A STUDY OF THE ROLE OF SPINAL PROSTAGLANDINS AND NITRIC OXIDE IN THE SPINAL NERVE LIGATION MODEL OF NEUROPATHIC PAIN

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A Study of the Role of Spinal Prostaglandins and Nitric Oxide in the Spinal Nerve Ligation Model of Neuropathic Pain

by

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A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Division of Basic Medical Sciences

Faculty of Medicine

Memorial University of Newfoundland

St. John's, Newfoundland

2004

ABSTRACT

Pain arising from nerve injury is often resistant to conventional analgesics and is believed to have mechanisms distinct from those of normal nociception. To determine if spinal prostaglandins (PG) contribute to tactile allodynia, male, Sprague-Dawley rats were fitted with either i.t. microdialysis or drug delivery catheters 3 days before tight ligation of the left lumbar 5/6 spinal nerves. Ligated rats developed tactile allodynia within 24 h, as evidenced by a decrease in paw withdrawal thresholds (PWT) in the affected hind paw (<4 g vs >15 g control). Allodynia was also characterized by a significant increase in the evoked release of PGE_2. Thus, brushing the plantar surface of the affected hind paw with a cotton-tipped applicator, up to 5 days post-ligation, increased the CSF level of PGE_2 (measured using intrathecal microdialysis - $[PGE_2]_{dialysate}$) compared to the pre-stimulus control period. *In vitro*, spinal cord slices were used to determine the effect of PGE_2 on glutamate release. The EC_{50} of PGE_2-evoked glutamate release ($2.4x10^{-11}$ M; control) was significantly decreased in affected spinal segments of allodynic rats ($8.9x10^{-15}$ M). In a separate group of rats and beginning 2 days after ligation, the acute i.t. injection of S(+)-ibuprofen (non-selective cyclooxygenase inhibitor), SC-51322 (prostaglandin E receptor antagonist), SC-236 (cyclooxygenase-2 selective inhibitor), or SC-560 (cyclooxygenase-1 selective inhibitor) significantly reversed allodynia. The nitric oxide synthase inhibitor L-NAME had a similar effect to S(+)-ibuprofen, and was subsequently tested using isobolographic analysis. The co-administration of both inhibitors resulted in an additive anti-allodynic effect. To investigate spinal PG as possible early triggers of allodynia, we studied the effects of early post-injury treatment with isozyme selective and non-selective COX inhibitors. Treatment with i.t. S(+)-ibuprofen or SC-560, beginning 2

h after ligation, prevented the decrease in PWT, the brush-evoked increase in $[PGE_2]_{dialysate}$, and the change in EC_{50} of PGE_2-evoked glutamate release. In contrast, i.t. R(-)-ibuprofen or SC-236 had no effect. The results of this study suggest that: a) spinal PG synthesis is triggered by spinal nerve ligation; b) pharmacological disruption of PG synthesis or signaling can transiently reverse established allodynia; c) spinal PG, synthesized by cyclooxygenase (COX)-1 in the first 4-8h after ligation, is a critical trigger of allodynia; d) spinal nitric oxide appears to act in concert with PG to mediate allodynia. Together, it appears that allodynia resulting from L5/L6 spinal nerve ligation is comprised of an initial, time-limited, PG-dependent stage (characterized by brush-evoked increases in $[PGE_2]_{dialysate}$ and sensitivity to COX-2 inhibitors) and long-term, PG-independent allodynia. The ability of appropriate COX inhibitors to prevent both stages suggests that the two are related.

ACKNOWLEDGEMENTS

I would like to thank Dr. Loomis for his help during my project and the writing of our publications and this thesis. My thanks are also accorded to the other members of my supervisory committee, Drs. Bieger and Harley.

My deepest thanks to the staff and faculty of the School of Pharmacy, particularly Denise and Heather and Dr. Linda Hensman.

I thank Janet Robinson for her unending help, friendship and support during my entire duration of graduate school and beyond. I would like to similarly thank Darren O'Rielly for his help, support, and friendship in and out of the lab.

I extend my appreciation for the help and friendship from the other undergraduate and graduate students in the Loomis lab, as well as the School of Pharmacy and Faculty of Medicine.

I would like to recognize the Rick Hansen Neurotrauma Institute for the financial support I received. As well as the financial support for my project from the Canadian Institutes of Health Research through operating grants to Dr. Loomis.

A special thanks is extended to my family, particularly my mother, for their overwhelming support, care, and concern during my graduate studies.

I offer my greatest appreciation to my friends for their support and distraction during my time as a graduate student and beyond.

I thank Drs. Yaksh and Marsala for their help with the spinal nerve ligation model and spinal microdialysis.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	analysis of variance
C fibre	a class of primary afferent neurons
CFA	Complete Freund's Adjuvant
CGRP	calcitonin gene-related peptide
CI	confidence interval
CNS	central nervous system
COX	cyclooxygenase
CSF	cerebrospinal fluid
DMSO	dimethyl sulfoxide, an organic solvent
ED_{50}	effective dose for 50 percent response
EP1	prostaglandin E receptor, subtype 1
GABA	-aminobutyric acid; -aminobutyrate
Gs	stimulatory G-protein
Gi	inhibitory G-protein
HD	hair deflection
iNOS	inducible nitric oxide synthase
i.t.	intrathecal
L5	lumbar vertebra number 5
L6	lumbar vertebra number 6
LI	-like immunoreactivity
L-NAME	L-N^G-Nitroarginine methyl ester

L-NOARG	*N*ω-nitro-l-arginine
NMDA	N-methyl-D-Aspartic acid
NS-398	N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide
NSAIDs	non-steroidal anti-inflammatory drugs
PE-10	size 10 polyethylene tubing (diameter~0.61mm)
PG	prostaglandin
SEM	standard error of the mean

CO-AUTHORSHIP STATEMENT:

For all experimental chapters, the author identified and designed the research proposal in consultation with Dr. C.W. Loomis. The author carried out all data analysis, and with assistance from Dr. C.W. Loomis, prepared the manuscript for publication. With the following exceptions, all experiments were performed by the author. Ms. Pamela Carter and Ms. Melissa Haley performed approximately 10% of the behavioural testing in the first experimental chapter. Regarding the second experimental chapter, Darren D. O'Rielly was provided with the appropriate tissue and protocol, and he performed the amino acid assay and provided the author with analyzed data.

1.0 INTRODUCTION

1.1 NEUROPATHIC PAIN

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as pain initiated or caused by a primary lesion or dysfunction in the nervous system (Merskey and Bogduk, 1994). It is an abnormal sensory state arising from trauma or disease affecting peripheral nerves, posterior spinal roots, the spinal cord itself, or certain regions of the brain. Examples include phantom limb pain, central post-stroke pain, diabetic, alcoholic, nutritional, traumatic or cancerous neuropathy, anterior spinal artery syndrome, post-herpetic neuralgia, reflex sympathetic dystrophy, plexus avulsion, post-cordotomy dysesthesia, and painful conditions associated with paraplegia and multiple sclerosis (Shibasaki and Kuroiwa, 1974; Boivie et al., 1989; Tasker, 1990; Portenoy and Hagen, 1990; Tanelian and Brose, 1991; Price et al., 1992; Triggs and Beric, 1992; Baron and Saguer, 1993; Portenoy, 2000). Neuropathic pain has also been described in several inherited diseases such as Charcot-Marie-Tooth disease (Charcot and Marie, 1886; Tooth, 1886), and other hereditary sensory/autonomic/motor neuropathies (see review, Kuhlenbaumer et al., 2002).

The absence of appropriate epidemiological studies makes it difficult to gauge the absolute prevalence of neuropathic pain. However, it has been estimated that up to 1.5% of the general population are affected (Chong and Bajwa, 2003). Prevalence also appears to vary with the causative event [e.g. post-herpetic neuralgia affects about 10% of all patients who contract the disease (Watson et al., 1995)] and the age at which nerve injury occurs (Beydoun et al., 1999). While a relatively rare and idiosyncratic outcome of nerve injury (Noordenbos and Wall, 1981; Arner and Meyerson, 1988; Tasker et al., 1990),

neuropathic pain can be extremely debilitating, is often intractable, and represents a major burden on the health and social systems (Arner and Meyerson, 1988; Rowbotham et al., 1991; Baron and Saguer, 1993; Schmader, 1998).

1.2 NEUROPATHIC PAIN DIFFERS FROM NOCICEPTIVE PAIN

Neuropathic pain differs from nociceptive pain in several important ways. Neuropathic pain is usually chronic in nature, sometimes persisting for years or even decades after the initial injury has healed. Its onset is delayed for weeks to months after the causative event (Tasker, 1990). For example, 82% of patients with spinal cord lesions experienced a delay in pain onset ranging from less than a month to more than one year after injury (Tasker et al., 1992). The sensations described by patients with neuropathic pain are also unique compared to non-neuropathic pain. The pain is generally described as a burning, ripping, and/or tearing sensation, yet patients are often unable to identify or locate the inciting stimulus. Radiation of the sensation, abnormal temporal summation, and after-sensations are also frequent sequelae of this syndrome (Lindblom and Verrillo, 1979 Noordenbos and Wall, 1981; Price et al., 1992; Kapur, 2003).

Another distinguishing feature of neuropathic pain and one of the most significant clinical problems is its poor response to current therapy. There remains a diversity of opinion on the best therapeutic strategies for treating neuropathic pain (Attal, 2001). They include but are not limited to opioid analgesics, tricyclic antidepressants, anticonvulsants, barbiturates, local anesthetics and various channel blockers. Therapeutic response is highly variable from patient to patient, and rarely do these drug classes effect

complete pain control, and likewise not all drugs from the same class necessarily show efficacy. Surgical interventions, intended to alleviate neuropathic pain, usually provide only incomplete and temporary relief, with the pain eventually returning (Tasker et al., 1992; Eide, 1998). Invasive electrical stimulation of specific spinal, thalamic, or sensorimotor cortical regions is another alternative, but this is also variable in outcome (Devulder et al., 2002). In short, the majority of patients with neuropathic pain are inadequately controlled, making this a serious clinical problem and a major therapeutic challenge (Eide, 1998).

Surveys of patients with neuropathic pain reveal their most common and troublesome symptom to be allodynia (Campbell et al., 1988; Raja et al., 1988). Allodynia is defined as "pain arising from a stimulus that does not normally evoke pain" (Merskey, 1986). Thus, normally innocuous stimuli such as a cold draft of air or the light touch of clothing may acquire the ability to evoke excruciating pain after nerve injury. Mechanical (tactile) allodynia is the most common type, occurring in 54% of patients with central neuropathic pain and 48% of patients with peripheral neuropathic pain (Nurmikko and Hietaharju, 1992). It represents the major form of allodynia in clinical neuropathic pain (Woolf and Doubell, 1994; Ma and Woolf, 1996).

1.3 ALLODYNIA IS MEDIATED BY Aβ-FIBERS

Clinical studies by Campbell et al. (1988) and Price et al. (1989) showed that in patients with neuropathic pain, both allodynia in the affected dermatome and the sensation of light touch in adjacent normal skin were mediated by the same neural

elements (i.e. Aβ-fibers). In contrast, temperature discrimination in the same region was unaffected, suggesting little if any involvement of Aδ- and C-fibers (Campbell et al., 1988). Similarly, conduction velocity for the detection of pain in nerve-injured limbs was similar to that for touch in normal limbs (Lindblom and Verrillo, 1979; Campbell et al., 1988; Gracely et al., 1992). Using the spinal nerve ligation model of neuropathic pain, Khan et al. (2002) recently showed that rats depleted of C-fibers with resiniferatoxin developed tactile allodynia comparable to that in control neuropathic animals. Moreover, whereas high-frequency, low-intensity electrical nerve stimulation normally attenuates clinical nociceptive pain, allodynia is triggered by the same treatment (Price et al., 1992). Neither did transcutaneous electrical nerve stimulation to the affected dermatome of nerve-injured rats, using the chronic constriction injury model, mitigate mechanical allodynia (Somers and Clemente, 1998). Since Aβ-fibers normally do not evoke pain, their ability to do so after peripheral or central nerve injury implies a change in somatosensory processing at the spinal and/or supraspinal level.

1.4 NERVE INJURY INDUCES CHANGES TO THE NERVOUS SYSTEM

Nerve injury triggers changes in both the peripheral and central nervous system. In the periphery, neuromas at the site of nerve transection exhibit increased sensitivity to mechanical and thermal stimulation that leads to increased Aβ-fiber discharges (Babbedge, 1996). In the ischemic sciatic nerve injury model, Aβ- and Aδ-fibers are most likely to display spontaneous, ongoing activity (Bulka et al., 2002). Various ion channels, particularly Na^+ channels, accumulate in the proximal stump of transected

nerves. Novel Na^+ channel subtypes have also been identified in these areas, at least one of which displays faster recovery than its counterparts (England et al., 1996). This characteristic would facilitate the repetitive firing of injured neurons and represents a possible explanation for the ectopic firing noted in many sites of nerve injury (Zimmermann, 2001). Chaplan et al. (2003) recently identified a hyperpolarization-activated, cation-nonselective, cyclic nucleotide-modulated channel at the site of injury as responsible for ongoing activity in Aβ- and Aδ-fibers.

Infiltration of inflammatory cells (e.g. macrophages) into myelin lamellae is a characteristic of inflammatory neuropathies (Nukada et al., 2000). The development of post-ganglionic sympathetic sprouts around type A dorsal root ganglion cells is a feature of certain neuropathic pain conditions that are exacerbated by sympathetic nervous system activity (McLachlan et al., 1993). Each of these mechanisms has the ability to generate sustained excitatory input to the spinal cord which, in turn, induces central hyperexcitability; a characteristic feature of hyperalgesic and allodynic conditions (Coderre, 1993; see review, Millan, 1999).

Nerve injury is also known to elicit pronounced changes in the spinal cord. Sprouting of large primary afferent (Aβ) terminals into lamina I and II of the spinal cord has been reported after peripheral nerve transection (Woolf et. al., 1992). Significant losses of opioid binding sites (18-53%) in the superficial dorsal horn were noted in three models of peripheral nerve injury (Besse et al., 1992). The up-regulation of several immediate early gene products such as c-fos and c-jun has been shown after nerve injury; many of these products are associated with the increased responsiveness of second-order

neurons (Herdegen et al., 1992; Molander et al., 1992). Following nerve ligation, large diameter sensory neurons begin to express neurotransmitters normally associated with nociceptive transmission (e.g. substance P and brain-derived neurotrophic factor; Noguchi et al,. 1994, 1995; Michael et al., 1999). Spinal afferent terminals have also been shown to express novel neurotransmitters such as neuropeptide Y, galanin, and vasoactive intestinal peptide after transection, loose ligation, or crushing of the sciatic nerve (Wakisaka et al., 1992).

In an extensive study of spinal inhibitory mechanisms, Moore et al. (2002) reported that $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) were decreased in two partial nerve injury models compared to naive animals. Analysis of unitary IPSCs suggested that presynaptic GABA release was reduced following chronic constriction- or spared nerve-injury. Moreover, there is recent evidence that glycinergic/GABAergic transmission can change from an inhibitory to an excitatory nature after nerve injury (Coull et al., 2003). Partial nerve injury induced neuronal apoptosis and decreased dorsal horn levels of glutamate decarboxylase (GAD65) ipsilateral to the injury (Moore et al., 2002). This builds upon earlier reports demonstrating the appearance of dark staining dorsal horn neurons (presumed to be deteriorating interneurons) after chronic constriction injury (Sugimoto et al., 1989; 1990; Mayer et al., 1999).

These presynaptic and postsynaptic changes suggest a major reorganization of spinal cord connections and/or nerve function after nerve injury, and are directly or indirectly correlated with the development of various types of hypersensitivity in the spinal cord (see review, Millan, 1999). Although the functional significance of these

changes with neuropathic pain remains to be determined, it is clear that the spinal cord is a major site of injury-induced adaptation and represents an obvious target for the treatment of neuropathic pain.

1.5 PROSTAGLANDIN SYNTHESIS

Prostaglandins belong to a family of lipid mediators derived from arachidonic acid, a 20-carbon fatty acid. They are not previously generated and stored in secretory granules but rather are synthesized de novo. The initial step in the biosynthesis of prostaglandins is the liberation of arachidonic acid from phospholipid membranes (Smith, 1992). This is commonly mediated by the phospholipase A_2 (PLA_2) family. Phospholipase C and D can have similar actions but their mechanisms differ from that of PLA_2 (Smith, 1992). Broadly, there are two subgroups within the PLA_2 family; a secretory ($sPLA_2$) and a cytosolic ($cPLA_2$) form. Expression of the former is induced by pro-inflammatory cytokines and inhibited by glucocorticoids. Secretory PLA_2 has been shown to have similar transcriptional regulation as other enzymes in the prostanoid synthetic pathway such as COX-2 (Murakami et al., 2000). Cytosolic PLA_2 appears to be involved in the generation of lipid mediators immediately following cell activation. Calcium-dependent translocation of $cPLA_2$ and subsequent binding to phospholipid membranes are required for enzymatic activity, often binding to the nuclear envelope or the endoplasmic reticulum – two common locations for the cyclooxygenases, lipooxygenases, and terminal PG synthases (Leslie, 1997; Murakami et al., 2000).

Once released from the lipid membranes, arachidonic acid is acted upon by cyclooxygenases (prostaglandin synthases) – this represents the first committed step in

prostanoid synthesis. Cyclooxygenase is a bifunctional enzyme that initially converts arachidonic acid to PGG_2. The same enzyme subsequently catalyzes this intermediate to PGH_2 via its peroxidase activity (see review, Smith et al., 2000; and section 1.6 for more detail). Specific synthases then utilize PGH_2 as a precursor to produce the most important biologically active products: PGD_2, PGE_2, PGF_2, PGI_2, and TxA_2 (Hara et al., 1994; Kuwamoto et al., 1997; Suzuki et al., 1997; Jakobsson et al., 1999). These are then free to diffuse out of the cell, or exit by means of a carrier-mediated process (Chan et al., 1998) and bind to prostanoid-specific receptors on the cell surfaces (Ushikubi et al., 1998; Sugimoto et al., 1998; Murata et al., 1997; also see review, Breyer et al., 2001) or to nuclear receptors (Lim et al., 1999; Kliewer et al., 1997; also see review Negishi et al., 1995).

While cyclooxygenase is inactivated during catalysis by suicidal inactivation, the biological relevance of this inactivation is unclear. The levels of COX normally exceed substrate concentration, and bursts of prostanoid synthesis do not result in a overall decline in COX activity. However, many agents that stimulate arachidonic acid release also initiate transcription of COX-2 (DeWitt, 1991). Transcription of the COX-2 gene is similar to that of highly regulated gene products, and contains promoter regions and transcription binding sites that can be activated immediately in response to external stimuli (Yamamoto et al., 1995). In contrast, the promoter region of the COX-1 gene has many characteristics of a housekeeper gene. Consistent with this observation is the fact that COX-1 mRNA and protein are expressed in most tissues under basal conditions and do not vary greatly (Wang et al., 1993). It is presumably through these mechanisms that

cells are able to regulate COX levels, control prostaglandin synthesis, and prevent the exhaustion of prostanoid generating capacity in the face of chronic stimulation.

1.6 CYCLOOXYGENASES AND SPECIFIC PROSTAGLANDIN SYNTHASES

Prostaglandin endoperoxide H synthase (commonly known as cyclooxygenase) was purified in 1976 and cloned in 1988. This enzyme is the key catalytic protein in the synthesis of prostaglandins from arachidonic acid, and is subject to inhibition by non-steroidal anti-inflammatory drugs (NSAIDS). In 1991, several laboratories identified a second gene product with COX activity, now termed COX-2. It is clear now that both isoforms, COX-1 and COX-2 are expressed in both peripheral tissues and several areas of the CNS (see reviews: Vanegas and Schaible, 2001; Kaufmann et al., 1997; Vane et al., 1998).

Both isoforms are membrane-associated enzymes having a molecular weight of 71 kD. Each exists as a homodimer, composed of three independent folding units: the active enzymatic domain within a long hydrophobic channel, a membrane-binding domain, and an epidermal growth factor-like domain (see review: Smith et al., 2000). They exhibit 60-65% amino acid sequence homology within the same species. Within each isoform, there is 85-90% homology across species. The most striking differences in amino acid sequence occur within the membrane-binding domains (Otto and Smith, 1996; Spencer et al., 1999). Each enzyme has two catalytic activities. The cyclooxygenase reaction occurs within a hydrophobic channel where arachidonic acid plus two molecules of oxygen are converted to PGG_2. Peroxidase activity, occurring at a separate site, leads to the reduction of PGG_2 to PGH_2.

Initially, COX-1 and COX-2 were thought to subserve physiological and pathophysiological functions, respectively. However, it is now recognized that both isoforms can fulfill either role, depending on the cell type and the prevailing conditions. For example, while COX-2 seems to be clearly involved in inflammatory responses and nociception, this isoform, along with COX-1, is also important in the homeostatic regulation of kidney function. A related over-simplification is their classification as either 'constitutive' or 'inducible'. Although COX-1 is normally present at a constant level in the cell types examined to date, and COX-2, which is often absent from several cell types, can rapidly be upregulated, there are exceptions. A notable example is the recent study by Zhu et al. (2003) who reported an increase in COX-1 immunoreactivity in glia of the dorsal horn and gracile nucleus in a post-operative pain model.

Conversion of the unstable endoperoxide intermediate PGH_2 to biologically-active prostaglandins is performed by cell-specific terminal prostaglandin isomerases and synthases. Individual enzymes exist for the conversion of PGH_2 to each class of prostanoid (e.g. PGD synthase converts PGH_2 to PGD_2).

A newly discovered variant of the cyclooxygenase family (so-called COX-3) is currently the subject of investigation. It was first reported by Chandraskharan et al. (2002) who described the presence of COX-3 mRNA in canine and human tissues, especially in the brain. This novel isoform may be a splice variant of COX-1 as it shares similar molecular properties with COX-1. Interestingly this study, and subsequent work by Ayoub et al. (2004), suggests that COX-3 (or some variant of COX-1) may be a target for acetaminophen. This may explain the disparity of actions observed with this drug. Since the first reports of COX-3 mRNA in canine and human tissue, COX-3 mRNA has

also been identified in rat and mouse CNS (Shaftel et al., 2003; Kis et al., 2004). Whether COX-3 mRNA actually encodes a protein capable of converting arachidonic acid to prostaglandins, and is thus a target for some NSAID drugs, remains to be elucidated (Dinchuk et al., 2003).

1.7 IMPORTANT COX PRODUCTS AND THEIR TARGETS

Prostaglandins are a family of autacoids comprising five primary biologically-active compounds: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, TXA_2 (see review, Smith, 1992). The first four are important in nociception. Because they are either chemically unstable or undergo rapid metabolism *in vivo*, prostanoids are thought to act locally, close to their site of production. Their biological actions are thought to be mediated by specific receptors corresponding to their prostanoid classification: prostaglandin D receptor (DP), prostaglandin E receptor (EP), prostaglandin F receptor (FP), prostaglandin I receptor (IP), and thromboxane A receptor (TP). The EP class is further subdivided into EP_1, EP_2, EP_3 and EP_4 subtypes (Coleman et al., 1990, 1994). Several splice variants have also been identified for the EP_1, EP_3, FP and TP classes, although the variation appears to be within the intracellular C-terminal region which does not seem to affect ligand-binding properties. Rather, this variation may affect G-protein coupling specificity, constitutive activity, and agonist-induced receptor phosphorylation, desensitization, and/or internalization. While each prostaglandin binds with highest affinity to its cognate receptor, considerable cross-reactivity has been observed between a particular prostaglandin and other prostaglandin receptors (see reviews: Breyer et al., 2001; Wise et al., 2002).

Prostaglandin receptors are coupled to G-proteins and thus have the usual seven-transmembrane-region architecture. They belong to family A G-protein coupled receptors and have been categorized into group V of this family according to phylogenetic examination. Group V also includes receptors for pituitary hormones and opioids. Prostaglandin receptors have the closest relation to the vasopressin receptor family. Within the prostaglandin receptor family, only about a 20-30% amino acid sequence homology exists, mostly found within the transmembrane regions.

1.7.1 PGD_2 AND DP RECEPTORS

A role for PGD_2 has been demonstrated in the sleep-wake cycle (Urade and Hayaishi, 1999), body temperature regulation (Sri Kantha et al., 1994), and mast cell activity (Lewis et al., 1982). PGD_2 also effects vasodilation, vasoconstriction, and the inhibition of platelet aggregation (Giles et al., 1989). Among the prostaglandin receptors, the DP receptor is the most recent to be cloned, and consequently the least characterized. Quantitatively, it also seems to be the least expressed. DP receptor mRNA is generally expressed in low levels, except in tissues like the retina and mucus-secreting cells of the GI tract (Hirata et al., 1994). DP receptor mRNA is also found in sensory and motor neurons in the rat spinal cord, suggesting some modulatory role of these functions (Wright et al, 1999). In the brain, DP receptor mRNA and protein have been localized in areas thought to be important in sleep. Thus, the infusion of PGD_2 into these areas, particularly the subarachnoid space below the basal forebrain, has been shown to induce sleep in experimental animals (Scammell et al., 1998).

The DP receptor class is coupled to Gs protein and is positively-coupled to adenylyl cyclase. The Ki value for PGD_2 at the DP receptor has been reported to be 21 nM using transfected CHO cells (Kiriyama et al. 1997).

1.7.2 PGE_2 AND EP RECEPTORS

Prostaglandin E_2 is the major product of COX-mediated arachidonic acid metabolism. Among all the prostanoids, PGE_2 exhibits a wide spectrum of physiological and pathophysiological roles. Many of these are apparently opposing actions. One example is on smooth muscle, where PGE_2's vasodilatory effect in both arterial and venous beds is well known. However, PGE_2 can induce powerful contractile responses in smooth muscle of the trachea and sections of the GI tract (Lydford et al., 1996; Coleman et al., 1990). That some structural analogs of PGE_2 can replicate the vasoconstrictor effect, but not the vasodilator action in other tissues where this effect is known to occur (the converse of this is also true), provides important functional evidence of subclasses of the EP receptor. PGE_2 can also stimulate or inhibit neurotransmitter release, and sodium and water reabsorption in the kidney providing further evidence for EP receptor subclasses and their distinct coupling to signal transduction pathways (Negishi et al., 1995).

1.7.2.1 EP_1 RECEPTORS

The distribution of EP_1 receptors is more restricted than the other EP classes. EP_1 mRNA has been identified in mouse kidney, lung, and stomach, where PGE_2 has been implicated in the regulation of water reabsorption and smooth muscle contraction (Watabe et al., 1993; Sugimoto et al., 1994). Within the CNS, EP_1 mRNA is localized in neurons of the thalamus (Y. Sugimoto, unpublished observations; see review, Narumiya et al., 1999), and in DRG cells (Oida et al., 1995). Signal transduction of EP_1 receptors is unclear, most likely because of the existence of splice variants in this subclass and the suggestion that these variants differ in their signaling pathways (Okuda-Ashitaka et al., 1996). While activation of human EP_1 receptors leads to increased IP_3 and $[Ca^{2+}]_i$, mouse EP_1 receptors mediate increases in $[Ca^{2+}]_i$ with little increase in IP_3 but require extracellular Ca^{2+}. These results suggest the lack of involvement of a Gq G-protein (Watabe et al., 1993; also see review, Narumiya et al., 1999). The exact G-protein to which the EP_1 receptor is coupled remains unclear. What is clear is that PGE_2 can induce increases in $[Ca^{2+}]_i$ via the EP_1 receptor.

1.7.2.2 EP_2 RECEPTORS

Although EP_2 receptors are the least abundant of the EP subclass, they can be upregulated in the face of certain stimuli (e.g. LPS application to macrophages) (Katsuyama et al., 1998). They are absent from many tissues, including the uterus, but are upregulated there during pregnancy. Receptor density peaks during blastocyst implantation suggesting physiological importance in this biological process (Katsuyama et al., 1997; Hizaki et al., 1999). EP_2 receptors mediate relaxation of airway smooth

muscle, and selective agonists were considered as anti-asthmatics (Coleman et al., 1994; Pavord et al., 1991). In the spinal cord, EP_2 receptor mRNA is present in neurons and endothelial cells of the dorsal and ventral horns (Kawamura et al., 1997).

