PAIN FACILITATING NEURONS IN THE BRAINSTEM MEDIATE CUTANEOUS ALLODYニア IN AN EXPERIMENTAL MODEL OF HEADACHE-RELATED PAIN

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‘...We know little, but that we must trust in what is difficult is a certainty that will never abandon us; it is good to be solitary, for solitude is difficult; that something is difficult must be one more reason for us to do it. It is also good to love: because love is difficult. For one human being to love another human being: that is perhaps the most difficult task that has been entrusted to us, the ultimate task, the final test and proof, the work for which all other work is merely preparation...’

Rainer Maria Rilke
# TABLE OF CONTENTS

LIST OF FIGURES.............................................................................................................8

ABSTRACT.......................................................................................................................10

CHAPTER 1: INTRODUCTION......................................................................................12

CHAPTER 2: MATERIALS & METHODS.....................................................................24

2.1 Animals....................................................................................................................24

2.2 Surgical Preparations.............................................................................................24

2.3 Behavioral Testing Protocols..................................................................................27

2.4 Injection Procedures & Reagents..........................................................................30

2.5 Immunolabeling......................................................................................................34

2.6 Imaging & Cell Counting......................................................................................37

2.7 Electrophysiological Studies..................................................................................38

2.8 Data Analysis..........................................................................................................39

CHAPTER 3: RESULTS..................................................................................................40

3.1 Dural IM Elicits Cutaneous Allodynia....................................................................40

3.2 FOS Expression in the TNC..................................................................................41

3.3 Systemic Drug Treatment of IM-Induced Cutaneous Allodynia.............................42

3.4 RVM Microinjection Studies..................................................................................44

3.5 IM-Related RVM Electrophysiology.....................................................................46

3.6 Glial Cell Immunofluorescence in the RVM.........................................................46

3.7 RVM 5-HT\textsubscript{1B/1D} Immunofluorescence..................................................48
**TABLE OF CONTENTS - Continued**

<table>
<thead>
<tr>
<th>Chapter 4: DISCUSSION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Cutaneous allodynia triggered by dural inflammation as a model for headache-related pain</td>
<td>49</td>
</tr>
<tr>
<td>4.2 IM-induced cutaneous allodynia and central sensitization</td>
<td>54</td>
</tr>
<tr>
<td>4.3 Descending facilitation of headache-related pain</td>
<td>55</td>
</tr>
<tr>
<td>4.4 Neuronal-glial interactions within the RVM</td>
<td>58</td>
</tr>
<tr>
<td>4.5 Implications for mechanisms of headache-related pain</td>
<td>61</td>
</tr>
<tr>
<td>4.6 Conclusion</td>
<td>63</td>
</tr>
</tbody>
</table>

**HUMAN/ANIMAL SUBJECTS APPROVAL** | 65 |

**FIGURES & LEGENDS** | 66 |

**REFERENCES** | 96 |
LIST OF FIGURES

FIGURE 1, Diagram of Dura Cannula Design..............................................................66
FIGURE 2, Location of Dura Cannula..........................................................................67
FIGURE 3, Ink Injection: Dura Cannula Verification..................................................68
FIGURE 4A, Dura + RVM Cannula Location.................................................................69
FIGURE 4B, RVM Cannula Location: Dorsoventral View............................................69
FIGURE 5, Dura + RVM Cannulation: Post Surgery...................................................70
FIGURE 6, RVM Cannula Verification........................................................................71
FIGURE 7, Experimental Testing Protocols.................................................................72
FIGURE 8, Cannula Injections.....................................................................................73
FIGURE 9, Dural IM Elicits Cutaneous Allodynia......................................................74
FIGURE 10, Inflammatory Mediator Dose Response....................................................75
FIGURE 11, FOS Expression in TNC............................................................................76
FIGURE 12, Subcutaneous Sumatriptan Treatment.......................................................77
FIGURE 13, Subcutaneous Naproxen Treatment...........................................................78
FIGURE 14, Subcutaneous Morphine Treatment...........................................................79
FIGURE 15, Intravenous CCGP Receptor Antagonist Treatment..................................80
FIGURE 16, Subcutaneous NK-1 Antagonist Treatment..............................................81
FIGURE 17, RVM Bupivacaine Treatment...................................................................82
FIGURE 18, RVM Dermorphin-Saporin Treatment....................................................83
FIGURE 19, RVM CCK Treatment..............................................................................84
LIST OF FIGURES - Continued

FIGURE 20, RVM CCK$_2$-Antagonist Treatment.................................................................85
FIGURE 21, RVM Sumatriptan and Minocyline Treatment..................................................86
FIGURE 22, IM-Related RVM Electrophysiology.................................................................87
FIGURE 23, Microglial Condition Comparison.......................................................................88
FIGURE 24, Astrocyte Condition Comparison.......................................................................90
FIGURE 25, RVM Sumatriptan or Minocyline Treatment vs. IM-Induced Microglial
  Activation.................................................................................................................92
FIGURE 26, RVM 5-HT$_{1B/1D}$ Immunoﬂuorescence.........................................................94
ABSTRACT

Migraine patients often demonstrate cutaneous allodynia, defined as a hypersensitivity of the skin to touch or mechanical stimuli that is considered non-noxious under normal circumstances. The allodynia sometimes begins intracranially and spreads, via unknown mechanisms, to extracranial regions. The goal of the study was to develop and validate a model of cutaneous allodynia triggered by dural inflammation for pain associated with headaches, and to explore neuronal and glial mechanisms underlying generalized allodynia. Inflammatory mediators (IM) were applied to the dura of unanesthetized rats via previously implanted cannulas and sensory thresholds of the face and hindpaws were characterized. IM elicited robust and time-related facial and hindpaw allodynia which peaked after approximately three hours as well as FOS expression in the trigeminal nucleus caudalis (TNC), indicative of central sensitization. These effects were reminiscent of cutaneous allodynia seen in patients with migraine or other primary headache conditions, and were reversed by agents used clinically in the treatment of migraine including sumatriptan, naproxen, CGRP-antagonist, and morphine. Consistent with clinical observations, the allodynia was unaffected by an NK-1 antagonist. Having established facial and hindpaw allodynia as a useful animal surrogate of headache-associated allodynia, we next showed that blocking pain-facilitating processes from the rostral ventromedial medulla (RVM) interfered with its expression. Inactivation of the RVM with local anesthetic, destruction of putative pain-facilitation cells, and blockade of cholecystokinin receptors all prevented or significantly attenuated IM-induced allodynia.
Electrophysiological studies confirmed activation of pain-facilitating “ON” cells and transient suppression of “OFF” cells in the RVM following IM. Additionally, microinjection of the RVM with a microglial inhibitor or sumatriptan also inhibited the expression of IM-induced cutaneous allodynia as well as microglial activation. Facial and hindpaw allodynia associated with dural stimulation is a useful surrogate of pain associated with primary headache including migraine and may be exploited mechanistically for the development of novel therapeutic strategies for headache pain. The data also demonstrate a requirement for activation of descending facilitation from the RVM, likely reliant on neuronal-glial interactions, for the expression of cranial and extracranial cutaneous allodynia. Consequently, the findings are consistent with a brainstem generator of allodynia associated with headache disorders.
CHAPTER 1
INTRODUCTION

Migraine headache is an episodic, multi-phasic, multi-symptom disorder. It is classically characterized by excruciating, incapacitating head pain and common symptoms such as aura, nausea, vomiting, and extreme sensitivity to light, sound or movement. Migraine is a worldwide problem that does not discriminate between race and socio-economic background. However, it does appear to choose its victims based on age, sex and genetics. According to many sources, this primary headache disorder generally affects 15-18% of women and 6-8% of men worldwide, with many patients claiming to have a close relative suffering from migraines. The World Health Organization maintains that migraine headache is most prevalent in people ranging in age from the late teens to the fifties. This incidence is problematic since these are the most industrious years in terms of societal influence and contribution, and the cost to the economy in lost wages, productivity, and health care are immense.

So, what are the barriers that keep us from effectively treating this disorder in patients? Many claim a lack of understanding of the disease that is shared in part by the physicians, researchers, sufferers, and society as a whole. Society’s perception of the disorder tends to lean towards the position of apathy. Reasonably, because migraine headaches are episodic, without an endpoint of disabling neurological degeneration or fatality, and sufferers can still lead a relatively ‘normal’ lifestyle during the intervals between attacks. Thus, migraine headache patients fall into the category of a niche market and are considered a non-lucrative business venture for those with the means to
invest. Researchers, as well, lack a clear understanding of the mechanisms underlying migraine headache and have trouble coming to a consensus on one fundamental strategy for pain management. In turn, physicians are left in the dark concerning current treatment options and new therapies for the individual patient. It has been said that migraine headache continues to be one of the most under-diagnosed and under-recognized neurological conditions.

The biggest challenge for migraine research begins with the variety of theories underlying the pathogenesis and origin of migraine headache. Cerebral vascular dysregulation, neurogenic inflammation within the dural meninges, genetic defect, cortical spreading depression as well as the possibility of a brainstem generator causing migraine-associated symptoms have all been put forward as potential theories fundamental to the genesis of migraine headache (Goadsby 2007). But none of these explanations alone has proven consistent with all of the available data.

Even though we do not understand the mechanism underlying the cause of the attacks, some of the essential anatomy important to migraine physiology has been agreed upon by researchers in the field. It is understood that the trigeminal nerve system, known as the fifth cranial nerve (V), is a key component for transmitting migraine-related pain information to the brain. The trigeminal nerve system relays mostly sensory information from the head; however, it does direct some aspects of voluntary motor control over the jaw. The organization of the trigeminal nerve is such that three main branches provide somatosensory innervation to distinct regions of the cranium. The ophthalmic branch (V₁) brings in sensory information from the dura, scalp, upper-forehead including certain eye
regions, the nose, nasal mucosa and sinuses. The maxillary branch (V₂) covers information from the lower eyelid and upper cheek to the upper lip and dorsal aspect of the mouth and palate. The mandibular division (V₃) carries sensory information from the lower lip and ventral areas of the mouth as well as the chin and jaw. It is the mandibular division that also carries motor input to the jaw.

Sensitization and activation of trigeminal nerves within the ophthalmic branch, particularly those of the dura, are thought to play an essential role in migraine (Pietrobon 2005). The dura, or the outermost membrane surrounding the brain, is innervated with a dense vasculature and a large quantity of sensory neurons that are components of the trigeminovascular system. The sensory neurons primarily responsible for pain in migraine are the peri-vascular nociceptive fibers located directly adjacent to the blood vessels within the dura. These afferent fibers can sense mechanical changes in dilatation and constriction of the vessels, as well as respond to stimuli via activation of receptors by pro-nociceptive transmitters like CGRP (calcitonin gene-related peptide), substance P, PGE₂ (prostaglandin E₂), and histamine (Ma et al. 2001; Goadsby et al. 2002; Potrebic et al. 2003; Schwenger et al. 2007).

Sensory information from trigeminal afferents travels to the trigeminal nuclei within the brainstem via the trigeminal ganglion (TG). It is within the TG where the distinct sensory branches from the cranium V₁, V₂, and V₃ first come together. The fibers synapse within the trigeminal nuclei in a somatotopic manner within the laminae and are also organized into nuclei based on the fiber’s primary modality. Neurons synapsing more rostrally in brainstem’s main trigeminal nucleus tend to bring in touch and position
information, and fibers synapsing more caudally in the spinal trigeminal nucleus or trigeminal nucleus caudalis (TNC) bring both pain and temperature information from the cranial regions.

The TNC is thought to be the most relevant trigeminal nucleus for transmission of headache related information (Buzzi 2001). Once sensory information reaches the TNC, it can then be disseminated centrally to numerous areas in the brain and brainstem through second-order and third-order neuronal connections. For sensory information to be perceived as painful or non-painful it must reach the highest levels of central integration, which are known as the somatosensory cortices. Most information that travels to the somatosensory cortex must first pass through the thalamus, an area of the brain known to integrate information from all over the body and the central nervous system (CNS). The somatosensory cortex is important for perception and discernment of stimuli; it is here that our brains decide how to handle the information. For example if a stimulus is perceived as noxious or painful, the somatosensory cortex will send the proper signal to our musculature to act in an aversive manner. In the case of migraine, one can sense that their head hurts and they logically take actions to alleviate such pain; typically these actions involve avoidance of painful triggers like light, sound, and movement as well as administration of pain relieving medications.

It is difficult to treat migraine because the medications available temporarily remedy the symptoms but do not resolve the underlying disorder. Depending on the patient’s diagnosis, which takes into consideration the frequency of attacks and severity of pain, the treatment usually falls within one of two pharmaceutical categories.
Sometimes patients will receive a combination of medications depending on the severity of their disorder.

