A STUDY OF THE ROLE OF SPINAL PROSTAGLANDINS AND NITRIC OXIDE EARLY AFTER NERVE INJURY

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A STUDY OF THE ROLE OF SPINAL PROSTAGLANDINS AND NITRIC OXIDE EARLY AFTER NERVE INJURY

by

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ABSTRACT/RESEARCH SUMMARY:

Allodynia is an abnormal state in which pain is triggered by innocuous sensory stimuli. Previous work in our laboratory has shown that L5/L6 spinal nerve ligation (SNL) induces robust mechanical allodynia in the rat which consists of two distinct phases: a) an early spinal prostaglandin (PG)-dependent phase lasting approximately seven to ten days; and b) a delayed PG-independent phase lasting at least seventy days. Importantly, the former is a pre-requisite to and a trigger for the more complex and time-dependent changes underlying long-term, irreversible allodynia. Interfering with critical signaling events early after nerve injury is a logical strategy by which chronic neuropathic pain might be prevented. Such an approach requires a clear understanding of the sequence, time-course and pharmacology of these early signals.

In light of the apparent pathogenic role of spinal PG early after nerve injury, the present research investigated the SNL-induced changes in spinal PG synthesis and signaling, their relevance to the development of spinal hyperexcitability and allodynia, and the mechanisms underlying the changes in PG synthesis and signaling in the spinal PG-dependent phase. The specific objectives were:

1. To characterize the effect of SNL on A- and C-fiber mediated reflex responses (AFRR and CFRR, respectively) in the affected hind limb, and to determine the temporal and spatial relationship of these changes to SNL-induced allodynia.

2. To determine if the SNL-induced changes in the AFRR and CFRR (i.e. spinal hyperexcitability) are spinal PG-dependent, and if so, to determine the relevant cyclooxygenase (COX) isoform(s).
3. To determine if SNL triggers the activation of nuclear factor kappa-B (NFκB) in the affected spinal cord which initiate the delayed induction of spinal COX-2.
4. To determine if the sensitivity to PG E type (PGE_2) is exaggerated during spinal PG-dependent allodynia, and if this abnormal state is limited to the affected spinal cord three days after SNL.
5. To determine if PG-dependent spinal hyperexcitability and mechanical allodynia are mediated by E-type prostaglandin (EP) receptors, and to investigate the changes in the expression of spinal EP_{1-3} receptor subtypes and the glycine-α3 receptor subunit (GLY-α3R) three days after SNL.
6. To determine if disrupting spinal PG synthesis/signaling immediately after SNL (i.e. pre-emptive treatment) prevents the development of PG-dependent spinal hyperexcitability and mechanical allodynia.
7. To determine if spinal PG-dependent hyperexcitability is affected by the SNL-induced generation of spinal nitric oxide (NO), and if so, to investigate relevant NO synthase (NOS) isoform(s).

8. To determine if, and how, spinal NO-mediated activity affects spinal PG-dependent spinal hyperexcitability and mechanical allodynia.

Male Sprague-Dawley rats were initially fitted with intrathecal (i.t.) catheters for drug delivery near the dorsal L1-L3 segments. Three days later, animals were anesthetized with halothane and the left L4 and L5 spinal nerves separated. In the SNL group, the L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham-controls, the L5 and L6 spinal nerves were isolated but not ligated. Allodynia, defined as a paw withdrawal threshold (PWT) of ≤4g, was confirmed using von Frey filaments. The decrease in PWT from baseline (>15g) was evident in the ipsilateral hind paw 24h after SNL, and remained stable for at least twenty days. It was also accompanied by the protective posturing (i.e. elevation above the cage floor and cupping) of the affected hind paw. Allodynic animals were otherwise healthy and exhibited normal behaviour and weight gain.

Objective 1. To characterize the effect of SNL on A- and C-fiber mediated reflex responses (AFRR and CFRR, respectively) in the affected hind limb.

Electrophysiologic responses evoked by transcutaneous A- or C-fiber electrical stimulation were recorded in the biceps femoris flexor reflex (AFRR and CFRR, respectively) one, three and ten days after SNL or sham surgery. PWT, measured immediately before the experiment, confirmed the presence and

absence of allodynia in SNL and sham-controls, respectively. The AFRR and CFRR were recorded using accepted activation and conduction criteria, and validated by their differential sensitivity to the inhibitory effects of i.t. morphine. These responses were highly reproducible within each treatment group, and unaffected by the presence of the spinal catheter. There were no significant electrophysiologic changes in sham-controls, with or without a spinal catheter, compared to naïve animals. In SNL (i.e. allodynic) animals, the AFRR and CFRR were characterized by a significant decrease in the respective activation threshold, and a corresponding increase in spike integration (i.e. response magnitude) in the ipsilateral, but not the contralateral hind limb. These exaggerated responses were evident one day after SNL, peaked at three days, and persisted for at least ten days, paralleling the time course-course of decreased PWT in the ipsilateral hind paw. The central sensitization induced by repeated A-fiber stimulation in SNL animals, indicative of abnormal sensory processing, is consistent with the known role of Aβ-afferent fibers in mechanical allodynia. These results demonstrate the reproducibility, reliability and validity of the AFRR and CFRR as quantitative measures of A- and C-fiber mediated spinal hyperexcitability (i.e. central sensitization), as well as their temporal and spatial relationships to mechanical allodynia in the SNL model.

Objective 2. To determine if the SNL-induced changes in the AFRR and CFRR (i.e. spinal hyperexcitability) are spinal PG-dependent, and if so, to determine the relevant COX isoform(s).

The active S(+)- or inactive R(-)-enantiomers of ibuprofen, a non-selective cyclooxygenase (COX) inhibitor, were given i.t. three days after SNL or sham surgery (i.e. time of maximum SNL-induced changes in the activation threshold and response magnitude of the AFRR and CFRR). S(+)-ibuprofen, but not the R(-)-enantiomer or vehicle, dose-dependently attenuated the changes in the AFRR (ID_{50}=36.3μg) and CFRR (50.8μg). This inhibitory effect was similar to the selective COX-2 inhibitor, SC-236. In contrast, the selective COX-1 inhibitor, SC-560 given three days after SNL had no significant effect on the AFRR and CFRR. When these experiments were repeated one day after SNL, SC-560 matched the effect of S(+)-ibuprofen while SC-236 was without effect. The results indicate that SNL-induced hyperexcitability is dependent on PG synthesis in the spinal cord up to three days after nerve injury. They also reveal an apparent shift in the dominant isoform responsible for spinal PG synthesis during the acute nerve injured state.

Given that both COX-1 and COX-2 are expressed constitutively in the spinal cord (COX-1 > COX-2), the apparent shift in the dominant COX isoform could reflect the delayed induction of COX-2 in affected spinal cord. To test this hypothesis, the expression of COX-1 and COX-2 protein was determined in the spinal cord one and three days after surgery. One day after SNL, Western

analysis revealed a significant increase in COX-1, but not COX-2 protein in the ipsilateral compared to the contralateral lumbar dorsal horn (L-DH). The expression of COX-1 and COX-2 was unchanged in the contralateral L-DH and bilateral ventral horns of SNL animals compared to sham-controls. Three days after SNL, COX-2 (but not COX-1) protein was significantly up-regulated in the ipsilateral L-DH. It is concluded that SNL affects the spinal expression of COX-1 and COX-2 in a manner which is temporally and spatially correlated with the emergence of PG-dependent spinal hyperexcitability and mechanical allodynia, and includes an early shift in the dominant enzyme responsible for PG synthesis.

Objective 3: To determine if SNL triggers the activation of nuclear factor kappa-B (NFκB) in the affected spinal cord which initiate the delayed induction of COX-2.

NFκB modulates the transcription of the COX-2 gene and is known to be activated by nerve injury. To determine if SNL triggers the activation of NFκB in the affected spinal cord, nuclear extracts were prepared from the left and right dorsal and ventral quadrants of the lumbar spinal cord twelve hours, one and three days after surgery. NFκB was significantly increased in the ipsilateral L-DH twelve hours after SNL compared to sham-controls. This effect preceded the increased expression of COX-2 and was still present three days later. NFκB was unchanged in the contralateral L-DH or bilateral ventral horns of SNL animals compared to sham-controls. Treatment with ammonium pyrrolidedithiocarbamate

(PDTC; 100μg i.t.), an inhibitor of NFκB activation, immediately after SNL, prevented the expression of spinal COX-2 in the ipsilateral L-DH three days later. The up-regulation of COX-2 was unchanged in vehicle-treated animals indicating that COX-2 expression in the affected spinal cord is secondary to NFκB activation, beginning immediately after nerve injury.

Objective 4. To determine if the sensitivity to PGE_2 is exaggerated in and limited to the affected spinal cord three days after SNL (i.e. during spinal PG-dependent allodynia) .

Slices from the lumbar, thoracic and cervical spinal cord were prepared three days after SNL or sham surgery for the determination of PGE_2-evoked glutamate release. PWT, measured immediately prior to spinal cord extraction, confirmed the presence and absence of allodynia in SNL and sham-controls, respectively. Addition of PGE_2 yielded a bell-shaped concentration-response curve (CRC) characteristic of prostanoids regardless of the spinal segment. With lumbar, but not cervical or thoracic slices from SNL animals, there was a marked, parallel leftward shift in the CRC compared to sham-controls. This corresponded to a 2855-fold decrease in the EC_{50} of PGE_2 three days after SNL. There were no differences in the peak effect of PGE_2 regardless of the surgery or spinal slice. In separate experiments, the concentration of PGE_2 determined in the incubation fluid containing lumbar slices from SNL animals was found to be up to 100-fold greater than the corresponding and verified concentration added to the bath.

These *in vitro* data suggest that PGE_2 can trigger its own synthesis in lumbar slices from allodynic animals. To test this hypothesis, lumbar slices prepared three days after SNL or sham surgery were pre-incubated for 20min with S(+)- or R(-)-ibuprofen. The disparity between the added versus bath concentration of PGE_2 was significantly attenuated using slices from SNL animals pretreated with S(+)- but not R(-)-ibuprofen or vehicle. In addition, the corresponding PGE_2 CRC exhibited a significant and parallel but incomplete rightward shift towards that of control. All three pre-treatments were without effect in slices prepared from sham-controls. Similar results were observed *in vivo*. There was a 362-fold decrease in the ED_{50} of i.t. PGE_2 on brush (BR)-evoked nociceptive-like behavior in allodynic animals compared to sham-controls. Pre-treatment (20min) with i.t. S(+)-ibuprofen three days after SNL shifted the PGE_2 dose-response curve significantly to the right compared to i.t. R(-)-ibuprofen or vehicle. These results, demonstrating a marked increase in the sensitivity to PGE_2 in the lumbar spinal cord, three days after SNL (i.e. time of maximum PG-dependent spinal hyperexcitability), are consistent with the SNL-induced up-regulation of COX-2. They also suggest the amplification of PG-signaling in the affected spinal cord.

Objective 5. To determine if PG-dependent spinal hyperexcitability and mechanical allodynia are mediated by E-type prostaglandin (EP) receptors, and to investigate changes in the expression of spinal EP_{1-3} receptor subtypes and the glycine-α3 receptor subunit (GLY-α3R) three days after SNL.

Animals were treated with the non-selective EP receptor antagonist, SC-51322 (100μg i.t.) or vehicle three days after nerve injury. All SNL-induced changes (i.e. PWT, the AFRR and CFRR, and the sensitivity to PGE_2 both *in vitro* and *in vivo*) were significantly attenuated in SC-51322- but not vehicle-treated rats. Subsequent Western blot analysis revealed a significant increase in the expression of EP_1, EP_2 and EP_3 receptors in the ipsilateral versus contralateral L-DH of SNL animals. There were no changes in the contralateral L-DH or bilateral ventral horns of SNL animals compared to sham-controls. The up-regulation of spinal EP_{1-3} receptors was evident one day after SNL and increased over the next two days. There was also a significant increase in the expression of GLY-α3R protein in the ipsilateral but not the contralateral L-DH three days after SNL. GLY-α3R expression was unchanged in the contralateral L-DH or bilateral ventral horns of SNL animals compared to sham-controls. These results demonstrate: a) the critical role of spinal EP receptor subtypes in established PG-dependent spinal hyperexcitability and mechanical allodynia; b) their progressive up-regulation three days after SNL; and c) the temporal and spatial similarities in the expression of EP receptors and COX-1/COX-2 in the affected spinal cord. They

are also consistent with increased spinal PGE_2/EP_2/GLY-α3R signaling in the acute nerve injured state. The latter is consistent with the robust allodynia induced by i.t. strychnine (a selective glycine receptor antagonist) in otherwise normal animals, and the depletion of glycine-receptor immunoreactive cells in the spinal cord following chronic constriction injury.

Objective 6. To determine if disrupting spinal PG synthesis/signaling immediately after SNL (i.e. pre-emptive treatment) prevents spinal PG-dependent allodynia.

S(+)-ibuprofen, R(-)-ibuprofen or vehicle were given i.t. immediately after SNL or sham surgery. S(+)-ibuprofen prevented the SNL-induced activation of NFκB, the up-regulation of COX-2, the increased sensitivity to PGE_2 in the affected spinal cord, the exaggeration of the AFRR and CFRR, and decrease in PWT. In contrast, the R(-)-isomer and vehicle had no effect on these SNL-induced changes. In separate experiments, identical treatment with PDTC, but not vehicle, blocked the exaggeration of A- and to a lesser extent C-fiber mediated responses, as well as the decrease in PWT three days after SNL. PDTC and vehicle were without effect in sham-controls. Thus, blocking the activation of spinal NFκB, either directly with PDTC or indirectly with S(+)-ibuprofen, prevented the SNL-induced expression of COX-2 and the development of PG-dependent spinal hyperexcitability and mechanical allodyina three days later. The results indicate that the SNL-induced activation of NFκB

and the induction of spinal COX-2 are critical antecedents in the development of PG-dependent spinal hyperexcitability and mechanical allodyina. They also suggest that spinal PG facilitate the activation of NFκB beginning immediately after SNL thereby explaining the pre-emptive effect of S(+)-ibuprofen (an inhibitor of COX activity) on spinal COX expression.

Objective 7. To determine if, and how spinal NO contributes to spinal PG-dependent allodynia.

NO is known to be recruited in the spinal cord by nerve injury. NFκB also modulates the transcription of NOS. SNL significantly increased nNOS and iNOS expression in the ipsilateral L-DH one and three later compared to sham-controls. Pre-emptive treatment with PDTC (100μg i.t.) blocked the increases in NFκB, nNOS, and iNOS three days later. These results indicate that NFκB activation not only produces COX up-regulation, but also NOS up-regulation in the affected spinal cord early after SNL. Given that SNL induced the up-regulation of NOS enzymes, an affect of blocking NOS activity on spinal hyperexcitability was examined. Treatment with *N*-nitro-L-arginine methylester (L-NAME), but not the D-isomer (D-NAME) or vehicle, dose-dependently attenuated the SNL-induced effect on PWT, AFRR and CFRR three days after surgery.

Likewise, pre-emptive treatment with L-NAME (100μg i.t.), but not the D-isomer (D-NAME) or vehicle, prevented the SNL-induced effect on PWT, AFRR and CFRR. These results indicated that NOS activity, in addition to COX activity,

was increased early after SNL and significantly contribute to spinal hyperexcitability and mechanical allodynia. In an attempt to determine if NO-signaling is enhanced early after SNL, the expression of cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) was investigated. Expression of cGMP and PKG protein was significantly increased in the ipsilateral L-DH of SNL animals compared to sham-controls. This result combined with the effect of L-NAME on spinal hyperexcitability and allodynia indicated that spinal NO-signaling is significantly enhanced early after SNL.

The results of this research support the hypothesis that spinal PG play an early pathogenic role in the emergence and early maintenance of spinal hyperexcitability and mechanical allodynia following SNL. They revealed a time-dependent shift in the COX isoform primarily responsible for spinal PG synthesis, identified the inter-relationships between NFκB, PG and NO synthesis/signaling in affected spinal cord, and characterized the nature of the interaction between PG and NO in this model of NP.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AFRR	A-fiber mediated reflex responses
ANOVA	Analysis of variance
BR	Brush
CFA	Complete Freud's Adjuvant
CFRR	C-fiber mediated reflex responses
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRC	Concentration-response curve
CSF	Cerebrospinal fluid
DH	Dorsal horn
DMSO	Dimethyl sulfoxide
D-NAME	*N*-nitro-D-arginine methylester
EC_{50}	Effective concentration 50%
ED_{50}	Effective dose 50%
EP	Prostaglandin E receptor
EP_1	Prostaglandin E receptor subtype 1
EP_2	Prostaglandin E receptor subtype 2

EP_3	Prostaglandin E receptor subtype 3
EP_4	Prostaglandin E receptor subtype 4
EPSCs	Excitatory postsynaptic currents
GABA	Gamma-aminobutyric acid
GLY-α3R	Glycine-α3 receptor subunit
i.p.	Intraperitoneal injection
i.t.	Intrathecal
i.v.	Intravenous
IASP	International Association for the Study of Pain
IBU	Ibuprofen
IKK	IκB kinase
iNOS	Inducible nitric oxide synthase
IR	Immunoareactivty
IκB	Inhibitor of nuclear factor kappa-B
L1-L3	1st three lumbar segments
L4	4th lumbar segment
L5	5th lumbar segment
L6	6th lumbar segment
L-DH	Lumbar dorsal horn
L-NAME	*N*-nitro-L-arginine methylester
min	Minute
mRNA	Messenger ribonucleic acid

NFκB	Nuclear factor kappa-B
NMDA	*N*-methyl-D-aspartic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Neuropathic pain
NSAIDs	Non-steroidal anti-inflammatory drugs
P-10-D	Ten days after surgery
P-1-D	One day after surgery
P-3-D	Three days after surgery
PDTC	Ammonium pyrrolidedithiocarbamate
PG	Prostaglandin(s)
PGE_2	Prostaglandin E type
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PWT	Paw withdrawal threshold
SC-236	4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1-pyrazol-1-l]
SC-51322	8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid,2-[3-[furanylmethyl)- thio]1-oxopropyl]hydrazide;
SC-560	5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole

sGC	Soluble guanylate cyclase
SNL	L5/L6 spinal nerve ligation

1.0 INTRODUCTION

1.1 PROSTAGLANDINS

Prostaglandins (PG) belong to a family of lipids derived from arachidonic acid (AA), a 20-carbon fatty acid. Unlike many biological mediators, PG are not stored in advance but are synthesized *de novo* as required. The initial and rate-limiting step in the biosynthesis of PG is the intracellular liberation of AA from the phospholipid membrane (Smith, 1992). This is normally accomplished by phospholipase A_2 (PLA_2), but phospholipase C and D serve a similar function (Smith, 1992). Once released, AA is available for conversion to PGG_2 by the enzyme, cyclooxygenase (COX). This bifunctional enzyme then catalyzes PGG_2 to PGH_2 via its peroxidase activity. The resulting biologically active products, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, or TxA_2 (Hara et al., 1994; Kuwamoto et al., 1997; Suzuki et al., 1997; Jakobsson et al., 1999; Smith et al., 2000) are then able to diffuse from the cell, or exit by means of a carrier-mediated process (Chan et al., 1998). They are then able to bind to PG-specific receptors on the plasma membrane (Ushikubi et al., 1998; Breyer et al., 2001). Some PG remain intracellularly where they are able to bind to PG receptors on the nuclear membrane (Negishi et al., 1995; Kliewer et al., 1997; Lim et al., 1999).

There are two primary isoforms of COX[†] (i.e. COX-1 and COX-2) which are both associated with intracellular membranes. COX-1 and COX-2 were

[†] Each isomer exists as a homodimer, composed of three independent folding units: a) the active enzymatic domain within a long hydrophobic channel; b) a membrane-binding domain; and c) an epidermal growth factor-like domain (Smith et al., 2000). The most striking differences in amino acid sequence occur within the membrane-binding domains (Otto and Smith, 1996; Spencer et al., 1999).

initially thought to subserve physiological and pathophysiological functions, respectively. It is now recognized that each can fulfill either role, depending on the cell type and the prevailing conditions. While COX-2, and to a lesser extent COX-1, contribute to inflammatory and nociceptive response (see Sections 1.2 and 1.4 below), both isoforms are known to play a role in homeostatic regulation. Likewise, the early classification of COX-1 and COX-2 as constitutive and inducible enzymes, respectively is now recognized to be an over-simplification. While COX-1 is the predominant constitutive enzyme in most cell types, its expression can increase in response to cellular conditions (see Sections 1.4 and 1.7 below). COX-2 is subject to rapid up-regulation (see Sections 1.4 and 1.7 below) but it is constitutively expressed in some cell types (e.g. central neurons and glia).

PG are chemically unstable and subject to rapid catabolism *in vivo* (Smith, 1992). As a result, PG exert their biological effects in close proximity to their site of synthesis; effects mediated by specific prostanoid receptors. For example, PGE_2 acts through E-type prostanoid (EP) receptors of which there are four subtypes - EP_1, EP_2, EP_3 and EP_4 (Coleman et al., 1990, 1994). While PG bind with highest affinity to their cognate receptor, cross-reactivity (e.g. PGE_2 signaling via F-type prostanoid receptors) is known to occur (Breyer et al., 2001; Wise et al., 2002). The most common product of COX-mediated AA metabolism is PGE_2 which has been implicated in many conditions, including pain and inflammation (Khanapure et al., 2007; Zeilhofer, 2007).

1.2 SPINAL PG AND NOCICEPTION

As early as 1971, studies suggested that non-steroidal anti-inflammatory drugs (NSAIDs) effect their analgesic and antihyperalgesic actions through the inhibition of peripheral PG synthesis (Vane, 1971; Smith and Willis 1971). Some twenty years later, PG were shown to also be synthesized in central nervous system (CNS) in response to peripheral tissue injury (Malmberg and Yaksh, 1992a, 1995a). That is, at central sites removed from the locus of injury and inflammation. For example, 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, 1995a). Importantly, spinal NSAIDs were 100 to 1000 times more potent than systemic administration in inhibiting the second phase of the formalin test (Malmberg and Yaksh, 1992a). These results indicated that NSAIDS can effect antinociception within the spinal cord using peripheral inflammatory pain models. The stereo-selective nature of this outcome (Malmberg and Yaksh, 1995a) further indicated that spinal antinociception was the result of COX inhibition.

That spinal PG synthesis is evoked by 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 ($[PGE_2]_{CSF}$). An increase in $[PGE_2]_{CSF}$ was also reported during the first- and second-phase of the rat formalin test

(Malmberg and Yaksh, 1995a, b); an effect triggered by C-fiber input (Hua et al., 1997). Both nociceptive behavior and the increase in $[PGE_2]_{CSF}$ were attenuated by i.t. S(+)-ibuprofen (IBU), but not the inactive R(-)-enantiomer (Malmberg, 1995b; Yang, 1996). Using an *in vitro* superfusion model, Dirig and Yaksh (1999) showed that spinal cord tissue, harvested from rats given kaolin/carrageenan in the knee joint, exhibited increased release of PGE_2 compared to age-matched naive cohorts. These results strongly suggested that peripheral inflammation could trigger the synthesis and release of PGE_2 from the spinal cord of experimental animals. The temporal correlation between spinal PG synthesis and release and nociceptive behaviour following peripheral inflammation further suggests that spinal PG may be critical in the induction of pain at the spinal level.

That PG modulate nociceptive signaling in the spinal cord was supported by the localization of COX in the dendrites of central excitatory neurons (Yamamoto, 1993), and the abundance of PGE_2 binding sites in the spinal dorsal horn (DH; Matsumura, 1992, 1995). The latter almost completely disappeared following dorsal rhizotomy indicating their location on the terminals of primary afferent fibers. This pattern of PGE_2 receptor-binding is consistent with the presynaptic facilitation of neuronal signaling. While it is known that the machinery for PG synthesis and signaling is located in key areas vital for transmission of nociceptive information in the spinal cord and COX inhibition results in antinociception, less is known regarding the cellular events underlying the critical role of spinal PG in the induction of pain.

1.3 CELLULAR EFFECTS OF SPINAL PG IN NOCICEPTION

The exact mechanisms by which spinal PG enhance nociceptive signaling have yet to be elucidated. One strong possibility is that PG exert their effects through excitatory receptors on primary afferent terminals that form synaptic connections with second order neurons and/or excitatory interneurons in the spinal DH (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. 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), and Gold et al. (1996) reported that PGE_2 increased tetrodotoxin-resistant Na^+ currents recorded from cultured DRG cells. These ion channel effects would be conducive to an overall increase in cellular excitability. 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 dorsal root ganglion (DRG) cells (Vasko, 1994) and rat spinal cord slices (Vasko et al., 1995). Perfusion with low concentrations of PGE_2 has also been shown to facilitate capsaicin- or bradykinin-evoked release of substance P or calcitonin gene-related peptide 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 modulate

neurotransmitter release from the spinal cord, as well as augment the release of neuropeptides from the spinal terminals of C-fibers.

PG may also enhance the excitability of DH neurons to afferent input through a direct postsynaptic effect. In an early study, injection 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). In addition, PGE_2 was shown to induce a long-lasting facilitation of evoked excitatory postsynaptic currents (EPSCs) in mouse DH neurons (Minami et al., 1999). Baba et al. (2001) showed that PGE_2 caused a direct activation of DH neurons *in vitro,* suggesting that this was a post-synaptic effect 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). These pharmacological studies provide further support for the modulatory effect of PG on spinal neurotransmission in nociception, raising the possibility of enhanced spinal PG synthesizing capacity in the acute pain state.

1.4 SPINAL PG BIOSYNTHESIS IN NOCICEPTION

COX-1 and COX-2 mRNA and protein are constitutively expressed in many regions of the central nervous system (CNS), including the dorsal and ventral grey matter of the spinal cord. 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 is present in neurons throughout the spinal cord, and is especially abundant in the superficial DH where nociceptive primary afferents are known to terminate. The presence of COX-1 in neurons is more controversial but COX-1-immunoareactivty (IR) was described in a subpopulation of putative nociceptive DRG neurons (Chopra et al., 2000). COX-1 and COX-2 are also expressed in astrocytes in the spinal cord (Vane et al., 1998).

The hypothesis that PG facilitate spinal nociceptive signaling is further supported by the rapid induction of COX-2 in the spinal cord following peripheral inflammation, secondary to tissue injury. A 2-fold increase in COX-2 mRNA and a smaller increase in COX-2 protein were detected bilaterally in the lumbar spinal cord of the rat following the acute injection of carrageenan in the footpad of the hind paw (Goppelt-Struebe et al., 1997; Hay and de Belleroche, 1997; Ichitani et al., 1997). Western analysis revealed a 1.6-fold increase in the level of COX-2 protein in the L-DH (lamina II-III) twenty-two days after the injection of Complete Freud's Adjuvant (CFA)(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 of COX-2 mRNA and protein were also noted in the same animals 12 h after injection (Samad et al., 2001). The increased expression of COX-2 was both neuronal and non-neuronal in nature (Beiche et al., 1998a, b; Maihöfner et al., 2000; Samad et al., 2001;

Tegeder et al., 2001). Where investigated, these increases coincided with a significant elevation in spinal PG concentration (Maihöfner et al., 2000; Samad et al., 2001; Tegeder et al., 2001). These results indicate that spinal COX-2 is not only inducible, and therefore influenced by peripheral inflammatory nociceptive events, but is likely responsible for the early increase in spinal PG synthesis and signaling.

In summary, it is well established that PG, synthesized in the spinal cord in response to noxious stimuli (i.e. at central sites removed from the locus of injury and inflammation), facilitate nociceptive transmission. That PG exert a central nociceptive action independent of their peripheral inflammatory and analgesic effects raises important questions about their possible role in neuropathic pain (NP) states.

1.5 NEUROPATHIC PAIN

NP is defined by the International Association for the Study of Pain (IASP) as pain initiated 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-neuropathy, post-herpetic neuralgia, and reflex sympathetic dystrophy (Wasner et al., 2003; Calcutt and Backonja, 2007; Giummarra et al., 2007; Dworkin et al., 2008; Henry et al., 2008). While

comprehensive epidemiological data are not available, current pooled estimates suggest that NP may affect as much as 3% of the population (Heliovaara et al., 1987; Schmader, 2000; Davis and Walsh, 2004; Verma et al., 2005). As a chronic and often debilitating condition, NP is a major burden on the healthcare system (Arner and Meyerson, 1988; Rowbotham et al., 1991; Baron and Saguer, 1993; Schmader, 1998). People with the condition have been found to generate 3-fold higher health care costs compared with matched controls (Berger et al., 2004). Moreover, the indirect costs associated with chronic pain conditions including NP (e.g. absenteeism, decreased productivity at work, etc.) are estimated to be more than $150 billion annually in the United States alone (Turk, 2002; McCarberg and Billington, 2006), of which almost $40 billion is attributable to NP.

The clinical features of NP are distinct from those of 'normal' nociceptive pain. The onset is often delayed for weeks to months after the causative event. Once established, NP is largely irreversible persisting for decades after the initial injury has healed (Tasker, 1990; Taylor, 2006). The stimuli eliciting NP are also unusual. Normally innocuous stimuli such as a cold draft of air or the light touch of clothing acquire the ability to evoke excruciating pain; a condition known as allodynia (Merskey, 1986). This is the most common and troublesome symptom of NP (Campbell et al., 1988; Raja et al., 1988). Allodynia is frequently described by patients with NP as a ripping, and/or tearing sensation; descriptors not normally associated with 'normal' nociceptive pain. Finally, NP generally

responds poorly to current pharmacotherapy, including drugs that exhibit analgesic effects only in NP states (e.g. tricyclic anti-depressants, anti-convulsants, barbiturates, various channel blockers, etc.). There is marked inter-individual variability in treatment response and even in patients who respond favorably, analgesia is incomplete. Surgical interventions intended to alleviate NP usually provide only temporary relief (Tasker et al., 1992; Eide, 1998).

Consequently, NP remains a serious clinical and socio-economic problem (Eide, 1998; McCarberg and Billington, 2006) whose unique clinical characteristics suggest complex and ultimately irreversible changes in somatosensory processing.

1.6 ALLODYNIA ARISES FROM ALTERED SOMATOSENSORY PROCESSING

Early clinical studies showed that allodynia in the affected dermatome(s) of patients with NP, as well as the sensation of light touch in adjacent normal skin, are both mediated by Aβ-fibers (Campbell et al., 1988; Price et al., 1989). Temperature discrimination in the same regions was unaffected (Campbell et al., 1988) suggesting minimal contributions from Aδ- and C-fibers in mechanical allodynia. Given that Aβ-fibers do not normally trigger pain, their participation in NP provided the first evidence of altered somatosensory processing following nerve injury.

In animal models, mechanical allodynia is generally measured as a reduction in the threshold force required to evoke withdrawal of the affected paw (paw withdrawal threshold; PWT). In nerve-injured animals, a PWT of ≤ 4 g from a baseline of 15 g is routinely observed along with protective posturing of the affected paw. This exaggerated reflex response to an otherwise innocuous peripheral stimulus supports the enhanced responsiveness of spinal neurons to low-threshold (i.e. Aβ-fiber) input. Peripherally, Aβ-fibers display spontaneous and persistent electrical activity (Bulka et al., 2002). Their central terminals have also been shown to sprout into lamina I and II of the spinal DH; laminae normally receiving input from smaller diameter nociceptive fibres (Woolf et. al., 1992; Bao et al., 2002; Shehab et al., 2003; Hu et al., 2004). Large diameter Aβ-fibers also begin to express neurotransmitters normally associated with nociceptive transmission, such as substance P and brain-derived neurotrophic factor (Noguchi et al,. 1994, 1995; Michael et al., 1999). The array of electrophysiologic, morphologic, and phenotypic changes occurring days to weeks after nerve injury reflect the complex, time-dependent and ultimately irreversible changes triggered by nerve injury (Beydoun and Backonja, 2003; Dworkin et al., 2003; Takeda et al., 2005). They also underlie the challenges in treating clinical NP effectively once it is established.

These time-dependent adaptations are initiated by signaling events in the spinal cord beginning immediately after nerve injury. Disrupting/inhibiting the latter provides, in principle, an opportunity to prevent the establishment and/or

severity of allodynia. If successful, this would represent a more advantageous and cost-effective strategy than trying to treat the outcomes of nerve injury weeks, months or even years later. However, any pre-emptive strategy requires a thorough understanding of the time-course, pharmacology and mechanisms underlying the development of long-term allodynia. Given the recognized role of spinal PG in 'normal' nociception, and the clinical availability of drugs with which to disrupt their effects, spinal PG represent an interesting and logical pre-emptive target. Indeed, there is growing evidence implicating spinal PG in the development of allodynia following experimental nerve injury.

1.7 SPINAL PG AND ALLODYNIA

The earliest and most direct evidence of a pro-allodynic effect elicited by spinal PG was provided by Uda et al. (1990) and Minami et al. (1992, 1994a, 1995a). They reported dose-dependent, touch-evoked agitation in conscious mice following the i.t. injection of PGE_2, PGD_2 or $PGF_{2\alpha}$; an effect blocked by the EP-receptor antagonist, NON-NT-012 (Minami et al. 1995b). Similarly, the delivery of PGE_2 to the rat spinal subarachnoid space triggered behaviorally-defined allodynia, whose onset and decline was temporally correlated with an increase and decrease in the concentration of glutamate, aspartate, taurine, glycine and gamma-aminobutyric acid (GABA) in spinal dialysate samples, respectively (Malmberg and Yaksh, 1995a). Thus, the introduction of exogenous PG into the spinal subarachnoid space of otherwise normal animals induced

temporary neurochemical and behavioral effects indicative of allodynia. Conversely, pretreatment with i.t. S(+)-IBU suppressed the brush (BR)-evoked neurochemical and physiological responses arising from spinal glycine-disinhibition in the i.t. strychnine model of allodynia (Hall et al., 1999). The R(-)-enantiomer was without effect suggesting the attenuation of these BR-evoked responses was related to the inhibition of COX in the lumbar spinal cord. In the GABA-disinhibitory model, focal application of NS-398 (COX-2 inhibitor) or SC-51322 (EP receptor antagonist) 20 minutes before bicuculline application to the dorsal surface of the rat spinal cord produced a highly localized anti-allodynia (Zhang et al., 2001). These results indicate that the inhibition of spinal PG synthesis or the blockade of spinal EP-receptors before the onset of glycine- or GABA-disinhibition in the rat L-DH prevents the development of allodynia.

Similar results have been reported using injury models of allodynia. For example, SNL elicits robust mechanical allodynia (PWT ≤ 4 g) in the ipsilateral hind paw (Kim and Chung, 1992); an effect fully expressed 24 h after injury (Hefferan et al., 2003a, b). Mild brushing on the plantar surface of the ipsilateral hind paw two days after SNL significantly increased $[PGE_2]_{CSF}$ compared to sham-controls (Hefferan et al., 2003a, b). The effect declined by day 5 and was absent by day 10, even though BR-evoked allodynia persisted for up to twenty days (i.e. independent of any detectable change in $[PGE_2]_{CSF}$). In the absence of brushing, or when brushing was applied away from the affected dermatome(s), there was no increase in $[PGE_2]_{CSF}$ at any time point after SNL (Hefferan et al.,

2003a, b). These results indicate that: a) spinal PGE_2 release is dependent on central synaptic activity evoked by brushing the affected dermatome(s); b) SNL does not effect a tonic increase in spinal PGE_2 synthesis; and c) BR-evoked release of PGE_2 is not sustained, in contrast to the allodynia arising from SNL.

SNL-induced allodynia was dose-dependently reversed by the daily injection of i.t. S(+)-IBU, SC-236 or SC-51322 (Hefferan et al., 2003a, b). This effect, beginning two days after injury, persisted for a further five days. In contrast, R(-)-IBU or SC-560 had no significant anti-allodynic effect, irrespective of the treatment day (Hefferan et al., 2003a, b). Systemic (i.v. or i.p.) administration of i.t. doses of S(+)-IBU, or i.t. delivery to the mid-cervical spinal cord also had no effect in SNL animals (Hefferan et al., 2003a, b). Thus, the stereo-selective inhibition of PG synthesis or the blockade of EP receptors in the lumbar spinal cord attenuated 'established' allodynia up to seven days after SNL. The results suggest that COX-2 is primarily, if not exclusively responsible for spinal PG synthesis beginning two days after SNL. The time-course of PG-dependent and PG-independent allodynia in the SNL model is summarized in Figure 1.1.

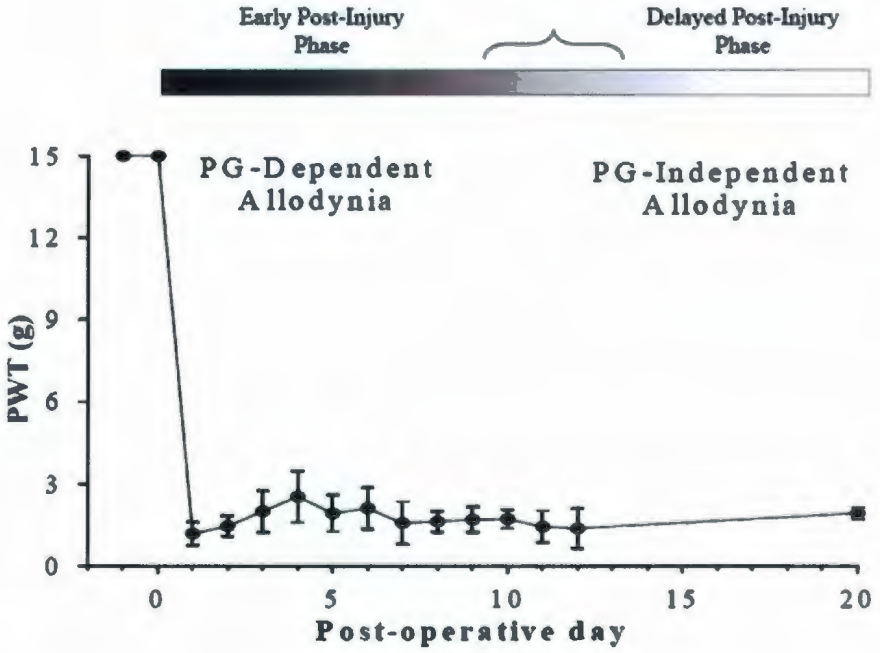

Figure 1.1. SNL-induced Allodynia consists of two phases: an early phase that is PG-dependent and a delayed phase that is PG-independent. Time course of PWT following tight ligation of the left L5/L6 spinal nerves (day 0) is also shown with allodynia being stable for at least twenty days. Data are presented as the mean ± SEM of seven animals. Shading represents the change in PG-dependence (Hefferan et al., 2003b).

1.8 SPINAL PG ARE CRITICAL TO THE DEVELOPMENT OF SNL-INDUCED, PG-INDEPENDENT ALLODYNIA

Treatment with i.t. S(+)-IBU or SC-560 (beginning 1h after SNL and repeated every 2 h for 8 h) completely prevented the SNL-induced decrease in PWT for up to twenty days (Hefferan et al., 2003b). Comparable treatment with R(-)-ibuprofen or SC-236 had no effect. The selective effect of i.t. S(+)-IBU in the SNL model supports the hypothesis that spinal PG are critical early signals in the acute nerve injured state, and their possibility as pre-emptive targets. This effect was matched by a COX-1 selective (SC-560), but not a COX-2 selective (SC-236) inhibitor, suggesting that pathogenic PG in the spinal cord are derived from COX-1, at least up to 8 h after SNL. That PG synthesis coincides with the onset of nerve injury is consistent with immediate afferent barrage triggered by peripheral nerve injury, the abnormal release of neurotransmitters (particularly glutamate) in the spinal DH (Farooque et al., 1996; Kawamata and Omote, 1996), the subsequent activation of postsynaptic NMDA receptors (Kajander et al., 1992; Yoon et al., 1996), and corresponding intracellular events effecting the release of AA and the early synthesis of spinal PG by constitutive enzymes (COX-1 ≥ COX-2). After diffusion into the extracellular space, spinal PG enhance the excitability of adjacent cells (Willingale et al., 1997; Baba et al., 2001) and the sustained activation of NMDA receptors. The latter underlies the central induction of COX-2 by primary afferent (i.e. non-immune) input (Vanegas and Schaible, 2001).

Transcription of the COX-2 gene is modulated by nuclear factor kappa-B (NFκB). Spinal COX-2 protein is also is known to be increased by peripheral nerve injury. This up-regulation of COX-2 may reflect the abnormal activation of NFκB in the affected spinal cord, consistent with exaggerated glutamate release and the subsequent increase in intracellular Ca^{2+} concentration in the acute nerve-injured state.

1.9 RELATIONSHIP BETWEEN SPINAL PG AND NFκB IN ALLODYNIA

NFκB is a ubiquitous modulator of gene transcription which is made up of the following proteins, p52, p50, RelB, c-Rel, RelA(p65). They reside in the cytoplasm of most cells as a complex with the inhibitory subunit, IκB. Phosphorylation of IκB by IκB kinase (IKK) results in polyubiquitination, the degradation of IκB by the proteasome, and the release of active NFκB (Perkins, 2000). The latter is then able to translocate into the nucleus where, in the presence of other transcription factors, it binds to the specific DNA fragments activating the expression of target genes. This process can be triggered by neurotransmitters (e.g. glutamate), cytokines, growth factors, drugs and chemicals, as well as conditions such as infection and stress (Grilli and Memo, 1999; Pahl, 1999; Shi et al., 1999; Perkins, 2000). Once activated, NFκB is known to regulate many genes including those coding for cytokines, apoptotic and survival proteins (e.g. Bcl-2, Bcl-xl, Bcl-xs, Bax, p53), adhesion molecules, COX-2, manganese superoxide dismutase, inducible NO synthase (iNOS), and

cyclin-D1 (Clemens et al., 2000). The fate of the cell appears to be governed by a host of factors including the nature of the activation and the set of target genes affected (Barkett and Gilmore, 1999).

In the CNS, NFκB influences survival, apoptosis and plasticity (Albensi & Mattson, 2000; Shishodia and Aggarwal, 2002; Bubici et al., 2006; Piva et al., 2006). NFκB is an important factor regulating gene expression triggered by Ca^{2+}-dependent synaptic transmission (Meffert et al., 2003), including that evoked by nerve injury. However, its exact role remains unclear (Marini et al., 2004). Its potential role in nerve injury-induced allodynia was first suggested in experimental studies using inhibitors of NFκB activation. Systemic or i.t. injection of ammonium pyrrolidedithiocarbamate (PDTC) or an oligonucleotide decoy attenuated the mechanical allodynia arising from chronic constriction of the sciatic nerve (Ebersberger et al., 2006), spinal cord injury (La Rosa et al., 2004; Jimenez-Garza et al., 2005), or treatment with i.t. dynorphin (Laughlin et al., 2000). However, their inhibiton of NFκB activation in the CNS, and the relevance of this mechanism to their anti-allodynic effect were not investigated. Moreoever, the relationship between NFκB activation, the subsequent induction of COX-2, the synthesis of spinal PG, and the development of hyperexcitability in the affected spinal cord are unkown. The possibility that spinal PG, generated immediately after SNL, facilitate the initial activation of NFκB and, in turn, its downstream transcriptional effect on COX-2 have not been investigated.

Given that NFκB also modulates iNOS gene expression, it is possible that spinal NO synthesis and signaling, like that of spinal PG, might be affected in the acute nerve injured state.

1.10 RELATIONSHIP BETWEEN SPINAL PG AND NITRIC OXIDE IN ALLODYNIA

Unlike other neuromodulators, NO is a diffusible messenger whose excitatory activity is neither mediated by membrane-bound cell surface receptors nor limited by their cellular distribution (Millan, 1999; Petersen-Zeitz & Basbaum, 1999). NO is also thought to subserve important neuromodulatory functions in the CNS (Millan, 1999). The effects mediated by NO include the modulation of neurotransmitter release, gene expression, learning, memory, and PG synthesis (Bredt and Snyder, 1992; Haley et al., 1992; Mollace et al., 1995; Wang and Robinson, 1997; Garthwaite et al., 1988).

Extensive evidence links spinal NO to nociceptive processing. For example, incubation of an astroglial cell line with NMDA 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 DH 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 NP is supported by pharmacological and molecular studies. Mechanical allodynia was attenuated in the spinal cord ischemia and the spinal cord ligation models by L-NAME (Hao and Xu, 1996; Yoon et al., 1998; Hefferan and Loomis, 2004). The amount of NO markedly increased after acute traumatic injury in the spinal cord (Nakahara et al., 2002) and nNOS staining in the superficial dorsal horn was dramatically increased after nerve injury (Ma and Eisenach, 2007). Given that NOS and COX are co-activated by NMDA receptor activation secondary to exaggerated glutamate release, that they exhibit similar changes in expression after injury, and that their expression is modulated by NFκB, raises the likelihood that spinal NFκB, NO and PG may work in concert to effect spinal hyperexcitability early after nerve injury.

1.11 RESEARCH HYPOTHESIS AND SPECIFIC OBJECTIVES

Research implicating spinal PG early in allodynia appears at odds with anecdotal reports suggesting that NSAIDs provide little, if any, benefit to patients with established allodynia. However, previous work in our laboratory (Hefferan et al., 2003a, b) indicates that the timing of treatment and the very purpose for which they are used are critical factors influencing the effectiveness of NSAIDs in allodynia. Specifically, they indicate that NSAIDs need to be given within hours of injury, not to relieve NP *per se* (i.e. as analgesics), but to mitigate its emergence.

To date, NSAIDs have been given long after nerve injury as adjuncts for symptom management. In short, NSAIDs have not been used clinically for the purpose suggested in this thesis research, and what limited experience that there is, does not refute the validity of the proposed research hypothesis. On the contrary, the implications of this research for NP are novel and very intriguing.

Arguably the most important clinical feature of neuropathic pain is allodynia, which is known to be triggered by input from low-threshold primary afferent (Aβ) fibers not normally involved in pain processing. While the mechanisms underlying this debilitating condition are poorly understood, there is a growing body of evidence that PG, generated in the spinal cord after nerve injury, may play an integral role in the early pathogenesis of mechanical allodynia. There is additional evidence that NO, generated in the spinal cord after nerve injury, works in concert with spinal PG to alter sensory processing, resulting in the miscoding of low-threshold input as pain.

Previous work in our laboratory has shown that SNL induces robust mechanical allodynia in the rat which consists of two distinct phases: a) an early spinal PG-dependent phase lasting approximately seven to ten days; and b) a delayed PG-independent phase lasting up to seventy days. Importantly, the former is a pre-requisite to and a trigger for the more complex and time-dependent changes underlying long-term, irreversible mechanical allodynia. The latter, which is long-term and largely irreversible, comprises very complex mechanisms, including gene transcription, phenotypic and anatomical changes in

both the peripheral and central nervous systems (Millan, 1999). Interfering with critical signaling events early after nerve injury is a logical strategy by which chronic neuropathic pain might be prevented. Such an approach requires a clear understanding of the sequence, time-course and pharmacology of these early signals. Investigating the potential advantage of intervening pharmacologically, before allodynia becomes chronic and irreversible in nature (I.e. pre-emptive treatment of allodynia), represents the ultimate goal of this thesis research.

In light of the apparent pathogenic role of spinal PG early after nerve injury, the present research investigated the SNL-induced changes in spinal PG synthesis and signaling, their relevance to the development of spinal hyperexcitability and mechanical allodynia, and the mechanisms underlying the changes in PG synthesis and signaling in the spinal PG-dependent phase. These hypotheses were tested using the L5/L6 spinal nerve ligation (SNL) model of NP (Kim and Chung, 1992) in which the L5 and L6 spinal nerves are tightly ligated (unilaterally) just distal to the DRG. This experimental NP model was selected because: a) mechanical allodynia develops within the first 24 h after injury and last for at least four months; b) it elicits pronounced mechanical allodynia while maintaining good animal health; and c) of the high reproducibility from one animal to another. This consistency, as well as absence of spontaneous recovery from allodynia, provided an excellent animal model to use in the present research. The specific objectives were:

1. To characterize the effect of SNL on A- and C-fiber mediated reflex responses (AFRR and CFRR, respectively) in the affected hind limb, and to determine the temporal and spatial relationship of these changes to SNL-induced allodynia.
2. To determine if the SNL-induced changes in the AFRR and CFRR (i.e. spinal hyperexcitability) are spinal PG-dependent, and if so, to determine the relevant COX isoform(s).
3. To determine if SNL triggers the activation of nuclear factor kappa B (NFκB) in the affected spinal cord which initiate the delayed induction of spinal COX-2.
4. To determine if the sensitivity to PGE_2 is exaggerated during spinal PG-dependent allodynia, and if this abnormal state is limited to the affected spinal cord three days after SNL.
5. To determine if PG-dependent spinal hyperexcitability and mechanical allodynia are mediated by E-type prostaglandin (EP) receptors, and to investigate the changes in the expression of spinal EP_{1-3} receptor subtypes and the glycine-α3 receptor subunit (GLY-α3R) three days after SNL.
6. To determine if disrupting spinal PG synthesis/signaling immediately after SNL (i.e. pre-emptive treatment) prevents the development of PG-dependent spinal hyperexcitability and mechanical allodynia.

7. To determine if spinal PG-dependent hyperexcitability is affected by the SNL-induced generation of spinal nitric oxide (NO), and if so, to investigate relevant NO synthase (NOS) isoform(s).
8. To determine if, and how, spinal NO-mediated activity affects spinal PG-dependent spinal hyperexcitability and mechanical allodynia.

2.0 INCREASED EXPRESSION OF CYCLOOXYGENASE AND NITRIC OXIDE SYNTHASE ISOFORMS, AND EXAGGERATED SENSITIVITY TO PROSTAGLANDIN E_2, IN THE RAT LUMBAR SPINAL CORD THREE DAYS AFTER L5/L6 SPINAL NERVE LIGATION

2.1 INTRODUCTION

There is growing evidence that PG, generated in the spinal cord following nerve injury, play a contributory role in the pathogenesis of NP. This was first suggested by the robust allodynia induced by i.t. PG in otherwise normal (uninjured) conscious mice (Minami et al., 1992,1994), an effect blocked by i.t. PG receptor antagonists (Minami et al., 1995; Kawahara et al., 2001), and later shown to occur in $EP_1^{(+/+)}$, $EP_3^{(+/+)}$ and $EP_3^{(-/-)}$, but not $EP_1^{(-/-)}$, mice (Minami et al., 2001). Other studies have reported increased EP-receptor-IR in injured nerves after partial sciatic nerve ligation (Ma and Eisenach, 2003), and BR-evoked increases in the concentration of PGE_2 in spinal dialysate two to eight days after SNL (Hefferan et al., 2003a). The latter were temporally and spatially linked to BR-evoked allodynia, and absent in sham-controls. Experimental allodynia was also attenuated by certain COX inhibitors or the EP-receptor antagonist, SC-51322, when given in the two to eight day window following SNL (Ma et al., 2002; Hefferan et al., 2003a).

SNL triggers early and sustained activation of spinal NMDA receptors (Ossipov et al., 2000); a critical trigger for central PG and NO (Sorkin and Moore,

1996). This is accompanied by the enhanced expression of COX (Resnick et al., 1998; Zhao et al., 2000) and NOS (Choi et al., 1996) in the spinal cord. Nerve injury-induced changes in spinal COX-1 and COX-2 expression or immunoreactivity have been described (Zhao et al., 2000; Zhu and Eisenach, 2003; Hefferan et al., 2003b), but little is known about the concurrent expression of nNOS and iNOS, or whether any of these changes are confined to the spinal segments affected by nerve injury. The expression of both iNOS and nNOS increase early after spinal cord injury (Xu et al., 2001; Nakahara et al., 2002). Furthermore, the genes coding for inducible NOS and COX have identical promoters and response elements to NFκB (Lowenstein et al., 1993), which also has increased expression after spinal cord injury (Bethea et al., 1998). In the present study, we compared the expression of COX-1, COX-2, nNOS and iNOS in the lumbar, thoracic and cervical spinal cord three days after SNL.

Anecdotal reports suggest that NSAIDs provide little, if any, clinical benefit in relieving NP. However, work in our laboratory has shown that the timing of drug administration after nerve injury, and the COX isoforms affected are important variables governing the effectiveness of these drugs in experimental neuropathy. For example, COX-1 selective inhibitors, given intrathecally two to four hours after SNL, prevented the emergence of allodynia in the rat for up to twenty-five days (Hefferan et al., 2003b), but were ineffective in reversing established allodynia. These results, and those of previous reports (Zhao et al., 2000; Hefferan et al., 2003a), strongly suggest that COX inhibitors need to be

given early after nerve injury, and not for the relief of neuropathic pain *per se*, but to prevent the emergence of allodynia. They, in turn, support a pathogenic role for spinal PG early after SNL, although the relationship between nerve injury, spinal PG activity, and allodynia remains unclear.

One possibility is that nerve-injured animals develop abnormal sensitivity to the pathophysiological effects of endogenous PG in the spinal cord. This could trigger, or at least facilitate (i.e. through a pathogenic cascade), further adaptations that effect long term disturbances in sensory processing. If correct, such a change in sensitivity should be greatest in, and possibly restricted to, those spinal segments most affected by nerve injury. It should also be attenuated by pharmacological agents that disrupt spinal PG activity. To test this hypothesis, we compared the concentration- and dose-response effects of PGE_2 in the lumbar and thoracic spinal cord of SNL, sham operated and naïve rats. We also investigated the effects of the EP-receptor antagonist, SC-51322, and the S(+)- and R(-)-enantiomers of IBU in this model.

2.2 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, NL, Canada.

Animals. Male Sprague-Dawley rats (~130 g on the day of experimentation) were obtained from the Vivarium of 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 after surgery. A 12 h light-dark cycle (lights on at 0700 h) was used throughout.

Intrathecal Catheterization. Intrathecal catheters (6.5 cm-length terminating near the lumbar enlargement or 4.0 cm-length terminating in the mid-thoracic segments) were implanted as previously described (Hefferan et al., 2003a). Rats with normal motor, grooming and feeding behavior were housed separately and allowed to recover for three days before SNL or sham surgery.

Neuropathy. Neuropathy was induced using the method of Kim and Chung (1994), as previously described (Hefferan et al., 2003a). Rats were anesthetized with halothane and the left L4 and L5 spinal nerves were isolated and separated. The L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham-controls, the L5 and L6 spinal nerves were isolated but not ligated. All animals were allowed to recover for three days before experimentation. Allodynia, defined as a PWT of ≤ 4 g, was confirmed using von Frey filaments.

