THE CONTRIBUTION OF DESCENDING PAIN FACILITATION TO THE MAINTENANCE OF NEUROPATHIC PAIN

by

Shannon Elizabeth Gardell

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Josephine Lai, Ph.D.	8/13/03 **Date**
Frank Porreca, Ph.D.	8/13/03 **Date**
T. Philip Malan, Jr., M.D., Ph.D.	8/13/03 **Date**
Robert Sloviter, Ph.D.	8/13/03 **Date**
Qin Chen, Ph.D.	8-13-2003 **Date**

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Josephine Lai, Ph.D. (Dissertation Director)	8/13/03 **Date**

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LIST OF ABBREVIATIONS

Mu opioid receptor	MOR
Rostral ventromedial medulla	RVM
Periaqueductal gray	PAG
Cholecystokinin	CCK
Dorsal root ganglion	DRG
Spinal nerve ligation	SNL
Ribosome-inactivating protein	RIP
Saporin	SAP
Dermorphin	DERM
Dermorphin-saporin	DERM-SAP
β-funaltrexamine	β-FNA
Dorsolateral funiculus	DLF
Calcitonin gene-related peptide	CGRP
In situ hybridization	ISH
Sham-operated	SHAM

ABSTRACT

This dissertation will examine the likelihood that tonic activation of descending facilitatory pathways may underlie chronic pain states arising from injuries to peripheral nerves, and that this activation is what maintains neuropathic pain. Activation of these pathways might be the result of plasticity in the RVM that is driven, in part, by release of CCK in response to pain signals from the injured nerve.

Hyperactivity of facilitatory ON cells in the RVM may contribute to neuropathic pain and lesions of these cells via the targeted toxin, DERM-SAP, may prevent or reverse neuropathic pain states in the injured animal. Rats treated with DERM or SAP and undergoing SNL exhibited tactile and thermal hypersensitivity. Rats receiving DERM-SAP showed similar responses to SHAM controls. Administration of RVM DERM-SAP to SNL rats fully reversed established allodynia/hyperalgesia by day 14. Thus, the targeted loss of cells expressing the MOR both prevents and reverses SNL-induced neuropathic pain.

Acutely, RVM lidocaine blocked tactile and thermal hypersensitivity on day 6 out to day 12 after SNL, but not on day 3. DERM-SAP pretreatment and DLF lesions did not prevent the onset of tactile and thermal hypersensitivity, but these neuropathic pain signs reversed toward baseline levels beginning on day 4 after SNL. These findings differentiate the mechanisms that initiate neuropathic pain as being independent of supraspinal influences needed to maintain such pain. These descending influences may underlie some of the SNL-induced changes at the spinal level, such as the upregulation of dynorphin and may be key to the maintenance of neuropathic pain.

Administration of RVM CCK produces hypersensitivity in naïve rats. Pretreatment with RVM DERM-SAP completely blocks the ability of RVM CCK to produce hypersensitivity. These results suggest that the action of CCK in the RVM may be mediated by activation of MOR expressing cells. Enhanced release of CCK in the RVM or upregulation of CCK receptors in RVM neurons after SNL may represent a mechanism of descending pain facilitation to maintain chronic pain states. An observed loss of both MOR and CCK_2 receptors after DERM-SAP treatment gives support to the notion that both receptor types may be co-expressed in RVM neurons, and these neurons may be critical for descending facilitatory input from the RVM to maintain neuropathic pain. Understanding the mechanisms of descending facilitation and the spinal effects of, CCK-activated discharge could provide new insight into the modulation of chronic pain, and, furthermore, provide new targets for the development of novel drug therapies.

CHAPTER I: INTRODUCTION

Pain Defined

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (International Association for the Study of Pain). The two primary types of pain are acute pain and chronic pain. Acute pain may be produced by inflammation, tissue damage, injury, illness, or a recent surgery. It is often characterized by redness, increased local temperature and swelling. In contrast to chronic pain, acute pain usually ends after the underlying cause is treated or has been resolved.

Chronic pain is pain that continues a month or more beyond the usual recovery period for an illness or injury or pain that goes on over months or years as a result of a chronic condition. It is an affliction that disables about 86 million Americans and it carries with it high economic costs; it is estimated that United States business and industry loses about $90 billion annually to sick time, reduced productivity, and direct medical and other benefit costs due to chronic pain among employees.

Nociception and Pain Transmission Systems

The first step in understanding chronic pain is to first comprehend how basic pain signals are transmitted. The fibers that are activated by innocuous and noxious stimuli are called cutaneous primary afferents and can be classified into three major groups on the basis of diameter, structure, and conduction velocity. The largest of these primary afferents fibers, which include Aα and Aβ fibers, have diameters ranging from 6-22 μm.

These fibers are heavily myelinated and fast conducting (30-100 m/sec). Aα fibers have the largest diameter of the myelinated primary afferents and include muscle proprioceptors (i.e, muscle spindles and Golgi tendon organs). Both Aα and Aβ fibers transmit information related to muscle stretch, light touch and position. The Aδ fibers, the second type of primary afferent, is medium-sized, with a diameter ranging from 2-6 μm. These fibers are myelinated, like the Aα and Aβ fibers, but possess a slower, intermediate conduction velocity (12-30 m/sec). The C fibers, the last type of primary afferent, are small sized, with a diameter ranging from 0.4-1.2 μm. In contrast to the other two types of primary afferents, these fibers are unmyelinated and have a slow conduction velocity (0.5-2.0 m/sec). Both Aδ and C transmit information related to noxious thermal, chemical and mechanical stimuli. Selective activation of these fibers by noxious, but not non-noxious, stimuli defines their role as nociceptors. For the experiments reported in this dissertation, primary afferent neurons will be classified as Aβ fibers and C fibers.

All primary afferent neurons that enter the spinal cord have their cell bodies in the DRG. These pseudounipolar cell bodies send out axons to both the periphery and the spinal cord. Upon entering the spinal cord, Aβ fibers primarily ascend the ipsilateral dorsal column to the nucleus gracilis, or the nucleus cuneatus in the brainstem, but also send collateral projections to lamina III-IV of the spinal dorsal horn, where they modulate the activity of wide dynamic range neurons. These fibers synapse onto 2nd order neurons in the dorsal column nuclei, and they cross the midline and project to 3rd order cells in the ventrobasal thalamus via the medial lemniscus. In contrast, C fibers enter the dorsal horn

and converge onto 2nd order neurons, which cross the midline, and ascend via the spinothalamic tract, to 3rd order cells in the ventrobasal thalamus. These cells then project to the somatosensory cortex, and other sites, where the perception of pain occurs.

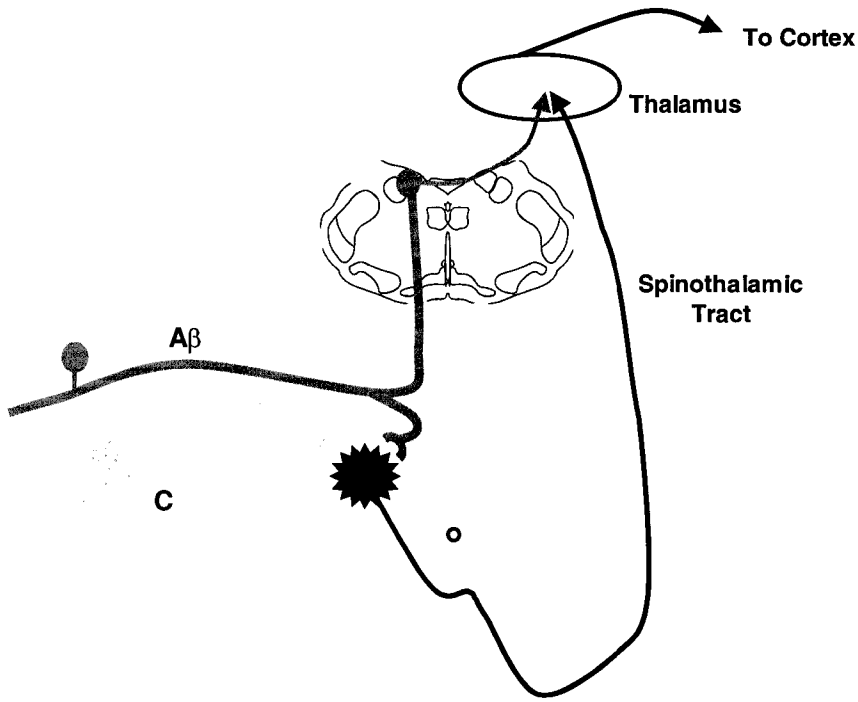

Figure 1.1. Ascending sensory fiber pathways. Depicted are the primary afferent Aβ and C fibers entering the spinal cord and ascending via the ipsilateral dorsal column to the dorsal column nuclei (n. gracilus or n. cuneatus) or contralateral spinothalamic tract to the thalamus, respectively. From the thalamus, these fibers project to the somatosensory cortex, and to other centers.

In addition to the ascending pain pathway, there exists an endogenous descending pain pathway, which serves to either inhibit or facilitate the processing of pain. Here, neurons found primarily in the PAG of the midbrain project to a number of areas, including the RVM, which serves as a relay station in the descending modulation of pain. Neurons in the RVM project their axons, via the DLF, to the different laminae of the spinal cord.

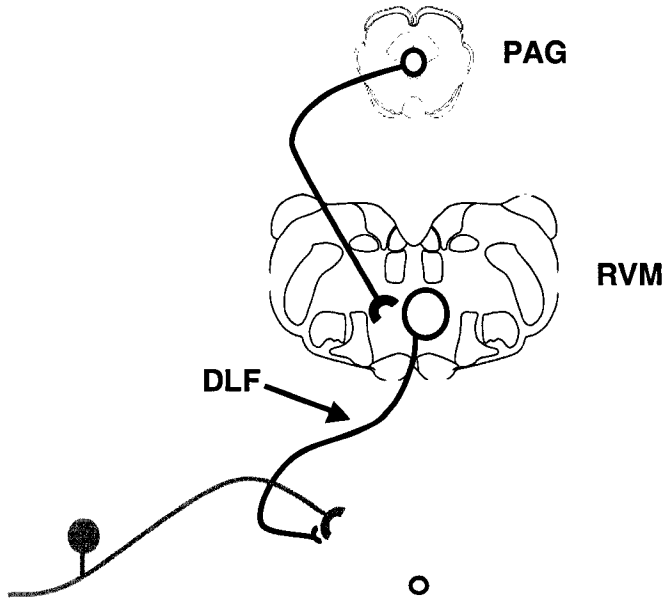

Figure 1.2. Descending pain modulatory pathway. Depicted are some of the major structures of the descending pain modulation system. Neurons from the PAG project to the RVM. Neurons from the RVM project to the spinal cord via the DLF.

Neuropathic Pain, A Closer Look

A common type of chronic pain is one that stems from an abnormal function in the nervous system. Neuropathic pain is a type of chronic pain that is initiated or caused by a primary lesion or dysfunction in the nervous system. In view of all its possible causes, the prevalence of neuropathic pain can be conservatively estimated at 0.6% of the U. S. population. To the extent that low-back pain can be sometimes considered neuropathic, the actual figure may be closer to 2% (Bennett 1998).

Neuropathic pain becomes possible whenever nerves are damaged, specifically, as a result of certain diseases, such as diabetes, herpes zoster, and late-stage cancer or from trauma, surgery, or amputation. In many cases, it occurs without any known injury or disease; nerves are transmitting pain signals, even though there is no identifiable cause

for them. Neuropathic pain comprises two main categories: spontaneous and evoked pain. Patients describe spontaneous or stimulus-independent neuropathic pain, as being shooting, stabbing, burning, lancinating or searing. Evoked, or stimulus-dependent neuropathic pain is characterized as allodynia, pain due to a stimulus that does not normally produce pain, and hyperalgesia, an increased response to a stimulus that is normally painful.

In addition to causing severe physical symptoms, neuropathic pain can lead to a constant state of suffering, sleeplessness, and sadness, termed the "terrible triad" (National Institute of Neurological Disorders and Stroke). When an individual becomes preoccupied with pain, he or she may become depressed and irritable. This depression and irritability often leads to insomnia and weariness, which compounds the problem by causing more irritability, depression and pain. Thus, since neuropathic pain influences all aspects of a patient's life, the primary goals of treatment are to reduce the pain as much as possible, balance the negative side effects of the treatment, and help patients manage any unresolved pain with various types of support.

Current Drug Therapies for the Treatment of Neuropathic Pain

Neuropathic pain is a very challenging condition to treat. Its symptoms can be described as being heterogeneous in nature and largely resistant to commonly prescribed analgesics. The four main reasons why treatments for neuropathic pain often fail are: inadequate diagnosis and a lack of appreciation of the mechanisms involved; insufficient management of comorbid conditions ("terrible triad"); incorrect understanding or selection of treatment options; and the use of inappropriate outcomes measures (Harden

and Cohen 2003). However, pain symptoms are still managed with techniques that fall into four main groups: drugs, physical therapy, injections and psychological therapies.

Current drug therapies for the treatment of neuropathic pain include tricyclic antidepressants, anticonvulsants, Gabapentin (Neurontin), antiarrythmics, topical drugs such as capsaicin, and non-steroidal anti-inflammatory drugs (NSAIDs) (Sah et al. 2003). Alternative strategies include electrical stimulation locally, to the brain, and to nerve endings under the skin (transcutaneous electrical nerve stimulation); acupuncture, surgery, local anaesthetic nerve blocks, and psychological treatments, which center on the belief that patients can do something on their own to control their pain. The current pharmacological mainstays (tricyclic antidepressants and certain anticonvulsants) of clinical management only achieve clinically significant (greater than 50%) pain relief in less than 50% of patients and, in addition, are associated with sub-optimal side effect profiles (McQuay et al. 1996; Sindrup and Jensen 1999).

In general, opioids are considered to be less effective for the treatment of neuropathic pain in comparison to inflammatory pain, with the dose response curve for neuropathic pain being shifted to the right of that for inflammatory pain, although the actual significance of this difference is controversial (Rowbotham 1999). Ultimately, since the majority of drug therapies have limited efficacy, current treatments are palliative, based more on controlling the symptoms, rather than curing the actual affliction.

Animal Models of Neuropathic Pain – SNL

Since human volunteer models of neuropathic pain are not readily available, it is advantageous to have an experimental model that mimics what is seen in humans. Indeed, various animal models of neuropathic pain may be utilized experimentally, including transection of the sciatic nerve, partial sciatic nerve ligation, the spared nerve injury model, diabetic neuropathy, the vincristine-induced neuropathy model, and the SNL model. The various experiments contained in this dissertation make use of the SNL model. In the SNL model, the L_5 and L_6 spinal nerves are tightly ligated distal to the DRG. The major advantage of this model is that it leaves intact input from the L_4 branch of the sciatic nerve, thus the individual contributions of the injured and intact nerve components may be examined. The two major hallmarks of neuropathic pain, occurring after SNL, are tactile and thermal hypersensitivity.

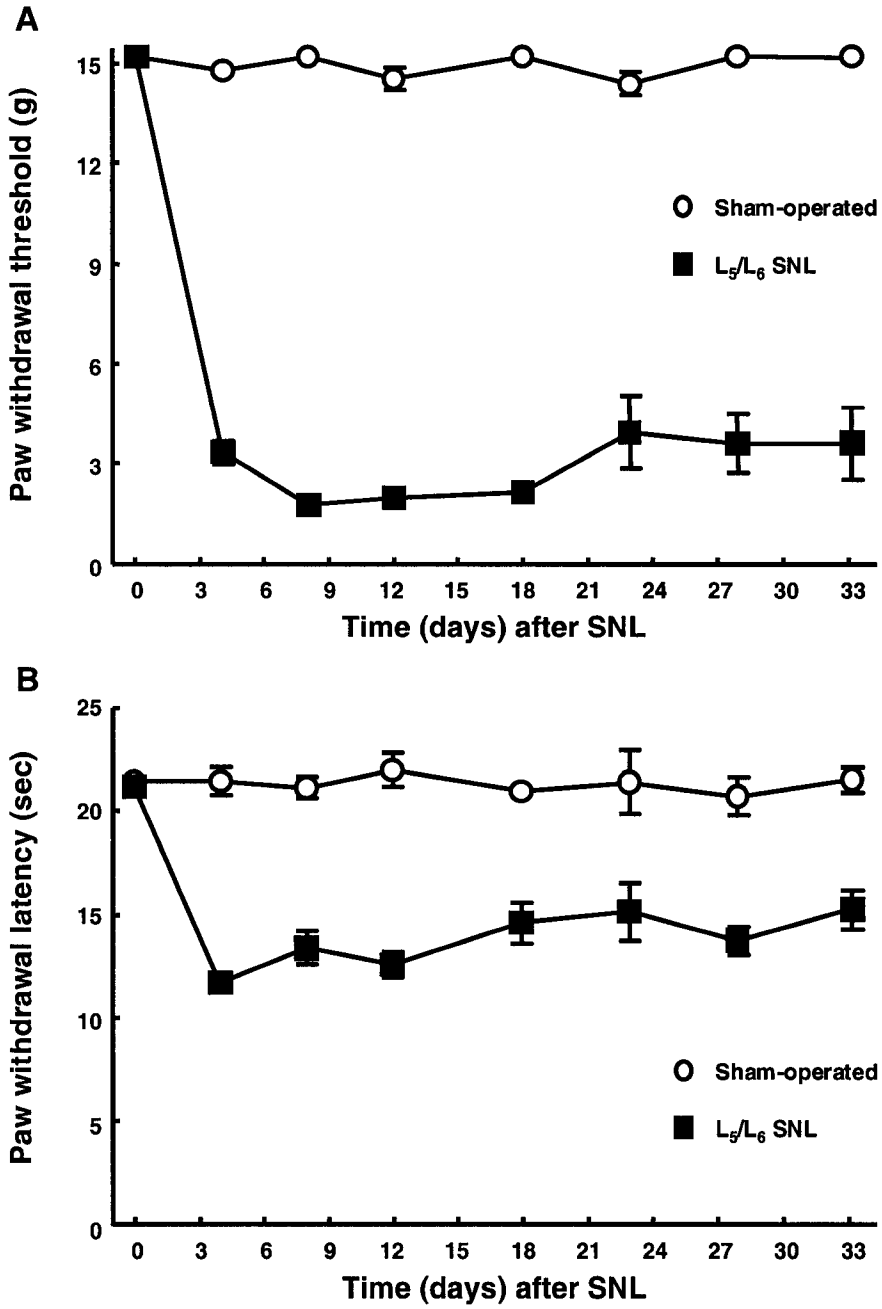

Figure 1.3. Time course of L_5/L_6 SNL-induced tactile and thermal hypersensitivity.

Ectopic Discharge

Ectopic afferent discharge is believed to be a major contributor to chronic pain resulting from peripheral nerve injury (Devor and Seltzer 1999). Within days of injuring

a peripheral nerve, damaged axons begin to behave in an abnormal manner. For example, some axons discharge action potentials spontaneously in the absence of any external stimuli and some respond to very moderate mechanical distortions of the injury site. These discharges have been found to result in plastic changes in the nervous system that lead to "central sensitization" of spinal neurons, which contributes to spontaneous pain and other abnormal sensations (Dubner and Ruda 1992; Gracely et al. 1992; Woolf and Salter 2000).

Ectopic discharge has been found to originate at the site of injury (the nerve end neuroma) and the DRG (Burchiel 1984; Govrin-Lippmann and Devor 1978; Kirk 1974; Study and Kral 1996; Wall and Devor 1983; Wall and Gutnick 1974a). Interestingly, it has been found that the contribution of the neuroma and the DRG is dependent upon the location of the nerve injury (Liu et al. 2000a; Liu et al. 1999). Following SNL, in which injury occurs close to the soma, the majority of the ectopic discharge originates in the DRG. In contrast, after sciatic nerve injury, which occurs farther away from the DRG, the neuroma is the major contributor of ectopic impulses. Further, it has been hypothesized that these sites are associated with the generation of discharges that can be initiated by noxious or non-noxious mechanical and chemical stimuli (Devor 1994; Fields and Rowbotham 1994).

Currently, the mechanisms that produce ectopic discharge are not well understood. A possible mechanism is the upregulation of ion channels after nerve injury. A number of researchers have found that blockers for sodium channels, potassium channels, and calcium channels all inhibit the generation of ectopic impulses (Devor et al.

1992; Kajander and Bennett 1992; Matzner and Devor 1994; Omana-Zapata et al. 1997a; Omana-Zapata et al. 1997b; Xiao and Bennett 1995; Xie et al. 1993). In particular, the increased density of sodium channels at the site of injury decreases the threshold for action potential generation, and, in turn, ectopic activity.

The onset of increased afferent discharge correlates well with the onset of tactile and thermal hypersensitivities in animal models (Han et al. 2000; Liu et al. 2000a; Liu et al. 2000b). An interesting observation made by Liu and colleagues (Liu et al. 2000a) demonstrated that ectopic discharge peaks 1 to 3 days after SNL and appears to return to control levels by days 7 to 9. In contrast, signs of neuropathic pain, including increased sensitivity to normally innocuous mechanical stimuli and to noxious thermal stimulation, continue at the same apparent intensity for many months after nerve injury, in spite of this decreased rate of ectopic discharge (Bian et al. 1999; Chaplan et al. 1994; Malan et al. 2000). These observations suggest that other mechanisms are likely to contribute to the maintenance of neuropathic pain (Sah et al. 2003), and more generally, that it is possible that the mechanisms responsible for the initiation of neuropathic pain are different than the mechanisms required to maintain it, or that perhaps, other mechanisms need to be evoked to maintain such pain.

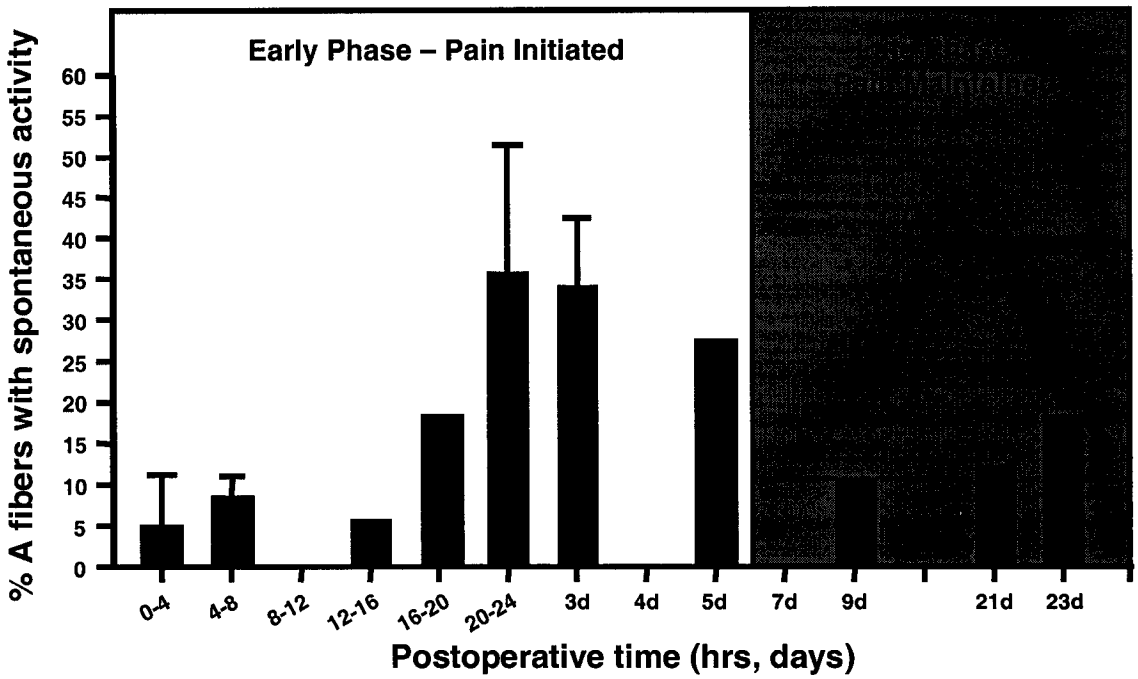

Figure 1.4. Time course of spontaneous A fiber activity following L_5/L_6 SNL. This figure has been redrawn from Devor and Colleagues (Liu et al. 2000a).

The RVM

As previously mentioned, persuasive evidence in the areas of electrophysiology, anatomy, and pharmacology has established the RVM as an integral relay station in the descending modulation of pain (Behbehani and Fields 1979; Fields et al. 1976; Fields and Basbaum 1978; Fields et al. 1977; Gebhart et al. 1983; Sandkuhler and Gebhart 1984). The RVM consists of various nuclei, specifically, the raphe magnus, the raphe pallidus, the lateral part of the paragigantocellular reticular nucleus, the magnocellular reticular nucleus, and the gigantocellular reticular nucleus. As a result of various studies, the RVM has been identified as a critical region in the processing and control of nociception (Fields and Heinricher 1989; Fields et al. 1995; Heinricher et al. 2001; Morgan et al. 1992).

A. ON and OFF Cells

Fields and colleagues first proposed the participation of three distinct cell types, localized within the RVM, in the modulation of nociceptive information. On the basis of individual neuronal firing responses to a noxious stimulus (see Figure 1.5), cells were classified as ON, OFF and neutral cells (Fields and Basbaum 1999; Fields et al. 1983; Fields and Heinricher 1985). Briefly, rats were anesthetized and placed in a stereotaxic apparatus. An electrode was placed in the RVM area to record the activity of single cells. The rat's tail was then placed above a projection heat lamp, which served as a radiant heat source. A transducer was attached to the tail and this provided a voltage pulse when the tail flick occurred. Neutral cells show a wide range of firing patterns, but do not appear to play a role in the modulation of pain transmission, however it is possible that they could be a subtype of ON or OFF cells. ON cells are normally silent and accelerate their firing immediately before the tail flick occurs. Lastly, OFF cells are normally active and exhibit an abrupt cessation of activity just prior to the nociceptive tail reflex.

