group.

Contusive lumbar spinal cord injury (SCI; L3-4 spinal contusion) resulted in a decrease in the number of cells expressing fos in all the columns of the PAG except for the dorsomedial column of the PAG (dmPAG). The remaining columns demonstrated a decrease in the

number of fos expressing cells to various degrees. The greatest change was observed in the lateral and ventrolateral columns of the PAG.

In the dlPAG the number of fos expressing cells was significantly reduced in the middle two-thirds of the column, at the 2nd-5th PAG levels. In the lPAG on the other hand the significant decrease in fos expression occurred more rostrally at the 1st - 4th PAG levels. Lastly the mean number of fos positive cells per 40μm sections in the vlPAG was decreased following SCI across most of the column, from the 2nd to the 6th PAG level (Figure 5.3). The mean number of fos positive cells at each level of the PAG in the dorsomedial, dorsolateral, lateral and ventrolateral column is given in Table 5.2. The percentage changes for these results are given in Table 5.3.

Thus the decrease in fos positive cells in these columns ranged from 17.31% at the 4th PAG level in the dlPAG (6.88-7.16mm behind bregma) to 42.40% in the 2nd PAG level of the vlPAG (6.16-6.44mm behind bregma). The changes in fos expression are illustrated in Figure 5.4 and 5.5.

5.2.3 NADPH-d

By far the largest number of NADPH-d positive cells in the PAG was contained in the dorsolateral column. This column was characterised by a dense plexus of NADPH-d positive cells and labelled fibres. The cells typically demonstrated a multipolar morphology and a Feret diameter that ranged from 9.2 - 13.5μm (Figure 5.6).

The entire rostrocaudal extent of the PAG was represented in approximately 18 forty-micron sections taken from a one in three series. These 18 sections were then examined in groups of three spanning 280μm each, to create six discrete levels, each spaced 80μm apart. The mean number of NADPH-d labelled cells in the first, most rostral level of the naïve group of animals was 27.8 (± 8.92 SEM) cells per 40μm section. Eighty microns caudal to that at the second PAG level, the mean number of cells per section rose to 133.7 (± 10.91) cells. The third PAG level demonstrated a mean of 195.6 (± 10.16) cells per section, the fourth 214.2 (± 8.95) cells, the fifth 163.9 (± 12.54) cells while the caudal most level of the PAG demonstrated a mean of 113.2 (± 10.97) cells per 40μm section.

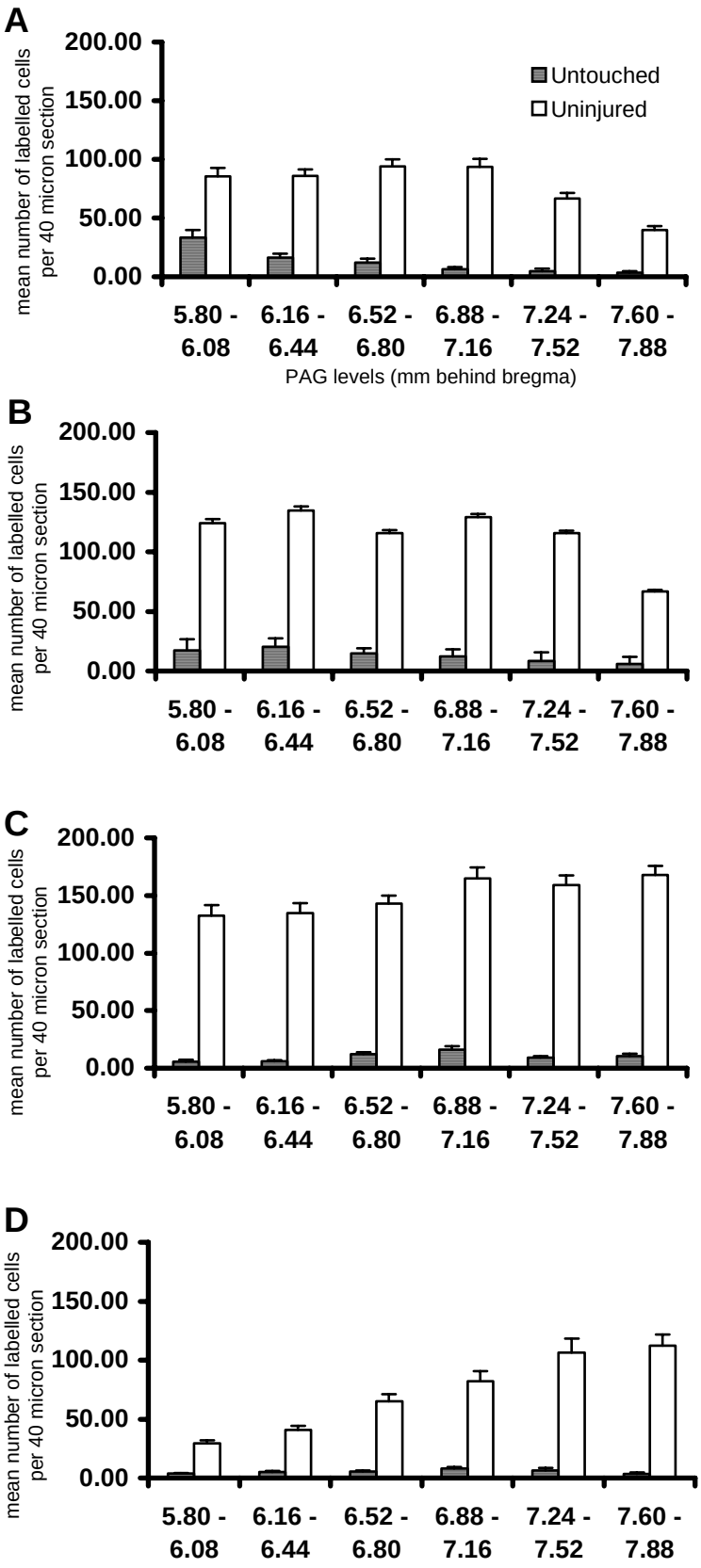


Figure 5.2 Graphs illustrating effect of behavioural testing on numbers of cells expressing fos in the PAG. Mean (plus SEM) number of fos positive cells per 40μm section plotted in rats that had been untouched prior to perfusion ("naïve untouched" group), and rats that had undergone behavioural testing prior to perfusion, but were otherwise unharmed ("uninjured" group). Four columns of the PAG were examined the dorsomedial column (A; dmPAG), the dorsolateral column (B; dlPAG), the lateral column (C; lPAG) and the ventrolateral column (D; vlPAG). Values given on the x axis are mm behind bregma.

Table 5.2 The mean number of fos positive cells (± SEM) per 40μm section at each level of the PAG examined. Counts are given for the dorsomedial, dorsolateral, lateral and ventrolateral column of the PAG, for both injured and uninjured groups of animals. The numbers in parentheses after the PAG level is the corresponding number of millimetres behind bregma of that level.

PAG level	dorsomedial cells/40μm section (±SEM)		dorsolateral cells/40μm section (±SEM)		lateral cells/40μm section (±SEM)		ventrolateral cells/40μm section (±SEM)	
	Uninjured	Injured	Uninjured	Injured	Uninjured	Injured	Uninjured	Injured
1 (5.80-6.08)	85.5 (± 7.30)	70.9 (± 15.01)	124.2 (±9.17)	122.9 (± 9.03)	132.6 (± 5.56)	88.9 (± 13.83)	29.4 (± 3.78)	22.4 (± 2.38)
2 (6.16-6.44)	85.9 (± 5.69)	84.8 (± 12.61)	134.4 (± 7.32)	109.4 (± 8.28)	134.5 (± 6.13)	92.5 (± 13.00)	40.8 (± 4.93)	23.5 (± 2.79)
3 (6.52-6.80)	94.2 (± 6.00)	94.3 (± 14.57)	115.6 (± 4.11)	98.5 (± 6.21)	142.8 (± 12.13)	90.8 (± 13.01)	65.2 (± 5.67)	42.6 (± 5.19)
4 (6.88-7.16)	93.5 (± 6.69)	106.2 (± 14.65)	128.9 (± 5.68)	106.6 (± 6.34)	164.7 (± 16.27)	117.2 (± 16.40)	82.3 (± 8.07)	50.1 (± 6.26)
5 (7.24-7.52)	66.7 (± 4.66)	82.2 (± 14.85)	115.7 (± 7.32)	86.9 (± 7.13)	158.9 (± 9.07)	129.6 (± 16.28)	106.5 (± 6.40)	63.9 (± 7.54)
6 (7.60-7.88)	39.6 (± 3.31)	49.5 (± 10.29)	66.5 (± 5.87)	66.8 (± 7.22)	167.9 (± 10.33)	152.9 (± 16.27)	112.1 (± 3.33)	79.4 (± 8.61)

Table 5.3 Percentage decrease in the number of fos expressing cells per 40μm section when comparing the uninjured group of animals to the injured group. Counts were taken throughout the 4 columns of the PAG examined; the dorsomedial column (dmPAG), the dorsolateral column (dlPAG), the lateral column (lPAG) and the ventrolateral column (vlPAG). NB Negative values indicate an increase in the number of fos expressing cells in the column following traumatic L4 spinal cord injury. The numbers in parentheses after the PAG level is the corresponding number of millimetres behind bregma of that level. * indicate significant changes.

PAG Level	dmPAG	dlPAG	lPAG	vlPAG
1 (5.80-6.08)	8.84%	9.08%	32.97%*	23.72%
2 (6.16-6.44)	1.27%	18.57%*	31.24%*	42.40%*
3 (6.52-6.80)	-0.14%	14.79%	36.43%*	34.73%*
4 (6.88-7.16)	-13.57%	17.31%*	28.84%*	39.15%*
5 (7.24-7.52)	-23.33%	24.88%*	18.42%	40.03%*
6 (7.60-7.88)	-24.96%	-0.40%	8.90%	29.21%*

Similar numbers were also found in the dlPAG of the uninjured group of animals. In these animals cell numbers were higher at 40.7 (± 9.05 SEM) cells per section in the first level, the mean number of cells was approximately the same in the second level at 133.8 (± 7.77) cells per section. At the third level there were fewer cells at 180.5 (± 4.98) cells per section,

at the fourth level there fewer cells at 185.7 (± 6.10) cells per section, fewer at 145.1 (± 10.88) cells per section in the fifth, while in the caudal most sixth level there were fewer cells at 63.5 (± 8.37) cells per 40μm section (Figure 5.7). The only significant change as a result of handling occurred in this caudal most level of the vlPAG. Here handling resulted in a significant decrease of 43.9% in the number of NADPH-d labelled cells per section ($p<0.01$).

Following spinal cord injury in animals that had undergone a light restraint for the purpose of sensory testing prior to perfusion there was little change in the number of NADPH-d producing cells per section. The first level demonstrating a mean of 39.4 (± 9.97 SEM) cells per section, the second 121.2 (± 7.98) cells, the third 185.5 (± 5.92) cells, the fourth 198.7 (± 5.85) cells, the fifth 164.6 (± 6.67) cells while the sixth PAG level demonstrated 68.7 (± 6.63) cells per 40μm section (Figure 5.8; see also Table 5.4 below for summary).

5.2.4 NADPH-d/ fos double label naïve

The dorsolateral column of the PAG was defined by the histochemical detection of NADPH-d. In control animals there were no cells observed which contained both NADPH-d and fos-immunoreactivity. NADPH-d containing neurons were also noted in the caudal part of the ventrolateral PAG where, unlike the dorsolateral column, NADPH-d positive cells were occasionally observed to express fos.

5.3 Discussion

The effects of SCI have been largely studied in the spinal cord, however various supraspinal structures have been implicated in the development of sensory dysfunction following this type of injury (Christensen et al., 1996; Drew et al., 2001; Gerke et al., 2003; Lin et al., 1999a; Lin et al., 1999b; Narita et al., 2003). One such structure is the PAG of the midbrain. The PAG is thought to be involved in the development of neuropathic pain following peripheral nerve lesion (Monhemius et al., 2001; Narita et al., 2003; Pertovaara et al., 1997), however little is known about how the PAG may be affected by contusive SCI. The aim of the present study therefore, was to examine the effect of contusive SCI on the activity of neurons in the PAG. The effect of handling and behavioural testing of the rats was also examined, as injured rats had been handled as part of their upkeep. The results

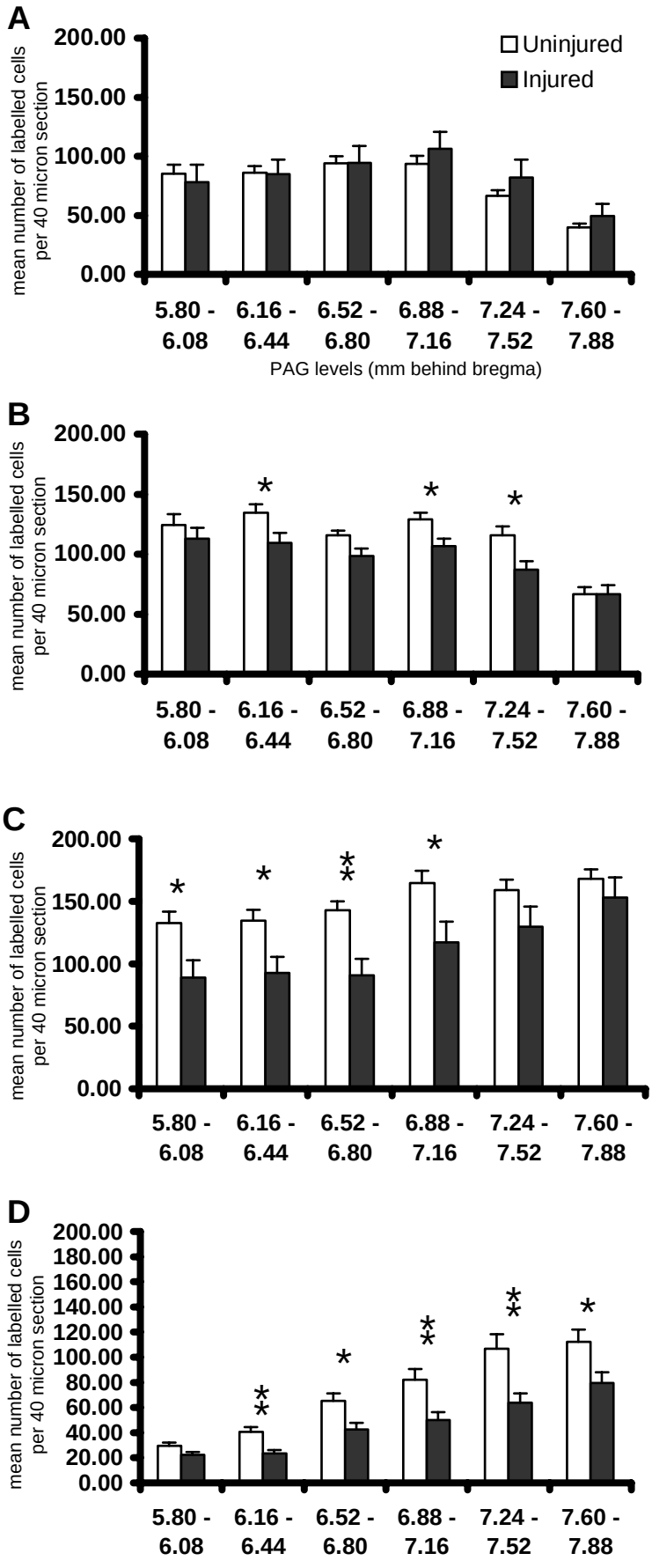


Figure 5.3 Graphs illustrating a decrease in fos expressing cells within the PAG following an L4 SCI. Mean (plus SEM) number of fos expressing cells per 40μm section is plotted over six equal divisions of the PAG. The columns studied were the dorsomedial column (A; dmPAG), the dorsolateral column (B; dlPAG), the lateral column (C; lPAG) and the ventrolateral column (D; vlPAG). Values given on the x axis are mm behind bregma.
* p< 0.05; ** p<0.01; Mann-Whitney U test.

