

THE ROLE OF GLIAL ACTIVATION IN DESCENDING FACILITATION FROM  
THE ROSTROVENTROMEDIAL MEDULLA (RVM) IN MODELS OF  
PERSISTENT PAIN

by

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## DEDICATION

To my parents

Mike and Jeanette

Thank you for your love, support and faith

## TABLE OF CONTENTS

<b>LIST OF FIGURES.....</b>	<b>8</b>
<b>LIST OF TABLES.....</b>	<b>10</b>
<b>ABSTRACT.....</b>	<b>11</b>
<b>CHAPTER 1: INTRODUCTION.....</b>	<b>13</b>
1.1 Chronic Pain.....	13
1.2 Pain Transmission System.....	14
1.3 Descending Modulation from the RVM.....	16
1.4 Neuropathic Pain.....	18
1.5 Opioid-induced Paradoxical Pain.....	20
1.6 Immune Cells.....	21
1.7 Activated Glial Cells in the Spinal Cord.....	23
1.8 Neuronal-Glial Communication.....	28
1.9 Hypothesis.....	31
<b>CHAPTER 2: MATERIALS AND METHODS.....</b>	<b>32</b>
2.1 Animals.....	32
2.2 Surgery and Injections.....	32
2.2.1 RVM Cannulation.....	32
2.2.2 RVM Microinjections.....	33
2.2.3 Carrageenan Injections.....	33
2.2.4 Osmotic Pump Implantation.....	33
2.2.5 Spinal Nerve Ligation Model.....	34
2.3 Drugs.....	34
2.4 Behavioral Tests.....	35
2.4.1 Thermal Hyperalgesia.....	35
2.4.2 Tactile Allodynia.....	35
2.5 Immunofluorescent Labeling and Imaging.....	36
2.6 Western Blots.....	37
2.7 Statistical Analysis.....	38
<b>CHAPTER 3: RESULTS.....</b>	<b>39</b>
3.1 RVM Cannula Placement.....	39
3.2 Carrageenan-induced Glial Activation in the RVM.....	39
3.3 Carrageenan-induced Hypersensitivity is Reversed by Microglia Inhibitor.....	40
3.4 Fluorocitrate in the RVM Reverses Carrageenan-induced Hypersensitivity.....	43

**TABLE OF CONTENTS - Continued**

3.5 Inhibition of p38 MAPK in the RVM Attenuates Inflammation-induced Hypersensitivity.....	45
3.6 ATP-induced Hypersensitivity and Inhibition of P2X Receptors.....	47
3.7 Sequestration of BDNF in RVM Attenuates Inflammation-induced Hyperalgesia.....	49
3.8 Inhibition of Microglia in the RVM Attenuates Morphine-induced Hypersensitivity.....	50
3.9 Inhibition of Astrocytes in the RVM Attenuates Nerve Injury-induced Hypersensitivity.....	52
<b>CHAPTER 4: DISCUSSION.....</b>	<b>55</b>
<b>REFERENCES.....</b>	<b>112</b>

## LIST OF FIGURES

Figure 1	Ascending pain pathways.....	73
Figure 2	Descending pain pathways.....	74
Figure 3	Two populations of neurons in RVM.....	75
Figure 4	RVM cannula location.....	76
Figure 5	Verification of cannula placement.....	77
Figure 6	Carrageenan-induced paw edema.....	78
Figure 7	Carrageenan-induced glial activation in RVM.....	79
Figure 8	Experimental design.....	80
Figure 9	Minocycline dose-response.....	81
Figure 10	Hypersensitivity attenuated by minocycline.....	82
Figure 11	Minocycline decreases microglia activation.....	83
Figure 12	Protein levels of Iba1.....	85
Figure 13	Hypersensitivity attenuated by fluorocitrate.....	86
Figure 14	Fluorocitrate decreases astrocyte activation.....	87
Figure 15	Protein levels of GFAP.....	89
Figure 16	Immunolabeling of p-p38 MAPK co-localization.....	90
Figure 17	SB 203580 dose-response.....	92
Figure 18	Hypersensitivity attenuated by SB 203580.....	93
Figure 19	SB 203580 decreases microglia activation.....	94
Figure 20	Pre-treatment with P2X receptor antagonists.....	96
Figure 21	Post-treatment with P2X receptor antagonists.....	97
Figure 22	Hypersensitivity attenuated with BDNF antiserum.....	98

**LIST OF FIGURES - Continued**

Figure 23 Morphine experimental design.....	99
Figure 24 Morphine-induced hypersensitivity with glial inhibitors.....	100
Figure 25 Sustained morphine activates microglia.....	101
Figure 26 Protein levels in the RVM with morphine treatment.....	102
Figure 27 Nerve injury experimental design.....	103
Figure 28 SNL-induced hypersensitivity with minocycline.....	104
Figure 29 SNL-induced hypersensitivity with fluorocitrate.....	106
Figure 30 Nerve injury activates astrocytes.....	108
Figure 31 SNL day 3 Western blots.....	109
Figure 32 SNL day 10 Western blots.....	110
Figure 33 SNL day 30 Western blots.....	111

**LIST OF TABLES**

Table 1	Pain models that lead to glial activation.....	72
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## ABSTRACT

Substantial evidence shows that activation of glial cells in the spinal cord may promote central sensitization and enhancement of pain. Descending facilitation from the rostroventromedial medulla (RVM) is also recognized as a critical component in the maintenance of chronic pain states, although the precise mechanisms driving this activity are unclear. Here, we investigated the possibility that glial activation in the RVM could promote descending facilitation from the RVM in states of enhanced pain. Peripheral inflammation was induced with carrageenan injected into the plantar aspect of the hindpaw of male Sprague-Dawley rats and behavioral responses to noxious thermal and light tactile stimuli were determined. Microinjection of the glial inhibitors minocycline or fluorocitrate, or of SB 203580, a p38 MAPK inhibitor, produced a significant and time-related reversal of behavioral hypersensitivity resulting from hindpaw inflammation. Moreover, carrageenan-induced inflammation appeared to produce an increase in immunolabeling for activated microglia and astrocytes in the RVM, as well as for phosphorylated p38 MAPK; the latter was localized to both microglia and neurons of the RVM. Microinjection of the glial inhibitors into the RVM appeared to diminish immunofluorescent labeling for activated RVM microglia and astrocytes. Carrageenan-induced inflammation also increased RVM protein levels of Iba1 and GFAP and administration of minocycline or fluorocitrate into the RVM attenuated this effect. To examine a possible mechanism of glial activation,  $\alpha,\beta$ -methylene-ATP was microinjected into the

RVM, inducing thermal hyperalgesia, and pre-treatment with the P2X antagonists, PPADS and TNP-ATP, delayed the initiation of ATP-induced hyperalgesia. Post-treatment with the antagonists had no effect on established ATP-induced or carrageenan-induced hypersensitivity. The activation of P2X receptors initiates a signaling cascade leading to the production and release of nociceptive mediators, including BDNF. The RVM microinjection of an anti-BDNF antibody reversed carrageenan-induced thermal hyperalgesia. A model of morphine-induced paradoxical pain was also used to examine the role of glial activation in the RVM. Sustained morphine administration induced tactile allodynia and RVM microinjection of minocycline, but not fluorocitrate, attenuated the behavioral hypersensitivity. Sustained morphine also induced morphological changes in microglia of the RVM, suggesting microglial activation. A third model of enhanced pain used to study medullary glial activation was the spinal nerve ligation (SNL) model of neuropathic pain. The SNL injury induced astrocyte activation within the RVM and microinjection of the astrocyte inhibitor fluorocitrate attenuated the nerve injury-induced tactile allodynia. Minocycline administered to the RVM did not attenuate the behavioral hypersensitivity, suggesting a role for astrocytes, not microglia, in nerve injury-induced enhanced pain. The data show that inflammatory, opioid-induced and neuropathic pain is associated with glial activation in the RVM which likely participates in driving descending pain facilitation via glial-neuronal communication. These findings reveal a novel site of glial modulation of pain.

## CHAPTER 1: INTRODUCTION

### 1.1 Chronic Pain

Pain is one of the largest health problems in the world. An estimated 75 million Americans suffer from pain caused by disease, accident or surgery (National Pain Survey, 1999) with two-thirds living with this pain for more than 5 years (Chronic Pain in America, 1999). The loss of productivity, daily activity and direct medical cost from chronic pain in the United States per annum is over \$150 billion (Tracey and Mantyh, 2007). Pain also diminishes individual's abilities to concentrate, do their job, exercise, socialize and sleep with untreated pain affecting both pain sufferer and family.

Pain is a conscious experience, an interpretation of the nociceptive input influenced by emotional, pathological, genetic, and cognitive factors. The behavioral response by a subject to a painful event is modified by what is appropriate or possible in any particular situation (Tracey and Mantyh, 2007) making pain a highly subjective experience. The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage." By its very nature, pain is difficult to assess, investigate, manage and treat, but clearly, many factors influencing pain are centrally mediated. Determining the balance between peripheral versus central influences and ascertaining which are due to pathological versus emotional or cognitive influences will guide treatment (Tracey and Mantyh, 2007).

## 1.2 Pain Transmission System

Primary afferent sensory neurons are the gateway by which sensory information from peripheral tissues is transmitted to the spinal cord and brain. These neurons innervate the skin and almost every organ in the body. At the peripheral level, primary afferent fibers can be classified based on structure, diameter and conduction velocity. The largest afferent fibers have diameters ranging from 5-20  $\mu\text{m}$ ; they include  $A\alpha$  and  $A\beta$  fibers, are heavily myelinated and rapidly conducting (30-120 m/sec). The large  $A\alpha$  fibers are exclusively the axons of muscle spindles and Golgi tendon organs. Both  $A\alpha$  and  $A\beta$  fibers are activated by innocuous stimuli, transmitting information related to muscle stretching, light touch and position. By contrast, most medium to small diameter (1-6  $\mu\text{m}$ ) fibers, designated as  $A\delta$  and C fibers, are specialized sensory neurons known as nociceptors which detect and convert environmental stimuli perceived as harmful into electrochemical signals transmitted to the central nervous system (CNS) (Abbadie et al., 1997). The  $A\delta$  fibers are poorly myelinated with an intermediate conduction velocity (5-30 m/sec), whereas C fibers are unmyelinated and poorly conducting (0.5-2 m/sec). Both  $A\delta$  and C fibers have the remarkable ability to detect a wide range of stimulus modalities, including those of a physical or chemical nature. To accomplish this, nociceptors express a diverse repertoire of receptors and transduction molecules that can sense forms of noxious stimulation (thermal, mechanical or chemical) with varying degrees of sensitivity. In addition to conveying information to the CNS, primary

afferent fibers uniquely release neurotransmitters peripherally, a process that underlies neurogenic inflammation (Snider and McMahon, 1998).

All primary afferent neurons that enter the spinal cord have their cell bodies in the dorsal root ganglion (DRG). Dorsal root ganglion neurons are pseudounipolar neurons with a central and peripheral branch. The peripheral branch terminates in the skin, muscle or other tissue and the central branch enters the spinal cord at the dorsal horn. Upon entering the spinal cord, A $\beta$  fibers ascend within the dorsal columns to the nucleus gracilis or nucleus cuneatus in the medulla and also send collateral projections to lamina III and IV in the dorsal horn. In the medulla, A $\beta$  neurons synapse on 2<sup>nd</sup> order neurons that cross the midline and project up to the thalamus via the medial lemniscus. In contrast, A $\delta$  and C fibers enter the spinal cord and synapse on 2<sup>nd</sup> order neurons in the superficial laminae in the dorsal horn. Axons of the 2<sup>nd</sup> order neurons cross the midline of the spinal cord and ascend via the spinothalamic tract to the thalamus where they synapse on 3<sup>rd</sup> order neurons. From the thalamus, signals from the spinothalamic tract and medial lemniscus reach the somatosensory cortex and this is where the perception of pain occurs (Fig. 1).

In addition to ascending pain pathways, endogenous descending pain pathways exist. The descending pain modulatory system is a well-characterized anatomical network modulating nociceptive processing in various circumstances to produce either facilitation (pro-nociception) or inhibition (anti-nociception) (Gardell et al., 2002; Julius and Basbaum, 2001; Gebhart, 2004). One of the

major sites in the brainstem known to play a role in the modulation of pain is the periaqueductal gray (PAG). Neurons project from the PAG to a number of areas including the rostroventromedial medulla (RVM), an important relay in descending pain processing. From the RVM, neurons project to various laminae in the spinal dorsal horn via the dorsolateral funiculus (DLF) (Vanderah et al., 2001; Ossipov and Porreca, 2005) (Fig. 2).

### **1.3 Descending Modulation from the RVM**

Overwhelming evidence shows that the rostroventromedial medulla (RVM) is an important relay in the modulation of nociceptive inputs at the level of the spinal cord. The RVM, including the nucleus gigantocellularis pars alpha and the nucleus raphe magnus (NRM) (Fields and Heinricher, 1985) is a major source of bulbospinal projections that terminate in laminae I, II and V of the spinal dorsal horn and these projections are in the dorsolateral funiculus (DLF). Lesions of the DLF block the inhibition of dorsal horn unit responses to noxious stimuli caused by electrical stimulation of the RVM (Basbaum and Fields, 1978; Fields and Basbaum, 1978), serving as the final relay in the descending inhibition of nociception.

The RVM is now being appreciated for its dual role in the inhibition and facilitation of nociception. These physiologically contradictory functions of the RVM are due to different classes of neurons with opposing functions. Based on their electrophysiological responses to noxious heat and correlated to the tail-flick reflex in the rat, three classes of neurons have been identified in the RVM: “on”,

“off” or “neutral” cells (Fields et al., 1983). On-cells increase their firing rate immediately before the nociceptive reflex occurs while off-cells are tonically active and pause in firing just before the animal withdraws its tail from the noxious stimulus (Fig. 3). Neutral-cells do not show changes with noxious stimuli and may play a role in homeostatic functions other than modulation of nociception (Fields et al., 1983;Fields and Heinricher, 1985). Because anti-nociceptive doses of morphine into the PAG or RVM increase off-cell activity and suppress on-cell activity (Fields et al., 1983;Heinricher et al., 1994), it is thought that the off-cells provide descending inhibition of nociception. The RVM has also been found to facilitate nociceptive inputs (Gebhart, 2004). Low intensity electrical stimulation facilitated, while high intensity inhibited, dorsal horn unit responses (Zhuo and Gebhart, 1992;Zhuo and Gebhart, 1990). On-cells display characteristics consistent with a pro-nociceptive role (Heinricher, 2003). Moreover, manipulations that increase responses to noxious stimuli also increase on-cell activity (Heinricher, 2003) and lidocaine in the RVM abolished the tail-flick reflex (Morgan and Fields, 1994). It appears that off-cells mediate descending inhibition, while on-cells mediate descending facilitation.

The facilitatory component of descending modulation has been studied as an important mediator in the maintenance of persistent pain states. Once an injury occurs (nerve injury, inflammation), an increase in the excitability of the afferent pathways from the site of injury maintains the subsequent pain (Lai et al., 2003). However, once the injury resolves, pain often still persists suggesting that

other factors must be involved in maintaining the enhanced pain state. Burgess et al. found that descending facilitation from the RVM is important for the maintenance, but not initiation, of nerve injury-induced pain as lidocaine in the RVM blocked such pain only when given at later time points (Burgess et al., 2002).

#### **1.4 Neuropathic Pain**

Neuropathic pain is a severely debilitating, chronic condition caused by injury or inflammation of the nervous system. Symptoms of neuropathic pain may include increased sensitivity to painful stimuli (hyperalgesia), the perception of innocuous stimuli as painful (allodynia) and spontaneous pain. A great deal has been written on neuropathic pain and its possible causes, but only with the development of animal models of pain has some real progress been made in understanding some of the mechanisms involved. The first widely used animal model of neuropathic pain was the chronic constriction injury of the rat sciatic nerve (Bennett and Xie, 1988), in which the sciatic nerve is encircled by four ligatures of chromic gut suture, leading to behavioral hypersensitivity. This animal model and others have made it clear that a number of mechanisms are involved in neuropathic pain with increasing evidence that inflammatory and immune mechanisms may play a role. In fact, pain may develop without any injury of sensory axons. This was shown in rats by placing segments of chromic gut suture next to the sciatic nerve, thereby inducing hyperalgesia (Maves et al., 1993). Enhanced pain was elicited without damage to sensory axons, implying

that sensitization or activation of sensory fibers by inflammatory mediators is sufficient to cause exaggerated pain.

When the primary pathogenic events of a pain state are unknown, the control of inflammation is the next best option. While the number of diseases considered “inflammatory” in origin may decline as infectious causes continue to be discovered, in several infectious diseases the inflammatory response may actually cause more damage than the primary pathogen. Although the search continues for possible infectious causes of multiple sclerosis, rheumatoid arthritis, type 1 diabetes mellitus and atherosclerosis, inflammation *per se* remains one of the main therapeutic targets (Nathan, 2002).

Today, we have animal models of pain in which mechanisms of hyperalgesia and allodynia can be analyzed. The injection of inflammatory agents such as carrageenan, complete Freund’s adjuvant (CFA) or zymozan in the hindpaw of the rat produces an intense inflammation characterized by edema, redness and hyperalgesia (Iadarola et al., 1988; Hargreaves et al., 1988; Chacur et al., 2004). The subcutaneous injection of formalin produces a biphasic inflammatory behavioral response (paw flinching or face rubbing) with an early and short-lasting first phase followed, after a quiescent period, by a second and prolonged phase (Dallel et al., 2003). A widely used model of neuropathic pain includes a ligation of the L5 and L6 spinal nerves (SNL) and is known to induce thermal and tactile hypersensitivity in rats (Kim and Chung,

1992). These models should help us understand the immune mechanisms in inflammatory and neuropathic pain.

### **1.5 Opioid-induced Paradoxical Pain**

Opioid analgesics represent the best option for the treatment of severe pain and for the management of chronic pain states. However, the clinical efficacy of these drugs is reduced by the rapid development of analgesic tolerance with repeated administration of opioids, such as morphine, requiring increased dosage to maintain pain control. Dose increase is problematic because it is associated with increased side effects such as constipation, nausea, respiratory depression, and impairment of mental alertness (Raith and Hochhaus, 2004). Tolerance may also be related to a state of hyperalgesia that results from the exposure of the opioid itself. Patients who receive long-term opioid therapy sometimes develop unexpected, abnormal pain (De et al., 1991a;De et al., 1991b;Sjogren et al., 1994b;Sjogren et al., 1994a;Doverty et al., 2001a;Doverty et al., 2001b). Furthermore, tolerance often leads to pain amplification when opioid administration is discontinued (Mao, 2002;Mao et al., 2002).

Many mechanisms have been proposed to account for opioid tolerance and withdrawal-induced pain enhancement. Some of these mechanisms involve adaptation within opioid or opioid receptor-expressing neurons, rendering opioid actions less effective. Such adaptations include internalization and/or downregulation of opioid receptors; upregulation of *N*-methyl-D-aspartate

(NMDA) receptor function; and downregulation of glutamate transporters (Raith and Hochhaus, 2004; King et al., 2005b). Other mechanisms activate pain facilitatory systems within the brain and spinal cord that actively oppose opioid analgesia. Such counter-regulatory adaptations include the release of cholecystokinin (CCK) (Watkins et al., 1984) and dynorphin (Vanderah et al., 2000).

New evidence suggests that immune cells play a pivotal role in morphine tolerance and in withdrawal-induced pain enhancement. Pro-inflammatory mediator transcription, translation and protein release are elevated in the spinal cord in response to chronic morphine (Raghavendra et al., 2002). Morphine tolerance and withdrawal-induced pain facilitation after chronic morphine are attenuated by intrathecal injections of anti-inflammatory mediators (Raghavendra et al., 2002; Johnston et al., 2004). These findings suggest that among its many actions, morphine triggers the activation of immune cells and that these in turn exert an anti-analgesic, homeostatic response, which not only limits the extent and duration of acute morphine analgesia, but also underlies the development of morphine tolerance following repeated administration.

### **1.6 Immune Cells**

Inflammation is a complex set of interactions among tissue factors in response to a variety of perturbations ranging from infection to tissue trauma (Dray, 2005), which lead to destruction of foreign bodies and assisted repair. If these interactions are not properly balanced, inflammation will lead to persistent

tissue damage (Nathan, 2002). Several inflammatory and immune-like glial cells, including mast cells, neutrophils, macrophages and T cells in the peripheral nervous system and microglia and astrocytes in the central nervous system (Moalem and Tracey, 2006a), have been implicated in the pathogenesis of neuropathic pain.

A resident population of mast cells in the peripheral nerve are degranulated at the site of injury (Olsson, 1967) with granules containing mediators such as histamine, proteases and cytokines (Galli et al., 2005), several of which are capable of sensitizing or activating neurons. The release of these mediators may elicit hyperalgesia by a direct action on the nociceptors (Rueff and Dray, 1993) or by initiating an inflammatory cascade. For example, the release of histamine may contribute to the recruitment of leukocytes (Gaboury et al., 1995; Malaviya and Abraham, 2000). The question of how mast cells are activated, however, remains unanswered.

Neutrophils, not observed in normal nerves, are found in significant numbers at the site of injury (Perkins and Tracey, 2000; Zuo et al., 2003) migrating towards the inflammatory site up a gradient of chemoattractants, where they may contribute to inflammatory hyperalgesia (Bennett et al., 1998; Levine et al., 1984). Giorgi et al. have found that neutrophils may also have an anti-inflammatory and anti-nociceptive role (Giorgi et al., 1998) as neutrophils may deactivate macrophages in vitro and suppress inflammatory pain in mice. This evidence for an anti-nociceptive role for neutrophils appears to contradict the pro-

nociceptive role stated above and may be due to differing models of persistent pain.

A third player in the inflammatory cascade is the macrophage, which phagocytose foreign particles and remove injured or dead tissue. There is a resident population of macrophages in the peripheral nerve and dorsal root ganglion and their equivalent in the CNS are the microglia. Clearance of debris is a prerequisite to regeneration (Correale and Villa, 2004; Perry and Brown, 1992) and defects which delay the recruitment of macrophages have shown a delay in Wallerian degeneration (Perry et al., 1992) and hyperalgesia (Myers et al., 1996) suggesting that macrophages may contribute to pain resulting from injury. This effect may be due to the release of pro-nociceptive molecules from macrophages, such as prostaglandins (Nathan et al., 1997), reactive oxygen species and cytokines (Sommer and Schafers, 1998). Lymphocytes are another class of cells that release cytokines modulating pain and can be classified as B cells or T cells. The T cells are divided into CD4<sup>+</sup> (helper) and CD8<sup>+</sup> (cytotoxic) cells, each with further divisions into type 1 and type 2 subsets. The subsets are defined by the cytokines they release; Th1 cells are pro-nociceptive and Th2 cells are anti-nociceptive with both subsets found to play a role in persistent pain.

### **1.7 Activated Glial Cells in the Spinal Cord**

While the modulation of immune cells in the peripheral nervous system is important, the role of glial cells in the central nervous system has been gaining attention. Glial cells surround and support neurons in the nervous system at

populations 10 to 50 times as numerous as neurons. In the CNS, glia are represented by astrocytes, oligodendrocytes and microglia. Microglia, a population of 5-10% of resident macrophages in the CNS, are normally quiescent with a basal function of surveillance for debris and pathogens. Astrocytes, the largest cell population in the CNS, are responsible for the regulation of extracellular ion and neurotransmitter concentrations, encapsulate synapses and actively participate in synaptic communication as well as other “housekeeping” functions. The influence of oligodendrocytes on neuronal function, apart from myelination, is the least understood among glial cells. In the discussion to follow, the term “glia” will be used to refer to both microglia and astrocytes.

Literature emerged in the 1970’s documenting that CNS microglia and astrocytes become activated following trauma to peripheral nerves (Sjostrand, 1971), but twenty years passed before work demonstrated that peripheral nerve damage that caused exaggerated nociceptive responses also activated spinal cord microglia and astrocytes, proposing a link between neuropathic pain and glial activation (Garrison et al., 1991; Garrison et al., 1994). Furthermore, the *N*-methyl-D-aspartate (NMDA) antagonist MK801, which blocks neuropathy-induced allodynia and hyperalgesia, also blocked glial activation. The term ‘activation’ refers to an enhanced ability of a cell to perform a function beyond that present in a basal state, leading to their involvement in a wide variety of functions. Glial activation is characterized by a specific morphology (retracted processes and hypertrophy), proliferation, increased expression of cell-surface

markers and/or changes in receptors, and changes in functional activities (increased production of pro-inflammatory substances) (Benveniste et al., 1995a; Benveniste and Benos, 1995; Benveniste et al., 1995b; Pekny, 2001; Perry, 1994). The manner in which activation is expressed is dependent on the type and intensity of the stimulus, and different patterns and time-courses of responses can occur.

The work of Garrison et al. was the first to link activation of glia to a functional outcome by using immunohistochemical techniques to visualize morphological changes in both astrocytes and microglia in the spinal dorsal horn after peripheral nerve damage (Garrison et al., 1994). Once activated, glia change from a ramified shape into a hypertrophied, amoeboid shape. Microglia upregulate expression of a variety of cell surface molecules, including complement receptor 3 (also known as cluster-determinant (CD) 11b, recognized by the antibody OX-42) and ionized calcium binding adaptor molecule 1 (Iba1) and astrocytes commonly upregulate expression of glial fibrillary acidic protein (GFAP). It is notable that while immunohistochemically-detected activation markers are useful as anatomical indices that glia have been activated, these glial activation markers are vastly different than neuronal activation markers such as Fos. Neuronal activation markers are transcription factors which are rapidly produced and have direct bearing on intracellular cascades. In contrast, glial activation markers are end products of intracellular cascades that provide

general anatomical “footprints” that some type of glial activation occurred (Watkins et al., 2001).

Whereas many studies have demonstrated an important role for astrocytes in neuropathic pain (Watkins and Maier, 2002; Ji et al., 2006; Moalem and Tracey, 2006b), by comparison, more literature describes the contribution of microglia to pathological pain. Using immunohistochemical procedures, spinal dorsal horn glial activation has been reported to occur in response to a variety of procedures known to enhance pain (Table 1). Immunohistochemical staining of the rat spinal cord using OX-42 antibodies and measurement of CD11b mRNA showed microglial activation after subcutaneous injection of zymozan or formalin into the paw. Microglial activation paralleled the development and/or maintenance of zymozan and formalin-induced mechanical allodynia, respectively (Colburn et al., 1997; Fu et al., 1999a; Sweitzer et al., 1999a; Wu et al., 2004). Studies show increases in glial activation in the spinal dorsal horn following intraplantar injections of carrageenan and complete Freund’s adjuvant (CFA) (Raghavendra et al., 2003b) and there is a consensus that spinal microglia show morphological signs of activation after injury to peripheral nerves and the spinal cord. Time of induction and duration of morphological changes vary among the models and investigators, but generally these changes have been reported to occur 1 hour to 7 weeks after injury.

These studies show that many pain models activate spinal microglia and astrocytes, but also suggest glial activation may be a causal factor in the

development of allodynia and hyperalgesia, which is a dramatic change from the classical view that exaggerated pain states are created and maintained solely by neurons. The question of whether glia are necessary for hyperalgesia and allodynia addresses whether exaggerated nociceptive responses will occur if neurons, but not glia, are present. To pharmacologically “remove” glia by disrupting their function, two glial inhibitors have been employed: minocycline and fluorocitrate. Minocycline is a second-generation tetracycline antibiotic with numerous immunomodulatory activities. Minocycline prevents microglial activation and is neuroprotective in models of ischemia (Yrjanheikki et al., 1998; Yrjanheikki et al., 1999), Parkinson’s disease (He et al., 2001; Wu et al., 2002), Huntington’s disease (Chen et al., 2000), amyotrophic lateral sclerosis (Zhu et al., 2002) and experimental autoimmune encephalitis (Brundula et al., 2002). Fluorocitrate, a gliotoxin relatively selective for astrocytes (Fonnum et al., 1997), blocks activity by inhibiting aconitase, an enzyme integral to the tricarboxylic acid cycle (Paulsen et al., 1987; Fonnum et al., 1997). Both inhibitors have been found to be effective in the spinal cord, blocking hyperalgesia and allodynia in a variety of pain models, such as subcutaneous irritants (Guo et al., 2007; Hua et al., 2005; Meller et al., 1994), spinal immune activation (Ledeboer et al., 2005; Milligan et al., 2000) and spinal nerve and cord trauma (Hains and Waxman, 2006; Peng et al., 2006; Popovich et al., 1997; Sweitzer et al., 2001).

To examine whether glial activation is sufficient for exaggerated pain processing, the strategy has been two-pronged. The first approach administered intrathecally the components of pathogens (viral envelope proteins, bacterial cell walls) known to bind to specific receptors expressed on glia. The resultant spinal glial activation induced exaggerated pain behaviors (Meller et al., 1994; Milligan et al., 2000). The second approach manipulated fractalkine, a protein expressed on the surface of neurons which breaks free upon strong neural excitation and binds to and activates microglia and astrocytes (Chapman et al., 2000). Intrathecal fractalkine creates both thermal hyperalgesia and mechanical allodynia, and blockade of the fractalkine receptor (CX3CR-1; expressed only on glia) blocks the hypersensitivity. Taken together, these lines of evidence support the conclusion that glial activation is necessary and sufficient to create exaggerated pain states and identifies a potential neuron-to-glia signal capable of driving pathological pain.