Preventative medications are taken on a daily basis with the hope of reducing the frequency of attacks in patients who experience more than two migraines per month. This therapy includes classes of drugs such as β-blockers, anticonvulsants, Ca^{2+}-channel blockers, and antidepressants. β-blockers are thought to decrease and stabilize blood pressure which keep the blood vessels in a relaxed state thereby decreasing the severity and duration of the migraine attacks. Anticonvulsant medications are thought to decrease abnormal neuronal excitation in the brain. If the patients are unresponsive to these first line preventative medications Ca^{2+}-channel blockers can also be prescribed; these compounds are considered to work for migraine by reducing reflex narrowing of the blood vessels after vasodilatation during an attack. Antidepressants of the tricyclic nature are also sometimes prescribed for migraine headache when other treatments fail.

Abortive medications are used to treat symptoms after the onset of an attack. Most of these medications must be taken as soon as the attack occurs; otherwise they may be ineffective in relieving symptoms. Abortive pharmaceuticals include triptans, ergot derivatives, opioids, corticosteroids, and over the counter medications like NSAIDs (non-steroidal anti-inflammatory drugs). Triptan medications are some of the most commonly prescribed therapies for migraine headache. These drugs are 5-HT agonists (serotonergic agonists) primarily selective for the 5-HT_{1B} and 5-HT_{1D} receptor subtypes. It is still mechanistically unclear exactly how these drugs effectively decrease migraine pain, but the most common current theory is that activation of the 5-HT_{1B} and 5-HT_{1D} receptor
subtypes decreases dural vasodilatation and neuronal excitability, respectively. Ergots, like triptans, are commonly prescribed for migraine. The therapeutic activity of ergot derivatives are also thought to be through activation of 5-HT receptors. Opioids such as morphine maintain their analgesic effects by working at the G-protein coupled opioid receptors located on sensory fibers throughout the trigeminovascular system. Opioids decrease neuronal excitability and neuropeptide release and the ultimate result is a decrease in the transmission of painful information. Corticosteroid therapy is generally prescribed for patients with long-lasting migraine attacks that persist for twenty-four to seventy-two hours, and can be used as a rescue therapy due to its anti-inflammatory properties. NSAIDs such as aspirin, ibuprofen, and naproxen are also taken for their anti-inflammatory effects. However the NSAIDs are considered the least effective at relieving the pain in most migraine patients.

In reality, many of these preventative and abortive medications are not very effective and are also not well tolerated by migraine patients. Most of the medications used to treat migraine pain come with the baggage of side effects and can often be contraindicated with other medications. The current therapies are not specific to the mechanisms of the disorder and most are off-label drugs prescribed with serendipitous effects. Better pharmaceuticals need to be developed for migraine-related pain. Nevertheless, for migraine therapy to advance there needs to be a way to test new medications for the disorder as well as study the mechanism behind the neurophysiology of the disorder. One of the best ways to advance migraine research is to develop an animal model of migraine or primary headache. Animal models are crucial for
translational science. They provide the congruent intermediate link between the basic science bench and the clinic. One of the main goals of this dissertation work was to develop a working animal model of migraine headache. To accomplish this mission it was important to first more clearly understand the symptoms of migraine headache and to exploit this knowledge for the advancement of our model.

The frequency of migraine headaches and the symptoms experienced can vary greatly from patient to patient. Depending on the individual, migraines can occur on a weekly or even a monthly basis. Generally the length of the attack lasts for four hours but in some cases can extend up to seventy-two hours or longer. The attack typically occurs in three characteristic phases: the premonitory or pre-headache phase, the headache itself, and the postdrome or post-headache phase. Sometimes the premonitory and postdrome phases can last for days, before and after an attack, with symptoms such as muscle tenderness, fatigue and mood changes.

A classic symptom experienced by migraineurs during an attack is the visual disturbances or what is known as an aura. The aura typically occurs during the premonitory phase less than one hour before the majority of the other symptoms set in, acting as a forewarning to the patient of an oncoming attack. These disturbances or distortions of the visual field have been described by patients as wavy lines, scintillations, flashes of light, tunnel vision, and blind spots. Not all patients experience the aura during the premonitory phase. One of the current theories behind the visual disturbances is the phenomenon of cortical spreading depression or CSD. CSD is defined as a wave of depolarization or neuronal activation that spreads across the surface of the cortex. CSD
has been linked to the aura experience through fMRI imaging studies (Hadjikhani et al. 2001); however, it has yet to be linked causally to the other symptoms of migraine, including the pain experienced by migraineurs.

The headache phase of the attack is characterized by a collection of symptoms that fall into the categories of headache features and non-headache features. The headache features include spontaneous pain in the head that has a pulsating throbbing quality. Some of the non-headache features include photophobia and phonophobia which are extreme sensitivity to light and sound stimuli. Nausea, vomiting, weariness, and fatigue are also common symptoms.

One of the most intriguing yet lesser recognized symptoms of the headache phase falls between the two categories. This is the spontaneous head pain that sometimes begins unilaterally on one side of the head and then spreads to other areas of the head, face, neck and other extracranial locations (Burstein et al. 2000b; Dodick and Silberstein 2006; Cooke et al. 2007). This symptom that migraineurs experience over their entire body is known as cutaneous allodynia; defined as a hypersensitivity of the skin to touch or mechanical stimuli that is considered non-noxious under normal circumstances. In some patients the pain and sensitivity is so extreme that daily activities like grooming and wearing jewelry, eye-glasses, or clothing can be severely irritating. The development of allodynia within the referred pain area occurs after a significant delay in the migraine attack (Burstein et al. 2000a; Ashkenazi et al. 2005), and is believed to reflect the sensitization of second-order neurons in the trigeminal system and higher structures.

Sensitization of the trigeminovascular system is thought to begin with the
activation of primary afferent nerves innervating the dural meninges and dural vasculature (Burstein et al. 2000a). It is still unclear if the phenomenon of headache begins with spontaneous activation of these neurons or if they are activated by something else within the brain. Nonetheless, these trigeminal afferents are thought to become sensitized (Strassman et al. 1996) with consequent activation and sensitization of second-order neurons within the brainstem trigeminal nuclei (i.e., central sensitization) (Strassman et al. 1996; Burstein et al. 1998). Both human and animal studies appear to support this neurophysiological progression, with the primary throbbing head pain occurring early in the attack apparently the result of activation and sensitization of primary afferent trigeminal fibers, followed by the development of central sensitization and referred pain after a significant time delay (Landy et al. 2004).

Our lab was particularly interested in this symptom of migraine headache. Testing for allodynia has been suggested as a strategy to optimize migraine therapy in humans (Burstein et al. 2000a; Burstein et al. 2000b; LoPinto et al. 2006; Ashkenazi et al. 2007), because its absence typically indicates a more robust response to abortive type treatments, presumably reflecting the correlation between headache pain and expression of central sensitization (Burstein et al. 2000b; Dodick and Silberstein 2006). Quantitative sensory testing has reliably demonstrated the presence of central sensitization in numerous experimental and clinical pain conditions in humans (Cruccu et al. 2004; Rolke et al. 2006). Because the mechanisms by which extracranial allodynia occurs remain largely unknown, it was hypothesized that an animal model of migraine headache which
produced reproducible and quantifiable cutaneous allodynia could be utilized to mechanistically study the development and maintenance of migraine-related pain.

For these studies, we explored the time-related development of cutaneous allodynia following inflammatory mediator (IM) application to the dura as a model for intracranial pain in non-anesthetized freely-moving animals. Dural IM has previously been used in acute electrophysiological studies in anesthetized preparations (Burstein et al. 1998) and in models of pain-induced loss of appetite (Malick et al. 2001) and recurrent headache (Oshinsky and Gomonchareonsiri 2007). The present studies were adapted from previously conducted studies by employing a chronically implanted cannula placed superficial to the dura. This minimized potential mechanical trauma to the dura, and allowed recovery from surgery with subsequent measurement of changes in tactile thresholds following dural stimulation. A single application of dural IM elicited time-related allodynia of the periorbital facial region in the animals. Critically, expression of allodynia following IM was generalized to other regions of the body including the hindpaws.

In order to verify that we had created a relevant model for headache-related pain, current clinically effective and ineffective medications were administered to the animals for treatment of the allodynia. Tissue from the TNC of IM treated animals was also examined to confirm that IM produces central sensitization and activation of related pain pathways. Having established dural IM-induced cutaneous allodynia as a useful model of headache-related pain, we began to look into ways to explain the phenomenon for the development of extracranial allodynia. It seems plausible if not provable that activation of
dural afferents would cause associated pain in the cranial dermatome, but how does activation of pain pathways in the trigeminal system causally relate to allodynia in extracranial regions not receiving sensory input from the trigeminal system.

One of the most interesting things about pain transmission is that the integrated efferent message traveling from the somatosensory cortex to the periphery can be modulated by areas within the brainstem before it reaches its final peripheral destination. This is known as descending modulation of pain (Gebhart 2004). Recent limited imaging data from human migraineurs suggests the possibility that the brainstem could play a critical role (Weiller et al. 1995; Diener and May 1996) in headache and associated pain. Similarly, an area of the brainstem known as the rostral ventromedial medulla (RVM) has been shown to facilitate as well as inhibit different types of pain under experimental conditions (Ossipov and Porreca 2006; Heinricher and Ingram 2008). So we decided to examine the possible contribution of the RVM in migraine-related pain.

Not only were we interested in the RVM’s neuronal influence on migraine pain, it was also hypothesized that the glial cells, the microglia and astrocytes within the RVM, may play a supportive role in establishing and or modulating headache-related intracranial and extracranial allodynia. Glial cells like microglia and astrocytes, once thought only to be essential for immune response and their supportive role as housekeeping cells, have recently been implicated as targets for clinical pain (Watkins and Maier 2003; McMahon et al. 2005). Specifically, it has been established that glial cells in the spinal cord can modulate behavioral pain responses (Meller et al. 1994). More recently it has been suggested that activated glia located in supraspinal sites within the
trigeminal nucleus and the RVM in the brainstem can contribute to mechanisms of pain facilitation (Guo et al. 2007; Wei et al. 2008). Thus, the following studies describe the development of an animal model for headache-related pain, and the relationship between central sensitization and descending facilitation of pain through both neuronal and glial cell pathways.
CHAPTER 2
MATERIALS AND METHODS

2.1 Animals

Male, Sprague-Dawley rats (250-300 g; Harlan, Indianapolis, IN) were maintained on a 12-hr light/dark cycle (lights on 7am / lights off 7pm) with food and water available *ad libitum*. All procedures were performed in accordance with the policies and recommendations of the International Association for the Study of Pain, the National Institutes of Health guidelines for the handling and use of laboratory animals, and by the Animal Care and Use Committees of the University of Arizona and Oregon Health and Science University.

2.2 Surgical Preparations

**Dura cannulation:** Anesthesia was induced with ketamine/xylazine (80 mg/kg and 12 mg/kg i.p., respectively). The top of the head was shaved using a rodent clipper (Oster Golden A5 w/size 50 blade) and cleaned with betadine and 70% ethanol. Rats were placed in a stereotactic headholder (model 51600, Stoelting, Wood Dale, IL), a 2 cm incision was made in the skin using a scalpel (#10 blade) and the underlying connective tissue and skin were retracted using hemostats to expose the skull. The location of the bregma and midline bone sutures were identified, and a small hole (1mm in diameter) was made 1 mm left of midline and 1 mm anterior to the coronal suture in the frontal
bone of the skull. The hole was drilled with a hand drill (DH-0 Pin Vise, Plastics One Inc., Roanoke, VA), to carefully expose the dura. A guide cannula (22 GA, #C313G, Plastics One Inc.), designed to extend 0.5 mm from the pedestal to avoid irritation of the dural tissue, was inserted into the hole and sealed in place with glue. The design of the cannula allowed delivery of solutions to the underlying dural membrane without penetration or damage to the membrane (Fig. 1). Two additional 1 mm holes were made on either side of the midline, approximately 4-5 mm caudal to the cannula, to mount stainless steel screws (#MPX-080-3F-1M, Small Parts Inc., Miami Lakes, FL). The screws were superficially placed in the skull to prevent damage to the underlying dural membrane and dental acrylic was used to secure the cannula and screws to the skull (Fig. 2). After the dental acrylic dried (5 to 10 min), a dummy cannula (#C313DC, Plastics One Inc.) was inserted and secured to prevent contaminants from entering the guide cannula during the recovery period. The skin was sutured (3-0 silk suture) closed around the dried acrylic and the antibiotic Amikacin C (5 mg/kg, i.m.) was administered. Following recovery from anesthesia animals were housed separately for a 6-8 day recovery period. Cannula placement and integrity of the dura were confirmed with microinjection of 10 µL of India ink, which in successful surgeries spread 3-5 mm on the dorsal aspect of the dura and did not penetrate the brain (Fig 3).