Western Blotting. The spinal cord was extracted hydraulically (de Sousa and Horrocks, 1979), immediately frozen in 2-methylbutane (Sigma Chemical, St. Louis, MO, USA), and stored at -80 °C. The spinal cord was separated into cervical, thoracic, and lumbar regions (L2 to L6), and further subdivided into the left and right, ventral and dorsal quadrants. Spinal cord was homogenized in ice cold lysis buffer (1% Nonidet-P40, 10% glycerol in TBS plus a protease inhibitor cocktail tablet - Roche Diagnostics, Laval, PQ, CA, 1 mM sodium vanadate, 1 mM sodium fluoride, and 0.025% SDS) and centrifuged at 10,000 rpm for 5 minutes (4 °C). Samples, diluted to achieve equal protein concentrations (30 µg), were separated by electrophoresis on 8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and incubated for 1.5 h in Tris buffer (Tris 25 mM, glycine 192 mM, 200 ml methanol, pH 8). Pre-stained protein markers were used for molecular weight determination. The blots were then stained with Ponceau Red to assess the equivalency of protein loading, and subsequently probed with the following antibodies: polyclonal rabbit anti-COX-1 (1:250), polyclonal rabbit anti-COX-2 (1:1000), polyclonal rabbit anti-nNOS (1:1000), and polyclonal rabbit anti-iNOS (1:1000) primary antibodies (Cayman Chemical, Ann Arbor, MI, USA). Membranes were incubated overnight at 4 °C with primary antibodies, and diluted in Tris buffer (containing 3% milk powder and 0.05% Tween-20). Protein bands were treated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Chemicon International Inc., Temecula, CA, USA) for 1 h at room temperature, washed for 30 minutes in Tris

buffer, visualized using enhanced chemiluminescence (PerkinElmer Life Sciences Boston, MA, USA), and exposed to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC, USA).

Spinal Glutamate Release. The spinal cord was extracted hydraulically (de Sousa and Horrocks, 1979) and the dura and arachnoid membranes carefully removed. The lumbar region (L2 to L6) was excised, mounted on cutting blocks and immersed in sucrose-modified artificial cerebrospinal fluid (aerated with 95% O_2 and 5% CO_2). Slices (600 to 800μm) were cut with a vibratome and placed in aerated artificial cerebrospinal fluid at room temperature. They were immersed in HEPES buffer 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 (Nicholls et al., 1987). The reduced form of nicotinamide adenine dinucleotide phosphate generated from this reaction was quantitated using spectrofluorometry with excitation at 335 nm and emission at 430 nm (Nicholls et al., 1987). Basal and PGE_2-evoked glutamate release was quantified using standard curves constructed with L-glutamate (0 to 360 pmol; Sigma-Aldrich, ON, CA) on each day of analysis. Basal glutamate release (i.e. release in the absence of any drug stimulus) was determined for 15 minutes before the introduction of drug into the cuvette. Drug-evoked release was defined as total release (i.e. after a drug stimulus) minus basal release. Protein content in each slice was determined

using a modified Lowry protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Behavioral Testing. Spontaneous (i.e. no brushing) and BR-evoked behavior were continuously graded using a modified scoring system (Malmberg et al., 1995). Spontaneous behavior (0 - normal behavior, bright, alert and exploring; 1 - huddling, burrowing, or hiding; 2 - one of the following: piloerection, occasional vocalization, or favoring the affected side; 3 - two or more of the following: piloerection, occasional vocalization, favoring the affected side; and 4 - any of the following: frequent or persistent vocalization, circling motion, licking or biting the affected dermatomes) was graded over a 4-min interval. The hair on the back, flanks, limbs, and hind paws was then brushed with a cotton-tipped applicator sufficient to deflect the pelage. This was continued for 1min and the behavior graded as follows: 0 - normal behavior, curious, alert, and exploring; 1 - avoidance of stimulus source or protection of the affected dermatomes; 2 - one of the following: piloerection, paw withdrawal, or occasional vocalization; 3 - two or more of the following: piloerection, paw withdrawal, or occasional vocalization; and 4 - any of the following: attacking the applicator, frequent or persistent vocalization, circling motion, licking or biting the affected dermatomes. This sequence was repeated every 5 minutes for the first hour and every 30 minutes thereafter up to 6 h or until no responses were detected. The investigator was

blinded to the nature of the treatment (i.e. vehicle, drug, concentration) in all behavioral experiments.

Drugs. PGE_2, SC-51322, S(+)- and R(-)-IBU were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Arachidonic acid was purchased from Cayman Chemical (Ann Arbor, MI, USA). PGE_2 solutions were prepared according to Nishihara et al. (1995). Briefly, PGE_2 was initially dissolved in ethanol and evaporated under nitrogen gas. It was then re-dissolved in normal saline and diluted with the same to yield the desired concentrations. All other drugs were dissolved in 100% DMSO and diluted with normal saline at the time of injection to yield a final DMSO concentration of 50%. For the *in vitro* experiments, drugs were added directly into the cuvette containing the slice using a microsyringe. Each concentration of PGE_2 was tested using a separate slice so that a full PGE_2 concentration–response curve (CRC) was determined in each animal. All equipment was sterilized with 70% alcohol and thoroughly rinsed with 0.9% sterile saline before injection. Intrathecal drugs were injected into conscious, unrestrained rats using a hand-held microsyringe. Drugs were delivered in a volume of 5 µl followed by 5 µl of sterile saline. The i.t. catheter was immediately resealed with a stainless steel plug. The position of the catheter tip was verified visually after sacrifice in randomly selected animals. In three animals, methylene blue (10 µl) was injected i.t. at the

mid-thoracic level (i.e. 2 cm rostral to the mid-lumbar enlargement) to assess the extent of staining below the thoracic cord.

Data Analysis. Data are reported as the mean ± SEM. Western blots were analyzed by optical density using ImageQuant® software (Amersham Biosciences Corp., Piscataway, NJ, USA). Expression data are presented as nanograms of protein, based on a single cyclooxygenase and NOS standard (50 ng), run in separate lanes on each gel, and corrected for background optical density. Area-under-the-curve (AUC) was calculated using trapezoidal integration. Concentration–response analysis was performed using methods from Tallarida and Murray (1987). Comparisons within each treatment group were performed using one-way, repeated measures ANOVA, followed by the Newman-Keuls test. Comparisons across all drug- and vehicle-treated groups at each time point were determined using one-way, completely randomized ANOVA, followed by the Newman-Keuls test (SigmaStat® 2.0; Systat Software, Inc., Point Richmond, CA, USA).

2.3 RESULTS

Rats undergoing SNL displayed a significant decrease in PWT from ≥ 15 g (baseline) to ≤ 4 g (Figure 2.1). This change in sensitivity remained stable for at least twenty days and confined to the plantar surface of the left hind paw (ipsilateral to nerve ligation). By day 20, a modest but significant ($p<0.01$)

decrease in PWT developed on the contralateral side of nerve-ligated animals (Figure 2.1). Generally, the affected hind paw was kept in an elevated and cupped position, thereby minimizing contact with the cage floor. All nerve-ligated rats were otherwise healthy, showed normal feeding and grooming behavior, and regular weight gain. Neither i.t. catheterization, nor sham-surgery had any effect on PWT compared to pre-surgical values (Figure 2.1).

Western analysis revealed a significant increase in the expression of COX-2 (45%), nNOS (25%) and iNOS (45%) compared to sham-controls in the lumbar cord three days after SNL (Figure 2.2). These changes were localized to the left dorsal horn (i.e. ipsilateral to SNL). In contrast, COX-2, nNOS, and iNOS expression remained unchanged across the dorso-ventral axis, and between the ipsilateral and contralateral sides in the thoracic and cervical segments (Figure 2.2A, 2.2C, 2.2D). Although COX-1 expression increased by 11% in the left L-DH of ligated rats, this was not statistically different ($p>0.05$) from sham-controls (Figure 2.2B). Neither was there a difference in COX-1 across the dorso-ventral axis, or between the ipsilateral and contralateral sides (Figure 2.2B). The relative expression of COX-1, COX-2, nNOS, and iNOS varied within each spinal region. However, there were no significant differences in the rostro-caudal expression of each protein ($p>0.05$) in SNL and sham operated animals, except in the left L-DH after SNL. Representative immunoblots of COX-2 and iNOS from the lumbar cord of sham operated and SNL animals, and their corresponding protein standards are shown in Figure 2.2E.

PGE_2 resulted in a concentration-dependent release of glutamate (EC_{50} = $2.37x10^{-11}$ M; Table 2.1) from lumbar slices harvested three days after sham surgery (Figure 2.3). PGE_2 was equi-effective using slices from naïve (no surgery) and sham operated animals (Figure 2.3A; Table 2.1). In contrast, the PGE_2 CRC using lumbar slices from nerve-ligated rats was significantly shifted to left compared to the sham and naïve groups (Figure 2.3A). The EC_{50} in the SNL group was 8.30×10^{-15} M (Table 2.1), representing a 2855- and 2807-fold increase in potency relative to sham- and naïve-controls, respectively. There was no significant difference in the peak effect of PGE_2 using SNL (3.12 ± 0.34 pmol/min/mg protein), sham operated (3.90 ± 0.62), and naive (3.98 ± 0.58) preparations. Neither was there a difference ($p>0.05$) in the potency of PGE_2 using thoracic slices from sham operated and SNL rats (Figure 2.3B; Table 2.1). Vehicle had no effect on glutamate release from lumbar slices of SNL animals (Figure 2.3A). Arachidonic acid evoked glutamate release from lumbar slices of naïve animals but was approximately 250-fold less potent, and 32% more efficacious than PGE_2 (Figure 2.3A; Table 2.1). All results were corrected for basal glutamate release, which was significantly greater ($p<0.05$) in nerve-ligated (2.94 ± 0.34 pmol/min/mg protein) as compared to sham-operated (1.50 ± 0.43) rats.

In slices from SNL animals, pretreatment with S(+)-IBU shifted the PGE_2 CRC to the right ($p<0.05$) relative to R(-)-IBU-treated, vehicle (DMSO)-treated or untreated preparations (Figure 2.4; Table 2.1). This corresponded to a 24-fold

increase in the EC_{50} of PGE_2 compared to DMSO pretreatment. R(-)-IBU and DMSO had no effect on the EC_{50} values. Neither S(+)- nor R(-)-IBU had any effect (p>0.05) on the potency of PGE_2 in sham operated preparations (Figure 2.4; Table 2.1). In separate experiments, pretreatment with the EP-receptor antagonist, SC-51322, increased the EC_{50} of PGE_2 in lumbar slices from nerve-ligated animals (Figure 2.5; Table 2.1). It also significantly increased the efficacy of PGE_2 relative to that in sham (41% increase), nerve-ligated (52% increase) and nerve-ligated + vehicle treated (42% increase) preparations (Figure 2.5).

To determine if the enhanced sensitivity to PGE_2 has relevance to allodynia, the dose-response effect of i.t. PGE_2 was compared in SNL and sham operated rats. Brushing the hind limbs of sham operated rats treated with i.t. PGE_2 evoked robust, nociceptive-like behavior (i.e. BR-evoked vocalizations, defensive posturing, licking the affected dermatomes, and biting the cotton-tipped applicator). Peak allodynia (90% of maximum possible score) was observed 10 min after injection and declined thereafter (Figure 2.6A). The duration of allodynia (0.1 µg) was 210 min. In SNL rats, the same dose of PGE_2 elicited peak allodynia (100% of maximum possible score) at 10 minutes and remained unchanged for 90min (Figure 2.6A). The duration of allodynia was >6 h. There were no significant differences between sham operated and SNL animals for spontaneous behavior (Figure 2.6, panels A and B). Dose-response analysis revealed a 362-fold decrease in the ED_{50} of PGE_2 in SNL compared to sham operated rats (Figure 2.6B; Table 2.2). In the absence of PGE_2, identical brushing of SNL +

vehicle-treated (302 ± 93 %MPS·min), SNL + untreated (262 ± 82), sham + vehicle-treated (154 ± 31) or sham + untreated (116 ± 50) rats had no significant effect (p>0.05; Figure 2.6B). In addition, there was no dose-response effect of PGE_2 on spontaneous behavior (p>0.05) in either SNL or sham operated animals (Figure 2.6B).

Area-under-the-curve analysis revealed a significant reduction in BR-evoked allodynia (63% decrease) and spontaneous behavior (53% decrease) in SNL animals when PGE_2 (0.1 µg) was injected into the thoracic as compared to the lumbar subarachnoid space (Figure 2.7). A similar trend was observed in sham-controls. Both BR-evoked and spontaneous behaviors were significantly lower after thoracic as compared to lumbar delivery in all groups. In three animals, methylene blue (10 µl) was injected intrathecally at the level of the mid-thoracic cord. No staining was observed below the thoracic cord.

In SNL rats, i.t. S(+)-IBU (100 µg) injected 20 min before PGE_2 significantly decreased BR-evoked behavior by 62% and spontaneous behavior by 46% compared to DMSO + PGE_2 (Figure 2.8). In sham operated rats, S(+)-IBU had no significant effect on either BR-evoked or spontaneous behaviors (p>0.05). R(-)-IBU was without effect in all treatment groups (Figure 2.8). In SNL animals, pretreatment with i.t. SC-51322 (100µg), 20 minutes before i.t. PGE_2 (0.01 µg), significantly attenuated BR-evoked (39%) and spontaneous behavior (28%) compared to DMSO + PGE_2 (Figure 2.9). SC-51322 had no effect on BR-evoked or spontaneous behaviors in sham operated animals (p>0.05).

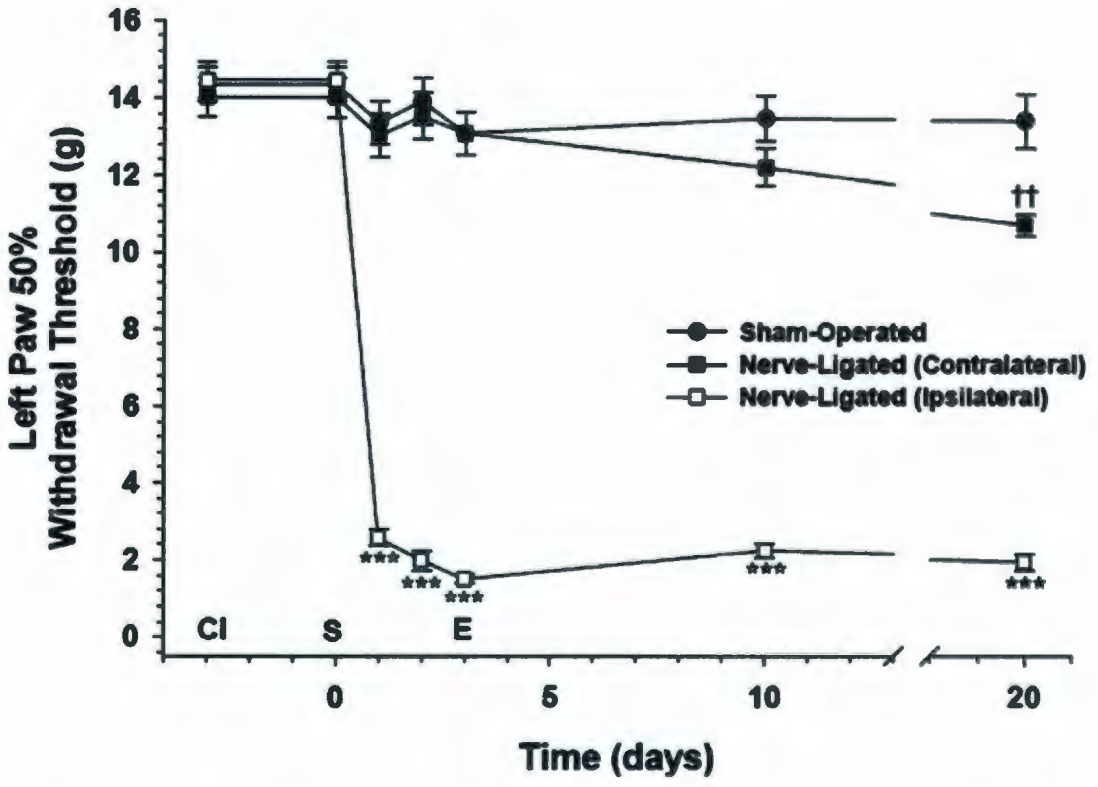

Figure 2.1. Tight ligation of the L5/L6 spinal nerves produced a significant reduction in PWT on the plantar surface of the ipsilateral hind paw. Animals were implanted with i.t. catheters (CI) three days before SNL or sham surgery (S) and all experiments (E) were conducted three days after surgery. Each point represents the mean ± SEM of ten animals. Asterisks indicate a significant difference from sham-controls or the contralateral side of nerve-ligated animals (***p<0.001). Daggers indicate a significant difference from sham-controls (††p<0.01). All paw withdrawal thresholds on the ipsilateral side of nerve-injured animals were significantly different from pre-ligation values.

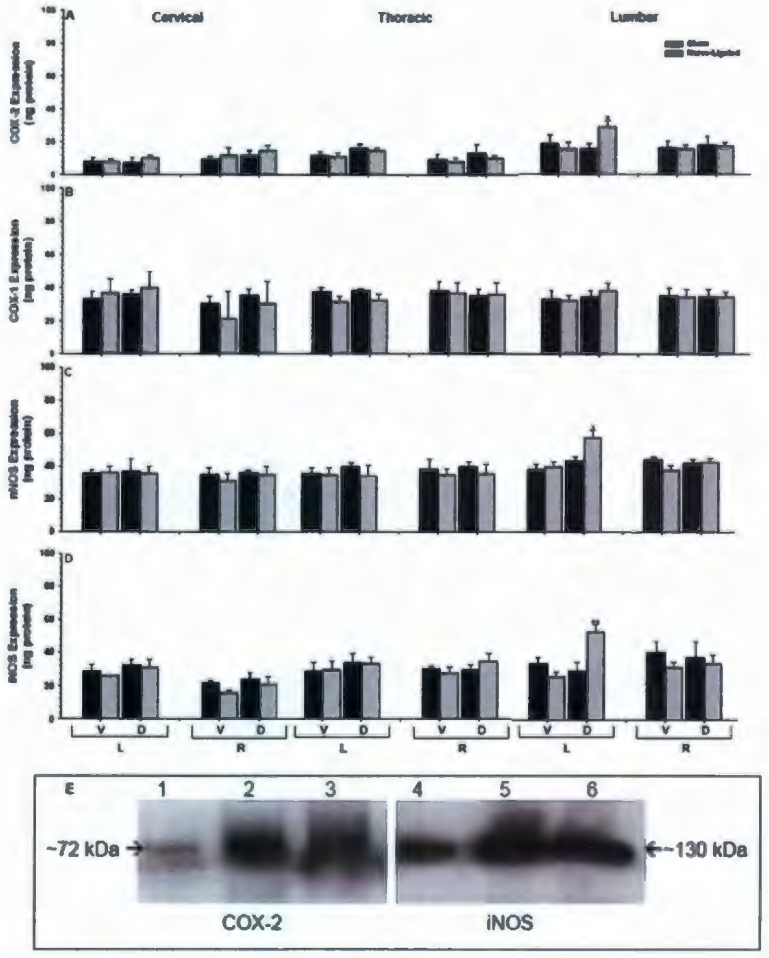

Figure 2.2. The expression of COX-2 (panel A), COX-1 (panel B), nNOS (panel C) and iNOS (panel D) in the left (L) and right (R) ventral (V) and dorsal (D) horns of the cervical, thoracic and lumbar spinal cord three days after SNL or sham surgery. Representative immunoblots (panel E) of COX-2 and iNOS in the lumbar spinal cord (lanes 1&4: sham-operated control; lanes 2&5: corresponding protein standard; lanes 3&6: SNL). Asterisks indicate a significant difference between sham-operated and SNL animals (*p<0.05, **p<0.01), and each bar represents the mean ± SEM of five to eight animals.

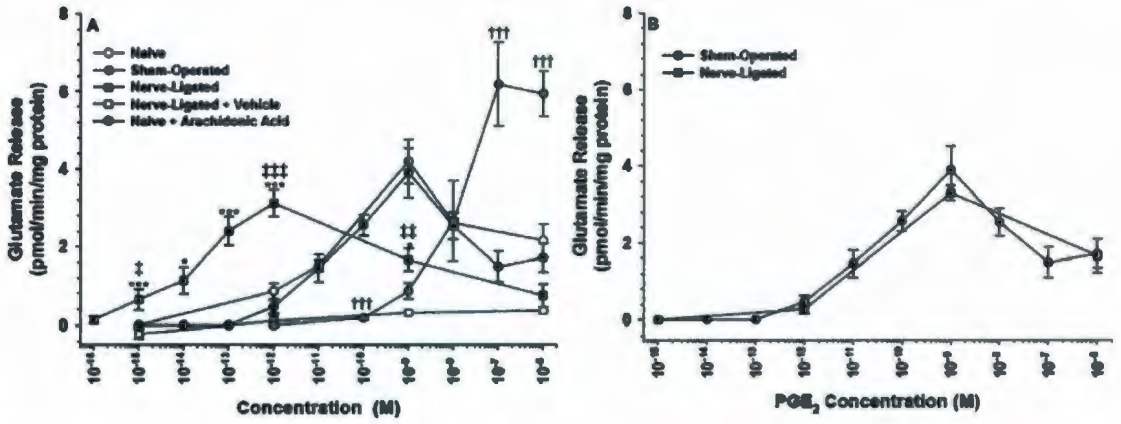

Figure 2.3. The effect of PGE_2 on glutamate release from lumbar slices of naïve, sham operated or SNL rats (panel A), and thoracic slices from SNL or lumbar slices from sham operated rats (panel B), three days after surgery. Each point represents the mean ± SEM of at least five animals (4 to 12 slices/dose) and asterisks indicate a significant difference between SNL, sham operated and/or naïve animals (*p<0.05, ***p<0.001) at the corresponding PGE_2 concentration. Daggers indicate a significant difference between naïve and sham operated animals following treatment with arachidonic acid (†p<0.05, †††p<0.001). Double daggers indicate a significant difference between the PGE_2-treated and vehicle-treated slices at the corresponding PGE_2 concentration (‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001).

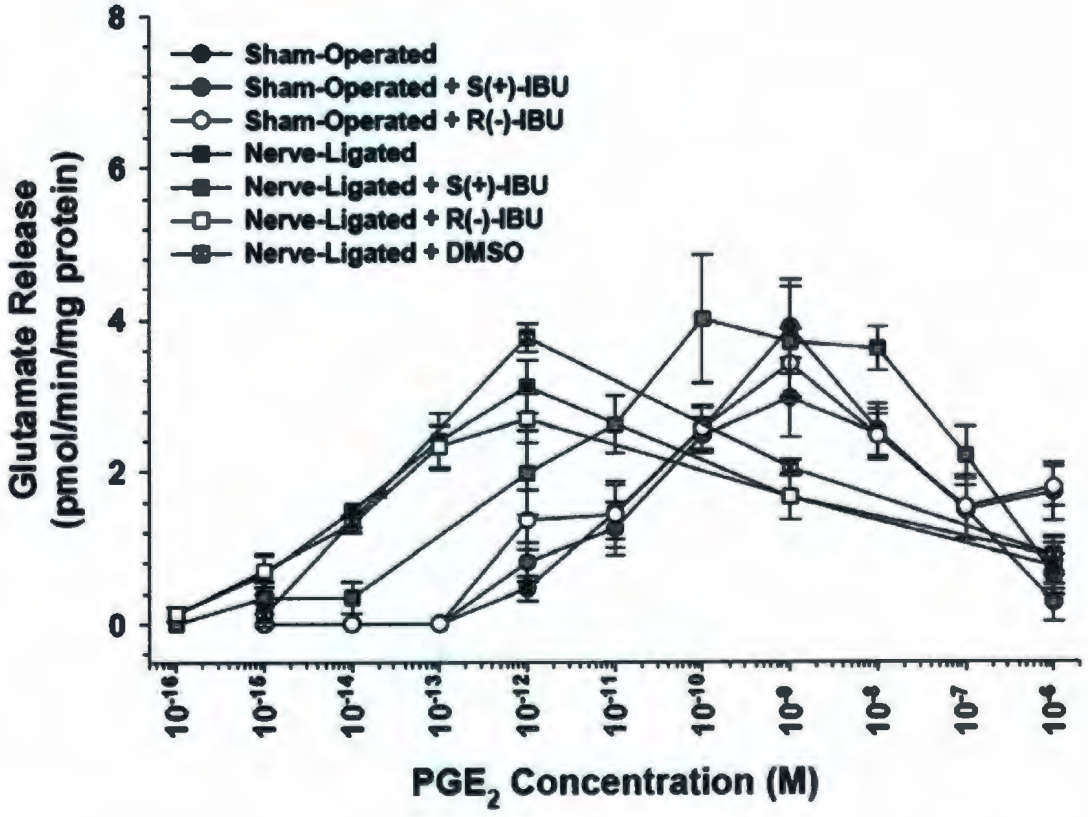

Figure 2.4. The effect of pretreatment with S(+)- or R(-)-IBU (1.0 μM) on PGE_2-evoked glutamate release. Spinal (lumbar) slices were harvested three days after SNL or sham surgery. Each point represents the mean ± SEM of at least six animals (4 to 10 slices/dose).

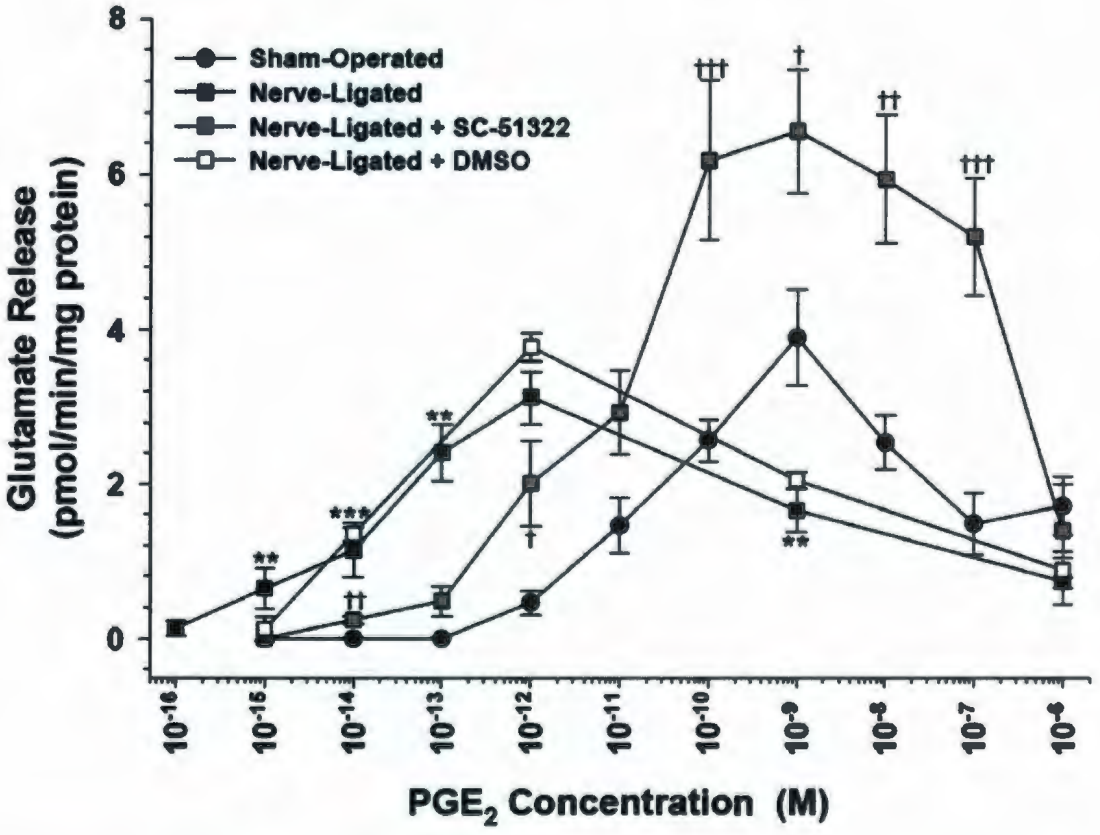

Figure 2.5. The effect of SC-51322 (1.0 μM) on PGE_2-evoked glutamate release from lumbar slices harvested three days after SNL. Each point represents the mean ± SEM of at least five animals (4 to 12 slices/dose). Asterisks indicate a significant difference from sham-controls at the corresponding PGE_2 concentration (**p<0.01, ***p<0.001). Daggers indicate a significant difference from sham-controls at the corresponding PGE_2 concentrations (†p<0.05, ††p<0.01, †††p<0.001).

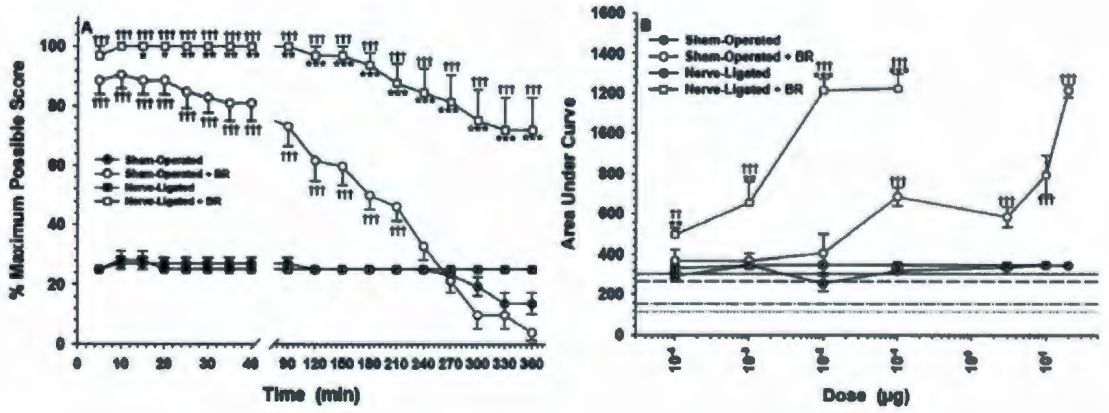

Figure 2.6. The time-course (panel A) and dose-response (panel B) curves of PGE_2 on BR-evoked and spontaneous behaviors in SNL or sham operated animals. For the time course experiments, rats were injected with i.t. PGE_2 (0.1 µg) three days after surgery. The horizontal lines in panel B represent mean BR-evoked results from vehicle-treated (___) and untreated (- - -) animals three days after SNL, and from vehicle-treated (- - -) and untreated (___) three days after sham surgery. None of these lines were significantly different from each other ($p>0.05$). Each point represents the mean ± SEM of six to fifteen animals. Asterisks indicate a significant difference from sham-controls at the individual time points (panel A) or doses (panel B) (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Daggers indicate a significant difference from their respective spontaneous (i.e. non-evoked) control (††$p<0.01$, †††$p<0.001$).

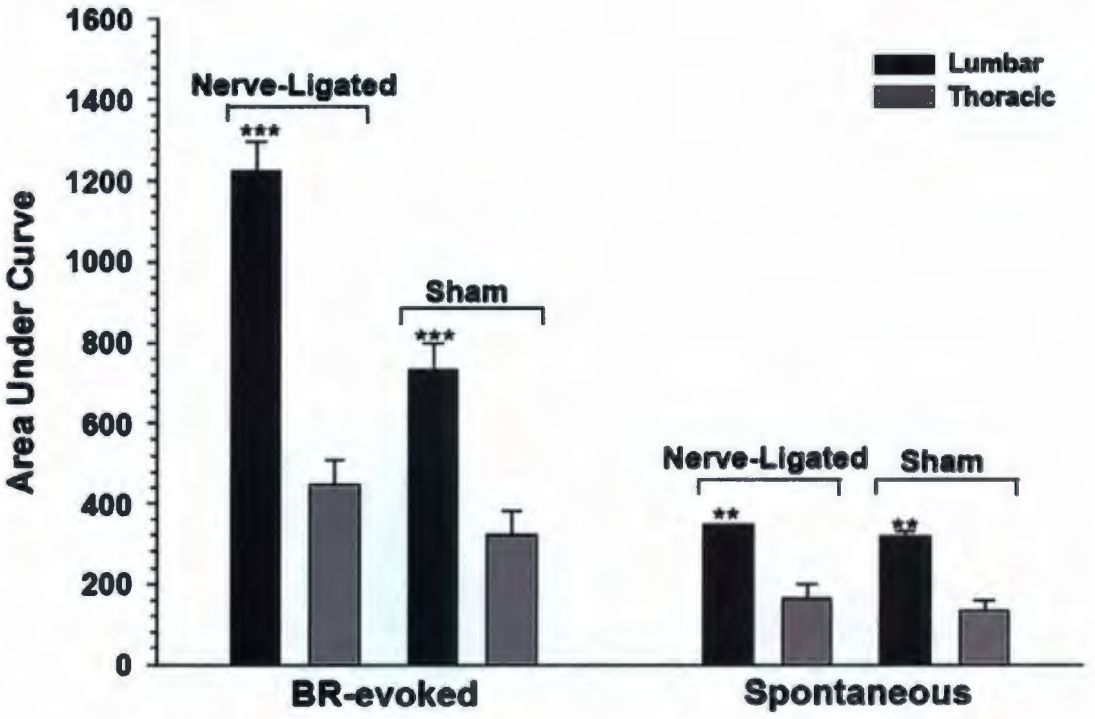

Figure 2.7. BR-evoked and spontaneous behaviors following i.t. PGE_2 (0.1 µg) into the lumbar or thoracic subarachnoid space three days after SNL or sham surgery. Each bar represents the mean ± SEM of six to fifteen animals. Asterisks indicate a significant difference from thoracic delivery within each treatment group (**p<0.01, ***p<0.001).

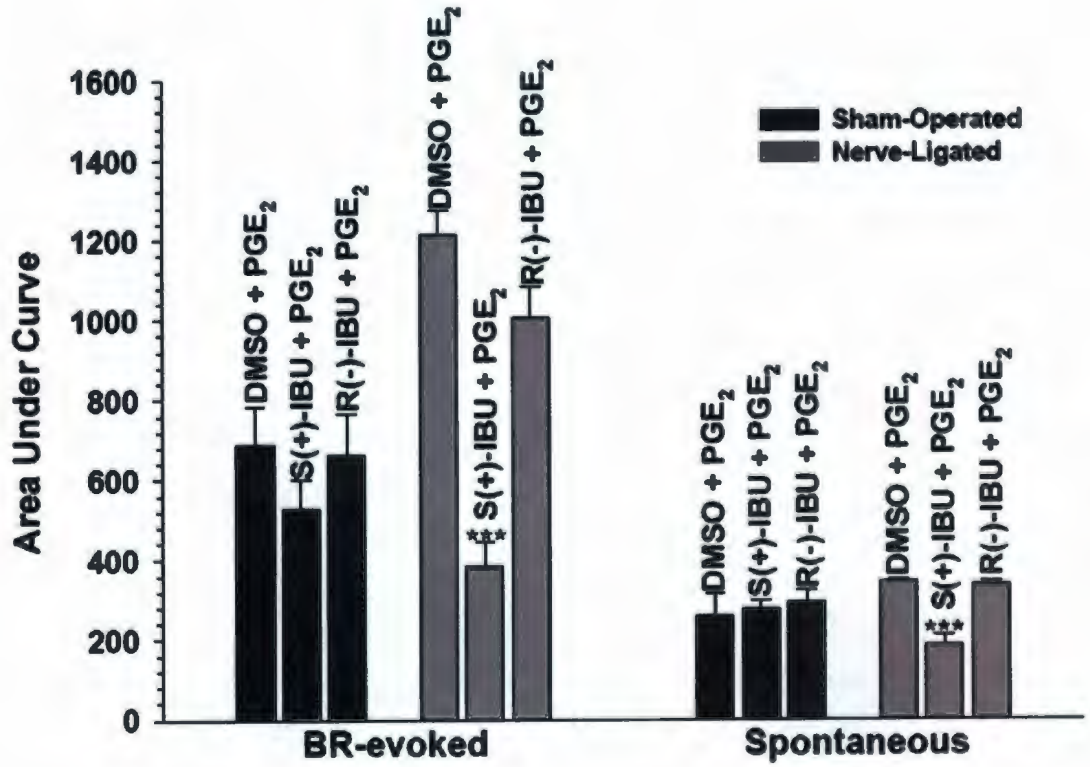

Figure 2.8. The effect of i.t. S(+)- or R(-)-IBU (100 µg) on BR-evoked and spontaneous behaviors induced by i.t. PGE_2 (0.01 µg). All drugs were injected three days after SNL or sham surgery. Each bar represents the mean ± SEM of six to twelve animals and asterisks indicate a significant difference (***p<0.001) from the both DMSO + PGE_2 or R(-)-IBU + PGE_2 groups.

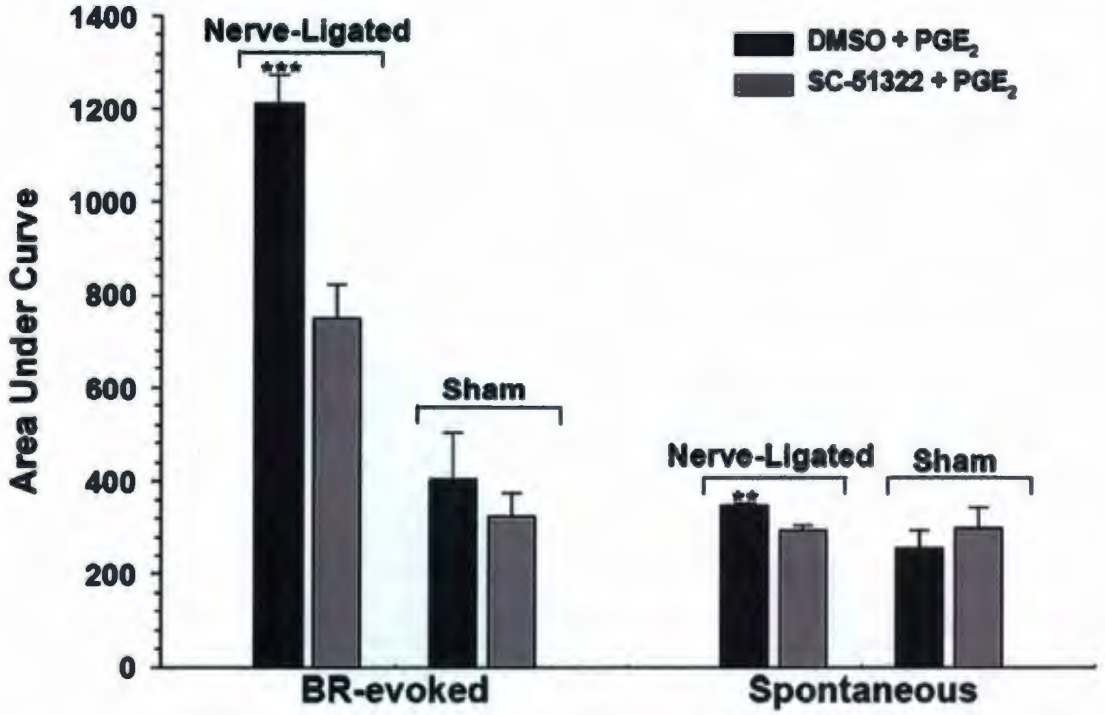

Figure 2.9. The effect of i.t. SC-51322 (100 µg) on BR-evoked and spontaneous behaviors elicited by i.t. PGE_2 (0.01 µg). All drugs were injected three days after SNL or sham surgery. Each bar represents the mean ± SEM of six to twelve animals and asterisks indicate a significant difference from the corresponding SC-51322 + PGE_2 treated group (**p<0.01, ***p<0.001).

2.4 DISCUSSION

Tight ligation of the left L5/L6 spinal nerves, but not sham surgery, induced localized hypersensitivity to both brushing and von Frey filaments. Allodynia was accompanied by the increased expression of COX-2, nNOS and iNOS, and an exaggerated sensitivity to spinal PGE_2 in the lumbar cord, three days after SNL. While the extent of change along the spinal cord was more difficult to define *in vivo* compared to the slice experiments, the behavioral data are consistent with a lumbar site of exaggerated sensitivity to PGE_2. This is supported by the absence of detectable pharmacodynamic changes in the thoracic cord of nerve-ligated (i.e. allodynic) animals, and the fact that increased dermatomal sensitivity to brushing and von Frey filaments remained confined to the left hind limb and plantar surface, respectively. The latter is in agreement with previous work using the SNL model (Hefferan et al., 2003b; Schafers et al., 2003).

The increase in COX-2 expression (45% above baseline three days after SNL) was confined to the L-DH ipsilateral to nerve injury. This effect is similar in magnitude to that reported by Zhao et al. (2000) but with a different time-course. They described a 45% increase 24 h after SNL which returned to baseline by day three. While the cellular localization of increased COX-2 immunoreactivity was not investigated in the present study, this isozyme is known to be expressed in macrophages and microglia (Durrenberger et al., 2004). There were also differences in COX-1, which is constitutively expressed in cells with glial

morphology in the DH and those with motor neuron morphology in the ventral horn (Hay et al., 1997a, b; Ebersberger et al., 1999; Maihofner et al., 2000; Zhu and Eisenach, 2003). Spinal COX-1 was unchanged from baseline in the present study, and exceeded that of COX-2, even in spinal segments where COX-2 was induced. In contrast, COX-1 expression was undetectable by Zhao et al. (2000). Zhu and Eisenach (2003) later reported an increase in the number of COX-1-IR cells in the deep and superficial laminae of the ipsilateral lumbar cord four days after SNL. This effect persisted in the superficial laminae for up to two weeks. The exact reasons for these discrepancies are unclear but could be due to differences in the immunoblotting protocol and/or nerve ligation pressure/injury. For example, the COX-1 primary antibody was incubated for 24 h at 4 °C in the present study compared to 1 h at room temperature by Zhao et al. (2000). Differences in nerve ligation pressure would explain the distinct onsets in allodynia (i.e. one day versus one week after SNL). Whatever the reasons, the results of the present study correlate temporally and spatially with: a) the pharmacological changes induced by SNL in this report; b) the preferential reversal of allodynia by COX-2 selective inhibitors given two to nine days after SNL (Ma et al., 2002; Hefferan et al., 2003a); and c) the prevention of allodynia for up to twenty-five days by an i.t. COX-1, but not a COX-2 selective inhibitor, given two to four hours after SNL (Hefferan et al., 2003b).

A significant increase in the spinal expression of nNOS and iNOS was also observed three days after SNL; an effect confined to the ipsilateral L-DH. In

a model of traumatic spinal cord injury, cells expressing nNOS increased immediately after injury and lasted for 12 h (Nakahara et al., 2002). Those cells expressing iNOS were evident from 12 h to three days after injury. A progressive increase in iNOS expression, beginning 24 h after spinal trauma and peaking on day 7, has also been reported (Xu et al., 2001). Neurons exhibiting increased NOS-immunoreactivity are located in the superficial laminae of L4 to L6 seggments ipsilateral to nerve (Lukacova et al., 2003) or spinal cord injury (Gonzalez et al., 2001). Collectively, the expression data indicate that COX and NOS isozymes are up-regulated in the spinal cord after SNL, but with different time-courses. nNOS and possibly COX-1 are affected within hours of injury (COX-1 expression is increased in the L-DH 12 h after SNL - unpublished results). COX-2 and iNOS exhibit a more delayed effect (i.e. days after injury). The genes coding for inducible NOS and COX have identical promoters and response elements to NFκB (Lowenstein et al., 1993); a factor known to be increased in the spinal cord after injury (Bethea et al., 1998). Experiments were subsequently conducted to investigate the connection between increased NFκB, NOS, COX, and EP-receptors in the SNL model.

In vitro, PGE_2 yielded a consistent bell-shaped CRC in all slice preparations. This was previously described using synaptosomes (Nishihara et al., 1995) and astrocytes (Bezzi et al., 1998) from normal animals, and may reflect the preferential activation of EP-receptors inhibiting glutamate release (e.g. EP_3) and/or the desensitization of excitatory EP-receptors (i.e. EP_1, EP_2 and

EP_4), at high PGE_2 concentrations. The former possibility seems most likely in view of the effect of SC-51322 to increase glutamate release at high PGE_2 concentrations. The blockade of EP_3 receptors, some of which are coupled to inhibitory G-proteins (Sonnenburg et al., 1990) on the central terminals of primary afferent fibers, would disinhibit this release. The parallel leftward shift in the PGE_2 CRC (including the descending phase) three days after SNL suggests that all EP-receptor subtypes eliciting or inhibiting glutamate release in the affected lumbar segments must have undergone comparable increases in sensitivity after SNL. Alterations in the spinal expression of EP receptors after SNL are currently being investigated in our laboratory. While AA also evoked glutamate release, it was significantly less potent than PGE_2. AA does not appear to activate inhibitory mechanisms at high concentrations as its peak effect was identical to that of PGE_2 in the presence of SC-51322.

Prostanoids are known to be active in a variety of nerve injury models. PGE_2 (10 μM), administered as part of an inflammatory cocktail, increased spontaneous activity in dorsal root fibers, and evoked activity in a subpopulation of previously 'silent' fibers, two to four weeks after chronic constriction injury (Song et al., 2003). In axotomized rats, a mixture containing PGE_2 (10 μM) enhanced ectopic mechanical excitability, reduced PWT and increased the response magnitude in most (77%) severed mechanosensitive C-fibers and some (46%) mechanosensitive A-fibers (Michaelis et al., 1998). Unlike the present study however, these reports provided no direct information on the

degree to which nerve injury alters the pharamcodynamic response to prostanoids.

The effect of COX inhibitors in NP models vary depending upon the time of administration (i.e. pre- or post-treatment). Isoform-selective and non-selective COX inhibitors, given systemically or intrathecally near the time of injury (i.e. before to one to two days after) attenuated thermal hyperalgesia and/or mechanical allodyina in various NP models (Zhao et al., 2000; Hefferan et al., 2003a; Ma and Eisenach, 2003; Suyama et al., 2004). In contrast, their effectiveness declined as the time of administration increased after nerve injury (i.e. greater than seven days). The results of the present study (i.e. three days after SNL) are consistent with this overall time-course profile.

The mechanisms underlying the marked sensitivity to PGE_2 remain to be determined but increased EP receptor-effector coupling in the affected spinal cord (i.e. receptor supersensitivity), and/or increased spinal EP receptor expression are obvious possibilities. Indeed, SNL resulted in a parallel leftward shift in the PGE_2 concentration-response curve, while pretreatment with SC-51322 yielded an opposite rightward shift. SC-51322 also inhibited BR-evoked and spontaneous behavior *in vivo* confirming that these effects are mediated by spinal EP-receptors. The sheer magnitude of the increase in potency of PGE_2 following SNL also argues strongly for amplification of the pharmacodynamic response at the EP-receptor-effector level. SC-51322 had no effect on spontaneous or BR-evoked behavioral responses in sham operated animals.

This is because an inactive dose of i.t. PGE_2 (0.01 µg) in sham-controls was selected for these experiments. Moreover, endogenous spinal PG have no effect on behavior in sham operated as compared to SNL rats early (i.e. post three days) after nerve injury (Hefferan et al., 2003a).

However, additional mechanisms appear to be involved which could also have important pathophysiological implications in the nerve-injured state. Pretreatment with S(+)-IBU, but not the R(-)-enantiomer, attenuated exogenous PGE_2-evoked glutamate release from slices of SNL animals, as well as BR-evoked and, to a lesser degree, spontaneous behavior *in vivo*. S(+)-IBU had a similar effect on electrically-evoked flexor afferent responses recorded from the rat biceps femoris three days after SNL (O'Rielly and Loomis, 2007). In every experiment, the effect of IBU was stereo-specific indicating that the attenuation of exogenous PGE_2 was related to COX inhibition. These results strongly suggest that spinal PG biosynthesis is activated by exogenous PGE_2 in the nerve-injured state, and that the resulting products contributed to the observed COX-dependent effects of spinal PGE_2 in this model. The up-regulation of COX and NOS, as suggested by the expression data three days after SNL, would provide the enhanced biosynthetic capacity in the lumbar spinal cord by which to generate such products. The preferential coupling and synergistic interaction between the inducible forms of COX-2 and membrane-bound PGE_2 synthase (Murakami et al., 2000) also represent a well regulated system by which the biosynthesis of PGE_2 could be specifically up-regulated after SNL.

In fact, the enzymes responsible for PGE_2 biosynthesis, and at least one of the EP receptor subtypes utilized by PGE_2, are strategically co-located within the cell. Functional EP_1-receptors, like those in the plasma membrane, are co-located with the inducible forms of COX-2 and membrane-bound PGE_2 synthase in the nuclear membrane and endoplasmic reticulum (Bhattacharya et al., 1998; Murakami et al., 2000). Moreover, COX-1 and COX-2 are also monotopically inserted in the endoplasmic reticulum and nuclear membrane with the substrate-binding pocket precisely orientated to take up AA as it is liberated from the membrane by PLA_2 or PLC (Ambs et al., 1995; Funk, 2001). PLA_2 isoforms are up-regulated after sciatic nerve injury (De et al., 2003), and PG rapidly increase the cytosolic $[Ca^{2+}]_{free}$ from internal stores in a concentration-dependent manner (Mene et al., 1987). The latter initiates Ca^{2+}-dependent phospholipase activity (Phillis and O'Regan, 2004) as evidenced by the effect of prostaglandin $F_{2\alpha}$ on PLC activity (Kelefiotis et al., 1995). These biochemical studies confirm the presence of the prostanoid catalytic cascade and a requisite receptor subtype within the cell by which PGE_2 could elicit its own synthesis in an up-regulated state[†]. Whether a similar relationship extends to NO remains to be determined, but it is noteworthy that: a) iNOS and nNOS are up-regulated in the ipsilateral L-DH three days after SNL; b) PG and NO are positively and reciprocally coupled

[†] Recent experiments using spinal slices from nerve-ligated animals showed a 100-fold or greater excess in the bath concentration as compared to the added concentration of PGE_2. These differences were blunted by pre-incubation with (S+)-IBU (unpublished results). Plotting the differences against the $[PGE_2]_{added}$ yielded a sigmoidal curve. This concentration-dependent effect would explain the parallel shift in the PGE_2 concentration-response curve after S(+)-IBU pretreatment in the present study.

in many cell types (Goodwin et al., 1999; Pérez-Sala and Lamas, 2001); and c) PGE_2 and NO exhibit additive pro-allodynic effects in the SNL model (Hefferan and Loomis, 2004).

In summary, the results of the present study support the hypothesis of early and abnormal sensitivity to spinal PGE_2 three days after SNL in the rat; effects which are mediated by spinal EP-receptors, COX-dependent, and localized to spinal segments most likely affected by SNL. They provide further evidence for an early pathogenic role of spinal PGE_2 in experimental allodynia, and indicate that spinal PG, in the nerve-injured state, are capable of triggering their own biosynthesis. These results have important pathophysiological implications, and are in accordance with previous reports on the functional relationship between spinal PG and experimental allodynia in the SNL model (Ma et al., 2002; Hefferan et al., 2003a, b).

3.0 SPINAL NERVE LIGATION-INDUCED NEURONAL HYPEREXCITABILITY MATCHES THE TIME-COURSE OF ALLODYNIA AND IS SENSITIVE TO SPINAL CYCLOOXYGENASE INHIBITION

3.1 INTRODUCTION

In the rat, L5/L6 SNL elicits stable allodynia, expressed as a marked reduction in the PWT and protective posturing of the affected dermatome(s) (Kim and Chung, 1994). This abnormality is preceded by ectopic discharges in injured nerves and sub-threshold oscillations recorded from the corresponding DRG (Liu et al., 2000a). These repetitive discharges coincide with the onset of allodynia (Liu et al., 2000b).

Exaggerated afferent input elicits molecular changes in the spinal cord giving rise to central sensitization (Attal and Bouhassira, 1999; Zimmermann, 2001). For example, up-regulation of NMDA receptor phosphorylation and enhanced expression of the $\alpha_2\delta$-1 calcium channel subunit occur in the ipsilateral DH after SNL (Li et al., 2004; Gao et al., 2005). This is accompanied by an induction of phosphoERK in neurons of the superficial DH (Zhuang et al., 2005). These up-stream effects are coupled to the increased expression of COX-1 and COX-2 protein in the ipsilateral L-DH early after SNL (Zhu and Eisenach, 2003; O'Rielly and Loomis, 2006); changes temporally correlated with the onset of allodynia (Zhu and Eisenach, 2003; Li et al., 2004; Gao et al., 2005; O'Rielly and Loomis, 2006).

Recent studies using SNL have demonstrated: a) an early PG-dependent phase of allodynia lasting approximately seven days after nerve injury (Hefferan et al., 2003b); b) increased expression of COX-1 and COX-2 in, and release of PGE_2 and PGI_2 from the injured spinal cord (Tonai et al., 1999; Ma and Eisenach, 2002, 2003; Hefferan et al., 2003a; O'Rielly and Loomis, 2006); c) a marked increase in the pharmacodynamic sensitivity to PGE_2 within the affected spinal segments (O'Rielly and Loomis, 2006); and d) the prevention of the neurochemical, pharmacological and behavioral features of allodynia by various COX inhibitors given intrathecally two to eight hours after nerve injury (Hefferan et al., 2003b). These studies support an early pathogenic role of spinal PG in the development of allodynia.

This hypothesis is further supported by the direct excitatory effect of PG on wide dynamic range DH neurons, and the ability of PG to sensitize them to afferent sensory input (Hingtgen et al., 1995; Ferreira et al., 1996; Willingale et al., 1997; Baba et al., 2001; Ahmadi et al., 2002). These effects, combined with an enhanced sensitivity to PG after SNL, should facilitate central hyperexcitabilty in the affected spinal segments. This outcome, which can be measured quantitatively using flexion reflexes (e.g. biceps femoris), and the corresponding contribution of spinal PG, have not been investigated in a nerve injury model.

In the present study, changes in the biceps femoris flexor reflex (Schouenborg and Sjölund, 1983; Herrero and Cervero, 1996a, b; Willingale et al., 1997) were investigated to determine: a) the effect of nerve injury on the

AFRR and CFRR, one, three and ten days after SNL; b) the relationship between these changes and the onset of allodynia in the SNL model; and c) if spinal PG synthesis/release is a necessary feature of SNL-induced hyperexcitability. A more detailed study of the effect of spinal PG on SNL-induced allodynia will be reported in a subsequent chapter.

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, NL, Canada.

Animals. Male Sprague-Dawley rats (~130 g on the day of experimentation) were obtained from the Vivarium of 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 after surgery. A 12 h light-dark cycle (lights on at 0700 h) was used throughout.

Intrathecal Catheterization. Intrathecal catheters (6.5 cm-length terminating near the lumbar enlargement or 4.0 cm-length terminating in the mid-thoracic segments) were implanted as previously described (Hefferan et al., 2003a). Rats with normal motor, grooming and feeding behavior were housed separately and allowed to recover for three days before SNL or sham surgery.