EP_2 receptors are positively coupled to adenylyl cyclase as evidenced by a 71-fold increase in intracellular cyclic AMP concentration ($[cAMP]_i$) following application of 1 μM PGE_2 to in EP_2-transfected human embryo kidney (HEK) cells as compared to untransfected cells (Fujino et al., 2003). This effect on cAMP is in agreement with the ability of PGE_2 to relax smooth muscle. While the EP_4 subclass exhibits agonist-induced desensitization, possibly due to phosphorylation of its C-terminal sequence, no such desensitization was noted with the EP_2 receptor (Nishigaki et al., 1996).

1.7.2.3 EP_3

EP_3 and EP_4 receptors are the most widely distributed subtypes within the EP class. Their mRNA has been identified in almost all mouse and human tissues examined, including kidney, uterus, adrenal gland, and stomach (Sugimoto et al., 1992; see also review Breyer et al., 2001). *In situ* hybridization studies in the kidney have shown EP_3 mRNA to be present in the outer medulla, thick ascending limb and cortical collecting ducts (Sugimoto et al., 1994a). These are probably responsible for mediating the regulatory effect of PGE_2 on ion transport. Similar distribution patterns were found in the human kidney (Breyer et al., 1996). The density of EP_3 receptors in the mouse uterus appear to be under considerable endocrine control, especially during early stages of pregnancy. This suggests their importance in fertilization and implantation (Katsuyama et al., 1997). In the mouse, EP_3 mRNA is most abundant in the brain (Sugimoto et al.,

1992) where it is found in neurons of the cortex, hippocampus, thalamus, hypothalamus, midbrain and lower brain stem (Sugimoto et al., 1994b). In the hypothalamus, EP_3 is expressed in the organum vasculosa lamina terminalis, a structure with a poor blood-brain barrier. As a result, it is more sensitive to factors in the peripheral circulation. Here, EP_3 receptors are believed to be responsible for the febrile response to PGE_2. Mice lacking this receptor exhibit a diminished febrile response (Ushikubi et al., 1998). Monoaminergic neurons arising from brainstem centers such as the locus ceruleus, substantia nigra and raphe nuclei also express EP_3 receptors. Their activation is thought to modulate the autoregulation of these cells. Momiyama et al. (1996) demonstrated the membrane-depolarizing effects of EP_3 receptor agonists on large serotonergic neurons in the dorsal raphe.

EP_3 mRNA has also been identified in DRG cells, where about half of all neurons (but not glia) were EP_3 positive. These were primarily small neurons suggesting a role for EP_3 receptors in PGE_2-mediated hyperalgesia (Oida et al., 1995; Sugimoto et al., 1994b). Many EP_3-positive DRG cells also express IP receptors (Oida et al., 1995). EP_3 receptor mRNA is also found in laminae I and II cells of the spinal dorsal horn, and on branches extending into deeper laminae (e.g. V; Beiche et al., 1998).

Because of splice variants in this receptor subclass (there are 4 major variants: EP_{3A-D}), the signal transduction pathways to which EP_3 receptors are coupled is not as clear as other PG receptors. The major pathway for EP_3 is the inhibition of adenylyl cyclase via a Gi-protein. Other variants utilize different signaling mechanisms (EP_{3A} inhibit adenylyl cyclase via Gi; EP_{3B} and EP_{3C} activate adenylyl cyclase via Gs). The EP_{3D} variant is unusually complex exhibiting coupling to Gq-, Gi- and Gs-proteins, and

can evoke a pertussis-toxin insensitive increase in phosphatidylinositol (Namba et al., 1993).

1.7.2.4 EP_4

In the mouse, EP_4 mRNA has been identified in most tissues studied, including the kidney glomerulus where it is thought to mediate the regulation of glomerular filtration by PGE_2 (Sugimoto et al., 1994). EP_4 mRNA is also found in neurons of the hypothalamus and lower brain stem (Y. Sugimoto, unpublished observations; see review, Narumiya et al., 1999), as well as in about 20% of DRG cells (Oida et al., 1995). Some of the latter cells also co-express IP receptors. EP_4 receptors, like the EP_2 and EP_3 subtypes, are expressed in the uterus, particularly the endometrium where their density can change significantly during the early stages of pregnancy (Katsuyama et al., 1997).

The EP_4 receptor is coupled to adenylyl cyclase via a Gs-protein. While the EP_2 and EP_4 receptors share the same signaling pathway, the latter appears to be less-efficiently coupled. The increase in cAMP following EP_4 receptor activation with PGE_2 was significantly less than at EP_2, despite the higher affinity of PGE_2 for the EP_4 receptor (Fujino et al., 2003). The basis of this difference remains unclear. The EP_4 receptor may also play an important role in gene regulation (Fujino et al., 2003) through a separate (cAMP-independent) transduction pathway. This appears to be a phosphatidylinositol 3-kinase dependent pathway involving extracellular signal-regulated kinases and early growth response factor-1 (Fujino et al., 2002).

1.7.3 $PGF_{2\alpha}$ AND FP RECEPTORS

$PGF_{2\alpha}$ was one of the first prostaglandins identified effecting potent constriction of vascular and non-vascular (e.g. bronchial) smooth muscle. $PGF_{2\alpha}$ plays a major role in female reproduction, being released from the endometrium, promoting luteolysis in several animal species, and causing regression of the corpus luteum. Its biological effects are mediated by the FP receptor whose tissue distribution has been less well studied than other prostaglandin receptors. To date, FP mRNA is most highly concentrated in the corpus luteum (Hasumoto et al., 1997). Its expression varies throughout the estrous cycle, suggesting a functional relationship between FP gene expression and luteolysis. This is in line with reports that FP receptor expression is critical for normal birth in mice (Sugimoto et al., 1997). FP mRNA is also present in the kidney, heart, lung, and stomach where its expression is stable throughout estrous (Kitanaka et al., 1994). Expression in the CNS has not been clear, but FP receptors have been detected in astrocytes (Negishi et al., 1995). FP receptors are coupled to a Gq -protein and linked to the activation of PLC (Ito et al., 1994). Interestingly, $PGF_{2\alpha}$ induced DNA synthesis in NIH 3T3 cells via the same FP receptor-effector pathway (Watanabe et al., 1994).

1.7.4 PGI_2 AND IP RECEPTORS

PGI_2, also known as prostacyclin, was originally derived from the vascular bed and identified as a vasodilator and inhibitor of platelet aggregation (Moncada et al., 1976). Endothelial cells are a major source of PGI_2 where it is responsible for vascular-related effects such as vasodilation and antithrombosis (Murata et al., 1997; Hoeper et al.,

2000). These biological effects directly oppose those of TXA_2 making PGI_2 an important factor in the maintenance of circulatory homeostasis.

Mouse IP receptor mRNA has been identified in several tissues, including arterial smooth muscle and the kidneys, particularly the glomerular afferent arterioles, where PGI_2 is known to serve important physiological functions (Oida et al., 1995). However, IP mRNA is found most abundantly in DRG cells where it is co-localized with the mRNA of a substance P precursor (Oida et al., 1995). In some DRG cells, IP receptor mRNA is co-expressed with mRNA of the EP receptor subtypes, suggesting possible overlap with the EP receptors, or perhaps a distinct role of IP receptors in pain transmission. IP mRNA was absent in glia of the DRG, and in neurons and glia of the spinal cord (Oida et al., 1995). PGI_2 binding sites have been identified in the superficial laminae of the spinal dorsal horn using autoradiography, consistent with a role for PGI_2 in pain signaling (Matsumura et al, 1995).

The IP receptor is known to activate adenylyl cyclase leading to increased cAMP production, as well as PLC via a Gq protein (Namba et al., 1994). The ability of the IP receptor to couple to different types of G-proteins based on cell type may explain the diverse biological actions of PGI_2.

1.8 CYCLOOXYGENASES AND PROSTAGLANDIN RECEPTORS ARE LOCALIZED IN THE SPINAL CORD

The concept that prostaglandins are synthesized in the CNS was strengthened by reports that COX is present in the brain and spinal cord. COX-1 and COX-2 mRNA and

protein are constitutively expressed in many regions of the CNS, including the dorsal and ventral grey matter of the spinal cord, as well as in the periphery in dorsal root ganglia (DRG). Immunocytochemical and autoradiographic studies confirmed the presence of COX-1 in spinal nociceptive pathways (Goppelt-Struebe et al., 1997; Willingale et al., 1997; Beiche et al., 1998a,b). COX-2-LI is present in neurons throughout the spinal cord, and is especially abundant in the superficial dorsal horn where the nociceptive primary afferent fibers are known to terminate. The presence of COX-1 in neurons is more controversial but COX-1 immunolabelling was recently described in a subpopulation of putative nociceptive DRG neurons (Chopra et al., 2000). COX-1 and COX-2 are also expressed in astrocytes (Vane et al., 1998).

Cyclooxygenase in the spinal cord is subject to induction. A 2-fold increase in COX-2 mRNA and a smaller increase in COX-2 protein was detected bilaterally in the lumbar spinal cord of the rat following acute carrageenan-induced, peripheral inflammation (Goppelt-Struebe et al., 1997; Ichitani et al., 1997; Hay and de Belleroche, 1997). Western blot analysis revealed a 1.6-fold increase in the level of COX-2 protein in the lumbar dorsal horn (lamina II-III) 22 days after the onset of adjuvant-induced arthritis in the rat (Goppelt-Struebe et al., 1997; Beiche et al., 1998a,b). Samad et al. (2001) reported a peak increase (16-fold) in COX-2 mRNA 6 h after CFA injection in the ipsilateral hind paw. Bilateral induction was noted 12 h after injection. Increases in COX-2 protein were also noted in the same animals. The increased expression of COX-2 is both neuronal and non-neuronal in nature (Samad et al., 2001; Beiche et al., 1998a,b; Maihöfner et al., 2000; Tegeder et al., 2001). Where investigated, these increases coincide with a significant elevation in spinal prostanoid concentration. These results

suggest that COX-2 is inducible and is likely responsible for the increase in spinal prostaglandin synthesis and release during peripheral inflammation.

The localization of COX in the dendrites of central excitatory neurons supports a role for prostaglandins in the modulation of synaptic signaling (Yamamoto, 1993). This hypothesis is further supported by the abundance of PGE_2 binding sites in the spinal dorsal horn (Matsumura, 1992; 1995). These appear to be located on the terminals of primary afferent fibers as the density of these binding sites almost completely disappeared after dorsal rhizotomy. The density on the operated side was only $4 \pm 4\%$ of the control side 8 days after surgery (Matsumura, 1995). This pattern of PGE_2 receptor-binding is consistent with the presynaptic facilitation of neuronal signaling.

In summary, there is substantial evidence that prostaglandins are synthesized and released in the spinal cord in response to nociceptive input. The temporal correlation of this central biochemical process with nociceptive behavior and peripheral inflammation indicates that spinal prostaglandins may be critical in the induction of a central pain state at the spinal level.

1.9 CELLULAR EFFECTS OF SPINAL PROSTAGLANDINS IMPORTANT IN NOCICEPTION

The exact mechanisms by which central prostaglandins enhance nociceptive signaling have yet to be elucidated. One strong possibility is that prostaglandins exert their effects through excitatory receptors on primary afferent terminals that form synaptic connections with second order neurons and/or excitatory interneurons in the dorsal horn (see review, Millan, 1999). Using cultured avian neurons, Nicol et al. (1992) showed that

1 μM PGE_2 yielded a 2-fold increase in substance P release; 5 μM PGE_2 resulted in a 4-fold increase similar to that evoked by 50 mM KCl. In the same study, 1 μM PGE_2 produced a 1.4-1.8 fold increase in Ca^{2+} current. Whole-cell patch clamp recordings of rat embryonic sensory neurons revealed a significant decrease in outward K^+ current following exposure to PGE_2 (Nicol et al., 1997). Gold et al. (1996) reported that PGE_2 increased tetrodotoxin-resistant Na^+ currents recorded from cultured DRG cells; an effect shared by other hyperalgesic agents. These ion channel effects would result in an overall increase in cell excitability and enhance the chance of cell firing.

PGE_2 has also been shown to evoke Ca^{2+}-dependent glutamate release from synaptosomes prepared from rat spinal cord (Nishihara, 1995), and substance P release from cultured rat DRG cells (Vasko, 1994) and rat spinal cord slices (Vasko et al., 1995). The effect of PGE_2 on substance P release was inhibited by the selective EP_1 receptor antagonist, SC-19220, and by guanosine-5'-[beta-thio] diphosphate, an inhibitor of stimulatory G-protein (Gs) to which the EP_1 receptor is coupled (Cui et al., 1995; White et al., 1996). Conversely, guanosine-5'-[gamma-thio] triphosphate, an activator of Gs protein, enhanced PGE_2-evoked release of substance P. Perfusion with low concentrations of PGE_2 has also been shown to facilitate capsaicin- or bradykinin-evoked release of substance P or CGRP from rat spinal cord slices (Hingtgen et al., 1995; Vasko et al., 1995). Collectively, these results indicate that PGE_2, acting through G-protein-coupled receptors, can directly effect neurotransmitter release from the spinal cord, as well as augment the release of neuropeptides from the spinal terminals of C-fibers evoked by known algogenic agents.

Prostaglandins may also enhance the excitability of dorsal horn neurons to afferent input through a direct postsynaptic effect. In an early study, microiontophoretic application of PGE_1 onto motor neurons and interneurons in the isolated spinal cord of the frog produced an abrupt excitatory effect (Coceani et al., 1975). More recently, PGE_2 was shown to induce a long-lasting facilitation of evoked excitatory postsynaptic currents (EPSCs) in mouse dorsal horn neurons (Minami et al., 1999). Baba et al. (2001) showed that the inclusion of PGE_2 in the bath caused a direct activation of dorsal horn neurons *in vitro*. They concluded that this was a post-synaptic effect, and probably mediated through the prostaglandin EP_2 receptor subclass. The "wind-up" of a spinal C-fiber nociceptive reflex, induced by repeated electrical stimulation of the sural nerve, was also dose-dependently inhibited by i.t. or i.v. indomethacin, and by i.v. administration of the selective COX-2 inhibitor, SC-58125 (Bustamante et al., 1997; Willingale et al., 1997). While these studies do not preclude a change in neuronal excitability secondary to an increase in glutamate and neuropeptide release, they do provide further support for the positive modulatory effect of prostaglandins on spinal neurotransmission.

1.10 EVIDENCE FOR THE ROLE OF PROSTAGLANDINS IN NORMAL PAIN AND HYPERALGESIA

Prostaglandins have long been linked to hyperalgesia and pain (see review, Lim, 1970). Indeed, early work by Vane (1971) and Smith and Willis (1971) suggested that non-steroidal anti-inflammatory drugs (NSAIDs) effect their analgesic and anti-hyperalgesic actions through the inhibition of peripheral prostaglandin synthesis. Later work by Yaksh and Malmberg (1993) pointed to a spinal site of action, in addition to, but

independent of, the more well-known role of peripheral prostaglandins in pain and inflammation.

The synthesis of prostaglandins within the central nervous system (CNS) in response to repeated C-fiber activity was first reported by Malmberg and Yaksh (1992a; also see McCormack 1994a,b for review). It was based on the observation that NSAIDs, injected directly into spinal subarachnoid space of conscious rats, dose-dependently inhibited the behavioral responses triggered by formalin injection in the foot pad (Malmberg and Yaksh, 1992a). When given spinally, NSAIDs were 100-1000 times more potent than systemic administration in inhibiting the second phase of the formalin test (Malmberg and Yaksh, 1992a). The antinociceptive effect of i.t. ibuprofen was also stereospecific (Malmberg and Yaksh, 1995a). Intrathecal NSAIDs inhibited glutamate- or substance P-induced hyperalgesia in the rat (Malmberg and Yaksh, 1992b), and reduced the spinal release of substance P and CGRP (Southall et al., 1998). These results strongly support a spinally mediated antinociceptive effect attributable to local COX inhibition.

That spinal prostaglandin synthesis is evoked by high-threshold (i.e. nociceptive) input was subsequently confirmed *in vivo* and *in vitro*. Using an i.t. microdialysis catheter, Yang et al. (1996) showed that the injection of carrageenan/kaolin into the knee joint of the rat evoked persistent nociceptive behavior and a time-dependent increase in the concentration of PGE_2 in spinal dialysate (146 ± 11% and 143 ± 18% above baseline 10 min and 24 h after injection, respectively). An increase in PGE_2 concentration (109 ± 10% and 83 ± 15% above baseline) was also reported during the first- and second-phase of the rat formalin test (Malmberg, 1995a,b); an effect triggered by C-fiber input (Hua et

al., 1997). Both nociceptive behavior and the increase in the PGE_2 concentration were attenuated by i.t. S(+)-ibuprofen, but not the inactive R(-)-enantiomer (Malmberg, 1995b; Yang, 1996). Intrathecal substance P, which induces hyperalgesia in the rat, also elicits a dramatic increase (362 ± 37% of baseline) in PGE_2 concentration in spinal CSF (Hua et al., 1999).

Using an *in vitro* spinal superfusion model, Dirig et al. (1999) showed that spinal cords harvested from rats pretreated with kaolin/carrageenan in the knee joint for 5-72 h exhibited increased release of PGE_2 compared to control. Further increases were induced with substance P (0.1-1.0 μM) or capsaicin (0.1-10 μM). These results indicate that spinal PGE_2 synthesis can be triggered by peripheral inflammation, or by directly exposing the cord to receptor ligands released in the CNS in response to peripheral inflammation.

1.11 CENTRAL PROSTAGLANDINS AND ALLODYNIA

The ability of prostaglandins to sensitize spinal cord neurons to high-threshold (C-fiber) somatosensory input raises an important question about their possible role in abnormal pain states. For example, do low threshold mechanoreceptive (Aβ) primary afferent fibers activate spinal prostanoid-mediated sensitizing mechanisms in allodynia? In turn, can central prostaglandins, synthesized in response to spinal or peripheral nerve injury, affect low-threshold input in a way that miscodes it as pain?

The most direct evidence supporting a role for prostaglandins comes from the studies of Uda et al. (1990) and Minami et al. (1992, 1994a, 1995a) who showed that the

i.t. injection of PGE_2, PGD_2 or PGF_2 in conscious mice elicited dose-dependent, touch-evoked agitation. The effect induced by PGE_2 was blocked by the receptor antagonist, NON-NT-012, suggesting that this was mediated by spinal EP_1-receptors (Minami et al. 1995b). Similarly, the delivery of PGE_2 through a microdialysis probe implanted in the spinal subarachnoid space of rats triggered an immediate increase in the concentration of glutamate, aspartate, taurine, glycine and GABA in dialysate samples (Malmberg and Yaksh, 1995a). These neurochemical responses were temporally correlated with the onset and decline of behaviorally-defined allodynia (Malmberg and Yaksh, 1995a). In the same study, the combination of PGE_2 (10 μM) and capsaicin (0.1 or 1.0 μM), concentrations that individually had no effect on neurotransmitter release, evoked a significant increase (60–100%) in the dialysate concentration of glutamate, aspartate, taurine, glycine and GABA, as well as tactile allodynia. These results indicate that introducing exogenous prostaglandins into the spinal subarachnoid space of otherwise normal animals induces short-term neurochemical and behavioral effects indicative of allodynia, and is consistent with the hypothesis that spinal prostaglandins contribute to the development of tactile allodynia in nerve injured animals.

Conversely, i.t. ketorolac or S(+)-ibuprofen suppressed the hair deflection-evoked neurochemical (i.e. increased catechol oxidation current in the locus coeruleus) and physiological (i.e. increased heart rate and blood pressure) responses in i.t. strychnine-treated rats (Hall et al., 1999). The inactive R(-) isomer of ibuprofen was without effect suggesting this attenuation was related to the inhibition of COX isoforms in the spinal cord. In a related disinhibitory model of allodynia (spinal bicuculline), focal application of the COX-2 inhibitor, NS-398, or the EP receptor antagonist, SC-51322, to the dorsal

surface of the rat spinal cord produced a highly localized anti-allodynic effect (Zhang et al., 2001). Intrathecal ketorolac (20 nmoles) also reduced thermal hyperalgesia and cold allodynia in the chronic constriction injury model (Parris et al., 1996).

Messenger RNA hybridization analysis reveals a 3-fold increase in COX-2 mRNA in the rat lumbar spinal cord compared to control 2-4 h after the unilateral intraplantar injection of Freund's complete adjuvant (FCA; Hay et al., 1997). This was accompanied by a significant, but delayed, increase (peak effect 8 h after FCA) in the spinal concentration of 6-keto PGF_1 and PGE_2. All changes were temporally correlated with a decrease in the weight-bearing capacity of the affected paw (a quantitative measure of allodynia). The s.c. administration of indomethacin, or the COX-2 selective inhibitor, flosulide, attenuated the increase in spinal prostaglandin concentration, and inhibited allodynia by 80-100%. Neither drug had any effect on mechanical hyperalgesia (Hay et al., 1997). Collectively, these studies provide convincing evidence that spinal prostaglandins contribute to the development of allodynia whether induced by peripheral inflammation, central disinhibition, or direct nerve injury.

1.12 PROSTAGLANDINS AND NEUROPROTECTION

While the role of prostaglandins as mediators of inflammation and normal nociception is well established, their role(s) in initiating mechanisms that lead to more permanent pathologies is less clear. However, there is growing evidence, including studies demonstrating the protective effects of COX inhibitors, that prostaglandins may indeed play such a role. For example, the application of LPS to a neuronal-glial culture induced neuronal loss in a dose-dependent manner as evidenced by decreased MAP-2

positive cells (an immunohistochemical marker of mature neurons) and the efflux of lactate dehydrogenase (an indicator of neuronal cell death) in culture media. Inclusion of NS-398, a selective COX-2 inhibitor, significantly attenuated neuronal cell death and the corresponding increase in PGE_2 concentration triggered by LPS (Araki et al., 2001). This attenuation was less pronounced using enriched neuronal cultures, suggesting an important contribution of glial cells. The role for glia in several central and peripheral pathologies is currently the subject of intense investigation. Similar results were described with 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)-phenyl-2(^{5}H)-furanone (DFU), a selective COX-2 inhibitor (Strauss and Marini, 2002). Pretreatment of cultured cerebellar granule neurons with DFU (10 nM) protected almost all vulnerable cells from glutamate-induced cell death, and reduced the concomitant increase in PGE_2 by 50%.

The protective effects afforded by inhibitors of prostaglandin synthesis have also been reported *in vivo* using several different neuropathological models. Subcutaneous injection of carrageenan into the plantar surface of the hind paw induces a hyperalgesic state that has both central and peripheral components (Dirig et al., 1998). Dirig and collegues (1998) showed that intrathecal pretreatment with either a non-selective COX inhibitor or a COX-2 selective inhibitor prevented the development of hyperalgesia. In a focal cerebral ischemia model, treatment with oral indomethacin (20mg/kg) before ischemia in the fronto-parietal region of rat brain reduced infarct volume by 43% and the concurrent increase in local PGD_2 concentration. Further support for a pathophysiological contribution of prostanoids is the observation that COX-2 knockout mice exhibit reduced susceptibility to focal cerebral ischemia induced by middle cerebral artery occlusion

(Iadecola et al., 2001). Homozygous knockouts showed the most significant reduction in infarct volume (~38%) while reduced protection was noted in hetereozygous knockouts (~20% reduction). In the same study, brain lesion volume induced by NMDA microinjected into the cerebral cortex was also reduced in COX-2 -/- mice; an effect mimicked by NS-398 (20mg/kg i.p.) in COX-2 +/+ mice, but not COX-2 -/- mice.

The deleterious effects of COX products have also been demonstrated in models of neuropathic pain. Zhao et al. (2000) reported that indomethacin (100μg), injected intrathecally 2h after unilateral spinal nerve ligation (L5 and L6), attenuated the development of tactile allodynia for up to 4 weeks after nerve injury. Identical treatment 1 day after nerve injury produced a similar effect, but resulted in a lesser degree of prevention of allodynia, suggesting a limited window of opportunity for post-injury protection. Comparable effects have been reported using knockout animals. Mice lacking one of the terminal prostaglandin synthases, namely membrane-associated PGE synthase (mPGES-1, the enzyme that converts PGH_2 to PGE_2), failed to develop mechanical allodynia or thermal hyperalgesia following unilateral L5 spinal nerve transection (Mabuchi et al., 2004). Interestingly, these same mice showed normal thermal and mechanical nociceptive responses. Overall, these studies support the hypothesis that prostaglandins, generated by nerve injury, are important mediators of several neuropathological states, including neuropathic pain. To date, emphasis has been directed at COX-2. The contributions of COX-1 and the putative COX-3 isozyme remain to be determined.

1.13 ROLE FOR SPINAL NITRIC OXIDE IN NOCICEPTION, HYPERALGESIA AND ALLODYNIA

Using cerebellar slices, Bredt and Snyder (1989) reported that glutamate, acting through the NMDA receptor complex, generated NO. This product appeared after an increase in intracellular Ca^{2+} concentration and the activation of NOS (Schuman and Madison, 1994; Brenman and Bredt, 1997). Nitric oxide is able to diffuse out of the cells from which it is generated and act on neighbouring elements (e.g. neurons and astrocytes) as a neurotransmitter. In this manner, NO is thought to subserve important neuromodulatory functions in the CNS (Snyder, 1992). Because of the high affinity that NO has for guanylyl cyclase, most NO-mediated effects are cGMP-dependent, and are therefore upstream from protein kinase G. These effects include the modulation of neurotransmitter release, gene expression (including those for COX), learning, memory, and prostaglandin synthesis (independent of that related to COX expression) (Wang and Robinson, 1997; Bredt and Snyder, 1992; Mollace et al., 1995; Haley et al., 1992; Garthwaite et al., 1988).

Extensive evidence links spinal NO to nociceptive processing. For example, incubation of an astroglial cell line with NMDA (10-600 μM) produced a dose-dependent increase in NO, PGE_2 and cGMP; effects inhibited by L-NAME (Mollace et al., 1995). The NO donor, sodium nitroprusside, evoked release of CGRP and substance P from dorsal horn slices (Garry et al., 1994). In contrast, L-NAME blocked thermal hyperalgesia in the rat tail flick and tail immersion tests (Radhakrishnan et al., 1995) as well as PGE_2-induced allodynia and hyperalgesia (Minami et al., 1995b; Park et al.,

2000). Dolan and Nolan (1999) reported that i.t. NMDA-induced allodynia in sheep is blocked by either L-NAME (2 μmol i.t.) or the COX-2 inhibitor, DFU (200 nmol i.t.). The relevance of spinal NO to neuropathic pain is supported by studies demonstrating attenuation of mechanical allodynia by i.p. L-NAME in the spinal cord ischemia and L5/L6 nerve ligation models (Hao and Xu, 1996; Yoon et al., 1998) Further support is drawn from the suppression of the neurochemical and cardiovascular indices of strychnine-allodynia by L-NOARG (Milne et al., 2001).

In summary, NOS and COX are co-activated by NMDA receptor activation secondary to exaggerated glutamate release immediately after nerve injury. Isoforms of NOS and COX appear to be up-regulated in the dorsal horn following nerve injury, and their inhibition is significantly anti-allodynic in a variety of experimental models. Thus, spinal NO and prostaglandins may work in concert to effect central sensitization early after nerve injury (see figure 1.1).