**Dura + RVM cannulation:** Identical surgical procedures were used as in dura cannulation, with the addition of a bilateral rostral ventromedial medulla (RVM) guide cannula. Following skull exposure and dura cannula placement, a craniotomy exposed the
dura superior to the RVM location. A bilateral guide cannula (26GA, #C235G-1.2mm, Plastics One Inc.) was directed toward the lateral portion of the RVM using the atlas of Paxinos and Watson (1998) (anteroposterior, -11.0 mm from bregma; dorsoventral, -7.5 mm from the dura mater; lateral, ± 0.6 mm on either side of the midline) (Fig. 4A,B). Bone wax (Ethicon Inc., Somerville, NJ) plugged the RVM craniotomy. The RVM guide cannula and the dura cannula were secured to the skull using stainless steel screws and dental acrylic (Fig. 5). A dummy cannula (#C235DC, Plastics One Inc.) was inserted and secured to prevent contaminants from entering the RVM guide cannula. The skin was sutured around the acrylic using 3-0 silk suture. Animals prepared for CCK experiments received only RVM cannulation without dura cannulation. Animals were given the antibiotic Amikacin C (5 mg/kg, i.m.) and removed from the stereotaxic apparatus. Following recovery from anesthesia, animals were housed separately for a 6-8 day recovery period. Cannula placement was confirmed histologically using microinjection of India ink (0.5µl per side) followed by microscopic examination of Nissl-stained medullary sections or microscopic examination of fluorescent staining following drug treatments (Fig. 6).

**Acute single RVM injection:** Identical surgical procedures were used in this preparation as in dura cannulation except that animals received an acute bilateral injection into the RVM 28 days before testing day without requiring cannulation. Following skull exposure, the locations of the bregma and midline bone sutures were identified, and a craniotomy was made to expose the dura superior to the RVM location. A 30 gauge microinjector
needle on a 5.0 µl Hamilton syringe was lowered into the RVM twice for the bilateral injections (anteroposterior, -11.0 mm from bregma; lateral, ± 0.6 mm; dorsoventral, -8.5 mm from the dura mater). A stereotaxic syringe pump (Stoelting Co., Wood Dale, IL) was used to slowly microinject drug into the RVM. Following the injections, the syringe was retracted and the craniotomy was plugged with bone wax. The skin was sutured using 3-0 silk suture. Animals were given an antibiotic injection and removed from the stereotaxic apparatus. Following recovery from anesthesia, animals were placed in clean rat cages for a 21 day recovery period. On day 21, following the acute RVM injections, animals received dura cannulation. Animals were again given an antibiotic injection and removed from the stereotaxic apparatus. Following recovery from anesthesia, animals were housed separately for an additional 7 day recovery period before testing.

2.3 Behavioral Testing Protocols

**Facial sensory testing of non-noxious tactile stimuli in rats:** Prior to surgery and on the day of testing, animals were acclimated to suspended plexiglass chambers (30cm L x 15cm W x 20cm H) with a wire mesh bottom (1cm²) for 30-60 min. The animals were allowed to freely move about their chambers during the entire testing protocol. The baseline facial response thresholds to tactile stimuli were determined in response to probing with calibrated von Frey filaments (model 58011, Stoelting). Each von Frey filament was applied for 3 to 6 sec, perpendicular to the midline of the forehead, within a 3 mm diameter area at the level of the eyes, until buckling slightly. A positive response
was indicated by a sharp withdrawal of the head, which sometimes included an attempt to grasp and/or bite the filament. Special care was taken when applying the filaments to the forehead to prevent a positive facial withdrawal response due to dynamic force and/or deflection of the facial hairs. The animals required some additional acclimatization to the experimenter’s hand inside the cage before testing could begin. Animals were allowed to smell and explore the filament and the gloved hand of the experimenter before the sequence of consecutive filaments was applied to the forehead.

**Hindpaw sensory testing of non-noxious tactile stimuli in rats:** Hindpaw measurements were always taken in the same animals that received the facial testing. The baseline hindpaw withdrawal thresholds to tactile stimuli were also determined in response to probing with calibrated von Frey filaments. Each von Frey filament was applied perpendicularly to the plantar surface of both hindpaws until it buckled slightly, and was held for 3 to 6 sec. A positive response was indicated by a sharp withdrawal of the hindpaw.

**Experimental testing protocols:** Baseline behavioral responses to probing of the face and hindpaws were obtained from all rats prior to drug administration. Rats then received either SIF or IM via the dura cannula, and behavioral responses were determined at 1 hr intervals for 5-6 hrs. Drug administration was performed either 10 min prior to dural cannula injection or at time points after dural injection by either systemic injection or
RVM microinjection (Fig. 7). Animals prepared for the CCK-8(s) experiment received facial and hindpaw testing over a time course of 2 hr at 15 min intervals.

**Calculation of facial and hindpaw tactile withdrawal thresholds:** The 50% facial/hindpaw withdrawal thresholds were determined using a non-parametric method (Dixon 1980). An initial probe equivalent to 1.00 g (facial) or 2.00 g (hindpaw) was applied. If the response was negative, the stimulus was increased one increment; otherwise a positive response resulted in a decrease of one increment. The stimulus was incrementally increased until a positive response was obtained, then decreased until a negative result was observed. This "up-down" method was repeated until three changes in behavior were determined. The pattern of positive and negative responses was tabulated. The 50% facial/hindpaw withdrawal threshold is determined with the help of FlashDixon, a visual basic-based program developed in-house by M.H.O.. Maximal filament strengths 8.0 g and 15.0 g were used as the maximal cut-off values for non-noxious tactile stimulus of the face and hindpaw, respectively. Only animals with baselines of 8.0 g (facial) and 15.0 g (hindpaw) were used in the experiments.

**Capsaicin induced guarding and licking behavioral measurements:** Animals were placed in a suspended plexiglass chamber (60cm L x 60cm W x 40cm H) with a wire mesh bottom (1cm²) for observation. The rats were pretreated 30 min before intraplantar capsaicin injection with a sub-cutaneous dose of NK-1 antagonist. The amount of time
spent guarding and licking the capsaicin injected hindpaw was recorded using a stopwatch over a period of 5 min at 1 min intervals.

### 2.4 Injection Procedures & Reagents

**Dura cannula injections:** Animals were removed from the testing chamber and their dummy cannulas were removed. An injection cannula (28GA, #C313I, Plastics One Inc.) cut to fit the dura guide cannula, connected to a 25 µl Hamilton Syringe (#1702SN) by Tygon tubing (95607-14, Cole-Parmer, Vernon Hills, IL) was used to slowly inject 10 µl of the inflammatory mediator cocktail or vehicle onto the dura (Fig. 8A). The inflammatory mediator (IM) and synthetic interstitial fluid (SIF) composition were adapted from solutions previously reported. The IM solution was formulated to yield a final concentration of 2 mM histamine, serotonin, bradykinin and 0.2 mM prostaglandin E2 in 10 mM Hepes buffer, pH 5.0, representing twice that used by Burstein and colleagues (Burstein et al. 1998; Malick et al. 2001). The SIF consisted of 10 mM Hepes, 5 mM KCl, 1 mM MgCl$_2$, 5 mM CaCl$_2$, and 135 mM NaCl, pH 7.3 (all reagents for IM and SIF purchased from Sigma, Saint Louis, MO). Pilot studies were performed with varying IM concentrations, and the formulation providing a robust and consistent, yet submaximal, behavioral response was employed. Following injection, the dummy cannula was replaced and the animals were returned to their corresponding testing chambers.
**Subcutaneous injections:** Subcutaneous (s.c.) injections were performed by manually holding the animal and inserting a 25 gauge disposable needle on a disposable 1 cc syringe into the abdominal region of the animal assuring that the needle remained between the muscle and the skin of the animal. Injections of compounds or vehicles were performed over a 5 sec period and were noted as positive by the development of an out-pocketing of the skin at the site of injection. Animals that received drug pretreatment were given s.c. injections 10 min prior to dura cannula injections, with the exception of the NK-1 antagonist control studies where rats were pretreated 30 min prior to capsaicin injection. Animals that received drug post-treatment were given s.c. injections at 30 min, 1.5 or 2.5 hr following dura cannula injections. The following drugs were used in these studies and doses used were kept within range of published literature as cited: sumatriptan succinate (GlaxoSmithKline) 600 µg/kg in saline (Johnson et al. 2001; Kayser et al. 2002; Ottani et al. 2004; Burstein et al. 2005); naproxen sodium (Sigma) 100 mg/kg in saline (Clarke et al. 1994; Jakubowski et al. 2007); L-732,138 (NK-1 antagonist, Tocris, Ellisville, MO) 10 mg/kg in 70% DMSO/dH₂O (Cascieri et al. 1994; Cahill and Coderre 2002; Gao et al. 2003; King et al. 2005) and morphine sulphate 3 and 10 mg/kg in saline (Craft et al. 1995). Following injection, the animals were returned to their corresponding testing chambers.

**Intravenous injections:** Intravenous (i.v.) injections (30 min post-treatment) were performed by placing the animal into a rat restrainer (VWR International, Tempe, AZ) and holding their tail in warm water for 5 sec to dilate the tail vein. A 30 gauge
disposable needle on a disposable 1 cc syringe was inserted into the tail vein and α-CGRP(8-37) (CGRP-antagonist, Bachem, Torrance, CA) 0.15 mg/kg (O'Shaughnessy and Connor 1994; Ambalavanar et al. 2006) or 0.45 mg/kg in saline was injected. The doses used were kept within range of published literature as cited. Injections of the compound or vehicle were performed over a 10 sec period and were noted as positive by the presence of blood in the tip of the syringe before injection and the absence of an out-pocketing of the tail-skin at the site of injection. Following injection, the animals were returned to their corresponding testing chambers.

**Intraplantar injections:** Intraplantar (i.pl.) injections were performed following NK-1 antagonist pretreatment by manually holding the animal and inserting a 30 gauge disposable needle on a disposable 1 cc syringe just beneath the plantar surface of the skin of the hindpaw. Animals received an injection of capsaicin 10 µg/100 µl in saline. Injections of compound or vehicle were performed over a 5 sec period and were noted as positive by the development of an out-pocketing of the skin at the site of injection. Following injection, the animals were returned to their corresponding testing chambers.

**RVM cannula injections:** Animals were removed from the testing chamber and their dummy cannulas were removed. An injection cannula (33GA, #C235I-SPC w/ 1 mm projection, Plastics One Inc.), that protruded an additional 1.0 mm into fresh brain tissue, connected to a 10 µl Hamilton Syringe (#1701SN) by tygon tubing (95607-14, Cole-Parmer) was used to slowly inject 1.0 µl of solution (0.5 µl/side) into the RVM double
guide cannula (Fig. 8B). Animals that received drug post-treatment were given RVM injections at 30 min, 1.5 or 2.5 hr following dura cannula injections. The following drugs were used in these studies and doses were kept within range of published literature as cited: bupivacaine hydrochloride (Sigma) 0.5% solution in saline (Kovelowski et al. 2000); YM022 (CCK$_2$-antagonist, Tocris) 0.5 ng/µl in saline (Kitano et al. 2000); CCK-8(s) (American Peptide Company Inc., Vista, CA) 0.06 µg/µl in dH2O (Kovelowski et al. 2000; Xie et al. 2005); sumatriptan succinate (GlaxoSmithKline) 2 µg/µl in saline; minocycline hydrochloride (Sigma) 25 µg/µl in saline, gently heated in water bath until dissolved (dose response performed in Porreca laboratory, unpublished data). Following injection, the animals were returned to their corresponding testing chambers.

**RVM acute injections:** Animals received injections under anesthesia ketamine/xylazine (80 mg/kg and 12 mg/kg i.p., respectively). Once the Hamilton syringe was lowered to the proper coordinate, drug was slowly expelled at 0.5 µl/min. Each rat received a total volume of 1.0 µl of solution (0.5 µl/side) into the RVM. The syringe was left in place following the injections for 1 min to allow the solution to penetrate the tissue and to prevent backflow up the syringe tract. The syringe was then slowly retracted and the animal’s incision was closed. The following drugs were used in these studies and doses used were kept within range of published literature as cited: dermorphin-saporin (Advanced Targeting Systems, San Diego, CA) 0.1 µg/µl in saline, and saporin (Advanced Targeting Systems) 0.1 µg/µl in saline (Porreca et al. 2001; Burgess et al. 2002).
2.5 Immunolabeling

**FOS immunohistochemistry:** Animals used for FOS immunohistochemistry received dura cannulation as described above and their hindpaw withdrawal thresholds were evaluated on testing day. Animals received no facial testing during this protocol so as not to confound the FOS immunolabeling by potentially inducing touch-evoked FOS. The animals received dural cannula injection and were tested for 2 hr before perfusion to verify behavioral responses to IM. In some cases, s.c. injections of sumatriptan succinate were given 10 min prior to dura cannula injections. Animals were anesthetized using ketamine/xylazine (80 mg/kg and 12 mg/kg i.p., respectively) and perfused transcardially at 3 hr following dura injection, with 400 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 500 ml of 4% paraformaldehyde in PBS. Brainstem tissue was fixed overnight in 4% paraformaldehyde (Sigma) and cryoprotected for 48 hrs in 30% sterile sucrose solution. The following DAB immunostaining protocol was used for FOS labeling. Sections 40 µm thick were collected serially through the caudal medulla at the level of trigeminal nucleus caudalis (TNC) in 0.1 M PBS for free-floating immunohistochemistry. After three 10 min washes in PBS, sections were pre-incubated for 30 min at room temperature in 0.3% hydrogen peroxide (Sigma). Following three 10 min washes in PBS, sections were incubated for 2 hrs at room temperature in blocking buffer (0.1 M PBS, 0.25% Triton X-100 (Sigma), 3.0% Normal Goat Serum). Sections were then incubated for 48 hours at 4ºC in FOS primary antibody in PBS (Ab-5; specific to the FOS N-terminal domain, 1:20,000, Oncogene Science, San Diego, CA). After three
10 min washes in PBS, sections were incubated with secondary antibody in PBS for 2 hrs at room temperature (biotinylated goat anti-rabbit IgG, 1:600, Vector Laboratories, Burlingame, CA). Following three 10 min washes in PBS, sections reacted with avidin-biotin complex in PBS for 1 hr at room temperature (1:200, Vector Elite Kit, Vector Laboratories). After three 10 min washes in PBS, sections reacted in dH$_2$O containing 0.7 mg/ml DAB, 0.17 mg/ml urea-hydrogen peroxide, and 0.06 M Tris buffer (DAB Sigma-Fast 3, 3’- diaminobenzidine Tablet Kit, Sigma). The reaction was terminated after 6 min with 3 washes in PBS. Sections were then mounted onto slides, air-dried and coverslipped using Permount mounting media (Fisher Scientific, Tustin, CA).