Neuropathy. Neuropathy was induced using the method of Kim and Chung (1994), as previously described (Hefferan et al., 2003a). Rats were anesthetized with halothane and the left L4 and L5 spinal nerves were isolated and separated. The L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham-controls, the L5 and L6 spinal nerves were isolated but not ligated. All animals were allowed to recover for three days before experimentation. Allodynia, defined as a PWT of ≤4 g, was confirmed using von Frey filaments.

Electrophysiological Recordings. Rats were anesthetized with halothane and cannulae were placed in the trachea, left carotid artery and right external jugular vein. Halothane anesthesia was then replaced by sodium thiobutabarbitone (1 $mg \cdot kg^{-1}$ i.v.; Inactin, Sigma-Aldrich, ON, CA). The depth of anesthesia was assessed by testing for hind limb withdrawal and corneal reflexes which had to be absent. Blood pressure was continuously monitored via a left carotid artery catheter and the mean arterial pressure maintained between 100 to 130 mmHg by additional anesthetic as required. Systolic blood pressure did not fall below 100 mmHg throughout the experiment. Core temperature was maintained close to 37 °C using a homeothermic blanket system. The animal preparation was allowed to stabilize for at least 30 minutes prior to recording.

Spinal flexor reflexes were evoked by subcutaneous electrical stimulation applied to the first toe of the hind paw. Needle location was based on the innervation pattern of the sural nerve (Wiesenfeld-Hallin, 1988) and square-wave pulses (0.2, 0.6, 1.0 Hz) of 1 ms duration were used. Stimulation at each frequency was repeated three times to ensure stability and extracellular electromyographic responses were recorded from the biceps femoris muscle using a pair of tungsten needle electrodes. Intervals of three to five minutes were introduced between successive stimulus trains to prevent a conditioning effect by the preceding stimulus. A low intensity supra-threshold stimulus refers to a voltage twice the AFRR activation threshold (five pulses at 0.2 Hz). A high intensity supra-threshold stimulus refers to a voltage twice the CFRR activation threshold (twenty pulses at 1.0 Hz).

AFRR and CFRR were distinguished on the basis of their response latencies. AFRR was classified as those appearing <100 ms after the stimulus artifact. CFRR was defined as those >100 ms up to a maximum of 600 ms. These criteria were confirmed by examining the effect of i.t. morphine (100 µg) and naloxone (100 µg) on the AFRR and CFRR of the biceps femoris reflex in naive animals. Based on the measured length of the sural nerve in rats weighing 130 to 150 g (i.e. 12 cm), afferent fibers mediating the late phase (>100 to 600 ms) had an average conduction velocity of 1.2 $m \cdot s^{-1}$; a value within the accepted range for C-fibers (Lynn and Carpenter, 1982). The A- and C-fiber intervals used in the present experiments are in agreement with previous reports on the rat

biceps femoris reflex (Lynn and Carpenter, 1982; Herrero and Cervero, 1996a, b).

To determine the AFRR activation threshold, stimulus trains of five pulses (0.2 to 1.0 Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least three of the five stimulus pulses produced a spike (i.e. amplitude >20 μV). To determine the CFRR activation threshold, stimulus trains of twenty pulses (0.2 to 1.0 Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least ten of the twenty stimulus pulses produced a spike (i.e. amplitude >20 μV).

Data were collected, stored and analyzed using a PowerLab® data acquisition system (ADInstruments, Inc., Colorado Springs, CO, USA). Off-line data analysis was initially performed using the data acquisition software provided by the manufacturer (Chart 5.0). The data were further analyzed using a customized computer program developed in collaboration with the Department of Computer Science, Memorial University.

Data Analysis. Electromyographic recordings in sham operated and SNL animals were compared using the mean responses elicited by three stimulus trains of 0.2, 0.6 and 1.0 Hz. Data were collected (4000 samples/sec) and high-pass digital filters (cut-off frequency 70 Hz) were used to remove noise and movement artifacts. Spontaneous activity was defined as the average spike

count integrated over a 60 s period prior to experimentation. In all cases, the magnitude of the AFRR and CFRR was calculated by integrating the spike count over the stimulation period (i.e. AUC analysis). Spikes were integrated from 0.5 to 99.9 ms and 100 to 600 ms for the AFRR and CFRR, respectively. Baseline reflex activity was defined as the response to the first stimulus in each impulse train. Windup was assessed by comparing the response evoked by the 20^{th} stimulus in each train of sham operated and SNL animals. Comparisons within and between the treatment groups were performed using one-way ANOVA, followed by the Newman-Keuls test. Data are reported as the mean ± SEM, and $p<0.05$ indicates a significant difference.

Drugs. S(+)- and R(-)-IBU were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, MI, USA). Drugs were dissolved in 100% DMSO and diluted with normal saline at the time of injection to yield a final DMSO concentration of 50%. All equipment was sterilized with 70% alcohol and thoroughly rinsed with 0.9% sterile saline before injection. Intrathecal drugs were injected into conscious, unrestrained rats using a hand-held microsyringe. Drugs were delivered in a volume of 5 µl followed by 5 µl of sterile saline. The i.t. catheter was immediately resealed with a stainless steel plug. The position of the catheter tip was verified visually after sacrifice in randomly selected animals.

3.3 RESULTS

After SNL, rats displayed a significant decrease in PWT from ≥15 g (baseline) to ≤4 g (data not shown; O'Rielly and Loomis, 2006). This change was apparent 12 h after SNL, was stable for at least twenty days, and remained confined to the plantar surface of the left hind paw (i.e. ipsilateral to SNL). By day 20, a modest but significant ($p<0.01$) decrease in PWT developed on the contralateral hind limb of SNL animals. Generally, the affected hind paw was kept in an elevated and cupped position to minimize contact with the cage floor. All SNL rats were otherwise healthy, showed normal feeding and grooming behavior, and regular weight gain. Sham surgery had no effect on PWT compared to pre-surgical values, as reported previously (O'Rielly and Loomis, 2006).

Electromyographic recordings of the AFRR and CFRR of the biceps femoris flexor reflex are shown in Figure 3.1A. Intrathecal morphine (100 µg) had no effect on evoked activity in the 0.5 to 99.9 ms range. In contrast, activity between 100 and 600 ms range was nearly eliminated (Figure 3.1B); an effect reversed by i.t. naloxone (100 µg; data not shown). The activation thresholds for both the AFRR and CFRR (ipsilateral to SNL) were significantly reduced compared to sham-controls (Figure 3.2). Three days after SNL (P-3-D), the AFRR exhibited a frequency-independent reduction ranging from 25 to 32% (Figure 3.2A). Otherwise, there were no significant differences across the stimulation frequencies, or across post-surgical days at a given stimulation

frequency. The reduction in the CFRR ranged from 54 to 62% and was also frequency-independent (Figure 3.2B). This effect was evident at P-1-D and persisted up to P-10-D. After SNL, CFRR was observed at voltages triggering AFRR activity in sham-controls. There were no significant differences across post-surgical days at a given stimulation frequency. The only difference between stimulation frequencies on a given experimental day was 0.2 Hz vs 0.6 Hz and 0.2 Hz vs 1.0 Hz (Figure 3.2B). Neither were there any significant differences between the contralateral hind limb of SNL animals and either side of sham-controls for the AFRR or CFRR (data not shown).

Compared to sham-controls, the magnitude of both AFRR and CFRR was significantly increased after SNL using either AFRR (Figures 3.3 and 3.4A&B; Table 3.1) or CFRR threshold stimulation (Figures 3.3 and 3.5A&B; Table 3.2). For example, the AFRR increased by 70.6% at AFRR threshold and by 49.4% at CFRR threshold at 1.0Hz (Table 3.1). Enhanced AFRR and CFRR were evident at P-1-D, peaked at P-3-D, and remained elevated at P-10-D, irrespective of stimulation frequency and activation threshold (Figures 3.4A&B and 3.5A&B). The only exceptions were the AFRR and CFRR evoked by a twenty pulse stimulus at 1.0 Hz (Figures 3.4B and 3.5B). Significant differences in AFRR and CFRR were observed across experimental days within each stimulation paradigm (e.g. 20 pulses, 0.2 Hz) (Figures 3.4A&B and 3.5A&B). The AFRR and CFRR did not vary significantly between respective stimulation paradigms on the same experimental day (Figure 3.4A&B). There were also no significant

differences in the response magnitude of the AFRR and CFRR (p>0.05) in sham-controls across the experimental days within each stimulation paradigm (Figures 3.4A&B and 3.5A&B), or between stimulation frequencies on a given experimental day (e.g. P-1-D at 5 pulses, 0.2 Hz versus P-1-D at 5 pulses, 1.0 Hz).

Similar results were observed using supra-threshold stimulation (Table 3.1). At twice the AFRR (Figure 3.7) or CFRR (Figure 3.8) activation threshold, the response magnitude of the AFRR was significantly increased in all nerve-ligated groups compared to their respective sham-controls. For a given stimulus paradigm (e.g. 5 pulses, 0.2 Hz), the response magnitude on P-10-D was significantly greater than P-3-D and/or P-1-D after SNL, except for the 20 pulse-1.0 Hz stimulation. There were no significant differences between P-1-D and P-3-D after SNL, or across all experimental days in sham-controls (Figure 3.7A&B). At twice the CFRR activation threshold, the magnitude of the CFRR was significantly increased compared to sham-controls on all experimental days (Figure 3.8B; Table 3.2). While there was a trend towards increased response magnitude over the ten day time-course after SNL, P-10-D was not significantly different from P-1-D and/or P-3-D (Figure 3.8B). AFRR supra-threshold activation had no effect on the CFRR irrespective of stimulus frequency or experimental day (Figure 3.8A).

At threshold activation, the AFRR had a pooled average latency of 12.3 ± 0.4 ms (AFRR activation threshold) and 9.7 ± 0.3 ms (CFRR activation

threshold). These latencies were unaffected by SNL irrespective of the stimulus frequency or experimental day (data not shown). The CFRR had a pooled average latency of 173.2 ± 9.9 ms at CFRR activation threshold regardless of the stimulation frequency. The latency of the CFRR evoked by a 20-pulse stimulus was significantly reduced at P-3-D and P-10-D compared to sham-controls at all frequencies tested (data not shown). For example, the CFRR latency decreased by 24.3% at 1.0 Hz compared to sham-controls on P-3-D. At supra-threshold stimulation, there were no significant differences in either the AFRR or CFRR of SNL animals compared to sham-controls (data not shown). In addition, there were no significant differences in the latencies of the AFRR or CFRR evoked by threshold or supra-threshold stimulation recorded from the contralateral hind limb of SNL animals (data not shown).

At activation threshold, the response duration of both the AFRR and CFRR was significantly increased after SNL compared to sham-controls (Figures 3.4C and 3.5C). This was evident at P-1-D, peaked at P-3-D, and remained elevated at P-10-D (Figures 3.4C and 3.5C). The only exception was the duration evoked by a 20 pulse stimulus at 0.2 Hz which did not reach statistical significance (Figure 3.5C). Supra-threshold stimulation had no effect on the duration of either the AFRR (Figure 3.7C) or CFRR (Figure 3.8C) components in SNL compared to sham operated animals. The response duration did not vary across experimental days within each treatment group, regardless of the stimulus paradigm.

Treatment with i.t. S(+)-IBU (100 μg), 30 minutes before supra-threshold CFRR activation threshold stimulation, significantly reduced the AFRR (Figure 3.9A) and CFRR (Figure 3.9B) in SNL rats for 2 h compared to three separate controls: S(+)-IBU+sham; R(-)-IBU+sham; and R(-)-IBU+SNL (Figures 3.9A and 3.9B). The inhibitory effect of S(+)-IBU on the AFRR and CFRR, determined 1 h after i.t. injection, was dose-dependent (Figures 3.9C and 3.9D).

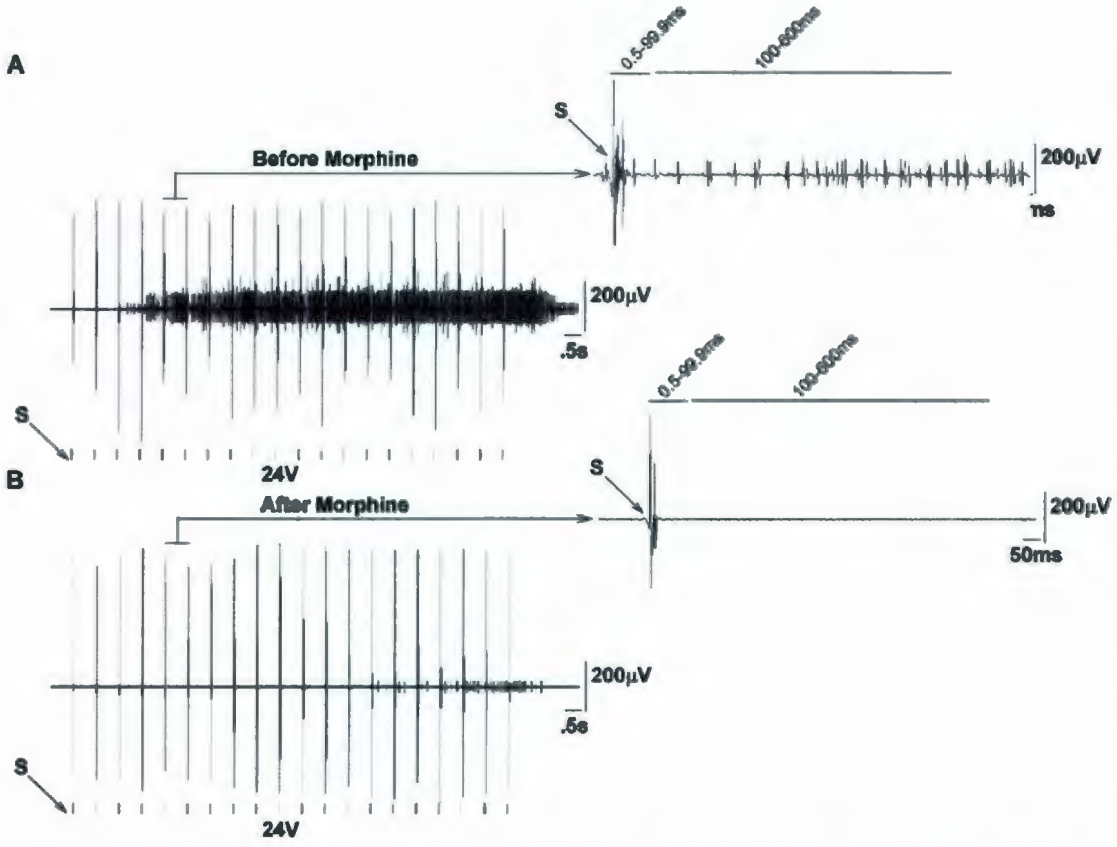

Figure 3.1. Representative tracings of the A- and C-fiber components of the biceps femoris flexor reflex in naïve animals. An electrical stimulus (S) at 1.5 Hz was applied to the hind paw before (A) and after (B) i.t. morphine (100 µg). The insert in panel A shows the A- and C-fiber components evoked by the 5th pulse. Whereas morphine had no effect on evoked activity in the 0.5 to 99.9 ms range, activity between 100 and 600 ms range was nearly eliminated (panel B). A pulse stimulus indicator bar is shown under each tracing.

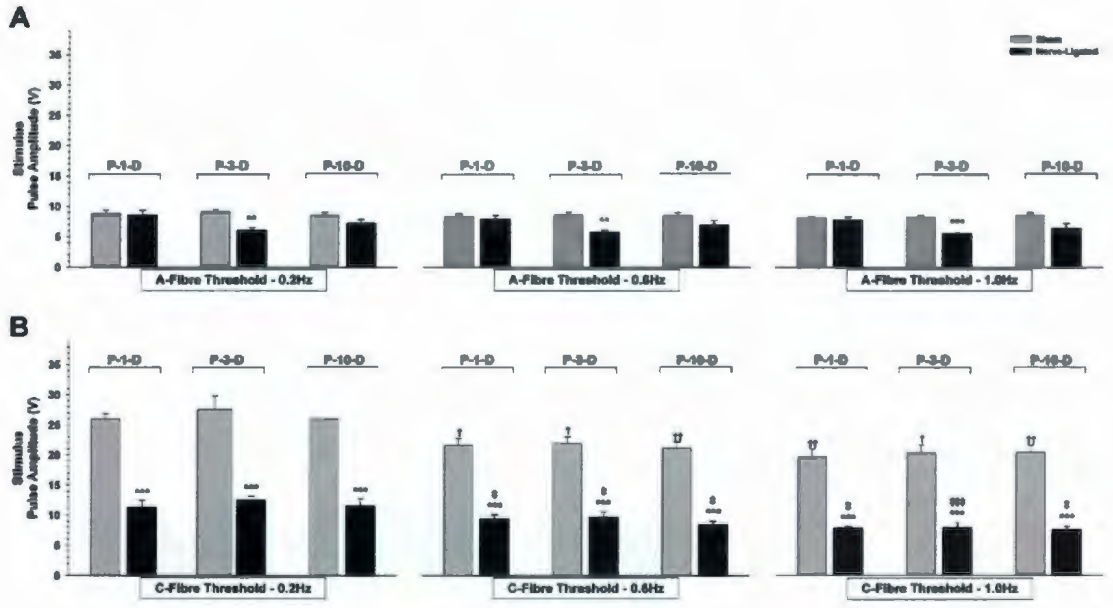

Figure 3.2. Threshold stimulation (V) required to activate the A-fiber (panel A) and C-fiber (panel B) components of the ipsilateral biceps femoris flexor reflex. Data were recorded one (P-1-D), three (P-3-D), and ten (P-10-D) days after SNL or sham surgery. Note that after SNL, C-fiber responses occur at voltages triggering A-fiber activity in sham-controls. Each bar represents the mean ± SEM of five to seven animals. Asterisks indicate a significant difference from the corresponding sham-control (**p<0.01; ***p<0.001). Daggers (†p<0.05; ††p<0.01) indicate a significant difference from 0.2 Hz on the same experimental day for sham-controls. Double daggers (‡p<0.05; ‡‡p<0.01; ‡‡‡p<0.001) indicate a significant difference from 0.2 Hz on the same experimental day for the SNL groups.

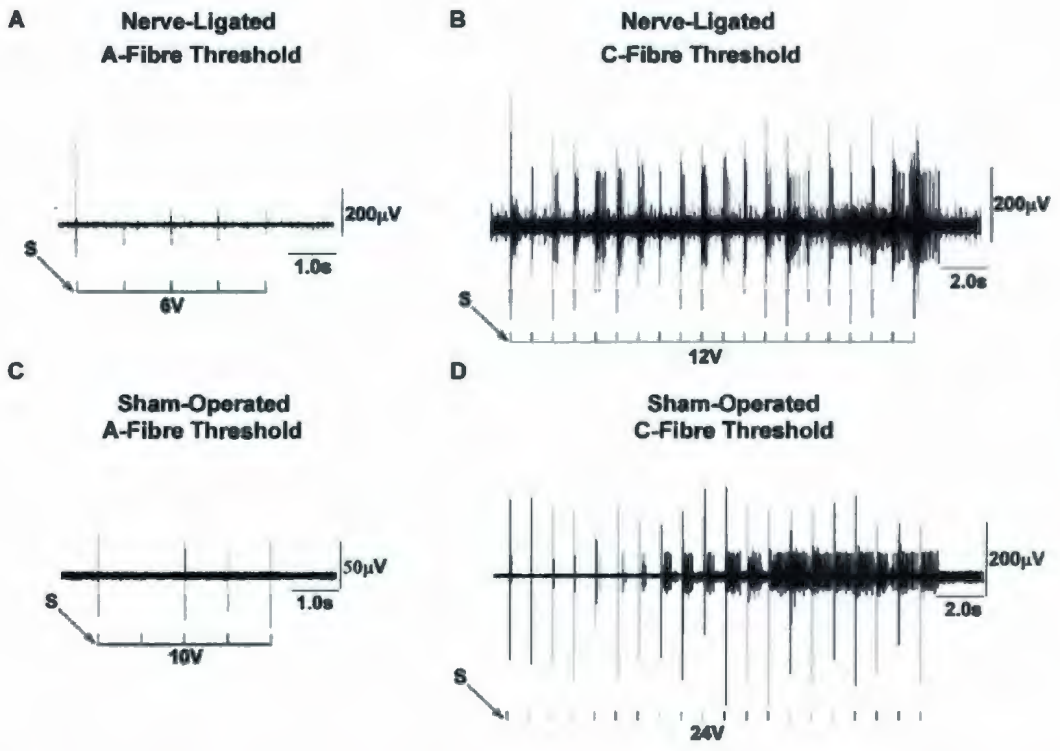

Figure 3.3. Representative tracings of A- and C-fiber components in the biceps femoris flexor reflex evoked by electrical stimulation (S) of the ipsilateral hind paw at corresponding activation thresholds. Tracings were recorded three days after SNL (panels A and B) or sham surgery (panels C and D) at a stimulus frequency of 1.0 Hz. A pulse stimulus indicator bar is shown under each tracing. Panels A and B, and panels C and D are from the same animal, respectively. Note the difference in scale in panel C (50 µV) compared to panels A, B and D (200 µV).

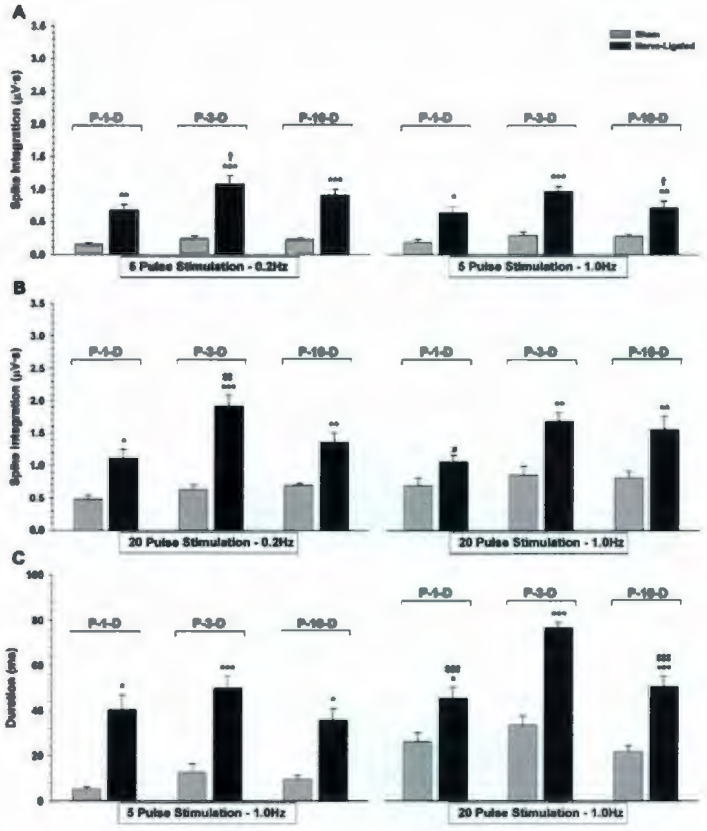

Figure 3.4. The A-fiber component of the biceps femoris flexor reflex evoked by electrical stimulation to the ipsilateral hind paw one (P-1-D), three (P-3-D) and ten (P-10-D) days after SNL or sham surgery. Spike-integration for A- and C-fiber activation thresholds are shown in panels A and B, respectively. The corresponding response duration at a frequency of 1.0 Hz is shown in panel C. Each bar represents the mean ± SEM of five to seven animals. Asterisks indicate a significant difference from the respective sham-control (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Daggers (†$p<0.05$) indicate a significant difference from P-1-D; double daggers (‡$p<0.05$; ‡‡‡ $p<0.001$) indicate a significant difference from both P-1-D and P-10-D; and pound (#$p<0.05$) indicates a significant difference from both P-3-D and P-10-D.

Table 3.1. Change in the A-fiber spike integration (μV·s) following SNL expressed as the percent of sham-controls. Spinal flexor reflex activity was recorded from the ipsilateral biceps femoris on days 1, 3 and 10 after surgery.

Post-Operative Day	Frequency (Hz)	A-Fiber Activity			
		Threshold Stimulation		Supra-threshold Stimulation	
		A-fiber	C-fiber	A-fiber	C-fiber
1	0.2 Hz	177.6%**	157.0%*	150.4%**	137.8%*
	1.0 Hz	163.5%*	135.0%	145.9%***	150.7%**
3	0.2 Hz	177.7%***	167.4%***	135.1%**	137.3%**
	1.0 Hz	170.6%***	149.4%**	145.5%***	139.1%***
10	0.2 Hz	175.3%***	148.8%**	146.1%***	143.8%***
	1.0 Hz	161.6%**	147.5%**	146.5%***	163.4%***

Asterisks indicate a significant difference from sham-controls (*p<0.05; **p<0.01; ***p<0.001). Threshold stimulation for A-fiber activity represents the voltage required to reach the firing threshold for A- and/or C-fiber activation. Supra-threshold stimulation for A-fiber activity refers to the voltage at twice the firing threshold for A- and/or C-fiber activation.

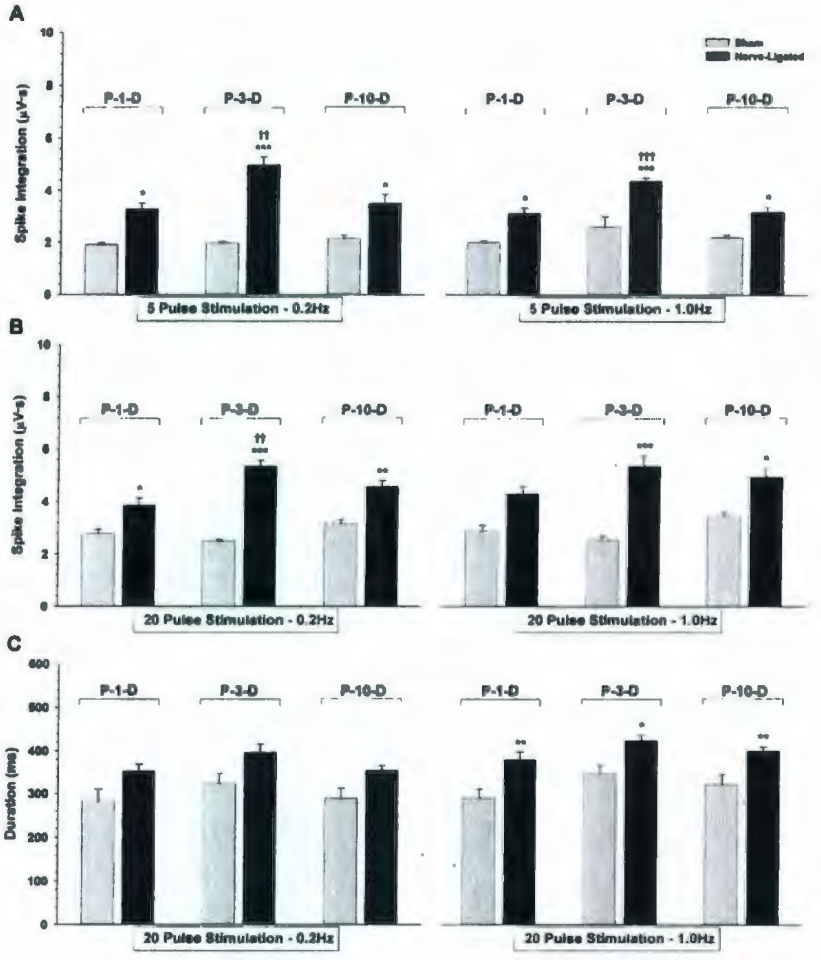

Figure 3.5. The C-fiber component of the biceps femoris flexor reflex evoked by electrical stimulation to the ipsilateral hind paw one (P-1-D), three (P-3-D) and ten (P-10-D) days after SNL or sham surgery. Spike-integration for A- and C-fiber activation thresholds are shown in panels A and B, respectively. The response duration of data in panel B are shown in panel C. Each bar represents the mean ± SEM of five to seven animals. Asterisks indicate a significant difference from the corresponding sham-control (*p<0.05; **p<0.01; ***p<0.001). Daggers (††p<0.05; ††† p<0.001) indicate a significant difference from both P-1-D and P-10-D.

Table 3.2. Change in the C-fiber spike integration (μV·s) following SNL expressed as the percent of sham-controls. Spinal flexor reflex activity was recorded from the ipsilateral biceps femoris on days 1, 3 and 10 after surgery.

Post-Operative Day	Frequency	C-fiber Activity			
		Threshold Stimulation		Supra-threshold Stimulation	
		A-fiber	C-fiber	A-fiber	C-fiber
1	0.2 Hz	141.55%*	128.36%*	115.61%	141.41%*
	1.0 Hz	135.84%*	132.29%	85.12%	141.78%**
3	0.2 Hz	160.19%***	153.66%***	91.85%	141.22%*
	1.0 Hz	140.48%***	152.40%***	123.47%	156.02%***
10	0.2 Hz	137.81%*	130.52%**	115.70%	152.59%***
	1.0 Hz	130.57%*	129.96%*	118.81%	156.55%***

Asterisks indicate a significant difference from sham operated animals (*p<0.05; **p<0.01; ***p<0.001). Threshold stimulation for C-fiber activity represents the voltage required to reach the firing threshold for A- and/or C-fiber activation. Supra-threshold stimulation for A-fiber activity refers to the voltage at twice the firing threshold for A- and/or C-fiber activation.

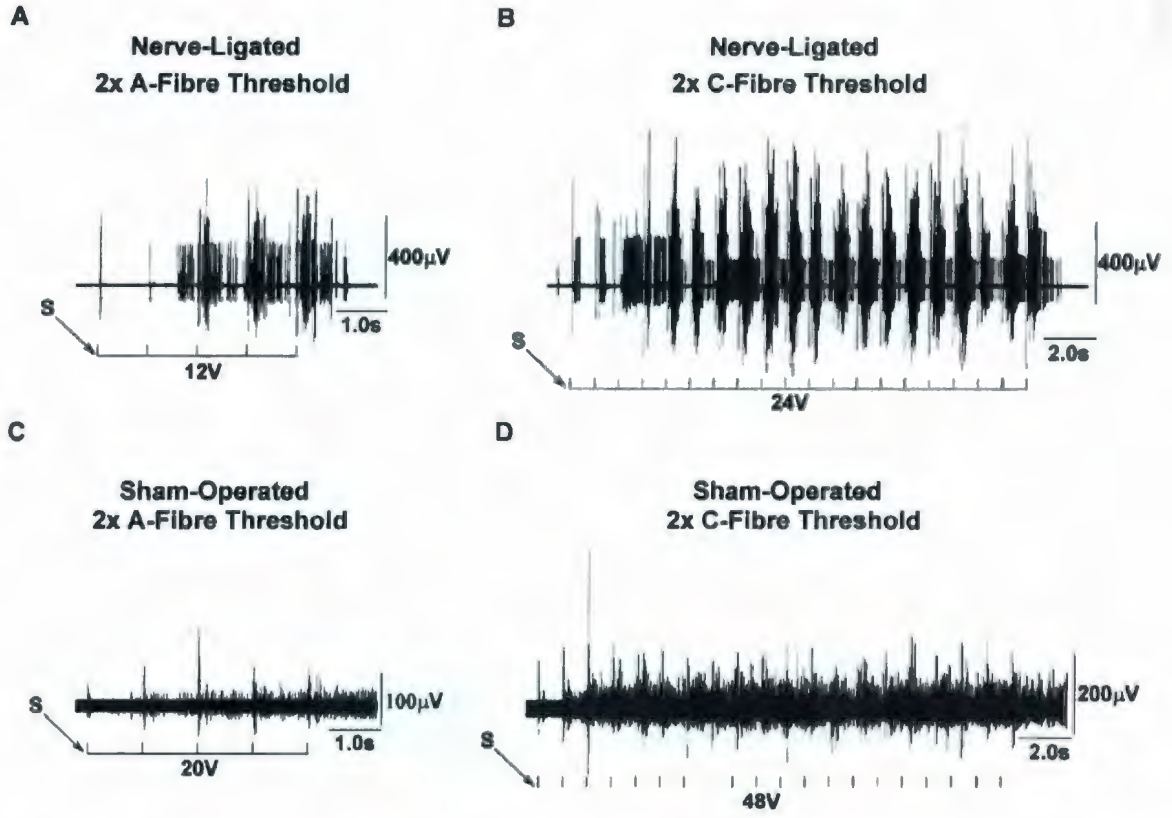

Figure 3.6. Representative tracings of A- and C-fiber components in the spinal flexor reflex (biceps femoris) evoked by an electrical stimulus (S) to the ipsilateral hind paw at the corresponding supra-threshold stimulation. Tracings were recorded three days after SNL (panels A and B) or sham surgery (panels C and D) at a stimulus frequency of 1.0 Hz. A pulse stimulus indicator bar is located under each tracing. Note the differences in stimulation (V) and scales used between SNL (panels A and B) and sham-controls (panels C and D).

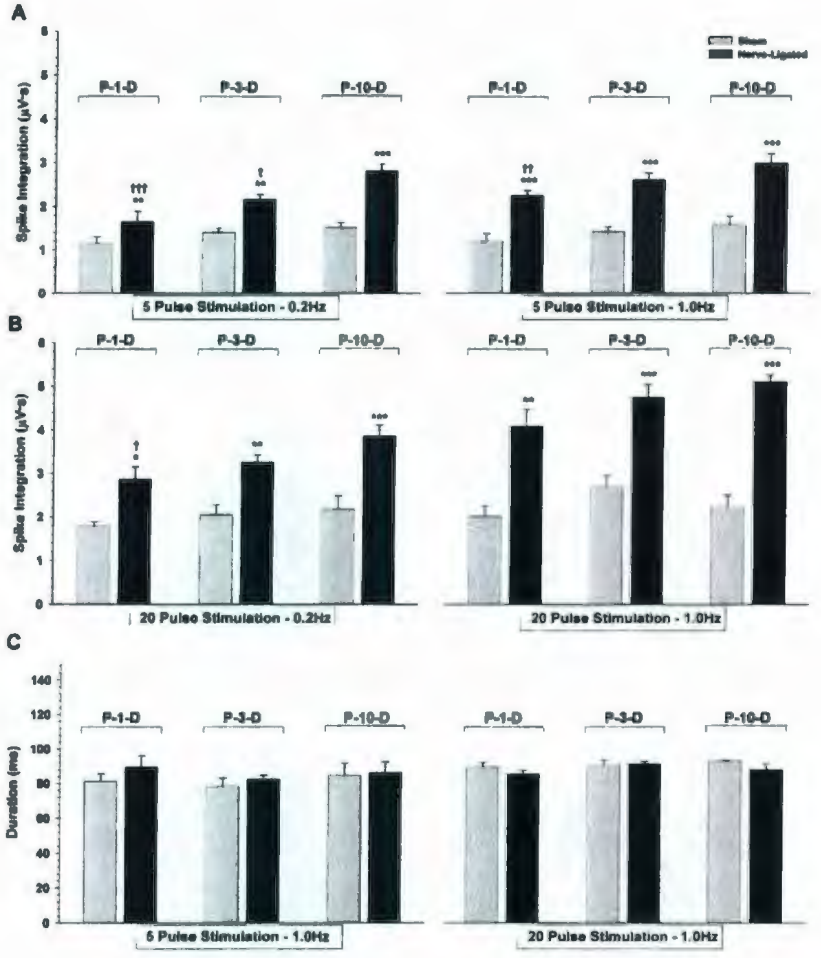

Figure 3.7. The A-fiber component of the spinal flexor reflex (biceps femoris) evoked by supra-threshold stimulation to the ipsilateral hind paw one (P-1-D), three (P-3-D) and ten (P-10-D) days after SNL or sham surgery. Spike-integration for A- (5 pulse) and C-fiber (20 pulse) activation thresholds are shown in panels A and B, respectively. The corresponding duration at a frequency of 1.0 Hz is shown in panel C (5 and 20 pulses). Each bar represents the mean ± SEM of five to seven animals. Asterisks indicate a significant difference from the respective sham-control (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Daggers (†$p<0.05$; ††$p<0.01$; †††$p<0.001$) indicate a significant difference from P-10-D.

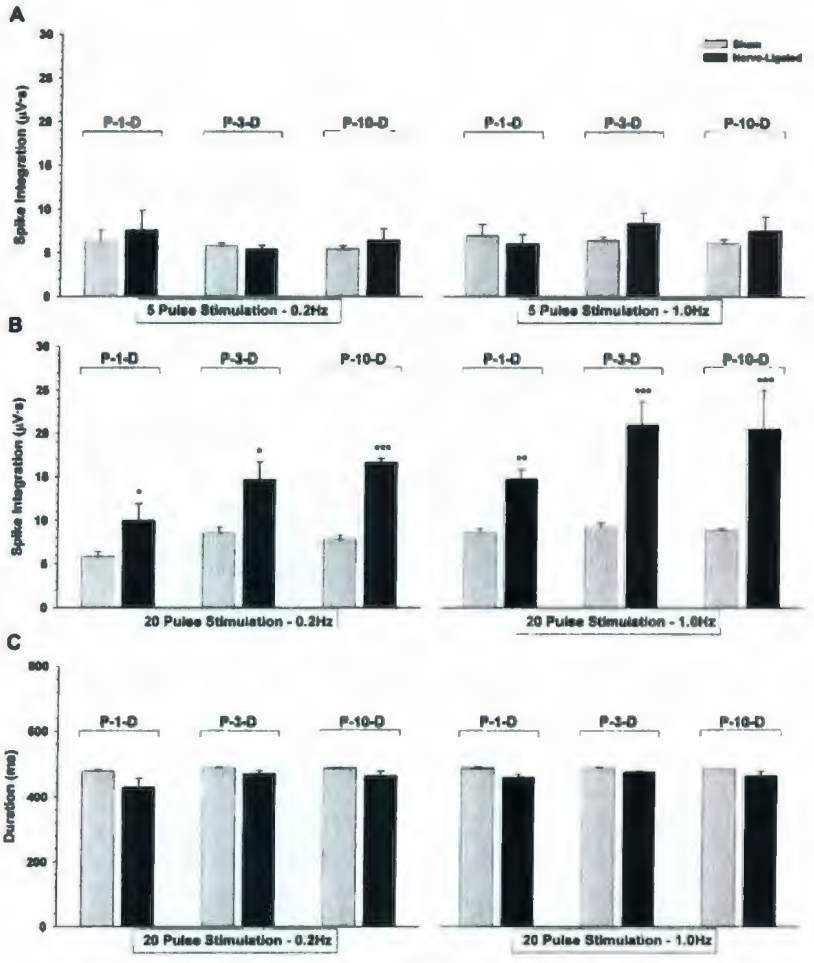

Figure 3.8. The C-fiber component of the spinal flexor reflex (biceps femoris) evoked by supra-threshold electrical stimulation to the ipsilateral hind paw one (P-1-D), three (P-3-D) and ten (P-10-D) days after SNL or sham surgery. Spike-integration for A- and C-fiber components are shown in panels A and B, respectively. The response duration of data in panel B are shown in panel C. Each bar represents the mean ± SEM of five to seven animals. Asterisks indicate a significant difference from the corresponding sham- control (*p<0.05; **p<0.01; ***p<0.001).

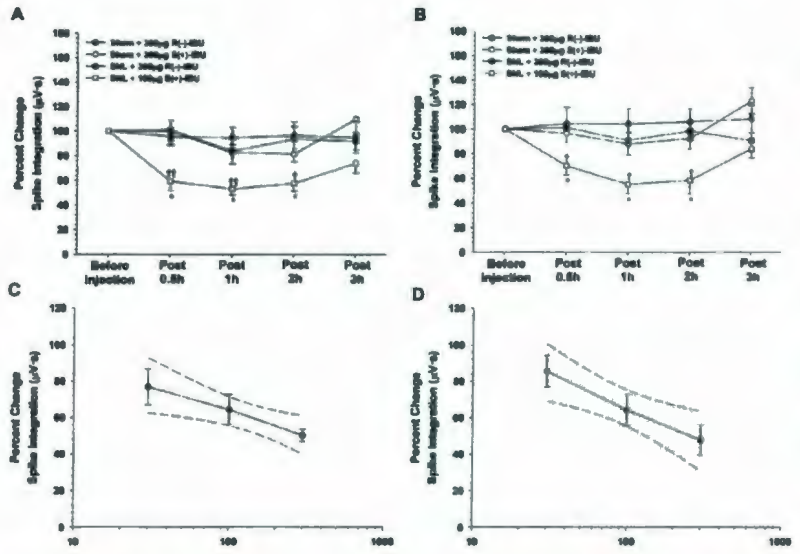

Figure 3.9. The time-course (top panel) and dose-response effect (bottom panel) of i.t. S(+)- and R(-)-IBU on the A- and C-fiber components of the biceps femoris flexor reflex. Electrical responses to supra-threshold C-fiber stimulation (1.0 Hz) were recorded from the ipsilateral biceps femoris three days after SNL or sham surgery. Each point represents the mean ± SEM of four to six animals. Treatment with i.t. S(+)-IBU (100 µg) 30 minutes before stimulation significantly reduced A-fiber (panel A) and C-fiber (panel B) responses in SNL rats compared to S(+)- and R(-)-IBU-treated sham and SNL animals, respectively. The inhibitory effect of S(+)-IBU on A-fiber (panel C) and C-fiber (panel D) responses, 1 h after injection, was dose-dependent. Asterisks indicate a significant difference between the S(+)- and R(-)-enantiomers in SNL animals (*p<0.05; **p<0.01; ***p<0.001). Daggers indicate a significant difference from the three control groups at individual time points (†p<0.05; †† p<0.01; †††p<0.001). The adjacent dashed lines in the bottom panel represent the 95% confidence interval around the corresponding DRC.

3.4 DISCUSSION

Changes in the excitability of spinal neurons after SNL were investigated using the transcutaneous-evoked biceps femoris flexor reflex. The AFRR and CFRR were defined using accepted activation and conduction velocity criteria (Hartell et al., 1991) and validated by their differential sensitivity to i.t. morphine. No attempt was made to further separate the A-fiber components because of the confounding effect of demyelination after SNL (Laird and Bennett, 1993). Evoked and baseline responses were highly reproducible within the treatment groups and across experimental days at each stimulus paradigm. The results indicate that neural excitability is significantly increased early after SNL and remains elevated for at least ten days.

The activation threshold of the AFRR and CFRR was significantly reduced after SNL; a characteristic feature of peripheral and central sensitization. The time course of these changes also matched the onset of allodynia in this model (Kim and Chung, 1992). Similar changes in activation threshold were described in the partial sciatic nerve ligation (Okamoto et al., 2001), sciatic nerve transection (Okamoto et al., 2001), and chronic constriction injury (Meyerson et al., 1995; Kohno et al., 2003) models. In contrast, Chapman et al. (1998) reported no such change in Aβ- or C-fibers seven to seventeen days after SNL. The latter may reflect differences in the stimulation/recording protocols, electrophysiological end-points (e.g. mean action potentials versus spike integration), fiber types investigated, and/or the time points studied after nerve

injury. That the experimental time course could be an important factor is suggested by evidence showing: a) a transition from spinal PG-dependent to PG-independent allodynia seven to ten days after SNL (Hefferan et al., 2003b); and b) the modulatory effect of endogenous spinal PG on the biceps femoris reflex.

The magnitude of evoked AFRR and CFRR was significantly increased after SNL, consistent with spinal sensitization and the onset of allodynia. In the SNT model, A-fiber EPSCs were significantly increased two to four weeks after injury (Okamoto et al., 2001). Likewise, Aβ- and C-fibers exhibited increased activity two to fifty-two weeks after spinal transection (Bennett et al., 2004). No such changes were reported seven to seventeen days after SNL (Chapman et al., 1998; see comments above) or nine to eleven days after chronic constriction injury (Laird and Bennett, 1993). The lack of effect in the latter model may reflect the preferential loss of large fibres.

Baseline activity in both the AFRR and CFRR was also enhanced in a stimulus-dependent manner. Previous studies reported increased compound potentials on the surface of the spinal cord (Colvin et al., 1996), and ectopic discharges in the injured DRG after SNL (Liu et al., 2000a, b). These ectopic discharges corresponded with the onset of allodynia. The results of the present study indicate that both baseline (i.e. before stimulus-evoked spatial and temporal summation are apparent) and evoked activity are significantly affected by SNL.

Exaggerated responses to repeated AFRR and CFRR stimulation also exhibited a windup-like effect in SNL animals. This feature was more prominent in the AFRR as compared to the CFRR. The latter was stimulus-dependent and only observed under activation threshold conditions. In an earlier study using spinal compression, windup of an electrically evoked spinal reflex was reported two weeks after injury (Bennett et al., 2004) but no attempt was made to distinguish between the A- and C-fiber components. Kimura et al. (2005) reported enhanced windup of a C-fiber mediated nociceptive flexor reflex in a mouse model of painful diabetic neuropathy. Others have reported no evidence of windup after experimental nerve injury (Laird and Bennett, 1993; Chapman et al., 1998). That A-fiber activity is exaggerated after SNL is consistent with the onset of mechanical allodynia in this model.

Unlike the AFRR, there was an apparent reduction in CFRR latency at threshold stimulation. This decrease was unexpected given the demyelination and degeneration reported one to two weeks after SNL (Senapati et al., 2005). Neurons exhibiting monosynaptic Aδ-fiber EPSCs decreased after CCI or SNT while those exhibiting polysynaptic Aδ-fiber EPSCs increased (Kohno et al., 2003). The apparent decrease in CFRR latency could reflect delayed and/or prolonged activation of injured Aδ-fibers as reported one week after chronic constriction injury (Bai et al., 1999). These early changes are unlikely to involve synaptic reorganization which normally occur weeks to months after nerve injury (Okamoto et al., 2001; Kohno et al., 2003).

To date, most electrophysiological studies of nerve injury models have focused on after-discharge activity making it impossible to analyze changes in the duration of the A- and C-fiber components. In the present study, the duration of both AFRR and CFRR activity was significantly increased by SNL. A similar effect was reported in rat pups irradiated with UV-light on the hind foot-pad (Thompson et al., 1994). The increase in duration, combined with the concurrent decrease in firing threshold, enhanced activity, and elevated baseline reflex clearly indicate an overall increase in spinal excitability early after SNL. This is likely to optimize the conditions necessary for the onset of allodynia in advance of the long term genotypic and phenotypic changes that sustain a neuropathic state.

Tactile allodynia arising from peripheral nerve injury is elicited by impulses carried along surviving Aβ-afferents (Kim and Chung, 1992). In the present study, both the AFRR and CFRR of the biceps femoris reflex exhibited a decrease in firing threshold and a corresponding increase in activity early after SNL. These changes coincide with the onset of behavioral allodynia (Kim and Chung, 1992). Indeed, enhanced electrophysiological activity was evident as early as one day after SNL. While A-fiber mediated windup has been described following peripheral inflammation (Ma and Woolf, 1996; Thompson et al,, 1994), this is the first report of such an effect early after nerve injury. The release of brain-derived nerve growth factor (BDNF), especially from injured A-fibers (Zhou et al., 2000), and nerve growth factor (NGF) are likely to be involved. Antibodies to each of

these neurotrophins, injected into the L5 DRG at the time of L5 SNL, significantly attenuated mechanical allodynia (Zhou et al., 2000). Conversely, BDNF and NGF injected into uninjured DRG increased mechanical sensitivity within 4h (Zhou et al., 2000). Enhanced C-fiber activity may also reflect the early pathogenic effect of substance P (sP) via NK-1 receptors (Pitcher and Henry, 2004). In this regard, NK-1 knockout mice did not display the increased mechanical sensitivity of wild type animals 24 h after SNL (Mansikka et al., 2000). BDNF, NGF and sP induce central sensitization through modulation of NMDA receptors. Functional reorganization and/or phenotypic changes within the spinal cord (Woolf et al., 1992; Kohno et al., 2003) are unlikely to contribute to allodynia so soon after SNL.

SNL-induced allodynia also features a PG-dependent phase beginning immediately after injury and lasting for seven to ten days (Hefferan et al., 2003b). The present study indicates that spinal PG modulation is reflected electrophysiologically in the biceps femoris reflex. Thus, i.t. S(+)-, but not R(-)-IBU, given three days after SNL, dose-dependently inhibited the nerve injury-induced exaggerated of the AFRR and CFRR. This stereo-selective effect mirrors the time-course of COX-1 and COX-2 expression in the ipsilateral lumbar cord (Zhu and Eisenach, 2003; O'Rielly and Loomis, 2005), the BR-evoked release of PGE_2 in spinal CSF (Hefferan et al., 2003a), and the marked increase in pharmacodynamic sensitivity to PGE_2 (O'Rielly and Loomis, 2006) in the lumbar cord after SNL. This is the first report of such an effect in a NP model and

corroborates, electrophysiologically, the early contribution of spinal PG in the development of allodynia. A detailed investigation of spinal PG on SNL-induced central hyperexcitability will be reported in a subsequent chapter.

This prostanoid modulation probably reflects the inhibition of glycine-mediated IPSCs in DH neurons; an effect mediated by spinal EP_2-receptors (Ahmadi et al., 2001). The resulting disinhibitory state would be especially relevant under conditions of exaggerated production (Ahmadi et al., 2001), release (Hefferan et al. 2003a) and sensitivity (O'Rielly and Loomis, 2006) to PGE_2; abnormalities present after SNL. Recent experiments in our laboratory indicate that EP_2-receptors are also up-regulated in the ipsilateral L-DH three days after SNL (unpublished results). This mechanism of SNL-induced allodynia also accords with the robust and highly selective allodynia induced by low dose i.t. strychnine in conscious, uninjured rats (Yaksh, 1989). A related SNL effect on motor neurons in the biceps femoris reflex cannot be completely excluded at this time. However, spasticity and/or seizure-like effects are unusual features of this model and were uniformly absent in the present study. Thus, afferent sensory input appears to be preferentially affected after SNL.

Electromyographic analysis of the biceps femoris flexor reflex revealed a significant increase in spinal excitability (i.e. decreased activation threshold, increased firing activity and response duration, increased baseline activity and windup) from one to ten days after SNL. The AFRR and CFRR were both affected but to varying degrees. The time course of these spinal changes

parallels the development of mechanical allodynia in the SNL model. Electrophysiological changes were stereo-selectively attenuated by i.t. IBU given three days after SNL providing further evidence for the early role of spinal PG in central sensitization and allodynia in this model.

4.0 SPINAL PROSTAGLANDINS EXAGGERATE A- AND C-FIBER EXCITABILITY AND ARE CRITICAL TO THE DEVELOPMENT OF MECHANICAL ALLODYNIA EARLY AFTER L5/L6 SPINAL NERVE LIGATION

4.1 INTRODUCTION

The mechanisms underlying NP (including allodynia) are known to be complex, multi-factorial, and subject to plasticity (Beydoun and Backonja, 2003; Dworkin et al., 2003; Takeda et al., 2005). Understanding the time-course and pharmacology of the mechanisms comprising the cascade leading to allodynia can enable the identification of interceptive measures that prevent acute nerve injury from becoming a chronic neuropathic state. In view of the complex and time-dependent changes triggered by nerve injury, targeting vital signaling events early after injury would be an obvious strategy. The early contribution of spinal PG in the development of experimental allodynia (Tonai et al., 1999; Ma and Eisenach, 2002, 2003; Zhu and Eisenach, 2003; O'Rielly and Loomis, 2006; Hefferan et al., 2003a, b), and the clinical availability of drugs with which to disrupt their effects, make prostanoids an interesting, potential target.

A role for spinal PG in allodynia is consistent with their direct excitatory effects on DH neurons, as well as their ability to exaggerate cellular responses to afferent sensory input (Hingtgen et al., 1995; Ferreira and Lorenzetti, 1996; Willingale et al., 1997; Baba et al., 2001; Ahmadi et al., 2002), and the early

induction of molecular changes, such as increases in NMDA receptor phosphorylation (Gao et al., 2005), α2δ-1 calcium channel subunit expression (Li et al., 2004), and the induction of phosphoERK (Zhuang et al., 2005), coupled to spinal COX-1 and COX-2 expression (Zhu and Eisenach, 2003; O'Rielly and Loomis, 2006). These changes are temporally correlated with the onset of allodynia (Zhu and Eisenach, 2003; Li et al., 2004; Gao et al., 2005; Zhuang et al., 2005; O'Rielly and Loomis, 2006), and support the hypothesis of an early pathogenic role of spinal PG.

Previous pharmacological studies with the L5/L6 SNL model of allodynia revealed a preferential sensitivity to COX-1 inhibitors given two to eight hours after injury (Hefferan et al., 2003b). Thus, i.t. SC-560 completely prevented the neurochemical, pharmacological and behavioral features of SNL-induced allodynia for at least twenty days. This result was comparable to that observed with i.t. S(+)-IBU (Hefferan et al., 2003b). In contrast, R(-)-IBU and SC-236 were without effect (Hefferan et al., 2003b). These data suggested that COX-1 is primarily responsible for the generation of spinal PG up to twenty-four hours after SNL which contribute to the development of allodynia in this model. However, the expression profile and time-course of the COX isoforms affecting allodynia, and their relevance (or not) to changes in central sensitivity early after SNL have not been characterized.

Treatment with i.t. SC-51322 starting two days after SNL significantly reversed tactile allodynia (Hefferan et al., 2003a) indicating that spinal EP

receptors mediate the pro-allodynic effects of PG in the spinal cord. EP receptor subtypes are known to be coupled to discrete signaling pathways, each of which could contribute to central sensitivity and allodynia. Increased EP_1, EP_2, EP_3 and EP_4 receptor-IR has been reported in the DRG following partial sciatic nerve ligation (Ma and Eisenach, 2003). However, the effect of experimental nerve injury, including SNL, on the expression of EP receptors in the spinal cord has not been investigated.

To further study the early contribution of spinal PG in experimental allodynia, we investigated: a) the effect of SNL on the expression of COX-1, COX-2, and EP_1, EP_2 and EP_3 receptors in the rat L-DH; and b) the temporal and pharmacological relationship of these changes to AFRR and CFRR and allodynia, twenty-four hours after SNL or sham surgery.

4.2 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, NL, Canada.

Animals. Male Sprague-Dawley rats (~130 g on the day of experimentation) were obtained from the Vivarium of 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 after surgery. A 12 h light-dark cycle (lights on at 0700 h) was used throughout.

Intrathecal Catheterization. Intrathecal catheters (6.5 cm-length terminating near the lumbar enlargement or 4.0 cm-length terminating in the mid-thoracic segments) were implanted as previously described (Hefferan et al., 2003a). Rats with normal motor, grooming and feeding behavior were housed separately and allowed to recover for three days before SNL or sham surgery.