### **1.8 Neuronal-Glial Communication**

The mechanisms by which glial activation enhances neuronal transmission of nociceptive information are only partially understood. Ultimately, activated glia must lead to hyperalgesia and allodynia by releasing substances that act on neurons in the pain pathway. Glia are activated by a variety of pain relevant substances released by neurons, such as bradykinin, calcitonin gene-related peptide (CGRP), cholecystokinin, excitatory amino acids, prostaglandins, substance P and adenosine 5'-triphosphate (ATP) (Hide et al., 2000; Muller et al.,

1997;Takuma et al., 1996). ATP release from central terminals of nociceptive afferents within the spinal cord has been documented (Bardoni et al., 1997), and ATP has been proposed to mediate both neuronal and injury-induced microglial activation. Studies by Tsuda et al. indicate that ATP, acting through P2X<sub>4</sub> receptors on spinal microglia, is necessary to induce tactile allodynia associated with neuropathic pain while blockade of these receptors with P2X<sub>4</sub> antisense significantly attenuated the pain behavior (Tsuda et al., 2003).

Activated glia release reactive oxygen species, nitric oxide, prostaglandins, excitatory amino acids, ATP and pro-inflammatory cytokines (Kreutzberg, 1996;Ridet et al., 1997) including tumour necrosis factor (TNF), interleukin-1 (IL-1) and IL-6. These cytokines are often sequentially formed in a cascade in which TNF is typically made first, causing the induction of IL-1, which in turn causes the induction of IL-6. The synergism of these pro-inflammatory cytokines produces more powerful effects than when only one cytokine is present (Dinarello, 1999;Watkins and Maier, 2003b). The injection of pro-inflammatory cytokines over the spinal cord has been shown to enhance nociception (DeLeo et al., 1996;Falchi et al., 2001;Reeve et al., 2000) while the blockade of pro-inflammatory cytokine function with the use of antagonists prevents and/or reverses allodynia and hyperalgesia in many animal models tested (Milligan et al., 2001;Milligan et al., 2003;Sweitzer et al., 2001;Watkins et al., 1997), including inflammation and/or injury to peripheral tissues, peripheral nerves, spinal nerves and spinal cord.

Pro-inflammatory cytokines can be controlled by blocking intracellular pathways leading to their production. Although multiple intracellular signaling cascades have been implicated in pro-inflammatory signaling and production, p38 MAP kinase is integrally involved in both (Clark et al., 2003). Recent work suggests that activation (phosphorylation) of p38 in dorsal root ganglia and spinal cord plays a critical role in inflammation-induced spinal pain processing and nerve injury (Ji et al., 2002; Jin et al., 2003; Schafers et al., 2003; Svensson et al., 2003; Tsuda et al., 2003; Svensson et al., 2005). Inflammation-induced p38 activation has also been found in the RVM (Imbe et al., 2007). Inhibition of p38 MAPK has disrupted a variety of exaggerated pain states including peripheral inflammation (Ji et al., 2002; Mizushima et al., 2005; Obata et al., 2005; Svensson et al., 2003), nerve injury (Inoue et al., 2003; Jin et al., 2003; Milligan et al., 2003; Obata et al., 2004) and intrathecal injection of substance P (Svensson et al., 2003).

Another potential mediator of neuropathic pain is brain-derived neurotrophic factor (BDNF). Intrathecal administration of BDNF induces an allodynic-like state in naïve rats and both function-blocking tyrosine kinase receptor B (trkB) antibodies and BDNF-sequestering TrkB-Fc fusion proteins reversed hypersensitivity induced by peripheral nerve injury (Coull et al., 2005) indicating that BDNF signaling is an important component of neuropathic pain. While the main focus of research has been on neuronally derived BDNF, new evidence shows that BDNF is also derived from microglia where, in vitro,

treatment of purified microglial cultures with ATP stimulated the secretion of BDNF (Beggs et al., 2009). The release of BDNF from activated microglia may then act on neuronal trkB receptors to modulate neuropathic pain.

Activation of the microglia P2X4 receptor via ATP initiates a cascade of signaling processes, including the phosphorylation of p38 MAPK leading to increased synthesis and secretion of BDNF which then acts on the trkB receptors located on neurons. While many factors play important roles in pain processing, this pathway is an example of the neuronal-glia communication which occurs during persistent pain states.

### **1.9 Hypothesis**

Evidence suggests that spinal microglia and astrocytes become activated in exaggerated pain states and modulation of glial activation can attenuate hyperalgesia and allodynia. Most studies of glial activation and sensitization to nociception have focused on the spinal cord. Microglial activation and phosphorylation of neuronal and glial p38 MAPK occurs at supraspinal sites in response to diverse stimuli. The possibility that neuronal-glia interactions may contribute to increased activation of descending facilitation from the RVM has not previously been examined. The present investigation was undertaken to investigate the hypothesis that peripheral inflammation, sustained morphine administration and peripheral nerve injury can result in the activation of glial cells within the RVM which in turn promote descending facilitation of nociception.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Animals

Male Sprague-Dawley rats (250-300 g; Harlan Labs) were housed in a climate-controlled room on a 12-h light/dark cycle and allowed food and water *ad libitum*. All testing was performed under an approved protocol from the Institutional Animal Care and Use Committee of the University of Arizona and was in accordance with the policies and recommendations of the International Association for the Study of Pain.

### 2.2 Surgery and Injections

#### 2.2.1 RVM Cannulation

Rats were anesthetized with a ketamine HCl/xylazine (80 mg/kg of ketamine and 12 mg/kg of xylazine) solution (100 mg/kg, i.p.; Sigma) and placed in a stereotaxic apparatus (Stoelting). A 2 cm incision was made in the scalp and the underlying connective tissue was retracted with hemostats to expose the skull. Paired guide cannulae 1.2 mm apart (26GA, #C235G-1.2 mm; Plastics One Inc.) were directed towards the RVM (10.8 mm caudal to bregma, 0.6mm to each side of the sagittal suture and 7.0 mm ventral to the dura mater surface); these coordinates were obtained from the rat atlas of Paxinos and Watson (1982). Bone wax was used to plug the hole around the cannula. The guide cannula was secured to the skull using stainless steel screws and dental acrylic. A dummy cannula (#C235DC; Plastics One Inc.) was inserted to prevent contaminants from entering the RVM guide cannula. Rats were given an

antibiotic injection (Amikacin C, 5 mg/kg, i.m.) and allowed to recover for 5 days. At the termination of the experiments, proper cannula placement was confirmed by injecting 0.5  $\mu$ l of India ink and verified histologically using 0.1% cresyl violet Nissl stain. Data from animals with incorrectly placed cannula were discarded.

### **2.2.2 RVM Microinjections**

Microinjection of drugs was performed through an injection cannula (33GA, #C235G; Plastics One Inc.) that extended 2 mm beyond the tip of the guide cannula into fresh brain tissue. The drug solutions were slowly expelled using a 10  $\mu$ l Hamilton Syringe connected to the injection cannula with Tygon tubing (Cole-Parmer Instrument Company). Microinjections were made in a volume of 0.5  $\mu$ l administered into each guide cannula. Drug doses are reported as the total amount administered bilaterally.

### **2.2.3 Carrageenan Injections**

Rats were briefly anesthetized with isoflurane (4% in 95% O<sub>2</sub>/5%CO<sub>2</sub> at a flow rate of 2-3 L/min). Subcutaneous injections of 100  $\mu$ l of a 3% suspension of  $\lambda$ -carrageenan (Sigma) were made into the plantar aspect of the hindpaw. Control injections were made as above using 0.9% saline solution. Paw volumes were measured before and 3 hours after carrageenan or saline injections using a plethysmometer (Ugo Basile) and expressed in milliliters (ml).

### **2.2.4 Osmotic Pump Implantation**

Osmotic pumps were used for continuous delivery of morphine (1.54 mg/day) or saline per manufacturer's instruction (model 2001, Alzet osmotic

pumps, Durect). The pumps were implanted subcutaneously in the area of the lower back.

### **2.2.5 Spinal Nerve Ligation Model**

Tight ligation of the L5/L6 spinal nerve was performed according to the method of Kim and Chung (Kim and Chung, 1992). The rats were maintained under anesthesia with isoflurane (4% in 95% O<sub>2</sub>/5%CO<sub>2</sub> at a flow rate of 2-3 L/min). After surgical preparation of the rats and exposure of the dorsal vertebral column from L4 to S2, the exposed L5 and L6 spinal nerves were tightly ligated with 4-0 silk suture. The incision was closed, and the animals were allowed to recover. Rats that exhibited motor deficiency or a lack of subsequent increased sensitivity to innocuous mechanical stimulation were excluded from additional testing. Sham control rats underwent the same operation and handling as the experimental animals, but without SNL.

### **2.3 Drugs**

Minocycline hydrochloride (Sigma) was freshly dissolved daily in dH<sub>2</sub>O and heated briefly in a water bath until completely dissolved into a clear solution. Fluorocitrate (DL-Fluorocitric acid barium salt; Sigma) was first dissolved in 1N HCl and then diluted in 0.9% sterile, isotonic saline. Fluorocitrate is a gliotoxin and has been shown to cause seizures in rats (Willoughby et al., 2003). Animals that exhibited seizure behavior after RVM microinjection were not used in the study. The following drugs were dissolved with dH<sub>2</sub>O: the p38 MAPK inhibitor, SB 203580 hydrochloride (EMD Biosciences), ATP ( $\alpha,\beta$ -methylene adenosine 5'-

triphosphate; Sigma), TNP-ATP triethylammonium salt (P2X<sub>1-4</sub> antagonist; Tocris), PPADS tetrasodium salt (P2X<sub>1-3,5,7</sub> antagonist; Tocris), BDNF antisera (Anti-Brain Derived Neurotrophic Factor Polyclonal Antibody; Chemicon) and morphine (for osmotic pump delivery of 1.54 mg/day, NIDA).

## **2.4 Behavioral Tests**

### **2.4.1 Thermal Hyperalgesia**

Rats were placed on a hot-plate at 52°C and latency to hindpaw lifting, stomping or licking was measured. A significant ( $p \leq 0.05$ ) reduction in hot-plate latency indicates thermal hyperalgesia.

### **2.4.2 Tactile Allodynia**

Rats were placed in elevated Plexiglass boxes (Plastics Plus) with wire-mesh floors. The paw withdrawal thresholds were determined by probing the hindpaw with a series of 8 von Frey filaments (Stoelting) calibrated in logarithmically spaced increments from 0.41 g to 15 g. Each filament was applied perpendicularly to the plantar surface of the hindpaw until it buckled. Measurements were taken before (baseline) and after drug administration. Mean paw withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength and analyzed by the Dixon non-parametric test (Chaplan et al., 1994; Dixon, 1980). A significant ( $p \leq 0.05$ ) reduction in paw withdrawal threshold indicates tactile allodynia.

## 2.5 Immunofluorescent Labeling and Imaging

At the termination of the experiment, the rats were sacrificed with CO<sub>2</sub> and decapitated. The brainstems were removed and fixed overnight in 10% formalin (Sigma). The tissue was then cryoprotected for 48 hours in sterile 30% sucrose-tris-buffered saline solution. Medullary sections 20 µm thick were cut in a -20°C cryostat and transferred serially to multi-well tissue culture plates containing 0.1 M tris-buffered saline (TBS). Free-floating sections were incubated in TBS containing 0.1% Triton X-100 (Sigma) and 8% bovine serum albumin (BSA; Sigma) at room temperature for 1 hour. The following primary antibodies were used: CD11b/c (OX-42, mouse monoclonal antibody, 1:500; BMA Biomedicals), glial fibrillary acidic protein (GFAP, mouse monoclonal antibody, 1:1000; Sigma), neuronal nuclei (NeuN, mouse monoclonal antibody, 1:1000; Chemicon), and phosphorylated-p38 MAPK (p-p38 MAPK, rabbit polyclonal antibody, 1:500; Cell Signaling Technology). All incubations with primary antibodies were performed in TBS with 3% BSA overnight at room temperature. Sections were then washed with TBS (3 times for 10 min each) and incubated with the secondary antibody for 2 hours at room temperature. Cy3-conjugated donkey anti-rabbit antibody (1:1000; Jackson ImmunoResearch) was used as the secondary antibody for p-p38 MAPK staining. FITC-conjugated donkey anti-mouse antibody (1:1000; Jackson ImmunoResearch) was used as the secondary antibody for OX-42, GFAP and NeuN staining. After incubation with secondary antibody, sections were washed (3 times for 10 min each) and mounted onto slides, air-dried and

coverslipped using Vectashield Hard Set Mounting Medium (Vector Laboratories). The fluorescent signals were detected using a Nikon E800 fluorescence microscope and images were acquired with a Hamamatsu C5810 color CCD camera and its proprietary Image Processor software (Hamamatsu Photonic Systems) or with a Zeiss LSM 510-Meta NLO confocal microscope (Zeiss).

## **2.6 Western Blots**

At the termination of the experiment, the animals were sacrificed with CO<sub>2</sub> and decapitated. The brainstem tissue was rapidly removed by hydraulic extrusion and immediately frozen in liquid nitrogen and stored at -80°C until processing. The tissue was homogenized and sonicated in cold tris buffer (pH 7.4) containing 10mM EDTA, 1% Triton X-100, 1% protease inhibitor, and 1% phosphatase inhibitor cocktail (Sigma) and centrifuged at 6500 rpm for 15 minutes at 4°C. Protein concentrations were determined and samples were prepared for electrophoresis. Proteins were separated using Bis-Tris or Tris-Acetate gel electrophoresis (4-12% Bis-Tris or 3-8% Tris-Acetate gels; Invitrogen), and electrophoretically transferred to polyvinylidene fluoride membranes (Invitrogen). Membranes were blocked with 5% bovine serum albumin (BSA Fraction V, Sigma) or non-fat dry milk in Tris-buffered saline containing 0.1% Triton X-100 (TBS-T) for at least 3 hours at room temperature, and then incubated overnight at 4°C with anti-GFAP (1:2500; Santa Cruz) or anti-Iba1 (1:1000; Wako). Membranes were washed in TBS-T and incubated with

appropriate secondary antibodies conjugated with horseradish peroxidase (goat anti-rabbit or goat anti-mouse, 1:5000; Thermo Scientific) at room temperature for 1 hour. The membranes were washed and immunoreactive proteins were detected by enhanced chemiluminescence (Pierce) and visualized by exposure to X-ray film. The membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) and re-blotted with anti- $\alpha$ -tubulin (1:10,000; Santa Cruz) to serve as a loading control. For the quantification of Western signals, the densities of specific GFAP, Iba1 and  $\alpha$ -tubulin bands were measured with a computer-assisted imaging analysis system (ImageJ; NIH). Target protein levels were normalized against the corresponding loading control levels and the relative protein levels were presented as the mean  $\pm$  SEM.

## **2.7 Statistical Analysis**

All data were expressed as means  $\pm$  SEM. Reversal of hyperalgesia or allodynia was indicated by significant increases from the post-carrageenan means. Changes in means within each group over time were detected by one-factor ANOVA followed by the Fisher's least significant difference post-hoc test. Significant differences between treatment groups over time were determined by two-factor ANOVA. Significance was set at  $p \leq 0.05$ .

## CHAPTER 3: RESULTS

### 3.1 RVM Cannula Placement

Rats were implanted with paired bilateral guide cannulae directed toward the rostroventromedial medulla (RVM; Fig. 4). The microinjection sites in the RVM were verified histologically at the termination of behavioral experiments and only data from rats with correctly placed injections were included in the analysis. Medullary sections were counterstained with Nissl stain and injection sites were visualized by the microinjection of 0.5  $\mu$ l of India ink through injection cannula inserted through each of the bilateral guide cannula (Fig. 5).

### 3.2 Carrageenan-induced Glial Activation in the RVM

Peripheral inflammation was induced by injecting 100  $\mu$ L of 3% carrageenan suspension into the plantar aspect of the hindpaw. Control animals received saline injections. Carrageenan injection caused swelling and redness of the ipsilateral hindpaw while saline injected hindpaws did not appear different from naïve. Paw volumes were measured using a plethysmometer; saline injected animals had paw volumes of  $1.03 \pm 0.04$  mls and carrageenan injected animals had paw volumes of  $2.29 \pm 0.03$  mls three hours after injection (Fig. 6).

Carrageenan injection produced behavioral signs of thermal hyperalgesia and tactile allodynia at three hours post-injection, as indicated by significant ( $p < 0.05$ ) reductions in 52°C hot-plate latencies to a mean of  $8.4 \pm 0.3$  sec from a baseline value of  $16.8 \pm 0.4$  sec ( $n=70$ ) and in paw withdrawal thresholds to  $4.4 \pm 0.4$  g from a baseline mean of  $12.6 \pm 0.5$  g ( $n=58$ ) (Fig. 7). Rats treated with saline

showed no significant difference compared to baseline. The rats were sacrificed three hours after carrageenan or saline injection and brainstem tissue was prepared for immunodetection of the microglial marker OX-42 or the astrocyte marker GFAP. Carrageenan-induced inflammation produced increased immunofluorescent labeling for both OX-42 and GFAP in the RVM, suggesting activation of microglia and astrocytes, respectively (Fig. 7). Notably, activated microglia and astrocytes exhibited hypertrophied cell bodies and shorter processes compared to tissue from rats treated with vehicle.

### **3.3 Carrageenan-induced Hypersensitivity is Reversed by Microglia Inhibitor**

In order to determine if the observed activation of microglia contributes to the development of behavioral signs of enhanced pain produced by inflammation, the specific microglia inhibitor, minocycline, was microinjected into the RVM. Baseline responses to hot-plate latencies and paw withdrawal thresholds were determined and carrageenan or saline was injected in the hindpaw (for experimental design, see Fig. 8). Carrageenan produced behavioral signs of thermal hyperalgesia and tactile allodynia within three hours after administration. Hot-plate latencies and paw withdrawal thresholds were significantly reduced in rats with inflammation of the hindpaw (Fig. 9). The rats were randomly divided into four groups and received RVM microinjections of vehicle or 10  $\mu$ g, 25  $\mu$ g or 50  $\mu$ g of minocycline and hot-plate latencies were determined at 10 minute intervals for 30 minutes (Fig. 9). Minocycline produced a dose-dependent

reversal of thermal hyperalgesia, indicated by significant ( $p < 0.05$ ) increases in hot-plate latencies, and reaching peak reversal 20 minutes after injection. As there was no significant difference in responses to either 25  $\mu\text{g}$  or 50  $\mu\text{g}$  of minocycline, the lower dose was employed in all subsequent experiments. Interestingly, the administration of minocycline into the RVM did not alter the hindpaw edema, indicated by non-significant changes in paw volume compared to carrageenan treated rats (Fig. 10C).

Microinjection of minocycline (25  $\mu\text{g}$ ) produced a significant ( $p < 0.05$ ) attenuation of carrageenan-induced thermal hyperalgesia within 15 minutes ( $14.1 \pm 1.2$ ;  $n=10$ ) and returned to post-carrageenan levels within 60 minutes ( $7.8 \pm 1.0$ ), indicating a reversible effect of minocycline (Fig. 10A). Minocycline also reversed tactile allodynia in a time-dependent manner, with a peak anti-allodynic effect observed 30 minutes after injection and indicated by a mean paw withdrawal threshold of  $9.8 \pm 1.3$  g ( $n=13$ ). Withdrawal thresholds returned to post-carrageenan levels within 60 minutes, indicated by a mean withdrawal threshold of  $4.9 \pm 0.8$  g (Fig. 10B). The administration of minocycline into the RVM of rats without hindpaw inflammation did not produce any significant changes in behavioral responses relative to baseline values (Fig. 10). These observations indicate that the inhibition of microglia in the RVM can reverse inflammation-induced thermal and tactile hypersensitivity.

To examine whether the reversal of behavioral signs of inflammatory hyperesthesia by minocycline was related to inhibition of microglial activation in

the RVM, medullary brain sections were obtained from control rats and rats with carrageenan-induced inflammation 15 to 30 minutes after microinjection of minocycline into the RVM. The sections were prepared for the immunolabeling of the microglia marker OX-42. Carrageenan-induced inflammation increased OX-42 immunofluorescence relative to the control section (Fig. 11B). Importantly, tissue obtained from rats treated with RVM minocycline after carrageenan-induced inflammation show decreased immunofluorescent labeling for OX-42 indicative of reduced microglial activation (Fig. 11C).

Activated microglia upregulate a variety of cell surface molecules, including OX-42 and Iba1 (Ito et al., 1998; Romero-Sandoval et al., 2008; Salter, 2005) and increased protein levels can be used as a measure of activation. Western blot analysis identified a 17 kDa band that corresponded to the molecular weight of Iba1, which showed increased levels after carrageenan administration (Fig. 12). The RVM microinjection of minocycline prevented an increase in Iba1 protein levels in animals treated with carrageenan (Fig. 12), indicating a decrease in microglia activation. These results suggest that minocycline diminishes carrageenan-induced microglia activation in the RVM, and that a decrease in microglia activation in the RVM correlates with an attenuation of inflammation-induced thermal and tactile hypersensitivity.

### **3.4 Fluorocitrate in the RVM Reverses Carrageenan-induced Hypersensitivity**

To determine if astrocytes as well as microglia play a role in inflammation-induced hypersensitivity, the general glial metabolic inhibitor, fluorocitrate, was microinjected into the RVM. Baseline responses to thermal and tactile stimuli were determined and carrageenan-induced inflammation was confirmed by significant reductions in hot-plate latencies and paw withdrawal thresholds, as described above. Either vehicle or fluorocitrate (1  $\mu$ g) was microinjected into the RVM. The microinjection of fluorocitrate into the RVM blocked carrageenan-induced thermal hyperalgesia within 60 minutes, indicated by a significant increase in hot-plate latency to  $15.2 \pm 2.3$  sec from a post-carrageenan mean of  $8.4 \pm 0.3$  sec (n=6) (Fig. 13A). Tactile allodynia was attenuated within 30 min of fluorocitrate administration, as indicated by a significant increase in paw withdrawal threshold to  $9.6 \pm 1.7$  g from a post-carrageenan mean of  $4.4 \pm 0.4$  g (n=10) (Fig. 13B). Hot-plate latencies and von Frey filament withdrawal thresholds returned to post-carrageenan levels within 120 and 180 min, as indicated by mean response values of  $10.6 \pm 0.3$  sec and  $5.7 \pm 0.3$  g, respectively, indicating reversibility of the effect of fluorocitrate (Fig. 13). Fluorocitrate was given to rats with hindpaw injections of vehicle and no significant difference compared to baseline was observed (Fig. 13). There was also no change in paw volume after the microinjection of fluorocitrate in the RVM (Fig. 13C).

Separate groups of rats received RVM microinjections of fluorocitrate three hours after the injection of saline or carrageenan. At the time of peak effect of the inhibitor (60 minutes after microinjection), the rats were sacrificed and brainstem tissue was prepared for immunofluorescent detection of the astrocyte marker, GFAP. Carrageenan-induced inflammation produced a visible intensification of immunofluorescent labeling for GFAP along with the morphologic changes indicative of astrocytic activation (Fig. 14B). The microinjection of fluorocitrate into the RVM resulted in decreased immunolabeling for GFAP indicative of a reduction in activated astrocytes (Fig. 14C). These results suggest that carrageenan-induced inflammation causes astrocyte activation and that application of fluorocitrate diminishes this activation in the RVM.

Activated astrocytes upregulate GFAP expression (Romero-Sandoval et al., 2008), and increased protein levels can be used as a measure of activation. Western blot analysis identified a 50 kDa band that corresponded to the molecular weight of GFAP, which showed increased levels after carrageenan administration (Fig. 15). The RVM microinjection of fluorocitrate prevented an increase in GFAP protein levels in animals treated with carrageenan (Fig. 15), indicating a decrease in astrocyte activation. These results suggest that fluorocitrate diminishes carrageenan-induced astrocyte activation in the RVM, and that a decrease in astrocyte activation in the RVM correlates with an attenuation of inflammation-induced thermal and tactile hypersensitivity.

### **3.5 Inhibition of p38 MAPK in the RVM Attenuates Inflammation-induced Hypersensitivity**

To investigate whether p38 MAPK activation occurs within the RVM following inflammatory injury and whether phosphorylated p38 MAPK is located in glia and/or neurons of the RVM, tissue of rats treated with saline or carrageenan was collected three hours after the hindpaw injection and prepared for immunohistochemical analysis. The sections were labeled with fluorescent marker for phosphorylated p38 MAPK (p-p38 MAPK) and co-labeled for OX-42 (microglial marker), GFAP (astrocytic marker) or NeuN (neuronal marker). Immunofluorescent label for p-p38 MAPK was found in labeled microglia and neurons of the RVM, but was not present in astrocytes (Fig. 16). In addition, there was an increase in co-labeled microglia after carrageenan-induced inflammation, which is consistent with microglial activation in the RVM (Imbe et al., 2007).

Baseline responses to thermal and tactile stimuli were determined and carrageenan-induced inflammation was confirmed by significant reductions in hot-plate latencies and paw withdrawal thresholds, as described above. Either vehicle or SB 203580, a p38 MAPK inhibitor, was microinjected into the RVM at various doses (1  $\mu$ g, 3  $\mu$ g, 10  $\mu$ g) and behavioral responses to a 52°C hot-plate were measured every 10 minutes for 30 minutes in order to identify a dose of inhibitor that did not produce overt adverse behavioral effects. RVM SB 203580 at 10  $\mu$ g increased hot-plate latencies to  $13.8 \pm 1.6$  sec from a post-carrageenan

mean of  $7.0 \pm 0.3$  sec (Fig. 17); this dose of SB 203580 was used for the remainder of this study.

The microinjection of SB 203580 (10  $\mu$ g) into the RVM blocked carrageenan-induced thermal hyperalgesia within 30 minutes, indicated by a significant increase in hot-plate latency to  $13.0 \pm 1.0$  sec from a post-carrageenan mean of  $8.4 \pm 0.3$  sec (n=13) (Fig. 18A). Thermal responses returned to post-carrageenan levels ( $10.4 \pm 0.8$  sec) within 90 minutes after microinjection (Fig. 18A). Tactile allodynia was attenuated within 30 minutes of SB 203580 administration, as indicated by a significant increase in paw withdrawal threshold to  $9.2 \pm 1.0$  g within 15 min of the injection (n=18) (Fig. 18B). Paw withdrawal thresholds returned to post-carrageenan levels ( $5.5 \pm 1.4$  g) within 90 minutes of administration (Fig. 18B). Administration of SB 203580 to rats with hindpaw injections of saline did not produce any significant changes in behavioral responses (Fig. 18).

Additional groups of rats received saline or carrageenan injections in the hindpaw. Thermal hyperalgesia was confirmed three hours after carrageenan and the rats received either vehicle or 10  $\mu$ g of SB 203580 in the RVM. At the time of peak behavioral effect following the microinjection of SB 203580 (30 min), the rats were sacrificed and medullary sections were obtained and immunofluorescently labeled for OX-42 in order to examine changes in microglial activation. Carrageenan-induced inflammation produced increased intensity in immunofluorescence for OX-42 relative to the control sections along with

morphological features indicative of microglial activation (Fig. 19B). Tissue obtained from rats treated with RVM SB 203580 after carrageenan-induced inflammation showed reduced intensity of immunofluorescent labeling for OX-42, as well as a reduction in the morphologic signs associated with microglial activation (Fig. 19C). The microinjection of SB 203580 did not appear to diminish immunofluorescent labeling for GFAP in tissue from rats treated with carrageenan (data not shown).

### **3.6 ATP-induced Hypersensitivity and Inhibition of P2X Receptors**

Studies suggest that ATP-induced activation of P2X receptors in the spinal dorsal horn induce behavioral hypersensitivity (Nakagawa et al., 2007; Tsuda et al., 2003). To examine a possible mechanism by which glia become activated in the RVM,  $\alpha,\beta$ -methylene-ATP (selective P2X agonist) was microinjected into the RVM of naïve rats at a dose of 1 nmol, inducing thermal hyperalgesia within 15 minutes, indicated by a significant decrease in hot-plate latency to  $9.1 \pm 1.4$  sec from a baseline mean of  $15.8 \pm 1.1$  sec ( $n=8$ ) that lasted for at least one week (Fig. 20A).

We tested the involvement of P2X receptors in the prevention of  $\alpha,\beta$ -methylene-ATP-induced hyperalgesia with TNP-ATP triethylammonium salt, an antagonist of P2X receptor subtypes 1-4. Baseline responses to thermal stimuli were determined and RVM microinjections of TNP-ATP were administered at a dose of 1 nmol 10 minutes prior to RVM microinjection of  $\alpha,\beta$ -methylene-ATP. Thermal hyperalgesia was not observed until the 30 minute time point, showing a

significant decrease in latency to  $12.1 \pm 1.6$  sec ( $n=7$ ), indicating a short-acting prevention of hyperalgesia (Fig. 20B). Pre-treatment with PPADS tetrasodium salt, an antagonist at receptor subtypes 1-3,5,7 but not 4, 10 minutes prior to RVM microinjection of  $\alpha,\beta$ -methylene-ATP prevented the development of thermal hyperalgesia until the 60 minute time point (latency of  $11.1 \pm 1.1$  sec decreased from a baseline value of  $15.8 \pm 1.1$  sec), suggesting blockade of these receptor subtypes blocks  $\alpha,\beta$ -methylene-ATP-induced hypersensitivity in a time-dependent manner (Fig. 20B). The RVM microinjection of TNP-ATP or PPADS only did not produce behavior different from baseline values (Fig. 20).