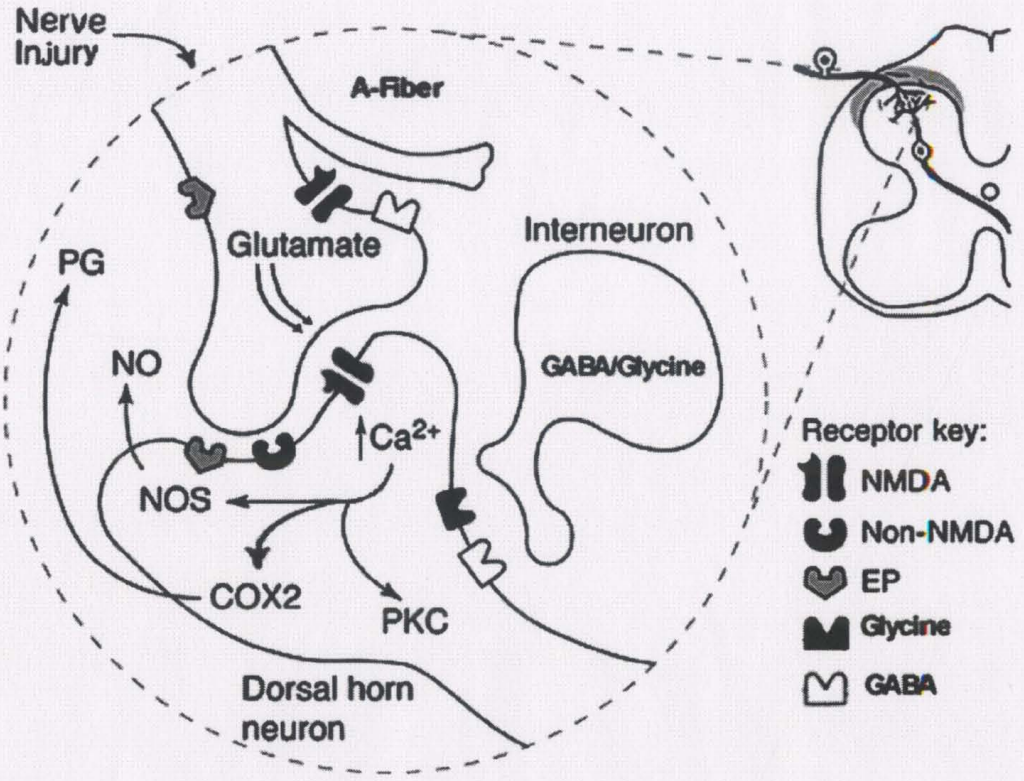

Figure 1.1 Schematic representation illustrating several dorsal horn systems that contribute to the processing of primary afferent input following nerve injury. Primary afferent A-fibers release excitatory amino acid (glutamate) products. Some postsynaptic elements contain nitric oxide synthase and are able to release NO upon a rise in intracellular Ca^{2+}. For glutamate, direct monosynaptic excitation is mediated by NMDA and non-NMDA receptors. Excitation in second-order neurons via a NMDA receptor leads to an increase in intracellular Ca^{2+}, activation of phospholipase A2, NOS, and phosphorylating enzymes. COX products (PG) and NO are formed and released. These agents diffuse extracellularly and facilitate transmitter release (retrograde transmission) from primary and nonprimary afferent terminals by either a direct cellular action or by an interaction with a specific class of receptors [e.g., PG type E (EP) receptors for prostanoids]. (Modified from Yaksh et al., 1999)

1.14 NEUROPATHIC PAIN MODELS

Animal models are used extensively in basic pain research. This is done on the premise that, by serving as surrogates for human pain states, the mechanisms and etiologies underlying these states can be determined and, in turn, that new drugs and treatment strategies can be reliably predicted. There are now a number of distinct models of neuropathic pain that mimic direct injury to the central or peripheral nervous system, metabolically-based neuropathies, and drug-induced pathologies.

1.14.1 PERIPHERAL NERVE INJURY MODELS

The three most commonly used models of peripheral nerve-injury are the chronic constriction injury (CCI), the partial sciatic nerve ligation (PSL), and the L5/L6 spinal nerve ligation (SNL) models.

1.14.1.1 CHRONIC CONSTRICTION INJURY MODEL

Chronic constriction injury was the first and probably the most widely-used model of neuropathic pain. Four loose chromic gut ligatures are placed unilaterally around the rat sciatic nerve at mid-thigh level eliciting a local inflammatory response (Bennett and Xie, 1988). As the nerve swells, it becomes tightly constricted by the ligatures leading to thermal and mechanical hyperalgesia, as well as cold and tactile allodynia. These sensory changes are evident within one week after ligation and last approximately two months, although the duration can vary (Bennett and Xie, 1988; Attal et al., 1990). Other outcomes included suppression of appetite, and frequent spontaneous

nocifensive behaviors. The authors also suggested that rats subjected to CCI have spontaneous pain as evidence by the presence of mild to moderate autotomy. This model has been used extensively to investigate: a) spinal and supraspinal sensitization after injury; b) the genetic basis of neuropathic pain; and c) pharmacological interventions for possible use in human pain states.

1.14.1.2 PARTIAL SCIATIC NERVE LIGATION MODEL

Seltzer et al. (1990) described a model of neuropathic pain that attempts to mimic partial peripheral nerve injury in humans. Injury is induced by the unilateral application of a loose ligature that narrows the sciatic nerve by 1/3-1/2 at the high-thigh level . Rats exhibit signs of mechanical allodynia and thermal and mechanical hyperalgesia within hours of ligation; effects which can persist for over seven months. These symptoms appear to have a sympathetically-mediated component similar to human causalgia. Spontaneous pain also seems to be present as rats sometimes lick the affected paw in the absence of stimulation. Autotomy is sometimes present but it is not a consistent feature of the model. Evoked pain can also develop a bilateral pattern of response suggesting the recruitment of central neuroplastic mechanisms.

1.14.1.3 L5/L6 SPINAL NERVE LIGATION (SNL) MODEL

In 1992, Kim and Chung reported another model resembling human causalgia in which the L5 and L6 spinal nerves are tightly ligated (unilaterally) just distal to the dorsal root ganglia. Allodynia and hyperalgesia develop within the first 24 h after injury and last for at least 4 months. Numerous modifications of SNL have since been reported,

including ligation of L5 only, as well as both ligation and transection of L5. These variations, while not commonly used and less well-characterized, result in a similar pattern of allodynia and hyperalgesia.

Of the three models, SNL elicits the most pronounced mechanical allodynia and is least likely to cause autotomy. This latter feature, along with the lack of spontaneous pain after SNL, is a major advantage of this model. The degree of hyperalgesia (thermal and mechanical) and bilateral development of NP are comparable among the three models. Another noteworthy advantage of SNL is the reproducibility of the injury from one animal to another. This is aided by the use of tight as compared to loose or partial ligation where consistency is more difficult to achieve.

Ligation of the L5/L6 spinal nerves does necessitate more extensive surgery than the other models making it somewhat more challenging to use. A related issue is the risk of damage to the L4 spinal nerve given its proximity to L5 and the need to separate the two before ligation. However, inadvertent injury can be detected by the emergence of significant motor deficits in the ipsilateral hindlimb since the L4 root contains an abundance of motor fibers (Kim and Chung, 1992).

1.14.1.4 PERIPHERAL NERVE TRANSECTION

The use of early models involving complete transection of a peripheral nerve, often the sciatic nerve, is generally in decline. These models most closely resemble conditions of limb amputation. Following transection, a neuroma forms at the proximal nerve stump consisting of regenerating axonal sprouts. This is thought to be intimately involved in the mechanisms underlying the NP that ensues (Fried et al., 1991; Devor et

al., 1976). Complete transection of a peripheral nerve almost always leads to tactile allodynia, thermal hyperalgesia, excessive grooming of the denervated limb, and autotomy (Wall et al., 1979). This latter behavior is now considered to be a reflection of NP arising from the nerve lesion and can be used as a behavioral correlate (Kauppila et al., 1998).

1.14.1.5 SPARED NERVE INJURY MODEL

Recently, Decosterd and Woolf (2000) described another model mimicking partial nerve injury. This involves axotomy and ligation of the tibial and common peroneal nerves, while leaving the sural nerve intact. This represents partial denervation of the ipsilateral limb, while preserving some sensory input, thus allowing investigation of both injured and uninjured fibers. In this model, injured animals exhibit robust mechanical and thermal sensitivity in the affected dermatomes within 24h; effects that persist for more than 6 months.

1.14.2 CHEMICAL- OR METABOLICALLY-INDUCED NP MODELS

Clinical NP can arise from metabolic disorders such as diabetic neuropathy, or treatment with anti-neoplastic drugs and immunotherapy. Diabetic neuropathy can be induced in experimental animals by a single injection of streptozocin (e.g. 75mg/kg i.p.) which is toxic to beta cells in the pancreas. This treatment also results in ultrastructural changes to nerve fibers that may explain, in part, the long-lasting (i.e. months) thermal and mechanical hyperalgesia, as well as cold and tactile allodynia that develop within 10 days of treatment (Courteix et al., 1993).

There are several models of chemotherapy-induced NP, including treatment with vincristine, cisplatin, or taxol. Each can cause dose- and treatment time-dependent peripheral neuropathies which often become the limiting factor in their clinical use (Strumberg et al., 2002; Aley et al., 1996; Cavaletti et al., 1995). In animals, treatment with the respective agent is required for several days resulting in treatment-dependent forms of hyperalgesia and/or allodynia. The exact mechanisms contributing to the resulting NP are unknown, but severe demyelination and axonal degeneration are known to occur.

Pediatric neuroblastomas are often treated with a chimeric human/mouse anti-GD2 (GD2 – a glycosphingolipid) antibody which can result in severe pain. Infusion of a monocolonal anti-ganglioside antibody (i.v. or i.t.) in experimental animals leads to quantifiable mechanical allodynia, but no thermal hyperalgesia (Slart et al., 1997; Sorkin et al., 2002). The exact mechanisms leading to these sensory changes are unknown. However, the antigen that binds to the antibody is known to be present on sensory nerves and is probably a relevant factor (Slart et al., 1997).

1.14.3 CENTRAL NEUROPATHIC PAIN MODELS

Neuropathic pain is a common problem in patients with spinal cord injury (see review, Yezierski, 1996). There are two common models of this central pain state: the spinal cord ischemia- and excitotoxic lesioning-models.

1.14.3.1 SPINAL CORD ISCHEMIC INJURY MODEL

Focal spinal cord ischemia can be induced using laser irradiation of the exposed spinal cord following the i.v. injection of a photosensitizing dye. The irradiated dye causes blood vessel occlusion in a highly restricted and circumscribed area leading to subsequent spinal cord injury (Watson et al., 1986; Prado et al., 1987; Hao et al., 1991). The resulting effects are mechanical and cold allodynia, hyperalgesia, and autotomy (Hao et al., 1991; Xu et al., 1992; Hao et al., 1992). The most common explanation for these sensory changes is based on the observation that small, inhibitory interneurons within the spinal cord are extremely sensitive to ischemic insult. As a result, their ability to modulate afferent sensory input is significantly impaired leading to its miscoding as pain. This hypothesis is strengthened by the fact that robust allodynia can be induced by pharmacological blockade of spinal $GABA_A$ or glycine receptors in otherwise normal (uninjured) animals (Yaksh, 1989).

1.14.3.2 EXCITOTOXIC SPINAL CORD INJURY MODEL

Local injection of a glutamate receptor agonist into the spinal cord induces excitotoxicity and the formation of a focal lesion. Normally, the AMPA receptor agonist, quisqualic acid is used. This is injected just above the lumbar segments, into areas of the spinal cord that control sensory function of hindlimb dermatomes. Long-lasting tactile allodynia and thermal hyperalgesia result in the dermatomes innervated by the spinal segments affected by the drug (Yezierski et al., 1998; Yezierski et al., 1993). Like the spinal cord ischemia model, the local injection of excitotoxic amino acids is thought to induce the relatively selective loss of inhibitory interneurons, thereby disrupting the

normal modulation of afferent sensory input. However, other theories have also been proposed (Yezierski et al., 1998) and it is likely that no single mechanism is totally responsible.

1.15 HYPOTHESIS AND SPECIFIC OBJECTIVES

Increased glutamatergic tone, acting through spinal NMDA receptors, leads to synthesis of spinal prostaglandins and NO; obligatory events preceding the facilitation of high-threshold (C-fiber) input and the development of hyperalgesia. Sustained C-fiber activity is also known to trigger rapid induction of COX-2 and iNOS in the spinal cord which could exacerbate this central sensitization process by catalyzing further prostanoid and NO synthesis. Because these changes are initiated by and thus dependent upon central NMDA-receptor activation, they are normally only recruited by sustained C-fibre (nociceptive) input.

One of the defining and clinically important features of neuropathic pain is allodynia. Allodynia is known to be triggered by input from low-threshold primary afferent (Aß) fibers not normally involved in pain. While the mechanisms underlying this debilitating condition are poorly understood, there is growing evidence that spinal prostaglandins, generated in the spinal cord after nerve injury, may play an integral role in the early pathogenesis of allodynia. There is additional evidence that NO, generated in the spinal cord after nerve injury (i.e. secondary to spinal NMDA-receptor activation), works in concert with prostaglandins to alter sensory processing, thereby leading to the miscoding of low-threshold input as pain.

The purpose of this research was to test the hypothesis that nerve injury triggers the synthesis of prostaglandins in the spinal segment(s) affected by that injury and that this early neurochemical response to nerve injury is a critical step in the development of long-term (prostaglandin-independent) allodynia. Increases in the cellular machinery required for spinal prostaglandin synthesis has already been reported after spinal nerve ligation (Zhao et al., 2000), along with increases in prostaglandin receptors (Ma and Eisenach, 2002). Interestingly, these changes were temporarily correlated with nerve injury, but were transient. Thus, it is possible that the role for spinal prostaglandins in neuropathic pain changes with time and this lead us to investigate the importance of these neuromodulators in a similar time frame that others have reported related changes.

It is further hypothesized that spinal NO interacts with prostaglandins to effect tactile allodynia early after nerve injury. This interaction has been suggested several times in the literature (e.g. Dolan and Nolan, 1999; Park et al., 2000), but has yet to be pharmacologically characterized by isobolographic analysis. Defining the nature of this interaction, whether it is synergistic, additive, or antagonistic could have a significant impact on the management of neuropathic pain. Characterization is necessary if simultaneous inhibition of COX and NOS is going to be considered, both of whose enzymatic products have been shown to be pro-allodynia (see sections 1.10, 1.11, 1.13).

These hypotheses were tested using the L5/L6 spinal nerve ligation model of neuropathic pain - a commonly used and well-characterized model of hyperalgesia and allodynia. Spinal nerve ligation elicits a pronounced mechanical allodynia, particularly in pre-pubescent rats, while being least likely to cause autotomy. This lack of autotomy as well as an apparent absence of spontaneous pain, are major advantages of this model.

Another significant advantage of the spinal nerve ligation model is the consistency of allodynia among animals. This is mostly due to the model's surgical endpoint - tight ligation of the spinal roots as opposed to to loose ligatures or partial nerve ligation, where consistency is more difficult to achieve. This consistency, as well as absence of spontaneous recovery from allodynia, provided an excellent animal model to use in this study. Accordingly, the following specific objectives were undertaken:

1. To determine if brushing the allodynic dermatome of nerve injured rats evokes the release of PGE_2 into lumbar CSF, and whether this effect is dependent on spinal COX activity.

2. To determine the time-course of prostaglandin-dependent allodynia following nerve injury, and its sensitivity to i.t. COX inhibitors or EP receptor antagonists.

3. To determine the COX isoforms responsible for prostaglandin synthesis in the prostaglandin-dependent phase of allodynia.

4. To determine if i.t. COX inhibitors, given hours after spinal nerve ligation, prevent the establishment of long term allodynia, and to identify the relevant COX isoform(s).

5. To determine the nature of the pharmacological interaction between spinal prostaglandins and NO in nerve-ligated (allodynic) animals.

2.0 SPINAL NERVE INJURY ACTIVATES PROSTAGLANDIN SYNTHESIS IN THE SPINAL CORD THAT CONTRIBUTES TO EARLY MAINTENANCE OF TACTILE ALLODYNIA

Michael P. Hefferan, Pamela Carter, Melissa Haley and Christopher W. Loomis, Pain 2003; 101:139-147.

2.1 INTRODUCTION

An intriguing and clinically important manifestation of neuropathic pain is allodynia. This is an abnormal condition in which innocuous stimuli (i.e. light touch) acquire the ability to evoke severe pain. Allodynia is known to be triggered by input from low-threshold primary afferent (A_β) fibers that are not normally involved in pain (Campbell et al., 1988; Price et al., 1989). However, the mechanisms underlying this change in sensory processing are poorly understood.

Prostaglandins, synthesized in the spinal cord in response to noxious (C-fiber) input, are known to facilitate nociceptive transmission (see McCormack 1994a,b for review). Thus, i.t. ketorolac (non-selective COX inhibitor) inhibited the second phase of the rat formalin test (Yaksh and Malmberg, 1993), and the spinal release of substance P and calcitonin gene-related peptide in a peripheral inflammatory model (Southall et al., 1998). Similarly, i.t. S(+)-ibuprofen, but not the R(-) isomer, reduced the concentration of PGE_2-LI in rat spinal CSF following subcutaneous (s.c.) formalin (Malmberg and Yaksh, 1995). Consistent with these reports are studies confirming the presence of cyclo-

oxygenase (COX) and prostaglandin receptors in the outer laminae of the spinal dorsal horn (Beiche et al., 1996; Willingale et al., 1997; Vane et al., 1998).

The observation that prostaglandins exert a *central* nociceptive action independent of their *peripheral* inflammatory and algesic effects raises questions about their contribution in abnormal (neuropathic) pain states. Indeed, there is growing evidence that spinal prostaglandins play a role in neuropathic pain. For example, i.t. ketorolac reduced thermal hyperalgesia and cold allodynia in the chronic constriction injury model (Parris et al., 1996). Intrathecal ibuprofen attenuated, in a stereospecific manner, the cardiovascular, behavioural and neurochemical indices of tactile allodynia in the i.t. strychnine model (Hall et al., 1999). Focal application of COX inhibitors to the dorsal surface of the rat spinal cord also produced a highly localized anti-allodynic effect in the spinal bicuculline model (Zhang et al., 2001). That spinal prostaglandins may be important in neuropathic pain is further supported by reports that COX-2 protein is up-regulated as early as one day after spinal nerve ligation (Zhao et al., 2000), and that the acute i.t. injection of PGE_2 induces hyperalgesia and allodynia in experimental animals (Uda et al., 1990; Z. Zhang, M.P. Hefferan and C.W. Loomis, unpublished results).

Preliminary work by Zhao et al. (2000) showed that indomethacin, given i.t. one day after spinal nerve ligation, partially inhibited the development of allodynia in the rat. However, the relationship between tactile stimulation and release of spinal prostaglandins in allodynic animals, the pharmacology of prostanoid modulation, and the effect of prostaglandins inhibition on *established* allodynia in the first two weeks of spinal nerve injury have not been reported. The present study provides the first report of brush-evoked changes in PGE_2 concentration in spinal CSF of allodynic rats using the spinal

nerve ligation model (Kim and Chung, 1992), and the anti-allodynic effects of stereo-specific, isoform-selective, and non-selective COX inhibitors, and a prostaglandin (EP) receptor antagonist, given i.t. 2-12 days after ligation.

2.2 METHODS

All studies were conducted in accordance with the guidelines of the Institutional Animal Care Committee of Memorial University of Newfoundland, St. John's, NF, Canada.

Animals: Male Sprague-Dawley rats (weights indicated below) were obtained from the Vivarium, Memorial University of Newfoundland and were housed in standard cages with woodchip bedding. Animals had free access to food and water and were housed singly following surgery. A 12/12 h light cycle (lights on at 0700 h) was used throughout.

Intrathecal catheterization: Intrathecal catheters were implanted in rats weighing 100-130 g as previously described (Sherman and Loomis, 1994). A 6.5-cm catheter was used for termination near the lumbar enlargement in these smaller animals (dead volume 6 μL). The catheter was sterilized with 70% alcohol and filled with sterile saline. Under halothane anesthesia, the catheter was inserted through an incision in the atlanto-occipital membrane of the cisterna magna. The catheter was externalized behind the head and sealed with a piece of stainless steel wire. Rats exhibiting normal gait, feeding and grooming behaviour were housed separately and allowed to recover for at least three, and not more than four days. Some animals were implanted with chronic i.t.

microdialysis catheters, or intravenous (i.v.) catheters (PE-50) in the right external jugular vein for i.v. injections. Microdialysis catheters (i.d. 240 μm, o.d. 290 μm, MW cutoff – 10 kDa) were constructed from triple lumen PE-5 tubing (Spectranetics, Colorado Springs, CO, USA) using a modified method of Marsala et al. (1995). Each animal was implanted with a new microdialysis catheter; no catheters were reused. Dialysate concentrations were equal to 31.7 ± 2.7% of the external PGE_2 concentration at a flow rate of 10 μl/ml *in vitro*. This value is similar to that reported by Malmberg and Yaksh (1995).

Spinal Nerve Ligation: Following recovery, neuropathy was induced using the method of Kim and Chung (1992), as modified by Chaplan et al. (1994). Rats were anesthetized with halothane and a dorsal midline incision made from L3-S2. Using blunt dissection, the left posterior interarticular process was located and resected to aid visualization of the L6 transverse process. This was partially removed to expose the L4 and L5 spinal nerves. Once separated, the L5 spinal root was tightly ligated with 6-0 silk thread. The L6 spinal root was then located medial and caudal to the sacroiliac junction and ligated in the same manner. The wound was closed with 4-0 silk sutures in two layers, cleaned with 70% alcohol and a 5-ml bolus of lactated Ringer's solution was injected intraperitoneally. The animal was placed under a warming light during recovery from anesthesia, and returned to the animal care facility for three days before experimentation. Sham surgery was performed identical to above, except each spinal nerve was identified, isolated in a similar fashion but not ligated.

Microdialysis: Intrathecal microdialysis (Marsala et al., 1995) was conducted 1 day before and 5 days after spinal nerve ligation in conscious animals. Briefly, the inflow channel of the microdialysis catheter was connected to a microsyringe pump. Artificial CSF, sterilized by filtration through a 0.22 μm pore filter (Micron Separations Inc., Edmonton, Canada) was perfused at a rate of 10 μl/min. This flow rate, selected on the basis of previous work (Malmberg and Yaksh, 1995), produced an *in vitro* recovery rate of 31.7±2.7% of the external PGE_2 concentration and has been established to balance adequate sample size, sampling time, and recovery rate (see chapters 2 and 3; also Malmberg and Yaksh, 1995; Marsala et al., 1995; Hefferan et al. 2003a,b). After a 30-min washout period, a baseline sample (100 μl) was collected (no paw stimulation). The plantar surface of the affected hind paw was then lightly brushed with a cotton-tipped applicator. This was repeated for 2 min followed by a 1 min rest over 10 min (6 min of stimulation in 10 min). The samples were collected on ice and immediately frozen at –80°C until assayed. The concentration of PGE_2 was determined using a commercially available ELISA kit (product # 514010, Cayman Chemical, Ann Arbor, MI).

Testing: Mechanical allodynia was quantified by determining the 50% paw withdrawal threshold using von Frey filaments (Chaplan et al., 1994). Briefly, rats were placed in a plastic cage with a wire-mesh bottom to allow access to the plantar surface of the left hind paw. Following a 20-min acclimatization period, a control threshold was measured. Drug or vehicle was then injected i.t. and thresholds were determined every 20 min for up to 3 h. Experiments were carried out during the daylight portion of the circadian cycle (0800-1800 h). All drug testing occurred 2-12 days after nerve ligation.

Drugs were administered on alternate days with the investigators (PC, MH, MPH) blinded to the drug treatment. No animal received more than 6 drug injections in total. For all experiments, allodynia was defined as a withdrawal threshold of <4 g. Animals with values ≥4 g were excluded (Chaplan et al., 1994).

Drugs: Due to their highly lipophilic nature, all drugs were dissolved in 100% DMSO and diluted with the same. They were injected i.t. using a Hamilton syringe and a hand-operated microsyringe pump. All equipment was sterilized with 70% alcohol before injection and thoroughly rinsed with 0.9% sterile saline. Drugs were injected i.t. in conscious, unrestrained rats in a volume of 5 μl followed by 8 μl of sterile saline. The i.t. catheter was immediately resealed with the stainless steel plug. S(+)-ibuprofen (active stereo-isomer) and R(-)-ibuprofen, (inactive isomer) were purchased from Research Biochemicals Int. (Natick, MA, USA); SC-51322 (an EP receptor antagonist with modest selectivity for the EP1 subtype) was purchased from Biomol (Plymouth Meeting, PA, USA); SC-236, (a selective COX-2 inhibitor) and SC-560 (a selective COX-1 inhibitor) were generous gifts from Searle (Skokie, IL, USA). The selectivity ratio, defined as the IC_{50} COX-1/IC_{50} COX-2, for SC-236 is 1780, while that of SC-560, defined as IC_{50} COX-2/IC_{50} COX-1, is 10700. These data were determined using human COX-1 and COX-2 and provided by Searle (Skokie, IL, USA).

Data Analysis: Results are presented as either raw withdrawal thresholds (g) or normalized as % anti-allodynia. The latter was calculated as follows: % anti-allodynia = (test value – control value)/(15 – control value) x 100 (Chaplan et al., 1997). In our experiments, 15 g was the approximate withdrawal threshold in naïve rats and was

therefore selected as the threshold representing complete reversal of allodynia (i.e. 100% anti-allodynia); this is consistent with previous reports (Chaplan et al., 1994). All dose-response curves were calculated using the peak effect of each dose taken from the time-course data. PGE_2 concentrations are presented as percent of baseline (defined as release in the absence of brushing). Absolute PGE_2 concentrations in pg/ml are also reported. All statistical testing was performed using Sigmastat® 2.03 for Windows®. Multiple comparisons were done using one-way analysis of variance followed by Student Newman-Keuls test. All results are shown as mean ± SEM. $P < 0.05$ was considered to be statistically significant.

2.3 RESULTS

Tight ligation of the L5/L6 spinal nerves produced mechanical allodynia manifested as protection of the affected hind paw ipsilateral to the nerve ligation (Fig. 2.1a/b) and a significant reduction in the paw withdrawal threshold (Fig. 2.2).

Thus, animals often kept the affected hind paw elevated above the cage floor and/or in a 'cupped' position presumably to prevent the mid-plantar surface from touching the cage. Apart from this abnormality, rats were healthy, exhibited normal grooming and feeding behavior, and regular weight gain. Withdrawal thresholds decreased from pre-surgical values of ≥15 g to 1-3 g after ligation. In a control group, this reduction was evident as early as 1 day post-ligation and remained stable for up to 18 days. Mechanical allodynia (defined as a withdrawal threshold ≤4 g) was observed in greater than 90% of ligated rats (55 of 60).

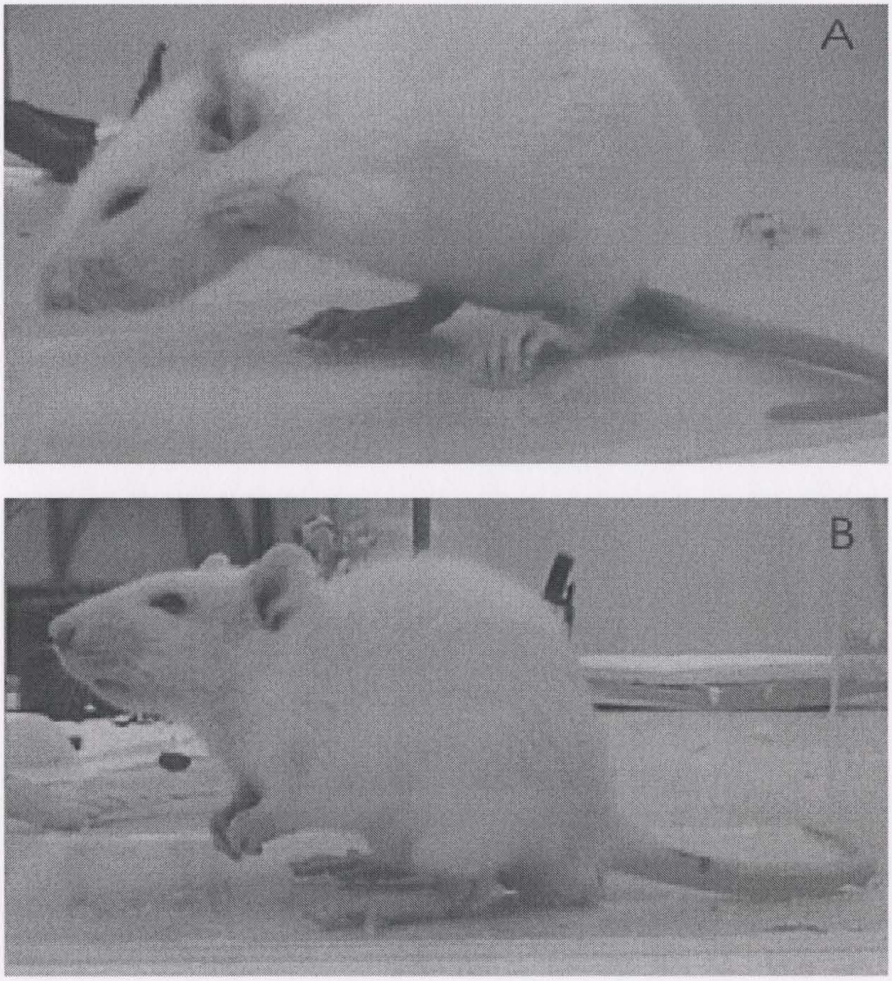

Figure 2.1. Tight ligation of the left L5/L6 spinal nerves results in tactile allodynia in the ipsilateral hind paw. This effect is observed as cupping of the affected paw (A), as compared to that of a sham-operated rat (B).