Depicted in Figure 1.6 is one proposed circuitry scheme for pain modulation in the RVM, based on electrophysiological responses to a noxious thermal stimulus. After the previously mentioned studies were done, it was found that activation of OFF cells (through disinhibition) correlated with inhibition of nociceptive input and nocifensive responses (Fields and Basbaum 1999; Fields et al. 1983; Fields and Heinricher 1985; Heinricher et al. 1992). By deduction, it is then unlikely that ON cells contribute to

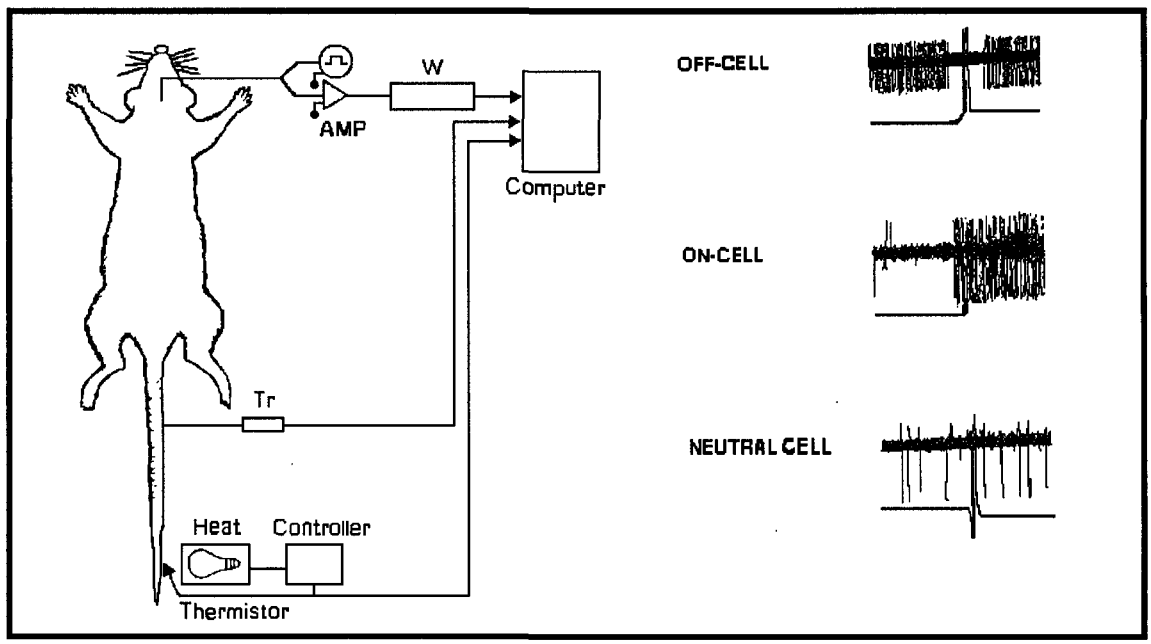

Figure 1.5. This figure depicts the experimental setup used by Fields in order to characterize the various cell types in the RVM (left side). To the right, representative electrophysiological traces of the three major cell types in the RVM are shown. This figure has been redrawn from Fields and Colleagues (Fields et al. 1983; Heinricher and McGaraughty 1999).

the inhibition of nociception, since their rate of firing increases prior to the tail flick response. It is rather likely that ON cells facilitate nociceptive processing through both local interactions within the RVM and descending systems projecting to the spinal cord (Fields et al. 1983; Fields and Heinricher 1985; Fields et al. 1991; Heinricher et al. 1992; Heinricher and Roychowdhury 1997). The terminals of projection neurons from the RVM, which comprise the DLF, are concentrated in dorsal horn laminae (I, II, and V) and contain both terminals of C fibers and cell bodies of spinothalamic tract neurons (Fields 1987). It is believed that opioids produce antinociception in the RVM through the disinhibition of OFF-cells (see (Fields and Basbaum 1999) for review). Inhibition of ON cells appears to be unnecessary for the antinociceptive effect of opioids administered in the RVM (Heinricher et al. 1994).

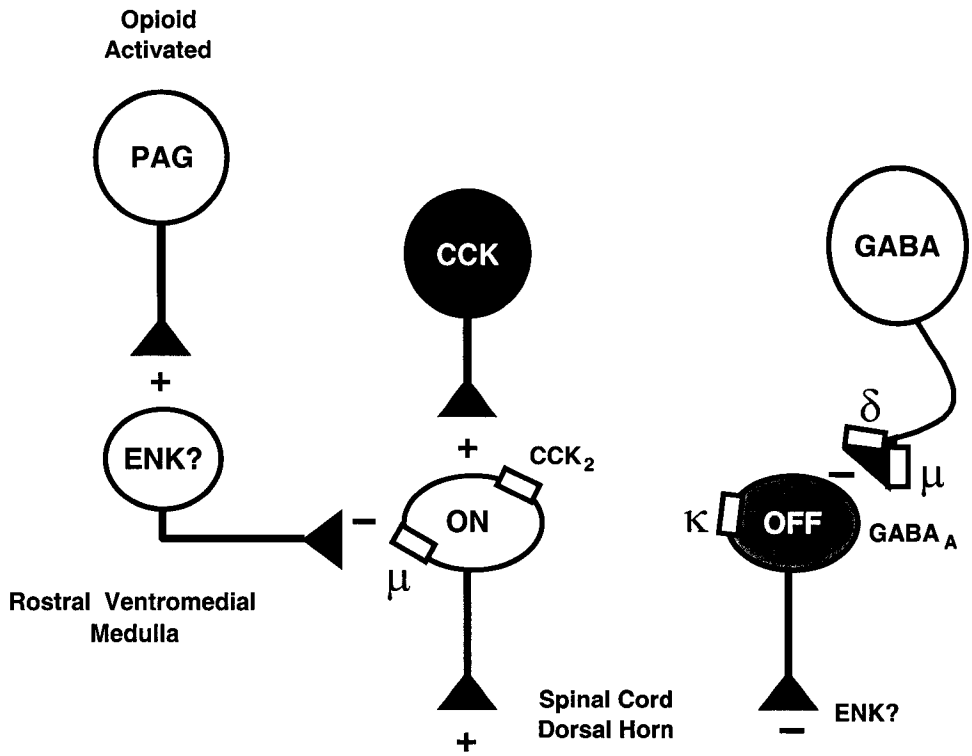

Figure 1.6. A proposed circuit in the RVM for pain modulation. OFF cells pause immediately prior to a withdrawal response to noxious stimulus and ON cells accelerate their firing. Neurons activated by opioids in the PAG may stimulate enkephalinergic projection neurons, which inhibit the firing of ON cells.

As part of the descending pain transmission system, ON cells project from the RVM to the spinal cord dorsal horn laminae and are likely to interact with a variety of neurons in the RVM (Fields et al. 1983; Fields and Heinricher 1985; Fields et al. 1991; Heinricher et al. 1992; McNally 1999). Fields and colleagues (Barbaro et al. 1989; Heinricher et al. 1989) also found that the pattern of ON and OFF cell firing is reciprocating and that, further, tail flick latency correlated with the activity of the RVM cells. When OFF cell activity was increased, the tail flick latency was longer, and when ON cell activity was increased, the tail flick latency was shorter. The results from these studies led Fields and colleagues to propose that ON cells facilitate and OFF cells inhibit

the transmission of nociceptive input from the spinal cord (Heinricher et al. 1989). Therefore, it would follow that manipulations that increase nociceptive responsiveness, indicating facilitation, would also increase ON cell activity. In fact, it was found that hyperalgesia and increased activity of RVM ON cells is associated with naloxone-precipitated opioid withdrawal (Bederson et al. 1990; Kim et al. 1990). Additionally, this hyperalgesia was blocked by intra-RVM injection of lidocaine, suggesting the involvement of neuronal activity in the RVM (Heinricher and Roychowdhury 1997; Kaplan and Fields 1991). These data suggest that supraspinal sites can contribute to the development or maintenance of chronic pain states.

B. Supraspinal modulation and descending facilitation of neuropathic pain

Anatomical and pharmacological evidence suggest that facilitatory and inhibitory pathways are distinct and may actually be activated at the same time in the transmission of acute pain signals. However, this balance may be broken in times of chronic pain, when plasticity in the RVM and other sites may lead to ON cell-mediated sustained facilitatory output, that drives the expression of exaggerated pain.

There is a myriad of evidence that supports the notion that manifestations of chronic pain require this supraspinal participation. For example, exaggerated responses to mechanical or cold, but not to noxious thermal, stimulation, in rats with peripheral nerve injury or hind paw inflammation were eliminated by thoracic spinal cord transection (Bian et al. 1998; Kauppila et al. 1997; Kauppila et al. 1998; Sun et al. 2001; Sung et al. 1998). In addition, selective disruption of the DLF ipsilateral to SNL abolished tactile and thermal hypersensitivity, while the same disruption did nothing in

SHAM rats (Ossipov et al. 2000a). Further, opioid- and SNL-induced abnormal pain were abolished by lesions of the DLF, as well as by RVM injection of lidocaine (Kovelowski et al. 2000; Pertovaara et al. 1996; Vanderah et al. 2001b). These observations give further support to the notion that behavioral signs of neuropathic pain depend on descending facilitation of spinal nociceptive input from the RVM, since it is the principal source of projections from the DLF (Fields and Basbaum 1999; Fields et al. 1991; Fields and Rowbotham 1994).

The role of the DLF in the transmission of descending inhibitory signals has been well established (Fields and Basbaum 1978; Fields and Basbaum 1999). Specifically, disruption of DLF fibers eliminates the antinociceptive effects of electrical stimulation or injections of morphine into the RVM or PAG (Fields et al. 1991; Fields and Rowbotham 1994). Since neurons in the RVM are both inhibitory and facilitatory, it is then reasonable that the DLF is also a conduit for descending facilitatory influences from the RVM. Electrical stimulation in the DLF can lead to excitement of lamina I dorsal horn neurons (McMahon and Wall 1983; McMahon and Wall 1988). Results from spinal cord block confirmed that part of the excitation is due to descending, not ascending, fiber activation (McMahon and Wall 1988). These results are contradictory to results from other studies that have suggested the importance of the ventrolateral funiculus (VLF) as a fiber tract mediator of RVM descending facilitation. For instance, RVM-mediated facilitation of nociceptive reflexes and neuronal activity in the dorsal horn is blocked by lesions of the VLF, but not by the DLF (Zhuo and Gebhart 1992; Zhuo and Gebhart 1997). Similarly, the RVM injection of neurotensin produced either inhibition or

facilitation of dorsal horn responses to thermal stimuli with DLF lesions blocking the inhibitory effect of neurotensin and VLF lesions blocking the facilitatory effect (Urban and Gebhart 1997). These conflicting experimental data may partially be explained by the observation that previously inhibitory stimulation in the RVM becomes facilitatory after DLF transection, suggesting that inhibitory input may dominate over facilitation (Zhuo and Gebhart 1992; Zhuo and Gebhart 1997). More anatomically derived studies need to be done, however, to elucidate individual contributions of the DLF and the VLF. However, from the results of the previous studies, it seems that descending inhibitory and facilitatory systems exist in both the ventral and dorsal parts of the spinal cord.

C. CCK as a mediator of descending facilitation from the RVM

CCK is a regulatory peptide hormone, predominantly found in the gastrointestinal tract, and a neurotransmitter present throughout the nervous system. In 1928, CCK was first discovered in the gastrointestinal tract (Ivy and Oldberg 1928) and was found to regulate motility, pancreatic enzyme secretion, gastric emptying, and gastric acid secretion. It was later found in the nervous system (Beinfeld et al. 1981; Beinfeld and Palkovits 1982; Hokfelt et al. 1985; Vanderhaeghen et al. 1975). In the nervous system, CCK is a mediator of anxiogenesis, satiety, nociception, thermoregulation, and memory and learning processes.

The biological actions of CCK are mediated by two G protein coupled receptor subtypes, whose names have recently been changed from CCK_A and CCK_B, to CCK_1 and CCK_2 (Vanhoutte et al. 1996). The CCK_1 subtype is predominant in the visceral organs of the digestive system and the CCK_2 subtype is predominant in the central nervous

system (Baber et al. 1989; Innis and Snyder 1980; Moran et al. 1986; Wank et al. 1994). In the nervous system, CCK is mainly present in the form of the carboxyterminal octapeptide CCK_8 (Rehfeld 1978) and CCK-like immunoreactivity and its distribution along with that of its receptor subtypes shows considerable overlap with that of endogenous opioids (Larsson and Stengaard-Pedersen 1981) and their receptors (Ghilardi et al. 1992) in the spinal cord and brain. As previously mentioned, one of the many functions of CCK is to mediate nociception. Indeed, immunoreactivity for CCK has been observed in the PAG, the raphe nuclei, and the medullary reticular formation (Baber et al. 1989; Hokfelt et al. 1988), structures known to modulate nociception.

With this in mind, it is possible that CCK can join substances such as neurotensin, excitatory amino acids, and nitric oxide as potential candidates that drive descending facilitation from the RVM. In earlier years, CCK was found to attenuate antinociception produced by β-endorphin and morphine (Faris et al. 1983; Itoh et al. 1992). This antiopioid effect of CCK seems to be tonic as CCK receptor antagonists enhance endogenous (Xu et al. 1997) and exogenous opioid-induced antinociception (Wiesenfeld-Hallin and Xu 1996). Recently, it has been observed that RVM injection of CCK_8 produces reversible tactile and thermal hypersensitivity in naïve rats (Kovelowski et al. 2000) and prevents morphine-induced antinociception and OFF cell activation (Heinricher et al. 2001). In addition, microinjection of a CCK_2 antagonist, L365,260, blocks SNL-induced tactile and thermal hypersensitivity (Kovelowski et al. 2000). These observations, along with the observation that binding of CCK_8 to the CCK_2 receptor reduces the binding affinity of MOR ligands (Wang and Han 1990), suggests that the

interaction between μ opioid and CCK systems may perhaps contribute to the activation of descending facilitatory systems to maintain SNL-induced, as well, as opioid-induced hypersensitivity.

Upregulation of dynorphin as a consequence of descending facilitation

Dynorphin was originally identified as an endogenous kappa opioid receptor agonist (Goldstein et al. 1979) with an antinociceptive role (Ossipov et al. 1996). There is considerable evidence, however, that spinal dynorphin and its fragments play a pronociceptive role in neuropathic pain states (Caudle and Isaac 1988; Cho and Basbaum 1989; Draisci et al. 1991; Dubner and Ruda 1992; Nahin et al. 1992; Stanfa and Dickenson 1995; Wang et al. 2001). Nerve injury results in an upregulation of spinal dynorphin that peaks ten days after injury, indicating a possible role of spinal dynorphin in the maintenance, rather than the initiation, of neuropathic pain.

Additionally, spinal administration of antiserum to dynorphin blocks tactile and thermal hypersensitivity in mice ten, but not two, days following nerve injury, which, again, supports the role of dynorphin in the maintenance of neuropathic pain. In response to nerve injury, mice with a deletion of the gene encoding prodynorphin initially develop neuropathic pain, but the pain resolves within seven days post-injury. Wild type mice exhibited enhanced pain throughout the course of the experiments. The experimental manipulations that block descending facilitation also block the upregulation of spinal dynorphin (Gardell et al. 2003; Gardell et al. 2002).

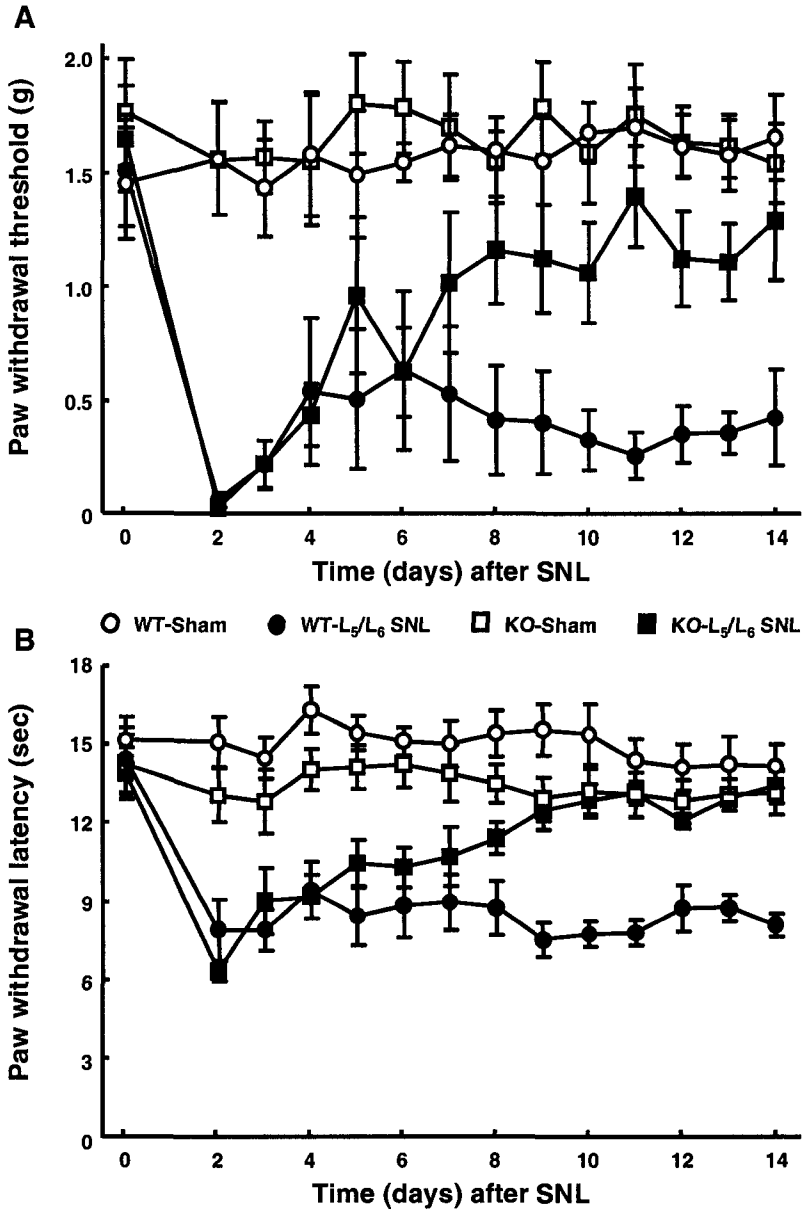

Figure 1.7. Response thresholds to innocuous mechanical (Panel A) and noxious thermal (Panel B) stimuli in wild-type (WT, circles) and prodynorphin knock-out (KO, squares) mice after sham (open symbols) or SNL surgery (closed circles). Neither WT nor KO mice show changes in mechanical or thermal thresholds from preinjury baselines after sham surgery throughout the test period. After SNL, both WT and KO mice developed decreased mechanical and thermal thresholds by post-surgery day 2. Whereas the decreased mechanical and thermal thresholds were maintained in WT mice (closed circles), thresholds in KO mice showed a progressive reversal to preinjury baseline levels (closed squares). By day 10 after SNL, both mechanical and thermal thresholds in KO mice were not significantly different from those exhibited by SHAM KO (or WT) mice done in parallel ($p<0.05$). (Figure contributed by Luis Gardell)

Use of a targeted toxin to destroy MOR-expressing cells

SAP is a large RIP derived from the seeds of the soapwort plant, *Saponaria officinalis*. The RIPs are divided into two types, 1 and 2. SAP, a type 1 RIP, irreversibly inhibits protein synthesis by rendering the 28S subunits of ribosomes unable to bind to elongation factor 2 (Endo et al. 1988; Stirpe et al. 1983). Specifically, it acts as an N-glycosidase catalytically cleaving a particular adenine (A^{4324} in the rat) of the 28S subunit ribosomal RNA (Endo et al. 1987; Endo and Tsurugi 1988).

Unlike type 2 RIPs, such as ricin, SAP lacks a cell-binding B chain, so it cannot cross the cellular membrane and, thus, it exhibits limited toxicity to whole cells. This property makes it considerably safer to handle and, additionally, it can be used in its native form without the toxicities associated with double-chain toxins, such as ricin. Thus, when targeted to cells, as in the synthesis of immunotoxins and ligand-toxin conjugates, SAP is a very efficient cytoxic agent.

In experiments contained in this dissertation, the RIP, SAP, was conjugated to DERM, a potent μ opioid agonist derived from the skin of South American frogs, belonging to the genus *Phyllomedusa* (Erspamer and Melchiorri 1980). In short, the MOR serves as a portal of entry for the bulky SAP protein. DERM binds to cells expressing the MOR, and, via a β-arrestin mediated internalization, the conjugate is internalized into clathrin-coated pits. Opioid μ receptors are then recycled or degraded and SAP is free to cause cytotoxicity.

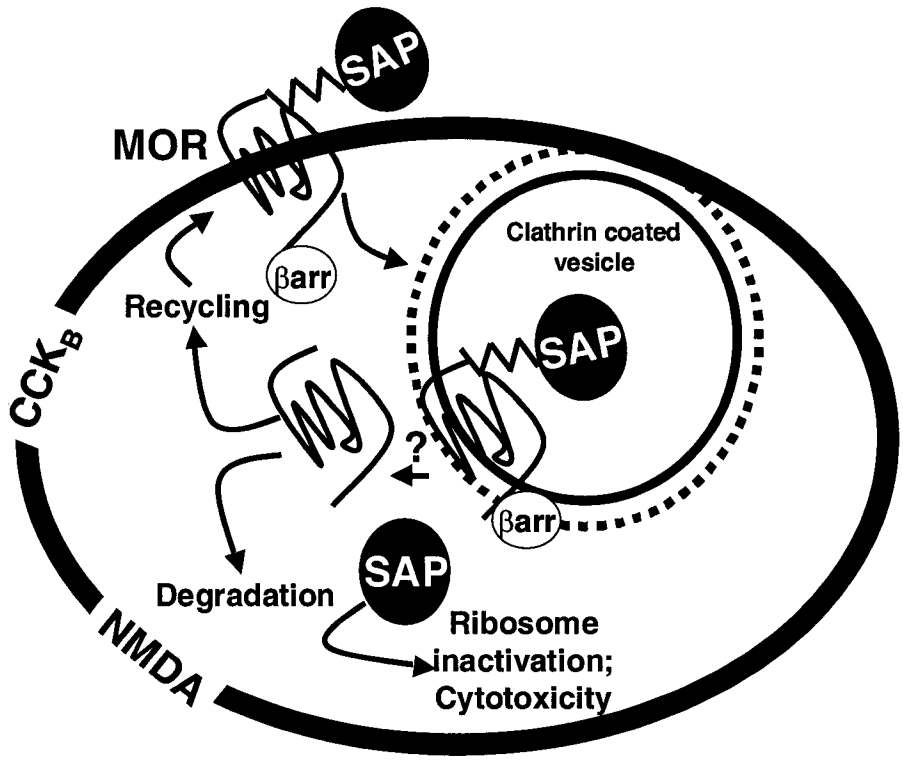

Figure 1.8. Schematic representation of the mechanism of action of DERM-SAP.

Summary of research goals

As discussed previously, treatments for chronic pain are rarely efficacious and, presently, there exists a huge need for the development of novel drug therapies. Data from recent studies have implicated the requirement of supraspinal participation in neuropathic pain. The development of cytotoxin conjugates that target specific cell types has enabled researchers to look at the contribution of certain cells to the development of conditions such as neuropathic pain. With this in mind, the focus of this dissertation will be to explore the role of descending facilitation from supraspinal sites, specifically the RVM, in the maintenance of neuropathic pain. Specifically, two hypotheses will be tested. First, the presence and activity of descending pain facilitation cells in the RVM are required for the expression of experimental neuropathic pain. Specifically, the

destruction of these cells, presumably those expressing the MOR, will both prevent and reverse SNL-induced neuropathic pain. Second, RVM CCK activates RVM descending facilitatory cells to drive the expression of neuropathic pain states.

CHAPTER II: MATERIALS AND GENERAL METHODS

Animals Used

Unless otherwise indicated the experiments contained in this dissertation were carried out using male Sprague-Dawley rats (225-300g; Harlan; Indianapolis, IN). All animals were maintained on a 12/12 hr light/dark cycle and provided food and water *ad libitum* except during the experimental procedures. All experiments, involving animals, were performed under an approved protocol in accordance with institutional guidelines and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

RVM Drug Microinjection

All rats were prepared for bilateral RVM drug administration as previously described (Kovelowski et al. 2000). Anesthetized (ketamine/xylazine 100 mg/kg, i.p.) animals were placed in a stereotaxic headholder. For intracranial bilateral drug administrations, the skull was exposed and two 26-gauge guide cannula separated by 1.2 mm (Plastics One Inc., Roanoke, VA) were directed toward the lateral portions of the RVM (AP-11.0 mm from bregma, L ± 0.6 mm, DV-8.5 mm from the base of skull). The guide cannulae were secured to the skull and the animals were allowed to recover for five days post-surgery prior to any drug administration. Drug administrations into the RVM was performed by slowly expelling 0.5 μL of drug solution through a 33-ga injection cannula inserted through the guide cannula and protruding an additional 1 mm into fresh brain tissue. DERM, SAP, and DERM-SAP were administered as a single dose of 3

pmol into the RVM (1.5 pmol in 0.5 μL each side). Lidocaine was given in a dose of 4%w/v in 0.5 μl. CCK_8 was given in a dose of 30 ng in 0.5 μl.

Intrathecal Drug Administration

Intrathecal (i.th.) injections (5.0 μl) were made into the subarachnoid space (L_5-L_7) in unanesthetized mice using a modified version (Porreca and Burks 1983) of a previously described method (Hylden and Wilcox 1980). While under halothane anesthesia, rats were implanted with i.th. catheters (PE-10, 7.5 cm) as described previously (Yaksh and Rudy 1976) for drug administration at the level of the lumbar spinal cord. Test compounds were injected through the i.th. catheter in a volume of 5.0 μl followed by a 9.0 μl saline flush.

Spinal Nerve Injury

SNL injury was induced using the procedure of Kim and Chung (Kim and Chung 1992). Rats were anesthetized with halothane vaporized in 95% O_2/5%CO_2 "to effect". After surgical preparation of the rats and exposure of the dorsal vertebral column from L_4 to S_2, the exposed L_5 and L_6 spinal nerves were tightly ligated with 4-0 silk suture. The incision was closed and the animals were allowed to recover for five days. Rats that exhibited motor deficiency or failure to exhibit subsequent increased sensitivity to innocuous mechanical stimulation were excluded from further testing (less than 5% of the animals were not used). Sham control rats underwent the same operation and handling as the experimental animals, but without SNL. Evaluation of response thresholds were performed on the hindpaw ipsilateral to SNL or sham-SNL using the procedures described in the Behavioral Assessment section.

Spinal DLF lesions

Bilateral spinal lesions at the T_8 level were performed in naive rats under halothane anesthesia. A laminectomy was made at the T_8 level to expose the spinal cord. Lesions of the DLF were performed by crushing the area with fine forceps. Sham DLF surgery was performed by exposing the vertebrae and performing the laminectomy, but without cutting neuronal tissue. Hemostasis was confirmed and the wound over the exposed spinal cord was packed with gelfoam and closed. All lesions were verified histologically at the termination of the experiment. Only rats with appropriately placed DLF lesions were included in the subsequent data analysis.

