NEUROPEPTIDES IN THE RVM PROMOTE DESCENDING FACILITATION AND ABNORMAL PAIN

By

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY

In Partial Fulfillment of the Requirement For the Degree of

DOCTOR OF PHILOSOPHY

With a Major in MEDICAL PHARMACOLOGY

In the Graduate College of

THE UNIVERSITY OF ARIZONA

2008

GRADUATE COLLEGE

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all of those who made the completion of this work possible. To begin, I would like to express my heartfelt thanks to Brent Pollitt for his technical expertise and surgical contribution to my studies, Dr. Shannon Holt and Konstantina Zuber for their many surgeries and hardwork, Qingmin Chen for her invaluable help and tutelage in many aspects of pharmacological research, and Dr. Michael Ossipov for his wide-ranging expertise in statistics and pharmacology. I would also like to offer a big thanks to the Porreca Lab, Lai Lab, and the Vanderah Lab, especially Beatriz Lindstrom, Tally Largent-Milnes, and Hamid Badghisi for all their help and support. I would like to offer a tremendous and sincere thank you to my advisor and mentor extraordinaire, Dr. Todd Vanderah, for all of the countless hours he devoted toward my education in this program, sharing with me his breadth of knowledge in the field of science and pharmacology, and his neverending commitment to helping me become a better scientist. And lastly, I would like to thank my committee members Dr. Glenn Sipes, Dr. Nathan Cherrington, Dr. Phil Malan, and Dr. Frank Porreca – who pushed me to be better.

DEDICATION

I would like to dedicate this work to my mom and dad, and friends and family, who have stood by me with tremendous support and love during these past four plus years and for the many years before. I wouldn't be here today if it wasn't for their neverending love, support, and encouragement through the years. And most importantly, I would like to thank God, the great universal power that binds us all, for all of the strength and continued guidance you have given me, and for which I am forever grateful. To all of those in my life, I thank you.

TABLE OF CONTENTS

LIST OF FIGURES..... 8

ABBREVIATIONS..... 13

ABSTRACT..... 14

CHAPTER ONE: Introduction..... 17

1.1 Origins and symptoms of neuropathic pain..... 20
1.2 Models of neuropathic pain..... 24
1.3 Mechanisms of neuropathic pain..... 29
1.3.1 Anatomical reorganization of the spinal cord in neuropathic pain..... 39
1.3.2 Central sensitization in neuropathic pain..... 40
1.4 The RVM and pain modulation..... 45
1.5 CCK and descending facilitation from the RVM..... 51
1.6 Serotonin and pronociception..... 53
1.7 Prostaglandin E2 and pronociception..... 61
1.8 Biochemistry and synthesis of prostaglandins..... 64
1.8.1 COX-1 and COX-2 origin, expression, and function..... 70
1.8.2 Important physiological functions of prostaglandins..... 71
1.8.3 NSAIDs as non-selective and selective COX inhibitors..... 73
1.8.4 Prostaglandins in disease..... 74
1.8.5 Interaction of nitric oxide and prostaglandins..... 77
1.9 Endogenous CCK is elevated in nerve-injury and chronic morphine..... 84
1.95 Dynorphin..... 87
1.99 Experimental objectives and hypothesis..... 97

CHAPTER TWO: Materials and Methods..... 101
2.0 Animals..... 101
2.1 Drugs and doses.....101
2.2 RVM cannulation..... 103
2.3 Spinal nerve ligation..... 104
2.4 Intrathecal catheterization..... 105
2.5 PGE2 analysis..... 107
2.6 5-HT analysis..... 108

2.7 Tactile thresholds..... 110
2.8 Quantitative RT-PCR..... 111
2.9 Data analysis..... 113

CHAPTER THREE
PART ONE: Results..... 116
3.1 CCK in the RVM evokes the release of PGE_2 in the lumbar spinal cord..... 116
3.2 RVM/CCK-induced tactile hypersensitivity is attenuated by the non-selective COX inhibitor naproxen (i.th.)..... 120
3.3 CCK in the RVM does not affect the release of 5-HT into the lumbar spinal cord..... 124
3.4 RVM/CCK-induced tactile hypersensitivity is attenuated by the $5\text{-}HT_3$ antagonist ondansetron (i.th.)..... 128
3.5 RVM/CCK-induced tactile hypersensitivity is attenuated by the pre-administration of the CCK_2 antagonist YM022 into the RVM..... 133

PART TWO: Results..... 137
3.6 Prodynorphin mRNA in the RVM is increased following nerve injury (SNL)..... 137
3.7 B1- and B2-receptor mRNA in the RVM are increased following nerve-injury (SNL)..... 137
3.8 The B1-antagonist DALBK attenuates dynorphin-induced abnormal pain..... 140
3.9 The B2-antagonist Hoe-140 attenuates dynorphin-induced abnormal pain..... 145
4.0 The B2-antagonist Hoe-140 reverses nerve-injury induced abnormal pain..... 150
4.1 Dynorphin A(1-13) anti-serum reverses nerve-injury induced abnormal pain..... 150
4.2 Bradykinin microinjected into the RVM produces abnormal pain..... 154
4.3 The B2-antagonist Hoe-140 blocks bradykinin-induced abnormal pain..... 155
4.4 Dynorphin microinjected into the RVM produces long-lasting tactile hypersensitivity..... 158

CHAPTER FOUR: Discussion..... 160
CHAPTER FIVE: References..... 178

LIST OF FIGURES

Part One **PAGE**

Figure 1 Graphical representation of hyperalgesia and allodynia..... 23

Figure 2 Schematic drawing of the different models of nerve-injury in neuropathic pain..... 27

Figure 3 Schematic representation of the reorganization of primary afferent fibers (C- and Aβ-fibers) into the spinal dorsal horn after peripheral nerve injury..... 43

Figure 4 Schematic drawing depicting the location of serotonergic cell body groups in the brainstem..... 46

Figure 5 Biosynthetic pathway of serotonin (5-HT) from dietary L-tryptophan, and formation of the oxidative 5-HT metabolite, 5-HIAA..... 55

Figure 6 Illustration of the RVM with important landmarks and nuclei designated..... 56

Figure 7 Illustration of the membrane phospholipids–arachidonic acid cascade..... 66

Figure 8 Illustration of prostaglandin pathway, and diagram of the biological functions of the COX-1 and COX-2 pathways..... 67

Figure 9 Illustration and description of COX-enzymes, and their ability for pharmacological manipulation…… 81

Figure 10 Functions and interdependency of constitutive and inducible forms of NOS and COX…….…… 82

Figure 11 Inflammatory stimuli induce both iNOS and COX-2 expression to further exacerbate inflammation…… 83

Figure 12 Schematic representation illustrating the pharmacology of bradykinin receptors…… 92

Figure 13 Diagram showing the basic organization of the spinal cord…… 115

Figure 14 CCK into the RVM evokes the release of PGE_2 in the lumbar spinal cord; CCK_2 antagonist, YM022 attenuates this release…….…… 118

Figure 15 RVM/CCK-induced tactile hypersensitivity is partially attenuated by the nonselective COX inhibitor, naproxen (i.th.)…… 121

Figure 16 Effect of i.th. naproxen, 45-min post-CCK injection into the RVM…… 122

Figure 17 A modified AUC graph of Figure 15 data…… 123

Figure 18 CCK administered into the RVM did not alter the release of 5-HT in the lumbar spinal cord..... 126

Figure 19 Data summary of Figure 18 data..... 127

Figure 20 RVM/CCK-induced tactile hypersensitivity is partially attenuated by the 5-HT_3 antagonist, ondansetron (i.th.)..... 130

Figure 21 Effect of i.th. ondansetron, 45-min post-CCK injection into the RVM..... 131

Figure 22 A modified AUC graph of Figure 20 data..... 132

Figure 23 RVM/CCK-induced tactile hypersensitivity is partially attenuated by the pre-administration of the CCK_2 antagonist, YM022 into the RVM..... 134

Figure 24 Effect of RVM YM022, 45-min post-CCK injection into the RVM..... 135

Figure 25 A modified AUC graph of Figure 23 data..... 136

Part Two

Figure 26 Prodynorphin mRNA in the RVM is increased following nerve-injury..... 138

Figure 27 B1 and B2-receptor mRNA in the RVM are increased following nerve-injury (SNL)..... 139

Figure 28 The B1-antagonist DALBK partially attenuates dynorphin-induced abnormal pain..... 142

Figure 29 Effect of RVM DALBK, 45-min post-dynorpin injection into the RVM..... 143

Figure 30 A modified AUC graph of Figure 28 data..... 144

Figure 31 The B2-antagonist Hoe-140 attenuates dynorphin-induced abnormal pain..... 147

Figure 32 Effect of RVM Hoe-140, 45-min post-dynorpin injection into the RVM..... 148

Figure 33 A modified AUC graph of Figure 31 data..... 149

Figure 34 The B2-antagonist Hoe-140 reverses nerve injury induced abnormal pain..... 152

Figure 35 Dynorphin A(1-13) anti-serum reverses nerve-injury induced abnormal pain..... 153

Figure 36 Bradykinin administered into the RVM induces abnormal pain..... 156

Figure 37 The B2-antagonist Hoe-140 blocks bradykinin-induced abnormal pain…… 157

Figure 38 Dynorphin administered into the RVM produces long-lasting tactile hypersensitivity…… 159

ABBREVIATIONS

B1	Bradykinin B1-receptor
B2	Bradykinin B2-receptor
CCI	Chronic Constriction Injury
CCK_2 receptor	Cholecystokinin type-2 receptor
CNS	Central Nervous System
DALBK	Des-Arg9,Leu8-Bradykinin
DRG	Dorsal Root Ganglion
Dyn	Dynorphin
GABA	Gamma-Aminobutyric Acid
5-HT	Serotonin; 5-Hydroxytryptamine
L5/L6	Lumbar 5, 6
NMDA	N-Methyl-D-Aspartate
PNL	Partial Nerve Ligation
RVM	Rostral Ventromedial Medulla
SNL	Spinal Nerve Ligation

ABSTRACT

The neuropeptides dynorphin and cholecystokinin (CCK), and their associated pronociceptive effects were investigated in the RVM. Utilizing a nerve-injury model (SNL), RT-PCR analysis revealed increases ($p<0.05$) of prodynorphin mRNA, and bradyinkin, B1- and B2-receptor mRNA, post-SNL, 14-days, 2-days, and 14-days, respectively. Administration of dynorphin into the RVM produced both acute and long-lasting (>30-days) tactile hypersensitivity. Administration of the B1-antagonist, DALBK and the B2-antagonist, Hoe-140, into the RVM significantly attenuated dynorphin-induced tactile hypersensitivity. Nerve-injury induced tactile hypersensitivity was significantly reversed by RVM administration of dynorphin antiserum or the B2-antagonist, Hoe-140. These data suggest that dynorphin is up-regulated in the RVM in nerve-injury, and via the activation of bradykinin receptors in the RVM, produces abnormal pain. Like dynorphin, CCK is up-regulated in the RVM in nerve-injury, with studies suggesting that elevated levels of CCK in the RVM mediate pronociceptive activity through CCK_2 receptor activation, resulting in enhanced spinal nociceptive transmission. At present, it is unknown

what key neurotransmitters are mediating this RVM CCK-driven effect at the level of the spinal cord. Here, spinal cerebrospinal fluid (CSF) levels of serotonin (5-HT) and prostaglandin E_2 (PGE_2) were measured in the lumbar spinal cord in naïve rats following CCK administration into the RVM. Following RVM CCK microinjection, an approximate 5-fold increase in spinal (CSF) PGE_2 levels was observed, as compared to baseline controls. PGE_2 levels showed a progressive increase with peak levels observed at the 80-minute post-CCK injection timepoint, whereas 5-HT levels in the spinal CSF remained unchanged following CCK administration into the RVM. This release of PGE_2 coincided with the timecourse for CCK-induced mechanical hypersensitivity. Administration of the CCK_2-antagonist YM022 prior to CCK into the RVM, significantly attenuated (>50%) the release of PGE_2 in the spinal cord. The non-selective COX-inhibitor naproxen and the $5\text{-}HT_3$ antagonist ondansetron, both administered intrathecally, significantly attenuated RVM CCK-induced hindpaw tactile hypersensitivity. In summary, these data suggest a bradykinin- or CCK_2-receptor antagonist could be used alone or in conjunction with current therapies in the treatment of chronic pain.

Key words: rostral ventromedial medulla; descending facilitation; cholecystokinin; dynorphin; bradykinin receptor; serotonin; PGE_2; tactile hypersensitivity

CHAPTER ONE

Introduction

The International Association for the Study of Pain (IASP), an international professional organization for doctors and other health professionals involved in the diagnosis, treatment and scientific study of pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey and Bogduk, 1984). Pain as an unpleasant sensation, can be classified into two primary types, acute or chronic pain. Acute pain is often pain that follows injury to the body, is self-limiting, and usually diappears when the injury heals. It is frequently associated with objective physical signs or symptoms of sympathetic nervous system involvement. In contrast, chronic pain is not normally associated with signs of sympathetic nervous system activity, and oftens lacks the outward, obvious expression of acute pain (American Pain Society, 1999). Since pain implies an affective component that is highly subjective and unmeasurable in animals, we are only able to measure what is referred to as nociception. Nociception, derived from the Latin prefix *nocere* "to hurt" and the suffix *ception* –

a truncation of the word perception, is defined as the sensation of pain, or the perception of physical pain (Encarta 2007). As defined in the scientific community, nociception is a measurable event of a type (ie. paw withdrawal using von Frey filaments) usually associated with pain.

In pharmacology, pain as a subjective experience with an emotional, affective component is separate and distinct from nociception. Nociception, lacking this immeasurable emotional component, is restricted to the response (ie. von Frey or Hargreaves tests) acquired through transmission of sensory information via high-threshold noxious receptors known as nociceptors. Nociceptors are sensory neurons (free nerve-endings) found outside of the central nervous system (CNS: brain and spinal cord) in such tissues as the cornea, skin, muscle, bone, and gut – with cell bodies located in either the dorsal root ganglia (DRG) or the trigeminal ganglia. As molecular transducers (ie. converting mechanical energy to electrochemical energy) of noxious information to the brain and spinal cord, nociceptors are classified according to the kind of stimulus to which they respond, such as chemical, mechanical, or thermal stimuli. With respect to their rate

of conductivity from the periphery to the central nervous system, peripheral nociceptors may have either slowly conducting unmyelinated C-fiber axons, or faster conducting Aδ-fiber axons.

Primary nociceptive afferents are fibers that travel from the periphery to the spinal cord and form synapses on physiologically distinct cell layers called laminae in the spinal dorsal horn. Peripheral afferent fibers in the skin can be classified into three main types based on their diameter, structure and conduction velocity: C-fibers are thin (0.4-1.2 μm in diameter), unmyelinated and slowly-conducting (0.5-2.0 m sec^{-1}); Aδ-fibers are medium (2-6 µm), myelinated and of intermediate conducting velocity (12-30 m sec^{-1}); and Aβ-fibers are large (>10 μm), myelinated and fast conducting velocity (30-100 m sec^{-1}) (Millan 1999). The different fiber types (ie. Aβ-fibers, Aδ-fibers, C-fibers) form synapses on specific laminae (ie. I-V) within the dorsal horn. For example, thin-unmyelinated C-fibers terminate primarily in lamina I and II, whereas intermediate-myelinated Aδ-fibers form synapses in laminae I and V, and those of the thickly-myelinated fast-conducting Aβ-fibers terminate in lamina I and III. Once

transmitted from the peripheral primary afferent neuron to a second-order neuron in the spinal cord, noxious sensory information is then relayed up the spinothalamic tract, to a third-order neuron in the thalamus, and onto higher order neurons in the somatosensory cortex in the brain, where nociceptive perception is ultimately experienced (Kandel 2000).

1.1 Origins and symptoms of neuropathic pain

Neuropathic pain is a form of chronic pain, and is defined as "abnormal pain initiated or caused by primary lesion or dysfunction in the nervous system" (Merskey et al., 1984). A primary characteristic of this type of pain is its ability to continue long after the original tissue injury has been repaired. It is often measured, especially in animal models, via two quantifiable behavioral parameters, thermal hyperalgesia and mechanical (tactile) allodynia. Hyperalgesia is defined as an enhanced response to a noxious stimulus (ie. hot plate), and similarly, allodynia is defined as a noxious or painful response to a normally non-noxious stimulus (ie. touch) (Figure 1).

Neuropathic pain is a common complication in HIV/AIDS, diabetes, alcoholism, vasculitis (Ziegler et al. 1992; Schmader 2002; Schiffito et al. 2002; Koike et al. 2003; Daousi et al. 2004; Wolfe and Trivedi 2004), and can also be a consequence of metabolic disease (ie. vitamin B deficiency), infection, ischemia, injury, entrapment, connective tissue disease, acquired, cancer, drugs, and toxins (Woolf and Mannion 1999; Dworkin 2002; Gonzales et al. 2003; Sommer 2003). It may also develop without any identifiable cause, as in intercostals neuralgia and idiopathic polyneuropathy, and the emergence of painful neuropathy may be the first sign of a systemic disease. Common signs associated with neuropathic pain, include positive signs such as mechanical allodynia and thermal hyperalgesia, and negative signs such as sensory loss, weakness, and muscle atrophy (Bowsher 1996; Baron 2000; Jensen et al. 2001; Jänig and Baron 2003). The clinical characteristics of this type of pain include electric shock-like pain, burning pain, pain paroxysms, dysesthesia, and paresthesia (Jensen et al. 2001; Dworkin 2002; Hansson 2002).

The significance of finding more effective, efficacious treatments for chronic neuropathic pain cannot

be overstated, as neuropathic pain is often very difficult to treat. The most successful treatments involve a variety of older medicines, which have been in use for decades and include tricyclic antidepressants such as amitriptyline and imipramine, anticonvulsants such as carbamazepine and valproic acid, and the TRPV1 receptor agonist/desensitizer capsaicin. An in-depth, systematic review of randomized controlled trials by Wong et al., 2007 found the older, traditional antidepressants and anticonvulsants to be more effective clinically than the newer generation selective-serotonin reuptake inhibitor (SSRI) citalopram, or the serotonin norepinephrine reuptake inhibitor (SNRI) duloxetine, and the newer anticonvulsants, pregabalin and gabapentin (Wong et al., 2007). With that said, there is still some evidence that both SSRIs and SNRIs are of some utility in the treatment of neuropathic pain. The SSRIs, paroxetine and citalopram have been shown to offer relief in steady and lancinating pain, and in diabetic neuropathy, respectively (Sindrup et al., 1990a, Sindrup et al., 1992).

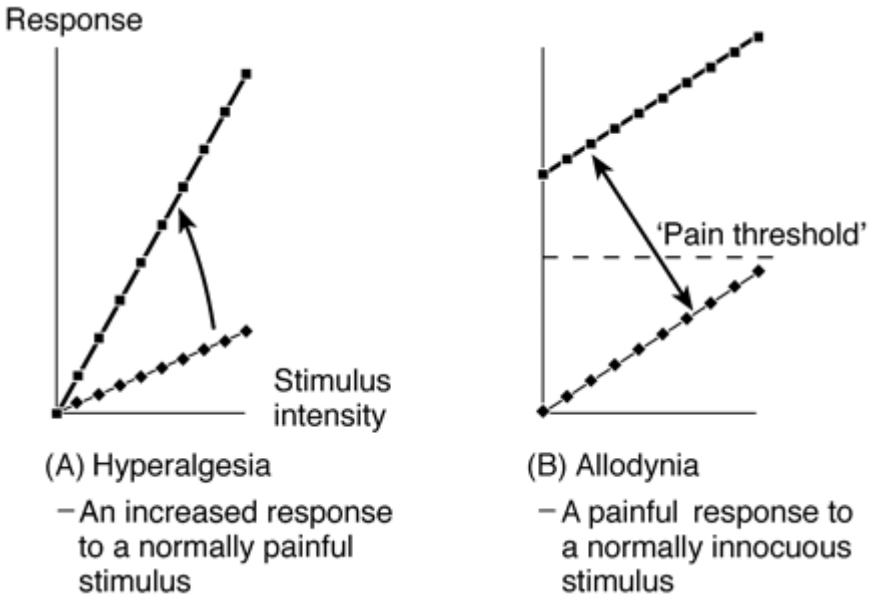

Figure 1 Graphical representation of hyperalgesia and allodynia: stimulus intensity *versus* response relationship for noxious and innocuous stimuli (Illustration from: Bridges et al., 2001).

1.2 Models of Neuropathic Pain

Several animal models of peripheral neuropathic pain have been developed over the years, particularly in rodents. These models of neuropathic pain have aptly demonstrated many features characteristic of neuropathic pain in an array of disease states in humans, and have one particular feature in common, which usually involves an alteration in hind-limb cutaneous sensory thresholds through injury to the sciatic nerve (ie. peripheral nerve). The most common physiological outcome of this nerve injury is hyperalgesia to noxious thermal stimuli (ie. hot plate test) and allodynia to mechanical (ie. von Frey test using calibrated filaments) or cold stimuli (ie. acetone or ice bath) (Bridges et al., 2001). At the present time, there are three commonly utilized models of neuropathic pain in research (Figure 2), and they include the partial sciatic nerve ligation (PNL) (Seltzer et al., 1990), the chronic constriction injury of sciatic nerve (CCI) (Bennett and Xie, 1988), and the spinal nerve ligation model (SNL) (Kim and Chung, 1992) which is singularly employed in my studies to provide a simulation of the neuropathic pain state. The existence and likewise availability of these models

provides the investigator the opportunity to explore the origins and mechanisms of neuropathic pain.

The following is a concise description of the aforementioned, most commonly employed animal models of neuropathic pain used to mimic the human condition: the PNL model is created through the tight ligation of one-third to one-half of the sciatic nerve at mid-thigh level. Guarding of the ipsilateral hind paw along with a licking behavior is suggestive of the existence of spontaneous pain (Seltzer et al., 1990). The CCI model is produced by the positioning of four loose ligatures comprised of chromic gut suture surrounding the sciatic nerve at mid-thigh level. This particular model induces an inflammatory reaction in response to the catgut suture, causing a loss of almost all A-fibers and some C-fibers (Tandrup et al., 2000), and subsequently resulting in the development of allodynia, hyperalgesia, and potential spontaneous pain (Bennett and Xie, 1988).

The last of these models is the SNL model, which mimics peripheral nerve injury, and is the sole model used throughout my studies. This model is produced by the use of tight ligatures around the L5 and L6 spinal nerves (distal to the DRG) prior to forming the sciatic nerve (Kim

and Chung, 1992). This produces destruction of the myelin sheaths and cell death to the nerves extending out to the periphery, distal to the nerve injury, while the cell bodies in the DRG and the afferents extending centrally remain functionally intact. Although intact and functional, due to the nerve injury, the cell bodies in the DRG experience aberrant ectopic activity. As a consequence of this nerve injury and the resulting abnormal ectopic discharges, spontaneous pain-like behavior develops immediately following this surgery, along with robust and long-lasting tactile hypersensitivity and thermal hyperalgesia. This model creates symptoms of painful neuropathy in much the same manner as the CCI model, but differs in that it yields a complete separation between injured and intact segments along with greater consistency of results.

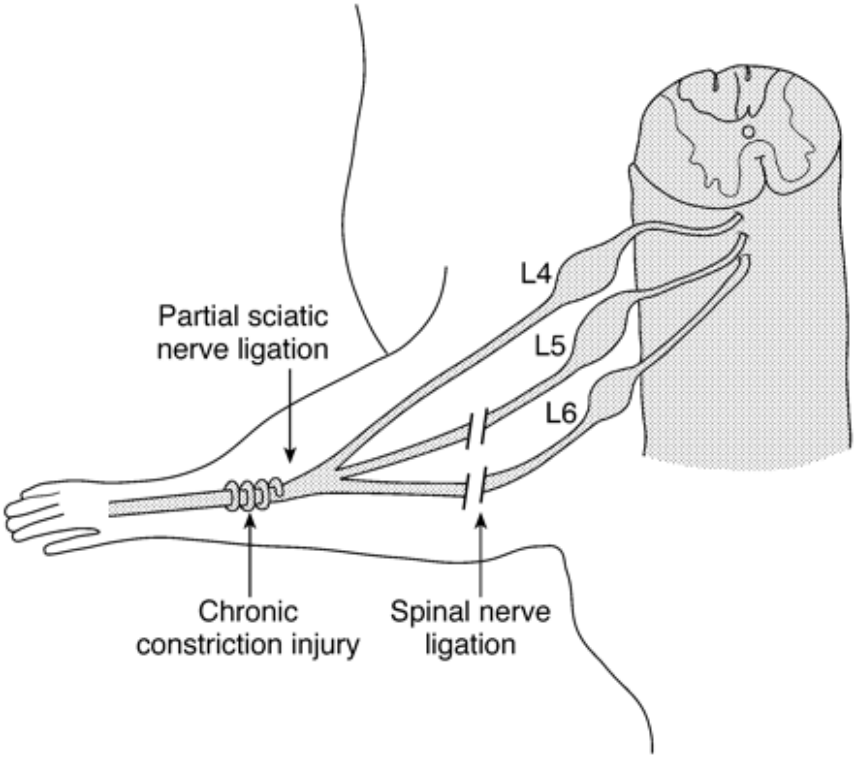

Figure 2 Schematic drawing of the partial sciatic nerve ligation (tight ligation of 33-50% of the sciatic nerve trunk), CCI (loose ligations of the sciatic nerve trunk), and SNL (tight ligation and transection of the L5 and L6 spinal nerves) animal models of neuropathic pain. (Illustration from: Bridges et al., 2001)

Given that the L4 spinal nerve also forms the sciatic nerve and is left intact, both injured and uninjured nerves from the sciatic nerve are left remaining. As a result, the nociceptive afferent pathway from the site of injury, extending from the paw and ectopic foci can be identified with relative ease.

In general, behavioral signs of both ongoing and evoked pain are produced in all of these three models. Though, comparisons taken between the PNL, CCI, and SNL models reveal that the SNL model shows the most pronounced indications of tactile allodynia, whereas the CCI model produced the most obvious behavioral signs of ongoing pain. With that said, it should be noted that because all of these models produce some form of neuropathic pain behavior, that despite their specific differences, it is probable that they share some fundamental mechanisms in common.

In addition to the previously discussed peripheral models of neuropathic pain, a variety of other animal models of neuropathic pain have been developed that more closely resemble specific disease states in humans. One of which is the streptozocin model of peripheral diabetic neuropathy, which involves a single injection of

streptozocin to produce diabetes accompanied with allodynia and hyperalgesia (Malcangio et al., 1998). Additional examples include models that utilize cytotoxic chemotherapeutic drugs such as paclitaxil and vincristine, which produce tactile and thermal hypersensitivities at relatively low doses (Aley et al., 1996; Authier et al., 1999, 2000, 2003; Flatters and Bennett, 2006; Siau and Bennett, 2006). The effects created by these drugs mimic the neuropathy and chronic inflammatory pain induced through long-term cancer treatment with chemotherapeutics, and also seen in such chronic inflammatory diseases as arthritis (Fields et al., 1994).

1.3 Mechanisms of Neuropathic Pain

During and immediately following peripheral injury, a host of inflammatory mediators such as bradykinin, PGE_2, substance P, and histamine are released into the site of injury, which act to promote pain and inflammation, both as a protective mechanism to warn of injury and to prevent further injury and encourage immobilization of the injured area. Due to the inflammation and inflammatory mediators released in the immediate and surrounding tissue, primary nociceptive afferents are activated resulting in primary

hyperalgesia, an amplified response to a noxious stimulus, and primary allodynia, a painful response to a normally non-noxious stimulus. Neighboring, but unaffected tissues can also become more sensitive, in what is referred to as secondary hyperalgesia (Vanegas 2004).

It has been well-documented that descending pain control systems in the brainstem (ie. RVM) play a strong role in modulating both acute and chronic peripheral sensitivity and pain (Porreca et al., 2002 Urban et al., 1999). The data suggests that descending inhibition, from serotonergic neurons located in the nucleus raphe magnus (NRM), predominates during acute pain episodes, and lessens the intensity of primary hyperalgesia, while descending facilitation predominates in secondary hyperalgesia. It has been shown that blocking descending control through bilateral lesioning of the dorsolateral funiculus or destruction of serotonergic neurons of the NRM, results in more intense primary hyperalgesia, induced through carrageenan or CFA hindpaw injection. This supports the presence and likewise predominance of descending inhibitory control during acute inflammation and pain (Ren and Dubner, 1996; Wei et al., 1999). Additional support to this was provided in earlier studies utilizing electrical

stimulation of the RVM through which the activation of glutamatergic synapses was shown to promote descending inhibition to the spinal cord. However, 3 hours after inducing inflammation with CFA, the ability of electrical stimulation in the RVM to produce spinal inhibition had decreased (Terayama et al., 2000). This suggested that along with inhibition, which predominated acutely, facilitatory mechanisms were also being activated, but with an apparent delay. From these observations, it is apparent that competition exists between descending inhibition and facilitation, with inhibition predominating in the initial stages of pain, and facilitation antagonizing and eventually outcompeting inhibition at later stages. Recent studies suggest that once the acute inflammation subsides, descending facilitation from the RVM serves an integral role in the maintenance of pain, with facilitation predominating during chronic pain conditions such as nerve-injury (SNL) and in visceral inflammatory states such as pancreatitis (Kovelowski, 2000; Porreca et al., 2001; Burgess et al., 2002; Sah et al., 2003; Vera-Portocarrero, 2006

Subsequent to nerve injury, a substantial increase in the level of spontaneous firing manifests in the afferent

neurons, in the area marked by and immediately surrounding the site of injury, and is termed ectopic discharge (Wall et al., 1974). Following peripheral nerve injury, it is believed that ectopic afferent discharge is a primary contributor to the development of chronic, neuropathic pain (Devor and Seltzer 1999). In primary afferent neurons that have been axotomized, the sites of origin with the most apparent ectopic discharge are the actual site of injury (neuroma) and the DRG (Burchiel 1984; Govrin-Lippmann and Devor 1978; Wall and Devor 1983; Wall and Gutnick 1974). Where the nerve injury occurs plays a governing role in where the ectopic activity actually originates. In sciatic nerve injury, which lies at a distance from the DRG, ectopic activity is seen at the site of injury, whereas in injury of the spinal nerves, spontaneous ectopic activity occurs in the DRG (Liu et al., 1999, 2000).

Ectopic activity and its progressive development may be especially important for the development of allodynia, hyperalgesia, and the unrelenting pain associated with nerve injury. Whereas, it is not entirely known what drives the pathogenesis of allodynia following peripheral nerve injury, it is known that continuous discharges from ectopic foci play a contributing role in the maintenance of

neuropathic pain (Sheen and Chung, 1993; Matzner and Devor, 1994; Ossipov et al., 1995). In addition, it has been proposed that ectopic discharge from injured peripheral afferent nerves acts as a driving force for the sensitization of spinal dorsal horn neurons ultimately leading to a state of central sensitization (Woolf 1983; Simone et al., 1991; Gracely et al., 1992; Yoon et al., 1996).

In animal nerve injury models, it is known that the inception of increased afferent discharge correlates well with the onset of both thermal and tactile hypersensitivities (Han et al. 2000; Liu et al., 2000). Both thermal and tactile hypersensitivities, two primary measures of neuropathic pain, persist for weeks following SNL surgery whereas the rate of ectopic discharge begins to diminish with time (Chaplan et al., 1994; Bian et al., 1999; Malan et al., 2000), displaying its greatest frequency within the first week following SNL surgery (Han et al., 2000).

Furthermore, it has been observed that ectopic discharge displays a peak in activity within 1 to 3 days following nerve injury, and a return to normal, control levels by day 7 to 9 (Liu et al., 2000). These

observations tend to suggest that the increased ectopic discharge seen in the first few days following nerve injury may be responsible for the initiation phase of neuropathic pain, while the mechanisms governing the maintenance phase are thought to involve more centrally mediated influences such as descending control from the RVM (Pertovaara et al., 1996; Kowelowski et al., 2000; Porreca et al., 2001; Burgess et al., 2002; Sah et al., 2003).

Moreover, it has been demonstrated that noxious or non-noxious mechanical and chemical stimuli can evoke the generation of ectopic discharge (Seltzer and Devor 1979; Tal and Devor 1992; Sheen and Chung 1993). It is known from electrophysiological studies of primary sensory neurons with transected peripheral axons, that their cell-bodies located in the DRG can become hyperexcitable, and that these cell-bodies can be a source of spontaneous ectopic discharge in the absence of external stimuli such as peripheral receptor activation or endogenous chemical mediators (Wall and Devor, 1983; Xie et al., 1995). Changes in the expression of sodium channels in the terminal neuroma of peripheral nerves and in their cell bodies, together are believed to be the source of nerve injury induced ectopic discharge. It was later revealed

that accumulation of sodium channels in the neuroma occurs (Devor et al., 1989), and subsequently demonstrated that the sodium channels were the cause of the ectopic activity (Matzner and Devor, 1994).

It has also been demonstrated that following nerve injury, changes occur in the distribution of TTX-resistant sodium channels (Novakovic et al., 1998; Porreca et al., 1999; Gold et al., 2003), which may lead to a state of neuronal hyperactivity by a lowering of the action potential threshold. A study conducted by Pan et al in 1998 found electrophysiological evidence that systemic gabapentin could inhibit peripheral ectopic afferent discharge in nerve-injured rats, which could contribute to and explain its anti-allodynic activity in treating neuropathic pain (Pan et al., 1998). It is thought that gabapentin's affect on neuroma ectopic discharge stems from its ability to inhibit impulse generation as opposed to impulse propagation (Matzner and Devor, 1994), which is similar to the mechanism of action of sodium channel blocking agents (Devor et al., 1992; Matzner and Devor, 1994).

In conjunction with changes occurring in voltage-gated sodium channels, it has also been observed that potassium

channels may serve an important role in influencing ectopic discharges. Potassium channel blocking agents applied either to the injury site (Matzner and Devor, 1994; Xie et al., 1993) or to the DRG (Liu et al., 2001) enhance ectopic discharge. Moreoever, application of potassium channel blockers at a low dose evokes discharges from quiescent axotomized neurons (Liu et al., 2000; Matzner and Devor, 1994; Xie et al., 1993; Kajander et al., 1992). From these data, one can surmise that hyperexcitability of axotomized DRG neurons is accompanied with the down regulation of potassium channels. Furthermore, it has been suggested that a decrease in potassium currents observed following peripheral nerve injury contributes to neuronal hyperexcitability (Devor 1983; Devor 1994), and following peripheral nerve axotomy, it has been demonstrated that a decrease of potassium currents in DRG neurons results (Liu 2000).

In addition to the influence of sodium and potassium channels in neuronal hyperexcitability, calcium channels have been shown to influence the neuropathic pain state through promoting the development of hyperalgesia and allodynia. The administration of neuronal N-type calcium channel antagonists have been shown to lessen thermal

hyperalgesia and tactile allodynia in the PNL (White and Cousins, 1998) and CCI models (Xiao and Bennett, 1995) of neuropathic pain. Studies utilizing electrophysiology have shown a decrease in N-type calcium current measured in the DRG following axotomy, and have also demonstrated alterations in non-specific calcium current after peripheral nerve injury (Baccei and Kocsis, 2000). Changes such as these, in both sodium and calcium channels, can lead to increases in both firing frequency and susceptibility, ultimately leading to the generation of spontaneous pain, central sensitization, and the development of chronic neuropathic pain.

Synthesized from the excitatory amino acid glutamic acid via a simple one-step decarboxylation reaction (catalyzed by the enzyme glutamic acid decarboxylase: GAD) – the biogenic amine gamma-amino butyric acid (GABA), acts as the primary inhibitory neurotransmitter in the CNS where it plays an important role in the modulation of pain. Extracellular GABA concentrations are known to decrease in nerve-injured rats as compared to normal rats (Stiller et al., 1996), and the administration of GABA agonists intrathecally have been shown to produce a dose-dependent reversal of allodynia in nerve-injured rats (SNL) (Hwang

and Yaksh, 1997). These data suggest that decreased levels or activity of GABA following nerve injury could play a significant role in the expression and severity of neuropathic pain. It is thought that a reduction in GABA activity could play a significant role in central sensitization.