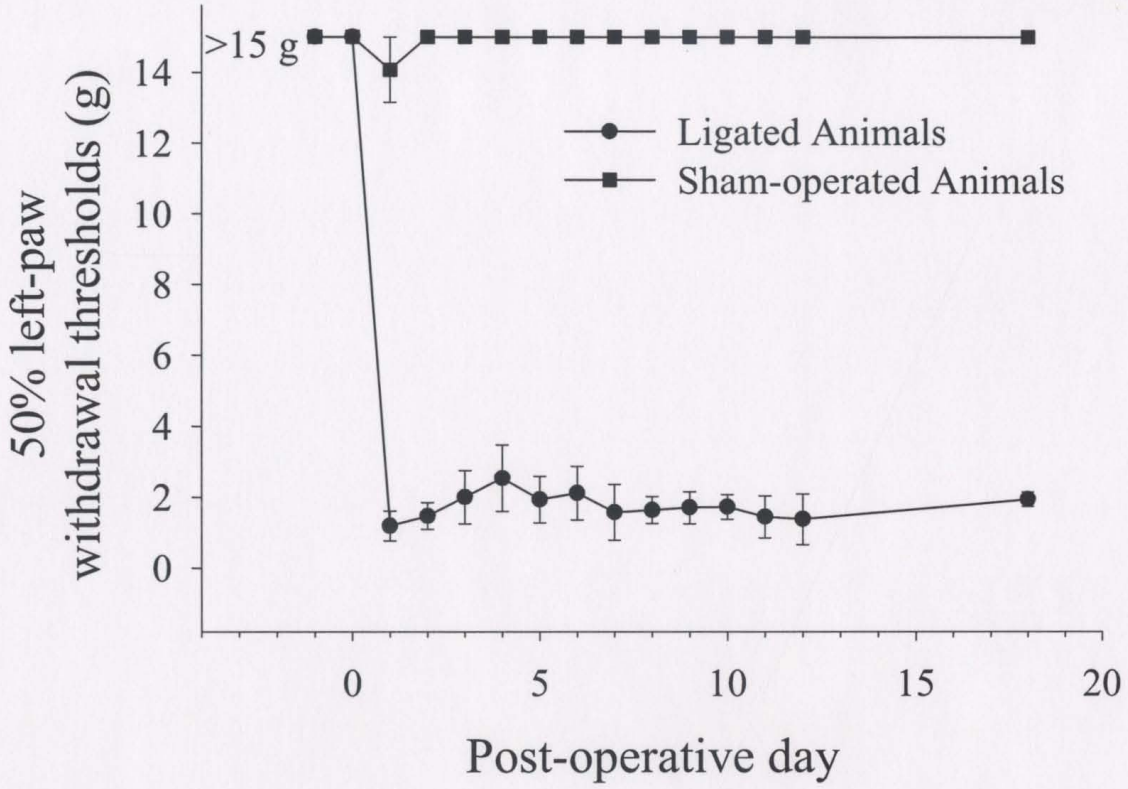

Figure 2.2 Tight ligation of the left lumbar 5/6 spinal nerves produced tactile allodynia in the plantar surface of the affected hind paw. Left-paw 50% withdrawal thresholds were determined over 18 days following spinal nerve ligation or sham-surgery (day 0). Each point represents the mean ± SEM of 6-8 experiments. All thresholds are significantly different from pre-ligation thresholds in ligated animals (p=0.017).

The withdrawal threshold in all sham-operated rats remained unchanged from pre-surgical values (fig. 2.2). The mean withdrawal thresholds after nerve ligation but before drug treatment were not statistically different across experimental days (data not shown).

Mechanical allodynia was accompanied by an increase in the evoked release of PGE_2 into spinal CSF. Figure 2.3 illustrates the concentration of PGE_2 in microdialysate samples collected from the i.t. space of ligated and sham-operated rats, 1 day before and 5 days after surgery. Brushing the affected hind paw evoked a significant increase (199 ± 34% of baseline; $p=0.042$) in allodynic rats as compared to sham-operated controls (92 ± 7%). Pretreatment with i.t. S(+)-ibuprofen (100 μg), but not i.t. R(-)-ibuprofen (100 μg), 60 min before brushing, decreased brush-evoked PGE_2 release to 142 ± 15% of baseline (fig. 2.3). This was not significantly different from baseline. Brushing the contralateral paw in ligated rats, or brushing either paw before surgery had no detectable effect on PGE_2 release in either group (fig. 2.3). There were no differences in the basal release of PGE_2 1 day before or 5 days after surgery in either ligated or sham-operated rats. The absolute concentrations of PGE_2 in spinal dialysate samples were as follows: Pre-ligation: 776 ± 235 pg/ml (no brushing) and 752 ± 180 pg/ml (brushing); Post-ligation: 672 ± 162 pg/ml (no brushing); 1337 ± 454 pg/ml (brushing).

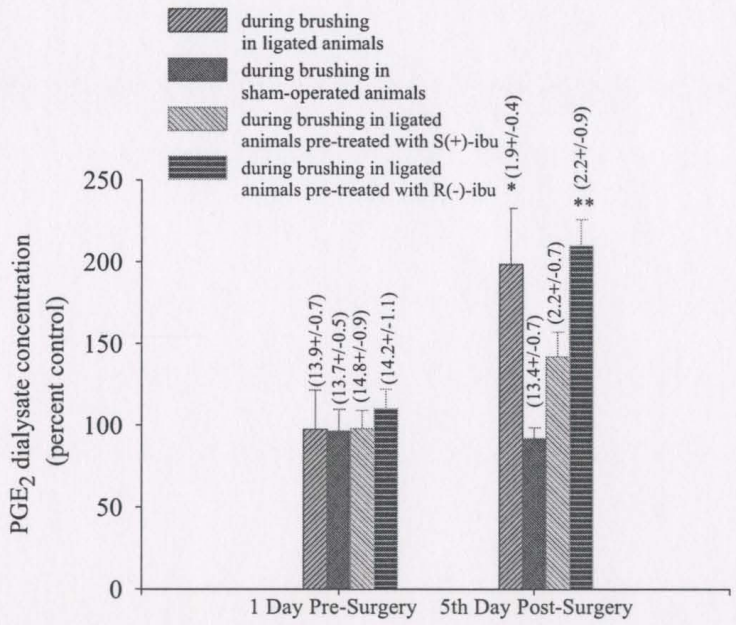

Figure 2.3. Brushing of the allodynic paw evoked spinal PGE_2 release. Vertical bars represent the concentration of PGE_2 in spinal dialysate samples collected in conscious, unrestrained rats, 1 day before and 5 days after either spinal nerve ligation or sham-surgery. Brushing the plantar surface of the affected hind paw after ligation evoked a significant increase in $[PGE_2]_{dialysate}$ (*p=0.040; n = 6-7 animals) compared to sham controls. Brushing before surgery was without effect. Intrathecal S(+)-ibuprofen (100 µg) administered 60 min before brushing significantly reduced the evoked PGE_2 release in ligated animals. ** Indicates a significant difference from the S(+)-ibuprofen-pretreated group (p=0.011). Numbers in brackets above the bars represent the mean ± SEM of 6-7 animals for the paw withdrawal threshold in the respective groups.

The i.t. injection of 10, 30, 60, or 100 μg of S(+)-ibuprofen significantly inhibited allodynia. Figure 2.4A shows the time-course of paw withdrawal threshold following the i.t. injection of 10 and 100 μg of S(+)-ibuprofen. The onset of effect was delayed, peaking between 60 and 100 min after injection (fig. 2.4A). A similar time course was observed with 30 and 60 μg of S(+)-ibuprofen (data not shown). The peak effect and duration of action were positively correlated with the i.t. dose. Dose-response analysis using the peak effect of each dose yielded a linear relationship over the range of 10-100 μg (fig. 2.4B). A similar observation was made using area-under-the-curve analysis (data not shown). In contrast, i.t. R(-)-ibuprofen (60 μg), DMSO, or normal saline had no effect on paw withdrawal threshold in ligated rats (fig. 2.5). The i.v. or i.p. injection of 100 μg of S(+)-ibuprofen was also without effect, as was i.t. delivery at the mid-cervical level. No adverse effect on motor function was noted.

The i.t. injection of SC-51322 produced a temporary blockade of mechanical allodynia (fig. 2.6A). As with S(+)-ibuprofen, the onset was delayed, and peaked at between 40 and 60 min. The maximum increase in withdrawal threshold was dose-dependent as indicated by the linear dose-response curve between 10 and 100 μg (fig. 2.6B). Dose-response analysis using the duration of action of SC-51322 yielded a similar result (not shown). Intrathecal SC-51322 did not appear to affect general motor activity.

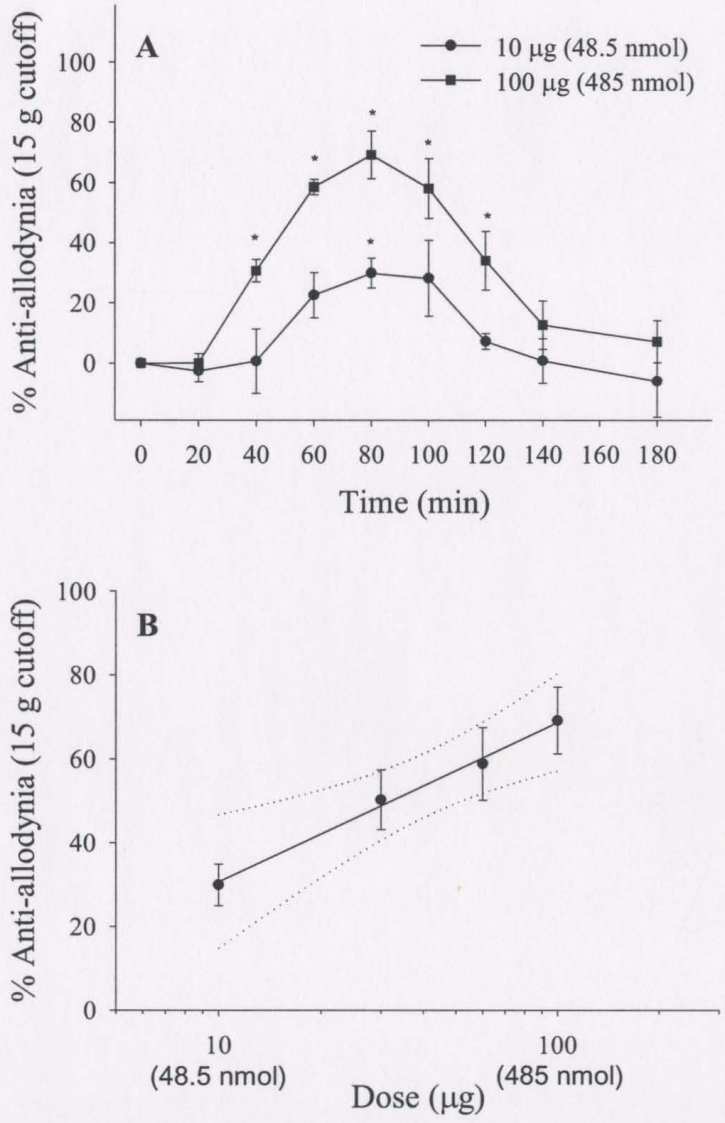

Figure 2.4. Intrathecal S(+)-ibuprofen significantly reversed allodynia in a dose-dependent manner. Time-course (A) and dose-response curve (B) for intrathecal S(+)-ibuprofen. Each point represents the mean ± SEM of 6-8 animals. Drug-induced changes in affected hind paw withdrawal thresholds are expressed as % anti-allodynia (see Methods). Dashed lines indicate the 95% confidence intervals. In panel A, each asterisk indicates a significant difference from control (time 0; p=0.017 for 10μg group; p<0.001 for 100 μg group).

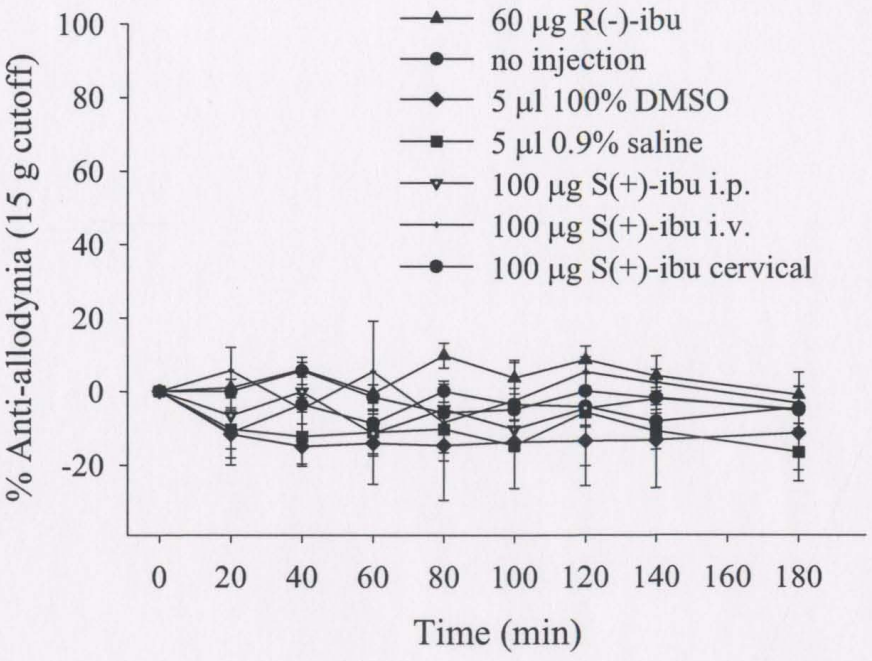

Figure 2.5. Intrathecal and systemic controls had no effect on allodynia. Time-course of changes in paw withdrawal thresholds (expressed as % anti-allodynia) following the injection of relevant controls or no treatment. Each point represents the mean ± SEM of 3-4 animals. No point is significantly different from time 0. (ibu – ibuprofen; DMSO – dimethyl sulfoxide). P values range: 0.090-0.926

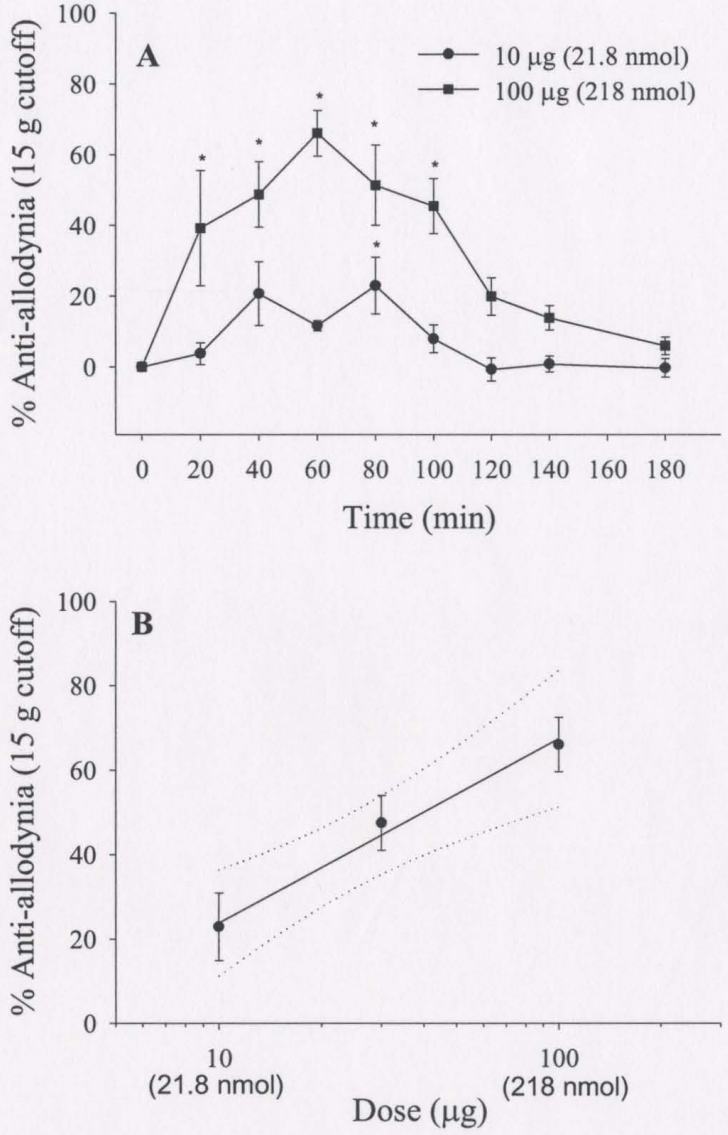

Figure 2.6. Intrathecal injection of the prostaglandin (EP) receptor antagonist, SC-51322 reversed allodynia. Time-course (A) and dose-response curve (B) for intrathecal SC-51322. Each point represents the mean ± SEM of 6-8 animals. Drug-induced changes in paw withdrawal thresholds are expressed as % anti-allodynia (see Methods). Dashed lines indicate the 95% confidence intervals. In panel A, each asterisk indicates a significant difference from control (time 0; $p<0.001$ for both 10 µg and 100 µg groups).

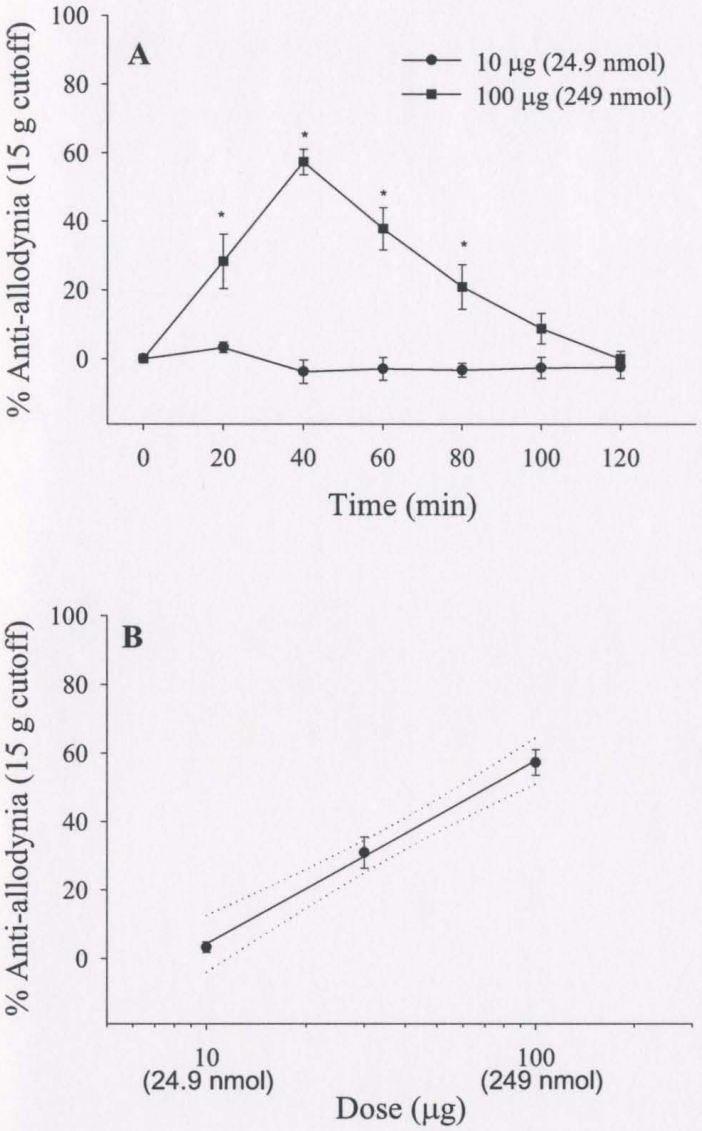

Figure 2.7. SC-236, a COX-2 selective inhibitor, significantly reversed allodynia. Time-course (A) and dose-response curve (B) for intrathecal SC-236. Each point represents the mean ± SEM of 6-11 animals. Drug-induced changes in the affected hind paw withdrawal thresholds are expressed as % anti-allodynia (see Methods). Dashed lines indicate the 95% confidence intervals. In panel A, each asterisk indicates a significant difference from control (time 0; p=0.510 for 10 µg group; p<0.001 for 100 µg group).

To investigate whether the prostaglandins implicated in this model are synthesized by spinal COX-2 and/or COX-1, the effects of i.t. SC-236 and SC-560 were determined. SC-236 significantly inhibited mechanical allodynia (fig. 2.7A) in a dose-dependent manner (fig. 2.7B). The maximum increase in paw withdrawal threshold, corresponding to 57.3 ± 3.7 % anti-allodynia, was achieved with the 100 μg dose. In contrast, SC-560 (up to 1000 μg) yielded only a modest increase in withdrawal threshold (fig. 2.8A). Although, this effect was dose-dependent (fig. 2.8B), the dose-response curve was considerably more shallow (slope = 0.19 ± 0.05%) as compared to SC-236 (slope = 0.51 ± 0.07%) ($P = 0.006$). Neither SC-236 nor SC-560 elicited any obvious adverse effects on motor function, even up to 1000 μg of SC-560.

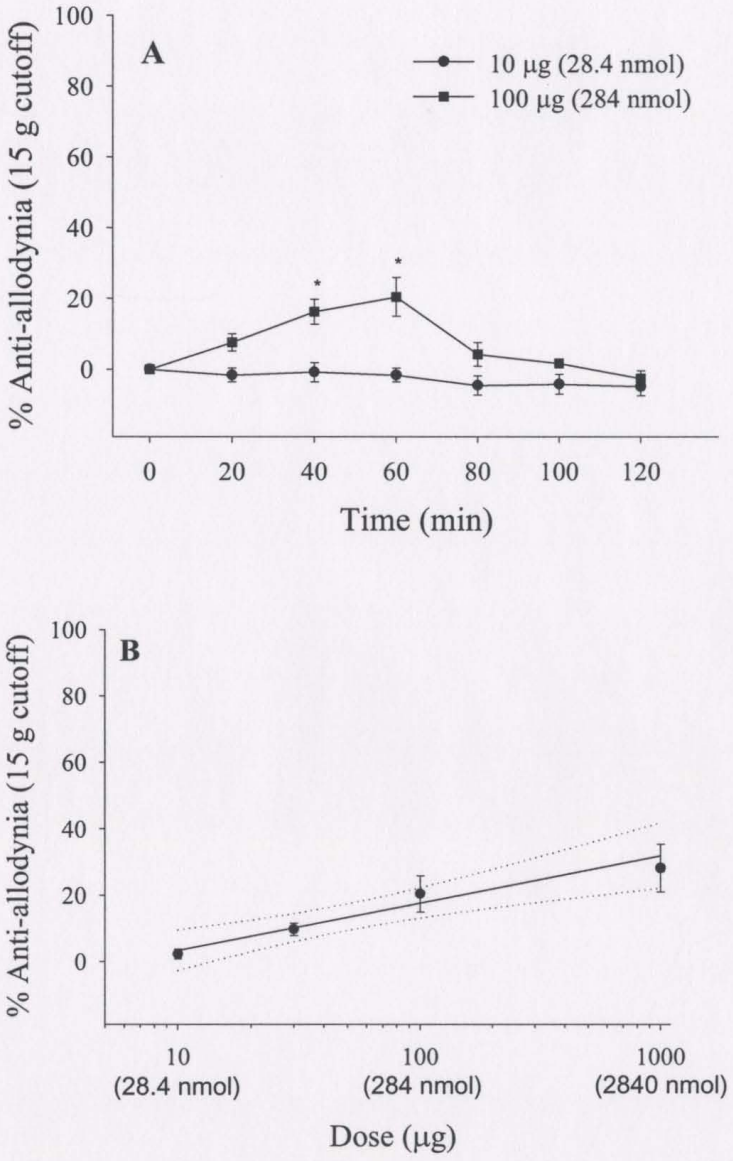

Figure 2.8. The COX-1 selective inhibitor SC-560 had minimal effect on allodynia. Time-course (A) and corresponding dose-response curve (B) for intrathecal SC-560. Data are shown as the mean ± SEM with each data point representing 6-8 animals. Drug-induced changes in left-paw withdrawal thresholds are expressed as % anti-allodynia (see methods). Dashed lines indicate the 95% confidence intervals. In panel A, each asterisk indicates a significant difference from control (time 0; $p=0.546$ for 10 µg group; $p<0.001$).

2.4 DISCUSSION

Tight ligation of the left L5 and L6 spinal nerves produced robust tactile allodynia in the plantar surface of the left-hind paw. This was evident from the marked reduction in withdrawal threshold 24 h after ligation; an effect that remained stable for at least 18 days. These observations are consistent with previous studies using the spinal nerve ligation model (Kim and Chung, 1992; Chaplan et al., 1994). The magnitude, stability and duration of the change in sensory threshold, and the absence of spontaneous recovery from allodynia, provided the requisite conditions for subsequent spinal dialysis and dose-response studies.

Spinal CSF concentrations of PGE_2 were determined before and after surgery, under basal (no brushing) and evoked (brushing) conditions. Brushing the plantar surface of the left hind paw enhanced PGE_2 release in allodynic, but not sham-operated animals. This effect was attenuated by i.t. pretreatment with the active isomer of ibuprofen, confirming its dependence on spinal COX activity. In contrast, basal release was unchanged from pre-surgical values in both the ligated- and sham (control)-groups. These data suggest that spinal prostaglandin release can increased by brushing the affected dermatome – likely triggered by primary afferent activity from that dermatome. This conclusion is supported by the absence of PGE_2 release when the contralateral dermatome was brushed, and an apparent temporal relationship between brushing, PGE_2 release and allodynic responses. The lack of change in basal PGE_2 concentration is also consistent with the absence of spontaneous pain behavior in this experimental model (Kim and Chung, 1992). Since prostaglandins are synthesized *de novo* following cell

activation (they are not stored; Rang et al., 1999), these results also suggest that spinal nerve ligation does not effect a tonic increase in prostanoid synthesis (as detected by changes in basal release). Rather, nerve ligation induces further cellular machinery required for prostaglandin synthesis in the spinal cord (Zhao et al, 2000), and thus increases its capacity to generate prostaglandins in response to low threshold sensory input from the affected dermatomes. Recent work by McCrory and Fitzgerald (2004) further supports a nerve injury-driven change in spinal prostaglandin systems. They report that human thorocotomy, a condition known to cause injury to intercostal nerves, results in an increase in CSF 6-keto-$PGF_{1\alpha}$ concentration; an effect that can be reduced by COX-2 selective or non-selective COX inhibitors.

Sustained C-fiber input induced by peripheral inflammation is known to trigger the synthesis of PGE_2 in rat spinal cord; an effect mediated by neuropeptides and glutamate released from the central terminals of nociceptive C-fibers (Yaksh et al., 1999). The finding that mild brushing elicits spinal prostaglandin synthesis in allodynic, but not control rats, indicates that low threshold input from the affected dermatome is able to recruit a central neurochemical mechanism (COX/prostaglandin) normally restricted to nociceptive input. Indeed, spinal PGE_2 is known to have pro-nociceptive effects including: a) the opening of voltage-sensitive Ca^{2+} channels; b) the enhanced release of neuropeptides from primary afferent terminals; and c) the possible modulation of gene transcription in sensitized primary afferent fibers (Hingtgen and Vasko, 1994; Millan, 1999). To determine if prostaglandins, synthesized in the spinal cord of nerve-injured rats, are relevant to the emergence of mechanical allodynia, pharmacological experiments were performed using COX inhibitors and a prostaglandin receptor antagonist.