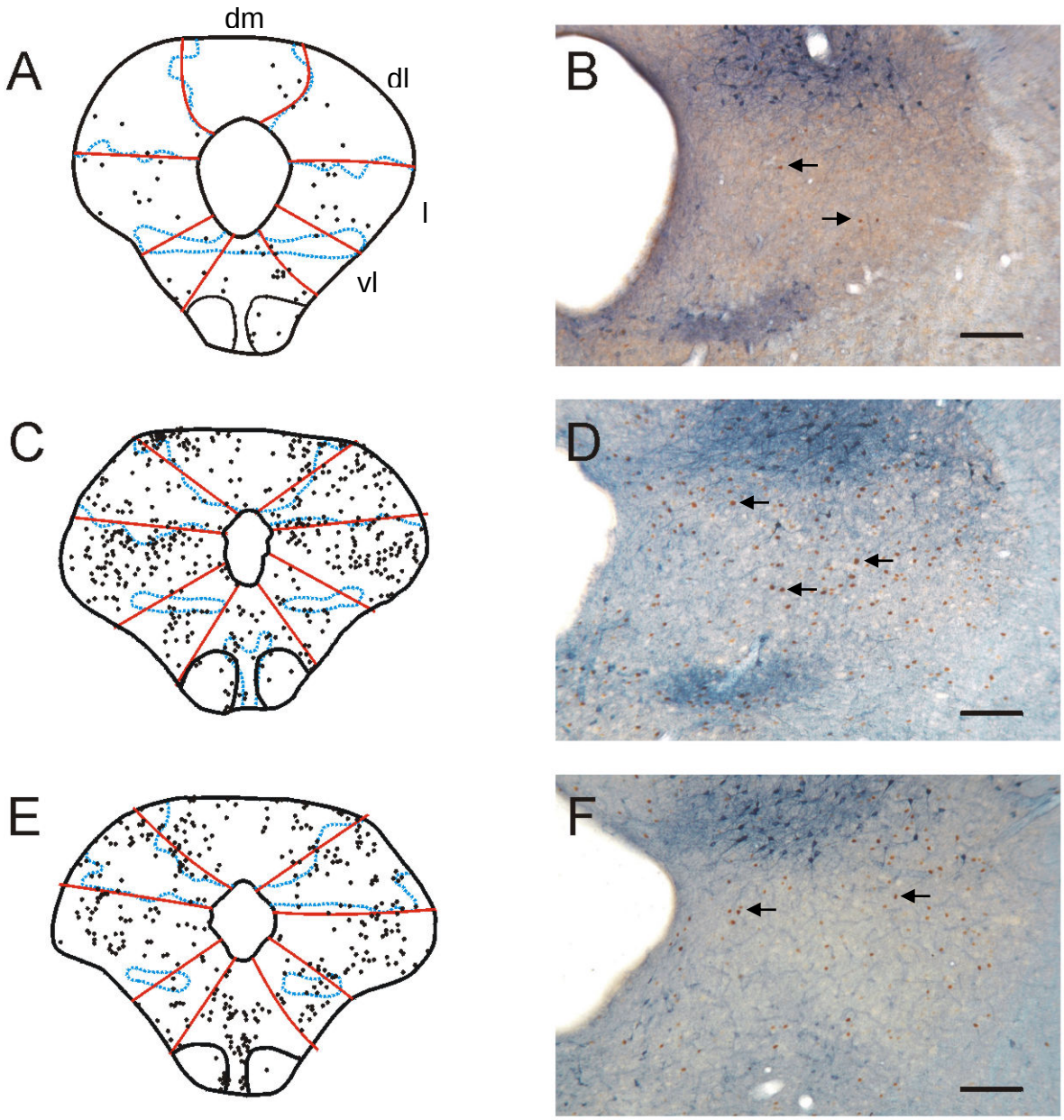


Figure 5.4 Representative camera lucidae of the number of fos labelled cells in the rostral PAG of naïve (A), uninjured (C) and injured (E) animals. The right panels are photomicrographs taken of the lateral PAG of equivalent sections at the same level illustrated (approximately 6.64mm behind bregma). Naïve animals demonstrate very low basal levels of fos expression (B), the handling and light restraint associated with behavioural testing levels however stimulates an increased level of fos expression (D). This is markedly reduced following SCI (F). Arrows indicate fos positive cells. Columns of the PAG examined are indicated in panel A; dm = dorsomedial column, dl = dorsolateral column, l = lateral column, vl = ventrolateral column. Scale bar represents 150μm.

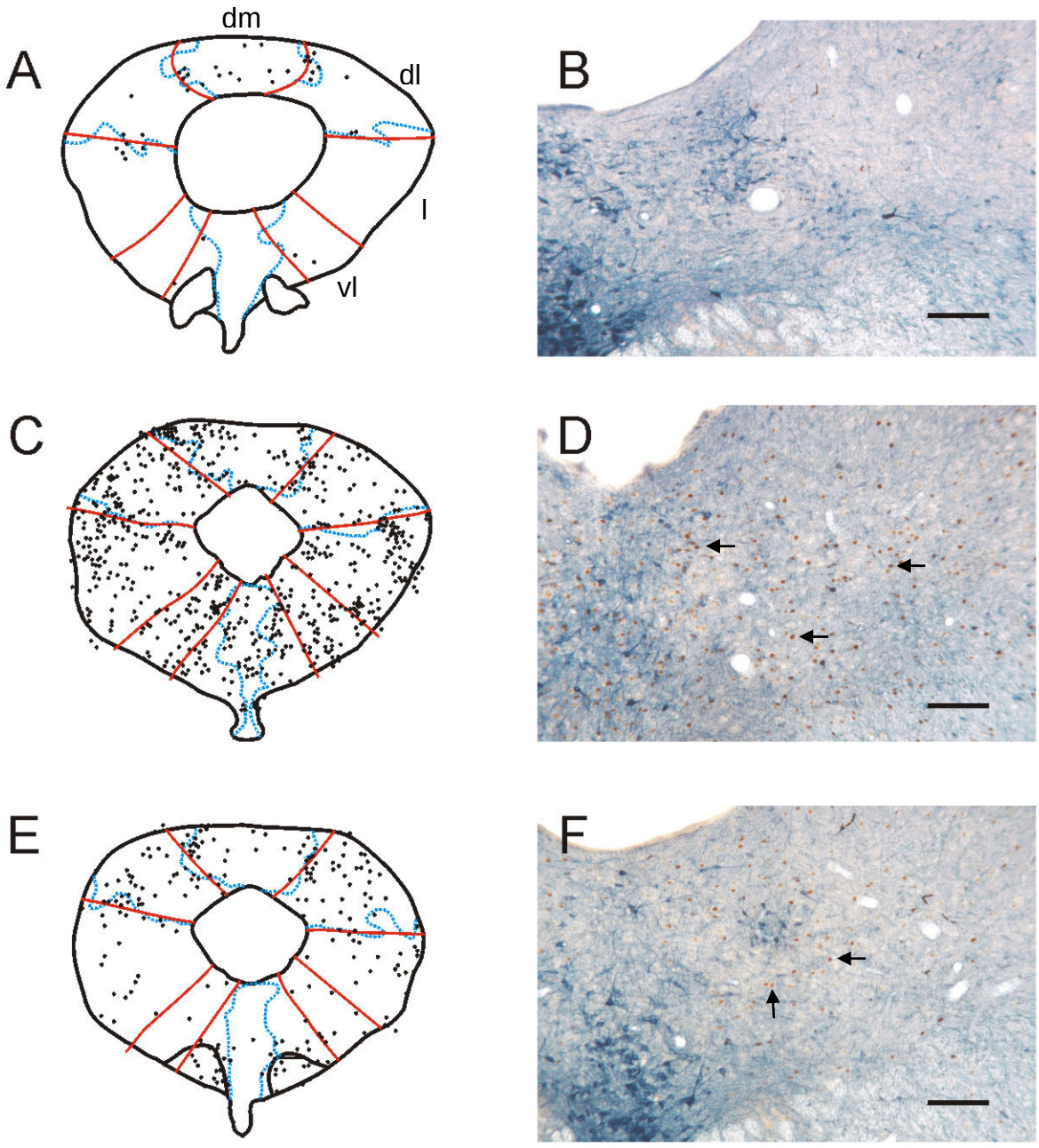


Figure 5.5 Representative camera lucidae of the number of fos labelled cells in the caudal PAG of naïve (A), uninjured (C) and injured (E) animals. The right panels are photomicrographs taken of the ventrolateral PAG of equivalent sections at the same level illustrated (approximately 7.48mm behind bregma). The naïve animal demonstrates very low basal levels of fos expression (B). Following the handling and light restraint associated with behavioural testing levels of fos expression are much increased (D). While SCI results in a marked decrease in the number of fos expressing cells (F). Arrows indicate fos positive cells. Columns of the PAG examined are indicated in panel A; dm = dorsomedial column, dl = dorsolateral column, l = lateral column, vl = ventrolateral column. Scale bar represents 150μm.

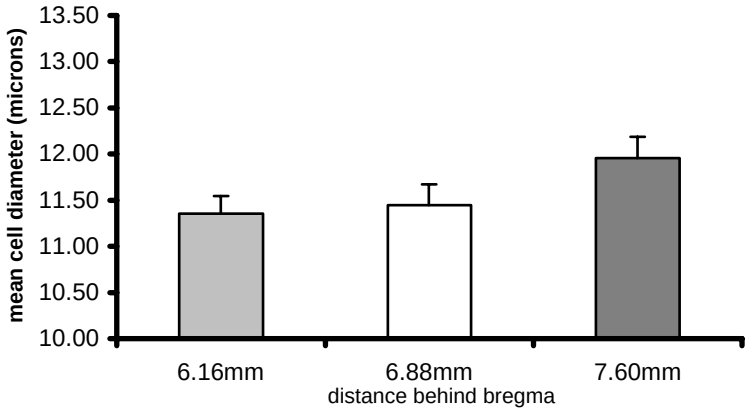


Figure 5.6 Mean Feret diameters (μm) of NO producing cells in the dorsolateral PAG of the uninjured group of animals. Cells were measured at approximately 6.16mm, 6.88mm and 7.60mm behind bregma.

of this study indicated that handling the rats alone resulted in a large increase in fos positive cells, of up to 33.6 fold, over naïve, unhandled rats. SCI on the other hand resulted in a notable *decrease* in fos expression over similarly handled uninjured rats. Significant reductions were observed in the middle two-thirds of the dlPAG, the rostral two-thirds of lPAG and over the entire vlPAG (see Figure 5.3).

5.3.1 Effect of handling and restraint on fos expression in the PAG

As indicated above, handling and behavioural testing of the rats resulted in a dramatic increase in the number of cells expressing fos. This was observed in all columns of the PAG. This increase ranged from a 2.6 fold increase in the rostral most division of dmPAG (5.8-6.08mm behind bregma), to a 33.6 fold increase in the caudal most division of the vlPAG (7.60-7.88mm behind bregma; see Figure 5.2).

Few studies have examined the effect of handling on fos expression in the PAG specifically. However, involuntary restraint of the rat has been reported to induce an upregulation of fos expression within various columns of the PAG (Cullinan et al., 1995; Otake et al., 2002). In particular these include the dlPAG which projects to the hypothalamic-adrenal axis. This projection has been implicated in the response to stressful stimuli (Otake et al., 2002). Furthermore antidepressants have been found to reduce fos expression in both the dorsolateral and ventrolateral PAG in response to restraint (Lino-de-Oliveira et al., 2001). This is likely due to relief of the psychological component of the stress response, which has been reported to be mediated particularly by the dlPAG (Keay and Bandler, 2001).

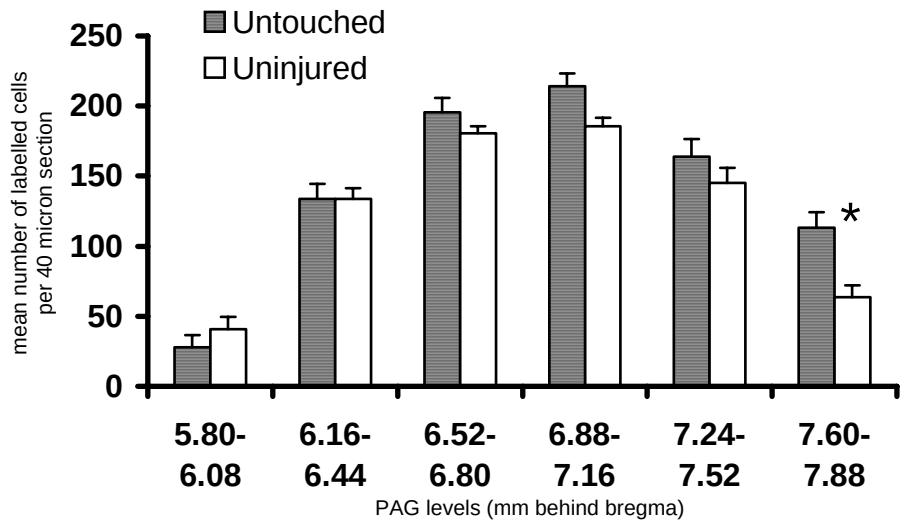


Figure 5.7 Effect of behavioural testing on expression of NADPH-diaphorase (NADPH-d) in the dorsolateral column of the PAG. X-axis shows the rostrocaudal extent of the PAG, from the most rostral sections of the PAG (5.80 – 6.08mm behind bregma) to the caudal most sections of the PAG (7.60-7.88mm behind bregma). Graph show the mean number (plus SEM) of NADPH-d expressing cells per 40μm thick section. See the methods section for a more detailed description of the sections counted. * P<0.01; Mann-Whitney U test.