**Glial cell immunofluorescence:** Animals used for glial immunofluorescence received dura + RVM cannulation as described above. Following behavioral evaluation, some rats were used to measure glial cell activation in the RVM. Fresh brainstem tissue was taken from the animals following dural injection of IM or SIF and one of the following treatments in the RVM: minocycline hydrochloride, sumatriptan, or saline. All compounds were administered 0.5 hrs following the dural injection, and tissue was taken between 1.5 and 2.5 hrs following dural injection. The brainstem tissue was fixed overnight in 10% formalin (Sigma) and cryoprotected for 48 hrs in 30% sterile sucrose solution. Medullary sections (20 µm thick, cut on a cryostat) were collected serially in 0.1 M tris-buffered saline (TBS, pH 7.4) for free-floating immunohistochemistry. After three 10 min washes in TBS, sections were incubated for 1 hr at room temperature in blocking buffer (0.1 M TBS, 0.5% Triton X-100 (Sigma), 5.0% Normal Donkey Serum (NDS,
Jackson ImmunoResearch, West Grove, PA). Sections were then incubated for 24 hours at room temperature in primary antibody. Two primary antibodies were used in TBS with 2% NDS: CD11b (OX-42 for microglia, mouse monoclonal antibody, 1:500, Serotec, Raleigh, NC), and glial fibrillary acidic protein (GFAP for astrocytes, mouse monoclonal antibody, 1:1000, Sigma). After three 10 min washes in TBS, sections were incubated with secondary antibody for 2 hrs at room temperature. FITC-conjugated donkey anti-mouse antibody (1:1000, Jackson ImmunoResearch) was used for OX-42 and GFAP secondary labeling. After three 10 min washes in TBS, sections were then mounted onto slides, air-dried and coverslipped using Vectashield hard-set mounting medium (Vector Laboratories).

5-HT₁B/₁D immunofluorescence: Naive brainstem tissues were processed to localize serotonin 1B and 1D (5-HT₁B and 5-HT₁D) receptors in the RVM. Trigeminal ganglion tissues were also taken and used as a control for 5-HT staining (Ma et al. 2001) in the RVM. The animals were anesthetized using ketamine/xylazine (100 mg/kg, i.p.) and perfused transcardially with 400 ml of phosphate-buffered saline (PBS; pH 7.4) followed by 500 ml of 4% paraformaldehyde in PBS. The brainstems were removed and post-fixed for 24 hr in 4% paraformaldehyde then cryoprotected for 48 hr in 30% sterile sucrose solution. Medullary sections and trigeminal ganglion (cut 20 µm thick on a cryostat) were collected serially in 0.1 M TBS for free-floating immunohistochemistry. After three 10 min washes in TBS, sections were incubated for 1 hr at room temperature in blocking buffer (0.1 M TBS, 0.5% Triton X-100 (Sigma), 5.0% NDS, (Jackson ImmunoResearch)).
Sections were initially incubated for 24 hrs at 4°C in primary antibodies for 5-HT\textsubscript{1B} or 5-HT\textsubscript{1D}. The primary antibodies for microglia, astrocytes, neuronal nuclei, and blood vessel endothelial cells were added to the solution for an additional 24 hrs of incubation at 4°C. The following primary antibodies were used in TBS with 2% NDS: serotonin receptor 1B; SR-1B(M-19) (5-HT\textsubscript{1B}, goat polyclonal antibody, 1:250, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and serotonin receptor 1D; SR-1D(S-18) (5-HT\textsubscript{1D}, goat polyclonal antibody, 1:250, Santa Cruz Biotechnology, Inc.) OX-42 (for microglia, mouse monoclonal antibody, 1:500, Serotec), GFAP (for astrocytes, mouse monoclonal antibody, 1:1000, Sigma), NeuN (for neuronal nuclei, mouse monoclonal antibody, 1:1000, Chemicon), and RECA-1 (for blood vessel endothelial cells, mouse monoclonal antibody, 1:500, Serotec). After three 10 min washes in TBS, sections were incubated with secondary antibodies for 2 hrs at room temperature. Texas Red, donkey anti-goat antibody (1:500, Santa Cruz Biotechnology) was used for 5-HT\textsubscript{1B} and 5-HT\textsubscript{1D} labeling. FITC-conjugated donkey anti-mouse antibody (1:1000, Jackson ImmunoResearch) was used for OX-42, GFAP, NeuN, and RECA-1 secondary labeling. After three 10 min washes in TBS, sections were then mounted onto slides, air-dried and coverslipped using Vectashield hard-set mounting medium.

2.6 Imaging & Cell Counting

The sections of RVM, trigeminal ganglion, and TNC were examined using a Nikon E800 microscope equipped with a digital Hamamatsu C4742-95 color camera along with
Wasabi software (version 1.5) for fluorescence imaging, and a digital Infinity 3 color camera that used Infinity Capture Application (version 3.7.5.) for light imaging. Images were prepared for presentation using CS2 Adobe Photoshop, version 9.02.

**FOS profile counting:** The location selected for FOS analysis was located 1 mm caudal to the trigeminal interpolar (V₁)/trigeminal caudalis (V₂) border. This location was based on a sampling of the TNC following IM injection to determine the area of the nucleus with the most robust change in FOS expression in comparison to naïve animals. FOS expression was analyzed from tissues under the following conditions: naïve, surgery only, SIF injection, IM injection, sumatriptan/SIF, and sumatriptan/IM. A total of 5 sections were counted from each rat, and 4 rats were examined from each treatment group.

### 2.7 Electrophysiological Studies

Male Sprague-Dawley rats (Taconic, Germantown, NY, 250-300g) were anesthetized with pentobarbital (60 mg/kg, i.p.) and prepared for RVM extracellular single unit recording as previously described (Heinricher and Ingram 2008). A cannula was implanted over the left frontal bone for infusion of IM as described above. Following surgery the animals were maintained in a lightly anesthetized state using a continuous infusion of methohexital (15-30 mg/kg per hr, i.v.). RVM neurons were classified as previously described (Heinricher and Ingram 2008). “OFF cells” showed an abrupt pause in ongoing activity just prior to paw withdrawal (PW). “ON cells” were identified by a
sudden burst of activity just prior to PW. “Neutral cells” were identified by no change in activity associated with PW. Following three baseline PW trials (5 min intervals), IM or vehicle was applied to the dura. Cell activity and PW latency were then monitored for an additional 105 min. Only one protocol was performed in each animal. At the conclusion of the experiments, recording sites were marked with an electrolytic lesion and patency of the dural cannula verified by dye injection. Recording sites were distributed in the RVM as in previous reports (Heinricher and Ingram 2008).

2.8 Data Analysis

Withdrawal thresholds to probing the face and hindpaws were determined at 1 hr intervals after administration of IM or SIF. Data were converted to area under the time-effect (AUC) curve and normalized as a percent of the vehicle-treated control group to allow for multiple comparisons. Comparisons among several treatment groups were performed by 2-factor ANOVA for repeated measures. Comparisons among treatment groups from the capsaicin induced guarding and licking study were made using Student’s t-test. Cell counts among treatment groups were evaluated with ANOVA followed by Student-Neuman-Keuls test. Electrophysiological data were analyzed using Friedman’s analysis of variance by ranks followed by Wilcoxon’s signed ranks test for post-hoc analysis. Significance was set at $P \leq 0.05$ for all data comparisons. Statistical analyses were performed with FlashCalc, a visual basic-based program developed in-house by M.H.O..
3.1 Dural IM Elicits Cutaneous Allodynia

Tactile withdrawal thresholds from both the face and the hindpaw were measured for deviations from baseline following dural application of IM or SIF. There were no significant differences in baseline tactile thresholds between naïve animals and animals which received dura cannulation 7 days earlier ($P > 0.05$) indicating that the surgery did not produce long-lasting effects on behavioral response thresholds (Fig. 9A,B). SIF injection did not produce significant tactile allodynia in either the face or the hindpaw ($P > 0.05$) (Fig. 9A,B). Preliminary experiments demonstrated that application of IM cocktail to the dura at either 1, 2 or 4 times the standard concentration originally reported produced significant allodynia of the face and hindpaws. However, the concentration 2 mM histamine, serotonin, bradykinin and 0.2 mM PGE$_2$, two times that originally reported, elicited the most robust and reproducible behavioral sensitivity and was used for all further experiments (Fig. 10). Animals receiving IM developed tactile allodynia of the face, as indicated by significant ($P < 0.0001$) reductions in facial response thresholds compared to SIF injection thresholds (Fig. 9A). The same animals with IM injections also developed tactile allodynia of the hindpaw, as indicated by significant ($P < 0.0001$) reductions in hindpaw response thresholds compared to SIF injection thresholds (Fig. 9B). The reductions in threshold were not observed immediately after dural IM but developed over time peaking at the 3 hr time point. Thresholds subsequently moved towards
baseline levels during the last 3 hrs of testing. A small number of animals injected with IM did not develop tactile allodynia in either the face or the hindpaw. Nevertheless, the data from these animals are included in the analysis of all experiments.

### 3.2 FOS Expression in the TNC

A quantitative assessment of FOS levels in the TNC showed that dural cannulation surgery alone elicited a significant ($P < 0.05$) increase in FOS expression, expressed as $9.0 \pm 0.7$ positive profiles per section, relative to naïve animals, which showed only $1.7 \pm 0.4$ FOS positive neuronal profiles per section (Fig. 11A). This result suggests surgery produces an inflammatory response with the likelihood of some associated sensitization in the TNC. The dural administration of SIF (Fig. 11A,D) produced a moderate $13.9 \pm 1.3$, but significant increase ($P < 0.05$ relative to the surgery only group) in FOS expression that was further doubled to $26.6 \pm 1.9$ profiles by dural IM ($P < 0.05$ as compared to SIF) (Fig. 11A,C). Animals pretreated with sumatriptan before IM or SIF injection expressed FOS positive profiles of $16.5 \pm 1.6$ and $13.7 \pm 1.0$ cells, respectively. Sumatriptan therefore, abolished IM-induced increase in FOS expression (Fig. 11A) indicated by no significant differences between sumatriptan pretreatment/IM, sumatriptan pretreatment/SIF, or SIF groups ($P > 0.05$).
3.3 Systemic Drug Treatment of IM-Induced Cutaneous Allodynia

Subcutaneous sumatriptan and naproxen: The possible effects of pretreatment or post-treatment with s.c. sumatriptan or naproxen on IM-induced cutaneous allodynia of the face and hindpaw were determined in separate groups of animals. Sumatriptan and naproxen significantly attenuated both facial (Fig. 12A & 13A) and hindpaw (Fig. 12B & 13B) IM-induced tactile allodynia when given as a pretreatment 10 min before or as a post-treatment 30 min after dural injection. Both facial and hindpaw thresholds were significantly \( P < 0.05 \) reduced in the presence of either sumatriptan or naproxen. However, post-treatment with sumatriptan administered at 1.5 hr and 2.5 hr after IM and naproxen given at 2.5 hr after IM did not significantly attenuate IM-induced tactile allodynia of the face or the hindpaw \( (P > 0.05) \) (Fig. 12A,B & 13A,B). Neither sumatriptan nor naproxen altered behavioral responses in SIF-treated animals over the 6 hr time course (Fig. 12 and 13).