Loss of GABAergic inhibition in the spinal dorsal horn was found to accompany ischemic injury to the spinal cord, creating a mechanical allodynia resembling that of peripheral nerve injury (Hao et al., 1992; Hao et al., 1996a and Hao et al., 1996b; Zhang et al., 1994a). Wide-dynamic range (WDR) neurons in the spinal dorsal horn exhibited a prolonged and enhanced response to electrical or innocuous, mechanical stimulation, while their response to thermal stimuli was found to be normal. Baclofen, a $GABA_B$ agonist, reversed the tactile allodynia along with the abnormal responsiveness of the WDR neurons, suggesting a loss of GABAergic inhibition in the dorsal horn. Consistent with the previous observations, GABA levels in the spinal cord and the number of GABAergic cells in lamina I and II were found to decrease in ischemia (Zhang et al., 1994a; Martiniak et al., 1991). However, the loss of GABAergic neurons is only transitory and they recover once

the allodynia subsides, which suggests that the neurons do not irreversibly degenerate (Hao et al., 1996a; Zhang et al., 1994a).

1.3.1 Anatomical reorganization of the spinal cord in neuropathic pain

Anatomical and neurochemical changes often occur within the spinal cord and brain subsequent to peripheral nerve-injury, and may serve an important role in the development of chronic, neuropathic pain (Tasker 1993). This “plasticity” of the CNS is thought to play an important role in the development of chronic, neuropathic pain. As is the case in the periphery, sensitization of neurons can occur centrally within the dorsal horn following peripheral tissue damage. This is marked by a decreased threshold and an increased responsivity to afferent input along with increased spontaneous activity of the dorsal horn neurons, and cell death in the spinal dorsal horn (Johnson and Parris 1994; Cameron et al., 1991; Garrison et al., 1991; Sugimoto et al., 1990).

After peripheral nerve injury, these large afferents gain access to spinal regions involved in transmitting high

intensity, noxious signals, instead of merely encoding low threshold information. These changes are referred to as central nervous system plasticity. Under normal, non-pathogical conditions, small unmyelinated C-fibers, which carry information about pain, temperature and itch, generally penetrate no deeper than lamina II in the spinal dorsal horn. Intermediate myelinated Aδ-fibers carry information about pain and temperature and terminate in lamina II also, while larger myelinated Aβ-fibers, which carry information regarding touch, penetrate the dorsal horn and travel ventrally to form synapses in lamina III and deeper. Though, under pathological conditions such as chronic nerve injury, Aβ-fibers are known to sprout dorsally from lamina III into the superficial (laminas I and II) dorsal horn (Woolf et al., 1992; Yaksh 1993; Koerber et al., 1994; Woolf and Doubell 1994; Woolf et al., 1995). Due to this anatomical reorganization in the spinal cord, functional synaptic contact with second-order neurons in the superficial lamina is established, thus allowing non-noxious low-threshold stimuli from Aβ-fibers to be interpreted as nociceptive (Figure 3). This however was not supported by later experiments, which show that optimal

Aβ-fiber sprouting does not occur until 2 weeks post-injury (Woolf et al., 1995; Lekan et al., 1996).

1.3.2 Central sensitization in neuropathic pain

The process of central sensitization, resulting from prolonged or excessive sensory input following peripheral tissue damage, nerve-injury or inflammation, can lead to the development of a sustained state of hyperexcitability of dorsal horn neurons in the spinal cord. Central sensitization has been called the underlying central mechanism for the generation of pain and allodynia and is distinguished by the phemomenon of “wind-up” (Wall and Woolf, 1986; Chung and Chung, 2002, Ji et al., 2003). Wind-up is characterized by an increasing response to repetitive noxious stimulation of C-fibers, and results in a progressive increase in the number of action potentials elicited per stimulus that occurs in dorsal horn neurons (Pockett 1995). C-fiber input is known to be critically important in triggering central sensitization (Woolf et al., 1991). Long-term potentiation (LTP), which involves a long-lasting enhancement in synaptic transmission, may be precipitated by repetitive episodes of "wind-up". While "wind-up" is thought to last from seconds to minutes, LTP

by definition lasts for hours and even months. Both "wind-up" and LTP are believed to underlie the process of central sensitization involved in many chronic pain states. Current studies have demonstrated that increased NMDA receptor activation is associated with sensitization in the spinal cord. In addition, following CCI, increased glutamate concentrations have been found in the ipsilateral dorsal horn (Kawamata and Omote, 1996), and studies utilizing the NMDA receptor antagonist, MK-801, have observed decreases in the behavioral signs of neuropathic pain (Davar et al., 1991; Mao et al., 1992; Sotgiu and Biella, 2000).

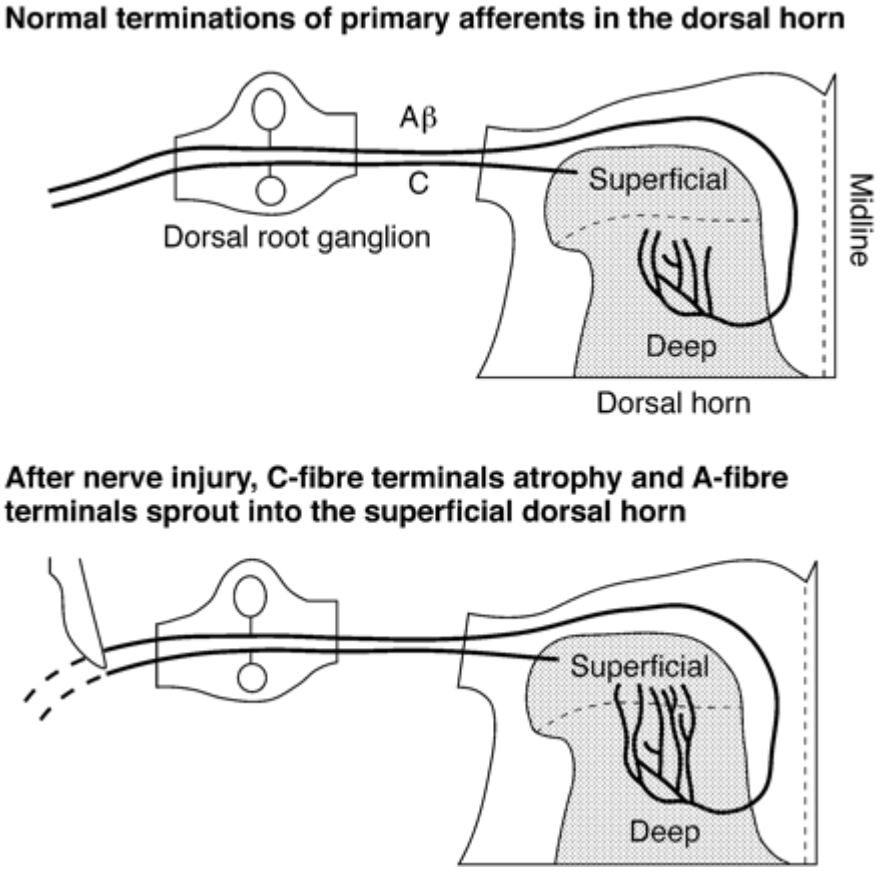

Figure 3 Schematic representation of the reorganization of primary afferent fibers (C- and Aβ-fibers) into the spinal dorsal horn observed after peripheral nerve injury (Illustration from: Bridges et al., 2001).

Central sensitization is known to involve two fundamental synaptic mechanisms, described as either, homosynaptic or heterosynaptic in origin (Magerl et al., 1998; Woolf et al., 1988). Homosynaptic sensitization occurs when the conditioning stimulus and the test stimulus involve the same type of input. This is evident in the aforementioned phenomenon "windup" where continual low-frequency stimulation of nociceptive C-fiber afferents in dorsal horn neurons, leads to an increasing response in the dorsal horn. As previously stated, "windup" is regarded as a short-term form of sensitization and lasts for seconds to minutes. Heterosynaptic sensitization refers to a situation where the conditioning stimulus and the test stimulus involve different sets of inputs. Heterosynaptic sensitization is seen in allodynia, wherein a non-noxious stimulus elicits a painful response. Electrophysiological evidence for heterosynaptic sensitization has been demonstrated in primate studies of dorsal horn cells where the response to light stroking of the skin was enhanced after capsaicin injection (Simone et al., 1991).

1.4 The RVM and pain modulation

The rostral ventromedial medulla (RVM)(Figure 4), a structure that includes the midline nucleus raphe magnus (NRM) and adjacent lateral reticular formation (Fields et al., 1983; Aimone and Gebhart, 1986) is a well-established region known to play a strong role in pain modulation (Basbaum and Fields, 1984; Fields et al., 1991; Heinricher and Morgan, 1999). In our study, the RVM was defined as the region containing the nucleus raphe magnus, nucleus gigantocellularis pars-alpha, and laterally adjacent reticular formation at the level of the facial nucleus (Heinricher and Neubert, 2004). With respect to its stereotaxic coordinates, the area was defined as: anteroposterior, 10–11.5 mm caudal from bregma; dorsoventral, 8.25–10.5 mm; mediolateral, 0–1.5 mm (Xie et al., 2005).

The RVM was first characterized as a supraspinal source of descending inhibitory influence, and later discovered to possess a prominent facilitatory influence on nociceptive transmission as well (Fields et al., 1992; Zhuo et al., 1997; Calejesan et al., 2000; Porreca et al., 2002). The nucleus raphe magnus (NRM) and adjacent structures within the RVM receive projections from another

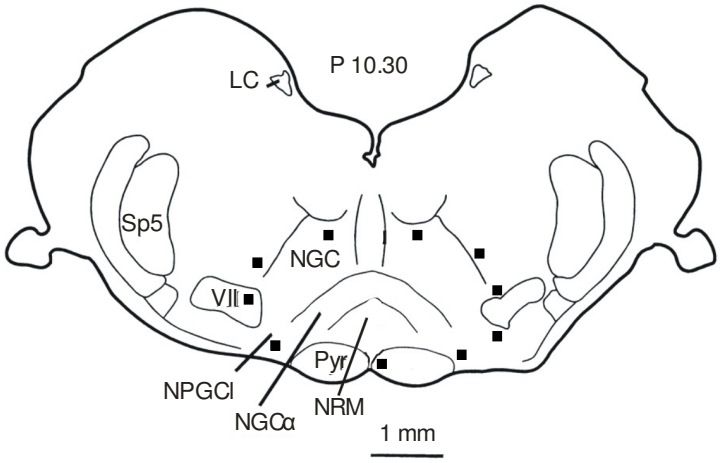

Figure 4 Important landmarks and nuclei in the RVM. The RVM was defined as the region containing the nucleus raphe magnus, nucleus gigantocellularis pars-alpha, and laterally adjacent reticular formation at the level of the facial nucleus. RVM area demarcated above, and defined as: AP, 10-11.5 mm caudal from bregma; dorsolateral, 8.25-10.5 mm; mediolateral, 0-1.5 mm). NRM, nucleus raphe magnus; NGC, nucleus gigantocellularis; NGCα, nucleus gigantocellularis pars-alpha; NPGCl, nucleus paragigantocellularis lateralis; Sp5, spinal trigeminal nucleus; VII, facial nucleus; Pyr, pyramidal tract. Illustration taken from: Zhuo, M. et al. J Neurophysiol 87: 2225-2236 2002.

pain-modulatory region in the brainstem, the periaqueductal grey (PAG) which lies further rostral in the midbrain. The RVM then projects to the spinal cord via pathways conducted primarily through the dorsolateral funiculus (DLF) to the spinal dorsal horn. This hierarchical modulatory system, comprised of the PAG and RVM, and even further rostral in the limbic system (ie. amygdala), together govern descending pain transmission through activating either inhibitory or facilitatory pathways to the spinal cord (Zimmermann, 1991; Fields and Basbaum, 1999; Marabese 2007). Concerning the effects of excitatory neurotransmitters in the RVM and its dual nature, glutamate microinjected into the RVM in naïve animals displays biphasic modulatory effects with facilitation at low doses and inhibition at higher doses (Zhuo and Gebhart, 1997).

At the present, three types of cells have been identified in the RVM. Two of which, the ON- and OFF-cells function directly in descending pain modulation and possess facilitatory and inhibitory activity, respectively. The third type, NEUTRAL-cells, represent all of the remaining cells in this region that are neither ON- nor OFF-cells. In response to noxious stimuli, the facilitatory ON-cell displays increase firing and is directly activated by the

prociceptive neurotransmitter CCK (Heinricher and Neubert, 2004) and inhibited by mu-opioid agonists (Bederson et al., 1990; Fields 1992; Heinricher et al., 1992; Pan et al., 2000), whereas in the presence of noxious stimuli, the inhibitory OFF-cell displays decrease firing and is indirectly activated by mu-opioids through GABAergic disinhibition (Fields et al., 1983b; Heinricher et al., 1994). Experimentally, it has been shown that CCK microinjection into the RVM results in ON-cell activation that coincides with behaviorally measurable features such as thermal hyperalgesia, which suggests that CCK activity in the RVM may drive descending pain facilitation (Heinricher and Neubert, 2004). In contrast, it has been shown that OFF-cell activation ensuing in antinociception and enhanced analgesia can be evoked by opioid peptides and blocked by microinjection of the "anti-opioid" CCK into the RVM (Heinricher and Morgan, 1999; Neubert et al., 2004).

Further studies have sought to uncover the mechanism whereby RVM-mediated descending facilitation exerts its effects further downstream in the spinal cord. RVM facilitated descending modulation has been previously characterized as playing an important role in spinal nociception (Fields et al., 1983; Fields and Heinricher,

1985; Morgan and Fields, 1994; Friedrich and Gebhart, 2003) and is mediated via bulbospinal projections traveling through the dorsolateral funiculus (DLF) (Zhou and Gebhart, 1992; Urban et al., 1996; Urban and Gebhart, 1997; Fields and Basbaum, 1999). Descending pain modulatory regions of the brainstem have also been shown to be important in both the manifestation and maintenance of certain chronic pain states (Ossipov et al., 2000; Millan, 2002). Previous studies have also shown that spinal transection blocks mechanical but not thermal responses in rats with injury to the L5/L6 (SNL) spinal nerves (Bian et al., 1998), implicating a supraspinal mechanism involving ascending and descending tracks for modulating tactile sensitivity whereas thermal sensitivity being predominately spinal mediated.

Considering the descending bulbospinal serotonergic system, antagonism of the 5-HT_3 receptor is known to decrease the peripheral response to mechanical stimuli (von Frey filaments), implying a strong supraspinal driven mechanism, while possessing much lesser effects on thermal responses. These observations are in agreement with a report showing that only 13% of 5-HT_3 afferents co-express

the heat-sensitive TRPV1 receptor (Suzuki et al., 2004; Zeitz et al., 2002).

In addition, experimentally produced neuropathic pain can be reversed by lidocaine microinjection into the RVM, offering further support to the critical role that descending facilitation plays as an important part in this type of pain (Pertovaara et al., 1996; Kovelowski et al., 2000). Stimulated at low electrical intensities, the RVM has been shown to promote increased dorsal horn neuronal activity along with increased tail flick reflex to nociceptive stimulus, indicative of enhanced nociceptive processing from this facilitatory region (Zhou and Gebhart, 1992, 1997). It has also been shown that selective ablation of mu-opioid receptor expressing cells in the RVM, thought to be important for descending facilitation, prevented the maintenance but not the initiation of experimental neuropathic pain, which supports the importance of the RVM in promoting and maintaining chronic pain states (Porreca et al., 2001; Burgess et al., 2002).

1.5 CCK and descending facilitation from the RVM

Cholecystokinin (CCK), a neuropeptide found both in the enteric and central nervous system, possesses contrasting digestive and nervous system activities along with displaying an unusually high degree of species-dependent, tissue heterogeneity. The major tissue difference is in the processing of pro-CCK in the brain and digestive tract. The major carboxyl-terminal amidated peptide in the brain is CCK-8, though some CCK-4 has also been detected in rat brain (Qureshi et al., 1993). With respect to the amino acid residues that confer CCK's biological activity, amidated sulfated CCK-7 is the smallest CCK peptide with full biological activity, while larger amidated, sulfated peptides like CCK-33 also display full activity. Electrophysiological studies of CCK provide evidence that CCK is an excitatory neurotransmitter or neuromodulator, often directly activating specific cells. However, the action of CCK can be blocked by antagonists of other neurotransmitter systems like DA, 5-HT, GABA or endogenous opiates, which suggests that CCK is releasing agents that are excitatory and perhaps even inhibitory (Albrecht 1995). CCK is known to excite neurons in the

periaqueductal gray (Liu et al., 1994), dorsal raphe (Boden and Woodruff, 1995), nucleus accumbens (Yim and Mogenson 1991), hippocampus (Bradwejn and deMontigny 1984), and spinal cord (Jeftinija et al., 1984). It also depolarizes oxytocin-containing neurons in the supraoptic nucleus of the hypothalamus (Beinfeld 1992) and serotonin-containing neurons in the dorsal raphe nucleus.

CCK has a well-documented antagonistic relationship with opioids in the RVM (Faris et al., 1983; Heinricher et al., 2001 Friedrich and Gebhart, 2003), thought in part as a result of their extensive overlapping distribution in the central nervous system (Stengaard-Pederson and Larsson, 1981; Ghilardi et al., 1992; Verge et al., 1993). CCK administered bilaterally into the RVM results in enhanced nociceptive input and attenuates the morphine induced reduction of ON-cell responses to nociception (Heinricher and McGaraughty, 1996). In addition to the widespread overlapping receptor distributions of CCK and opioids, it is possible that a portion of their antagonistic relationship could be mediated by the postsynaptic co-localization of the excitatory CCK_2 receptor with the inhibitory mu-opioid receptor on ON-cells in the RVM

(unpublished observations; Lai et al., 2006). Along with the study by Heinricher and Neubert in 2004, which showed that CCK microinjected into the RVM induces ON-cell activity, there is also evidence suggesting that endogenous CCK drives descending facilitation from the RVM (Kovelowski et al., 2000; Friedrich and Gebhart, 2003). Recent studies have also demonstrated that CCK antagonists can enhance opioid analgesia in humans (McCleane et al., 1998, 2000, 2003).

1.6 Serotonin and pronociception

Serotonin (5-hydroxytryptamine; 5-HT), is an important monoamine (indoleamine) neurotransmitter in the CNS, derived from dietary tryptophan and synthesized via a two-step enzymatic reaction involving a ring hydroxylation (tryptophan hydroxylase) followed by a decarboxylation (L-amino acid decarboxylase) step (Foye 1995; Figure 5). Serotonergic neurons and nuclei are distributed throughout and along the midline of the brainstem, and are particularly concentrated in the raphe nuclei, along with the following nuclei and regions of the brainstem: nucleus raphe pallidus and nucleus raphe obscurus in the rostral medulla, the superior central nucleus in the rostral pons,

the dorsal raphe nucleus in the caudal midbrain, and the nucleus raphe magnus in the caudal pons. These nuclei have extensive projections that innervate virtually all parts of the CNS (Figure 6), and play a major role in descending pain modulation (Yaksh et al., 1981; Baumgarter and Gothert, 1997; Millan, 1997; Nolte 2002). Nearly all of the serotonergic neurons projecting from the raphe-nuclei to the spinal cord are unmyelinated in the rat (Dahlstrom and Fuxe, 1965), and raphe-spinal axons from the nucleus raphe magnus have been shown to be concentrated in the dorsolateral funiculus (Basbaum and Fields, 1979). Due to the widespread release of 5-HT from nerve terminals throughout the CNS, the serotonergic system plays an important role in many physiological functions such as the regulation of body temperature, blood pressure, hormone release, appetite, emotional behavior, and the perception of pain (Fink and Gothert, 2007).

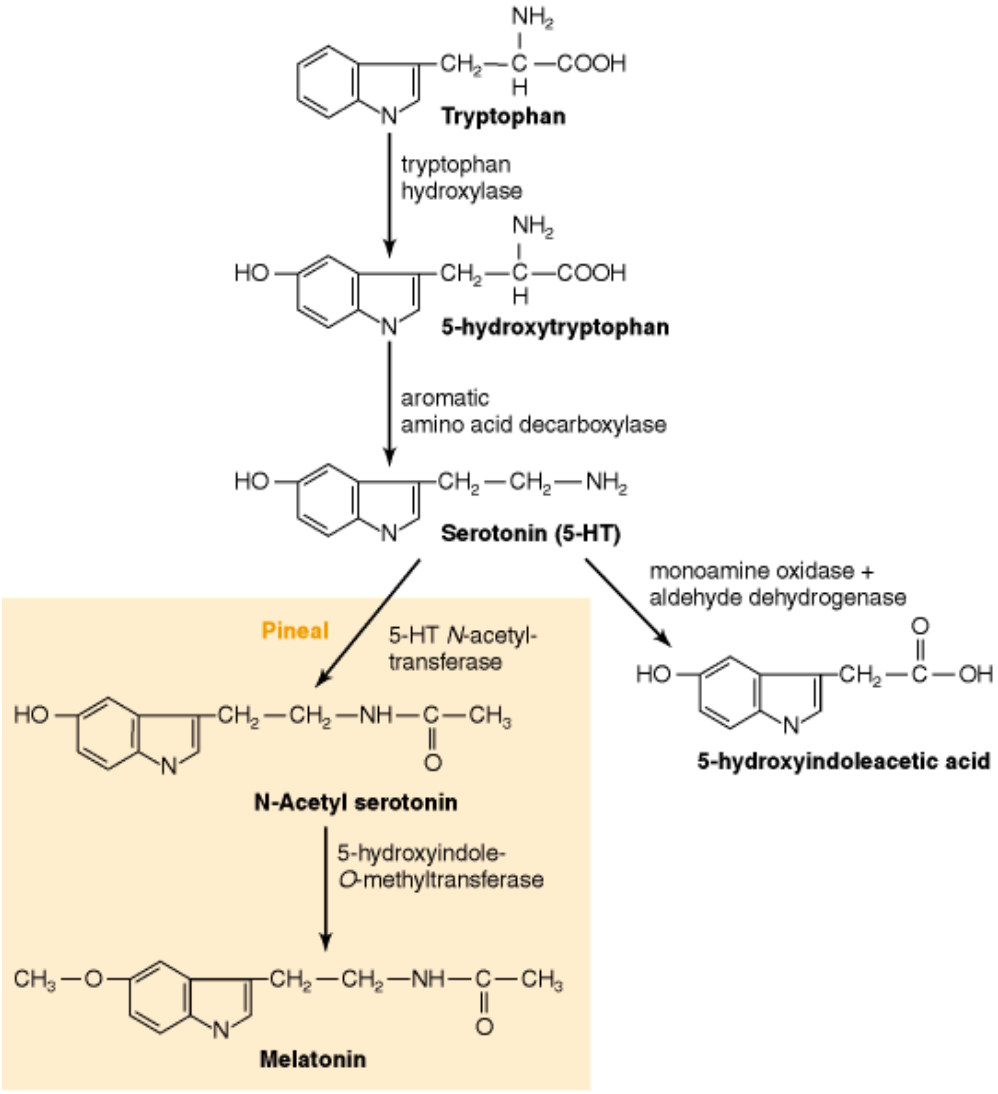

Figure 5 Biosynthetic pathway of serotonin (5-HT) from dietary L-tryptophan, and formation of the oxidative 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA). In the pineal gland, 5-HT is converted enzymatically to melatonin.

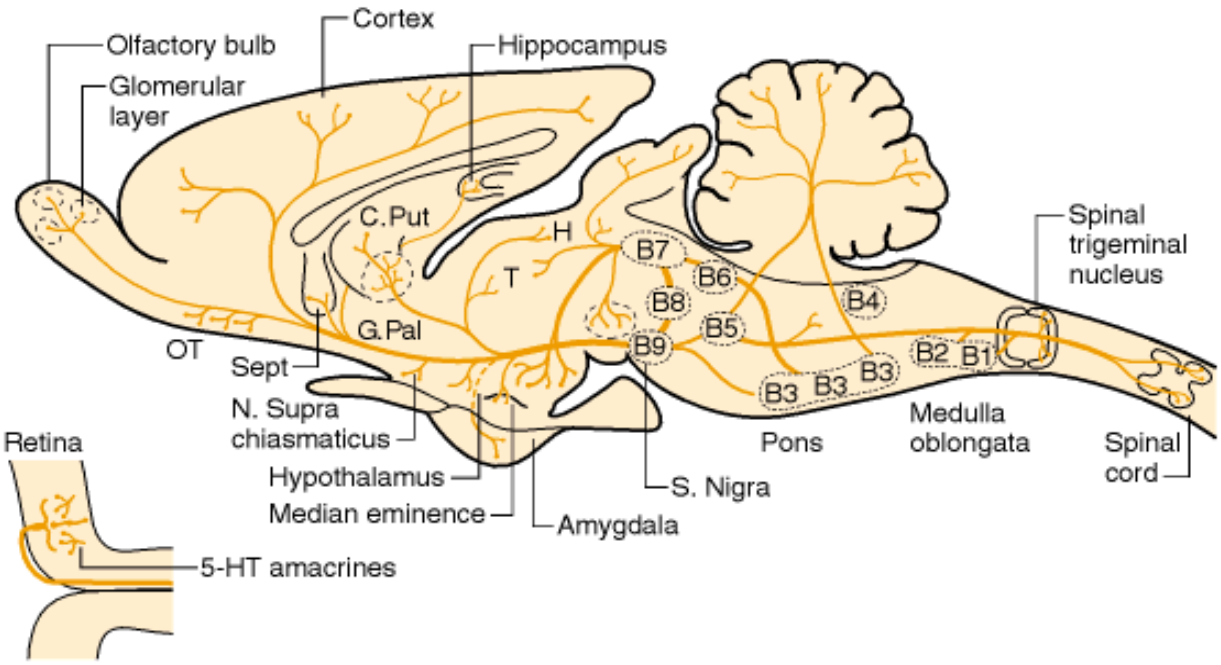

Figure 6 Schematic drawing depicting the location of the serotonergic cell body groups in a sagittal section of the rat central nervous system and their major projections. *OT*, olfactory tuberculum; *Sept*, septum; *C. Put*, nucleus caudate-putamen; *G. Pal*, globus pallidus; *T*, thalamus; *H*, habenula; *S. Nigra*, substantia nigra (Figure taken from: George J. Siegel's, Basic Neurochemistry, 1999).

5-HT is a well-characterized neurotransmitter, with wide-ranging effects due to activity at a large and diverse collection of 5-HT receptors. Fourteen genetically and pharmacologically distinct cell surface serotonergic receptors have been identified, and are classified into seven discrete families, 5-HT_1 to 5-HT_7, according to their structural diversity and mode of action (Langer, 1980; Hoyer et al., 1994, 2002; Baumgarten and Gothert, 1997; Barnes and Sharp, 1999; Sari, 2004; Bockaert et al., 2006). All, but one of the seven receptor subtypes, reside within the G-protein-coupled receptor (GPCR) family of receptors, with that particular distinction belonging to the excitatory 5-HT_3 receptor – the sole ligand-gated ion-channel receptor in the 5-HT family of receptors. Through its activation of GPCRs ($G_{q/o}$, G_i, and G_s), 5-HT can activate a variety of second messenger systems, and as a result, indirectly modulate the activity of ion-channels. It can also activate ion-channels directly through activation of the ionotropic 5-HT_3 receptor, which implicates its involvement in fast, excitatory responses (Maricq et al., 1991).

The serotonin 5-HT_3 receptor belongs to the super-family of ligand-gated ion-channel receptors that includes

the excitatory nicotinic acetylcholine receptor, and the $GABA_A$ receptor. When activated by 5-HT, the $5\text{-}HT_3$ receptor's cation channel is nonselective for K^+/Na^+ and Ca^{2+} ions, and mediates a transient inward current with resulting membrane depolarization (Ronde and Nichols, 1998; van Hooft and Vijverberg, 2000).

Employing autoradiographic studies, the $5\text{-}HT_3$ receptor was found to be concentrated (50-200 fmol/mg tissue equivalent) in specific brainstem nuclei (area postrema, nucleus tractus solitarius, spinal trigeminal nucleus), with lower concentrations (4-17 fmol/mg tissue equivalent) in the forebrain (nucleus accumbens, hippocampus, putamen, caudate; Parker et al., 1996). As alluded to previously, the $5\text{-}HT_3$ receptor is concentrated centrally in a clinically important, key emetogenic center in the brainstem (medulla) referred to as the chemoreceptor trigger zone of the area postrema (Kilpatrick et al., 1988) residing within the floor of the 4^{th} ventricle. The $5\text{-}HT_3$ receptor is also localized spinally to a subgroup of small diameter primary afferents in the superficial dorsal horn, where it functions as an excitatory, pronociceptive receptor in the spinal cord (Green et al., 2000; Zietz et al., 2002). Considering its localization on central terminals of

primary sensory neurons, the 5-HT_3 receptor is thought to function in facilitating the release of pronociceptive peptides such as substance P, neurokinin A, or calcitonin gene related peptide from these presynaptic sites (Saria et al., 1990).

Due to 5-HT_3 distribution in the regions previously mentioned above, the 5-HT_3 receptor plays a strong role in mediating the pronociceptive activity of 5-HT along with the severe emesis induced by toxic chemotherapeutic agents (ie. cisplatin, doxorubicin) - whose side-effects include the release of large quantities of serotonin through the undesirable destruction of enterochromaffin cells lining the gastrointestinal tract. These cells produce and store greater than 90% of the body's total 5-HT in the periphery, whereas nuclei (ie. raphe nuclei) in the brainstem are responsible for supplying serotonin to the brain and spinal cord. As a consequence of this large release of serotonin by toxic chemotherapeutics, prophylactic administration of 5-HT_3 antagonists such as ondansetron have proved to be invaluable in countering the debilitating nausea and vomiting produced by high-to-moderate emetogenic chemotherapeutic agents, and also in preventing the emesis

produced from the post-operative administration of opioids (Roila et al., 1997; Hesketh 2000).

With respect to the role of 5-HT_3 receptors in pain mediation, it is known that neuropathic pain states are associated with an enhanced descending facilitatory control, mediated through the spinal release of 5-HT and activation of excitatory, spinal 5-HT_3 receptors (Suzuki et al., 2004). Furthermore, recent studies have demonstrated that in addition to their anti-emetic (Roila et al., 1997) properties, 5-HT_3 antagonists such as ondansetron, possess significant antinociceptive (Suzuki et al., 2004) and clinically significant, pain-relieving properties as well (McCleane et al., 2003). In the last ten years, clinical studies have demonstrated that the 5-HT_3 antagonist ondansetron can attenuate both acute and chronic pain states (Ambesh et al., 1999; McCleane et al., 2003). In a randomized, double-blinded controlled clinical study, ondansetron pretreatment was shown to significantly reduce the pain of propofol injection used to induce surgical anesthesia (Ambesh et al., 1997). In all, these studies implicate a strong role for the 5-HT_3 receptor and its endogenous ligand, 5-HT, in enhanced spinal pronociceptive processing and central sensitization.

1.7 Prostaglandin E_2 and pronociception

Another spinal pronociceptive mediator of equal interest is that of prostaglandin E_2 (PGE_2), a key pro-inflammatory prostaglandin that sensitizes and excites dorsal horn neurons, playing a strong role in central sensitization (Yaksh et al., 1984; Baba et al., 2001; Koetzner et al., 2003), and mediating its effects through the activation of G-protein-coupled prostaglandin EP1-EP4 receptor subtypes (Oida et al., 1995; Sugimoto et al., 1994; Beiche et al., 1998a; Stock, J.L., et al 2001; Minami, T., et al 2001; Reinold, et al, 2005). With respect to the role of prostaglandins in pain mediation, much of the research in this area has focused on PGE_2 where it functions as an important mediator in the development of both peripheral inflammation and sensitization (Vane, 1971; Julius and Basbaum, 2001), and when applied directly to the spinal cord, produces an allodynic and hyperalgesic state (Taiwo and Levine, 1988; Uda et al., 1990; Minami et al., 1994, 1996; Malmberg and Yaksh, 1995a,b; Ferreira and Lorenzetti, 1996). In the spinal cord, PGE_2 has been shown

to directly activate rat spinal dorsal horn neurons (Baba et al., 2001), and spinal inflammatory hyperalgesia is known to be mediated by PGE_2-induced EP2, EP1, and EP3 receptor activation (Reinold, et al, 2005; Stock, J.L., et al 2001).

In general, the pronociceptive effects of PGE_2 are known to be mediated by the entire spectrum (EP1-EP4) of prostaglandin receptors, with all four receptors residing in the spinal cord. It has been reported that the EP1, EP3, and EP4 receptors are expressed in dorsal root ganglion (Oida et al., 1995; Sugimoto et al., 1994; Beiche et al., 1998a), suggesting that prostaglandins influence the release of neurotransmitters from primary afferent terminals. Autoradiographic studies have also revealed that the highest density of spinal PGE_2 binding sites lie within the substantia gelatinosa (SG), in laminae II, where PGE_2 binding is decreased but not abolished by dorsal rhizotomy (results in the destruction of central primary afferents via a lesion/disconnect from their life-sustaining cell bodies in the DRG), which suggests that receptor sites are located on both presynaptic terminals of unmyelinated C-fibers and on postsynaptic dorsal horn neurons (Matsumura et al., 1995). Thus, PGE_2 may act

presynaptically to facilitate neurotransmitter release (Nicol et al., 1992; Hingtgen et al., 1995; Vasko, 1995), and postsynaptically to directly excite dorsal horn neurons (Baba et al., 2001). Moreover, the expression of EP2 receptors in spinal cord neurons (Kawamura et al., 1997) and their direct depolarization underlies the potential for postsynaptic effects of PGE_2 (Baba et al., 2001; Ahmadi et al., 2002), and its role in modulating sensory processing through altering neuronal excitability in the spinal dorsal horn. Furthermore, topical application of PGE_2 to the exposed spinal cord induces a state of hyperexcitability in dorsal horn neurons analogous to peripheral inflammation, and the application of anti-inflammatory cyclooxygenase (COX) inhibitors spinally, suppresses C-fiber reflexes, inhibits dorsal horn neuronal sensitization, and attenuates the generation of inflammation-evoked inflammatory pain (Bianchi and Panerai, 1996; Bustamante et al., 1997; Herrero et al., 1997; Malmberg and Yaksh, 1992; McCormack, 1994; Willingale and Grubb, 1996; Willingale et al., 1997) and central sensitization (Vasquez et al., 2001).

Mechanistically, through the activation of the protein kinase A (PKA)/cAMP pathway, prostaglandins enhance

tetrodotoxin-resistant sodium currents, inhibit voltage-dependent potassium currents, and increase voltage-dependent calcium inflow in nociceptive afferents. Through these events, prostaglandins decrease firing thresholds, increase firing rate, and promote the release of excitatory amino acids, substance P, calcitonin gene-related peptide (CGRP), and NO. In contrast, glutamate, substance P, and CGRP promote prostaglandin release. Prostaglandins also act to facilitate membrane currents and promote the release of substance P and CGRP induced by bradykinin, capsaicin (Malmberg and Yaksh 1994), and low pH. With that said, the elicitation and synaptic transfer of pain signals in the spinal cord ought to presumably be enhanced by the diversity of effects produced by prostaglandins (Yamamoto and Nozaki-Taguchi, 1996; Mollace et al., 2005).

1.8 Biochemistry and synthesis of prostaglandins

Prostaglandins such as PGE_2 are synthesized via a multi-step process beginning with the enzyme cyclooxygenase (COX), which catalyzes the formation of prostaglandins from arachidonic acid, a ubiquitous 20-carbon fatty acid that is generated from the action of phospholipase-A_2 on membrane phospholipids. A two-step conversion is involved, first

with the action of PG endoperoxide synthase (PGHS; ie. COX), which transforms arachidonic acid into the unstable, cyclic endoperoxide, PGG_2, followed by PGG_2 peroxidase, to yield the unstable prostaglandin intermediate, PGH_2. PGH_2 serves as the final common precursor for prostaglandins PGD_2, $PGF_{2\alpha}$, PGE_2, prostacyclin, and thromboxane A2 (Flower and Vane, 1972; Smith and Lands, 1972; Needleman et al., 1986; Smith et al., 1991) (Figure 7).