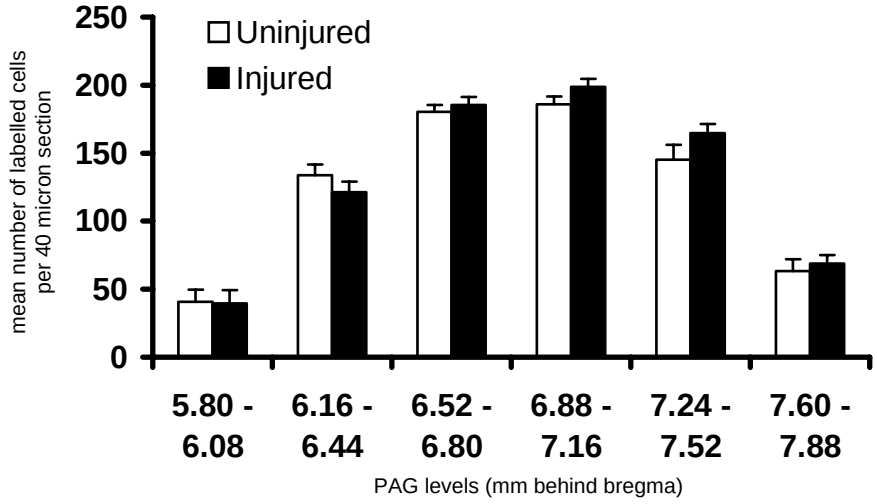


Figure 5.8 Effect of L3-4 spinal injury on expression of NADPH-diaphorase in the dorsolateral column of the PAG. X-axis shows the rostrocaudal extent of the PAG, from the most rostral sections of the PAG (5.80 – 6.08mm behind bregma) to the caudal most sections of the PAG (7.60-7.88mm behind bregma). Graph show the mean number (plus SEM) of NADPH-d expressing cells per 40μm thick section. See the methods section for a more detailed description of the sections counted.

Table 5.4 A comparison of the number of NADPH-d labelled cells per 40μm section within the dorsolateral column of the PAG (dlPAG), at each of the levels of the PAG examined. Each level represents approximately a sixth of the length of the dlPAG. Each level consists of approximately three 40μm sections from each rat within the group. The number of cells per section for each level is the mean number of cells per section for the number of sections that comprise that level across all the rats of each experimental group. The numbers in parentheses after the PAG level is the corresponding number of millimetres behind bregma of that level.

PAG Level	**Naïve** (n=5) **cells/40μm section**	**Uninjured** (n=5) **cells/40μm section**	**Injured** (n=7) **cells/40μm section**
1 (5.80-6.08)	27.8 (± 8.92)	40.7 (± 9.05)	39.4 (± 9.97)
2 (6.16-6.44)	133.7 (± 10.91)	133.8 (± 7.77)	121.2 (± 7.98)
3 (6.52-6.80)	195.6 (± 10.16)	180.5 (± 4.98)	185.5 (± 5.92)
4 (6.88-7.16)	214.2 (± 8.95)	185.7 (± 6.10)	198.7 (± 5.85)
5 (7.24-7.52)	163.9 (± 12.54)	145.1 (± 10.88)	164.6 (± 6.67)
6 (7.60-7.88)	113.2 (± 10.97)	63.5 (± 8.37)	68.7 (± 6.63)

In light of these results then, it would appear that the increased fos expression observed in the PAG following handling and behavioural testing is likely due to the anxiety induced by the handling. The increase in fos expression in the PAG as a result of restraint-induced anxiety may reflect the role of the PAG in mediating behavioural responses to stressful situations (Bandler and Shipley, 1994).

5.3.2 Effect of SCI on fos expression in the PAG

Following SCI, while no significant changes were observed within the dmPAG, there were significant *reductions* in the number of cells expressing fos in the dlPAG, lPAG and vlPAG columns of the PAG. This finding suggests that the activity of cells within these columns had been reduced as a result of lumbar spinal cord injury. The reason for this decrease in activity is not known. However, it has been reported previously that there is a strong spinal projection to the PAG in the rat (Clement et al., 2000; Keay et al., 1997; Menetrey et al., 1982; Yezierski, 1988). A possible explanation for the reduced neuronal activity in the PAG as a result of SCI then may be a loss of inputs from the spinal cord.

It has been noted however, that the projections from the spinal cord to the lPAG at least are arranged somatotopically. That is, projections from more caudal regions of the spinal cord project to more caudal regions of the lPAG, while more rostral spinal neurons project to more rostral parts of the column (Bandler et al., 1991b; Carrive, 1993; Wiberg et al., 1987). Spinal projections to the vlPAG, on the other hand, are convergent and do not appear to project somatotopically (Bandler and Shipley, 1994). The injury-induced decreases in fos expression within the PAG of the present study were also observed to occur with particular rostrocaudal distributions.

The injury performed in the present study was made at approximately the L4 spinal level, and thus should reduce the lumbosacral input to the PAG. Ascending input from more rostral spinal levels should remain intact. The decrease in fos expression observed in the vlPAG was observed over the entire rostrocaudal extent of the column which is consistent with the non-somatotopically arranged spinal inputs to this column. However the decrease in fos expression observed in the lPAG occurred in the rostral two thirds of the column (see Figure 5.3). This corresponds to the region of the column in which the spinal inputs are likely to be intact following SCI. This result suggests that the reduced neuronal activity observed in the PAG following lumbar SCI may not be due to a simple loss of spinal input. It is suggested here that the change in activity may instead reflect a functional reorganisation of the PAG as a result of injury.

The neuronal activity in the dlPAG also appears to be reduced over a distinct rostrocaudal distribution (middle two-thirds of the column). However dlPAG does not appear to receive direct somatic input from the spinal cord (Bandler and Shipley, 1994) so it is difficult to draw a conclusion as to the significance of the pattern of fos expression in this column.

In addition to handling/restraint stress as discussed here, neurons of the PAG have been found to become activated by noxious stimuli (Clement et al., 1996; Keay et al., 2002; Monnikes et al., 2003; Ohtori et al., 2000) in addition to a range of non-noxious stimuli including swim stress (Bellchambers et al., 1998; Cullinan et al., 1995), sexual activity (Pfaus and Heeb, 1997; Struthers, 2001), airpuff startle (Palmer and Printz, 2002), loud ultrasonic noise (Neophytou et al., 2000), light running (Ichiyama et al., 2002; Iwamoto et al., 1996), predating a live food source (Comoli et al., 2003), the presence of a predator (Comoli et al., 2003; Dielenberg et al., 2001; Dielenberg and McGregor, 2001), open spaces

(Silveira et al., 1994; Silveira et al., 2001), and electro acupuncture (Lee and Beitz, 1993). Activation of the PAG has also been found to be involved in antinociception (Sandkühler, 1996). These results indicate that the PAG may mediate the behavioural responses to a large range of external stressors, as has been suggested previously (Bandler et al., 1991b; Bandler and Shipley, 1994; de Oliveira et al., 2001; Keay and Bandler, 2001). For this reason, any event which alters neural activation within the PAG may well impact on the ability of an animal to respond to those stressors. The reduced activity observed in the PAG as a result of SCI then may reduce the normal behavioural or physiological responses mediated by this structure.

In support of this suggestion are studies that demonstrate that the PAG is responsible for mediating strong defensive behaviours (Bandler and Carrive, 1988; Bandler et al., 1985; Bandler and Keay, 1996; Bandler et al., 2000a; Bandler et al., 2000b; Bandler and Shipley, 1994; Depaulis et al., 1989; Depaulis and Vergnes, 1986; Di Scala et al., 1984). According to the results of the present study, it is likely that SCI would therefore result in a reduced ability to react appropriately to an external threat. While no study has been made of traumatic SCI, recent work of Keay and colleagues demonstrate that rats receiving a chronic constriction injury to the sciatic nerve lose the ability to respond to the threat of a foreign rat introduced into their home cage (Monassi et al., 2003). While the animal model used in this instance was a peripheral nerve injury model, similar sensory and behavioural changes were observed to occur compared to the present model, thus it is likely to be comparable. In particular the changes in defensive behaviour observed in these studies appear to support the present findings.

5.3.3 Effect of handling on NADPH-d in the dlPAG

In addition to examining the effect of SCI on neuronal activity in the PAG, the present study also sought to examine the effect of injury on NO synthesis. The dlPAG is characterised by a dense plexus of nitric oxide synthesising neurons and their processes along the entire rostrocaudal extent of the column (Guimaraes et al., 1994; Onstott et al., 1993; Traub et al., 1994). Since the neuronal activity of the spinal cord was altered as a result of SCI, it is a possibility that molecules involved in the normal function of this structure may also be altered.

The major finding of the present study in regards to nitric oxide expression was that handling the rats, the equivalent of light restraint, resulted in a decrease in the number of NADPH-d positive cells in the caudal most segment of the dlPAG (7.6 - 7.88mm behind bregma; see Figure 5.7). As described in this study and others, the nitric oxide producing cells appear as a network of medium sized (9.2 - 13.5μm), multipolar neurons with a dense reticulum of NADPH-d positive processes that run the rostrocaudal extent of the column (Guimaraes et al., 1994; Onstott et al., 1993; Traub et al., 1994). The NO producing cells of the dlPAG are interspersed with non-NO producing neurons (Lovick and Key, 1996; Onstott et al., 1993; Vincent and Kimura, 1992). The observation that NOS was localised predominantly within a longitudinal column of the PAG was some of the first evidence to suggest that the PAG is biochemically arranged in columns. These are postulated to underlie the functional organization of this region (Bandler and Shipley, 1994; Onstott et al., 1993). Why handling should result in a decrease in the caudal dlPAG is not yet known.

The dlPAG is a region of the brain involved in the behavioural response to anxiety (Guimaraes et al., 1991; Guimaraes et al., 1994; Matheus et al., 1994). The high density of NO synthesising cells in this column suggests that NO may be involved in this behavioural response. Evidence to support this suggestion has been obtained from studies examining the role of NO in the function of this column. Stimulation of the dlPAG with EAAs was found to induce flight reactions, while EAA antagonists had a calming (anxiolytic) effect. These effects appear to be mediated at least in part by NO since injection of nitric oxide donors into the dlPAG can produce the same oriented flight/escape response as the EAA injections (de Oliveira et al., 2001; de Oliveira et al., 2000b). Injection of NOS inhibitors, on the other hand, produced an anxiolytic response, similar to injection of NMDA receptor antagonists (de Oliveira et al., 2001; Guimaraes et al., 1994). In addition, injection of the membrane permeable cGMP analogue, 8-Bromo-cGMP into the dlPAG induced a brief flight reaction followed by increased locomotion, again similar to the injection of EAAs or NO donors (de Oliveira et al., 2001). Lastly, the anxiolytic effect of nitric oxide synthase inhibitor L-NAME was antagonized by a pre-treatment with microinjections of L-arginine, the substrate used by the synthetic enzyme for NO (Guimaraes et al., 1994). These results suggest that NO may play a role in the flight-escape responses mediated by this column of the PAG.

The findings of the present study indicated that frequent handling, including light restraint (3-5 sessions over 1 week prior to sacrifice), resulted in a decrease in NADPH-d positive

cells in the caudal most level of the dlPAG. It was noted that over the handling period escape attempts by the rats became less frequent and were less intense. The rats appeared noticeably calmer as a result of frequent handling. As indicated above the release of NO in the dlPAG appears to give rise to strong escape attempts (de Oliveira et al., 2001; de Oliveira et al., 2000b) while NO in either the rostral or caudal dlPAG and the lPAG leads to tachycardia and hypertension (de Oliveira et al., 2000b; Wang et al., 2001). The decrease in the number of NADPH-d labelled cells within the dlPAG observed in the present study then may reflect handling acclimatisation. The reduced NO synthesis, indicated by a reduced number of NADPH-d positive cells, is likely to result in the reduced escape attempts observed over the week of handling.

In support of these findings, it has been found previously that following seven days of restraint, at two hours of restraint a day, there was less nNOS expression in the dlPAG than after only a single day of restraint (de Oliveira et al., 2000a). This was also interpreted as reflecting the development of a tolerance towards the stress of restraint.

5.3.4 Effect of injury on NADPH-d expression

The findings of the present study indicated that traumatic SCI does not have any effect on the number of NADPH-d positive cells in the dlPAG at seven days following injury. No similar studies have been performed following traumatic SCI, although the behavioural and anatomical connections of the PAG have been examined. At earlier time points however it has been noted that strong nociceptive stimuli such as ip acetic acid resulted in an increased expression of NADPH-d in the vlPAG at 12 hours after injection (Rodella et al., 1998). This column of the PAG was not was not examined in the present study.

It was reported in Chapter 2 that SCI did alter NADPH-d labelling but only at spinal levels adjacent to the injury. This indicated that NADPH-d was likely altered by the local injury-induced environment. At spinal levels distant to the injury SCI did not have an effect on NADPH-d labelling. As the PAG is also distant to the injury it is possible that it, similarly, is not affected by the injury at seven days.

5.4 Conclusion

The present study examined the effect of a lumbar spinal cord injury on the expression of c-fos and nitric oxide within the PAG of the rat. Since the injured rats were handled as part of

their normal upkeep, an uninjured group was handled in a similar way. For that reason the effect of handling on the expression of these two molecules was also obtained. The activity of neurons in the PAG (as determined by fos expression) was found to be increased across all columns of the PAG by handling and behavioural testing. A number of studies have found that fos expression is increased within the PAG following a period of forced restraint likely due to the role that the PAG plays in mediating behaviours associated with fear and anxiety. It was interpreted then that the increase in fos observed in the present study following testing was for a similar reason, and was in support of these previous studies.

Following traumatic lumbar SCI however, fos expression was found to be reduced within the dlPAG, lPAG and vlPAG. One possibility was that this decrease in activity was due to reduced input from the spinal cord. It was noted however, that the decrease in fos positive cells occurred in regions of the PAG that received surviving fibres from more rostral spinal levels. It was suggested that perhaps the reduction in neuronal activity induced by SCI may instead reflect functional changes in the PAG associated with SCI.