Neuropathy. Neuropathy was induced using the method of Kim and Chung (1994), as previously described (Hefferan et al., 2003a). Rats were anesthetized with halothane and the left L4 and L5 spinal nerves were isolated and separated. The L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham-controls, the L5 and L6 spinal nerves were isolated but not ligated. All animals were allowed to recover for three days before experimentation. Allodynia, defined as a PWT of ≤ 4 g, was confirmed using von Frey filaments.

Electrophysiological Recordings. Rats were anesthetized with halothane and cannulae were placed in the trachea, left carotid artery and right external jugular vein. Halothane anesthesia was then replaced by sodium thiobutabarbitone (1 $mg \cdot kg^{-1}$ i.v.; Inactin, Sigma-Aldrich, ON, CA). The depth of

anesthesia was assessed by testing for hind limb withdrawal and corneal reflexes which had to be absent. Blood pressure was continuously monitored via a left carotid artery catheter and the mean arterial pressure maintained between 100 to 130 mmHg by additional anesthetic as required. Systolic blood pressure did not fall below 100 mmHg throughout the experiment. Core temperature was maintained close to 37 °C using a homeothermic blanket system. The animal preparation was allowed to stabilize for at least 30 minutes prior to recording.

Spinal flexor reflexes were evoked by subcutaneous electrical stimulation applied to the first toe of the hind paw. Needle location was based on the innervation pattern of the sural nerve (Wiesenfeld-Hallin, 1988) and square-wave pulses (0.2, 0.6, 1.0 Hz) of 1 ms duration were used. Stimulation at each frequency was repeated three times to ensure stability and extracellular electromyographic responses were recorded from the biceps femoris muscle using a pair of tungsten needle electrodes. Intervals of three to five minutes were introduced between successive stimulus trains to prevent a conditioning effect by the preceding stimulus.

AFRR and CFRR were distinguished on the basis of their response latencies. AFRR was classified as those appearing <100 ms after the stimulus artifact. CFRR was defined as those >100 ms up to a maximum of 600 ms. These criteria were confirmed by examining the effect of intrathecal morphine (100 μg) and naloxone (100 μg) on the AFRR and CFRR of the biceps femoris reflex in naive animals. Based on the measured length of the sural nerve in rats

weighing 130 to 150 g (i.e. 12 cm), afferent fibers mediating the late phase (>100-600 ms) had an average conduction velocity of 1.2 $m \cdot s^{-1}$; a value within the accepted range for C-fibers (Lynn and Carpenter, 1982). The A- and C-fiber intervals used in the present experiments are in agreement with previous reports on the rat biceps femoris reflex (Lynn and Carpenter, 1982; Herrero and Cervero, 1996a, b).

To determine the AFRR activation threshold, stimulus trains of five pulses (0.2 to 1.0 Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least three of the five stimulus pulses produced a spike (i.e. amplitude >20 μV). To determine the CFRR activation threshold, stimulus trains of twenty pulses (0.2 to 1.0 Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least ten of the twenty stimulus pulses produced a spike (i.e. amplitude >20 μV).

Data were collected, stored and analyzed using a PowerLab® data acquisition system (ADInstruments, Inc., Colorado Springs, CO, USA). Off-line data analysis was initially performed using the data acquisition software provided by the manufacturer (Chart 5.0). The data were further analyzed using a customized computer program developed in collaboration with the Department of Computer Science, Memorial University.

Immunohistochemistry. Animals were deeply anesthetized with urethane (1.2 mg/ kg i.p.) and perfused intracardially with 0.1 M phosphate buffer (PBS) followed by 4% paraformaldehyde in ice cold 0.1 M PBS buffer. The lumbar region (L2 to L6) was subsequently removed, post-fixed for 18 to 24 h and cryoprotected in 50% sucrose overnight at 4 °C. Sections (20 µm) were cut on a cryostat and stored in 0.1 M PBS at 4 °C. Cryostat sections were blocked in normal goat serum (5% in 0.1 M PBS) for 1 h at room temperature, and sections were washed extensively in 0.3 M PBS between each of the following steps. Sections were incubated overnight with the COX-1 primary antibody (1:5000). Binding sites were visualized with Alexa Fluor secondary antibodies diluted in 0.1 M PBS. Specifically, anti-COX-1 was detected with goat anti-rabbit antibody (1:1000; Molecular Probes, Burlington, ONT, CA). To determine the specificity of antibody staining, the COX-1 antibody was preabsorbed with 1 µg/ml of a blocking peptide (Cayman Chemical, Ann Arbor, MI, USA). A monoclonal antibody to vertebrate neuron-specific nuclear protein was used to identify most neuronal cell types throughout the spinal cord (NeuN; 1:1000; Upstate Cell Signaling, Charlotteville, VA, USA). Anti-Integrin alphaM, clone OX-42 was used to identifiy glial cells and macrophages (1:1000; Upstate Cell Signaling, Charlotteville, VA, USA). Anti-Macrophages/Monocytes, clone ED-1 was used to identify infiltrating macrophages and monocytes (ED-1; 1:1000; Upstate Cell Signaling, Charlotteville, VA, USA). Binding sites were visualized with a goat anti-rabbit antibody (1:1000; Molecular Probes, Burlington, ON, CA). Sections were

then mounted on slides, coverslipped, and assessed by confocal laser scanning microscopy (Leica Microsystems Inc., Richmond Hill, ON, CA). Double labeled sections were imaged using filter combinations appropriate for the specific visualization of Alexa Fluor secondary antibodies (488 and 594 nm; Molecular Probes, Burlington, ON, CA).

Western Blotting. Animals were deeply anesthetized with urethane (1.2 mg/kg i.p.) and perfused intracardially with ice cold saline (0.9% NaCl). The spinal cord was extracted hydraulically (de Sousa and Horrocks, 1979), immediately frozen in 2-methylbutane (Sigma Chemical, St. Louis, MO, USA), and stored at -80 °C. The lumbar region (L2 to L6) of the spinal cord was isolated, removed and subsequently divided into the left and right, ventral and dorsal quadrants. Spinal cord was homogenized in ice cold lysis buffer (1% Nonidet-P40, 10% glycerol in TBS plus a protease inhibitor cocktail tablet - Roche Diagnostics, Laval, PQ, CA, 1mM sodium vanadate, 1 mM sodium fluoride, and 0.025% SDS); and centrifuged at 10,000 rpm for 5 min (4 °C). Samples, diluted to achieve equal protein concentrations (30µg), were separated by electrophoresis on 8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and incubated for 1.5 h in Tris buffer (Tris 25 mM, glycine 192 mM, 200 ml methanol, pH 8). Pre-stained protein markers were used for molecular weight determination. Blots were initially stained with Ponceau Red, and later probed with an antibody directed against the cytosolic protein, actin

(1:1000; Sigma, Oakville, ONT, CA) to assess the equivalency of protein loading. Blots were subsequently probed with the following primary antibodies: polyclonal rabbit COX-1 antibody (1:250); polyclonal rabbit COX-2 antibody (1:1000); polyclonal rabbit EP_1 receptor antibody (1:1000); polyclonal rabbit EP_2 receptor antibody (1:2000); and a polyclonal EP_3 receptor antibody (1:1000). These primary antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA). Protein standards for COX-1, COX-2 and EP receptors were probed with antibodies that were preabsorbed with their corresponding blocking peptide (Cayman Chemical, Ann Arbor, MI, USA) to determine the antibody specificity. Western blots were corrected for non-specific binding by subtracting the optical density in the presence of the corresponding blocking peptide from that of each test sample. The membranes were incubated overnight at 4 °C with the primary antibodies, and diluted in Tris buffer (containing 3% milk powder and 0.05% Tween-20). Protein bands were treated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Chemicon International Inc., Temecula, CA, USA) for 1 h at room temperature, washed for 30 minutes in Tris buffer, visualized using enhanced chemiluminescence (PerkinElmer Life Sciences Boston, MA, USA), and exposed to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC, USA).

Drugs. SC-236 and SC-560 were generous gifts from Searle (Skokie, IL, USA). SC-51322 was purchased from Biomol Research Laboratories, Inc.

(Plymouth Meeting, MI, USA). Drugs were dissolved in 100% DMSO and diluted with normal saline at the time of injection to yield a final DMSO concentration of 50%. All equipment was sterilized with 70% alcohol and thoroughly rinsed with 0.9% sterile saline before injection. Intrathecal drugs were injected into anesthetized rats using a hand-held microsyringe. Drugs were delivered in a volume of 5 µl followed by 5 µl of sterile saline. The i.t. catheter was immediately resealed with a stainless steel plug. The position of the catheter tip was verified visually after sacrifice in randomly selected animals.

Data Analysis. Electromyographic recordings in sham operated and SNL animals were compared using the mean responses elicited by three stimulus trains of 0.2, 0.6 and 1.0 Hz. Data were collected (4000 samples/sec) and high-pass digital filters (cut-off frequency 60 Hz) were used to remove noise and movement artifacts. Spontaneous activity was defined as the average spike count integrated over a 60 s period prior to experimentation. In all cases, the magnitude of AFRR and CFRR was calculated by integrating the spike count over the stimulation period (i.e. area-under-the-curve analysis). Spikes were integrated from 0.5 to 99.9 ms and 100 to 600 ms for the AFRR and CFRR, respectively. Windup was assessed by comparing the response evoked by the last stimulus in each train of sham operated and SNL animals. Area-under-the-curve was calculated using trapezoidal integration. Western blots were analyzed by optical density using ImageQuant® software (Amersham Biosciences Corp.,

Piscataway, NJ, USA). Expression data are presented as nanograms of protein relative to COX (50 ng) or EP receptor (25 ng) standards which were corrected for background optical density and normalized using the cytosolic protein, actin. Dose–response analysis was performed using methods from Tallarida and Murray (1988). Comparisons within each treatment group were performed using one-way, repeated measures ANOVA, followed by the Newman–Keuls test. Comparisons across all drug- and vehicle-treated groups at each time point were determined using one-way, completely randomized ANOVA, followed by the Newman–Keuls test (SigmaStat® 2.0).

4.3 RESULTS

After SNL, rats displayed a significant decrease in PWT from ≥ 15 g (baseline) to ≤ 4 g (data not shown). This change was apparent 24 h after SNL, was stable for at least twenty days, and remained confined to the plantar surface of the left hind paw (i.e. ipsilateral to nerve ligation). Generally, the affected hind paw was kept in an elevated and cupped position to minimize contact with the cage floor. All SNL rats were otherwise healthy, showed normal feeding and grooming behavior, and regular weight gain. Sham-surgery had no effect on PWT compared to pre-surgical values, as reported previously.

Western analysis revealed a 4.2-fold increase ($p<0.05$) in the expression of COX-1 in the ipsilateral L-DH of SNL rats compared to sham-controls 24 h after surgery (Figure 4.1A). In contrast, COX-2 was unchanged from sham-

controls (p>0.05; Figure 4.1C). In addition, the expression of COX-1 or COX-2 in the contralateral lumbar cord was unchanged from their respective ipsilateral or contralateral sham-controls (p>0.05; Figure 4.1A&C). At this time point, COX-1-immunoreactivity was clearly evident in the cytoplasm of neurons (Figure 4.2A-C), glial cells (Figure 4.2D-F) and macrophages (resident and infiltrating) of SNL rats (Figure 4.2G-I).

Electromyographic recordings of the AFRR and CFRR of the biceps femoris flexor reflex in naïve animals are shown in Figure 4.3A. Intrathecal morphine (100μg) had no significant effect on evoked activity in the AFRR. In contrast, activity in the CFRR was nearly eliminated (Figure 4.3). There was no change in the activation threshold for the AFRR. However, the activation threshold for the CFRR (ipsilateral to SNL) was significantly reduced compared to sham-controls (Figure 4.4B). The reduction in the CFRR ranged from 56 to 60% evoked at voltages triggering AFRR activity in sham-controls (Figure 4.4A&B). Neither were there any significant differences between the contralateral hind limb of SNL animals and either side of sham-controls for AFRR or CFRR activity (data not shown).

The magnitude of both AFRR and CFRR evoked by supra-threshold stimulation was significantly increased 24 h after SNL compared to sham-controls (Figure 4.5A-C). This increase in evoked activity was greater in A-fibers (51.9%) compared to C-fibers (41.8%). Repeated low- and high-intensity supra-threshold stimulation yielded significant amplification (i.e. a windup-like effect) of

the AFRR in SNL as compared to sham operated animals (Table 4.1). A comparable effect was observed with CFRR activity (Table 4.1). The AFRR and CFRR activity to the first evoked stimulus was significantly increased in nerve-ligated compared to sham operated animals using supra-threshold stimulation (Table 4.1). Spontaneous activity was also increased in SNL rats compared to sham-controls but failed to reach statistical significance ($p>0.05$). There was no increase in spontaneous activity on the contralateral hind limb (data not shown). At supra-threshold activation, the AFRR had a pooled average latency of 12.3 ± 0.4 ms, whereas the CFRR had a pooled average latency of 173.2 ± 9.9 ms. These latencies were unaffected by SNL irrespective of the stimulus intensity (data not shown). In addition, there were no significant differences in the latencies of the AFRR or CFRR evoked by supra-threshold stimulation recorded from the contralateral hind limb of SNL animals (data not shown).

Treatment with SC-560 (100 µg), 30 minutes before low- or high-intensity stimulation significantly reduced AFRR activity in nerve-ligated rats, 24 h after SNL (Figure 4.6A). Whereas SC-560 also significantly inhibited CFRR activity evoked by high-intensity stimulation, no such effect was observed using low-intensity stimulation (Figure 4.6B). SC-236 had no effect on AFRR or CFRR activity using either paradigm (Figure 4.6A&B). The duration of action of SC-560 was 2 h (Figure 4.6C&D). SC-560, but not SC-236 significantly reduced A- and C-fibre windup for up to 2 h (Table 4.2). The AFRR to the first evoked stimulus was inhibited for 1 h by SC-560, but not SC-236 (Table 4.2). There was no

significant effect (p>0.05) on the C-fiber response to the first evoked stimulus by either SC-560 or SC-236. Both SC-560 and SC-236 had no significant effect on spontaneous activity, response latency or duration after SNL or sham surgery (data not shown). DMSO had no significant effect (p>0.05) on any evoked responses except for the exaggeration of AFRR activity at high-intensity stimulation (Figure 4.6A&B). The inhibitory effect of SC-560 on AFRR and CFRR activity, determined 30 minutes after i.t. injection, was dose-dependent (Figure 4.7A&B). SC-560 had no effect in sham-controls (p>0.05; data not shown). There is a lack of a dose-response effect of SC-236 on the AFRR and CFRR, determined after i.t. injection, respectively (data not shown). SC-560 and SC-236 had no effect in sham-controls (p>0.05).

Treatment with i.t. SC-51322 (100 μg), 30 minutes before low- and high-intensity stimulation significantly reduced AFRR activity in nerve-ligated rats, 24 h after SNL (Figure 4.8A). Whereas SC-51322 also significantly inhibited CFRR activity evoked by high-intensity stimulation, no such effect was observed using low-intensity stimulation (Figure 4.8B). While, the duration of action of SC-51322 on AFRR activity was 2 h, the inhibitory effect on CFRR activity was 1 h (Figure 4.8C&D). The inhibitory effect of SC-51322 on A- and C-fiber windup produced a similar time-course (data not shown). SC-51322 had no effect on AFRR or CFRR activity in sham-controls (p>0.05; data not shown). Furthermore, SC-51322 had no effect on AFRR and CFRR latencies, duration and response to the first evoked stimulus in either nerve-ligated animals or sham-controls (data not

shown). Spontaneous activity was unaffected with i.t. SC-51322. DMSO had no significant effect ($p>0.05$) on any evoked responses except for the exaggeration of AFRR activity at high-intensity stimulation (Figure 4.8A&B).

Western analysis revealed a significant increase in the expression of EP_1 (2.0-fold), EP_2 (2.2-fold) and EP_3 (2.5-fold) in nerve ligated animals compared to sham-controls in the ipsilateral L-DH 24 h after SNL (Figure 4.9). In addition, the expression of all EP receptor subtypes in the contralateral lumbar cord was unchanged from their respective ipsilateral or contralateral sham-controls ($p>0.05$; Figure 4.9A-C).

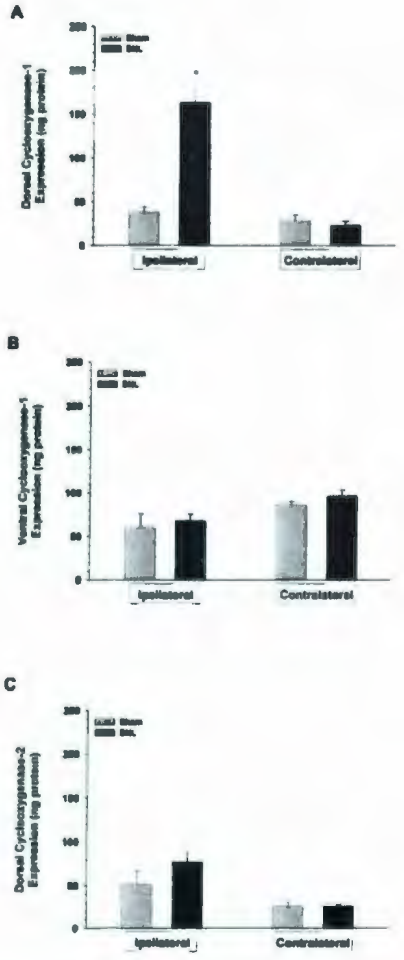

Figure 4.1. The expression of COX-1 (panels A&B) and COX-2 (panel C) in the dorsal or ventral horns of the lumbar spinal cord 24 h after SNL or sham surgery. Data were normalized using total protein content, corrected for non-specific immunoreactivity, and expressed as ng of protein (see Methods). Asterisks indicate a significant difference from sham-operated animals (*p<0.05). Each bar represents the mean ± SEM of three to five animals.

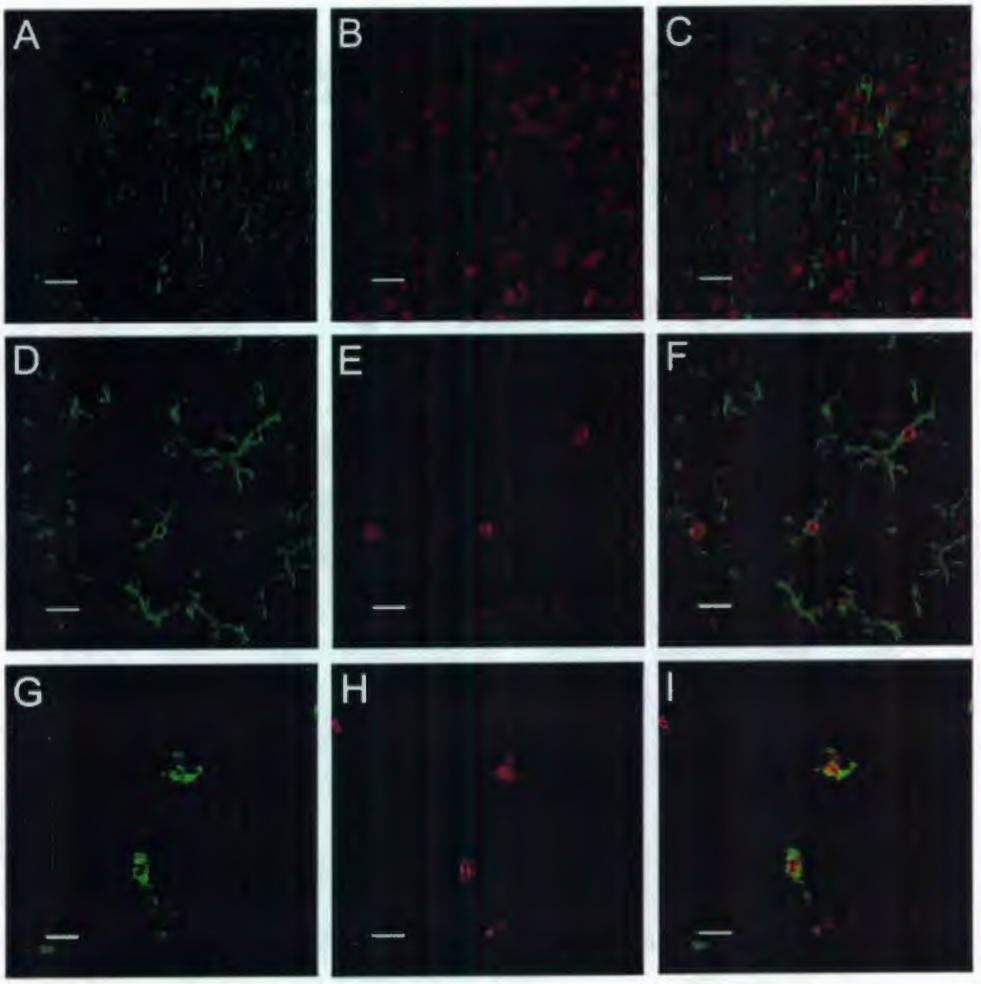

Figure 4.2. COX-1-IR was co-localized in neurons (panels A-C; green=neurons; red=COX-1), microglia (panels D-F; green=microglia; red=COX-1) and macrophages (panels G-I; green=macrophages; red=COX-1) 24h after SNL. Sections were probed with anti-COX-1 and subsequently doubled-labeled using neuN, anti-OX-42 and anti-ED-1. The last column (panels C, F & I) represents a merge of confocal images from each row, respectively (scale bar of 20μm).

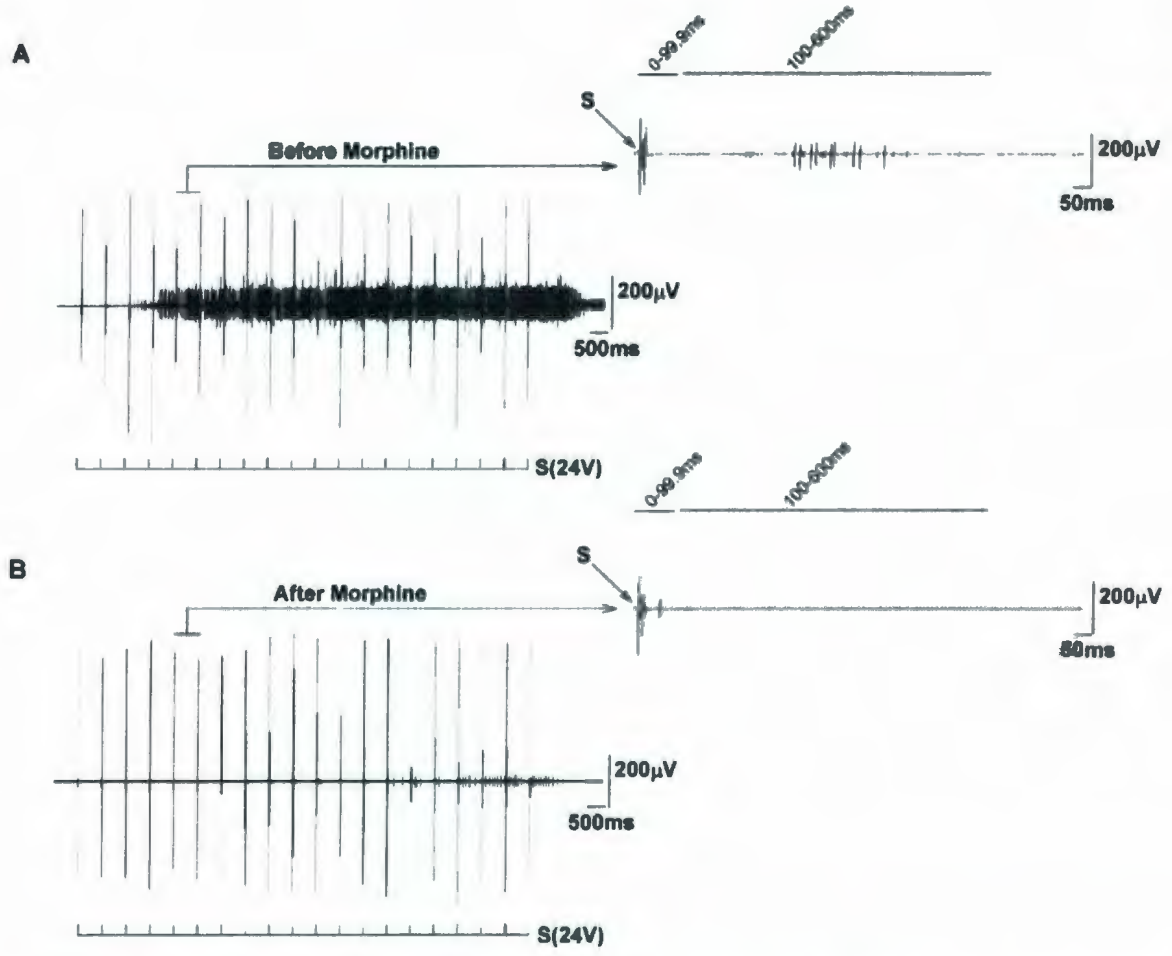

Figure 4.3. Representative electromyographic tracings of the AFRR and CFRR from naïve animals. An indicator bar (S) illustrating individual stimuli (1.5 Hz; 24 V) is included below each tracing. Stimuli were applied to the hind paw before (panel A) and after (panel B) i.t. morphine (100 µg). Inserts represent the AFRR (0.5 to 99.9 ms) and CFRR (100 to 600 ms) evoked by a single stimulus. Note the virtual absence of evoked activity between 100 and 600 ms after i.t. morphine; the 0.5 to 99.9 ms interval remained unchained (panel B).

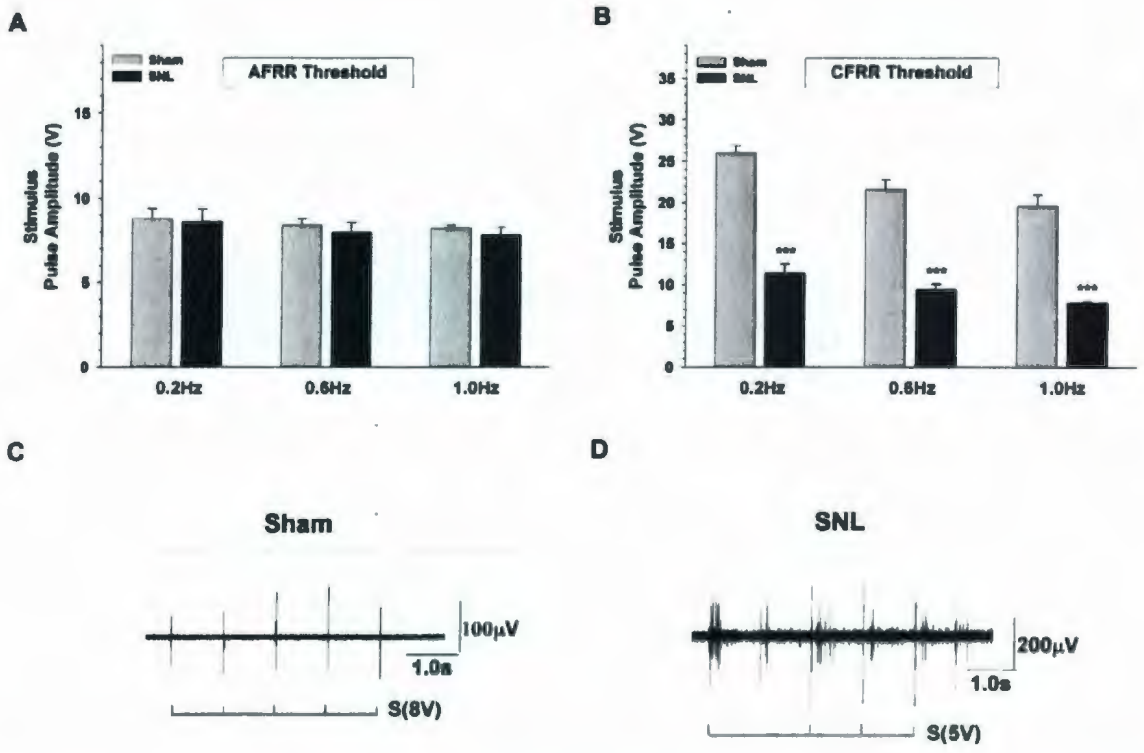

Figure 4.4. Activation threshold (V) eliciting the AFRR (Panel A) and CFRR (panel B) in the ipsilateral biceps femoris 24 h after SNL or sham surgery. Each bar represents the mean ± SEM of five to seven animals and asterisks indicate a significant difference from the respective sham-control (***p<0.001). Note the differences in activation threshold (V) for the AFRR and CFRR. Representative electromyographic tracings of the AFRR and CFRR after sham surgery (panel C) and SNL (panel D). Note the differences in both the stimulus (S) and response voltages between SNL and sham-controls, and the presence of the CFRR evoked by threshold stimulation of the AFRR after SNL.

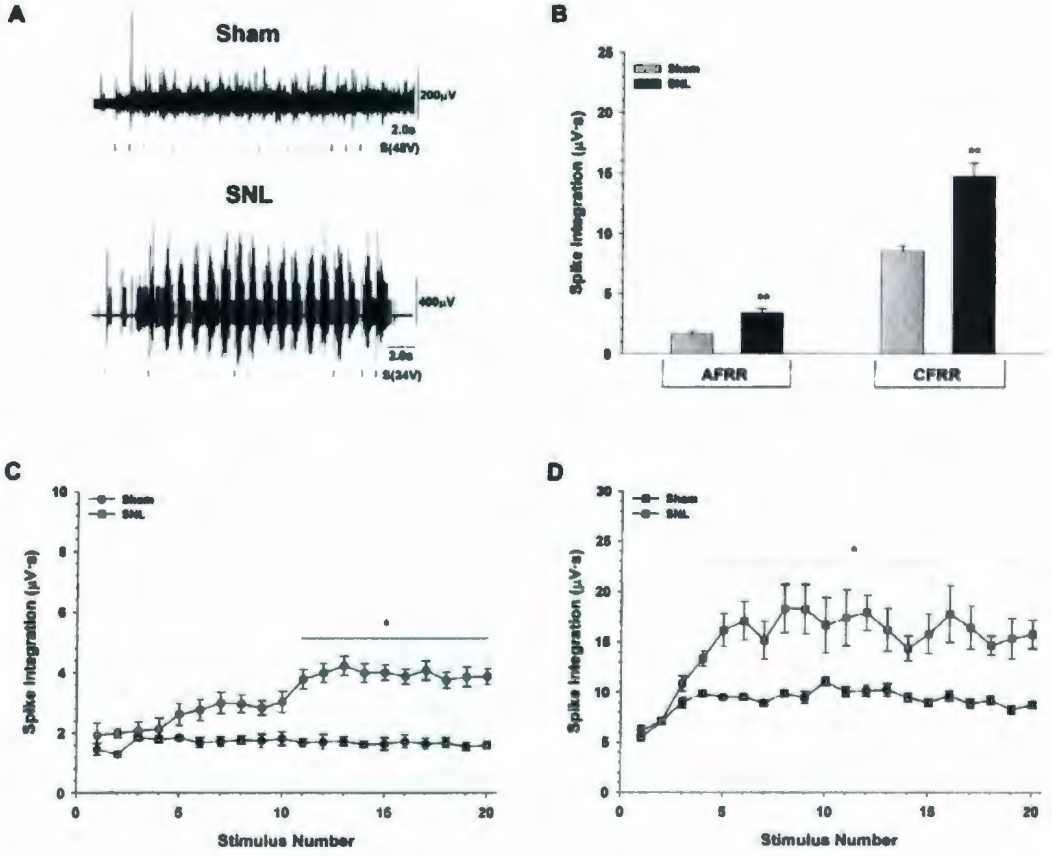

Figure 4.5. Individual electromyographic tracings (panels A), spike integration of the AFRR and CFRR in the ipsilateral biceps femoris (panel B) and representative wind-up versus stimulus number curves for the AFRR (panel C) and CFRR (panel D). EMG responses were evoked by high intensity supra-threshold stimulation (1.0 Hz) 24 h after SNL or sham surgery. Data are presented as the mean ± SEM of four to six animals and asterisks indicate a significant difference from sham-controls (*p<0.05; **p<0.01). Note the differences in the stimulation (S) and response voltages between SNL (24 V & 400 μV) and sham-controls (48V & 200 μV) in panel A. Also note the differences in spike integration scale for AFRR (panel C) and CFRR (panel D).

Table 4.1. Spike integration of reflex responses recorded from the ipsilateral biceps femoris 24 h after SNL or sham surgery.

Pulse Number	Stimulus Intensity	A-Fiber Mediated Reflex Response (μV·s)		C-Fiber Mediated Reflex Response (μV·s)	
		Sham	SNL	Sham	SNL
1st	Low	1.01 ± 0.25	2.70 ± 0.60*	5.30 ± 0.25	5.45 ± 0.41
	High	1.45 ± 0.29	2.96 ± 0.56*	6.60 ± 0.79	6.14 ± 0.74
20th	Low	1.07 ± 0.15	2.02 ± 0.22***	5.21 ± 0.14	7.44 ± 0.58**
	High	1.60 ± 0.12	3.23 ± 0.26*	8.62 ± 0.48	13.62 ± 2.07*

Asterisks indicate a significant difference from sham operated animals (*p<0.05; **p<0.01 ***p<0.001). Reflex responses were evoked by supra-threshold stimulation (see Methods).

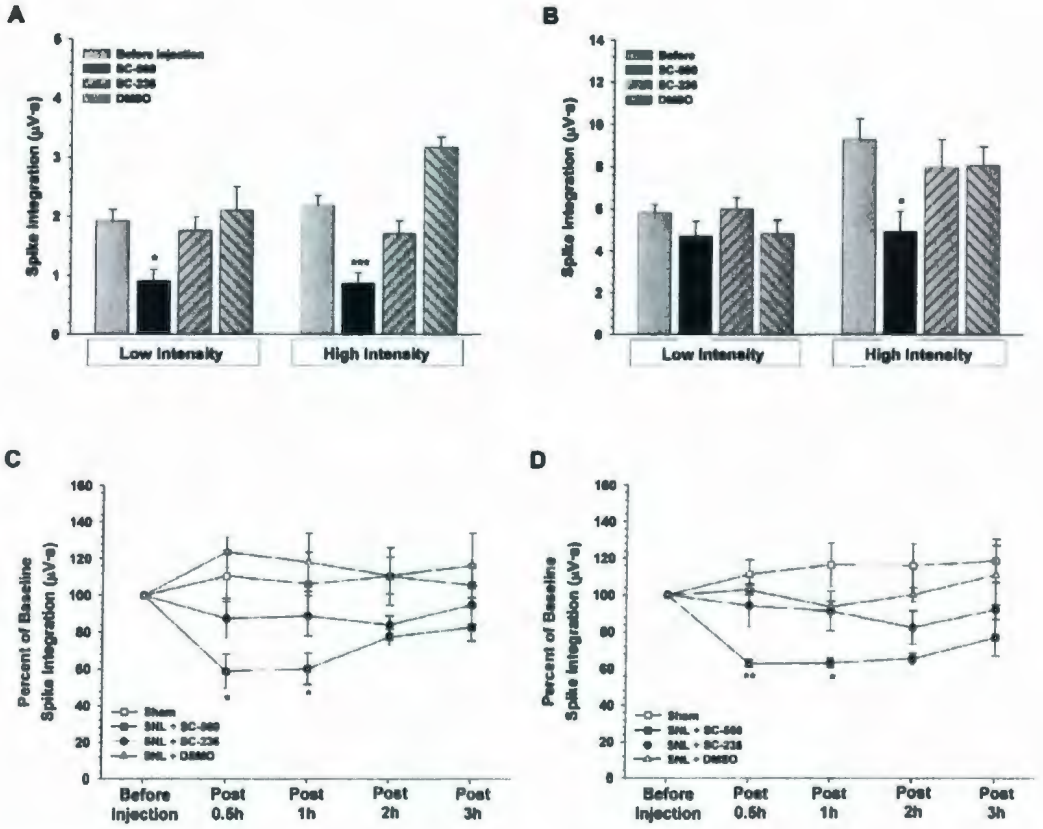

Figure 4.6. The effect of i.t. SC-560 and SC-236 on SNL-induced exaggeration of the AFRR (panels A&C) and CFRR (panels B&D). Electromyographic responses were recorded ipsilateral biceps femoris, 24 h after SNL. All data are presented as the mean ± SEM of four to six animals. Panels A and B represent the peak effect (i.e. 30 minutes after injection) of SC-560 and SC-236, respectively. Asterisks indicate a significant difference from before injection (*p<0.05; ***p<0.001). Note the differences in scale in panels A and B. The time-course of drug effects on the AFRR and CFRR evoked by high intensity supra-threshold stimulation are shown in panels C and D, respectively. Asterisks indicate a significant difference from sham-controls (*p<0.05; **p<0.01).

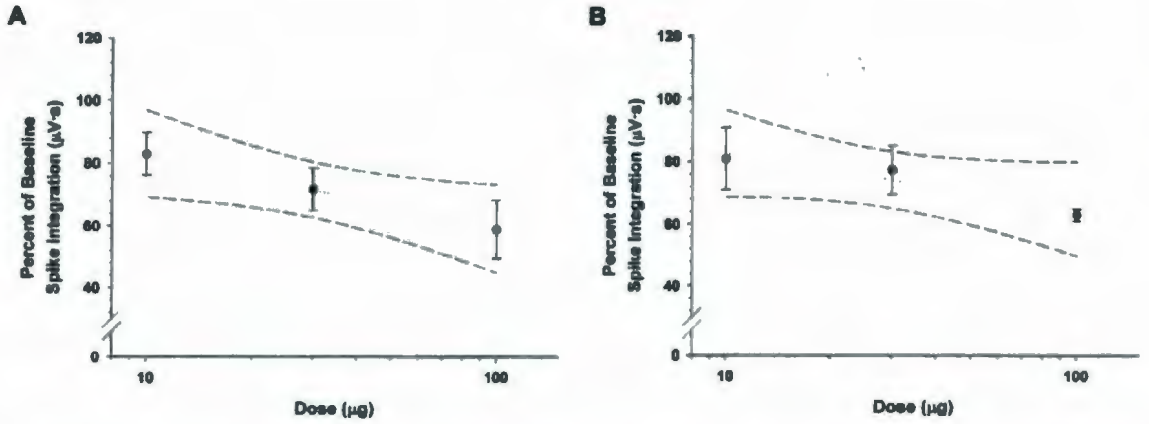

Figure 4.7. The dose-response effect of i.t. SC-560 on the AFRR (panel A) and CFRR (panel B) in the biceps femoris. Electromyographic responses to high intensity supra-threshold stimulation were recorded ipsilaterally, 24 h after SNL. Data are expressed as the mean ± SEM of four to six animals and represent the peak effect of SC-560 (30 minutes after injection). The linear regressions (r^2 values) for AFRR and CFRR were 0.25 and 0.15, respectively. Dashed lines indicate the 95% confidence intervals around the regression (dotted) line.

Table 4.2. Effect of i.t. SC-560 (100 µg) on reflex responses recorded ipsilaterally 24 h after SNL or sham surgery.

Pulse Number	Hours After Injection	A-Fiber Mediated Reflex Response (% baseline)		C-Fiber Mediated Reflex Response (% baseline)	
		Sham	SNL	Sham	SNL
1st	0.5	120.15%	60.48%*	128.94%	59.85%*
	1.0	116.52%	62.53%*	123.43%	59.58%*
	2.0	126.23%	80.91%*	131.18%	59.19%*
	3.0	115.77%	93.47%	110.40%	68.30%
20th	0.5	109.29%	42.72%*	118.81%	85.68%
	1.0	110.29%	46.68%*	106.77%	82.25%
	2.0	110.80%	64.06%	110.69%	93.00%
	3.0	111.73%	70.57%	102.33%	101.30%

Asterisks indicate a significant difference (*p<0.05; **p<0.01; ***p<0.001) from before i.t. drug injection (i.e. baseline). Reflex responses were evoked by supra-threshold stimulation (see Methods).

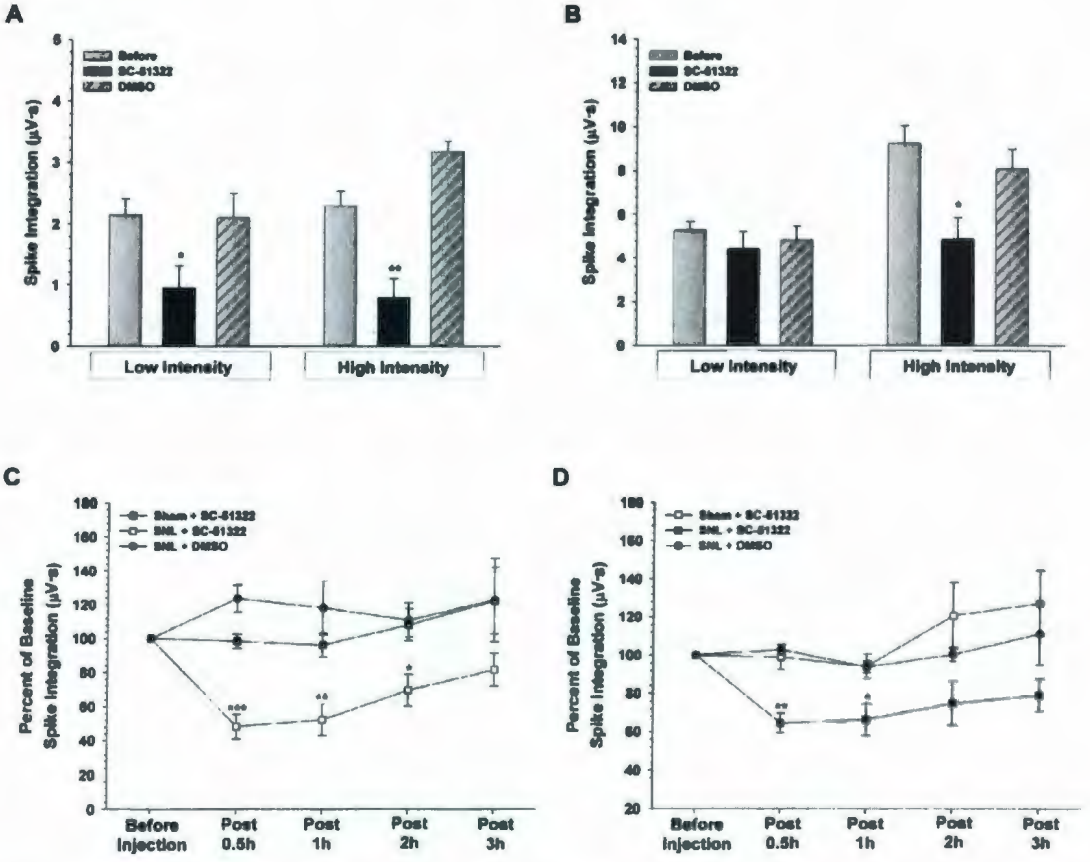

Figure 4.8. The effect of i.t. SC-51322 on the AFRR (panels A&C) and CFRR (panels B&D) of the biceps femoris. Electromyographic responses to supra-threshold stimulation were recorded ipsilaterally, 24 h after SNL. All data are presented as the mean ± SEM of four to six animals. In Panels A and B, asterisks indicate a significant difference from before injection (*p<0.05; **p<0.01). Note the differences in scale in panels A and B. The time-courses of SC-51322 on the AFRR and CFRR evoked by high-intensity supra-threshold stimulation are shown in panels C and D, respectively. Asterisks indicate a significant difference from sham-controls (*p<0.05; **p<0.01; ***p<0.001).

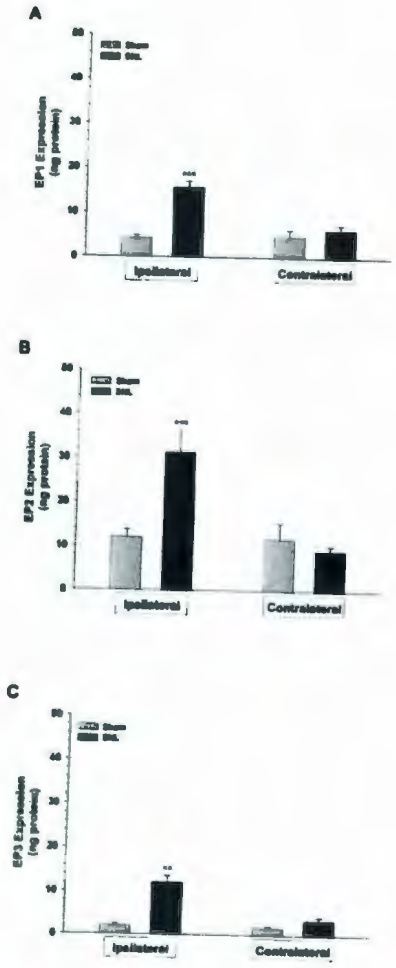

Figure 4.9. The expression of EP_1- (panel A), EP_2- (panel B) and EP_3- (panel C) receptors in the ipsilateral and contralateral L-DH 24 h after SNL or sham surgery. Each bar represents the mean ± SEM of three to five animals and asterisks indicate a significant difference from the corresponding sham-control (**p<0.01; ***p<0.001).

4.4 DISCUSSION

Changes in the excitability of spinal neurons were investigated 24 h after SNL using the biceps femoris flexor reflex. EMG analysis revealed a significant increase in excitability as evidenced by the decrease in activation threshold, increase in evoked activity to single (i.e. activity before spatial and temporal summation) and repetitive stimulation, and the presence of windup; sustained input known to trigger the neural generation of spinal prostanoids (Millan, 1999; Vanegas and Schaible, 2001). These changes, characteristic of peripheral and central sensitization, paralleled the development of mechanical allodynia. The effect on AFRR activity is especially interesting given that SNL-induced mechanical allodynia is elicited by impulses carried along surviving Aβ-afferents (Kim and Chung, 1992). To our knowledge, this is the first report of A-fibre mediated windup after SNL. While an effect on motor neurons cannot be completely excluded at this time, spasticity and/or seizure-like effects are highly unusual in the SNL model and were uniformly absent in these experiments. Thus, afferent sensory input appears to be preferentially affected by SNL.

The AFRR and CFRR were investigated using accepted activation and conduction criteria (Lynn and Carpenter, 1982; Herrero and Cervero, 1996a, b), and further validated by their differential sensitivity to the inhibitory effects of i.t. morphine. Responses were highly reproducible within the treatment groups at low- and high-intensity stimulation. No attempt was made to separate the A-fiber subtypes primarily because of altered conductivity to injured and uninjured A-

fibers (Laird and Bennett, 1993). The absence of detectable changes in AFRR or CFRR latencies was not surprising since synaptic reorganization in the spinal cord is delayed, normally taking weeks to months after nerve injury (Okamoto et al., 2001; Kohno et al., 2003). The results of the present study illustrate the influence of SNL on the excitability of spinal neurons to both single and repetitive stimulation 24 h after nerve injury.

Comparable increases in spinal excitability have been described in other nerve injury models including chronic constriction injury (Meyerson et al., 1995; Colvin et al., 1996; Kohno et al., 2003), complete sciatic nerve transection (Okamoto et al., 2001; Kohno et al., 2003), partial sciatic nerve ligation (Meyerson et al., 1995), spared nerve injury (Kohno et al., 2003) and spinal cord transection (Bennett et al., 2004). In contrast, Chapman et al. (1998) reported no change in the excitability of Aβ- and C-fibers, seven to seventeen days after SNL. While this result may be due, in part, to methodological differences from the present study (e.g. degree of constriction injury), there is evidence that the time points investigated after SNL are important. For example, previous work (Hefferan et al., 2003b) demonstrated a transition from spinal PG-dependent to PG-independent allodynia seven to ten days after SNL[†]. Moreover, the central sensitization described in the present study was significantly attenuated by i.t. SC-51322 or SC-560, but not SC-236. Thus, the hyperexcitability recorded 24 h after SNL was not only spinal PG-dependent and EP receptor-mediated, but also

[†] Early results in our laboratory indicate that central sensitization also exhibits a transition to PG-independence seven to ten days after SNL (unpublished results).

consistent with the preferential increase in spinal COX-1 protein at the same time point. COX-1 expression was evident in neurons and glia as well as macrophages of the ipsilateral L-DH, indicating that this was not an inflammatory response. This finding is in agreement with others which reported increased expression of COX-1 following experimental nerve injury (Garrison et al., 1991; Zhu and Eisenach, 2003; Abbadie, 2005). That spinal COX-2 was not up-regulated at 24 h does not mean it is unaffected by SNL. A significant increase in COX-2 expression is evident three days after SNL (O'Rielly and Loomis, 2006), suggesting that the dominant isoform undergoes a time-dependent shift from COX-1 (24 h) to COX-2 (72 h) in affected spinal segments.

Western analysis revealed a significant increase in the expression of EP_1, EP_2 and EP_3 receptors in the ipsilateral L-DH 24 h after SNL. Unfortunately, reliable and selective staining of EP_4 receptors could not be achieved with the commercially available antibodies precluding further investigation in this study. EP_1 receptors, located on primary afferent terminals, facilitate neurotransmitter release (Vanegas and Schaible, 2001) and likely mediate, at least in part, the central sensitizing effect of spinal PG. Post-synaptic EP_2 receptors in the DH (Baba et al., 2001) attenuate glycine-mediated IPSCs (Ahmadi et al., 2002). That the resulting disinhibition is conducive to allodynia is indicated by the robust and selective allodynia following low dose i.t. strychnine in conscious rats (Yaksh, 1989). The EP_2-mediated disinhibitory state would also be exaggerated by the enhanced production/release of and sensitivity to PGE_2; abnormalities known to

occur in the affected spinal cord early after SNL (Hefferan et al., 2003a,b; O'Rielly and Loomis, 2006). The contribution of the EP_3 receptor subtype to central sensitization is more complex, reflecting its multiple variants and signaling pathways. Some EP_3 receptors in the DH are positively coupled to NOS with the resulting NO (Matsumura et al., 2005) amplifying PG synthesis and PG-dependent allodynia (Bredt and Snyder, 1992; Mollace et al., 1995; Hefferan MP and Loomis, 2004). The up-regulation of EP_3 variants which are negatively coupled to adenylyl cyclase explains the previously reported increase in glutamate release from slices of SNL rats pretreated with SC-51322 and evoked by high concentrations of PGE_2 (O'Rielly and Loomis, 2006).

In summary, SNL triggered a significant increase in A- and C-fiber input as well as COX-1, EP_1, EP_2 and EP_3 expression in the spinal cord 24 h after injury. These SNL-induced effects: a) paralleled the development of allodynia; b) were only evident in animals exhibiting this abnormality; and c) were significantly attenuated by i.t. SC-560 or SC-51322 (but not SC-236) given 24 h after nerve injury. The results demonstrate for the first time the significant influence of spinal PG on exaggerated A- and C-fiber excitability after SNL, and identify COX-1 as the dominant isoform in the affected spinal cord 24 h after nerve injury. They also corroborate earlier evidence for the contribution of spinal PG in the development of SNL-induced allodynia including: a) the increased expression of COX-1 and COX-2 in, and the release of PGE_2 and PGI_2 from the injured spinal cord (Tonai et al., 1999; Ma and Eisenach, 2002, 2003; Hefferan et al., 2003a; Zhu and

Eisenach, 2003; O'Rielly and Loomis, 2006); b) the brush-evoked release of PGE_2 in spinal CSF from SNL rats but not sham-controls (Hefferan et al., 2003a); c) the ability of i.t. COX inhibitors, given two to eight hours after SNL, to prevent the development of allodynia for at least twenty days (Hefferan et al., 2003b); d) the marked increase (2855-fold *in vitro* and 362-fold *in vivo*) in pharmacodynamic sensitivity to PGE_2 in SNL (i.e. allodynic) rats (O'Rielly and Loomis, 2006); e) the increase in EP receptor immunoreactivity in the injured nerve following partial sciatic nerve ligation (Ma and Eisenach, 2003), and f) the reversal of tactile allodynia (i.e. ~2 h duration) by SC-51322 starting two days after SNL (Hefferan et al., 2003a).

5.0 SPINAL NERVE LIGATION-INDUCED ACTIVATION OF NUCLEAR FACTOR KAPPAB IS FACILITATED BY PROSTAGLANDINS IN THE AFFECTED SPINAL CORD AND IS A CRITICAL STEP IN THE DEVELOPMENT OF MECHANICAL ALLODYNIA

5.1 INTRODUCTION

NFκB is constitutively expressed in glia and neurons (Lu et al., 2003; Lee et al., 2004; Yan et al., 2004; Tsatsanis et al., 2006) where it is stored in the cytoplasm as an inactive complex with the inhibitory factor, IκB (Egan & Toruner, 2006). Following cell activation, IκB is phosphorylated by protein kinases such as IκB kinase alpha, releasing NFκB from the complex. NFκB is then free to translocate into the nucleus where it is activated and binds to specific response elements of the DNA initiating gene transcription.

Experimental nerve injuries such as partial ligation, complete transection or chronic constriction of the sciatic nerve, or spinal cord compression trigger the activation of NFκB (Sakaue et al., 2001; Pollock et al., 2005; Bethea et al., 1998). As a modulator of gene transcription (Lu et al., 2003; Lee et al., 2004; Yan et al., 2004; Tsatsanis et al., 2006), NFκB plays a critical role in cell survival, apoptosis, and synaptic plasticity (Shishodia & Aggarwal, 2002; Piva et al., 2006; Bubici et al., 2006; Albensi & Mattson, 2000). Thus, it is well positioned to affect the functional outcomes induced by nerve injury. Consistent with this hypothesis are reports that blocking the transcriptional effects of NFκB with PDTC or its decoy

significantly attenuated the mechanical allodynia normally elicited by chronic constriction of the sciatic nerve (Ebersberger et al., 2006), spinal cord injury (La Rosa et al., 2004; Jimenez-Garza et al., 2005), or treatment with i.t. dynorphin (Laughlin et al., 2000). Understanding the pharmacology of these acute signaling events provides a scientific basis for safe and effective interventions to mitigate the chronic and often debilitating outcomes of nerve injury.

L5/L6 spinal nerve ligation is a widely used nerve injury model inducing mechanical allodynia in experimental animals (Kim and Chung, 1992; Hefferan et al., 2003a, b). This model is characterized by early changes in and dependence on spinal PG signaling seven to ten days after SNL (Hefferan et al., 2003b; O'Reilly and Loomis, 2006; 2007). These changes include the up-regulation of COX-2 and $EP_{1\text{-}3}$ receptors in the affected DH, a marked increase in pharmacodynamic sensitivity to PGE_2 in the lumbar cord (O'Rielly and Loomis, 2006; 2007), and the onset of BR-evoked (i.e. non-noxious) release of PGE_2 into spinal CSF (Hefferan et al., 2003b). Transcription of the COX-2 gene, which reaches maximum protein expression three days after SNL (O'Rielly and Loomis, 2006), is known to be modulated by NFκB (Tsatsanis et al., 2006), and COX-2-derived spinal PG play a critical role in the development of SNL-induced allodynia (O'Rielly and Loomis, 2006, 2007; Hefferan et al., 2003a, b). Thus, SNL represents a logical NP model in which to determine if the activation of NFκB in the affected spinal cord is an immediate and necessary step in the development of allodynia.