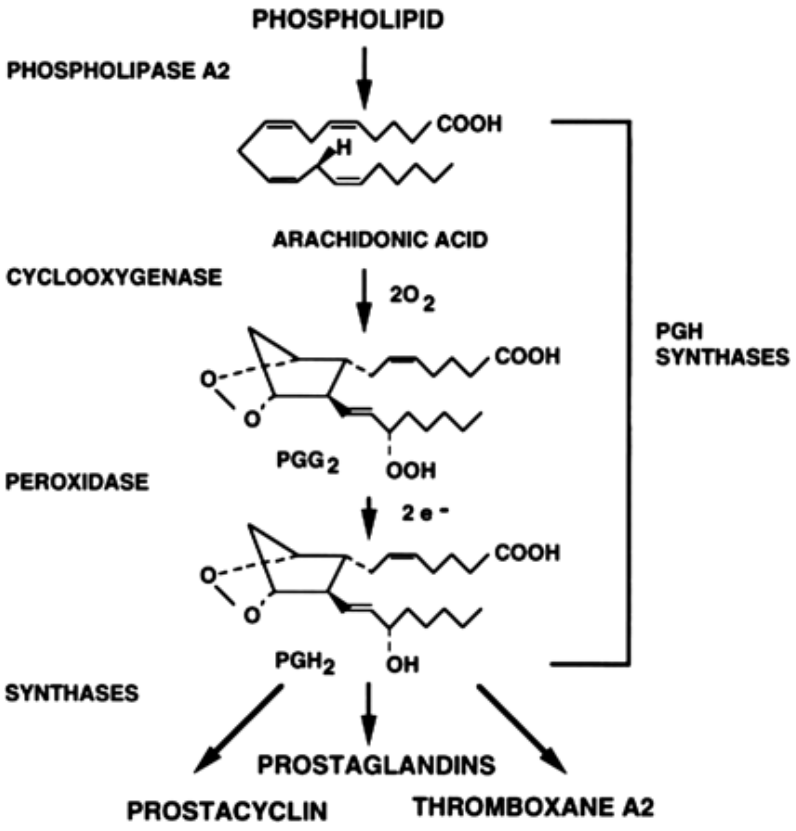

Figure 7 The membrane phospholipids—arachidonic acid cascade (Illustration from Mollace et al., 2005).

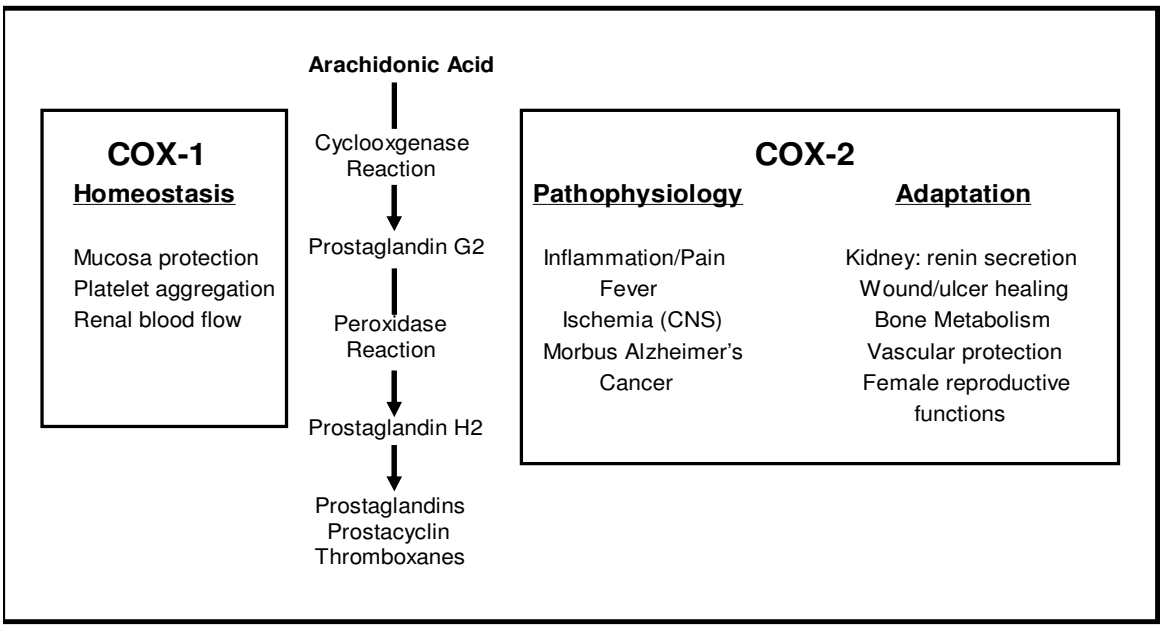

Figure 8 Bi-functional role of the COX enzyme (prostaglandin H synthase) in the biosynthesis of prostaglandins and thromboxanes, and physiological and pathophysiological effects of COX-1 and COX-2. (Taken from: http://www.albany.edu/faculty/cs812/bio366/Cyclooxygenase_ppt.pdf.)

1.8.1 COX-1 and COX-2 origin, expression, and function

Prostaglandins are found in all tissues and body fluids with the exception of red blood cells, and as local hormone mediators with a broad spectrum of effects, act to modulate a multitude of biological and cellular functions. The COX enzyme, responsible for the production of prostanoids (ie. prostaglandins and prostracyclins) was first isolated in 1976 from the seminal vesicles of sheep, where it is found in high concentrations (Hemler and Lands, 1976; Miyamoto et al., 1976; Van der Ouderaa et al., 1977), and exists as a homodimer with a molecular mass of 71 kDa. The COX enzyme was later cloned from the same tissue in 1988 (DeWitt and Smith, 1988; Merlie et al., 1988; Figure 8). The COX-2 isoform was discovered in 1991 by Xie and colleagues (Xie et al., 1991), and was determined to be an "inducible" isoform, expressed in response to a variety of pro-inflammatory stimuli and found in the brain, spinal cord, reproductive organs, the kidneys, and in osteoblasts (Feldman et al., 2000).

Unlike COX-1, COX-2 expression is usually minimal, however constitutive COX-2 expression has been reported in the brain and spinal cord where it modulates fever and neurotransmission (Vane et al., 1998). When activated,

COX-2 regulates prostaglandin production primarily within inflammatory cells, where this inflammatory response is a vital part of the healing and repair process when appropriately regulated. The COX-1 gene, located on human chromosome 9, is a constitutively expressed “housekeeping” gene responsible for maintaining biological homeostasis (ie. kidney and platelet function, gastric mucosal integrity), and in line with its constitutive biological role, lacks a regulatory TATA box (Kraemer et al., 1992). In contrast, the COX-2 gene, located on human chromosome 1, is rapidly up-regulated in response to inflammatory stimuli, and possesses a promotor region that contains a TATA box and the binding sites for a variety of transcription factors (Kraemer et al., 1992), including nuclear factor-kappa-beta (NF-kB), cyclic AMP response element binding protein, and the nuclear factor for interleukin-6 expression (NF-IL-6) (Appleby et al., 1994). As such, COX-2 expression is thus modulated via a broad spectrum of mediators involved in inflammation. It is also known that lipopolysaccharide, growth factors, and pro-inflammatory cytokines such as IL-1β all promote COX-2 expression, whereas IL-4, IL-13, glucocorticoids, and the anti-inflammtory IL-10 have all been shown to inhibit COX-2

expression (Lee et al., 1992; Onoe et al., 1996; Niiro et al., 1997).

Furthermore, the data suggests that COX-2 localization correlates closely with glutamatergic neurotransmission. With COX-2 primarily expressed by glutamatergic neurons that are immunolocalized in the cell body, proximal and distal dendrites, and dendritic spines (Kaufmann et al., 1996). COX is also expressed by cell types other than neurons including astrocytes, oligodendrocytes, and microglia (Minghetti et al., 1998). It has been shown that use of the non-competitive NMDA (glutamate receptor) antagonist, MK-801 can block both the basal expression of COX-2 and seizure-induced COX-2 overexpression, suggesting the existence of constitutive COX-2 expression and activity in glutamatergic mechanisms (Yamagata et al., 1993).

It is also known that products of COX-2 metabolism such as PGE_2 may serve a regulatory feedback role on the expression of the COX-2 enzyme. A recent study found that the non-selective COX inhibitor indomethacin, through inhibition of prostaglandin synthesis at the site of injury, blocked COX-2 expression in the inflamed paw in a rat model of carrageenan-induced inflammation (Nantel et

al., 1999) – suggesting that prostaglandins produced at the site of injury, could induce COX-2 expression, and thus through a positive feedback loop increase inflammation. In a variety of similar experiments, it was found that PGE_2, a major COX-2 product, was found to upregulate the expression of COX-2 in various cell types, including rat microglia (Minghetti et al., 1997), human blood monocytes (Hinz et al., 2000a), murine macrophages (Hinz et al., 2000b), and murine keratinocytes (Maldve et al., 2000), through its ability to increase intracellular cAMP levels.

1.8.2 Important physiological functions of prostaglandins

Prostaglandins serve a variety of biological and physiological roles. In addition to their fundamental role in signaling infection and tissue injury, via fever and acute pain and inflammation, respectively, protective prostaglandins produced by the COX-1 enzyme (ie. PGE_2 and PGI_2) are involved in the maintenance of heart and kidney function through vasodilatation to ensure proper blood flow in these vital organs. With respect to their role in gastric function, prostaglandins decrease acid secretion, stimulate mucus and duodenal bicarbonate for acid neutralization, and promote vasodilatation of vessels in

the gastric mucosa. These effects in their entirety promote gastrointestinal function, health, and integrity. The COX-1 enzyme is the only isoform present in platelets, with its functional activity blocked with a small (ie. 81 mg) daily dose of aspirin. Due to a special property of aspirin, namely an acetyl-group whose presence separates it from the other NSAIDs, produces through COX acetylation, an irreversible loss of COX activity resulting in the therapeutic aim in its treatment for protection against thromboembolic disease. This particular prophylaxis is accomplished through the inhibition of COX-1, which results in diminished thromboxane A_2 production, essential for platelet aggregation.

With respect to the side-effect profile of NSAIDS, many of the side effects such as gastrointestinal ulceration and bleeding are due to the above mentioned suppression of COX-1 activity, whereas inhibition of COX-2-derived prostanoids facilitates the analgesic, anti-inflammatory, and antipyretic effects of this class of drugs. The COX-2 enzyme, well-known for its inducible nature, is also constitutively expressed in the brain and spinal cord, where it plays a role in fever and the neurotransmission of pain (Vane et al., 1998). PGI_2 through

its antithrombogenic activity is critical for vascular health and function, and along with PGE_2, another product of the COX-1 enzyme, plays an important cytoprotective role in preserving the integrity of the stomach lining (Moncada et al., 1976; Whittle et al., 1978).

1.8.3 NSAIDs as non-selective and selective COX inhibitors

Non-selective, non-steroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid (aspirin), ibuprofen, and naproxen inhibit both forms of cyclooxygenase to reduce pain and inflammation, whereas drugs such as acetaminophen (N-acetyl-para-aminophenol; APAP) (Hinz 2007) and celecoxib act as selective inhibitors of peripheral COX-2 enzymes to accomplish the same feat minus the gastrointestinal irritation characteristic of non-selective COX inhibitors (Figure 9; Ehrich 1999). With respect to acetaminophen's role in COX-2 inhibition and its long-time standing elusive mechanism of action, though believed for sometime, it looks less probable that it decreases fever and pain through a central COX-3 splice variant of COX-1 as was once thought (Graham and Scott, 2005; Hersh et al., 2005; Kis et al., 2005). A recent study by Hinz and colleagues in 2007 demonstrated that acetaminophen possessed strong COX-2

inhibitory activity, which is quite contrary to the belief held for years, that it lacked the anti-inflammatory activity of the NSAID drugs and therefore was ineffective in the treatment of inflammatory conditions. It should be noted that selective COX-2 inhibitors, while devoid of gastrointestinal side-effects such as bleeding and ulceration, pose a significant cardiovascular risk for chronic users of these drugs by lowering the critical vasodilatory to vasoconstricting, PGI_2 / TXA_2 ratio in the bloodstream (Hersh et al., 2005).

1.8.4 Prostaglandins in disease

The rapidly inducible and tightly regulated COX-2 isoform, is up-regulated and activated by proinflammatory cytokines, lipopolysaccharide, growth factors, and capable of producing large and pathological amounts of prostaglandins (Sano et al., 1992; Vane et al., 1998). Together, with the "housekeeping" COX-1 isoform, these enzymes are responsible for the production of vasoconstricting thromboxanes such as TXA_2 and powerful vasodilatory prostaglandins, PGE_2 and PGI_2, which synergize with inflammatory mediators such as histamine and

bradykinin to promote increased vasodilation and inflammation (Rang et al., 2003).

A study by Riendeau and colleagues concluded that the COX-1 enzyme is the predominant isoform modulating the perception of acute non-inflammatory pain episodes, whereas due to the time lapse (ie. minutes in transcriptional modulation) required for COX-2 gene induction, COX-2 is recruited during an inflammatory response and serves a predominant role in the mediation of inflammatory pain (Riendeau et al., 1997). While, the COX-2 isoform shows some constitutive activity in neurons, and in the spinal cord along with COX-1 (Svensson and Yaksh, 2002), it can be heavily affected by synaptic events, which can markedly up-regulate its functional status. For example, the activation of NMDA receptors coupled with an increase in $[Ca^{2+}]_I$ accelerates the synthesis of prostaglandins (Beiche et al., 1996; Breder et al., 1995; Willingale et al., 1997; Yamagata et al., 1993). Furthermore, recent support to the strong role that prostaglandins play in neuronal excitability suggests that spinal NMDA receptors initiate a cascade involving the release of prostaglandins, whereby acting through prostanoid receptors, facilitate spinal responses to afferent stimuli (Svensson and Yaksh, 2002).

With respect to the distribution of COX enzymes in the central nervous system (CNS), it is known that COX-1 is found in the DRG of peripheral afferent fibers and the spinal cord (Beiche et al., 1996; Willingale et al., 1997; Inoue et al., 1999), while COX-2 enzymes predominate in several regions of the spinal cord receiving nociceptive input, and is especially concentrated in laminae I, II, and X. The COX-2 isoform also shows marked up-regulation during peripheral inflammation (Beiche et al., 1996, 1998b; Hay and de Belleroche, 1997; Hay et al., 1997; Goppelt-Struebe and Beiche, 1998; Samad et al., 2001) coinciding with enhanced intraspinal release of PGE_2 (Yang et al., 1996; Ebersberger et al., 1999).

Furthermore, inflammation through its effect in inducing COX-2 activity and prostaglandin biosynthesis, has been shown to result in the sensitization of presynaptic peripheral terminals, thereby increasing peripheral pain sensitivity (Beiche et al., 1996). In addition, behavioral studies suggest that PGE_2 may assist nociceptive transmission in the spinal cord (Uda et al., 1990; Minami et al., 1994), serving as a major contributing factor to central sensitization – an amplification of central sensory outflow from the spinal cord and responsible for the spread

of sensitivity extending outside of the site of injury (Woolf and King, 1990).

1.8.5 Interaction of nitric oxide and prostaglandins

In particular, immunohistochemical studies have detailed the localization and regulation of COX-1 and COX-2 and neuronal NOS in the lumbar spinal cord under basal conditions as well as following induction of painful peripheral stimuli (Maihofner et al., 2001). Recently, immunohistochemical studies have identified the co-localization of the COX-1/COX-2 enzymes with neuronal NOS in the lumbar spinal cord under both basal and noxious stimuli induced conditions. The existence of constant cross-talk between NO and prostaglandin release, occurring at many levels (ie. transcription, post-translation) has emerged with these studies documenting the role NO plays in the modulation of the COX pathway. The effect of NO appears to depend on the basal levels of NO and NO donors (ie. arginine, bradykinin), which can, depending on their concentration, turn either on or off the COX pathway. The reaction of NO with the superoxide (O_2^-) radical also leads to the formation of the highly unstable peroxynitrite radical ($ONOO^-$), which in addition to NO, acts to modulate

the activity of COX enzymes. Recent evidence also suggests that coupling of NO donors with COX inhibitors such as aspirin and ibuprofen to form nitro-aspirin and nitro-ibuprofen, respectively, may have a clinical role in the treatment of both non-inflammatory and inflammatory disorders.

In terms of the chemistry and functionality of these newly developed bi-functional NSAIDs, this offers further support for the complementary association of the NO/COX interaction, which as it seems by their physiochemical properties (ie. vasodilatory activity, pro-inflammatory effects), may involve a great deal of overlap in their influence on the mechanisms governing a variety of disease states (ie. hypertension, vascular disorders, etc) (Mollace et al., 2005).

NO and prostaglandins share a number of similarities, both being vasodilatory and pro-inflammatory mediators, and as previously alluded to, possess an interdependent modulatory relationship with the other. It is known that nitric oxide synthase (NOS) enzymes in their production of NO, modulate COX activity and prostaglandin biosynthesis. The highly regulated biosynthetic enzymes NOS and COX,

acting on their respective substrates, arginine (NO) and arachidonic acid (prostaglandins), produce nanomolar concentrations of these biological effectors to produce a variety of effects in the vasculature and organs. Constitutive basal activity of these two biosynthetic pathways serves a critically important role in maintaining proper cardiovascular (ie. vasodilation, antiplatelet activity), gastric (ie. stomach lining), and organ function (ie. kidney, brain).

In the event of inflammation, NO and prostaglandins are concurrently released in large, micromolar concentrations, primarily due to the activation of the inducible NOS and COX isoforms (ie. iNOS, COX-2). In inflammatory conditions where both iNOS and COX-2 are induced, there is a NO-mediated induction of COX-2 leading to increased formation of pro-inflammatory prostaglandins, resulting in an exacerbated inflammatory response (Figure 11). Under these circumstances, dual inhibition of NO and prostaglandins account for the anti-inflammatory effects of NO synthase inhibitors (ie. L-*N*-monomethylarginine or L-*N*-arginine methylester). Furthermore, in pathophysiological states such as hypertenstion, kidney disease, or ischemia of the

brain or heart, regulation of NOS and COX activity is even more critical due to their powerful effects on vascular tone, platelet function, and inflammation (Mollace et al., 2005).

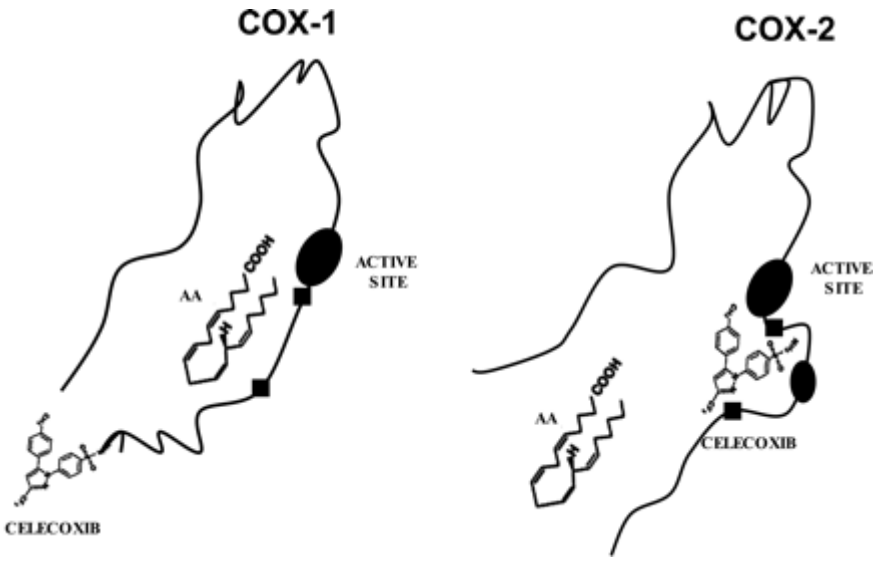

Figure 9 With the exception of aspirin, which irreversibly inactivates the COX enzymes by acetylation of an active site serine residue, most NSAIDs such as indomethacin, ibuprofen, and naproxen compete with arachidonic acid for binding to the active site.

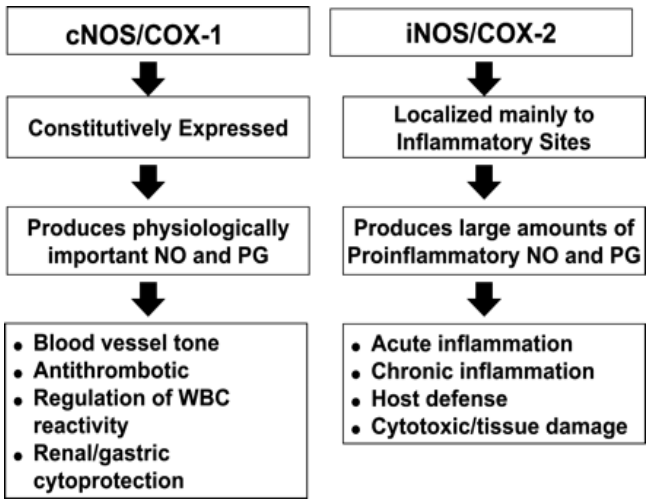

Figure 10 Functions and interdependency of constitutive and inducible forms of NOS and COX in normal and aberrant cellular function. Inducible forms of NOS and COX (iNOS and COX-2) are localized primarily to inflammatory sites in the body, whereas constitutive forms (cNOS and COX-1) play a homeostatic role in regulating blood vessel tone, white-blood cell reactivity, platelet aggregation, and renal and gastric function.

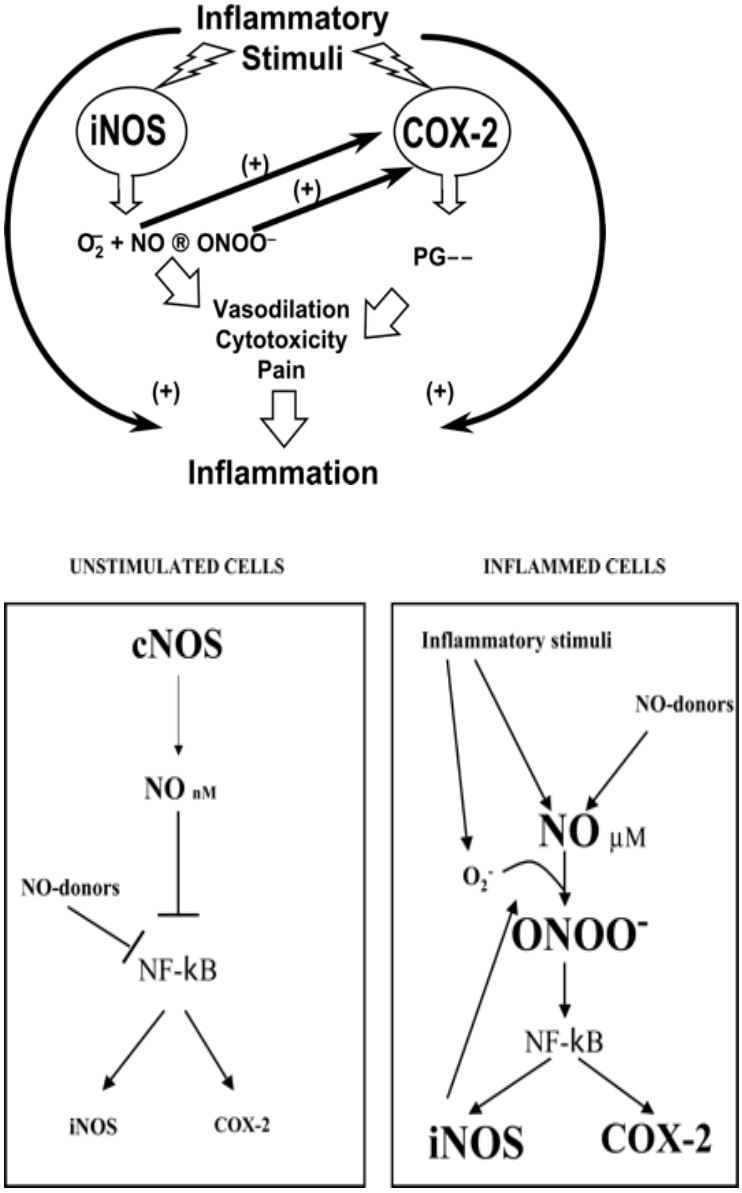

Figure 11 Inflammatory stimuli induce both iNOS and COX-2 expression to further exacerbate inflammation. The transcription factor, NF-kβ, increases the expression of both iNOS and COX-2. NO/COX interaction in resting and inflamed cells. NF-kB seems to play a crucial role in both conditions. NO in unstimulated cells exists in nanomolar (nM) concentrations, whereas in inflamed cells, NO is produced in pathogenic micromolar (µM) concentrations. NO formation occurring after inflammatory conditions further potentiates both iNOS and COX-2 activity.

1.9 Endogenous CCK is elevated in nerve-injury and chronic morphine

Utilizing pharmacological manipulation, previous studies suggest CCK is up-regulated in the RVM in chronic nerve injury (SNL) (Kovelowski 2000). Following SNL surgery, administration of a CCK_2 receptor antagonist into the RVM resulted in a partial reversal of nerve-injury induced tactile hypersensitivity and thermal hyperalgesia – two primary behavioral parameters commonly used to assess the nociceptive state of the animals tested. Pharmacologic and microdialysis studies have demonstrated that cholecystokinin (CCK), a pronociceptive peptide, is increased in the rostral ventromedial medulla (RVM) following nerve-injury (Kovelowski et al., 2000) and chronic morphine administration (Xie et al., 2005), respectively. The administration of a CCK_2 receptor antagonist into the RVM reversed opioid-induced hyperalgesia and antinociceptive tolerance, suggesting pharmacologically that an up-regulation of endogenous CCK was acting as an "antiopioid" (Faris et al., 1983), and counteracting opioid analgesia (Xie et al., 2005). CCK exogenously administered into the RVM produced tactile and thermal hypersensitivity, which was blocked by the CCK_2

receptor antagonist L365,260, but not by the CCK_1 antagonist L364,718 which suggests a CCK_2 mediated mechanism in promoting abnormal pain (Xie et al., 2005). Morphine given by either continuous subcutaneous administration (pellets) or via osmotic minipump resulted in increased tactile and thermal hypersensitivity (Vanderah et al., 2000, 20001a,b). As described previously, chronic morphine administration results in opioid-induced pain (and subsequently was found to result in an increase in endogenous CCK in the RVM), though the role of descending facilitation from the RVM to the spinal dorsal horn remained in question. Regarding descending facilitation from the RVM, tactile and thermal hypersensitivity induced by chronic opioid administration was reversed by lesioning of the dorsolateral funiculus (DLF), the primary conduit for descending fibers from the RVM to the spinal dorsal horn, or by lidocaine administration into the RVM (Vanderah et al., 2001b). Thus, implicating descending pain facilitation from the RVM to the spinal cord as necessary for the manifestation of opioid-induced hypersensitivity to noxious and non-noxious stimuli (Xie et al., 2005; Vanderah et al., 2001a,b).

Concerning the spinal neurotransmitters known to promote pain and inflammation, our interest lies in measuring the levels of 5-HT and PGE_2 in the spinal CSF levels, following CCK microinjection in the RVM. Ne**u**ropathic pain states are known to be associated with an enhanced descending facilitatory control, mediated through the spinal release of 5-HT and activation of excitatory, spinal $5\text{-}HT_3$ receptors (Suzuki et al., 2004). Clinical studies have demonstrated that the $5\text{-}HT_3$ antagonist, ondansetron can attenuate both acute and chronic pain states (Ambesh et al., 1999; McCleane et al., 2003). These studies implicate a strong role for the $5\text{-}HT_3$ receptor and its endogenous ligand, 5-HT, in enhanced spinal pronociceptive processing and central sensitization. Similarly, PGE_2 – has been shown to directly activate spinal dorsal horn neurons, suggesting a role in central sensitization (Baba et al., 2001), mediating its effects through the activation of G-protein coupled prostaglandin EP1-EP4 receptor subtypes (Oida et al., 1995; Sugimoto et al., 1994; Beiche et al., 1998a; Stock, J.L., et al 2001; Minami, T., et al 2001; Reinold, et al, 2005). It is our hypothesis that activation of descending facilitatory pathways originating in the RVM result in the evoked

release of 5-HT and PGE_2 in the spinal cord, ultimately leading to a heightened degree of spinal nociceptive processing.

1.95 Dynorphin

The other primary neuropeptide of interest in this study is dynorphin, whose functional presence was investigated in the RVM. In humans and animals there exists a family of endogenous opioid peptides consisting of endorphins, enkephalins, and dynorphins that function to modulate a diversity of pain, motor, and autonomic activities along with responses to stress (Beaumont and Hughes, 1979). One in particular, dynorphin, has been a great subject of interest in our group, and is widely distributed in the central nervous system in such regions at the hippocampus, amygdala, striatum, and spinal cord (Fallon and Leslie, 1986; Khachaturian et al., 1982; Mansour et al., 1994; Pierce et al., 1999; Van Bockstaele et al., 1994; Vanderah et al., 2000). Recent studies in our group have documented a newly discovered pronociceptive interaction involving direct activation of bradykinin receptors, B1 and B2, by dynorphin A(2-13) in sensory

neurons (Lai et al., 2006). Dynorphin A(1–17) occurs endogeneously as a primary proteolytic fragment of prodynorphin (Goldstein et al., 1979; Quiron and Pilapil., 1984), and with the first tyrosine residue intact, possesses high-affinity for the three opioid receptor subtypes: mu, kappa, and delta (Chavkin et al., 1982; Corbett et al., 1982; Zhang et al., 1998). In contrast, des-tyrosyl fragments of dynorphin (ie. dynorphin A(2–13) lack opioid activity, but retain potent pronociceptive effects.

In the spinal cord, intrathecal injection of dynorphin and its non-opioid fragments induce long-lasting abnormal pain states characterized as allodynia and hyperalgesia (Vanderah et al., 1996; Laughlin et al., 1997), and evoke the spinal release of prostaglandin E_2 and excitatory amino acids (Koetzer et al., 2004). The pronociceptive effects of spinal dynorphin are not blocked by opioid antagonists (Laughlin et al., 1997). Spinal dynorphin levels are increased after peripheral nerve injury with its up-regulation notably secondary to the initiation of descending facilitatory processes (Kajander et al., 1990; Gardell et al., 2002). In cultured neurons, dynorphin A evokes the release of excitatory amino acids (Skilling et

al., 1992), while inducing an increase in intracellular calcium (Tang et al., 2000) and being potentially excitotoxic at high doses (Hauser et al., 1999). Following nerve injury and peripheral inflammation, endogenous dynorphin is up-regulated, and dynorphin antiserum blocks nerve injury induced neuropathic pain (Lai et al., 2006). It has also been shown that antisera to dynorphin can diminish the pathological manifestations of spinal cord trauma, implicating a potential role of dynorphin in neurological dysfunctions of the spinal cord (Faden 1990; Shukla and LaMaire 1994).

The biological half-life of dynorphin and its fragments vary considerably depending on the fragment of consideration and its surrounding biological environment (ie. enzymes, pH). One particular carboxy-terminal truncated metabolite of dynorphin, dynorphin A(1-13), was found to possess a half-life of 2.5 hours in the cerebrospinal fluid (CSF) in humans (Miiller et al., 1996). In a different study, this time by Chou and colleagues found that endogenous dynorphin A(1-13) in human blood had a half-life of less than 1 minute, whereas the larger fragment, dynorphin A(1-17), possessed a half-life of close to 3 hrs with the main transformation product being the

nonopioid fragment dynorphin A(2–17). The authors concluded that the rate of biotransformation of the different dynorphin fragments varied tremendously, and was dependent on the last four carboxy-terminal amino acid residues [comparing Dyn A(1–17) vs Dyn A(1–13)], which functioned to protect and thereby increase the stability of the peptide from biotransforming enzymes (Chou et al., 1996).

An *in vitro* study measuring the half-life of Dynorphin A(1–13) in rat-brain membranes found a much more rapid degradation by membrane-bound enzymes, with a half-life of 5 minutes or less (Leslie and Goldstein, 1982). Dynorphin A(1–13) administered intravenously in human volunteers was found to have a half-life of less than 1 minute (Gambus et al., 1998), which is in agreement with the findings reported by Chou et al., 1996. Thus, fragments such as dynorphin A(1–13), display different rates of bio-transformation, which is a function of peptide length, and their immediate biological environment – whether in CSF (2.5 hrs), blood (< 1 min), or in the brain (≤ 5 min).

Along with dynorphin, and its recently identified activation of the bradykinin receptors, kinins such as bradykinin and its de-arginated metabolite des-arg9-bradykinin were the first endogenous ligands identified to

activate the bradykinin receptors. The two kinin receptors, consisting of the constitutive B2 and the inducible B1, function in modulating the cellular actions of kinins, which include inflammation, vasodilatation, and calcium influx. These cellular functions are mediated by all of the known second messenger systems (ie. PKA/cAMP, PLC/PKC) and have been shown to localize in glial tissue and in parts of the brain important in the regulation of cardiovascular homeostasis and nociception, providing evidence for the functional role the kinin system plays in the nervous system. The two kinin receptors, in addition to their role in vascular homeostasis and pain modulation, are also involved in the release of excitatory neurotransmitters, and modulating cellular immunity in the brain (Raidoo and Bhoola 1998).

The B2-receptor, which is constitutively expressed (Regoli and Barabe 1980) and widely distributed in most tissues, mediates the majority of kinin effects. The receptor is activated by bradykinin (the first endogenously identified ligand for the B2-receptor), along with its newly discovered activation by dynorphin and its fragments (Lai et al., 2006). In the central nervous system of the rat, the B2 receptors have been shown to localize

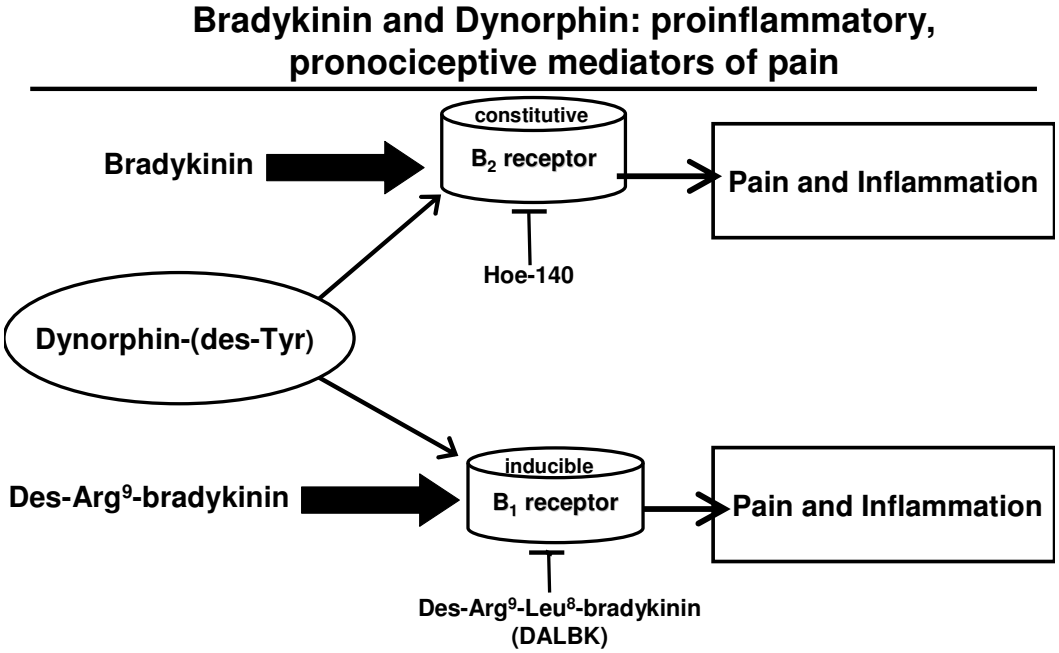

Figure 12 Bradykinin and dynorphin mediate their cellular effects (proinflammation and pronociception) through the activation of bradykinin receptors (B1 and B2). Bradykinin receptor specific antagonists (B1: DALBK; B2: Hoe-140) are used to pharmacologically verify the presence and function of these receptors. Taken from: Lai et al., 2006.

predominately in the superficial laminae in the dorsal horn (substantia gelatinosa, laminae I and II) of the spinal cord, providing anatomical evidence for the role of bradykinin as a spinal modulator of nociceptive information (Lopes *et al.*, 1993a).