NO synthesising cells in the dlPAG, on the other hand, were found to be reduced in the caudal most part of this column by handling and behavioural testing. Due to the excitatory nature of NO in this part of the PAG it was thought that reduced NO may be related to handling acclimatisation.

The findings of this study into fos and nitric oxide in the PAG extends those of the previous chapters in which the same molecules were examined in the spinal cord. As discussed in this chapter extensive connections exist between the spinal cord and the PAG. The findings of Chapter 2, in regards to nitric oxide within the spinal cord following lumbar SCI, were that nitric oxide was altered from control values only in areas of the cord adjacent to the injury. This suggests that nitric oxide synthesis was altered in response to biochemical changes in the cord as a result of the injury. Fos expression on the other hand was altered even at levels of the spinal cord distant to the injury. This was interpreted to suggest that fos expression was altered, perhaps by global changes to the level of neural activity within the CNS, possibly descending from supraspinal structures.

The present study has demonstrated that the neuronal activity in the supraspinal PAG was also reduced following traumatic lumbar SCI. This indicated that perhaps it too may be

affected by the same global suppression of activity as the spinal cord. The mechanisms by which this might occur are still not known.

If the neuronal activity of supraspinal structures such as the PAG at the level of the mid brain are affected by lumbar SCI, it is interesting to determine whether any higher structures might be similarly affected. The parietal cortex in the rat is the equivalent of the sensory and motor cortex in the human. Thus it is involved in both the reception of sensation as well as motor outflow. As SCI is characterised by both sensory disruption and loss of voluntary motor control it is likely that this structure may also be affected by SCI. This is the focus of the next chapter of this thesis.

Chapter 6

The effect of traumatic SCI on fos expression in the parietal cortex of the rat

In humans, the part of the cortex responsible for the reception and high order processing of sensory information is the somatosensory cortex. This structure is located posterior to the central sulcus in the parietal lobe. While the brain of the rat has no sulci, it has been mapped into frontal, parietal, temporal and occipital regions according to functional studies and cytoarchitecture. The equivalent of the somatosensory cortex in the rat occurs in the parietal region as it does in humans (Zilles and Wree, 1995). It also appears to perform equivalent functions, receiving inputs from cutaneous mechanoreceptors, thermocepotors and proprioceptive fibres (e.g. De Ryck et al., 1992; Wang et al., 2003; Wiest and Nicolelis, 2003).

In rats, both electrical stimulation of peripheral nerves (Ito, 2002; Wright et al., 2001) and whisker deflection (Lindauer et al., 1993) result in activation of the sensorimotor cortex. Furthermore, tactile function is disrupted by lesions of the parietal cortex (De Ryck et al., 1992; Napieralski et al., 1998). These findings are similar to those found in humans and non-human primates (Burton et al., 1999; Human: Gandevia et al., 1983; Jousmaki and Forss, 1998; Pause et al., 1989; Squirrel monkey: Tommerdahl et al., 1996)

In addition, the parietal cortex has also been shown to become active following painful stimulation in both rats and humans (Coghill et al., 1994; Derbyshire et al., 1997; Porro et al., 2003; Talbot et al., 1991), which is possibly linked to the observation that lesions of the parietal cortex have been associated with central pain syndromes (Peyron et al., 2000).

Previous chapters of this thesis examined the effect of SCI on neuronal activity (as determined by fos expression) in the spinal cord and in the PAG. In both of these structures, neuronal activity was found to have been reduced at seven days following traumatic injury to the lumbar spinal cord. These results demonstrated that the change in

neuronal activity as a result of SCI was not dependant on proximity to the injury, as sections taken from either the mid cervical enlargement or the PAG were similar to those adjacent to the injury. Further to this, it was demonstrated in Chapter 5 that the reduction in neuronal activity in functional columns of the PAG corresponded somatotopically with regions of the intact cord, above the injury. This indicated that it was not simple deafferentation that resulted in the reduction in neuronal activity.

Collectively these results appear to indicate that traumatic lumbar SCI results in an alteration in function of CNS structures distant to the actual injury. If this is true then it is likely that other structures in the CNS may be similarly affected. To follow the examination of the somatosensory system discussed to date by this thesis further, it was the aim of the present chapter then to examine the effect of SCI on neuronal activity in the parietal cortex of the rat. Due to the role of the parietal cortex in the processing of sensation, any change in the pattern of neuronal activity in this structure as a result of SCI may give rise to changes throughout the somatosensory system. This would help explain the results of the previous studies reported here. Study of the parietal cortex would also be important for extending these previous findings by examining the effect of SCI on structures at the highest level of the somatosensory pathway.

6.1 Methods

6.1.1 Experimental groups

A total of 16 female Wistar rats (200-300g) were used in the present study. Of these, 6 were subjected to contusive SCI, 5 served as uninjured controls which underwent behavioural testing (uninjured), while another 5 served as uninjured controls that had undergone no behavioural testing (naïve, unhandled). These groups were described extensively in the previous chapter. All experiments were conducted following approval of the study protocol by the institutional animal care and ethics committee, and observed the animal welfare guidelines of the National Health and Medical Research Council of Australia and the International Association for the Study of Pain (Zimmermann, 1983).

6.1.2 Spinal cord injury

Rats underwent a traumatic spinal cord injury under halothane anaesthesia, receiving a weight drop of 2, 3 or 5cm as described in Chapter 2. Sacrifice and perfusion were also performed as described in Chapter 2.

6.1.3 Handling and restraint

Rats undergoing spinal cord injury also underwent regular behavioural testing as part of their normal upkeep. This included light restraint, in which the rat was held by the experimenter during testing. Behavioural tests included both sensory and motor testing and were described in detail in Chapter 4. The benefit of testing was two fold. Firstly it allowed the animals to be monitored for any undue suffering or behavioural deficits as a result of SCI, while secondly it provided a tactile and emotional stimulus allowing the effect of injury on the somatosensory cortex to be examined. The "uninjured" group of animals underwent the same testing, while the "naïve, unhandled" group of animals were neither tested nor handled.

6.1.4 Tissue processing

While the number of NADPH-d positive cells was not examined in the present study, NADPH-d histochemistry was performed as a counterstain, allowing good contrast of brain tissue. The brain was blocked prior to microtomy coronally through the level of the optic chiasm, and through the anterior third of the cerebellum. A one in three series of 40µm coronal sections was cut on a freezing microtome (Leica). Free-floating sections from each animal were then processed for NADPH-d histochemistry and fos immunohistochemistry which were described in Chapter 2.

6.1.5 Plotting

Sections were observed under the light microscope and plotted using a camera-lucida attachment. Sections were plotted from three levels of the parietal cortex, 0.7mm, 1.4mm and 2.3mm behind bregma, using coordinates from Paxinos and Watson to determine the boundaries of the structures examined (Paxinos and Watson, 1998). Fos positive cells in area 1 and 2 of the parietal cortex were plotted and counted at 4x objective at each of the three levels (See Figure 6.1 for schematic diagram of region of cortex examined).

6.1.6 Statistical analysis

Data was analysed using a Mann-Whitney U test with a level of significance of 0.05.

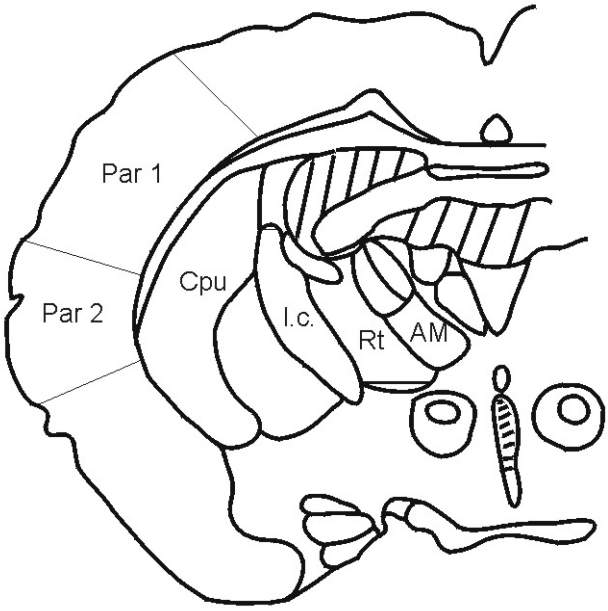


Figure 6.1 Schematic diagram of forebrain indicating area 1 (Par 1) and area 2 (Par 2) of the parietal cortex. These areas of the cortex were examined at 0.7mm, 1.4mm and 2.3mm behind bregma. CPu, caudate putamen; l.c., internal capsule; Rt, reticular thalamic nucleus; AM, anteromedial thalamic nucleus.

6.2 Results

6.2.1 Fos in the parietal cortex of the rat

Very few fos labelled cells were observed in area 1 of the parietal cortex throughout the extent of this area in the "naïve, unhandled" groups of rats (uninjured, unhandled). At 0.7mm behind bregma there was a mean of 20.7 (±20.17 SEM) fos labelled cells per 40μm section. At 1.4mm behind bregma there was a mean of 5.0 (±2.65) cells and at 2.3mm behind bregma there was a mean of 5.60 (±2.73) cells per section. Area 2 demonstrated similarly very low levels of fos expression per 40μm section at each of the levels, demonstrating a mean of 0.67 (±0.67 SEM), 4.20 (±2.46) and 10.20 cells (±6.02) at the same three levels respectively (Figure 6.2).

6.2.2 Effect of handling on cortical fos

Handling and light restraint of the uninjured rat resulted in a vast increase in the number of fos expressing cells in both area 1 and 2 of the parietal cortex (Par 1 and 2). At 0.7mm behind bregma in Par 1 there was an almost 95 fold increase in the number of cells expressing fos increasing from 20.7 (±20.17 SEM) cells per 40μm section in the naïve, unhandled rats to 1959.5 (±412.31) cells per section in the behaviourally tested uninjured rat. At 1.4mm behind bregma there was a 223 fold increase to a mean of 1117.3 (±230.34)

cells while at 2.3mm behind bregma there was a 520 fold increase in fos expressing cells to a mean of 2910.8 (±416.11) cells per 40μm section.

Par 2 showed similar changes, increasing 613 fold to 410.8 (±93.18 SEM) cells per 40μm section at 0.7mm behind bregma, 122 fold to 511.6 (±89.51) cells per section at 1.44mm behind bregma, and 83 fold to 850.2 (±114.03) cells per 40μm section at 2.3mm behind bregma. Fos expression was most prominent in layers IVa and IVc, with scattered labelling through layers II and III and sparse labelling in layer VI (See figure 6.3 and 6.4).

6.2.3 Effect of SCI on cortical fos

A contusive T13 vertebral (~L4 spinal level) spinal cord injury, resulted in a dramatic decrease in the number of fos expressing cells in both Par 1 and 2 when compared with behaviourally tested uninjured rats. SCI resulted in a mean of 319.3 (±251.34) cells at 0.7mm behind bregma, 230.7 (±79.49) cells at 1.4mm behind bregma and 649.7 (±222.75)

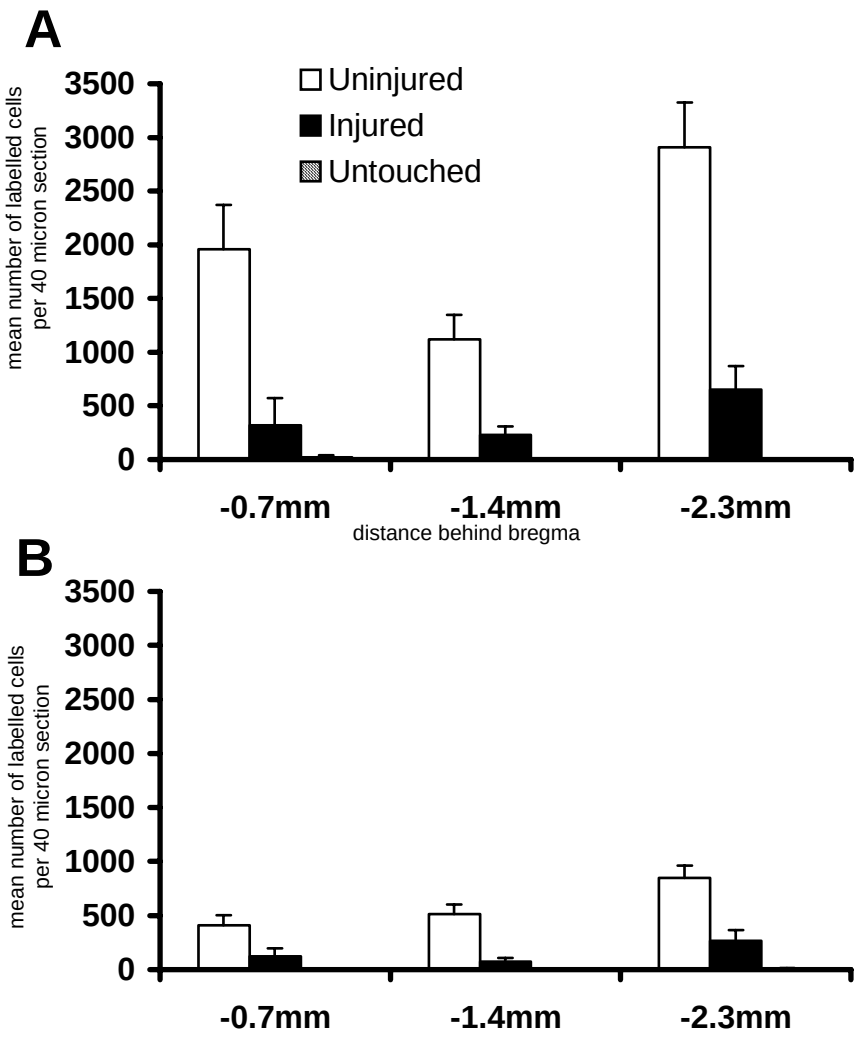


Figure 6.2 Comparisons of number of fos expressing cells in the parietal cortex of the rat. The uninjured groups were handled in a similar fashion to the injured group, while the naïve, unhandled group received no handling at all. The injured group received a L4 spinal level contusive SCI. The numbers shown represent the mean number of fos labelled nuclei per 40μm section (plus SEM) in parietal cortex area 1 (A) and parietal cortex area 2 (B). Basal level of fos expression in the parietal cortex of naïve, unhandled animals was negligible at the scale shown here. The x-axis indicates the levels of coronal sections examined (0.7mm, 1.4mm and 2.3mm behind bregma). Significant differences were found between all animal groups ($p<0.05$) when examined with a Mann-Whitney U test.

cells at 2.3 mm behind bregma in Par 1. This corresponded to decreases of 83.7%, 79.4% and 77.7% respectively. In Par 2 the mean number of fos positive neurons was 121.3 (±76.64 SEM) cells, 76.3 (±33.92) cells and 265.0 (±99.98) cells per 40μm section at 0.7mm, 1.4mm and 2.3mm behind bregma (see Figures 6.2 - 6.4). This corresponded to a 70.5%, 85.1% and 68.8% decrease respectively. Fos is decreased generally across all cortical layers, but in particular there is no prominent labelling in layer IVa and IVc, as in the uninjured group of animals.