We next tested the effects of the two P2X receptor antagonists on the attenuation of  $\alpha,\beta$ -methylene-ATP-induced thermal hypersensitivity. Four days after the RVM microinjection of  $\alpha,\beta$ -methylene-ATP, TNP-ATP (1 nmol) or PPADS (1 nmol) were microinjected into the RVM and behavioral responses to thermal stimuli were measured every 15 minutes for 45 minutes. There was no significant difference between the group that received saline in the RVM and those that received the antagonist (Fig. 21A). Finally, we examined the role of P2X receptors in carrageenan-induced inflammation. Baseline responses to hot-plate latencies were measured and carrageenan was injected into the plantar aspect of the hindpaw. The development of thermal hyperalgesia was observed three hours after the injection of carrageenan and RVM microinjections of TNP-ATP (1 nmol) or PPADS (1 nmol) did not attenuate the established hypersensitivity, indicated by TNP-ATP or PPADS latency values of  $8.8 \pm 0.5$  sec

or  $8.3 \pm 0.7$  sec, respectively, from post-carrageenan values of  $7.5 \pm 0.9$  sec (Fig. 21B). Taken together, these data suggest  $\alpha, \beta$ -methylene-ATP acts through multiple P2X receptors (not only through P2X<sub>4</sub>) in the RVM to induce hyperalgesia and other receptors and pathways become activated to maintain the enhanced pain.

### **3.7 Sequestration of BDNF in RVM Attenuates Inflammation-induced Hyperalgesia**

The activation of microglia through stimulation with ATP has been shown to evoke the release of brain-derived neurotrophic factor (BDNF) (Coull et al., 2005). BDNF then acts at trkB receptors located on neurons to induce exaggerated pain states. To determine if BDNF in the RVM plays a role in carrageenan-induced hypersensitivity, endogenous BDNF was neutralized by the microinjection of an anti-BDNF antibody. Carrageenan injection produced behavioral signs of thermal hyperalgesia within three hours of administration. The animals were randomly divided into four groups and received RVM microinjections of vehicle or 50 ng, 100 ng, or 200 ng of BDNF antiserum and hot-plate latencies were measured at 15 minutes intervals for 2 hours (Fig. 22). The BDNF antiserum produced a dose-dependent reversal of thermal hyperalgesia, indicated by significant ( $p < 0.05$ ) increases in hot-plate latencies, and reaching peak reversal 30 minutes after injection (Fig. 22). Hot-plate latencies returned to post-carrageenan levels approximately two hours after microinjection, indicating this effect is reversible. Animals with hindpaw injections

of saline received RVM microinjections of the BDNF anti-serum and there was no difference from baseline latencies (data not shown). These results suggest inflammation-induced activated microglia in the RVM may release BDNF and sequestration of endogenous BDNF in the RVM reverses thermal hypersensitivity.

### **3.8 Inhibition of Microglia in the RVM Attenuates Morphine-induced Hypersensitivity**

Sustained administration of morphine can induce behavioral hypersensitivity. To determine if microglia or astrocytes contribute to the development of hypersensitivity, rats were implanted with osmotic pumps to continuously infuse morphine (1.54 mg/day) or saline systemically for six days (see experimental design Fig. 23) as well as implantation of RVM cannulas. The development of tactile allodynia was observed after six days of morphine administration (Fig. 24) and the RVM microinjection of minocycline (25  $\mu$ g) significantly attenuated the allodynia within 15 minutes after administration to a value of  $15.0 \pm 0.0$  from a post-morphine value of  $4.24 \pm 0.49$  (Fig. 24A). Responses returned to post-morphine values ( $4.53 \pm 1.17$ ) within 60 minutes after RVM administration of minocycline. The RVM microinjection of the astrocyte inhibitor, fluorocitrate (1  $\mu$ g), showed no significant differences from post-morphine values at any of the time points tested (Fig. 24B). Animals that received sustained administration of saline did not show behavior different from baseline values and saline-treated animals that received minocycline or

fluorocitrate in the RVM did not show differences from baseline (Fig. 24). These results suggest that microglial activation plays a role in morphine-induced nociception.

Additional groups of rats received sustained saline or morphine administration and tactile allodynia was confirmed six days after administration. The rats were sacrificed and medullary sections were obtained and immunofluorescently labeled for OX-42 in order to examine changes in microglial activation. Sustained morphine administration produced increased intensity in immunofluorescence for OX-42 relative to the control sections along with morphological features indicative of microglial activation (Fig. 25). The administration of morphine did not appear to induce astrocyte activation, as no morphological changes were observed (data not shown).

To quantify RVM protein levels of Iba1 or GFAP, additional groups of rats received chronic saline or morphine and on day six, the rats received RVM microinjections of either minocycline (25  $\mu$ g) or fluorocitrate (1  $\mu$ g) and at the peak effect of the inhibitor, tissue was collected for Western blot analysis. Protein levels of Iba1 from animals that received morphine or morphine plus minocycline in the RVM were not significantly different than those that received saline (Fig. 26A). There was also no significant difference in GFAP protein levels from animals that received morphine or morphine plus fluorocitrate in the RVM in comparison to those that received saline (Fig. 26B). While these results indicate there is no increase in Iba1 protein levels in the RVM with sustained

administration of morphine, it does not mean there is no microglial activation in the RVM, as morphological cell changes were observed and the use of the microglial inhibitor attenuated the behavioral hypersensitivity.

### **3.9 Inhibition of Astrocytes in the RVM Attenuates Nerve Injury-induced Hypersensitivity**

To examine whether RVM glial activation plays a role in neuropathic pain, a spinal nerve ligation (SNL) model of nerve injury was employed. Animals received either sham or SNL injury as well as implantation of an RVM cannula (see experimental design Fig. 27) and on day three after injury, behavioral tactile allodynia was observed, as there was a significant decrease in paw withdrawal threshold ( $3.2 \pm 0.6$  g) compared to baseline values ( $15.0 \pm 0.0$  g) (Fig. 28A). Tactile allodynia was also observed on day 10 ( $3.0 \pm 0.5$  g) and day 30 ( $3.1 \pm 0.5$  g) in animals that received SNL injury (Fig. 28B and C). The RVM microinjection of minocycline (25  $\mu$ g) did not attenuate the behavioral hypersensitivity when given on day 3, 10 or 30, indicated by non-significant changes from post-SNL values (Fig. 28). The administration of the astrocyte inhibitor, fluorocitrate (1  $\mu$ g), into the RVM on days 3, 10 and 30 resulted in the attenuation of tactile allodynia at all three time points (Fig. 29). Fluorocitrate attenuated the post-SNL hypersensitivity ( $3.0 \pm 0.4$ ) within 15 minutes on day 3 ( $11.8 \pm 2.0$ , Fig. 29A), day 10 ( $9.3 \pm 2.0$ , Fig. 29B) and on day 30 ( $15.0 \pm 0.0$ , Fig. 29C). Responses returned to post-SNL values within 60 minutes after administration (Fig. 29). Animals that received sham injury with saline, minocycline or fluorocitrate in the

RVM did not show behavior different from baseline values (Fig. 28, 29). These data indicate that inhibition of astrocytes in the RVM can attenuate nerve injury-induced tactile allodynia in a time-dependent manner.

Additional groups of rats received sham or SNL injury and tactile allodynia was confirmed ten days later. The rats were sacrificed and medullary sections were obtained and immunofluorescently labeled for GFAP in order to examine changes in astrocyte activation. SNL injury produced increased intensity in immunofluorescence for GFAP relative to the control sections along with morphological features indicative of astrocyte activation (Fig. 30). The nerve injury did not appear to induce microglial activation, as no morphological changes were observed (data not shown).

To quantify the glial activation in the RVM, Western blot analysis was performed on tissue from a separate group of animals that received either sham or SNL injury. The tissue was collected at the peak effect of fluorocitrate (approximately 30 minutes) administration into the RVM or 15 minutes after the microinjection of minocycline on days 3, 10 and 30 after injury. Protein levels of GFAP from animals that received SNL or SNL plus fluorocitrate in the RVM were not significantly different than those with sham injury on day 3 (Fig. 31B), day 10 (Fig. 32B) or day 30 (Fig. 33B). There was also no significant difference in Iba1 protein levels from animals that received SNL or SNL plus minocycline in the RVM in comparison to those with sham injury on day 3 (Fig. 31A), day 10 (Fig. 32A) or day 30 (Fig. 33A). The lack of increase in GFAP protein levels with SNL

injury does not mean there is no astrocyte activation in the RVM. The above results indicate a role for astrocyte activation in nerve injury-induced hypersensitivity due to morphological changes visualized in the RVM tissue and the attenuation of tactile hypersensitivity with the RVM microinjection of fluorocitrate.

## CHAPTER 4: DISCUSSION

The present study demonstrated that chronic pain syndromes lead to activation of microglia and astrocytes in the RVM. The administration of the selective inhibitor of microglia, minocycline, into the RVM attenuated behavioral signs of thermal hyperalgesia and tactile allodynia, and inhibited the activation of microglia in the RVM. Intraplantar injections of carrageenan increased RVM protein levels of Iba1 compared to saline treated animals and administration of minocycline into the RVM decreased Iba1 levels. Likewise, fluorocitrate, the nonspecific glial inhibitor, reversed the behavioral signs of inflammatory pain and attenuated the evidence of activation of astrocytes, as indicated by the decrease in RVM immunolabeling and protein levels of GFAP. Additionally, phosphorylation of p38 MAPK mediated, at least in part, behavioral signs of inflammatory pain since the microinjection of the p38 MAPK inhibitor, SB 203580, into the RVM attenuated both carrageenan-induced thermal hyperalgesia and tactile allodynia. Activation of microglial p38 MAPK may be partly responsible for the behavioral expression of hyperesthesia due to inflammation, since the administration of SB 203580 into the RVM decreased OX-42 labeling of RVM tissue. To examine the role of P2X receptors in glial activation in the RVM,  $\alpha,\beta$ -methylene-ATP was microinjected into the RVM, inducing thermal hyperalgesia, and pre-treatment with the P2X antagonists, PPADS and TNP-ATP, delayed the initiation of ATP-induced hyperalgesia. Post-treatment with the antagonists had no effect on established ATP-induced or carrageenan-induced hypersensitivity.

The activation of P2X receptors initiates a signaling cascade leading to the production and release of nociceptive mediators, including BDNF (Beggs et al., 2009). The present study also indicated that sequestration of BDNF, an endogenous modulator of nociception (Coull et al., 2005; Mannion et al., 1999; Thompson et al., 1999), with an RVM microinjection of an anti-BDNF antibody attenuated carrageenan-induced thermal hyperalgesia. The sustained administration of morphine was found to activate microglia within the RVM and the microinjection of minocycline into the RVM attenuated the corresponding morphine-induced allodynia. On the flip side, spinal nerve ligation was found to activate astrocytes within the RVM and the administration of fluorocitrate attenuated the nerve injury-induced allodynia in a time-dependent manner. The results of the present investigation indicate that peripheral inflammation induces microglial and astrocyte activation, chronic morphine induces microglial activation and nerve injury induces astrocyte activation in the RVM. Activated glia of the RVM may contribute to enhanced pain, likely through glial-neuronal communication, leading to descending facilitation from the RVM.

Converging lines of evidence indicate the RVM is a central component of descending facilitation of nociceptive inputs at the level of the spinal cord (Gebhart, 2004; Porreca et al., 2002; Urban and Gebhart, 1999). Considerable evidence has been generated to show that descending facilitatory influences from the RVM can contribute to chronic pain states (Fields, 2000; Urban and Gebhart, 1999) and such contributions are important for the maintenance of

thermal and tactile hypersensitivity. Administration of lidocaine into the RVM and lesions of the dorsolateral funiculus (DLF) have been shown to block behavioral signs of neuropathic and inflammatory nociception (Burgess et al., 2002; Xie et al., 2005; Pertovaara et al., 1996; Pertovaara et al., 1998). Microinjection of the cytotoxin saporin conjugated to dermorphin selectively destroyed RVM neurons expressing  $\mu$ -opioid receptors, likely facilitation cells (Burgess et al., 2002; Porreca et al., 2001). This manipulation prevented or reversed behavioral signs of enhanced pain (Burgess et al., 2002; Porreca et al., 2001). Finally, pharmacologic and surgical manipulations that abolish descending facilitation from the RVM have also abolished enhanced capsaicin-evoked release of excitatory transmitters from primary afferent terminals and the upregulation of spinal dynorphin to pathologically elevated pro-nociceptive levels (Burgess et al., 2002; Porreca et al., 2001). Whereas activation of descending facilitation has been considered to be a neuronal phenomenon, we now present evidence that activation of glial cells in the RVM contributes to descending facilitation of enhanced pain.

Although the neuronal processes that contribute to central sensitization in the spinal cord have been examined, the potential contribution of glial cells to this process is only recently coming to light. It is now appreciated that peripheral nerve injury as well as inflammation results in the activation of spinal glia (Svensson et al., 2005; Sweitzer et al., 1999b; Garrison et al., 1991). Several studies have now shown that sensitization of peripheral nociceptors by

subcutaneous injection of irritants, peripheral inflammation or nerve injury, as well as intrathecal injection of sensitizing agents such as NMDA all result in the activation of microglia and astrocytes in the spinal cord, and that glial activation correlates with enhanced nociceptive responses (Tanga et al., 2005;Fu et al., 1999b;Fu et al., 2000;Raghavendra et al., 2004;Scholz and Woolf, 2007;Sweitzer et al., 1999b). Studies performed with genetically modified mice or by selective knockdown of receptors and peptides with antisense oligodeoxynucleotides further revealed that activated spinal microglia promote enhanced pain due to nerve injury and inflammation (Abbadie et al., 2003;Tanga et al., 2005;Tsuda et al., 2003;Tsuda et al., 2005). Inhibition of spinal microglial or astrocytic activity with intrathecal glial inhibitors attenuated behavioral signs of hyperalgesia and allodynia induced by formalin, carrageenan-induced inflammation, and spinal injection of NMDA (Raghavendra et al., 2003a;Tsuda et al., 2005;Hua et al., 2005;Ledeboer et al., 2005). Whereas the preponderance of such studies have focused on the role of spinal glia in enhanced pain states, little work exists at present that examines changes in glial function at supraspinal sites. Thus, the results of the present investigation extend the role of glial activation to supraspinal sites, and specifically to the RVM, which is prominent in descending control of nociception. An earlier study had indicated that CFA-induced inflammation caused supraspinal glial activation, indicated by increased expression of glial markers in the medulla, pons, midbrain and thalamus (Raghavendra et al., 2004). In that study, markers of microglial activity were

elevated in the brainstem and forebrain within 4 hours of CFA-induced inflammation and remained elevated up to 14 days afterwards, whereas astrocyte activation was not evident until 4 days after CFA administration (Raghavendra et al., 2004). However, there was no attempt to attenuate supraspinal microglial or astrocytic activation and to correlate such inhibition of glial activation with changes in inflammatory pain in this study (Raghavendra et al., 2004). In the present investigation, we have extended these observations to include the RVM and to correlate glial activation with inflammatory pain.

Carrageenan-induced inflammation led to evidence of activation of microglia and astrocytes in the RVM. Inhibitors of activation of microglia and astrocytes administered into the RVM reduced carrageenan-induced activation of these glial cells, and abolished tactile allodynia and thermal hyperalgesia. Minocycline is a second-generation tetracycline antibiotic that has been shown to possess several immunomodulatory functions, including blockade of microglial activation. Intrathecal administration of minocycline blocks spinal microglial activation and behavioral signs of enhanced pain induced by inflammation and nerve injury (Ledeboer et al., 2003; Milligan et al., 2000; Raghavendra et al., 2003b; Watkins et al., 1997). The mechanisms by which minocycline block microglial activation remain unclear. Minocycline has been shown to work on both microglia and T cells, resulting in decreased cytokine levels produced in T cell-microglia interactions (Giuliani et al., 2005). Spinal administration of minocycline or fluorocitrate, a gliotoxin relatively selective for astrocytes,

decrease pro-inflammatory cytokine production and corresponding hyperalgesia and allodynia (Hua et al., 2005;Ledeboer et al., 2005;Raghavendra et al., 2004). In the present study, inflammation-induced glial activation in the RVM may result in increased production of pro-inflammatory cytokines, resulting in the sensitization of neurons and facilitation of nociception.

Evidence suggests that spinal microglial activation precedes that of any other immune cell type. It has been shown in models of enhanced pain that microglia activation initiates, while astrocyte activation maintains, hypersensitivity (Raghavendra et al., 2004;Watkins and Maier, 2003a). Interestingly, minocycline administration into the RVM attenuated thermal hyperalgesia and tactile allodynia approximately 30 minutes after injection, while behavioral effects of fluorocitrate administration were not observed until approximately 60 minutes after injection. The time course of action of these two inhibitors may be due to an initial activation of microglia in the RVM followed by the activation of astrocytes to maintain inflammation-induced hypersensitivity.

Upon activation, microglia and astrocytes are known to upregulate the expression of OX-42, Iba1 and GFAP in the CNS (Romero-Sandoval et al., 2008). Immunohistochemistry has been used extensively to monitor the morphological and biological transformation of activated glia in the spinal cord after injury(Guo et al., 2007;Ledeboer et al., 2005;Romero-Sandoval et al., 2008). However, in order to quantify microglia and astrocyte activation in the RVM, protein levels of markers for these cell types were examined using

Western blot. Carrageenan-induced inflammation increased Iba1 and GFAP protein levels, indicating microglia and astrocyte activation, respectively, and the microinjection of glial inhibitors into the RVM returned these protein levels to near baseline values. The effects of minocycline and fluorocitrate on glial activation and behavioral hypersensitivity are due to direct administration into the RVM and not a decrease in peripheral inflammation, as the administration of the glial inhibitors had no effect on paw edema.

Glial activation leads to the phosphorylation of p38 MAP kinase which, in turn, promotes the production of inflammatory mediators such as prostaglandin E2 and cytokines (Shi and Gaestel, 2002; Takeda and Ichijo, 2002; Obata et al., 2000). It has been shown that peripheral inflammation and nerve injury provoke phosphorylation (activation) of p38 MAPK in DRG neurons and in both microglia and neurons in the dorsal horn of the spinal cord (Kim et al., 2002). Importantly, however, whereas spinal microglia demonstrate phosphorylation of p38 MAPK after nerve injury, inflammation, subcutaneous injection of algogenic substances or spinal injection of NMDA or substance P, increased phosphorylation of neuronal p38 MAPK is inconsistent and is not detected after peripheral injection of formalin or intrathecal injection of substance P (Hua et al., 2005; Svensson et al., 2007; Tsuda et al., 2005). It was also found that the p38 $\alpha$  isoform of p38 MAPK is found predominantly in neurons whereas the p38 $\beta$  isoform is found in microglia (Svensson et al., 2005). Antisense experiments that produced selective knock-down of either isoform of p38 MAPK showed that the p38 $\beta$

isoform, and not the p38 $\alpha$  isoform, mediates hyperalgesia due to intraplantar formalin or spinal substance P (Svensson et al., 2005). These observations indicate that, at least in the spinal cord, microglial, but not neuronal, p38 MAPK contributes to enhanced sensitivity in chronic pain states. In the present investigation, phosphorylated p38 MAPK was localized in both microglia and neurons in the RVM in the resting state, with increased phosphorylation of p38 MAPK in microglia after carrageenan-induced inflammation. The relative contribution of neuronal p38 MAPK to the expression of enhanced pain requires further investigation.

While inhibitors of p38 MAPK activation do not alter basal responses to acute noxious stimuli (Hua et al., 2005), pretreatment with intrathecal injections of p38 MAPK inhibitors has been shown to block hyperalgesia induced by formalin, capsaicin or carrageenan injected into the hindpaw, peripheral nerve injury or intrathecal injections of inflammatory mediators such as IL-6 $\beta$  (Svensson et al., 2005; Svensson et al., 2007; Sweitzer et al., 1999b; Mizushima et al., 2005). In addition, p38 MAPK inhibitors blocked substance P-induced evoked release of PGE<sub>2</sub> into the CSF (Svensson et al., 2005). One of the most commonly used compounds among various p38 MAPK inhibitors is SB 203580. We report that microinjection of SB 203580 into the RVM attenuated behavioral hyperalgesia and allodynia as well as immunofluorescent evidence of activated microglia in the RVM. Interestingly, the time course of action of both SB 203580 and minocycline on thermal hyperalgesia and tactile allodynia was approximately

30 minutes, suggesting the activation of p38 MAPK correlates with the activation of microglia. Others have reported that peripheral inflammation induced by injection of CFA into the hindpaw resulted in phosphorylation of p38 MAPK that was predominant in RVM neurons but also present in some microglia of the RVM within 30 min of the injection (Imbe et al., 2007). However, in that study, the functional significance of phosphorylated p38 MAPK in non-neuronal cells was not investigated (Imbe et al., 2007). The phosphorylation of p38 MAPK diminished within 7 hours, which corresponded with increased activation of ERK, which itself might be transcriptionally related to the activation of p38 MAPK (Imbe et al., 2007). In that study, it was also found that approximately 40% of neurons expressing phosphorylated p38 MAPK were serotonergic, and it was hypothesized that p38 MAPK activation could lead to neuroplastic changes in descending pain modulation, in this case, by increasing transcription of tryptophan hydroxylase and promoting serotonergic transmission from the brainstem (Imbe et al., 2007). Transcriptional events produced by activated p38 MAPK could also lead to increased expression of pro-nociceptive mediators in the RVM; it is known that p38 MAPK activates basic fibroblast growth factor (bFGF)-mediated transcription of cholecystokinin (CCK) (Hansen et al., 1999). Previous reports indicate increased CCK in the RVM mediates enhanced descending facilitation (Xie et al., 2005).

A growing body of evidence suggests that neurotrophic factors in the CNS can act as algogenic mediators in chronic pain states. In particular, brain-derived

neurotrophic factor (BDNF) has been implicated as a key mediator of central nociceptive processes, although the focus of research has been neuronally derived BDNF. Evidence suggests that inflammatory pain is indeed mediated by neuronal BDNF (Kerr et al., 1999; Mannion et al., 1999) and investigations of a conditional knock-out mouse where BDNF was selectively removed from nociceptive sensory neurons showed reduced pain-related behaviors, both at basal levels and in response to inflammation (Zhao et al., 2006). Intrathecal administration of BDNF induces allodynia in naïve rats and administration of BDNF-sequestering fusion proteins or trkB receptor (BDNF receptor located on neurons) antagonists reversed behavioral hypersensitivity induced by nerve injury, indicating BDNF-trkB signaling is an important component of enhanced pain (Coull et al., 2005). Recent investigations report that BDNF is released from cultured microglia upon stimulation with ATP (Coull et al., 2005) and intrathecal administration of microglia induce behavioral hypersensitivity and a shift in the anion reversal potential in spinal neurons. Pretreatment of microglia with siRNA directed against BDNF prior to stimulation and spinal administration inhibited the effects of these cells on the withdrawal threshold and anion reversal potential (Coull et al., 2005). Increased levels of BDNF and trkB phosphorylation in the RVM after inflammation has been reported (Guo et al., 2006), and our present investigations show that intra-RVM sequestration of BDNF attenuates carrageenan-induced thermal hyperalgesia. This data indicates that BDNF is an

important component of descending facilitation, but the cell type origin of BDNF release in the RVM is unclear and requires further investigation.

An important mediator of both neuronal and glial activation is adenosine triphosphate (ATP), which is released from damaged and/or inflamed tissue, from central terminals of nociceptive afferents within the spinal cord, and from astrocytes during calcium wave propagation (Bardoni et al., 1997). ATP is an endogenous ligand for the P2 purinergic receptor family which can be divided into two subgroups: the G-protein-coupled P2Y receptors and the ATP-gated cation channels of the P2X receptors. To date at least seven P2X- and eight P2Y-subtypes have been cloned and most are expressed on primary afferent neurons or spinal dorsal horn neurons. Recently, however, P2X and P2Y receptor expression has been found on spinal microglia and astrocytes and are now known to have an important role in pain processing. The role of P2Y receptors for more than chemotaxis and migration is only now coming to light, as the P2Y<sub>12</sub> receptor on microglia has been found to be important for the pathogenesis of enhanced pain (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). To this point, investigations have focused on the role of P2X receptors in the facilitation of enhanced pain.

Previous studies had suggested that P2X<sub>2</sub> and P2X<sub>3</sub> receptors are abundantly expressed specifically on nociceptive neurons (Vulchanova et al., 1997). However, pharmacological blockade of these two receptors by intrathecal injection of a known antagonist, PPADS, had no effect on the established

allodynia, whereas treatment with an alternative antagonist, TNP-ATP, produced a reversal of nerve injury-induced enhanced pain (Tsuda et al., 2003). The pharmacological profiles of these two antagonists suggested P2X<sub>4</sub> receptors were being targeted and further examinations revealed an increase in P2X<sub>4</sub> protein in microglia of the spinal dorsal horn (Tsuda et al., 2003). These findings indicate that ATP, acting through P2X<sub>4</sub> receptors on microglia, is necessary to induce the tactile allodynia associated with nerve injury. Activation and up-regulation of P2X<sub>4</sub> receptors in microglia has also been implicated in the induction of long-term potentiation of C-fiber-evoked field potentials and the release of BDNF in the spinal dorsal horn (Gong et al., 2008;Ulmann et al., 2008).

In the present investigation, the administration of the P2X-receptor agonist  $\alpha,\beta$ -methylene-ATP into the RVM of naïve rats produced long-lasting hyperalgesia. This model is useful to stimulate chronic pain without tissue damage or peripheral nerve injury and was used to examine the role of P2X receptors in the RVM. Pre-treatment with PPADS (P2X<sub>1-3,5,7</sub> antagonist) or TNP-ATP (P2X<sub>1-4</sub> antagonist) prior to  $\alpha,\beta$ -methylene-ATP administration produced a short-acting anti-hyperalgesia, suggesting that ATP acts at multiple receptors. McGaraughty et al. found an intrathecal injection of the selective P2X<sub>3</sub>/P2X<sub>2/3</sub>-receptor antagonist, A-317491, produced an anti-hyperalgesic effect in an inflammatory model of pain (McGaraughty et al., 2003). Co-administration of ATP and A-317491 was found to prevent tactile allodynia induced by spinal ATP

injection, however, post-administration of the antagonist had little or no effect on allodynia (Nakagawa et al., 2007). RVM microinjection of PPADS or TNP-ATP four days after the RVM administration of  $\alpha,\beta$ -methylene-ATP did not attenuate the established hyperalgesia and, importantly, did not attenuate carrageenan-induced inflammation. Taken together, these data suggest  $\alpha,\beta$ -methylene-ATP acts through multiple P2X receptors (not only through P2X<sub>4</sub>) in the RVM to induce hyperalgesia, and other receptors (such as P2Y receptors) then become activated to maintain the enhanced pain. Antagonism of supraspinal P2X receptors alone after the establishment of the enhanced pain is not enough to attenuate the behavioral signs of hypersensitivity. The use of more selective antagonists will need to be used to examine the roles of receptors in a variety of cell types within the RVM in future investigations.

To this point, opioid analgesics represent the best option for the treatment of severe pain and for the management of chronic pain states. However, the clinical efficacy of these drugs is reduced by the rapid development of analgesic tolerance with repeated administration of opioids, such as morphine. Patients who receive long-term opioid therapy sometimes develop unexpected, abnormal pain (De et al., 1991a; De et al., 1991b; Sjogren et al., 1994b; Sjogren et al., 1994a; Doverty et al., 2001a; Doverty et al., 2001b). Many mechanisms have been proposed to account for the development of this morphine-induced paradoxical pain, but the concept that glial activation may play a role is only now being examined. Chronic administration of morphine has been shown to activate

glia and upregulate pro-inflammatory cytokines in the spinal cord (Raghavendra et al., 2002) and inhibition of glia in the spinal cord attenuated morphine-induced hypersensitivity (Song and Zhao, 2001). The present investigation examines glial activation within the RVM after sustained administration of morphine and shows that chronic morphine induces morphological changes in microglia in the RVM, suggesting microglial activation. Furthermore, inhibition of microglia with the RVM microinjection of minocycline attenuates morphine-induced tactile allodynia. While there was no significant increase in Iba1 protein levels in the RVM with sustained morphine administration, this does not mean there is no microglial activation. Increases in pro-inflammatory cytokines within the RVM are possible, along with other functional changes not examined here. Further investigation needs to be performed to determine if these changes occur. Importantly, the behavioral changes observed with the inhibition of microglia in the RVM suggest a role for microglia in morphine-induced hypersensitivity.