To test this hypothesis, NFκB was determined in nuclear extracts from the ipsilateral and contralateral L-DH twelve hours, one day and three days after SNL or sham surgery. To assess the transcriptional effect of NFκB, the expression of COX-2 protein was determined using Western analysis. PDTC, an inhibitor of NFκB, was used to probe the functional relationship between NFκB in the affected spinal cord, the induction of spinal COX-2, and the development of mechanical allodynia and spinal hyperexcitability three days after SNL. The latter was investigated using AFRR, CFRR, and PWT was used to confirm mechanical allodynia. The possibility that spinal PG, generated immediately after SNL, might facilitate the initial activation of NFκB and, in turn, its downstream transcriptional effect on COX-2 was also investigated.

5.2 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, NL, Canada.

Animals. Male Sprague-Dawley rats (130 to 150 g) were obtained from the Vivarium of Memorial University of Newfoundland (St. John's, NL, CA) and housed in standard cages with woodchip bedding. Animals had free access to food and water. A 12 h light-dark cycle (lights on at 0700 h) was used throughout.

Intrathecal Catheterization. Intrathecal catheters (6.5 cm-length terminating near the lumbar enlargement) were implanted according to method of Yaksh and Rudy (1976) as modified by Hefferan et al. (2003a). Catheters were 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. Intrathecal catheters (inner diameter, i.d. 240 μm, outer diameter, o.d. 290 μm) were constructed from triple lumen PE-5 tubing (Spectranetics, Colorado Springs, CO, USA) using the modified method of Marsala et al. (1995). Rats with normal motor, grooming and feeding behavior were housed separately and allowed to recover for two days before SNL or sham surgery.

Neuropathy. Neuropathy was induced using the method of Kim and Chung (1992) as previously described (Hefferan et al., 2003a). Briefly, rats were anesthetized with halothane, the left L4 and L5 spinal nerves were isolated and separated, and the L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham-controls, the L5 and L6 spinal nerves were isolated but not ligated. Except for NFκB Western analysis, which was determined 12 and 24 h after surgery, all animals were allowed to recover for three days before experimentation.

Paw Withdrawal Threshold. Mechanical allodynia was quantified by determining the PWT using von Frey filaments (Chaplan et al., 1994). Briefly, rats were placed in a plastic cage with a wire-mesh bottom allowing access to the plantar surface of the left hindpaw. Following a 20-min acclimatization period, a control threshold was determined. Allodynia was defined as a PWT of ≤ 4 g (Chaplan et al. 1994; Hefferan et al., 2003a,b).

Electrophysiological Recordings. Rats were anesthetized with halothane and cannulae were placed in the trachea, left carotid artery and right external jugular vein. Halothane anesthesia was then replaced by sodium thiobutabarbitone (1 $mg \cdot kg^{-1}$ i.v.; Inactin, Sigma-Aldrich, Oakville, ON, CA). Adequate depth of anesthesia was assessed by testing for hind limb withdrawal and corneal reflexes which had to be absent. Blood pressure was continuously monitored via a left carotid artery catheter and the mean arterial pressure maintained between 100 to 130 mmHg by additional anesthetic as required. Systolic blood pressure did not fall below 100 mmHg throughout the experiment. In the event that the blood pressure dropped and consistently remained below 100 mmHg, the experiment was stopped and the animal euthenized. Core temperature was maintained close to 37 °C using a homeothermic blanket system. The animal preparation was allowed to stabilize for at least 30 minutes prior to data collection.

Spinal flexor reflexes were evoked by subcutaneous electrical stimulation applied to the first toe of the hind paw. Needle location was based on the innervation pattern of the sural nerve (Wiesenfeld-Hallin, 1988) and square-wave pulses (0.2, 0.6, 1.0 Hz) of 1ms duration were used. Stimulation at each frequency was repeated three times to ensure stability and extracellular electromyographic responses were recorded from the biceps femoris muscle using a pair of tungsten needle electrodes. Intervals of three to five minutes were introduced between successive stimulus trains to prevent a conditioning effect by the preceding stimulus. A low intensity supra-threshold stimulus refers to a voltage twice the AFRR activation threshold (five pulses at 0.2 Hz). A high intensity supra-threshold stimulus refers to a voltage twice the CFRR activation threshold (twenty pulses at 1.0 Hz).

The AFRR and CFRR were distinguished on the basis of activation threshold and response latency. Electrical stimulation sufficient to activate the CFRR resulted in two distinct components; an early and late phase separated by a quiescent period of variable duration. The AFRR and CFRR were classified as those appearing <100 ms after the stimulus artifact, and >100 ms up to a maximum of 600 ms, respectively. These criteria were confirmed by examining the effect of i.t. morphine (100 μg) and naloxone (100 μg) on the AFRR and CFRR of the biceps femoris reflex in naive animals (O'Rielly and Loomis, 2007). Assuming a motor neuron conduction distance of 11 cm, a conduction velocity of 60 $m \cdot s^{-1}$ (Chamberlain and Lewis, 1989), a synaptic delay of 0.5 ms at the

neuromuscular junction, a minimum central synaptic delay of 0.5 ms, and an afferent conduction distance of 12 cm, the fastest afferent fibers mediating the late component of the reflex response had conduction velocities of approximately 1.2 $m \cdot s^{-1}$ (Lynn and Carpenter, 1982). The AFRR and CFRR intervals used in the present experiments are in agreement with previous reports on the rat biceps femoris reflex (Lynn and Carpenter, 1982; Herrero and Cervero, 1996a, b). No attempt was made to separate the A-fiber subtypes primarily because of altered conductivity to injured and uninjured A-fibers (Laird and Bennett, 1993).

To determine the AFRR activation threshold, stimulus trains of five pulses (0.2 to 1.0 Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least three of the five stimulus pulses produced a spike (i.e. amplitude >20 μV). To determine the CFRR activation threshold, stimulus trains of twenty pulses (0.2 to 1.0 Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least ten of the twenty stimulus pulses produced a spike (i.e. amplitude >20 μV). For the purposes of this study, spinal hyperexcitability is defined as a significant decrease ($p<0.05$) in the activation threshold and an increase in the response magnitude of the AFRR and/or CFRR following SNL.

Data were collected, stored and analyzed using a PowerLab data acquisition system (ADInstruments, Inc., Colorado Springs, CO, USA). Off-line data analysis was initially performed using the data acquisition software provided

by the manufacturer (Chart 5.0). The data were further analyzed using a customized computer program developed in collaboration with the Department of Computer Science, Memorial University of Newfoundland (St. John's, NL, CA).

Western Blotting. Animals were deeply anesthetized with urethane (1.2 $mg \cdot kg^{-1}$ i.p.) and perfused intracardially with ice cold saline (0.9% NaCl). The spinal cord was extracted hydraulically (de Sousa and Horrocks, 1979), immediately frozen in 2-methylbutane (Sigma Chemical, Oakville, ON, CA), and stored at -80 °C. The lumbar region (L2 to L6) of the spinal cord was isolated, removed and subsequently divided into the left and right ventral and dorsal quadrants. The quadrants were homogenized in ice cold lysis buffer (1% Nonidet-P40, 10% glycerol in TBS plus a protease inhibitor cocktail tablet - Roche Diagnostics, Laval, PQ, CA, 1 mM sodium vanadate, 1 mM sodium fluoride, and 0.025% SDS) and centrifuged at 10,000 rpm for 5 minutes (4 °C). Preparation of nuclear extracts was performed using special homogenization buffers and additional centrifugation as previously described (Bethea et al., 1998). The Coomassie Plus Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA) was used to determine the protein quantity of all samples. Samples, diluted to achieve equal protein concentrations (30 µg), were separated by electrophoresis on 8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and incubated for 1.5 h in Tris buffer (Tris 25 mM, glycine 192 mM, 200 ml methanol, pH 8). Pre-stained protein markers were used

for molecular weight determination. Blots were initially stained with Ponceau Red, and later probed with an antibody directed against the cytosolic protein, actin (1:1000; Sigma, Oakville, ON, CA) to assess the equivalency of protein loading. Blots were subsequently probed with the following primary antibodies: polyclonal rabbit COX-1 antibody (1:250); polyclonal rabbit COX-2 antibody (1:1000); and polyclonal rabbit anti-NFκB-p65 (1:2000). The latter antibody is selective for NFκB-p65 when used in combination with a control (blocking) peptide provided by the supplier. All primary antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA) except the NFκB-p65 antibody which was purchased from Chemicon International, Inc. (Temecula, CA, USA). Protein standards for COX-1, COX-2, and NFκB (non-stimulated A431 cell lysate; Upstate Cell Signaling Solutions, Charlottesville, VA, USA) were pre-absorbed with their corresponding blocking peptide to determine the antibody specificity. Since most antibodies have some degree of cross-reactivity, western blots were corrected for non-specific binding by subtracting the optical density in the presence of the corresponding blocking peptide from that of each test sample. The membranes were incubated overnight at 4 °C with the primary antibodies, and diluted in Tris buffer (containing 3% milk powder and 0.05% Tween-20). Protein bands were treated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Chemicon International Inc., Temecula, CA, USA) for 1 h at room temperature, washed for 30 minutes in Tris buffer, visualized using enhanced chemiluminescence (PerkinElmer Life Sciences Boston, MA, USA),

and exposed to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC, USA).

Drugs. S(+)-IBU and R(-)-IBU were purchased from Sigma-Aldrich (Oakville, ON, CA). PDTC was purchased from Sigma-Aldrich (Oakville, ON, CA). All drugs were dissolved in 100% DMSO and diluted with normal saline at the time of injection to yield a final DMSO concentration of 50%. All equipment was sterilized with 70% alcohol and thoroughly rinsed with 0.9% sterile saline before injection. Intrathecal drugs were injected into anesthetized rats using a hand-held microsyringe. Drugs were delivered in a volume of 5 µl followed by 5 µl of sterile saline. The i.t. catheter was immediately resealed with a stainless steel plug. The position of the catheter tip was verified visually after sacrifice in randomly selected animals.

Data Analysis. Electromyographic recordings in sham operated and SNL animals were compared using the mean responses elicited by three stimulus trains of 0.2, 0.6 and 1.0 Hz. Data were collected (4000 samples/sec) and high-pass digital filters (cut-off frequency 60 Hz) were used to remove noise and movement artifacts. Spontaneous activity was defined as the average spike count integrated over a 60 s period prior to experimentation. In all cases, the magnitude of the AFRR and CFRR was calculated by integrating the spike count over the stimulation period (i.e. area-under-the-curve analysis). Spikes were

integrated from 0.5 to 99.9 ms and 100 to 600 ms for the AFRR and CFRR, respectively. Western blots were analyzed by optical density using ImageQuant software (Amersham Biosciences Corp., Piscataway, NJ, USA). Expression data are presented as micrograms of protein relative to an NFκB protein standard (10 μg), and as nanograms of protein relative to COX-1 or COX-2 (50 ng). Expression data were corrected for background optical density and normalized using the cytosolic protein, actin. Dose-response analysis was performed using methods from Tallarida and Murray (1987). Comparisons within each treatment group were performed using one-way, repeated measures ANOVA, followed by the Newman–Keuls test. Comparisons across all drug- and vehicle-treated groups at each time point were determined using one-way, completely randomized ANOVA, followed by the Newman–Keuls test (SigmaStat 2.0; Systat Software Inc., San Jose, CA, USA).

5.3 RESULTS

There was a progressive and significant increase in the amount of NFκB in the ipsilateral L-DH, twelve hours (35%), one day (55%) and three days (59%) after SNL compared to their respective sham-controls (Figure 5.1). NFκB in the contralateral DH (Figure 5.1) or in either ventral horn (data not shown) was unchanged from sham-controls. There was a corresponding increase in the expression of COX-2 protein ($p<0.05$) in the ipsilateral L-DH of SNL animals compared to sham-controls three days after surgery (Figure 5.2). The PWT was

significantly reduced (<4 g) in all SNL rats (Figure 5.3A); an effect confined to the plantar surface of the ipsilateral hind paw. Generally, the affected paw was kept in an elevated and cupped position to minimize contact with the cage floor. Allodynic animals were otherwise healthy, and exhibited normal grooming behavior except for the left hind paw. Sham-surgery had no effect on PWT compared to pre-surgical values (Figure 5.3A).

To further investigate the enhanced sensitivity to mechanical stimulation and its possible connection to the SNL-induced changes in spinal NFκB, A- and C-fiber mediated responses were recorded from the biceps femoris flexor reflex three days after SNL or sham surgery. The threshold voltages eliciting the AFRR (Figure 5.3B) and CFRR (Figure 5.3C) were significantly reduced compared to sham-controls. This effect was independent of the stimulation frequency (0.2 to 1.0 Hz) and still evident ten days after nerve injury (Figure 5.3B&C). In contrast, the threshold voltages eliciting the AFRR and CFRR in the contralateral hind limb were unchanged ($p>0.05$) from sham-controls three or ten days after surgery (data not shown). Consistent with reduction in firing thresholds of the AFRR and CFRR were the exaggerated responses to high intensity supra-threshold stimulation (Figure 5.4B) compared to sham-controls (Figure 5.4A). As shown in Figure 5.4C, the mean response magnitudes of the AFRR and CFRR in SNL rats were increased ($p<0.05$) by 110% and 30%, respectively at their respective supra-threshold stimulation voltages (see Methods). In addition, spontaneous (i.e. non-evoked) activity increased by 104% ($p<0.05$) compared to sham-

controls (Figure 5.4C) or the contralateral hind limb of SNL animals (data not shown). Response latencies were unaffected by SNL at all stimulus intensities (data not shown).

To determine if the development of mechanical allodynia (i.e. changes in PWT, firing threshold and response magnitude of the AFRR and CFRR) is related to the SNL-induced activation of spinal NFκB, rats were given PDTC (100μg i.t.), vehicle (50% DMSO) or no drug immediately after SNL or sham surgery. Three days later, the PWT in the left hind paw of SNL+PDTC treated animals was not significantly different from sham-controls (≥15 g; Figure 5.5A). In contrast, the PWT was <4 g in all SNL rats receiving either vehicle or no drug treatment (Figure 5.5A). Likewise, PDTC completely prevented the SNL-induced decrease in the firing threshold of the AFRR (Figure 5.5B) and partially prevented the decrease in the CFRR (Figure 5.5C) compared to sham-controls. The average firing thresholds were 88% and 65% of control for the AFRR and CFRR, respectively (Figure 5.5B&C). Neither of the control groups (i.e. vehicle or no drug treatment) had any effect on the SNL-induced decreases in activation threshold (Figure 5.5B&C). PDTC also prevented the SNL-induced increase in spike integration of the AFRR and CFRR compared to the SNL + vehicle or SNL+ no drug groups (Figure 5.5D). The effect of i.t. PDTC on spinal hyperexcitability was dose-dependent (e.g. IC_{50}=25.4 μg for the response magnitude of AFRR; IC_{50}=41.5 μg for the CFRR). The response magnitudes of

the AFRR and CFRR in the SNL + PDTC group were unchanged (p>0.05) from sham-controls (Figure 5.5D).

If the SNL-induced expression of spinal COX-2 (Figure 5.2) is also initiated by NFκB activation, then animals treated with PDTC (100 μg i.t.) immediately after SNL should exhibit little or no change in COX-2 protein three days later (i.e. the time of maximum COX-2 expression in this nerve injury model). PDTC, which blocked the activation of NFκB in these animals (Figure 5.6A), not only prevented the increase in spinal COX-2 (Figure 5.6C), but decreased it below that of sham-controls. While this was more prominent in the ipsilateral cord, a bilateral effect was apparent (Figure 5.6B&C). Neither PDTC nor vehicle had any effect in sham controls (Figure 5.6A-C).

To investigate the possibility that spinal PG, generated immediately after SNL, might promote the activation of NFκB and, in turn, the induction of spinal COX-2, spinal hyperexcitability and allodynia, S(+)-IBU, R(-)-IBU or DMSO were given immediately after SNL or sham surgery. S(+)-IBU (100 μg i.t.) not only blocked the SNL-induced increase in NFκB activation (Figure 5.7A) and COX-2 protein (Figure 5.7C) three days later, but reduced COX-1 and COX-2 expression (p<0.05) below that in sham-controls (Figure 5.7B&C). Likewise, S(+)-IBU prevented the concurrent SNL-induced changes in PWT (Figure 5.8A), firing threshold (Figure 5.8B&C) and response magnitude (Figure 5.8D) of the AFRR and CFRR. R(-)-IBU and DMSO had no effect on the SNL-induced expression of NFκB (Figure 7A) and COX-2 (Figure 5.7B) or the development of spinal

hyperexcitability and allodynia (Figure 5.8A-D). The effect of i.t. S(+)-IBU on spinal hyperexcitability was dose-dependent (e.g. IC_{50}=36.3 μg for the response magnitude of AFRR; IC_{50}=50.8 μg for the CFRR). S(+)-IBU, R(-)-IBU and DMSO were without effect in sham controls or the contralateral DH of SNL rats (Figure 5.7&5.8).

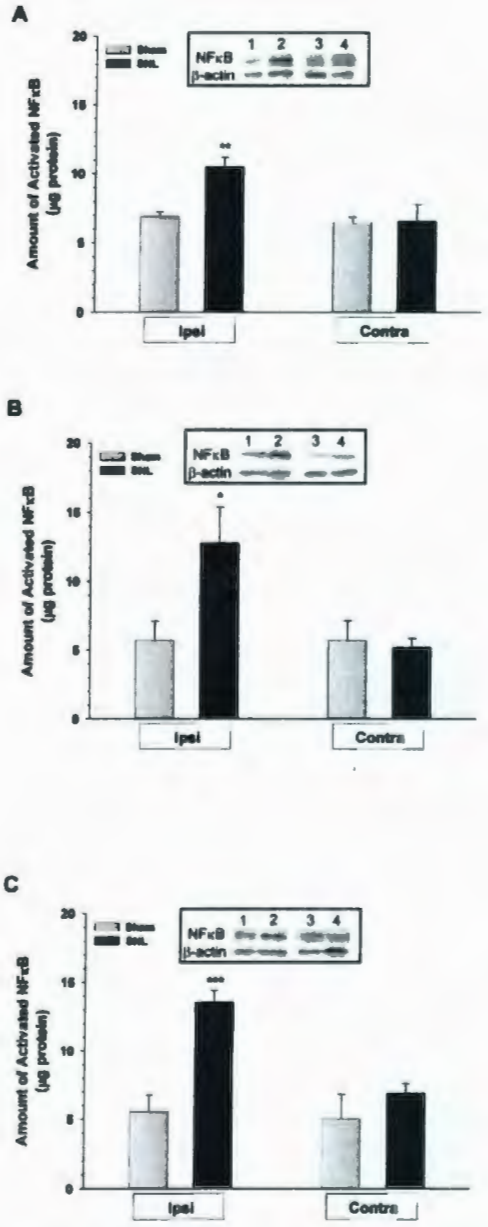

Figure 5.1. The amount of activated NFκB in the ipsilateral and contralateral L-DH twelve hours (panel A), one day (panel B) and three days (panel C) after SNL or sham surgery. Data were normalized using total protein content, corrected for non-specific immunoreactivity and expressed as μg of protein (see Methods). Each bar represents the mean ± SEM of three to five animals and asterisks indicate a significant difference from sham-operated animals (*p<0.05; **p<0.01; ***p<0.001). Representative immunoblots of activated NFκB and β-actin in the ipsilateral (ipsi) or contralteral (contra) L-DH are shown in the inserts above each panel. Lane 1: sham (ipsi); Lane 2: SNL (ipsi); Lane 3: sham (contra); Lane 4: SNL (contra).

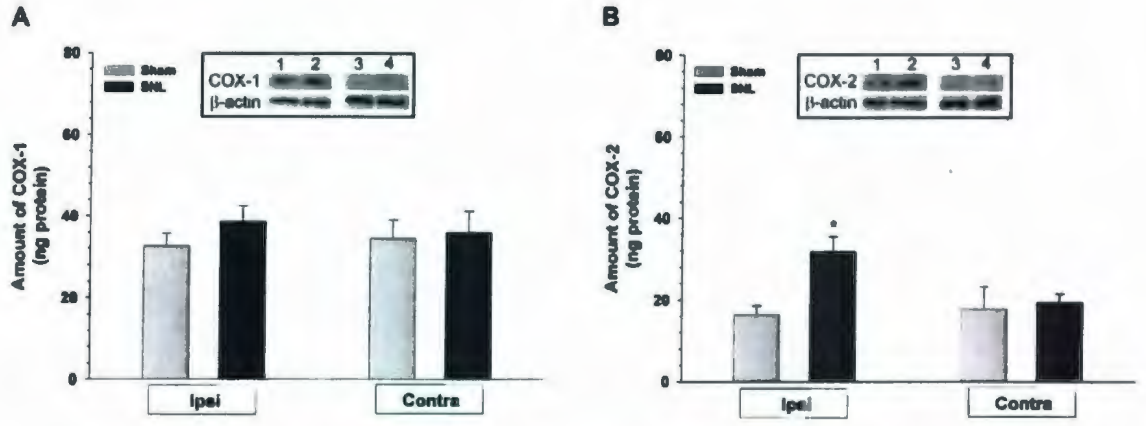

Figure 5.2. The amount of COX-1 (panel A) and COX-2 (panel B) in the ipsilateral and contralateral L-DH three days after SNL or sham surgery. Data were normalized using total protein content, corrected for non-specific immunoreactivity and expressed as ng of protein (see Methods). Each bar represents the mean ± SEM of three to five animals and asterisks indicate a significant difference from sham-controls (*p<0.05). Representative immunoblots of COX-1 and COX-2 in the ipsilateral (ipsi) or contralteral (contra) L-DH are shown in the inserts above each panel. Lane 1: sham (ipsi); Lane 2: SNL (ipsi); Lane 3: sham (contra); Lane 4: SNL (contra).

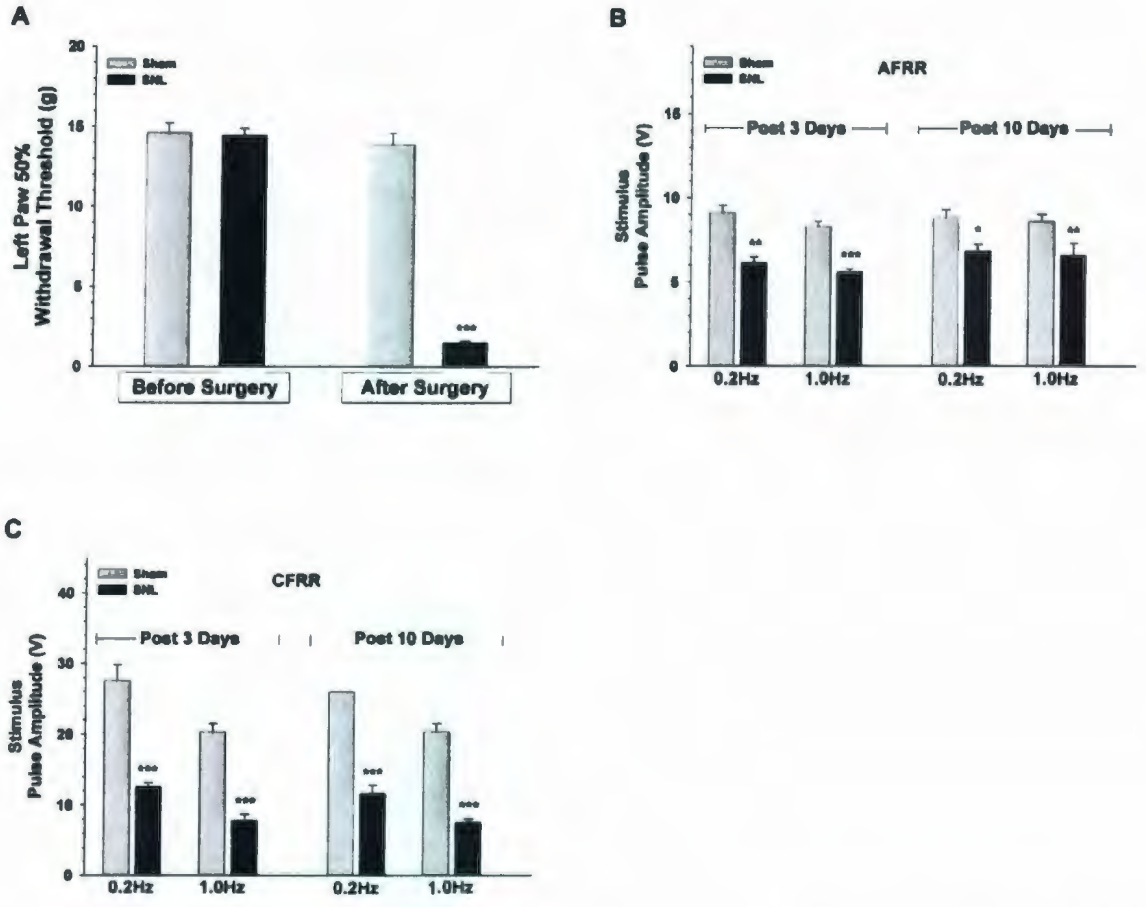

Figure 5.3. The effect of SNL on PWT (panel A) and activation threshold (V) of the AFRR (panel B) and CFRR (panel C). While PWT was assessed three days after surgery, evoked responses in the ipsilateral biceps femoris flexor reflex were recorded three and ten days after surgery. Each bar represents the mean ± SEM of five to seven animals and asterisks indicate a significant difference from the corresponding sham-control (*p<0.05; **p<0.01; ***p<0.001). Note the differences in pulse amplitude (V) between panels B and C.

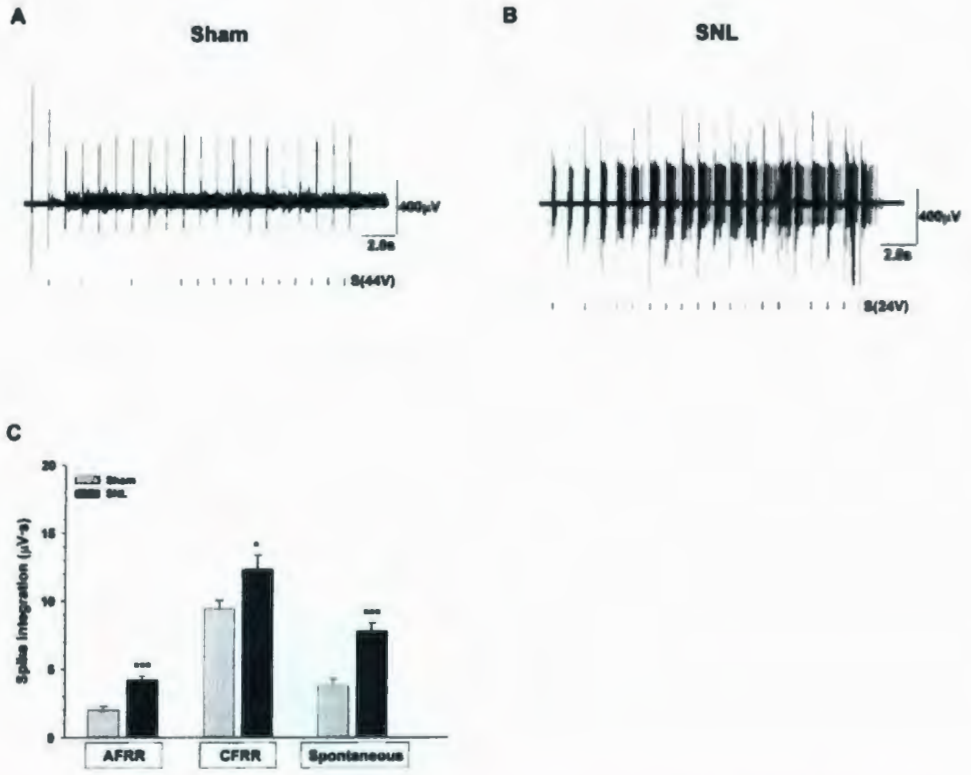

Figure 5.4. Electromyographic (EMG) recordings and spike integration of the ipsilateral biceps femoris flexor reflex three days after sham surgery or SNL. EMG responses were evoked by high intensity, supra-threshold stimulation (1.0 Hz). A pulse stimulus indicator bar is shown under each tracing (panels A&B). Note the differences in the stimulation (S) and response voltages between SNL (24 V & 400 μV) and sham-controls (44 V & 400 μV). Spike integration analysis of the AFRR and CFRR and spontaneous (i.e. non-evoked) activity three days after SNL or sham surgery is summarized in panel C. Each bar represents the mean ± SEM of five to seven animals and asterisks indicate a significant difference from the corresponding sham-control (*p<0.05; ***p<0.001).

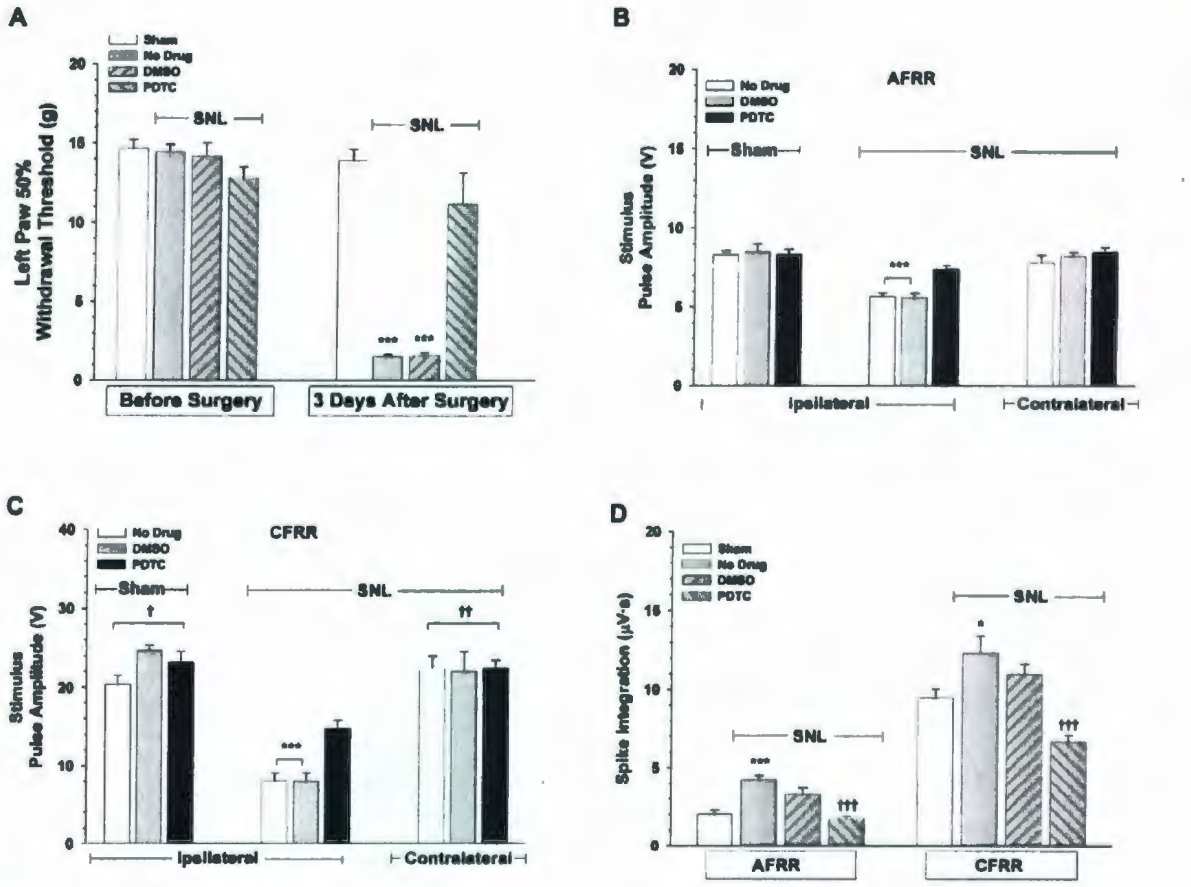

Figure 5.5. The effect of PDTC, vehicle (50% DMSO) or no drug treatment on PWT (panel A), the activation threshold of the AFRR (panel B) and CFRR (panel C), and spike integration (panel D) three days after SNL or sham surgery. Note the scale difference in panel C. PDTC (100 μg) or DMSO was injected i.t. immediately after surgery. Data are presented as the mean ± SEM of five to seven animals and asterisks in all panels indicate a significant difference from corresponding sham-controls (*p<0.05; ***p<0.001). Daggers in panels C indicate a significant difference from the SNL + PDTC group (††p<0.01) and those in panel D indicate a significant difference from the SNL + no drug and SNL + DMSO groups (†p<0.05; ††p<0.01; †††p<0.001).

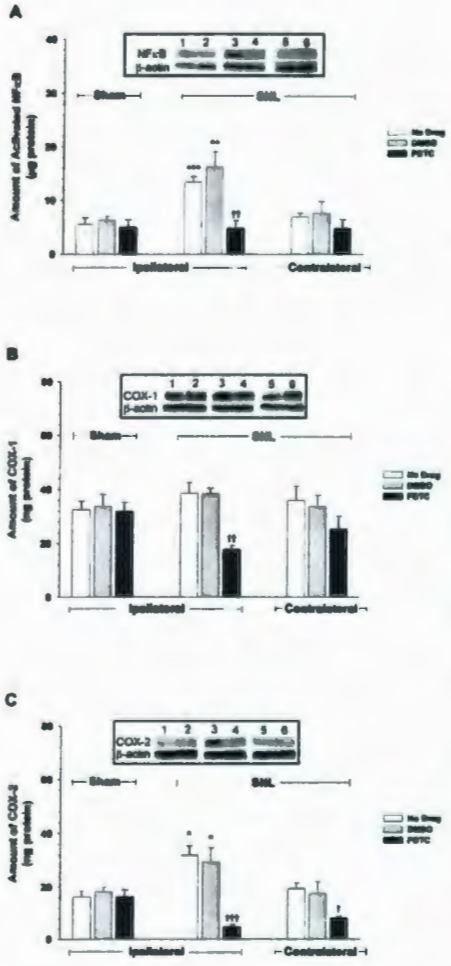

Figure 5.6. The effect of PDTC, vehicle (50% DMSO), or no drug treatment on the amount of activated NFκB (panel A), COX-1 (panel B), and COX-2 (panel C) in the ipsilateral and contralateral L-DH, three days after SNL or sham surgery. PDTC (100 μg) or DMSO was injected i.t. immediately after surgery. Data were normalized using total protein content, corrected for non-specific immunoreactivity and expressed as the amount of protein (see Methods). Note the scale difference in panel A. Each bar represents the mean ± SEM of three to five animals. Asterisks indicate a significant difference from the corresponding sham-control (*p<0.05; **p<0.01; ***p<0.001) and daggers indicate a significant difference from SNL animals receiving either no drug or DMSO (†p<0.05; ††p<0.01; †††p<0.001). Representative immunoblots of activated NFκB, COX-1, COX-2, and β-actin in the ipsilateral (ipsi) or contralteral (contra) L-DH are shown in the inserts above each panel. Lane 1: sham + no drug (ipsi); Lane 2: sham + PDTC (ipsi); Lane 3: SNL + DMSO (ipsi); Lane 4: SNL + PDTC (ipsi); Lane 5: SNL + DMSO (contra); Lane 6: SNL + PDTC (contra).

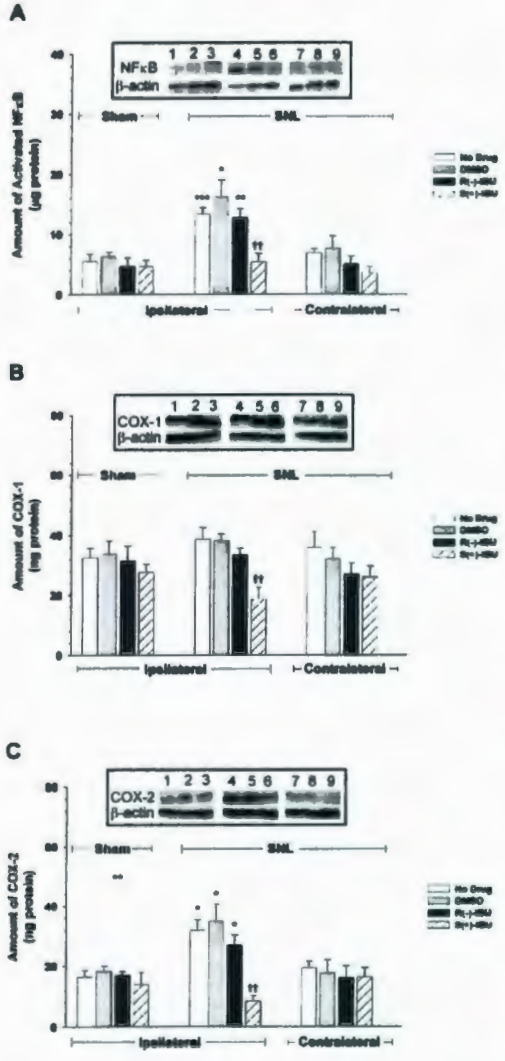

Figure 5.7. The effect of i.t. S(+)-IBU (100 μg), R(-)-IBU (100μg), DMSO or no drug treatment on the amount of activated NFκB (panel A), COX-1 (panel B) and COX-2 (panel C) in the ipsilateral and contralateral L-DH three days after SNL or sham surgery. All drugs were given immediately after surgery. Data were normalized using total protein content, corrected for non-specific immunoreactivity and expressed as the amount of protein (see Methods). Note the scale difference in panel A. Each bar represents the mean ± SEM of three to five animals and asterisks indicate a significant difference from all other treatment groups (**p<0.01). Representative immunoblots of activated NFκB, COX-1, COX-2 and β-actin in the ipsilateral (ipsi) or contralteral (contra) L-DH are shown in the inserts above each panel. Lane 1: sham + no drug (ipsi); Lane 2: sham + R(-)-IBU (ipsi); Lane 3: sham + S(+)-IBU (ispi); Lane 4: SNL + DMSO (ipsi); Lane 5: SNL + R(-)-IBU (ipsi); Lane 6: SNL + S(+)-IBU (ipsi); Lane 7: SNL + DMSO (contra); Lane 8: SNL + R(-)-IBU (contra); Lane 9: SNL + S(+)-IBU (contra).

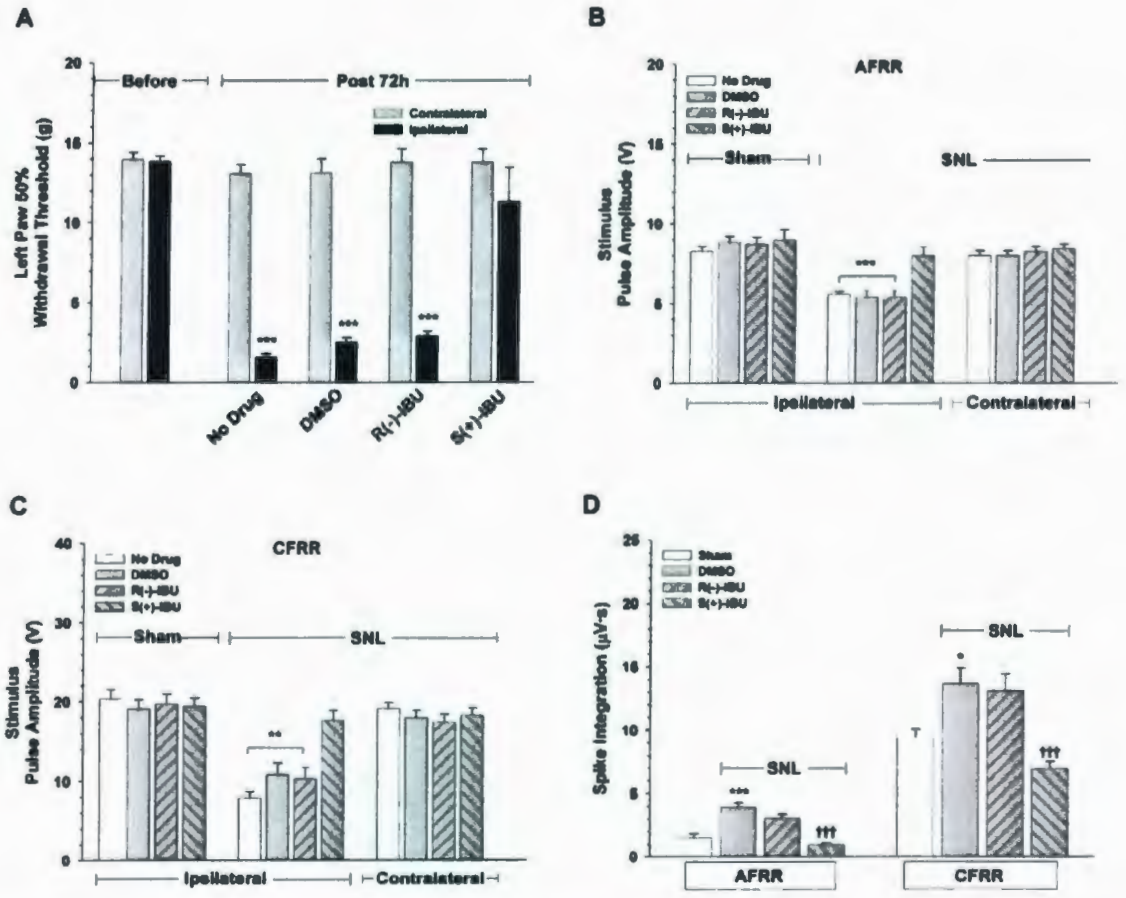

Figure 5.8. The effect of i.t. S(+)-IBU, R(-)-IBU, DMSO or no drug treatment on PWT (panel A), the activation threshold of the AFRR (panel B) and CFRR (panel C), and spike integration (panel D), three days after SNL or sham surgery. Note the scale difference in panel C. S(+)-IBU (100 μg), R(-)-IBU (100 μg) or DMSO was injected i.t. immediately after surgery. Data are presented as the mean ± SEM of five to seven animals and asterisks in all panels indicate a significant difference from the corresponding sham-controls (*p<0.05; ***p<0.001). Daggers in panel D indicate significant differences from the SNL + R(-)-ibuprofen and the SNL + DMSO groups (†††p<0.001).

5.4 DISCUSSION

NFκB, measured in nuclear extracts from the left and right dorsal and ventral quadrants was significantly up-regulated in the ipsilateral, but not contralateral L-DH twelve hours after SNL. This effect preceded the induction of spinal COX-2, the development of spinal hyperexcitability, and the reduction in PWT in the affected hind paw. This time-course of NFκB activation is comparable to that reported in the peripheral nervous system following partial (Ma and Bisby, 1998) or complete sciatic nerve transection (Pollock et al., 2005), or in the spinal cord of animals with spinal contusion injury (Bethea et al., 1998). In a recent study using SNL, the overall amount and nuclear localization of NFκB in the ipsilateral DRG were shown to exceed that in the contralateral DRG (Wu et al., 2006). However, changes in the spinal cord were not investigated. The present study is the first report of nerve injury-induced changes in NFκB activation in the affected spinal cord.

All SNL animals displayed significant decreases in PWT (<4 g), increased spontaneous activity in the ipsilateral biceps femoris reflex, and reduced activation thresholds and corresponding increases in the response magnitudes of the AFRR and CFRR to transcutaneous electrical nerve stimulation. These changes, indicative of spinal hyperexcitability, are comparable to those reported in other nerve (Meyerson et al., 1995; Colvin et al., 1996; Okamoto et al., 2001; Kohno et al., 2003) or spinal cord (Bennett et al., 2004) injury models one or more weeks after nerve injury. A comparison of the present results to those

determined one day after SNL (O'Rielly and Loomis, 2007) revealed an exaggeration of the AFRR and CFRR on day three. For example, the response magnitude of the AFRR was increased nearly two-fold compared to day one (O'Rielly and Loomis, 2007), matching the near two-fold increase in COX-2 expression in the affected DH (O'Rielly and Loomis, 2006). These results indicate an early progression of the mechanisms underlying spinal hyperexcitability in the SNL model; mechanisms preceded by the activation of spinal NFκB and previously shown to involve spinal PG derived primarily from spinal COX-2 (O'Rielly and Loomis, 2007; Hefferan et al., 2003a, b).

To determine if the activation of NFκB is critical to the development of spinal hyperexcitability and mechanical allodynia, and not just a coincidental event, i.t. PDTC was given immediately after SNL. PDTC is a highly effective inhibitor of NFκB, disrupting its translocation from the cytoplasm into the nucleus, the binding of NFκB to specific response elements on the DNA, and the subsequent transcription of relevant effector genes (Karin & Ben-Neriah, 2000; Tergaonkar, 2006). In the present study, PDTC not only blocked the activation of NFκB, but prevented the induction of spinal COX-2, the increase in spinal hyperexcitability (i.e. AFRR and CFRR), and the reduction in PWT in the ipsilateral hind paw. The effect of PDTC on COX-2 protein was evident in both the ipsilateral and contralateral lumbar cord, resembling its effect on COX-2 mRNA (Lee et al., 2004), and corroborating, *in vivo*, the modulatory role of NFκB activation on the COX-2 gene in the nerve-injured state. The effect of PDTC on

A- and C-fiber mediated responses in the present study extends the results with PDTC in other nerve injury models in which only changes in PWT were determined (Laughlin et al., 2000; Tegeder et al., 2004). The present results demonstrate an important functional relationship between the early activation of spinal NFκB and the subsequent exaggeration of A- and to a lesser extent C-fiber mediated responses in the affected hind limb. The changes in the AFRR and their sensitivity to PDTC are especially relevant given the predominant role of A-fibers in mechanical allodynia (Zimmerman, 2001).

PG promote neuronal depolarization by modulating the kinetics of Na^+ and K^+ channels, and by increasing intracellular Ca^{2+} levels (Vasko, 1995; Baba et al., 2001; Chen & Bazan, 2005; Sugimoto & Narumiya, 2007). Their synthesis by constitutive and inducible COX at the onset of nerve injury could promote the initial activation of NFκB and its downstream transcriptional effects. S(+)-IBU, given i.t. immediately after SNL, blocked the activation of NFκB, the induction of spinal COX-2 and the development of spinal hyperexcitability and allodynia three days later, mimicking the effect of PDTC. In contrast, identical treatment with the inactive R(-)-enantiomer was without effect. These results are consistent with a locus of action of i.t. IBU on spinal COX (Hefferan et al, 2003a, b), and indicate that spinal PG synthesis begins at the onset of nerve injury. In turn, PG facilitate the activation and transcriptional effect of NFκB in the affected spinal cord. This is the first report of COX inhibitors attenuating NFκB activation in a nerve-injury model and could explain how i.t. S(+)-IBU, given up to 2 h after SNL, prevented

the development of mechanical allodynia for as long as twenty-one days (Hefferan et al., 2003a, b). A similar effect on NFκB activation has been reported *in vitro* with flurbiprofen (Tegeder et al., 2001) and rofecoxib (Niederberger et al., 2003) in cultured macrophages. The observed decrease in COX-2 protein below sham-controls is not due to a decrease in binding of COX-2 to the antibody in the presence of ibuprofen. Rather, it likely reflects increased enzyme catabolism (i.e. turnover) in nerve-injured and/or drug-treated animals. The COX inhibitor, paracoxib accelerated the degradation of COX-2 by the ubiquitin proteosome system in cultured HeLa cells (Neuss et al., 2007).

Notwithstanding the clear evidence for an early role of spinal PG in this and other nerve injury models, the activation of NFκB following nerve injury is bound to be multi-factorial. For example, NO promotes cellular excitability by soluble guanylate cyclase (sGC)-dependent and sGC-independent mechanisms, including the enhanced release of calcitonin gene-related peptide and PGE_2 (Holzer et al., 1995). These mediators (especially PGE_2) exert a positive feedback effect on NOS to drive further NO production (Salvemini et al., 1993; Tsai et al., 1994; Aley et al., 1998; Sakai et al., 1998; Matsumura et al., 2005; Dudhgaonkar et al., 2006). In addition, NFκB is known to modulate the transcription of iNOS (Lowenstein et al., 1993; Mabuchi et al., 2003). Preliminary work in our laboratory has shown a PDTC-sensitive up-regulation of iNOS in the spinal cord three days after SNL (unpublished results). The concurrent up-regulation of NOS and COX following nerve injury, and a corresponding increase

in NO- and PG-signaling could have an additive or even synergistic effect on the activation of NFκB and, based on the results of the present study, the development of spinal hyperexcitability and allodynia. This is the focus of a later chapter.

In summary, blocking the activation of spinal NFκB, either directly with PDTC or indirectly with S(+)-IBU, prevented the SNL-induced expression of spinal COX-2 and the development of spinal hyperexcitability and allodynia three days later. These results demonstrate that NFκB is not only activated by SNL, but facilitated by PG (and undoubtedly other mediators) generated in the affected spinal cord from the onset of nerve injury. Interrupting critical signaling events early after nerve injury could reduce the chronic and debilitating features of NP, and would be more effective than intervening weeks to months after nerve injury. While the potential of this strategy has been demonstrated in preliminary studies of experimental nerve injury (Araujo et al., 2003; Takahashi et al., 2005; Marabese et al., 2007; O'Rielly and Loomis, 2007), the results of the present study provide a scientific basis for the early disruption of spinal PG signaling and its relationship to NFκB.

6.0 THE SPINAL INTERACTION BETWEEN NFKB AND NITRIC OXIDE IN THE DEVELOPMENT OF HYPEREXCITABILITY AND TACTILE ALLODYNIA FOLLOWING L5/L6 SPINAL NERVE LIGATION

6.1 INTRODUCTION

Tight ligation of the L5/L6 spinal roots triggers the activation of NFκB and the up-regulation of iNOS and nNOS, respectively in the affected spinal cord (Mabuchi et al., 2003; O'Rielly and Loomis, 2006, 2008). This is consistent with the known increase in NFκB in the peripheral and central nervous system following experimental nerve injury (Bethea et al., 1998; Sakaue et al., 2001; Pollock et al., 2005; O'Rielly and Loomis, 2008), the direct modulation of iNOS transcription by NFκB (Lowenstein et al., 1993; Mabuchi et al., 2003), and the positive correlation between the NFκB activation and nNOS expression (Miscusi et al., 2006).

The early activation of NFκB, the up-regulation of NOS protein and activity (Choi et al., 1996; Gordh et al., 1998; Mabuchi et al., 2003), and the enhancement of NO signaling in the affected spinal cord may be critical steps in the development of allodynia following SNL. This is further supported by reports that blockers of NFκB significantly attenuated mechanical allodynia and thermal hyperalgesia in several nerve injury models (Laughlin et al., 2000; La Rosa et al., 2004; Jimenez-Garza et al., 2005; Ebersberger et al., 2006). In this chapter, we

tested this hypothesis by investigating the relationship between these molecular, cellular and functional changes using a range of experimental techniques.

Unlike other neuromodulators, NO is a diffusible messenger whose excitatory activity is neither mediated by membrane-bound cell surface receptors nor limited by their cellular distribution (Millan, 1999; Petersen-Zeitz & Basbaum, 1999). Given the marked effect of NO on cellular excitability and the fact that NO is not stored but must be synthesized *de novo*, it is possible that spinal NO, generated by constitutive NOS isozymes at the onset of SNL, promotes the activation of NFκB. The subsequent up-regulation of NOS isozymes and corresponding increase in NO signaling could sustain, at least in part, the activation of NFκB through a positive feedback effect (i.e. vicious cycle). The hypothesis that spinal NO affects the expression of enzymes responsible for its synthesis/signaling in the acute nerve-injured state was therefore investigated.

To test these hypotheses, we investigated the effect of i.t. PDTC, a selective inhibitor of NFκB, on the expression of nNOS and iNOS protein in the lumbar dorsal horn three days after SNL. The contribution of NO signaling to the development of spinal hyperexcitability and allodynia was investigated using molecular (i.e. cGMP and PKG protein) and pharmacological approaches (i.e. L-NAME). The electrophysiological properties of the AFRR and CFRR in the biceps femoris flexor reflex were used as measures of spinal hyperexcitability elicited by A- and C-fiber input, respectively. Allodynia was confirmed and quantified using von Frey filaments.

6.2 MATERIALS AND METHODS

Studies were approved by, and experiments conducted in accordance with the guidelines of the Institutional Animal Care Committee of Memorial University of Newfoundland (St. John's, NL, CA).

Animals. Male Sprague-Dawley rats (130 to 150 g) were obtained from the Vivarium of Memorial University of Newfoundland (St. John's, NL, CA) and housed in standard cages with woodchip bedding. Animals had free access to food and water. A 12 h light-dark cycle (lights on at 0700 h) was used throughout.

Intrathecal Catheterization. Intrathecal catheters (6.5 cm-length terminating near the lumbar enlargement) were implanted according to the method of Yaksh and Rudy (1976) as previously modified (Hefferan et al., 2003a, b). Catheters were 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. Intrathecal catheters (inner diameter, i.d. 240 μm, outer diameter, o.d. 290 μm) were constructed from triple lumen PE-5 tubing (Spectranetics, Colorado Springs, CO, USA) using the modified method of Marsala et al (1995). Rats with normal motor,

grooming and feeding behavior were housed separately and allowed to recover for two days before SNL or sham surgery.

Neuropathy. Neuropathy was induced using the method of Kim and Chung (1992) as previously described (Hefferan et al., 2003a, b; O'Rielly and Loomis, 2006-2008). Briefly, rats were anesthetized with halothane and the left L4 and L5 spinal nerves were isolated and separated. The L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham controls, the L5 and L6 spinal nerves were isolated, but not ligated. Animals were allowed to recover for three days before experimentation except for the NOS expression experiments which were investigated one and three days after surgery.

Paw Withdrawal Threshold. Mechanical allodynia was quantified by determining the PWT using von Frey filaments (Chaplan et al., 1994). Briefly, rats were placed in a plastic cage with a wire-mesh bottom allowing access to the plantar surface of the left hindpaw. Following a 20-min acclimatization period, a control threshold was determined. Allodynia was defined as a PWT of ≤4 g (Chaplan et al. 1994; Hefferan et al., 2003a, b).