In the present study, R(-)-ibuprofen was used as a control for the active S(+) enantiomer (Smith et al., 1994). Although R(-)-ibuprofen is known to interfere with NFκB (Scheuren et al., 1998), a regulator of COX-2 gene expression, it had no effect on paw withdrawal threshold, or basal and brush-evoked release of PGE_2 in this study. In contrast, S(+)-ibuprofen (10-100 μg i.t.) produced a dose-dependent reversal of allodynia for up to 2 h. This stereo-specific effect strongly suggests that the attenuation of allodynia is due to COX inhibition and thus a decrease in spinal prostaglandin synthesis. Indeed, S(+)-ibuprofen, but not the R(-) enantiomer, has been shown to: a) lower PGE_2-LI in CSF dialysate samples following formalin-paw injection (Malmberg and Yaksh, 1995); b) inhibit the autonomic, motor, and neurochemical responses in the i.t. strychnine model of allodynia (Hall et al., 1999); and c) to block the hair deflection-evoked increase in PGE_2-LI in spinal CSF during strychnine-allodynia (Milne et al., 2001). In addition, focal application of COX inhibitors to the dorsal surface of the spinal cord produces significant and highly localized inhibition of bicuculline-allodynia in the rat (Zhang et al., 2001). Although others have reported i.t. COX inhibitors to be ineffective in reversing *established* allodynia in the spinal nerve ligation model (Lashbrook et al., 1999; Zhao et al., 2000), these inhibitors were administered 14 or more days after nerve injury. We also have tested i.t. COX inhibitors more than 14 days after ligation, and see little or no anti-allodynic effect (unpublished observation). In light of this temporal change, retrospective data analysis suggests that there is a difference in the anti-allodynic effect of S(+)-ibuprofen if the time-course data (2-12 days) are subdivided into 2-7 and 8-12 day periods. In this case, 100 μg i.t. produced approximately 80% anti-allodynia in the early

period, whereas during the 8-12 day period, maximal anti-allodynia was approximately 35%. Unfortunately, an insufficient number of animals were used on each experimental day to permit a more detailed day-to-day analysis of the change in anti-allodynic efficacy. Regardless, to the best of our knowledge, the present study is the first to demonstrate that pharmacological disruption of spinal prostaglandin signalling early after spinal nerve ligation, using either COX inhibitors or a prostaglandin receptor antagonist, reverses *established* allodynia for up to 120 min after injection.

Systemic (i.v. or i.p.) administration of S(+)-ibuprofen, at doses that were effective when administered i.t., had no effect on paw withdrawal threshold. These results strongly suggest that the site of action of S(+)-ibuprofen in reversing tactile allodynia is within the central nervous system, and that the effect is unrelated to any peripheral actions arising from systemic redistribution. The spinal segments affected by nerve ligation, and thus the sites most likely to exhibit brush-evoked prostanoid activity, are in the caudal spinal cord. In this study, the tip of the i.t. catheter was positioned adjacent to the L5-L6 spinal segments to achieve maximal drug concentration in the affected region. When S(+)-ibuprofen was delivered to the mid-cervical region, there was no effect on withdrawal threshold. The bulk flow of CSF within the spinal subarachnoid space moves rostrally thereby carrying i.t. drugs away from the relevant sites of action in this model. Although i.t. drugs can also undergo redistribution through the vascular system (i.e. absorption into the spinal microvasculature and subsequent movement into the systemic circulation), direct i.v. injection of the maximum i.t. dose of ibuprofen was without effect. These data, and that of a previous paper (Zhang et al, 2001), support the conclusion that prostanoids exert a highly localized allodynic effect

within the affected spinal segments. They also indicate that the delayed onset of action of i.t. ibuprofen was not due to migration to rostral sites of action within the CNS.

Delays in the onset of action of i.t. drugs are not uncommon (Lee and Yaksh, 1995; Hwang and Yaksh, 1997) and, in the present study, probably reflect the time to achieve adequate COX inhibition before behavioral effects were detected. This is further supported by the more rapid onset of action of the prostaglandin receptor antagonist, SC-51322. The significant, dose-dependent inhibition of allodynia by SC-51322 is consistent with reports that: a) EP receptors are densely localized in the spinal dorsal horn of the rat (Beiche et al., 1998); b) the EP_1 receptor antagonist, ONO-NT-012, blocked the allodynia evoked by i.t. PGE_2 in mice (Minami et al., 1994, 1995); and c) PGE_2 , a major prostanoid in spinal nociceptive transmission (Malmberg et al., 1994), has greatest affinity for EP receptors (Coleman et al., 1994).

A role for spinal prostaglandins in allodynia is also supported by reports that i.t. PGD_2, PGE_2 or $PGF_{2\alpha}$ produce hyperalgesia and/or allodynia in conscious mice (Uda et al., 1990; Minami et al., 1992, 1994), the effect of i.t. PGE_2 is attenuated in EP_1 receptor knock-out mice (Minami et al., 2001), and that spinally administered COX inhibitors reduce thermal hyperalgesia and cold allodynia in the chronic constriction injury model (Parris et al., 1996) and mechanical allodynia in the strychnine- and bicuculline models (Hall et al., 1999; Zhang et al., 2001). In the present study, i.t. SC-236 and S(+)-ibuprofen have comparable effects at the highest doses tested (maximum inhibition of 57.3 ± 3.7% and 69.1 ± 7.9%, respectively). In contrast, the maximum inhibition achieved with SC-560, using near equimolar doses and the same post-ligation interval, was approximately 20.4 ± 5.5%. These data indicate that COX-2 is the predominant

isoform responsible for the *de novo* synthesis of spinal prostaglandins. This is supported by reports of COX-2 mRNA and protein in spinal laminae known to be important in nociceptive processing (Beiche et al., 1996; Willingale et al., 1997; Vane et al., 1998), and the up-regulation of COX-2, but not COX-1 protein, as early as one day after spinal nerve ligation (Zhao et al., 2000). Spinal COX-2 is also the major isoform associated with thermal hyperalgesia induced by the intraplantar injection of carrageenan in the rat (Yamamoto and Nozaki-Taguchi, 1997).

The mechanism(s) by which spinal prostaglandins contribute to the early maintenance of allodynia is unknown. However, studies of their effects on normal nociceptive neurotransmission provide some clues. For example, prostaglandins have been shown to: a) directly stimulate wide-dynamic range neurons in the rat dorsal horn (Baba et al., 1999); b) sensitize wide-dynamic range neurons in the rat dorsal horn to noxious mechanical stimulation of the ankle or knee joint (Willingale et al., 1997); c) enhance glutamate and substance P release from primary afferent terminals in the spinal cord (Hingtgen et al., 1995; Ferreira and Lorenzetti, 1996); and d) induce COX-2 activity in primary afferents terminals through the EP receptor subclass (Appleton, 1997). That these effects might have relevance to the spinal nerve ligation model is supported by the observation that the tactile allodynia arising from spinal nerve ligation is highly sensitive to spinal NMDA receptor blockade (Lee and Yaksh, 1995). NMDA receptor activation is an essential trigger of central prostaglandin synthesis (Dirig and Yaksh, 1999), but is not normally involved in low threshold neurotransmission (Urban et al., 1994). The emergence of both NMDA- and prostaglandin-properties in tactile allodynia suggests a convergence of cellular events by which low- and high-threshold input are processed.

In summary, brushing the dermatomes affected by L5/L6 spinal nerve ligation evoked COX-dependent PGE_2 release from the spinal cord. Treatment with i.t. COX inhibitors or an EP receptor antagonist up to 12 days after injury temporarily reversed *established* allodynia. These results support the hypothesis that spinal prostaglandins are critical in the early maintenance of allodynia. Based on these data, we propose that that allodynia induced by L5/L6 ligation in the rat has at least two phases (fig. 2.9); an early PG-dependent phase of approximately 10-12 days duration, and a delayed PG-independent phase that follows. COX-2 appears to be the predominant isoform, and the prostaglandin effects are mediated by spinal EP receptors. The contribution of prostaglandin metabolites and/or non-prostanoid neuromodulators, and their interaction with spinal prostaglandins, remains to be determined. These results suggest that disruption of spinal prostaglandin synthesis and/or their receptor-mediated effects may be of benefit in the early management of allodynia, however it is clear from the present data that their efficacy recedes with time, and detailed analysis of exactly when this treatment would become ineffective is necessary.

We are unaware of any controlled clinical studies either confirming or refuting the value of NSAIDS in neuropathic pain. However, our results indicate that the timing of NSAID administration is likely to be critical in determining their clinical effectiveness. That is, NSAIDS would likely need to be given early after nerve injury to be effective. Such an intervention would need to be deliberate given that the symptoms of clinical neuropathic pain are delayed in onset (weeks to months after nerve injury).

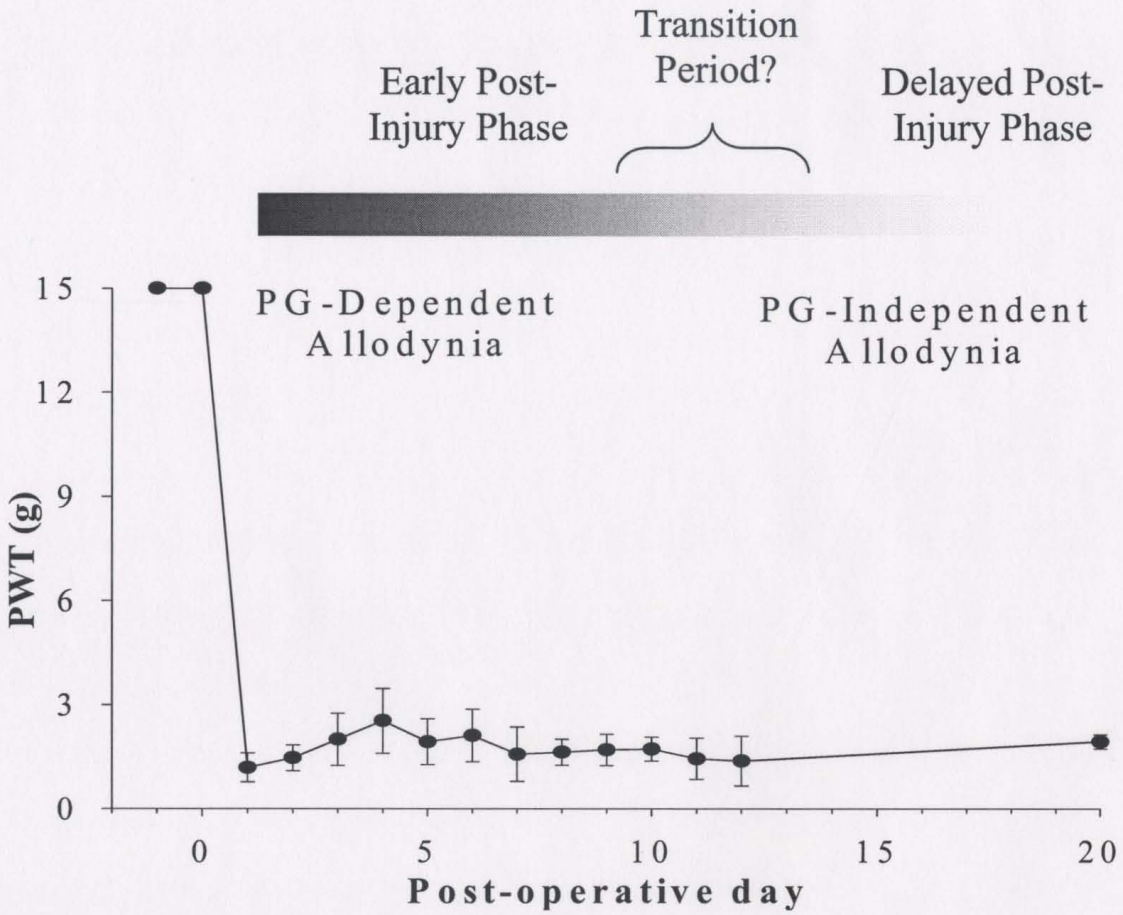

Figure 2.9. Time course of paw withdrawal thresholds (PWT) following tight ligation of the left L5/L6 spinal nerves (day 0). Allodynia is stable for at least 20 days, and has two phases–an early phase that is PG-dependent and a delayed phase that is PG-independent. Data are presented as the mean+/-SEM of 7 animals. Shading represents the change in PG-dependence.

3.0 INHIBITION OF SPINAL PROSTAGLANDIN SYNTHESIS EARLY AFTER L5/L6 NERVE LIGATION PREVENTS THE DEVELOPMENT OF PROSTAGLANDIN-DEPENDENT AND PROSTAGLANDIN-INDEPENDENT ALLODYNIA IN THE RAT

Michael P. Hefferan, Darren O'Rielly and Christopher W. Loomis, Anesthesiology 2003; 99:1180-1188

3.1 INTRODUCTION

Prostaglandins are synthesized in the spinal cord in response to repetitive C-fiber (noxious) input (Malmberg and Yaksh, 1992; Yaksh and Malmberg, 1993; McCormack, 1994a,b; Southall et al, 1998) an effect independent of their role in pain and inflammation in the periphery. This finding is consistent with the localization of cyclooxygenase and prostaglandin binding sites in spinal laminae known to receive nociceptive afferent input (Matsumura et al., 1995; Goppelt-Struebe and Beiche, 1997; Willingale et al., 1997; Vane et al, 1998), and the ability of i.t. non-steroidal anti-inflammatory drugs to block both nociceptive behaviours and the concurrent release of prostaglandins into spinal cerebrospinal fluid (Malmberg and Yaksh, 1995).

The facilitatory effect of spinal prostaglandins on nociceptive transmission (see review, Millan, 1999) suggests that prostaglandin synthesis early after nerve injury could be important in the development of allodynia; a neuropathic condition in which pain is evoked by a stimulus that does not normally evoke pain (e.g. cold breeze or light touch; Merskey, 1986). Indeed, studies of the effects of spinal prostaglandins on normal nociceptive neurotransmission provide clues as to how this could occur. Prostaglandins directly stimulate wide-dynamic range neurons in the rat dorsal horn (Baba et al., 2001),

sensitize these same neurons to noxious mechanical stimulation (Willingale et al., 1997), enhance glutamate and substance P release from primary afferent terminals in the spinal cord (Ferreira and Lorenzetti, 1996; Hingtgen et al., 1995), and reduce glycine-mediated inhibition in the spinal cord (Ahmadi et al., 2002).

Certainly, there is growing experimental evidence linking spinal prostaglandins to allodynia. Intrathecal prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) evoke robust allodynia in conscious mice and rats; an effect blocked by prostaglandin receptor antagonists (Ferreira and Lorenzetti, 1996; Uda et al., 1990) Minami et al., 1995; Minami et al., 1995). Allodynia induced by i.t. strychnine Yaksh, 1989) features brush-evoked increases in the concentration of PGE_2 in spinal dialysate (Milne et al., 2001) and attenuation by i.t. cyclooxygenase inhibitors (Hall et al., 1999). Bicuculline-induced allodynia is also blocked by cyclooxygenase inhibitors given locally to the affected spinal segments (Zhang et al, 2001). In the spinal nerve ligation model (Kim and Chung, 1992), significant up-regulation of the cyclooxygenase-2 protein has been reported in the spinal cord as early as one day after ligation (Zhao et al., 2000), and allodynia can be temporarily reversed by an i.t. cyclooxygenase inhibitor or EP receptor antagonist (see chapter 2; also Hefferan et al., 2003a). Experimental allodynia is also sensitive to spinal N-methyl D-aspartate (NMDA) receptor blockade (Zhang et al, 2001; Lee and Yaksh, 1995; Khandwala et al., 1997; Leung et al., 2001; Bennett et al., 2000; Hao et al., 1996; Minami et al, 2001). NMDA receptor activation, an essential trigger for central prostaglandin synthesis (Dirig and Yaksh, 1999), is not normally involved in low threshold neurotransmission (Urban et al., 1994). The emergence of both NMDA- and prostaglandin-properties in allodynia suggests a convergence of cellular

events by which low- and high-threshold input are processed. Thus, the enhancement of: a) glutamate release; b) NMDA-mediated Ca^{2+} influx; c) activation of cyclooxygenase; and d) prostaglandin synthesis may comprise synaptic events beginning immediately after nerve injury, which initiate the subsequent and more complex changes leading to permanent (prostaglandin-independent) allodynia.

In the present study, we used the rat spinal nerve ligation model to test the hypothesis that mechanical allodynia is comprised of an early spinal prostaglandin-dependent phase, the disruption of which prevents the establishment of delayed (prostaglandin-independent) allodynia.

3.2 MATERIALS AND METHODS

All studies were conducted in accordance with the guidelines of the Institutional Animal Care Committee of Memorial University of Newfoundland, St. John's, NF, Canada.

In Vivo Experiments

Animals: As described in section 2.2.

Intrathecal Catheterization: Intrathecal microdialysis catheters were constructed and implanted as described in section 2.2. Drugs were delivered intrathecally through a separate channel of the microdialysis catheter. Rats displaying normal motor behaviour were housed separately and allowed to recover for three days before spinal nerve ligation. A laminectomy was performed at the end of the experiments to visually confirm the

position of the i.t. catheter (except for rats used in the *in vitro* experiments where this was not feasible). No animal had to be excluded from the results because of an improperly positioned catheter.

Spinal Nerve Ligation: As described in section 2.2.

Microdialysis: As described in section 2.2

Testing: Quantification of mechanical allodynia was performed as described in section 2.2. The investigator (MPH) was blinded to the identity of the treatments throughout the study.

Drugs: Drugs, dilutions and injection techniques were similar to that described in section 2.2., except SC-51322 was not used here.

In Vitro Experiments

Tissue Preparation: Rats were anesthetized with urethane (1.5 g/kg intraperitoneal) and killed by decapitation. The spinal cord was removed immediately using the hydraulic technique (Meikle and Martin, 1981). After careful removal of the dural and arachnoid membranes, the lumbar region was visually identified, excised, and mounted on cutting blocks. The tissue was submersed in sucrose-modified artificial cerebrospinal fluid aerated with 95% O_2/5% CO_2 and sectioned using a vibratome.

Spinal cord slices (600-800 μm) were placed in artificial cerebrospinal fluid aerated with 95% O_2/5% CO_2 and stored at room temperature.

Determination of Glutamate Concentration: The basal and PGE_2-evoked release of glutamate from spinal cord slices was determined using a modified enzymatic assay (Nicholls et al., 1987; Bezzi et al., 1998). Briefly, the slices were transferred from the storage buffer to a 1 x 1 cm cuvette containing (mM): NaCl (120), KCl (3.1), NaH_2PO_4 (1.25), HEPES (25), glucose (4), $MgCl_2$ (1), $CaCl_2$ (2), glutamate dehydrogenase (40 U/ml; BioVectra, Charlottetown, Canada), and nicotinamide adenine dinucleotide phosphate (1); pH 7.4. The cuvette was placed in a temperature-controlled Shimadzu RF-1501 spectrofluorometer. The contents of the cuvette were continuously mixed using a magnetic stirrer and the temperature held at 37°C. HEPES buffer containing the tissue slice was oxygenated with 100% O_2 throughout the experiment. Glutamate released from the tissue was immediately oxidized to α-ketoglutarate by glutamate dehydrogenase thereby preventing neuronal reuptake of glutamate (Bezzi et al., 1998; Vesce et al., 1997). The reduced form of nicotinamide adenine dinucleotide phosphate generated from this reaction, was quantitated using spectrofluorometry (excitation: 335 nm; emission: 430 nm; delay <1 s). Prostaglandin E_2 (PGE_2; Biomol) was initially dissolved in ethanol and evaporated under 100% nitrogen gas. It was then re-dissolved in normal saline, diluted with the same to yield the desired concentrations, and added directly to the cuvette using a microsyringe. Each concentration of PGE_2 was

tested using a separate slice such that a full PGE_2 concentration-response curve was determined in each animal. Standard curves were prepared on each day of analysis.

Statistical Analysis: All data are reported as the mean ± SEM. Paw withdrawal thresholds are presented in grams. The concentration of PGE_2 in microdialysis samples ($[PGE_2]_{dialysate}$) is presented as either pmol/ml or the percent of baseline. Glutamate release is reported as pmol/min/mg protein. Statistical testing was performed using Sigmastat® 2.03 for Windows® (SPSS Inc., Chicago, USA). Pre- and post-treatment values were compared within each treatment group using one-way repeated measures analysis of variance followed by the Newman-Keuls test. Comparisons were also made across all drug- and vehicle-treated groups at each time point using one-way completely randomized analysis of variance followed by the Newman-Keuls test. Concentration-response analysis was performed using methods from Tallarida and Murray (1987). $P < 0.05$ was considered to be statistically significant.

3.3 RESULTS

Rats undergoing L5/L6 spinal nerve ligation displayed a significant decrease in paw withdrawal threshold (from ≥15 g to ≤4 g) beginning one day later (fig. 3.1A). This enhancement in mechano-sensitivity remained stable for at least 20 days, and was confined to the plantar surface of the hind paw ipsilateral to nerve ligation. In addition, the affected hind paw was often kept in an elevated and cupped position presumably to minimize contact with the cage floor. These rats were otherwise healthy, exhibiting

normal feeding behaviour and regular weight gain. The paw withdrawal thresholds of all sham-operated rats remained unchanged from pre-surgical values (fig. 3.1A). The basal concentration of PGE_2 in spinal dialysate samples, which ranged from 1.6 ± 0.4 to 4.1 ± 1.4 pmol/ml, was unchanged from pre-ligation values up to 20 days after nerve ligation (fig. 3.1B).

Brushing the plantar surface of the left hind paw had no effect on PGE_2 concentration prior to nerve ligation (fig. 3.2A). In contrast, brushing evoked a significant increase in PGE_2 concentration in spinal dialysate on days 1 (259 ± 59% of baseline; fig. 3.2B), 2 (257 ± 62%; fig. 3.3A), and 5 (199 ± 34%; fig. 3.3B) after nerve ligation as compared to baseline or the corresponding sham-control. PGE_2 concentration peaked during brushing and declined gradually (up to 30 min) thereafter. Brushing was without effect 10 and 20 days after nerve ligation (fig. 3.4A,B). The overall time-course of PGE_2 concentration in spinal dialysate from nerve-ligated and sham-operated rats is summarized in figure 3.5.

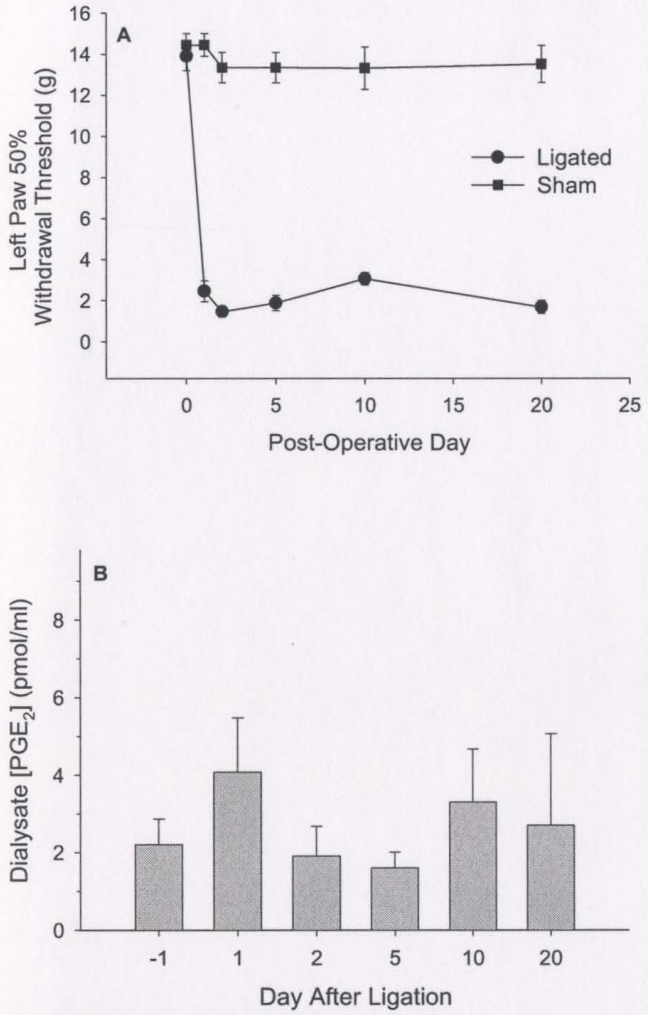

Figure 3.1 A) Left hind paw withdrawal thresholds of animals used in the microdialysis experiments. All values for the ligated group are significantly different from pre-ligation values ($p < 0.001$ for ligated group; $p=0.659$ for sham group). Each point represents mean ± SEM of 6-7 animals. B) Basal PGE_2 concentrations in spinal dialysate before and after nerve ligation. Spinal dialysate samples were collected immediately before brushing the left hind paw (see Methods). Each bar represents the mean ± SEM of 6-8 animals. There were no significant differences among the groups ($p=0.190$).

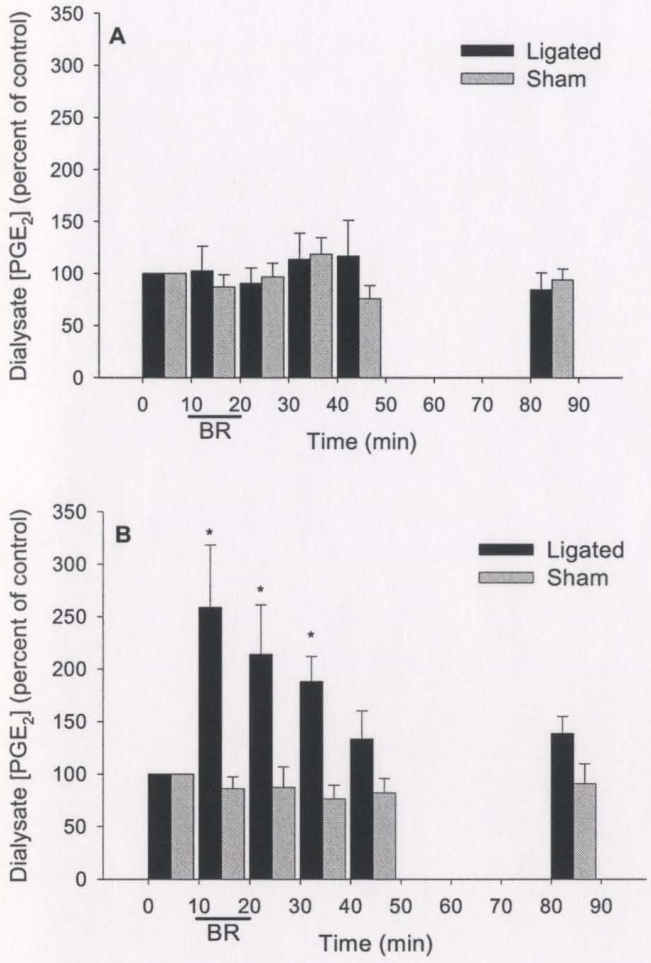

Figure 3.2. PGE_2 concentration in spinal dialysate before and after brushing (BR) the left hind paw of nerve-ligated or sham-operated rats. Before ligation, brushing of the plantar surface of the left hindpaw evoked no detectable increase in the PGE_2 concentration in spinal dialysate (A; p=0.889 for ligated group; p=0.219 for sham group). In contrast, 1 day after nerve ligation the same stimulus applied to the left hind paw evoked a significant increase in PGE_2 concentration compared to baseline or the corresponding sham controls (B; p=0.037 for ligated group; p=0.894 for sham group). Each bar represents the mean ± SEM of 6-7 animals.