Opioid tolerance and neuropathic pain conditions share features of diminished  $\mu$ -opioid analgesia and abnormal pain (Mayer et al., 1999) and it is suggested that the mechanisms of morphine-induced hypersensitivity are reminiscent of those associated with inflammatory pain (King et al., 2005a). These common features have led to suggestions of common mechanisms between these pain syndromes. Evidence indicates that nerve injury induces glial activation in the spinal cord and inhibition of glia attenuates nerve injury-induced hypersensitivity (Scholz and Woolf, 2007; Garrison et al., 1991; Tanga et

al., 2005). In the present study, spinal nerve ligation (SNL) injury was used as a model of nerve injury and glial activation in the RVM induced by this injury was examined. SNL induced tactile allodynia on days 3, 10 and 30 after injury and RVM microinjection of the astrocyte inhibitor, fluorocitrate, attenuated SNL-induced hypersensitivity at all three time points. Morphological changes in astrocytes within the RVM were also observed, and these results indicate a role of astrocytes in nerve injury-induced hypersensitivity. While there was no observable increase in GFAP protein levels in the RVM, other pro-inflammatory mediators may be upregulated and further investigation will determine if these changes take place within the RVM. A previous study also suggests glial activation in the RVM in a nerve injury model (chronic constriction injury) of the infraorbital nerve (Wei et al., 2008). While they found a role of both astrocytes and microglia in the RVM in a model of CCI, it is possible that different nerve injury conditions may produce different patterns of activation.

## **Conclusions**

In the present investigation, we demonstrated that peripheral inflammation induced with carrageenan activated microglia and astrocytes in the RVM as well as the phosphorylation of p38 MAPK in RVM neurons and microglia.

Microinjection of inhibitors of glial activation or of p38 MAPK attenuates both carrageenan-induced thermal hyperalgesia and tactile allodynia.

Immunofluorescent examination of medullary sections shows an intensification in labeling for OX-42, GFAP and p-p38 MAPK in tissue obtained from rats treated

with carrageenan. Microinjection of minocycline, fluorocitrate and SB 203580 decreased the activation of microglia and astrocytes in the RVM. Activation of P2X receptors in the RVM via  $\alpha,\beta$ -methylene-ATP initiates a cascade of signaling processes, including the phosphorylation of p38 MAPK, leading to increased production and release of BDNF which act on the trkB receptors located on neurons.

It was also demonstrated that sustained morphine administration induces behavioral hypersensitivity and inhibition of microglia in the RVM can attenuate this behavior, suggesting an important role for microglia in opioid-induced enhanced pain. Astrocyte activation was found to be important for nerve injury-induced behavioral hypersensitivity, as fluorocitrate attenuated the tactile allodynia induced by SNL.

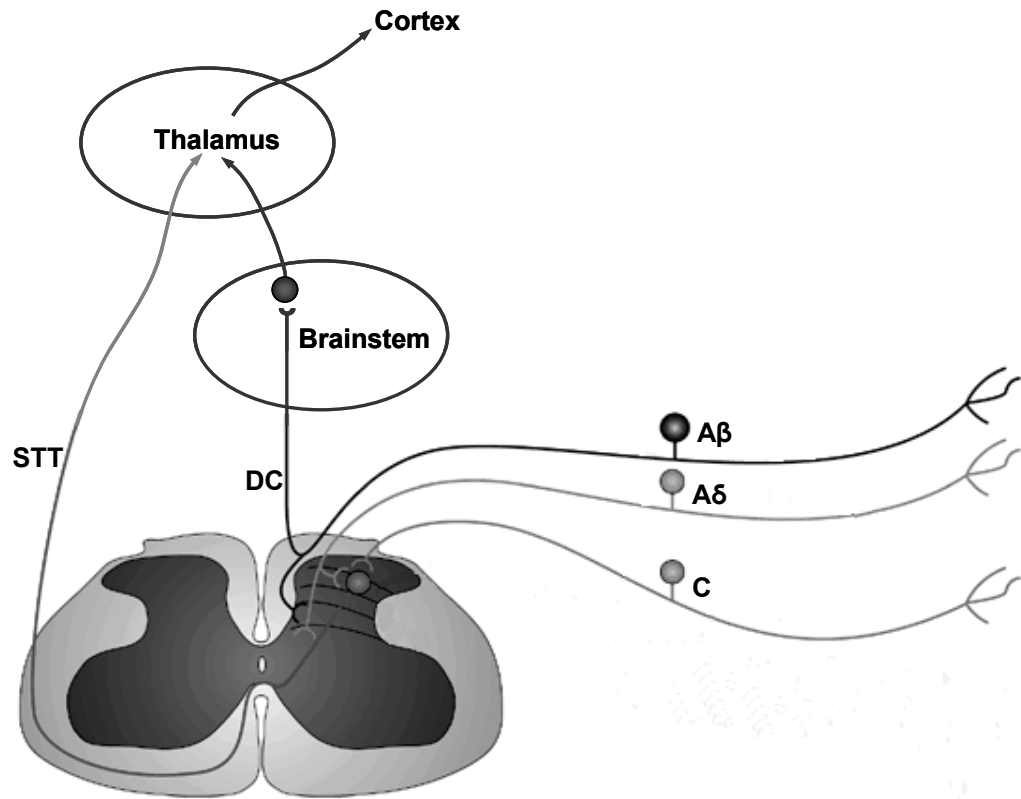
While many factors are important for pain processing, we now show that glial activation within the RVM is a crucial player in the descending facilitation of nociception. Within the RVM, three different pain syndromes have been found to activate glia, but with varying patterns. The concept that different pain syndromes, such as inflammation, opioid-induced pain and neuropathic pain, can activate different cell types is important for our understanding of the mechanisms of chronic pain. Each of these cell types can produce and release a variety of mediators that can act on the neurons of the RVM to facilitate pain. This glial-neuronal communication within the RVM may lead to a novel mechanism that

may represent a novel target for the development efforts aimed at the control of chronic pain states.

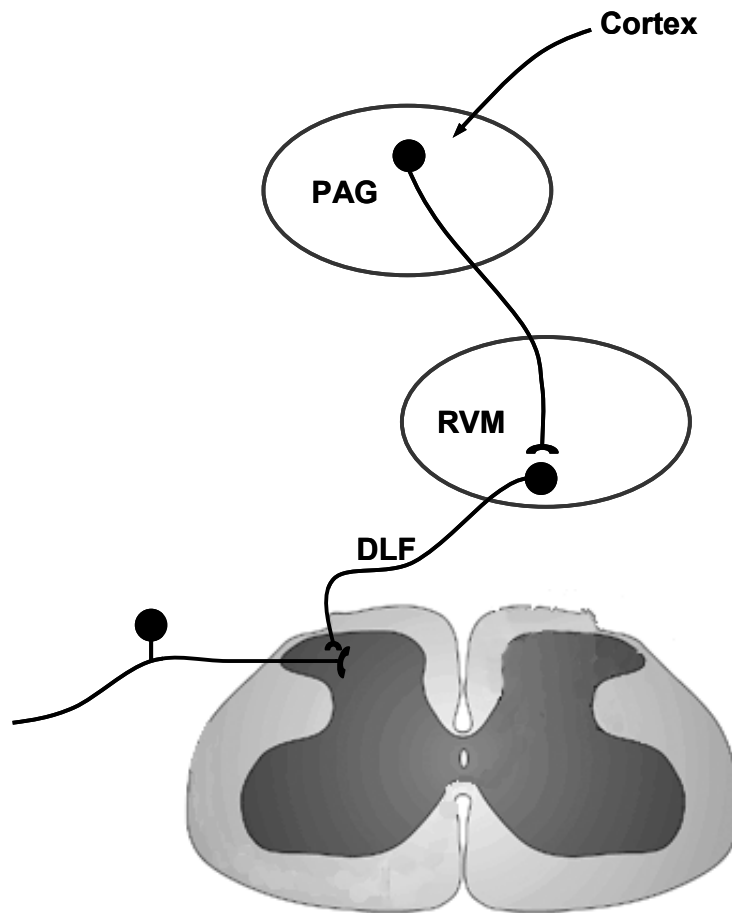
**Table 1**  
Pain-enhancing procedures known to activate\* glia in spinal dorsal horn

Model	Reference
Subcutaneous irritants	Colburn et al. 1997 Fu et al. 1999 Sweitzer et al. 1999 Raghavendra et al. 2004 Hua et al. 2005
Spinal nerve ligation (SNL)	Colburn et al. 1997 Zhang et al. 2003 Narita et al. 2006
Partial SNL	Clark et al. 2007
SNL + transection	Colburn and DeLeo 1999 Tsuda et al. 2004
Chronic constriction injury	Colburn et al. 1997 Zhang et al. 2003 Durrenberger et al. 2004
Spinal nerve transection	Arruda et al. 2000 Raghavendra et al. 2003 Colburn et al. 1999
Nerve root ligation	Hashizume et al. 2000
Spinal cord injury	Hains et al. 2006 Peng et al. 2006 Popovich et al. 1997
Bone cancer	Schwei et al. 1999

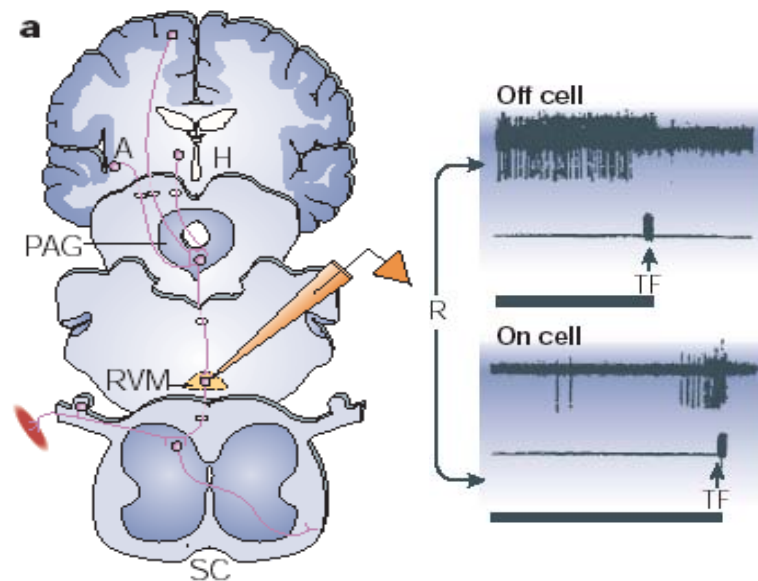
\* Activation is based on morphological changes and increased expression of glial markers assessed by immunohistochemistry.



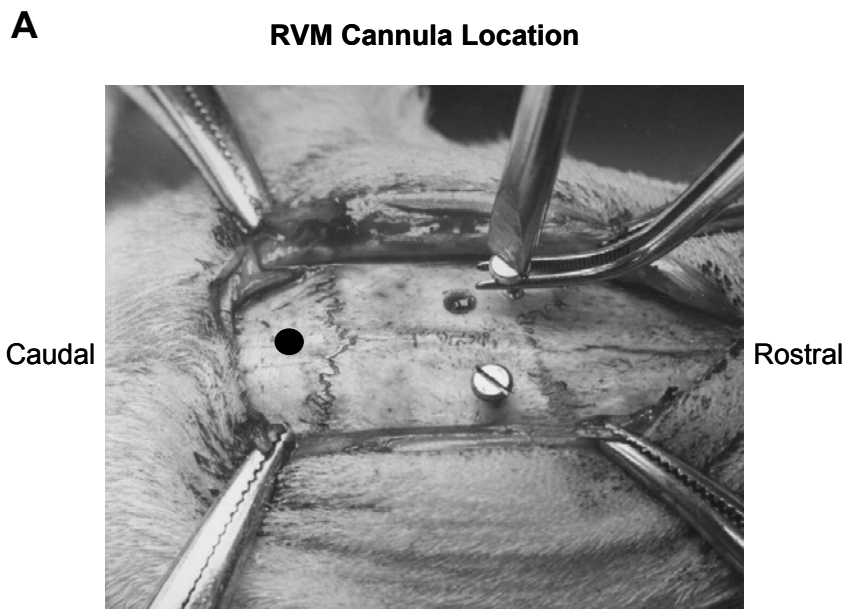
**Figure 1:** Ascending pain pathways. The primary afferent A $\beta$ , A $\delta$  and C fibers enter the spinal cord. The A $\beta$  fibers ascend via the ipsilateral dorsal column (DC) to the dorsal column nuclei in the brainstem. The A $\delta$  and C fibers ascend via the contralateral spinothalamic tract (STT). Fibers continue to the thalamus and then to the somatosensory cortex.



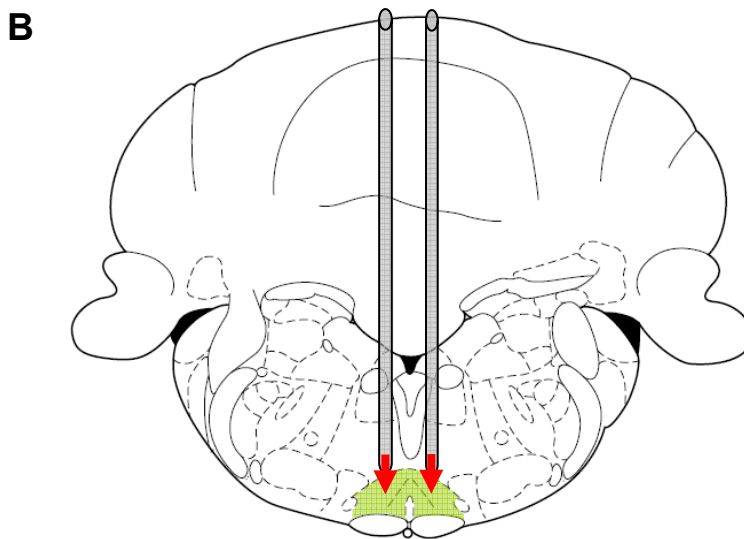
**Figure 2:** Descending pain pathways. Depicted are some of the major structures of the descending pain modulation system. Neurons from the periaqueductal gray (PAG) project to the rostroventromedial medulla (RVM) in the brainstem. Neurons from the RVM project to the dorsal spinal cord via the dorsolateral funiculus (DLF).



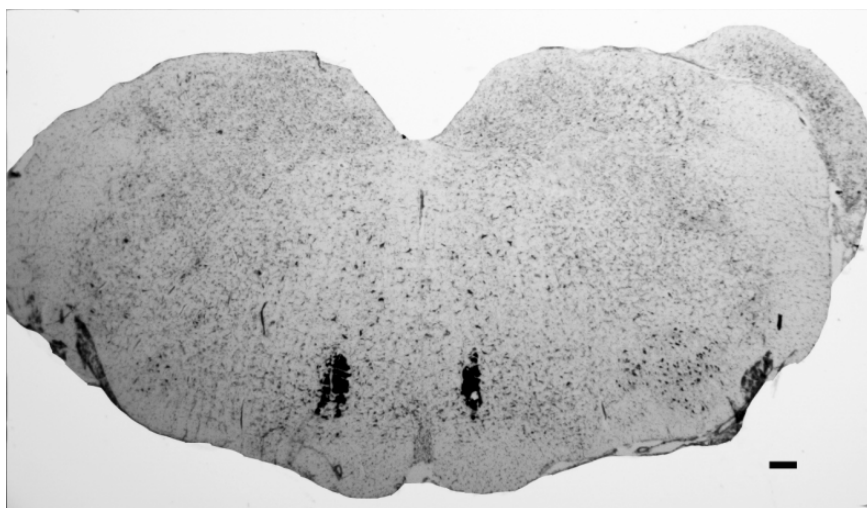
**Figure 3:** Opposing modulatory actions by two populations of neurons. Recording from neurons in the RVM during the application of noxious stimuli reveals one class that pauses (off cells) and one that bursts (on cells) just prior to withdrawal. A = amygdala; H = hypothalamus; PAG = periaqueductal gray; R = recording electrode; SC = spinal cord. Image from Fields 2004.



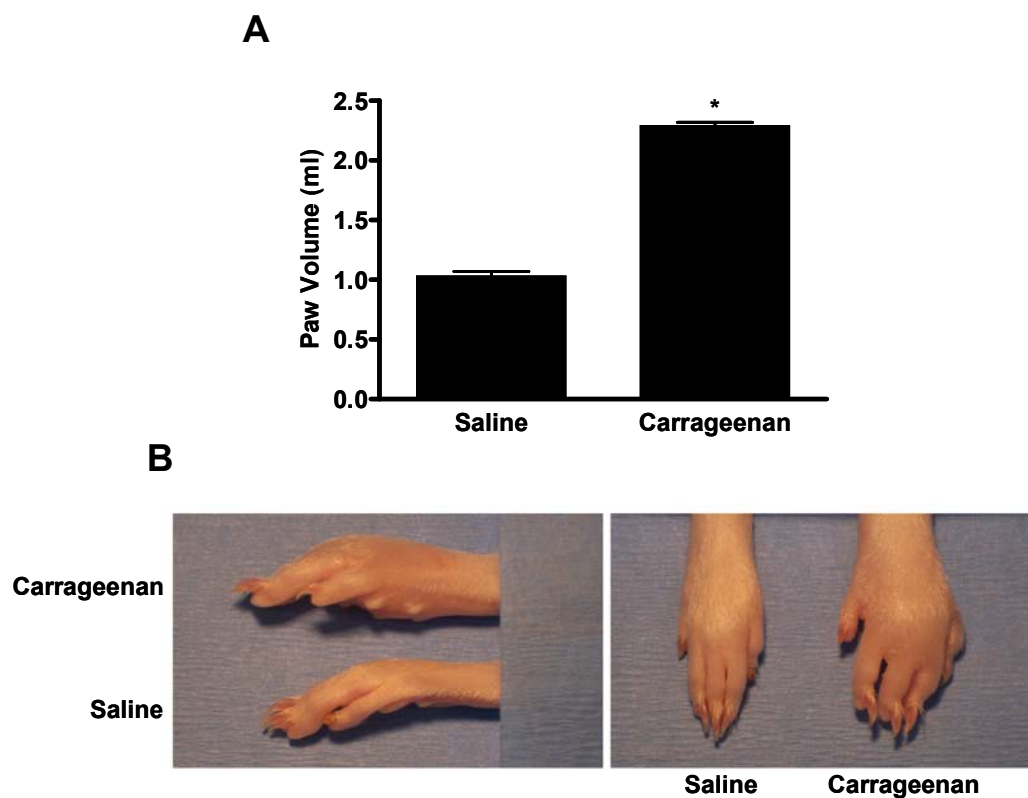
Adapted from: © Stereotaxic Surgery in the Rat:  
A Photographic Series. [www3.sympatico.ca/ajkirbyco/](http://www3.sympatico.ca/ajkirbyco/)



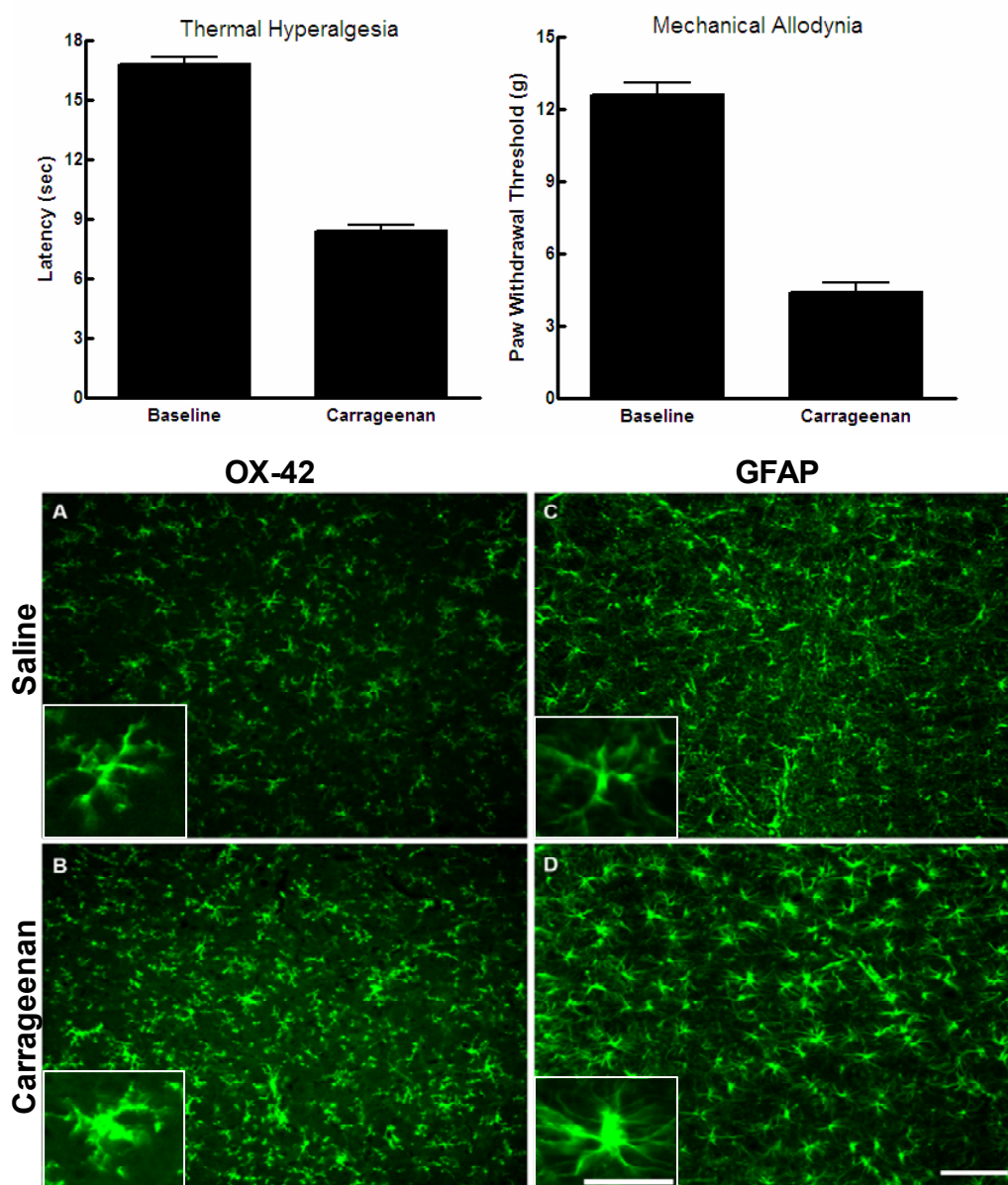
**Figure 4:** Location of paired guide cannulae directed toward the RVM. A) Black circle indicates placement of cannula (10.8 mm caudal to bregma, 0.6mm to each side of the sagittal suture and 7.0 mm ventral to the dura mater surface). B) Diagram shows position of guide cannulae (gray bars) in reference to the RVM (green shaded area). Diagram from Paxinos and Watson (1992).



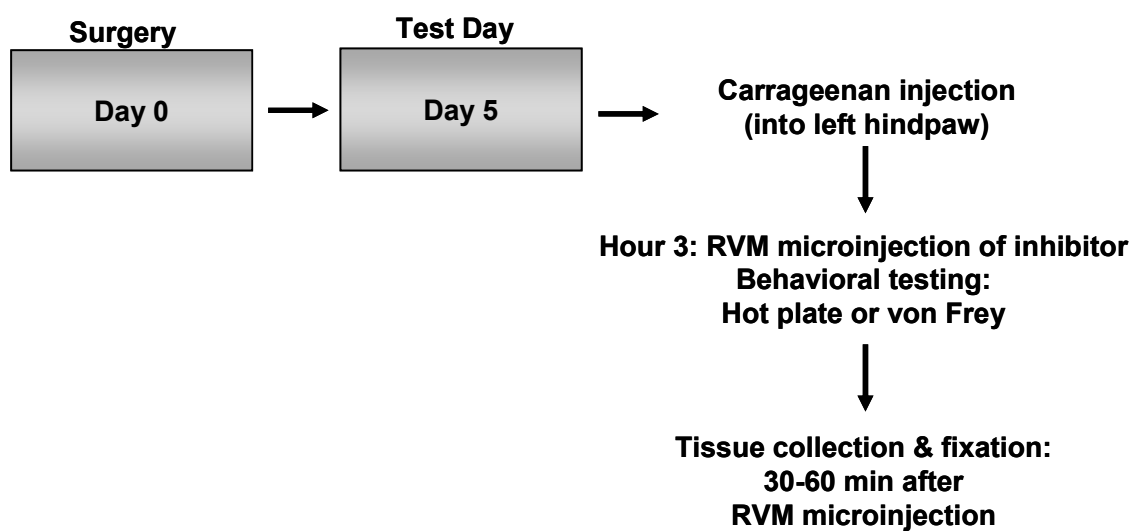
**Figure 5:** Photomicrograph of medullary section counterstained with Nissl stain. The microinjection sites in the RVM were visualized with the microinjection of India ink to verify proper cannula placement. Scale bar = 100  $\mu$ m.



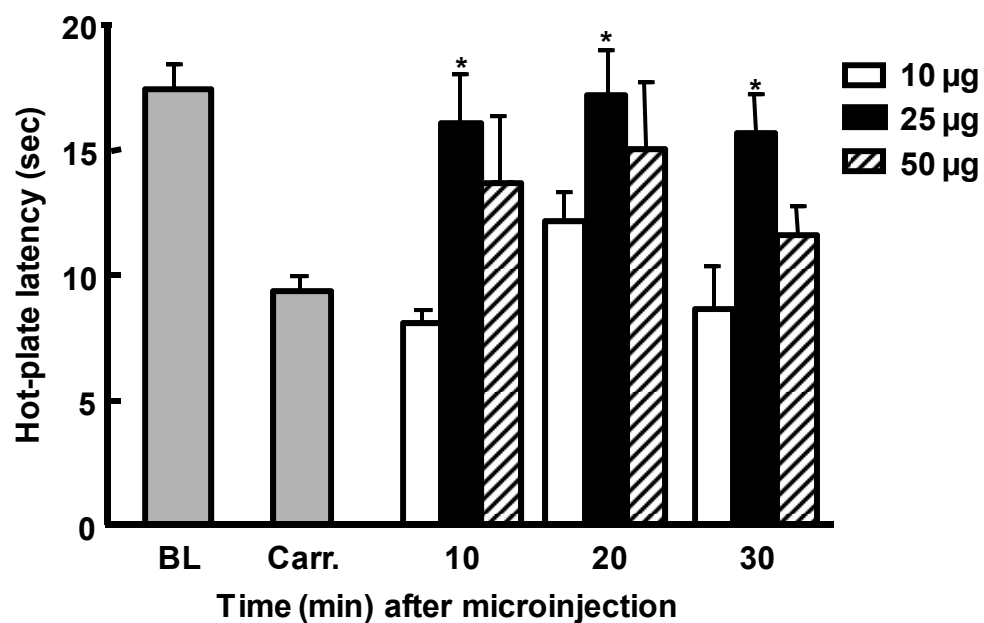
**Figure 6:** Carrageenan-induced paw edema. A) Paw volumes (ml) were measured three hours after saline or carrageenan intraplantar injection. \*  $p \leq 0.05$  compared to saline. B) Images of rat hindpaws injected with saline or carrageenan. A pronounced swelling of the rat paw in the animals receiving carrageenan was observed compared to the saline group.