Electrophysiological Recordings. Rats were anesthetized with halothane and cannulae were placed in the trachea, left carotid artery and right external jugular vein. Halothane anesthesia was then replaced by sodium

thiobutabarbitone (1 mg•kg^{-1} i.v.; Inactin, Sigma-Aldrich, Oakville, ON, CA). The depth of anesthesia was assessed by testing for hind limb withdrawal and corneal reflexes which had to be absent. Blood pressure was continuously monitored via a left carotid artery catheter and the mean arterial pressure maintained between 100 to 120 mmHg by additional anesthetic as required. Systolic blood pressure did not fall below 100 mmHg throughout the experiment. In the event that the blood pressure dropped and consistently remained below 100mmHg, the experiment was stopped and the animal euthanized. Core temperature was maintained close to 37 °C using a homeothermic blanket system. The animal preparation was allowed to stabilize for at least 30 minutes prior to data collection.

Spinal flexor reflexes were evoked by subcutaneous electrical stimulation applied to the first toe of the hind paw. Needle location was based on the innervation pattern of the sural nerve (Wiesenfeld-Hallin, 1988) and square-wave pulses (0.2, 0.6, 1.0 Hz) of 1 ms duration were used. Stimulation at each frequency was repeated three times to ensure stability and extracellular electromyographic responses were recorded from the biceps femoris muscle using a pair of tungsten needle electrodes. Intervals of three to five minutes were introduced between successive stimulus trains to prevent a conditioning effect by the preceding stimulus. A low intensity supra-threshold stimulus refers to a voltage twice the A-fiber mediated reflex response (AFRR) activation threshold (five pulses at 0.2 Hz). A high intensity supra-threshold stimulus refers to a

voltage twice the C-fiber mediated reflex response (CFRR) activation threshold (twenty pulses at 1.0 Hz).

The AFRR and CFRR were distinguished on the basis of activation threshold and response latency. Electrical stimulation sufficient to activate the CFRR resulted in two distinct components; an early and late phase separated by a quiescent period of variable duration. The AFRR and CFRR were classified as those appearing <100 ms after the stimulus artifact, and >100 ms up to a maximum of 600 ms, respectively. These criteria were confirmed by examining the effect of i.t. morphine (100 μg) and naloxone (100 μg) on the AFRR and CFRR of the biceps femoris reflex in naive animals (O'Rielly and Loomis, 2007). Based on the measured length of the sural nerve in rats weighing 130 to 150 g (i.e. 12 cm), afferent fibers mediating the late phase (>100 to 600 ms) had an average conduction velocity of 1.2 $m \cdot s^{-1}$; a value within the accepted range for C-fibers (Lynn and Carpenter, 1982). The AFRR and CFRR intervals used in the present experiments are in agreement with previous reports on the rat biceps femoris reflex (Lynn and Carpenter, 1982; Herrero and Cervero, 1996a, b). No attempt was made to separate the A-fiber subtypes primarily because of altered conductivity of injured and uninjured A-fibers (Laird and Bennett, 1993).

The threshold voltages eliciting the AFRR and CFRR were investigated using accepted criteria for A- and C-fibers and validated by their differential sensitivity to the inhibitory effects of i.t. morphine (O'Rielly and Loomis, 2007). To determine the AFRR activation threshold, stimulus trains of five pulses (0.2 to 1.0

Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least three of the five stimulus pulses produced a spike (i.e. amplitude >20 μV). To determine the CFRR activation threshold, stimulus trains of twenty pulses (0.2 to 1.0 Hz) were applied at increasing voltages until a positive response was obtained. A positive response was deemed to have occurred when at least ten of the twenty stimulus pulses produced a spike (i.e. amplitude >20 μV). For the purposes of this study, spinal hyperexcitability is defined as a significant decrease ($p<0.05$) in the activation threshold and an increase in the response magnitude of the AFRR and/or CFRR following SNL.

Data were collected, stored and analyzed using a PowerLab data acquisition system (ADInstruments, Inc., Colorado Springs, CO, USA). Off-line data analysis was initially performed using the data acquisition software provided by the manufacturer (Chart 5.0). The data were further analyzed using a customized computer program developed in collaboration with the Department of Computer Science, Memorial University of Newfoundland (St. John's, NL, CA).

Western Blotting. Animals were deeply anesthetized with urethane (1.2 mg/kg i.p.) and perfused intracardially with ice cold saline (0.9% NaCl). The spinal cord was extracted hydraulically (de Sousa and Horrocks, 1979), immediately frozen in 2-methylbutane (Sigma-Aldrich, Oakville, ON, CA), and stored at -80 °C. The lumbar region (L2 to L6) of the spinal cord was isolated,

removed and subsequently divided into the left and right, ventral and dorsal quadrants. Spinal cord was homogenized in ice cold lysis buffer (1% Nonidet-P40, 10% glycerol in TBS plus a protease inhibitor cocktail tablet - Roche Diagnostics, Laval, PQ, CA, 1 mM sodium vanadate, 1 mM sodium fluoride, and 0.025% SDS), and centrifuged at 10,000 rpm for 5 minutes (4 °C). Preparation of nuclear extracts was performed using special homogenization buffers and additional centrifugation as previously described (Bethea et al., 1998). The Coomassie Plus Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA) was used to determine the protein quantity of all samples. Samples, diluted to achieve equal protein concentrations (30 µg), were separated by electrophoresis on 8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and incubated for 1.5 h in Tris buffer (Tris 25 mM, glycine 192 mM, 200 ml methanol, pH 8). Pre-stained protein markers were used for molecular weight determination. Blots were initially stained with Ponceau Red, and later probed with an antibody directed against the cytosolic protein, actin (1:1000; Sigma-Aldrich, Oakville, ONT, CA) to assess the equivalency of protein loading. Blots were subsequently probed with the following primary antibodies: polyclonal rabbit anti-nNOS (1:1000), polyclonal rabbit anti-iNOS (1:1000), polyclonal anti-cGMP (1:2000), and polyclonal anti-PKG (1:500), and polyclonal rabbit anti-NFκB-p65 (1:2000). Anti-nNOS and polyclonal rabbit anti-iNOS were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-NFκB-p65 was purchased from Chemicon International, Inc. (Temecula, CA, USA) and anti-cGMP and anti-PKG

were purchased from Upstate Cell Signaling Solutions (Temecula, CA, USA) and Stressgen Bioreagents (Ann Arbor, MI, USA), respectively. Standards for nNOS, iNOS and NFκB (non-stimulated A431 cell lysate; Upstate Cell Signaling Solutions, Charlottesville, VA, USA) were preabsorbed with their corresponding blocking peptide to determine the antibody specificity. With the exception of anti-cGMP or anti-PKG, all western blots were corrected for non-specific binding by subtracting the optical density in the presence of the corresponding blocking peptide from that of each test sample. In the case of anti-cGMP or anti-PKG where commercially available blocking peptides were unavailable, non-specific binding was assessed using samples incubated without the primary antibody. The membranes were incubated overnight at 4 °C with the primary antibodies, and diluted in Tris buffer (containing 3% milk powder and 0.05% Tween-20). Protein bands were treated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Chemicon International Inc., Temecula, CA, USA) for 1 h at room temperature, washed for 30 minutes in Tris buffer, visualized using enhanced chemiluminescence (PerkinElmer Life Sciences Boston, MA, USA), and exposed to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC, USA).

Drugs. L-NAME, and D-NAME were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). These were appropriate choices given L-NAME's comparable and stereo-specific inhibition of nNOS and iNOS;

isozymes, and the value of the inactive D-isomer as a pharmacological control. PDTC was purchased from Sigma-Aldrich (Oakville, ON, CA). PDTC was dissolved in 100% DMSO and diluted with normal saline at the time of injection to yield a final DMSO concentration of 50%, whereas L-NAME and D-NAME were dissolved in saline (0.9% NaCl). All equipment was sterilized with 70% alcohol and thoroughly rinsed with 0.9% sterile saline before injection. Intrathecal drugs were injected into anesthetized rats using a hand-held microsyringe. Drugs were delivered in a volume of 5 µl followed by 5 µl of sterile saline. The i.t. catheter was immediately resealed with a stainless steel plug. The position of the catheter tip was verified visually after sacrifice in randomly selected animals.

Data Analysis. Electromyographic recordings in sham operated and SNL animals were compared using the mean responses elicited by three stimulus trains of 0.2, 0.6 and 1.0 Hz. Data were collected (4000 samples/sec) and high-pass digital filters (cut-off frequency 60 Hz) were used to remove noise and movement artifacts. Spontaneous activity was defined as the average spike count integrated over a 60 s period prior to experimentation. In all cases, the magnitude of the AFRR and CFRR was calculated by integrating the spike count over the stimulation period (i.e. area-under-the-curve analysis). Spikes were integrated from 0.5 to 99.9 ms and 100 to 600 ms for the AFRR and CFRR, respectively. Area-under-the-curve was calculated using trapezoidal integration. Western blots were analyzed by optical density using ImageQuant software

(Amersham Biosciences Corp., Piscataway, NJ, USA). Expression data are presented as micrograms of protein relative to an NFκB protein standard (10 μg), and as nanograms of protein relative to nNOS/iNOS (50 ng) standards. Expression data were corrected for background optical density except for cGMP and PKG (see Western Blotting above), and all expression data were normalized using the cytosolic protein, β-actin. The absence of available standards for cGMP and PKG meant that optical density could not be converted to the amount of protein and therefore are presented as % of sham-control. Dose-response analysis was performed using methods from Tallarida and Murray (1987). Comparisons within each treatment group were performed using one-way, repeated measures ANOVA, followed by the Newman-Keuls test. Comparisons across all drug- and vehicle-treated groups at each time point were determined using one-way, completely randomized ANOVA, followed by the Newman-Keuls test (SigmaStat 2.0; Systat Software Inc., San Jose, CA, USA).

6.3 RESULTS

Expression of nNOS and iNOS protein was significantly increased in the ipsilateral L-DH compared to sham-controls or the contralateral DH of SNL animals, one and three days after surgery (Figure 6.1). The amount of nNOS protein (Figure 6.1A) exceeded that of iNOS (Figure 6.1B) by 39 ± 9% in the ipsilateral L-DH one day after SNL. By day three, this pattern had changed with the amount of iNOS protein (Figure 6.1D) nearly matching that of nNOS (Figure

6.1C). This reflected a 41 ± 7% increase ($p<0.05$) in the amount of iNOS protein (Figure 6.1D) compared to day 1 (Figure 6.1B), and the absence of any significant increase in nNOS protein (Figure 6.1C vs 6.1A). There was a corresponding increase ($p<0.05$) in the expression of cGMP and PKG protein in the ipsilateral L-DH of SNL animals compared to sham-controls (Figure 6.1E). SNL had no effect ($p>0.05$) on cGMP and PKG in the contralateral L-DH (Figure 6.1E).

Three days after SNL, there was also a significant increase in the amount of activated NFκB in the ipsilateral L-DH compared to sham-controls or the contralateral DH of SNL animals (Figure 6.2A). PDTC (100 μg i.t.), given immediately after SNL, prevented the increase in activated NFκB (Figure 6.2A), nNOS (Figure 6.2B) and iNOS (Figure 6.2C). These amounts were unchanged from their respective sham-control or the contralateral side of SNL animals (Figure 6.2). In contrast, vehicle had no effect on the SNL-induced increase in activated NFκB, nNOS or iNOS protein three days after injury similar to that observed in ligated animals receiving no drug treatment (Figure 6.2). For ease of comparison, the quantitative data shown in Figure 6.1C&D (i.e. SNL without drug treatment) are replicated in Figure 6.2B&C, respectively.

In the absence of drug treatment, all SNL animals were clearly allodynic as indicated by the decrease in PWT from a baseline of 15 g to ≤4 g (Figure 6.3A), and the corresponding reduction in firing threshold of the AFRR (Figure 6.3B) and CFRR (Figure 6.3C), ipsilateral to SNL. Moreover, the affected hind

paw was kept in an elevated and cupped position to minimize contact with the cage floor. To determine if the SNL-induced increases in NOS protein are relevant to allodynia at three days, i.t. L-NAME (100 µg), D-NAME (100 µg) or vehicle were injected three days after SNL (i.e. at the onset of maximum allodynia). L-NAME significantly attenuated the SNL-induced decrease in PWT such that there were no significant differences from either pre-surgical values or the contralateral hind-limb (Figure 6.3A). In contrast, neither D-NAME (100 µg i.t.) nor vehicle had any effect on PWT in SNL animals. Both groups were unchanged from nerve ligated-untreated animals (Figure 6.3A).

In view of the marked effect of SNL on PWT and its sensitivity to i.t. L-NAME, the possible contribution of spinal NO signaling to changes in the excitability of A- and C-fibers three days after SNL was investigated using the biceps femoris flexor reflex. The threshold voltages eliciting the AFRR (Figure 6.3B) and CFRR (Figure 6.3C) were significantly reduced in the ipsilateral hind-limb compared to sham-controls. As illustrated in Figure 6.4, CFRR activity was evoked by a threshold stimulus triggering AFRR activity in SNL animals. Consistent with the results on PWT, treatment with L-NAME (100 µg i.t.) three days after nerve injury (i.e. 30minutes before electrical stimulation) significantly prevented the SNL-induced decrease in activation threshold of the AFRR (Figure 6.3B) and CFRR (Figure 6.3C). D-NAME (100µg i.t.) and vehicle were without effect (Figure 6.3).

There was also a significant increase in the response magnitude (i.e. spike integration) of the AFRR (panel A) and CFRR (panel B) three days after SNL, irrespective of stimulus intensity (Figure 6.4). L-NAME (100 μg i.t.) given 30 minutes before electrical stimulation on day 3, significantly attenuated the SNL-induced increase in spike integration of the AFRR (Figure 6.5A) and CFRR (Figure 6.5B) compared to control, vehicle or treatment with D-NAME (Figure 6.5A&B). The inhibitory effect of i.t. L-NAME peaked 30min after injection, lasted for 2 h (Figure 6.5C&D) and was dose-dependent over the range 10 to 100 μg (ID_{50}=58.4 μg AFRR; ID_{50}=49.8 μg CFRR). These results indicate that spinal NO is not only an important contributor to the functional changes in A- and C-fiber mediated responses characteristic of allodynia three days after SNL, but that these events are triggered by the SNL-induced activation of spinal NFκB.

To determine if spinal NO, generated at the onset of SNL, also facilitates the initial activation of spinal NFκB, L-NAME, D-NAME or vehicle were injected immediately after SNL or sham surgery. In the absence of treatment (i.e. no drug), the amount of activated NFκB, nNOS, and iNOS protein were significantly increased in the ipsliateral L-DH three days after SNL (Figure 6.6). Treatment with either vehicle or D-NAME (100 μg i.t.) had no effect compared to SNL animals without drug treatment (Figure 6.6). However, L-NAME (100 μg i.t.) significantly prevented the SNL-induced increase in NFκBa, nNOS, and iNOS protein in the ipsilateral L-DH three days later (Figure 6.6). These values were unchanged from those of the contralateral side (Figure 6.6). Similarly, L-NAME

prevented the reduction in PWT (Figure 6.7A) and the corresponding increase in response magnitude of the AFRR and CFRR (Figure 6.7B) three days after SNL. D-NAME (100 μg i.t.) and vehicle were without effect (Figure 6.7A&B). These results indicate the additional role of NO-signaling at the onset of SNL, including the activation of spinal NFκB and subsequent downstream effects leading to allodynia.

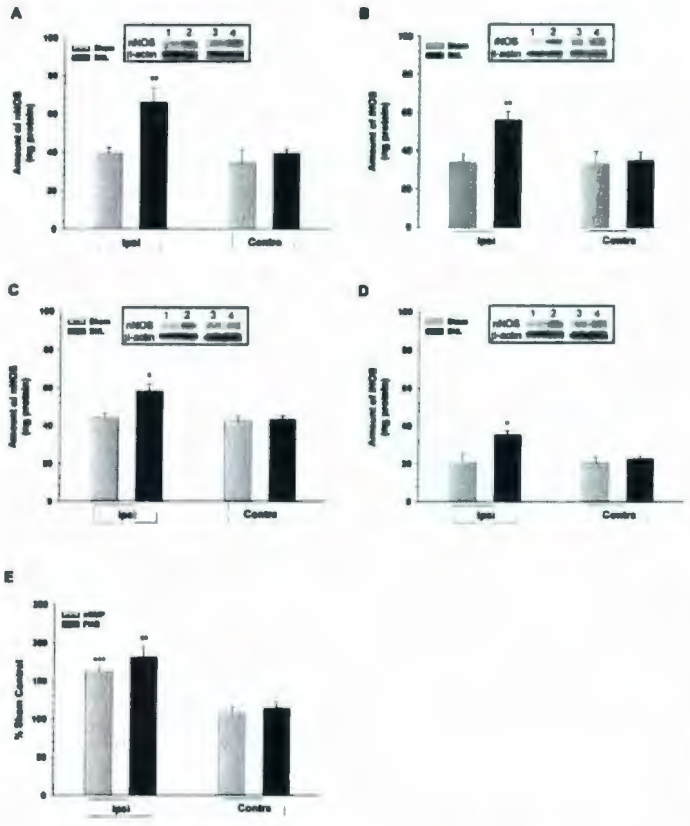

Figure 6.1. The effect of SNL or sham surgery on the expression of enzymes involved in NO synthesis and signaling in the L-DH. Panels A&B illustrate the expression of nNOS and iNOS protein one day after surgery, and panels C&D illustrate the expression of nNOS and iNOS protein three days after surgery. Each bar represents the mean ± SEM of three to five animals and asterisks indicate a significant difference from sham-controls or the contralateral DH of SNL animals (*p<0.05; **p<0.01). The expression of cGMP and PKG protein is illustrated in panel E. Data are expressed as % of sham-control, based on units of optical density and normalized using total protein content (see Methods). Asterisks indicate a significant difference between the ipsilateral and contralateral lumbar dorsal horns of SNL animals (**p<0.01; ***p<0.001). Representative immunoblots of nNOS, iNOS, and β-actin in the ipsilateral (ipsi) or contralteral (contra) L-DH are shown in the inserts above each panel. Lane 1: sham (ipsi); Lane 2: SNL (ipsi); Lane 3: Sham (contra); Lane 4: SNL (contra).

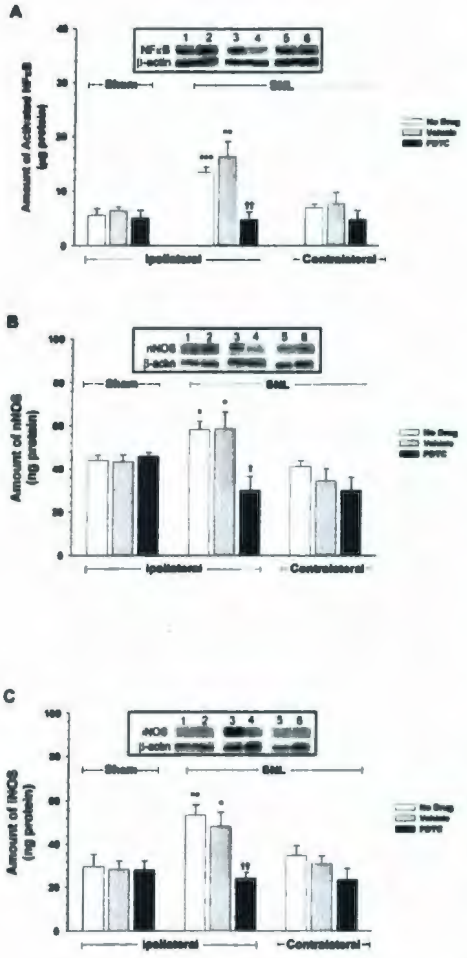

Figure 6.2. The effect of PDTC, vehicle (50% DMSO) or no drug treatment on the amount of activated NFκB (panel A), nNOS (panel B) and iNOS (panel C) in the ipsilateral and contralateral L-DH, three days after SNL or sham surgery. PDTC (100μg) or DMSO was injected i.t. immediately after surgery. Data were normalized using total protein content, corrected for non-specific immunoreactivity and expressed as the amount of protein (see Methods). Note the scale difference in panel A. Each bar represents the mean ± SEM of three to five animals and asterisks indicate a significant difference from sham animals receiving no drug, DMSO or PDTC (*p<0.05; **p<0.01; ***p<0.001). Daggers indicate a significant difference from SNL animals receiving no drug or DMSO (†p<0.05; ††p<0.01). Representative immunoblots of activated NFκB, nNOS, iNOS and β-actin in the ipsilateral (ipsi) or contralteral (contra) L-DH are shown in the inserts above each panel. Lane 1: sham + no drug (ipsi); Lane 2: sham + PDTC (ipsi); Lane 3: SNL + DMSO (ispi); Lane 4: SNL + PDTC (ipsi); Lane 5: SNL + DMSO (contra); Lane 6: SNL + PDTC (contra).

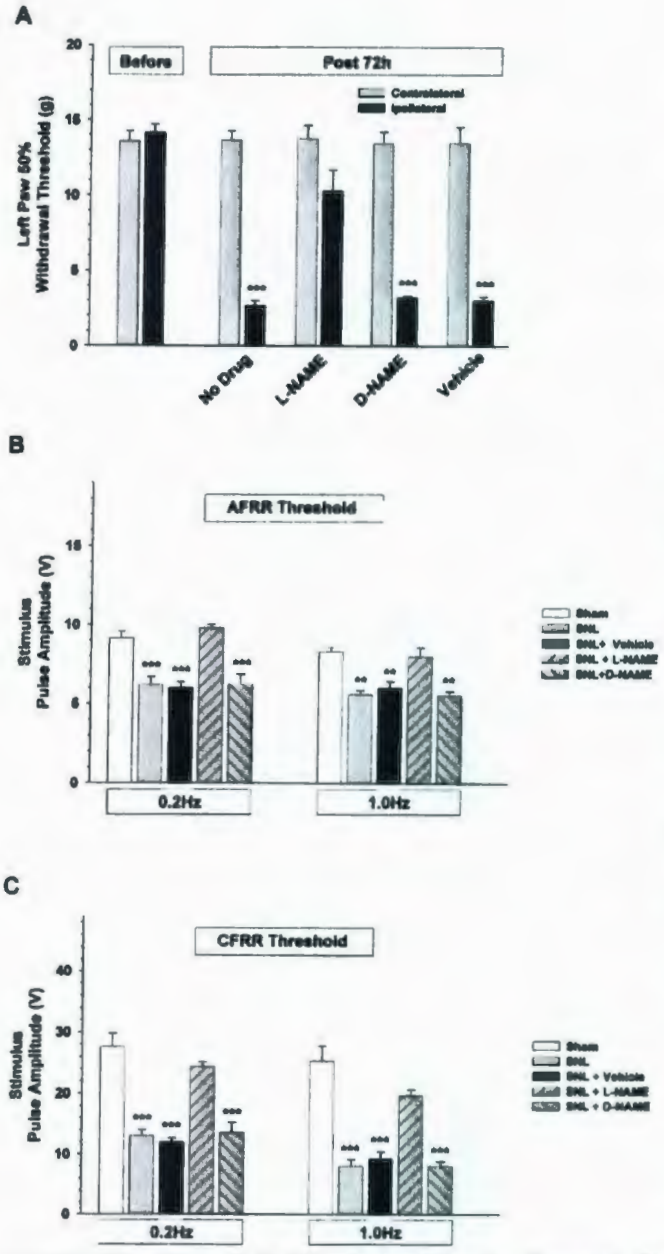

Figure 6.3. The effect of L-NAME (100 µg), D-NAME (100 µg) or vehicle (i.e. saline) on PWT (Panel A) and activation threshold of the AFRR (panel B) and CFRR (panel C) three days after surgery. All drugs were injected three days after SNL or sham surgery and responses recorded as described in the Methods. Data are presented as the mean ± SEM of five to seven animals. Asterisks in Panel A indicate a significant difference from the corresponding contralateral control after SNL (**p<0.01; ***p<0.001). Asterisks in Panels B and C indicate a difference from sham-controls or SNL animals treated with L-NAME (**p<0.01; ***p<0.001).

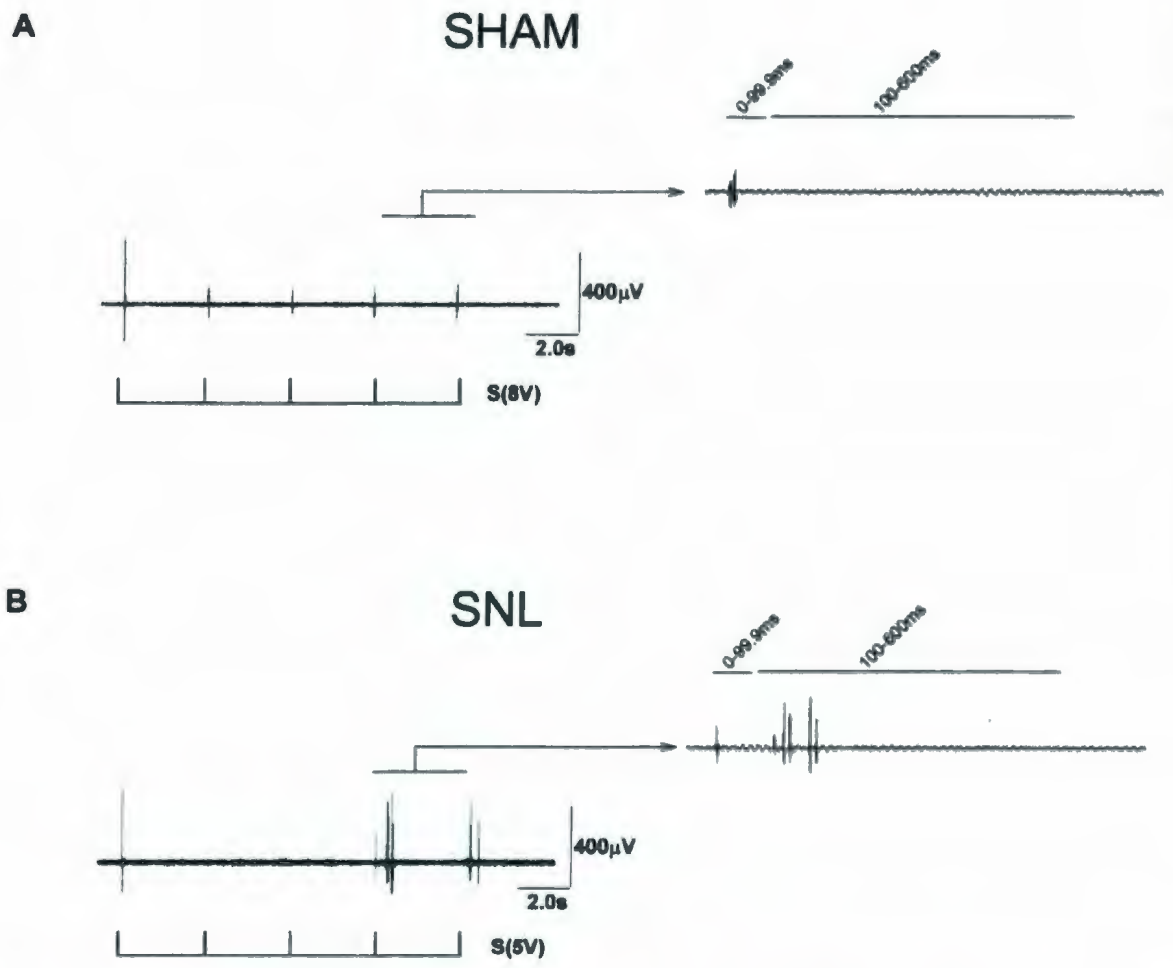

Figure 6.4. Representative electromyographic tracings evoked at AFRR threshold three days after SNL (Panel A) or sham surgery (Panel B). Each panel contains a magnification of the activity evoked by a single (i.e. 4^{th}) stimulus at AFRR threshold. A stimulus indicator bar is shown below each tracing. Note the presence of CFRR activity evoked at AFRR threshold after SNL (Panel A). See methods for the selection and activation criteria of the AFRR and CFRR.

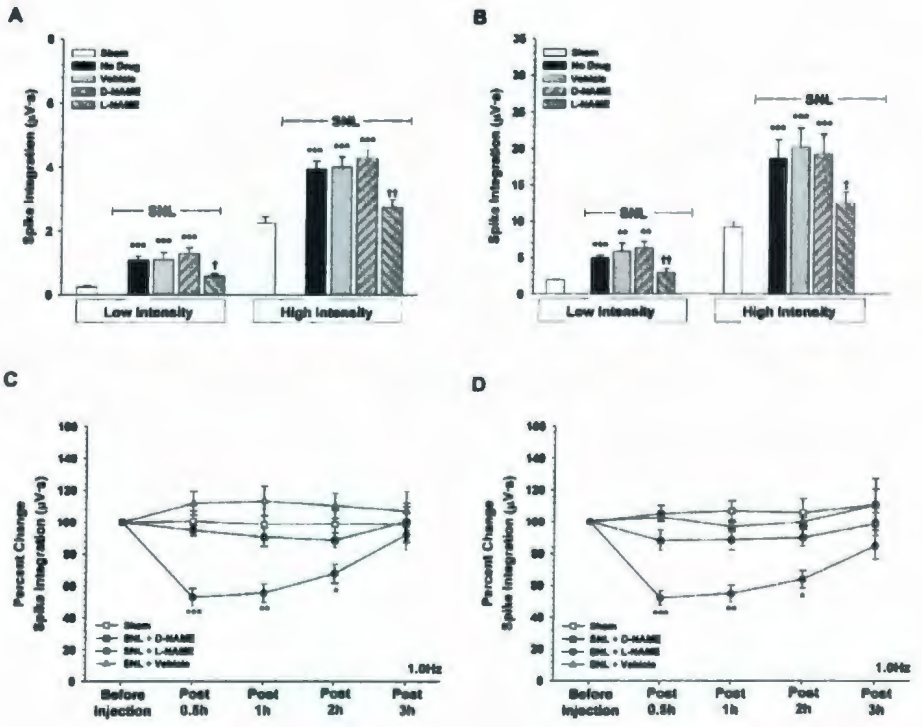

Figure 6.5. The effect of L-NAME (100 µg), D-NAME (100 µg) or vehicle (i.e. saline) on the magnitude of the AFRR (Panel A) and CFRR (Panel B) three days after SNL. All drugs were injected three days after surgery and responses were evoked by low or high-intensity stimulation (see methods). Each bar represents the mean ± SEM of five to seven animals. In panels A&B, asterisks indicate a significant difference from sham animals (**p<0.01; ***p<0.001) and daggers indicate a significant difference from SNL animals with or without vehicle or D-NAME (†p<0.05; ††p<0.01). The time-course of L-NAME (100 µg), D-NAME (100 µg) or vehicle on the response magnitude of the AFRR and CFRR are shown in Panels C and D. Asterisks indicate a significant difference from sham animals, and SNL animals with or without vehicle or D-NAME (*p<0.05; **p<0.01; ***p<0.001).

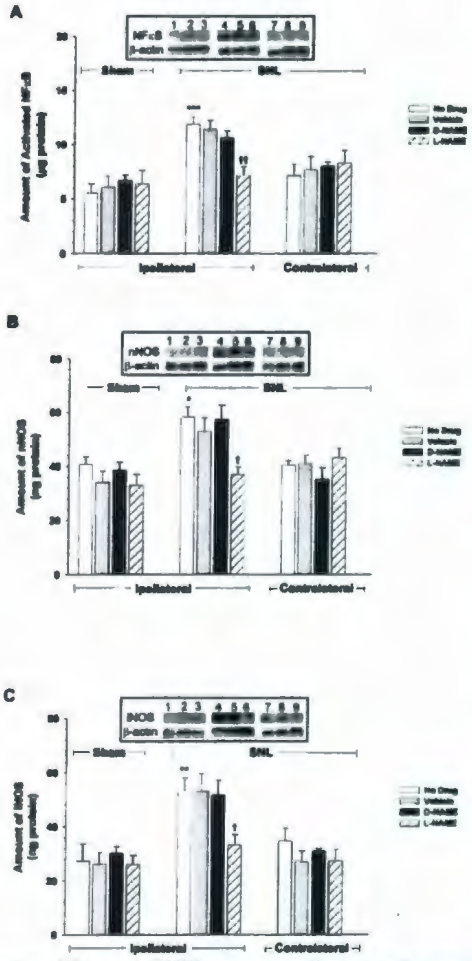

Figure 6.6. The effect of L-NAME (100 µg), D-NAME (100 µg), or vehicle (i.e. saline) on the amount of activated NFκB (panel A), nNOS (panel B) and iNOS (panel C) in the ipsilateral and contralateral L-DH, three days after SNL or sham surgery. All drugs were injected i.t. immediately after surgery. Data were normalized using total protein content, corrected for non-specific immunoreactivity and expressed as ng of protein (see Methods). Note the scale difference in panel A. Each bar represents the mean ± SEM of three to five animals. Asterisks indicate a significant difference from sham animals receiving no drug, saline, D-NAME, or L-NAME (*p<0.05; **p<0.01; ***p<0.001) and daggers indicate a significant difference from SNL animals receiving no drug, saline or D-NAME (†<0.05; ††p<0.01). Representative immunoblots of activated NFκB, nNOS, iNOS and β-actin in the ipsilateral (ipsi) or contralteral (contra) L-DH are shown in the inserts above each panel. Lane 1: sham + no drug (ipsi); Lane 2: sham + D-NAME (ipsi); Lane 3: sham + L-NAME (ispi); Lane 4: SNL + Saline (ipsi); Lane 5: SNL + D-NAME (ipsi); Lane 6: SNL + L-NAME (ipsi); Lane 7: SNL + Saline (contra); Lane 8: SNL + D-NAME (contra); Lane 9: SNL + L-NAME (contra).

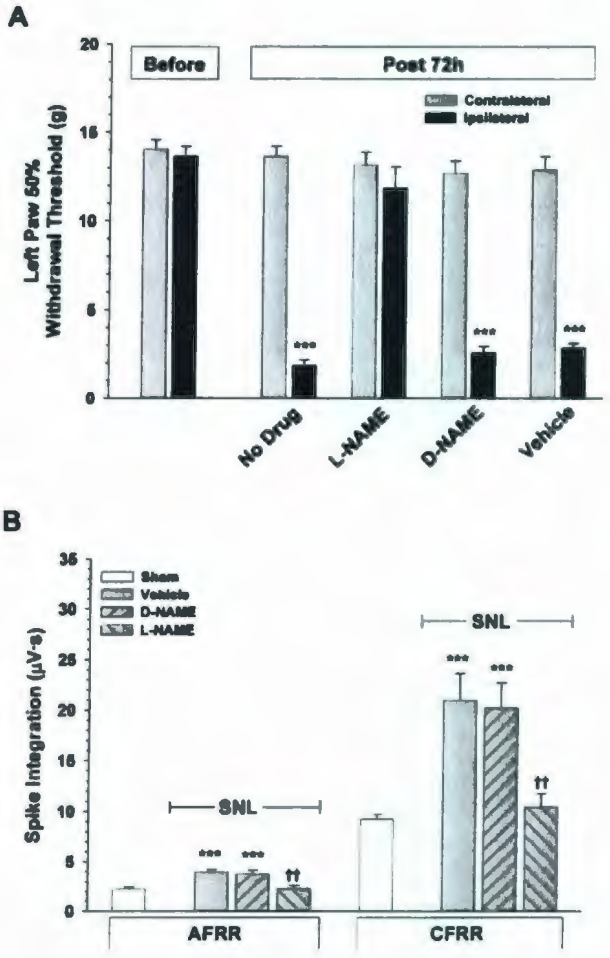

Figure 6.7. The effect of L-NAME (100 μg), D-NAME (100 μg) or vehicle (i.e. saline) on PWT (Panel A) and response magnitude of the AFRR and CFRR (Panel B) three days after SNL or sham surgery. All drugs were injected immediately after surgery. The response magnitude of the AFRR and CFRR were evoked using high-intensity stimulation (see Methods). Each bar represents the mean ± SEM of five to seven animals and asterisks. Asterisks in Panel A indicate a significant difference from the corresponding contralateral control after SNL (***p<0.001). Asterisks in Panel B indicate a significant difference from sham animals (***p<0.001) and daggers indicate a significant difference from SNL animals receiving vehicle or D-NAME (††p<0.01).

6.4 DISCUSSION

Molecular, electrophysiological and behavioural outcomes reflecting changes to spinal NO-synthesis/signaling were observed three days after SNL. These changes were completely absent in sham-controls (with or without i.t. catheters), unaffected by i.t. catheterization in SNL animals (O'Rielly and Loomis, unpublished observations), and only evident ipsilateral to nerve injury. Consequently, the molecular and functional effects reported in this study are directly attributable to nerve ligation and not surgery *per se*. As such, they appear to be critical steps in the development of allodynia early after SNL.

Expression of nNOS and iNOS protein was significantly increased in the lumbar dorsal horn ipsilateral to nerve ligation. nNOS, the more constitutive and predominant isoform in neurons (Szabó, 1996), exceeded that of iNOS in both absolute and relative terms one day after SNL. By three days, this pattern had changed reflecting the time-dependent induction of iNOS in cells of the affected dorsal horn. Significant increases in NFκB protein were also found in the nuclear fraction prepared from the same DH segments. SNL had no effect on NFκB in the ipsilateral or contralateral ventral horns of the lumbar cord (O'Rielly and Loomis, 2008). The increase in NFκB was completely prevented by i.t. PDTC given immediately after SNL. PDTC, a selective inhibitor of NFκB, blocks the translocation of NFκB from the cytoplasm into the nucleus, its binding to corresponding promoter regions of the DNA, and the promotion of genes coding for NOS and other proteins (Karin & Ben-Neriah, 2000; Tergaonak, 2006). The

parallel increases in NFκB and NOS protein in the affected DH, and their mutual attenuation by i.t. PDTC indicate that the activation of NFκB triggered by SNL is a critical antecedent to the up-regulation of nNOS and iNOS protein in the early nerve-injured state. These results are consistent with the direct modulation of iNOS transcription by NFκB (Lowenstein et al., 1993; Mabuchi et al., 2003), and the positive correlation between the activation of NFκB and nNOS expression following spinal cord injury (Miscusi et al., 2006).

Surprisingly, i.t. L-NAME given immediately after SNL mirrored the effect of PDTC on NFκB, nNOS and iNOS protein. In contrast, i.t. L-NAME given three days after SNL had no significant effect on NFκB or NOS expression (O'Rielly and Loomis, unpublished observations), but did normalize the SNL-induced changes in A- and C-fiber mediated reflex and PWT responses for 2 h (discussed below). The results with L-NAME given immediately after SNL suggest that spinal NO, generated by constitutive NOS at the onset of nerve injury, promotes the injury-induced activation of NFκB. It also explains how L-NAME, a stereo-specific inhibitor of NOS activity, affects the downstream expression of NOS isoforms. The latter was not an artifact of the immunoblotting technique as the binding of NOS to either antibody was unaffected by the presence of L-NAME, and indistinguishable from that with D-NAME (data not shown). This is the first evidence that NO affects the activation of NFκB and the downstream expression of enzymes responsible for NO synthesis/signaling in the acute nerve-injured state. Whether this is a direct or indirect effect remains to be determined. Either

way, it enables rapid amplification of spinal NO-synthesis/signaling in the affected dorsal horn and the exaggeration of effects facilitated by spinal NO early after nerve injury.

To further assess whether the up-regulation of NOS isozymes translated into increased NO-signaling, two important elements in the NO-signaling cascade were investigated. Western analysis provided a convenient method for probing SNL-induced changes in cGMP and PKG protein in the same spinal cord segments described above. The concurrent up-regulation of cGMP, PKG, nNOS and iNOS in the ipsilateral L-DH three days after SNL suggests an increase in both NO synthesis and signaling in the affected lumbar cord. This is consistent with effects of i.t. L-NAME on spinal hyperexcitability and allodynia (described below), and previous reports implicating the NO-cGMP-PKG pathway in the development of nerve injury-induced tactile allodynia (Hua et al., 1999; Sung et al., 2004, 2006). Whereas others have reported changes in cGMP and PKG in the DRG or in the spinal cord weeks after nerve injury (Siegan et al., 1996; Shi et al., 1998), this is the first study describing changes in spinal cGMP and PKG protein early after experimental nerve injury, and the first using the SNL model.

The molecular changes induced by SNL in this and a previous report (O'Rielly and Loomis, 2008) were accompanied by spinal hyperexcitability and mechanical allodynia ipsilateral to nerve injury. All SNL animals featured exaggerated reflex responses (AFRR and CFRR) in the biceps femoris flexor reflex of the affected hind-limb, and hypersensitivity to von Frey filaments on the

plantar surface of the same hind-paw. The threshold voltages eliciting the AFRR and CFRR were significantly reduced in the ipsilateral hind limb. Indeed, CFRR activity was evoked by threshold stimuli normally triggering AFRR activity. The more pronounced effect of SNL on the firing threshold of the CFRR compared to the AFRR probably reflects the greater sensitivity of small diameter, unmyelinated fibers to nerve constriction. There was also a corresponding increase in the response magnitude (i.e. spike integration) of the AFRR and CFRR. It is unlikely that the spinal hyperexcitability observed after SNL is explained by supersensitivity of the denervated portion of the biceps femoris. Since the SNL model utilizes tight ligation of the L5/L6 spinal roots leaving many afferent and efferent fibres intact (i.e. uninjured and functional), evident by the significant decrease activation thresholds of the AFRR and CFRR (and PWT). These electrophysiological changes, which are evident one day after SNL (O'Rielly and Loomis, 2007), were exacerbated at three days (i.e. an approximate 2-fold increase compared to day one) and detectable for at least ten days (O'Rielly and Loomis, 2007, 2008). They were also highly reproducible within treatment groups, and qualitatively similar to those reported in other experimental nerve (Meyerson et al., 1995; Colvin et al., 1996; Okamoto et al., 2001; Kohno et al., 2003), and spinal cord (Bennett et al., 2004) injury models. The latter suggests broader applicability of the present results to the early nerve-injured state.

In a recent study, PDTC given i.t. immediately after SNL consistently prevented the development of spinal hyperexcitability and mechanical allodynia three days later (O'Rielly and Loomis, 2008). There is no evidence that PDTC affects electrophysiological activity or PWT directly in either nerve-injured animals or sham-controls, indicating that the molecular and functional effects induced by SNL begin with the activation of spinal NFκB. In the present study, L-NAME given i.t. immediately after SNL also prevented the up-regulation of NFκB, nNOS and iNOS protein (see above), and the development of spinal hyperexcitability and allodynia. Together, these results underscore the importance of the up-regulation of nNOS and iNOS in the spinal cord, and the corresponding increase in spinal NO-synthesis/signaling to the development of SNL-induced spinal hyperexcitability and allodynia. That these outcomes continue to depend on NO-synthesis/signaling is indicated by the temporary, dose-dependent normalization of the AFRR, CFRR and PWT by i.t. L-NAME given three days after SNL. Overall, the results highlight the role of spinal NO in the onset, development and early maintenance of spinal hyperexcitability and allodynia.

The molecular, electrophysiological and behavioural changes described in this study appear to represent an important set of coordinated responses contributing to spinal hyperexcitability and mechanical allodynia early after SNL. That these outcomes reflect increased NO-synthesis/signaling in the affected DH is in agreement with previous studies reporting the normalization of PWT by i.t. L-

NAME after nerve injury (Hefferan and Loomis, 2004), the facilitatory effect of NO on neuronal excitability (Wilson, 2000; Ahern et al., 2002; González-Forero et al., 2007), and the recognized role of low-threshold A-fibers in mechanical allodynia (Bulka et al., 2002). Considering the degree of hyperexcitability that develops rapidly in the acute nerve-injured state, including the range of neuromodulators that are unleashed (Millan, 1999), the outcomes attributed to spinal NO-signaling in the present study are unlikely to be mediated by NO alone. One obvious family is the prostanoids, especially PGE_2. There is an extensive literature documenting: a) the role of spinal PG in acute nerve-injured states (Zhao et al., 2000; Kawahara et al., 2001; Ma et al., 2002; Hefferan et al., 2003a, b; Ma and Eisenach, 2003; Zhu and Eisenach 2003; O'Rielly and Loomis, 2006-2008); b) interactions between PG and NO (Salvemini et al., 1993, 1995; Tsai et al., 1994; Salvemini et al., 1995; Landino et al., 1996; Mollace et al., 1998; Kim et al., 2005; Matsumura et al., 2005; Dudhgaonkar et al., 2007); c) the modulation of COX-2 and iNOS gene transcription by NFκB (Lowenstein et al., 1993; Mabuchi et al., 2003); and d) the parallel changes in COX and NOS activity following nerve injury (Dolan and Nolan, 1999; Milne et al., 2001). The nature of the interaction between spinal NO and PG early after SNL is the subject of the next chapter.

In summary, the present study describes the differential up-regulation of nNOS and iNOS protein in the ipsilateral L-DH one and three days after SNL; effects occurring secondary to the activation of NFκB. The activation of NFκB appears to be facilitated by spinal NO at the onset of ligation and persists for at

least three days. Functionally, the results demonstrate the relevance of increased NO synthesis and signaling on A- and C-fiber mediated reflex responses and PWT in the SNL model. Pharmacological disruption of NO-synthesis/signaling immediately after SNL prevented the electrophysiological and behavioural features of allodynia. Overall, the results support the hypothesis that spinal NO is a critical early signal in the complex events underlying allodynia, and the rationale for early interventions to mitigate the chronic and often debilitating outcomes of nerve injury.

7.0 NITRIC OXIDE MEDIATES PROSTAGLANDIN SIGNALING IN THE DEVELOPMENT OF ALLODYNIA FOLLOWING L5/L6 SPINAL NERVE LIGATION

7.1 INTRODUCTION

Nerve ligation of the L5/L6 spinal roots triggers the immediate activation of NFκB in the L-DH ipsilateral to nerve injury (O'Rielly and Loomis, 2008). This initiates a cascade of events amplifying PG signaling in the spinal cord including the up-regulation of COX isoforms, the increased expression of PGE-type receptors (EPR_{1-3}) and a marked increase in pharmacodynamic sensitivity to PGE_2 (O'Rielly and Loomis, 2006-2008). These molecular and physiological changes are evident 24h after SNL, are temporally and spatially correlated with the onset of spinal PG-dependent hyperexcitability and mechanical allodynia, and persist for approximately ten days (Kim and Chung, 1992; O'Rielly and Loomis, 2006, 2007). Importantly, they are also prerequisite steps in the development of long term, PG-independent allodynia in the SNL model (Hefferan et al., 2003a, b; O'Rielly and Loomis, 2006-2008).

The SNL-induced activation of NFκB also affects the expression of nNOS and iNOS in the same affected segments (O'Rielly and Loomis, 2006-2008; Mabuchi et al., 2003). This observation is consistent with the direct modulation of COX-2 and iNOS gene transcription by NFκB (Lowenstein et al., 1993; Mabuchi et al., 2003), and the positive correlation between the activation of NFκB and the

expression of nNOS (Miscusi et al., 2006). It is also in accord with the concurrent up-regulation of COX and NOS protein in the central and/or peripheral nervous system (Dolan and Nolan, 1999; Milne et al., 2001; O'Rielly and Loomis, 2006, 2007, 2008) and the corresponding increase in NOS activity after nerve injury (Choi et al., 1996; Gordh et al., 1998; Mabuchi et al., 2003). Like spinal PG, NO signaling is critical in the development and early maintenance of SNL-induced spinal hyperexcitability and allodynia (O'Rielly and Loomis, 2008). This includes the facilitation of NFκB activation in the affected dorsal horn beginning at the onset of nerve injury (O'Rielly and Loomis, 2007, 2008). The parallel changes in spinal PG- and NO-signaling, and their comparable effects on spinal hyperexcitability and allodynia (Dolan and Nolan, 1999; Milne et al., 2001; O'Rielly and Loomis, 2006-2008) indicate that both signaling pathways are triggered at the onset of nerve injury. These pathways may operate dependently or independently in the acute nerve injured state.

In a previous study using selective COX and NOS inhibitors, fixed-molar isobolographic analysis revealed an additive anti-allodynic interaction in the SNL model (Hefferan and Loomis, 2004). This result points to a convergence of spinal PG- and NO-pathways in the development of allodynia, and thus a dependency in their modulation of sensory processing. NO is a readily diffusible, excitatory neuromodulator which is known to release arachidonic acid from the plasma membrane, to increase COX activity through a variety of mechanisms, and to evoke the release of neurotransmitters and other neuromodulators, including PG

(Davidge et al., 1995; Landino et al., 1996; Chalimoniuk et al., 2006; Egan et al., 1976; Salvemini et al., 1995; Salvemini et al., 1993; Tsai et al., 1994; Dudhgaonkar et al., 2006; Sakai et al., 1998; Matsumura et al., 2005). Therefore, we tested the hypothesis that spinal NO-signaling is critical to the development of enhanced sensitization to spinal PG and thus the PG-dependent phase of SNL-induced allodynia. In turn, we investigated the effect of NO-signaling on COX-1 and COX-2, the glycine-α3 receptor (GLY-α3R), and EP_{1-3} receptors in the affected dorsal horn; molecular targets governing PG synthesis, signaling and spinal sensitization.

7.2 MATERIALS AND METHODS

Studies were approved by, and experiments conducted in accordance with the guidelines of the Institutional Animal Care Committee of Memorial University of Newfoundland (St. John's, NL, Canada).

Animals. Male Sprague-Dawley rats (130 to 150 g) were obtained from the Vivarium of Memorial University of Newfoundland (St. John's, NL, CA) and housed in standard cages with woodchip bedding. Animals had free access to food and water. A 12 h light-dark cycle (lights on at 0700 h) was used throughout.

Intrathecal Catheterization. Intrathecal catheters (6.5 cm-length terminating near the lumbar enlargement) were implanted according to method of

Yaksh and Rudy (1976) as previously modified (Hefferan et al., 2003a). Catheters were 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. Intrathecal catheters (inner diameter, i.d. 240 μm, outer diameter, o.d. 290 μm) were constructed from triple lumen PE-5 tubing (Spectranetics, Colorado Springs, CO, USA) using the modified method of Marsala et al. (1995). Rats with normal motor, grooming and feeding behavior were housed separately and allowed to recover for two days before SNL or sham surgery.

Neuropathy. Neuropathy was induced using the method of Kim and Chung (1992) as previously described (Hefferan et al., 2003a, b; O'Rielly and Loomis, 2006, 2007a, b). Briefly, rats were anesthetized with halothane and the left L4 and L5 spinal nerves were isolated and separated. The L5 and L6 spinal nerves were tightly ligated with 6-0 silk thread. In sham-controls, the L5 and L6 spinal nerves were isolated, but not ligated. All animals were allowed to recover for 3 days before experimentation. Allodynia, defined as a paw withdrawal threshold (PWT) of ≤4 g, was confirmed using von Frey filaments prior to experimentation.

Western Blotting. Animals were deeply anesthetized with urethane (1.2 mg/kg i.p.) and perfused intracardially with ice cold saline (0.9% NaCl). The spinal cord was extracted hydraulically (de Sousa and Horrocks, 1979), immediately frozen in 2-methylbutane (Sigma-Aldrich, Oakville, ON, CA), and stored at -80 °C. The lumbar region (L2-L6) of the spinal cord was isolated, removed and subsequently divided into the left and right, ventral and dorsal quadrants. Spinal cord was homogenized in ice cold lysis buffer (1% Nonidet-P40, 10% glycerol in TBS plus a protease inhibitor cocktail tablet - Roche Diagnostics, Laval, PQ, CA, 1mM sodium vanadate, 1mM sodium fluoride, and 0.025% SDS), and centrifuged at 10,000rpm for 5 min (4 °C). Samples, diluted to achieve equal protein concentrations (30 µg), were separated by electrophoresis on 8% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and incubated for 1.5 h in Tris buffer (Tris 25mM, glycine 192mM, 200ml methanol, pH 8). Pre-stained protein markers were used for molecular weight determination. Blots were initially stained with Ponceau Red, and later probed with an antibody directed against the cytosolic protein, actin (1:1000; Sigma-Aldrich, Oakville, ONT, CA) to assess the equivalency of protein loading.

Blots were subsequently probed with the following primary antibodies: polyclonal rabbit COX-1 antibody (1:250); polyclonal rabbit COX-2 antibody (1:1000); polyclonal rabbit EP_1 receptor antibody (1:1000); polyclonal rabbit EP_2 receptor antibody (1:2000); and a polyclonal EP_3 receptor antibody (1:1000), and polyclonal rabbit Glyα3 (GLY-α3R) receptor antibody (1:1000). All primary

antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA) except for the GLY-α3R receptor antibody (Invitrogen, Carlsbad, California, USA). Standards for COX-1, COX-2, and EP receptors were preabsorbed with their corresponding blocking peptide to determine the antibody specificity with the exception of a cGMP standard (Sigma-Aldrich, Oakville, ONT, CA). Antibody specificity was not preformed using anti-Glyα3R due to a lack of a commercially available blocking peptide. Where appropriate, the western blots were corrected for non-specific binding by subtracting the optical density in the presence of the corresponding blocking peptide from that of each test sample. The membranes were incubated overnight at 4°C with the primary antibodies, and diluted in Tris buffer (containing 3% milk powder and 0.05% Tween-20). Protein bands were treated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Chemicon International Inc., Temecula, CA, USA) for 1h at room temperature, washed for 30min in Tris buffer, visualized using enhanced chemiluminescence (PerkinElmer Life Sciences Boston, MA, USA), and exposed to X-ray film (Cronex MRF Clear base, Agfa Corp, Greenville, SC, USA).

Spinal Glutamate Release. The spinal cord was extracted hydraulically (de Sousa and Horrocks, 1979) and the dura and arachnoid membranes carefully removed. The lumbar region (L2 to L6) was excised, mounted on cutting blocks and immersed in sucrose-modified artificial cerebrospinal fluid (aerated with 95% O_2 and 5% CO_2). Slices (600 to 800 μm) were cut with a vibratome and placed in

aerated artificial cerebrospinal fluid at room temperature. They were immersed in HEPES buffer 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 (Nicholls et al., 1987). The reduced form of nicotinamide adenine dinucleotide phosphate generated from this reaction was quantitated using spectrofluorometry (excitation: 335 nm; emission: 430 nm; Nicholls et al., 1987). Basal and PGE_2-evoked glutamate release was quantified using standard curves constructed with L-glutamate (0 to 360 pmol; Sigma-Aldrich, Oakville, ON, CA) on each day of analysis. Basal glutamate release (i.e. release in the absence of any drug stimulus) was determined for 15 minutes before the introduction of drug into the cuvette. Drug-evoked release was defined as total release (i.e. after a drug stimulus) minus basal release. Protein content in each slice was determined using a modified Lowry protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Behavorial Testing. Spontaneous (no brushing) and BR-evoked behavior were continuously graded using a modified scoring system (Malmberg et al., 1995), as previously described (O'Rielly and Loomis, 2006). Spontaneous behavior (0 - normal behavior, bright, alert and exploring; 1 - huddling, burrowing, or hiding; 2 - one of the following: piloerection, occasional vocalization, or favoring the affected side; 3 - two or more of the following: piloerection,

occasional vocalization, favoring the affected side; and 4 - any of the following: frequent or persistent vocalization, circling motion, licking or biting the affected dermatomes) was graded over a 4-min interval. The hair on the back, flanks, limbs, and hind paws was then brushed with a cotton-tipped applicator sufficient to deflect the pelage. This was continued for 1min and the behavior graded as follows: 0 - normal behavior, curious, alert, and exploring; 1 - avoidance of stimulus source or protection of the affected dermatomes; 2 - one of the following: piloerection, paw withdrawal, or occasional vocalization; 3 - two or more of the following: piloerection, paw withdrawal, or occasional vocalization; and 4 - any of the following: attacking the applicator, frequent or persistent vocalization, circling motion, licking or biting the affected dermatomes. This sequence was repeated every 5 minutes for the first hour and every 30min thereafter up to 6 h or until no responses were detected. The investigator was blinded to the nature of the treatment (i.e. vehicle, drug, concentration) in all behavioral experiments.