Radioligand Binding

Radioligand competition analysis to determine the affinity of DERM-SAP for the rat mu and delta opioid receptors was carried out using crude membrane preparations from NG 108-15 (expresses mouse opioid delta receptors) and from HN9.10 transfected cells that express the MOR. All radioligand binding assays were carried out in duplicate in 50 mM Tris, pH 7.4, in the presence of 0.5 mg/ mL bovine serum albumin (BSA) and protease inhibitors (30 μM bestatin, 10 μM captopril, 0.37 U/mL bacitracin and 0.1 mM phenylmethylsulfonylfluoride (PMSF)). All reactions were carried out at 25°C for 3 hr in a total reaction volume of 1 mL. At least 10 concentrations of DERM (10^{-14} M to 10^{-5} M) or DERM-SAP (10^{-14} M to $10^{-7.5}$ M) were used in the competition analysis. The concentrations of [^{3}H]DAMGO (2.2 nM) and of [^{3}H]pCl-DPDPE (1.8 nM) to label mu receptors in the transfected HN9.10 cells and delta receptors in the transfected NG-108 cells were based on the K_D values of the radioligands determined from saturation

analysis. The reaction was terminated by rapid filtration through Whatman GF/B filters presoaked in polyethyleneimine, and washed with 3 X 4 mL of ice-cold PBS. Non-specific binding was defined as that in the presence of 10 μM naloxone. Radioactivity on the filters was determined by liquid scintillation counting. Data were analyzed by non-linear regression analysis using GraphPad Prism (GraphPad, Inc. San Diego, CA). The K_i value(s) for each ligand was calculated from the IC_{50} value(s) based on the Cheng and Prusoff equation from at least 3 independent experiments.

Tissue Preparation

For the ISH experiments using cDNA probes, rats were deeply anesthetized with ketamine and perfused transcardially with phosphate-buffered saline (PBS) treated with 0.1% diethyl pyrocarbonate (DEPC), followed by 4% paraformaldehyde. Whole rat brains were removed and postfixed in fixative overnight, cryoprotected in 30% sucrose in PBS treated with 0.1% DEPC, and stored at 4°C. Frozen frontal sections (20 to 40 μm) were prepared from the brain stem caudal to the site of incision of the cannulae and mounted on positively charged slides.

For the ISH experiments using riboprobes, there were a few slight differences in the tissue preparation protocol. Whole rat brains were removed and postfixed in fixative for 1 hour, instead of overnight and frozen frontal sections were cut strictly at a thickness of 20 μm and stored at -80°C.

For the immunohistochemistry experiments, treated rats were deeply anesthetized with ketamine and perfused transcardially with 200 ml of PBS, pH 7.4, containing heparin (1500 IU/ l), followed by 500 ml of cold 4% paraformaldehyde. After perfusion

the spinal cords were isolated and post-fixed for 4 hr in 4% paraformaldehyde and then cryoprotected with 30% sucrose in PBS overnight at 4°C. Frontal frozen sections (40 μm) were prepared from the lumbar enlargement of the spinal cord.

ISH of MOR mRNA using cDNA probes

A single-stranded, fluorescein (FL)-labeled partial cDNA probe corresponding to nucleotides 628-965 of the coding region of the rat opioid mu receptor cDNA was synthesized using the polymerase chain reaction (PCR) by using a 100:1 ratio of antisense (3') to sense (5') primers and a mixture of FL-labeled dUTP/ unlabeled dNTP. The PCR reaction was carried out for 30 cycles (45sec @ 95°C/60sec @ 60°C/2 min@ 72°C), the product purified by ethanol precipitation. The probe was reconstituted in RNase free 1X TE and analyzed by agarose gel electrophoresis and Southern transfer to determine the yield. A corresponding sense probe was synthesized under the same conditions except using a 1:100 ratio of antisense to sense primers, with or without fluorescein-dUTP. ISH was carried out using both standard and proprietary reagents from InnoGenex (San Ramon, CA) under conditions that were carefully optimized for our cDNA probe.

Briefly, mounted tissue sections were post-fixed with 1% formaldehyde, then deproteinated with proteinase K (100 μg/mL). Following a second 1% formaldehyde treatment, the sections were heated to 80°C for 5 min in a hybridization buffer containing formamide, dextran sulfate and the FL-labeled probe (typically 1:2 to 1:5 dilution), allowed to cool to 37°C and the hybridization continued for 16 hr at 37°C. The sections were washed extensively with PBS/ 0.1% Tween-20, pre-blocked with a buffer

containing casein and sodium-azide, then incubated with a biotinylated anti-FL antibody. The sections were washed, followed by incubation with a 1:3 dilution of streptavidin-alkaline phosphatase conjugate. The sections were developed by incubating with Fast Red for 1 hr, counter-stained with Mayer's Hematoxylin, and mounted with SuperMount. RNase pretreatment was carried out by incubating sections with 200μg/mL RNase A in 100 mM Tris, pH 8.0 and 0.5M NaCl for 60 min at 37°C, followed by extensive washing prior to hybridization.

Computer-assisted mapping was performed under bright field illumination of coronal sections using an image-combining computer microscope using Neurolucida software (Microbrightfield Inc., Baltimore, MD). The boundaries of the facial nuclei and pyramidal tracts were manually traced using a Nikon 4X plan apo objective. The sections were then systematically scanned for labeled neurons using a Merzhauser motorized stage and a Nikon 40X plan apo objective. Bright-field images of tissue sections were acquired with a Nikon E800 microscope outfitted with a Hamamatsu C5810 color CCD camera and a 40X plan fluor 0.75 NA objective lens. The digitized output of the camera was acquired through a SCSI interface to a microcomputer.

Subcloning and *in vitro* transcription of riboprobes

Sense and antisense FL-labeled cRNA probes were prepared from a 338-bp fragment of rat MOR cDNA (nucleotides 628 to 965 of the coding region) that was subcloned into a pCRII vector (Invitrogen). The plasmid DNA was linearized with BAMHI for in vitro syntheses of the antisense probe (T3 promoter) or sense probe (T7 promoter), respectively, in the presence of FL-labeled UTP.

Sense and antisense digoxigenin (DIG)-labeled cRNA probes were prepared from a 187-bp fragment of rat CCK_2 receptor cDNA (nucleotides 117 to 302 of the coding region) that was subcloned into a pCRII vector (Invitrogen). The plasmid DNA was linearized with EcoRI for in vitro syntheses of the antisense probe (T3 promoter) or sense probe (T7 promoter), respectively, in the presence of DIG-labeled UTP.

The labeling efficiency of both probes was routinely checked by spotting a series of dilutions onto a positively charged nylon membrane and comparing the intensity of the spots to the known concentrations of control RNA. The ISH procedures were performed as described in the Nonradioactive ISH Application Manual (Roche Diagnostic Corp., Indianapolis, IN) with minor modifications.

ISH of Opioid Mu Receptor and CCK_2 receptor mRNA using riboprobes

Briefly, mounted tissue sections were thawed and then incubated consecutively with PBS (twice, 5 min each), PBS containing 0.1M glycine (twice, 5 min each), and PBS containing 0.3% Triton X-100 (15 min). Sections were washed once again with PBS (twice, 5 min each) and then deproteinated for 30 min at 37°C with proteinase K (1 μg/mL) in 100 mM Tris-HCl buffer containing 50 mM of EDTA (pH 8.0). Following a postfixation step with 4% formaldehyde, the sections were washed with PBS and then acetylated with 0.1 M triethanolamine (TEA) buffer, pH 8.0 containing 0.25% (v/v) acetic anhydride. The sections were prehybridized in a buffer containing formamide and tween-20 for at least 10 min. They were then incubated with a 1:50 dilution of probe for 16 hrs at either 42°C (mu riboprobe) or 50°C (CCK_2 riboprobe) in a hybridization buffer containing 0.1M Tris-HCl (pH 7.6), 4 x SSC, 50% deionized formamide, 1 x denhardt,

10% dextran sulphate, 150 μg/mL yeast tRNA and 150 μg/mLsheared salmon sperm DNA.

Coverslips were removed from slides by immersing in 2 x SSC. The sections were then washed consecutively with 2 x SSC and 1 x SSC for 30 min each at 37°C. To digest any single-stranded RNA probe, sections were then incubated with 20 μg/mL Rnase A in 10 mM Tris buffer containing 500 mM NaCl and 1 mM EDTA (pH 8.0) for 30 min at 37°C. Sections were then washed for 1 hr with 0.1 x SSC at 37°C.

For visualization of FL- and DIG-labeled mRNA, sections were washed twice (5 min each) in 100 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl 1 and then blocked in the above buffer containing 0.1% Triton X-100 and 2% normal sheep serum. The blocking solution was decanted and the sections were incubated for 2 hrs with a 1:500 dilution of either sheep anti-FL conjugated to alkaline phosphatase (AP) (for sections hybridized with the mu riboprobe) or sheep anti-DIG conjugated to AP (for sections hybridized with the CCK_2 riboprobe) in the same buffer. The slides were washed twice with 100 mM Tris-HCl buffer (ph 7.5) containing 150 mM NaCl for 10 min each, equilibrated in substrate buffer containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$ for 10 min, and then incubated overnight in the dark with Fast Red in Tris buffer. The color development was stopped with 10 mM Tris-HCl buffer containing 1 mM EDTA. Nuclear counterstaining was done with Mayer's Hematoxylin. The slides were mounted with Immunomount (ThermoShandon).

Dynorphin immunoassay

Tissues that were stored at -70°C were thawed and placed in 1.0 N acetic acid, disrupted using a Polytron homogenizer and incubated for 30 min at 95°C. After centrifugation at 14,000 x g for 20 min (4°C) the supernatant was lyophilized and then stored at -70°C. Protein concentrations were determined using the bichinchoninic acid method with bovine serum albumin as a standard. Immunoassay was performed with a commercial enzyme immunoassay system using an antibody specific for dynorphin $A_{(1\text{-}17)}$ (Bachem, San Carlos, CA). Standard curves were constructed and the dynorphin content determined using GraphPad Prizm (GraphPad Inc, San Diego, CA).

CGRP release assay

Immunoreactive CGRP release was measured using a modified version (Gardell et al. 2003; Gardell et al. 2002) of a previously described method (Chen et al. 1996). Minced tissue samples were placed in a 1 cc chamber and continuously superfused with oxygenated modified Kreb's buffer (135 mM NaCl, 3.5 mM KCl, 1 mM MgCl2, 20 mM NaHCO3, 1 mM NaHPO4, 2.5 mM CaCl2, 3.3 mM dextrose, 0.1 mM ascorbic acid, 10 mM thiorphan and 0.1 % bovine serum albumin) maintained at 37°C, pH 7.4. The buffer was maintained at a flow rate of 0.5 ml/min with a Brandel Superfusion Pump (Brandel, Gaithersburg, MD). The tissue was allowed to equilibrate for 45 minutes. Superfusate was collected in 3 minute intervals into test tubes using a fraction collector (Gilson, Middleton, WI). A total of 5 fractions (15 min) were collected prior to the addition of capsaicin to the perfusate. Capsaicin was added to the perfusate to result in a

concentration of 1 μM for 6 minutes (2 fractions). The superfusate was then collected for an additional 27 minutes (9 fractions).

Radioimmunoassay for CGRP in superfusate

Each tube of superfusate obtained from the release assay was pre-incubated with 100 μl of a C-terminally directed anti-CGRP antibody (Peninsula/Bachem, San Carlos, CA) for 24 hr at 4°C. The samples were each mixed with 100 μl of [^{125}I-Tyr0]-CGRP$_{28-37}$ (at 20,000–25,000 cpm per assay tube) and 50 μl of goat anti-rabbit antiserum coupled to ferric beads and incubated for an additional 24 hr. The [^{125}I]CGRP bound to the CGRP antibody was separated from the free tracer through immunomagnetic separation (PerSeptive Diagnostics, Cambridge, MA). The immunoprecipitates were determined by gamma counting. Standard curves were generated and CGRP content was determined through logit-log analysis. This assay has a minimal detection limit of 1 to 3 fmol/tube. The CGRP antiserum used in these experiments binds near the C-terminal end of CGRP and does not cross-react with CCK, neuropeptide Y, or other peptides with similar C-terminal residues (Gardell et al. 2003; Gardell et al. 2002). The CGRP-ir concentrations were plotted against time in 3 minute intervals. Evoked release was calculated as the total amount of CGRP released (i.e., CGRP-ir) in response to the capsaicin infusion above the basal release of CGRP-ir.

Immunostaining for the MOR

The sections were immunolabeled with a rabbit antiserum against the rat MOR (courtesy of Dr. Robert Elde). Briefly, the spinal cord sections were rinsed twice for 5 min each in PBS and then preincubated with PBS containing 4% normal goat serum,

0.3% Triton X-100, and 1% bovine serum albumin for 30 min at room temperature. The sections were then incubated with the primary antiserum diluted in the preincubation buffer overnight at 4°C at a 1:20,000 dilution). The sections were washed three times for 10 min each in PBS, followed by incubation with a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at 1:1000 in PBS with 0.25% bovine serum albumin and 0.1% Triton X-100 (PBS-BT) for 60 min at room temperature. Sections were washed three times for 10 min each in PBS and stained with the avidin-biotin complex (ABC kit; Vector Laboratories); sections were incubated with an avidin-biotinylated horseradish peroxidase complex diluted 1:500 in PBS-BT for 2 hr at room temperature, washed three times for 10 min each in PBS, and developed with a solution of diaminobenzidine and H_2O_2 (FAST DAB SETS; Sigma, St. Louis, MO) maintained uniformly throughout the experiments. Sections from control and treated animals were processed in parallel under identical experimental conditions. The sections were washed and mounted on glass slides, air-dried overnight, rinsed in histological clearing solvent, and coverslipped with DPX.

Behavioral Assessments

Assessment of sensitivity to non-noxious mechanical stimuli

Rats were allowed to acclimate within plexiglass enclosures for approximately 20 min. A series of calibrated (1.56-15.0 g for rats, logarithmically spaced) von Frey filaments were applied to the plantar aspect of a hindpaw until a response was elicited. Paw withdrawal thresholds to probing were determined according to the method previously described (Chaplan et al. 1994). These data were represented as mean paw

withdrawal threshold ± SEM. Paw withdrawal thresholds were determined to the nearest 0.1 g before treatment (baseline) and again, either daily during, or after the termination of, the treatment regimen.

Assessment of sensitivity to noxious thermal stimuli

Radiant heat paw flick test

Rats were allowed to acclimate within plexiglass enclosures, on a surface maintained at 30°C, for approximately 20 min. A radiant heat source (i.e., high intensity projector lamp) was activated with a timer and focused onto the plantar surface of the hindpaw. A motion detector that halted both lamp and timer when the paw was withdrawn determined paw withdrawal latency. A maximal cut-off of 40 sec was employed to prevent tissue damage. A significant reduction in paw withdrawal latency from baseline value was interpreted as thermal hypersensitivity. Pairwise comparisons were performed with Student's t-test and significance was set at $p \leq 0.05$.

Tail flick test

Nociceptive testing was also performed using the tail-flick test with water at 52°C employed as the noxious stimulus. Tail flick latencies were determined to the nearest 0.1 s before treatment and in response to a challenge dose of test compound. A cut-off latency of 10 sec was employed in order to prevent tissue injury.

CHAPTER IH: SELECTIVE ABLATION OF μ OPIOID RECEPTOR-EXPRESSING CELLS IN THE RVM BY DERMORPHIN-SAPORIN PREVENTS AND REVERSES SNL-INDUCED NEUROPATHIC PAIN

Introduction

The presence of endogenous pain-modulating mechanisms is well established. Although pain inhibitory systems projecting from the brainstem to the spinal cord offer obvious survival advantages, the role of descending projections that facilitate pain transmission is less clear (Mason 1999; Watkins and Mayer 1982; Wiertelak et al. 1992). Nevertheless, the presence of pain facilitation mechanisms raises the possibility that their abnormal sustained activity may underlie chronic pain.

Injury to nerves may elicit neuropathic pain characterized, in part, by increased sensitivity to normally non-noxious and noxious stimuli (allodynia and hyperalgesia, respectively) (Chaplan and Sorkin 1997; Payne 1986). Aspects of this human condition have been modeled by injury to spinal nerves in animals (Bennett and Xie 1988; Kim et al. 1997; Kim and Chung 1992; Seltzer et al. 1990). Increased spontaneous and persistent afferent discharge may be critical in eliciting hypersensitivity of spinal neurons (i.e., central sensitization) (Devor 1991; Devor 1994; Kajander et al. 1992; Kirk 1974; Wall and Gutnick 1974b). Both injured as well as adjacent uninjured fibers become spontaneously active after injury (Li et al. 2000; Wu et al. 2001). Manipulations designed to interfere with ascending, large-fiber projections to brainstem nuclei, including spinal transection, ipsilateral and contralateral hemisections, and selective

lesions of the ipsilateral or contralateral (relative to the side of peripheral nerve injury) dorsal columns block nerve injury-induced pain (Bian et al. 1998; Sun et al. 2001; Sung et al. 1998). Lidocaine injection into the ipsilateral (relative to the side of peripheral nerve injury), but not contralateral, nucleus gracilis also blocks nerve injury-induced pain (Sun et al. 2001). These studies support a role for central processes in the mediation of experimental neuropathic pain.

Blockade of established nerve injury-induced pain is also produced by lidocaine microinjection into the RVM, indicating additionally the importance of descending modulatory pathways (Kovelowski et al. 2000; Pertovaara et al. 1996) and the possibility of tonic discharge of cells mediating descending facilitation (Fields 1992; Fields et al. 1991). The importance of descending facilitation is also supported by observations that lesions of the DLF block nerve injury-induced pain (Ossipov et al. 2000a). One characteristic of RVM cells that may mediate descending facilitation is their sensitivity to opioid μ receptor agonists (Fields et al. 1983; Fields and Heinricher 1989; Heinricher et al. 1994; Pan et al. 1990). These findings suggest that RVM cells that may mediate descending facilitation in chronic pain states might be identified by their expression of this opioid receptor.

The present experiments tested this hypothesis by targeting RVM μ receptor-expressing cells with the cytotoxin SAP, by conjugating the toxin to a potent opioid μ receptor agonist, DERM (Braga et al. 1984; Broccardo et al. 1981). The goal was to determine the role of RVM MOR-expressing cells in preventing or reversing nerve injury-induced pain. A similar approach has been successfully used to lesion spinal cord

lamina I projection cells with a substance P-SAP conjugate (Mantyh et al. 1997; Nichols et al. 1999).

Characterization of the targeted toxin, DERM-SAP

Competition Studies

The radioligand binding studies in membrane preparations from HN9.10 transfected cells that express rat opioid μ receptors demonstrated that the conjugation of SAP to DERM did not significantly alter the affinity of DERM for MORs (Figure 3.1). As expected, DERM alone demonstrated a high affinity for the MOR and a K_i value of 0.7 nM. The K_i value for DERM-SAP was 0.1 nM. In addition, the conjugation of SAP to DERM did not alter the selectivity of DERM for MORs. Both DERM alone and the DERM-SAP conjugate demonstrated approximately 100-fold selectivity for the MOR over the δ opioid receptor.

	MOR [^{3}H]DAMGO K_i nM	**DOR [^{3}H]pCl-DPDPE K_i nM**
DERM	0.70 (0.59 - 0.78)	67.7 (64.8 - 73.4)
DERM-SAP	0.10 (0.09 - 0.11)	9.56 (0.17 - 24.1)

Figure 3.1. Radioligand binding was performed in HN9.10 transfected cells expressing the rat MOR and NG-108 cells expressing the rat δ receptor. At least 10 concentrations of DERM (10^{-14} to 10^{-5}M) or DERM-SAP (10^{-14} to $10^{-7.5}$M) were used. Data were analyzed using GraphPad Prism. The K_I value(s) for each ligand was calculated from the IC_{50} value(s) based on the Cheng and Prusoff equation from at least three independent experiments.

Antinociceptive activity

The bilateral microinjection of 3 pmol of DERM or of DERM-SAP directly into the RVM produced a robust antinociceptive effect in the 52°C hot water tail flick test (Figure 3.2). The peak antinociceptive effect of DERM, 78 ± 13.2 % MPE, was not significantly different from that of DERM-SAP conjugate, which was 59 ± 4.7 % MPE ($p > 0.5$, Student's t-test).

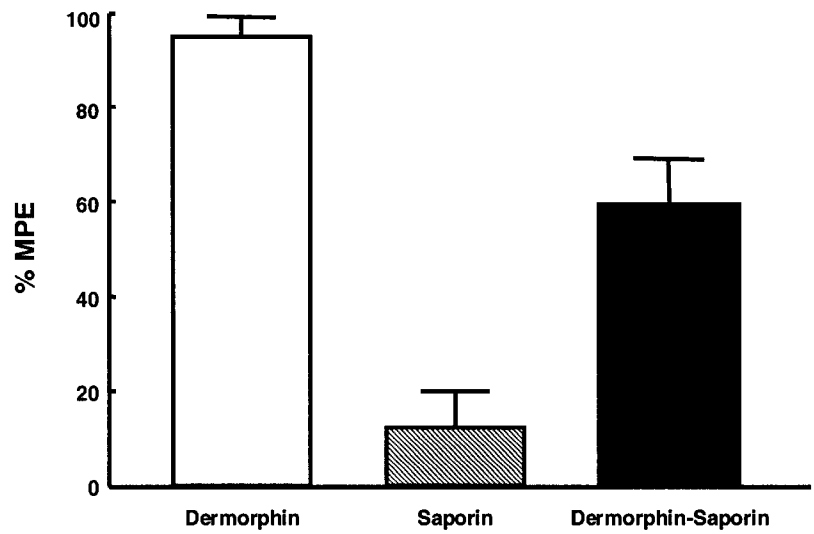

Figure 3.2. The tail flick test was performed by determining latency to withdrawal from a 52°C water bath. Data were expressed as percentage of maximal possible effect (%MPE), which is 100 x (test – baseline)/(15 – baseline). A 15 second cutoff was used. The peak antinociceptive effect of DERM, 78 ± 13.2 % MPE, was not significantly different from that of DERM-SAP conjugate, which was 59 ± 4.7 % MPE ($p>0.05$, Student's t-test). The RVM microinjection of unconjugated SAP did not elicit any changes in tail flick latency

Immunohistochemical Analysis

Decreased staining of MOR-expressing cells was seen in superficial dorsal horn 28 days after intrathecal injection of DERM-SAP, but not after SAL, DERM or SAP (Figure 3.3).

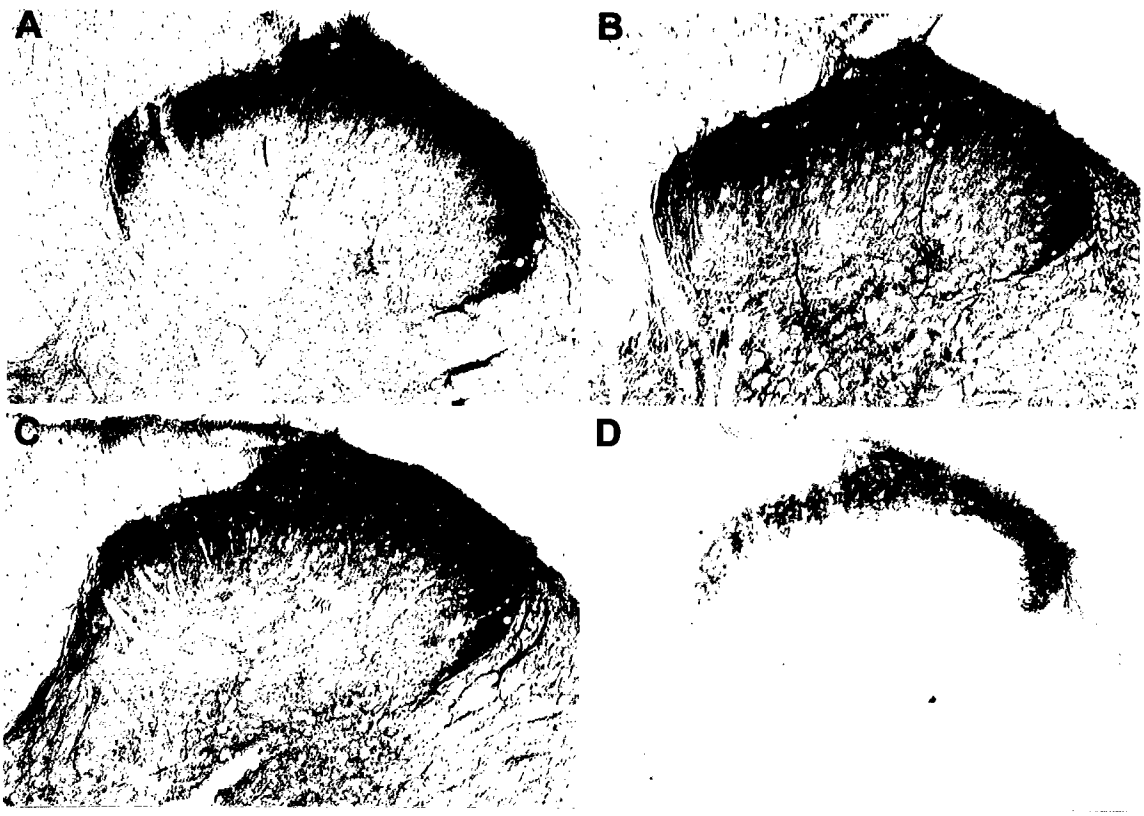

Figure 3.3. Localization of the MOR in frontal sections (40 μm) of the lumbar dorsal horn of the spinal cord. The sections were immunolabeled with a rabbit antiserum against the rat MOR. Decreased staining of the MOR was seen in rats given an intrathecal injection of DERM-SAP (D), but not those that received a single injection of SAL (A), DERM (B), SAP (C), 28 days previously. (Figure contributed by En-Tan Zhang)

Effect on baseline sensory thresholds

The microinjection of unconjugated SAP into the RVM did not elicit any changes in tail flick latency. Furthermore, the nociceptive tail flick reflex to 52°C water immersion was also unaffected in these experimental animals (Figure 3.4).

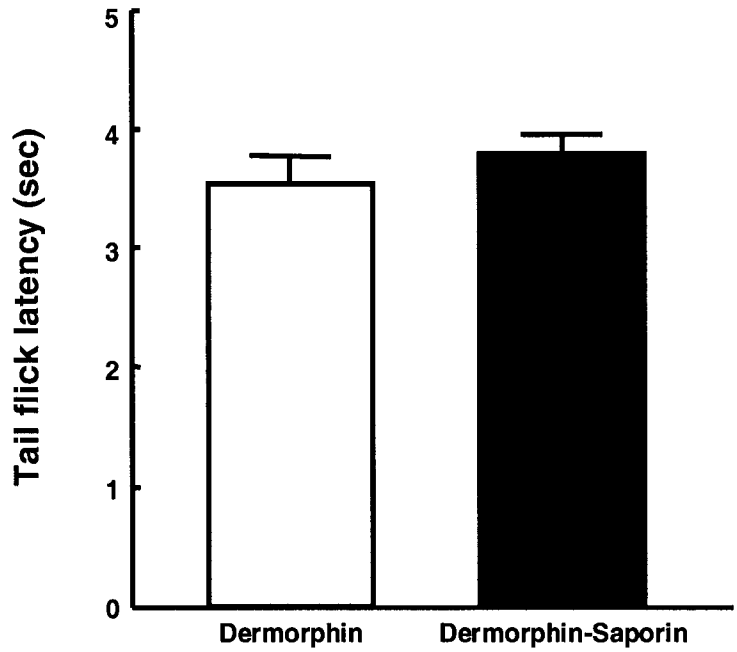

Figure 3.4. The baseline tail flick latency was unaltered in rats that received a single injection of DERM-SAP, when compared to DERM-treated controls.