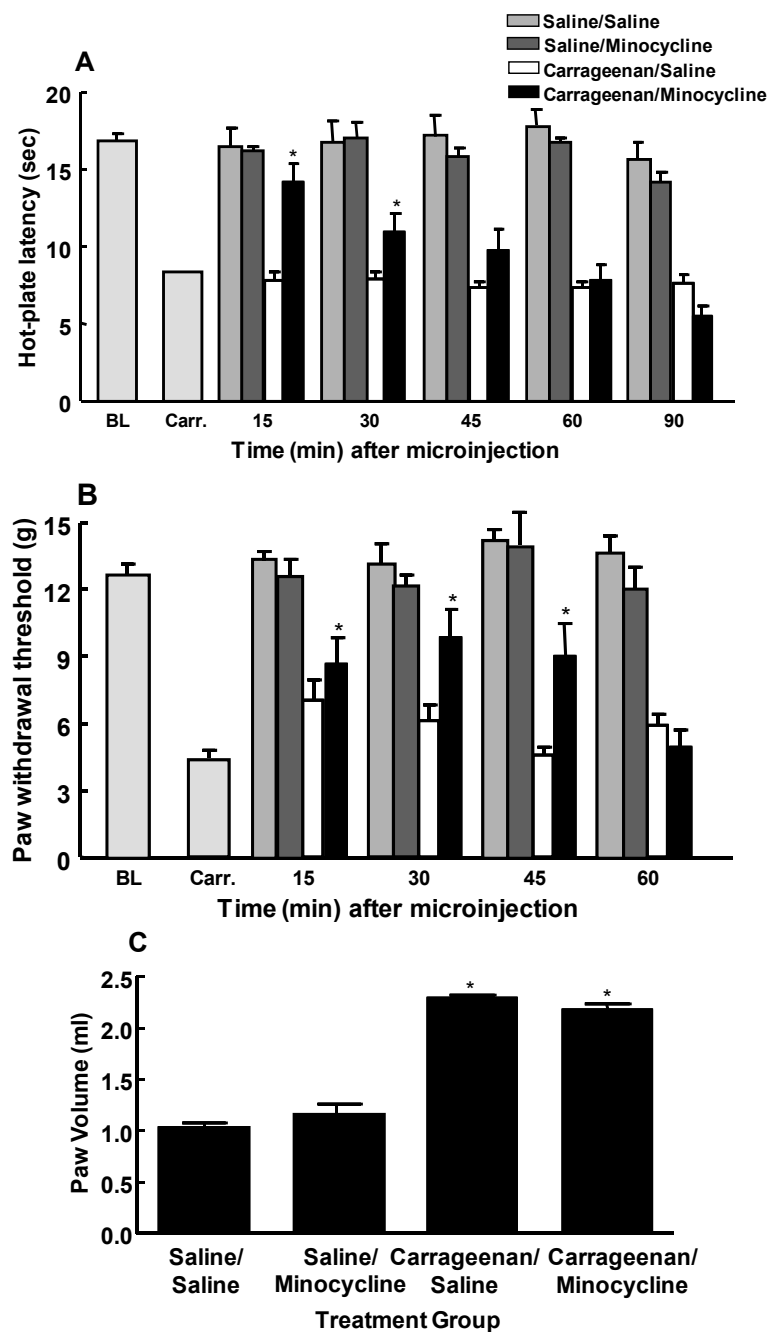
**Figure 7:** Carrageenan in the hindpaw induces thermal hyperalgesia and tactile allodynia three hours after injection, as indicated by decreased hot-plate latencies and paw withdrawal thresholds. Medullary sections were obtained from rats treated with saline (A,C) or with carrageenan (B,D) and immunolabeling for OX-42 (microglia, A,B), and GFAP (astrocytes, C,D) are shown. Rats with inflammation indicate an intensification in labeling for OX-42 (B) and GFAP (D) along with changes in morphology indicative of activation of glia. Scale bar = 100  $\mu$ m. Inset images are individual cells magnified to show morphology. Scale bar = 20  $\mu$ m.



**Figure 8:** Flow chart depicting the experimental design used for testing the effects of drugs microinjected into the RVM on carrageenan-induced hypersensitivity.

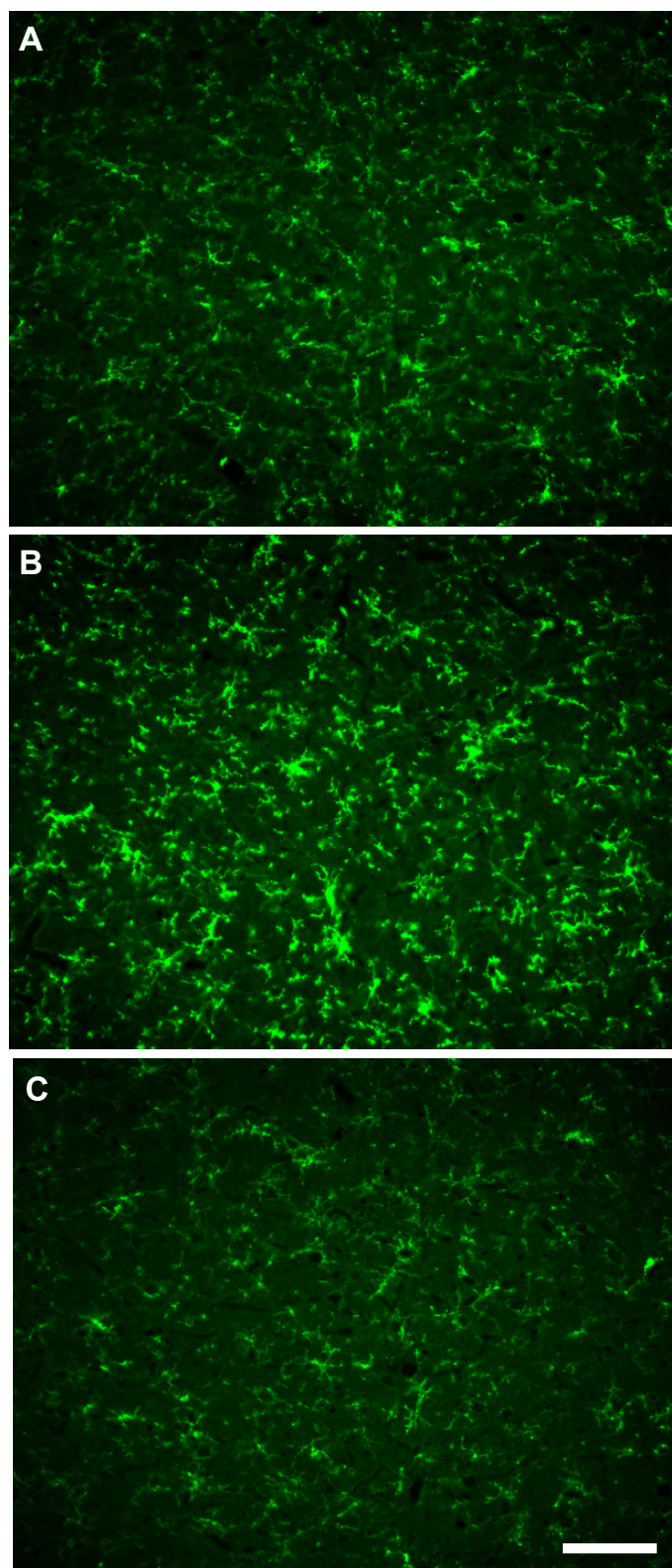


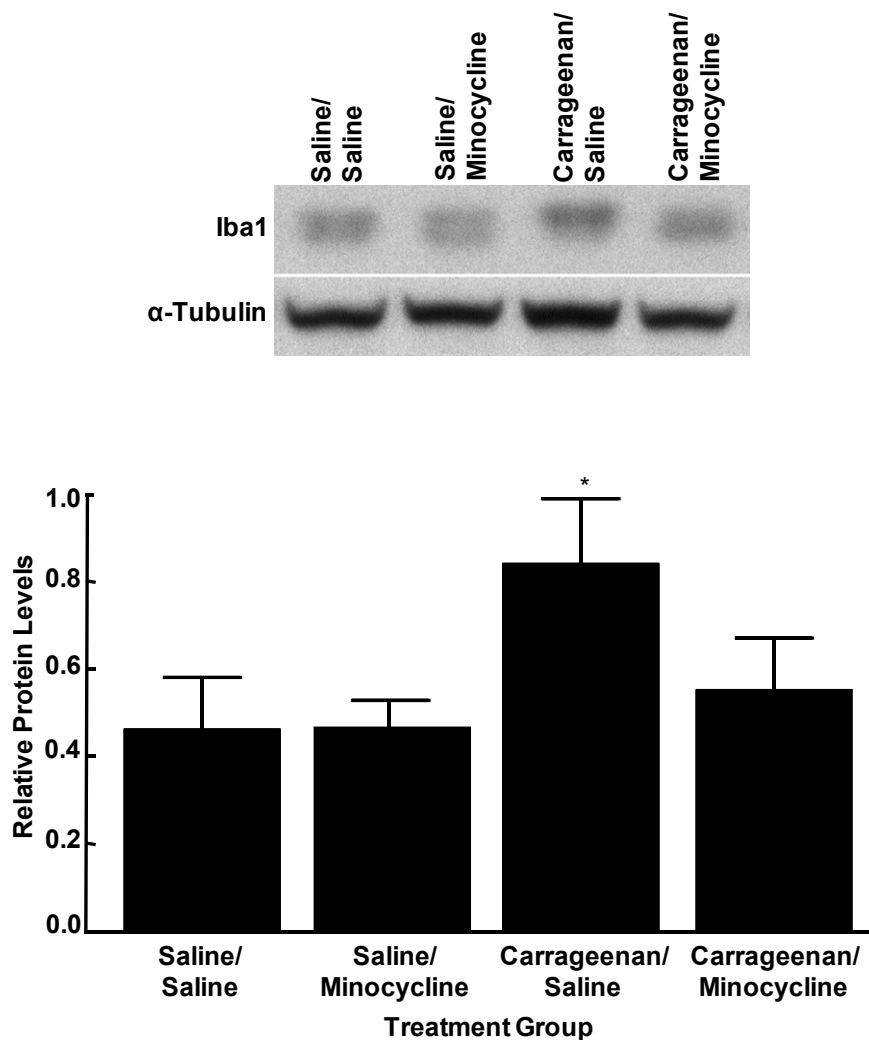
**Figure 9:** Minocycline dose-dependent attenuation of carrageenan-induced thermal hyperalgesia. Carrageenan in the hindpaw induces decreased latency to 52°C hot-plate three hours after injection and minocycline microinjected into the RVM at 10, 25 and 50 µg reversed the inflammation-induced hypersensitivity in a dose- and time-dependent manner. BL indicates mean baseline responses, Carr. indicates the mean post-carrageenan responses. \* indicates significant ( $p \leq 0.05$ ) increases in responses relative to the post-carrageenan values.



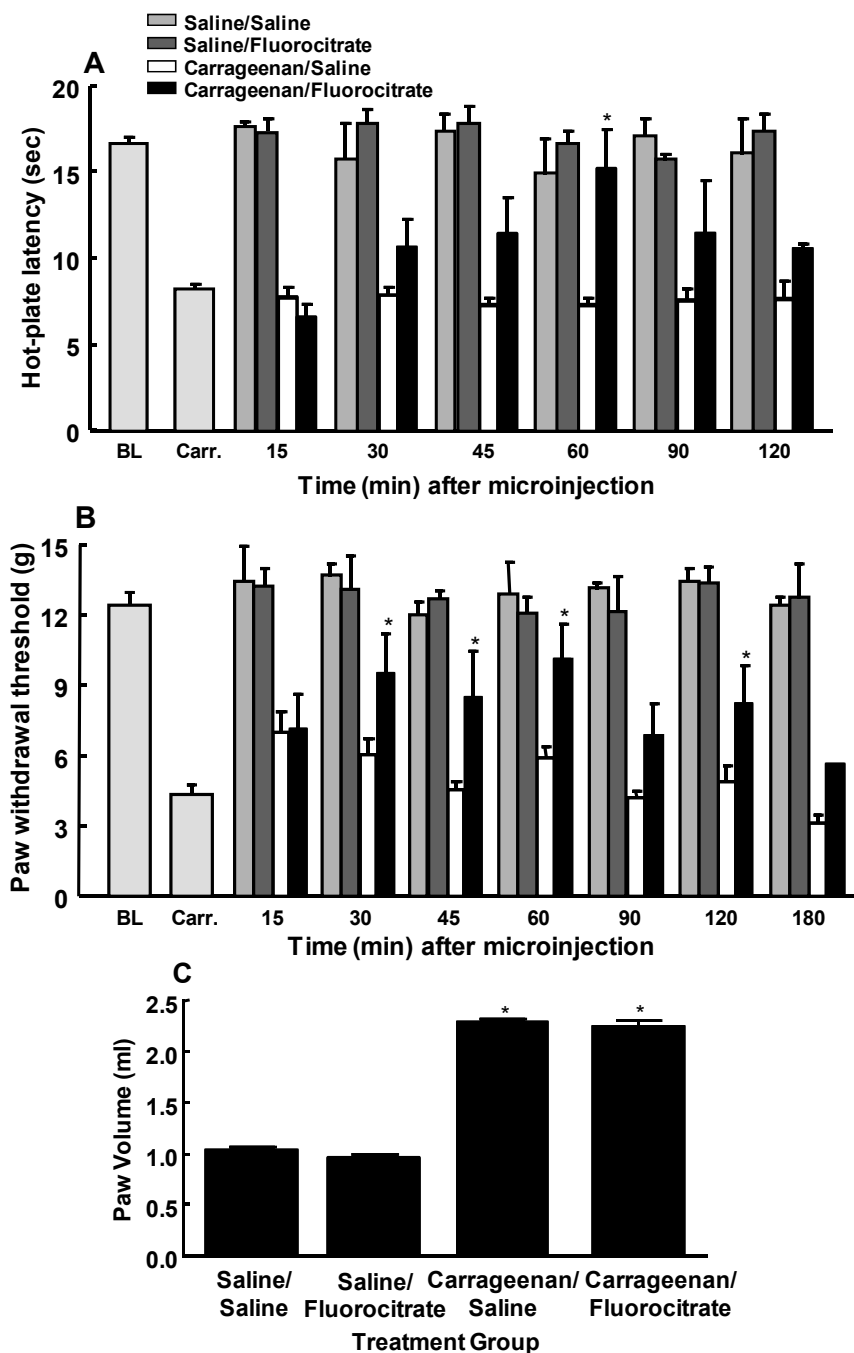
**Figure 10:** Minocycline (25  $\mu$ g) microinjection produced a time-dependent reversal of carrageenan-induced thermal hyperalgesia (A) and tactile allodynia (B). Paw volume (ml) did not change with the administration of minocycline into the RVM compared to post-carrageenan values (C). BL indicates mean baseline responses, Carr. indicates the mean post-carrageenan responses. \* indicates significant ( $p \leq 0.05$ ) increases in responses relative to the post-carrageenan values (of those that received carrageenan).

**Figure 11:** Medullary sections were obtained from rats treated with hindpaw injections of saline (A) or carrageenan and receiving microinjections of vehicle (B) or 25  $\mu\text{g}$  of minocycline (C) in the RVM and were labeled with OX-42 for immunofluorescent visualization of microglia. Microinjection of the microglial inhibitor minocycline into the RVM three hours after hindpaw injection of carrageenan produced an apparent reduction in immunofluorescence for OX-42 along with morphologic changes suggesting reduced microglial activation. Scale bar = 100  $\mu\text{m}$ .



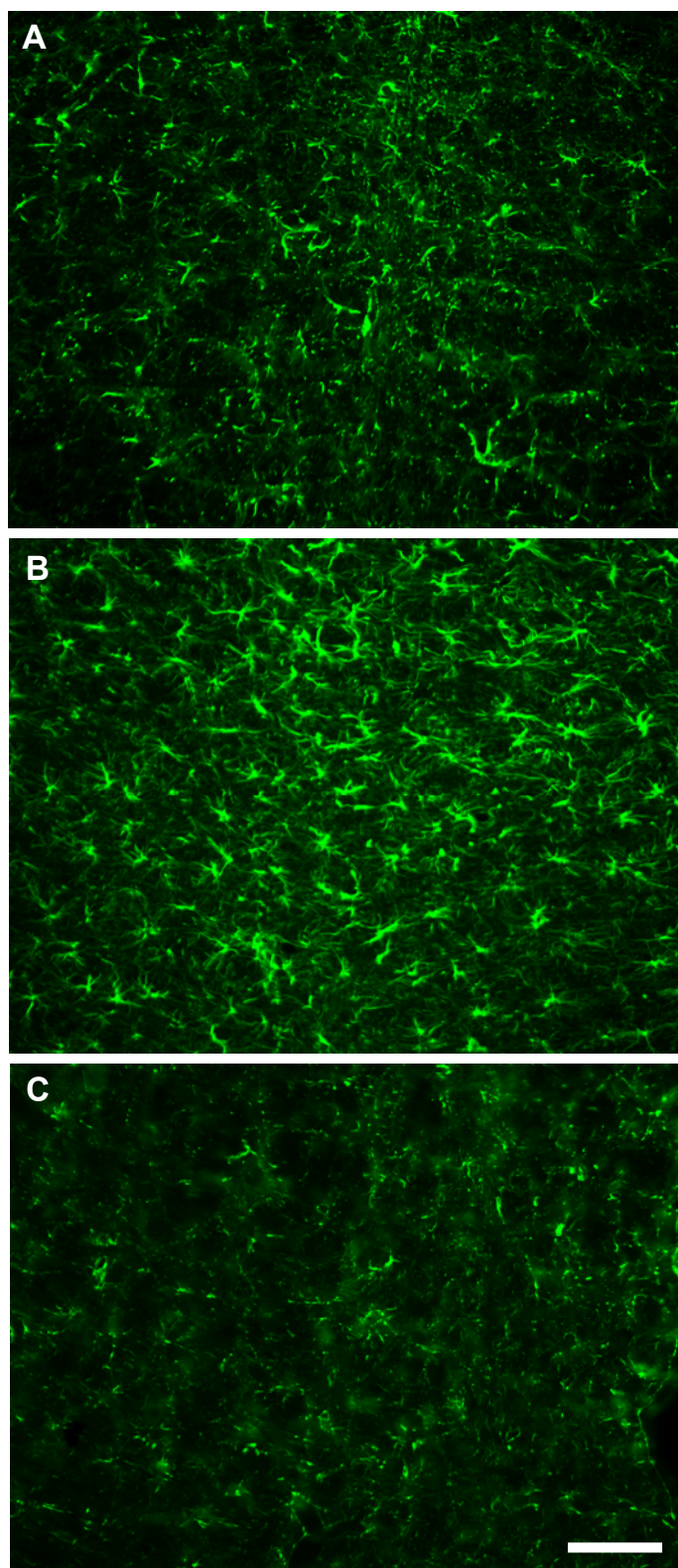


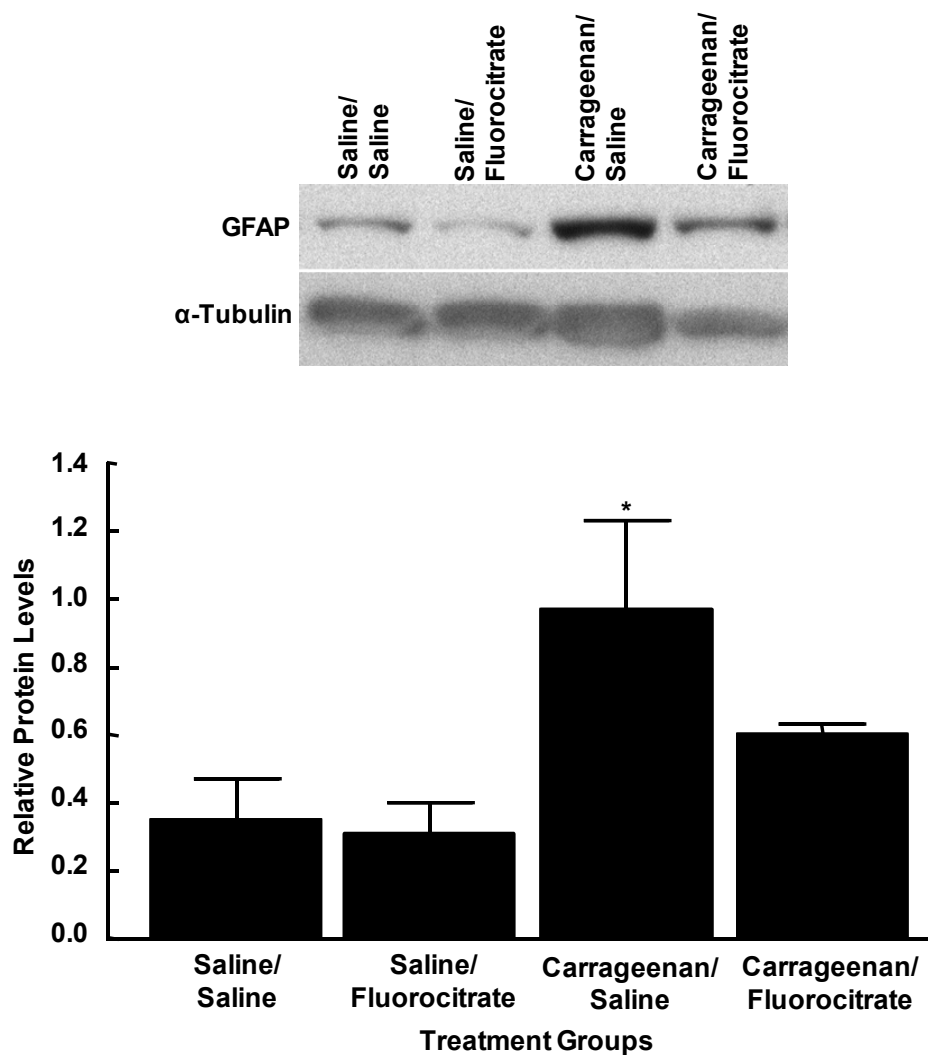
**Figure 12:** Inflammation-induced upregulation of Iba1 in the RVM. Top blot shows examples of the immunoreactive bands against anti-Iba1. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of Iba1 normalized to tubulin. Carrageenan administration increased the relative Iba1 levels (mean  $\pm$  SEM) and RVM microinjection of minocycline decreased Iba1 protein levels. \* indicates significant ( $p \leq 0.05$ ) increases in relative protein levels compared to the saline/saline treated group.



**Figure 13:** Fluorocitrate (1  $\mu$ g) microinjection produced a time-dependent reversal of carrageenan-induced thermal hyperalgesia (A) and tactile allodynia (B). Paw volume (ml) did not change with the administration of fluorocitrate into the RVM compared to post-carrageenan values (C). BL indicates mean baseline responses, Carr. indicates the mean post-carrageenan responses. \* indicates significant ( $p \leq 0.05$ ) increases in responses relative to the post-carrageenan values (of those that received carrageenan).

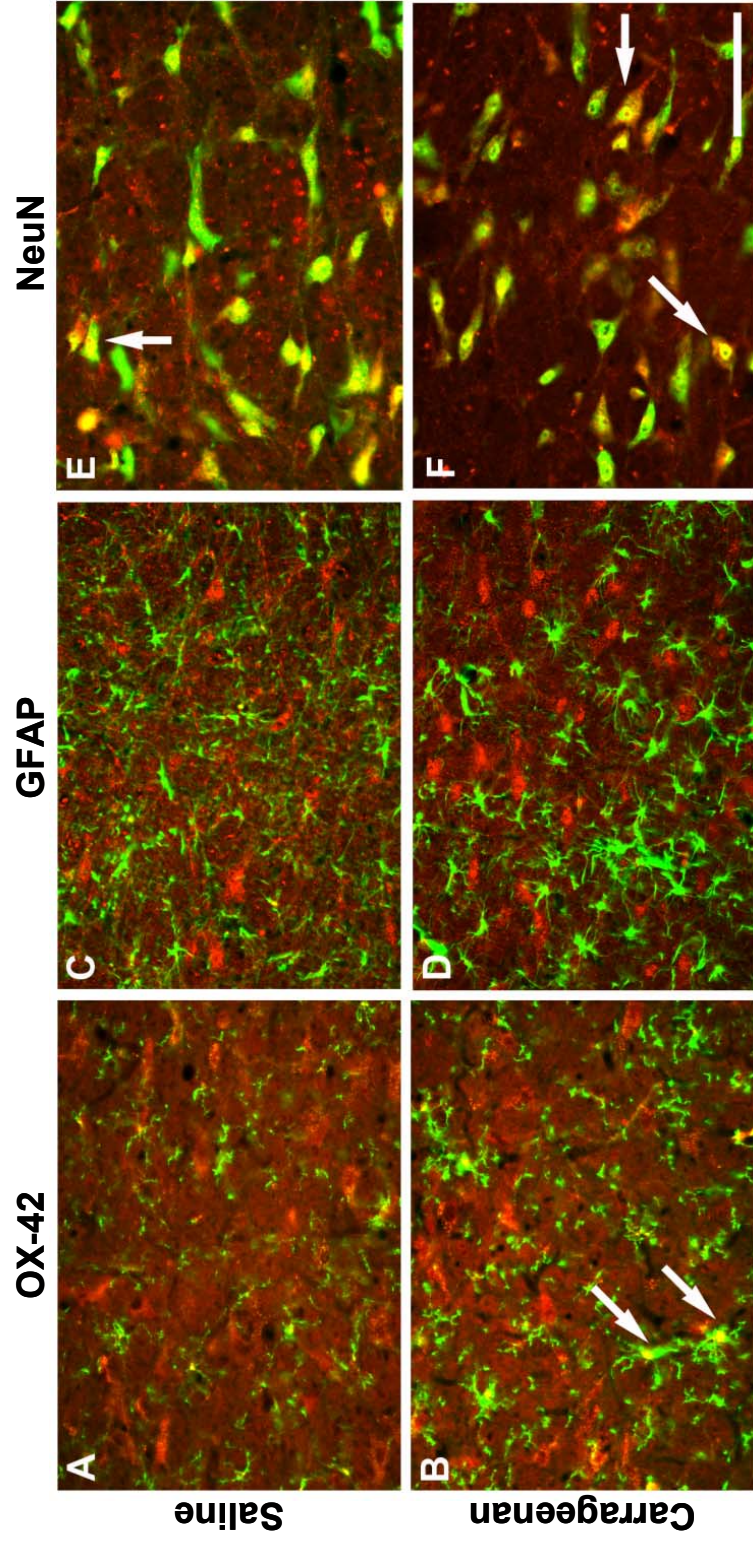
**Figure 14:** Medullary sections were obtained from rats treated with hindpaw injections of saline (A) or carrageenan and receiving microinjections of vehicle (B) or 1  $\mu\text{g}$  of fluorocitrate (C) in the RVM and were labeled with GFAP for immunofluorescent visualization of astrocytes. Microinjection of the glial inhibitor fluorocitrate into the RVM three hours after hindpaw injection of carrageenan produced an apparent reduction in immunofluorescence for GFAP along with morphologic changes suggesting reduced astrocyte activation. Scale bar = 100  $\mu\text{m}$ .





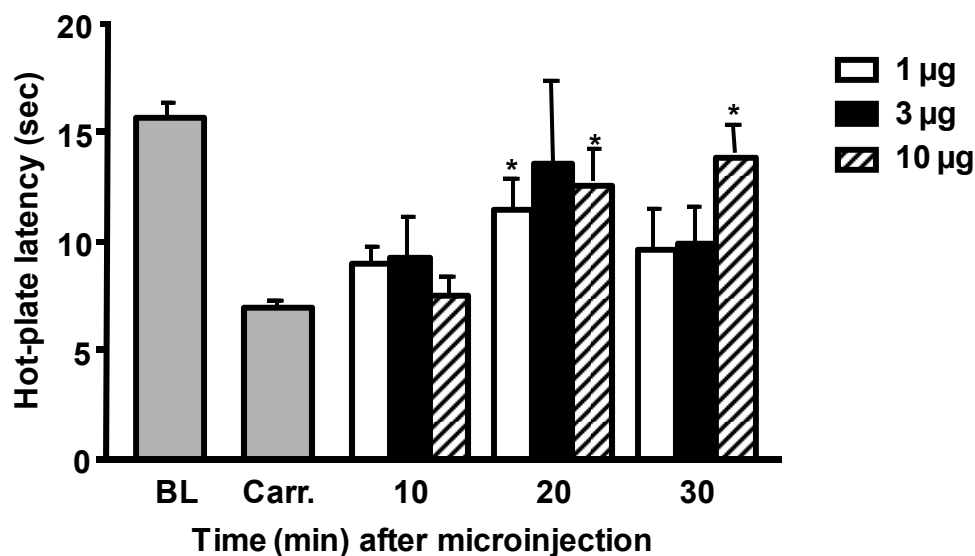
**Figure 15:** Inflammation-induced upregulation of GFAP in the RVM. Top blot shows examples of the immunoreactive bands against anti-GFAP. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of GFAP normalized to tubulin. Carrageenan administration increased the relative GFAP levels (mean  $\pm$  SEM) and RVM microinjection of fluorocitrate decreased GFAP protein levels. \* indicates significant ( $p \leq 0.05$ ) increases in relative protein levels compared to the saline/saline treated group.

**Figure 16:** Medullary sections were obtained from saline-treated control rats (A,C,E) and rats with carrageenan-induced inflammation (B,D,F) three hours after injection. Sections were immunolabeled for phosphorylated-p38 MAPK (red) and co-labeled (green) with OX-42 (microglia, A,B), GFAP (astrocytes, C,D) and NeuN (neurons, E,F). Label for phosphorylated-p38 MAPK is co-localized with microglia and neurons, but not astrocytes. Additionally, there is a visible increase in expression of microglia co-expressing p-p38 MAPK, whereas there is no observable change in neurons expressing this marker. Scale bar = 100  $\mu$ m.