In contrast, the B1-receptor has limited expression under normal conditions, but is rapidly up-regulated during inflammation or tissue injury (Regoli et al., 1981; Deblois et al., 1989; Marceau, 1995; Marceau et al., 1998) and is activated by the endogenously produced bradykinin metabolite, des-arg9-bradykinin. The biotransformation of bradykinin to des-arg9-bradykinin and arginine by the kininase I group of enzymes, either in synaptic clefts or in close proximity to or within endothethial cells, provides the substrate (ie. arginine) for the enzyme nitric oxide synthase (NOS) to produce another strong vasodilatory molecule, nitric oxide (NO; endothelium-derived relaxing factor) (Bhoola *et al.*, 1992; Erdös, 1990). The kinin B1 receptor, was first identified as mediating the contractile effect of kinins (Regoli *et al.*, 1977), and is known to be induced by a host of inflammatory mediators such as the cytokine interleukin-1β, the endotoxin lipopolysaccharide (LPS), and various growth promoters (Marceau 1995). With

respect to its structural and functional characteristics, the B1-receptor is composed of 353 amino acids, has a 36% amino acid sequence homology to the B2-receptor, and is in the family of G-protein-coupled receptors (GPCRs) (Menke *et al.*, 1994).

The constitutive B2-receptor, which accounts for the majority of the physiological effects of bradykinin, belongs to the superfamily of G-protein-coupled (G_α and G_q) rhodopsin-like receptors characterized by seven transmembrane domains connected by three extracellular and three intracellular loops (Burch and Axelrod, 1987). The receptor protein consists of 364 amino acids, is highly glycosylated, and exists in multiple isoforms at 69 kDa with isoelectric points of pH 6.8-7.1, and contains the bradykinin binding site at the amino-terminal part of the third extracellular loop (Abd Alla *et al.*, 1993, 1996).

Due to the ability of kinins to activate peripheral nociceptive receptors located on the terminals of C-fibers, clear evidence exists for their fundamental involvement in the transmission and modulation of pain. The demonstration of [3H]-bradykinin binding to the dorsal root and trigeminal ganglia and substantia gelatinosa in the rat (Fugiwara *et al.*, 1989; Steranka *et al.*, 1988) and the

localization of immunoreactive B1 receptors in the dorsal horn of the human spinal cord (Raidoo and Bhoola, 1997; Raidoo *et al.*, 1993) support a role for bradykinin in central pain modulation, as these areas are involved in nociception.

Moreoever, a recent study by Dalmolin et al., in 2006 showed that bradykinin (0.25 nmol) injected into the amygdala of rats induced behavioral signs of pain (thermal hyperalgesia), which could be blocked by the co-administration of the B2-antagonist (5 pmol) Hoe-140, demonstrating the presence of a bradykinin receptor mediated mechanism in promoting descending facilitation from the amygdala to the periphery. Dalmolin and colleagues in this same study also demonstrated that co-administration of bradykinin along with the COX inhibitor indomethacin (10 nmol), or the NMDA antagonist MK-801 (5 nmol) into the amygdala, abolished bradykinin-induced algesia, implicating a prostaglandin- and glutamatergic-mediated component in the pain behavior observed (Dalmolin et al., 2006).

In addition, the nona- and deca-peptides bradykinin and kallidin (lys-bradykinin) when cleaved from their protein precursor tissue kallikrein (a serine protease

enzyme), have been described as playing a pathophysiological role in the mammalian brain (Bhoola et al., 1992). Kininases, the enzymes that hydrolyse and inactivate kinins (Erdös, 1979), have been demonstrated in various areas of the rat brain by both bio- and fluorometric assay systems (Iwaki *et al.*, 1984; Kariya *et al.*, 1981).

A recent study confirmed that more than 75% of the total amount of kallikrein in extracts of the rat cerebral cortex and brainstem, determined by ELISA, is in the form of prokallikrein, suggesting that the greater part of the enzyme exists in the form of pro-enzyme in the CNS, and furthermore, immunohistochemical examination revealed that the immunoreactive substances preferentially locate in the neuronal cell bodies and their processes in both the cerebral cortex and brainstem (Kizuki *et al.*, 1994). Using specific cDNA probes, tissue kallikrein mRNA has been detected in the hypothalamus, cerebral cortex, cerebellum, brainstem, and pineal gland of the rat (Chao *et al.*, 1987), thus implicating the physiological role of kinins in these regions. In tissues, active tissue kallikrein, when released from tissue-prokallikrein by proteolytic enzymes,

liberates the decapeptide lysine-bradykinin from low molecular weight kininogen (LMWK). This activation occurs following tissue injury, disease, infection, and inflammation. Also, kinin production may occur independent of these systems, as cellular proteases released from mast cells and basophils during acute inflammation also stimulate kinin formation (Lupke *et al.*, 1982; Proud *et al.*, 1985).

It is our opinion, that in addition to its well-documented activity in the spinal cord, dynorphin mediates its pronociceptive effects through bradykinin receptor activation in the RVM.

1.99 Experimental objectives and hypothesis

The purpose of this study is comprised of two parts. The first part is to further define the CCK-induced RVM-mediated descending facilitatory pathway in terms of the neurotransmitters (CCK, PGE_2, 5-HT) and receptors (CCK_2, $5\text{-}HT_3$) mediating this activity, and to measure endogenous levels of pronociceptive neurotransmitters (5-HT, PGE_2) in the lumbar spinal cord. The second part is to investigate the role of dynorphin in the RVM, a primary pain modulatory

region in the brainstem known to play an integral role in modulating descending pathways to the spinal cord. Based on results from previous studies with dynorphin, it is our hypothesis that dynorphin in the RVM acts as a driving force in activating descending facilitatory pathways, is up-regulated in the RVM in nerve-injury (SNL), and through bradykinin receptors in the RVM, mediates its pain-promoting effects to the periphery. The following is a list of the specific aims this study sought to accomplish:

RVM-CCK Descending Facilitation Study

Specific Aim 1:

Part I - Using intrathecal microdialysis, measure PGE_2 and 5-HT levels in the spinal cord following CCK microinjection into the RVM. Analyze spinal CSF samples for PGE_2 and 5-HT using EIA and HPLC-fluorometric detection, respectively.

Part II - Administer CCK into the RVM, along with a selective CCK_2 receptor antagonist, and measure spinal levels of both PGE_2 and 5-HT to determine if the spinal

release of these pronociceptive neurotransmitters is a CCK_2 mediated process, and if their release can be blocked by a CCK_2-R antagonist.

Specific Aim 2: Administer CCK into the RVM accompanied with the $5\text{-}HT_3$ receptor antagonist, ondansetron administered intrathecally, and test for tactile hypersensitivity (von Frey test). Establish a dose-response curve for intrathecal ondansetron.

Specific Aim 3: Administer CCK into the RVM accompanied with the non-selective COX inhibitor, naproxen administered intrathecally, and test for tactile hypersensitivity (von Frey test). Establish a dose-response curve for intrathecal naproxen.

RVM-Dynorphin Descending Facilitation Study

Specific Aim 1: Using RT-PCR, determine prodynorphin and B1- and B2-receptor mRNA levels in the RVM in sham and nerve-injured (SNL) rats.

Specific Aim 2: Administer dynorphin A(2-13) into the RVM in naïve rats, and test for both acute and long-term behavioral effects (tactile hypersensitivity).

Specific Aim 3: Administer dynorphin antiserum or Hoe-140 into the RVM in nerve-injured animals (10-days post-SNL) and test for tactile hypersensitivity.

Specific Aim 4: Determine if the B1-antagonist, DALBK or the B2-antagonist, Hoe-140, administered into the RVM, can block dynorphin-induced tactile hypersensitivity.

Specific Aim 5: Verify presence of bradykinin receptors in the RVM by administering bradykinin, with and without the B2-antagonist, Hoe-140, into the RVM and test for tactile hypersensitivity.

CHAPTER TWO

Materials and Methods

2.0 Animals. Male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), weighing 250-350g at the time of testing, were maintained in a climate-controlled room on a 12-hr light/dark cycle (lights on at 7:00 A.M.), and food and water were available *ad libitum*. All of the testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain (IASP) and the National Institutes of Health (NIH) guidelines for the handling and use of laboratory animals and received approval from the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona.

2.1 Drugs and doses. Prior to administering drugs into the RVM, baseline measurements were taken using calibrated von Frey filaments. Pre-experimental baseline values were established, and naïve animals abnormally sensitive (paw withdrawal threshold < 11.982g = 4.93 filament) were removed from the testing group. All drugs administered into the RVM were microinjected bilaterally (0.5 µL of drug per each side for a total of 1.0 µL) into a dual cannula

secured on the animal's (Sprague-Dawley rat) skull as described previously in the section, RVM cannulation. The following drugs were used experimentally:

RVM: the CCK_2 receptor agonist cholecystokinin sulfated octapeptide (26-33)-CCK-8(s) was used at a dose of 60 ng/µL and obtained from American Peptide Company, Inc; primary sequence Asp-Tyr (SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2, MW 1143.2 amu, peptide purity: 96.5%, peptide content: 85.4%. YM022, a highly potent and selective non-peptide CCK_2 receptor antagonist was used at a dose of 50 pg/µL, K_i value is 68 pM at CCK_2 and 63 nM at CCK_1, long duration of action, MW 516.60, obtained from Tocris Bioscience. Dynorphin A(2-13), Anaspec Inc.; Bradykinin acetate 100 mg, Bachem Bioscience Inc.; Dynorphin A(1-13) antiserum (porcine) rabbit serum, Peninsula Laboratories; Lys-(Dys-Arg9, Leu8)-bradykinin (DALBK), Bradykinin-1 antagonist, American Peptide Company, Inc.; Hoe-140 (D-Arg[Hyp3, Thi5, D-Tic7, Oic8]bradykinin, B2-antagonist, American Peptide Company Inc. and Sigma Chemical Company.

Intrathecal: Ondansetron (Zofran®) hydrochloride USP, for injection, 2 mg/mL, MW 365.9, obtained from GlaxoSmithKline

(GSK). The i.th. doses of ondansetron used were 0.1, 1, and 10 µL. Naproxen Sodium, MW 252.2, Sigma-Aldrich Company. The i.th. doses of naproxen used were 100 and 200 µg. The intrathecal administration of drugs was accomplished with a 1 µL air bubble separating the drug, dissolved in 5 µL, followed by a 9 µL saline flush.

HPLC analysis: 5-hydroxytryptamine (5-HT, Serotonin) hydrochloride, MW 212.7 amu, and 5-hydroxyindole-3-acetic acid (5-HIAA), MW 191.19 amu, obtained from Sigma-Aldrich Company.

2.2 RVM cannulation. Rats were anesthetized with ketamine-xylazine (100 mg/kg, i.p.) for stereotaxic placement of bilateral cannula in the RVM. The skull was exposed, and two 26-gauge guide cannulas separated by 1.2 mm (Plastics One, Roanoke, VA) were directed toward the lateral portions of the RVM using the atlas of Paxinos and Watson (1986) (anteroposterior, -11.0 mm from bregma; lateral, ±0.6 mm; dorsoventral, -6.5 mm from the dura mater). The guide cannulas were cemented in place and secured to the skull by small stainless steel machine screws. The animals were allowed to recover for 4-7 days after surgery before any

pharmacological manipulations were applied. Drug administrations into the RVM were performed by slowly expelling 0.5 µl of drug solution through a 33-gauge injection cannula inserted through the guide cannula and protruding an additional 2 mm into fresh brain tissue (for a depth of −8.5 mm from the dura mater or −9.5 from the top of the skull) to prevent backflow of drug into the guide cannula. Pontamine blue was injected into the RVM injection site at the termination of the experiments, and cannula placement was verified histologically. Data from animals with incorrectly placed cannula were not included within the data analysis. Histological data from animals with misplaced cannulas were utilized as off-site controls.

2.3 Spinal nerve ligation (SNL). Tight ligation of the L5/L6 spinal nerves was performed in accordance with the method described by Kim and Chung (Kim and Chung, 1992). Anesthesia was induced and maintained with intraperitoneal or intramuscular injections of a ketamine (80 mg/kg)/xylazine (12 mg/kg) mixture. Animals were shaven dorsally (back hairs), and placed in a prone position on a surgical platform. Following surgical preparation of the rats and exposure of the dorsal vertebral column via a 2 cm

paraspinal incision from L4 to S2, the exposed L5 and L6 spinal nerves were tightly ligated with 4-0 silk suture. The incisions were then closed, and the animals were given at least 5 days recovery before further use. Sham control animals were subjected to the same operating procedure minus the L5/L6 nerve ligation (Burgess et al, 2002). Animals demonstrating motor difficulties or a lack of nerve injury induced tactile hypersensitivity were excluded from the study.

2.4 Intrathecal catheterization. While under ketamine (80 mg/kg) and xylazine (12 mg/kg) anesthesia/parasthesia, rats were implanted with permanently indwelling catheters (polyethylene 10, 7.8 cm from atlanto-occipital membrane at the base of the dorsal neck) into the spinal subarachnoid space for drug delivery at the L5/L6 level of the lumbar spinal cord (Yaksh 1976). Animals were allowed 4-7 days of recovery before experiments were conducted. ***In vivo* intrathecal microdialysis.** 1-hour before the experiment, rats were placed in a recording chamber with access to food and water *ad libitum*. After acclimation, the microdialysis system was connected to the animals with inflow and outflow tubing comprised of polyethylene 10 tubing connected to a

polyethylene 50 joint connected to the exposed polyethylene 10 (PE-10) microdialysis catheters extending out from the base of the rat's neck. The externalized PE-10 catheter tubing was then connected to a 2.5 mL Hamilton syringe containing artificial cerebrospinal fluid (CSF) attached to a BAS (Bioanalytical Systems, Inc) syringe pump, and served as the inflow. The other externalized PE-10 tubing served as the outflow, with the tubing terminating in a 1.7 mL microcentrifuge tube (Corning Inc.) residing in a refrigerated (4°C) fraction collector (Univentor Ltd., High Precision Engineering, 810 Microsampler) for dialysate collection.

Microdialysis of the cerebrospinal fluid (CSF) in the lower lumbar spinal cord was performed in freely moving, non-anesthetized, awake animals, as based on previous studies, which have utilized *in vivo* microdialysis to analyze endogenous levels of norepinephrine, 5-HT, and PGE_2 in the spinal cord (Malberg et al., 1995a,b; Hammond et al., 1985). At a flow rate of 10 µl/min, the microdialysis probe was continuously perfused with artificial CSF (in mM: 138 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 11 $NaHCO_3$, and 1 NaH_2PO_4, pH 7.4) containing 0.2% bovine serum albumin, 0.2% glucose, and

0.03% of the peptidase inhibitor Bacitracin. To allow the analyte levels to reach a steady state, the probe was perfused for a 30-minute washout period before collection of the first baseline sample (Malmberg and Yaksh, 1995b). Forty-minute fraction collections began after the washout period to establish baseline levels of PGE_2 or 5-HT in the lumbar spinal cord. Microdialysate was then collected for 40-minute fraction intervals over the entire 160-minute collection period. In all experiments, four dialysate fractions of 400 µl (40-min of perfusion each) were collected at 4°C and then immediately frozen at -80°C until their PGE_2 and 5-HT content could be analyzed via EIA or HPLC-fluorometric detection, respectively. At no point during the microdialysis did the animals exhibit any behavioral signs of discomfort, abnormal behavior, or motor hyperactivity.

2.5 PGE_2 analysis. PGE_2 concentrations were assayed using a commercially available kit (Amersham Biosciences RPN222, Buckinghamshire, England). This kit uses a monoclonal antibody to PGE_2 (anchored to the plate via anti-IgG antibody) and PGE_2-peroxidase conjugate. The PGE_2 antibody has the following cross-reactivities: 7.0% with PGE_1, 4.3%

with $PGF_{2\alpha}$ and 5.4% with 6-Keto $PGF_{1\alpha}$; no other cross-reactivities exceeding 2% are known (manufacturer's specifications). After incubation of the reagents and spinal CSF sample in the EIA plate, activity bound to the solid phase is measured as directly proportional to the HRP-TMB (3,3',5,5'-tetramethylbenzidine) substrate reaction as absorbance at 450 nm. Due to the competition between the PGE_2 in the sample and the HRP-conjugate reagent, the amount of PGE_2 can be calculated by an inversely proportional relationship to the absorbance measured. The limit of detection of the kit for PGE_2 was 2.5 pg/50µL sample, using the standard sensitivity protocol.

2.6 5-HT analysis. 5-HT concentrations were determined using a modified HPLC-fluorometric detection method (Kalen et al., 1988) developed to improve sensitivity for the naturally fluorescing indoleamines, 5-HT and its primary metabolite, 5-HIAA - found at femtomole (picogram) levels in spinal CSF. HPLC conditions: flow rate was optimized to 0.6 mL/min using a 3.0 x 100 mm C18 analytical Waters/Atlantis HPLC-column (3-micron particle-size) on a Waters 2695 HPLC coupled to a Waters 2475 Fluorescence Detector; column temperature was set to 30°C. An isocratic

gradient was used with a mobile phase consisting of 8% ammonium acetate (100 mM, pH 5), 4% acetonitrile (J.T. Baker HPLC-grade), and 88% diH_2O (Millipore). An injection volume of 10 µL per sample was used. The excitation, emission wavelengths used for fluorescence detection of the indoleamines was 285nm and 335nm, respectively. Internal standards for both 5-HT and 5-HIAA were used to create standard curves for quantification, and to confirm the molecular identity of chromatographic peaks of interest in analyzed spinal CSF samples. A standard curve was created with the following range of standard (5-HT or 5-HIAA) values: 290 fmol, 145 fmol, 72.5 fmol, 36.25 fmol, 18.125 fmol – converted to mass of analyte, 5-HT: 51.1 pg, 25.5 pg, 12.8 pg, 6.39 pg, 3.19 pg; 5-HIAA, 55.4 pg, 27.7 pg, 13.9 pg, 6.93 pg, 3.46 pg. The standard curve was created to determine the linear range of detection of our method, and the associated limit of detection, for 5-HT and 5-HIAA. The curve generated from the mole (mass) range of analytes listed gave a linear increase in response, measured as peak height, corresponding to the amount of indoleamine injected onto the column. The sensitivity of the method was defined as the amount of indoleamine producing a peak twice the basal noise, and was found to be approximately 3-5 pg

(bottom standard for standard/calibration curve used) per standard 10 µL injection volume.

Masses of interest in unknown spinal CSF samples were quantified using peak height, and calculated as percent of change from baseline values measured; absolute mass quantification was not used nor relevant to the experimental design being used. Further mass confirmation of the analyte being measured was obtained through the use of electrospray-ionization mass spectrometry (ESI-MS).

2.7 Tactile thresholds. The withdrawal threshold of the hindpaw in response to probing with a series of eight calibrated von Frey filaments (Stoelting, Wood Dale, IL) in logarithmically spaced increments ranging from 0.41 to 15 g (4-150 N) was determined. Each filament was applied perpendicularly to the plantar surface of the left hindpaw of rats kept in suspended wire-mesh cages. Measurements were taken both before and after administration of drug or vehicle. Withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength (“up and down” method), analyzed using a Dixon nonparametric test (Chaplan et al., 1994), and expressed as

the mean withdrawal threshold. Significant changes from baseline control values were detected by ANOVA, followed by Fisher's least significant difference test. These evaluations were all performed with the aid of the statistics package FlashCalc. Significance was set at $p < 0.05$.

2.8 Quantitative RT-PCR. Quantitation was performed using the iCycler iQ Multicolor Real-Time PCR Detection System with iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (Bio-Rad). Total RNA was isolated from the RVM by the TRIzol method (Invitrogen, CA, USA). Two step reverse transcription (RT) was performed using 1 µg total RNA and the Retroscript kit (Ambion, Austin, TX). Real-Time PCR analysis was performed on an iCycler MyiQ Single Color Real-Time PCR detection system (Bio-Rad, CA, USA). The sequences of the genes of interest were taken from the GeneBank: access number; B1 receptor NM_030851, B2 receptor X69681, and prodynorphin NM_019374. Gene-specific primers for the amplification were obtained from the Midland Certified Reagent Company. Real-Time Polymerase Chain Reaction primers sequences for the amplification were:

B1 receptor/forward primer: 5' GCATCTTCCTGGTGGTGG 3'
(nucleotides: 540~557)
B1 receptor/reverse primer: 5' CAGAGCGTAGAAGGAATGTG 3'
(nucleotides: 682~701)
B2 receptor/forward primer: 5' CTTTGTCCTCAGCGTGTTC 3'
(nucleotides: 364~382)
B2 receptor/reverse primer: 5' CAGCACCTCTCCGAACAG 3'
(nucleotides: 506~523)
Prodynorphin/forward primer: 5' GCAAATACCCCAAGAGGAG 3'
(nucleotides: 670~688)
Prodynorphin/reverse primer: 5' CGCAGAQAAACCACCATAGC 3''
(nucleotides: 817~835)
GAPDH/forward primer: 5' ATCATCCCTGCATCCACTG 3'
GAPDH/reverse primer: 5' GCCTGCTTCACCACCTCC 3'

The Real-Time PCR reactions were carried out in a total reaction volume of 20 µL containing the final concentration of: 1x SYBR Green Master Mix (Bio-Rad, Hercules, CA), 300 nM of forward and reverse primers, and 200 ng of cDNA from the RT step. All samples from different animals were run in triplicate using an annealing temperature of 60°C. The expression of target genes was normalized to that of GAPDH. The differences of target

mRNA expression between treatment and control were analyzed using the Comparative CT Method. The threshold cycle (CT) is defined as the cycle at which the fluorescence generated from a certain amount of amplified PCR product reaches a fixed threshold. For each sample, the CT value for the control gene (GAPDH) was subtracted from the CT value for the gene of interest (B1 receptor, B2 receptor and prodynorphin) to obtain a ΔCT value. The control sample ΔCT value was then subtracted from the treated animal ΔCT value to obtain the ΔΔCT. The relative fold change from our control was expressed by a calculation of 2- ΔΔCT for each sample. The level of expression of each target gene is converted to the copy number of that same target gene which is relative to 500,000 copies of GAPDH.

All samples were run in triplicate using an annealing temperature of 60°C. For real-time RT-PCR, the following primers and probes were selected: data are expressed as the mean ± SEM.

2.9 Data analysis. PGE_2 and 5-HT baseline concentrations were measured and normalized to 100%, and used as a basis of comparison for all post-baseline measurements, which

were calculated as percent change of baseline values. Significant changes from control values were detected by ANOVA, followed by Fisher's least significant difference post hoc test. These evaluations were all performed with the aid of the statistics package FlashCalc. Statistical significance was set at $p < 0.05$.

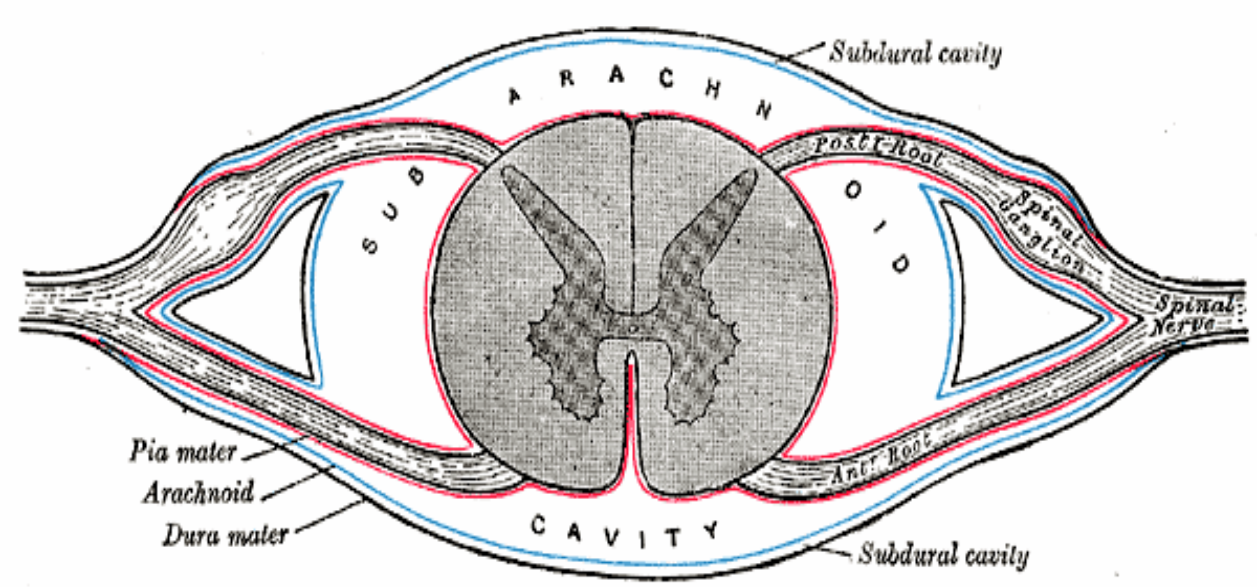

Figure 13 Diagram showing the basic organization of the spinal cord. Posterior and dorsal spinal roots are shown, along with the meninges and subarachnoid cavity in the spinal cord (Illustration taken from Gray's Anatomy online source, website ref).

CHAPTER THREE

Results – Part One

3.1 CCK in the RVM evokes the release of PGE_2 in the lumbar spinal cord

Administration of CCK-8(s)[30 ng/0.5µL] via a single, bilateral microinjection into the RVM resulted in a time-dependent increase in the release of PGE_2 in the lumbar spinal cord. Release of PGE_2 into the spinal CSF showed peak levels, 40-min post-CCK injection into the RVM, and continued at this level through 80-min, with no statistical difference ($p=0.385$) in PGE_2 release between the 40- and 80-min timepoints. Post-baseline, percent of baseline measurements of PGE_2 in the spinal CSF were as follows: 40-min, 198 ± 20.1; 80-min, 469 ± 93.0; 120-min, 555 ± 79.7%. CCK-8(s) administered bilaterally into the RVM resulted in a significant ($p=0.075$) increase in spinal PGE_2 release with a mean PGE_2 release (from baseline levels) of 508 ± 108% for the two post-CCK injection timepoints mathematically pooled (80-min collection period; mean calculated; n = 6) (Fig. 1.2A). This release of PGE_2 was significantly attenuated ($p=0.025$) with the pre-administration of the CCK_2 receptor

antagonist, YM022, given 10-minutes prior to CCK, both administered into the RVM (Fig. 1.2B).

CCK into the RVM evokes the release of PGE_2 in the lumbar spinal cord; CCK_2 antagonist, YM022 attenuates this release

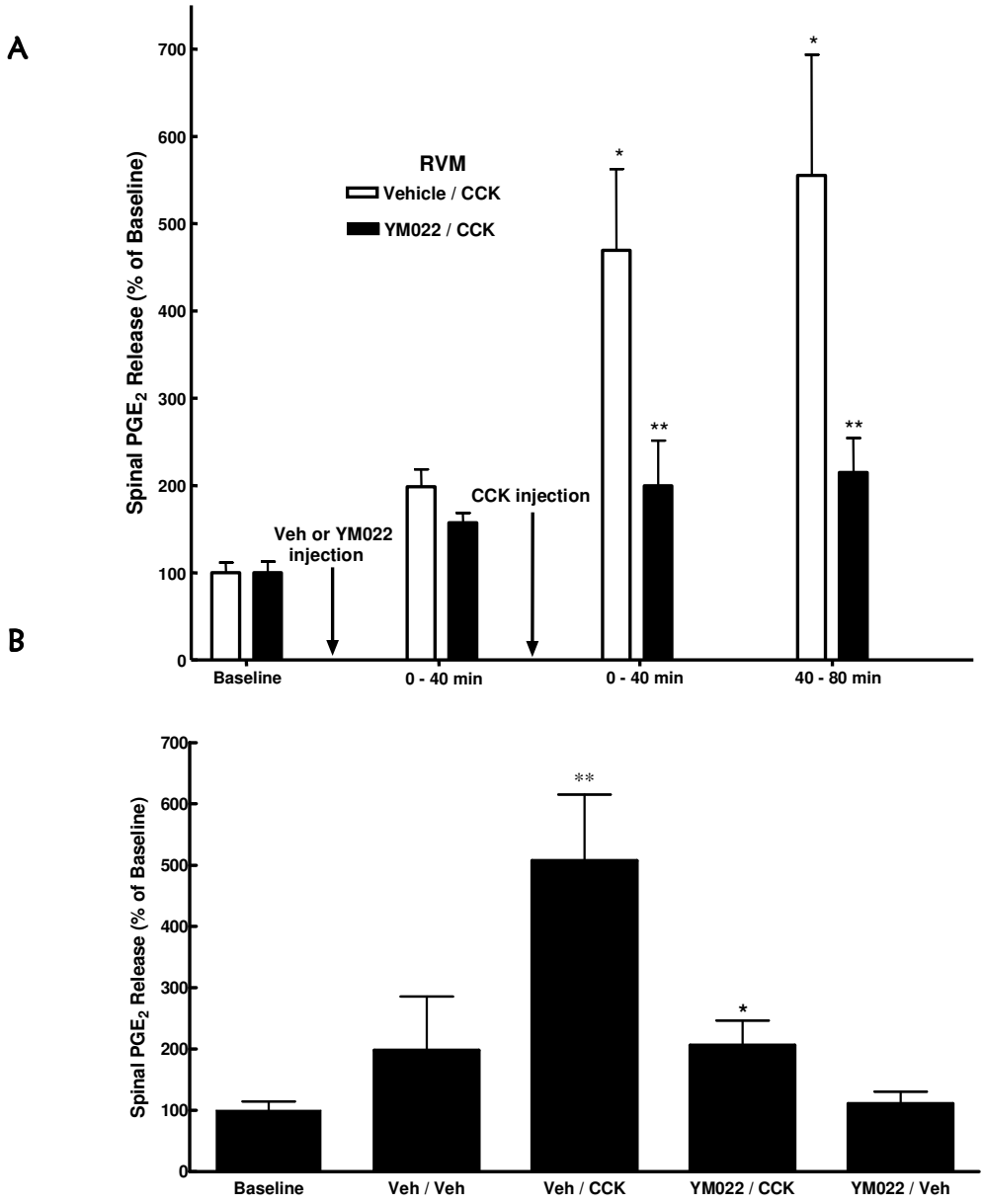

Figure 14 Male Sprague Dawley rats (250–350g) received CCK-8(s)[30 ng/0.5µL] bilaterally into the RVM and PGE_2 levels were determined through intrathecal microdialysis collection of the spinal CSF in the lumbar spinal cord.

(Figure A) Microdialysis collections were taken at 40-min intervals for 160-min. CCK-8(s) administered bilaterally into the RVM resulted in a significant increase in spinal PGE_2 release from baseline and vehicle levels (n = 5; **p < 0.05). YM022 into the RVM, 10-min prior to RVM CCK-8(s) resulted in a significant attenuation of CCK-8(s) induced spinal release of PGE_2 for the two post-CCK injection timepoints (80-min collection period)(n = 6; *p < 0.05). (Figure B) Summary of Figure (A) data, post-injection timepoints were mathematically pooled (mean calculated for each group).

3.2 RVM/CCK-induced tactile hypersensitivity is attenuated by the non-selective COX inhibitor, naproxen (i.th.)

CCK-8(s)[30 ng/0.5µL] administered bilaterally into the RVM in conjunction with saline vehicle (i.th.) produced a pronounced decrease in the paw withdrawal threshold, indicating an increase in tactile hypersensitivity. Administration of CCK-8(s) into the RVM produced its maximum nociceptive effect ($p=0.032$) at 45-min with the following paw withdrawal threshold(g): 3.88 ± 0.595g, which was significantly ($p=0.005$) attenuated by the pre-administration (i.th.; 15-min prior to CCK) of the non-selective COX inhibitor naproxen, at both the 100 µg and 200 µg doses into the spinal cord. Paw withdrawal thresholds (g) for the 30- and 45-min timepoints (where naproxen demonstrated its peak activity), respectively, were as follows for the two doses used: 100 µg dose, 12.7 ± 0.358g, and 13.9 ± 0.283g; 200 µg dose, 14.4 ± 0.302g, and 14.4 ± 0.302g. A dose-responsive attenuation of CCK-induced nociception was observed for naproxen, with the greatest effect (45 min; 14.4 ± 0.302g) observed at the 200 µg dose.

RVM/CCK-induced tactile hypersensitivity is attenuated by the non-selective COX inhibitor, naproxen (i.th.)

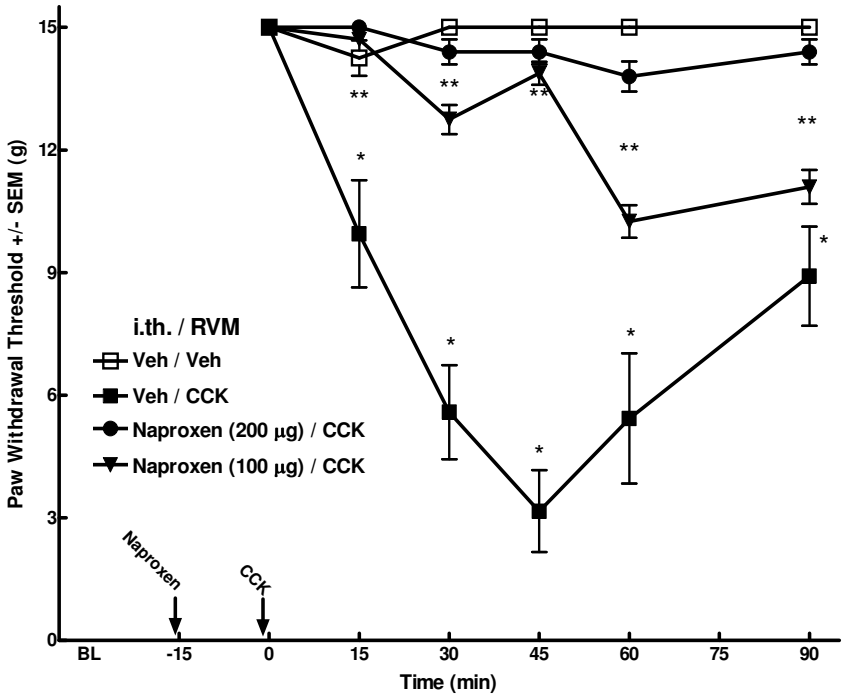

Figure 15 Male Sprague-Dawley rats (250-350g) received naproxen (100 or 200µg) intrathecally followed by CCK-8(s)[30 ng/0.5µL] administered bilaterally into the RVM. CCK-8(s) administered into the RVM resulted in a significant increase in tactile hypersensitivity (*$p < 0.05$, $n = 5$), which was significantly attenuated at all timepoints by the pre-administration of the non-selective COX inhibitor naproxen at the two doses tested (**$p < 0.05$; *n* = 5 per group).

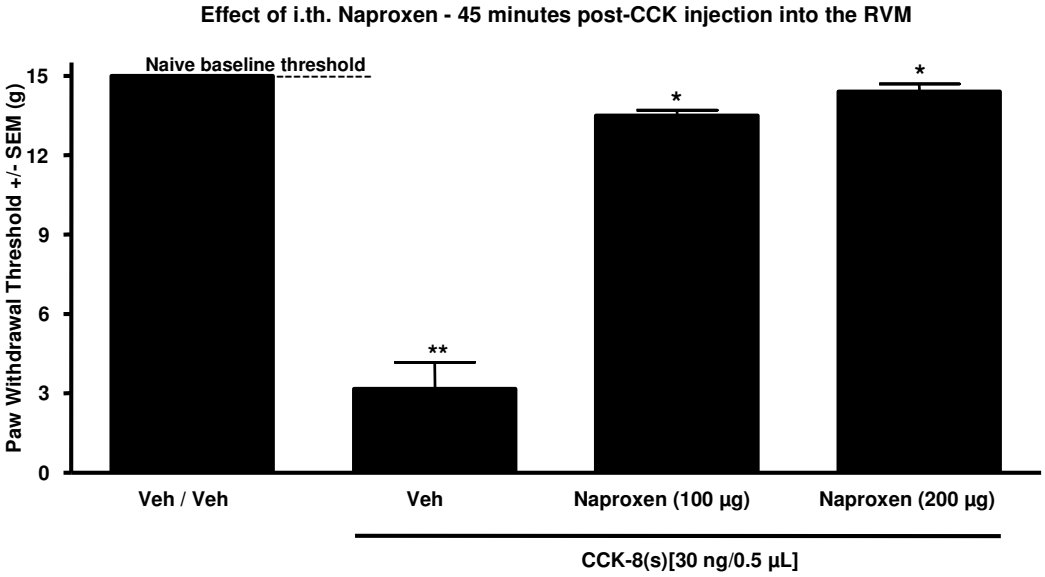

Figure 16 Male Sprague-Dawley rats (250-350g) received naproxen (100 or 200µg) intrathecally followed by CCK-8(s)[30 ng/0.5µL] administered bilaterally into the RVM. CCK-8(s) administered into the RVM resulted in a significant increase (**$p < 0.05$) in tactile hypersensitivity, which was significantly attenuated by the pre-administration of the non-selective COX inhibitor naproxen ($p < 0.05$; $n = 5$ per group).