The bilateral microinjection of 3 pmol of DERM, SAP or of DERM-SAP into the RVM of naive rats produced no observable behavioral changes over a period of 28 days. Paw withdrawal thresholds remained unchanged over this time period (Figure 3.5, Panel A). Similarly, the paw withdrawal latencies to radiant heat applied to the plantar aspect of the hindpaw also did not change over this 28 day period (Figure 3.5, Panel B).

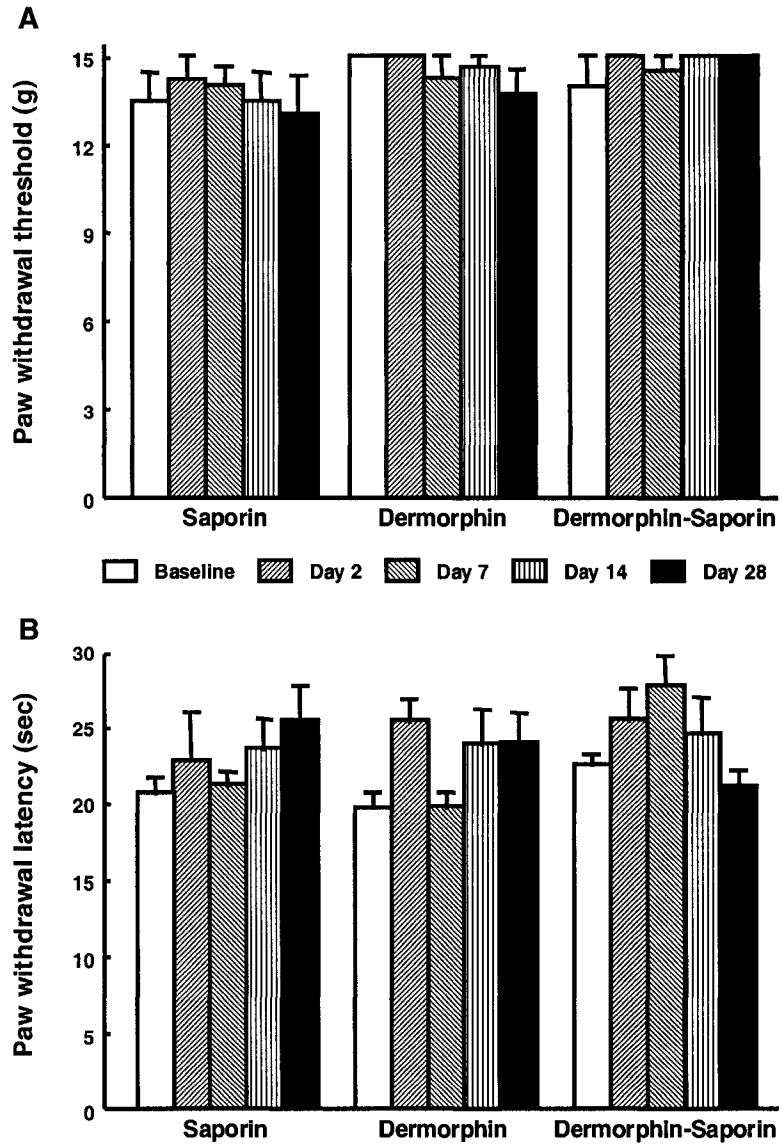

Figure 3.5. Rats received a bilateral microinjection of SAP, DERM, or DERM-SAP into the RVM. The animals were tested for responses to non-noxious mechanical stimuli (Panel A) and to noxious radiant heat (Panel B) on days 0 (baseline), 2, 7, 14, and 28 after the microinjection. No significant changes ($p > 0.05$) were observed in any of the groups over this observation period. n = 5-6 rats per group.

Prevention of Neuropathic Pain

The same group of rats then underwent either sham or SNL surgery and were tested again 7 days after surgery. The rats that initially received either SAP or DERM alone demonstrated a clear development of behavioral signs of neuropathic pain

associated with SNL (Figure 3.6). Rats receiving SNL and either RVM DERM or SAP alone displayed tactile allodynia and thermal hyperalgesia as evidenced by significant reduction in paw withdrawal thresholds (Figure 3.6, Panel A). Likewise, the paw withdrawal latencies for the DERM-pretreated and SAP-pretreated groups were significantly ($p \leq 0.05$) decreased 7 days after SNL (Figure 3.6, Panel B). In contrast, the DERM-SAP pretreated SNL group showed responses to non-noxious or noxious stimuli which did not differ significantly from the pre-SNL baseline values (Figure 3.6). The responses of the SHAM rats did not demonstrate any evidence of neuropathic pain behavior against either non-noxious mechanical or noxious radiant heat.

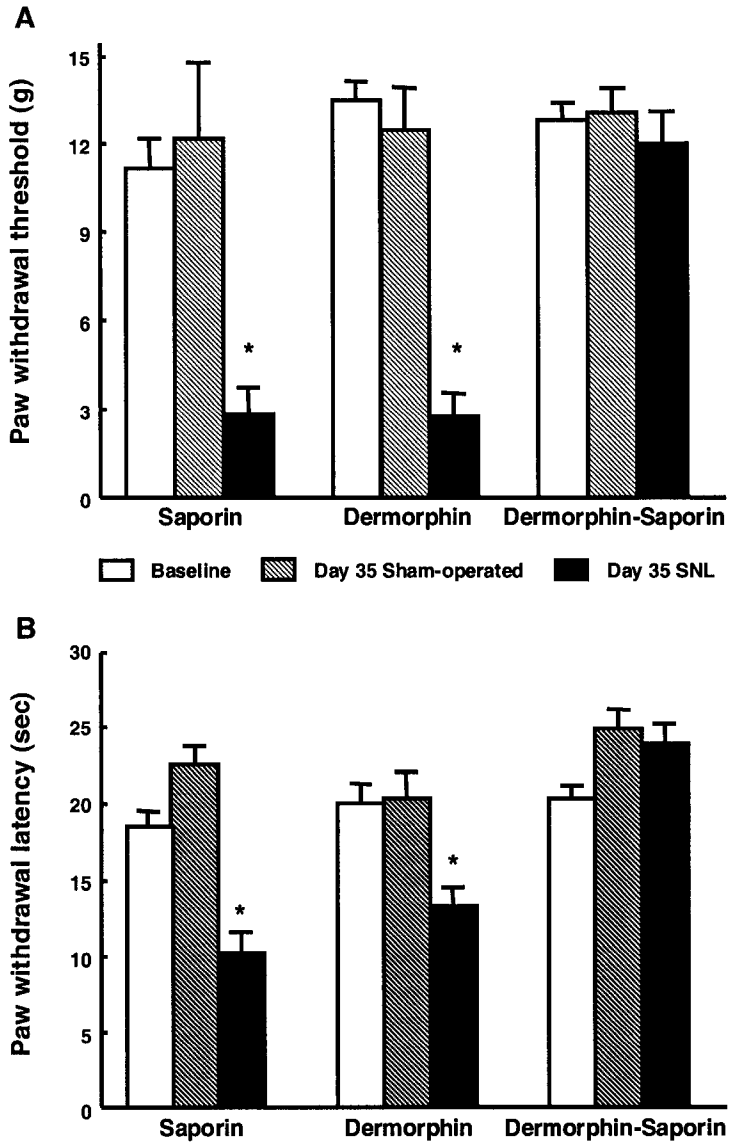

Figure 3.6. Rats received a bilateral microinjection of SAP, DERM, or DERM-SAP into the RVM. After 28 days, rats were subjected to either SNL or sham surgery. Seven days after surgery (i.e., 35 days following RVM injection), the animals were tested for their responsivity to non-noxious mechanical (Panel A) or noxious thermal (Panel B) stimuli. Rats that had received RVM pretreatment of either SAP or DERM showed both tactile and thermal hypersensitivity after SNL. However, rats with SNL that were pre-treated with RVM DERM-SAP showed response thresholds to mechanical or thermal stimuli that were not significantly different from baseline values ($p>0.05$). None of the sham SNL groups demonstrated any appreciable changes in behavioral responses when compared with the pretreatment baseline after any RVM pretreatment.

Reversal of Neuropathic pain

The paw withdrawal thresholds to probing with von Frey filaments and the latencies to withdrawal from noxious radiant heat were determined prior to any manipulations. The pooled paw withdrawal thresholds were 12.6 ± 0.88 g and the pooled paw withdrawal latencies were 20 ± 1.15 sec (Figure 3.7). Rats with either SNL or sham surgery were randomly separated into 3 groups each destined for microinjection of either DERM, SAP or DERM-SAP into the RVM. Rats with SNL showed clear behavioral signs of neuropathic pain on the 5^{th} day after surgery. Tactile allodynia was indicated by the significant ($p \leq 0.05$) reductions in the paw withdrawal thresholds of rats with SNL to innocuous mechanical light touch (Figure 3.7, Panel A). Thermal hyperalgesia was indicated in rats with SNL by the significant ($p \leq 0.05$) reduction in paw withdrawal latencies to noxious radiant heat (Figure 3.7, Panel B).

After these baseline determinations were made, rats received 3 pmol of either DERM, SAP, or DERM-SAP bilaterally into the RVM. Animals were tested at 2, 7, 14 and 28 days after the microinjections. Rats with SNL that received the DERM-SAP conjugate demonstrated a gradual loss of heightened sensitivity to both innocuous mechanical and noxious thermal stimuli over the 28 day observation period (Figure 3.7). On day 28, the paw withdrawal thresholds to probing with von Frey filaments was significantly ($p \leq 0.05$) elevated and not significantly different ($p > 0.05$) to pre-ligation values (Figure 3.7, Panel A) in rats with SNL. Similarly, paw withdrawal latencies to noxious radiant heat were radiant heat were not significantly ($p \leq 0.05$) different from pre-SNL values 28 days after the microinjection of DERM-SAP (Figure 3.7, Panel B). The

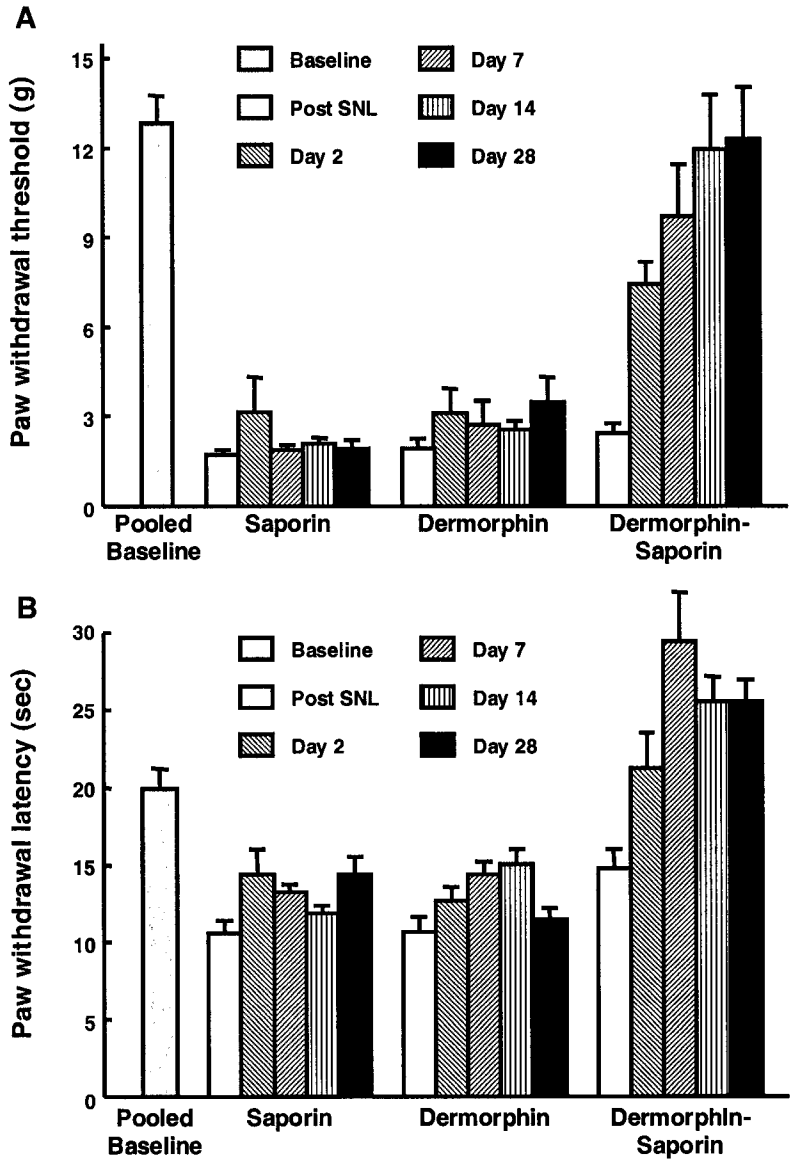

Figure 3.7. Baseline responses to non-noxious mechanical and to noxious thermal stimuli were determined in rats. These rats were then subjected to SNL. After tactile (panel A) and thermal (panel B) hypersensitivity was clearly established in the SNL rats, they received RVM microinjections of SAP, DERM, or DERM-SAP. The rats were tested for the response threshold to non-noxious mechanical and noxious thermal stimuli over a 28 day period. By day 2 after the injection, all groups of SNL rats clearly demonstrated tactile and thermal hypersensitivity. By postinjection day 8, however, and continuing to the end of the experiment, the SNL rats that received an RVM microinjection of DERM-SAP exhibited a progressive reversal of both mechanical and thermal thresholds to levels that did not differ significantly from baseline ($p>0.05$). SNL rats that received DERM or SAP retained both behavioral signs of experimental neuropathic pain. n = 5 6 rats per group.

microinjection of 3 pmol of either DERM or SAP alone did not effect any changes in the behavioral responses to light tactile or noxious thermal stimuli. On day 28 after the microinjection of DERM or SAP into the RVM, the paw withdrawal thresholds and latencies of the rats with SNL were essentially unchanged and significantly lower than pre-SNL values ($p>0.05$) (Figure 3.7).

Blockade with β-FNA

Groups of 6 male S-D rats were tested for acute antinociception using the 52°C warm-water tail-flick test after RVM DERM (3 pmol), U69,593 (60 nmol), or [D-Ala^2,Glu^4]deltorphin (60 nmol). These opioids produced an acute antinociceptive effect of 95 ± 4.4 % MPE, 54 ± 14 % MPE and 63 ± 9 % MPE, respectively (data not shown). The rats were allowed to rest for 7 days, and each then given an injection of an MOR-selective dose of 18.8 nmol β-FNA bilaterally (9.4 nmol in 0.5 μL each side) into the RVM. Each group of rats was then tested 24 hr later for acute antinociception with the same dose of receptor-selective opioid as before. Pretreatment with β-FNA did not affect baseline latencies to tail flick when measured 24 hr after administration but significantly inhibited the acute antinociception of RVM DERM as indicated by an antinociceptive index of only 9.7 ± 5.6 % MPE (data not shown). In contrast, the antinociceptive effects of RVM U69,593 or of RVM [D-Ala^2,Glu^4]deltorphin were not changed by pretreatment with β-FNA. The antinociceptive indices obtained were 48 ± 8.8 and 61 ± 10 % MPE after β-FNA, respectively, values not significantly different from control ($p>0.05$) (data not shown). These data assure that the dose of β-FNA employed was selective for the μ opioid, but not the κ or δ opioid receptors.

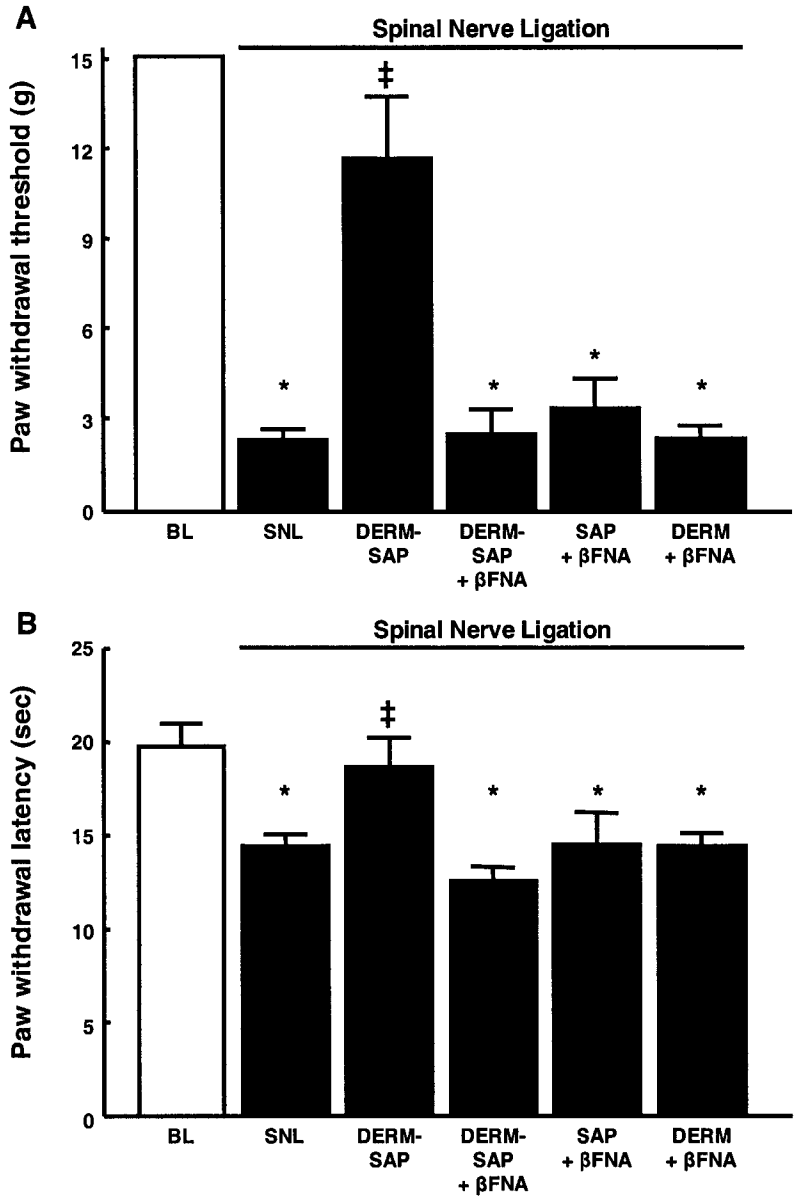

Figure 3.8. Rats were tested for threshold responses to non-noxious mechanical or noxious thermal stimuli (baseline values) and subsequently received either the noncompetitive opioid μ receptor antagonist β-FNA or saline into the RVM 24 hr before the administration of SAP, DERM, or DERM-SAP into the RVM. All groups were subjected to SNL 28 days after RVM DERM, SAP, or DERM-SAP and tested for their response thresholds to non-noxious mechanical or noxious thermal stimuli 7 days after SNL surgery. Pretreatment with β-FNA before RVM DERM-SAP abolished the ability of RVM DERM-SAP to prevent SNL-induced tactile and thermal hypersensitivity but did not alter the effects of SNL in SAP- or DERM-pretreated rats. n = 5-6 rats per group. BL = Baseline; DERM = dermorphin; SAP = saporin. *$p < 0.05$ from baseline; ‡$p < 0.05$ from SNL.

The same dose, 18.8 nmol of β-FNA was microinjected bilaterally into the RVM of naive rats. After 24 hr, the rats received RVM microinjections of 3 pmol of either DERM, SAP, or DERM-SAP conjugate. After 28 days, the animals were subjected to SNL and evaluated for responses to innocuous mechanical and noxious thermal stimuli 7 days later. The groups that received β-FNA pretreatment prior to SAP, DERM or DERM-SAP all exhibited increased sensitivity to innocuous mechanical (Figure 3.8, Panel A) and noxious thermal (Figure 3.8, Panel B) stimuli as expected with SNL. In contrast, SNL rats that received saline prior to DERM-SAP conjugate did not show any behavioral signs of neuropathic pain. The paw withdrawal thresholds to von Frey filaments (Figure 3.8, Panel A) and the latencies to noxious radiant heat (Figure 3.8, Panel B) were not significantly different from the pretreatment baseline values.

ISH of MOR transcripts

Our initial analysis using coronal sections from the brain stem region of naive rats (taken between -11.0 to -13.2 mm from bregma) showed that the localization of cells expressing MOR transcripts in the caudal brainstem including the RVM was highly consistent with that previously reported based on autoradiography for the receptor or on ISH for message (Bowker et al. 1988; Bowker and Dilts 1988; Mansour et al. 1994a; Mansour et al. 1994b; Peckys and Landwehrmeyer 1999). In the RVM, cells that expressed MOR transcripts were highly localized to the raphe magnus and the magnocellular reticular nucleus, and also found at a lower cell density in the paragigantocellular reticular nucleus-lateral part and the gigantocellular reticular nucleus. Caudal to the RVM, MOR transcripts were highly localized to cells in the raphe

obscurus, the raphe pallidus, the inferior olivary complex, the external nucleus cuneatus, the medial solitary nucleus, the hypoglossal nucleus, the dorsal motor nucleus of the vagus, the spinal trigeminal tract, and the nucleus ambiguus. A low density of discrete cell bodies in the nucleus gigantoreticular nucleus were also labeled. The localization of the cells that express MOR mRNA is consistent with that previously described.

For the experiments, coronal sections 20 μm thick were obtained 28 days after the bilateral microinjection of DERM, SAP or DERM-SAP into the RVM. The brain slices obtained from rats that had been pretreated with DERM or SAP alone showed similar densities of labeling for mRNA for the MOR in the RVM (Figure 3.9). The RVM of the DERM-SAP pretreated rats consists of significantly fewer labeled cells when compared with that of DERM or SAP pretreated rats (Figure 3.9). This regional loss of MOR transcripts in DERM-SAP pretreated tissues was not due to differences in experimental conditions because the processing of tissues and the ISH procedures were carried out in parallel with tissues from DERM or SAP pretreated rats. Furthermore, brainstem sections caudal to the RVM (between -12.2 mm to -13.2 mm from bregma) taken from DERM, SAP, or DERM-SAP pretreated animals showed similar distributions and densities of labeled cells, suggesting that the loss of labeling correlates with the stereotaxic delivery of DERM-SAP to the RVM and that this loss is specific to DERM-SAP treatment (data not shown). The immunolabeling could be abolished by pre-treatment of the tissue sections with RNase, or by an excess of the complementary sense DNA during the hybridization reaction. The histological staining of the tissue sections indicates that tissue necrosis was negligible. Thus, a selective degeneration of MOR-

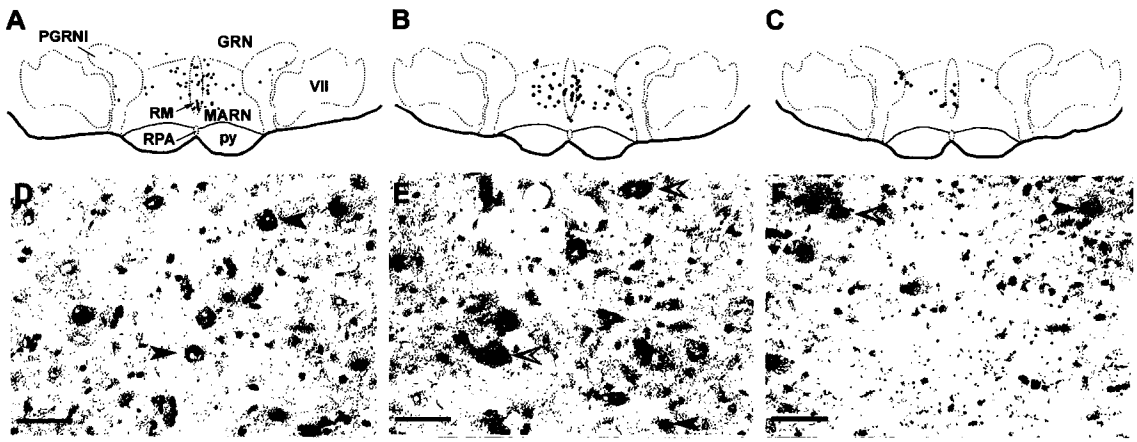

Figure 3.9. Localization of MOR mRNA in frontal sections (20 μm) of the brainstem at the level of the caudal raphe nuclei. Bright-field micrographs (*D-F*) show the distribution of the probe as a red stain (fast red). The sections were counterstained with Mayer's hematoxylin (blue nuclei). Computer-assisted mapping of representative sections shows the location of MOR mRNA-labeled cells (*dots*) in the RVM of rats that had been pretreated with DERM (*A*), SAP (*B*), or DERM-SAP (*C*) and the corresponding high-magnification, bright-field micrographs (*D-F*) taken from the same area in the raphe magnus (denoted by *arrow* in *A*) of *A-C*, respectively. The cDNA probe labeled discrete neuronal cell bodies. Two types of staining by fast red are seen, perinuclear staining (*closed arrowheads*) and cytoplasmic staining (*open arrowheads*). Unlabeled cells can be seen as blue nuclei with negligible or very light blue cytoplasmic stain. In the RVM, cells that expressed MOR transcripts were highly localized to the *RM* and the *MARN* and also found at a lower cell density in the *PGRNl* and the *GRN*. The localization of the cells that express MOR mRNA is consistent with that described previously. The RVM of DERM-SAP-pretreated rats (*C, F*) consists of significantly fewer labeled cells when compared with that of DERM-pretreated (*A, D*) or SAP-pretreated (*B, E*) rats. The specificity of labeling for MOR transcripts is also supported by the consistency in the distribution of labeling across multiple brainstem sections and sections from multiple animals. This labeling could be abolished by RNase A pretreatment of the sections or by the presence of an unlabeled, corresponding sense DNA during hybridization. Abbreviations: *VII*, facial nucleus; *GRN*, gigantocellular reticular nucleus; *MARN*, magnocellular reticular nucleus; *PGRNl*, paragigantocellular reticular nucleus-lateral part; *py*, pyramidal tract; *RM*, raphe magnus; *RPA*, raphe pallidus. Scale bars = 50 μm.

expressing cells in the RVM by DERM-SAP prevented abnormal pain resulting from SNL, without affecting normal sensory responses.