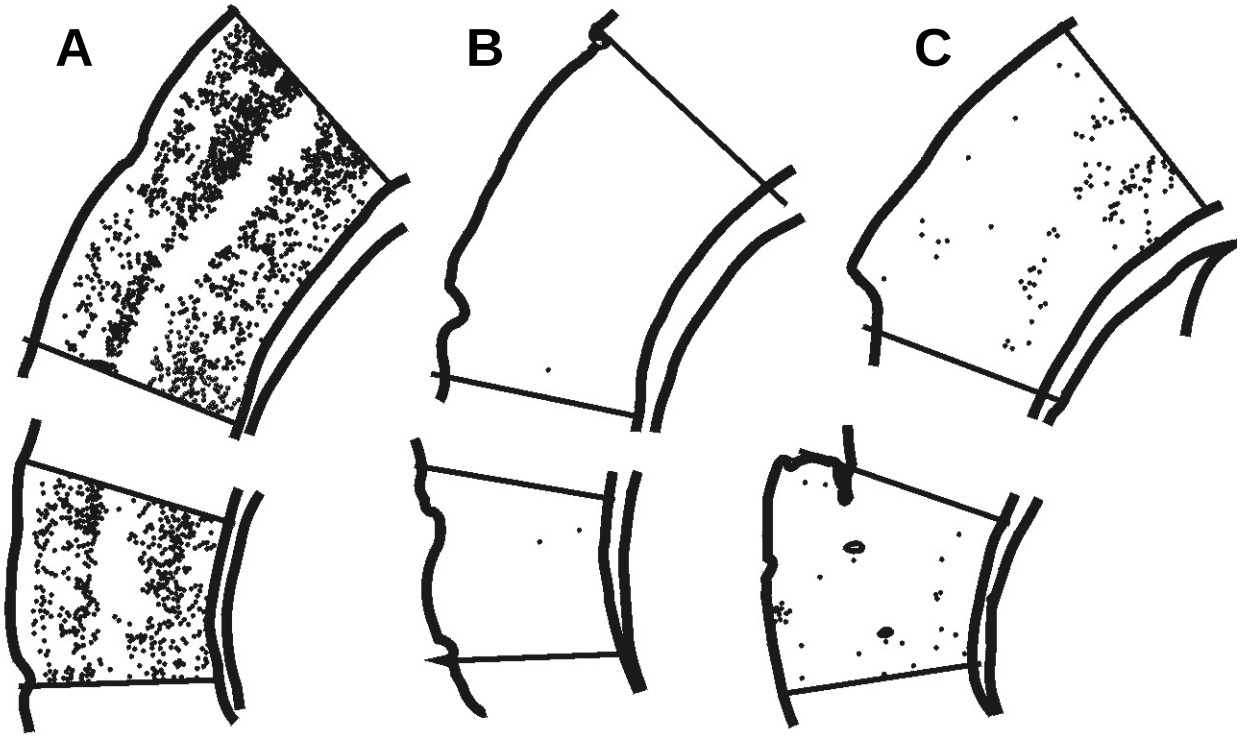


Figure 6.3 Camera lucidae of parietal cortex area 1 (Par 1; top panel) and parietal cortex area 2 (Par 2; bottom panel) at approximately 1.4mm behind bregma. A; shows fos expression in rats that were uninjured but had been behaviourally tested in the same manner as the injured rats. B; shows fos expression in the cortex of the naïve, unhandled group of rats. Fos expression is noticeably absent in this group of animals. C; shows fos expression in the injured group of animals, demonstrating an intermediate number of fos expressing cells.

6.2.4 Statistical significance

The results of the Mann Whitney U test indicated that all animal groups, at all levels of the parietal cortex examined, were statistically different at a confidence level of $p<0.05$.

6.2.5 Effect of SCI on the number of NADPH-d cells in the parietal cortex

There were no changes in the number of NADPH-d cells in the parietal cortex following either handling or SCI.

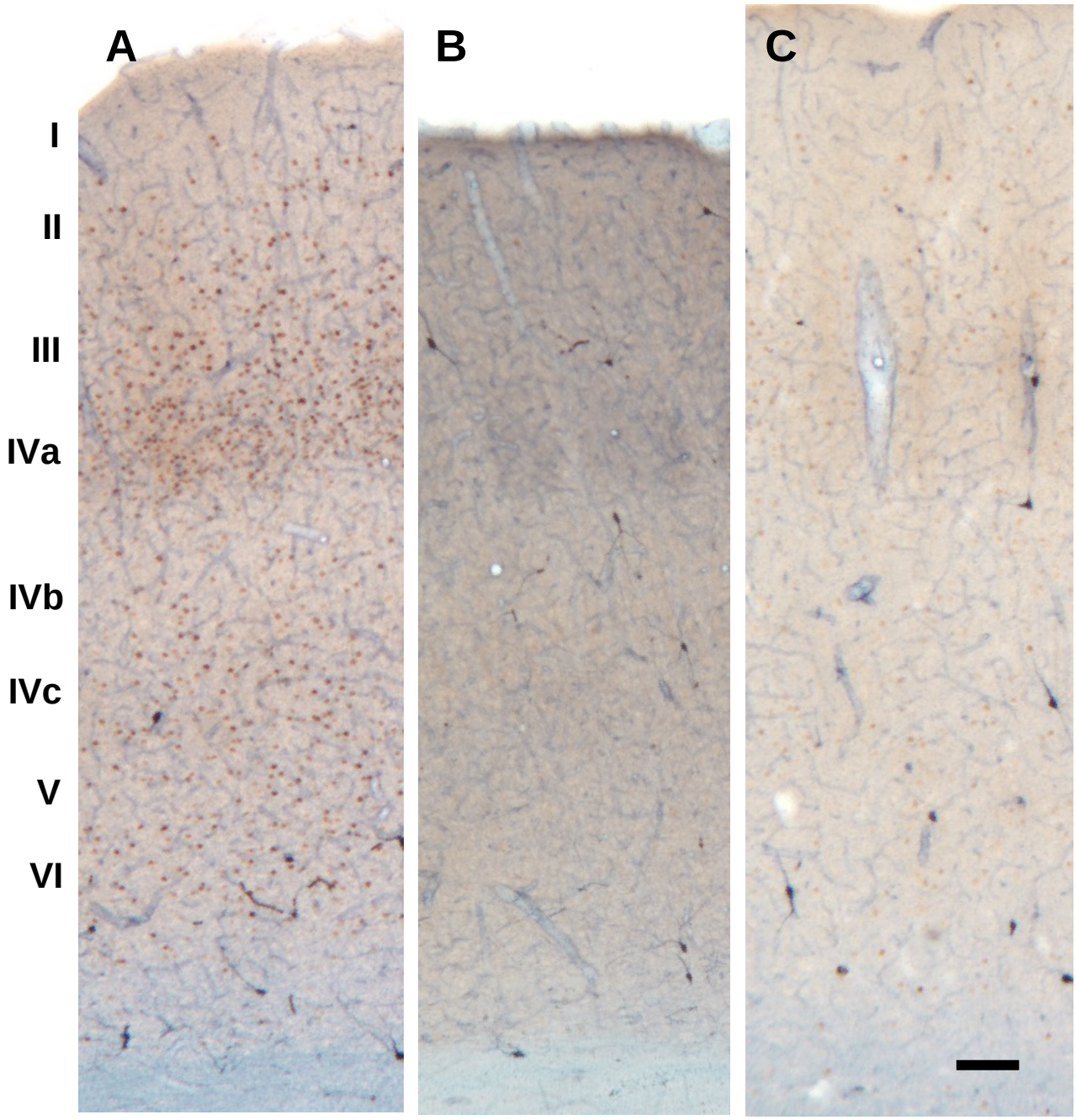


Figure 6.4 Representative photomicrographs of parietal cortex area 1 (Par 1) taken from an uninjured, behaviourally tested animal, A; a naïve, unhandled animal, B; and a spinally injured, behaviourally tested animal, C. Micrographs were taken at 4x objective. Cortical laminae are labelled to the left of panel A. Micrographs are shown at equal magnifications, differing apparent cortical thickness is artifactual from tissue processing. Scale bar represents 110μm.

6.3 Discussion

It was the aim of the present study to examine the effect of lumbar SCI on neuronal activity in the parietal cortex of the rat. Previous chapters of this thesis indicated that such an injury resulted in changes to neuronal activity in both spinal and supraspinal structures, despite these being remote to the actual injury site. As discussed in Chapter 4, one of the complications of SCI is the development of neuropathic pain. While the specific mechanisms for this development are not yet known, any changes in neuronal activity that affect sensory processing may represent important possibilities.

Chapter 4 reported changes to the spinal processing of tactile stimuli in animals with neuropathic pain. The results reported in Chapter 2 provides some evidence to suggest that this may have been due to disruption of inhibitory tone within spinal laminae receiving sensory information. It was speculated that supraspinal structures may also be involved. Changes in neuronal activity in the PAG were reported in Chapter 5, although the involvement of these in the development of neuropathic pain was not examined. These results did, however, indicate that supraspinal structures distant from the injury itself were also affected.

The ultimate target of the somatosensory pathway is the somatosensory cortex. As sensory processing at the level of the spinal cord appears to be disrupted following SCI, it is likely that the activity of the somatosensory cortex would also be altered. Neuronal activity as a result of SCI has not been examined histologically in this region of the cortex. The present study therefore represents a first step in isolating components of the sensory neuraxis that may be involved in the development of neuropathic pain using histology as an adjunct to the physiology and behaviour reported elsewhere. Clearly other structures will also be involved and these will likely be examined in the future.

The major findings of the present study indicate that the handling and light restraint of rats resulted in a vast increase in the levels of fos expression in the parietal cortex. This increase was between 83 and 613 fold over unhandled levels. In contrast it was found that a contusive lumbar SCI resulted in a reduction of 70-85% of the number of fos expressing

cells in the cortices of rats that had been similarly handled. This decrease in neuronal activity was despite the parietal cortex being remote from the injury site.

6.3.1 Handling and light restraint

The primary function of handling and behavioural testing was to monitor the health and wellbeing of the spinally injured rats. Handling and light restraint of the rats however, had an additional benefit. As reported in this and in previous chapters, handling and light restraint resulted in a dramatic increase in the number of fos labelled cells in the spinal cord, the PAG and in the cortex. In the previous chapter and this one, the numbers of fos positive cells in the PAG and the cortex of the unhandled, naïve rat were also counted. In these animals fos expression was extremely low. Thus, in the unhandled rat it would not be possible to observe how SCI altered neuronal activity, as there is little activity to begin with. Increasing the basal level of neuronal activity with a mild, non-invasive stimulus allowed any change in activity as a result of lumbar SCI to be observed.

Light restraint has been shown previously to result in acute increases in fos expression in multiple forebrain structures, including the somatosensory cortex (Chowdhury et al., 2000; Yokoyama and Sasaki, 1999). The present study confirmed these previous findings. While restraint has most often been used to examine structures related to stress, it is also a highly tactile stimulus. During restraint large areas of hairy skin are contacted often involving deflection of the vibrissae. Similar moderate stimuli have been shown to induce fos expression in the parietal cortex (Filipkowski et al., 2000). It is therefore likely that it is this tactile nature of the stimulus that has resulted in activation of the somatosensory cortex following handling in the present study.

6.3.2 Effect of SCI on cortical fos expression

When compared with uninjured animals that had been similarly handled, SCI resulted in a dramatic decrease (70-85%%) in the number of cells expressing fos. It was observed that the principal cortical layers in which fos was induced following handling were the granular layers IVa and IVc, which are primarily involved in the reception of sensation (Lamour et al., 1983a; Lamour et al., 1983b). Following injury, prominent labelling in layer IV was no longer present, and was generally reduced throughout Area 1 and 2. This result indicated that the effect of injury is to prevent the handling-induced increase in fos expression

observed in the uninjured group of rats. This finding has not previously been reported but the mechanism underlying the reduced level of fos expression may be postulated.

Injury to the spinal cord has been reported to decrease the passage of neurons from beneath the lesion to supraspinal structures including the sensorimotor cortex (Joshi and Fehlings, 2002). Cortical projections going to areas beneath the lesion, as well, have been found to be largely abolished following SCI due to the dorsal nature of these columns (Ferguson et al., 2001; Kim et al., 2002). Thus the most likely explanation of reduced neuronal activity in the somatosensory cortex as a result of SCI is a reduced afferent drive to these areas.

The decrease in cortical activity, however, occurred across the whole of Area 1 and Area 2 of the parietal cortex. These areas included somatotopic representations of regions of the body for which supraspinal contact should not have been disrupted, transmitted by the more rostral cord. The cortex in the rat, however receives no direct sensory projection from the spinal cord, instead sensory information is relayed to the somatosensory cortex via a range of subcortical structures including the thalamus the raphae nuclei, locus coeruleus and other areas of the cortex (Chapin et al., 1987; Tracey, 2004). Thus, the disruption of supraspinal projections by SCI may indirectly affect a larger range of inputs to the sensory cortex than would be suggested by the extent of the injury.