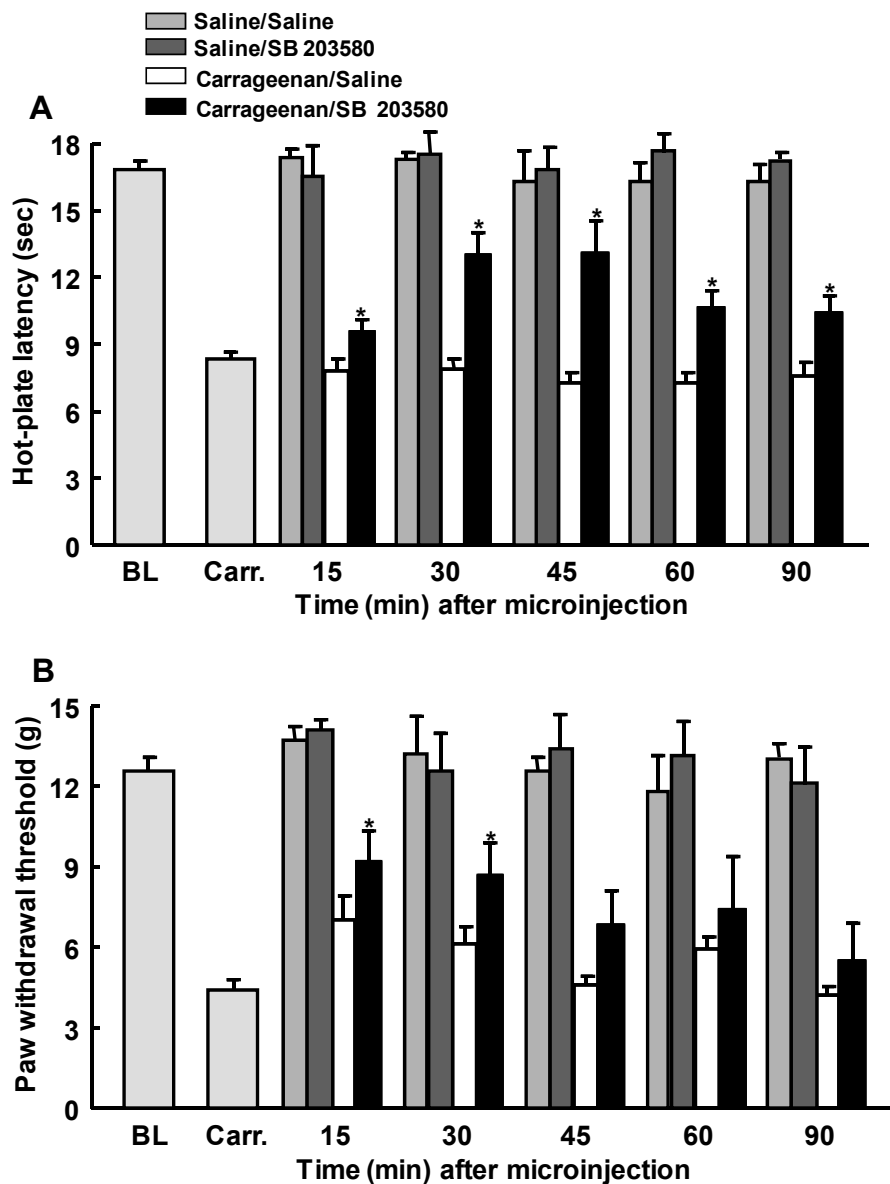


Saline

Carrageenan

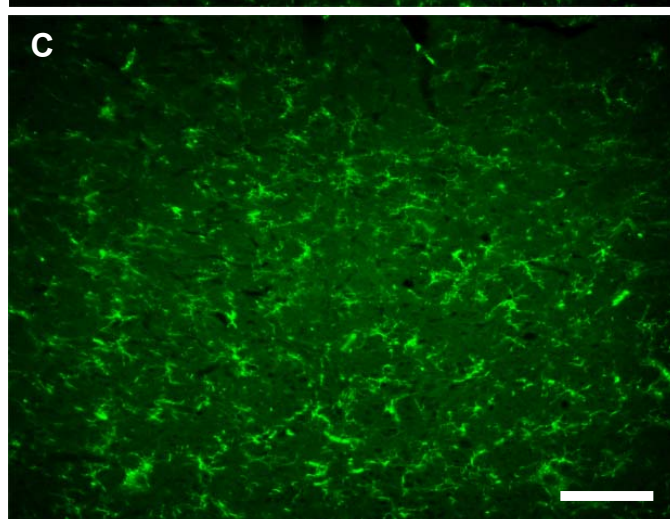
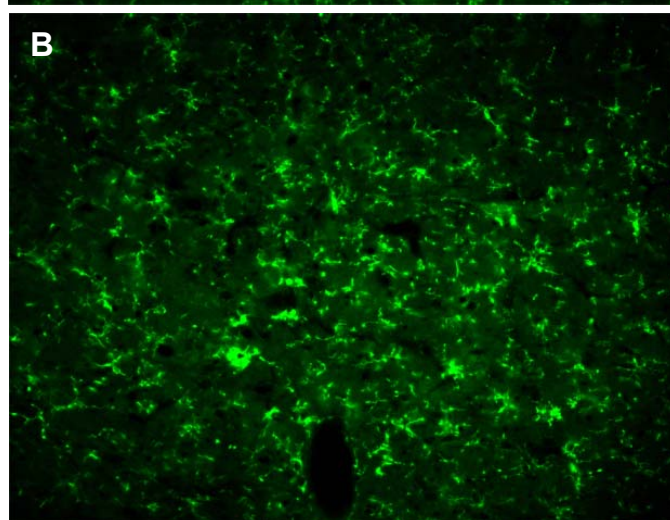
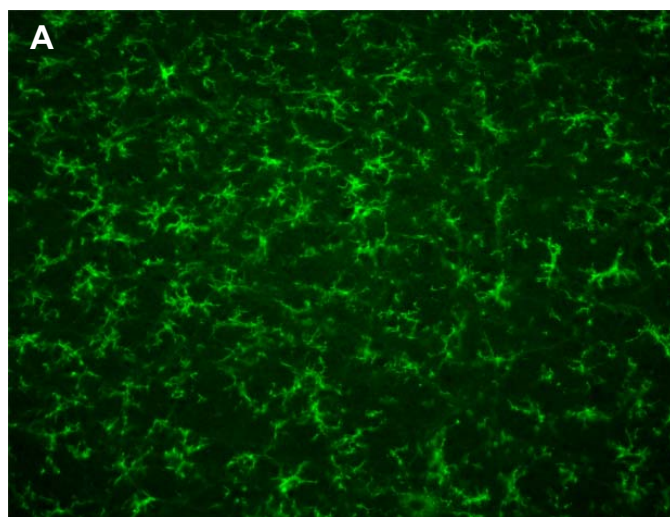


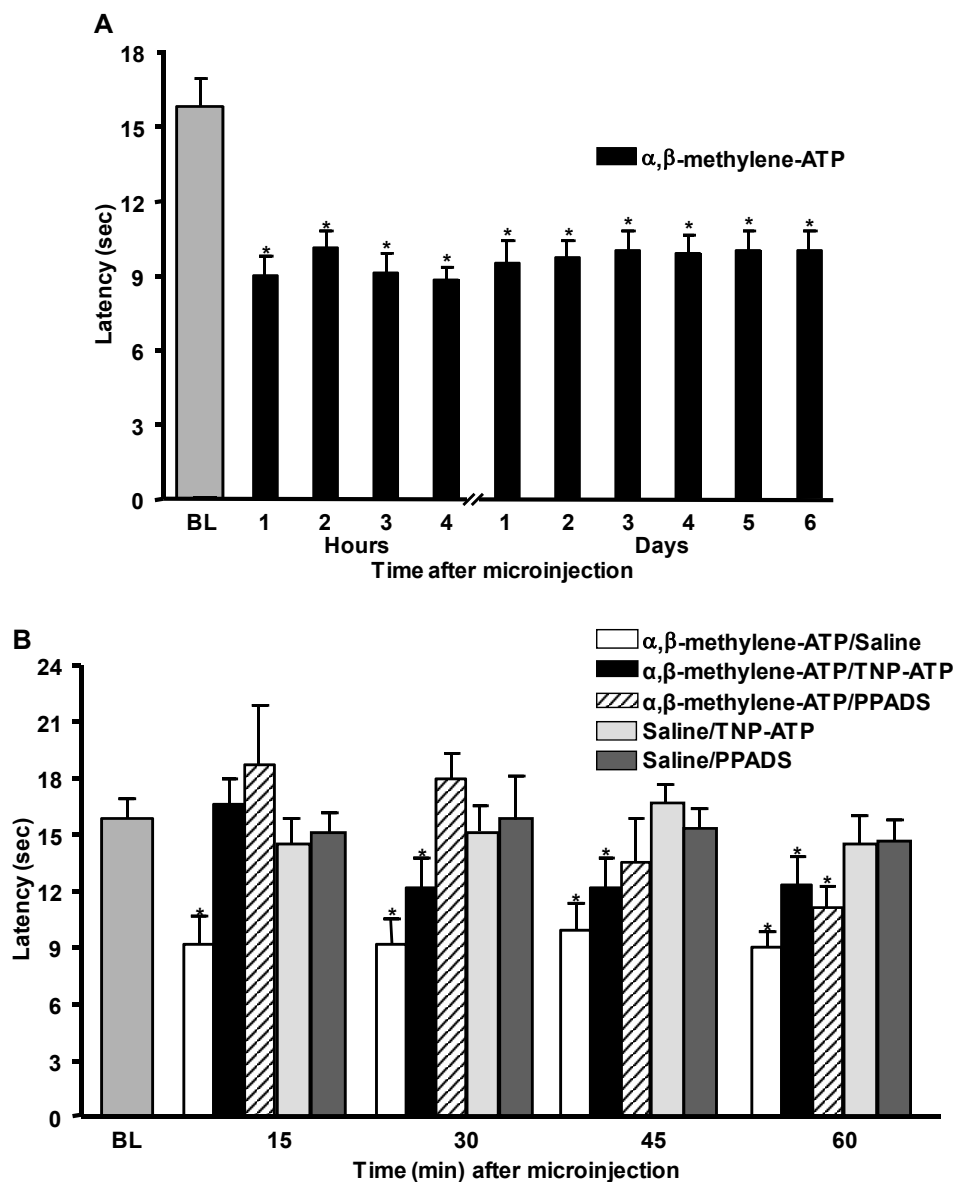
**Figure 17:** SB 203580, a p38 MAPK inhibitor, causes a dose-dependent attenuation of carrageenan-induced thermal hyperalgesia. Carrageenan in the hindpaw induces decreased latency to 52°C hot plate three hours after injection and SB 203580 microinjected into the RVM at 1, 3 and 10 µg reverses the inflammation-induced hypersensitivity in a dose- and time-dependent manner. BL indicates mean baseline responses, Carr. indicates the mean post-carrageenan responses. \* indicates significant ( $p \leq 0.05$ ) increases in responses relative to the post-carrageenan values.



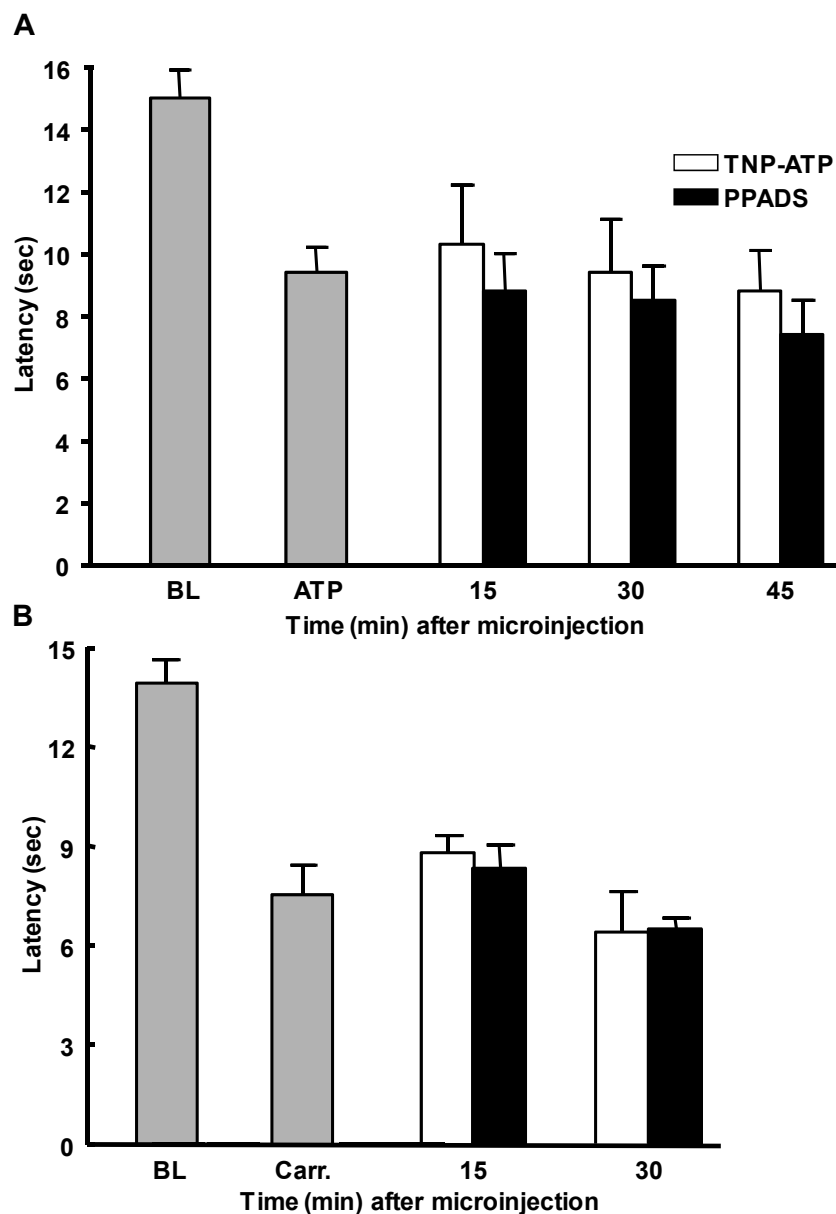
**Figure 18:** SB 203580 microinjection produced a time-dependent reversal of carrageenan-induced thermal hyperalgesia (A) and tactile allodynia (B). BL indicates mean baseline responses, Carr. indicates the mean post-carrageenan responses. \* indicates significant ( $p \leq 0.05$ ) increases in responses relative to the post-carrageenan values (of those that received carrageenan).

**Figure 19:** Medullary sections were obtained from rats treated with hindpaw injections of saline (A) or carrageenan and receiving microinjections of vehicle (B) or 10  $\mu\text{g}$  of SB 203580 (C) in the RVM and were labeled with OX-42 for immunofluorescent visualization of microglia. Microinjection of the p38 MAPK inhibitor SB 203580 into the RVM three hours after hindpaw injection of carrageenan produced an apparent reduction in immunofluorescence for OX-42 along with morphologic changes suggesting reduced microglia activation. Scale bar = 100  $\mu\text{m}$ .

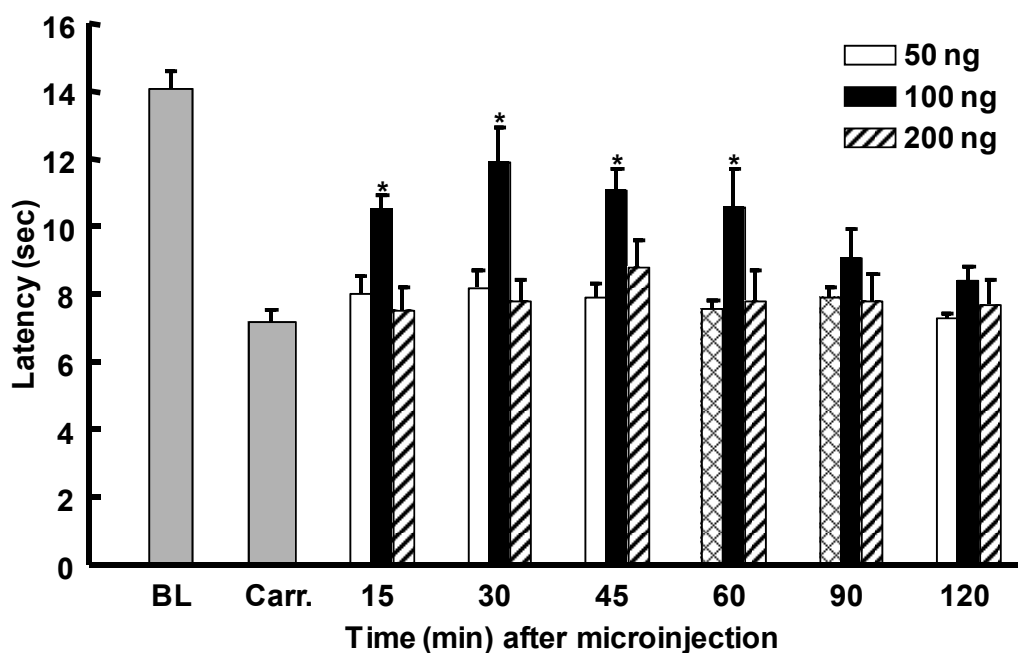




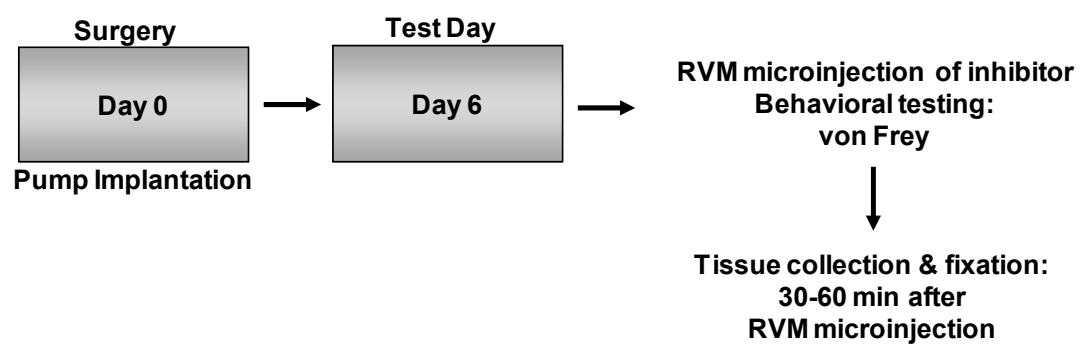
**Figure 20:** RVM microinjection of  $\alpha,\beta$ -methylene-ATP produced long-lasting thermal hyperalgesia and P2X antagonists delayed the onset of behavioral hypersensitivity. A)  $\alpha,\beta$ -methylene-ATP (1 nmol) microinjected into the RVM of naïve rats induced thermal hyperalgesia that lasted at least one week. B) RVM microinjection of TNP-ATP (P2X<sub>1-4</sub> antagonist; 1 nmol) 10 minutes prior to  $\alpha,\beta$ -methylene-ATP (1 nmol) prevented thermal hyperalgesia at the 15 minute time point. RVM microinjection of PPADS (P2X<sub>1-3,5,7</sub> antagonist; 1 nmol) 10 minutes prior to  $\alpha,\beta$ -methylene-ATP (1 nmol) prevented thermal hyperalgesia at the 15, 30 and 45 minute time points. BL indicates mean baseline responses. \* indicates significant ( $p \leq 0.05$ ) decreases in responses relative to the baseline values.



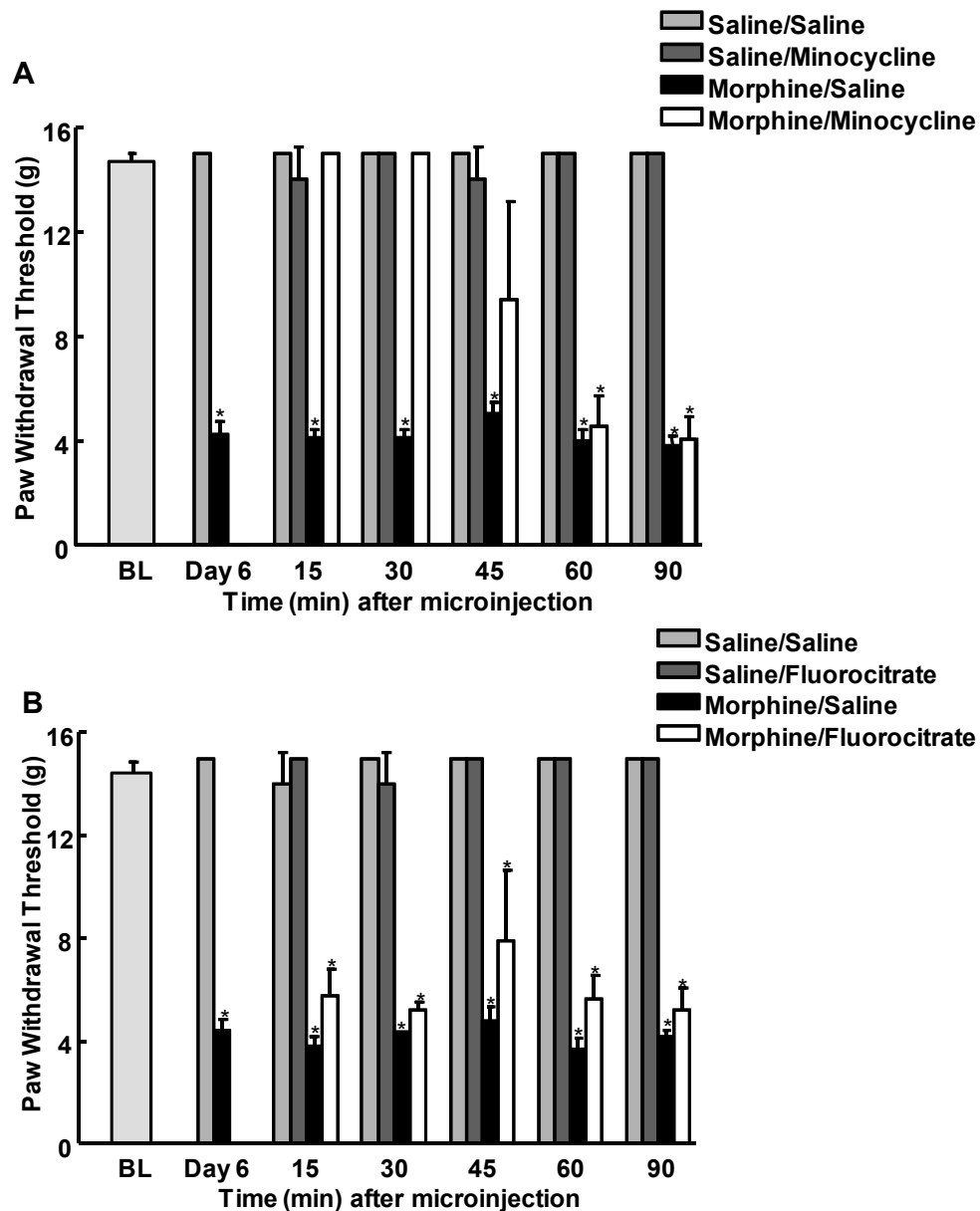
**Figure 21:** Post-treatment with P2X antagonists does not attenuate ATP- or carrageenan-induced hypersensitivity. A) RVM microinjection of TNP-ATP (P2X1-4 antagonist; 1 nmol) or PPADS (P2X1-3,5,7 antagonist; 1 nmol) four days after RVM administration of  $\alpha,\beta$ -methylene-ATP (1 nmol) does not reverse thermal hyperalgesia. B) RVM microinjection of TNP-ATP (1 nmol) or PPADS (1 nmol) three hours after carrageenan-induced inflammation does not reverse thermal hyperalgesia. BL indicates mean baseline responses, Carr. indicates the mean post-carrageenan responses.



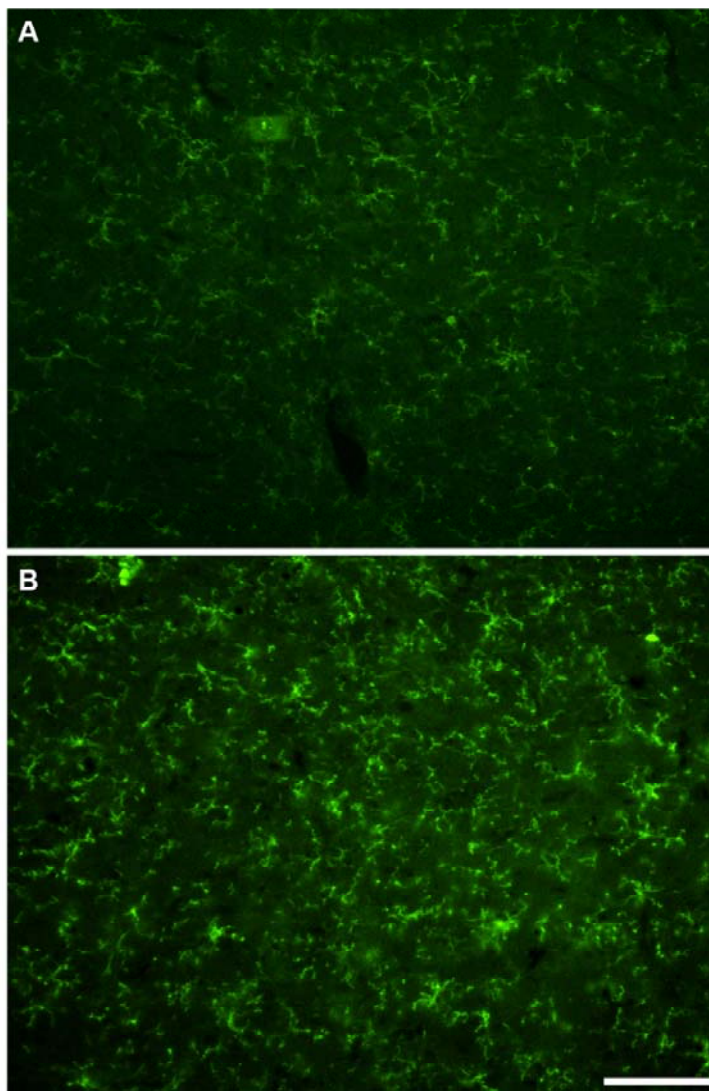
**Figure 22:** BDNF antiserum dose-dependent attenuation of carrageenan-induced thermal hyperalgesia. Carrageenan in the hindpaw induces decreased latency to 52°C hot-plate three hours after injection and anti-BDNF antibody microinjected into the RVM at 50, 100 and 200 ng reverse the inflammation-induced hypersensitivity in a dose- and time-dependent manner. BL indicates mean baseline responses, Carr. indicates the mean post-carrageenan responses. \* indicates significant ( $p \leq 0.05$ ) increases in responses relative to the post-carrageenan values.



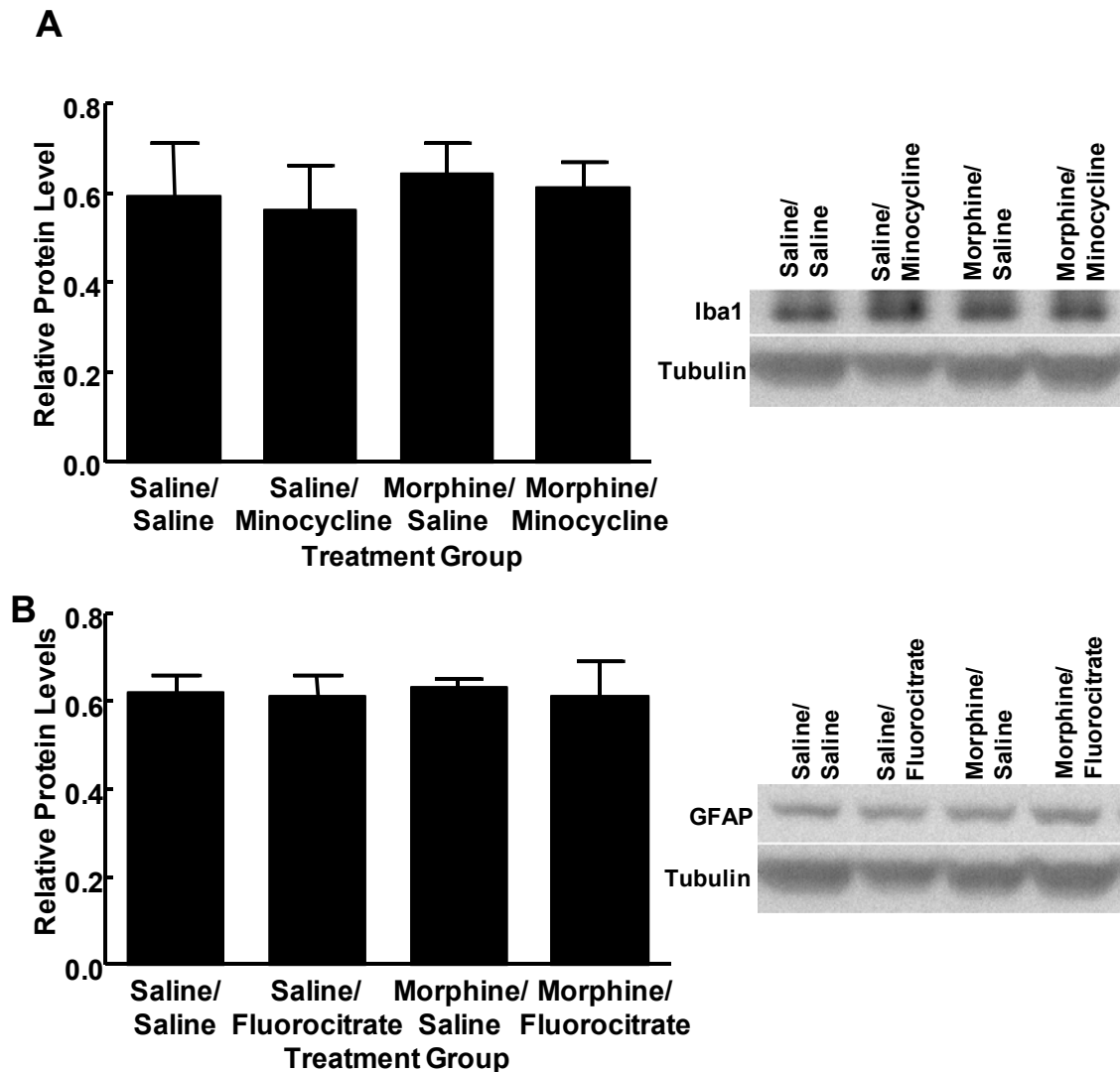
**Figure 23:** Flow chart depicting the experimental design used for testing the effects of drugs microinjected into the RVM on morphine-induced hypersensitivity.