Discussion

Mechanistic interpretation of the neuropathic state has generally focused on injury-induced changes in peripheral nerves and in the spinal dorsal horn. Spinal sensitization is believed to occur as a consequence of the increased firing of primary afferent fibers and has been thought to be a key element in nerve injury-induced pain (Devor 1991; Fields et al. 1997; Kajander et al. 1992; Wall and Gutnick 1974b). The time course of increased discharge from injured nerves does not appear to correlate perfectly, however, with the sustained nature of nerve injury-induced pain. Although nerve injury-induced pain is sustained essentially unchanged for many weeks (Bian et al. 1995; Chaplan et al. 1994), data from several groups show that the discharge rate of injured afferents declines significantly over just a few days after the injury (Han et al. 2000; Liu et al. 2000b). Large myelinated fibers are spontaneously active in the postinjury state, and uninjured adjacent fibers also discharge tonically (Boucher et al. 2000; Li et al. 2000; Wu et al. 2001). The known projections of these fibers to the brainstem, along with the observations of plasticity at levels as far rostral as the midbrain after injury to peripheral nerves (Kovelowski et al. 2000), point to a role of supraspinal sites in the nerve injury-induced pain state. This concept is supported by various lesion studies of ascending pathways (Houghton et al. 1999; Sun et al. 2001) as well as by the emergence of evidence of the critical importance of descending pain facilitation pathways in nerve injury-induced pain. Specifically, blockade of nerve injury-induced pain is seen

after RVM microinjection of lidocaine (Kovelowski et al. 2000; Pertovaara et al. 1996) as well as by lesions of the DLF (Ossipov et al. 2000a). The results of the present study suggest that a specific population of RVM neurons, namely, those expressing MORs, is critical in the behavioral expression of experimental neuropathic pain.

These RVM cells display characteristics consistent with those characterized previously as facilitatory or pronociceptive. The electrophysiologic characteristics of the neurons of this region have been well characterized and strongly point to this region as a likely source of facilitation of nociceptive input (Fields 1992; Fields et al. 1983; Fields and Heinricher 1985). One neuronal class, labeled ON cells because of a firing burst recorded just before activation of a nocifensive response, is believed to be responsible for descending facilitation of nociception via both local interactions within the RVM and descending systems projecting to the spinal cord (Fields et al. 1991; Heinricher et al. 1992; Heinricher and Roychowdhury 1997). Manipulations that increase nociceptive responsiveness, thus indicating facilitation, also increase ON cell activity (Fields and Basbaum 1999; Heinricher et al. 1989; Kaplan and Fields 1991; Morgan and Fields 1994). Importantly, this class of neurons is hypothesized to represent the population MOR-expressing cells. In agreement with this hypothesis, systemic or RVM morphine produces a naloxone-sensitive depression in spontaneous and evoked firing rates of identified ON cells (Heinricher et al. 1992), whereas the firing characteristics of other RVM neurons are not affected by the opiate (Heinricher et al. 1992). Analogous studies in vitro show that RVM MOR agonists directly hyperpolarize "secondary cells" (Pan et

al. 1990). Such findings suggest that ON cells are likely to be the only MOR-expressing cells in the RVM (Heinricher et al. 1992).

Our data show that conjugated DERM–SAP retains affinity and efficacy at the MOR, suggesting that this molecule is suitable for targeting cells that express these receptors. Selective loss of MOR-containing neurons was demonstrated by a significant decrease in the number of cells positively labeled for MOR transcripts in animals pretreated with DERM-SAP but not with DERM or SAP. Rats treated with RVM DERM or SAP demonstrated an equivalent presence of labeling for MOR mRNA. Neurons that were not in the vicinity of the site of DERM–SAP injection were spared, however, suggesting that the loss of MOR-expressing cells was not the result of a nonselective cytotoxicity or because of trauma resulting from cannula implantation or microinjection.

RVM microinjection of DERM–SAP, or of unconjugated SAP or DERM, did not produce any significant changes in response thresholds to normally non-noxious mechanical or noxious thermal stimuli of the paw when evaluated over a 28 day time course. Because a significant depletion of cells expressing MOR transcripts was seen in animals injected with RVM DERM–SAP, it appears that MOR-expressing cells do not participate in the response to non-noxious or noxious sensory thresholds. Subsequent experimental nerve injury, however, showed that although rats pretreated with RVM DERM or SAP developed the expected increased sensitivity to normally non-noxious mechanical and noxious thermal stimuli, those animals pretreated with RVM DERM–SAP did not. These findings indicate that depletion of RVM cells MORs, presumably cells that mediate descending facilitation to the spinal dorsal horn, prevents the expected

neuropathic pain state. In the absence of electrophysiological investigation, it is unknown whether such lesioned cells represent the previously characterized population of RVM cells referred to as ON cells. The specificity of DERM–SAP for RVM cells expressing MORs was confirmed by the use of β-FNA, a selective and irreversible MOR antagonist (Jiang et al. 1990; Ward et al. 1982). RVM microin-jection of β-FNA was shown to antagonize the antinociceptive effects of a receptor-selective opioid μ, but not selective δ or κ, agonist indicating that the dose administered selectively blocked MORs; the selectivity of the antagonist is supported by similar results in previous studies (Melchiorri et al. 1991; Tiberi et al. 1988). Administration of RVM DERM–SAP in rats pretreated with β-FNA showed that the expected prevention of nerve injury-induced pain was blocked; β-FNA pretreatment did not alter the development of nerve injury-induced pain in groups pretreated with either DERM or SAP.

In addition to the observation of prevention of nerve injury-induced pain by RVM pretreatment with DERM–SAP, our data also demonstrate that the behavioral signs of experimental neuropathic pain can be reversed by targeting MOR-expressing cells in this region. Administration of DERM–SAP, but not of DERM or SAP, showed a time-related return to normal levels of sensitivity to non-noxious mechanical or to noxious thermal stimuli in nerve-injured rats. The reversal of established experimental neuropathic pain demonstrates the importance of descending facilitation in sustaining pain. Together, these observations suggest a requirement for RVM MOR-expressing cells for the expression and maintenance of nerve injury-induced pain. The data indicate the importance of such cells under pathological, but not normal physiological, conditions

because reactions to light touch or to acute noxious stimuli are unaltered in uninjured or SHAM rats. These findings are consistent with previous observations indicating a role for supraspinal mechanisms of neuropathic pain (Bian et al. 1998; Kovelowski et al. 2000; Mansikka and Pertovaara 1997; Ossipov et al. 2000c; Pertovaara et al. 1996; Sun et al. 2001) and are in agreement with data resulting from experiments with more generalized and reversible blockade of RVM activity such as lidocaine injection (Kovelowski et al. 2000; Pertovaara et al. 1996). Finally, the data of the present study are consistent with observations showing reversal of nerve injury-induced pain by physical disruption of the DLF (Ossipov et al. 2000c).

The results presented here are consistent with the hypothesis that the presence and activity of descending pain facilitation cells in the RVM are required for the expression of experimental neuropathic pain. Furthermore, these neurons are likely to be MOR-expressing cells of the RVM. Because DERM–SAP was shown to be effective in reversing established experimental pain, the MOR-expressing neurons should therefore represent an appropriate target for the development of strategies for the treatment of abnormal pain states. It is clearly more important to be able to treat established neuropathic pain, because one cannot anticipate its development. Critically, targeting mechanisms of descending pain facilitation offers a novel approach to the alleviation of chronic, pathological pain that does not alter normal sensitivity to innocuous or noxious sensations. These observations raise many important questions including the nature of the tonic activation in the RVM that drives descending facilitatory systems and the spinal circuitry by which such projections facilitate the transmission of pain. Increased

understanding of underlying mechanisms that may drive abnormal pain would be relevant to the formulation of novel treatment protocols.

CHAPTER IV: TIME-DEPENDENT DESCENDING FACILITATION FROM THE RVM MAINTAINS, BUT DOES NOT INITIATE, NEUROPATHIC PAIN

Introduction

Neuropathic pain may result from increased excitability of injured nerves (Kirk 1974; Wall and Gutnick 1974a; Wall and Gutnick 1974b). Persistent spontaneous afferent input also results in sensitization of spinal neurons to promote enhanced pain (Devor 1991; Woolf 1991; Woolf and Thompson 1991). Agents that diminish spontaneous afferent activity are effective in clinical and experimental neuropathic pain (Chaplan et al. 1995; Chapman et al. 1998; Devor and Seltzer 1999). Spontaneous afferent activity also correlates with expression of neuropathic pain (Han et al. 2000; Liu et al. 2000a; Liu et al. 2000b), and the onset of tactile hypersensitivity occurs with the development of afferent discharge (Liu et al. 2000a). Discharges are most pronounced 1 week after injury but diminish significantly and rapidly over time (Han et al. 2000). Nerve injury elicits a fourfold to sixfold increase in spontaneous ectopic discharge within 24 hr but is largely reduced by postinjury day 5 (Liu et al. 2000a). Notably, however, once developed, behavioral signs of neuropathic pain remain constant for many weeks (Bian et al. 1995; Chaplan et al. 1994; Malan et al. 2000) despite the diminished rate of afferent discharge. These observations suggest the possibility that although the enhanced discharge associated with nerve injury may be critical in the initiation of neuropathic pain, such increased afferent activity may be insufficient to maintain neuropathic pain in the absence of other mechanisms.

Descending facilitation arising from neuroplastic changes occurring in the RVM and projecting to the spinal dorsal horn through the DLF has been suggested to be necessary for expression of neuropathic pain (Ossipov et al. 2001). Blocking descending facilitation by lesions of the DLF, RVM microinjection of lidocaine, or CCK_2 antagonists all block neuropathic behavior (Kovelowski et al. 2000; Ossipov et al. 2000b; Pertovaara et al. 1996). Selective lesioning of RVM cells expressing MORs also blocks neuropathic behaviors (Chapter IV). However, the role of descending facilitation in the processes that initiate or maintain the expression of neuropathic pain is not known.

Peripheral nerve injury is known to elevate spinal dynorphin content, which may promote nociception (Bian et al. 1999; Claude et al. 1999; Kajander et al. 1990; Malan et al. 2000). Spinal dynorphin content is maximal by post-SNL day 10 (Malan et al. 2000). Prodynorphin knock-out mice demonstrated tactile and thermal hypersensitivity that fully reversed to preinjury baselines within 8 d after SNL, whereas the wild-type mice sustained pain (Wang et al. 2001). Neuropathic behaviors were reversed by dynorphin antiserum in wild-type mice in late but not early periods after injury, suggesting that dynorphin is required for sustained expression of pain (Wang et al. 2001). The late time course of SNL-induced spinal dynorphin upregulation suggests the novel possibility that some of the nerve injury-induced spinal plasticity may be secondary to neuroplasticity in other parts of the nervous system. One possibility is that spinal dynorphin upregulation may depend on developing neuroplasticity in the RVM. The present experiments explore the hypothesis that descending facilitation from the RVM develops over time and

influences the spinal upregulation of dynorphin. These processes may be essential in the maintenance of experimental neuropathic pain.

RVM lidocaine

Behavioral signs of tactile hypersensitivity and thermal hyperalgesia were clearly evident within 3 days after SNL (Figure 4.1). The preligation baseline paw withdrawal threshold to probing with von Frey filaments was 14.25 ± 0.30 g and the paw withdrawal latency to noxious radiant heat was 21.0 ± 0.19 sec (Figure 4.2). After SNL, the paw withdrawal threshold was significantly ($p \leq 0.05$) reduced to 3.67 ± 0.44 g and the paw withdrawal latency was significantly ($p \leq 0.05$) reduced to 14.5 ± 0.20 sec (Figure 4.2). In contrast, sham surgery had no significant effect on behavioral signs of neuropathic pain; the post-surgical paw withdrawal tactile threshold and thermal latency were 14.5 ± 0.37 g and 20.5 ± 0.38 sec, respectively (Figure 4.2).

The bilateral microinjection of lidocaine (4% w/v; 0.5 µl) or saline into the RVM on day 3 after SNL did not elicit any changes in paw withdrawal thresholds to probing with von Frey filaments (Figure 4.1, Panel A) or to noxious radiant heat (Figure 4.1, Panel B) over the 60 minute observation period. The paw withdrawal threshold to von Frey filaments was 4.13 ± 0.77 g 10 minutes after lidocaine (Figure 4.1, Panel A), and the paw withdrawal latency to noxious heat was 14.3 ± 0.45 sec 10 minutes after lidocaine (Figure 4.1, Panel B). However, behavioral manifestations of neuropathic pain were reversed when lidocaine was microinjected into the RVM on the 6^{th} day after SNL (Figure 4.1, Panels C and D). The maximal effect of lidocaine was observed 10 minutes

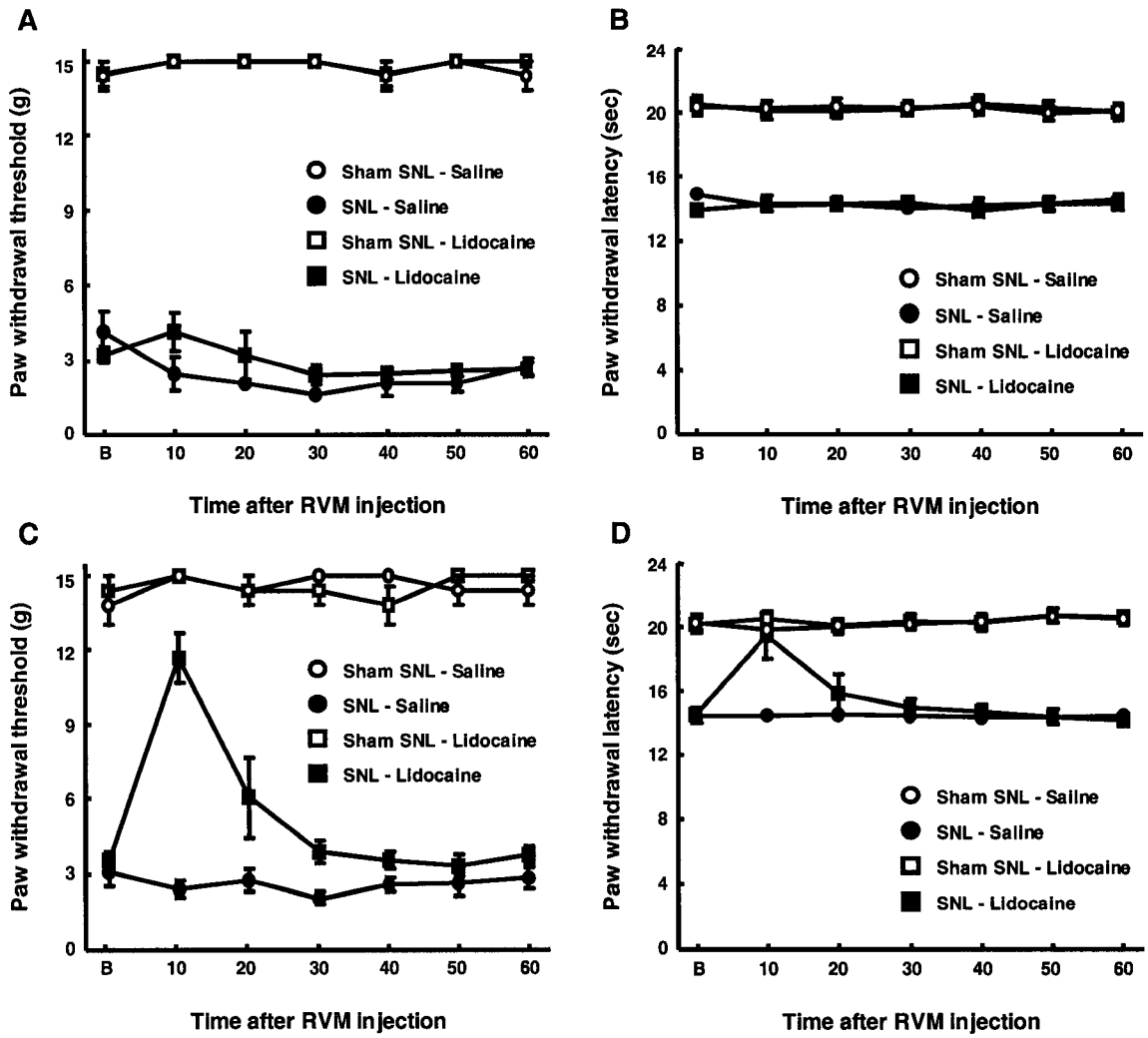

Figure 4.1. Lidocaine (4% w/v) or saline was microinjected bilaterally into the RVM of SHAM male Sprague-Dawley rats and of rats with L_5/L_6 SNL 3 and 6 days after nerve injury. Baseline responses to tactile and thermal stimuli were determined in SHAM and SNL rats before injections (B). Tactile hypersensitivity (A,C) and thermal hyperalgesia (B,D), indicated by significant decreases in the response thresholds, were measured at 10 minute intervals for 60 minutes after lidocaine or saline microinjection. Lidocaine did not reverse tactile hypersensitivity and thermal hyperalgesia on post-SNL day 3 (A and B), but was effective on day 6 (C and D). Behavioral responses were not altered by lidocaine in SHAM rats or by saline in either group.

after microinjection into the RVM, significantly ($p \leq 0.05$) raising paw withdrawal thresholds to light tactile stimuli to 11.7 ± 1.0 g (Figure 4.1, Panel C) and mean paw withdrawal latencies to radiant heat to 19.4 ± 1.46 sec (Figure 4.1, Panel D). The

blockade of tactile hypersensitivity and thermal hyperalgesia by RVM lidocaine rapidly returned to baseline values within 30 minutes of the injection.

Similarly, lidocaine microinjected into the RVM also reversed signs of neuropathic pain on the 9^{th} and 12^{th} day after SNL (Figure 4.2). The paw withdrawal thresholds to probing with von Frey filaments were significantly ($p \leq 0.05$) elevated to 12.1 ± 0.77 g and 12.4 ± 0.76 g, respectively on those days (Figure 4.2, Panel A). Similarly, the paw withdrawal latencies to radiant heat were significantly ($p \leq 0.05$) elevated to 19.8 ± 1.63 sec and 20.0 ± 0.73 sec on the same days (Figure 4.2, Panel B). The effects of lidocaine against behavioral signs of neuropathic pain on post-SNL days 9 and 12 were similar to the data shown for post-SNL day 6 with maximal reversal at 10 minutes after microinjection and return to baseline values by 30 minutes (data not shown). Microinjection of lidocaine into the RVM did not alter responses to either tactile or thermal stimuli in the SHAM rats over the entire course of the study. Furthermore, the microinjection of saline into the RVM of either SHAM or SNL rats did not produce any changes in either tactile or thermal responses over the time course of this study (Figure 4.2).

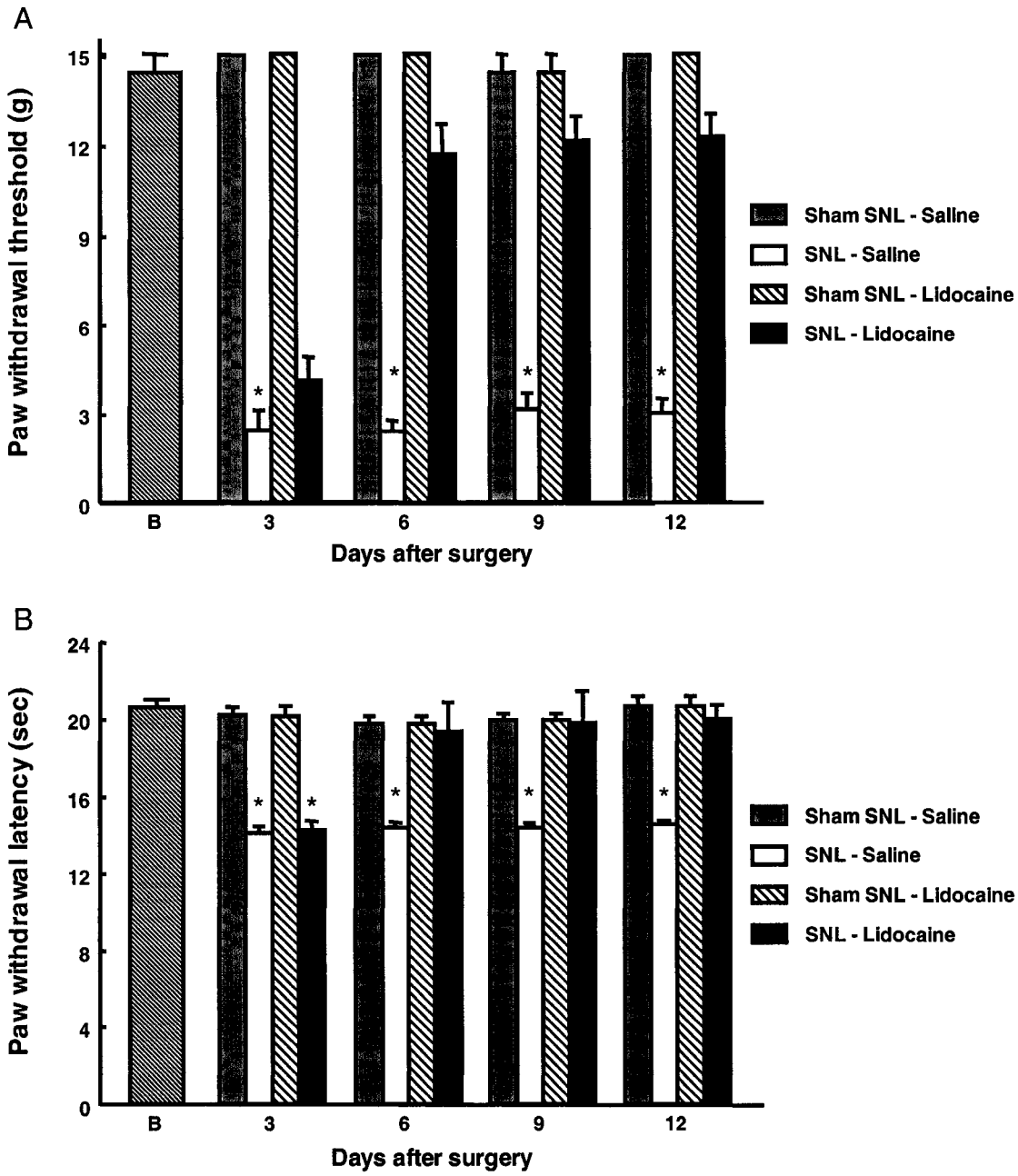

Figure 4.2. Lidocaine (4% w/v) or saline was microinjected bilaterally into the RVM of SHAM rats and of rats with L_5/L_6 SNL 3, 6, 9, and 12 days after nerve injury. Baseline responses to tactile and thermal stimuli were determined before surgery (*pre*-SNL) and on day 3 after surgery before injections (BL). Tactile hypersensitivity (A) and thermal hyperalgesia (B), indicated by significant decreases in the response thresholds, were measured 10 minutes after each lidocaine or saline microinjection. Lidocaine did not reverse tactile hypersensitivity and thermal hyperalgesia on post-SNL day 3 but was effective thereafter. Behavioral responses were not altered by lidocaine in SHAM rats or by saline in either group.

DERM-SAP microinjection

Rats received a single bilateral injection of saline (0.5 μl), DERM (3 pmol), SAP (3 pmol) or of the DERM-SAP conjugate (3 pmol) into the RVM. Experiments conducted in Chapter III revealed that this protocol of DERM-SAP treatment elicited a selective loss of RVM neurons expressing the MOR at post-injection day 28. These microinjections did not produce any changes in the baseline hindpaw responses to probing with von Frey filaments (Figure 4.3) or to noxious radiant heat (Figure 4.4) when evaluated after 28 days. On the 28^{th} day after the RVM microinjections, each of the pretreated groups were divided into 2 groups, one receiving L_5/L_6 SNL and the other receiving sham surgery.

The behavioral responses to light tactile and noxious heat stimuli were measured on a daily basis. None of the groups of rats with sham surgery demonstrated any significant decreases in behavioral responses to either tactile or thermal stimuli over the entire 14 day observation period (Figures 4.3 and 4.4, respectively). All groups of rats with SNL demonstrated tactile hypersensitivity and thermal hyperalgesia evident by the second day after SNL. Paw withdrawal thresholds to light tactile stimuli ranged between 14.4 ± 0.6 g and 15 ± 0 g prior to SNL and were significantly ($p \leq 0.05$) reduced to between 3.7 ± 0.84 g and 4.9 ± 1.85 g (Figure 4.3). Similarly, the paw withdrawal latencies to noxious heat ranged from 19.9 ± 0.56 sec to 20.3 ± 0.23 sec prior to SNL and were significantly ($p \leq 0.05$) reduced to between 14.0 ± 0.38 sec and 15.8 ± 0.46 sec by the second day after SNL (Figure 4.4). Tactile and thermal hypersensitivity remained evident throughout the 14-day observation period in the rats with SNL that were

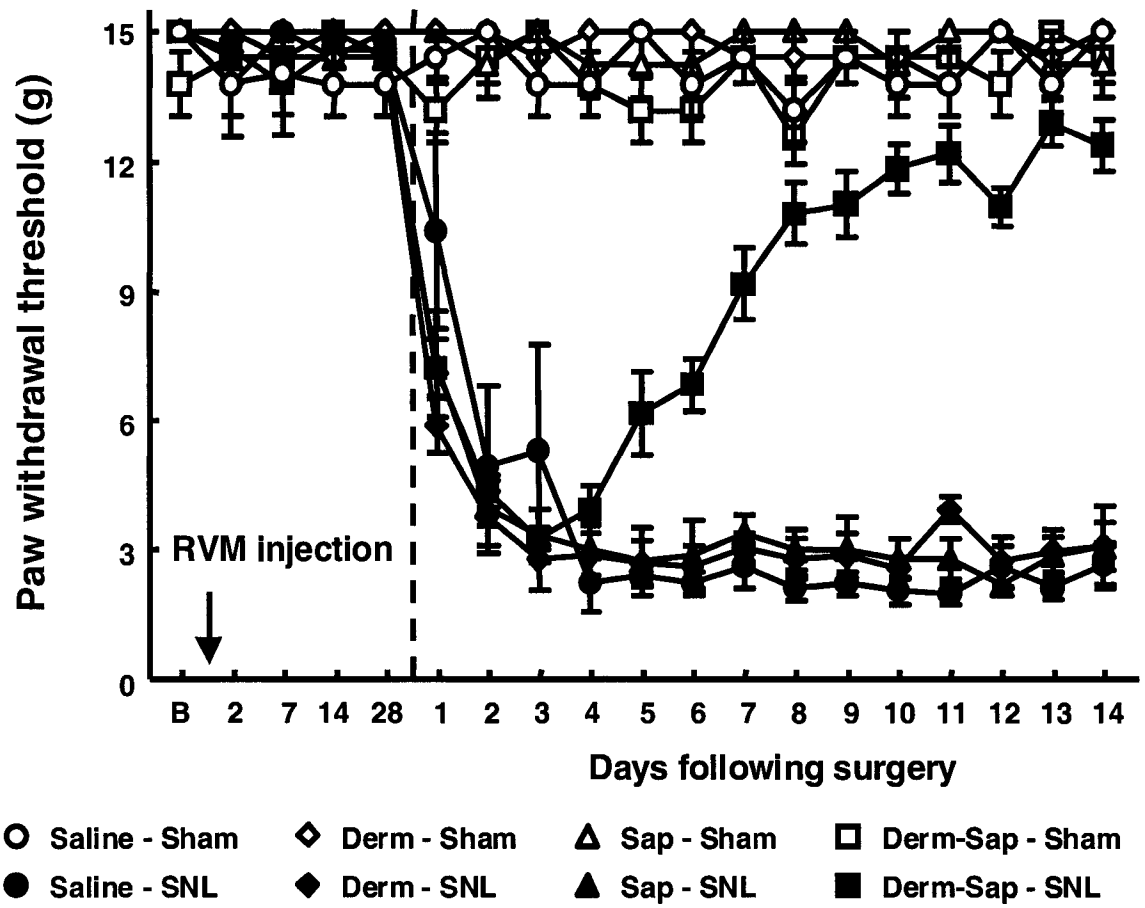

Figure 4.3. Male Sprague-Dawley rats received bilateral microinjections of saline or of SAP, DERM or the DERM-SAP conjugate (1.5 pmol on each side of the RVM). After 28 days, the rats were subjected to either L_5/L_6 SNL or sham surgery. Paw withdrawal thresholds to light tactile stimuli were determined before microinjections (B), weekly after the microinjections, and daily for 14 days after SNL or sham-surgery. Tactile hypersensitivity was evident in all groups with SNL during the initial 4 days of testing, as indicated by the significant decreases in response thresholds. However, the rats pretreated with the DERM-SAP conjugate demonstrated clear reversal of SNL-induced tactile hypersensitivity commencing at post-surgery day 5.

pretreated with saline, DERM or SAP microinjected into the RVM (Figures 4.3 and 4.4, respectively). In contrast, the rats that were pretreated with DERM-SAP demonstrated a time-related reversal of heightened sensitivity to tactile and thermal stimuli which was seen beginning at the 5^{th} day after SNL and ultimately these thresholds were not significantly different from pre-SNL baseline values (Figures 4.3 and 4.4, respectively).