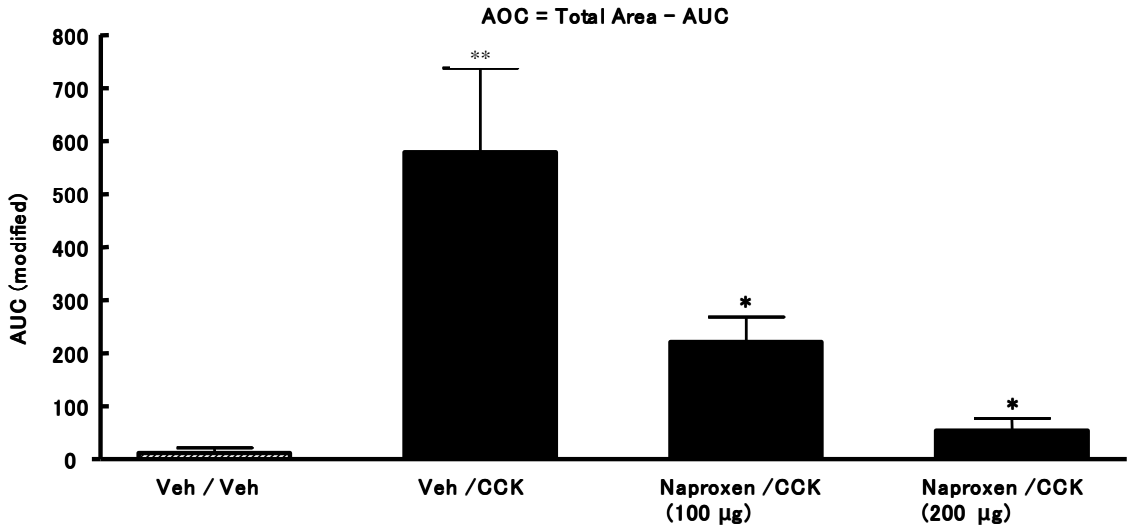

Figure 17 A modified AUC graph (AOC = Total Area – AUC) of Figure 15 data was used to confer a more intuitive graphical representation of the antagonist data, with a summary of the data across the total experimental timecourse (*p < 0.05 compared to Veh/CCK group; **p < 0.05 compared to Veh/Veh group).

3.3 CCK in the RVM does not affect the release of 5-HT into the lumbar spinal cord

CCK-8(s)[30 ng/0.5µL] administered bilaterally into the RVM did not result in a significant change in the spinal CSF release of 5-HT with a mean 5-HT release (from baseline levels) of 111 ± 10.3% for the two post-injection timepoints mathematically pooled (80-min collection period; mean calculated; n = 7). Administering the CCK_2 receptor antagonist, YM022 (25 pg/0.5µL) into the RVM, 10-min prior to CCK resulted in a small, but non-significant (p=0.663) decrease in spinal 5-HT release with a mean 5-HT (percent of baseline) level of 109 ± 13.5% for the two post-CCK injection timepoints mathematically pooled (80-min collection period; mean calculated; n = 8). The administration of YM022 (25 pg/0.5µL) coupled with a diH_2O vehicle injection (YM022/diH_2O; first injection/second injection) into the RVM resulted in a significant (p=0.003) decrease in 5-HT from vehicle control levels (108 ± 19.3%) with a mean 5-HT (percent of baseline) release of 73.1 ± 4.21% for the post-YM022/diH_2O injection, 120-min collection period (n = 6). The administration of vehicle (diH_2O) into the RVM did not significantly (p=0.106) alter 5-HT release

in the spinal cord from non-injection baseline controls levels. ESI-Mass Spec analysis of CSF samples analyzed for 5-HT and 5-HIAA, confirmed the presence of both indoleamines. Parent masses for 5-HT and 5-HIAA were identified at 176.6 amu and 192.9 amu, respectively. Homodimers of 5-HT were identified in analyzed samples with a mass of 337.5 amu (2 x 5-HT – H_2O + $2H^+$) and heterodimers formed between the parent mass, 5-HT, and its metabolite (5-HT + 5-HIAA; 176 + 191 = 367 amu).

CCK administered into the RVM did not alter the release of 5-HT in the lumbar spinal cord

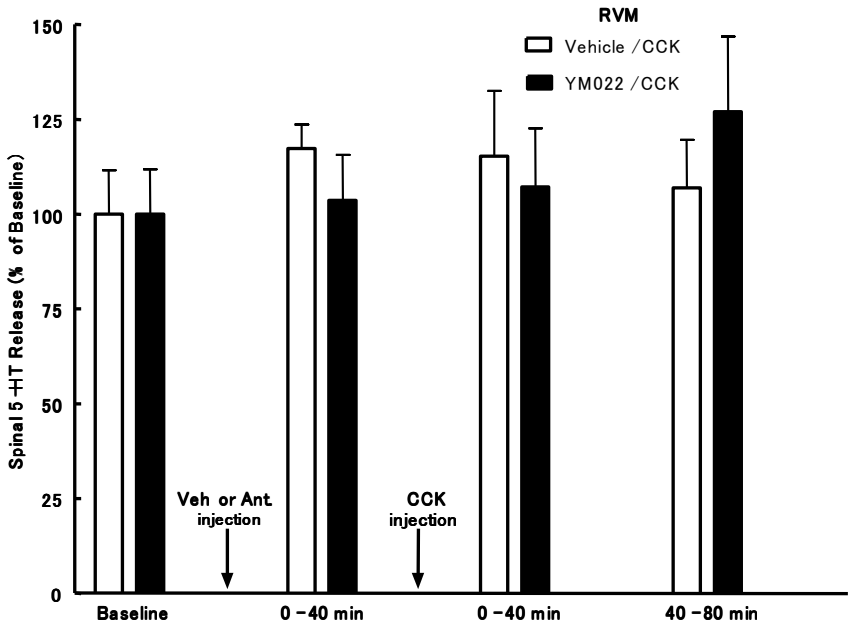

Figure 18 Male Sprague Dawley rats (250-350g) received CCK-8(s)[30 ng/0.5µL] bilaterally into the RVM and 5-HT levels were determined through intrathecal microdialysis collection of spinal CSF in the lumbar spinal cord. CCK-8(s) administered into the RVM resulted in a non-significant ($p > 0.05$) increase in spinal 5-HT release for the 80-min collection period following injection ($n = 6$). Administering the CCK_2 receptor antagonist, YM022 (25 pg/0.5µL) into the RVM, 10-min prior to CCK did not significantly ($p > 0.05$) reduce 5-HT release compared to baseline, vehicle, or CCK only injections.

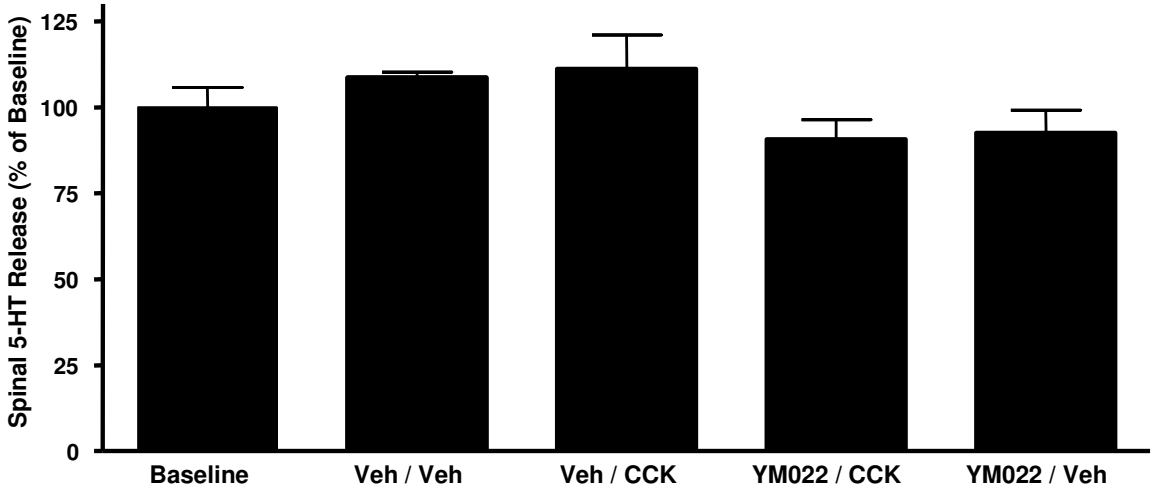

Figure 19 Male Sprague Dawley rats (250–350g) received CCK-8(s)[30 ng/0.5µL] bilaterally into the RVM and 5-HT levels were determined through intrathecal microdialysis collection of spinal CSF in the lumbar spinal cord. CCK-8(s) administered into the RVM resulted in a small, but non-significant ($p > 0.05$) increase in spinal 5-HT release for the post-CCK injection timepoints mathematically pooled (80-min collection period; mean calculated; $n = 6$). Administering the CCK_2 receptor antagonist, YM022 (25 pg/0.5µL) into the RVM, 10-min prior to CCK did not significantly ($p > 0.05$) reduce 5-HT release compared to baseline, vehicle, or CCK only injections.

3.4 RVM/CCK-induced tactile hypersensitivity is attenuated by the 5-HT_3 antagonist, ondansetron (i.th.)

CCK-8(s)[30 ng/0.5µL] administration into the RVM in conjunction with saline vehicle (i.th.) produced a pronounced decrease in the paw withdrawal threshold, indicating an increase in tactile hypersensitivity. Administration of CCK via bilateral microinjection into the RVM resulted in a significant ($p=0.003$) increase in tactile hypersensitivity, which was significantly attenuated by the 5-HT_3 antagonist, ondansetron, into the spinal cord. Administration of CCK into the RVM produced its maximum nociceptive effect at 45- min with the following paw withdrawal threshold 5.23 ± 0.744g, which was significantly ($p<<0.05$) blocked by the post-administration (i.th.) of ondansetron at all three doses tested: 0.1, 1, and 10 µg. Paw withdrawal thresholds(g) for the 30- and 45-min timepoints (where ondansetron demonstrated its peak activity), respectively, were as follows for the three doses used: 0.1 µg dose: 10.0 ± 0.721g, and 8.74 ± 0.799g; 1 µg dose: 10.3 ± 0.724g, and 10.1 ± 0.813g; 10 µg dose: 12.0 ± 0.862g, and 10.3 ± 0.725g. A dose-responsive attenuation of CCK-induced nociception was observed for

ondansetron, with significance achieved (p=0.033) for both the 1 and 10 µg doses over the entire timecourse (AOC data = AUC - Total Area), and the greatest effect observed at the 10 µg dose.

RVM/CCK-induced tactile hypersensitivity is partially attenuated by the 5-HT_3 antagonist, ondansetron (i.th.)

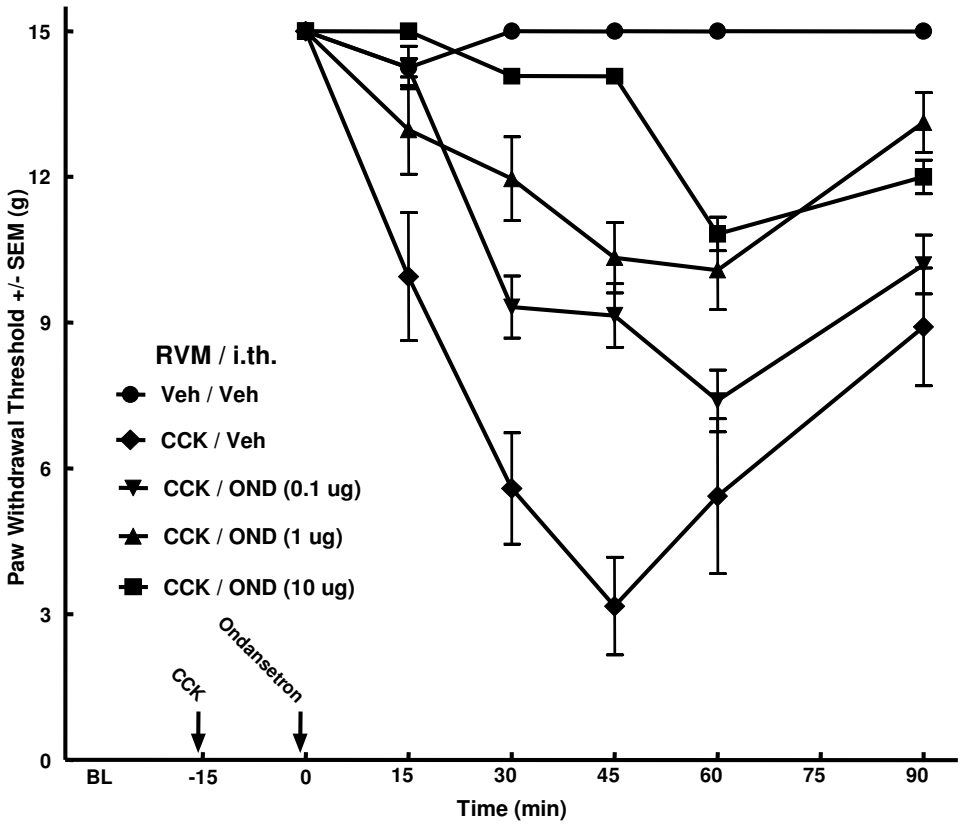

Figure 20 Male Sprague Dawley rats (250-350g) received CCK-8(s)[30 ng/0.5µL] bilaterally into the RVM, followed by either saline or ondansetron (0.1, 1, 10 µg/5µL) delivered intrathecally. CCK administered into the RVM resulted in a significant increase in tactile hypersensitivity ($p < 0.05$; $n = 6-7$) at all timepoints, which was significantly attenuated (15-45 min interval, all doses tested) by the post-administration of the 5-HT_3 antagonist, ondansetron ($p < 0.05$; $n = 6-7$).

RVM/CCK-induced tactile hypersensitivity is partially attenuated by the 5-HT_3 antagonist, ondansetron (i.th.)

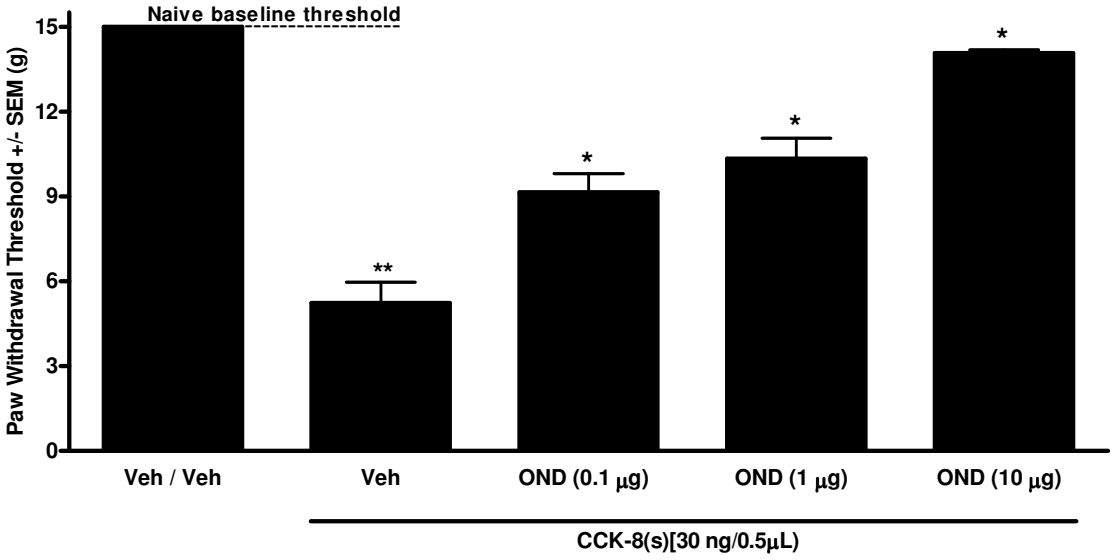

Figure 21 Effects of graded doses (0.1, 1, 10 µg/5µL) of intrathecal (i.th.) ondansetron on tactile hypersensitivity induced by CCK-8(s)[30 ng/0.5µL] microinjection into the RVM. The i.th. injection of the 5-HT_3 antagonist ondansetron (*p < 0.05; *n* = 6) significantly attenuated the effects of RVM CCK (**p < 0.05; n = 5), 45-min post-CCK administration into the RVM.

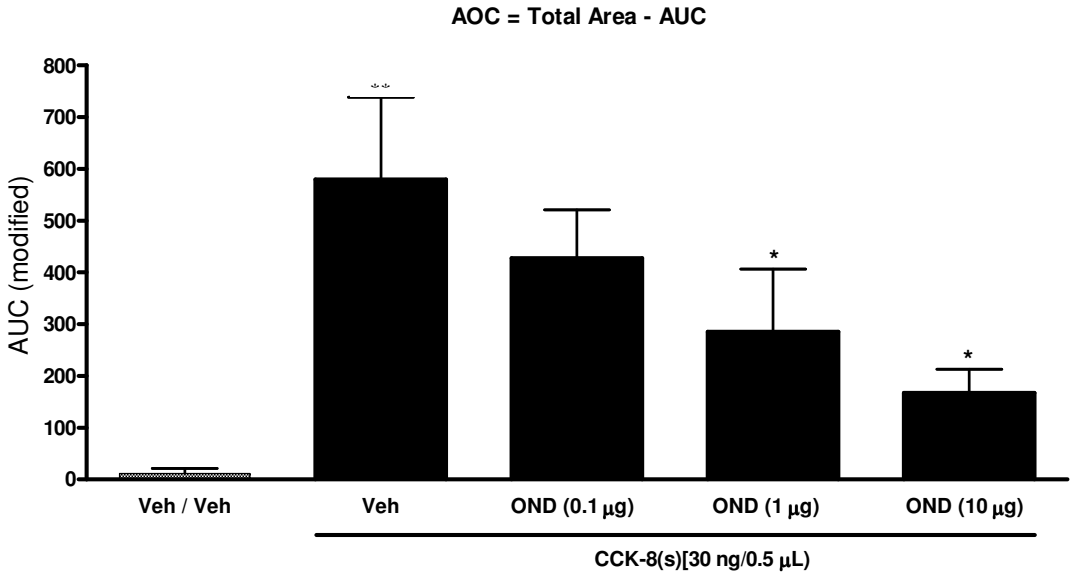

Figure 22 A modified AUC graph (AOC = Total Area – AUC) of Figure 20 data was used to confer a more intuitive graphical representation of the antagonist data (*p < 0.05 compared to Veh/CCK group; **p < 0.05 compared to Veh/Veh group).

3.5 RVM/CCK-induced tactile hypersensitivity is attenuated by the pre-administration of the CCK_2 antagonist, YM022 into the RVM

Administration of CCK-8(s)[30 ng/0.5µL] via bilateral microinjection into the RVM resulted in a significant ($p=0.03$) increase in tactile hypersensitivity. The CCK_2 receptor antagonist, YM022 (25 pg/0.5µL) administered 10-min prior to CCK into the RVM, achieved a significant block of CCK-induced tactile allodynia at the 30-min ($p=0.026$), and 45-min ($p=0.004$) timepoints. Administration of CCK into the RVM produced its maximum nociceptive effect at 45-min with the following paw withdrawal threshold 7.02 ± 1.98g, which was significantly ($p=0.004$) attenuated by YM022 injection into the RVM.

RVM/CCK-induced tactile hypersensitivity is partially attenuated by the pre-administration of the CCK_2 antagonist, YM022 into the RVM

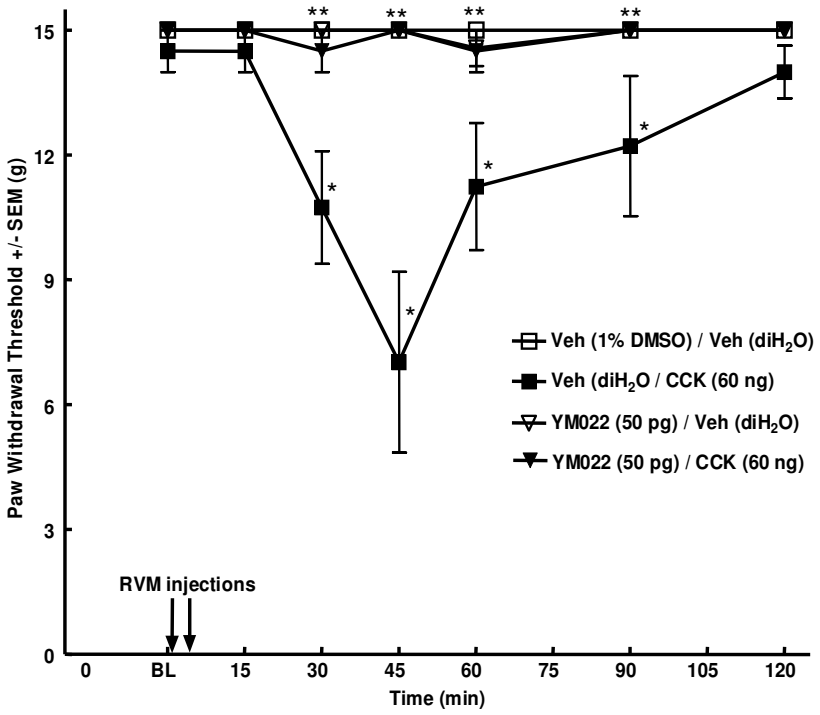

Figure 23 Male Sprague Dawley rats (250-350g) received CCK-8(s)[30 ng/0.5µL] bilaterally into the RVM with either vehicle (diH_2O; n = 6) or the CCK_2 antagonist YM022 (n = 6) pre-administered into the RVM. CCK-8(s) administered bilaterally into the RVM resulted in a significant increase in tactile hypersensitivity (*p < 0.05; *n* = 6), which was significantly attenuated by the pre-administration of YM022 (**p < 0.05; *n* = 6). Vehicle (1% DMSO or diH_2O) injection into the RVM had no observable effect.

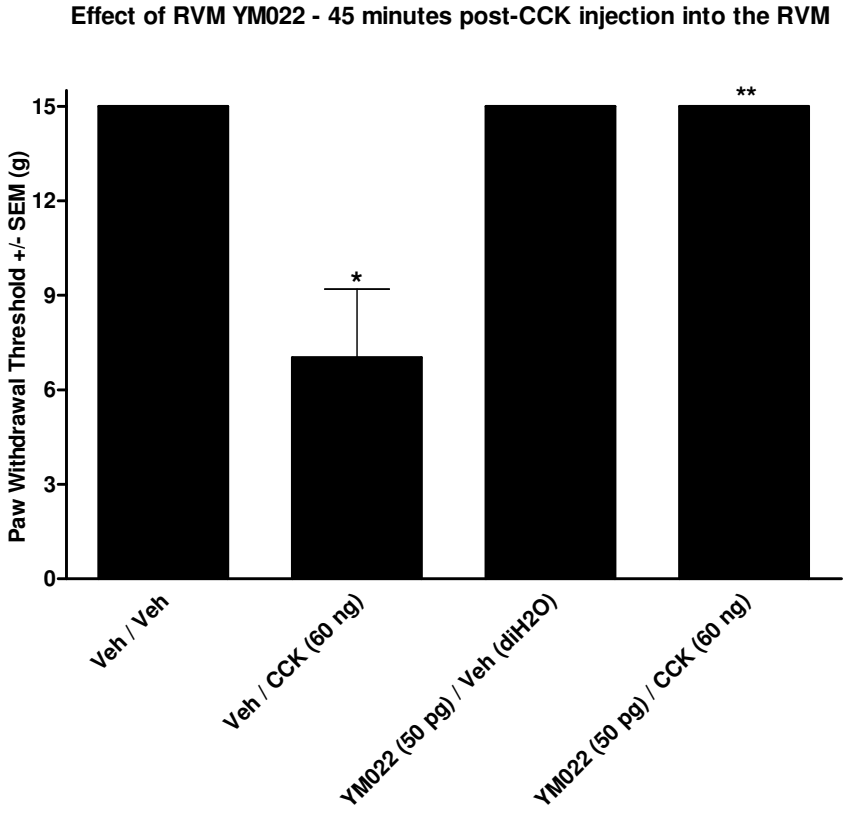

Figure 24 Effect of the CCK_2 receptor antagonist, YM022 (25 pg/0.5µL) on tactile hypersensitivity induced by CCK-8(s)[30 ng/0.5µL](*$p < 0.05$) microinjection into the RVM in male Sprague-Dawley rats (250-350g). The RVM injection of YMO22 (**$p < 0.05$;*n* = 6 per group) significantly attenuated tactile hypersensitivity induced by CCK-8(s), 45-min post-CCK administration into the RVM.

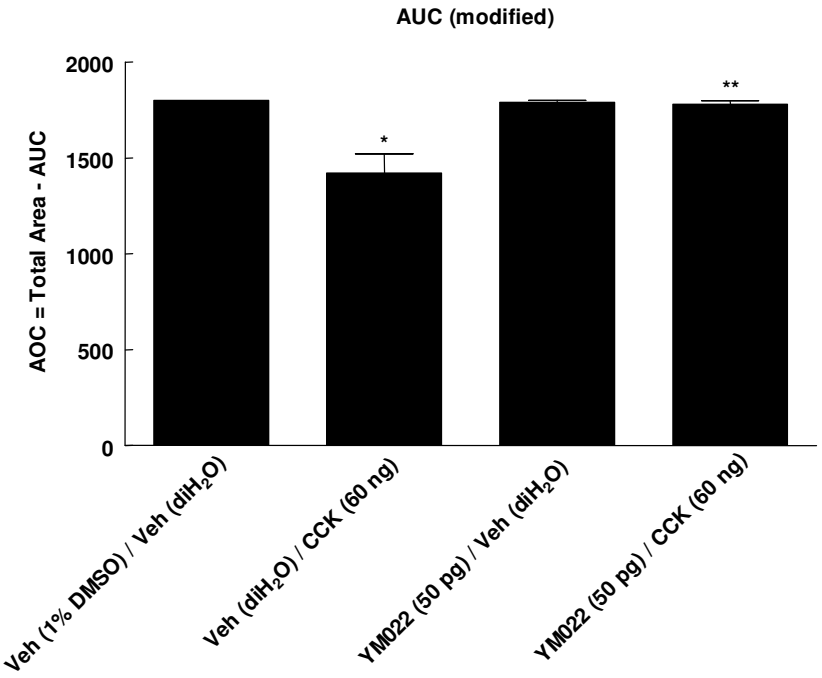

Figure 25 A modified AUC graph (AOC = Total Area – AUC) of Figure 23 data. Male Sprague Dawley rats (250–350g) received CCK-8(s)[30 ng/0.5µL] bilaterally into the RVM with either vehicle (diH_2O; n = 6) or the CCK_2 antagonist YM022 pre-administered into the RVM. CCK-8(s) administered into the RVM resulted in a significant increase in tactile hypersensitivity (*p < 0.05; *n* = 6–7), which was significantly attenuated by the pre-administration of YM022 (**p < 0.05; *n* = 6–7). Vehicle (1% DMSO or diH_2O) injection into the RVM had no observable effect.

Results – Part Two

3.6 Prodynorphin mRNA in the RVM is increased following nerve-injury (SNL)

RT-PCR was employed to determine levels of prodynorphin mRNA in the RVM of nerve-injured (SNL) and sham animals. Prodynorphin mRNA levels were significantly increased (Figure 26; p=0.009) 14-days post SNL, but not 2-days post SNL.

3.7 B1 and B2-receptor mRNA in the RVM are increased following nerve-injury (SNL)

RT-PCR was also employed to determine levels of B1- and B2-receptor mRNA in the RVM of nerve-injured (SNL) and sham animals. B1-receptor mRNA levels were significantly increased 2-days post-SNL (Figure 27; (p=0.003), but not 14-days post SNL. B2-receptor mRNA was significantly (p=0.046) increased 14-days post SNL, but not 2-days post SNL.

Prodynorphin mRNA in the RVM is increased following nerve-injury

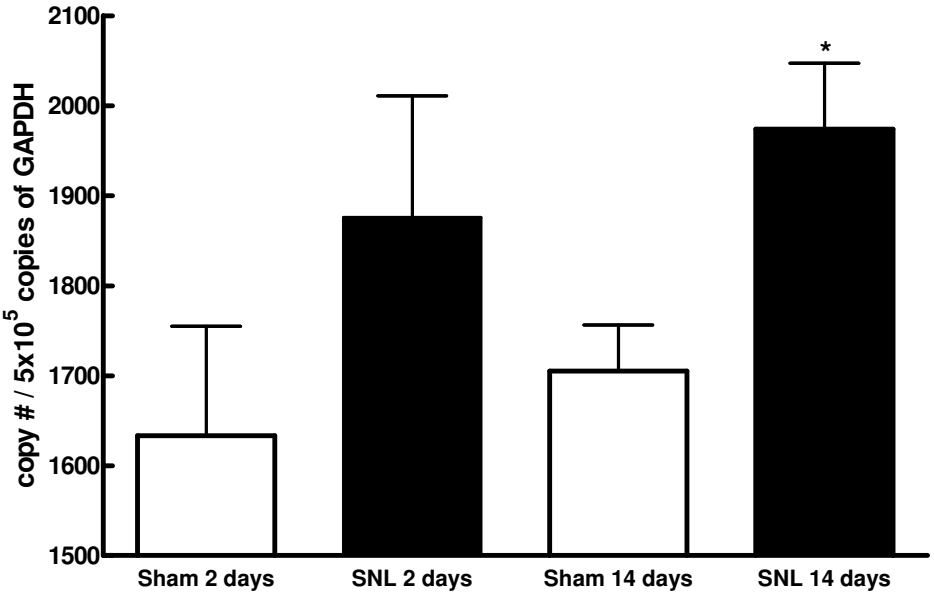

Figure 26 RT-PCR analysis of prodynorphin mRNA expression in the RVM of nerve-injured (SNL) and sham male Sprague Dawley rats (250-350g) at either 2-days or 14-days post-SNL surgery. Data are represented as the mean ± SEM. The sample size was *n* = 4 for each group, with each RVM tissue sample run in triplicate.

B1 and B2-receptor mRNA in the RVM are increased following nerve-injury (SNL)

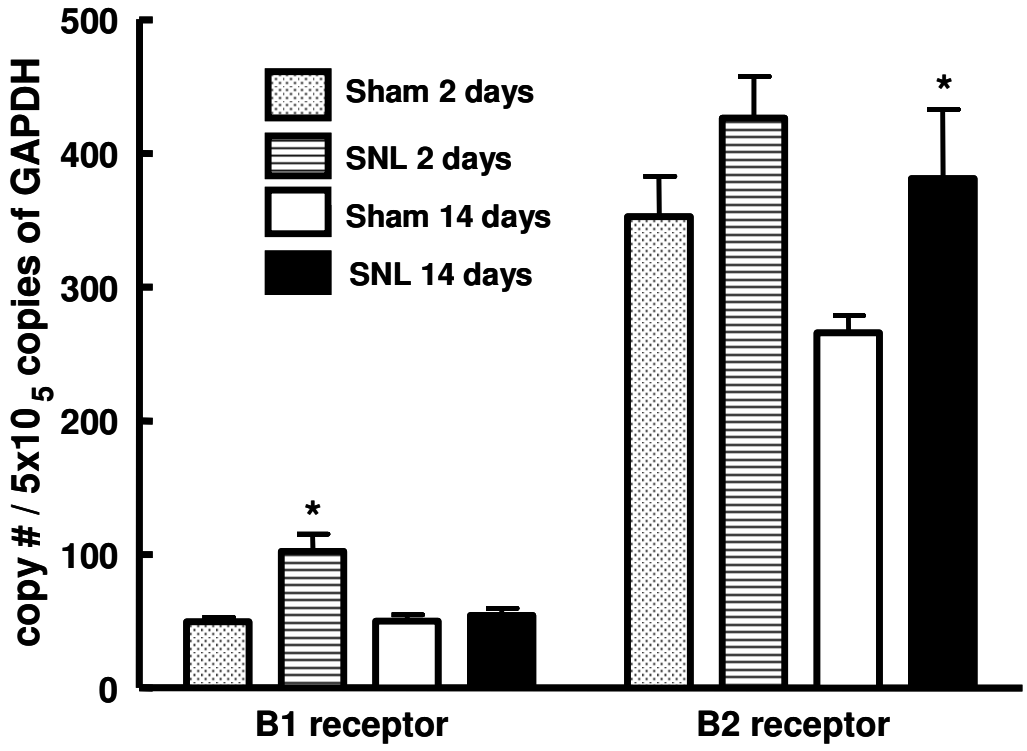

Figure 27 Levels of B1 and B2-receptor mRNA in the RVM of nerve-injured (SNL) and sham male Sprague Dawley rats (250-350g) at either 2-days or 14-days post surgery were assayed via RT-PCR. Data are represented as the mean ± SEM. Sample size was n = 4 for each group, with each RVM tissue sample run in triplicate.

3.8 The B1-antagonist DALBK attenuates dynorphin-induced abnormal pain

A single, bilateral microinjection of 6 nmol (total dose) dynorphin A(2-13) was administered into the RVM and elicted a strong pronociceptive response as indicated by a significant (45-min: $p<<0.05$) decrease in the paw withdrawal threshold (Figure 29). Administration of dynorphin A(2-13) into the RVM produced its maximum nociceptive effect at 45-min with the following paw withdrawal threshold (g) 6.89 ± 0.395g, which was significantly ($p=0.003$) attenuated by the pre-administration of the B1-antagonist DALBK at the 2, 6, and 20 nmol doses (Figure 29). Paw withdrawal thresholds (g) for the 45-min timepoint, where DALBK demonstrated its peak activity, were as follows for the three doses tested: 2 nmol dose, 11.5 ± 0.667g; 6 nmol dose, 10.4 ± 0.427g; 20 nmol dose, 13.1 ± 0.27g. A dose-responsive attenuation of dynorphin-induced nociception was observed for DALBK, with the greatest effect seen at the 20 nmol dose. Graphically representing a summary of the total timecourse data (Figure 2.9) as a modified AUC graph (AOC = Total Area - AUC), a dose-response trend (20 nmol > 6 nmol) of the DALBK

antagonist data is evident. In reference to the data across the entire timecourse (Fig 30; AOC data), significance was achieved at the 6 nmol ($p=0.044$), and 20 nmol ($p=0.001$) doses tested.