Subcutaneous morphine: Systemic morphine was given as a 0.5 hr post-treatment for the IM-induced cutaneous allodynia. Administration of s.c. morphine at 10 mg/kg and 3 mg/kg morphine both significantly attenuated facial \( (P < 0.05) \) and hindpaw \( (P < 0.05) \) tactile allodynia (Fig. 14). However, the 10 mg/kg dose produced behavioral signs of sedation, known to be an effect of high doses of opiates. The 3 mg/kg dose was able to attenuate the IM-induced allodynia without the behavioral manifestations of sedation.
Systemic morphine did not alter behavioral thresholds in SIF-treated animals over the 5 hr time course (Fig. 14).

**Intravenous CGRP receptor antagonist:** The effects of a CGRP receptor antagonist on IM-induced cutaneous allodynia were explored by administration of i.v. α-CGRP\textsubscript{(8-37)}. The 0.45 mg/kg dose of α-CGRP\textsubscript{(8-37)} significantly attenuated ($P < 0.05$) both facial and hindpaw IM-induced tactile allodynia when given 30 min following dural injection, however, the dose 0.15 mg/kg had no effect on behavioral allodynia ($P > 0.05$) (Fig. 15). Systemic α-CGRP\textsubscript{(8-37)} did not alter sensory thresholds in SIF-treated animals over the 6 hr time course (Fig. 15).

**Subcutaneous NK-1 antagonist:** In contrast, pretreatment 10 min prior to IM with L-732,138, an NK-1 antagonist, failed to prevent IM-induced cutaneous allodynia of the face or the hindpaw ($P > 0.05$) (Fig. 16A). L-732,138 given to SIF-treated rats did not cause any significant changes in facial or hindpaw withdrawal threshold over the 6 hr time course (Fig. 16A). A separate study was conducted to test that the dose of NK-1 antagonist employed was sufficient to block NK-1 agonist activity. Rats were pretreated with either vehicle or L-732,138 and then challenged with an intraplantar injection of capsaicin. The time spent licking or guarding the hindpaw was determined over a 10 min period. Capsaicin treatment following vehicle injection produced a mean cumulative response time of 133 ± 33.3 sec. Rats injected with L-732,138 showed a significantly ($P$
< 0.05) reduced mean cumulative response time of 46 ± 14.7 sec, suggesting that the dose of L-732,138 was sufficient to block the NK-1 mediated nociceptive activity (Fig. 16B).

### 3.4 RVM Microinjection Studies

**Microinjection of bupivacaine:** The bilateral microinjection of 0.5 µl of 0.5% w/v bupivacaine, a short-acting local anesthetic, into the RVM 30 min after dural IM reversibly prevented, or significantly reduced ($P < 0.05$), facial and hindpaw allodynia (Fig 17A, B). Administration of bupivacaine 1.5 hr after IM significantly ($P < 0.05$) and reversibly attenuated facial and hindpaw allodynia (Fig. 17C, D). RVM bupivacaine did not cause any significant changes in the behavioral responses of rats treated with SIF over the 6 hr time course ($P > 0.05$) (Fig. 17A-D).

**Microinjection of dermorphin-saporin:** The µ-opioid receptor agonist dermorphin conjugated to the cytotoxin saporin (Derm-Sap) was used to selectively lesion RVM µ-opioid receptor expressing (i.e., putative pain-facilitation) cells, as previously described (Porreca et al. 2001; Burgess et al. 2002). The unconjugated saporin (Sap) was used as a control. Twenty-eight days following RVM Derm-Sap or Sap treatment, rats were challenged with a dural injection of IM or SIF. While dural IM elicited allodynia in Sap pretreated rats, Derm-Sap significantly ($P < 0.05$) abolished or diminished IM-induced facial and hindpaw allodynia (Fig. 18). Pretreatment with Derm-Sap or Sap along with
dural SIF did not cause any significant changes in baseline facial or hindpaw withdrawal thresholds (Fig. 18).

**Microinjection of CCK:** Microinjection of cholecystokinin: CCK-8(s) into the RVM of otherwise untreated rats produced time-dependent and significant ($P < 0.05$) hindpaw and facial allodynia in comparison to dH$_2$O microinjection (Fig 19A,B). The reductions in hindpaw response thresholds were consistent with previous observations. The reductions in both facial and hindpaw thresholds peaked within 30-45 min of injection and subsequently moved back towards baseline levels by the 2 hr time-point of testing.

**Microinjection of CCK$_2$-antagonist:** To determine whether endogenous CCK in the RVM participates in IM-induced cutaneous allodynia, YM022 (a CCK$_2$-antagonist) was microinjected 30 min following dural injection of IM or SIF. Administration of YM022 significantly attenuated both facial and hindpaw allodynia ($P < 0.05$) without affecting thresholds in SIF-treated animals over the 6 hr time course (Fig. 20).

**Microinjection of sumatriptan and minocycline:** The behavioral effects of early vs. late post-treatment with RVM sumatriptan or RVM minocycline (a selective microglial inhibitor) were explored in separate groups of animals following dural IM. RVM sumatriptan or minocycline given 30 min after dural injection significantly ($P < 0.05$) attenuated both facial (Fig. 21A) and hindpaw (Fig. 21B) IM-induced allodynia. However, post-treatment with sumatriptan or minocycline 1.5 hr after IM did not significantly
attenuate \((P > 0.05)\) or reverse behavioral allodynia of the face (Fig. 21A) or the hindpaw (Fig. 21B). Neither RVM sumatriptan nor RVM minocycline altered behavioral responses in SIF-treated animals over the 6 hr time course (Fig. 21).

3.5 IM-Related RVM Electrophysiology

Responses of physiologically identified “ON” cells, “OFF” cells and neutral cells were recorded following dural IM or SIF (Fig. 22). “ON” cells were potently activated during IM application, and this was followed by a progressive increase in ongoing firing rate which was maintained for 105 min (Fig. 22B). Reflex-related firing rate was increased in parallel with ongoing activity (Fig. 22A). By contrast, “OFF” cells displayed a transient inhibition during and immediately after the IM infusion, but this was not maintained and activity recovered to baseline within 10 min (Fig. 22B). The firing of neutral cells was unaffected by IM application (Fig. 22B). Infusion of SIF had no effect on the discharges of “ON” or “OFF” cells (Fig. 22C).

3.6 Glial Cell Immunofluorescence in the RVM

**IM-induced microglial activation in the RVM:** An initial survey was conducted to explore IM-dependent glial activation in the RVM following IM or SIF treatment. Five separate groups of tissue were taken for the analysis: Naïve, SIF-treated or IM-treated (taken at 1.5 hrs post injection), and SIF-treated or IM-treated (taken at 2.5 hrs post
injection). All conditions were processed for OX-42 (microglia) and GFAP (astrocytes) fluorescence and then evaluated by using morphological change in cellular structure as the variant; hypertrophy of the microglia and astrocytes could indicate glial cell activation. In comparison to naïve animals, the SIF-treated tissues indicated a slight increase in hypertrophy of microglial cells at both the 1.5 and 2.5 hr time-points (Fig. 23). However, the findings indicate a greater increase in microglial hypertrophy following IM-treatment at both the 1.5 and 2.5 hr time-points (Fig. 23). The microglia of the IM-treated animals appear to be more amoeboid-like in shape with shorter processes than those of naïve animals. In contrast, there does not appear to be any morphological difference in astrocyte structure following treatment with either SIF or IM in comparison to naïve animals (Fig. 24).

**RVM sumatriptan or minocycline treatment vs. IM-induced microglial activation:**
The bilateral RVM microinjection of sumatriptan appears to attenuate the hypertrophy in microglia seen 2.5 hrs following dural-IM treatment (Fig. 25). In addition, administration of RVM minocycline also appears to reverse the hypertrophy in microglia seen following dural-IM treatment (Fig. 25). The tissue of sumatriptan and minocycline treated animals resembles that of the saline treated and/or naïve animals. Neither sumatriptan nor minocycline appear to have any effect on microglial cellular structure in SIF-treated animals in comparison to naïve (Fig. 25).
3.7 RVM 5-HT$_{1B/1D}$ Immunofluorescence

Naïve RVM tissue was analyzed for localization of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. The trigeminal ganglion tissue was used as a control for the receptor colocalization based on the study by (Ma et al. 2001), where they found weak staining for 5-HT$_{1B}$ and 5-HT$_{1D}$ localized within neurons of the trigeminal ganglion tissue. RVM tissues stained for 5-HT$_{1B}$ showed a significant amount of punctuate staining co-localized with the RECA-1 antibody for blood vessels (Fig. 26). However 5-HT$_{1B}$ labeling was not co-localized with neuronal cells in either the trigeminal ganglion or the RVM. In addition, 5-HT$_{1B}$ labeling did not show any co-localization with either the microglia or the astrocytes in the RVM (data not shown). RVM tissues stained for 5-HT$_{1D}$ receptors showed a very small amount of punctuate staining co-localized with the RECA-1 antibody for blood vessels (Fig. 26). Similarly, 5-HT$_{1D}$ receptors were not co-localized with neurons in either the RVM or trigeminal tissues, nor the microglia and astrocytes within the RVM (data not shown).
4.1 Cutaneous allodynia triggered by dural inflammation as a model for headache-related pain

The present studies evaluated the behavioral consequences of application of IM to the dura of the rat in an effort to explore the mechanisms associated with headache pain. We have demonstrated that chemical inflammation of the dura elicits cutaneous allodynia that extends beyond the trigeminal dermatome. Specifically, IM applied to the dorsal surface of the dura produced a pronounced and significant decrease in both the facial and hindpaw withdrawal thresholds in response to tactile stimulation. These responses occur over several hours following IM, consistent with the time-course for the development of cutaneous allodynia seen in migraine patients (Jakubowski et al. 2005; Landy et al. 2007) and complementing previously reported acute electrophysiological responses (Burstein et al. 1998; Jakubowski et al. 2005).

The pharmacology of IM-induced allodynia demonstrated many important parallels with the clinical pharmacology of pain associated with migraine headache. The triptans are very effective in the acute treatment of migraine, yet their site of action both anatomically and pharmacologically is still a matter of debate. Binding studies conducted in both human and rat brain tissues have revealed complimentary evidence of both 5-HT_{1B}, 5-HT_{1D}, and to some extent 5-HT_{1F} receptors within the brain, brainstem and spinal cord (Bruinvels et al. 1994; Castro et al. 1997). It is still uncertain whether or not the receptors are located pre- or post-synaptically on neurons, but the studies suggest a
predominance of 5-HT$_{1B}$ receptors localized in the brain tissue as well as blood vessels (Jazayeri et al. 1989; Hamel et al. 1993). The significant levels of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors within brain tissues responsible for pain control implicate a functional role for them in antinoceptive mechanisms (Castro et al. 1997). A growing body of evidence pharmacologically suggests that triptans, acting primarily as an agonist at 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors within the trigeminovascular system to decrease vasodilatation in the dura and neuronal activation, are the most effective at relieving migraine associated symptoms when the treatment is given early following the initial onset of the attack (Burstein et al. 2004; Landy et al. 2007). Our findings suggest that this is certainly true in the case of IM-induced cutaneous allodynia. Pretreatment and early post-treatment with sumatriptan, a current first-line therapy for migraine headache, was effective against IM-induced allodynia. However, sumatriptan appears to lose efficacy when the systemic treatment is delayed following dural inflammation with IM.

The NSAID naproxen, a non-specific cyclooxygenase (COX1/COX2) inhibitor, does not readily cross the blood-brain barrier (Phelan and Bodor 1989) and it is difficult to elucidate naproxen’s peripheral versus central effects in migraine specific pathophysiology. It has been proposed that systemic administration of naproxen alleviates central activation of second-order medullary dorsal horn neurons (Jakubowski et al. 2007) and that systemic and topical administration of naproxen to the dura will decrease activation of primary afferent trigeminal neurons (Levy et al. 2008). Nevertheless, naproxen’s general mechanism of action is inhibition of prostaglandin synthesis, a product of COX enzyme activity. Prostaglandins released by inflamed tissues
do not generally activate nociceptive fibers directly; they tend to sensitize both pre- and post-synaptic sensory fibers to the presence of other inflammatory mediators (Rang et al. 1991; Clarke et al. 1994). Our finding that systemic treatment with naproxen significantly inhibits IM-induced cutaneous allodynia infers that prostaglandin inhibition blocks sensitization of the trigeminovascular system. From these studies, it is still unclear whether or not naproxen is working through a peripheral or a central site of action. However, because naproxen treatment is more successful when given early versus late following IM the time-course of efficacy would suggest blockade of peripheral COX mediated primary afferent sensitization, which may be sufficient to block central sensitization and decrease alldynia. The most recent suggestion for migraine therapy is a sumatriptan/naproxen combination treatment. The theory behind this is that the drugs work at two different sites of action to decrease neuronal sensitization resulting in long lasting relief for patients (Cleves and Tepper 2008).