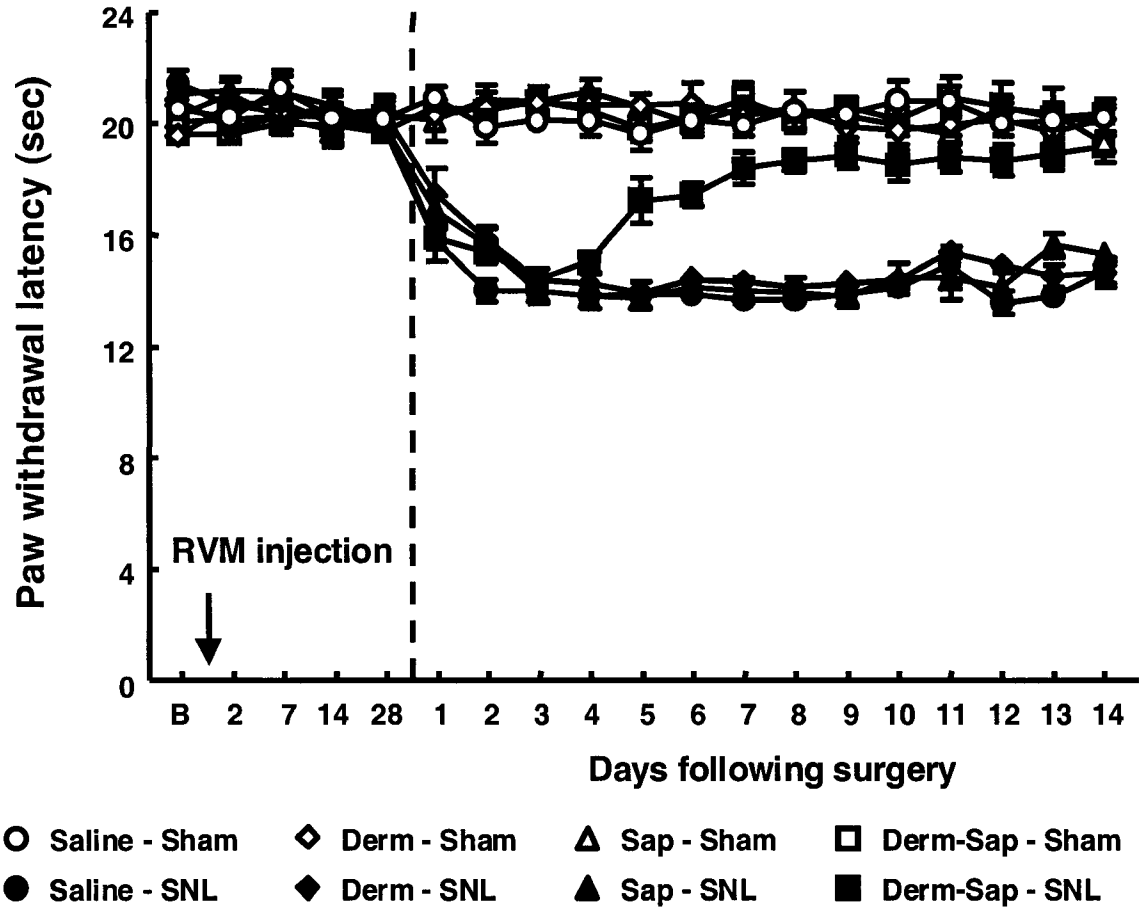

Figure 4.4. Male Sprague-Dawley rats received bilateral microinjections of saline or of SAP, DERM or the DERM-SAP conjugate (1.5 pmol on each side of the RVM). After 28 days, the rats were subjected to either L_5/L_6 SNL or sham surgery. Paw withdrawal latencies in response to noxious radiant heat were determined before microinjections (B), weekly after the microinjections, and daily for 14 days after SNL or sham-surgery. Thermal hypersensitivity was evident in all groups with SNL during the initial 4 days of testing, as indicated by the significant decreases in response thresholds. However, the rats pretreated with the DERM-SAP conjugate demonstrated clear reversal of SNL-induced tactile hypersensitivity commencing at post-surgery day 5.

DLF lesions

Rats received either lesions of the DLF or sham surgery at T_8. Each group was further subdivided and received either sham surgery or L_5/L_6 SNL after a further 7 days. Neither sham DLF nor DLF lesions caused any changes in behavioral responses to tactile or thermal stimuli. Paw withdrawal thresholds to light touch were 15 ± 0 g before and

after spinal surgery and the paw withdrawal latencies to noxious radiant heat were 20.2 ± 0.17 sec before spinal surgery and 20.2 ± 0.23 sec after sham DLF and 20.1 ± 0.25 sec after DLF lesions (Figures 4.5 and 4.6, respectively). Sham operation did not produce any significant decreases in behavioral responses to either light tactile or noxious radiant heat over the entire 14-day observation period (Figures 4.5 and 4.6, respectively). Both

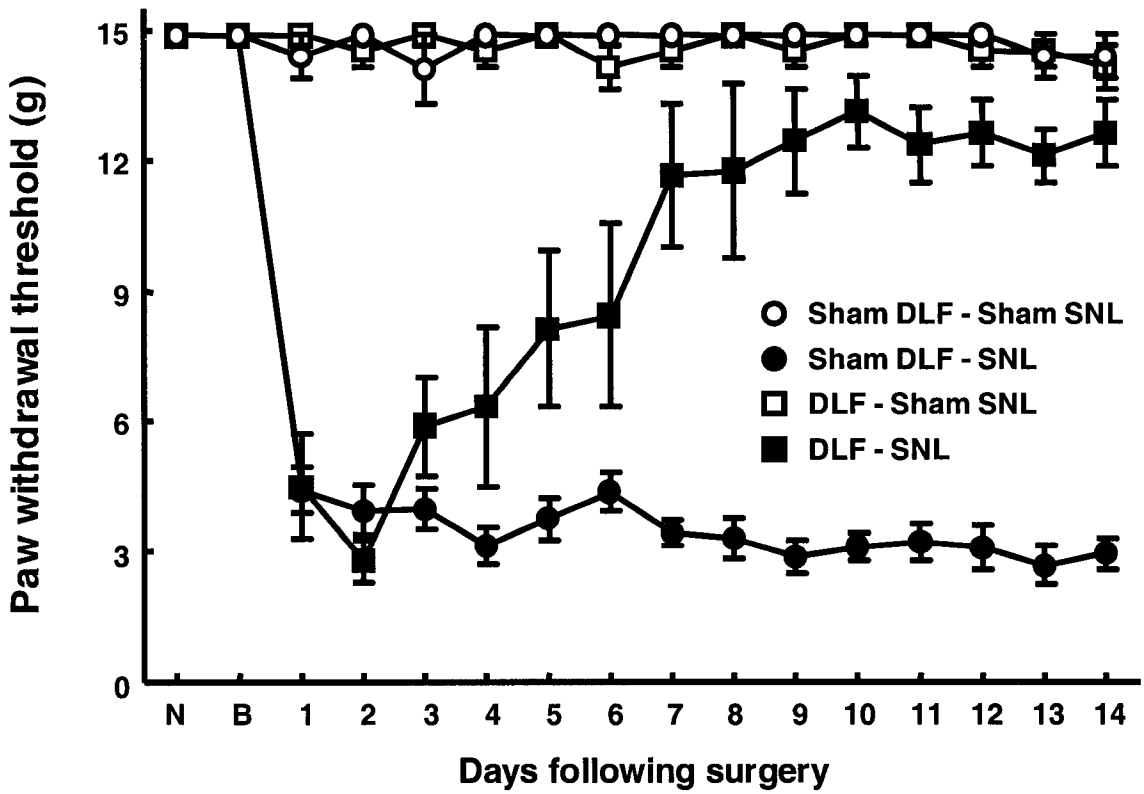

Figure 4.5. Rats received bilateral surgical lesions of the DLF or sham DLF surgery at T_8. After 7 days, the rats were subjected to either L_5/L_6 SNL or sham surgery. Paw withdrawal thresholds to light tactile stimuli were determined before spinal surgery (N), prior to SNL (B) and daily for 14 days after SNL- or sham-surgery. Tactile hypersensitivity was evident in all groups with SNL during the initial 4 days of testing, as indicated by the significant decreases in behavioral responses. However, the rats that received both L_5/L_6 SNL and lesions of the DLF demonstrated a clear reversal of SNL-induced threshold changes commencing at post-surgery days 4-5.

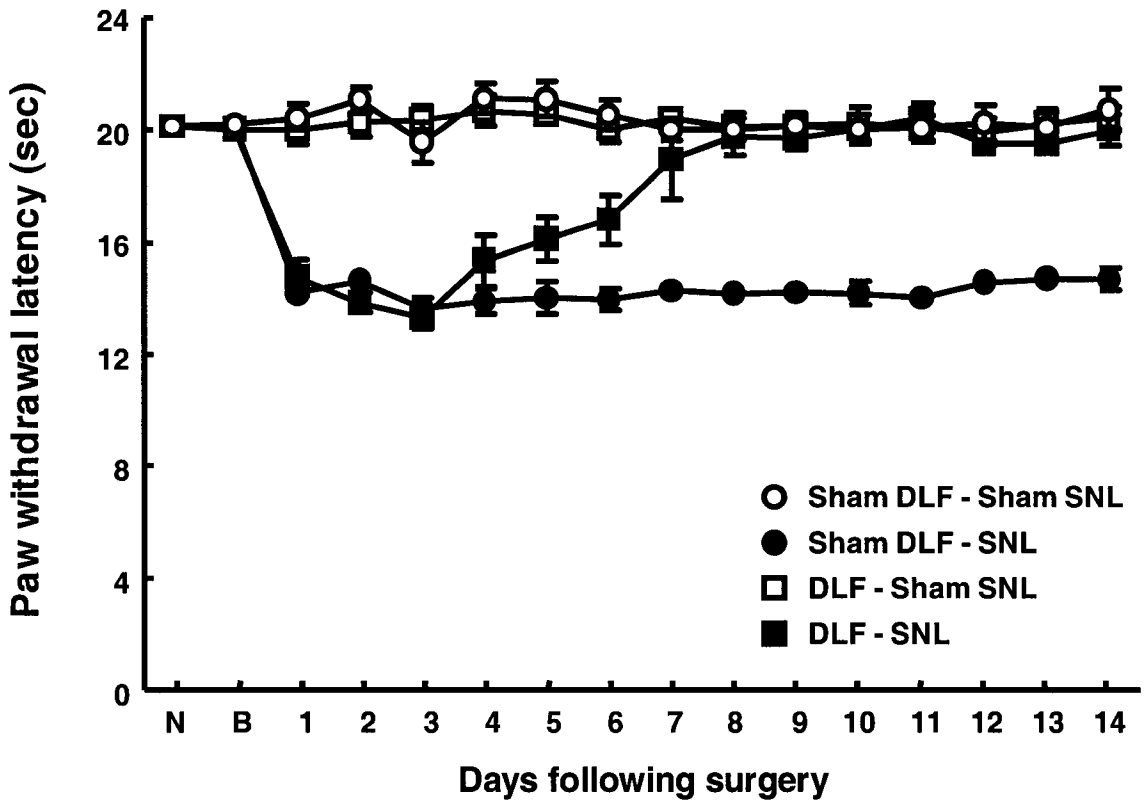

Figure 4.6. Rats received bilateral surgical lesions of the DLF or sham DLF surgery at T_8. After 7 days, the rats were subjected to either L_5/L_6 SNL or sham surgery. Paw withdrawal latencies in response to noxious radiant heat were determined before spinal surgery (N), prior to SNL (B) and daily for 14 days after SNL- or sham-surgery. Thermal hypersensitivity was evident in all groups with SNL during the initial 4 days of testing, as indicated by the significant decreases in behavioral responses. However, the rats that received both L_5/L_6 SNL and lesions of the DLF demonstrated a clear reversal of SNL-induced threshold changes commencing at post-surgery days 4-5.

groups of rats with SNL demonstrated tactile hypersensitivity and thermal hyperalgesia by the 2nd day after SNL. Paw withdrawal thresholds of the SHAM and DLF-lesioned rats to light tactile stimuli were significantly ($p \leq 0.05$) reduced to 3.9 ± 0.6 g and 2.8 ± 0.5 g, respectively (Figure 4.5). Similarly, the paw withdrawal latencies of the SHAM and DLF-lesioned rats to noxious heat were significantly ($p \leq 0.05$) reduced to 14.6 ± 0.3 sec and 13.8 ± 0.4 sec, respectively, on the second day after SNL (Figure 4.6). The behavioral responses of the rats with SNL and sham DLF surgery remained constant

throughout the 14-day observation period. In contrast, the heightened sensory responses of the rats with SNL and DLF lesions began to return to pre-SNL baseline values within 4-5 days after SNL (Figures 4.5 and 4.6, respectively). The paw withdrawal threshold to light tactile stimuli was significantly ($p \leq 0.05$) increased to 11.7 ± 1.7 g and the paw withdrawal latency to radiant heat was significantly ($p \leq 0.05$) increased to 18.9 ± 1.4 sec by the 7th day after SNL (Figures 4.5 and 4.6, respectively).

Spinal Dynorphin Content in DLF-lesioned Rats

Spinal cords were extruded and assayed for dynorphin content in the dorsal quadrant ipsilateral to SNL or sham ligation on the 10th day after surgery. This time point was chosen because our previous investigations demonstrated that dynorphin levels were maximally increased at this time point (Malan et al. 2000). Rats with sham DLF lesions and L_5/L_6 SNL demonstrated a significant ($p \leq 0.05$) elevation in spinal dynorphin content 10 days after SNL. The spinal dynorphin content of rats with sham DLF lesion and with SNL was 991 ± 63 pg dynorphin/mg protein, whereas that of rats with sham DLF lesion and sham SNL was 698 ± 53 pg dynorphin/mg protein (Figure 4.7). In contrast, lesions of the DLF prevented the elevation in spinal dynorphin content. The spinal dynorphin content in the rats with SNL and DLF lesions was 656 ± 22 pg dynorphin/mg protein, which was not significantly different ($p > 0.05$) from that of the control group (Figure 4.7). Rats with DLF lesions and sham SNL surgery also had a spinal dynorphin content of 667 ± 93 pg dynorphin/mg protein) which was similar to that of the rats with sham DLF and sham SNL surgery.

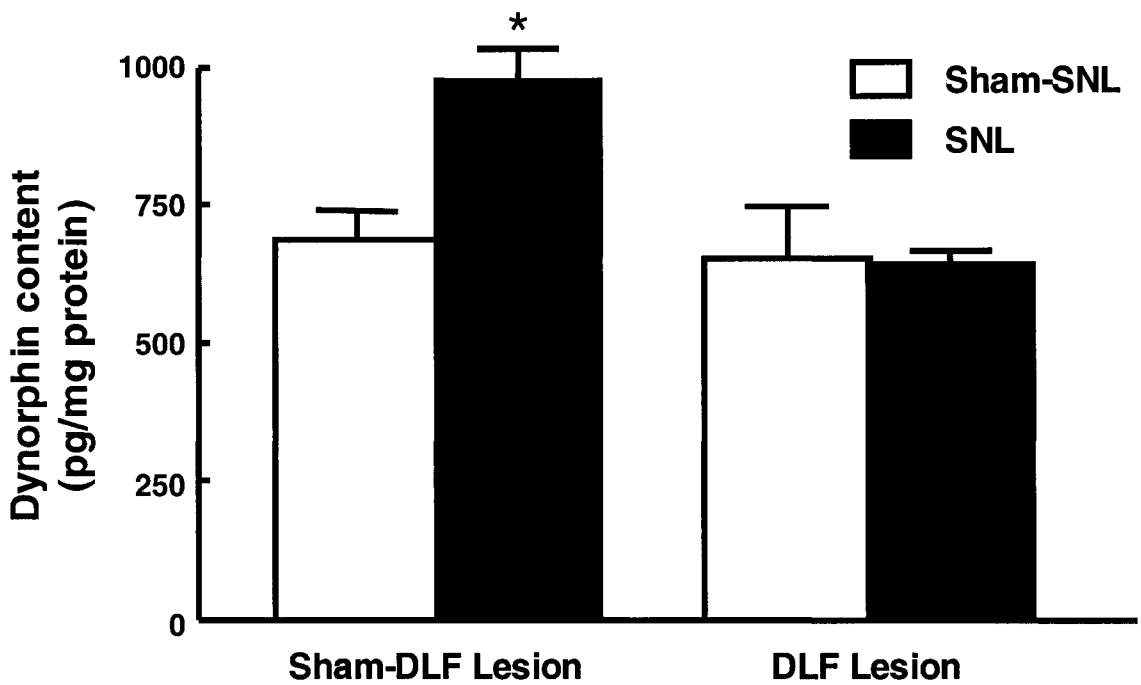

Figure 4.7. The spinal cords of SHAM (Sham-SNL) and rats with L_5/L_6 SNL and that had received either lesions of the DLF or sham spinal surgery (Sham-DLF) were removed on day 10 after surgery. The ispsilateral (relative to SNL or Sham-surgery) dorsal quadrant of the lumbar cord was isolated and assayed for dynorphin content by enzyme immunoassay. The rats with SNL and that had also received Sham-DLF surgery showed a significant (*; $p \leq 0.05$; Student's t-test) increase in spinal dynorphin content when compared to the Sham-DLF/Sham-SNL group. In contrast, lesions of the DLF prevented the upregulation of spinal dynorphin content in rats with SNL. The spinal dynorphin content of this group was not significantly different ($p > 0.05$, Student's t-test) than that of the Sham-DLF/Sham-SNL group, and was significantly ($p \leq 0.05$; Student's t-test) less than that of the Sham-DLF/SNL group. Finally, the spinal dynorphin content of the rats with Sham-SNL and with lesions of the DLF was not significantly different ($p > 0.05$, Student's t-test) than that of the Sham-DLF/Sham SNL group.

Spinal Dynorphin Content in Rats Pretreated with DERM-SAP

Rats were pretreated with a single microinjection of either SAP, DERM or the DERM-SAP conjugate (3 pmol) into the RVM. After 28 days, rats from each group received either SNL or sham surgery. On the 10^{th} day after surgery (i.e., 38 days after RVM injections), the rats were euthanized and the spinal cords were dissected into quadrants for determination of spinal dynorphin content. The spinal dynorphin content of

the sham-SNL groups receiving RVM treatments did not differ significantly (ANOVA, $F_{3,36} = 1.72$, $P = 0.18$) from each other or from a naive untreated group indicating that pretreatment with either SAP, DERM or the DERM-SAP conjugate in the RVM did not alter spinal dynorphin content of animals without nerve injury. Since these assays were performed separately, but under identical conditions, the data for each experimental group were normalized in terms of percent of the corresponding SHAM control group. Thus, the dynorphin content of the SHAM control group pretreated with SAP, DERM

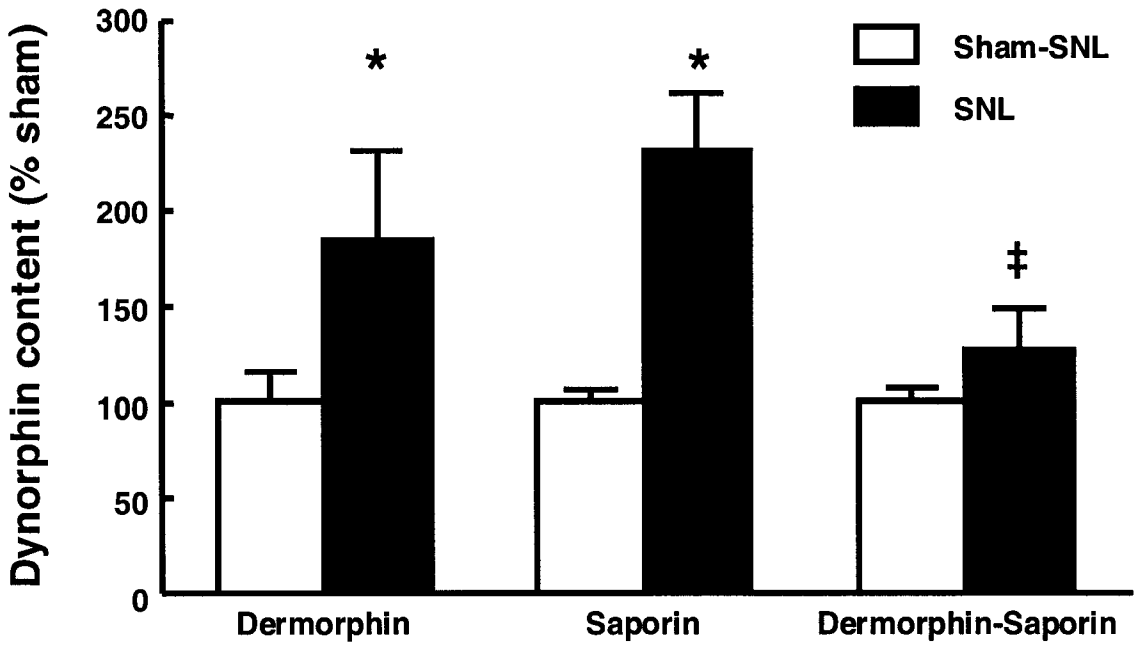

Figure 4.8. The spinal cords of SHAM (Sham-SNL) and rats with L_5/L_6 SNL and that had received a single RVM pretreatment with DERM, SAP or DERM-SAP conjugate. The ispsilateral (relative to SNL or Sham-surgery) dorsal quadrant of the lumbar cord was isolated and assayed for dynorphin content by enzyme immunoassay. The rats with SNL that had also received either DERM or SAP showed a significant (*; $p \leq 0.05$; Student's t-test) increase in spinal dynorphin content when compared to their respective Sham-SNL groups. In contrast, pretreatment with DERM-SAP prevented the upregulation of spinal dynorphin content in rats with SNL. The spinal dynorphin content of this group was not significantly different ($p > 0.05$, Student's t-test) than that of the Sham-SNL group.

and DERM-SAP conjugate was normalized as 100 ± 6%, 100 ± 15% and 100 ± 7%,

respectively (Figure 4.8). Rats pretreated with either SAP or DERM and subsequently receiving L_5/L_6 SNL showed significant ($p \leq 0.05$) increases in spinal dynorphin content to 230 ± 31 % and 183 ± 47% of the corresponding SHAM control groups. In contrast, the spinal dynorphin content of the group pretreated with the DERM-SAP conjugate was 140 ± 22% of the corresponding SHAM group, a value which was not significantly different (Figure 4.8).

CGRP release in tissues from SNL rats with DLF lesions

Tissues were taken from rats undergoing DLF or sham-DLF lesion (3 days prior to peripheral nerve injury) at day 10 following SNL or sham-SNL surgery. No differences in baseline release were observed when DLF and sham-DLF tissues were compared in sham-SNL tissues. Spinal cord tissue obtained from rats with SNL and sham DLF lesions showed capsaicin-evoked release of 173 ± 16% of the corresponding control group (i.e., sham-DLF and sham-SNL), which was 100 ± 27%, demonstrating a significant ($p \leq 0.05$) enhancement of capsaicin-evoked CGRP release (Figure 4.9). Capsaicin-evoked release of CGRP after DLF lesion and SNL was 115 ± 7% of the sham-SNL and DLF group, which was 100 ± 23% (Figure 4.9); these values were not significantly different.

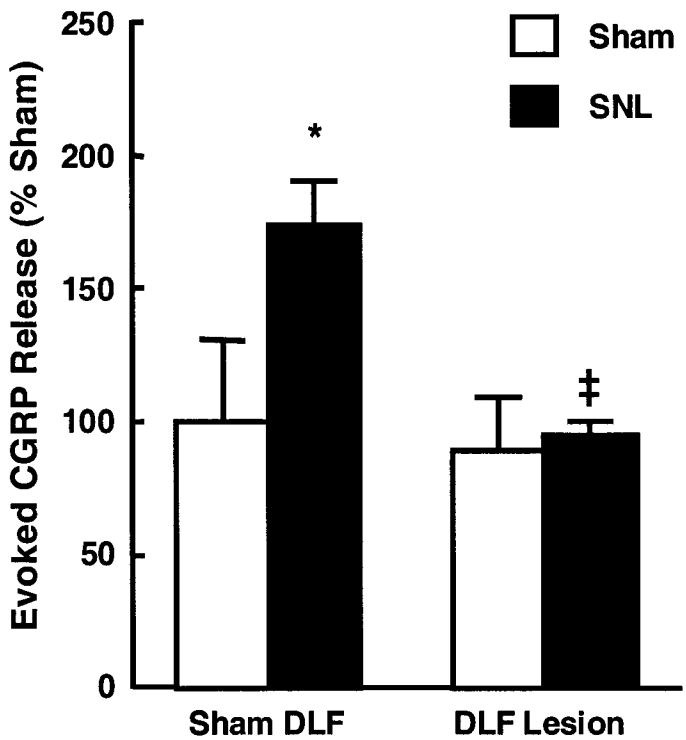

Figure 4.9. Capsaicin-evoked CGRP release was determined in minced lumbar sections obtained from the ipsilateral dorsal quadrants of sham- or SNL-operated rats 10 days after peripheral nerve injury in rats previously undergoing either sham- or DLF lesion (3 days prior to sham- or SNL surgery) at T8. The capsaicin-evoked release of CGRP from the sham DLF/SNL group was significantly (*; $p \leq 0.05$) greater than that of the sham-DLF/sham-SNL group. Capsaicin-evoked CGRP release in DLF/SNL rats was not significantly different from that of the sham-SNL/DLF lesions. Capsaicin evoked CGRP release in DLF/sham-SNL lesion did not differ from that in sham-DLF/sham-SNL rats. The data were converted to percent of the evoked release from SHAM rats in order to account for inter-assay variability. n = 6 to 8 rats per group.