Drugs. Prostaglandin E_2 (PGE_2), *N*-nitro-L-arginine methylester (L-NAME), and *N*-nitro-D-arginine methylester (D-NAME) were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). PGE_2 solutions were prepared according to Nishihara et al (1995). Briefly, PGE_2 was initially dissolved in ethanol and evaporated under nitrogen gas. It was then re-dissolved in normal saline and diluted with the same to yield the desired

concentrations. PDTC was dissolved in 100% DMSO and diluted with normal saline at the time of injection to yield a final DMSO concentration of 50%, whereas L-NAME and D-NAME were dissolved in saline (0.9% NaCl). For the *in vitro* glutamate release experiments, exogenous PGE_2 was added directly into the cuvette containing the slice using a microsyringe. Each concentration of PGE_2 was tested using a separate slice so that a full PGE_2 concentration–response curve was determined in each animal. All equipment was sterilized with 70% alcohol and thoroughly rinsed with 0.9% sterile saline before injection. Intrathecal drugs were injected into anesthetized rats using a hand-held microsyringe. Drugs were delivered in a volume of 5 µl followed by 5 µl of sterile saline. The i.t. catheter was immediately resealed with a stainless steel plug. The position of the catheter tip was verified visually after sacrifice in randomly selected animals.

Data Analysis. All data are displayed as mean ± SEM. Western blots were analyzed by optical density using ImageQuant software (Amersham Biosciences Corp., Piscataway, NJ, USA). Expression data are presented as nanograms of protein relative to COX-1/COX-2 (50 ng), or EP receptor (25 ng) standards. Expression data was corrected for background optical density and normalized using the cytosolic protein, actin. The absence of available standards for GLY-α3R meant that optical density could not be converted to the amount of protein and therefore are presented as % of sham-control. Dose-response

analysis was performed using methods from Tallarida and Murray (1987). Comparisons within each treatment group were performed using one-way, repeated measures ANOVA, followed by the Newman-Keuls test. Comparisons across all drug- and vehicle-treated groups at each time point were determined using one-way, completely randomized ANOVA, followed by the Newman-Keuls test (SigmaStat 2.0; Systat Software Inc., San Jose, CA, USA).

7.3 RESULTS

All ligated rats displayed a significant decrease in PWT (≤4 g) three days after SNL which remained confined to the plantar surface of the left hind paw (i.e. ipsilateral to SNL). Generally, the affected hind paw was kept in an elevated and cupped position to minimize contact with the cage floor. These rats were otherwise healthy, showed normal feeding and grooming behavior, and regular weight gain. Neither intrathecal catheterization, nor sham-surgery had any effect on PWT compared to pre-surgical values (data not shown).

To investigate the effect of spinal NO on the SNL-induced expression of COX-1 and COX-2 protein in the L-DH, animals were treated with i.t. L-NAME (100 µg i.t.), D-NAME (100 µg i.t.) or vehicle (i.e. saline) immediately after surgery. Three days after SNL, there was a significant increase in COX-2 expression in the ipsilateral L-DH in D-NAME, vehicle or untreated animals compared to sham-controls (Figure 7.1A). There was also a modest increase in COX-1 expression but this was not statistically different ($p>0.05$) from sham-

controls (Figure 7.1B). In contrast, SNL animals treated with i.t. L-NAME (100 µg) prevented the increased expression of COX-1 and COX-2 in the ipsilateral L-DH (Figure 7.1). The SNL-induced expression of COX-1 and COX-2 in the contralateral L-DH (Figure 7.1) or lumbar ventral horns (data not shown) was unchanged from sham-controls regardless of drug treatment.

To examine whether the SNL-induced synthesis of NO exacerbates the PGE_2-evoked release of glutamate from the affected spinal cord, animals were treated with i.t. L-NAME (100 µg), D-NAME (100 µg) or saline immediately after sham surgery or SNL. Lumbar spinal cord slices were harvested three days later and PGE_2 concentration-response curves (CRC) determined. There were marked differences in the potency of PGE_2 (Figure 7.2; Table 7.1) depending on drug treatment. L-NAME prevented the increased sensitivity to PGE_2 (i.e. 2855-fold decrease in EC_{50}) in slices harvested from SNL animals compared to sham-controls (Figure 7.2; Table 7.1). Treatment with D-NAME or vehicle had no effect on PGE_2-evoked glutamate release from lumbar slices of SNL animals (Figure 7.2, Table 7.1). There was no significant difference in the peak effect of PGE_2 regardless of drug treatment (Figure 7.2). All results were corrected for basal glutamate release, which was significantly greater ($p<0.05$) in SNL (2.94 ± 0.34 pmol/min/mg protein) as compared to sham operated (1.50 ± 0.43) rats.

The effect of spinal NO on the sensitivity of PGE_2 *in vivo* was investigated using BR-evoked behavior as a measure of tactile allodynia. Animals were treated with i.t. L-NAME (100 µg), D-NAME (100 µg) or saline immediately after

SNL or sham surgery and i.t. PGE_2 injected three days later. For these experiments, sub-threshold doses of PGE_2 in naïve or sham-animals were chosen from earlier dose-response experiments (O'Rielly and Loomis, 2006). Initially, a single dose of PGE_2 (0.01 μg) was used. Mild brushing of the ipsilateral hind limb evoked comparable nociceptive-like behavior (i.e. vocalization, defensive posturing, licking the affected dermatomes, and biting the cotton-tipped applicator) in SNL animals treated with either D-NAME- or vehicle (Figure 7.3A). In contrast, nociceptive-like behavior in SNL animals given L-NAME was significantly reduced ($p<0.05$) compared to D-NAME- and vehicle-treated animals, and similar in magnitude to sham controls regardless of drug treatment; experimental groups in which brushing had no effect (Figure 7.3A). To verify the apparent NO effect on PGE_2 *in vivo*, these experiments were repeated using PGE_2 dose-response analysis (Figure 7.3B; Table 7.2). SNL animals displayed a significant increase in sensitivity to i.t. PGE compared to sham-controls as indicated by a 230-fold decrease in ED_{50} (Table 7.2). This SNL-induced effect was significantly attenuated by pre-emptive treatment with L-NAME compared to that with D-NAME or vehicle (Figure 7.3B).

To explore possible mechanism(s) underlying the effect of spinal NO on PGE_2 pharmacodynamics triggered by SNL, the effect of pre-emptive treatment with L-NAME, D-NAME or vehicle on the SNL-induced expression of EP receptors in the L-DH was determined three days after surgery. There was a significant increase in the expression of EP_1, EP_2 and EP_3 in the ipsilateral L-DH

of SNL animals versus sham-controls (Figure 7.4). In contrast, EP_1, EP_2 and EP_3 receptor expression in the contralateral L-DH or lumbar ventral horns was unchanged ($p>0.05$) from sham-controls (Figure 7.4). Pre-emptive treatment with i.t. L-NAME, but not D-NAME or vehicle, significantly attenuated the SNL-induced increase in EP_1, EP_2, and EP_3 receptor expression three days later (Figure 7.4). There were also related changes in spinal Glyα3R of SNL animals (Figure 7.5). The expression of Glyα3R was significantly decreased in the ispilateral L-DH three days after SNL compared to the contralateral L-DH (Figure 7.5) or lumbar ventral horns (data not shown) of SNL animals, or the dorsal (Figure 7.5) and ventral horns (data not shown) of sham-controls (Figure 7.5). Pre-emptive treatment with L-NAME, but not D-NAME or vehicle, significantly reversed the SNL-induced attenuation of Glyα3R expression in the ipsilateral L-DH (Figure 7.5). Glyα3R expression in the contralateral L-DH and lumbar ventral horns of SNL animals, or the lumbar dorsal and ventral horns was unaffected by drug treatment in this study (Figure 7.5 and data not shown).

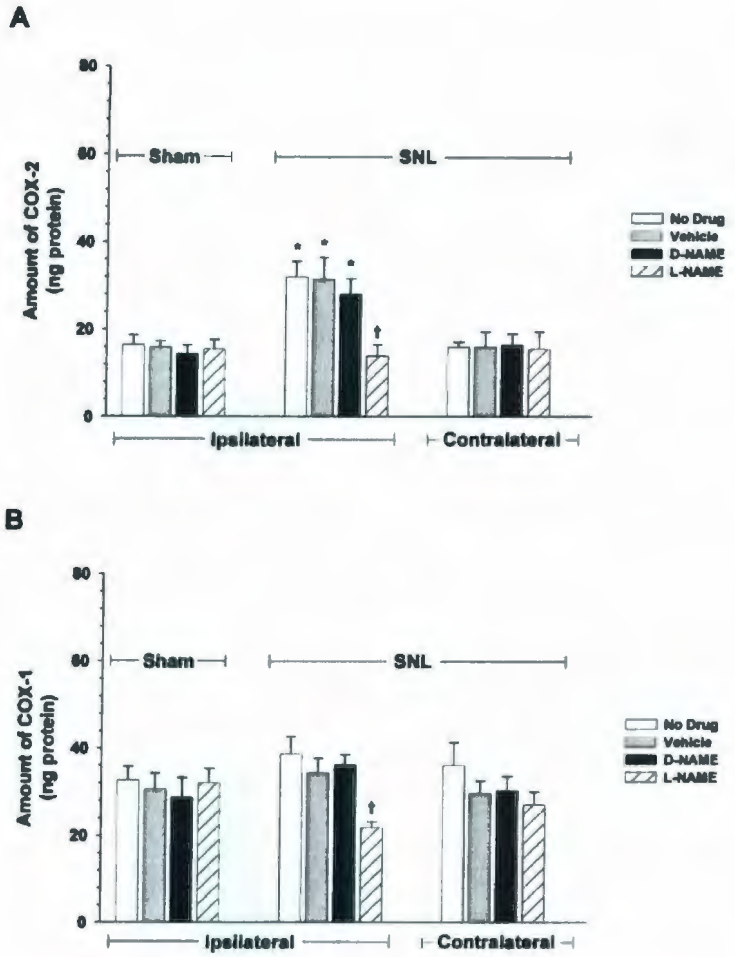

Figure 7.1. The effect of L-NAME (100µg), D-NAME (100µg), or vehicle (i.e. saline) on the expression of COX-1 (panel A) and COX-2 (panel B) in the ipsilateral (ipsi) and contralateral (contra) L-DH, three days after SNL. All drugs were injected i.t. immediately after SNL and analyzed three days later. Data were normalized using total protein content, corrected for non-specific immunoreactivity and expressed as ng of protein (see Methods). Each bar represents the mean ± SEM of three to five animals. Asterisks indicate a significant difference from sham-controls (*$p<0.05$) and daggers indicate a significant difference from the SNL + L-NAME group (†$p<0.05$).

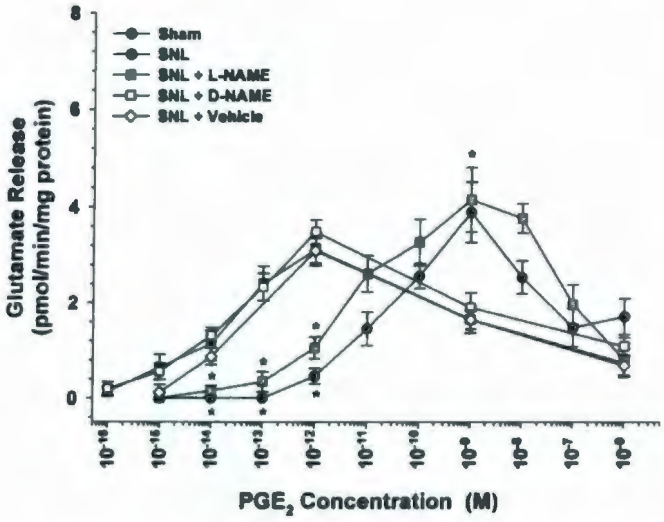

Figure 7.2. The effect of pretreatment with L-NAME (100µg), D-NAME (100µg), or vehicle on PGE_2-evoked spinal glutamate release. All drugs, except PGE_2, were injected i.t. immediately after surgery and spinal lumbar slices were harvested three days after SNL or sham surgery. Exogenous PGE_2 was added to the spinal cord slices using a microsyringe (see Methods). Data are presented as the mean ± SEM of four to six animals. Asterisks indicate a significant difference (*p<0.05) from SNL-controls with or without vehicle or D-NAME.

Table 7.1. EC_{50} values and 95% confidence intervals of PGE_2 on glutamate release from lumbar or thoracic spinal cord slices from SNL or sham-controls.

Experimental Treatment	EC_{50} (M)	95% C.I. (M)
Naïve + PGE_2	2.33×10^{-11}	$1.23 \times 10^{-11} - 4.41 \times 10^{-11}$
Sham + PGE_2	2.37×10^{-11}	$9.25 \times 10^{-12} - 6.08 \times 10^{-11}$
SNL + PGE_2	8.30×10^{-15}	$1.65 \times 10^{-15} - 4.18 \times 10^{-14}$
SNL + L-NAME + PGE_2	4.13×10^{-12}	$1.80 \times 10^{-12} - 9.45 \times 10^{-12}$
SNL + D-NAME + PGE_2	1.45×10^{-14}	$8.69 \times 10^{-15} - 2.41 \times 10^{-14}$
SNL + vehicle (saline) + PGE_2	3.03×10^{-14}	$9.37 \times 10^{-15} - 9.77 \times 10^{-14}$

EC_{50}=concentration producing 50% of maximum response; M=molar concentration; 95% C.I.=95% confidence interval.

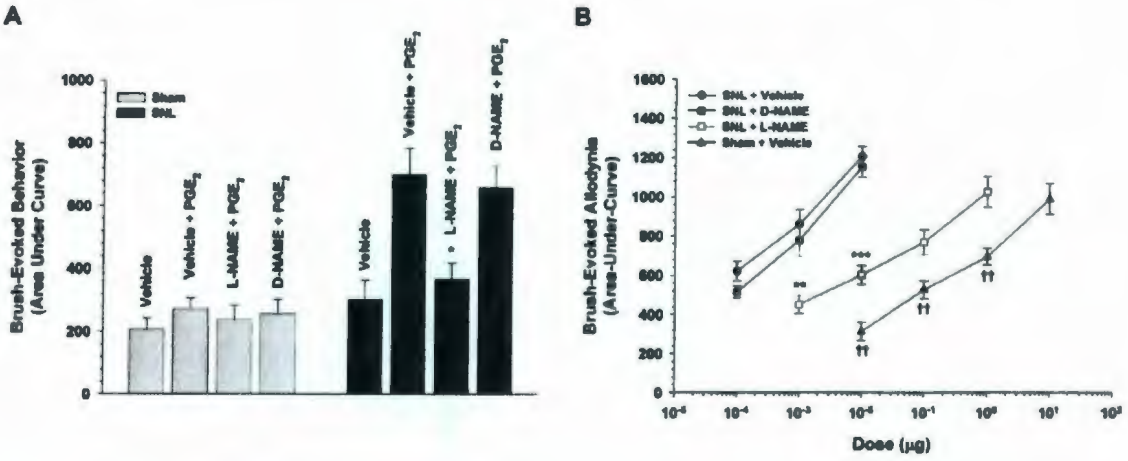

Figure 7.3. The effect of L-NAME (100μg), D-NAME (100μg), or vehicle on BR-evoked behavior in the presence of PGE_2. Drugs were injected i.t. immediately after SNL or sham surgery and behavioral experiments conducted three days later (see Methods). Panel A represents a single dose while panel B illustrates a dose-response-curve for PGE_2. Each bar represents the mean ± SEM of five to seven animals. Asterisks indicate a significant difference (*p<0.05; **p<0.01; ***p<0.001) from both SNL + vehicle and SNL + D-NAME groups. Daggers indicate a significant difference from the sham + vehicle group (††p<0.01).

Table 7.2. ED_{50} values and 95% confidence intervals of PGE_2 on BR-evoked allodynia after SNL or sham surgery.

Experimental Treatment	ED_{50} (µg)	95% C.I. (µg)
SNL + L-NAME + PGE_2	1.5×10^{-2}	$6.1 \times 10^{-3} - 3.9 \times 10^{-2}$
SNL + D-NAME + PGE_2	5.9×10^{-4}	$8.2 \times 10^{-5} - 4.3 \times 10^{-3}$
SNL + vehicle + PGE_2	7.4×10^{-4}	$8.7 \times 10^{-5} - 6.3 \times 10^{-3}$
Sham + vehicle + PGE_2	1.7×10^{-1}	$1.0 \times 10^{-1} - 3.0 \times 10^{-1}$

ED_{50}=dose producing 50% of maximum response; 95% C.I.= 95% confidence interval.

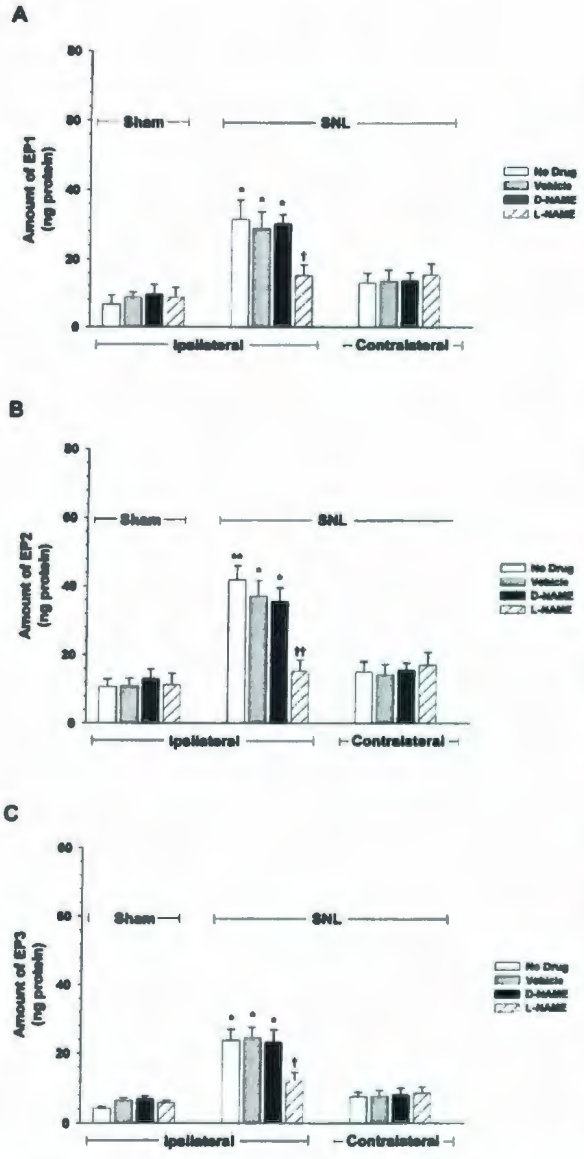

Figure 7.4. The effect of L-NAME (100µg), D-NAME (100µg), or vehicle (i.e. saline) on the expression of EP-1 (panel A), EP-2 (panel B), and EP-3 (panel C) receptors in the ipsilateral and contralateral L-DH, three days after SNL. All drugs were injected i.t. immediately after SNL and analyzed three days later. Data were normalized using total protein content, corrected for non-specific immunoreactivity and expressed as ng of protein (see Methods). Each bar represents the mean ± SEM of three to five animals. Asterisks indicate a significant difference from sham-controls (*p<0.05; **p<0.01; ***p<0.001), and daggers indicate a significant difference from the SNL + L-NAME group (†p<0.05; ††p<0.01).

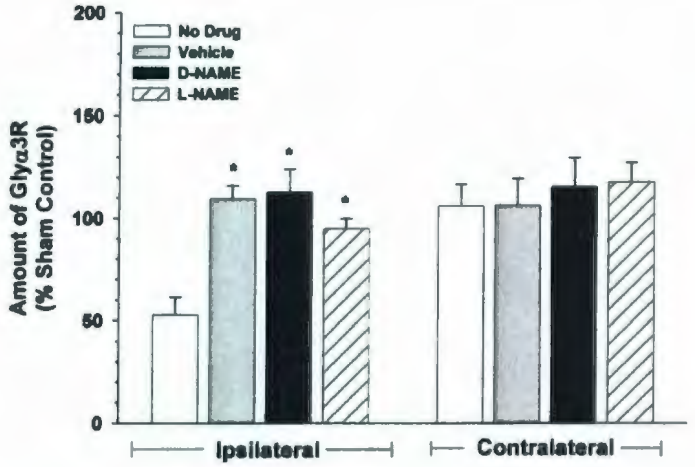

Figure 7.5. The effect of L-NAME (100µg), D-NAME (100µg), or vehicle (i.e. saline) on the expression of the glycine receptor in the ipsilateral and contralateral L-DH, three days after SNL. All drugs were injected i.t. immediately after SNL and analyzed three days later. Data were normalized using total protein content, and expressed as percent of sham-controls (see Methods). Each bar represents the mean ± SEM of three to five animals and asterisks indicate a significant difference from the SNL + no drug group (*$p<0.05$).

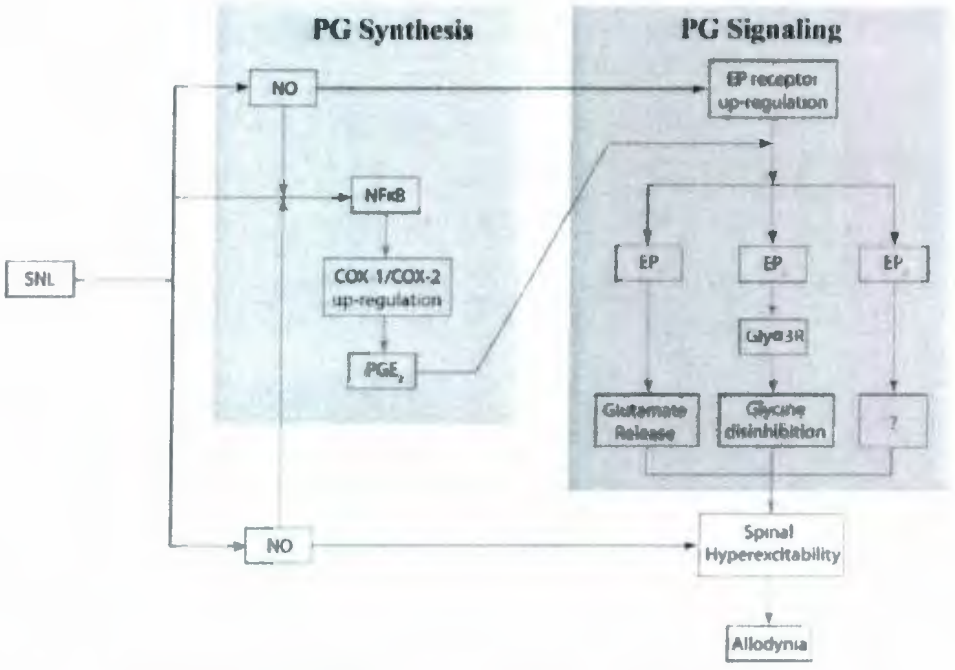

Figure 7.6. A schematic illustrating the relevant changes to spinal PG- and NO-synthesis/signaling early after SNL. SNL triggers the up-regulation of constitutive COX-1 and nNOS expression, as well as, NFκB in the ipsilateral L-DH one day after injury. The increase in NFκB induces the up-regulation of COX-2 and iNOS expression in the ipsilateral L-DH three days after injury. The resulting increase in PG- and NO-signaling further increases the SNL-induced: a) activation of spinal NFκB; b) EP_1 receptor expression/signaling resulting in increased spinal glutamate release, and c) EP_2 receptor expression/signaling resulting in decreased glycinergic tone. These changes in NO- and PG-synthesis/signaling comprise a viscous cycle early after SNL culminating in spinal hyperexcitibility and mechanical allodynia.

7.4 DISCUSSION

SNL has been shown to trigger the up-regulation of NFκB (O'Rielly and Loomis, 2008) and, in turn, the expression of COX and NOS enzymes (O'Rielly and Loomis, 2006) in the ipsilateral L-DH three days after injury. Moreover, the pharmacological inhibition of spinal NFκB immediately after SNL prevented the increase in COX (COX-2 > COX-1) and NOS (iNOS > nNOS) expression three days later (O'Rielly and Loomis, 2008). These results reflect the shared modulatory effect of NFκB on COX and NOS gene transcription (Lowenstein et al., 1993) and its activation at the onset of SNL. In the present study, pre-emptive treatment with i.t. L-NAME to inhibit spinal NO-synthesis prevented the SNL-induced expression of COX-1 and COX-2 in the ipsilateral L-DH three days later. This stereo-specific effect, like the SNL-induced expression of COX-1 and COX-2, was confined to the ipsilateral L-DH. This is the first report of an NOS inhibitor influencing the expression of enzymes responsible for PG synthesis in the acute nerve injured state. Spinal NO, generated by constitutive NOS at the onset of SNL, has been shown to promote the activation of NFκB in the ipsilateral L-DH (O'Rielly and Loomis, 2008) and would explain the results with i.t. L-NAME on COX expression in the same area. These results suggest a critical role of spinal NO in enhanced PG-signaling early after SNL.

The influence of NO on spinal PG after nerve injury was not limited to effects on COX expression. Pre-emptive treatment with i.t. L-NAME significantly attenuated the SNL-induced increase in sensitivity to PGE_2 both *in vitro* (i.e.

PGE_2-evoked glutamate release from lumbar slices) and *in vivo* (i.e. brush-evoked behaviorally-defined allodynia) determined three days after surgery. These results, which were also stereo-specific for the active isomer, indicate a significant normalization of the sensitivity to PGE_2 three days after pre-emptive treatment with L-NAME. The parallel nature and magnitude of the rightward shift of the PGE_2 CRC and DRC three days after L-NAME strongly indicate a change in EP receptor-effector coupling.

To explore this possibility, EP receptor expression in the ipsilateral L-DH was determined three days after surgery. EP_1, EP_2 and EP_3 receptors were significantly up-regulated in SNL animals compared to sham-controls. EP receptor expression for all three subtypes was significantly greater three days as compared to one day after SNL (O'Rielly and Loomis, 2007). This suggests a sustained and progressive effect of SNL on EP receptor expression over this time period. Spinal EP_1 receptors, which are located on presynaptic primary afferent terminals, are known to facilitate neurotransmitter release through an effect on intracellular calcium (Vanegas and Schaible, 2001). Spinal EP_2 receptors, which are located post-synaptically, affect the inhibition of glycine-mediated IPSCs in DH neurons (Baba et al., 2001; Ahmadi et al., 2001). The contribution of EP_3 receptors is more complex due to multiple variants of this receptor subtype (i.e. EP_{3A}, EP_{3B}, EP_{3C}) and signaling pathways. Some of EP_3 receptors in the DH are positively coupled to NOS with the resulting NO (Matsumura et al., 2005) amplifying PG synthesis and PG-dependent allodynia

(Bredt and Snyder, 1992; Mollace et al., 1995; Hefferan and Loomis, 2004). The up-regulation of EP_3 variants negatively coupled to adenylyl cyclase explains the previously reported increase in glutamate release from slices of SNL animals pretreated with the EP receptor antagonist, SC-51322 and evoked by high concentrations of PGE_2 (O'Rielly and Loomis, 2006). The reduced expression of EP_{3C} receptors in neuronal cultures using antisense oligonucleotides also abolished PGE_2-evoked cAMP production and the corresponding release of substance P and calcitonin gene-related peptide (Southall and Vasko, 2001); known excitatory neuromodulators. The time-course and pattern of SNL-induced changes in the present study correspond with the increased sensitivity to PGE_2, especially the up-regulation of the EP_1 (i.e. increased neurotransmitter release) and EP_2 (i.e. glycinergic disinhibition) receptor subtypes.

Pre-emptive treatment with i.t. L-NAME, but not D-NAME or vehicle, significantly attenuated the SNL-induced increase in EP_1, EP_2, and EP_3 receptors and explains, at least in part, the normalization of pharmacodynamic sensitivity to PGE_2 three days later. This stereo-specific effect, like that observed with the SNL-induced expression of COX-1 and COX-2, was confined to the ipsilateral L-DH. This is the first report demonstrating that spinal NO, generated at the onset of SNL, effects the early expression of both the enzymes responsible for PG synthesis and the receptor subtypes mediating the actions of PGE_2 in the affected spinal cord. This points to the robust up-regulation of those elements essential for PG-signaling early after SNL and explains: a) the abrupt onset of

spinal PG-dependent allodynia (Hefferan et al., 2003a, b; O'Rielly and Loomis, 2006-2008); b) the consistent and dramatic pre-emptive effect of i.t. L-NAME (O'Rielly and Loomis, 2008); c) the similar effectiveness of L-NAME and S(+)-ibuprofen as pre-emptive treatments (O'Rielly and Loomis, 2007, 2008); and d) their additive interaction in the SNL model, suggesting a convergence of spinal NO- and PG-signaling in the affected spinal cord (Hefferan and Loomis, 2004). The exact mechanism by which spinal NO affects the SNL-induced expression of EP receptors in the L-DH remains to be determined.

Spinal PGE_2/EP_2 signaling inhibits glycinergic neurotransmission in the DH (Baba et al., 2001) via the protein kinase A-dependent phosphorylation of the GlyRα3 subunit (Ahmadi et al., 2001). That the resulting disinhibition yields a robust and selective allodynic state is clearly evident after the i.t. injection of low (i.e. sub-convulsive) doses of strychnine (Yaksh, 1989). There is also evidence that glycine receptor density in the spinal DH is significantly decreased in allodynic animals following sciatic nerve constriction injury (Simpson & Huang, 1998). If the SNL-induced changes in EP_2 receptor expression using Western analysis are directly related to the increased sensitivity to PGE_2 (and not just a non-specific response to SNL), and the normalization of EP_2 expression is functionally linked to the pre-emptive effect of i.t. L-NAME, then corresponding inverse changes in the expression of GlyRα3 would be predicted. Consistent with this hypothesis, Glyα3R expression was significantly decreased three days after SNL compared to sham-controls. As with other SNL-induced changes, this

effect was confined to the ipsilateral L-DH. These results are in accord with spinal glycinergic control of low-threshold afferent input in the DH (Keller et al., 2001; Lynch, 2004; Legendre, 2001), and the early development of spinal PG-dependent hypersensitivity and allodynia in the SNL model (Hefferan et al., 2003a, b; O'Rielly and Loomis, 2006-2008). This functional relationship is further supported by the pre-emptive effect of i.t. L-NAME on the SNL-induced expression of spinal EP_2 and Glyα3R. This is the first report demonstrating the down-regulation of Glyα3R in the L-DH in the SNL model, and the critical role of spinal NO in the development of abnormal PGE_2/EP_2/Glyα3R signaling early after nerve injury.

The results of the present study are summarized in Figure 6. Spinal NO, generated at the onset of SNL, facilitates the SNL-induced activation of NFκB in the ipsilateral L-DH (O'Rielly and Loomis, 2008). As a modulator of COX gene transcription, NFκB triggers the up-regulation of COX-2 and, in turn, the increased synthesis of PGE_2. Spinal NO also contributes to the increased allodynic effect of PGE_2 following SNL. This is related to the up-regulation of EP receptors (EP_1, EP_2, and EP_3), corresponding changes in effector molecules (e.g. Glyα3R) and exaggerated spinal excitability (e.g. enhanced glutamate release, glycine disinhibition). The direct (i.e. PG-independent) effect of NO on spinal excitability is also depicted. Clearly, spinal NO represents a critical factor in the development of PG-dependent allodynia early after SNL.

8.0 DISCUSSION

The results of this research support the hypothesis that spinal PG play an early pathogenic role in the emergence and early maintenance of spinal hyperexcitability and mechanical allodynia following SNL. They revealed a time-dependent shift in the COX isoform primarily responsible for spinal PG synthesis, identified the inter-relationships between NFκB, PG and NO synthesis/signaling in affected spinal cord, and characterized the nature of the interaction between PG and NO in this model of NP. The major results of this research are illustrated in Figure 8.1.

Previous work has indicated that SNL elicits stable allodynia, expressed as a marked reduction in the PWT and protective posturing of the affected dermatomes (Kim and Chung, 1992; Hefferan et al., 2003a, b). However, it was impossible to ascertain which fibre types are affected by SNL based on PWT alone. To this end, A- and C-fiber mediated reflex responses were recorded from the ipsilateral biceps femoris of SNL animals. EMG analysis revealed a significant increase in excitability as evidenced by the decrease in activation threshold, increase in evoked activity to single (i.e. activity before spatial and temporal summation) and repetitive stimulation, and the presence of windup; sustained input known to trigger the neural generation of spinal prostanoids (Millan, 1999; Vanegas and Schaible, 2001). These electrophysiological changes, characteristic of peripheral and central sensitization, paralleled the development of mechanical allodynia. This thesis research indicated that the

effect of SNL, including its modulation by spinal PG is reflected electrophysiologically in the biceps femoris reflex. However, the duration of these electrophysiological abnormalities are unknown as longest time investigated after SNL was ten days. The effect on A-fiber activity is especially interesting given that SNL-induced mechanical allodynia is elicited by impulses carried along surviving Aβ-afferents (Kim and Chung, 1992). The results were highly reproducible within treatment groups, and qualitatively similar to those reported in other experimental nerve (Meyerson et al., 1995; Colvin et al., 1996; Okamoto et al., 2001; Kohno et al., 2003), and spinal cord (Bennett et al., 2004) injury models. The latter suggests broader applicability of the present results to the early nerve-injured state.

The AFRR and CFRR were evoked by electrical stimulation using a stimulation paradigm designed to activate low-threshold A-fibers. No attempt was made to separate the A-fiber subtypes in the present study as altered conductivity to injured and uninjured A-fibers (Laird and Bennett, 1993) make it difficult, if not impossible, to accurately determine the contribution of surviving Aβ-afferents to the AFRR and CFRR. Future studies should include the use of mechanical stimuli (i.e. von Frey filaments, mild brushing) to activate low-threshold A-fibers.

The spinal hyperexcitability (and mechanical allodynia) induced by SNL was unlikely to be the result of "denervation" supersensitivity. SNL involves tight ligation of the L5/L6 spinal roots and, unlike nerve transection, leaves many

afferent and efferent fibres intact (i.e. uninjured and functional) (Kim and Chung, 1992). Moreover, SNL primarily affects smaller diameter primary afferent fibers compared to larger fibers including those innervating muscle. This is consistent with the protective posturing of the affected hind paw which remained responsive to low-threshold mechanical stimulation following SNL. An abrupt and coordinated response to von Frey filaments argues against the development of significant motor deficits. While an effect of SNL on motor neurons cannot be completely excluded, afferent sensory input appears to be preferentially affected in this model.

SNL-induced allodynia consists of two distinct phases: a) an early PG-dependent phase lasting approximately seven to ten days; and b) a delayed PG-independent phase lasting up to seventy days (Hefferan et al., 2003b). The former was first investigated using the active S(+)- and inactive R(-)-enantiomers of IBU, as well as COX-1 and COX-2 selective inhibitors given immediately after SNL (i.e. pre-emptive treatment) or three days later (i.e. time of maximum PG-dependent spinal hyperexcitability and mechanical allodynia). These experiments confirmed the critical role of spinal COX activity in the development and early maintenance of spinal hyperexcitability and allodynia, beginning at the onset of SNL, and are consistent with a time-dependent shift in the dominant isoform responsible for increased PG synthesis (i.e. COX-1 to COX-2) in the affected spinal segments (Hefferan et al., 2003a, b). As with all such experiments, drug selectivity is an issue requiring caution in the interpretation of the data.

The IC_{50} values for SC-560, SC-236 and S(+)-IBU against COX-1 and COX-2 are summarized in Table 8.1. *In vitro*, SC-560 and SC-236 are equipotent against COX-1 and COX-2, respectively, and exhibit comparable potency ratios for their preferred target (Gierse et al., 1996; Yaksh et al., 2001).

Table 8.1. IC50 values determined against recombinant human cyclooxygenases (hCOX).

Inhibitor	hCOX-1	hCOX-2	hCOX-1:hCOX-2	hCOX-2:hCOX-1
S(+)-IBU	3.3 μM	3.8 μM	0.87	1.15
SC-560	~5 μM	160 μM	0.03	32
SC-236	17 μM	5 nM	3400	0.0003

In contrast, S(+)-IBU is equipotent against both COX isoforms and considerably less potent than either SC-560 or SC-236. Accordingly, similar effective i.t. doses of SC-560 and SC-236 were used throughout this research. Given that both significantly inhibited spinal hyperexcitability and mechanical allodynia, albeit on different days after SNL, the results indicate that: a) effective COX inhibition was achieved in the spinal cord by each drug; and b) their selectivity for COX-1 and COX-2 was preserved at the i.t. doses used. The doses of S(+)-IBU, SC-560 and SC-236 selected for our experiments were comparable to those used by other laboratories (Dirig et al., 1997; Hall et al., 1999; Deleo et al., 2000; Yaksh et al., 2001; Yamamoto and Nozaki-Taguchi, 2002). Thus, we conclude that the

difference in effect with SC-560 and SC-236 reflects the distinct contributions of COX-1 and COX-2 in the emergence and early maintenance of mechanical allodynia, respectively.

These results were subsequently corroborated by expression studies of COX-1 and COX-2 protein in the affected spinal cord. Western analysis confirmed the preferential expression of COX-1 one day after SNL and COX-2 three days after SNL in ipsilateral L-DH. This validated the results with SC-560 and SC-236, and is in accord with earlier reports of SNL-induced changes in spinal COX expression (Zhao et al., 2000). Recognizing that increased protein expression does not necessarily reflect increased enzyme activity (i.e. PG synthesis), efforts to measure spinal COX-1 and COX-2 activity directly were made. Ultimately, these proved unsuccessful largely due to the composition of spinal tissue. However, changes in COX expression are frequently associated with changes in PG synthesis. For example, 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). Likewise, the concentration of brush-evoked PGE_2 in spinal CSF was significantly increased three days after SNL (Hefferan et al., 2003a) corresponding to the time of maximum COX-2 expression in the L-DH. While COX activity data would certainly strengthen the connection between enhanced COX expression and spinal PG synthesis in the SNL model, we believe the

current body of evidence supports the critical role of spinal PG in the development of spinal hyperexcitability and mechanical allodynia.

Apart from its constitutive expression, the dominant role of COX-1 in spinal PG synthesis is also explained by enzyme kinetics and the intracellular conditions favouring COX-1 activity immediately following nerve injury. While high concentrations of arachidonic acid (e.g. >10 μM) shunt production through the COX-1 pathway (Murakami et al., 1999), low concentrations (e.g. <1 μM) favour PG synthesis via COX-2. The rapid and repetitive firing of injured nerves immediately following SNL (Chaplan et al, 2003; Han et al, 2000) would be expected to generate high intracellular concentrations of free arachidonic acid in affected spinal cord cells, including neurons. In this regard, cell activation studies using cytokines or lipopolysaccharide showed an initial burst in COX-1 activity (10 to 30 min duration) followed by delayed COX-2 activity (Smith et al., 2000). This, of course, does not obviate the importance of other factors such the subcellular distribution and, as demonstrated in the present study, the time-course of expression of COX1 and COX-2 protein.

The ability of PG to effect responses at exceptionally low concentrations is well documented. Indeed, discrepancies between the active concentrations of PG and their Kd values at corresponding receptors are not uncommon (Garcia-Perez and Smith, 1984; Sugimoto et al., 1993; Namba et al., 1994). While the mechanisms underlying this phenomenon remain unclear, PG routinely elicit biologic responses at concentrations well below their reported Kd values, even

under normal conditions. The results of the present study suggest that PG can trigger, either directly or indirectly, their own synthesis in the affected spinal cord three days after SNL. Likewise, there was clear evidence of exaggerated sensitivity to spinal PGE_2 *in vitro* and *in vivo* which paralleled the development of spinal hyperexcitability and mechanical allodynia in the acute nerve injured state. From a pharmacodynamic perspective, the marked increase in potency of PGE_2 argues strongly for amplification at the EP-receptor-effector level. The latter is supported by the increased expression of $EP_{1\text{-}3}$ receptors and Glyα3R in the ipsilateral L-DH three days after SNL. Although the signal transduction mechanisms coupled to these receptors were not measured in this study, the concurrent normalization of both receptor expression and sensitivity to spinal PGE_2 (described below) strongly suggests a cause and effect relationship. Spinal EP_2 receptors, which are functionally linked to the Glyα3R, represent a specific target by which spinal PG disrupt endogenous glycinergic tone (i.e. disinhibition). Collectively, the *in vitro* and *in vivo* results of the present research are consistent with exaggerated PG synthesis and signaling in the ipsilateral L-DH three days after SNL. They highlight the robust up-regulation of elements essential for increased PG-synthesis/signaling, and explain the abrupt onset of spinal PG-dependent allodynia in the SNL model (Hefferan et al., 2003a, b).

NFκB has a well documented role in neuronal cell survival, apoptosis, and synaptic plasticity (Shishodia & Aggarwal, 2002; Piva et al., 2006; Bubici et al., 2006; Albensi & Mattson, 2000). It also modulates transcription of the genes

coding for COX and NOS (Tsatsanis et al., 2006), and is affected by nerve injury (Sakaue et al., 2001; Pollock et al., 2005; Bethea et al., 1998). SNL induced a sustained increase in activated NFκB in the ipsilateral L-DH which preceded the: a) induction of spinal COX-2; b) development of spinal PG-dependent hyperexcitability; and c) reduction in PWT in the affected hind limb. Pharmacological blockade of spinal NFκB activity demonstrated the critical role of spinal NFκB in the subsequent up-regulation of spinal COX-2, and the exaggeration of A- and to a lesser extent C-fiber mediated responses in the affected hind limb. The SNL-induced changes in the AFRR, and their sensitivity to the blockade of spinal NFκB activity, are especially relevant given the predominant role of functional A-fibers in mechanical allodynia (Zimmerman, 2001).

PG promote neuronal depolarization by modulating the kinetics of Na^+ and K^+ channels, and by increasing intracellular Ca^{2+} levels (Vasko, 1995; Chen & Bazan, 2005; Baba et al., 2001; Sugimoto & Narumiya, 2007); factors known to promote the liberation of NFκB from IkB in the cytoplasm. Thus, PG synthesized at the onset of nerve injury could promote the SNL-induced activation of NFκB and its downstream transcriptional effects. In this regard, the pre-emptive blockade of COX activity with i.t. S(+)-IBU not only attenuated the spinal activation of NFκB, but it prevented the induction of spinal COX-2 and the development of spinal hyperexcitability and allodynia three days later. These results further illustrate the importance of spinal PG to the development of SNL-

induced allodynia, and indicate possible mechanisms by which this occurs. Promoting the activation and transcriptional effect of NFκB in the affected spinal cord also explains, at least in part, how pre-emptive treatment with i.t. S(+)-IBU prevented the up-regulation of COX-2 protein in the affected L-DH of SNL animals, and how i.t. S(+)-IBU, given up to 2 h after SNL, prevented mechanical allodynia for at least twenty-one days (Hefferan et al., 2003a,b).

Notwithstanding the clear evidence for an early role of spinal PG in the SNL model, the activation of NFκB inevitably triggers many other effects. In particular, NFκB directly and indirectly modulates the transcription of iNOS and nNOS expression following spinal cord injury (Lowenstein et al., 1993; Mabuchi et al., 2003; Miscusi et al., 2006). Their product, NO, is known to positively interact with spinal PG early after SNL (Hefferan and Loomis, 2004). The parallel increase in NFκB and NOS protein in the affected L-DH, and their mutual attenuation by i.t. PDTC indicate that the activation of NFκB is a critical antecedent to the SNL-induced up-regulation of nNOS and iNOS protein in the acute nerve-injured state. Like spinal PG synthesis/signaling, the stereo-selective inhibition of NOS activity blocked the activation of NFκB and the up-regulation of NOS isoforms in the affected spinal cord, and prevented the development of spinal hyperexcitability and mechanical allodynia in the SNL model. The results suggest that spinal NO, generated by constitutive NOS at the onset of nerve injury, also promotes the injury-induced activation of NFκB, and underscores the importance of spinal NO-synthesis/signaling to the development and early

maintenance of SNL-induced spinal hyperexcitability and mechanical allodynia. They also explain how i.t. L-NAME, a stereo-specific inhibitor of NOS activity, affected the downstream expression of spinal NOS.

A striking feature of this thesis research is the similarity in spinal PG- and NO-synthesis/signaling early after SNL, their comparable but independent effects on NFκB in the spinal cord, and their influence on the subsequent development of spinal hyperexcitability and mechanical allodynia. One obvious question arising from these observations is whether they are simply coincidental, and possibly redundant outcomes to nerve injury, or whether they comprise a coordinated (i.e. interdependent) response. That pre-emptive treatment with i.t. L-NAME significantly attenuated all PG-dependent features of SNL-induced allodynia supports the latter proposition. It suggests a convergence of spinal NO- and PG-signaling in the affected spinal cord, consistent with and building upon previous demonstrating an additive interaction in the SNL model (Hefferan and Loomis, 2004). It also illustrates the importance of spinal NO, generated at the onset of SNL, to spinal PG synthesis/signaling in the acute nerve injured state. However, the mechanism(s) by which spinal NO affects the expression of spinal EP receptors in the SNL model remains to be determined.

The results of this research raise a number of other questions. For example, can pre-emptive treatment with a systemic (preferably oral) COX inhibitor prevent the development of mechanical allodynia, and would it be as affective as the i.t route? If so, what is the optimal dosing schedule, and how long

after nerve injury does pre-emptive treatment with these drugs remain effective? Given that systemic administration requires higher drug dosing, would the resulting adverse effects limit their therapeutic effectiveness? Using a combination of systemic low-dose COX and NOS inhibitors (or other agents such as free radical scavengers) represents an alternate and perhaps more effective pharmacologic strategy. Including a non-selective EP receptor antagonist in the cocktail would also warrant consideration based on the results of this thesis research.

The evidence for a PG-dependent phase in SNL-induced spinal hyperexcitability and mechanical allodynia is largely pharmacologic (Hefferan et al., 2003a, b). It is unknown, whether COX-1 or COX-2 deficient mice develop PG-dependent spinal hyperexcitability and mechanical allodynia following SNL, and how this compares to the results obtained using the rat. Given the striking similarity of SNL on spinal PG- and NO-synthesis/signaling early after nerve injury, it's possible that spinal NO effects spinal hyperexcitability and allodynia indirectly by enhancing spinal PG-synthesis/signaling. In future studies, this and related hypotheses could also be tested using COX-deficient mice.

The cellular actions attributed to spinal PG in the acute nerve injured state also require further elucidation. Apart from Glyα3R, what other downstream targets meditate the pro-allodynic effects of spinal PG, and on what cell types are they expressed? What other spinal PG contribute to the development of spinal hyperexcitability and allodynia in the acute nerve injured state? Two obvious

possibilities are $PGF_{2\alpha}$ and PGI_2 which have been implicated in normal pain processing in the spinal cord. Given that the cellular effects of NFκB are influenced by specific co-factors (including those which control apoptosis and cell survival), what co-factors are up-regulated after SNL, and how are they affected by pre-emptive treatment with COX and NOS inhibitors?

A more complete understanding of the critical signaling events occurring early after nerve injury, their interaction and relevant pharmacology is essential for effective pre-emptive treatment strategies in humans presenting with nerve injury.

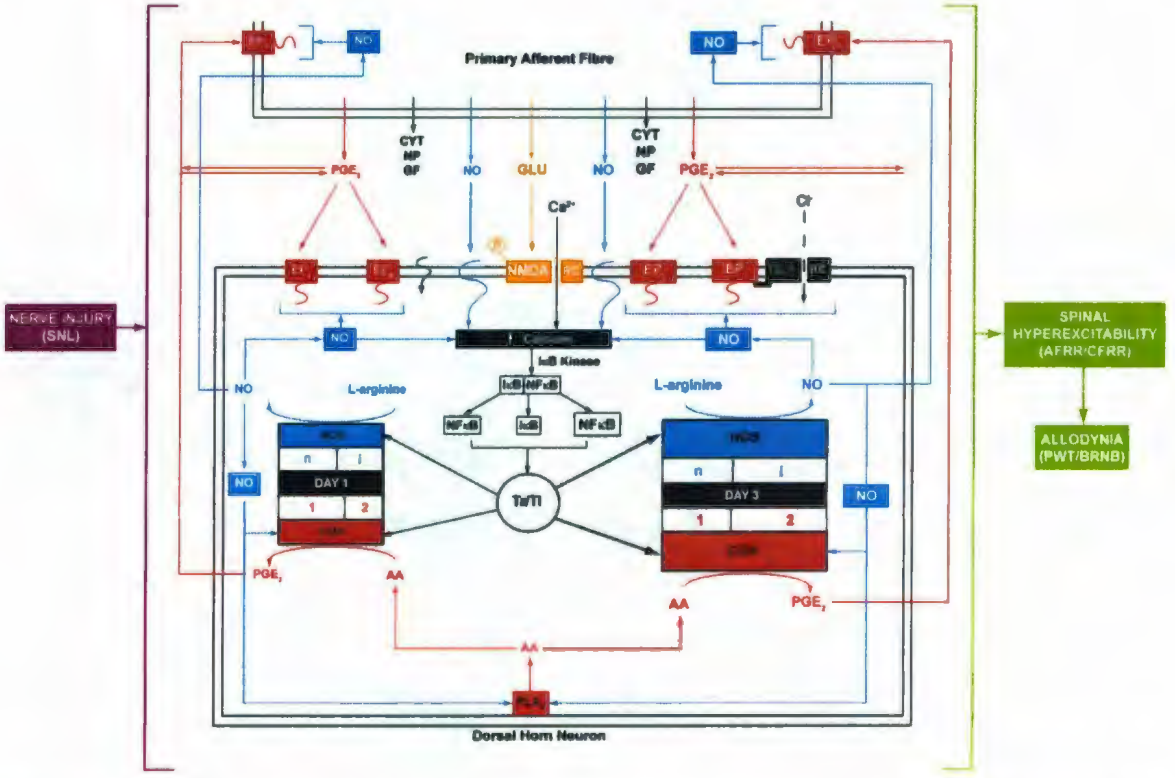

Figure 8.1. A summary of the SNL-induced changes to spinal PG and NO effecting early hyperexcitability and allodynia in the rat. Nerve injury-induced synthesis/release of PG, NO, glutamate and other mediators (e.g. cytokines, growth factors and neuropeptides) from presynaptic elements in the spinal cord drive dorsal horn cells through their respective signaling pathways. This includes the: a) activation of NFκB-mediated transcription and subsequent induction of COX and NOS; b) concurrent up-regulation of spinal $EP_{1\text{-}3}$ receptors; c) the brush-evoked release of spinal PGE_2; and d) a marked increase in the pharmacodynamic sensitivity to PGE_2. These adaptations to nerve injury underlie the early development of spinal PG-dependent hyperexcitability and allodynia in this model. Acting in concert, these adaptations serve to amplify afferent sensory

input and are conducive to an early "vicious cycle" between presynaptic and dorsal horn cells. The exaggerated synthesis and release of PG and NO from both presynaptic and dorsal horn cells initiate an early "vicious cycle"; an outcome reinforced by the concurrent up-regulation of spinal $EP_{1\text{-}3}$ receptors and a marked increase in pharmacodynamic sensitivity to PGE_2. The time-dependent increase in spinal PG/NO, COX/NOS, and EP receptors is depicted by the enlarged font or box size. Also shown is the transition from primarily COX-1 to COX-2 and nNOS to iNOS synthesis of PG and NO, respectively three days after SNL. Comparable SNL-induced changes in presynaptic cells have been omitted for clarity.

9.0 REFERENCES

Abbadie C: Chemokines, chemokine receptors and pain. Trends Immunol 2005; 26: 529-534.

Ahern GP, Klyachko VA, Jackson MB: cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. Trends Neurosci 2002; 25: 510-517.

Ahmadi S, Lippross S, Neuhuber WL, Zeilhofer HU: PGE(2) selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. Nat Neurosci 2002; 5: 34-40.

Albensi BC, Mattson MP: Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. Synapse 2000; 35: 151-159.

Aley KO, McCarter G, Levine JD: Nitric oxide signaling in pain and nociceptor sensitization in the rat. J Neurosci 1998; 18: 7008-7014.

Ambs P, Baccarini M, Fitzke E, Dieter P: Role of cytosolic phospholipase A_2 in arachidonic acid release of rat-liver macrophages: regulation by Ca^{2+} and phosphorylation. Biochem J 1995; 311: 189-195.

Araujo MC, Sinnott CJ, Strichartz GR: Multiple phases of relief from experimental mechanical allodynia by systemic lidocaine: responses to early and late infusions. Pain 2003; 103: 21-29.

Arner S, Meyerson BA: Lack of analgesic effect of opioids on neuropathic and idiopathic forms of pain. Pain 1988; 33: 11-23.

Attal N, Bouhassira D: Mechanisms of pain in peripheral neuropathy. Acta Neurol Scand Suppl 1999; 173: 12-24; discussion 48-52.

Baba H, Kohno T, Moore KA, Woolf CJ: Direct activation of rat spinal dorsal horn neurons by prostaglandin E2. J Neurosci 2001; 21: 1750-1756.

Bai YH, Takemitsu M, Atsuta Y, Matsuno T: Peripheral mononeuropathy induced by loose ligation of the sciatic nerve in the rat: behavioral, electrophysiological and histopathologic studies. Exp Anim 1999; 48: 87-94.

Bao L, Wang HF, Cai HJ, Tong YG, Jin SX, Lu YJ, Grant G, Hökfelt T, Zhang X: Peripheral axotomy induces only very limited sprouting of coarse myelinated afferents into inner lamina II of rat spinal cord. Eur J Neurosci 2002; 16: 175-185.

Barkett M, Gilmore TD: Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene 1999; 18: 6910-6924.

Baron R, Saguer M: Postherpetic neuralgia: Are C-nociceptors involved in signaling and maintenance of tactile allodynia? Brain 1993; 116: 1477-1496.