It is well known that deafferentation of the cortex can result in extensive reorganisation of the cortex (for reviews see Buonomano and Merzenich, 1998; Churchill et al., 1998; Curt et al., 2002; Jones, 2000; Kaas, 2000; and see also Merzenich et al., 1983). Since primary afferents or their second order spinal targets do not synapse directly with the cortex it is likely that such reorganisation occurs as a result of altered inputs from sub cortical structures. This indicates that the activity of the cortex may be altered by changes in the sensory system in general. Indeed this network driven mechanism is the underlying principal behind cortical reorganisation. The model was first proposed in 1949 by Donald Hebb (Hebb, 1949), who was trying to account for brain responses to both external and internal stimuli. He suggested that groups of neurons were connected together by largely excitatory synapses such that if one or a couple of the neurons in a group, what he termed a cell assembly, were excited, then all the cells of the assembly would fire. He proposed that continued excitation of this pathway would result in its reinforcement, making it more

likely to fire as a result of the next stimulation. Disruption to one part of the network then results in an imbalance of inputs and changes in activity across the whole network. This has been observed experimentally and is thought to be due to the unmasking of synapses that ordinarily are unable to induce a cellular response due to other more dominant connections (Eysel et al., 1981; Garraghty and Kaas, 1991b; Kolarik et al., 1994; Recanzone et al., 1993). This model forms the basis of the currently accepted theory of memory and learning

Lastly, the proposition made here, that network reorganisation across multiple levels of the neuraxis may be responsible for the alterations in neuronal activity observed, would also explain why changes in fos expression were found to occur at all levels of the neuraxis examined. In support of this it has been suggested previously that the activity of the cortex may be involved in setting the level activity for the nervous system from the top down, and that this control may be altered following traumatic SCI (Jasmin et al., 2003). Indeed, as in the present study, SCI has been found to result in changes in activity in not only the cortex but also in subcortical structures, particularly the thalamus (Davis et al., 1998; Florence and Kaas, 1995; Gerke et al., 2003; Jones and Pons, 1998; Morrow et al., 2000). The dependence of cortical reorganisation on concurrent reorganisation of sub cortical structures has previously been noted (Faggin et al., 1997; Garraghty and Kaas, 1991a).

These results indicate an interdependence of the levels of activity throughout the CNS. Thus SCI appears to result in an imbalance in synaptic input to the network that comprises the CNS, resulting in changes in neuronal activity throughout the entire system, not just locally.

6.3.3 Functional significance of cortical changes

Having observed a reduced level of neuronal activity in the parietal cortex following SCI, the functional upshot of this may be discussed. The specific nature of any functional changes will be related to the structures in which alterations of neuronal activity occurred. In the parietal cortex these are likely to involve sensory processing, as well as spatial localisation and cognitive function.

In the rat, the parietal cortex receives sensory information from cutaneous and deep peripheral touch receptors and pain fibres (Zilles and Wree, 1995). Lesion of the parietal

cortex results in reduced ability at foot placement. This was interpreted as due to lesion-induced impairment of touch and proprioception (De Ryck et al., 1992), indicating the importance of the parietal cortex in these functions. The parietal cortex is also activated by noxious peripheral stimulation (Porro et al., 2003).

In humans too, both non-noxious and noxious peripheral stimulation has been found to activate the parietal cortex in areas associated with the extremity stimulated (Casey et al., 1994; Coghill et al., 1994; Derbyshire et al., 1997; Nelson et al., 2004; Talbot et al., 1991; Torquati et al., 2002; Xu et al., 1997). These results support the notion that the parietal cortex is likely involved in sensory processing. The reduced level of neuronal activity observed in the in the parietal cortex of the rat following SCI may therefore result in altered sensory processing.

While this suggestion awaits experimental verification in the rat, it is supported by human studies which indicate that lesion of the parietal lobe also produces various sensory deficits that appear similar to those induced by spinal cord injury (for review see Freund, 2003). Also, there is evidence to suggest that cortical reorganisation may be in part responsible for the development of neuropathic pain conditions such as allodynia (Maihofner et al., 2003). Indeed, in a rat model of SCI, that is typified by the development of neuropathic pain, injection of quisqualic acid into the spinal cord resulted in the activation of a number of forebrain structures. These included the parietal cortex in addition to the arcuate nucleus and a number of thalamic nuclei. It was observed that these structures were all involved in the processing of sensory information (Morrow et al., 2000).

Thus it appears that alteration in neuronal activity in the parietal cortex as a result of traumatic SCI may indicate a reduced functional state for this region of the cortex. This may include a processing of sensation.

The other major role of the parietal cortex is the processing of allocentric (external) sensory cues, or more specifically how those cues exist in space in relation to the body (Save et al., 2001; Soblosky et al., 1996). Furthermore it is thought that each of these representations are stored in terms of a series of actions that can be carried out in relation to that object, e.g. reaching out and grasping an object, avoiding an object, navigating around a room in relation to an object (Colby and Duhamel, 1996).

Much of our knowledge of the function of the parietal cortex comes from injury studies, particularly lesion studies in which a traumatic brain injury (TBI) is performed and the behavioural or cognitive effects are noted. In rat models of TBI, the sensorimotor cortex is frequently injured (Soblosky et al., 1996). Neurological impairment is commonly observed following injury to the parietal cortex including sensori-motor impairment (Dixon et al., 1991; Piot-Grosjean et al., 2001), and spatial memory deficits (Lyeth et al., 1990; Piot-Grosjean et al., 2001). In rats, severe deficits in allocentric (external cuing) spatial localisation occur following lesion of the parietal cortex or the hippocampus (Kesner et al., 1989; Soblosky et al., 1996). Injury to the parietal cortex can also lead to somatosensory neglect of the contralateral side of the body (Holm and Mogensen, 1993).

These indicate that, just as lesion to this region of the cortex may disrupt the processing of allocentric cues by the parietal cortex, it is a possibility that an SCI-induced reduction in neuronal activity may also result in alterations to this function. This notion awaits more direct experimental verification.

6.4 Conclusion

In conclusion it was found that contusive lumbar SCI resulted in a profound reduction in the number of cells activated within the parietal cortex of the rat as a result of handling and light restraint. It was proposed that the changes in activation were likely due to synaptic reorganisation which has been shown to occur as a result of deafferentation. The reductions in neuronal activation observed in the present study was similar to those reported at other levels of the neuraxis in previous chapters. These alterations occurred despite the structures being remote to the actual injury site. This was likely due to the interdependence of CNS structures on the levels of activity across the whole network. Due to the function of the parietal cortex, it is possible that that both sensory processing and the cognitive function related to allocentric processing would be altered due to the SCI-induced changes in neuronal activity.

Chapter 7

Conclusions

From the simplest concepts of the nervous system, piece by piece we have built up an idea of how this most complex of systems works. However, despite the work done, particularly in recent times a complete understanding still eludes us. A portion of this can be attributed to what it is we are trying to identify. Are we trying to work out what the brain is, or what chemicals are made by its neurons, or how those neurons are interconnected? Are we trying to work out what the different parts of the brain do or how they do it? The present thesis examined the effect of lumbar spinal cord injury on NO synthesis at levels of the neuraxis both adjacent to the injury as well as distant to it. It also examined how this was related to changes in neuronal activity associated with the injury. However apart from the surface findings reported here of the various changes in the various proteins. What remains interesting however is what this tells us about the central nervous system its self.

This thesis may be viewed at two levels then, in terms of the molecular changes that were observed, but also underlying that, how this information fits in with out understanding of the CNS as a whole.

In the front of the fourth edition of Kandel, Schwartz and Jessell's, Principles of neural science (McGraw Hill), there is the Egyptian hieroglyph for the word Brain.

This hieroglyph was taken from a papyrus, today named after the American Egyptologist Edwin Smith, who bought the papyrus from a dealer in Luxor in January of 1862. The papyrus is dated from 1700BC and consists of a list of surgical presentations, possibly to the Egyptian surgeon Imhotep. The papyrus is significant as it contains the first written record of the brain, spinal cord, meninges and cerebrospinal fluid. Also of interest, is that

amongst the cases described was one individual who had suffered an injury to the side of the head, over the area we would now describe as the temporal bone. It was reported that this individual could not talk and thus likely represents the first description of aphasia some 3500 years before the condition was described by neurologist Paul Broca. This function was not yet attributed to the brain however.

No description of nerves were given in the papyrus though it was recognised that the brain was likely involved somehow with movement. Nerves were not discovered until 1200 years later when the Greek physician Alcmaeon of Crotona, a student of philosopher and mathematician Pythagoras, first dissected human tissue. He described veins and arteries and noted what he described as “passages” from the eyes to the brain, in effect providing the first description of the optic nerves.

Ascribing particular functions to structures began later with Hippocrates who in the 4th century BC described epilepsy as a condition originating from the brain. Though he described the condition as due to cold phlegm from the brain flowing into the warm blood, it was the first step in associating observable functions of the body with the brain. Though Plato in the early 3rd century BC described the brain as the seat of mental processes, 50 years later Aristotle was still teaching that the heart was the centre for cognition.

At about this time argument had started as to where the human soul was located, a concept that was closely tied with human intelligence. Despite the teachings of Aristotle it had become well established that the brain was responsible for intelligence and therefore the location of the human soul, however where in the brain these faculties were located was much debated. For the next two thousand years the debate raged centring largely around the ventricles. Galen, for instance suggested that the fourth ventricle was the seat of the soul, St Augustine on the other hand believed that soul resided in the middle (third) ventricle. Certainly the Arabian philosophers also held that the ventricles were the seat of the soul and consciousness.

It was believed that nerves from the senses brought stimuli into the ventricles where it was processed. It was recognised for example that the senses of hearing and sight could both be received by an individual, and attended to, at the same time. Similarly touch, taste and pain could all somehow be brought into our consciousness at once. It was thought that

there must therefore be some common sensory centre to which they were all sent. It was from this hypothesis of a common sensory centre that the term common sense originated, meaning someone who is sensible and considers all his options. The common sensory centre was thought to be the centre in the brain where all the senses were integrated into consciousness.

This idea of the integration of neuronal function to a single whole is an idea that has developed over the centuries and today forms the basis of our understanding of how the brain works. Thus it was the search for the human soul which lead to the first proposition of the integrative nature of neural processing.

The picture we get is that the brain is greater than the sum of its parts. Consciousness for instance does not exist in the thalamus, or in the cortex, or the pons, or the basal ganglia. Rather once all these nuclei are joined together in a vast network consciousness appears to be the product of the whole system.

In terms of the sensory system we know that sensory information is processed somehow by the CNS such that we perceive and react to our environment. As reviewed in Chapter 1 of this thesis, the first step in sensory processing is the division of different primary afferents into different fibre types. These then carry their different modalities to the CNS for separate processing. Far from feeding into three discrete centres however, they instead feed into a network of processing units which are in constant balance. The arrival of a signal then affects that balance and results in a pattern of activation across the network. One current theory is that it is this pattern, rather than a given stimulus its self activating a particular centre, that forms our perception of the stimulus.

There are 20,000,000,000 neurons in the adult human brain for instance and each one receives synapses from up to 5000 other neurons, forming a system of some 10^{14} (100 million million) synapses. By the middle of the 20th Century it had been noted that neurons formed an interconnected network and, importantly, synaptic connections between neurons appeared to be dynamic, that is to say their state of activity may be modified through use. In 1949, the psychologist, Donald Hebb proposed that neurons that were in close proximity with each other were able to assist each other in firing. The more often call A caused cell B to fire the greater the likelihood that cell B would fire the next time that cell A fired. He

suggested that some metabolic changes must occur with in the cells that gives rise to this synaptic strengthening. In doing so networks between neurons are formed and it is this theory which underlies the one of the dominant concepts currently held of learning and memory, and more broadly how the CNS as a whole functions.

7.1.1 How this relates to the present thesis?

At the most superficial level, the present thesis examined changes in nitric oxide synthesis as a result of SCI. It was observed that NO was one of the most widely utilised signalling molecules in the body. In addition to this its anatomical localisation in regions of the spinal cord associated with sensory processing suggested that any alterations in this level of synthesis may inturn affect sensation. Alterations in sensation is a well reported consequence of spinal cord injury. Far from resulting in a simple *cessation* of sensation spinal cord injury more often than not results in altered sensation. SCI results in conditions ranging from reductions in sensation of touch, vibration, joint position, two point discrimination to dysaesthesias, hypersensitivities and neuropathic pain. Rather than simply blocking incoming nerve impulses then, spinal cord injury appears to result in functional changes to the sensory system such that sensory information is miss-processed and therefore misperceived.

Previous studies have indicated that a range of nervous system injures can result in changes to NO synthesis, just as they result in a range sensory deficits. Thus it was likely that the contusive spinal cord injury used here would find a change in NO synthesis. However rather than simply looking at injury-induced changes to the CNS it was one of the principal aims of the present thesis to relate any changes observed to the functional nature of NO. That is, how might alterations in NO synthesis affect neuronal function? An histological method for examining neural activity (fos protein imunohistochemsitry) was selected in order to better examine widespread changes throughout the tissue studied. It was felt that this would allow better observation of the relationship between these two molecules.

It was found that the number of NO synthesising cells was only altered in spinal segments adjacent to the injury. From this it was inferred that NO synthesis was therefore likely affected by the local injury-induced environment, which includes lipid damage, release of

inflammatory peptides, free radicals, and a loss of calcium regulation and neuronal energy depletion.

It was suggested that NO synthesising cells in the superficial laminae of the dorsal horn, particularly lamina II, are a population of inhibitory interneurons. A reduction in NO synthesis in these neurons, would interrupt the role of NO its self on sensation which is generally to dampen background activity of dorsal horn neurons. In addition to this however, the change also indicated that the biochemistry and therefore perhaps the function of the inhibitory neurons themselves may have also been altered.

It was supposed that this effect of SCI may give rise to changes in sensory processing resulting in the neuropathic pains commonly associated with SCI. When neural activity was examined however it was noted that activity (as determined by fos immunohistochemistry) was altered not only at spinal levels adjacent to the injury, but also many segments distant. The change observed was a decrease in neural activity, which was surprising if SCI had resulted in a reduction in inhibitory drive.

Similar wide ranging reductions in neural activity were noted in the midbrain and in the somatosensory cortex. Indicating that while changes in NO synthesis was restricted to the site of injury, SCI had resulted in changes in neural activity across the entire neuraxis. It is not known what benefit to the organism a reduction in neural activity following SCI would be. However this thesis provides evidence to support the suggestion that changes in sensation following SCI may be related to functional changes to the neuronal networks that constitute the sensory system as a whole. The theory proposed by Hebb suggests that the nervous system exists not as a static series of nuclei waiting to be stimulated by an incoming transmission, but rather that the whole network is in a constant state of balance.

Thus the inputs of the system are just as much part of the system as the components of the system. By altering the inputs to the sensory system the whole somatosensory network is imbalanced resulting in changes in the connectivity between neurons in the network. Evidence for this suggestion lies in studies into cortical reorganisation as a result of peripheral nerve deafferentation. In these, areas of the cortex begin to respond to inputs that they that would not normally respond to, demonstrating the formation of new active synapses. It is proposed here then that the disruption of sensory processing observed

following injury and the development of syndromes such as neuropathic pains are alterations in the balance of activity across the sensory network. These imbalances inturn allow sensory impulses to travel to inappropriate parts of the network through newly formed synapses, resulting in the miss-perception of the sensation.