Opioids are extremely effective as an acute treatment for migraine headache in the clinic (Dodick and Freitag 2006). The peripheral and central actions of opioids are similar to those of triptans and could account for their efficacy in treating acute migraine pain. Studies indicate that opioids have the ability to block neurogenic dural vasodilatation through peripheral sites of action on perivascular trigeminal afferents as well as decrease nociceptive neurotransmission within the TNC (Williamson et al. 2001). Although morphine has the ability to interact with the three opioid receptor subtypes µ, δ and κ, its analgesic effects in migraine-related pathology appear to be through a µ-opioid receptor mechanism, demonstrated by the inability of selective δ- and κ-agonists to block dural
vasodilatation (Williamson et al. 2001). Our finding that systemic morphine treatment easily suppresses IM-induced cutaneous allodynia parallels its efficacy seen in the clinic. One of the newest drug targets for migraine-related therapy is the inhibition of calcitonin gene related peptide (CGRP) activity within the nervous system. CGRP is a known vasodilator as well as a potent pro-inflammatory neuropeptide. Recent studies have shown that the release of CGRP into the blood stream (Goadsby et al. 1990; Goadsby and Edvinsson 1994) and the perivascular space from trigeminal nerve endings (Knyihar-Csillik et al. 2001; Zimmermann et al. 2002) may play a primary role in migraine pathophysiology. The peptide CGRP receptor antagonist α-CGRP(8-37), though not suitable for human use, was used as a tool in these studies to examine the role of CGRP in IM-induced cutaneous allodynia. The finding that early systemic post-treatment with this compound is able to significantly block IM-induced cutaneous allodynia of both the face and the hindpaw correlates with the drug’s ability to block α-CGRP-induced dilatation of the middle cerebral artery (Edvinsson et al. 2007) and block plasma extravasation and head and hindlimb mechanical allodynia following deep tissue inflammation (Ambalavanar et al. 2006). The data are consistent with clinical trials (Doods et al. 2007; Ho et al. 2008) and support the ongoing investigation of CGRP antagonists for the treatment of migraine related pain.

It has been hypothesized that the neuropeptide substance P plays a role in migraine pain by inducing neurogenic inflammation within the dural meninges through activation of NK-1 receptors on the perivascular terminals of trigeminal afferents (Markowitz et al. 1987). The non-peptide NK-1 receptor antagonist L-732,138 was found
to have extremely high affinity for the human NK-1 receptor, with a 200 fold lower affinity for rat NK-1 receptor (Cascieri et al. 1994). Nevertheless, animal studies that have used the NK-1 antagonist L-732,138 as a treatment for opioid-induced hyperalgesia, neuropathic pain, and inflammation have demonstrated relief of pain symptoms (Cahill and Coderre 2002; Gao et al. 2003; King et al. 2005). Studies involving noxious chemical inflammation of the dura have shown that the NK-1 antagonist RPR 100893 can decrease the expression of FOS within the trigeminal nucleus caudalis (TNC) (Cutrer et al. 1995). And the NK-1 antagonist RP 67580 has been shown to decrease protein plasma extravasation (PPE) induced by trigeminal nerve stimulation (Moussaoui et al. 1993). All of this evidence, in part, supports the development of NK-1 antagonists for the treatment of migraine pain. Yet many reports have found that the NK-1 antagonists lack efficacy in the clinic for this manifestation of pain. Recent studies have shown that NK-1 antagonist Lanepitant was not effective as an acute (Goldstein et al. 1997) or prophylactic (Goldstein et al. 2001) therapy for migraine pain nor did it change the severity of migraine associated symptoms. Thus, L-732, 138 was used as a negative control for our present study. The finding that this compound does not attenuate IM-induced tactile allodynia in our behavioral model yet was able to significantly attenuate nocifensive guarding and licking behavior when given as a pretreatment to capsaicin injection into the hindpaw, suggests that cutaneous allodynia associated with dural inflammation is more complicated than blockade of substance P alone. Consequently, this observation is consistent with the clinical failure of NK-1 antagonists in treatment of migraine headache.
These pharmacological data help to establish dural IM-induced facial and hindpaw allodynia as a useful animal surrogate of migraine headache-associated pain, as well as for allodynia associated with primary headaches including cluster headache, SUNCT, and other paroxysmal types of headaches (Goadsby and Edvinsson 1994; Pareja and Sjaastad 1997; Sjaastad and Bakketeig 2007).

4.2 IM-induced cutaneous allodynia and central sensitization

As mentioned, IM-induced allodynia developed over several hours and was found not only on the face, which like the dura is innervated by the trigeminal system (Bereiter et al. 2008), but also extrasegmentally on the hindpaws. These observations suggest that the presenting allodynia is not a direct effect of the chemical or mechanical stimulus from the injection, but most likely related to central activation of pain pathways considering the delayed onset. This is also consistent with electrophysiological data following dural inflammation. In previous experiments, application of the local anesthetic lidocaine to the dura attenuated responses of sensitized trigeminal neurons to stimulation of the dura but not of the skin, indicating that sensitization of central trigeminal pathways was not dependent on ongoing activity from the dura (Burstein et al. 1998). The expression of the proto-oncogene product FOS in the TNC is related to neuronal excitation and correlates with trigeminal activation (Moskowitz et al. 1993; Malick et al. 2001; Schuh-Hofer et al. 2006). Studies have also shown that enhanced behavioral responses to light touch and increased FOS expression are indicators of central sensitization in pain models (Hunt et al. 1987).
To validate that our model of dural inflammation was producing central activation of the trigeminal system, FOS expression was measured in the TNC following IM application. Dural IM produced a significant increase in FOS expression in TNC, consistent with previous work (Malick et al. 2001) which was blocked by administration of sumatriptan. IM-induced central sensitization is supported by the effectiveness of sumatriptan in suppressing allodynia only when given as a pretreatment or early post-treatment. These data parallel some (Burstein et al. 2004; Landy et al. 2007) but not all (Cady et al. 2007; Goadsby et al. 2008) clinical studies showing that triptan administration blocked cutaneous allodynia when administered upon migraine onset, but are relatively less effective when administered two hours later. A similar time course was seen in electrophysiological studies (Burstein and Jakubowski 2004), suggesting that triptans block transmission from primary afferents to second-order neurons by actions on presynaptic 5-HT\textsubscript{1B/1D} receptors in this pathway. If this is true, the triptans would not be expected to reverse sensitization of second-order neurons in the trigeminal system once it had been established (Burstein and Jakubowski 2004; Levy et al. 2004). Early intervention with triptans has generally been more successful than late intervention, regardless of presence of allodynia (Cady et al. 2007; Goadsby et al. 2008).

4.3 Descending facilitation of headache-related pain

The present studies extend the idea of sensitization beyond the trigeminal sensory system to include pain-facilitating systems arising from the RVM (Heinricher and Ingram 2008). Because the RVM has been identified as the final common output for descending
influences on pain transmission from sites within the brain (Gebhart 2004), we
investigated the mechanism for the development of extracranial hypersensitivity through
manipulation of RVM influence.

Initial electrophysiological studies profiling RVM cellular activity demonstrated
that dural IM elicits a slowly developing but prolonged activation of RVM “ON” cells, a
cell class known to facilitate nociceptive processes at the level of the dorsal horn. Notably, RVM “OFF” cells, which suppress nociceptive transmission, showed only a
transient suppression of firing during and immediately after dural IM application. This is
consistent with the literature that shows an increase in “ON” cell activity correlates with
nociceptive stimulation (Morgan and Fields 1994). The SIF application alone did not
produce any profound effect on RVM cellular activity. Not only do these findings
indicate a specific effect by the IM application but they confirm that the resulting
cutaneous allodynia could be a consequence of central sensitization of the trigeminal
system as well as activation of descending facilitatory pathways.

Pharmacological inactivation of the RVM with a local short-acting anesthetic
significantly, but reversibly, attenuated the development of the IM-induced cutaneous
allodynia during the time-course of action of the drug. Permanent destruction of putative
pain facilitation cells, those expressing the µ-opioid receptor, and a group likely to
include “ON” cells was conducted using the µ-opioid receptor cell toxin dermorphin-
saporin. This treatment prevented the development of allodynia in both the face and the
hindpaw, indicating a probable role for the RVM in the initiation of IM-induced
cutaneous allodynia.
Descending pain-facilitatory projections may be driven, in part, by RVM CCK (Heinricher and Neubert 2004; Xie et al. 2005). Previous studies have shown that microinjection of CCK into the RVM of uninjured rats can elicit allodynia of the hindpaw without the need for afferent input (Kovelowski et al. 2000; Heinricher et al. 2001). For this reason, we questioned whether microinjection of CCK into the RVM of otherwise untreated rats would produce similar changes in tactile withdrawal thresholds as seen with IM application to the dura. Our findings indicate that RVM CCK, which activates “ON” cells (Heinricher and Neubert 2004), mimicked the effects of dural IM administration in evoking widespread allodynia and parallels the time-course previously reported for hindpaw allodynia (Kovelowski et al. 2000). This experiment led us to question whether or not endogenous CCK release in the RVM played a role in facilitating IM-induced allodynia. We observed that RVM microinjection of CCK-receptor antagonist, which inhibits CCK activity at receptor sites presumably localized to “ON” cells (Heinricher and Neubert 2004), was able to attenuate IM-induced facial and hindpaw allodynia. This suggests one possible mechanism for RVM influence on both cranial and extracranial hypersensitivity related to headache associated pain.

In search of a central site of action for triptan therapy within nociceptive control centers of the brain, we attempted to show that microinjection of sumatriptan into the RVM could block IM-induced cutaneous allodynia. Early post-treatment with this drug was successful in decreasing allodynia, however late post-treatment was unable to attenuate the hypersensitivity, similar to systemic treatment observations. These data further support the idea that, once established, processes of central sensitization do not
depend on ongoing dural input, and confirm a functional role for the presence of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors within brainstem pain control centers (Castro et al. 1997).

### 4.4 Neuronal-glial interactions within the RVM

A burgeoning theory in the field of pain research is the concept of neuronal-glial cell communication as a contributing factor for the establishment and maintenance of clinical pain states (Watkins and Maier 2003; McMahon et al. 2005; Thalakoti et al. 2007). During ‘normal’ pain states glia within the CNS are mostly quiescent, however in ‘abnormal’ or pathological pain states where the nervous system endures a constant influx of nociceptive transmission, likely to include scenarios of central sensitization, glia can become activated (Watkins and Maier 2003). When astrocytes become activated beyond their basal level of function, which normally includes regulation of extracellular pH, ions and neurotransmitters, they can morphologically change through increased production of the cyto-structural protein GFAP (Colburn et al. 1997) as well as increase synthesis of pro-inflammatory substances, ultimately leading to alteration in their function (Milligan and Watkins 2009). Microglial activation, similar to astrocytes, can produce a change in morphology as well as release of pro-inflammatory factors (Milligan and Watkins 2009). It is worth mentioning that activation of glia can also occur in the absence of morphological change (Watkins and Maier 2003; Lin et al. 2007), but their release of pro-inflammatory and excitatory substances can, in part, enhance neuronal drive of pain transmission.
Recent studies demonstrating neuronal-glial communication in the trigeminal ganglion via gap junctions and paracrine signaling are relevant for the development of peripheral sensitization and possibly central sensitization in headache-related pain (Thalakoti et al. 2007). In addition, mechanical allodynia associated with a rat model of orofacial pain was decreased through RVM microinjection of glial inhibitors (Wei et al. 2008) suggesting a site of glial influence within the RVM for trigeminal associated pain. Naturally, glial cell influence on IM-induced cutaneous hypersensitivity became a matter of interest for our studies.

To determine the presence of glial activation in our model, initial studies were conducted comparing RVM tissues from animals treated with IM or SIF for variations in glial cell morphology. The presence of microglial hypertrophy following IM treatment, in absence of comparable astrocyte changes, indicated a possible role for microglia in the facilitation of migraine pain. Follow-up behavioral studies using the microglial inhibitor minocycline injected into the RVM revealed that early post-treatment, in contrast to late post-treatment, could attenuate IM-induced facial and hindpaw allodynia. These observations were similar to the time-course of efficacy in our studies with RVM sumatriptan treatment. In addition, both minocycline and sumatriptan RVM treatment had the ability to decrease IM-induced microglial hypertrophy.

Together, these results led to a new hypothesis in regulation of pain facilitation. If neuronal blockade with a local anesthetic can be effective in decreasing IM-induced allodynia at later time-points, why would sumatriptan, supposedly working through a neuronal mechanism, have a time-course of action more similar to that of a glial
inhibitor? Furthermore, why would local sumatriptan treatment cause a decrease in glial hypertrophy, a marker for microglial activation? Two different theories have evolved implicating a modulatory role for triptans in effecting neuronal-glial interactions within the RVM. It is feasible that triptans have a direct site of action on microglia and thus, through inhibition of glial activation, they can decrease the excitatory influence on neurons. Another possible theory proposes that triptan activity at neuronal sites of action leads to an indirect decrease in glial activation through a decrease in excitatory neuronal activity.