Beiche F, Brune K, Geisslinger G, Goppelt-Struebe M: Expression of cyclooxygenase isoforms in the rat spinal cord and their regulation during adjuvant-induced arthritis. Inlamm Res 1998b; 47: 482-487.

Beiche F, Klein T, Nusing R, Neuhuber W, Goppelt-Struebe M: Localization of cyclooxgenase-2 and prostaglandin E2 receptor EP3 in the rat lumbar spinal cord. J Neuroimmunol 1998a; 89: 26-34.

Bennett DJ, Sanelli L, Cooke CL, Harvey PJ, Gorassini MA: Spastic long-lasting reflexes in the awake rat after sacral spinal cord injury. J Neurophysiol 2004; 91: 2247-2258.

Berger A, Dukes EM, Oster G: Clinical characteristics and economic costs of patients with painful neuropathic disorders. J Pain 2004; 5: 143-149.

Bethea JR, Castro M, Keane RW, Lee TT, Dietrich WD, Yezierski RP: Traumatic spinal cord injury induces nuclear factor-kappaB activation. J Neurosci 1998; 18: 3251-3260.

Beydoun A, Backonja MM: Mechanistic stratification of antineuralgic agents. J Pain Symptom Manage 2003; 25: S18-S30.

Bezzi P, Carmignoto G, Pasti L, Vesce S, Rossi D, Rizzini BL, Pozzan T, Volterra A: Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. Nature 1998; 391: 281-285.

Bhattacharya M, Peri KG, Almazan G, Ribeiro-da-Silva A, Shichi H, Durocher Y, Abramovitz M, Hou X, Varma DR, Chemtob S: Nuclear localization of prostaglandin E_2 receptors. Proc Natl Acad Sci USA 1998; 95: 15792-15797.

Bredt DS, Snyder SH: Nitric oxide, a novel neuronal messenger. Neuron 1992; 8: 3-11.

Breyer RM, Bagdassarian CK, Myers SA, Breyer MD: Prostanoid receptors: subtypes and signaling. Annu Rev Pharmacol Toxicol 2001; 41: 661-690.

Bubici C, Papa S, Dean K, Franzoso G: Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. Oncogene 2006; 25: 6731-6748.

Bulka A, Hao JX, Wiesenfeld-Hallin Z: Response characteristics of cutaneous mechanoreceptors in neuropathic rats. Neurosci Lett 2002; 317: 89-92.

Bustamante D, Paeile C, Willer JC, Le-Bars D: Effects of intrathecal or intracerebroventricular administration of nonsteroidal anti-inflammatory drugs on a C-fiber reflex in rats. J Pharmacol Exp Ther 1997; 281: 1381-1391.

Calcutt NA, Backonja MM: Pathogenesis of pain in peripheral diabetic neuropathy. Curr Diab Rep 2007; 7: 429-434.

Campbell JN, Raja SN, Meyer RA, Mackinnon SE: Myelinated afferents signal the hyperalgesia associated with nerve injury. Pain 1988; 32: 89-94.

Chalimoniuk M, Glowacka J, Zabielna A, Eckert A, Strosznajder JB: Nitric oxide alters arachidonic acid turnover in brain cortex synaptoneurosomes. Neurochem Int 2006; 48: 1-8.

Chamberlain S, Lewis DM: Contractile characteristics and innervation ratio of rat soleus motor units. J Physiol 1989; 412: 1-21.

Chan BS, Satriano JA, Pucci M, Schuster VL: Mechanism of prostaglandin E2 transport across the plasma membrane of HeLa cells and Xenopus oocytes expressing the prostaglandin transporter "PGT". J Biol Chem 1998; 273: 6689-6697.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994; 53: 55-63.

Chapman V, Suzuki R, Dickenson AH: Electrophysiological characterization of spinal neuronal response properties in anaesthetized rats after ligation of spinal nerves L5-L6. J Physiol 1998; 507: 881-894.

Chen C, Bazan NG: Lipid signaling: sleep, synaptic plasticity, and neuroprotection. Prostaglandins Other Lipid Mediat 2005; 77: 65-76.

Choi Y, Raja SN, Moore LC, Tobin JR: Neuropathic pain in rats is associated with altered nitric oxide synthase activity in neural tissue. J Neurol Sci 1996; 138: 14-20.

Chopra B, Giblett S, Little JG, Donaldson LF, Tate S, Evans RJ, Grubb BD: Cyclooxygenase-1 is a marker for a subpopulation of putative nociceptive neurons in rat dorsal root ganglia. Eur J Neurosci 2000; 12: 911-920.

Clemens JA: Cerebral ischemia: gene activation, neuronal injury, and the protective role of antioxidants. Free Radic Biol Med 2000; 28: 1526-1531.

Coceani F, Viti A: Responses of spinal neurons to iontophoretically applied prostaglandin E1 in the frog. Can J Physiol Pharmacol 1975; 53: 273-284.

Coleman RA, Kennedy I, Humphrey PPA, Bunce K, Lumley P: Prostanoids and their receptors. In Comprehensive Medicinal Chemistry, ed. JC Emmet, pp. 643-714, 1990; Oxford, UK: Pergamon.

Coleman RA, Smith WL, Naruimya S: International Union of Pharmacology classification of prostanid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994; 46: 205-229.

Colvin LA, Mark MA, Duggan AW: Bilaterally enhanced dorsal horn postsynaptic currents in a rat model of peripheral mononeuropathy. Neurosci Lett 1996; 207: 29-32.

Davidge ST, Baker PN, Laughlin MK, Roberts JM: Nitric oxide produced by endothelial cells increases production of eicosanoids through activation of prostaglandin H synthase. Circ Res 1995; 77: 274-283.

Davis MP, Walsh D: Epidemiology of cancer pain and factors influencing poor pain control. Am J Hosp Palliat Care 2004; 21: 137-142.

De S, Trigueros MA, Kalyvas A, David S: Phospholipase A_2 plays an important role in myelin breakdown and phagocytosis during Wallerian degeneration. Mol Cell Neurosci 2003; 24: 753-765.

de Sousa BM, Horrocks LA: Development of rat spinal cord. Dev Neurosci 1979; 2: 115-121.

Dolan S, Nolan AM: N-methyl D-aspartate induced mechanical allodynia is blocked by nitric oxide synthase and cyclooxygenase-2 inhibitors. Neuroreport 1999; 10: 449-452.

Dudhgaonkar SP, Tandan SK, Kumar D, Naik AK, Raviprakash V: Ameliorative effect of combined administration of inducible nitric oxide synthase inhibitor with

cyclooxygenase-2 inhibitors in neuropathic pain in rats. Eur J Pain 2007; 11: 528-534.

Durrenberger PF, Facer P, Gray RA, Chessell IP, Naylor A, Bountra C, Banati RB, Birch R, Anand P: Cyclooxygenase-2 (Cox-2) in injured human nerve and a rat model of nerve injury. J Peripher Nerv Syst 2004; 9: 15-25.

Dworkin RH, Backonja M, Rowbotham MC, Allen RR, Argoff CR, Bennett GJ, Bushnell MC, Farrar JT, Galer BS, Haythornthwaite JA, Hewitt DJ, Loeser JD, Max MB, Saltarelli M, Schmader KE, Stein C, Thompson D, Turk DC, Wallace MS, Watkins LR, Weinstein SM: Advances in neuropathic pain: diagnosis, mechanisms, and treatment recommendations. Arch Neurol 2003; 60: 1524-1534.

Dworkin RH, Gnann JW Jr, Oaklander AL, Raja SN, Schmader KE, Whitley RJ: Diagnosis and assessment of pain associated with herpes zoster and postherpetic neuralgia. J Pain 2008; 9: S37-S44.

Ebersberger A, Buchmann M, Ritzeler O, Michaelis M, Schaible HG: The role of spinal nuclear factor-kappa B in spinal hyperexcitability. Neuroreport 2006; 17: 1615-1618.

Ebersberger A, Grubb BD, Willingale HL, Gardiner NJ, Nebe J, Schaible HG: The intraspinal release of prostaglandin E_2 in a model of acute arthritis is accompanied by an up-regulation of cyclo-oxygenase-2 in the spinal cord. Neuroscience 1999; 93: 775-781.

Egan LJ, Toruner M: NF-kappaB signaling: pros and cons of altering NF-kappaB as a therapeutic approach. Ann N Y Acad Sci 2006; 1072: 114-122.

Egan RW, Paxton J, Kuehl FA Jr: Mechanism for irreversible self-deactivation of prostaglandin synthetase. J Biol Chem 1976; 251: 7329-7935.

Eide PK: Pathophysiological mechanisms of central neuropathic pain after spinal cord injury. Spinal Cord 1998; 36: 601-612.

Ernfors P, Rosario CM, Merlio JP, Grant G, Aldskogius H, Persson H: Expression of mRNAs for neurotrophin receptors in the dorsal root ganglion and spinal cord during development and following peripheral or central axotomy. Brain Res Mol Brain Res 1993; 17: 217-226.

Fabi F, Calabrese R, Stati T, del Basso P: Nitric oxide (NO) modulation of PAF-induced cardiopulmonary action: interaction between NO synthase and cyclo-oxygenase-2 pathways. Br J Pharmacol 2001; 134: 777-788.

Farooque M, Hillered L, Holtz A, Olsson Y: Changes of extracellular levels of amino acids after graded compression trauma to the spinal cord: An experimental study in the rat using microdialysis. J Neurotrauma 1996; 13: 537-548.

Ferreira SH, Lorenzetti BB: Intrathecal administration of prostaglandin E_2 causes sensitization of the primary afferent neuron via the spinal release of glutamate. Inflamm Res 1996; 45: 499-502.

Foster E, Robertson B, Fried K: trkB-like immunoreactivity in rat dorsal root ganglia following sciatic nerve injury. Brain Res 1994; 659: 267-271.

Funk CD: Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 2001; 294: 1871-1875.

Gao X, Kim HK, Chung JM, Chung K: Enhancement of NMDA receptor phosphorylation of the spinal dorsal horn and nucleus gracilis neurons in neuropathic rats. Pain 2005; 116: 62-72.

Garrison CJ, Dougherty PM, Kajander KC, Carlton SM: Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. Brain Res 1991; 565: 1-7.

Garry MG, Richardson JD, Hargreaves KM: Sodium nitroprusside evokes the release of immunoreactive calcitonin gene-related peptide and substance P from dorsal horn slices via nitric oxide-dependent and nitric oxide-independent mechanisms. J Neurosci 1994; 14: 4329-4337.

Garthwaite J, Charles SL, Chess-Williams R: Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intracellular messenger in the brain. Nature 1988; 336: 385-387.

Giummarra MJ, Gibson SJ, Georgiou-Karistianis N, Bradshaw JL: Central mechanisms in phantom limb perception: the past, present and future. Brain Res Rev 2007; 54: 219-232.

Gold MS, Reichling DB, Shuster MJ, Levine JD: Hyperalgesic agents increase a tetrodotoxin-resistant Na+ current in nociceptors. Proc Natl Acad Sci USA 1996; 93: 1108-1112.

Gonzalez S, Labombarda F, Deniselle MCG, Saravia FE, Roig P, De Nicola AF: Glucocorticoid effects of Fos immunoreactivity and NADPH-diaphorase histochemical staining following spinal cord injury. Brain Res 2001; 912: 144-153.

González-Forero D, Portillo F, Gómez L, Montero F, Kasparov S, Moreno-López B: Inhibition of resting potassium conductances by long-term activation of the NO/cGMP/protein kinase G pathway: a new mechanism regulating neuronal excitability. J Neurosci 2007; 27: 6302-6312.

Goodwin DC, Landino LM, Marnett LJ: Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis. FASEB J 1999; 13: 1121-1136.

Goppelt-Struebe M, Beiche F: Cyclooxygenase-2 in the spinal cord: localization and regulation after a peripheral inflammatory stimulus. Adv Exp Med Biol 1997; 433: 209-212.

Gordh T, Sharma HS, Alm P, Westman J: Spinal nerve lesion induces upregulation of neuronal nitric oxide synthase in the spinal cord. An immunohistochemical investigation in the rat. Amino Acids 1998; 14: 105-112.

Gracely RH, Lynch SA, Bennett GJ: Painful neuropathy: altered central processing maintained dynamically by peripheral input. Pain 1992; 51: 175-194.

Grilli M, Memo M: Nuclear factor-kappaB/Rel proteins: a point of convergence of signalling pathways relevant in neuronal function and dysfunction. Biochem Pharmacol 1999; 57: 1-7.

Haley JE, Dickenson AH, Schachter M: Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociception in the rat. Neuropharmacology 1992; 31: 251-258.

Hall SR, Milne B, Loomis C: Spinal action of ketorolac, S(+)- and R(-)-ibuprofen on non-noxious activation of the catechol oxidation in the rat locus coeruleus: evidence for a central role of prostaglandins in the strychnine model of allodynia. Anesthesiology 1999; 90: 165-173.

Hao JX, Blakeman KH, Yu W, Hultenby K, Xu XJ, Wiesenfeld-Hallin Z: Development of a mouse model of neuropathic pain following photochemically induced ischemia in the sciatic nerve. Exp Neurol 2000; 163: 231-238.

Hao JX, Xu XJ: Treatment of a chronic allodynia-like response in spinally injured rats: effects of systemically administered excitatory amino acid receptor antagonists. Pain 1996; 66: 279-285.

Hara S, Miyata A, Yokoyama C, Inoue H, Brugger R, Lottspeich F, Ullrich V, Tanabe T: Isolation and molecular cloning of prostacyclin synthase from bovine endothelial cells. J Biol Chem 1994; 269: 19897-19903.

Hartell NA, Headley PM: The effect of naloxone on spinal reflexes to electrical and mechanical stimuli in the anaesthetized, spinalized rat. Physiol 1991; 442: 513-526.

Hay C, de Belleroche J: Carrageenan-induced hyperalgesia is associated with increased cyclo-oxygenase-2 expression in spinal cord. Neuroreport 1997; 8: 1249-1251.

Hay CH, de Belleroche JS: Dexamethasone prevents the induction of COX-2 mRNA and prostaglandins in the lumbar spinal cord following intraplantar FCA in parallel with inhibition of oedema. Neuropharmacology 1998; 37: 739-744.

Hay CH, Trevethick MA, Wheeldon A, Bowers JS, de Belleroche JS: The potential role of spinal cord cyclooxygenase-2 in the development of Freund's

complete adjuvant-induced changes in hyperalgesia and allodynia. Neuroscience 1997; 78: 843-850.

Hefferan MP, Carter P, Haley M, Loomis CW: Spinal nerve injury activates prostaglandin synthesis in the spinal cord that contributes to early maintenance of tactile allodynia. Pain 2003a; 101: 139-147.

Hefferan MP, Loomis CW: Interaction of spinal nitric oxide and prostaglandins after L5-L6 spinal nerve ligation in the rat: an isobolographic analysis. Anesthesiology 2004; 100: 1611-1614.

Hefferan MP, Loomis CW: Interaction of spinal nitric oxide and prostaglandins after L5-L6 spinal nerve ligation in the rat: an isobolographic analysis. Anesthesiology 2004; 100: 1611-1614.

Hefferan MP, O'Rielly DD, Loomis CW. Inhibition of spinal prostaglandin synthesis early after L5/L6 nerve ligation prevents the development of prostaglandin-dependent and prostaglandin-independent allodynia in the rat. Anesthesiology 2003b; 99: 1180-1188.

Heliövaara M, Impivaara O, Sievers K, Melkas T, Knekt P, Korpi J, Aromaa A: Lumbar disc syndrome in Finland. J Epidemiol Community Health 1987; 41: 251-258.

Henry JL, Lalloo C, Yashpal K: Central poststroke pain: an abstruse outcome. Pain Res Manag. 2008; 13: 41-49.

Herrero JF, Cervero F: Changes in nociceptive reflex facilitation during carrageenan-induced arthritis. Brain Res 1996; 717: 62-68.

Herrero JF, Cervero F: Supraspinal influences on the facilitation of rat nociceptive reflexes induced by carrageenan monoarthritis. Neurosci Lett 1996; 209: 21-24.

Herrero JF, Headley PM: Functional evidence for multiple receptor activation by kappa-ligands in the inhibition of spinal nociceptive reflexes in the rat. Br J Pharmacol 1993; 110: 303-309.

Herrero JF, Headley PM: The effects of sham and full spinalization on the systemic potency of mu- and kappa-opioids on spinal nociceptive reflexes in rats. Br J Pharmacol 1991; 104: 166-170.

Hingtgen CM, Waite KJ, Vasko MR: Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 3',5'-cyclic monophosphate transduction cascade. J Neurosci 1995; 15: 5411-5419.

Holzer P, Jocic M, Peskar BA: Mediation by prostaglandins of the nitric oxide-induced neurogenic vasodilatation in rat skin. Br J Pharmacol 1995; 116: 2365-2370.

Hu J, Mata M, Hao S, Zhang G, Fink DJ: Central sprouting of uninjured small fiber afferents in the adult rat spinal cord following spinal nerve ligation. Eur J Neurosci 2004; 20: 1705-1012.

Hua XY, Calcutt NA, Malmberg AB: Neonatal capsaicin treatment abolishes formalin-induced spinal PGE2 release. Neuroreport 1997; 8: 2325-2329.

Hua XY, Chen P, Yaksh TL: Inhibition of spinal protein kinase C reduces nerve injury-induced tactile allodynia in neuropathic rats. Neurosci Lett 1999; 276: 99-102.

Ichitani Y, Shi T, Haeggstrom JZ, Samuelsson B, Hokfelt T: Increased levels of cyclooxygenase-2 mRNA in the rat spinal cord after peripheral inflammation: an in situ hybridization study. Neuroreport 1997; 8: 2949-2952.

Inoue M, Rashid MH, Fujita R, Contos JJ, Chun J, Ueda H: Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. Nat Med 2004; 10: 712-718.

Jakobsson PJ, Thoren S, Morgenstern R, Samuelsson B: Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. Proc Natl Acad Sci USA 1999; 96: 7220-7225.

Jiménez-Garza O, Camacho J, Ibarra A, Martínez A, Guízar-Sahagún G: Early effects of modulating nuclear factor-kappaB activation on traumatic spinal cord injury in rats. Ann N Y Acad Sci 2005; 1053: 148-150.

Kajander KC, Wakisaka S, Bennett GJ: Spontaneous discharge originates in the dorsal root ganglion at the onset of a painful peripheral neuropathy in the rat. Neurosci Lett 1992; 138: 225-228.

Karin M, Ben-Neriah Y: Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 2000; 18: 621-663.

Kawahara H, Sakamoto A, Takeda S, Onodera H, Imaki J, Ogawa R: A prostaglandin E_2 receptor subtype EP_1 receptor antagonist (ONO-8711) reduces hyperalgesia, allodynia, and c-fos gene expression in rats with chronic nerve constriction. Anesth Analg 2001; 93: 1012-1017.

Kawamata M, Omote K: Involvement of increased excitatory amino acids and intracellular Ca2+ concentration in the spinal dorsal horn in an animal model of neuropathic pain. Pain 1996; 68: 85-96.

Kelefiotis D, Bresnahan BA, Stratidakis I, Lianos EA: Eicosanoid-induced growth and signaling events in rat glomerular mesangial cells. Prostaglandins 1995; 49: 269-283.

Khanapure SP, Garvey DS, Janero DR, Letts LG: Eicosanoids in inflammation: biosynthesis, pharmacology, and therapeutic frontiers. Curr Top Med Chem. 2007; 7: 311-340.

Kim SF, Huri DA, Snyder SH: Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. Science 2005; 310: 1966-1970.

Kim SH, Chung JM: An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 1992; 50: 355-363.

Kimura S, Tanabe M, Honda M, Ono H: Enhanced wind-up of the C-fiber-mediated nociceptive flexor reflex movement following painful diabetic neuropathy in mice. J Pharmacol Sci 2005; 97: 195-202.

Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM: Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci USA 1997; 94: 4318-4323.

Koerber HR, Mirnics K, Kavookjian AM, Light AR: Ultrastructural analysis of ectopic synaptic boutons arising from peripherally regenerated primary afferent fibers. J Neurophysiol 1999; 81: 1636-1644.

Kohama I, Ishikawa K, Kocsis JD: Synaptic reorganization in the substantia gelatinosa after peripheral nerve neuroma formation: aberrant innervation of lamina II neurons by Abeta afferents. J Neurosci 2000; 20: 1538-1549.

Kohno T, Moore KA, Baba H, Woolf CJ: Peripheral nerve injury alters excitatory synaptic transmission in lamina II of the rat dorsal horn. J Physiol 2003; 548: 131-138.

Kuwamoto S, Inoue H, Tone Y, Izumi Y, Tanabe T: Inverse gene expression of prostacyclin and thromboxane synthases in resident and activated peritoneal macrophages. FEBS Lett 1997; 409: 242-246.

La Rosa G, Cardali S, Genovese T, Conti A, Di Paola R, La Torre D, Cacciola F, Cuzzocrea S: Inhibition of the nuclear factor-kappaB activation with pyrrolidine dithiocarbamate attenuating inflammation and oxidative stress after experimental spinal cord trauma in rats. J Neurosurg Spine 2004; 1: 311-321.

Laird JM, Bennett GJ: An electrophysiological study of dorsal horn neurons in the spinal cord of rats with an experimental peripheral neuropathy. J Neurophysiol 1993; 69: 2072-2085.

Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ: Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. Proc Natl Acad Sci USA 1996; 93: 15069-15074.

Laughlin TM, Bethea JR, Yezierski RP, Wilcox GL: Cytokine involvement in dynorphin-induced allodynia. Pain. 2000; 84: 159-167.

Lee SH, Seo GS, Sohn DH: Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase by butein in RAW 264.7 cells. Biochem Biophys Res Commun 2004; 323: 125-132.

Li CY, Song YH, Higuera ES, Luo ZD: Spinal dorsal horn calcium channel alpha2 delta-1 subunit upregulation contributes to peripheral nerve injury-induced tactile allodynia. J Neurosci 2004; 24: 8494-8499.

Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM, Dey SK: Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. Genes Dev 1999; 13: 1561-1574.

Liu CN, Michaelis M, Amir R, Devor M: Spinal nerve injury enhances subthreshold membrane potential oscillations in DRG neurons: relation to neuropathic pain. J Neurophysiol 2000b; 84: 205-215.

Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M: Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 2000a; 85: 503-521.

Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, Murphy WJ: Macrophage nitric oxide synthase gene: two upstream regions

mediate induction by interferon gamma and lipopolysaccharide. Proc Natl Acad Sci USA 1993; 90: 9730-9734.

Lu K, Liang CL, Chen HJ, Chen SD, Hsu HC, Chen YC, Hsu FF, Cho CL: Nuclear factor-kappaB-regulated cyclooxygenase-2 expression in surgery-associated paraspinal muscle injury in rats. J Neurosurg 2003; 98: 181-187.

Lukacova N, Cizkova D, Krizanova O, Pavel J, Marsala M, Marsala J: Peripheral axotomy affects nictotinamide adenine dinucleotide phosphate diaphorase and nitric oxide synthases in the spinal cord of the rabbit. J Neurosci Res 2003; 71: 300-313.

Lynn B and Carpenter SE: Primary afferent units from the hairy skin of the rat hind limb. Brain Res 1982; 238: 29-43.

Ma QP, Tian L: A-fibres sprouting from lamina I into lamina II of spinal dorsal horn after peripheral nerve injury in rats. Brain Res 2001; 904: 137-140.

Ma QP, Woolf CJ: Progressive tactile hypersensitivity: an inflammation-induced incremental increase in the excitability of the spinal cord. Pain 1996; 67: 97-106.

Ma W, Bisby MA: Increased activation of nuclear factor kappa B in rat lumbar dorsal root ganglion neurons following partial sciatic nerve injuries. Brain Res 1998; 797: 243-254.

Ma W, Du W, Eisenach JC: Role for both spinal cord COX-1 and COX-2 in maintenance of mechanical hypersensitivity following peripheral nerve injury. Brain Res 2002; 937: 94-99.

Ma W, Eisenach JC: Cyclooxygenase 2 in infiltrating inflammatory cells in injured nerve is universally up-regulated following various types of peripheral nerve injury. Neuroscience 2003; 121: 691-704.

Ma W, Eisenach JC: Four PGE_2 EP receptors are up-regulated in injured nerve following partial sciatic nerve ligation. Exp Neurol 2003; 183: 581-592.

Ma W, Eisenach JC: Morphological and pharmacological evidence for the role of peripheral prostaglandins in the pathogenesis of neuropathic pain. Eur J Neurosci 2002; 15: 1037-1047.

Ma W, Eisenach JC: Neuronal nitric oxide synthase is upregulated in a subset of primary sensory afferents after nerve injury which are necessary for analgesia from alpha2-adrenoceptor stimulation. Brain Res 2007; 1127: 52-58.

Mabuchi T, Matsumura S, Okuda-Ashitaka E, Kitano T, Kojima H, Nagano T, Minami T, Ito S: Attenuation of neuropathic pain by the nociceptin/orphanin FQ antagonist JTC-801 is mediated by inhibition of nitric oxide production. Eur J Neurosci 2003; 17: 1384-1392.

Maihofner C, Tegeder I, Euchenhofer C, deWitt D, Brune K, Bang R, Neuhuber W, Geisslinger G: Localization and regulation of cyclo-oxygenase-1 and -2 and neuronal nitric oxide synthase in mouse spinal cord. Neuroscience 2000; 101: 1093-1108.

Malmberg AB, Yaksh TL: Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J Pharmacol Exp Ther 1992a; 263: 136-146.

Malmberg AB, Yaksh TL: Cyclooxygenase inhibition and the spinal release of prostaglandin E2 and amino acids evoked by paw formalin injection: a microdialysis study in unanaesthetized rats. J Neurosci 1995a; 15: 2768-2776.

Malmberg AB, Yaksh TL: The effect of morphine on formalin-evoked behaviour and spinal release of excitatory amino acids and prostaglandin E2 using microdialysis in conscious rats. Br J Pharmacol 1995b; 114: 1069-1075.

Malmberg AB, Hamberger A, Hedner T: Effects of prostaglandin E_2 and capsaicin on behaviour and cerebrospinal fluid amino acid concentrations of unanesthetized rats: A microdialysis study. J Neurochem 1995; 65: 2185-2193.

Mansikka H, Sheth RN, DeVries C, Lee H, Winchurch R, Raja SN: Nerve injury-induced mechanical but not thermal hyperalgesia is attenuated in neurokinin-1 receptor knockout mice. Exp Neurol 2000; 162: 343-349.

Marabese I, de Novellis V, Palazzo E, Scafuro MA, Vita D, Rossi F, Maione S: Effects of (S)-3,4-DCPG, an mGlu8 receptor agonist, on inflammatory and neuropathic pain in mice. Neuropharmacology 2007; 52: 253-262.

Marini AM, Jiang X, Wu X, Tian F, Zhu D, Okagaki P, Lipsky RH: Role of brain-derived neurotrophic factor and NF-kappaB in neuronal plasticity and survival: From genes to phenotype. Restor Neurol Neurosci 2004; 22: 121-130.

Marsala M, Malmberg AB, Yaksh TL: The spinal loop dialysis catheter: characterization of use in the unanesthetized rat. J Neurosci Methods 1995; 62: 43-53.

Matsumura K, Watanabe Y, Imai Matsumura K, Connolly M, Koyama Y, Onoe H and Watanabe Y: Mapping of prostaglandin E2 binding sites in rat brain using quantitative autoradiography. Brain Res 1992; 581: 292-298.

Matsumura K, Watanabe Y, Onoe H, Watanabe Y: Prostacyclin receptor in the brain and central terminals of the primary sensory neurons: an autoradiographic study using a stable prostacyclin analogue [3H]iloprost. Neuroscience 1995; 65: 493-503.

Matsumura S, Abe T, Mabuchi T, Katano T, Takagi K, Okuda-Ashitaka E, Tatsumi S, Nakai Y, Hidaka H, Suzuki M, Sasaki Y, Minami T, Ito S: Rho-kinase mediates spinal nitric oxide formation by prostaglandin E2 via EP3 subtype. Biochem Biophys Res Commun 2005; 338: 550-557.

McCarberg BH, Billington R: Consequences of neuropathic pain: quality-of-life issues and associated costs. Am J Manag Care 2006; 12: S263-S268.

Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D: NF-kappa B functions in synaptic signaling and behavior. Nat Neurosci 2003; 6: 1072-1078.

Mene P, Dubyak GR, Scarpa A, and Dunn MJ: Stimulation of cytosolic free calcium and inositol phosphates by prostaglandins in cultured rat mesangial cells. Biochem Biophys Res Commun 1987; 142: 579-586.

Merskey H (Ed.): Classification of chronic pain, Part II. Pain Suppl 1986; 3: S215-S221.

Merskey H, Bogduk N: In Classification of Chronic Pain, Second Edition, IASP Task Force on Taxonomy, IASP Press, Seattle,1994; 209-214.

Meyerson BA, Ren B, Herregodts P, Linderoth B: Spinal cord stimulation in animal models of mononeuropathy: effects on the withdrawal response and the flexor reflex. Pain 1995; 61: 229-243.

Michael GJ, Averill S, Shortland PJ, Yan Q, Priestley JV: Axotomy results in major changes in BDNF expression by dorsal root ganglion cells: BDNF expression in large trkB and trkC cells, in pericellular baskets, and in projections to deep dorsal horn and dorsal column nuclei. Eur J Neurosci 1999; 11: 3539-3551.

Michaelis M, Vogel C, Blenk KH, Arnarson A, Janig W: Inflammatory mediators sensitize acutely axotomized nerve fibers to mechanical stimulation in the rat. J Neurosci 1998; 18: 7581-7587.

Millan MJ: The induction of pain: an integrative review. Prog Neurobiol 1999; 57: 1-164.

Milne B, Hall SR, Sullivan ME, Loomis C: The release of spinal prostaglandin E2 and the effect of nitric oxide synthetase inhibition during strychnine-induced allodynia. Anesth Analg 2001; 93: 728-733.

Minami T, Nakano H, Kobayashi T, Sugimoto Y, Ushikubi F, Ichikawa A, Narumiya S, Ito S: Characterization of EP receptor subtypes responsible for prostaglandin E2-induced pain responses by use of EP_1 and EP_3 receptor knockout mice. Br J Pharmacol 2001; 133: 438-444.

Minami T, Nishihara I, Ito S, Sakamoto K, Hyodo M and Hayaishi O: Nitric oxide mediates allodynia induced by intrathecal administration of prostaglandin E2 or prostaglandin F2 in conscious mice. Pain 1995a; 61: 285-290.

Minami T, Nishihara I, Sakamoto K, Ito S, Hyodo M and Hayaishi O: Blockade by NON-NT-012, a unique prostanoid analogue, of prostaglandin E2-induced allodynia in conscious mice. Br J Pharmacol 1995b; 115: 73-76.

Minami T, Okuda-Ashitaka E, Hori Y, Sakuma S, Sugimoto T, Sakimura K, Mishina M and Ito S: Involvement of primary afferent C-fibers in touch-evoked pain (allodynia) induced by prostaglandin E2. Eur J Neurosci 1999; 11: 1849-1856.

Minami T, Uda R, Horiguchi S, Ito S, Hyodo M and Hayaishi O: Allodynia evoked by intrathecal administration of prostaglandin E2 to conscious mice. Pain 1994a; 57: 217-223.

Minami T, Uda R, Horiguchi S, Ito S, Hyodo M, Hayaishi O: Allodynia evoked by intrathecal administration of prostaglandin F2 alpha to conscious mice. Pain 1992; 50: 223-229.

Minami T, Uda R, Horiguchi S, Ito S, Hyodo M, Hayaishi O: Allodynia evoked by intrathecal administration of prostaglandin E_2 to conscious mice. Pain 1994b; 57: 217-223.

Miscusi M, Ebner F, Ceccariglia S, Menegazzi M, Mariotto S, Berra L, Del Fa A, Gangitano C, Lauretti L, Missori P, Delfini R, Suzuki H: Early nuclear factor-kappaB activation and inducible nitric oxide synthase expression in injured spinal cord neurons correlating with a diffuse reduction of constitutive nitric oxide synthase activity. J Neurosurg Spine 2006; 4: 485-493.

Mollace V, Colasanti M, Muscoli C, Lauro GM, Iannone M, Rotiroti D, Nistico G: The effect of nitric oxide on cytokine-induced release of PGE2 by human cultured astroglial cells. Br J Pharmacol 1998; 124: 742-746.

Mollace V, Colasanti M, Rodino P, Lauro GM, Rotiroti D, Nistico G: NMDA-dependent prostaglandin E2 release by human cultured astroglial cells is driven by nitric oxide. Biochem Biophys Res Commun 1995; 215: 793-799.

Mollace V, Muscoli C, Masini E, Cuzzocrea S, Salvemini D: Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. Pharmacol Rev 2005; 57: 217-252.

Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Oh S, Kudo I: Regulation of prostaglandin E_2 biosynthesis by inducible membrane-associated prostaglandin E_2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 2000; 275: 32783-32792.

Nakahara S, Yone K, Setoguchi T, Yamaura I, Arishima Y, Yoshino S, Komiya S: Changes in nitric oxide and expression of nitric oxide synthase in spinal cord after acute traumatic injury in rats. J Neurotrauma 2002; 19: 1467-1474.

Negishi M, Sugimoto Y, Ichikawa A: Molecular mechanisms of diverse actions of prostanoid receptors. Biochim Biophys Acta 1995; 1259: 109-119.

Neuss H, Huang X, Hetfeld BK, Deva R, Henklein P, Nigam S, Mall JW, Schwenk W, Dubiel W: The ubiquitin- and proteasome-dependent degradation of COX-2 is regulated by the COP9 signalosome and differentially influenced by coxibs. J Mol Med 2007; 85: 961-970.

Nicholls DG, Sihra TS, Sanchez-Prieto J: Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. J Neurochem 1987; 49: 50-57.

Nicol GD, Klingberg DK, Vasko MR: Prostaglandin E2 increases calcium conductance and stimulates release of substance P in avian sensory neurons. J Neurosci 1992; 12: 1917-1927.

Nicol GD, Vasko MR, Evans AR: Prostaglandins suppress an outward potassium current in embryonic rat sensory neurons. J Neurophysiol 1997; 77: 167-176.

Niederberger E, Tegeder I, Schafer C, Seegel M, Grosch S, Geisslinger G: Opposite effects of rofecoxib on nuclear factor-kappaB and activating protein-1 activation. J Pharmacol Exp Ther 2003; 304: 1153-1160.

Nishihara I, Minami T, WatanabeY, Ito S, Hayaishi O: Prostaglandin E_2 stimulates glutamate release from synaptosomes of rat spinal cord. Neurosci Lett 1995; 196: 57-60.

Noguchi K, Dubner R, De Leon M, Senba E, Ruda MA: Axotomy induces preprotachykinin gene expression in a subpopulation of dorsal root ganglion neurons. J Neurosci Res 1994; 37: 596-603.

Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K: Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. J Neurosci 1995; 11: 7633-7643.

O'Rielly DD, Loomis CW: Spinal prostaglandins facilitate exaggerated A- and C-fiber-mediated reflex responses and are critical to the development of allodynia early after L5-L6 spinal nerve ligation. Anesthesiology 2007; 106: 795-805.

O'Rielly DD, Loomis CW: Rat L5/L6 spinal nerve ligation (SNL) triggers multiple changes supporting an early pathogenic role for spinal prostaglandins (PG) in neuropathic pain. Abstract #4783, Society for Neuroscience, 34th Annual Meeting, San Diego, CA, USA, 2004.

O'Rielly DD, Loomis CW: Spinal nerve ligation-induced activation of nuclear factor kappaB is facilitated by prostaglandins in the affected spinal cord and is a critical step in the development of mechanical allodynia. Neuroscience. 2008 May 21. [Epub ahead of print].

O'Rielly DD, Loomis CW: Spinal prostaglandins facilitate exaggerated A- and C-fibre mediated reflex responses and are critical to the development of allodynia early after L5/L6 spinal nerve ligation. Anesthesiology 2007; 106: 795-805.

Okamoto M, Baba H, Goldstein PA, Higashi H, Shimoji K, Yoshimura M: Functional reorganization of sensory pathways in the rat spinal dorsal horn following peripheral nerve injury. J Physiol 2001; 532: 241-250.

O'Rielly DD, Loomis CW: Increased expression of cyclooxygenase and nitric oxide isoforms, and exaggerated sensitivity to prostaglandin E_2, in the rat lumbar

spinal cord 3 days after L5-L6 spinal nerve ligation. Anesthesiology 2006; 104: 328-337.

Ossipov MH, Lai J, Malan TP, Porreca F: Spinal and supraspinal mechanisms of neuropathic pain. Ann NY Acad Sci 2000; 909: 12-24.

Otto JC, Smith WL: Photolabeling of prostaglandin endoperoxide H synthase-1 with 3-trifluoro-3-(m-[125I]iodophenyl)diazirine as a probe of membrane association and the cyclooxygenase active site. J Biol Chem; 1996; 271: 9906-9910.

Pahl HL: Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999; 18: 6853-6866.

Park YH, Shin CY, Lee TS, Huh IH, Sohn UD: The role of nitric oxide and prostaglandin E2 on the hyperalgesia induced by excitatory amino acids in rats. J Pharm Pharmacol 2000; 52: 431-436.

Pérez-Sala D, Lamas S: Regulation of cyclooxygenase-2 expression by nitric oxide in cells. Antioxid Redox Signal 2001; 3: 231-248.

Perkins ND: The Rel/NF-kappa B family: friend and foe. Trends Biochem Sci 2000; 25: 434-440.

Petersen-Zeitz KR, Basbaum AI: Second messengers, the substantia gelatinosa and injury-induced persistent pain. Pain 1999; Suppl 6: S5-S12.

Phillis JW, O'Regan MH: A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders. Brain Res Brain Res Rev 2004; 44: 13-47.

Pitcher GM, Henry JL: Nociceptive response to innocuous mechanical stimulation is mediated via myelinated afferents and NK-1 receptor activation in a rat model of neuropathic pain. Exp Neurol 2004; 186: 173-197.

Piva R, Belardo G, Santoro MG: NF-kappaB: a stress-regulated switch for cell survival. Antioxid Redox Signal 2006; 8: 478-486.

Pollock G, Pennypacker KR, Memet S, Israel A, Saporta S: Activation of NF-kappaB in the mouse spinal cord following sciatic nerve transection. Exp Brain Res 2005; 165: 470-477.

Price DD, Bennett GJ, Rafii A: Psychophysical observations on patients with neuropathic pain relieved by a sympathetic block. Pain 1989; 36: 273-288.

Radhakrishnan V, Yashpal K, Hui-Chan CW, Henry JL: Implication of a nitric oxide synthase mechanism in the action of substance P: L-NAME blocks thermal hyperalgesia induced by endogenous and exogenous substance P in the rat. Eur J Neurosci 1995; 7: 1920-1925.

Raja SN, Meyer RA, Campbell JN: Peripheral mechanisms of somatic pain. Anesthesiology 1988; 68: 571-590.

Ren B, Linderoth B, Meyerson BA: Effects of spinal cord stimulation on the flexor reflex and involvement of supraspinal mechanisms: an experimental study in mononeuropathic rats. J Neurosurg 1996; 84: 244-249.

Resnick DK, Graham SH, Dixon CE, Marion DW: Role of cyclooxygenase 2 in acute spinal cord injury. J Neurotrauma 1998; 15: 1005-1013.

Rowbotham MC, Reisner-Keller LA, Fields HL: Both intravenous lidocaine and morphine reduce the pain of postherpetic neuralgia. Neurology 1991; 41: 1024-1028.

Sakai M, Minami T, Hara N, Nishihara I, Kitade H, Kamiyama Y, Okuda K, Takahashi H, Mori H, Ito S: Stimulation of nitric oxide release from rat spinal cord by prostaglandin E2. Br J Pharmacol 1998; 123: 890-894.

Sakaue G, Shimaoka M, Fukuoka T, Hiroi T, Inoue T, Hashimoto N, Sakaguchi T, Sawa Y, Morishita R, Kiyono H, Noguchi K, Mashimo T: NF-kappa B decoy suppresses cytokine expression and thermal hyperalgesia in a rat neuropathic pain model. Neuroreport 2001; 12: 2079-2084.

Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P: Nitric oxide activates cyclooxygenase enzymes. Proc Natl Acad Sci USA 1993; 90: 7240-7244.

Salvemini D, Settle SL, Masferrer JL, Seibert K, Currie MG, Needleman P: Regulation of prostaglandin production by nitric oxide; an in vivo analysis. Br J Pharmacol 1995; 114: 1171-1178.

Samad TA, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, Woolf CJ: Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 2001; 410: 471-475.

Schafers M, Lee DH, Brors D, Yaksh TL, Sorkin LS: Increased sensitivity of injured and adjacent uninjured rat primary sensory neurons to exogenous tumor necrosis factor-alpha after spinal nerve ligation. J Neurosci 2003; 23: 3028-3038.

Schmader K: Postherpetic neuralgia in immunocompetent elderly people. Vaccine 1998; 16: 1768-1770.

Schmader KE: Epidemiology and impact on quality of life of postherpetic neuralgia and painful diabetic neuropathy. Clin J Pain 2002; 18: 350-354.

Schouenborg J, Sjőlund BH: Activity evoked by A- and C-afferent fibers in rat dorsal horn neurons and its relationschip to a flexion reflex. J Neurophysiol 1983; 50: 1108-1121.

Senapati AK, Radhakrishnan H, Liu H, Peng YB: Detection of degeneration in rat sciatic nerve by in vivo near infrared spectroscopy. Brain Res Brain Res Protoc 2005; 14: 119-125.

Seybold VS, Jia YP, Abrahams LG: Cyclo-oxygenase-2 contributes to central sensitization in rats with peripheral inflammation. Pain 2003; 105: 47-55.

Shehab SA, Spike RC, Todd AJ: Evidence against cholera toxin B subunit as a reliable tracer for sprouting of primary afferents following peripheral nerve injury. Brain Res 2003; 964: 218-227.

Shi TJ, Holmberg K, Xu ZQ, Steinbusch H, de Vente J, Hökfelt T: Effect of peripheral nerve injury on cGMP and nitric oxide synthase levels in rat dorsal root ganglia: time course and coexistence. Pain 1998; 78: 171-180.

Shi X, Dong Z, Huang C, Ma W, Liu K, Ye J, Chen F, Leonard SS, Ding M, Castranova V, Vallyathan V: The role of hydroxyl radical as a messenger in the activation of nuclear transcription factor NF-kappaB. Mol Cell Biochem 1999; 194: 63-70.

Shishodia S, Aggarwal BB: Nuclear factor-kappaB activation: a question of life or death. J Biochem Mol Biol. 2002; 35: 28-40.

Siegan JB, Hama AT, Sagen J: Alterations in rat spinal cord cGMP by peripheral nerve injury and adrenal medullary transplantation. Neurosci Lett 1996; 215: 49-52.

Smith JB and Willis AL: Aspirin selectively inhibits prostaglandin production in human platelets. Nat New Biol 1971; 231: 235-237.

Smith W: Prostanoid biosynthesis and mechanisms of action. Am J Physiol 1992; 263 (Pt 2): F181-191.

Smith WL, DeWitt DL, Garavito RM: Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem 2000; 69: 145-182.

Song XJ, Zhang JM, Hu SJ, LaMotte RH: Somata of nerve-injured sensory neurons exhibit enhanced responses to inflammatory mediators. Pain 2003; 104: 701-709.

Sonnenburg WK, Zhu JH, Smith WL: A prostaglandin E receptor coupled to a pertussis toxin-sensitive guanine nucleotide regulatory protein in rabbit cortical collecting tubule cells. J Biol Chem 1990; 265: 8479-8483.

Sorkin LS, Moore JH: Evoked Release of Amino Acids and Prostanoids in Spinal Cords of Anesthetized Rats: Changes During Peripheral Inflammation and Hyperalgesia. Am J Ther 1996; 3: 268-275.

Spencer AG, Thuresson E, Otto JC, Song I, Smith T, DeWitt DL, Garavito RM, Smith WL: The membrane binding domains of prostaglandin endoperoxide H

synthases 1 and 2. Peptide mapping and mutational analysis. J Biol Chem 1999; 274: 32936-32942.

Sugimoto Y, Narumiya S: Prostaglandin E receptors. J Biol Chem 2007; 282: 11613-11617.

Sung YJ, Ambron RT: Pathways that elicit long-term changes in gene expression in nociceptive neurons following nerve injury: contributions to neuropathic pain. Neurol Res 2004; 26: 195-203.

Sung YJ, Chiu DT, Ambron RT: Activation and retrograde transport of protein kinase G in rat nociceptive neurons after nerve injury and inflammation. Neuroscience 2006; 141: 697-709.

Suyama H, Kawamoto M, Gaus S, Yuge O: Effect of etodolac, a COX-2 inhibitor, on neuropathic pain in a rat model. Brain Res 2004; 1010: 144-150.

Suzuki T, Watanabe K, Kanaoka Y, Sato T, Hayaishi O: Induction of hematopoietic prostaglandin D synthase in human megakaryocytic cells by phorbol ester. Biochem Biophys Res Commun 1997; 241: 288-293.

Szabó C: Physiological and pathophysiological roles of nitric oxide in the central nervous system. Brain Res Bull 1996; 41: 131-141.

Takahashi M, Kawaguchi M, Shimada K, Nakashima T, Furuya H: Systemic meloxicam reduces tactile allodynia development after L5 single spinal nerve injury in rats. Reg Anesth Pain Med 2005; 30: 351-355.

Takeda K, Sawamura S, Tamai H, Sekiyama H, Hanaoka K: Role for cyclooxygenase 2 in the development and maintenance of neuropathic pain and spinal glial activation. Anesthesiology 2005; 103: 837-844.

Tallarida RJ, Murray RB: Manual of Pharmacologic Calculations with Computer Programs, 2nd edition. Springer-Verlag, New York, 1987.

Tasker RR, DeCarvalho GT and Dolan EJ: Intractable pain of spinal cord origin: clinical features and implications for surgery. J Neurosurg 1992; 77: 373-378.

Tasker RR: Pain resulting from central nervous system pathology (central pain). In: Bonica (Ed.), The Management of Pain, Vol. 1, Lea & Febiger, Philadelphia, 1990; 264-286.

Taylor RS: Epidemiology of refractory neuropathic pain. Pain Pract 2006; 6: 22-26.

Tegeder I, Niederberger E, Israr E, Guhring H, Brune K, Euchenhofer C, Grosch S, Geisslinger G: Inhibition of NF-kappaB and AP-1 activation by R- and S-flurbiprofen. FASEB J 2001; 15: 2-4.

Tegeder I, Niederberger E, Schmidt R, Kunz S, Guhring H, Ritzeler O, Michaelis M, Geisslinger G: Specific Inhibition of IkappaB kinase reduces hyperalgesia in inflammatory and neuropathic pain models in rats. J Neurosci 2004; 24: 1637-1645.

Tegeder I, Niederberger E, Vetter G, Brautigam L, Geisslinger G: Effects of selective COX-1 and -2 inhibition on formalin-evoked nociceptive behaviour and prostaglandin E(2) release in the spinal cord. J Neurochem 2001; 79: 777-786.

Tergaonkar V: NFkappaB pathway: a good signaling paradigm and therapeutic target. Int J Biochem Cell Biol 2006; 38: 1647-1653.

Thompson SW, Dray A, Urban L: Injury-induced plasticity of spinal reflex activity: NK1 neurokinin receptor activation and enhanced A- and C-fiber mediated responses in the rat spinal cord in vitro. J Neurosci 1994; 14: 3672-3687.

Tonai T, Taketani Y, Ueda N, Nishisho T, Ohmoto Y, Sakata Y, Muraguchi M, Wada K, Yamamoto S: Possible involvement of interleukin-1 in cyclooxygenase-2 induction after spinal cord injury in rats. J Neurochem 1999; 72: 302-309.

Tsai AL, Wei C, Kulmacz RJ: Interaction between nitric oxide and prostaglandin H synthase. Arch Biochem Biophys 1994; 313: 367-372.

Tsatsanis C, Androulidaki A, Venihaki M, Margioris AN: Signalling networks regulating cyclooxygenase-2. Int J Biochem Cell Biol 2006; 38:1654-1661.

Turk DC: Clinical effectiveness and cost-effectiveness of treatments for patients with chronic pain. Clin J Pain 2002; 18: 355-365.

Uda R, Horiguchi S, Ito S, Hyodo M, Hayaishi O: Nociceptive effects induced by intrathecal administration of prostaglandin D2, E2, or F2 alpha to conscious mice. Brain Res 1990; 510: 26-32.

Ushikubi F, Segi E, Sugimoto Y, Murata T, Matsuoka T, Kobayashi T, Hizaki H, Tuboi K, Katsuyama M, Ichikawa A, Tanaka T, Yoshida N, Narumiya S: Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. Nature 1998; 395: 281-284.

Vane JR, Bakhle YS, Botting RM: Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998; 38: 97-120.

Vane JR: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 1971; 231: 232-235.

Vanegas H, Schaible HG: Prostaglandins and cyclooxygenases in the spinal cord. Prog Neurobiol 2001; 64: 327-363.

Vasko M, Campbell W, Waite K: Prostaglandin E2 enhances bradykinin stimulated release of neuropeptides from rat sensory neurons in culture. J Neurosci 1994; 14: 4987-4997.

Vasko MR: Prostaglandin-induced neuropeptide release from spinal cord. Prog Brain Res 1995; 104: 367-380.

Verma S, Estanislao L, Simpson D: HIV-associated neuropathic pain: epidemiology, pathophysiology and management. CNS Drugs 2005; 19: 325-334.

Wang X, Robinson PJ: Cyclic GMP-dependent protein kinase and cellular signaling in the nervous system. J Neurochem 1997; 68: 443-456.

Wasner G, Schattschneider J, Binder A, Baron R: Complex regional pain syndrome--diagnostic, mechanisms, CNS involvement and therapy. Spinal Cord. 2003 Feb;41: 61-75.

Wiesenfeld-Hallin Z: Partially overlapping territories of nerves to hindlimb foot skin demonstrated by plasma extravasation to antidromic C-fiber stimulation in the rat. Neurosci Lett 1988; 84: 261-265.

Willingale HL, Gardiner NJ, McLymont N, Giblett S, Grubb BD: Prostanoids synthesized by cyclo-oxygenase isoforms in the rat spinal cord and their contribution to the development of neuronal hyperexcitability. Br J Pharmacol 1997; 122: 1593-1604.

Wilson LB: Spinal modulation of the muscle pressor reflex by nitric oxide and acetylcholine. Brain Res Bull 2000; 53: 51-58.

Wise H, Wong YH, Jones RL: Prostanoid signal integration and cross talk. Neurosignals 2002; 11: 20-28.

Woolf CJ, Doubell TP: The pathophysiology of chronic pain-increased sensitivity to low threshold A beta-fibre inputs. Curr Opin Neurobiol 1994; 4: 525-534.

Woolf CJ, Shortland P, Coggeshall RE: Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 1992; 355: 75-78.

Woolf CJ, Thompson SW: The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. Pain 1991; 44: 293-299.

Wu LC, Goettl VM, Madiai F, Hackshaw KV, Hussain SR: Reciprocal regulation of nuclear factor kappa B and its inhibitor ZAS3 after peripheral nerve injury. BMC Neurosci 2006; 7: 4.

Xu J, Kim GM, Chen S, Yan P, Ahmed SH, Ku G, Beckman JS, Xu XM, Hsu CY: iNOS and nitrotyrosine expression after spinal cord injury. J Neurotrauma 2001; 18: 523-532.

Yaksh TL, Rudy TA: Chronic catheterization of the spinal subarachnoid space. Physiol Behav 1976; 17: 1031-1036.

Yaksh TL: Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists. Pain 1989; 37: 111-123.

Yamamoto T, Yaksh TL: Effects of intrathecal strychnine and bicuculline on nerve compression-induced thermal hyperalgesia and selective antagonism by MK-801. Pain 1993; 54: 79-84.

Yan Z, Stapleton PP, Freeman TA, Fuortes M, Daly JM: Enhanced expression of cyclooxygenase-2 and prostaglandin E2 in response to endotoxin after trauma is dependent on MAPK and NF-kappaB mechanisms. Cell Immunol 2004; 232: 116-126.

Yang LC, Marsala M, Yaksh TL: Characterization of time course of spinal amino acids, citrulline and PGE2 release after carrageenan/kaolin-induced knee joint inflammation: a chronic microdialysis study. Pain 1996; 67: 345-354.

Yoon YW, Na HS, Chung JM: Contributions of injured and intact afferents to neuropathic pain in an experimental rat model. Pain 1996; 64: 27-36.

Yoon YW, Sung B, Chung JM: Nitric oxide mediates behavioral signs of neuropathic pain in an experimental rat model. Neuroreport 1998; 9: 367-372.

You HJ, Morch CD, Chen J, Arendt-Nielsen L: Differential antinociceptive effects induced by a selective cyclooxygenase-2 inhibitor (SC-236) on dorsal horn neurons and spinal withdrawal reflexes in anesthetized spinal rats. Neuroscience 2003; 121: 459-472.

Yu W, Kauppila T, Hultenby K, Persson JK, Xu XJ, Wiesenfeld-Hallin Z: Photochemically-induced ischemic injury of the rat sciatic nerve: a light- and electron microscopic study. J Peripher Nerv Syst 2000; 5: 209-217.

Zeilhofer HU: Prostanoids in nociception and pain. Biochem Pharmacol. 2007; 73: 165-174.

Zhang Z, Hefferan MP, Loomis CW: Topical bicuculline to the rat spinal cord induces highly localized allodynia that is mediated by spinal prostaglandins. Pain 2001; 92:351-361.

Zhao Z, Chen SR, Eisenach JC, Busija DW, Pan HL: Spinal cyclooxygenase-2 is involved in development of allodynia after nerve injury in rats. Neuroscience 2000; 97: 743-748.

Zhou XF, Chie ET, Deng YS, Zhong JH, Xue Q, Rush RA, Xian CJ: Injured primary sensory neurons switch phenotype for brain-derived neurotrophic factor in the rat. Neuroscience 1999; 92: 841-853.

Zhou XF, Deng YS, Xian CJ, Zhong JH: Neurotrophins from dorsal root ganglia trigger allodynia after spinal nerve injury in rats. Eur J Neurosci 2000; 12: 100-105.

Zhu X, Eisenach JC: Cyclooxygenase-1 in the spinal cord is altered after peripheral nerve injury. Anesthesiology 2003; 99: 1175-1179.

Zhuang ZY, Gerner P, Woolf CJ, Ji RR: ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. Pain 2005; 114: 149-159.

Zimmermann M: Pathobiology of neuropathic pain. Eur J Pharmacol 2001; 429: 23-37.