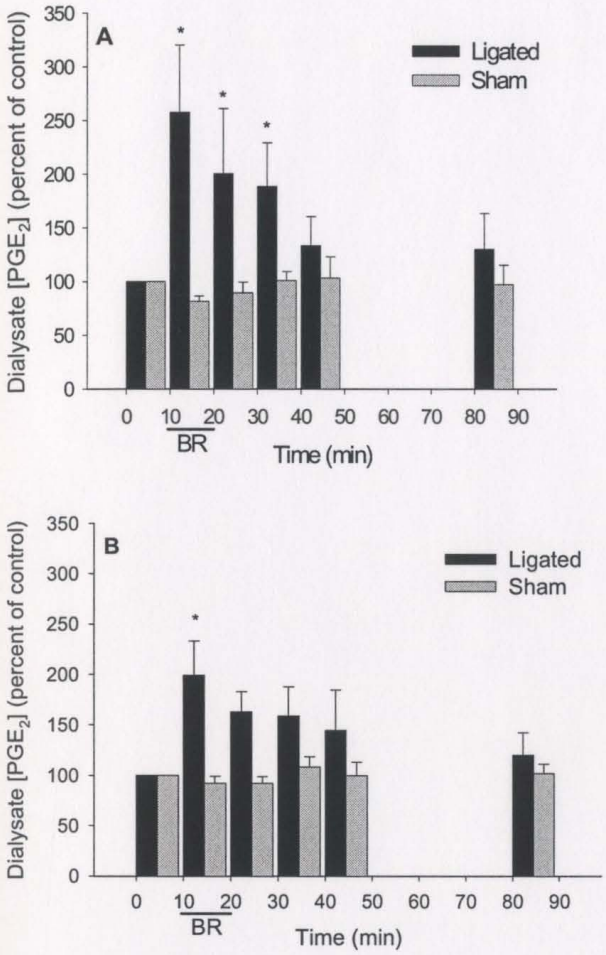

Figure 3.3. PGE_2 concentration in spinal dialysate before and after brushing (BR) the left hind paw of nerve-ligated or sham-operated rats. Brushing the left hind paw evoked a significant increase in PGE_2 concentration compared to baseline or the corresponding sham controls on day 2 (A; p=0.044 for ligated group; p=0.333 for sham group), and 5 (B; p=0.029 for ligated group; p=0.718 for sham group) after nerve ligation. Each bar represents the mean ± SEM of 6-7 animals.

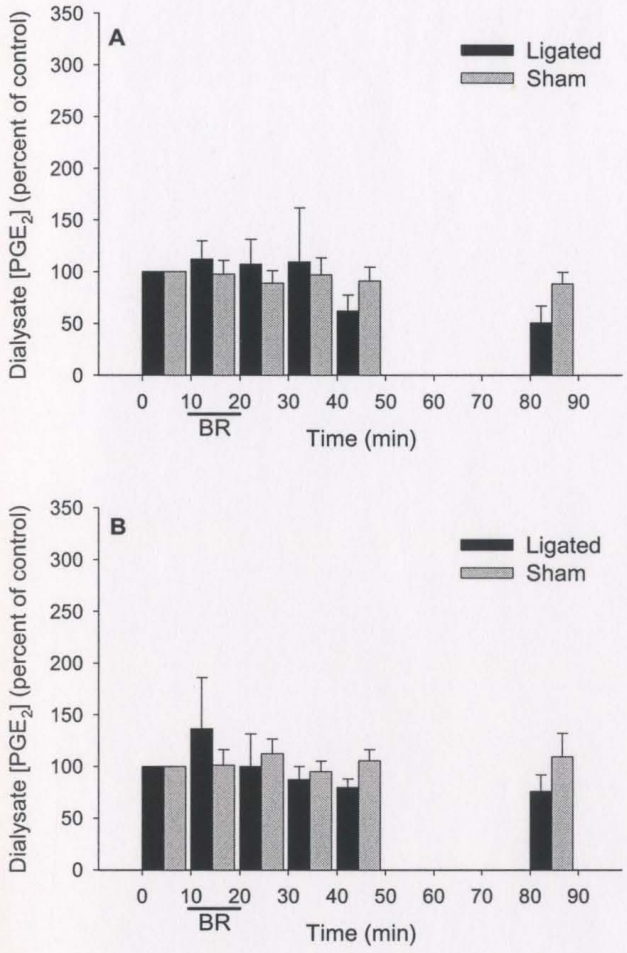

Figure 3.4. PGE_2 concentration in spinal dialysate before and after brushing (BR) the left hind paw of nerve-ligated or sham-operated rats. Brushing had no effect on days 10 (A; p=0.177 for ligated group; p=0.971 for sham group) or 20 (B; p=0.589 for ligated group; p=0.952 for sham group). Each bar represents the mean ± SEM of 6-7 animals.

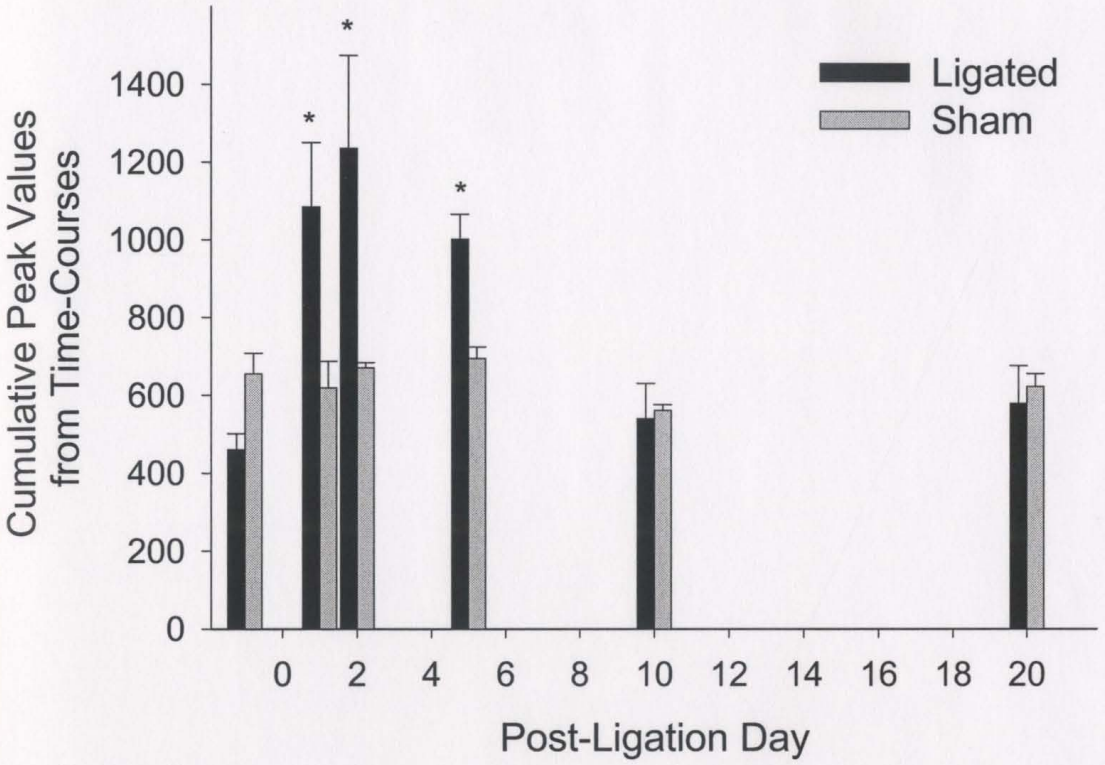

Figure 3.5. The time-course of the brush-evoked changes in spinal dialysate PGE_2 concentration from nerve-ligated or sham-operated rats. Each bar represents the sum of the $[PGE_2]_{dialysate}$ from 0-90 min (see fig. 3.2) for each experimental day. The brush-evoked changes in $[PGE_2]_{dialysate}$ on days 1, 2 and 5, but not 10 and 20 post-ligation, were significantly different from both pre-ligation and the corresponding sham control values (* p = 0.005 for ligated group; p=0.303 for sham group).

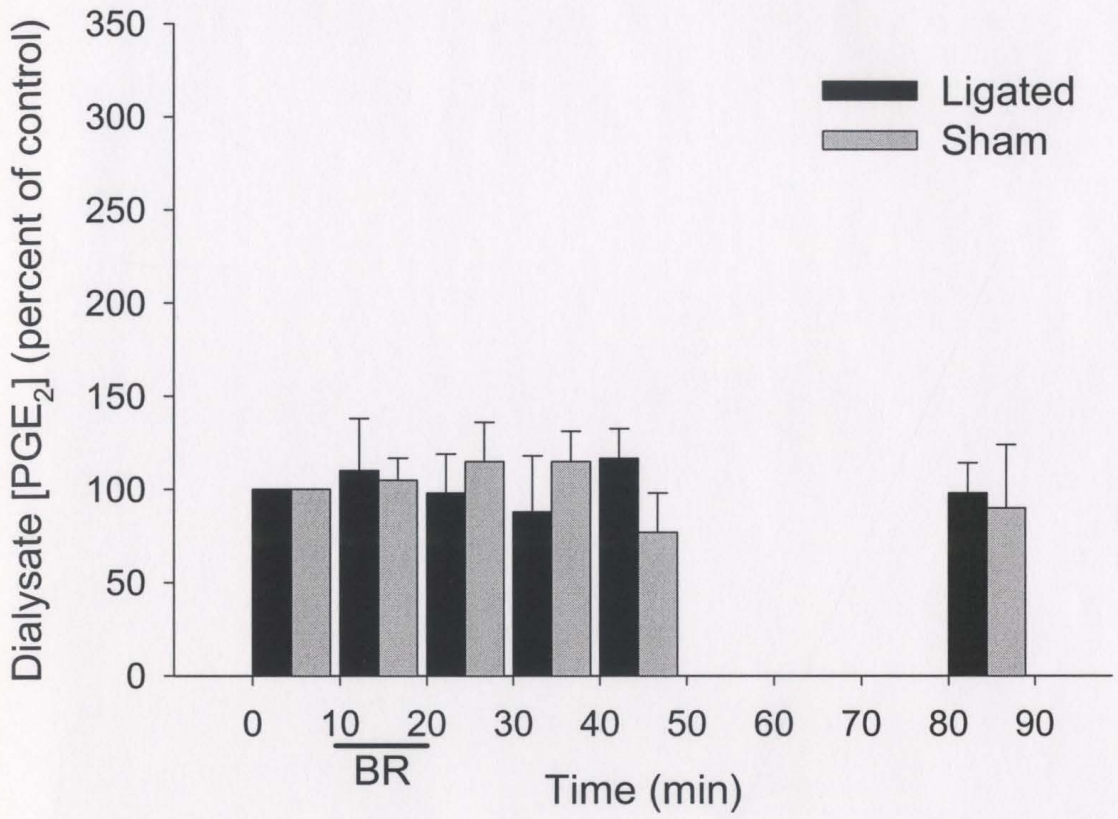

Figure 3.6. PGE_2 concentration in spinal dialysate before and after brushing (BR) the contralateral (right) hind paw of nerve-ligated or sham-operated rats 5 days after surgery. The $[PGE_2]_{dialysate}$ was unchanged from baseline in both groups ($p=0.947$ for ligated group; $p=0.741$ for sham group). Each bar represents the mean ± SEM of 6-7 animals.

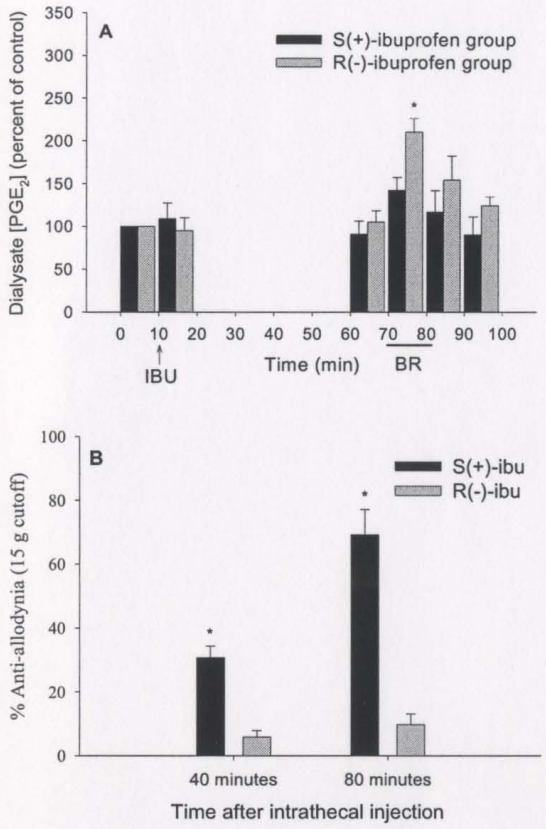

Figure 3.7. The effect of intrathecal S(+)- and R(-)-ibuprofen (IBU) on the brush (BR)-evoked change in PGE_2 concentration in spinal dialysate (A) and mechanical allodynia (B) using nerve-ligated and sham-operated rats. All experiments were performed 5 days after surgery, and each bar represents the mean ± SEM of 6-8 animals. In panel A, S(+)- or R(-)-ibuprofen (100 μg) was injected 60 min before brushing the left hind paw. Asterisks indicate a significant difference from baseline or the corresponding control ($p<0.001$ for ligated group; $p=0.309$ for sham group). In panel B, S(+)- or R(-)-ibuprofen (100 μg) was injected at time 0 followed by von Frey testing at 40, 60 and 80 min. Asterisks indicate a significant difference from R(-)-ibuprofen group ($p<0.001$ for both 40 min and 80 min groups).

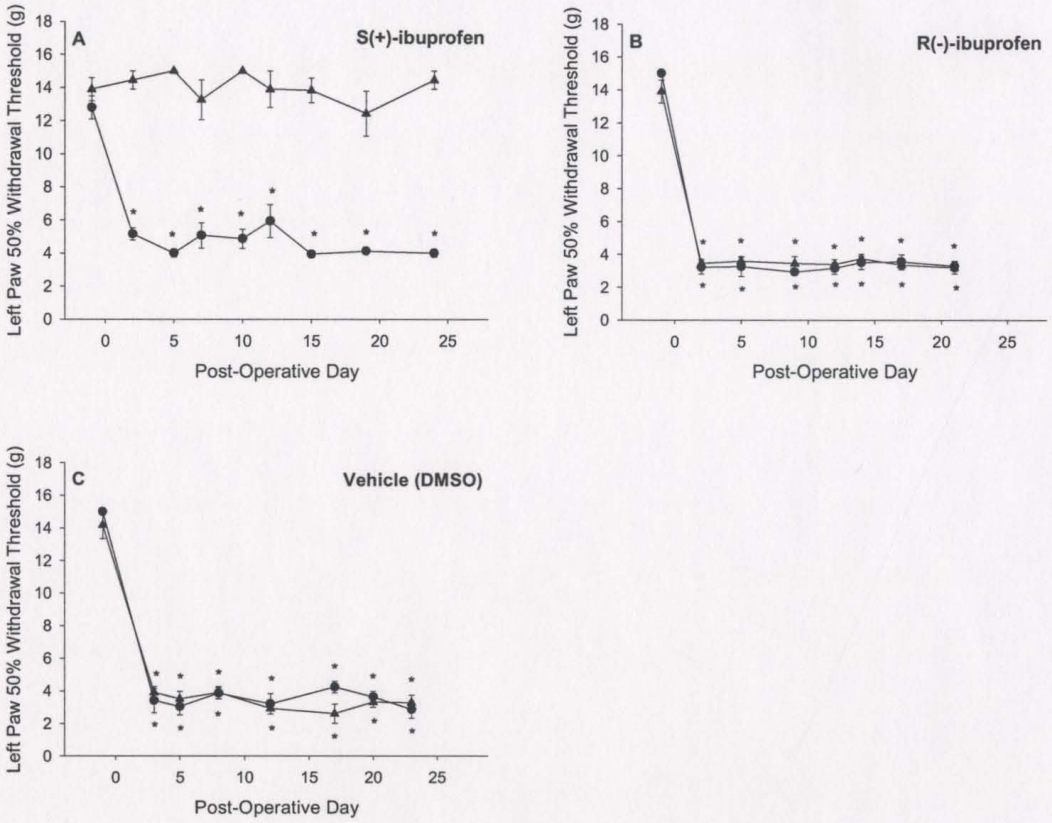

Figure 3.8. Left hind paw withdrawal thresholds up to 24 days after spinal nerve ligation. Animals either received 2 (4 hour treatment; •) or 4 (8 hour treatment; ▲) post-operative doses of 100 µg S(+)-ibuprofen (A), 100 µg R(-)-ibuprofen (B), or 5 µl dimethyl sulfoxide (C) given intrathecally beginning 2 h after spinal nerve ligation was completed. Each point represents the mean ± SEM of 6 animals. All values for the 4 h S(+)-ibuprofen treatment group are significantly different from pre-ligation values and the corresponding 8 h treatment group ($p<0.001$). No value for the 8 h S(+)-ibuprofen treatment group was different from the pre-ligation value ($p=0.429$). All values for the R(-)-ibuprofen ($p<0.001$ for 4h and 8h groups) and dimethyl sulfoxide ($p<0.001$ for 4h and 8h groups) treated groups were significantly different from pre-ligation values.

Brushing the contralateral (right) hind paw of either nerve-ligated or sham-operated rats had no effect on PGE_2 concentrations 5 days after surgery (fig. 3.6). Pretreatment with i.t S(+)-ibuprofen (100 μg), a dose that reversed tactile allodynia 5 days after nerve ligation (fig. 3.7B), significantly attenuated the brush-evoked increase in PGE_2 concentration in nerve-ligated rats (fig. 3.7A). R(-)-ibuprofen (100 μg) had no effect on either the brush-evoked concentration of PGE_2 (fig. 3.7A) or tactile allodynia (fig. 3.7B).

To test the relevance of spinal prostanoid synthesis to allodynia, separate groups of rats were treated with 100 μg of i.t. S(+)-ibuprofen, R(-)-ibuprofen, or vehicle beginning 2 h after spinal nerve ligation (fig. 3.8, 3.9). Rats treated with i.t. S(+)-ibuprofen over an 8-h period (4 i.t. injections of 100 μg given every 2h) exhibited normal posture (no cupped hind paw) and paw withdrawal thresholds (≥15g) for at least 24 days after nerve injury. Brushing the ipsilateral hind paw had no effect on the $[PGE_2]_{dialysate}$ in these same animals (fig. 3.9A/B).

In contrast, ligated rats treated with i.t. S(+)-ibuprofen over 4h (100 μg every 2h for a total of 2 injections; fig. 3.8A), R(-)-ibuprofen over 4 h or 8 h (100 μg given every 2 h for a total of 2 or 4 injections, respectively; fig. 3.8B), or vehicle using the same treatment schedules (fig. 3.8C) developed allodynia that was indistinguishable from untreated, ligated rats in terms of paw withdrawal threshold (Figure 1A), and brush-evoked changes in behaviour and $[PGE_2]_{dialysate}$ (fig. 3.9A-C). Area-under-the-curve analysis revealed no difference ($p=0.65$) in the brush-evoked $[PGE_2]_{dialysate}$ of the R(-)-ibuprofen treated group (fig. 3.9B) versus ligated, untreated rats (fig. 3.3A). The ability

of brushing to increase $[PGE_2]_{dialysate}$ in the R(-)-ibuprofen (allodynic) group was lost by day 10 (fig. 3.9C), similar to that in ligated, untreated (allodynic) rats (fig. 3.4A).

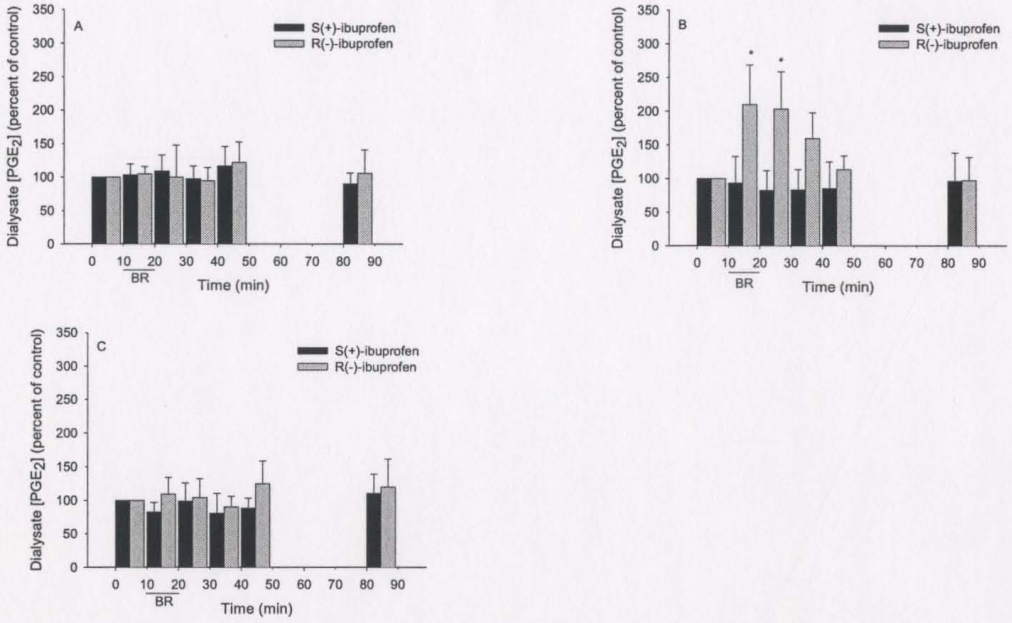

Figure 3.9. PGE_2 concentration in spinal dialysate before and after brushing (BR) the left hind paw of nerve-ligated rats. Before ligation, BR of the plantar surface of the left hindpaw evoked no detectable increase in the PGE_2 concentration in spinal dialysate (A; p=0.954 for S(+)-ibuprofen group; p=0.987 for R(-)-ibuprofen group). In contrast, on day 2 post-ligation, the same stimulus applied to the left hind paw of rats treated post-operatively with R(-)-ibuprofen evoked a significant increase in PGE_2 concentration compared to baseline or the corresponding S(+)-ibuprofen-treated animals (B; * p=0.010); no detectable increase was observed in rats treated post-operatively with S(+)-ibuprofen (B; p=0.555). Brushing had no effect on day 10 in either group (C; p=0.912 for S(+)-ibuprofen group; p=0.966 for R(-)-ibuprofen group). Each bar represents the mean ± SEM of 6-7 animals.

To determine if allodynic animals also exhibit pharmacodynamic changes to the response evoked by spinal prostaglandins, separate groups of rats undergoing spinal nerve ligation were treated with i.t. R(-)- or S(+)-ibuprofen over 8 h (100 μg given every 2 h for a total of 4 injections). The concentration-response effect of PGE_2 on glutamate release was compared using spinal cord slices prepared from allodynic and non-allodynic animals. PGE_2 evoked release from the slices of sham-operated [non-allodynic] rats in a concentration-dependent manner, resulting in a bell-shaped concentration-response curve (fig. 3.10; Table 3.1). Whereas the PGE_2 concentration-response curves of ligated [untreated] and ligated [R(-)-ibuprofen treated] rats displayed dramatic leftward shifts from control, there was no difference between sham-operated [non-allodynic] and ligated [S(+)-ibuprofen (8 h) treated, non-allodynic] rats (fig. 3.10; Table 3.1).

Table 3.1. EC_{50} values (95% confidence intervals) of PGE_2 on Glutamate Release from Spinal Cord Slices

Sham-operated (non-allodynic)	**S(+)-ibuprofen treated (non-allodynic)**
$2.4x10^{-11}$ ($9.3x10^{-12}$ – $6.1x10^{-11}$) M	$1.1x10^{-11}$ ($5.0x10^{-12}$ – $2.5x10^{-11}$) M
Ligated (allodynic)	**R(-)-ibuprofen treated (allodynic)**
$8.9x10^{-15}$ ($4.3x10^{-15}$ – $1.9x10^{-14}$) M	$2.0x10^{-14}$ ($1.1x10^{-14}$ – $3.7x10^{-14}$) M

Note: All animals underwent L5/L6 spinal nerve ligation except the sham-operated group. Spinal cord tissue was harvested from rats 3 days after spinal nerve injury.

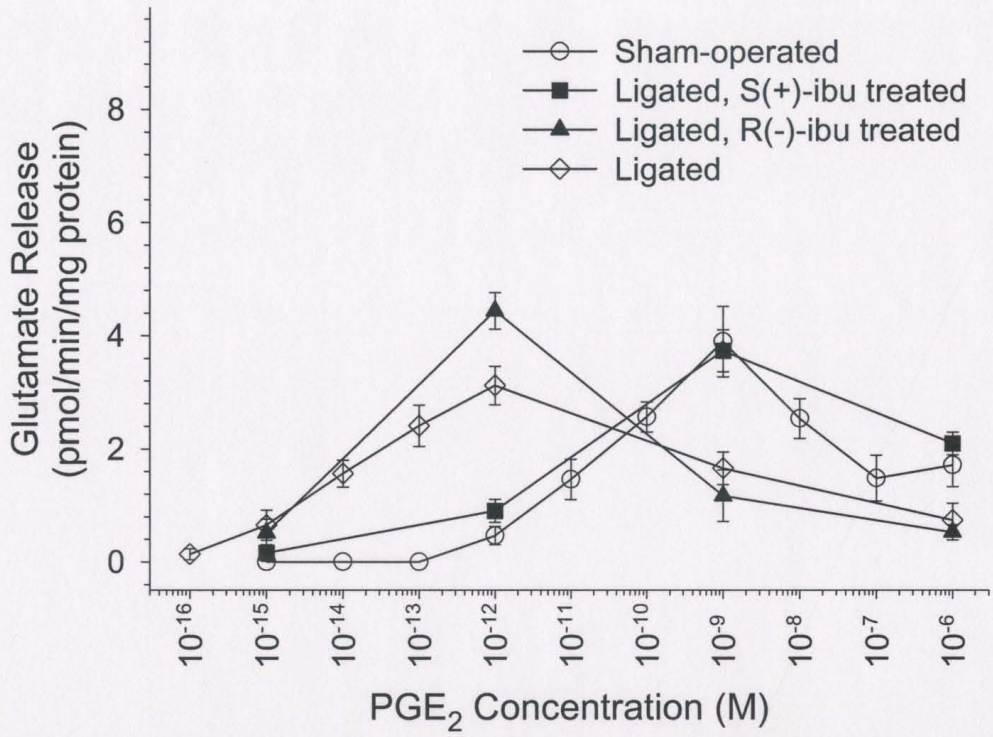

Figure 3.10. PGE_2-evoked glutamate release using spinal cord slices from sham-operated and nerve-ligated rats 3 days after surgery. The addition of PGE_2 produced concentration-dependent glutamate release from spinal cord slices of sham-operated (non-allodynic) animals (see Table 3.1 for EC_{50} and 95% confidence intervals of each group). This effect was significantly enhanced (leftward shift) in slices from ligated (allodynic) rats. The concentration-response curve using slices from ligated rats treated with S(+)-ibuprofen (4 intrathecal doses of 100 μg every 2 h beginning 2 h after ligation), which prevented allodynia (see fig. 3.8), was not significantly different from the sham-operated group. In contrast, slices from ligated rats treated with R(-)-ibuprofen, which had no effect on allodynia (see fig. 3.8) yielded a concentration-response curve that was not significantly different from ligated, untreated (allodynic) rats. Each point represents the mean ±SEM of at least 5 animals (5-14 slices).

To determine whether this protective effect resulted from early cyclooxygenase-1 and/or cyclooxygenase-2 inhibition, rats were treated with i.t. SC-560 or SC-236 (100 μg) beginning 2 h after spinal nerve ligation. Paw withdrawal thresholds in ligated rats treated with either an 8h regimen of i.t. SC-560 were slightly but not significantly decreased compared to pre-ligation values for up to 20 days (fig. 3.11A). For the 4 h treatment, paw withdrawal thresholds on days 2 and 5 were statistically different from pre-ligation values but never fell below 9 g. In contrast, animals treated with i.t. SC-236 (4 or 8 h regimen) displayed a time course of paw withdrawal threshold (fig. 3.11B) unchanged from ligated, untreated rats (fig. 3.1A); all time points were significantly different from pre-ligation values.