Further studies need to be conducted to confirm the location of the serotonergic receptors responsible for the actions of triptans in centers of pain control. Exhaustive immunohistochemical studies were performed with little success using antibodies for 5-HT$_{1B}$, and 5-HT$_{1D}$ to co-localize with neurons, glia and blood vessels within the RVM tissues. Positive staining for 5-HT$_{1B}$ was visualized in the RVM co-localized with cerebral blood vessels, which is consistent with studies indicating these are the most ubiquitous receptors in pain control centers of the CNS (Bruinvels et al. 1994; Castro et al. 1997). However, the consensus on the commercially available antibodies for these two specific receptors is that they are unreliable, and that a molecular method for verification would be more optimal.

Additional studies are underway to determine more definitively the relationship between triptans and microglia. Molecular studies that have been suggested to verify the theory include running a real-time PCR on cultured microglia samples in order to localize 5-HT$_{1B/1D}$ receptors. In addition, preliminary cell culture studies have shown that
morphological activation of cultured cortical microglia can be induced through application of ATP. We would like to look at release of pro-inflammatory cytokines including TNF or IL-1 from these cultures as a marker for activation, and to measure the effects of sumatriptan on the release of these substances. If sumatriptan does not have the ability to decrease hypertrophy or decrease the release of pro-inflammatory factors, then we may be able to assume that the inhibitory actions of triptans on glia are indirect and through a neuronal influence. If real-time PCR of microglia samples shows the presence of 5-HT\textsubscript{1B/1D} receptors and sumatriptan has the ability to inhibit activation in culture, then it is possible sumatriptan has a direct effect on microglial cell inhibition. Either scenario could implicate an interactive relationship between neurons and glia in headache-related pain, and could shed light on the debate surrounding the mechanism of triptans in the CNS. As in injury-induced pain states, allodynia as a surrogate for migraine headache-associated pain could depend on the activation of a neuronal-glial pain facilitating system arising from the RVM (Wei et al. 2008).

4.5 Implications for mechanisms of headache-related pain.

Despite the high prevalence of migraine and other types of primary headache in the general population, our understanding of the underlying mechanisms remains incomplete. For migraine, theories of pain include neurogenic inflammation, in which activation of trigeminal meningeal afferents evokes plasma extravasation and vasodilatation via interactions within the neurovascular unit (Moskowitz 1992; Williamson and Hargreaves 2001). Additionally, theories of cortical spreading
depression, parasympathetic vasodilatation, and activation of meningeal mast cells have all been put forward (Bolay et al. 2002; Levy et al. 2006; Zhang et al. 2007). Whereas these theories of migraine pain address important aspects associated with migraine headaches, a unified mechanistic concept has not yet emerged. Another proposal is that migraine represents a dysfunction of brainstem mechanisms of pain modulation, or more generally, sensory gating (Goadsby 2007). This idea is attractive because a central dysfunction could potentially explain the multiple triggers for migraine attacks and the range of symptoms associated with migraine including nausea, photophobia, and phonophobia (May and Goadsby 1999; May and Matharu 2007). Functional imaging studies performed on migraineurs demonstrated activation of brainstem structures with the onset of a migraine headache. These areas remained active even after the pain was resolved with triptans, suggesting the possible existence of a “brainstem generator” or a “migraine center” (Diener and May 1996; May and Goadsby 1999; Goadsby 2007; May and Matharu 2007).

Although not universally accepted, the concept of a brainstem generator of migraine is intriguing in that the proposed sites such as the periaqueductal grey (PAG), reticular formation/RVM, and locus coeruleus are prominent components of pain modulatory pathways (Fields and Basbaum 1999). The RVM receives inputs from the PAG and exerts bi-directional control over nociception under different physiological and pathophysiological conditions (Fields and Basbaum 1999). A facilitating influence from the RVM has been implicated in models of hyperalgesia and persistent pain, acute opiate withdrawal, opioid-induced hyperalgesia, inflammation, and neuropathic pain (Fields and
Based on our observations, we propose that the pronociceptive role of the RVM in sensitized pain states is critical for cutaneous allodynia associated with headache pain. Glial-neuronal interactions within the RVM may also contribute to the pronociceptive function of this pain modulatory center, which not only poses a new theory for migraine pathology, but generates potential approaches for therapeutic intervention in migraine therapy as well. It is extremely likely that the pharmaceuticals effective for migraine headache, in addition to their known actions, also have the ability to disrupt neuronal-glial signaling within the CNS.

4.6 Conclusion

Animal models of chronic pain are critical in order to aid our understanding of fundamental mechanisms and to further development of novel and effective therapies. To this end, the present study explored allodynia resulting from application of IM to the dura as a possible surrogate for migraine-associated pain. Dural inflammation gave rise to a slowly developing allodynia expressed not only in the trigeminal dermatome, but in extracranial locations like the hindpaws. Therapies clinically effective in migraine headache including early triptans, CGRP-antagonists, NSAIDS and opioids were efficacious in this model, whereas those proven to be less or ineffective in clinical situations such as late triptans or NK-1 antagonists were unsuccessful here. The slow development and extensive distribution of the tactile allodynia are consistent with development of central sensitization following dural inflammation that is dependent on a
brainstem generator of cutaneous allodynia associated with headache-related pain. Facial and hindpaw allodynia associated with dural inflammation is thus a useful surrogate of migraine-associated pain and could be used in the future to explore novel mechanisms for therapeutic intervention.
Verification of Participation  
For Dissertation Files  
By The Institutional Animal Care and Use Committee (IACUC)  
The University of Arizona  
PHS Assurance No. A-3248-01 -- USDA No. 86-3

Name of Participant/Certification Number:  
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IACUC Assigned Protocols - PI/Department:  
#05-123 “Antinociceptive Effects of Opioids”  
#05-175 “Effects of Sustained Administration of Opiates on Pain”  
#08-040 “Behavioral and Neurochemical Aspects of Migraine Pain-CSD Model”  
#08-051 “Rodent Model of Migraine Pain- Migraine and Pain Fund”  
#08-046 “Rodent Model of Migraine Pain-GSK”

PI: Dr. Frank Porreca, Pharmacology Department

Participation Status was Confirmed: July 9, 2008

Mary Durham, IACUC Office  
Institutional Official: Leslie P. Tolbert, Ph.D.  
Vice President for Research

DATE: July 9, 2008  
DATE: July 9, 2008
Diagram of Dura Cannula Design

Figure 1. Diagram of Dura Cannula Design. A coronal section of the rodent brain displays the level of the dura cannula coordinates. A 1 mm hole was made 1 mm left of midline and 1 mm anterior to the coronal suture in the frontal bone of the skull with a hand drill to carefully expose the dura. A guide cannula extending 0.5 mm from the pedestal to avoid irritation of the dural tissue was inserted into the hole and sealed in place with super-glue. The design of the cannula allowed delivery of solutions to the underlying dural membrane without penetration of or damage to the membrane. A dummy cannula was inserted and secured to prevent contaminants from entering the guide cannula during the recovery period.
Location of Dura Cannula

Figure 2. Location of Dura Cannula. The location of the bregma and midline bone sutures were identified, and as indicated by arrow and black circle, a 1 mm hole was drilled for the dura cannula 1 mm left of midline and 1 mm anterior to the coronal suture in the frontal bone of the skull. Two additional 1 mm holes were made on either side of the midline approximately 4-5 mm caudal to the cannula to mount stainless steel screws. The screws were placed in the skull superficially to prevent damage to the underlying dural membrane with dental acrylic used to secure the cannula and screws to the skull. Rostal = toward the nose. Caudal = toward the tail.
Ink Injection: Dura Cannula Verification

Figure 3. Ink Injection: Dura Cannula Verification. Cannula placement and integrity of the dura was confirmed with microinjection of 10 µL of India ink as seen on skull dissection on the left. The black ink spreads 3-5 mm on the dorsal aspect of the dura. As pictured in the sample brain on the right, the ink does not penetrate through the dura indicated by the lack of ink on the tissue of the cortex. Additionally, no underlying damage to the cortical tissue is caused by the surgery.
Figure 4A. Dura + RVM Cannula Location. As indicated by arrow and circle on the left, a bilateral guide cannula was directed toward the lateral portions of the RVM (anteroposterior, -11.0 mm from bregma; dorsoventral, -7.5 mm from the dura mater; lateral, ± 0.6 mm on either side of the midline). The dura cannula location is indicated by arrow and circle on the right. Rostal = toward the nose. Caudal = toward the tail.

Figure 4B. RVM Cannula Location: Dorsoventral View. The bilateral guide cannula is directed towards the lateral portions of the RVM using the coordinates -7.5 mm from the dura mater. The RVM area is shaded in green.
Figure 5. Dura + RVM Cannulation: Post Surgery. The RVM guide cannula (located back-left, in photo) and the dura cannula (located front-right, in photo) were secured to the skull using silk suture and dental acrylic. Dummy cannulas were inserted and secured in the dura cannula and the RVM cannula to prevent contaminants from entering. Following recovery from anesthesia, animals were housed separately for a 6-8 day recovery period.
RVM Cannula Verification

Figure 6. RVM Cannula Verification. Pictured above is a sample section for cannula placement verification. This animal received a saline injection into the RVM (0.5µl per side). Following fluorescent immunohistochemical staining, the cannula tracts and injection sites were confirmed with microscopic examination. The injection site lies within the lateral portions of the RVM dorsal to the pyramids (corticospinal tract). Additional methods of cannula verification were used throughout the studies, including microinjection of India ink (0.5µl per side) and microscopic examination of Nissl-stained medullary sections.
Experimental Testing Protocols

Figure 7. Experimental Testing Protocols. Following recovery from surgery, the baseline behavioral responses to probing of the face and hindpaws were obtained from all rats prior to drug administration. Rats then received either SIF or IM via the dura cannula and behavioral responses were determined at 1 hr intervals for 5-6 hrs. Drugs were administered either 10 min prior to dural cannula injection or at time points after dural injection by either systemic injection or RVM microinjection.
Cannula Injections

Figure 8. Cannula Injections. (A) Dura cannula injection: an injection cannula, cut to fit the dura guide cannula and connected to a 25 µl Hamilton syringe by Tygon tubing, was used to slowly inject 10 µl of the inflammatory mediator cocktail or vehicle onto the dura. (B) RVM cannula injection: an injection cannula, protruding an additional 1.0 mm into fresh brain tissue and connected to a 10 µl Hamilton syringe by Tygon tubing, was used to slowly inject 1.0 µl of solution (0.5 µl/side) into the RVM double guide cannula.
Dural IM Elicits Cutaneous Alloodynia

Figure 9. Dural IM Elicits Cutaneous Alloodynia. Withdrawal thresholds to tactile stimuli applied to the face (A) and the hindpaw (B) were measured in rats before any surgical manipulations (naïve) and immediately before (BL; baseline) dural application of inflammatory mediators (IM; circles) or synthetic interstitial fluid (SIF; squares). No significant differences \( (P > 0.05) \) between the responses of naïve rats and those receiving SIF indicated that surgery alone did not produce a sensitization of the face or hindpaws to tactile stimuli. Withdrawal responses to tactile stimuli applied to the face and hindpaws developed slowly over time and reached a maximal decrease in threshold 3 hours after administration. Withdrawal responses to stimuli applied to the face and hindpaws approached baseline values 5 and 6 hours, respectively, after IM administration. For both the face and hindpaw, two-factor analysis of variance indicated that response thresholds of IM-treated rats were significantly \( (P < 0.0001) \) less than those of SIF-treated rats.
Inflammatory Mediator Dose Response

Figure 10. Inflammatory Mediator Dose Response. Preliminary experiments demonstrated that application of IM cocktail to the dura at either 1x (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE₂), 2x (2 mM histamine, serotonin, bradykinin and 0.2 mM PGE₂), or 4x (4 mM histamine, serotonin, bradykinin and 0.4 mM PGE₂) times the concentration originally reported produced significant allodynia of the face and hindpaws. However, the concentration 2x (two times that originally reported) elicited the most robust and reproducible behavioral sensitivity and was used for all further experiments. SIF (Synthetic Interstitial Fluid) = Vehicle.
Figure 11. FOS Expression in TNC. (A) Trigeminal nucleus caudalis (TNC) sections were analyzed from naïve rats, rats with dural cannulation only, and rats receiving either synthetic interstitial fluid (SIF) or inflammatory mediators (IM) in the absence or presence of systemic sumatriptan. The numbers of FOS-positive profiles within the TNC were counted. The results indicate that cannulation surgery alone produced a significant increase ($P < 0.05$) in trigeminal FOS expression compared with the naïve rats. The dural administration of SIF produced a moderate, but significant (*$P < 0.05$) increase in FOS expression relative to the surgery only group. This was further doubled by dural IM (**$P < 0.05$ compared to SIF). IM expression was reduced to the same level as the SIF-treated group by administration of sumatriptan (***$P < 0.05$ compared to IM). (B) Medullary sections (40 µm thick) at the level of the TNC were harvested. Shaded kidney shaped green region represents the borders of the TNC, and shaded gray box represents external border of photographs taken for counting and sample images C and D. (C,D) Sections were prepared for DAB staining to visualize FOS expression. C represents IM treated animals, D represents SIF treated animals. Note that the majority of labeled cells in the IM section are located within the superficial ventrolateral laminae of the TNC.
Subcutaneous Sumatriptan Treatment