The B1-antagonist DALBK attenuates dynorphin-induced abnormal pain

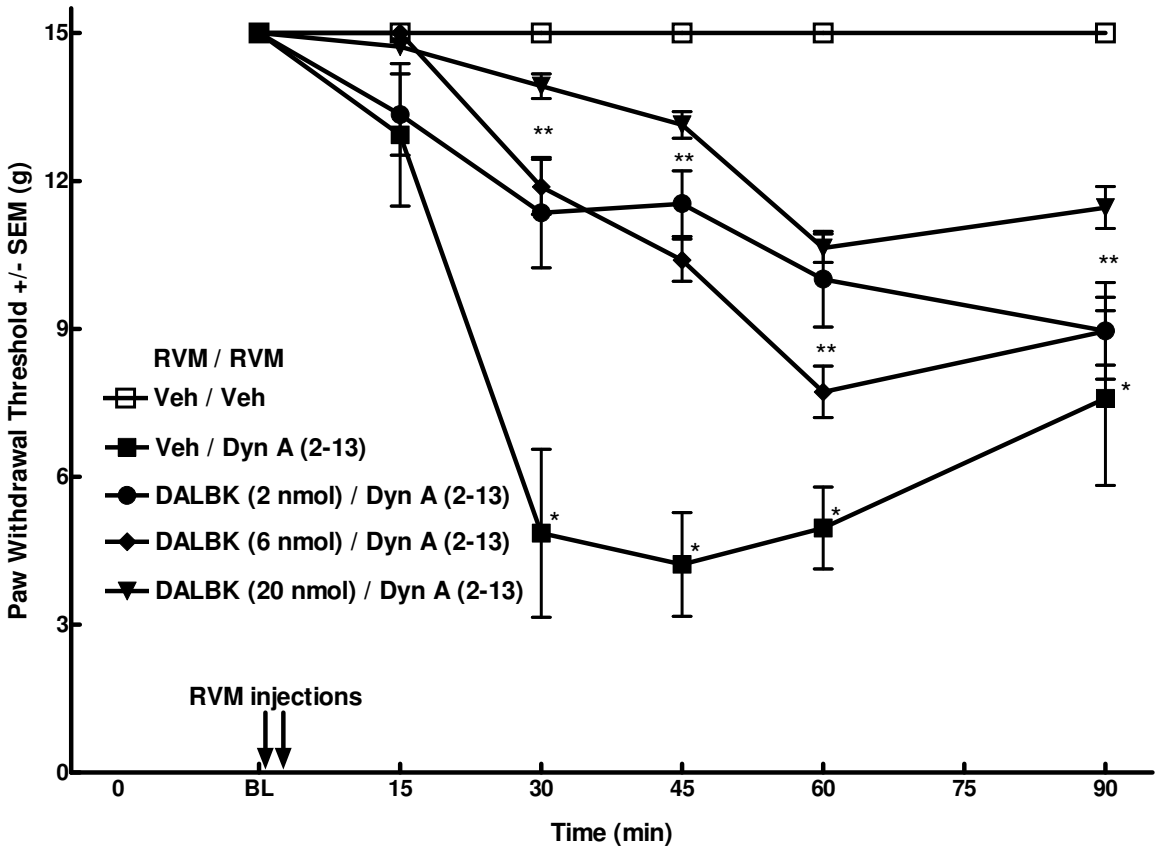

Figure 28 Male Sprague Dawley rats (250-350g) received the B1-antagonist, Des-Arg9-[Leu8]-bradykinin (DALBK) or vehicle (diH_20) followed by dynorphin A(2-13)[6 nmol] bilaterally into the RVM, and then tested for tactile hypersensitivity using von Frey filaments. Dynorphin A(2-13) administered bilaterally into the RVM resulted in a significant increase in tactile hypersensitivity (*$p < 0.05$; $n = 8$), which was significantly attenuated by the pre-administration of DALBK (**$p < 0.05$; $n = 5-11$).

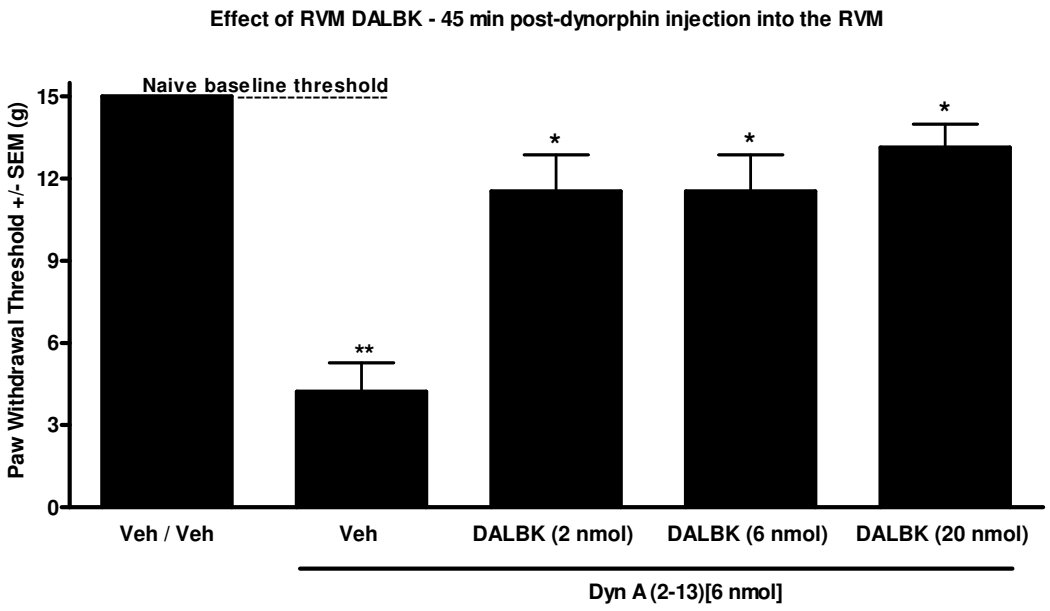

Figure 29 Male Sprague–Dawley rats (250–350g) received DALBK (2, 6, or 20 nmol) followed by dynorphin A(2–13)[6 nmol] bilaterally into the RVM. The data plotted represent the effect of DALBK at 45–min post–dynorphin injection into the RVM – where it demonstrated its peak effect. Dynorphin A(2–13) administration into the RVM resulted in a significant increase in tactile hypersensitivity (**$p < 0.05$; $n = 6$), which was partially blocked by the pre–administration of the B_1–antagonist DALBK (*$p < 0.05$; $n = 11$).

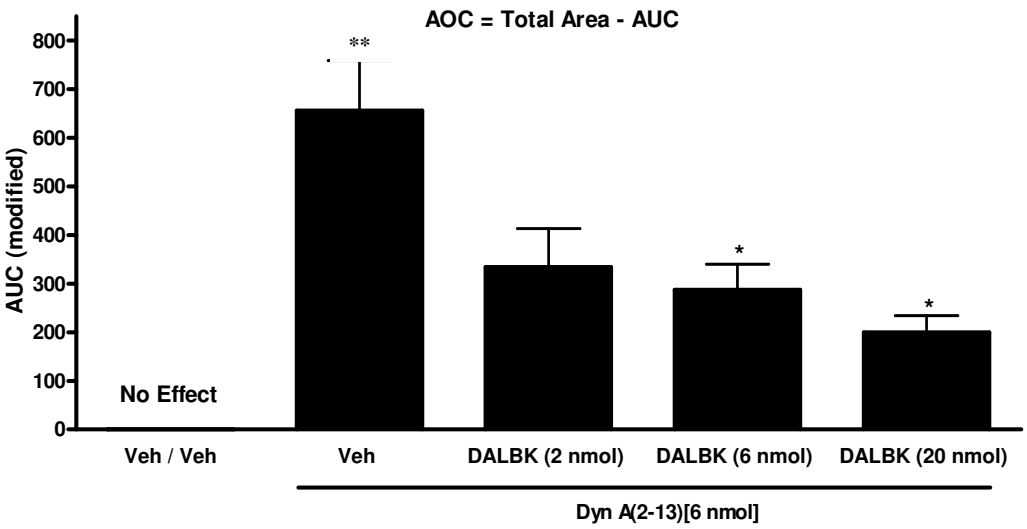

Figure 30 A modified AUC graph (AOC = Total Area – AUC) of Figure 28 data was used to confer a more intuitive graphical representation of the antagonist data, with a summary of the data across the total experimental time course (*p < 0.05 compared to Veh/Dyn group; **p < 0.05 compared to Veh/Veh group).

3.9 The B2-antagonist Hoe-140 attenutes dynorphin-induced abnormal pain

Administration of a single, bilateral microinjection of the B2-antagonist Hoe-140 at the 0.02 nmol (20 pmol), 0.1 nmol (100 pmol), 1 nmol (1000 pmol), and 2 nmol (2000 pmol) doses, 10-min prior to dynorphin A(2-13)[6 nmol] into the RVM, produced a dose-dependent attenuation of the dynorphin-induced increase in tactile hypersensitivity with significance (AUC data: ($p<<0.05$) achieved at the three doses tested. The 20 pmol dose of Hoe-140 demonstrated significance (30-min: $p=0.057$, 45-min: $p=0.009$, 60-min: $p<<0.05$, 90-min: $p=0.006$) at all timepoints tested, with the exception of the first 15-min timepoint. The 100 pmol dose of Hoe-140 demonstrated significance (30-min: $p<<0.05$, 45-min: $p<<0.05$, 60 min: $p=0.037$) at the 30-, 45-, and 60-min timepoints. Increasing the Hoe-140 dose 10-fold to 1000 pmol resulted in no observable increase in antinociceptive response relative to the 100 pmol dose tested. In the interest of achieving a full block in dynorphin-induced tactile hypersensitivity, a 2-fold increase in dose to 2 nmol (2000 pmol) of Hoe-140 was tested, and demonstrated significance ($p<<0.05$) at all

timepoints tested. This full block is most likely the result of Hoe-140 acting as an antagonist at both the B1 and B2 receptors, in knowledge of its IC50 value of 1000 nmol/L at the B1 receptor, and 0.148 nmol/L at the B2 receptor (Fraser et al., 2003). Dyn A(2-13)[6 nmol] administration into the RVM produced its maximum nociceptive effect at 45-min with a paw withdrawal threshold (g) of 4.22 ± 1.05g. This peak pronociceptive effect was significantly (see statistics above) attenuated by the pre-administration of Hoe-140 at the 20 pmol, 100 pmol, and 2 nmol (2000 pmol) doses tested.

The B2-antagonist Hoe-140 attenuates dynorphin-induced abnormal pain

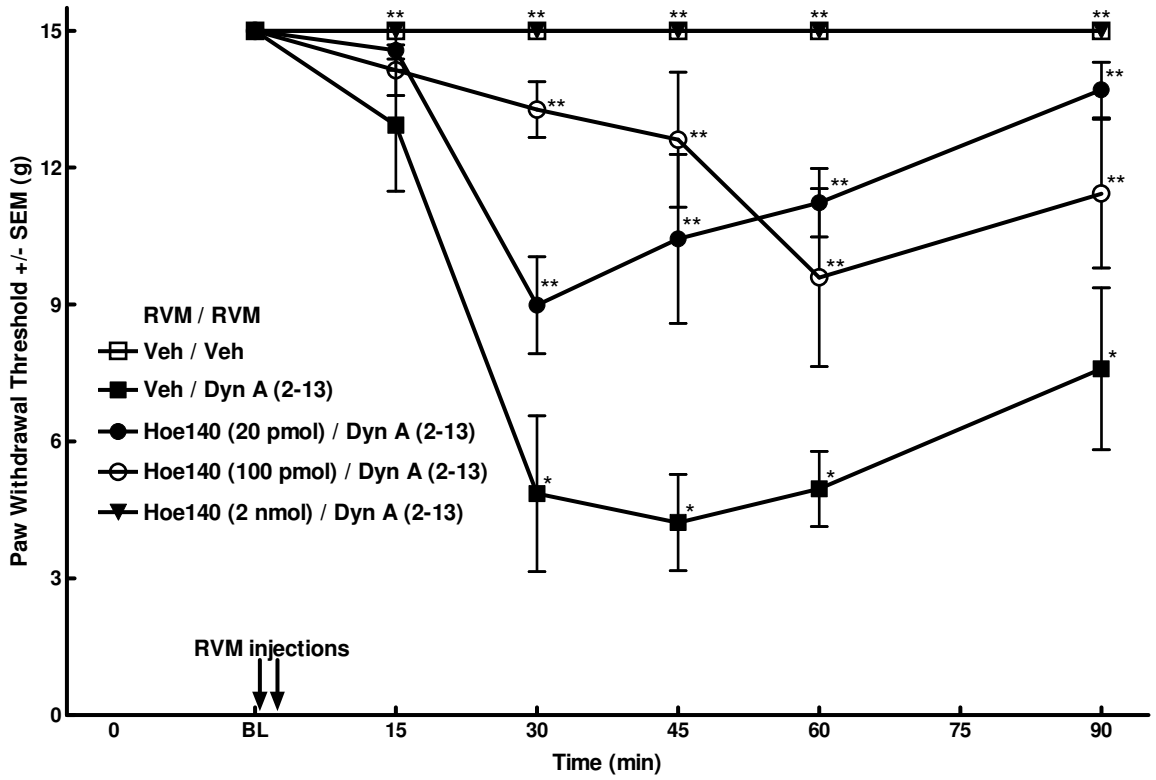

Figure 31 Male Sprague Dawley rats (250-350g) received vehicle (diH_20) or the B2-antagonist, Hoe-140 followed by dynorphin A(2-13)[6 nmol] bilaterally into the RVM. Dynorphin A(2-13) microinjected into the RVM resulted in a significant increase in tactile hypersensitivity (*p < 0.05; *n* = 8), which was significantly attenuated by the pre-administration of Hoe-140 (**p < 0.05; *n* = 5-9) into the RVM.

The B2-antagonist Hoe-140 attenuates dynorphin-induced abnormal pain

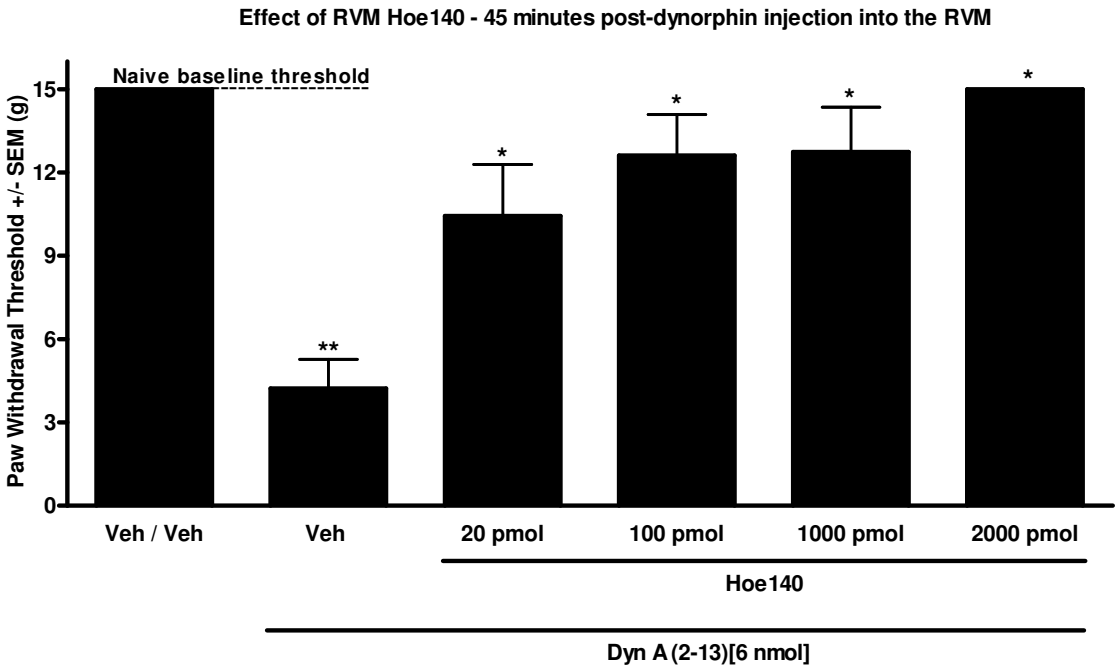

Figure 32 Male Sprague-Dawley rats (250-350g) received Hoe-140 followed by dynorphin A(2-13)[6 nmol] bilaterally into the RVM. Dynorphin A(2-13) administration into the RVM resulted in a significant increase in tactile hypersensitivity (**p < 0.05; *n* = 8), which was significantly attenuated by the pre-administration of the B2-antagonist Hoe-140 (10, 100, 1000, 2000 pmol: *p < 0.05; *n* = 5-9) into the RVM.

The B2-antagonist Hoe-140 attenuates dynorphin-induced abnormal pain

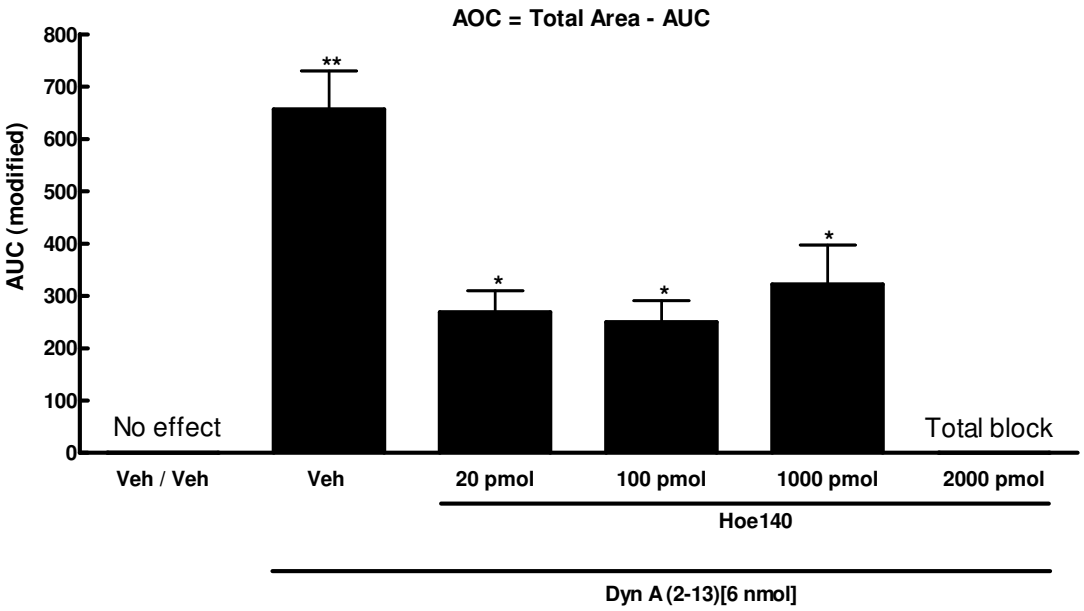

Figure 33 A modified AUC graph (AOC = Total Area – AUC) of Figure 31 data was used to confer a more intuitive graphical representation of the antagonist data, with a summary of the data across the total experimental timecourse (*p < 0.05 compared to Veh/Dyn group; **p < 0.05 compared to Veh/Veh group).

4.0 The B2-antagonist Hoe-140 reverses nerve injury-induced abnormal pain

Administration of a single, bilateral microinjection of the B2-antagonist Hoe-140 into the RVM of nerve-injured animals produced a dose-dependent reversal in SNL-induced tactile hypersensitivity (Figure 34). Nerve injury resulted in a mean post-SNL baseline paw withdrawal threshold (g) of 2.46 ± 0.287g. The 0.2 nmol (200 pmol) dose achieved significance with a paw withdrawal threshold of 7.12 ± 1.05g at the 10-min timepoint ($p=0.045$), and at the 30-min timepoint ($p=0.029$) with a paw withdrawal threshold of 4.74 ± 0.480g. The ten-fold higher dose of 2 nmol dose achieved significance ($p<<0.05$) at the 10-, 20-, 30-, and 40-minute timepoints.

4.1 Dynorphin A(1-13) anti-serum reverses nerve-injury induced abnormal pain

Administration of a single, bilateral microinjection of dynorphin A(1-13) antiserum (40 µg) into the RVM, produced a partial, but significant (Fig 35; $p=0.007$) reversal in nerve injury-induced (3.61 ± 1.15g) tactile hypersensitivity compared to the normal rabbit serum

control. Significance was achieved across the four 15-min timepoints (ie. 15- to 60-min), with antiserum affects subsiding and returning to post-SNL baseline levels by 120-min. RVM administration of normal rabbit serum, which contains a variety of nonspecific antibodies, possessed no significant ($p > 0.05$) change from post-SNL baseline thresholds.

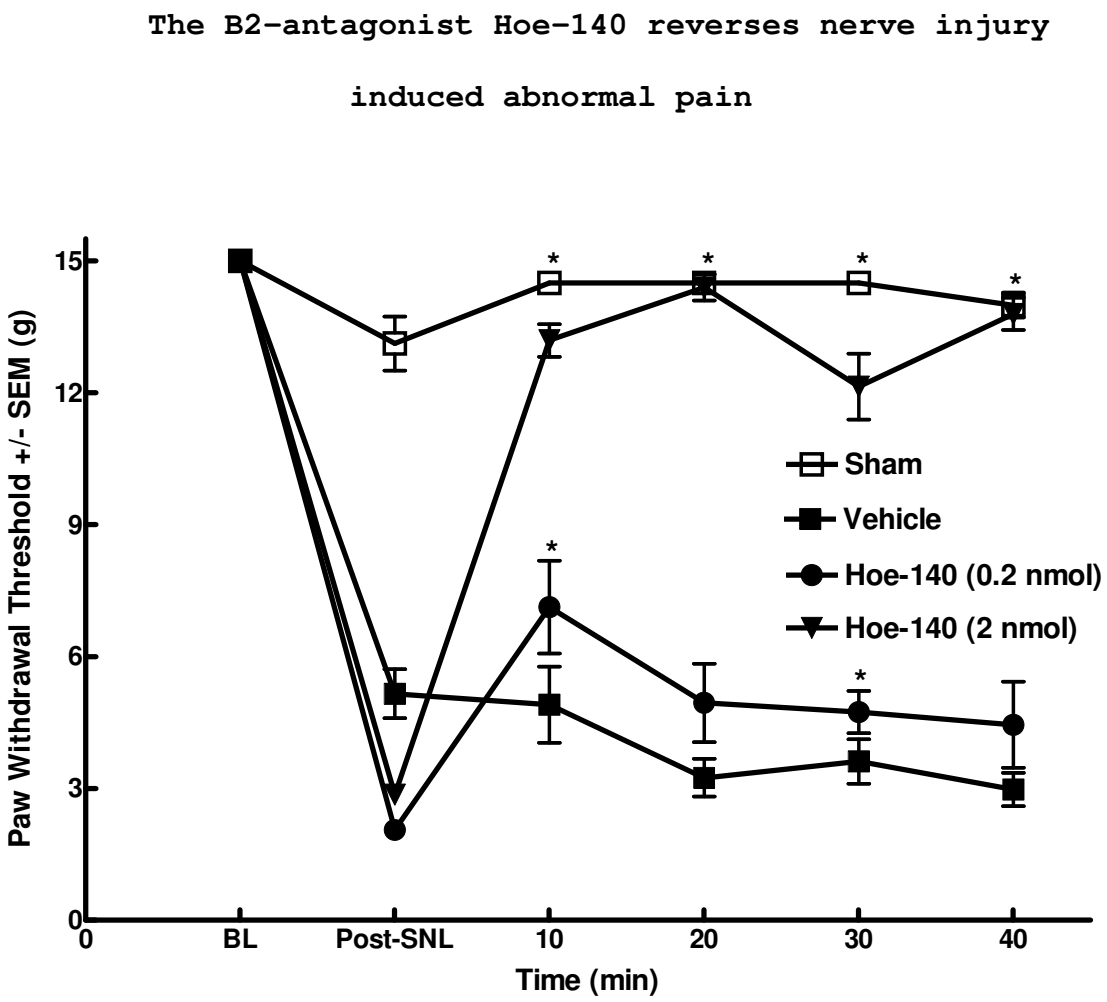

Figure 34 Nerve-injured male Sprague-Dawley rats (250-350g) received vehicle (diH_20), or the B2-antagonist, Hoe-140 (0.2 or 2 nmol) into the RVM, and then tested for tactile hypersensitivity. Hoe-140 administered bilaterally into the RVM resulted in a significant (*$p < 0.05$; $n = 5$) reversal of the nerve-injury evoked increase in tactile hypersensitivity at the 0.2 nmol (10- and 30-min timepoints) and 2 nmol dose (all timepoints). Nerve-injury induced tactile allodynia was not significantly affected by the administration of the diH_20 water control ($p > 0.05$; $n = 4$).

Dynorphin A(1–13) anti–serum administered into the RVM reverses nerve–injury induced abnormal pain

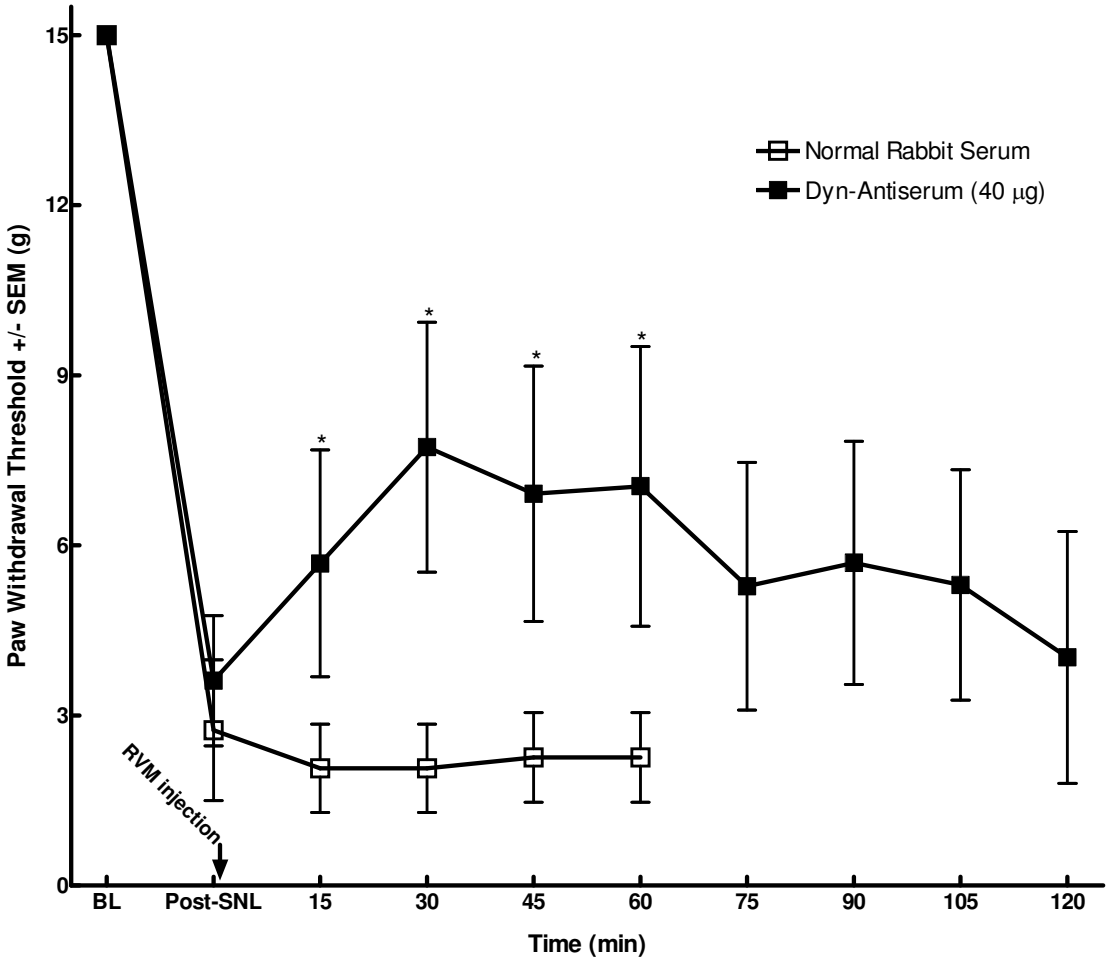

Figure 35 Nerve–injured male Sprague–Dawley rats (250–350g) received dynorphin A(1–13) antiserum (40 μg) or normal rabbit serum (control) into the RVM, and then tested for tactile hypersensitivity. Dynorphin A(1–13) antiserum administered into the RVM elicited a significant reversal (15–60 min; *$p < 0.05$, $n = 6$) of the nerve–injury evoked increase in tactile hypersensitivity. Nerve–injury induced tactile hypersensitivity was not significantly affected by the administration of normal rabbit serum ($p > 0.05$; $n = 5$).

4.2 Bradykinin microinjected into the RVM produces abnormal pain

The dose of bradykinin used in this experiment (0.28 nmol) was comparable to the dose used (0.25 nmol) by Dalmolin et al., 2007 in their study of bradykinin in the amygdala (Dalmolin et al., 2007). Bradykinin (0.28 nmol) microinjected bilaterally into the RVM produced a significant ($p=0.003$) increase in tactile hypersensitivity, with an onset of effect beginning at 30-min and lasting through the 90-min timecourse. This follows the same timecourse as another B2-agonist, dynorphin A(2-13). This was a proof of concept experiment with the sole objective to verify the presence of bradykinin receptors in the RVM that function in descending facilitation. Bradykinin produced a strong pronociceptive effect by 30-min post-RVM injection (10.1 ± 0.537g), continuing through the remainder of the timecourse, with a paw withdrawal threshold of 10.2 ± 0.498g at 90-min. Pre-administration of the B2-antagonist, Hoe-140, 10-min prior to bradykinin into the RVM significantly attenuated bradykinin-induced tactile hypersensitivity (peak effect of agonist/antagonist, 45-min; ($p=0.002$). As a basis for comparison, dynorphin A(2-

13) microinjection into the RVM, conducted in a separate experiment, was graphed alongside the bradykinin data (Figure 36).

4.3 The B2-antagonist Hoe-140 blocks bradykinin-induced abnormal pain

Bilateral microinjection of the B2-receptor antagonist Hoe-140 (2 nmol) prior to bradykinin (0.28 nmol) into the RVM resulted in a marked attenuation of bradykinin-induced tactile hypersensitivity. Significance (Figure 37; $p < 0.05$) for the Hoe-140 was achieved at 30-, 45-, and 60-min post-RVM injection of both antagonist and agonist. Vehicle (diH_2O) administration into the RVM produced no observable effect. Both bradykinin and Hoe-140 produced their maximum effect by 45-min with a paw withdrawal threshold of 9.35 ± 0.475g, and 13.1 ± 0.749g, respectively.

Bradykinin administered into the RVM induces tactile hypersensitivity

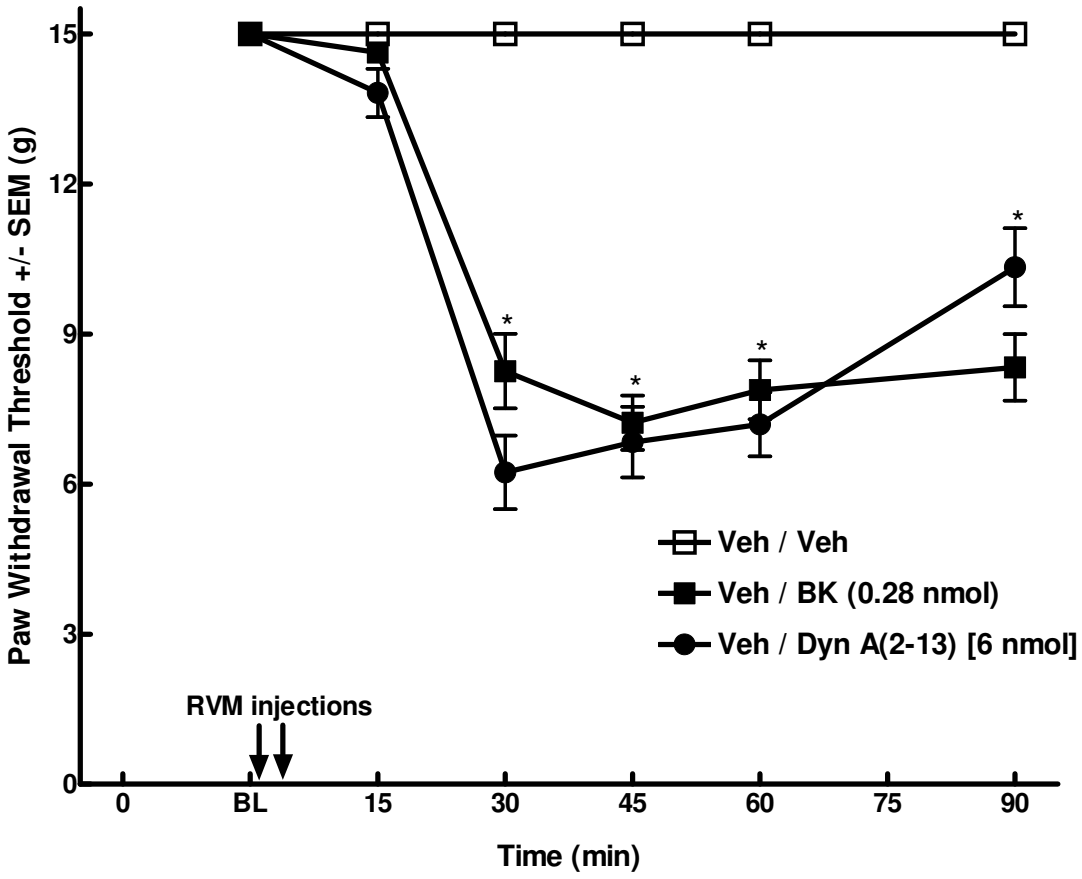

Figure 36 Male Sprague-Dawley rats (250-350g) received bradykinin (0.28 nmol), dynorphin A(2-13)[6 nmol], or vehicle (diH_20) microinjected bilaterally into the RVM, and were then tested for tactile hypersensitivity. Both bradykinin and dynorphin A(2-13) administered into the RVM resulted in a significant increase in tactile hypersensitivity (*$p < 0.05$; $n = 8$).

The B2-antagonist Hoe-140 blocks bradykinin-induced abnormal pain

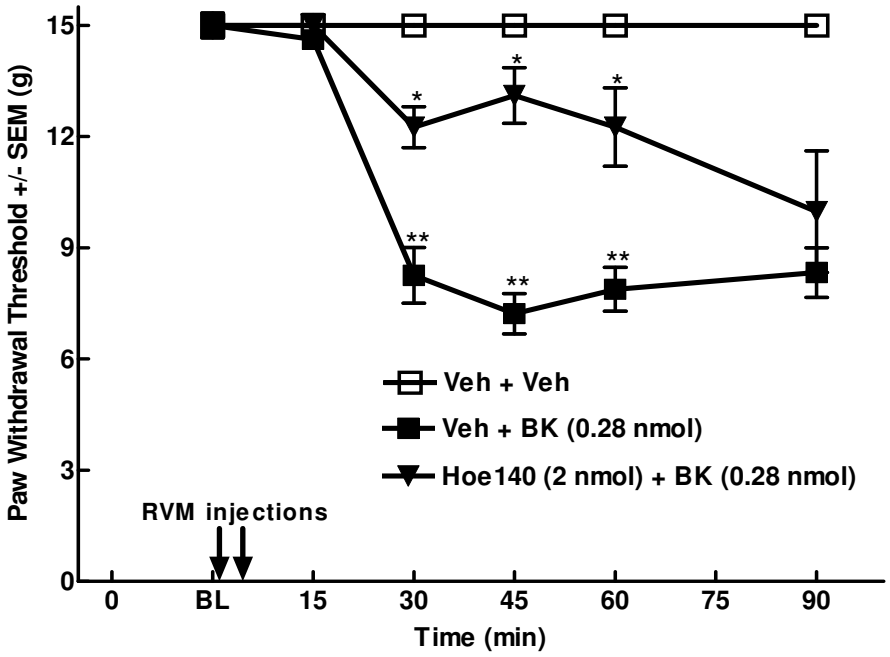

Figure 37 Acute effects of RVM, bradykinin (0.28 nmol) and the B2-antagonist Hoe-140 (2 nmol) in male Sprague-Dawley rats (250-350g), and then tested for tactile hypersensitivity. Hoe-140 microinjected bilaterally into the RVM resulted in a significant reversal (*$p < 0.05$; $n = 8$) of the bradykinin-induced (**$p < 0.05$; $n = 11$) increase in tactile hypersensitivity, which was not significantly attenuated by the administration of the diH_20 water control ($p > 0.05$; $n = 8$).

4.4 Dynorphin microinjected into the RVM produces long-lasting tactile hypersensitivity

Bilateral microinjection of dynorphin A(2-13[6 nmol] into the RVM produced a significant, long-lasting tactile hypersensitivity (Figure 36; $p<<0.05$) with its peak pronociceptive effect displayed 9-days post-RVM injection, while continuing to produce robust tactile allodynia for greater than 30-days (day 30, PWT: 7.01 ± 1.45g). A one-half lower dose (3 nmol) of dynorphin was subsequently tested, and displayed a dose-responsive behavior with peak activity and significance ($p<<0.05$) observed by day 2 (PWT: 3.85 ± 0.63), post RVM-injection and a gradual return to baseline levels starting at day 20. Comparing the two-doses at 9-days, where the high-dose (6 nmol) had its maximum effect, revealed a statistically significant difference ($p=0.013$) between the two doses (pronociceptive activity: 6 nmol > 3 nmol) tested. Vehicle (diH_2O) injection into the RVM produced an acute, although relatively minor pronociceptive effect that peaked at 240-minutes, but returned to normal naïve baseline (approx. 15 g) levels for the remainder of the testing.