7.2 The novel findings of this thesis

In the process of performing the experimental work for this thesis a number of important findings have been made. These have addressed, and have achieved the aims of this thesis. While the various effects of spinal cord injury have been well studied, the approach of this study was new and resulted in some valuable information. The principal novel findings will be outlined here.

While a number of studies have examined various injury-induced pathologies occurring within the spinal cord as a result of SCI, relatively few have examined these mechanisms over an extended length of the cord. Even fewer studies have examined the effect of SCI on neural mechanisms over the entire neuraxis. The present series of studies then is unique in that it examines the effects of traumatic SCI at a number of sites through out the CNS and relates them, in particular, to sensation. It is becoming increasingly apparent that alterations in sensation due to spinal injury may involve alterations in the activity of supraspinal structures (e.g. Abraham et al., 2000; Abraham et al., 2001; Gerke et al., 2003; Lenz, 1991; Lin et al., 1997; Lin et al., 1999a; Morrow et al., 2000; Vierck, 1991). Thus, in order to explain SCI-induced alterations in sensation, it may not be sufficient to examine neural mechanisms of altered sensation only in close proximity to the injury site.

In addition, the present study made a comparison between nitric oxide and alterations in neuronal activity. While the results suggest that neural activity was not affected either directly by the injury or by changes in nitric oxide, it was important to make this distinction.

One of the more important differences between this series of studies and others was a methodological one. The rats of the present study were handled daily and behaviourally tested through out the experimental period. This resulted in a level of fos expression that was considerably higher than that found in naïve rats. Thus it was discovered that lumbar SCI resulted in altered neuronal activity at all levels of the neuraxis, a finding which would not have been observable otherwise.

The somatic stimulation technique used in Chapter 4 allowed the active sensory system to be examined anatomically. This allowed examination of changes to laminar processing in the spinal cord which has not previously been reported.

7.3 Final remarks

This study has provided an extensive examination of the functioning rat nervous system both in the normal rat, but most importantly following lumbar SCI. It has related such alterations in neural function that may occur following injury to alterations in sensation that have been reported here and elsewhere. A number of unique methodologies were utilised. Due to the novel approach and its wide ranging examination of the CNS, this thesis has drawn together considerable information regarding the neural effects of spinal cord injury.

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Appendix

Future Directions

As discussed in the preceding chapters, the work reported here represents a large body of data examining a number of important pathological mechanisms set into motion by traumatic SCI. These were largely concerned with sensory and motor dysfunction. However a number of interesting observations were made during the course of this thesis that were not immediately relevant to the principal discussion. These may indicate underlying pathology, and represent good starting points for future work.

8.1 Fos Clusters

In addition to the injury induced changes in NADPH-d activity and fos expression in the spinal cord, reported in Chapter 2, it was noted that occasionally clusters of small (<10μm) fos positive nuclei would be apparent within the tissue. These were only ever found in spinally injured animals but were not observed in all animals (only 10-20%). The size of the nuclei suggested these cells were non-neuronal in origin. The clusters of fos cells appeared to be induced most often in quadrants around lamina X (see Figure 1).

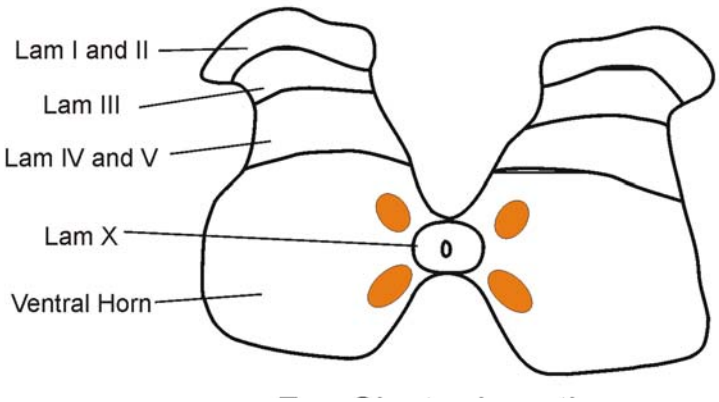

Figure 1 Usual locations of fos clusters, indicated by the orange ovals.

However they were observed in most other laminae of the grey matter as well as the white matter of the spinal cord (see also Figures 2 and 3).

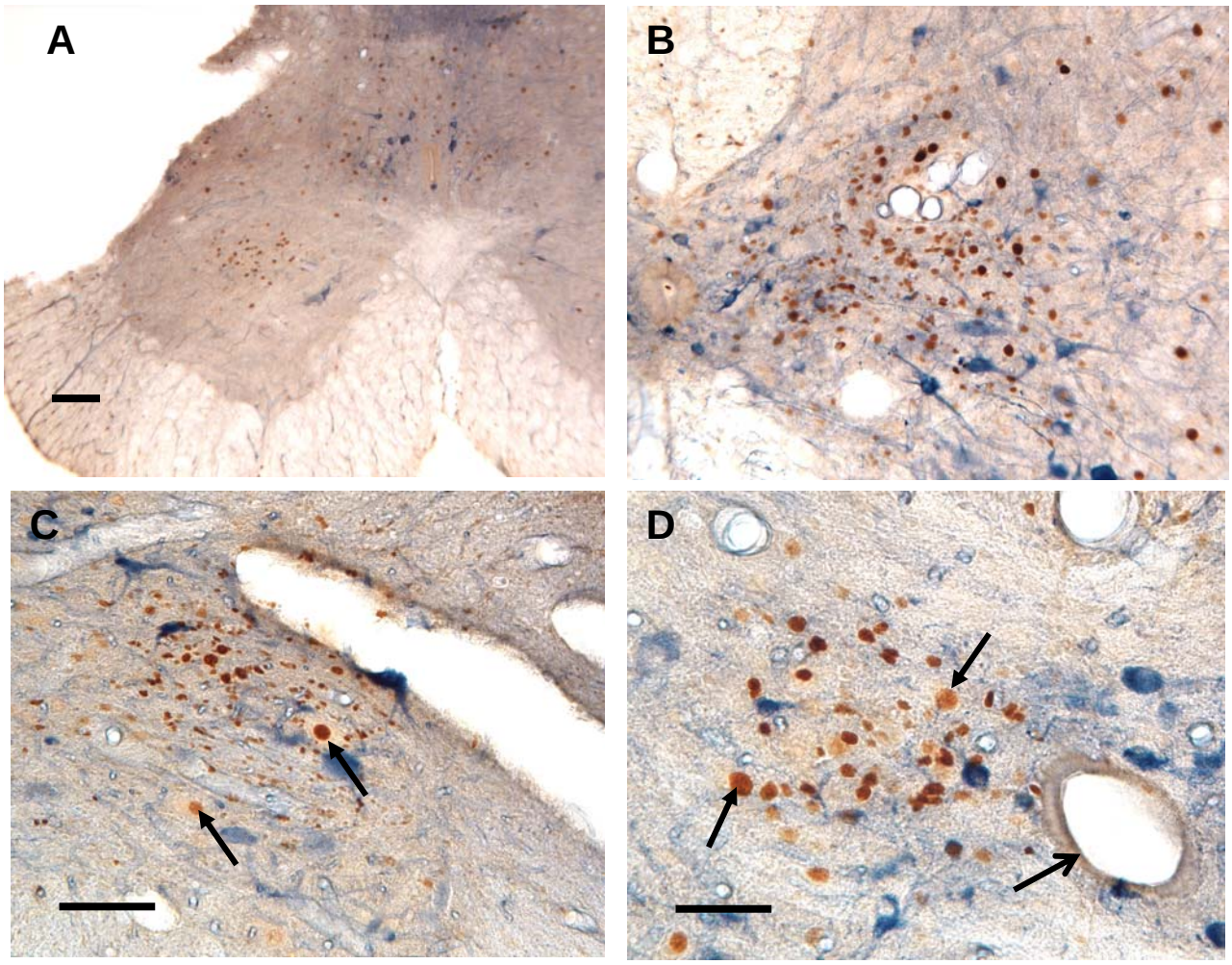

Figure 2 Photomicrographs of clusters of fos cells with in the grey matter of the spinal cord of the spinally injured rat. Panel A was taken from the sacral spinal cord at 4x objective B was from the lumbar spinal cord just rostral to the injury at 10x objective. Blue NADPH-d positive cells are visible in the ventral horn. Intense fos labelling can be observed in cells that appear to be both neuronal and non-neuronal based on the size of the nuclei. The circle in panel A indicate a cluster of fos positive cells. Panel C was taken at 10x objective while panel D was taken at 20x objective. Arrows indicate likely neuronal profiles. Nuclei are large and rounded in shape. D, open arrow indicates central canal (Scale bar A 225μm; B, C 220μm; D 110μm).

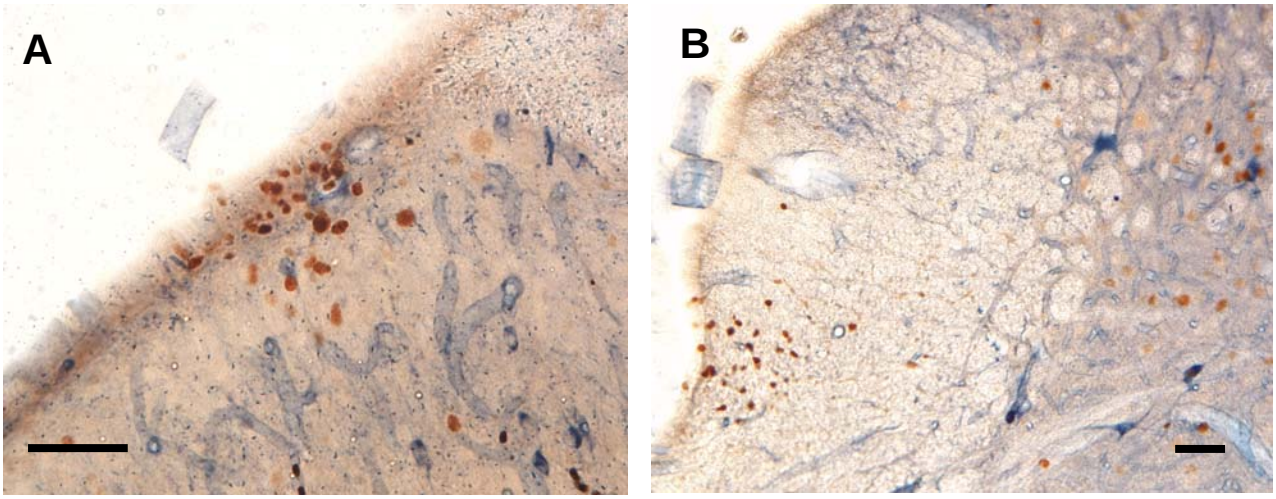

Figure 3 Photomicrographs of fos clusters in other locations within the spinal cord. A, image taken at 20x objective, cluster in lamina I of the grey matter. B, image taken at 10x cluster in lateral white matter. Scale bars represent 110μm.

It was also observed that, when close enough to the central canal, the activation of these small polymorphic profiles may also include the ependymal cells surrounding the canal (Figure 4), further reinforcing the conclusion that these cells are largely of a non-neuronal origin.

It is not known what causes the clusters of small nuclear profiles to express fos. It can occasionally be observed, however, that these small cells appear to line up along the somata and processes of NADPH-d positive cells (Figure 5). However, the fos clusters are more often observed without any obvious association with NADPH-d positive cells. Also the small fos positive profiles can occasionally be observed to be closely approximated to a non-NADPH-d positive cell (Figure 6), or non-NADPH-d labelled fos positive motoneurons (Figure 7).

Lastly, amongst the larger, rounded fos positive profiles that are likely to represent neuronal nuclei, occasionally the section passed though a nucleus undergoing nuclear cleavage (Figure 8). This process is thought to be a characteristic of cells undergoing apoptosis.

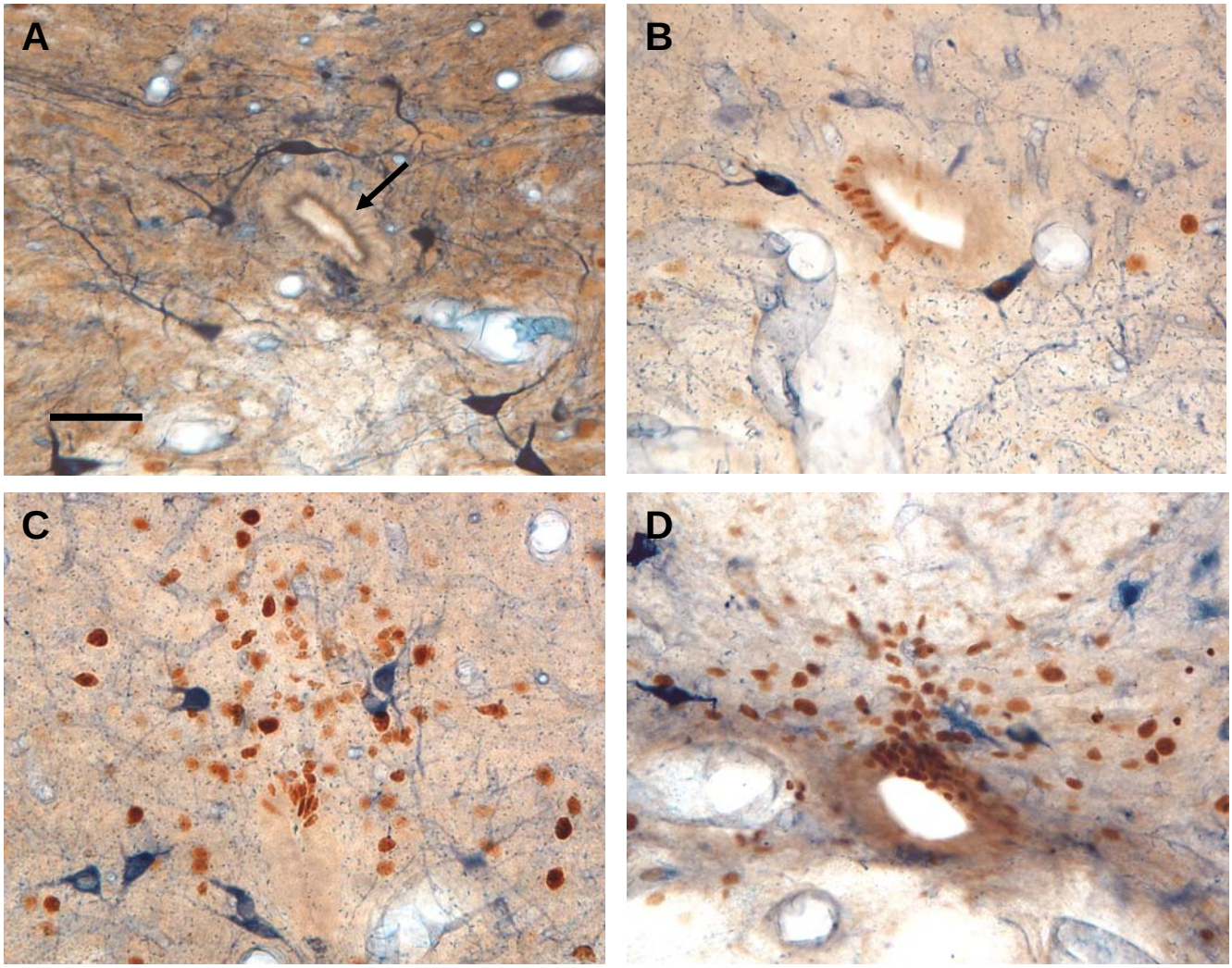

Figure 4 Photomicrographs of lamina X including the central canal taken at 20x objective. A, shows typical central canal as it appears in the uninjured animal (arrow). Clustering of small fos positive cells may include the ependymal cells surrounding the central canal, B, C and D. Scale bar 110μm.