CGRP release in tissues from SNL rats with RVM DERM-SAP pretreatment

Twenty-eight days after receiving a single RVM SAP, DERM or DERM-SAP conjugate pretreatment, rats received either sham or L_5/L_6 SNL surgery. Tissues were taken for evaluation of evoked CGRP release after a further 10 days, i.e., 38 days after the RVM pretreatment. Spinal cord sections at the L_5 level were removed and capsaicin-evoked release of CGRP performed as described above. Each set of data within each of the pretreatment groups were compared to the SHAM control of the corresponding group.

Baseline release of CGRP was unaltered by any of the RVM pretreatments. The RVM pretreatment with either unconjugated SAP or DERM did not alter the expected enhanced capsaicin-evoked release of CGRP resulting from peripheral nerve injury. The capsaicin-evoked CGRP release of SAP or DERM pretreated, SHAM groups were 100 ± 33% and

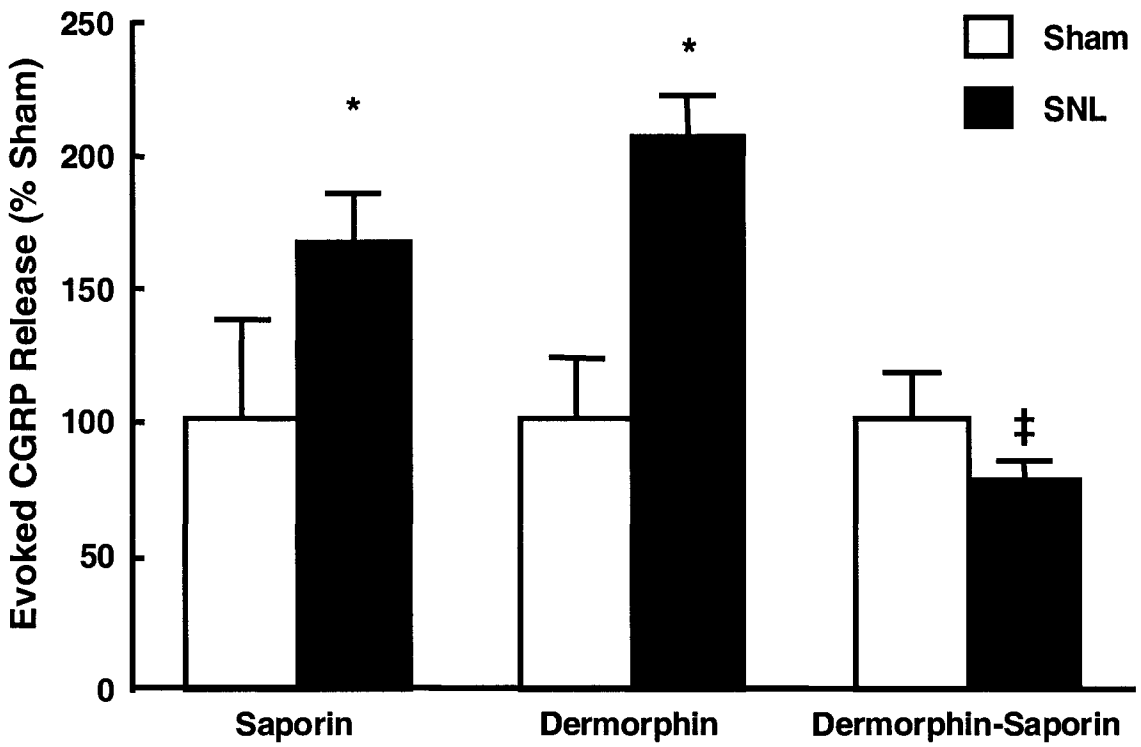

Figure 4.10. Capsaicin-evoked CGRP release was determined in minced lumbar sections obtained from the ipsilateral dorsal quadrants of sham- or SNL-operated rats 10 days after peripheral nerve injury in rats pretreated 38 days previously with RVM SAP, DERM or DERM-SAP conjugate. Tissues from rats with SNL that were pretreated with either SAP or DERM in the RVM demonstrated a significantly greater (*; $p \leq 0.05$) capsaicin-evoked CGRP release than those tissues taken from rats with the same RVM pretreatment and sham surgery. In contrast, RVM pretreatment with DERM-SAP prevented the SNL-induced enhancement of capsaicin-evoked CGRP release in tissues from SNL rats. The capsaicin-evoked release of CGRP from the SNL group pretreated with the DERM-SAP conjugate was not different from that of the SHAM group and was not different from that seen in tissues from RVM saline pretreated SHAM rats. The data were converted to percent of the evoked release from SHAM rats in order to account for inter-assay variability. n = 6 to 8 rats per group.

100 ± 21%, respectively (Figure 4.10). Both the DERM or SAP pretreated groups with SNL demonstrated significant ($p \leq 0.05$) increases in capsaicin evoked CGRP release of 223 ± 21% and 167 ± 18%, respectively (Figure 4.10). In contrast, the selective lesioning of RVM neurons that express the MOR by microinjection of the DERM-SAP conjugate prevented the injury-induced enhancement of capsaicin-evoked CGRP release. The evoked release obtained from the SNL group was 78 ± 6%, which was not significantly different from the value of 100 ± 16% obtained from the corresponding SHAM control group (Figure 4.10).

Discussion

The results of the present experiments provide supporting evidence for the hypothesis that mechanisms that initiate neuropathic pain differ from those that maintain such pain. In addition, the data support the hypothesis that some of the nerve injury-induced plasticity occurring at the spinal level may be secondary to developing plasticity in other regions of the neuroaxis. Although the initiation of neuropathic pain is likely to be mediated by increased afferent drive occurring shortly after the injury, such enhanced activity is insufficient to maintain the neuropathic state in the absence of time-related development of descending facilitation arising in the RVM and an attendant elevation in spinal dynorphin content. The need for descending modulatory influences and enhancement of spinal dynorphin does not exclude the possibility of other mechanisms that may also be important in maintaining the neuropathic state.

Considerable evidence supports the importance of afferent drive as a mechanism of neuropathic pain (for review, see Devor et al. 1992; Dickenson et al. 2001).

Behavioral signs of neuropathic pain in nerve-injured mice were blocked by spinal (+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801) at postinjury day 2, although they were insensitive to dynorphin antiserum at this time, suggesting the importance of excitatory transmission possibly arising in part from increased afferent input (Wang et al. 2001). Tactile and thermal hypersensitivity in nerve-injured rats was significantly attenuated by the application of lidocaine directly at the injury site (Kovelowski et al. 2000; Malan et al. 2000; Ossipov et al. 1995) and local lidocaine application has been used to successfully treat postherpetic neuralgia (Rowbotham et al. 1995; Rowbotham et al. 1996). These observations suggest that enhanced afferent discharge is an important component of the neuropathic state at both the initial stage and at subsequent stages after injury. Experimental observations also confirm, however, that although maintained above preinjury baselines, spontaneous ectopic activity diminishes quite rapidly within the first week after injury (Han et al. 2000; Liu et al. 2000a; Liu et al. 2000b). Despite the diminishing afferent input over time, the behavioral hypersensitivity remains unchanged for many weeks once it is established, suggesting that other mechanisms may be also necessary to maintain the neuropathic state.

Previous work has shown that the behavioral expression of neuropathic pain is dependent on descending facilitatory systems that arise in the RVM. Such facilitation may be time dependent, resulting from plasticity in the RVM and may act to further enhance the now diminished afferent input from injured (Devor and Seltzer 1999) or adjacent (Tal and Bennett 1994; Wu et al. 2001; Yoon et al. 1996) fibers. Evidence

supports a role for pontine–medullary sites in the manifestation of experimental neuropathic pain (Kovelowski et al. 2000; Pertovaara et al. 2001; Pertovaara et al. 1996). Manipulations that disrupt communication between the brain and spinal cord have been shown to block the expression of tactile and thermal hypersensitivity. Spinal transection and hemisection eliminate nerve injury-induced tactile hypersensitivity, indicating the critical contribution of a supraspinal component in the expression of neuropathic pain (Bian et al. 1998; Kauppila et al. 1998; Sun et al. 2001). Similarly, enhanced responses of wide dynamic-range neurons to tactile stimuli induced by mustard oil are blocked by transection of the spinal cord (Mansikka and Pertovaara 1997; Pertovaara 1998).

The RVM has been well characterized in regard to spinopetal modulatory control of nociception mediating both inhibition and facilitation of nociception (Fields 1992; Zhuo and Gebhart 1992; Zhuo and Gebhart 1997). Persistent input from injured or adjacent fibers to supraspinal sites (Sun et al. 2001) may ultimately elicit neuroplastic changes within the RVM that might elicit a time-related activation of descending facilitation. One possibility for such descending facilitation is the class of RVM neurons identified as ON cells, because they accelerate firing immediately before a nociceptive reflex occurs (Fields 1992; Fields et al. 1983; Fields and Heinricher 1985; Heinricher et al. 1992; Heinricher and Roychowdhury 1997). Enhanced nociceptive sensitivity has been noted when ON cell activity is increased (Bederson et al. 1990; Heinricher et al. 1989; Kim et al. 1990). Consistent with this, RVM lidocaine blocks both SNL-induced enhanced activity of spinal dorsal horn units and neuropathic behavior, suggesting the presence of a facilitatory influence from this region (Kovelowski et al. 2000; Mansikka

and Pertovaara 1997; Pertovaara 1998; Pertovaara et al. 2001; Pertovaara et al. 1996; Porreca et al. 2002). The possible time dependency of neuropathic pain on such descending facilitation has not been explored previously. The present studies reveal that descending influences are not apparent for the first 3 days after injury but are clearly present by day 6, when RVM lidocaine blocks both SNL-induced tactile and thermal hypersensitivity. Critically, RVM lidocaine was inactive at postinjury day 3, suggesting that at this time, tonic activity of cells in this region is unlikely.

Evidence supports the possibility that RVM cells that mediate descending facilitation may express MORs (Fields et al. 1983; Fields and Heinricher 1989; Heinricher et al. 1994; Pan et al. 1990). It has been shown previously that DERM–SAP produced a partial lesion of MOR-expressing neurons in the RVM and prevented as well as reversed the behavioral manifestation of neuropathic pain when evaluated at postinjury day 7 (Chapter III). Similarly, selective ablation of the DLF, which includes the spinopetal projections from the RVM, also prevented and reversed experimental neuropathic pain behavior when evaluated at postinjury day 7 (Ossipov et al. 2000a). Together, these observations provide strong evidence that descending facilitation from the RVM is a critical factor in the expression of pain. The present studies show that such descending facilitation does not play a role in the early phase of the postinjury state but seems to be critical to the maintenance of the neuropathic condition. Lesions of the DLF or of MOR-expressing cells in the RVM show a reversal of SNL-induced behavior that is apparent by approximately postinjury day 5 and a return to preinjury baselines by approximately day 8. The time course of the reversal of both tactile and thermal

hypersensitivity after lesion of the DLF or of RVM cells with DERM–SAP is remarkably similar, suggesting that RVM plasticity over this time period and later is crucial to the neuropathic state. These data are also consistent with the observed reversible blockade of nerve injury-induced pain by RVM lidocaine.

The time course over which descending facilitation develops is also consistent with the time course of nerve injury-induced upregulation of spinal dynorphin content, which may provide insights into spinal mechanisms by which facilitation may occur. The relatively late peak in expression of spinal dynorphin after nerve injury (Malan et al. 2000; Wang et al. 2001) suggests the possibility that upregulation depends on the time-related development of descending modulatory influences and may ultimately function to maintain the neuropathic state. This possibility is supported by the data, because manipulations that blocked the maintained state of neuropathic pain also blocked the SNL-induced elevation of spinal dynorphin content. For example, disruption of the spinopetal tracts from the RVM through the DLF and obliteration of descending facilitatory cells expressing the MOR with DERM-SAP, prevented SNL-induced upregulation levels of spinal dynorphin. Because neither DLF lesion or dorsal rhizotomy blocks basal expression of spinal dynorphin, it is highly likely that upregulation of dynorphin results from local interneurons (Cho and Basbaum 1988). Significantly, lidocaine in the RVM also did not block neuropathic pain behaviors at postinjury day 3, a time at which spinal dynorphin is not significantly elevated, suggesting the presence of a transitional period for descending influence (Malan et al. 2000; Wang et al. 2001). Dynorphin antiserum was shown to abolish tactile and thermal hypersensitivity at

postinjury day 14, but not at day 2, whereas MK-801 was effective at both time points (Wang et al. 2001). Finally, mice with deletions of the prodynorphin gene displayed the behavioral signs of neuropathic pain only up to postinjury day 5, with complete reversal by day 8, whereas wild-type littermates maintained pain for the entire 14 day observation period (Wang et al. 2001). These data are all consistent with the view that upregulation of spinal dynorphin is a mechanism that maintains the neuropathic state, perhaps through a nonopioid action, to enhance release of excitatory neurotransmitters such as glutamate or excitatory peptides from primary afferents (Arcaya et al. 1999; Claude et al. 1999; Faden 1992; Gardell et al. 2003; Skilling et al. 1992).

These data provide evidence for the presence of time-related descending facilitatory influences arising in the RVM that are critical to the maintenance but not the initiation of experimental neuropathic pain. In addition, the data show the importance of descending influences in eliciting plasticity at the spinal level. It is not known whether other changes observed in the spinal dorsal horn after nerve injury similarly depend on descending influences. Together, these and possibly other events appear to be established by the initial processes of peripheral nerve injury to maintain the expression of abnormal pain. Patients experiencing neuropathic pain are likely to require intervention at time points substantially long after the precipitating injury has occurred, suggesting that the understanding of the processes that maintain neuropathic pain will be critically important in the development of rational approaches for therapeutic interventions.

CHAPTER V: DERMORPHIN-SAPORIN TARGETS DESCENDING FACILITATION IN THE RVM TO BLOCK CCK-INDUCED ABNORMAL PAIN

Introduction

There is a significant body of evidence which suggests that the RVM may be a key source for descending facilitation of nociceptive inputs (Calejesan et al. 1998; Fields 1992; Heinricher and Roychowdhury 1997; Kaplan and Fields 1991; McNally 1999; Porreca et al. 2002; Urban et al. 1999; Urban and Gebhart 1999; Zhuo and Gebhart 1992; Zhuo and Gebhart 1997). Microinjection of glutamate or electrical stimulation in the RVM elicits increased dorsal horn unit activity and enhances nociceptive reflexes (Urban and Gebhart 1997; Zhuo and Gebhart 1992; Zhuo and Gebhart 1997). Furthermore, lidocaine in the RVM blocks enhanced pain behaviors in rats with SNL or inflammatory pain (Kovelowski et al. 2000; Mansikka and Pertovaara 1997; Pertovaara et al. 1996) (Chapter IV). Electrical stimulation of the DLF, which conducts the bulbospinal projections from the RVM, elicits an excitation of dorsal horn units in lamina I of the spinal dorsal horn, demonstrating a clear descending facilitation through this pathway (McMahon and Wall 1983; McMahon and Wall 1988). Finally, lesions of the DLF block SNL-induced tactile and thermal hypersensitivity (Ossipov et al. 2000a) (Chapter IV).

The activation of descending pain facilitation arising from the RVM in response to a peripheral nerve injury suggests that the implementation of processes is time-related. This is supported by data presented in Chapter IV. For example, the microinjection of lidocaine into the RVM did not block tactile or thermal hypersensitivity on day 3 after

SNL, but did so effectively on days 6, 9 and 12 after SNL. These findings suggest that the initial manifestation of neuropathic pain is mediated by mechanisms that are independent of RVM activity, which, once initiated, become critical in the persistence of the pain states. This interpretation is supported by the finding that DLF lesions performed 2 days prior to SNL block only the later (or maintenance) phase of neuropathic pain behaviors, without preventing the initial onset of abnormal pain during the first 4 days after SNL. Finally, virtually identical results were obtained when the selective μ opioid DERM conjugated to the cytotoxin SAP was microinjected into the RVM 28 days prior to SNL (Chapter III). Taken together, these results demonstrate that processes that initiate the behavioral expression of neuropathic pain differ from those necessary to maintain such pain.

The relative distribution and possible co-expression of opioid receptors and CCK receptors in the brain, unlike that in the DRG and spinal cord, is largely unknown. Recent findings show that RVM CCK attenuates opioid activation of OFF cell firing and inhibition of the tail flick response without significantly altering the opioid suppression of ON cell firing (Heinricher et al. 2001). These data suggest that during acute nociception, the anti-opioid effect of CCK is predominantly due to inhibiting OFF cell firing rather than promoting ON cell firing. The inhibition of OFF cell firing is perhaps due to the excitatory effect of CCK on GABA release, which overcomes the opioid mediated excitation of OFF cells by disinhibition. The source of GABA remains to be established. There exists in the RVM a population of GABA interneurons (Marinelli et al. 2002) and many of these are regulated by opioids. Alternatively, GABAergic terminals projecting

to the RVM could be regulated by RVM CCK and opioid presynaptically. In both cases, one would predict the co-localization of CCK receptors and opioid receptors particularly of the μ type.

Early studies hinted at an antinociceptive role for CCK, but such reports have been inconsistent (Baber et al. 1989). In contrast, the supraspinal or spinal administration of CCK induces pronounced hyperalgesic activity and increased dorsal horn unit activity. These actions are suggestive of a pronociceptive role of CCK in the CNS (Hong and Takemori 1989; Jeftinija et al. 1981; Pittaway and Hill 1987; Pittaway et al. 1987). Our recent study found that microinjection of CCK into the RVM of naive rats provoked tactile hypersensitivity and thermal hyperalgesia (Kovelowski et al. 2000). In addition, the application of the CCK_2 antagonist, L365,260, into the RVM reversed both tactile hypersensitivity and thermal hyperalgesia in rats with SNL injury, indicating a CCK-mediated descending facilitation from this site (Kovelowski et al. 2000). The infusion of CCK into the RVM attenuated the antinociceptive effect of systemic morphine, but the mechanism through which this effect was mediated was not determined (Heinricher et al. 2001). Thermal hyperalgesia elicited by neurotensin in the RVM was blocked by spinal CCK antagonists, indicating that CCK is released spinally by a descending facilitatory input from the RVM (Urban et al. 1996).

Here, we explore the hypothesis that time-dependent neuroplastic changes in the RVM act to maintain neuropathic pain, and that such pain is "driven" by increased activity of CCK in the RVM.

Time course of CCK_8 –induced tactile and thermal hypersensitivity

Paw withdrawal thresholds to probing of the hindpaw with von Frey filaments and paw withdrawal latencies from noxious radiant heat were determined immediately prior to dosing and for 60 minutes, at 15 minute intervals, after the microinjection of saline (0.5 μL per side) or 30 ng/0.5 μL of CCK_8. The microinjection of CCK_8 produced a significant ($p<0.05$) reduction (at 30 min) in paw withdrawal thresholds from a baseline value of 15 ± 0 g to 4.1 ± 1.0 g, as well as a significant ($p<0.05$) reduction in paw

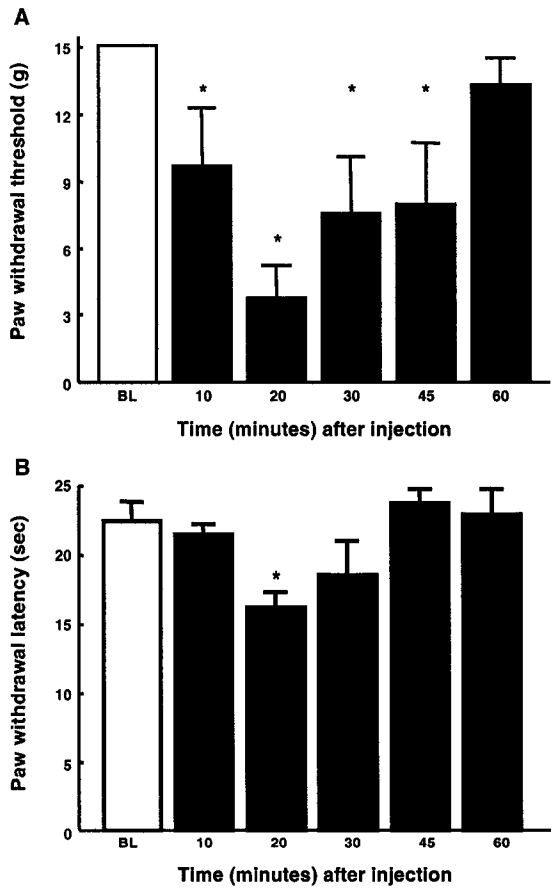

Figure 5.1. Rats were tested for threshold responses to non-noxious mechanical (Panel A) and noxious thermal stimuli (Panel B). Rats then received an RVM injection of CCK_8 (60 ng total). Rats exhibited time-related tactile and thermal hypersensitivity that peaked at 20-30 minutes after injection.

withdrawal latencies from a baseline value of 21.6 ± 4.0 sec to 14.6 ± 2.10 sec (Figure

5.1, Panels A and B, respectively). Microinjection of vehicle did not produce any change in these behavioral endpoints (data not shown).

Inhibition of CCK_8–induced tactile and thermal hypersensitivity by L365,260

The microinjection into the RVM of the CCK2 antagonist L365,260 (2.5 ng/0.5 μL) 5 minutes prior to CCK_8 administration inhibited the CCK-induced hypersensitivity to tactile and thermal stimuli (Figure 5.2). The administration of L365,260 or its vehicle

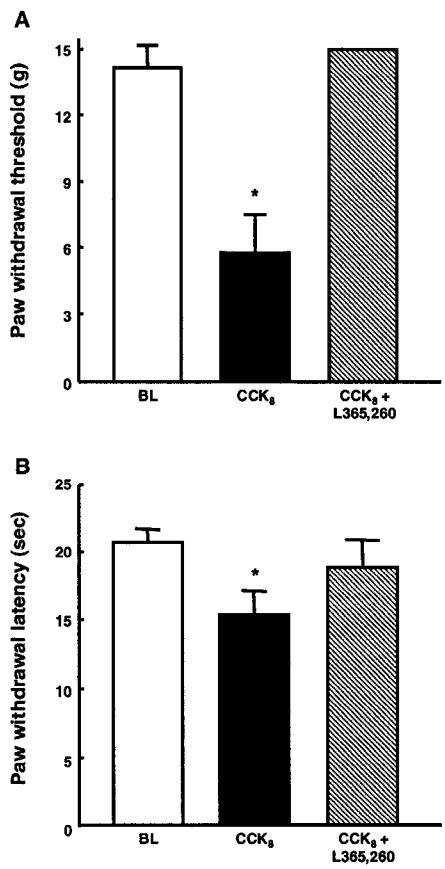

Figure 5.2. Rats were tested for threshold responses to non-noxious mechanical (Panel A) and noxious thermal stimuli (Panel B). Rats then received and RVM injection of CCK_8 or CCK_8 preceded by the CCK_2 antagonist, L365,260. Rats that received an injection of CCK_8 alone exhibited the expected development of tactile and thermal hypersensitivity. However, injection of L365,260 blocked the development of CCK_8-induced hypersensitivity. (Figure contributed by Yanhua Xie)

(10% DMSO in water) alone did not produce any change in these behavioral endpoints

(data not shown). These results indicate that CCK in the RVM may act to elicit nociceptive behaviors through activation of CCK_2 receptors.

Inhibition of CCK_8–induced tactile and thermal hypersensitivity by lesions of the DLF

In order to determine whether RVM CCK-induced tactile and thermal hypersensitivity is mediated through descending pain facilitatory pathways via the DLF, rats received either bilateral lesions of the DLF or sham lesions. Following a recovery period, CCK was administered into the RVM and rats were subsequently tested for possible tactile and thermal hypersensitivity.

Rats with sham lesions that were administered CCK into the RVM developed robust and transient tactile and thermal hypersensitivty, which peaked between 30 and 45 min. Paw withdrawal thresholds decreased significantly ($p<0.05$) from 12.7 ± 1.3 g to 5.8 ± 1.7 g following RVM CCK administration (data not shown). Similarly, paw withdrawal latencies decreased significantly ($p<0.05$) from 20.7 ± 1.4 sec to 13.8 ± 0.4 sec following RVM CCK administration (data not shown). In contrast, CCK-induced tactile and thermal hypersensitivity was completely blocked in rats with bilateral DLF lesions (Figure 5.3, Panels A & B, respectively). Paw withdrawal thresholds were not altered significantly as none of the time points were different than the baseline level of 12.7 ± 1.3 g following RVM CCK administration (all $ps>0.05$, Figure 5.3 Panel A). Similarly, paw withdrawal latencies were not altered significantly as none of the time points were different than the baseline level of 20.7 ± 1.4 sec following RVM CCK administration (all $ps>0.05$, Figure 5.3 Panel B).

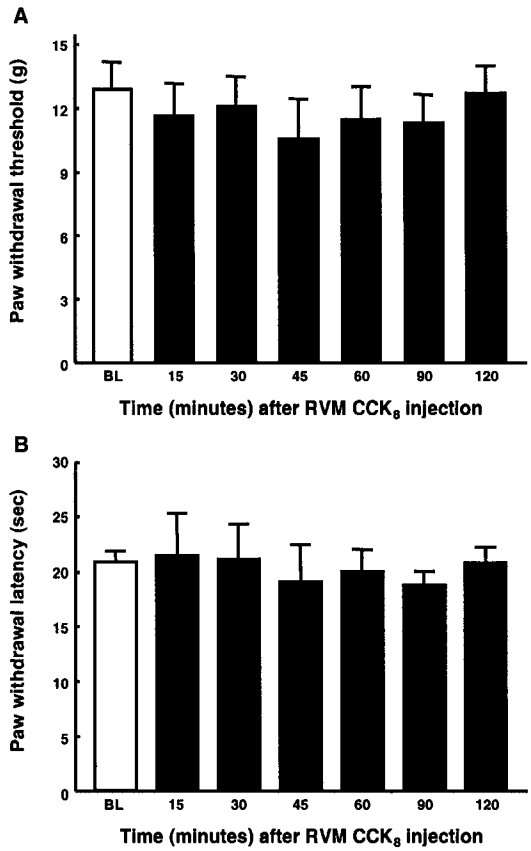

Figure 5.3. Rats received bilateral lesions of the DLF. Following a period of recovery, rats were baselined and then administered CCK (60 ng total) into the RVM. Following RVM CCK microinjection, the animals were tested for the presence of possible tactile (Panel A) and thermal (Panel B) hypersensitvity. Whereas rats with sham-DLF lesions demonstrated a robust and transient tactile and thermal hypersensitivity (data not shown), rats with DLF lesions failed to exhibit CCK-induced hypersensitivity. (Figure contributed by Yanhua Xie)

Inhibition of CCK_8 –induced tactile and thermal hypersensitivity by pretreatment with RVM DERM-SAP

A separate group of rats were pretreated with a single bilateral microinjection of either saline or 3 pmol DERM, SAP, or DERM-SAP into the RVM. Administration of DERM-SAP into the RVM produces a selective loss of MOR-expressing neurons in the RVM within 28 days (Chapter III).