Dynorphin administered into the RVM produces long-lasting tactile hypersensitivity

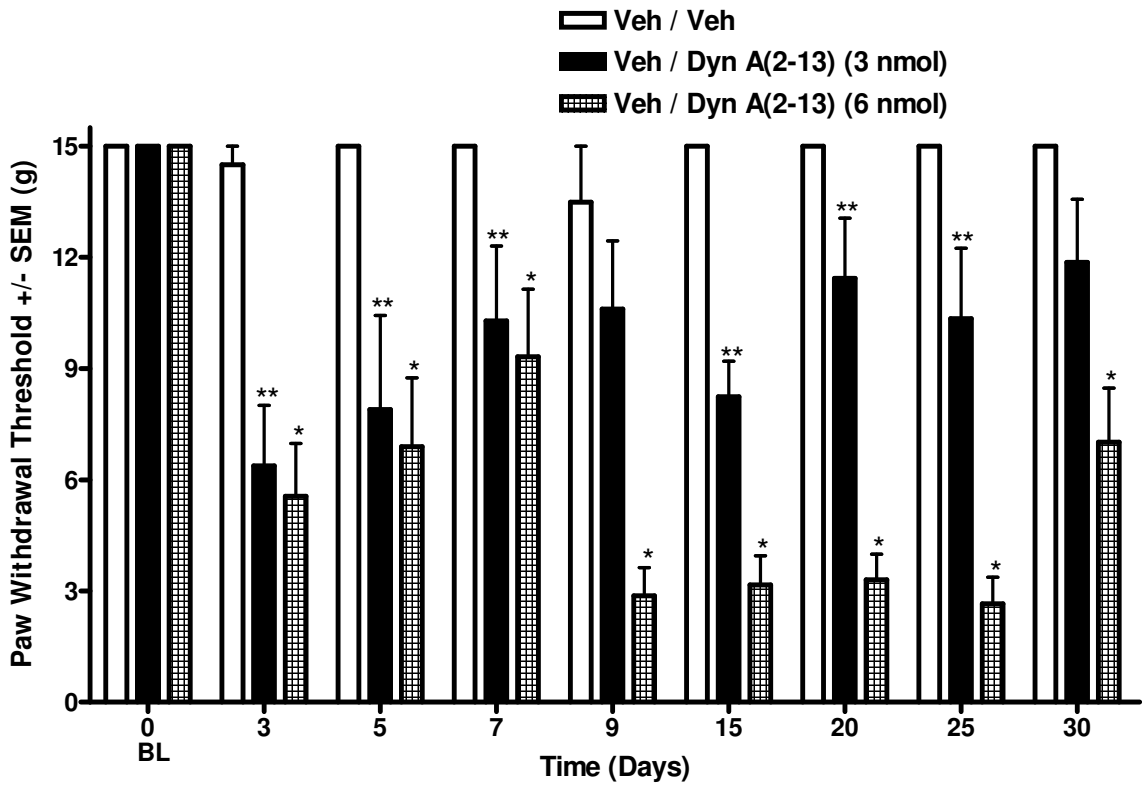

Figure 38 Male Sprague Dawley rats (250-350g) received vehicle (diH_20) or dynorphin A(2-13)[3 or 6 nmol] microinjected bilaterally into the RVM, and were tested for acute and chronic tactile hypersensitivity. Dynorphin A(2-13) administered bilaterally into the RVM resulted in a dose-dependent, long-lasting tactile hypersensitivity ($p < 0.05$; $n = 5-7$), which persisted for more than 30 days.

CHAPTER FOUR

Discussion

In the first part of this two-part study, pharmacological and behavioral models were used to further elucidate the role of CCK in the RVM, a key pain-modulatory region of the brainstem, and identify what neurotransmitters (ie. 5-HT, PGE_2) at the level of the spinal cord mediate this descending influence. In the second part, the role of the pronociceptive peptide dynorphin was investigated in the RVM, in conjunction with the identification of receptor(s) involved in mediating its effects to the spinal cord to promote abnormal pain. The CCK study will first be addressed, followed by the dynorphin study, and then the overall significance and findings of the two will be discussed.

Recent studies have demonstrated the role of RVM CCK in promoting neuropathic pain (Heinricher and Neubert, 2004), opioid-induced tactile and thermal hypersensitivity, and opioid antinociceptive tolerance (Xie et al., 2005). As mentioned previously, two pain-modulatory cell types have

been identified in the RVM: two of which, the ON- and OFF-cells are believed to function directly in descending pain modulation and possess facilitatory and inhibitory activity, respectively. CCK microinjection into the RVM has been shown to promote ON-cell activation that coincides with behaviorally measurable features such as thermal hyperalgesia, suggesting that CCK activity in the RVM may drive descending pain facilitation (Heinricher and Neubert, 2004). Conversely, it has been shown that OFF-cell activation ensuing in antinociception and enhanced analgesia can be evoked by opioid peptides and blocked by microinjection of the "anti-opioid" CCK into the RVM (Heinricher et al., 1999; Neubert et al., 2004). Recent studies have also demonstrated that CCK_2 receptor antagonists can enhance opioid analgesia in humans (McCleane, 1998, 2000, 2003).

In a recent study in our group, we found that in spinal nerve ligation injury, RVM CCK is increased more than 200% over baseline controls (Vanderah Lab, unpublished obervation. This supports previous observations, showing a reversal in both tactile and thermal hypersensitivity in nerve-injured rats with the use of the CCK_2 antagonist, L365,260 microinjected into the RVM (Xie et al., 2005;

Kovelowski et al., 2000). More importantly, studies have shown that exogenous administration of CCK into the RVM of naïve rats closely mimics the nerve-injured state, eliciting the same hypersensitivity of the hindpaws seen in nerve-injury, and that by blocking the CCK_2 receptor, the abnormal pain can be attenuated (Xie et al., 2005). In total, these data support the idea that CCK_2 receptor activation in the RVM plays a major role in promoting descending facilitation and abnormal pain.

RVM facilitated descending modulation has been previously characterized as playing an important role in spinal nociception (Fields et al., 1983; Fields and Heinricher, 1985; Morgan and Fields, 1994; Friedrich and Gebhart, 2003) and is mediated via the DLF by bulbospinal projections (Zhou and Gebhart, 1992; Urban et al., 1996; Urban and Gebhart, 1997; Fields and Basbaum, 1999). Descending pain modulatory regions of the brainstem have also been shown to be important in both the manifestation and maintenance of certain chronic pain states (Ossipov et al., 2000; Millan, 2002). Experimentally produced neuropathic pain can be reversed by lidocaine microinjection into the RVM, which offers further support to the critical role that descending facilitation from the

RVM plays in this type of pain (Pertovaara et al., 1996; Kovelowski et al., 2000).

Concerning serotonin's effects at the 5-HT_3 receptor, recent studies have demonstrated that 5-HT_3 antagonists such as ondansetron, possess significant antinociceptive (Suzuki et al., 2004) and clinically significant pain-relieving properties as well (McCleane et al., 2003). A clinical study by Ambesh and colleagues found that ondansetron pretreatment significantly reduced the pain of propofol injection used to induce surgical anesthesia (Ambesh et al., 1997).

In our present study, we by-passed the blood-brain-barrier and delivered ondansetron directly to its peripherally modulating confirmed site of action in the spinal cord (Zeitz et al, 2002; Maxwell et al., 2003), following a pronociceptive dose of CCK into the RVM, and observed a significant attenuation in the CCK-induced tactile hypersensitivity response in naïve rats. These data suggest that when CCK is elevated in the RVM, 5-HT is being released spinally and is acting at the 5-HT_3 receptor in the spinal cord to promote abnormal pain. Blocking activation of the 5-HT_3 receptor by ondansetron, attenuated this abnormal pain. For the purposes of our study, this

offers further support that CCK in the RVM, acting through the excitatory CCK_2 receptor, activates descending facilitatory pathways leading to the release of 5-HT in the spinal cord. Although, we were unable to detect a change in levels of 5-HT in the spinal CSF with our detection method, in light of ondansetron's antinociceptive activity when given by intrathecal injection, it appears that 5-HT is being released spinally and that a significant portion of RVM CCK-induced pronociceptive activity is mediated through the activation of spinal $5\text{-}HT_3$ receptors by, an as of yet, not fully characterized mechanism.

Addressing our HPLC-Fluorometric detection method that was utilized for 5-HT/5-HIAA quantitation, a possible explanation as to why we were unable to measure a change in 5-HT levels in the spinal cord, is that our method was simply not sufficiently sensitive in its ability to detect 5-HT, as all of our quantitation was based on the metabolite 5-HIAA, and not 5-HT itself. If there were femtogram to picogram levels of 5-HT remaining in the dialysate that we were simply unable to detect, that would constitute one explanation as to why we didn't see a significant change in 5-HT levels following CCK injection in the RVM. It may be that levels of the metabolite 5-HIAA

formed in the spinal CSF are not entirely representative of 5-HT levels acting within the spinal cord.

Another possibility is that 5-HT is not up-regulated to a significant extent in the spinal CSF following elevated levels of CCK into the RVM, and that measuring levels in the spinal subarachnoid space may not be indicative of actual levels of 5-HT acting within the spinal cord to promote 5-HT_3 receptor activation.

Our current study in concert with previous findings further substantiates the role of the RVM and supraspinal mechanisms in the expression of peripheral nociception, and the presence of both a strong 5-HT_3 and prostanoid mediated component in the lumbar spinal cord. The data suggests that RVM CCK-evoked release of PGE_2 and possibly 5-HT in the spinal cord may be the primary mechanism whereby the RVM exerts its pronociceptive effects to the periphery. An approximate 500% increase in PGE_2 in the spinal CSF, and a non-detectable change in 5-HT levels in the spinal CSF was observed after CCK microinjection into the RVM, with the resulting tactile hypersensitivity abolished with the intrathecal injection of either the non-selective COX inhibitor naproxen, or the 5-HT_3 receptor antagonist ondansetron. With spinal ondansetron demonstrating

efficacy in blocking RVM CCK tactile hypersensitivity, it is not known as to the origin of the 5-HT that is apparently working spinally to activate spinal 5-HT_3 receptors. In view of the data, it appears that our analytical method was perhaps unable to sufficiently detect the apparently small yet profound changes in 5-HT levels occurring within the spinal cord following CCK injection into the RVM. In summary, our data suggests that RVM CCK mediates its pronociceptive effects, in part through enhanced prostaglandin release, and through a yet to be elucidated activation of 5-HT_3 receptors and the associated pharmacologically apparent release of 5-HT at the level of the spinal cord.

In the second part of this study, we used pharmacological and behavioral models to determine the role of dynorphin in the RVM. From previous studies, it is known that elevated spinal dynorphin is associated with states of chronic inflammation and peripheral nerve injury bearing physical manifestations of abnormal pain such as allodynia, hyperalgesia, and spontaneous pain. Increased spinal expression of dynorphin is pronociceptive and appears to promote facilitated pain states (Kajander et

al., 1990; Draisci et al., 1991; Dubner and Ruda, 1992). In addition, sustained opioid administration, either systemically or spinally, leads to the development of tolerance and abnormal pain, in conjunction with elevations of spinal dynorphin content (Vanderah et al., 2001a, 2001b; Gardell et al., 2002). Bilateral lesions of the DLF have been shown to prevent this up-regulation of spinal dynorphin along with blocking the development of abnormal pain and antinociceptive tolerance to morphine (Gardell 2003).

In previous studies in our group, we have shown that a single injection of dynorphin A and associated non-opioid fragments administered spinally produce long-lasting (> 70 days) tactile allodynia (Vanderah et al., 1996b; Laughlin et al., 1997), and can thereby simulate and create a state of chronic neuropathic pain. This aptly demonstrates the role descending facilitation plays in spinal dynorphin release - by effectively disconnecting bulbospinal tracts from the RVM (ie. DLF lesion), the up-regulation of dynorphin is prevented and the corresponding abnormal pain state is likewise averted.

In our present study, we observed long-lasting (> 30-days) tactile allodynia from a single, bilateral injection of dynorphin A(2-13) into the RVM. Two doses of dynorphin A(2-13) were tested, and found to display a dose-responsive effect on the tactile hypersensitivity induced. The lower of the two doses (3 nmol) of dynorphin A(2-13) displayed peak pronociceptive activity (PWT: 7.01g ± 1.45) on day 2 post-injection into the RVM, and began a gradual reversal in effect (ie. progressing toward naïve baseline levels) by day 20 (PWT: 10.6 ± 2.17) onward. In contrast, the 6 nmol dose displayed a more pronounced peak effect, manifesting on day 9 (PWT: 2.87g ± 0.756) and continuing through day 30 (PWT: 7.01g ± 1.45) post-injection into the RVM. Neuronal excitoxicity and tissue necrosis at the site of injection in the RVM and possibly the spinal cord as well, are likely contributing factors to the long-term dynorphin-induced tactile hypersensitivity observed.

In an effort to determine the receptor(s) mediating dynorphin's effect in the RVM, and on to the periphery, antagonists targeted against the bradykinin, B1- and B2-receptors were employed. RVM injection of dynorphin A(2-13) produced significant tactile hypersensitivity, which

was attenuated with pre-administration (RVM) of the B1-(DALBK), and B2-antagonist, Hoe-140. Administering dynorphin A(1-13) antiserum into the RVM reversed nerve-injury induced tactile hypersensitivity, offering evidence for the up-regulaton of dynorphin in the RVM following nerve-injury (SNL). This pharmacologically apparent increase in dynorphin observed in nerve-injury is in conjunction with RT-PCR data showing a significant increase in prodynorphin, B_1- and B_2-receptor mRNA in the RVM following nerve-injury. In total, these data strongly support the hypothesis that dynorphin is increased in the RVM in nerve-injury, and through bradykinin receptor activation, promotes descending facilitation in this key pain-modulatory region of the brainstem.

Previous studies in our group have documented the intrathecal effects of bradykinin injections, which consist of a highly transient (less than 30 sec) but immediate anxiogenic effect, without any measurable increase in either tactile or thermal sensitivity (Lai et al., 2006). In contrast, in the present study we observed that bradykinin microinjection in the RVM results in a pronounced increase in tactile hypersensitivity, and

interestingly enough, follows the same timecourse of effect (ie. onset of activity at 30 min post-RVM injection) as dynorphin A(2-13) into the RVM, as one might predict given that the B2-receptor largely mediates the pro-inflammatory, pronociceptive activities of both bradykinin and the newly discovered dynorphin B2-receptor interaction. Injecting bradykinin into the RVM was a proof of concept experiment, and provided further verification for the presence of bradykinin receptors, and their pronociceptive role in mediating ligand (ie. dynorphin) effects in this pain modulatory region.

Peripheral nerve injury-induced abnormal pain states are associated with increased spinal dynorphin levels, and treatments or procedures that prevent (ie. DLF lesions) or antagonize (ie. anti-serum or antagonists) elevated dynorphin content or activity also abolish this abnormal pain (Malan et al., 2000; Porreca et al., 2001; Wang et al., 2001; Burgess et al., 2002; Gardell et al., 2003). The exact mechanism through which increased spinal dynorphin produces abnormal pain and opioid antinociceptive tolerance is still being investigated, though three contributing mechanisms have been strongly implicated by

the present data in playing a strong role in dynorphin-induced allodynia, excitoxicity, and central sensitization.

Addressing the first of these proposed mechanisms, it is known that elevated levels of spinal dynorphin promote excitatory neurotransmitter release from primary afferent terminals, and acting in the manner of a positive feedback loop, amplifies further sensory input. Exogenous dynorphin administered into the spinal cord and hippocampus has been shown to elicit localized, dose-dependent release of glutamate and aspartate as measured via microdialysis collection (Faden 1992; Skilling et al., 1992), an effect which could lead to excitotoxicity. This is consistent with previous observations that dynorphin A increases capsaicin-evoked, substance P release from primary afferent C-fibers (Arcaya et al., 1999), and potentiates capsaicin-evoked release of calcitonin gene-related peptide (CGRP) in spinal cord slices in vitro (Claude et al., 1999; Gardell et al., 2002, 2003) - both supporting a presynaptic mechanism for dynorphin-induced abnormal pain.

Secondly, ischemia may result from dynorphin-induced decreases in blood flow (Long et al., 1987; Thornhill et

al., 1989). Transient ischemia on its own has been shown to induce allodynia in rats (Hao et al., 1991). Furthermore, the *in vitro* application of dynorphin to cultured spinal cord neurons was absent of the cell death observed *in vivo* (Long et al., 1994), implicating a reduction in blood flow induced by dynorphin, as the mechanism of its neurotoxicity. Administration of dynorphin with the vasodilatory drug, hydralazine, resulted in the inhibition of dynorphin-induced paralysis (Long et al, 1994). It is known that ischemia results in the increased production of lactic acid and free radicals along with an increased release of fatty acids and excitatory amino acids, together with the functional collapse of the blood-brain barrier, these effects can ultimately lead to cell death.

Lastly, dynorphin-induced paralysis is linked with an increase in nicotinamide adenine dinucleotide phosphate (NADPH-diphorase), a biochemical marker for nitric oxide producing cells, suggesting that nitric oxide levels in the spinal cord can be increased by dynorphin (Hu et al., 1996).

Recent *in vitro* binding studies in our group have demonstrated that dynorphin A binds with moderate, micromolar affinity (K_D: μM) to bradykinin receptors, and may produce its excitatory effects via calcium influx through the activation of L-type and P/Q type voltage-sensitive calcium channels (VSCC) by a PKA-dependent pathway (Lai et al., 2006).

In this dissertation, I have demonstrated the following:

Part I:

1) CCK-8(s) microinjection into the RVM increases PGE_2 levels in the spinal cord, suggesting CCK produces its pronociceptive effects in the periphery partly through the spinal release of PGE_2.
2) CCK-8(s) microinjection into the RVM evokes tactile hypersensitivity, which is attenuated by the RVM administration of the CCK_2 receptor antagonist YM022, providing behavioral data to coincide with the increase in PGE_2 levels observed while utilizing *in vivo* microdialysis under the same experimental conditions.

3) CCK-8(s)-induced tactile hypersensitivity is attenutated by intrathecal administration of the non-selective COX inhibitor naproxen, providing further support that RVM CCK promotes prostaglandin release.
4) CCK-8(s)-induced tactile hypersensitivity is attenuated by intrathecal administration of the 5-HT_3 antagonist ondansetron, suggesting spinal 5-HT levels are evoked by RVM CCK.

Part II:

5) Prodynorphin is significantly increased in the RVM 14-days post nerve-injury. B1-receptor mRNA is significantly increased 2-days post nerve-injury. B2-receptor mRNA is increased 14-days post nerve injury.
6) Dynophin A(2-13) injection into the RVM evokes significant tactile hypersensitivity by 30-min post-injection, with a strong duration lasting through 60-min, suggesting RVM dynorphin evokes abnormal pain in the periphery.
7) Dynorphin A(2-13)-induced tactile hypersensitivity is significantly attenuated by the B1-antagonist DALBK,

suggesting an RVM B1-receptor mediated contribution in peripheral pain.

8) Dynorphin A(2-13)-induced tactile hypersensitivity is significantly attenuated by the B2-antagonist, Hoe-140, suggesting an RVM B2-receptor mediated contribution in peripheral pain.

9) Dynorphin A(2-13)[3 or 6 nmol] microinjection into the RVM evokes long-lasting (> 30 days), dose-dependent tactile hypersensitivity, suggesting the presence of tissue excitotoxicity at the site of injection in the RVM and possibly spinal cord.

10) Dynorphin A(1-13) antiserum microinjection into the RVM attenuates nerve-injury evoked tactile hypersensitivity, suggesting dynorphin is increased in the RVM in nerve-injury.

11) The B2-antagonist Hoe-140 dose-dependently reverses nerve-injury induced tactile hypersensitivity, suggesting an RVM B2-receptor mediated contribution in neuropathic pain.

12) Bradykinin microinjection into the RVM evokes tactile hypersensitivity, which is attenuated by the B2-antagonist Hoe-140, providing further confirmation of

peripheral nociception mediated through bradykinin receptors in the RVM.

In summary, the data presented herein strongly suggest that dynorphin and CCK (Vanderah Lab, unpublished observations) are elevated in the RVM following nerve-injury, and their pronociceptive activity mediated through bradykinin receptor and CCK_2 receptor activation, respectively. Furthermore, these data suggest that dynorphin activity in the RVM, and the associated bradykinin receptor activation, is responsible for the maintenance of long-lasting pain states in the periphery. This study provides further support to previous studies documenting dynorphin's role spinally (Laughlin et al., 1997; Vanderah et al., 2000) as a pain-promoting excitatory neuropeptide, now with strong evidence demonstrating dynorphin's pain-promoting activity in the RVM, and in dynorphin-driven descending facilitation.

Concerning the clinical significance of the data presented herein, these data suggest the development of a bradykinin or CCK_2 receptor antagonist that is able to gain access to the RVM as a potential therapy for the treatment

of neuropathic pain. Blocking the pronociceptive effects of CCK in the RVM with a CCK_2 receptor antagonist, along with the inherent anxiolytic activity of this class of drugs, could be employed to complement current pain therapies. Bradykinin receptor antagonists, in particular, could be implemented as single or adjunctive therapy to opioid treatments used in pain, to counter the pronociceptive effects of increased dynorphin seen with chronic opioid use.

Possible future studies involving dynorphin in the RVM could investigate the relationship of dynorphin to CCK in the RVM. One study could investigate whether dynorphin injection into the RVM increases endogenous CCK levels, and whether RVM dynorphin increases dynorphin in the spinal cord. In another study, one could administer a CCK_2 receptor antagonist (ie. YM022) into the RVM followed by dynorphin, and determine if that blocks dynorphin-induced abnormal pain in the periphery. And lastly, utilizing this same design, one could inject a bradykinin receptor antagonist such as Hoe-140 or DALBK into the RVM followed by CCK, and see if this antagonism blocks CCK-induced abnormal pain in the periphery.

CHAPTER FIVE

References

1. Aimone LD, and Gebhart GF. Stimulation-produced spinal inhibition from the midbrain in the rat is mediated by an excitatory amino acid neurotransmitter in the medial medulla. *J Neurosci.* 1986; 66:1803-1813.
2. Albrecht D, Zippel U, and Henklein P. Inhibitory effects of cholecystokinin on rat's geniculate activity can be blocked by GABA-antagonists. *Neurosci Lett.* 1995; 197:69-71.
3. Aley KO, Reichling DB, and Levine JD. Vincristine hyperalgesia in the rat: a model of painful vincristine neuropathy in humans. *Neuroscience*. 1996; 73:259-265.
4. The American Heritage Stedman's Medical Dictionary, 2nd Edition, Houghton Mifflin, 2004.
5. American Pain Society. Principles of analgesics use in the treatment of acute pain and cancer pain (4th ed.), 1999, Glenview, IL: Author.

6. Appleby SB, Ristimaki A, Neilson K, Narko K, and Hla T. Structure of the human cyclo-oxygenase-2 gene. *Biochem J. 1994;* 302:723-727.

7. Arcaya JL, Cano G, Gómez G, Maixner W, and Suárez-Roca H. Dynorphin A increases substance P release from trigeminal primary afferent C-fibers. *Eur J Pharmacol*. 1999 Jan 29; 366(1):27-34.

8. Authier N, Coudore F, Eschalier A, and Fialip J. Pain related behaviour during vincristine-induced neuropathy in rats. *NeuroReport*. 1999; 10:965-968.

9. Authier N, Gillet JP, Fialip J, Eschalier A, and Coudore F. Description of a short-term taxol-induced nociceptive neuropathy in rats. *Brain Res*. 2000; 887:239-249.

10. Authier N, Gillet JP, Fialip J, Eschalier A, and Coudore F. A new animal model of vincristine-induced nociceptive peripheral neuropathy. *Neurotoxicology*. 2003; 24:797-805.

11. Barnes NM, and Sharp T. A review of central 5-HT receptors and their function. *Neuropharmacology*. 1999; 38:1083-1152.

12. Baron R. Peripheral neuropathic pain: from mechanisms to symptoms. *Clin J Pain*. 2000; 16(2 Suppl):S12-20.

13. Basbaum AI, and Fields HL. Endogenous pain control systems: Brainstem spinal pathways and endorphin circuitry. *Annu Rev Neurosci*. 1984; 7:309-338.

14. Basbaum AI, and Fields HL. The origin of descending pathways in the dorsolateral funiculus of the spinal cord of the cat and rat: further studies on the anatomy of pain modulation. *J Comp Neurol.* 1979 Oct 1; 187(3):513-31.

15. Baumgarten HG, and Gothert M. Serotoninergic Neurons and 5-HT Receptors in the CNS Handbook of Experimental Pharmacology, 1997, vol 129, Springer, Berlin.

16. Beaumont A, and Hughes J. Biology of opioid peptides. *Annu Rev Pharmacol Toxicol*. 1979; 19:245-67.

17. Bhoola KD, Figueroa CD, and Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev*. 1992; 44:1-80.

18. Bian D, Ossipov MH, Zhong C, Malan TP Jr, and Porreca F. Tactile allodynia, but not thermal hyperalgesia, of the hindlimbs is blocked by spinal transection in rats with nerve injury. *Neurosci Lett*. 1998 Jan 30; 241(2-3):79-82.

19. Burch RM, and Axelrod J. Dissociation of bradykin in stimulated arachidonic acid and release from inositol phosphate formation in Swiss 3T3 fibroblasts. Evidence for G-protein regulation of phospholiphase A2. *Proc Natl Acad Sci USA*. 1987; 84:6374-6378.

20. Burgess SE, Gardell LR, Ossipov MH, Malan TP Jr, Vanderah TW, Lai J, and Porreca F. Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. *J Neurosci*. 2002 Jun 15; 22(12):5129-36.

21. Ahmadi S, Lippross S, Neuhuber WL, and Zeilhofer HU. PGE_2 selectively blocks inhibitory glycinergic neurotransmission onto rat superficial dorsal horn neurons. *Nat Neurosci*. 2002 Jan; 5(1):34-40.

22. Bederson JB, Fields HL, and Barbaro NM. Hyperalgesia during naloxone-precipitated withdrawal from morphine is associated with increased on-cell

activity in the rostral ventromedial medulla. *Somatosen Mot Res.* 1990; 7:185–203.

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

24. Beinfeld MC. CCK mRNA expression, pro-CCK processing, and regulated secretion of immunoreactive CCK peptides by rat insulinoma (RIN 5F) and mouse pituitary tumor (AtT-20) cells in culture. *Neuropeptides* 1992; 22:213–217.

25. Beitz AJ. The sites of origin brain stem neurotensin and serotonin projections to the rodent nucleus raphe magnus. *J Neurosci*. 1982 Jul; 2(7):829–4.

26. Bennett GJ, and Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain*. 1988; 3:33–107.

27. Boden PR, and Woodruff GN. Benzodiazepine/cholecystokinin interactions at

functional CCK receptors in rat brain. *Br J Pharmacol*. 1994; 112:429-434.

28. Bockaert J, Claeysen S, Becamel C, Dumuis A, and Marin P. Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. *Cell Tissue Res.* 2006; **326**:353-372.

29. Bowsher D. Central pain: clinical and physiological characteristics. *J Neurol Neurosurg Psychiatry*. 1996 Jul; 61(1):62-69.

30. Bradwejn J, and deMontigny C. Benzodiazepines antagonize cholecystokinin-induced activation of rat hippocampal neurones. *Nature*. 1984; 321:363-364.

31. Brodin E, Lindefors N, and Ungerstedt U. Potassium evoked in vivo release of substance P in rat caudate nucleus measured using a new technique of brain dialysis and an improved substance P-radioimmunoassay. *Acta Physiol Scand Suppl.* 1983; 515:17-20.

32. Burchiel K. Spontaneous impulse generation in normal and denervated dorsal root ganglia: sensitivity to alpha-adrenergic stimulation and hypoxia. *Exp Neurol.* 1984; 85:257-272.

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

34. Calejesan AA, Kim SJ, and Zhuo M. Descending facilitatory modulation of a behavioral nociceptive response by stimulation in the adult rat anterior cingulate cortex. *Eur J Pain*. 2000; 4:83-96.

35. Cameron AA, Cliffer KD, and Dougherty PM. Changes in lectin, GAP-43 and neuropeptide staining in the rat dorsal horn following experimental peripheral neuropathy. *Neurosci Lett.* 1991; 131:249-252.

36. Carter GT, and Galer BS. Advances in the management of neuropathic pain. *Phys Med Rehabil Clin N Am.* 2001; 12:447-459.

37. Chao J, Chao L, Christopher C, Swain CC, Tsai J, and Margolius HS. Tissue kallikrein in rat brain and pituitary: regional distribution and estrogen induction in the anterior pituitary, *Endocrinology*. 1987; 120:475-482.

38. Chaplan SR, and FW Bach. Quantitative assessment of tactile allodynia in the rat paw. *Journal of Neuroscience Methods*. 1994; 53(1):55-63.

39. Chavkin C, and James IF, Goldstein A. Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science*. 1982 Jan 22; 215(4531):413-5.

40. Chen L, and Y Gu. The mechanism of action for the block of NMDA receptor channels by the opioid peptide dynorphin. *J Neurosci*. 1995; 15(6):4602-4611.

41. Chen L, and Y Gu. The opioid peptide dynorphin directly blocks NMDA receptor channels in the rat. *J Physiol* (Lond). 1995; 482(3):575-581.

42. Chou JZ, Chait BT, Wang R, and Kreek MJ. Differential biotransformation of dynorphin A(1-17) and dynorphin A (1-13) peptides in human blood, ex vivo. *Peptides*. 1996; 17:983-990.

43. Corbett AD, Paterson SJ, McKnight AT, Magnan J, and Kosterlitz HW. Dynorphin and dynorphin are ligands for the kappa-subtype of opiate receptor. *Nature*. 1982 Sep 2; 299(5878):79-81. Chung JM. Ectopic discharges and adrenergic sensitivity of sensory neurons after spinal nerve injury. *Brain Res*. 1999; 849:244-247.

44. Chung JM, and Chung K. Importance of Hyperexcitability of DRG Neurons in Neuropathic Pain. *Pain Practice.* 2002; 2(2):87-97.

45. Claude P, Gracia N, Wagner L, and Hargreaves KM. Effect of dynorphin on ICGRP release from capsaicin-sensitive fibers. *International Association for the Study of Pain. 9th World Congress Abstracts*, 1999, p. 262.

46. Dahlstrom A, and Fuxe K. Evidence for the existence of monoamine neurons in the central nervous system. II. Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems. *Neurosci. Lett.* 27:335-339.

47. Deblois D, Bouthillier J, and Marceau F. Pharmacological modulation of the upregulated responses to desArg9 bradykinin in vivo and in vitro. *Immunopharmacology*. 1989; 17:187-198.

48. Decosterd I, and Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain.* 2000; 87(2):149-58.

49. Devor M. Potassium channels moderate ectopic excitability of nerve end neuromas in rats. *Neurosci Lett*. 1983; 40:181-186.

50. Devor M, Wall PD, and Catalan N. Systemic lidocaine silences ectopic neuroma and DRG discharge without blocking nerve conduction. *Pain*. 1992; 48:261–268.

51. Devor M. The pathophysiology of damaged peripheral nerve. In: Wall PD, Melzack R (eds). *Textbook of Pain.* New York, NY: Churchill Livingstone; 1994: 79–100.

52. Devor M, and Seltzer Z. Pathophysiology of damaged nerves in relation to chronic pain. In: *Textbook of Pain* (4th ed.), edited by Wall PD, and Melzack R. London: Churchill Livingston, 1999, p. 129–164.

53. Draisci G, Kajander KC, Dubner R, Bennett GJ, and Iadarola MJ. Up–regulation of opioid gene expression in spinal cord evoked by experimental nerve injuries and inflammation. *Brain Res*. 1991; 560:186–192.

54. Drube S, and Liebmann C. In various tumour cell lines the peptide bradykinin B_2 receptor antagonist, Hoe 140 (Icatibant), may act as mitogenic agonist. *Br J Pharmacol*. 2000; 131:1553–1560.

55. Dubner R, and Ruda MA. Activity-dependent neuronal plasticity following tissue injury and inflammation. *Trends Neurosci*. 1992; 15:96-103.

56. Dworkin RH. An overview of neuropathic pain: syndromes, symptoms, signs and several mechanisms. *Clin J Pain*. 2002; 18:343-349.

57. Ehrich EW, Dallob A, De Lepeleire I, Van Hecken A, Riendeau D, Yuan W, Porras A, Wittreich J, Seibold JR, De Schepper P, Mehlisch DR, and Gertz BJ. Characterization of rofecoxib as a cyclooxygenase-2 isoform inhibitor and demonstration of analgesia in the dental pain model. *Clin Pharmacol Ther.* 1999 Mar; 65(3):336-47.

58. Faden, AI. Opioid and nonopioid mechanisms may contribute to dynorphin's pathophysiological actions in spinal cord injury. *Ann Neurol*. 1990; 27:67-74.

59. Faden AI. Dynorphin increases extracellular levels of excitatory amino acids in the brain through a non-opioid mechanism. *J Neurosci*. 1992; 12:425-429.

60. Fallon JH, and Leslie FM. Distribution of dynorphin and enkephalin peptides in the rat brain. *J Comp Neurol.* 1986 Jul 15; 249(3):293-336.

61. Feldman M, and McMahon AT. Do cyclooxygenase-2 inhibitors provide benefits similar to those of traditional nonsteroidal anti-inflammatory drugs, with less gastrointestinal toxicity? *Ann Intern Med.* 2000; 132(2):134-43.

62. Feletou M, Germain M, Thurieau C, Fauchere JL, and Canet E. Agonistic and antagonistic properties of the bradykinin B_2 receptor antagonist, Hoe 140, in isolated blood vessels from different species. *Br J Pharmacol*. 1994; 112:683-689.

63. Fields HL, and Rowbotham M. Multiple mechanisms of neuropathic pain: a clinical perspective. In processing of the 7^{th} world congress on pain. Gebhart G, Hammond D, and Jensen T, eds. (seattle: IASP), pp. 437-454.

64. Fields HL, Bry J, Hentall I, and Zorman G. The activity of neurons in the rostral medulla of the rat during withdrawal from noxious heat. *J Neurosci.* 1983; 3:2545-2552.

65. Fields HL, Vanegas H, Hentall ID, and Zorman G. Evidence that disinhibition of brain stem neurones contributes to morphine analgesia. *Nature.* 1983; 306: 684-686.

66. Fields HL, Heinricher MM, and Mason P. Neurotransmitters in nociceptive modulatory circuits. *Annu Rev Neurosci*. 1991; 14:219–45.

67. Fields HL. Is there a facilitating component to central pain modulation. *APS J.* 1992; 1:71–78.

68. Fields HL, Basbaum AI. Central nervous system mechanisms of pain modulation, in: PD wall, R. Melsack (Eds), Textbook of Pain, 999, Churchill Livingstone, London, 1999, pp. 309–329.

69. Fior DR, Martins DT, and Lindsey CJ. Tissue kallikrein–kinin system in the brain and the regulation of arterial pressure. *Agents Actions 38.* 1992; (Part 3):31–38.

70. Flatters SJL, Bennett GJ. Studies of peripheral sensory nerves in paclitaxel–induced painful peripheral neuropathy: evidence for mitochondrial dysfunction. *Pain*. 2006; 122:247–257.

71. Flower RJ, and Vane JR. Inhibition of PG synthetase in brain explains the anti–pyretic activity of paracetamol (4–acetamidophenol). *Nature (Lond)*. 1972; 240:410–411.

72. Foye WO, Lemke TL, and Williams DA. Williams & Wilkins. Principles of Medicinal Chemistry. Fourth Edition, 1995.

73. Gambus PL, Schnider TW, and Minto CF. Pharmacokinetics of intravenous dynorphin A(1-13) in opioid-naive and opioid-treated human volunteers. *Clin Pharmacol Ther*. 1998; 64:27-38.

74. Gardell LR, Vanderah TW, Gardell SE, Wang R, Ossipov MH, Lai J, and Porreca F. Enhanced evoked excitatory transmitter release in experimental neuropathy requires descending facilitation. *J Neurosci*. 2003 Sep 10; 23(23):8370-9.

75. Gardell LR, Ibrahim M, Wang R, Wang Z, Ossipov MH, Malan TP Jr, Porreca F, and Lai J. Mouse strains that lack spinal dynorphin up-regulation after peripheral nerve injury do not develop neuropathic pain. *Neuroscience*. 2004; 123:43-52.

76. Gardell LR, Wang R, Burgess SE, Ossipov MH, Vanderah TW, Malan TP, Lai J, and Porreca F. Sustained Morphine Exposure Induces a Spinal Dynorphin-Dependent Enhancement of Excitatory Transmitter Release from Primary Afferent Fibers. *The*

Journal of Neuroscience. August 1, 2002; 22(15):6747-6755.