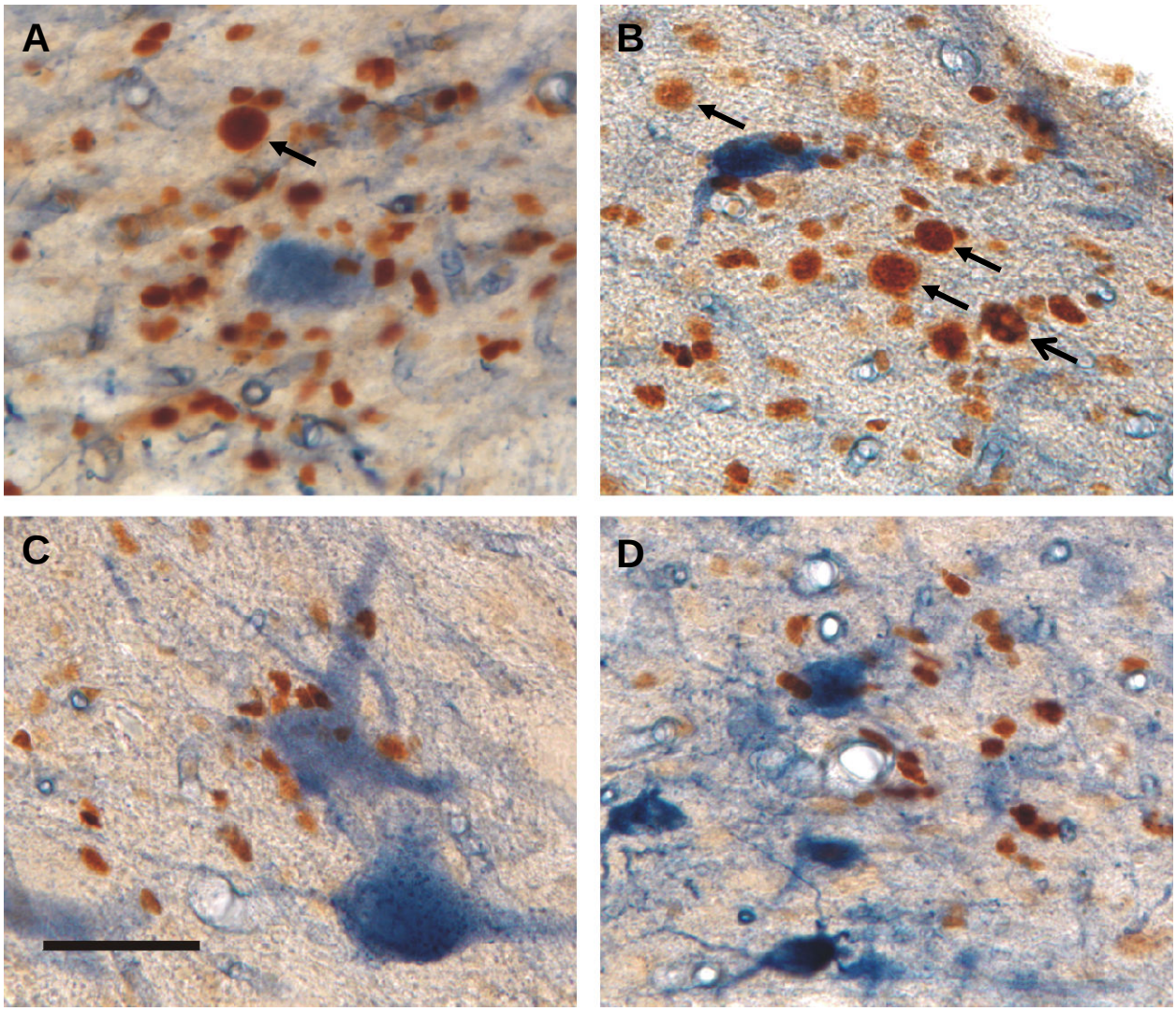

Figure 5 Photomicrographs of small fos positive cells that appear to be in close apposition to NADPH-d positive cells of the ventral horn. Photos taken at 40x objective. A & B, large, rounded fos-positive profiles likely to be of a neuronal origin can also be observed (arrows), outline of non-fos positive nucleolus can also be observed, indicating a nucleus of neuronal origin (B, open arrow). Scale bar 50μm.

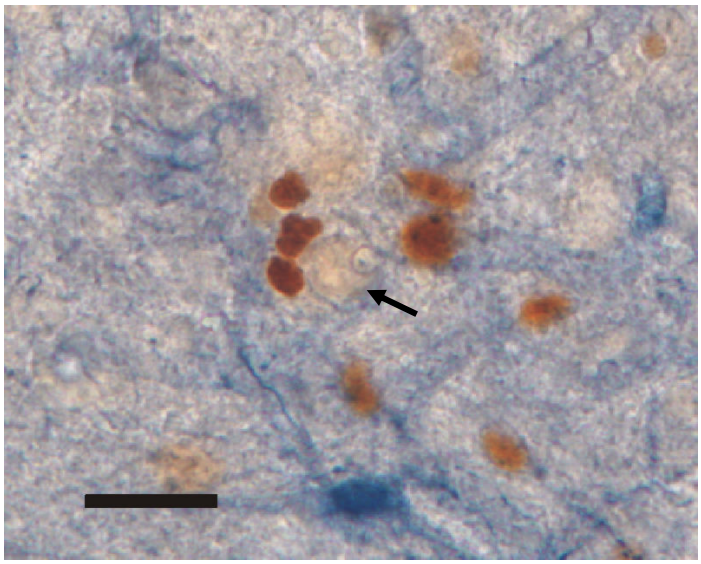

Figure 6 Micrograph of small fos positive cells surrounding a cell that appears to be devoid of both NADPH-d and fos labelling (arrow). Scale bar 25μm.

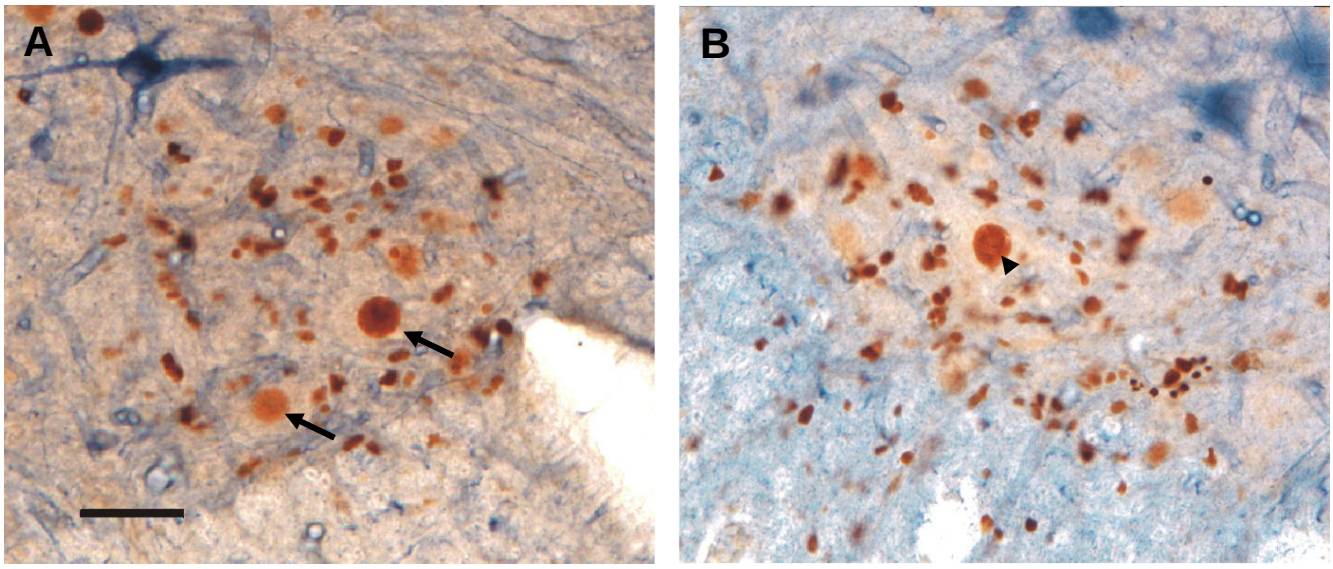

Figure 7 Micrographs taken at 40x objective of small fos positive profiles surrounding large fos positive nuclei (A, arrow). These nuclei were located within lamina IX of the spinal gray matter. B, presence of nucleolus (arrow head) indicates neuronal origin of cells, which are likely motor neurons. Scale bar 25μm.

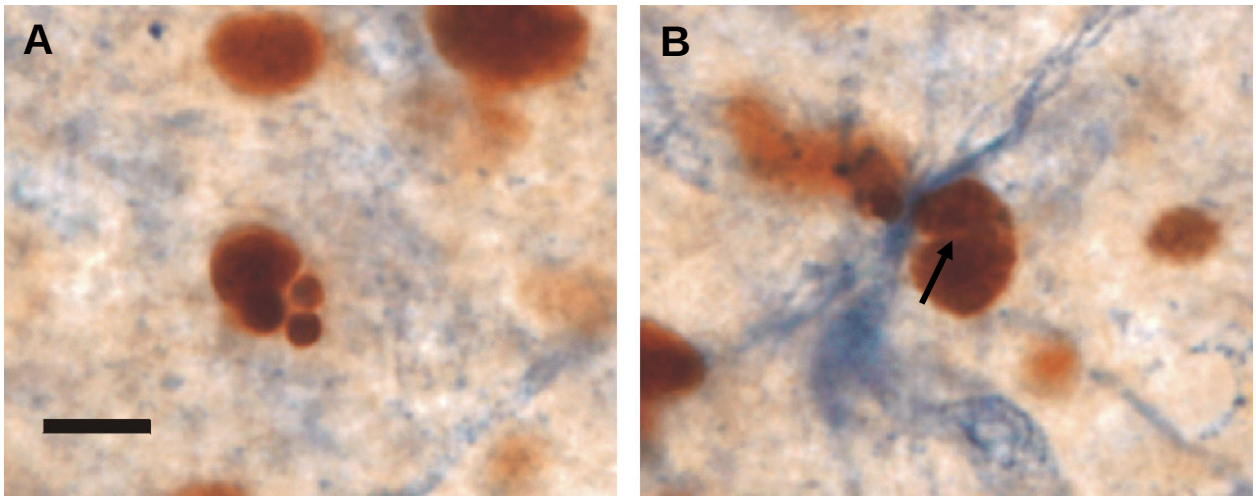

Figure 8 Photomicrograph of fos positive nuclei undergoing nuclear cleavage, a characteristic feature of apoptosis. Nucleus in panel B is caught as a cleavage furrow is forming (arrow). Scale bar 10μm.

From these observations then, a question that arises is what is the identity of these small clusters of fos positive nuclei? It has been observed previously that injury may induce the activation of glial cells {e.g. \Kreutzberg, 1996 #201;Coyle, 1998 #66;Cheng, 2003 #947;Du, 1999 #949}, and in particular it has been noted that microglia are activated and may be involved in the survival of motoneurons following de-afferentation {Kreutzberg, 1989 #200;Yuan, 2003 #946;Kobbert, 2000 #948;Streit, 1988 #951}.

So while it is likely that the small clusters of fos positive cells are glial cells, it would be an interesting extension of the present work to identify these cells immunohistochemically with glial cell markers. In addition the clusters of fos cells are at times observed to extend over 300μm through the grey matter, suggesting the activating agent may be highly diffusible. As nitric oxide is such a highly diffusible agent {Gally, 1990 #746;Lancaster, 1997 #742;Philippides, 2000 #724} and the clustering of fos cells in the present series of studies were occasionally observed to surround NADPH-d positive cells (Figure 5), one possibility is that the NO induced in the ventral horn as a result of traumatic injury may be involved in the activation of these cells. Since the work in this thesis indicated that NO may be involved in the pathological and behavioural consequences of SCI, it would be interesting to determine whether NO is capable of producing these observed clusters of fos cells. This could be done through the endogenous application of NO and the concurrent labelling for fos protein.

It has been reported previously that traumatic SCI may induce apoptosis in spinal neurons {e.g. \Springer, 1999 #942;Hayashi, 1998 #943;Wingrave, 2003 #677}. As some evidence of apoptosis has been observed here (Figure 8), it would be interesting to determine the extent to which the loss of neurons observed in the present work may be due to this process. This may reflect a more active process set into motion by the injury, as apposed to a reactive one where the physical disruption, or the environment produced by the injury, destroys the cells.

Lastly there are a number of ways in which the experiments reported in this thesis could be extended. The behavioural study reported in chapter 4 was incomplete due to insufficient allodynic animals. It would be good to complete this study as the alterations in laminar processing of sensation in allodynic animals may potentially be a very interesting finding.

More time could also be devoted to the cortical chapter, examining a number of additional supraspinal structures to confirm and extend the observations made in this thesis. These may include additional regions of the parietal cortex, such as the hind limb and forelimb areas as well as insula and cingulate cortex. It would be interesting to also examine additional nuclei at other levels of the neuraxis which are also involved in sensory processing. These may include the thalamus, locus coeruleus, the raphae nuclei and maybe the ventrolateral medulla. These experiments for now must remain in the future.