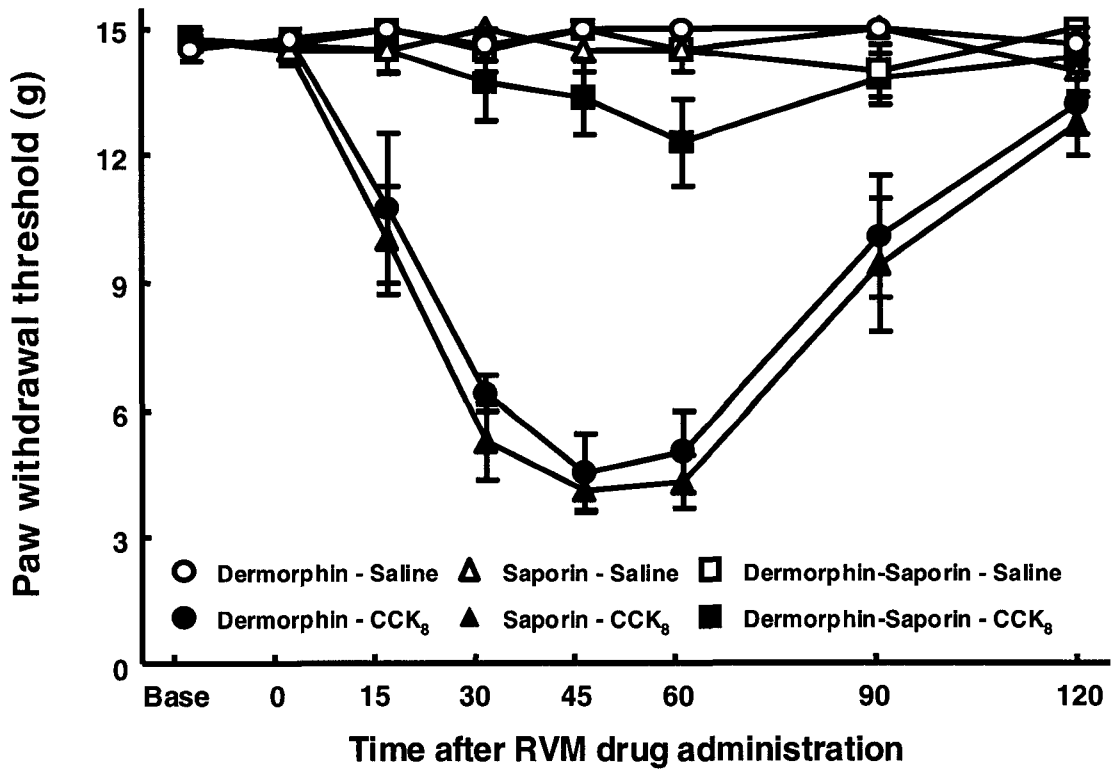

Figure 5.4. Rats were pretreated with injections of DERM, SAP, or DERM-SAP 28 days previously. Paw withdrawal thresholds to light tactile stimuli were determined before before and after injections of either saline or CCK_8. Rats that were pretreated with DERM (filled circles), or SAP (filled triangles), exhibited tactile hypersensitivity that peaked at around 30 min following an RVM microinjection of CCK. However, rats that were pretreated with RVM DERM-SAP (filled squares) demonstrated response thresholds, following an RVM microinjection of CCK, that were not significantly different ($p>0.05$) from those observed in saline-injected control rats.

Baseline responses to tactile or thermal stimuli were also unchanged by these treatments (Chapter III). On the 28th day after the microinjection, rats received bilateral microinjection of either 30 ng/0.5 μL of CCK_8 or of saline into the RVM. Paw withdrawal thresholds to probing the hindpaw with von Frey filaments and paw withdrawal latencies from noxious radiant heat were then monitored over 120 min time-course.

The behavioral response to either tactile or thermal stimuli remained unchanged over time in all of the rats that received a RVM microinjection of saline (open symbols) (Figures 5.4 and 5.5, respectively). Rats that were pretreated with DERM (filled circles), or SAP (filled triangles), exhibited both tactile and thermal hypersensitivity that peaked at around 30 min following a RVM microinjection of CCK.

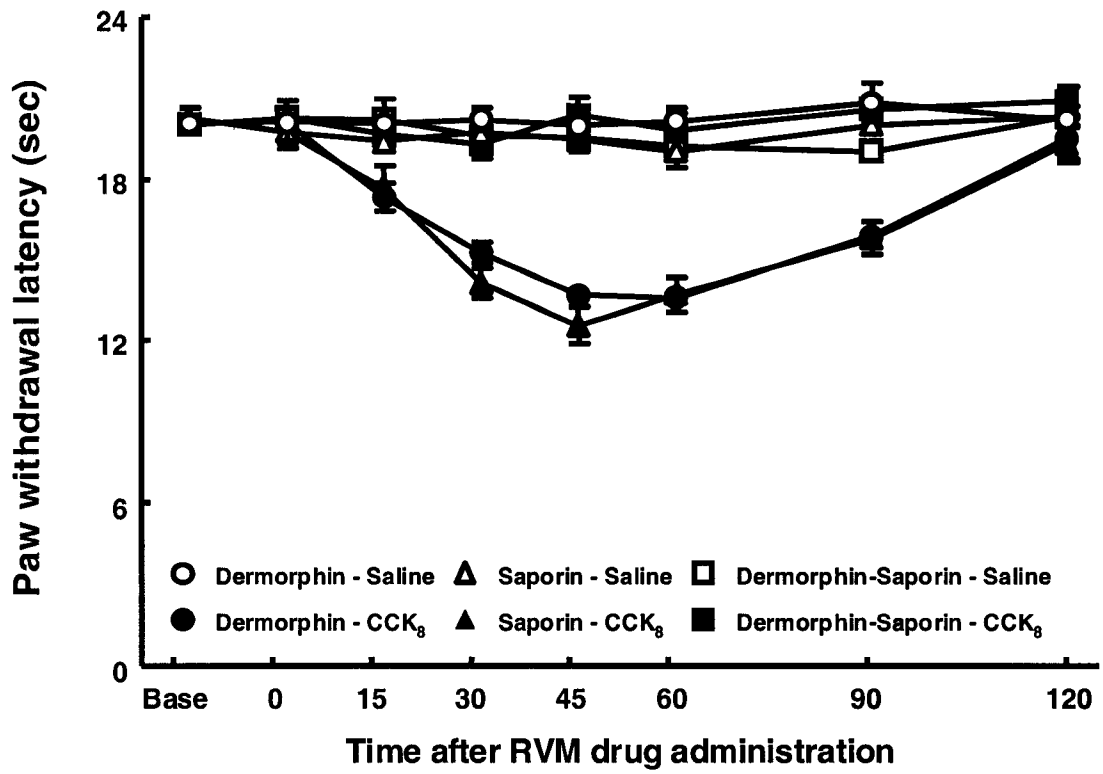

Figure 5.5. Rats were pretreated with injections of DERM, SAP, or DERM-SAP 28 days previously. Paw withdrawal latencies in response to noxious radiant heat were determined before and after injections of either saline or CCK_8. Rats that were pretreated with DERM (filled circles), or SAP (filled triangles), exhibited thermal hypersensitivity that peaked at around 30 min following an RVM microinjection of CCK. However, rats that were pretreated with RVM DERM-SAP (filled squares) demonstrated paw withdrawal latencies, following an RVM microinjection of CCK, that were not significantly different ($p>0.05$) from those observed in saline-injected control rats.

The CCK-induced tactile and thermal hypersensitivity gradually returned to baseline levels by 120 min. In contrast, the rats that were pretreated with RVM DERM-

SAP (filled squares) demonstrated response thresholds, following a RVM microinjection of CCK, that were not significantly different ($p>0.05$) from those observed in saline-injected control rats. These results suggest that the MOR-expressing cells in the RVM are critical for the pronociceptive actions of RVM CCK.

ISH of μ opioid and CCK_2 receptor transcripts in rats treated with either RVM SAP or DERM-SAP

In additional studies, DERM-SAP pretreatment, but not SAP control treatment, resulted in a reduction in CCK_2-receptor expressing cells in the RVM (Figure 5.6). In particular, it seems that DERM-SAP treatment selectively killed large-sized CCK_2-receptor expressing neurons. This concomitant loss of both μ opioid (seen first in Chapter III and repeated with MOR riboprobe in Figure 5.6) and CCK_2 receptors after DERM-SAP treatment gives support to the notion that RVM CCK directly (or indirectly) activates RVM descending facilitatory cells, some of which may express both types of receptors.

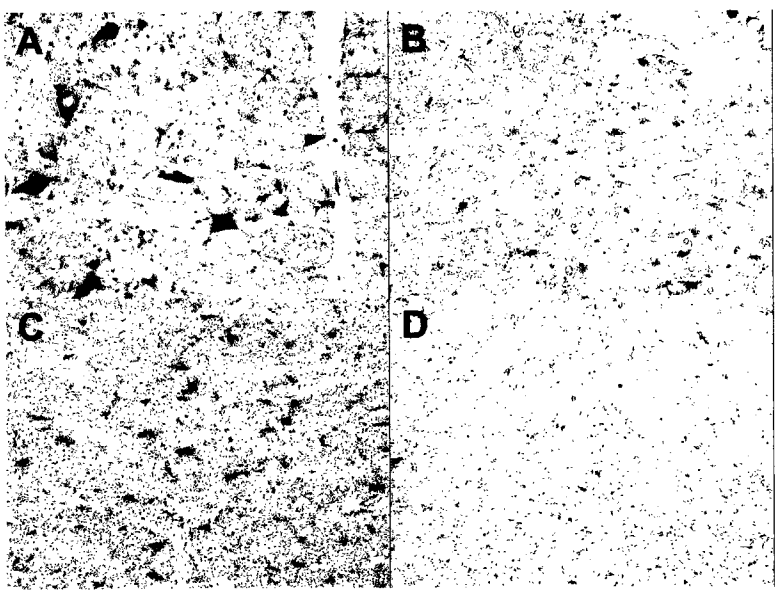

Figure 5.6. ISH of μ opioid and CCK_2 receptor mRNA in frontal sections (20 μm) of the brainstem at the level of the caudal raphe nuclei by a colorimetric immunodetection method using fluorescein- or digoxigenin-labeled riboprobes generated from the coding region of rat μ opioid and CCK_2 receptors, respectively. Hybridized probes were detected using either anti-fluorescein (μ opioid) or anti-digoxigenin (CCK_2) antibodies. High-magnification, bright-field micrographs (*A-D*) show the distribution of the probes as a red stain (fast red). The localization of the cells that express MOR mRNA (*C*) is consistent with that described in Chapter III. The distribution of CCK_2 receptor transcripts (*A*) was similar to the MOR distribution. The RVM of DERM-SAP-pretreated rats (*B & D*) consists of significantly fewer labeled cells when compared with that of SAP-pretreated (*A & C*) rats. Scale bars = 50 μm.

Discussion

The data presented in this Chapter provides evidence for a functional relationship between the pronociceptive actions of CCK and the MOR-expressing cells in the RVM (some of which are ON cells). Previous work from our laboratory demonstrated that microinjection of CCK into the RVM of naive rats produces a pronounced and transient increase in sensitivity to both non-noxious mechanical and noxious thermal stimuli (Kovelowski et al. 2000). This effect of CCK was also observed in the present Chapter. These actions of CCK are likely mediated through either the direct or indirect activation of RVM ON cells. Lesioning of the DLF blocks the ability of RVM CCK to produce tactile and thermal hypersensitivity. Further, selective ablation of MOR-expressing cells with DERM-SAP abolishes the pronociceptive actions of RVM CCK. Moreover, the DERM-SAP mediated lesion, which produced a significant loss of MOR-expressing cells, also results in a marked loss of cells positive for CCK_2 receptor mRNA. Taken together, these data suggest that MOR-expressing cells in the RVM mediate the pronociceptive actions of CCK. It is likely that a subpopulation of RVM neurons co-express μ opioid and CCK_2 receptors, and this may be a phenotype of the functionally defined ON cells. It is our contention that over-activation of these RVM ON cells may provide a mechanism for the maintenance of neuropathic pain (Porreca et al. 2002). These data represent the first phenotypic characterization of the RVM neurons that are critical for neuropathic pain.

CCK may thus represent a key mediator in maintaining descending pain facilitation from the RVM. To date, two CCK receptor subtypes have been identified,

CCK_1 and CCK_2. CCK_1 receptors are found predominantely in visceral organs of the digestive system, whereas CCK_2 receptors are generally localized in the CNS (Baber et al. 1989; Innis and Snyder 1980; Moran et al. 1986; Wank et al. 1994). Both receptors are G-protein coupled primarily through the $G_{\alpha q}$/PLC pathway (Wank 1995). Activation of CCK receptors produces a transient increase in intracellular calcium (Wank 1995). Recent data from our laboratory has shown that pharmacological administration of CCK can potentiate capsaicin-evoked CGRP release from primary afferent terminals in spinal dorsal horn tissue, which is selectively blocked by the CCK_2 antagonist, L365,260. Therefore, it is likely that CCK_2 receptors are the functionally relevant CCK receptor subtype in the RVM since the CCK_2 antagonist, L365,260 blocks CCK_8-induced tactile and thermal hypersensitivity. However, the potential role of CCK_1 cannot be ruled out without confirming its contribution by looking at the effect of CCK_1 receptor antagonists on CCK_8 or SNL-induced tactile and thermal hypersensitivity.

In addition, it is of particular interest whether CCK_8 acts presynaptically or postsynaptically. Evidence from our laboratory has found that intrathecal administration of DERM-SAP does not result in a decreased MOR population in the DRG (Zhang and Porreca, unpublished observations). Thus, it appears that DERM-SAP is not retrogradely transported and is likely to lesion local MOR-expressing cells, but not those that project to the RVM. Therefore, in accordance with the ISH results presented in this Chapter, DERM-SAP is likely to eliminate postsynaptic μ opioid and CCK_2 receptors associated with RVM neurons. Due to the transport limitations of DERM-SAP, it is not technically feasible to look at the potential contribution of presynaptic μ opioid and CCK_2 receptor-

expressing cells in the RVM to the maintenance of neuropathic pain.

The relative distribution and possible co-expression of opioid receptors and CCK receptors in the brain, unlike that in the DRG and spinal cord, is largely unknown. Results from the ISH experiments from the present Chapter reveal at least 2 morphologically distinct populations of CCK_2 receptor positive cells that can be preliminarily distinguished based on size. It appears that treatment with RVM DERM-SAP, which resulted in a significant loss of MOR-expressing cells, also results in a marked loss of cells positive for the CCK_2 receptor. Of particular interest, it seems DERM-SAP selectively ablates the subpopulation of CCK_2 receptor positive cells that appear to be of larger-size, while staining for smaller cells remains relatively intact. Presently, the phenotype(s) of these two different cell populations remains unclear. Further investigation will be required in order to characterize these two distinct cell populations.

Taken together, these data suggest that MOR-expressing cells in the RVM mediate the pronociceptive actions of CCK and cells which co-express both μ opioid and CCK_2 receptors are likely to be ON cells. It is our contention that over activation of these RVM ON cells may provide a mechanism for the maintenance of neuropathic pain (Porreca et al. 2002).

CHAPTER VI: CONCLUSIONS

The focus of this dissertation was to explore the role of descending facilitation from supraspinal sites, specifically the RVM, in the maintenance of neuropathic pain. Specifically, three hypotheses were tested. First, the presence and activity of descending pain facilitation cells in the RVM are required for the expression of experimental neuropathic pain. Specifically, the destruction of these cells, presumably those expressing the MOR, will both prevent and reverse SNL-induced neuropathic pain. Second, mechanisms that initiate neuropathic pain are separate from those that maintain such pain. Lastly, RVM CCK directly activates RVM descending facilitatory cells to drive the expression of neuropathic pain and may represent a critical mechanism of such pain.

Pain has important physiological functions. It may warn us of actual or impending tissue damage. However, it is hard to provide justification for the importance of pain facilitation mechanisms. In times of persistent injury, these mechanisms may exist to protect the injured area by restricting use of it. However, neuropathic pain exists long after the initial injury has healed, serving no protective mechanism and thus, is more difficult to understand. We can postulate that the protective nature of pain changes after nerve injury, and can become a source of prolonged and exaggerated abnormal pain.

Data from Chapter III demonstrated that the selective obliteration of cells expressing the MOR resulted in a block and a reversal of SNL-induced tactile and thermal hypersensitivity. It is well established that the increased firing of primary

afferent fibers was thought to be a key element in nerve injury-induced pain (Devor 1991; Devor 1994; Fields et al. 1997; Kajander et al. 1992; Wall and Gutnick 1974b). The time course of this afferent discharge does not correlate, however, with the continuous nature of nerve injury-induced pain. Although this pain continues for many weeks (Bian et al. 1995; Chaplan et al. 1994), recent observations show that this discharge from injured afferents declines significantly a few short days after injury (Han et al. 2000; Liu et al. 2000b). With the knowledge that large, myelinated primary afferents project to the brainstem and that plastic changes occur in the RVM and other sites (Kovelowski et al. 2000), we can postulate that supraspinal sites play a major role in the nerve injury-induced pain state. This is again supported by the results of several lesion studies of ascending pain pathways (Houghton et al. 1999; Sun et al. 2001) and by the blockade of SNL-induced pain after RVM lidocaine (Kovelowski et al. 2000; Pertovaara et al. 1996) and lesions of the DLF (Ossipov et al. 2000a).

The data from Chapter III also suggest that it is specifically MOR-expressing neurons in the RVM that are critical in the behavioral expression of neuropathic pain. These cells exhibit characteristics that are consistent with those characterized previously as facilitatory or pronociceptive, and are presumably ON cells. It has been shown that manipulations that increase nociceptive responsiveness, thus indicating facilitation, also increase ON cell activity (Fields and Basbaum 1999; Heinricher et al. 1989; Kaplan and Fields 1991; Morgan and Fields 1994). We have further shown that the presence and activity of these descending facilitatory cells (ON cells) in the RVM are required for the expression of experimental neuropathic pain. Since targeting of these particular neurons

with DERM-SAP resulted in a block and a critical reversal of SNL-induced pain, which is most clinically relevant, these MOR-expressing cells may represent a novel target for the development of strategies to treat this abnormal pain.

Data from Chapter IV provide support for the hypothesis that mechanisms that initiate neuropathic pain differ from those that maintain such pain and that the descending influences responsible for the maintenance phase of neuropathic pain may also be responsible for plastic spinal changes, such as the elevation of spinal dynorphin. As discussed previously, the initiation of neuropathic pain is probably mediated by increased afferent discharge, but this enhanced activity is not sufficient to maintain such pain. We have demonstrated this by targeting MOR-expressing cells, presumably ON cells, in the RVM to eliminate descending facilitation and observing that neuropathic pain was initiated, but not maintained, and that elevation in spinal dynorphin content as well as a corresponding increase in CGRP release from primary afferent neurons, was blocked.

Interestingly, the time course over which descending facilitation develops correlates with the time course of SNL-induced upregulation of spinal dynorphin. The peak in the expression of spinal dynorphin occurs relatively late after nerve injury (Malan et al. 2000; Wang et al. 2001) and suggests that upregulation may depend on the time-related development of descending modulatory influences and may function to maintain neuropathic pain. This postulation is supported by data in Chapter IV demonstrating that manipulations that blocked the neuropathic state also blocked SNL-induced elevation of spinal dynorphin. Additionally, it was shown that lidocaine in the RVM did not block tactile and thermal hypersensitivity at postinjury day 3, a time at which dynorphin levels

are not elevated, indicating a transitional type of phase for descending influence (Malan et al. 2000; Wang et al. 2001). Additional work from our laboratory found that antiserum to dynorphin abolished SNL-induced hypersensitivity at postinjury day 14, but not day 2, although MK-801, an NMDA receptor antagonist, was effective at both time points (Wang et al. 2001). Lastly, mice with deletions of the prodynorphin gene exhibited neuropathic pain behaviors only up to postinjury day 5, with a complete reversal by day 8, a time course consistent with that of descending facilitation (Wang et al. 2001). These data all support the notion that the upregulation of dynorphin is an additional mechanism that maintains the neuropathic pain state, perhaps through its non-opioid actions, enhancing excitatory neurotransmitters such as glutamate or excitatory peptides from primary afferents (Arcaya et al. 1999; Claude et al. 1999; Faden 1992; Gardell et al. 2003; Gardell et al. 2002; Skilling et al. 1992; Vanderah et al. 2001a).

Data from Chapter V provide us with a possible activator of RVM descending facilitatory cells, CCK, that serves to drive the expression of neuropathic pain and may represent a critical mechanism of such pain. To review, consistent with its pronociceptive role, RVM microinjection of CCK excites dorsal horn unit activity (Hong and Takemori 1989; Jeftinija et al. 1981; Pittaway and Hill 1987) and produces tactile and thermal hypersensitivity when microinjected into the RVM of rats. We have shown that pretreatment with RVM DERM-SAP, but not DERM or SAP alone, blocks the proalgesic effect of CCK, suggesting that its actions may be mediated by activation of MOR-expressing cells. Enhanced release of CCK in the RVM after SNL may assist in maintaining neuropathic pain states. Additionally, DERM-SAP pretreatment, but not

SAP control treatment, resulted in a reduction in CCK_2 receptor expressing cells in the RVM. This concomitant loss of both μ opioid and CCK_2 receptors after DERM-SAP treatment gives support to the notion that RVM CCK activates RVM descending facilitatory cells and may represent a critical mechanism of neuropathic pain. Data from one future study would provide us with further support. This study would evaluate the extent of co-localization of the MOR and CCK_2 receptor transcripts in the RVM. Cells that expressed both receptor transcripts would provide us with a "biomarker" of sorts for descending facilitatory ON cells.

As a whole, the data included in this dissertation suggest that processes that initiate SNL-induced pain are different from those that are required for the maintenance of such pain. Furthermore, this maintenance phase is pertinent in that it is probably the time that clinical intervention in humans would occur. The possibility that spinal plasticity is critical in the maintenance of neuropathic pain and that these spinal changes may be, at least in part, the result of CCK-driven activation of descending facilitation from the brainstem provides us with a basis for the development of new treatment therapies for neuropathic pain. (SEE FIGURE)

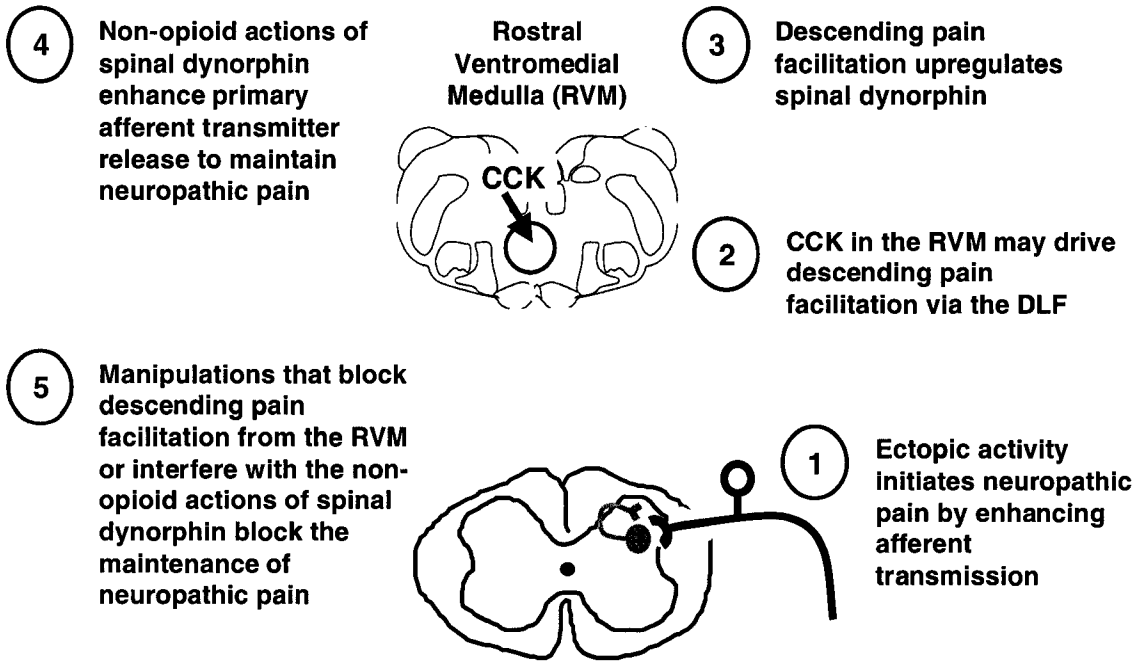

Figure 6.1 Modulation of injury-induced enhanced afferent activity by descending systems

APPENDIX - LIST OF PUBLICATIONS

1. Gardell LR, Reid ML, Cavallaro CA, **Burgess SE**, Wallace RF, Hubbell CL, & Reid LD. Amlodipine, a calcium channel inhibitor, and cocaine and ethanol's reinforcing effects. *Pharmacology Biochemistry and Behavior, 64,* 567-572, 1999.

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3. Porreca F, **Burgess SE**, Gardell LR, Vanderah TW, Malan Jr., TP, Ossipov MH, Lappi DA, & Lai J. Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the mu opioid receptor. *Journal of Neuroscience, 21,* 5281-5288. 2001.

4. Gardell LR, **Burgess SE**, Dogrul A, Ossipov MH, Malan Jr., TP, Lai J, & Porreca F. Pronociceptive effects of spinal dynorphin promote cannabinoid-induced pain and antinociceptive tolerance. *Pain, 98,* 79-88. 2002.

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6. Gardell LR, Wang R, **Burgess SE**, Ossipov MH, Vanderah TW, Malan Jr., TP, Lai J, & Porreca F. Sustained morphine exposure induces enhancement of evoked excitatory transmitter release which is mediated by spinal dynorphin. *Journal of Neuroscience, 22,* 6747-6755. 2002.

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