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

78. Goldstein A, Tachibana S, Lowney LI, Hunkapiller M, and Hood L. Dynorphin-(1-13), an extraordinarily potent opioid peptide. *Proc Natl Acad Sci U S A*. 1979 Dec; 76(12):6666-70.

79. Gonzales GR, and Casey KL. Central pain syndromes. In: Jensen TS, Wilson PR, Rice ACR (Eds). *Clinical Pain Management: Chronic Pain.* London: Arnold, 2003, pp 403-416.

80. Govrin-Lippmann R, and Devor M. Ongoing activity in severed nerves: Source and variation with time. *Brain Res*. 1978; 159:406-410.

81. Gracely R, Lynch S, and Bennett G. Painful neuropathy: altered central processing, maintained dynamically by peripheral input. *Pain*. 1992; 51:175-194.

82. Graham GG, and Scott KF. Mechanism of action of paracetamol. *Am J Ther*. 2005 Jan-Feb; 12(1):46-55.

83. Gustafsson H, de Araujo Lucas G, Schott E, Stiller CO, Alster P, Wiesenfeld-Hallin Z, and Brodin E. Measurement of cholecystokinin release in vivo in the rat spinal dorsal horn. *Brain Res Protoc*. 1999; 4:1192-2000.

84. Hammond DL, Tyce GM, and Yaksh TL. Efflucts of 5-hydroxytryptamine and noradrenaline into spinal cord superfusates during stimulation of the rat medulla. *J Physiol*. 1985; 359:151-162.

85. Hansson P. Neuropathic pain: clinical characteristics and diagnostic workup. *Eur J Pain*. 2002; 6(Suppl A):47-50.

86. Hauser KF, Foldes JK, Turbek CS. Dynorphin A (1-13) neurotoxicity in vitro: opioid and non-opioid mechanisms in mouse spinal cord neurons. *Exp Neurol*. 1999 Dec; 160(2):361-75.

87. Heinricher MM, Morgan MM, and Fields HL. Direct and indirect actions of morphine on medullary neurons that modulate nociception. *Neuroscience. 1992;* 48:533-543.

88. Heinricher MM, Morgan MM, Tortorici V, and Fields HL. Disinhibition of off-cells and antinociception

produced by an opioid action within the rostral ventromedial medulla. *Neuroscience*. 1994; 63:279–288.

89. Heinricher MM, and Morgan MM. Supraspinal mechanisms of opioid analgesia. In: *Opioids and Pain Control*, edited by Stein C. Cambridge, UK: Cambridge Univ Press, 1999, p. 46–69.

90. Hemler M, and Lands WE. Purification of the COX that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J Biol Chem.* 1976; 251:5575–5579.

91. Hersh EV, Lally ET, and Moore PA. Update on cyclooxygenase inhibitors: has a third COX isoform entered the fray? *Curr Med Res Opin*. 2005 Aug; 21(8):1217–26.

92. Hingtgen CM, Waite KJ, and Vasko MR. Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosine 39,59-cyclic monophosphate transduction cascade. *J Neurosci*. 1995; 15:5411–5419.

93. Hinz B, Brune K, and Pahl A. Cyclooxygenase-2 expression in lipopolysaccharide-stimulated human monocytes is modulated by cyclic AMP, prostaglandin

E_2 and nonsteroidal anti-inflammatory drugs. *Biochem Biophys Res Commun.* 2000a; 278:790-796.

94. Hinz B, Brune K, and Pahl A. Prostaglandin E_2 up-regulates cyclooxygenase-2 expression in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Biochem Biophys Res Commun.* 2000b; 272:744-748.

95. Hinz B, Cheremina O, and Brune K. Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. *FASEB J.* 2007 Sep 20; Epub ahead of print.

96. Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke S, Breipohl G, König W, Knolle J, and Scholkens BA. Hoe 140 a new potent and long acting bradykinin-antagonist: in vitro studies. *Br J Pharmacol*. 1991; 102:769-773.

97. Hoyer D, Hannon JP, and Martin GR. Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol Biochem Behav.* 2002; **71**:533-554.

98. Hu WH, Lee FC, Wan XS, Chen YT, Jen MF. Neurosci Lett. 1996 Jan 12;203(1):13-6. Dynorphin neurotoxicity induced nitric oxide synthase expression in ventral horn cells of rat spinal cord.

99. Inoue A, Ikoma K, Morioka N, Kumagai K, Hashimoto T, Hide I, and Nakata Y. Interleukin-1β induces substance P release from primary afferent neurons through the cyclooxygenase-2 system. J Neurochem. 1999; 73:2206-2213.

100. IASP Pain Terminology Encarta® World English Dictionary [North American Edition] © and (P)2007 Microsoft Corporation. All rights reserved. Developed for Microsoft by Bloomsbury Publishing Plc.

101. Jänig W, and Baron R. Complex regional pain syndrome: mystery explained? *Lancet Neurol.* 2003; 2:687-695.

102. Jeftinija S, Miletic V, and Randic M. Cholecystokinin octapeptide excites dorsal horn neurons both in vivo and in vitro. *Brain Res* 1981; 213:231-236.

103. Jensen TS, Gottrup H, Sindrup SH, and Bach FW. The clinical picture of neuropatic pain. *Eur J Pharmacol*. 2001; 429:1-11.

104. Ji R, Kohno, T, Moore KA, and Woolf CJ. Central sensitization and LTP: do pain and memory share

similar mechanisms? *Trends in Neurosciences*. 2003; 26(12):696-705.

105. Johnson BW, and Parris WCV. Mechanisms of Neuropathic Pain. In Current Review of Pain, Raj PP, (ed.) Current Medicine, 1994.

106. Kajander KC, Sahara Y, Iadarola MJ, and Bennett GJ. Dynorphin increases in the dorsal spinal cord in rats with a painful peripheral neuropathy. *Peptides*. 1990; 11:719-728.

107. Kajander KC, Wakisaka S, and 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.

108. Kandel ER, Schwartz JH, and Jessell TM. *Principles of Neural Science*, 4th ed., pp.472-479. New York: McGraw-Hill, 2000.

109. Kaufmann WE, Worley PF, Pegg J, Bremer M, and Isakson P. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. *Proc Natl Acad Sci USA. 1996;* 93:2317-2321.

110. Kawamura T, Yamauchi T, Koyama M, Maruyama T, Akira T, and Nakamura N. Expression of prostaglandin EP2 receptor mRNA in the rat spinal cord. *Life Sci*. 1997; 61:2111–2116.

111. Khachaturian H, Watson SJ, Lewis ME, Coy D, Goldstein A, Akil H. Dynorphin immunocytochemistry in the rat central nervous system. *Peptides*. 1982 Nov-Dec;3(6):941–54.

112. Kilpatrick GJ, Jones BJ, and Tyers MB. The distribution of specific binding of the 5-HT_3 receptor ligand [3H]GR65630 in rat brain using quantitative autoradiography. *Neurosci Lett*. 1988; 94:156–60.

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

114. Kis B, Snipes JA, and Busija DW. Acetaminophen and the cyclooxygenase-3 puzzle: sorting out facts, fictions, and uncertainties. *J Pharmacol Exp Ther*. 2005 Oct; 315(1):1–7. Epub 2005 May 6. Review.

115. Kovelowski CJ, Ossipov MH, Sun H, Lai J, Malan TP, and Porreca F. Supraspinal cholecystokinin may drive tonic descending facilitation mechanisms to

maintain neuropathic pain in the rat. *Pain*. 2000; 87:265-273.

116. Kraemer SA, Meade EA, and DeWitt DL. Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5'-flanking regulatory sequences. *Arch Biochem Biophys*. 1992; 293:391-400.

117. Heinricher MM, and Morgan MM. Supraspinal mechanisms of opioid analgesia. In: *Opioids and Pain Control*, edited by Stein C. Cambridge, UK: Cambridge Univ Press, 1999, p. 46-69.

118. Hesketh PJ. Comparative review of 5-HT3 receptor antagonists in the treatment of acute chemotherapy-induced nausea and vomiting. *Cancer Invest*. 2000; 18(2):163-73.

119. Huang YL, Saljo A, Suneson A, and Hansson HA. Comparison Among Different Approaches for Sampling Cerebrospinal Fluid in Rats. *Brain Research Bulletin*. 1996; 41(5):273-279.

120. Lai J, and Ossipov MH. Neuropathic Pain: The Paradox of Dynorphin. *Mol Interv*. 2001; 1(3):160-167.

121. Lai J, Luo MC, Chen Q, Ma S, Gardell LR, Ossipov MH, and Porreca F. Dynorphin A activates bradykinin

receptors to maintain neuropathic pain. *Nat Neurosci*. 2006; 9(12):1465-1467.

122. Langer SZ. Presynaptic regulation of the release of catecholamines. *Pharmacol Rev*. 1980; **32**:337-362.

123. Laughlin TM, Vanderah TW, Lashbrook J, Nichols ML, Ossipov M, Porreca F, and Wilcox GL. Spinally administered dynorphin A produces long-lasting allodynia: involvement of NMDA but not opioid receptors. *Pain*. 1997 Aug; 72(1-2):253-60.

124. Leeb-Lundberg LMF, Mathis SA, and Herzig MC. Antagonists of bradykinin that stabilize a G-protein-uncoupled state of the B_2-receptor act as inverse agonists in rat myometrial cells. *J Biol Chem*. 1994; 269:25970-25973.

125. Leslie FM, and Goldstein A. Degradation of dynorphin A1-13 by membrane-bound rat brain enzymes. *Neuropeptides*. 1982; 2:185-196.

126. Long JB, Kinney RC, Malcolm DS, Graeber GM, Holaday JW. Intrathecal dynorphin A1-13 and dynorphin A3-13 reduce rat spinal cord blood flow by non-opioid mechanisms. *Brain Res*. 1987 Dec 15; 436(2):374-9.

127. Long JB, Rigamonti DD, Oleshansky MA, Wingfield CP, Martinez-Arizala A. Dynorphin A-induced rat

spinal cord injury: evidence for excitatory amino acid involvement in a pharmacological model of ischemic spinal cord injury. *J Pharmacol Exp Ther*. 1994 Apr; 269(1):358-66.

128. Lopes P, Regoli D, Couture R. Cardiovascular effects of intrathecally administered bradykinin in the rat: characterization of receptors with antagonists. *Br J Pharmacol*. 1993 Dec; 110(4):1369-74.

129. Lee B, Won R, Baik E, Lee S, and Moon C. An animal model of neuropathic pain employing injury to the sciatic nerve branches. *Neuroreport*. 2000; 11(4):657-61.

130. Lee SH, Soyoola E, Chanmugam P, Hart S, Sun W, Zhong H, Liou S, Simmons D, and Hwang D. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J Biol Chem.* 1992; 267:25934-25938.

131. Liu H, Chandler S, Beitz AJ, Shipley MT, and Behbehani MM. Characterization of the effect of cholecystokinin (CCK) on neurons in the periaqueductal gray of the rat: immunocytochemical

and in vivo and in vitro electrophysiological studies. *Brain Res*. 1994; 642:83-94.

132. Liu X, Chung K, Merskey H, and Bogduk N, Eds. Classification of Chronic Pain, Second Ed. Seattle, WA: IASP Press, 1994.

133. Liu X, Eschenfelder S, Blenk K-H, Jänig W, and Häbler H-J. Spontaneous activity of axotomized afferent neurons after L5 spinal nerve injury in rats. *Pain*. 2000; 84:309-318.

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

135. Liu X, Zhou J, Chung K, and Chung JM. Ion channels associated with the ectopic discharges generated after segmental spinal nerve injury in the rat. *Brain Research*. 2001; 900:119-127.

136. Low PA, Opfer-Gehrking TL, Dyck PJ, Litchy WJ, and O'Brien PC. Doubleblind, placebo-controlled study of the application of capsaicin cream in chronic distal painful polyneuropathy. *Pain*. 1995; 62:163-168.

137. Lydiard RB. Neuropeptides and anxiety: focus on cholecystokinin. Clin Chem. 1994; 40:315–318.

138. Maihofner C, Schlotzer-Schrehardt U, Guhring H, Zeilhofer HU, Naumann GO, Pahl A, Mardin C, Tamm ER, and Brune K. Expression of COX-1 and -2 in normal and glaucomatous human eyes. *Investig Ophthalmol Vis Sci.* 2001; 42:2616–2624.

139. Malan TP Jr, Ossipov MH, Gardell LR, Ibrahim M, Bian D, Lai J, and Porreca F. Extraterritorial neuropathic pain correlates with multisegmental elevation of spinal dynorphin in nerve-injured rats. *Pain*. 2000; 86:185–194.

140. Malcangio M, and Tomlinson DR. A pharmacologic analysis of mechanical hyperalgesia in streptozocin/diabetic rats. *Pain*. 1998; 76:151–157.

141. Maldve RE, Kim Y, Muga SJ, and Fischer SM. Prostaglandin E_2 regulation of cyclooxygenase expression in keratinocytes is mediated via cyclic nucleotide–linked prostaglandin receptors. *J Lipid Res.* 2000; 41:873–881.

142. Malmberg AB, and Yaksh TL. Hyperalgesia mediated by spinal glutamate or substance P receptor blocked

by spinal cyclooxygenase inhibition. *Science.* 1992; 257:1276-1279.

143. Malmberg AB, and 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* 1995a; 114:1069-1075.

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

145. Malmberg A, and Basbaum A. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. *Pain.* 1998; 76(1-2):215-22.

146. Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ. Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. *J Comp Neurol*. 1994 Dec 15; 350(3):412-38.

147. Marabese I, Rossi F, Palazzo E, de Novellis V, Starowicz K, Cristino L, Vita D, Gatta L, Guida F, Di

Marzo V, Rossi F, Maione S. Periaqueductal gray metabotropic glutamate receptor subtype 7 and 8 mediate opposite effects on amino acid release, rostral ventromedial medulla cell activities, and thermal nociception. *J Neurophysiol*. 2007 Jul; 98(1):43-53.

148. Marceau, F. Kinin B1 receptors: a review. *Immunopharmacology*. 1995; 30:1-26.

149. Marceau F, Hess F, Bachvarov DR, 1998. The B1 receptors for kinins. *Pharmacol Rev.* 50, 357-386.

150. Maricq AV, Peterson AS, Brake AJ, Myers RM, and Julius D. Primary structure and functional expression of the 5HT3 receptor, a serotonin-gated ion channel. *Science*. 1991 Oct 18; 254(5030):432-7.

151. Marie J, Koch C, Pruneau D, Paquet JL, Grobleswski T, Larguier R, Lombar C, Deslauriers B, Maigret B, and Bonnafous JC. Constitutive activation of the human bradykinin B_2 receptor induced mutations in transmembrane helices III and VI. *Mol Pharmacol*. 1999; 55:92-101.

152. Matzner O, and Devor M. Hyperexcitability at sites of nerve injury depends on voltage-sensitive Na^+ channels. *J Neurophysiol*. 1994; 72:349-359.

153. McCleane GJ. The cholecystokinin antagonist proglumide enhances the analgesic efficacy of morphine in humans with chronic benign pain. Anesth Analg. 1998 Nov; 87(5):1117-20.

154. McCleane GJ. The cholecystokinin antagonist proglumide has an analgesic effect in chronic pancreatitis. Pancreas. 2000 Oct; 21(3):324-5.

155. McCleane GJ. A phase 1 study of the cholecystokinin (CCK) B antagonist L-365,260 in human subjects taking morphine for intractable non-cancer pain. Neurosci Lett. 2002 Nov 8; 332(3):210-2.

156. McCleane, GJ, Suzuki R, and Dickenson AH. Does a Single Intravenous Injection of the 5HT3 Receptor Antagonist Ondansetron Have an Analgesic Effect in Neuropathic Pain? A Double-Blinded, Placebo Controlled Cross-Over Study. *Anesth Analg*. 2003; 97(5):1474-1478.

157. McCormack K. Non-steroidal anti-inflammatory drugs and spinal nociceptive processing. *Pain*. 1994; 59:9-43.

158. McQuay HJ, Tramer M, Nye BA, Carroll D, Wiffen P, and Moore A. A systematic review of antidepressants in neuropathic pain. *Pain*. 1996; 68:217–227.

159. Meuser T, Pietruck C, Gabriel A, Xie GX, Lim KJ, Pierce Palmer P. 5-HT_7 receptors are involved in mediating 5-HT-induced activation of rat primary afferent neurons. *Life Sci*. 2002; 71:2279–2289.

160. Millan MJ. Descending control of pain. *Prog Neurobiol*. 2002 Apr; 66(6):355–474. Review.

161. Minghetti L, Polazzi E, Nicolini A, Creminon C and Levi G. Up-regulation of cyclooxygenase-2 expression in cultured microglia by prostaglandin E_2, cyclic AMP and non-steroidal anti-inflammatory drugs. *Eur J Neurosci*. 1997; 9:934–940.

162. Minghetti L and Levi G. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. *Prog Neurobiol*. 1998. 54:99–125.

163. Miyamoto T, Ogino N, Yamamoto S, and Hayaishi O. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem*. 1976; 251:2629–2636.

164. Mollace V, Muscoli C, Masini E, Cuzzocrea S, and Salvemini D. Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. *Pharmacol Rev*. 2005 Jun; 57(2):217-52.

165. Müller S, Grundy BL, and Hochhaus G. Metabolism of Dynorphin A1-13 in Human CSF. *Neurochemical Research*. 1996 Oct; 21(10):1213-1219.

166. Nantel F, Denis D, Gordon R, Northey A, Cirino M, Metters KM, and Chan CC. Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation. *Br J Pharmacol.* 1999; 128:853-859.

167. Needleman P, Turk J, Jakschik BA, Morrison AR, and Lefkowith JB. Arachidonic acid metabolism. *Annu Rev Biochem.* 1986; 55:69-102.

168. Neubert MJ, Kincaid W, and Heinricher MM. Nociceptive facilitating neurons in the rostral ventromedial medulla. *Pain*. 2004; 110:158-165.

169. Niiro H, Otsuka T, Izuhara K, Yamaoka K, Ohshima K, Tanabe T, Hara S, Nemoto Y, Tanaka Y, Nakashima H and Niho Y. Regulation by interleukin-10 and interleukin-4 of cyclooxygenase-2 expression in human neutrophils. *Blood.* 1997; 89:1621-162.

170. Nolte J. The Human Brain: An Introduction to its functional anatomy. Fifth Edition, Mosby Inc., an affiliate of Elsevier, pp. 282-284, 2002.

171. Onoe Y, Miyaura C, Kaminakayashiki T, Nagai Y, Noguchi K, Chen QR, Seo H, Ohta H, Nozawa S, Kudo I and Suda T. IL-13 and IL-4 inhibit bone resorption by suppressing cyclooxygenase-2-dependent prostaglandin synthesis in osteoblasts. *J Immunol.* 1996; 156:758-764.

172. Ossipov MH, Lopez Y, Nichols ML, Bian D and Porreca F. The loss of antinociceptive efficacy of spinal morphine in rats with nerve ligation injury is prevented by reducing spinal afferent drive. *Neurosci Lett.* 1995; 199:87-90.

173. Ossipov MH, Lai J, Malan TP Jr, and Porreca F. Spinal and supraspinal mechanisms of neuropathic pain. *Ann N Y Acad Sci*. 2000; 909:12-24. Review.

174. Pan Z, Hirakawa N, and Fields HL. A cellular mechanism for the bidirectional pain-modulating actions of orphanin FQ/nociceptin. *Neuron.* 2000; 26:515-522.

175. Pertovaara A, Wei H, Hämäläinen MM. Lidocaine in the rostroventromedial medulla and the periaqueductal

gray attenuates allodynia in neuropathic rats. *Neurosci Lett*. 1996 Nov 1; 218(2):127-30.

176. Pierce JP, Kurucz OS, and Milner TA. Morphometry of a peptidergic transmitter system: dynorphin B-like immunoreactivity in the rat hippocampal mossy fiber pathway before and after seizures. *Hippocampus*. 1999; 9(3):255-76.

177. Pockett S. Spinal cord sympathetic plasticity and chronic pain. *Anesth. Analg*. 1995; 80:173-179.

178. Porreca F, Burgess SE, Gardell LR, Vanderah TW, Malan TP Jr, Ossipov MH, Lappi DA, and Lai J. Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the mu-opioid receptor. *J Neurosci*. 2001 Jul 15;21(14):5281-8.

179. Porreca F, Ossipov MH, and Gebhart GF. Chronic pain and medullary descending facilitation. *Trends Neurosci*. 2002; 25:319-25.

180. Privitera PJ, Thibodeaux H, and Yates P. Rostral ventrolateral medulla as a site for the central hypertensive action of kinins. *Hypertension*. 1994; 23:52-58.

181. Quirion R, and Pilapil C. Opioid receptor binding profile of [3H]dynorphin A-(1-8) in rat and guinea pig brain. *Eur J Pharmacol*. 1984 Apr 6; 99(4):361-3.

182. Qureshi GA, Bednar I, and Min Q. Quantitation and identification of two cholecystokinin peptides, CCK-4 and CCK-8s, in rat brain by HPLC and fast atom bombardment mass spectrometry. *Biomed Chromatogr.* 1993; 7:251-255.

183. Racké K, Reimann A, Schwörer H, and Kilbinger H. Regulation of 5-HT release from enterochromaffin cells. *Behav Brain Res*. 1996; 73(1-2):83-7.

184. Raidoo DM, and Bhoola KD. Pathophysiology of the kallikrein-kinin system in mammalian nervous tissue. *Pharmacology and Therapeutics*. 1998 Aug; 79(2):105-127.

185. Rang HP, Dale MM, Ritter JM, Moore PK. Pharmacology, Fifth Edition, Churchill Livingstone Publisher - Elsevier Science Limited, pp. 232-234, 2003.

186. Regoli D, Barabé J, and Park WK. Receptors for bradykinin in rabbit aortae. *Can J Physiol Pharmacol*. 1977; 55:855-867.

187. Regoli D, and Barabe J. Pharmacology of bradykinin and related kinins. *Pharmacol*. 1980; Rev. 32, 1-46.

188. Regoli D, Marceau F, and Lavigne J. Induction of B1 receptors for kinins in the rabbit by a bacterial lipopolysaccharide. *Eur J Pharmacol*. 1981; 71:105-115.

189. Ren K, and Dubner R. Enhanced descending modulation of nocieption in rats with persistent hindpaw inflammation. *J Neurophysiol*. 1996; 76:3025-3037.

190. Riendeau D, Percival MD, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, Evans J, Falgueyret JP, and Ford-Hutchinson AW. Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. *Br J Pharmacol*. 1997; 121:105-117.

191. Roila F, Ballatori E, Tonato M, and Favero A. 5-HT3 receptor antagonists: differences and similarities. Eur J Cancer. 1997; 33:1364-1370.

192. Rondé P, and Nichols RA. High calcium permeability of serotonin 5-HT_3 receptors on

presynaptic nerve terminals from rat striatum. *J Neurochem*. 1998; 70(3):1094-103.

193. Ruat M, Traiffort E, Leurs R, Tardivel-Lacombe J, Diaz J, Arrang J-M, and Schwartz J-C. Molecular Cloning, Characterization, and Localization of a High-Affinity Serotonin Receptor (5-HT Activating cAMP Formation. *Proceedings of the National Academy of Sciences of the United States of America*. Sept 1993; 90(18):8547-8551

194. Salvemini D, Settle SL, Masferrer JL, Seibert K, Currie MG, and Needleman P. Regulation of prostaglandin production by nitric oxide; an in vivo analysis. *Br J Pharmacol.* 1995 Mar; 114(6):1171-8.

195. Sano H, Hla T, and Maier JA. In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis *J Clin Invest. 1992.* 89; 97-108.

196. Sari Y. Serotonin1B receptors: from protein to physiological function and behavior. *Neurosci Biobehav Rev.* 2004; 28:565-582.

197. Saria A, Javorsky F, Humpel C, Gamse R. 5-HT_3 receptor antagonists inhibit sensory neuropeptide

release from the rat spinal cord. *Neuroreport.* 1990 Oct; 1(2):104-6.

198. Scicli AG, Forbes G, Nolly H, Dujovny M, and Carretero OA. Kallikrein-kinins in the central nervous system, *Clin Exp Hypertens., Part A, Theory and Practice*. 1984; 6:1731-1738.

199. Seltzer Z, Dubner R, and Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain.* 1990; 43:205-218.

200. Sheen K, and Chung JM. Signs of neuropathic pain depend on signals from injured nerve fibers in a rat model. *Brain Res*. 1993; 610:62-68.

201. Shisheva AC, Printz MP, Herman K, and Ganten D. Kinin-forming activity in rat brain, *Neurochem Internat.* 1985; 7:621-629.

202. Shukla VK, and Lemaire S. Non-opioid effects of dynorphins: possible role of the NMDA receptor. *Trends Pharmacol Sci*. 1994; 15:420-424.

203. Skilling SR, Sun X, Kurtz HJ, and Larson AA. Selective potentiation of NMDA-induced activity and release of excitatory amino acids by dynorphin:

possible roles in paralysis and neurotoxicity. *Brain Res*. 1992; 575:272-278.

204. Siau C, and Bennett GJ. Dysregulation of neuronal calcium homeostasis in chemotherapy-evoked painful peripheral neuropathy. *Anesth Analg*. 2006; 102:1485-1490.

205. Siegel GJ. Basic Neurochemistry. Understanding the neuroanatomical organization of serotonergic cells in the brain provides insight into the functions of this neurotransmitter, 1999, Bernard W. Agranoff, Stephen K. Fisher, R. Wayne Albers, Michael D. Uhler, Sixth, Lippincott Williams and Wilkins.

206. Sindrup SH, Gram LF, Brùsen K, Eshùj O, and Mogensen EF. The selective serotonin reuptake inhibitor paroxetine is effective in the treatment of diabetic neuropathy symptoms. *Pain*. 1990; 42:135-144.

207. Sindrup SH, Bjerre U, Dejgaard A, Brssen K, Aaes-Jùrgensen T, and Gram LF. The selective serotonin reuptake inhibitor citalopram relieves the symptoms of diabetic neuropathy. *Clin Pharmacol Ther.* 1992; 52:547-552.

208. Sindrup SH, and Jensen TS. Efficacy of pharmacological treatments of neuropathic pain: an

update and effect related to mechanism of drug action. *Pain*. 1999 Dec; 83(3):389-400.

209. Smith WL, and Lands WE. Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular gland. *Biochemistry*. 1972; 11:3276-3285.

210. Smith WL, Marnett LJ, and DeWitt DL. Prostaglandin and thromboxane biosynthesis. *Pharmacol Ther*. 1991; 49:153-179.

211. Soberman RJ, and Christmas P. The organization and consequences of eicosanoid signaling". *J Clin Invest*. 2003; 111:1107-1113.

212. Sommer C. Painful neuropathies. *Curr Opin Neurol*. 2003; 16:623-628.

213. Sugimoto T, Bennett GJ, Kajander KC. Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: Effects of a chronic constriction injury, transection and strychnine. *Pain*. 42:205-213, 1990.

214. Sung B, Na H, Kim Y, Yoon Y, Han H, Nahm S, and Hong S. Supraspinal involvement in the production of mechanical allodynia by spinal nerve injury in rats. *Neurosci Lett*. 1998; 246(2):117-9.

215. Suzuki R, Rahman W, Hunt SP, and Dickenson AH. Descending facilitatory control of mechanically evoked responses is enhanced in deep dorsal horn neurones following peripheral nerve injury. *Brain Research*. 2004; 1019(1-2):68-76.

216. Tang Q, Lynch RM, Porreca F, and Lai J. Dynorphin A elicits an increase in intracellular calcium neurons via a non-opioid, non-NMDA mechanism. *J Neurophysiol*. 2000; 83:2610-2615.

217. Tasker RR. Central Pain Syndromes. In Principles and Practice of Pain Management, Warfield, CA (ed.) McGraw Hill, Inc. New York, 1993.

218. Terayama R, Guan Y, Dubner R and Ren K. Activity-induced plasticity in brain stem pain modulatory circuitry after inflammation. *Neuroreport.* 2000; 11:1915-1919.

219. Thornhill JA, Gregor L, Mathison R, Pittman Q. Intrathecal dynorphin A administration causes pressor responses in rats associated with an increased resistance to spinal cord blood flow. *Brain Res*. 1989 Jun 19; 490(1):174-7.

220. Urban MO, Gebhart GF. Characterization of biphasic modulation of spinal nociceptive

transmission by neurotensin in the rat rostral ventromedial medulla. *J Neurophysiol*. 1997; 78:1550–62.

221. Vadakkan K, Jia Y, and Zhuo M. A behavioral model of neuropathic pain induced by ligation of the common peroneal nerve in mice. *The Journal of Pain: official journal of the American Pain Society.* 2005; 6(11):747–56.

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

223. Vanderah TW, Gardell LR, Burgess SE, Ibrahim M, Zhong CM, Zhang ET, Malan TP Jr, Ossipov MH, Lai J, and Porreca F. Dynorphin promotes abnormal pain and spinal opioid antinociceptive tolerance. *J Neurosci*. 2000; 18:7074–7079.

224. Van Bockstaele EJ, Sesack SR, and Pickel VM. Dynorphin-immunoreactive terminals in the rat nucleus accumbens: cellular sites for modulation of target neurons and interactions with catecholamine afferents. *J Comp Neurol*. 1994 Mar 1; 341(1):1–15.

225. Van der Ouderaa FJ, Buytenhek M, Nugteren DH, and Van Dorp DA. Purification and characterisation of PG

endoperoxide synthetase from sheep vesicular glands. *Biochim Biophys Acta.* 1977; 487:315-331.

226. van Hooft JA, and Vijverberg HPM. 5-HT_3 receptors and neurotransmitter release in the CNS: a nerve ending story? *Trends in Neurosciences.* 2000 Dec; 23(12):605-610

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

228. Vera-Portocarrero LP, Xie JY, Kowal J, Ossipov MH, King T, and Porreca F. Descending facilitation from the rostral ventromedial medulla maintains visceral pain in rats with experimental pancreatitis *Gastroenterology.* 2006 Jun; 130(7):2155-64.

229. Wall PD, and Gutnick M. Ongoing activity in peripheral nerves: the physiology and pharmacology of impulses originating from a neuroma. *Exp Neurol.* 1974; 43:580-593.

230. Wall PD, and Devor M. Sensory afferent impulses originate from dorsal root ganglia as well as from the periphery in normal and nerve injured rats. *Pain.* 1983; 17:321-339.

231. Wang Z, Gardell LR, Ossipov MH, Vanderah TW, Brennan MB, Hochgeschwender U, Hruby VJ, Malan TP Jr, Lai J, and Porreca F. Pronociceptive actions of dynorphin maintain chronic neuropathic pain. *J Neurosci*. 2001; 21:1779–1786.

232. Warner TD, and Mitchell JA. Cyclooxygenase–3 (COX–3): Filling in the gaps toward a COX continuum. *PNAS*. 2002 Oct 8; 99(21):13371–13373.

233. Watson CPN, and Babul N. Efficacy of oxycodone in neuropathic pain: a randomized trial in postherpetic neuralgia. *Neurology*. 1998; 50:1837–1841.

234. Wei F, Dubner R, Ren K. Nucleus reticularis gigantocellularis and nucleus raphe magnus in the brainstem exert opposite effects on behavioral hyperalgesia and spinal FOS protein expression after peripheral inflammation. *Pain*. 1999; 80:127–141.

235. Willingale HL. and Grubb BD. Evidence that prostaglandins may modulate wind-up in a spinal nociceptive reflex in anaesthetised rats. *Br J Pharmac*. 1996; 118:49.

236. Willingale HL, Gardiner NJ, McLymont N, Giblett S, and Grubb BD. Prostanoids synthesized by cyclo-oxygenase isoforms in rat spinal cord and their

contribution to the development of neuronal hyperexcitability. *Br J Pharmac*. 1997; 122:1593-1604.

237. Wirth K, Hock FJ, Albus U, Linz W, Alpermann HG, Anagnostopoulos H, Henk S, Breipohl G, König W, Knolle J, and Schölkens BA. Hoe 140 a new potent and long acting bradykinin antagonist: in vivo studies. *Br J Pharmacol*. 1991; 102:774-777.

238. Witherow FN, Dawson P, Ludlam CA, Webb DJ, Fox KAA, and Newby DE. Bradykinin Receptor Antagonism and Endothelial Tissue Plasminogen Activator Release in Humans. *Arterioscler Thromb Vasc Biol.* 2003; 23:1667-1670.

239. Wong MC, Chung JW, and Wong TK. Effects of treatments for symptoms of painful diabetic neuropathy: systematic review. *BMJ.* 2007; 335(7610):87.

240. Woolf CJ. Evidence for a central component of post-injury pain hypersensitivity. *Nature.* 1983; 306:686-688.

241. Woolf CJ, & Mannion RJ. Neuropathic pain: Aetiology, symptoms, mechanisms, and management [Review]. *Lancet.* 1990; 353:1959-1964.

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

243. Woolf CJ, and Doubell TP. The pathophysiology of chronic pain--increased sensitivity to low threshold A beta-fibre inputs. *Curr Opin Neurobiol*. 1994 Aug;4(4):525-34. Review.

244. Woolf CJ, Shortland P, Reynolds M, Ridings J, Doubell T, and Coggeshall RE. Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. *J Comp Neurol.* 1995 Sep 11; 360(1):121-34.

245. Xie Y., Zhang J., Petersen M, and Lamotte RH. Functional changes in dorsal root ganglion cells after chronic nerve constriction in the rat. *J. Neurophysiol.* 1995; 73:1811-1820.

246. Xie Y-K, Xiao WH, and Li HQ. The relationship between new ion channels and ectopic discharges from a region of nerve injury. Science in China (Series B). 1993; 36:68-74.

247. Xie W, Chipman JG, Robertson DL, Erikson RL and Simmons DL. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci USA.* 1991; 88:2692-2696.

248. Xu XM, Sansores-Garcia L, Chen XM, Matijevic-Aleksic N, Du M, and Wu KK. Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc Natl Acad Sci USA.* 1999; 96:5292-5297.

249. Yamagata K, Andreasson KI, Kaufmann WE, Barnes CA, and Worley PF. Expression of a mitogen-inducible COX in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron.* 1993; 11:371-386.

250. Yamamoto T and Nozaki-Taguchi N. Analysis of the effects of cyclooxygenase (COX)-1 and COX-2 in spinal nociceptive transmission using indomethacin, a non-selective COX inhibitor and NS-398, a COX-2 selective inhibitor. *Brain Res.* 1996; 739:104-110.

251. Yaksh TL and Rudy TA. Chronic catherization of the spinal subarachnoid space. *Physiol Behav.* 1976; 17: 1031-1036.

252. Yaksh TL: The spinal pharmacology of facilitation of afferent processing evoked by high-threshold

afferent input of the post injury pain state Curr *Opin Neurol Neurosurg*. 1993; 6:250–256.

253. Yim CC, and Mogenson GJ. Electrophysiological evidence of modulatory interaction between dopamine and cholecystokinin in the nucleus accumbens. *Brain Res.* 1991; 541:12–20.

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

255. You ZB, Herrera-Marschitz M, Brodin E, Meana JJ, Morini P, Hökfelt T, Silveira R, Goiny M, and Ungerstedt U. On the origin of striatal cholecystokinin release: studies with in vivo microdialysis. *J Neurochem*. 1994; 62:76–85.

256. Zeitz KP, Guy N, Malmberg AB, Dirajlal S, Martin WJ, Sun L, Bonhaus DW, Stucky CL, Julius D, and Basbaum AI. The 5-HT_3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors. J *Neurosci.* 2002; 22:1010–1019.

257. Zhang S, Tong Y, Tian M, Dehaven RN, Cortesburgos L, Mansson E, Simonin F, Kieffer B, and Yu L. Dynorphin A as a potential endogenous ligand for four

members of the opioid receptor gene family. *J Pharmacol Exp Ther.* 1998 Jul; 286(1):136-41.

258. Zhuo M and Gebhart GF. Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. *J Neurosci*. 1997; 97:746-758.

259. Zhuo M, Gebhart GF. Characterization of descending inhibition and facilitation from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. *Pain*. 1990; 42:337-50.

260. Zhuo M, Gebhart GF. Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. *J Neurophysiol*. 1992; 67:1599-614.

261. Zimmermann M. Central nervous mechanisms modulating pain-related information: do they become deficient after lesions of the peripheral or central nervous system? In: KL Casey (Ed.), Pain and Central Nervous System Disease: The Central Pain Syndromes, Raven, New York, 1991, pp. 183-199.