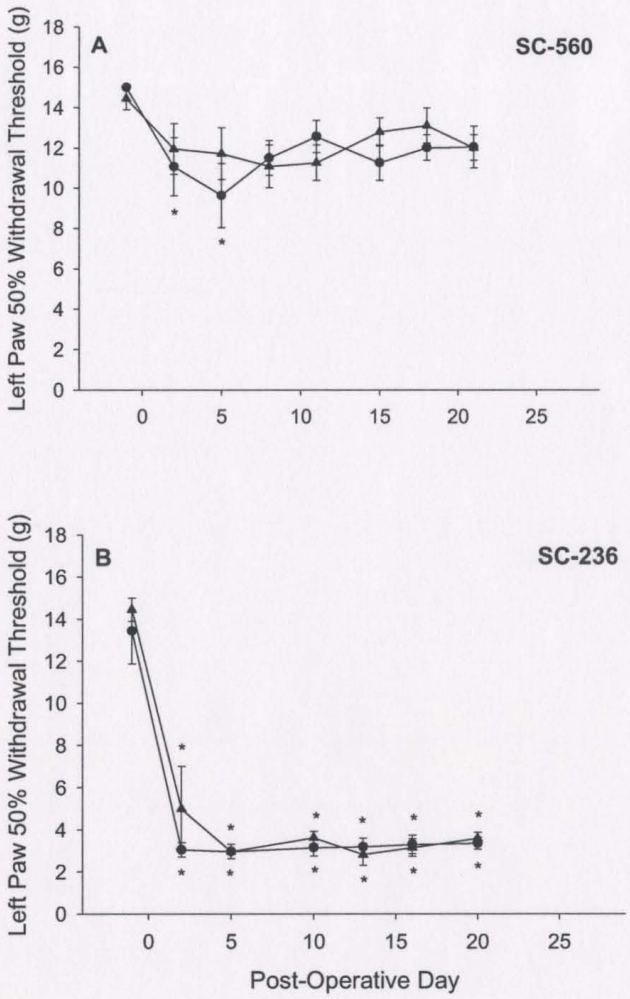

Figure 3.11. Left hind paw withdrawal thresholds up to 21 days after spinal nerve ligation. Animals either received 2 (4 hour treatment; ●) or 4 (8 hour treatment; ▲) post-operative doses of 100 µg SC-560 (A) or 100 µg SC-236 (B) given intrathecally beginning 2 h after spinal nerve ligation. Each point represents the mean ± SEM of 6 animals. Asterisks indicate significant difference from pre-ligation values (p =0.013 for 4h SC-236 group; p=0.009 for 8h SC-236 group; p=0.022 for 4h SC-560 group; p=0.235 for 8h SC-560 group).

3.4 DISCUSSION

Ligation of the left L5/L6 spinal nerves produced a robust sensitization to innocuous mechanical stimulation on the plantar surface of the left hind paw beginning 1 day later. This was indicated by the: 1) marked decrease in the paw withdrawal threshold (<4 g); 2) brush-evoked nociceptive-like behaviour; and 3) concurrent stimulus-evoked increase in $[PGE_2]_{dialysate}$. The area of sensitization remained highly circumscribed over time, and was absent in sham-operated controls. Brushing the hind paws of sham-operated animals, or outside the affected dermatomes (including the contralateral hind paw) of ligated rats was without effect. These results indicate that prostanoid synthesis is recruited in the spinal cord of L5/L6 nerve-ligated animals: a response that appears to be triggered by brushing (a stimulus that elicited allodynic behaviour in the same animals during the prostaglandin-dependent phase of allodynia). This brush-evoked release of PGE_2, which declines by day 5, was absent on day 10 even though brush-evoked allodynia persisted for up to 20 days independent of any detectable change in $[PGE_2]_{dialysate}$.

The connection between allodynia and spinal prostaglandins appears to involve more than just the emergence of brush-evoked prostanoid synthesis. There was also a marked decrease in the EC_{50} of PGE_2-evoked glutamate release in slices prepared from the affected (L5/L6) segments of allodynic rats compared to either thoracic slices from the same animals (data not shown), or slices of L5/L6 spinal cord from sham-operated controls. The concentration-response curve for PGE_2-evoked glutamate release was bell-shaped, similar to that reported by Nishihara et al. (1995) using spinal cord

synaptosomes and by Uda and collegues (1990) who reported this phenomenon with prostaglandin-evoked nociceptive behavior in mice and by that. Whether this decrease in response to high PGE_2 concentrations has physiological importance is yet to be determined. Regardless, glutamate has has a recognized role in neuropathic states, including the NMDA-receptor coupled activation of phopholipase A_2, NOS, COX, and various kinases (Yaksh et al., 1999; Yaksh, 1999; Yang et al., 1996), many of whose products diffuse into the extracellular space to enhance neurotransmitter release. The mechanism(s) underlying the increased potency of PGE_2 are currently being investigated but could include the amplification of spinal prostaglandin synthesis in nerve-injured segments where increased expression of cyclooxygenase-2 has been shown to occur (Zhao et al., 2000). Such a pharmacodynamic change in the affected spinal segments of demonstrably allodynic animals has not, to our knowledge, been previously reported.

Allodynia and spinal prostaglandins appear to be functionally linked in the early post-injury period. Brush-evoked spinal PGE_2 release and allodynia were significantly attenuated by i.t. S(+)-ibuprofen (a cyclooxygenase-1/cyclooxygenase-2 inhibitor), but not R(-)-ibuprofen, in the spinal nerve ligation model (see chapter 2; also Hefferan et al., 2003a). The relevance of spinal prostaglandins to the development of allodynia is further strengthened by the results of the present study. When given early after ligation, all rats treated with 100 μg of S(+)-ibuprofen developed none of the characteristic features of allodynia for up to 25 days. Paw withdrawal thresholds remained normal (≥15 g), brushing had no effect on the $[PGE_2]_{dialysate}$, and there was no change in PGE_2-evoked glutamate release from spinal cord slices, compared to sham-operated controls. In

contrast, R(-)-ibuprofen, an inactive cyclooxygenase inhibitor and control treatment in these experiments, had no protective effect whatsoever. R(-)-ibuprofen has been shown to inhibit the transcription factor, NFκB *in vitro* (Scheuren et al., 1998; Grilli et al., 1996); a factor known to regulate the expression of cyclooxygenase-2 in macrophages and human gingival fibroblasts (Mestre et al., 2001; Nakao et al., 2000). To the extent that up-regulation of cyclooxygenase-2 after injury is important in the establishment of allodynia (a factor yet to be confirmed), then the inhibition of NFκB by R(-)-ibuprofen would be predicted to attenuate allodynia. No such effect was observed but further studies are required to address this possibility. Overall, the results of these experiments support the hypothesis that spinal prostaglandins are critical factors in the initiation of changes (e.g. central synaptic excitability and neuronal sensitization) leading to both prostaglandin-dependent and prostaglandin-independent allodynia. The protective effect of S(+)-ibuprofen was also dependent on the duration of treatment (only the 8 h regimen was effective) suggesting that prostaglandin synthesis must be inhibited for a minimum period of time following ligation to effectively prevent/interrupt the events leading to allodynia. The results of this study are in general agreement with and build upon the preliminary work of Zhao et al. (2000) who showed that the non-selective cyclooxygenase inhibitor, indomethacin, given spinally 2 h after spinal nerve ligation, partially attenuated the development of allodynia for up to 4 weeks in the rat.

To extend this work, cyclooxygenase-1 and cyclooxygenase-2 selective inhibitors were utilized to provide information about the isozyme(s) catalyzing spinal prostaglandin synthesis in the nerve-injured cord. The doses of SC-236 (cyclooxygenase-2 selective)

and SC-560 (cyclooxygenase-1 selective) were chosen from previous dose-response studies in our laboratory (see chapter 2; also Hefferan et al., 2003a). In that study, we showed a dramatic difference in the inhibitory effect of SC-236 and SC-560 on established allodynia using near equi-molar i.t. doses suggesting that isozyme-selectivity is maintained at the doses used. In the present study, the contrast in effects with SC-560 and SC-236 was also striking. Whereas SC-560 (both 4 and 8 h treatments) prevented the emergence of all features of allodynia, an effect comparable to that of S(+)-ibuprofen (8h treatment), SC-236 (4 or 8 h treatment) was totally ineffective. These results indicate that cyclooxygenase-1, known to be constitutively expressed in the spinal cord (Vanegas and Schaible, 2001), catalyzes the synthesis of prostaglandins in the period immediately following ligation. It is this early synthesis that appears necessary for the development of both prostaglandin-dependent and prostaglandin-independent allodynia. This early role for COX-1 is also suggested by the work of Zhu and Eisenach (2003) who report increases in COX-1 IR within 4 days after peripheral nerve injury. In contrast, inducible cyclooxygenase-2, which is known to be up-regulated 24h after ligation (Zhao et al., 2001), appears to be important in maintaining prostaglandin-dependent allodynia; a stage when cyclooxygenase-1 inhibitors are largely ineffective (see chapter 2; also Hefferan et al., 2003a). Thus, cyclooxygenase-2 may need to achieve sufficient expression over time. The differential localization of constitutive cyclooxygenase-1 and constitutive cyclooxygenase-2 reported in primary afferent terminals of the rat (Willingale et al. 1997) might also explain the early sensitivity to cyclooxygenase-1 inhibitors.

An obvious question arising from these experiments is whether allodynia would have developed without drug treatment (e.g. unsuccessful nerve ligation). This outcome

is unlikely considering that the protective effect was stereo-specific, cyclooxygenase-1 selective, and treatment duration-dependent. Moreover, greater than 90% of naïve animals undergoing L5/L6 spinal nerve ligation in our laboratory develop allodynia within 1 day of surgery; a result comparable to that reported by Chaplan et al. (1994). Thus, the uniform failure of nerve ligation within an experimental group is improbable and inconsistent with the results of this study. These data indicate that treatment with spinal cyclooxygenase inhibitors early after ligation prevents, or at the very least significantly delays (>25 days), the emergence of prostaglandin-dependent and prostaglandin-independent allodynia.

Figure 3.12 is a proposed model of the early (synaptically-mediated) events effecting prostaglandin-dependent and prostaglandin-independent allodynia following L5/L6 spinal nerve ligation. Glutamate, released from the central terminals of primary afferent fibres beginning immediately after nerve injury (Kawamata and Omote, 1996; Farooque et al., 1996), activates post-synaptic NMDA receptors; an essential step in central sensitization and allodynia (Kajander et al., 1992; Yoon et al., 1996). Subsequent intracellular events, including a rise in Ca^{2+} concentration and the release of arachidonic acid via PLA_2, trigger the early synthesis of spinal prostaglandins by constitutive enzymes (cyclooxygenase-1>cyclooxygenase-2). This immediate release of prostaglandins following injury is probably represented by the near 2-fold increase in resting $[PGE_2]_{dialysate}$ one day after injury (see fig. 3.1b), although this increase did not reach statistical significance ($p=0.190$). Upon diffusion to the extracellular space, spinal prostaglandins enhance the excitability of adjacent cells (Willingale et al., 1997; Baba et al., 2001), and feed back to reinforce glutamate release (Ferreira and Lorenzetti, 1996;

Hingtgen et al., 1995; Vasko et al., 1994). Sustained NMDA receptor activation initiates the expression of inducible cyclooxygenase-2 (Vanegas and Schaible, 2001), thereby enhancing the capacity for stimulus-evoked prostaglandin synthesis in the spinal cord (cyclooxygenase-2>cyclooxygenase-1) and the emergence of prostaglandin-dependent allodynia (see chapter 2; also Hefferan et al., 2003a,b). Over the next few weeks, prostaglandin-dependent allodynia recedes leaving long-term, prostaglandin-independent allodynia (Lashbrook et al., 1999 ; Zhao et al., 2000). The latter may reflect phenotypic changes in primary afferents, sprouting of primary afferents into outer laminae, and/or altered gene regulation (Wakisaka et al., 1992; LaMotte et al., 1991; Woolf et al., 1992; Herdegen et al., 1992; Cameron-Curry et al., 1991). The ability of cyclooxygenase inhibitors to prevent the emergence of both prostaglandin-dependent and prostaglandin-independent allodynia strongly suggests that these two states are related.

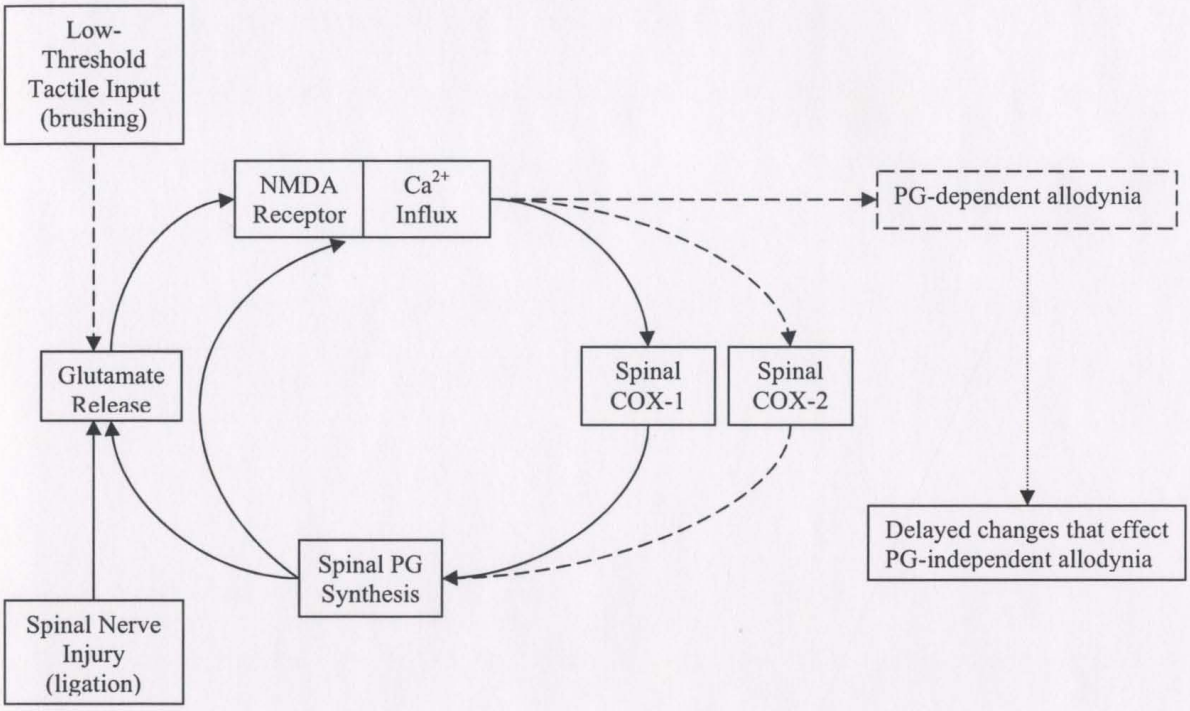

Figure 3.12. A proposed model of the early (synaptically-mediated) events in the spinal cord effecting allodynia following L5/L6 spinal nerve ligation. Glutamate, released from the central terminals of primary afferent fibres beginning immediately after ligation, activates post-synaptic NMDA receptors. Subsequent intracellular events (omitted for clarity), including a rise in the Ca^{2+} concentration and activation of PLA_2, trigger the early synthesis of spinal prostaglandins by constitutive activity (cyclooxygenase-1>cyclooxygenase-2). In the extracellular space, spinal prostaglandins diffuse to adjacent cells to enhance glutamate release and cell excitability (post-synaptic effect). Sustained NMDA receptor activation initiates the delayed expression of inducible cyclooxygenase-2 in the spinal cord (——). Within 24 h of ligation, central sensitization combined with the enhanced

capacity for prostaglandin synthesis (cyclooxygenase-2>cyclooxygenase-1) results in the emergence of temporary, prostaglandin-dependent allodynia. Over the next few weeks (·····), prostaglandin-dependent allodynia recedes leaving long-term allodynia that is independent of spinal prostaglandins. This may reflect phenotypic changes in primary afferents, sprouting of primary afferents into outer laminae, altered gene regulation, and changes in expression of critical receptors, ion channels, and/or enzymes.

In conclusion, the results of this study provide evidence that spinal prostaglandins, synthesized immediately after spinal nerve ligation, are critical in the development of prostaglandin-dependent and prostaglandin-independent allodynia, and that their early pharmacological disruption affords protection against this neuropathic state. They build upon and are consistent with previous work demonstrating the prostaglandin-dependent nature of allodynia in this model (see chapter 2; also Zhao et al., 2000; Hefferan et al., 2003a), but are seemingly at odds with anecdotal reports that non-steroidal anti-inflammatory drugs provide little, if any, benefit to patients with allodynia. How then can this apparent contradiction be resolved? Our data indicate that the timing of treatment, the type of cyclooxygenase inhibitor used, and perhaps the route of administration, are critical factors influencing the effectiveness of non-steroidal anti-inflammatory drugs, not in relieving neuropathic pain *per se,* but in preventing its emergence. They suggest that non-steroidal anti-inflammatory drugs would need to be given early (hours) after injury, following which their clinical value would decline. The latter prediction is seemingly consistent with current clinical experience. It should also

be noted that since the current research examined evoked nociceptive responses, not indices of chronic pain, it is possible that the clinical implications for the results would only benefit patients with evoked, and not chronic pain. While it is clear that further research is needed to clarify these issues, the potential implications of this research for clinical neuropathic pain are exciting.

4.0 INTERACTION OF SPINAL NITRIC OXIDE AND PROSTAGLANDINS AFTER L5/L6 SPINAL NERVE LIGATION IN THE RAT: AN ISOBOLOGRAPHIC ANALYSIS

Michael P. Hefferan and Christopher W. Loomis, Anesthesiology 2004; 100:1611-1614

4.1 INTRODUCTION

Spinal prostaglandins appear to be important in the pathogenesis and early maintenance (hours-days after nerve injury) of experimental allodynia (see chapters 2 and 3; also Parris et al., 1996; Zhao et al., 2000; Hefferan et al., 2003a,b). This relationship is consistent with: a) cyclooxygenase and prostanoid receptor localization in the outer laminae of the spinal dorsal horn (Beiche et al., 1996; Willingale et al., 1997; Vane et al., 1998); b) exogenous prostaglandins (i.e. i.t.) eliciting allodynia-like behaviour in otherwise normal animals (Uda et al., 1990; Minami et al., 1995; Ferreira SH, Lorenzetti, 1996); and c) brush-evoked allodynia and spinal PGE_2 release in nerve-injured, but not sham-operated, rats (see chapters 2 and 3; also Hefferan et al, 2003a,b). It is also supported by recent studies showing that i.t. cyclooxygenase inhibitors, given 2-8 h after L5/L6 spinal nerve ligation, prevented the development of allodynia for at least 20 days in the rat (see chapter 3; also Zhao et al., 2000; Hefferan et al., 2003b). A similar connection between spinal NOS and neuropathic pain has emerged (Hao and Xu, 1996; Yoon et al., 1998; Dolan and Nolan, 1999; Park et al., 2000; Milne et al., 2001), including the increased expression of neuronal NOS in the spinal cord after L5/L6 spinal nerve ligation (Dolan and Nolan, 1999). These reports, combined with the apparent regulation of cyclooxygenase expression and prostaglandin synthesis by NO (Bredt and

Snyder, 1992; Mollace et al., 1995; Haley et al., 1992; Garthwaite et al., 1988), raise questions about the interaction between spinal NO and prostaglandins in abnormal pain states. The nature of this interaction could have significant implications in the treatment of neuropathic pain. An additive or a synergistic interaction would most likely require different treatment regimens. In the present study, we investigated this interaction using selective NOS- and cyclooxygenase-inhibitors in the L5/L6 spinal nerve ligation model.

4.2 METHODS

All studies were conducted in accordance with the guidelines of the Institutional Animal Care Committee of Memorial University of Newfoundland, St. John's, NF, Canada.

Animals: As described in section 2.2.

Intrathecal catheterization: As described in section2.2.

Spinal Nerve Ligation: As described in section2.2.

Testing: Quantification of mechanical allodynia and drug-induced changes in paw withdrawal thresholds were performed as described in section 2.2.

Experimental Protocols: Dose-response curves for S(+)-ibuprofen and L-NAME were determined in separate groups of rats. For combination studies, a molar ratio of 1:5 was used based on the ED_{40} values of each agent [S(+)-ibuprofen=87 nmol (18 μg); L-NAME=445 nmol (120 μg)] (see chapter 2; also Hefferan et al., 2003a). Dose ratios of S(+)-ibuprofen:L-NAME were: 10:50, 30:150, 100:500, or 300:1500 nmol (2:13, 6:40, 21:135, or 62:404 μg, respectively). Each drug was delivered intrathecally,

with a 20-min delay between S(+)-ibuprofen and L-NAME (to correct for the difference in the time of peak effect). In a separate experiment, the order was reversed to control for the sequence of administration. S(+)-ibuprofen (active stereo-isomer) was dissolved in 100% DMSO and diluted with saline upon injection for a final DMSO concentration of 50%; L-NAME and aminoguanidine were dissolved in saline and diluted with the same. All drugs were purchased from Research Biochemicals International (Natick, MA, USA).

Data Analysis: As described in section 2.2.

4.3 RESULTS

Tight ligation of the L5/L6 spinal nerves produced touch-evoked allodynia manifested as a significant reduction in the PWT (from ≥15 g to 1-3 g) of the ipsilateral hind paw, and posturing to protect the sensitized area. PWTs in all sham-operated rats remained unchanged from pre-surgical values (≥15 g).

Intrathecal L-NAME produced dose-dependent but incomplete reversal of allodynia (57% of maximum) over the 30-300 μg range. Figure 4.1A shows the time-course of paw withdrawal threshold following the i.t. injection of L-NAME. The onset of effect was 20-40 min after injection and peaked at 40-60 min. Dose-response analysis using the peak effect of each dose yielded a linear relationship over the range of 30-300 μg (fig. 4.1B); no change in efficacy of L-NAME was noted over the 2-12 day experiment period. A dose of 1000 μg yielded no further reversal of allodynia (data not shown). Intrathecal aminoguanidine (an inhibitor of iNOS; 30-300 μg) was largely ineffective in reversing allodynia (fig. 4.2A/B). Neither lumbar injections of D-NAME

(100 μg; fig. 4.1A/B), mid-cervical injections of L-NAME (100 μg; fig. 4.1B), DMSO nor saline had any effect on PWT in ligated rats (see chapter 2; also Hefferan et al., 2003a). While not systematically tested, no drug tested seemed to elicit adverse motor effects, with injected rats having normal gait and level of alertness.

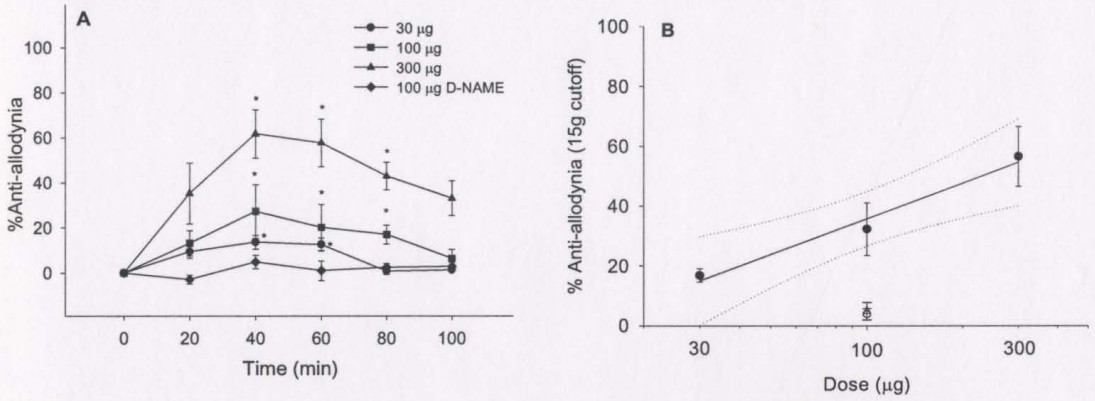

Figure 4.1. Intrathecal L-NAME dose-dependently reversed allodynia in the L5/L6 spinal nerve ligation model. Time-course (A) and dose-response curve (B) for intrathecal L-NAME. In panel A, each asterisk indicates a significant difference from control (time 0; $p<0.001$ for 30 μg group; $p=0.009$ for 100 μg group; $p=0.003$ for 300 μg group). Each point represents the mean ± SEM of 6-8 animals. Dose-response experiments were conducted 2-12 days after nerve ligation. Drug-induced changes in left-paw withdrawal thresholds are expressed as % anti-allodynia (see Methods). Dashed lines indicate the 95% confidence intervals. The ED_{50} (95% confidence intervals) of L-NAME = 212 (112-402) μg. The effects of intrathecal D-NAME (○; lumbar injection) and L-NAME (▽; mid-cervical injection) are also shown as single points.

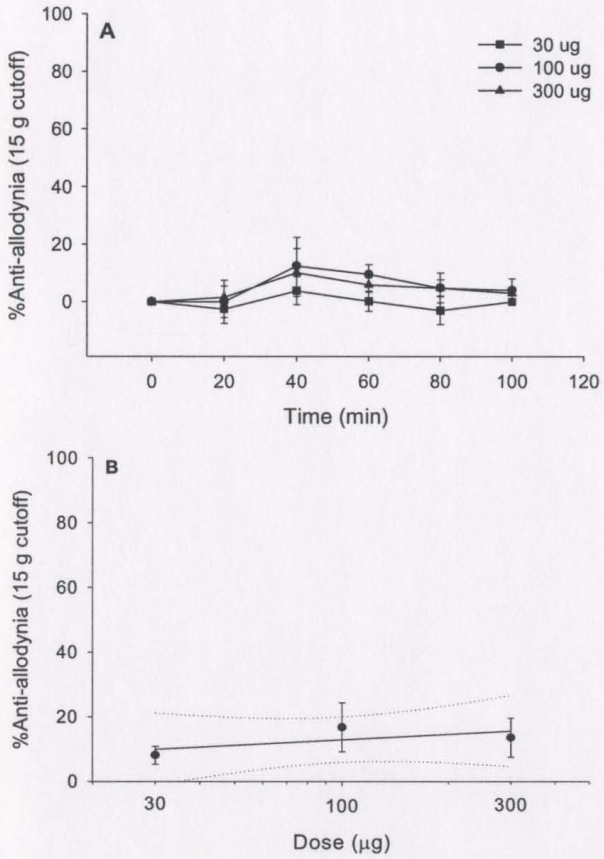

Figure 4.2. Intrathecal aminoguanidine had no dose-dependent effect on allodynia in the L5/L6 spinal nerve ligation model (p=0.795 for 30 μg group; p=0.610 for 100 μg group; p=0.597 for 300 μg group). Time-course (A) and dose-response curve (B) for intrathecal aminoguanidine. Each point represents the mean ± SEM of 4-5 animals. Dose-response experiments were conducted 2-12 days after nerve ligation. Drug-induced changes in left-paw withdrawal thresholds are expressed as % anti-allodynia (see Methods). Dashed lines indicate the 95% confidence intervals. The slope of the line was not significantly different from zero (p=0.589).

The combination of i.t. S(+)-ibuprofen and L-NAME attenuated allodynia in a dose-dependent manner, irrespective of treatment order. Figure 4.3A shows sample time-courses of both injections orders; the dose-response curves were statistically indistinguishable. S(+)-ibuprofen significantly reduced the ED_{50} of L-NAME from 212 to 69 μg (L-NAME followed by ibuprofen), and increased the maximum anti-allodynic effect to 89% (fig. 4.3B). Isobolographic analysis revealed an additive interaction with the ED_{50}s of the combination falling within the 95% confidence intervals of the theoretical additive line (fig. 4.4). Unlike their sequential injection, the co-injection of S(+)-ibuprofen and L-NAME yielded inconsistent results (data not shown), possibly reflecting their incompatibility in solution, while the 20 min delay between the sequential injections would allow the local CSF concentration of the first agent to be sufficiently low not to significantly intereact with the second agent. The inactive R(-)-isomer of ibuprofen was without effect (data not shown).