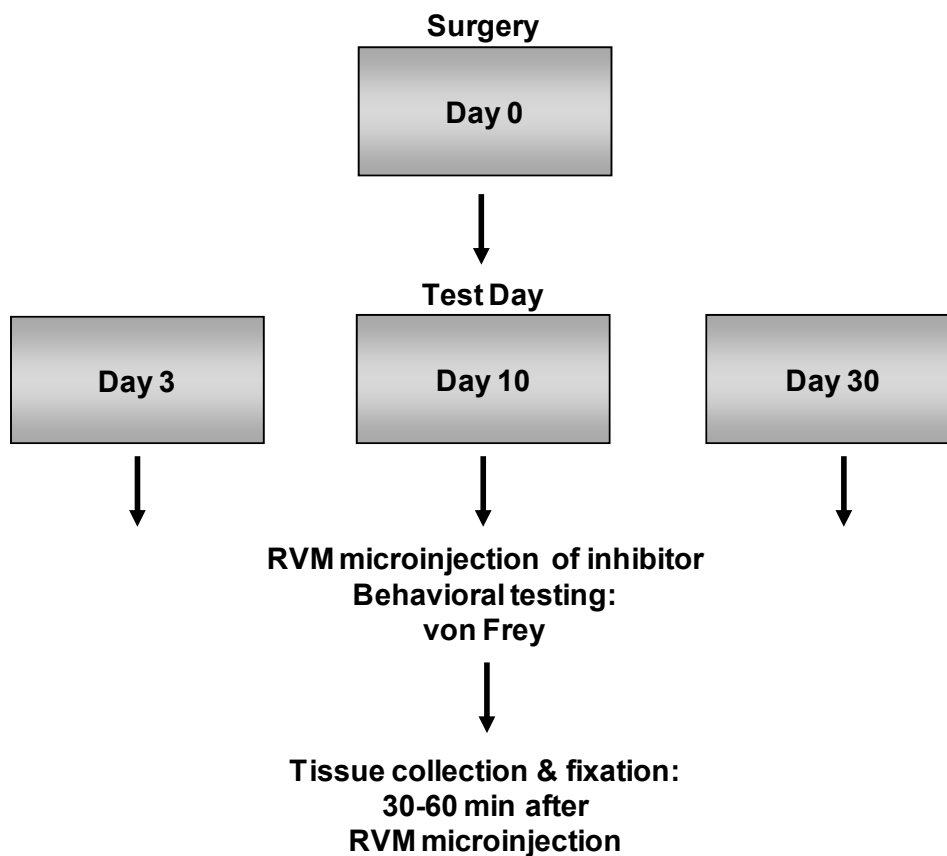
**Figure 24:** Microglial inhibitor in the RVM attenuates morphine-induced behavioral hypersensitivity. A) Day 6 after saline or morphine sustained systemic administration, minocycline (25  $\mu$ g) microinjected into the RVM attenuated tactile allodynia. B) Day 6 after saline or morphine sustained systemic administration, fluorocitrate (1  $\mu$ g) microinjected into the RVM did not attenuate the tactile allodynia. BL indicates mean baseline responses. \* indicates significant ( $p \leq 0.05$ ) decreases in responses relative to the baseline values.



**Figure 25:** Medullary sections were obtained from rats treated with systemic saline (A) or morphine (1.54 mg/day) (B) and were labeled with OX-42 for immunofluorescent visualization of microglia. Sustained morphine administered for six days induced apparent morphological changes, indicating microglia activation. Scale bar = 100  $\mu$ m.

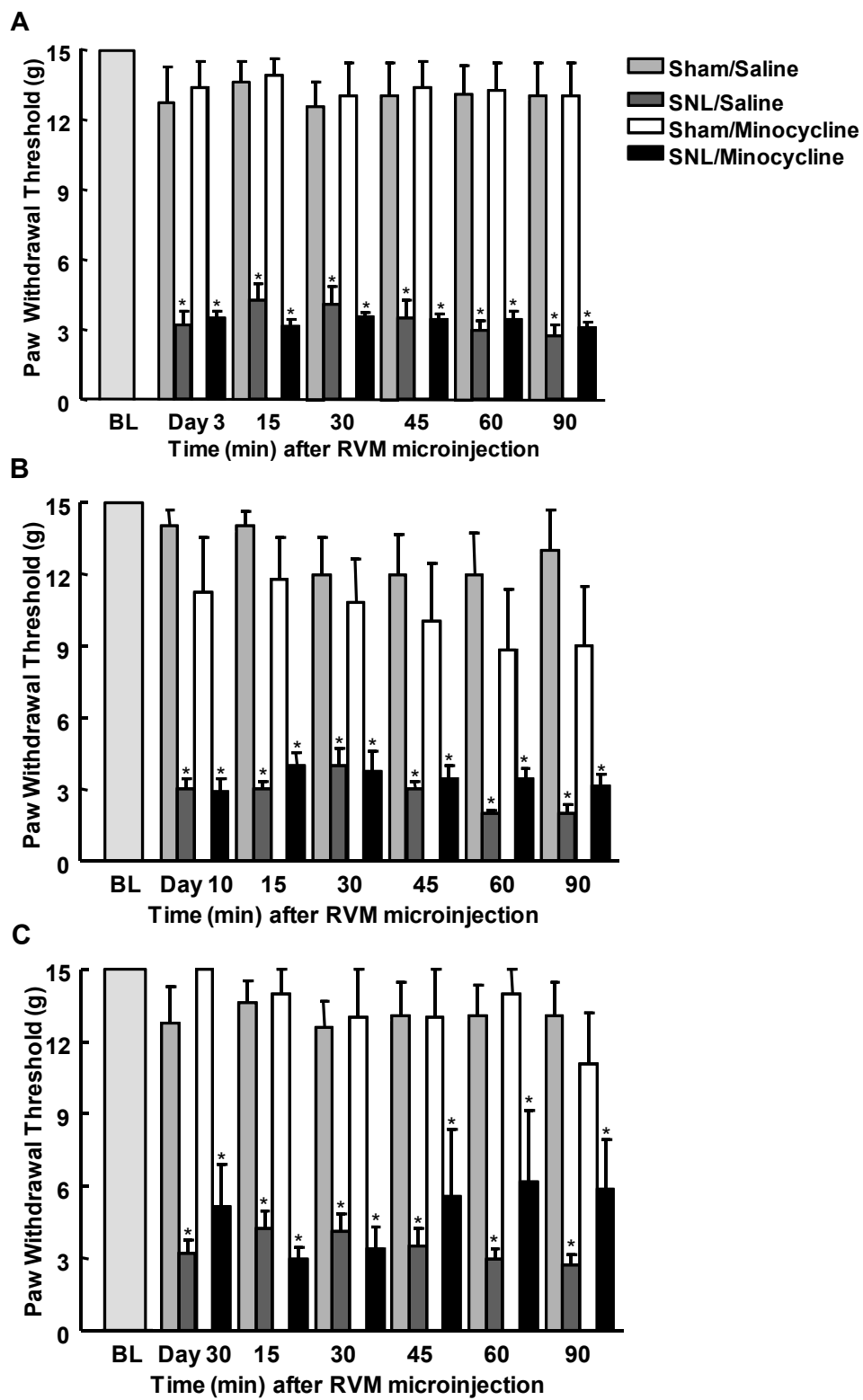


**Figure 26:** Protein levels in the RVM after six days of sustained systemic morphine or saline administration. (A) Minocycline treatment: top blot shows examples of the immunoreactive bands against anti-Iba1. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of Iba1 normalized to tubulin. (B) Fluorocitrate treatment: top blot shows examples of the immunoreactive bands against anti-GFAP. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of GFAP normalized to tubulin.

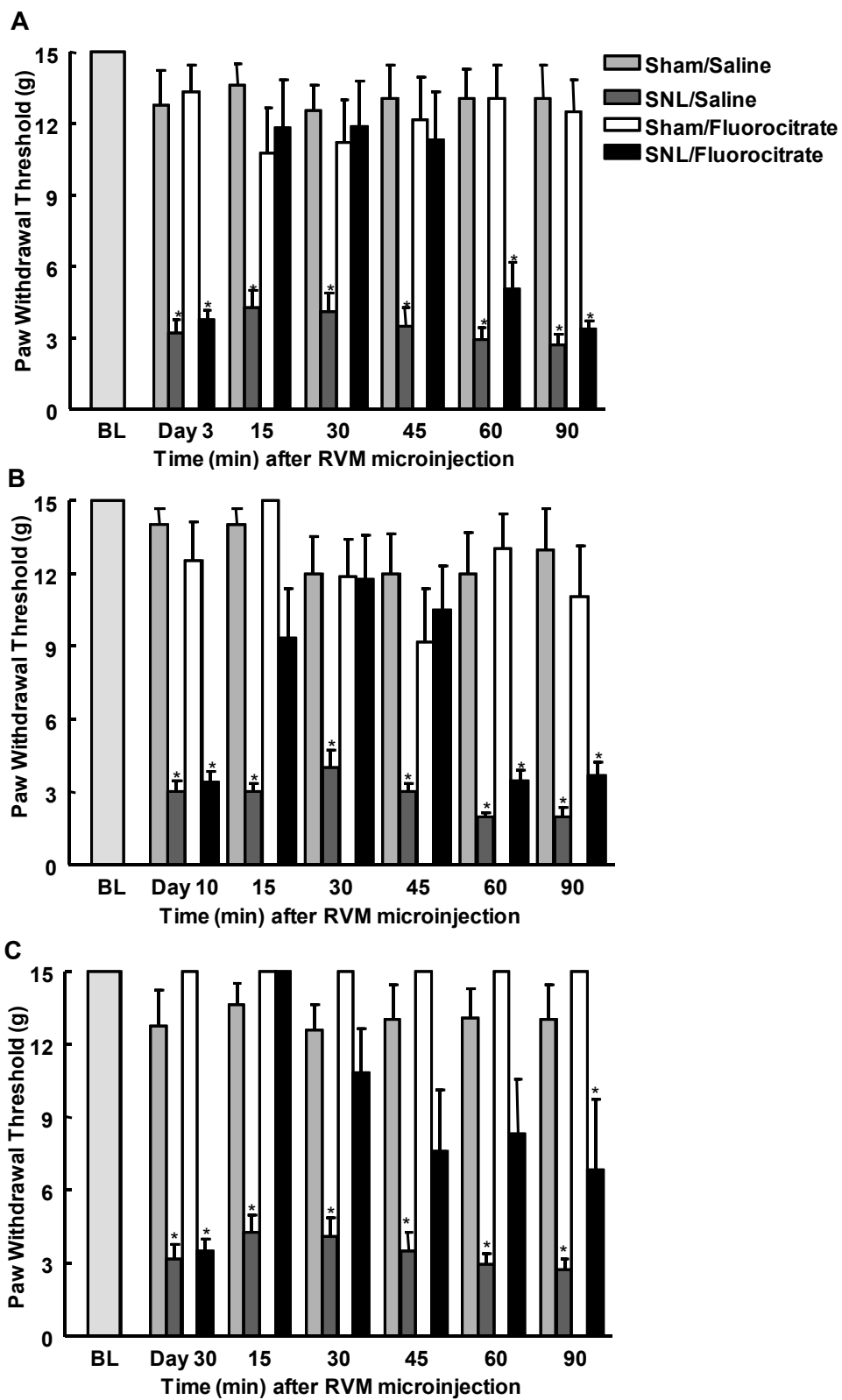


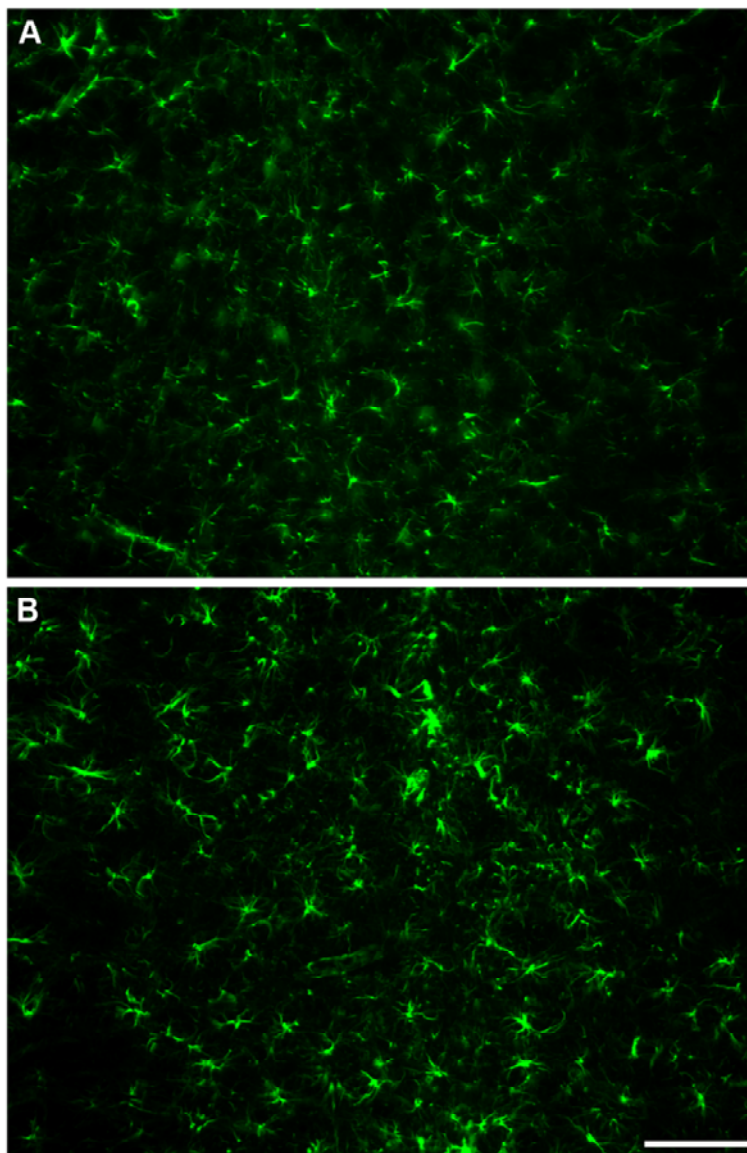
**Figure 27:** Flow chart depicting the experimental design used for testing the effects of drugs microinjected into the RVM on nerve injury-induced hypersensitivity.

**Figure 28:** Minocycline RVM microinjection did not produce an attenuation of nerve injury-induced tactile allodynia on (A) Day 3, (B) Day 10 or (C) Day 30 after injury. BL indicates mean baseline responses. \* indicates significant ( $p \leq 0.05$ ) decreases in responses relative to the baseline values.

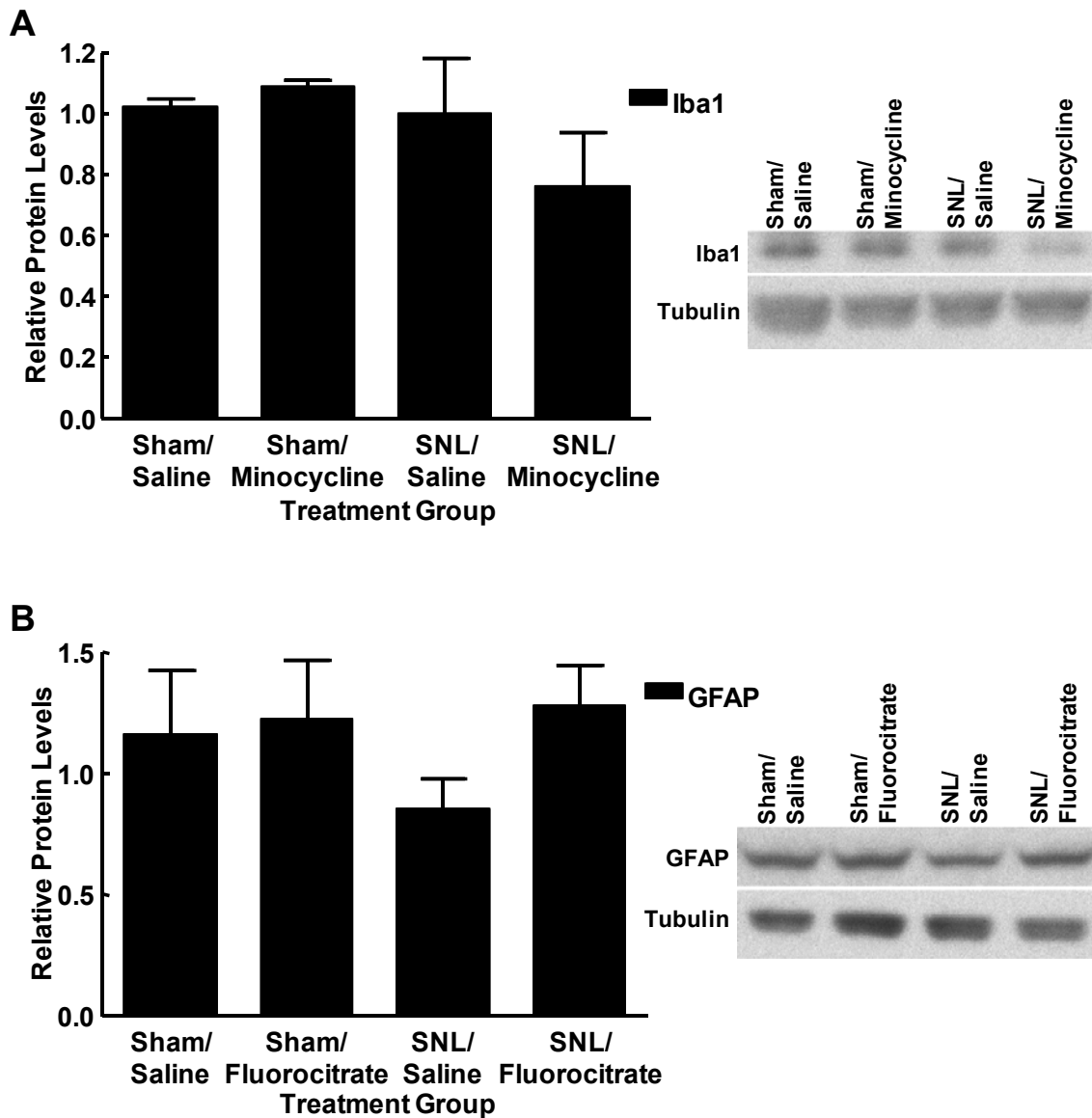


**Figure 29:** Fluorocitrate RVM microinjection produced a time-dependent reversal of nerve injury-induced tactile allodynia on (A) Day 3, (B) Day 10 and (C) Day 30 after injury. BL indicates mean baseline responses. \* indicates significant ( $p \leq 0.05$ ) decreases in responses relative to the baseline values.

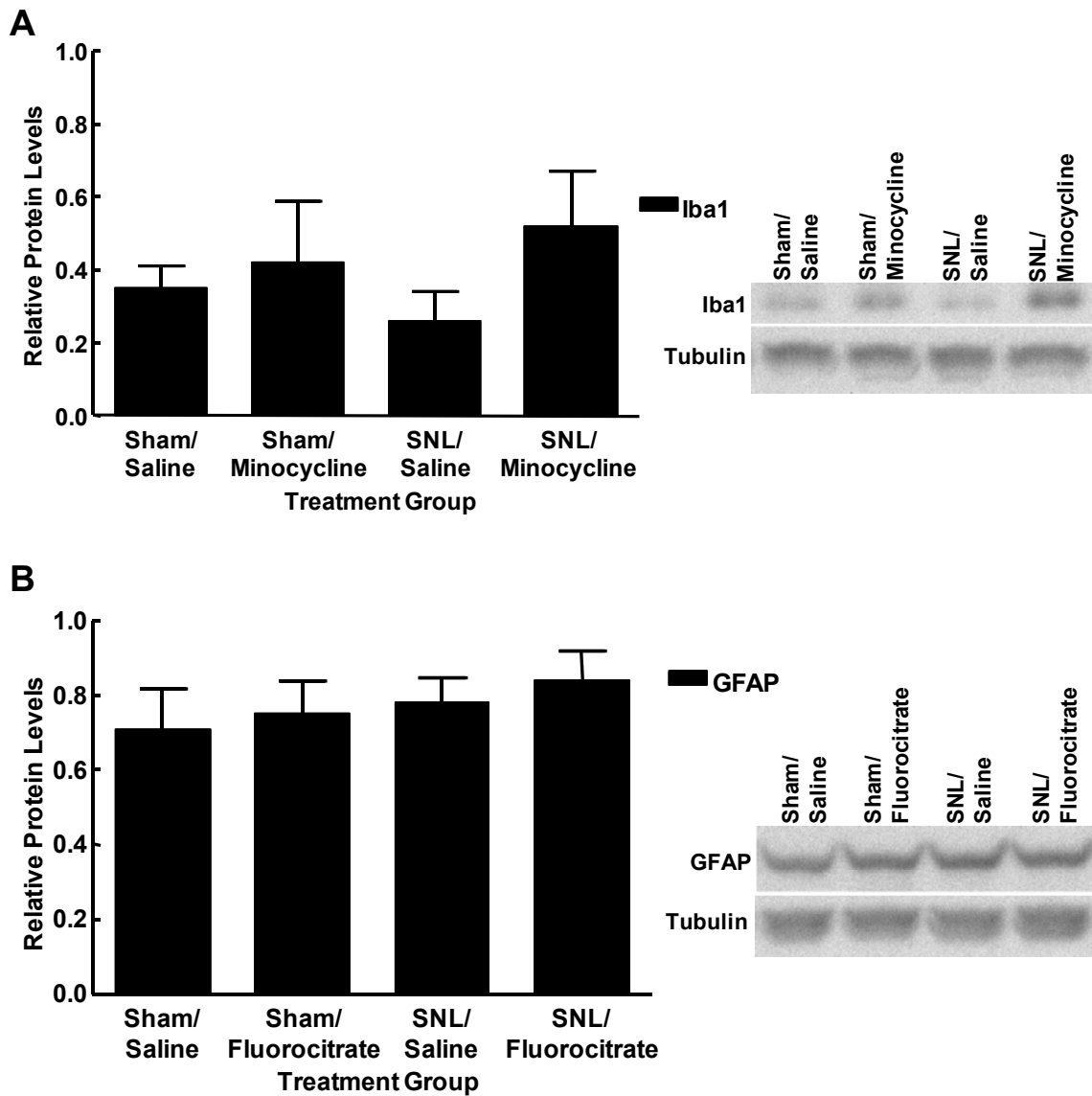




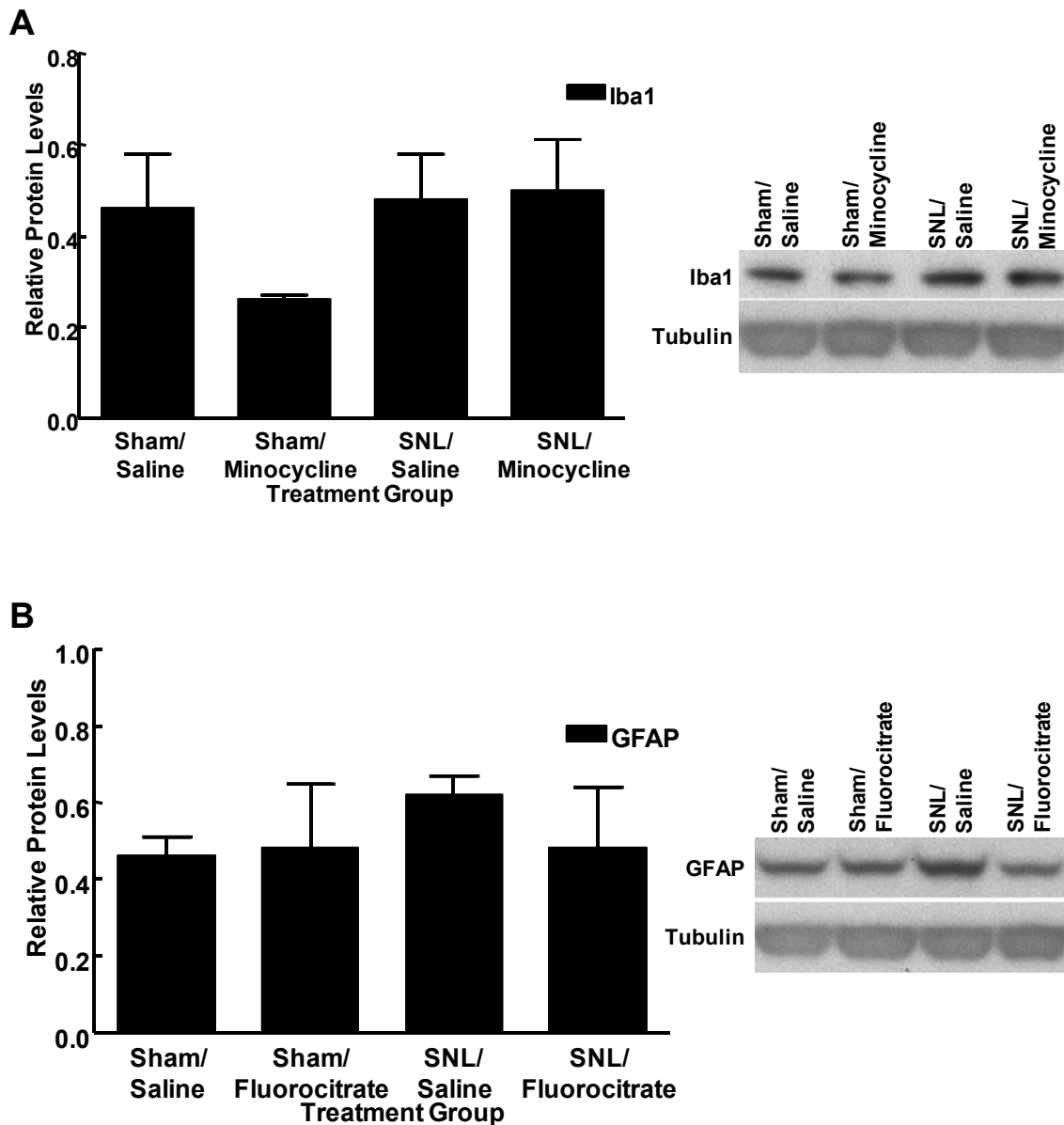
**Figure 30:** Nerve injury-induced astrocyte activation. Medullary sections were obtained from rats with sham (A) or SNL (B) injury and were labeled with GFAP for immunofluorescent visualization of astrocytes. On day 10 after SNL injury, there is an apparent morphological change in the astrocytes, indicating astrocyte activation. Scale bar = 100  $\mu\text{m}$ .



**Figure 31:** Protein levels in the RVM on day 3 after spinal nerve ligation or sham injury. (A) Top blot shows examples of the immunoreactive bands against anti-Iba1. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of Iba1 normalized to tubulin. (B) Top blot shows examples of the immunoreactive bands against anti-GFAP. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of GFAP normalized to tubulin.



**Figure 32:** Protein levels in the RVM on day 10 after spinal nerve ligation or sham injury. (A) Top blot shows examples of the immunoreactive bands against anti-Iba1. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of Iba1 normalized to tubulin. (B) Top blot shows examples of the immunoreactive bands against anti-GFAP. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of GFAP normalized to tubulin.



**Figure 33:** Protein levels in the RVM on day 30 after spinal nerve ligation or sham injury. (A) Top blot shows examples of the immunoreactive bands against anti-Iba1. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of Iba1 normalized to tubulin. (B) Top blot shows examples of the immunoreactive bands against anti-GFAP. The bottom blot shows immunoreactive bands against anti- $\alpha$ -tubulin after stripping and reprobing the same membrane. The bar graph shows the mean levels of GFAP normalized to tubulin.

## REFERENCES

- Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, Forrest MJ (2003) Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc Natl Acad Sci U S A* 100:7947-7952.
- Abbadie C, Trafletton J, Liu H, Mantyh PW, Basbaum AI (1997) Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation. *J Neurosci* 17:8049-8060.
- Bardoni R, Goldstein PA, Lee CJ, Gu JG, MacDermott AB (1997) ATP P2X receptors mediate fast synaptic transmission in the dorsal horn of the rat spinal cord. *J Neurosci* 17:5297-5304.
- Basbaum AI, Fields HL (1978) Endogenous pain control mechanisms: review and hypothesis. *Ann Neurol* 4:451-462.
- Beggs S, Trang T, Samad TA (2009) The Role of ATP and Microglia in Enhanced Pain States. In: *Immune and Glial Regulation of Pain* (DeLeo JA, Sorkin LS, Watkins LR, eds), pp 283-295. Seattle: IASP Press.
- Bennett G, al-Rashed S, Hoult JR, Brain SD (1998) Nerve growth factor induced hyperalgesia in the rat hind paw is dependent on circulating neutrophils. *Pain* 77:315-322.
- Bennett GJ, Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33:87-107.
- Benveniste EN, Benos DJ (1995) TNF-alpha- and IFN-gamma-mediated signal transduction pathways: effects on glial cell gene expression and function. *FASEB J* 9:1577-1584.
- Benveniste EN, Huneycutt BS, Shrikant P, Ballestas ME (1995a) Second messenger systems in the regulation of cytokines and adhesion molecules in the central nervous system. *Brain Behav Immun* 9:304-314.
- Benveniste EN, Tang LP, Law RM (1995b) Differential regulation of astrocyte TNF-alpha expression by the cytokines TGF-beta, IL-6 and IL-10. *Int J Dev Neurosci* 13:341-349.
- Brundula V, Rewcastle NB, Metz LM, Bernard CC, Yong VW (2002) Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. *Brain* 125:1297-1308.