Figure 12. Subcutaneous Sumatriptan Treatment. Rats received dural administration of inflammatory mediators (IM) or synthetic interstitial fluid (SIF) and also received subcutaneous (s.c.) sumatriptan or saline. Animals were injected s.c. either 10 minutes before the dural administration or 0.5-, 1.5-, or 2.5-hrs after dural administration. Pretreatment and 0.5-hr post-treatment with sumatriptan prevented the development of tactile allodynia of the face (A) and hindpaws (B), indicated by significant (*$P < 0.05$) reductions in normalized allodynic responses. In contrast, administration of sumatriptan 1.5- and 2.5-hrs after IM did not significantly alter the withdrawal thresholds to light tactile stimuli applied to the face (A) or hindpaws (B). Animals treated with SIF, and then with s.c. saline or sumatriptan did not demonstrate tactile allodynia of the face or hindpaws.
Subcutaneous Naproxen Treatment

Figure 13. Subcutaneous Naproxen Treatment. Rats received dural administration of inflammatory mediators (IM) or synthetic interstitial fluid (SIF) and also received subcutaneous (s.c.) naproxen or saline. Animals were injected s.c. either 10 minutes before the dural administrations or 0.5-, 1.5-, or 2.5-hrs after dural administration. Pretreatment and 0.5-hr post-treatment with naproxen prevented the development of tactile allodynia of the face (A) and hindpaws (B), indicated by significant (*P < 0.05) reductions in normalized allodynic responses. In contrast, administration of naproxen 1.5- and 2.5-hrs after IM did not significantly alter the withdrawal thresholds to light tactile stimuli applied to the face (A) nor at 2.5-hrs in the hindpaws (B). Animals treated with SIF, and then treated with s.c. saline or naproxen did not demonstrate significant evidence of tactile allodynia of the face or hindpaws.
Subcutaneous Morphine Treatment

Figure 14. Subcutaneous Morphine Treatment. Rats received dural administration of inflammatory mediators (IM) or synthetic interstitial fluid (SIF) followed by subcutaneous (s.c.) morphine or saline. Animals were injected s.c. 0.5-hrs after dural administration. Administration of 10 mg/kg (black bars) and 3 mg/kg (white bars) morphine both significantly attenuated facial (*$P < 0.05$) and hindpaw (*$P < 0.05$) tactile allodynia, however, the 10 mg/kg dose produced behavioral signs of sedation. The 3 mg/kg dose was able to attenuate the IM-induced allodynia without the behavioral manifestations of sedation. Systemic morphine did not alter behavioral thresholds in SIF-treated animals over the 5 hr time course.
**Intravenous CGRP Receptor Antagonist Treatment**

![Graph showing % of IM-induced Allodynia](image)

**Figure 15. Intravenous CGRP Receptor Antagonist Treatment.** Rats received dural administration of inflammatory mediators (IM) or synthetic interstitial fluid (SIF) followed by intravenous (i.v.) CGRP\(_{(8-37)}\) or saline. Animals were injected i.v. 0.5-hrs after dural administration. Administration of the 0.45 mg/kg dose of \(\alpha\)-CGRP\(_{(8-37)}\) (black bars) significantly attenuated (*P < 0.05) both facial and hindpaw IM-induced tactile allodynia, however, the 0.15 mg/kg (white bars) dose had no effect on behavioral allodynia (*P > 0.05). Systemic \(\alpha\)-CGRP\(_{(8-37)}\) did not alter sensory thresholds in SIF-treated animals over the 6 hr time course.
**Subcutaneous NK-1 Antagonist Treatment**

**Figure 16. Subcutaneous NK-1 Antagonist Treatment.** (A) The NK-1 antagonist L-732,138 (black bars) was administered 10 min before dural administration of inflammatory mediators (IM) or synthetic interstitial fluid (SIF). Pretreatment with L-732,138 failed to prevent development of behavioral signs of tactile allodynia of the face or hindpaws ($P > 0.05$). (B) A separate study tested that the dose of NK-1 antagonist employed was sufficient to block NK-1 agonist activity. Rats were pretreated with either vehicle or L-732,138, challenged with an intraplantar injection of capsaicin and the time spent licking or guarding the hindpaw was determined over a 10 min period. Capsaicin treatment following vehicle injection (white bar) produced a mean cumulative response time of $133 \pm 33.3$ sec. Rats injected with L-732,138 (black bar) showed a significantly ($*P < 0.05$) reduced mean cumulative response time of $46 \pm 14.7$ sec, suggesting that the dose of L-732,138 was sufficient to block NK-1 mediated nociceptive activity.
RVM Bupivacaine Treatment

Figure 17. RVM Bupivacaine Treatment. Rats received dural administration of inflammatory mediators (IM) or synthetic interstitial fluid (SIF) followed by bupivacaine (0.5% w/v) microinjected into the rostral ventromedial medulla (RVM) as a 0.5- or 1.5-hr post-treatment. Bupivacaine given 0.5-hrs after IM blocked the appearance of behavioral signs of facial allodynia (A) and attenuated the development of hindpaw allodynia in a time-dependent, reversible manner ($P < 0.05$) (B). Bupivacaine given 1.5-hrs after dural administration of IM significantly ($P < 0.05$) and reversibly attenuated behavioral signs of allodynia in both the face (C) and hindpaws (D). Behavioral responses were not altered by bupivacaine in SIF rats or vehicle in SIF rats over the 6 hr time course.
Figure 18. RVM Dermorphin-Saporin Treatment. Rats were pretreated with dermorphin-saporin (Derm-Sap) or saporin (Sap) before behavioral testing. Twenty-eight days following RVM Derm-Sap or Sap treatment rats were challenged with a dural injection of inflammatory mediators (IM) or synthetic interstitial fluid (SIF). While dural IM elicited allodynia in Sap pretreated rats (gray bars), Derm-Sap significantly (*$P < 0.05$) diminished IM-induced facial and hindpaw allodynia (black bars). Treatment with Derm-Sap or Sap along with dural SIF did not significantly change baseline facial or hindpaw withdrawal thresholds.
Figure 19. RVM CCK-8(s) Treatment. Tactile allodynia of the face (A) and hindpaws (B), indicated by significant decreases in response thresholds, were measured at 15-minute intervals for 2 hours after RVM microinjection of cholecystokinin (CCK-8(s)) or vehicle. Microinjection of CCK-8(s) into the RVM of otherwise untreated rats produced time-dependent and significant ($P < 0.05$) tactile allodynia of both the face (A) and hindpaws (B) that peaked at the 30- to 45-min time point and moved toward baseline by 2 hours. Behavioral responses were not significantly altered by vehicle injection.
RVM CCK$_2$-Antagonist Treatment

Figure 20. RVM CCK$_2$-Antagonist Treatment. Rats received dural administration of inflammatory mediators (IM) or synthetic interstitial fluid (SIF) and also received a CCK$_2$-antagonist (YM022) or vehicle microinjected into the RVM 0.5-hr later. YM022 (black bars) significantly attenuated facial and hindpaw (*$P < 0.05$) allodynia compared with the vehicle-treated group (gray bars). Behavioral responses were not altered by YM022 in SIF-treated rats over the 6 hr time course.
RVM Sumatriptan and Minocycline Treatment

Animals received dural administration of inflammatory mediators (IM) or synthetic interstitial fluid (SIF) followed by sumatriptan or minocycline or vehicle microinjected into the RVM as a 0.5- or 1.5-hr post-treatment. RVM sumatriptan or minocycline given 0.5 hr after dural injection significantly (*$P < 0.05$) attenuated both facial (A) and hindpaw (B) IM-induced allodynia. However, post-treatment with sumatriptan or minocycline 1.5 hr after IM did not significantly attenuate ($P > 0.05$) or reverse behavioral allodynia of the face (A) or the hindpaw (B). Neither RVM sumatriptan nor RVM minocycline altered behavioral responses in SIF-treated animals over the 6 hr time course.

Figure 21. RVM Sumatriptan and Minocycline Treatment.
Figure 22. IM-Related RVM Electrophysiology. (A) The effect of dural IM on the discharge of an identified RVM “ON” cell. The cell responded vigorously during the infusion of IM, but then showed a progressive increase in overall firing to approximately three times baseline 60 min after infusion. IM was injected at time indicated by underscore; triangles indicate paw withdrawal trials. Data were collected in 1-sec bins. (B) Ongoing firing of RVM “ON” and “OFF” cells and neutral cells after IM infusion, represented by the mean ± standard error of overall ongoing discharge of RVM neurons at baseline (B) and after infusion of IM. “OFF” cells (n = 9; squares) displayed a transient inhibition with IM application, whereas “ON” cells (n = 8; diamonds) showed a gradual increase in firing peaking 1 to 2 hours after IM. Neutral cell firing (n = 6; open triangles) was unchanged after IM. *P < 0.05 compared with baseline. (C) SIF-Vehicle infusion did not alter “ON” or “OFF” cell firing. *P < 0.05 compared with baseline.
Figure 23. Microglial Condition Comparison. Naïve, SIF-treated or IM-treated RVM tissue was taken at 1.5-hrs post injection, and SIF-treated or IM-treated tissue was taken at 2.5-hrs post injection. All conditions were processed for OX-42 (microglia are green) fluorescence, and then evaluated by using morphological change in cellular structure as the variant: hypertrophy of the microglia could indicate glial cell activation. In comparison to naïve animals, the SIF-treated RVM tissues indicate a slight increase in hypertrophy of microglial cells at both the 1.5- and 2.5-hr timepoint. However, the findings indicate a greater increase in microglial hypertrophy following IM-treatment at both the 1.5- and 2.5-hr timepoints. The RVM microglia of the IM-treated animals appear to be more amoeboid-like with shorter processes than those of naïve animals. Photographs taken at 20x magnification.
Figure 23. Microglial Condition Comparison
Figure 24. Astrocyte Condition Comparison. Naïve, SIF-treated or IM-treated RVM tissue was taken at 1.5-hrs post injection, and SIF-treated or IM-treated tissue was taken at 2.5-hrs post injection. All conditions were processed for GFAP (astrocytes are green) fluorescence, and then evaluated by using morphological change in cellular structure as the variant: hypertrophy of the astrocytes could indicate glial cell activation. In comparison to naïve animals, there is no indication of morphological change in the SIF-treated and IM-treated RVM tissues at either the 1.5- or 2.5-hr timepoints. Photographs taken at 20x magnification.
Figure 24. Astrocyte Condition Comparison
Figure 25. RVM Sumatriptan or Minocycline Treatment vs. IM-Induced Microglial Activation. All conditions were processed for OX-42 (microglia are green) fluorescence, and then evaluated by using morphological change in cellular structure as the variant. The findings indicate an increase in RVM microglial hypertrophy following IM-treatment at the 2.5-hr timepoint. The RVM microglia of the IM-treated animals appear to be more amoeboïd-like with shorter processes than those of naïve animals. The bilateral RVM microinjection of sumatriptan appears to attenuate the hypertrophy in microglia seen 2.5-hrs following dural-IM treatment. In addition, administration of RVM minocycline also appears to reverse the hypertrophy in RVM microglia seen following dural-IM treatment. The RVM tissue of sumatriptan and minocycline treated animals resembles that of the saline treated and/or naïve animals. Neither sumatriptan nor minocycline appear to have any effect on RVM microglial cellular structure in SIF-treated animals in comparison to naïve. Photographs taken at 20x magnification. All drug treatments were administered 0.5-hrs post dura-injection. Tissue samples were taken at the 2.5-hr timepoint, following 2 hours of behavioral testing to confirm drug efficacy.
Figure 25. RVM Sumatriptan or Minocycline Treatment vs. IM-Induced Microglial Activation.
**Figure 26. RVM 5-HT\textsubscript{1B/1D} Immunofluorescence.** Naïve RVM tissue was analyzed for localization of 5-HT\textsubscript{1B} and 5-HT\textsubscript{1D} receptors. RVM tissues stained for 5-HT\textsubscript{1B} (red) showed a significant amount of punctuate staining co-localized with the RECA-1 antibody for blood vessels (green). RVM tissues stained for 5-HT\textsubscript{1D} receptors (red) showed a very small amount of punctuate staining co-localized with the RECA-1 antibody for blood vessels (green). Arrows indicate transverse section of blood vessels, arrowheads indicate sagital section of blood vessels. Yellow color on merge sections indicates co-localization of antibodies.
Figure 26. RVM 5-HT\textsubscript{1B/1D} Immunofluorescence.
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