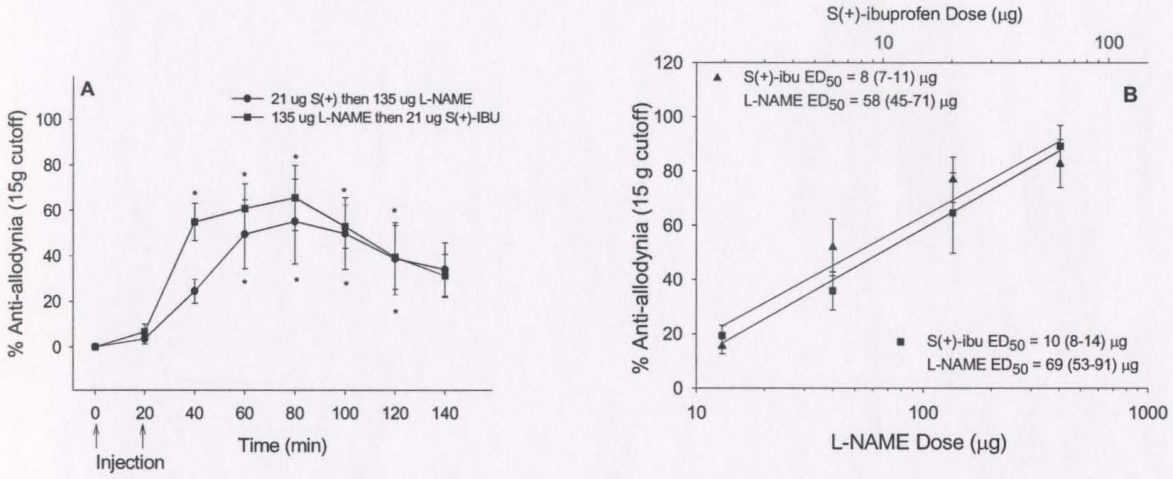

Figure 4.3. Addition of intrathecal S(+)-ibuprofen enhanced the anti-allodynic effect of L-NAME in the L5/L6 spinal nerve ligation model. Panel A is an example of one time course (21 μg S(+)-ibu & 135 μg L-NAME; others omitted for clarity) and panel B is the dose-response curves for the combination studies. Each point represents the mean ± SEM of 6-8 animals. Asterisks represent significant difference from baseline (time 0; p=0.002 for 'S(+)-ibuprofen then L-NAME' group; p<0.001 for 'L-NAME then S(+)-ibuprofen' group). Dose-response experiments were conducted 2-12 days after nerve ligation. S(+)-ibuprofen was administered either 20 min before (■) or 20 min after (▲) intrathecal L-NAME. Drug-induced changes in left-paw withdrawal thresholds are expressed as % anti-allodynia (see Methods). The ED_{50}s and corresponding 95% confidence intervals for each dose-response curve are shown in the figure.

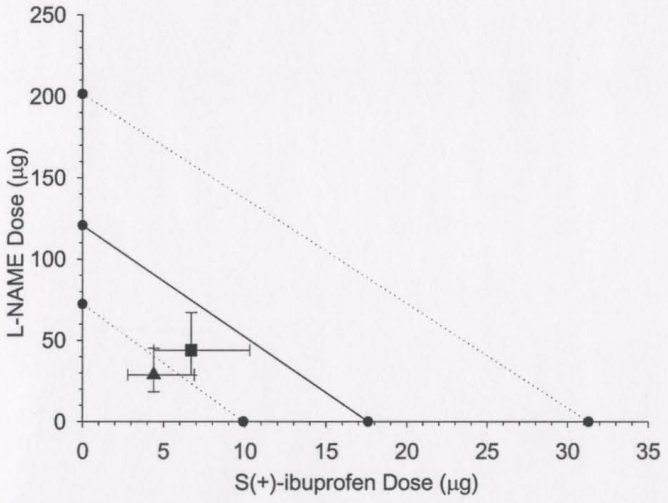

Figure 4.4. Isobologram illustrating the additive interaction between intrathecal L-NAME and S(+)-ibuprofen in the L5/L6 spinal nerve ligation model. The points on the x- and y-axes are the ED_{40} and 95% confidence intervals of S(+)-ibuprofen and L-NAME, respectively. The ▲ indicates the ED_{40} and corresponding 95% confidence intervals for the combination of L-NAME followed by S(+)-ibuprofen. The ■ indicates the ED_{40} and corresponding 95% confidence intervals for the combination of S(+)-ibuprofen followed by L-NAME.

4.4 DISCUSSION

S(+)-ibuprofen enhanced the effect of L-NAME in the L5/L6 spinal nerve ligation model as indicated by the significant increase in both the potency of L-NAME (>3-fold) and the maximum reversal of allodynia (57% to 89%) as compared to L-NAME alone. The stereo-specificity of L-NAME and S(+)-ibuprofen suggests that their anti-allodynic effects can be attributed to NOS- and cyclooxygenase-inhibition, respectively. Isobolographic analysis revealed the interaction to be additive at a fixed molar ratio of

1:5 (ibuprofen:L-NAME). That the interaction was not significantly affected by the order of drug injection indicates that cyclooxygenase- and NOS-inhibition was of sufficient duration to correct for the difference in peak effects, and that spinal NOS and cyclooxygenase pathways were both activated early after spinal nerve injury.

A spinal NO-prostanoid interaction is consistent with reports of their individual effects in neuroapthic pain models. For example, allodynia induced by i.t. NMDA was blocked by NOS and cyclooxygnease-2 inhibitors (Dolan and Nolan, 1999). Intrathecal L-NAME attenuates both the hyperalgesia elicited by spinal PGE_2 (Park et al., 2000), and the allodynia induced by spinal strychnine (Milne et al., 2001). Allodynia is also partially reversed by spinal cyclooxygenase inhibitors or prostaglandin receptor antagonists in the chronic constriction-, i.t. strychnine-, i.t. bicuculline-, and the spinal nerve ligation-models (see chapter 2; also Parris et al., 1996; Hefferan et al., 2003a; Hall et al., 1999; Zhang et al., 2001). In addition, normally innocuous brushing elicited spinal prostaglandin synthesis and nocifensive behaviours in allodynic, but not sham-operated animals; both effects were blocked by i.t. S(+)-ibuprofen (see chapters 2 and 3; also Hefferan et al., 2003a,b). These studies underscore the relevance of spinal prostanoids and NO to experimental allodynia, and thus their ability to effect, in combination, this abnormal state.

A spinal NO-prostanoid interaction is also supported by studies investigating the relationship between NO- and prostanoid-pathways (O'Banion, 1999; Perez-Sala and Lamas, 2001). The genes coding for NOS and cyclooxygenase have identical promoters and response elements to NF-κB and NF-IL6 (Lowenstein et al., 1993; Xie et al., 1993), and their expression is triggered by similar mediators (Vane et al., 1994; Swierkosz et al.,

1995). Prostaglandin synthesis was triggered in human fetal fibroblasts, bovine endothelial cells, and mouse macrophages exposed to NO donors (Salvemini et al., 1993; Davidge et al., 1995), while NO potentiated the IL-1β induced upregulation of COX-2 mRNA and protein in cultured DRG cells (Morioka et al., 2002). Inducible NOS has also been implicated in the regulation of hepatic COX-2 (Ahmad et al., 2002). Conversely, NOS activity and nitrite levels were increased in murine macrophages treated with PGE_2 (Milano et al., 1995), and ibuprofen inhibited the synthesis of iNOS (mRNA and protein) in glial cell cultures at concentrations blocking PGE_2 production (Stratman et al., 1997). These reports indicate a bi-directional relationship between distinct pathways that contribute to allodynia early after nerve injury, and are consistent with the additive interaction reported in the present study. The latter may include the exacerbation of nerve injury-induced cell damage through the local formation of peroxynitrite (Beckmann et al., 1994; Dawson et al., 1996), the reaction product of NO + superoxide anion (derived from cyclooxygenase).

In summary, the interaction between spinal NO and prostanoids has been characterized in a well-established model of neuropathic pain using isobolographic analysis. While it has been well-known that spinal NO and prostanoids exert their pro-allodynic effects via distinct pathways, interaction, if any, between these pathways has not been previously investigated. The additive interaction reported here indicates a convergence in their alteration of sensory processing at the spinal level. If similar spinal mechanisms are at work in clinical neuropathic pain, then a low-dose combination of NOS- and cyclooxygenase-inhibitors could be effective in the early treatment of allodynia.

5.0 GENERAL DISCUSSION

There are a number of important results arising from this investigation of experimental allodynia.

- Following L5/L6 spinal nerve ligation, PGE_2 concentrations in spinal CSF increased significantly in response to brushing of the allodynic dermatomes; basal concentrations remained unchanged. There was a clear temporal and spatial relationship between low-threshold afferent stimulation, PGE_2 release, and allodynic responses in nerve-injured animals. The results support the hypothesis that spinal prostaglandin release and allodynia depend upon central synaptic activity that is driven by input from affected dermatomes.
- Tactile allodynia depended upon spinal prostaglandin activity for 10-12 days after spinal nerve ligation. COX-2 was the predominant isoform responsible for the *de novo* prostaglandin synthesis during this phase. Thereafter, pharmacological disruption of spinal prostaglandin activity was without effect leaving long-term, prostaglandin-independent allodynia.
- Spinal nerve ligation triggered pharmacodynamic changes to the spinal prostaglandin system that are also temporally and spatially correlated to allodynia. PGE_2-evoked glutamate release was markedly enhanced in the affected spinal segments (L5/L6) of allodynic rats during prostaglandin-dependent allodynia. Possible explanations for this effect include an increase in EP receptor-effector coupling, and amplification of prostaglandin synthesis in the nerve-injured segments where increased expression of COX-2 has been demonstrated.

Preliminary experiments in our laboratory showed that pretreating spinal cord slices with COX inhibitors before exposure to PGE_2 blocked glutamate release (unpublished results). Similarly, pretreatment with intrathecal COX inhibitors before spinal prostaglandin administration attenuated PGE_2-induced allodynia in conscious rats (unpublished data). Thus, exogenous PGE_2, or prostaglandins synthesized in response to exaggerated afferent input may, through a positive feedback effect, trigger excess prostaglandin production in the affected spinal segments where up-regulation of cyclooxygenase is known to occur.

- Treatment with i.t. COX-1 or non-selective COX inhibitors, beginning 2h after nerve ligation, consistently blocked the emergence of allodynia. COX-2 inhibitors were without effect. Thus, spinal prostanoid synthesis appears to be an early, obligatory step in the development of this allodynic state, and COX-1 is the relevant isoform catalyzing prostaglandin synthesis in the hours immediately following nerve injury. This early effect is in addition to the role served by spinal prostaglandins once allodynia is established (i.e. during the prostaglandin-dependent phase).
- Spinal prostanoids and NO, which have discrete pro-allodynic effects, interact positively in the spinal nerve ligation model. Intrathecal S(+)-ibuprofen enhanced the anti-allodynic effect of L-NAME. Isobolographic analysis showed this interaction to be additive, suggesting a possible convergence of actions at the spinal level.

5.1 DIFFERENTIAL EFFECTS OF SC-236, SC-560 AND S(+)-IBUPROFEN

In these experiments, selective inhibitors of COX-1 or COX-2, and non-selective COX inhibitors were used to probe the spinal pharmacology of spinal nerve ligation-induced allodynia. As with all pharmacological approaches, it is possible that the apparent differences in the contributions of COX-1 and COX-2 in this model may simply reflect differences in the potency of SC-236 and SC-560, and thus the degree of enzyme inhibition achieved with each drug under the conditions used in this study. As summarized in table 2, SC-560 and SC-236 have virtually identical IC_{50} values against COX-1 and COX-2, respectively, and are 3400 and 32000 times less potent against the opposite COX isoform. S(+)-ibuprofen is equipotent against both COX-1 and COX-2 but considerably less potent than SC-560 and SC-236 at their respective COX isoforms.

Table 2. IC_{50} Values Determined Against Recombinant Human Cyclooxygenases (hCOX)

INHIBITOR	hCOX-1	hCOX-2
SC-560	~5 nM	160 μM
SC-236	17 μM	5 nM
S(+)-ibuprofen	3.3 μM	3.8 μM

(From Gierse et al., 1996; Yaksh et al., 2001)

On the basis of these data, similar doses of SC-560 and SC-236, delivered identically into the lumbar intrathecal space, were used throughout this study. The marked temporal differences in sensitivity to these equi-potent drugs following spinal nerve ligation,

combined with the observation that S(+)-ibuprofen, a less potent, non-selective COX inhibitor, was similarly effective against allodynia, strongly suggest that differences in drug potency and therefore enzyme inhibition do not account for these results.

Indeed, the doses of SC-560 and SC-236 selected for our experiments were comparable to those used by other laboratories. Intrathecal SC-560 (1-100ug) inhibited the behavioral responses of the formalin test (Yamamoto and Nozaki-Taguchi, 2002), substance P-induced hyperalgesia (Yaksh et al., 2001), and a model of post-operative pain (Zhu et al., 2003). While Dirig et al. (1997) found 100μg of SC-236 to be ineffective in reducing the rise in PGE_2 concentration in spinal dialysate during the formalin test, Deleo et al. (2000) reported that 0.2mg/kg of SC-236 (approximately 25μg/125g rat) alleviated nerve injury pain in an experimental model of radiculopathy. Likewise, the ibuprofen doses are in line with those reported by others (Hall et al., 1999; Dirig et al., 1997). Thus, the choice of 10-100μg appears to be a reasonable dose range, especially considering that 1000μg exhibited no greater anti-allodynic effect than a 100μg-dose.

We conclude that the difference in effect noted with SC-560 and SC-236 in the spinal nerve ligation model reflects the distinct contributions of COX-1 and COX-2 in the emergence and early maintenance of tactile allodynia, respectively.

5.2 COX EXPRESSION, COX ACTIVITY AND PG SYNTHESIS

Preliminary results using Western analysis in our laboratory indicated detectable changes in spinal COX and NOS expression early after nerve injury. These results are in

agreement with earlier reports of changes in COX expression after spinal nerve ligation (Zhao et al., 2000). While the data are consistent with the other results of the thesis research and support the overall working hypothesis, increased expression does not necessarily translate into changes in enzyme activity and, therefore increased PG synthesis. To address this issue directly, attempts were made to measure COX-1 and COX-2 activity in spinal cord harvested from nerve-ligated and sham-operated rats using a commercially available kit (Cayman Chemical, product #760151). Unfortunately, efforts to eliminate background interference, thought to be caused by the high lipid content of spinal tissue, while retaining enzymatic activity proved unsuccessful. As a result, we were unable to generate reliable and reproducible COX activity data.

In spite of this limitation, changes in COX levels are often used as indicators of increased prostanoid participation and are frequently correlated with changes in PG synthesis. For example, Eibl et al., (2003) reported a correlation between COX-2 expression and both basal and arachidonic acid-stimulated PGE_2 synthesis in pancreatic cells. Samad et al. (2001) also reported a correlation between PGE_2 concentration in cisternal CSF samples and the spinal expression of COX-2 following inflammation in the hindpaw. Concurrent increases in COX-2 expression and COX-mediated PGE_2 synthesis have also been reported in the spinal cord of rats with experimental diabetic neuropathy (Freshwater et al., 2002).

While the inclusion of spinal COX activity data would have undoubtedly strengthened the connection between enhanced COX expression and spinal prostanoid synthesis early after spinal nerve ligation, we believe the body of evidence presented in

this thesis is sufficiently convincing to support further studies of this relationship, and its functional consequences in the establishment of allodynia.

5.3 DISTINCT EFFECTS OF SPINAL COX-1 AND COX-2 IN ALLODYNIA

An interesting and important result arising from the present research is the differential role for COX-1 and COX-2 in the allodynia following spinal nerve ligation. Considering the fact that both isoforms are constitutively expressed in the spinal cord, an obvious and related question is why the early inhibition of COX-1 is more effective in preventing the emergence of allodynia than that of COX-2.

Studies with knockout mice expressing only COX-1 or COX-2 indicate that each isozyme subserves particular physiological functions. For example, COX-1 knockouts showed significantly impaired hemostatic function, which was not affectd by COX-2 knockout. Also, COX-2 knockout mice developed renal impairments, and the females were infertile, although this infertilitiy was reversible by PGE_2 treatment (DeWitt, 1999; Langenbach et al., 1999). It has also been shown that cells expressing both isozymes can selectively synthesize prostanoids through either pathway, suggesting a segregation of metabolic pathways (Kuwata et al., 1998; Fujishima et al., 1999). This could be achieved through differential gene expression, and/or differences in the subcellular localization of each isozyme. Morita et al. (1995) showed COX-2 to be more concentrated in the nuclear envelope of murine 3T3 cells and human and bovine endothelial cells, while COX-1 was equally distributed between the endoplasmic reticulum and nuclear envelope. This pattern raises the possibility that prostanoids originating from COX-1 mediate their effects primarily through cell surface receptors, while products of COX-2 operate

primarily through nuclear receptors. However, the subcellular distribution of these isozymes also appears dependent upon the cell type. For example, in DRG cells, COX-1 is found in the cytoplasm and nuclear membrane as well as axonal processes (Willingale et al., 1997).

Another explanation for the segregation of COX-1 and COX-2 activity and function arises from the differences in their individual kinetics. Studies using many cell types (including macrophages and mast cells) have shown that when these are stimulated with cytokines or lipopolysaccharide, there is an initial burst in prostanoid synthesis that is mediated by COX-1, lasts for 10-30 min, and is associated with high concentrations of arachidonic acid. This is followed by delayed prostanoid synthesis which is catalyzed by COX-2 and is associated with lower concentrations of arachidonic acid (see review, Smith et al., 2000). Interestingly, while low concentrations of arachidonic acid (e.g. <1 μM) favor prostanoid synthesis via COX-2, high concentrations (e.g. >10 μM) shunt production through the COX-1 pathway (Murakami et al., 1999). They reported that concentrations of arachidonic acid greater than 25 μM resulted in a 5-fold increase in PGE_2 in cells only expressing COX-1 as compared to cells only expressing COX-2. This differential catalytic activity was also observed in cells expressing both isozymes concurrently (Kuwata et al., 1998; Murakami et al. 1994).

Ionophore-based cell stimulation is known to produce non-specific activation of Ca^{2+}-dependent phospholipases. This, in turn, yields high intracellular concentrations of free arachidonic acid. The rapid and repetitive firing of injured nerves following spinal nerve ligation (Chaplan et al, 2003; Han et al, 2000) would be expected to generate a similar increase in arachidonic acid concentration in spinal cord neurons subject to this

afferent input. Based on the kinetic characteristics described above, this input would favour COX-1 mediated PG synthesis immediately after nerve injury; a prediction consistent with the results of present research. In the days following injury, exaggerated afferent input abates as other adaptations and response mechanisms (e.g. changes in gene expression, ion channel populations, receptor population density) take effect (see review, Smith et al., 2000). The intracellular concentration of free arachidonic acid presumably declines correspondingly and PG synthesis is shunted through the COX-2 pathway (see review, Smith et al., 2000). This is also in agreement with the time course of COX-2 mediated PG synthesis and allodynia following spinal nerve ligation.

As yet another explanation for differential roles for COX-1 and COX-2 could be based on the animals' age. Recent work by Ririe et al. (2004) suggests an age-dependent behavioral response to selective COX-1 inhibitors, at least in the acute post-operative pain model of allodynia. If this is the case in the spinal nerve ligation model, it is possible that the efficacy of COX-1 or COX-2 inhibitors might change as the animal ages. In fact this is an important issue considering the present results were obtained from pre-pubescent rats. The spinal nerve ligation model was originally described in young animals (4-6 weeks old; Kim and Chung, 1992), and it was reported that performing the nerve injury in young verses adult rats resulted in more robust tactile allodynia with greater consistency (Kim and Chung, 1992). It was for these reasons that the young animals were used in the present study. However, others have successfully used older rats, but with seemingly different absolute values for withdrawal thresholds (Zhu and Eisenach, 2003). If there are in fact age-dependent behavioral responses to the isozyme-selective COX inhibitors, and apparent age-dependent differences in development of

allodynia, an important issue is raised - that is, are the mechanisms surrounding the development and maintenance of allodynia in young rats different than that in adult rats.

Attributing any or all of these possibilities (i.e. gene expression, isozyme kinetics, subcellular distribution patterns) to the differential effects of COX-1 and COX-2 early after spinal nerve ligation will obviously require further investigation. Nevertheless, these diverse studies provide feasible explanations and documented evidence of differential COX-1 and COX-2 mediated PG synthesis even within the same cell under conditions comparable to those expected after nerve injury.

5.4 REQUIREMENT FOR ALTERED AFFERENT DRIVE IN THE DEVELOPMENT OF ALLODYNIA

The application of tight ligatures to the peripheral segment of the L5/L6 spinal nerves in the juvenile rat elicits a cascade of central neurochemical events that enable the miscoding of low-threshold input from the affected dermatome as a nociceptive event (i.e. allodynia). One of the early, critical steps in this cascade is the abnormal synthesis of spinal PG, especially PGE_2, followed by enhanced pharmacodynamic sensitivity to their effects in the affected spinal cord. These central responses to peripheral nerve injury, which underlie the emergence of an allodynic state, must be triggered by altered afferent drive into the spinal cord. Indeed, there is ample evidence that spinal PG synthesis is extensively regulated by neuronal input and mediated by glutamate operating through spinal NMDA receptors (Dirig and Yaksh, 1999). Thus, blocking input from injured nerve fibers should prevent, or at the very least disrupt the development of allodynia, including the synthesis of spinal PG.

Indeed, this has been tested in the spinal nerve ligation model previously. Abdi et al. (2000) applied lignocaine (2%) or bupivicaine (0.5%) solution directly to L5 and L6 spinal nerves for 10 min prior to nerve ligation. A separate experiment was also conducted in which local anesthetic was applied 4 d after ligation. Neither treatment prevented development of NP. This is not surprising given the relatively short duration of nerve blockade (single dose). Moreover, the results of this thesis research indicate that pharmacological treatment after nerve injury must be sustained for a minimum period of time (at least 8 h with i.t. COX inhibitors) if any effect on developing NP is to be achieved. While the exact duration of nerve blockade using local anesthetics is unknown in this model, the expected duration of action following the topical application of local anesthetics used by Abdi et al. (2000) appears to be insufficient in this model.

A more comprehensive study of altered afferent drive following nerve injury was performed by Suter and colleagues (2003). Using the spared-nerve injury model, they applied bupivicaine directly to the sciatic nerve, proximal to the site of injury, using microspheres thereby providing slow release of the anesthetic over several days. Interestingly, nerve block that was present before and up to 6-10 days after nerve injury (confirmed by sensory-motor testing) failed to prevent development of NP, suggesting that blockade of afferent input may only delay development of NP. While this may seem contradictory to the theory formulated in this thesis, there are at least two possible explanations for this. Firstly, a different model of NP (i.e. the spared-nerve injury model) was used in the study by Suter et al. (2003) whose pathophysiological mechanisms are likely to differ from those of L5/L6 spinal nerve ligation. Indeed, the pharmacological profile of this model is different than that of other animal models, particularly the spinal

nerve ligation model. For example, Decosterd et al. (2004) and Erichsen and Blackburn-Munro (2002) reported the tactile allodynia in the spared-nerve injury model to be insensitive to MK-801, an agent that is known to be effective in temporarily reversing tactile allodynia in the spinal nerve ligation model (Lee and Yaksh, 1995). Secondly, application of local anesthetics to peripheral nerves may itself cause nerve damage (see review, Borgeat and Ekatodramis, 2001). Suter and colleagues did not conduct any ultrastructural examination of the targeted nerve after anesthetic blockade to eliminate the possibility of this confounding effect. Clearly, the issue of and dependency on altered afferent drive to trigger early central adaptations that manifest as NP, including allodynia, require further and better investigation.

5.5 PROSTAGLANDINS ARE BIOLOGICALLY ACTIVE AT VERY LOW CONCENTRATIONS

Even under normal conditions, prostaglandins are known to effect biological responses at exceptionally low concentrations, and discrepancies between their effective concentrations and Kd values in experimental preparations are not uncommon. For example, iloprost induced increases in cAMP in Chinese hamster ovary (CHO) cells transfected with IP receptors at an EC_{50} of 100 pM (Namba et al., 1994). PGE_2 decreased cAMP production in EP_3-transfected CHO cells with an IC_{50} of 100 pM (Sugimoto et al, 1993). These values are at least 30-45 fold lower than the reported binding affinities at their respective prostanoid receptors. Similarly, PGE_2 inhibited cAMP levels and antagonized the actions of vasopressin in canine cortical collecting tubules at picomolar concentrations (Garcia-Perez and Smith, 1984).

Differences have also been reported in the efficiency of signaling by different transduction mechanisms coupled to the same receptor. While picomolar concentrations of iloprost initiated cAMP production at the IP receptor, phosphatidylinositol turnover required concentrations of iloprost three orders of magnitude higher (Narumiya et al., 1999). Variations in coupling efficiency at the IP receptor have been reported in platelets from many species (Armstrong et al., 1989). Taken together, these reports suggest that prostaglandins can elicit responses even at concentrations well below their reported Kd values, even though the mechanism of this phenomenon remains unclear. Preliminary experiments in our laboratory suggest one possibility: prostaglandin-driven prostaglandin synthesis, or the ability of exogenous PGE_2 to sufficiently increase cell activity to activate further prostaglandin synthesis. We have noted this both *in vitro* and *in vivo*.

Firstly, when PGE_2 was applied to spinal cord slices, a concurrent assay of bath media for glutamate and PGE_2 resulted in the well-documentated increase in glutamate concentration (see chapter 3), but also an unexpected increase in bath concentration of PGE_2 – indeed a concentration higher than that applied to the tissue (personal communication, D. O'Rielly and C. W. Loomis). This appears to depend on COX activity since incubation of the tissue with S(+)-ibuprofen significantly reduced the increases in bath concentration of both glutamate and PGE_2 (personal communication, D. O'Rielly and C. W. Loomis) Secondly, a similar result was found in vivo, when the pretreatment of naïve rats with i.t. S(+)-ibuprofen reduced i.t. PGE_2-induced allodynia (personal communication, J. Robinson and C. W. Loomis). Whether this effect is a direct action mediated by PGE_2, through EP receptors and activation of COX, or a secondary effect mediated by the glutamate release from the cells first activated by PGE_2, is

currently being investigated. Regardless, the result in both cases appears to be a higher concentration of PGE_2 in contact with the tissue (whether in vitro or in vivo) than actually administered. This is yet another possible explanation of how the administration of picomolar (or lower) concentrations of PGE_2 could appear to have cellular effects.

5.6 SUMMARY

The results of this thesis research affirm a role for spinal prostaglandins in the emergence and early expression of brush-evoked allodynia following L5/L6 spinal nerve ligation. In addition, they define the period after nerve injury in which spinal prostanoid synthesis is critically important for development of allodynia, and characterize the nature of the interaction between PG and NO in this model of NP. In turn, they raise a number of important questions which could be the subject of future studies. At what point after nerve injury do COX inhibitors lose their anti-allodynic effect? It seems they become ineffective about 14 days or later, but clearly, detailed experiments are needed to characterize this waning effect. What other spinal prostanoids are relevant in this model of allodynia? Two obvious possibilities are $PGF_{2\alpha}$ and PGI_2 - both of which have been implicated in normal pain processing in the spinal cord. What prostaglandin receptor subtypes mediate the pro-allodynic effects of spinal PG, where are they localized in spinal dorsal horn, and on what cell types are they expressed? For example, all four EP receptor subtypes were shown to be over-expressed in the injured nerve after partial sciatic nerve ligation (Ma and Eisenach, 2003). Whether a similar effect and receptor profile occurs in the spinal cord after spinal nerve ligation has yet to be determined.

Finally, the cellular mechanisms by which prostaglandins elicit these effects remain to be determined. These could include the direct activation of spinal neurons leading to central sensitization and hyperexcitability (Baba et al., 2001). However, their effects on sensory processing are likely to be far more complex. For example, Ahmadi and collegues (2001) reported that PGE_2 reduces glycine-mediated inhibition in rat spinal cord slices; this effect was further characterized by Harvey et al. (2004). This may be directly related to a recent report by Coull et al. (2003) who show that the reversal potential of the membrane currents evoked by glycine and GABA became more positive after peripheral nerve injury. As a result, physiological mechanisms normally suppressing lamina I neurons now amplified their output. Finally, the possible contributions of COX-3, a recently identified isoform whose physiological relevance remains unknown (Chandrasekharan et al., 2002), in this model have not been investigated. These future studies will better define the role(s) of PG in allodynia, thereby contributing to more effective treatment strategies for humans, including the prevention of abnormal pain states collectively known as neuropathic pain.

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