Burgess SE, Gardell LR, Ossipov MH, Malan TP, Jr., Vanderah TW, Lai J, Porreca F (2002) Time-dependent descending facilitation from the rostral ventromedial medulla maintains, but does not initiate, neuropathic pain. *J Neurosci* 22:5129-5136.

Chacur M, Gutierrez JM, Milligan ED, Wieseler-Frank J, Britto LR, Maier SF, Watkins LR, Cury Y (2004) Snake venom components enhance pain upon subcutaneous injection: an initial examination of spinal cord mediators. *Pain* 111:65-76.

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

Chapman GA, Moores K, Harrison D, Campbell CA, Stewart BR, Strijbos PJ (2000) Fractalkine cleavage from neuronal membranes represents an acute event in the inflammatory response to excitotoxic brain damage. *J Neurosci* 20:RC87.

Chen A, Kumar SM, Sahley CL, Muller KJ (2000) Nitric oxide influences injury-induced microglial migration and accumulation in the leech CNS. *J Neurosci* 20:1036-1043.

Clark AR, Dean JL, Saklatvala J (2003) Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. *FEBS Lett* 546:37-44.

Colburn RW, DeLeo JA, Rickman AJ, Yeager MP, Kwon P, Hickey WF (1997) Dissociation of microglial activation and neuropathic pain behaviors following peripheral nerve injury in the rat. *J Neuroimmunol* 79:163-175.

Correale J, Villa A (2004) The neuroprotective role of inflammation in nervous system injuries. *J Neurol* 251:1304-1316.

Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De KY (2005) BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature* 438:1017-1021.

Dallel R, Villanueva L, Woda A, Voisin D (2003) [Neurobiology of trigeminal pain]. *Med Sci (Paris)* 19:567-574.

De CF, Caraceni A, Martini C, Spoldi E, Salvetti M, Ventafridda V (1991a) Hyperalgesia and myoclonus with intrathecal infusion of high-dose morphine. *Pain* 47:337-339.

De CF, Ripamonti C, Sbanotto A, Barletta L, Zecca E, Martini C, Ventafridda V (1991b) A clinical study on the use of codeine, oxycodone, dextropropoxyphene,

buprenorphine, and pentazocine in cancer pain. *J Pain Symptom Manage* 6:423-427.

DeLeo JA, Colburn RW, Nichols M, Malhotra A (1996) Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. *J Interferon Cytokine Res* 16:695-700.

Dinarello CA (1999) IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J Allergy Clin Immunol* 103:11-24.

Dixon WJ (1980) Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* 20:441-462.

Doverly M, Somogyi AA, White JM, Bochner F, Beare CH, Menelaou A, Ling W (2001a) Methadone maintenance patients are cross-tolerant to the antinociceptive effects of morphine. *Pain* 93:155-163.

Doverly M, White JM, Somogyi AA, Bochner F, Ali R, Ling W (2001b) Hyperalgesic responses in methadone maintenance patients. *Pain* 90:91-96.

Dray A (2005) Pharmacology of Pain. In: *The Paths of Pain 1975-2005* (Merskey H, Loeser JD, Dubner R, eds), pp 177-190. Seattle: IASP Press.

Falchi M, Ferrara F, Gharib C, Dib B (2001) Hyperalgesic effect of intrathecally administered interleukin-1 in rats. *Drugs Exp Clin Res* 27:97-101.

Fields HL (2000) Pain modulation: expectation, opioid analgesia and virtual pain. *Prog Brain Res* 122:245-253.

Fields HL, Basbaum AI (1978) Brainstem control of spinal pain-transmission neurons. *Annu Rev Physiol* 40:217-248.

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

Fields HL, Heinricher MM (1985) Anatomy and physiology of a nociceptive modulatory system. *Philos Trans R Soc Lond B Biol Sci* 308:361-374.

Fonnum F, Johnsen A, Hassel B (1997) Use of fluorocitrate and fluoroacetate in the study of brain metabolism. *Glia* 21:106-113.

Fu KY, Light AR, Maixner W (2000) Relationship between nociceptor activity, peripheral edema, spinal microglial activation and long-term hyperalgesia induced by formalin. *Neuroscience* 101:1127-1135.

- Fu KY, Light AR, Matsushima GK, Maixner W (1999a) Microglial reactions after subcutaneous formalin injection into the rat hind paw. *Brain Res* 825:59-67.
- Gaboury JP, Johnston B, Niu XF, Kubes P (1995) Mechanisms underlying acute mast cell-induced leukocyte rolling and adhesion in vivo. *J Immunol* 154:804-813.
- Galli SJ, Kalesnikoff J, Grimbaldston MA, Piliponsky AM, Williams CM, Tsai M (2005) Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 23:749-786.
- Gardell LR, Burgess SE, Dogrul A, Ossipov MH, Malan TP, Lai J, Porreca F (2002) Pronociceptive effects of spinal dynorphin promote cannabinoid-induced pain and antinociceptive tolerance. *Pain* 98:79-88.
- Garrison CJ, Dougherty PM, Carlton SM (1994) GFAP expression in lumbar spinal cord of naive and neuropathic rats treated with MK-801. *Exp Neurol* 129:237-243.
- Garrison CJ, Dougherty PM, Kajander KC, Carlton SM (1991) Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. *Brain Res* 565:1-7.
- Gebhart GF (2004) Descending modulation of pain. *Neurosci Biobehav Rev* 27:729-737.
- Giorgi R, Pagano RL, Dias MA, guiar-Passeti T, Sorg C, Mariano M (1998) Antinociceptive effect of the calcium-binding protein MRP-14 and the role played by neutrophils on the control of inflammatory pain. *J Leukoc Biol* 64:214-220.
- Giuliani F, Hader W, Yong VW (2005) Minocycline attenuates T cell and microglia activity to impair cytokine production in T cell-microglia interaction. *J Leukoc Biol* 78:135-143.
- Gong QJ, Li YY, Xin WJ, Zang Y, Ren WJ, Wei XH, Li YY, Zhang T, Liu XG (2008) ATP induces long-term potentiation of C-fiber-evoked field potentials in spinal dorsal horn: The roles of P2X(4) receptors and p38 MAPK in microglia. *Glia*.
- Guo W, Robbins MT, Wei F, Zou S, Dubner R, Ren K (2006) Supraspinal brain-derived neurotrophic factor signaling: a novel mechanism for descending pain facilitation. *J Neurosci* 26:126-137.
- Guo W, Wang H, Watanabe M, Shimizu K, Zou S, LaGraize SC, Wei F, Dubner R, Ren K (2007) Glial-cytokine-neuronal interactions underlying the mechanisms of persistent pain. *J Neurosci* 27:6006-6018.

Hains BC, Waxman SG (2006) Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci* 26:4308-4317.

Hansen TV, Rehfeld JF, Nielsen FC (1999) Mitogen-activated protein kinase and protein kinase A signaling pathways stimulate cholecystikinin transcription via activation of cyclic adenosine 3',5'-monophosphate response element-binding protein. *Mol Endocrinol* 13:466-475.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32:77-88.

He Y, Appel S, Le W (2001) Minocycline inhibits microglial activation and protects nigral cells after 6-hydroxydopamine injection into mouse striatum. *Brain Res* 909:187-193.

Heinricher MM (2003) Orphanin FQ/nociceptin: from neural circuitry to behavior. *Life Sci* 73:813-822.

Heinricher MM, Morgan MM, Tortorici V, Fields HL (1994) Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. *Neuroscience* 63:279-288.

Hide I, Tanaka M, Inoue A, Nakajima K, Kohsaka S, Inoue K, Nakata Y (2000) Extracellular ATP triggers tumor necrosis factor- $\alpha$  release from rat microglia. *J Neurochem* 75:965-972.

Hua XY, Svensson CI, Matsui T, Fitzsimmons B, Yaksh TL, Webb M (2005) Intrathecal minocycline attenuates peripheral inflammation-induced hyperalgesia by inhibiting p38 MAPK in spinal microglia. *Eur J Neurosci* 22:2431-2440.

Iadarola MJ, Brady LS, Draisci G, Dubner R (1988) Enhancement of dynorphin gene expression in spinal cord following experimental inflammation: stimulus specificity, behavioral parameters and opioid receptor binding. *Pain* 35:313-326.

Imbe H, Okamoto K, Aikawa F, Kimura A, Donishi T, Tamai Y, Iwai-Liao Y, Senba E (2007) Effects of peripheral inflammation on activation of p38 mitogen-activated protein kinase in the rostral ventromedial medulla. *Brain Res* 1134:131-139.

Inoue K, Koizumi S, Tsuda M, Shigemoto-Mogami Y (2003) Signaling of ATP receptors in glia-neuron interaction and pain. *Life Sci* 74:189-197.

Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S (1998) Microglia-specific localisation of a novel calcium binding protein, Iba1. *Brain Res Mol Brain Res* 57:1-9.

Ji RR, Kawasaki Y, Zhuang ZY, Wen YR, Decosterd I (2006) Possible role of spinal astrocytes in maintaining chronic pain sensitization: review of current evidence with focus on bFGF/JNK pathway. *Neuron Glia Biol* 2:259-269.

Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ (2002) p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* 36:57-68.

Jin SX, Zhuang ZY, Woolf CJ, Ji RR (2003) p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci* 23:4017-4022.

Johnston IN, Milligan ED, Wieseler-Frank J, Frank MG, Zapata V, Campisi J, Langer S, Martin D, Green P, Fleshner M, Leinwand L, Maier SF, Watkins LR (2004) A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. *J Neurosci* 24:7353-7365.

Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. *Nature* 413:203-210.

Kerr BJ, Bradbury EJ, Bennett DL, Trivedi PM, Dassan P, French J, Shelton DB, McMahon SB, Thompson SW (1999) Brain-derived neurotrophic factor modulates nociceptive sensory inputs and NMDA-evoked responses in the rat spinal cord. *J Neurosci* 19:5138-5148.

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

Kim SY, Bae JC, Kim JY, Lee HL, Lee KM, Kim DS, Cho HJ (2002) Activation of p38 MAP kinase in the rat dorsal root ganglia and spinal cord following peripheral inflammation and nerve injury. *Neuroreport* 13:2483-2486.

King T, Gardell LR, Wang R, Vardanyan A, Ossipov MH, Malan TP, Jr., Vanderah TW, Hunt SP, Hruby VJ, Lai J, Porreca F (2005a) Role of NK-1 neurotransmission in opioid-induced hyperalgesia. *Pain* 116:276-288.

King T, Ossipov MH, Vanderah TW, Porreca F, Lai J (2005b) Is paradoxical pain induced by sustained opioid exposure an underlying mechanism of opioid antinociceptive tolerance? *Neurosignals* 14:194-205.

Kobayashi K, Yamanaka H, Fukuoka T, Dai Y, Obata K, Noguchi K (2008) P2Y12 receptor upregulation in activated microglia is a gateway of p38 signaling and neuropathic pain. *J Neurosci* 28:2892-2902.

Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 19:312-318.

Lai J, Hunter JC, Porreca F (2003) The role of voltage-gated sodium channels in neuropathic pain. *Curr Opin Neurobiol* 13:291-297.

Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, Watkins LR (2005) Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain* 115:71-83.

Ledeboer A, Wierinckx A, Bol JG, Floris S, Renardel de LC, De Vries HE, van den Berg TK, Dijkstra CD, Tilders FJ, van dam AM (2003) Regional and temporal expression patterns of interleukin-10, interleukin-10 receptor and adhesion molecules in the rat spinal cord during chronic relapsing EAE. *J Neuroimmunol* 136:94-103.

Levine JD, Lau W, Kwiat G, Goetzl EJ (1984) Leukotriene B4 produces hyperalgesia that is dependent on polymorphonuclear leukocytes. *Science* 225:743-745.

Malaviya R, Abraham SN (2000) Role of mast cell leukotrienes in neutrophil recruitment and bacterial clearance in infectious peritonitis. *J Leukoc Biol* 67:841-846.

Mannion RJ, Costigan M, Decosterd I, Amaya F, Ma QP, Holstege JC, Ji RR, Acheson A, Lindsay RM, Wilkinson GA, Woolf CJ (1999) Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity. *Proc Natl Acad Sci U S A* 96:9385-9390.

Mao J (2002) Opioid-induced abnormal pain sensitivity: implications in clinical opioid therapy. *Pain* 100:213-217.

Mao J, Sung B, Ji RR, Lim G (2002) Chronic morphine induces downregulation of spinal glutamate transporters: implications in morphine tolerance and abnormal pain sensitivity. *J Neurosci* 22:8312-8323.

Maves TJ, Pechman PS, Gebhart GF, Meller ST (1993) Possible chemical contribution from chronic gut sutures produces disorders of pain sensation like those seen in man. *Pain* 54:57-69.

Mayer DJ, Mao J, Holt J, Price DD (1999) Cellular mechanisms of neuropathic pain, morphine tolerance, and their interactions. *Proc Natl Acad Sci U S A* 96:7731-7736.

McGaraughty S, Wismer CT, Zhu CZ, Mikusa J, Honore P, Chu KL, Lee CH, Faltynek CR, Jarvis MF (2003) Effects of A-317491, a novel and selective P2X3/P2X2/3 receptor antagonist, on neuropathic, inflammatory and chemogenic nociception following intrathecal and intraplantar administration. *Br J Pharmacol* 140:1381-1388.

Meller ST, Dykstra C, Grzybycki D, Murphy S, Gebhart GF (1994) The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. *Neuropharmacology* 33:1471-1478.

Milligan ED, Mehmert KK, Hinde JL, Harvey LO, Martin D, Tracey KJ, Maier SF, Watkins LR (2000) Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120. *Brain Res* 861:105-116.

Milligan ED, O'Connor KA, Armstrong CB, Hansen MK, Martin D, Tracey KJ, Maier SF, Watkins LR (2001) Systemic administration of CNI-1493, a p38 mitogen-activated protein kinase inhibitor, blocks intrathecal human immunodeficiency virus-1 gp120-induced enhanced pain states in rats. *J Pain* 2:326-333.

Milligan ED, Twining C, Chacur M, Biedenkapp J, O'Connor K, Poole S, Tracey K, Martin D, Maier SF, Watkins LR (2003) Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. *J Neurosci* 23:1026-1040.

Mizushima T, Obata K, Yamanaka H, Dai Y, Fukuoka T, Tokunaga A, Mashimo T, Noguchi K (2005) Activation of p38 MAPK in primary afferent neurons by noxious stimulation and its involvement in the development of thermal hyperalgesia. *Pain* 113:51-60.

Moalem G, Tracey DJ (2006a) Immune and inflammatory mechanisms in neuropathic pain. *Brain Res Rev* 51:240-264.

Morgan MM, Fields HL (1994) Pronounced changes in the activity of nociceptive modulatory neurons in the rostral ventromedial medulla in response to prolonged thermal noxious stimuli. *J Neurophysiol* 72:1161-1170.

Muller W, Heinemann U, Berlin K (1997) Cholecystokinin activates CCKB-receptor-mediated Ca-signaling in hippocampal astrocytes. *J Neurophysiol* 78:1997-2001.

Myers RR, Heckman HM, Rodriguez M (1996) Reduced hyperalgesia in nerve-injured WLD mice: relationship to nerve fiber phagocytosis, axonal degeneration, and regeneration in normal mice. *Exp Neurol* 141:94-101.

Nakagawa T, Wakamatsu K, Zhang N, Maeda S, Minami M, Satoh M, Kaneko S (2007) Intrathecal administration of ATP produces long-lasting allodynia in rats: differential mechanisms in the phase of the induction and maintenance. *Neuroscience* 147:445-455.

Nathan C (2002) Points of control in inflammation. *Nature* 420:846-852.

Nathan N, Denizot Y, Cornu E, Jauberteau MO, Chauvreau C, Feiss P (1997) Cytokine and lipid mediator blood concentrations after coronary artery surgery. *Anesth Analg* 85:1240-1246.

Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Tominaga M, Noguchi K (2005) TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. *J Clin Invest* 115:2393-2401.

Obata K, Yamanaka H, Kobayashi K, Dai Y, Mizushima T, Katsura H, Fukuoka T, Tokunaga A, Noguchi K (2004) Role of mitogen-activated protein kinase activation in injured and intact primary afferent neurons for mechanical and heat hypersensitivity after spinal nerve ligation. *J Neurosci* 24:10211-10222.

Obata T, Brown GE, Yaffe MB (2000) MAP kinase pathways activated by stress: the p38 MAPK pathway. *Crit Care Med* 28:N67-N77.

Olsson Y (1967) Degranulation of mast cells in peripheral nerve injuries. *Acta Neurol Scand* 43:365-374.

Ossipov MH, Porreca F (2005) Descending Modulation of Pain. In: *The Paths of Pain 1975-2005* (Merskey H, Loeser JD, Dubner R, eds), pp 117-130. Seattle: IASP Press.

Paulsen RE, Contestabile A, Villani L, Fonnum F (1987) An in vivo model for studying function of brain tissue temporarily devoid of glial cell metabolism: the use of fluorocitrate. *J Neurochem* 48:1377-1385.

Pekny M (2001) Astrocytic intermediate filaments: lessons from GFAP and vimentin knock-out mice. *Prog Brain Res* 132:23-30.

Peng XM, Zhou ZG, Glorioso JC, Fink DJ, Mata M (2006) Tumor necrosis factor- $\alpha$  contributes to below-level neuropathic pain after spinal cord injury. *Ann Neurol* 59:843-851.

Perkins NM, Tracey DJ (2000) Hyperalgesia due to nerve injury: role of neutrophils. *Neuroscience* 101:745-757.

- Perry VH (1994) Modulation of microglia phenotype. *Neuropathol Appl Neurobiol* 20:177.
- Perry VH, Brown MC (1992) Macrophages and nerve regeneration. *Curr Opin Neurobiol* 2:679-682.
- Perry VH, Brown MC, Tsao JW (1992) The Effectiveness of the Gene Which Slows the Rate of Wallerian Degeneration in C57BL/Ola Mice Declines With Age. *Eur J Neurosci* 4:1000-1002.
- Pertovaara A, Hamalainen MM, Kauppila T, Panula P (1998) Carrageenan-induced changes in spinal nociception and its modulation by the brain stem. *Neuroreport* 9:351-355.
- Pertovaara A, Wei H, Hamalainen MM (1996) Lidocaine in the rostroventromedial medulla and the periaqueductal gray attenuates allodynia in neuropathic rats. *Neurosci Lett* 218:127-130.
- Popovich PG, Wei P, Stokes BT (1997) Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. *J Comp Neurol* 377:443-464.
- Porreca F, Burgess SE, Gardell LR, Vanderah TW, Malan TP, Jr., Ossipov MH, Lappi DA, Lai J (2001) Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the mu-opioid receptor. *J Neurosci* 21:5281-5288.
- Porreca F, Ossipov MH, Gebhart GF (2002) Chronic pain and medullary descending facilitation. *Trends Neurosci* 25:319-325.
- Raghavendra V, Rutkowski MD, DeLeo JA (2002) The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. *J Neurosci* 22:9980-9989.
- Raghavendra V, Tanga F, DeLeo JA (2003a) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J Pharmacol Exp Ther* 306:624-630.
- Raghavendra V, Tanga F, Rutkowski MD, DeLeo JA (2003b) Anti-hyperalgesic and morphine-sparing actions of propentofylline following peripheral nerve injury in rats: mechanistic implications of spinal glia and proinflammatory cytokines. *Pain* 104:655-664.
- Raghavendra V, Tanga FY, DeLeo JA (2004) Complete Freund's adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. *Eur J Neurosci* 20:467-473.

Raith K, Hochhaus G (2004) Drugs used in the treatment of opioid tolerance and physical dependence: a review. *Int J Clin Pharmacol Ther* 42:191-203.

Reeve AJ, Patel S, Fox A, Walker K, Urban L (2000) Intrathecally administered endotoxin or cytokines produce allodynia, hyperalgesia and changes in spinal cord neuronal responses to nociceptive stimuli in the rat. *Eur J Pain* 4:247-257.

Ridet JL, Malhotra SK, Privat A, Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci* 20:570-577.

Romero-Sandoval A, Chai N, Nutile-McMenemy N, DeLeo JA (2008) A comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. *Brain Res* 1219:116-126.

Rueff A, Dray A (1993) Sensitization of peripheral afferent fibres in the in vitro neonatal rat spinal cord-tail by bradykinin and prostaglandins. *Neuroscience* 54:527-535.

Salter MW (2005) Cellular signalling pathways of spinal pain neuroplasticity as targets for analgesic development. *Curr Top Med Chem* 5:557-567.

Schafers M, Svensson CI, Sommer C, Sorkin LS (2003) Tumor necrosis factor- $\alpha$  induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *J Neurosci* 23:2517-2521.

Scholz J, Woolf CJ (2007) The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci* 10:1361-1368.

Shi Y, Gaestel M (2002) In the cellular garden of forking paths: how p38 MAPKs signal for downstream assistance. *Biol Chem* 383:1519-1536.

Sjogren P, Banning AM, Christensen CB, Pedersen O (1994a) Continuous reaction time after single dose, long-term oral and epidural opioid administration. *Eur J Anaesthesiol* 11:95-100.

Sjogren P, Jensen NH, Jensen TS (1994b) Disappearance of morphine-induced hyperalgesia after discontinuing or substituting morphine with other opioid agonists. *Pain* 59:313-316.

Sjostrand J (1971) Neuroglial proliferation in the hypoglossal nucleus after nerve injury. *Exp Neurol* 30:178-189.

Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron* 20:629-632.

Sommer C, Schafers M (1998) Painful mononeuropathy in C57BL/Wld mice with delayed wallerian degeneration: differential effects of cytokine production and nerve regeneration on thermal and mechanical hypersensitivity. *Brain Res* 784:154-162.

Song P, Zhao ZQ (2001) The involvement of glial cells in the development of morphine tolerance. *Neurosci Res* 39:281-286.

Svensson CI, Fitzsimmons B, Azizi S, Powell HC, Hua XY, Yaksh TL (2005) Spinal p38beta isoform mediates tissue injury-induced hyperalgesia and spinal sensitization. *J Neurochem* 92:1508-1520.

Svensson CI, Marsala M, Westerlund A, Calcutt NA, Campana WM, Freshwater JD, Catalano R, Feng Y, Protter AA, Scott B, Yaksh TL (2003) Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. *J Neurochem* 86:1534-1544.

Svensson CI, Yaksh TL, Sorkin LS (2007) The Role of p38 in Microglial Regulation of Spinal Pain Processing. In: *Immune and Glial Regulation of Pain* (DeLeo JA, Sorkin LS, Watkins LR, eds), pp 297-317. Seattle: IASP Press.

Sweitzer S, Martin D, DeLeo JA (2001) Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. *Neuroscience* 103:529-539.

Sweitzer SM, Colburn RW, Rutkowski M, DeLeo JA (1999a) Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behavior in the rat. *Brain Res* 829:209-221.

Takeda K, Ichijo H (2002) Neuronal p38 MAPK signalling: an emerging regulator of cell fate and function in the nervous system. *Genes Cells* 7:1099-1111.

Takuma K, Matsuda T, Hashimoto H, Kitanaka J, Asano S, Kishida Y, Baba A (1996) Role of Na(+)-Ca<sup>2+</sup> exchanger in agonist-induced Ca<sup>2+</sup> signaling in cultured rat astrocytes. *J Neurochem* 67:1840-1845.

Tanga FY, Nutile-McMenemy N, DeLeo JA (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* 102:5856-5861.

Thompson SW, Bennett DL, Kerr BJ, Bradbury EJ, McMahon SB (1999) Brain-derived neurotrophic factor is an endogenous modulator of nociceptive responses in the spinal cord. *Proc Natl Acad Sci U S A* 96:7714-7718.

Tozaki-Saitoh H, Tsuda M, Miyata H, Ueda K, Kohsaka S, Inoue K (2008) P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *J Neurosci* 28:4949-4956.

Tracey I, Mantyh PW (2007) The cerebral signature for pain perception and its modulation. *Neuron* 55:377-391.

Tsuda M, Inoue K, Salter MW (2005) Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends Neurosci* 28:101-107.

Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K (2003) P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature* 424:778-783.

Ulmann L, Hatcher JP, Hughes JP, Chaumont S, Green PJ, Conquet F, Buell GN, Reeve AJ, Chessell IP, Rassendren F (2008) Up-regulation of P2X4 receptors in spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain. *J Neurosci* 28:11263-11268.

Urban MO, Gebhart GF (1999) Supraspinal contributions to hyperalgesia. *Proc Natl Acad Sci U S A* 96:7687-7692.

Vanderah TW, Gardell LR, Burgess SE, Ibrahim M, Dogrul A, Zhong CM, Zhang ET, Malan TP, Jr., Ossipov MH, Lai J, Porreca F (2000) Dynorphin promotes abnormal pain and spinal opioid antinociceptive tolerance. *J Neurosci* 20:7074-7079.

Vanderah TW, Suenaga NM, Ossipov MH, Malan TP, Jr., Lai J, Porreca F (2001) Tonic descending facilitation from the rostral ventromedial medulla mediates opioid-induced abnormal pain and antinociceptive tolerance. *J Neurosci* 21:279-286.

Vulchanova L, Riedl MS, Shuster SJ, Buell G, Surprenant A, North RA, Elde R (1997) Immunohistochemical study of the P2X2 and P2X3 receptor subunits in rat and monkey sensory neurons and their central terminals. *Neuropharmacology* 36:1229-1242.

Watkins LR, Kinscheck IB, Mayer DJ (1984) Potentiation of opiate analgesia and apparent reversal of morphine tolerance by proglumide. *Science* 224:395-396.

Watkins LR, Maier SF (2002) Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. *Physiol Rev* 82:981-1011.

Watkins LR, Maier SF (2003b) Glia: a novel drug discovery target for clinical pain. *Nat Rev Drug Discov* 2:973-985.

- Watkins LR, Martin D, Ulrich P, Tracey KJ, Maier SF (1997) Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. *Pain* 71:225-235.
- Watkins LR, Milligan ED, Maier SF (2001) Glial activation: a driving force for pathological pain. *Trends Neurosci* 24:450-455.
- Wei F, Guo W, Zou S, Ren K, Dubner R (2008) Supraspinal glial-neuronal interactions contribute to descending pain facilitation. *J Neurosci* 28:10482-10495.
- Willoughby JO, Mackenzie L, Broberg M, Thoren AE, Medvedev A, Sims NR, Nilsson M (2003) Fluorocitrate-mediated astroglial dysfunction causes seizures. *J Neurosci Res* 74:160-166.
- Wu DC, Jackson-Lewis V, Vila M, Tieu K, Teismann P, Vadseth C, Choi DK, Ischiropoulos H, Przedborski S (2002) Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. *J Neurosci* 22:1763-1771.
- Wu Y, Willcockson HH, Maixner W, Light AR (2004) Suramin inhibits spinal cord microglia activation and long-term hyperalgesia induced by formalin injection. *J Pain* 5:48-55.
- Xie JY, Herman DS, Stiller CO, Gardell LR, Ossipov MH, Lai J, Porreca F, Vanderah TW (2005) Cholecystikinin in the rostral ventromedial medulla mediates opioid-induced hyperalgesia and antinociceptive tolerance. *J Neurosci* 25:409-416.
- Yrjanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J (1998) Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc Natl Acad Sci U S A* 95:15769-15774.
- Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J (1999) A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc Natl Acad Sci U S A* 96:13496-13500.
- Zhao J, Seereeram A, Nassar MA, Levato A, Pezet S, Hathaway G, Morenilla-Palao C, Stirling C, Fitzgerald M, McMahon SB, Rios M, Wood JN (2006) Nociceptor-derived brain-derived neurotrophic factor regulates acute and inflammatory but not neuropathic pain. *Mol Cell Neurosci* 31:539-548.
- Zhu S, Stavrovskaya IG, Drozda M, Kim BY, Ona V, Li M, Sarang S, Liu AS, Hartley DM, Wu DC, Gullans S, Ferrante RJ, Przedborski S, Kristal BS,

Friedlander RM (2002) Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* 417:74-78.

Zhuo M, Gebhart GF (1990) Characterization of descending inhibition and facilitation from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. *Pain* 42:337-350.

Zhuo M, Gebhart GF (1992) Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. *J Neurophysiol* 67:1599-1614.

Zuo Y, Perkins NM, Tracey DJ, Geczy CL (2003) Inflammation and hyperalgesia induced by nerve injury in the rat: a key role of mast cells. *Pain* 105:467-479.