SEX DIFFERENCES IN GENE EXPRESSION AND PAIN-RELATED BEHAVIORS IN A PRECLINICAL MODEL OF MIGRAINE

BY

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Abstract

Many laboratory animal studies of migraine have employed electrophysiological techniques to assess neuronal sensitization, but few have examined behaviors using International Headache Society criteria, which are based on behavioral changes including duration and intensity of pain and avoidance of routine activity. Fewer still have attempted to correlate the appearance of these diagnostic symptoms with changes in the activity of pain-related neurotransmitters and neuromodulators, such as calcitonin gene related peptide (CGRP). A vasoactive neuropeptide, CGRP might contribute to the vasodilatory component of migraine, and to the pain associated with this condition as it is present in nociceptors, including those in the trigeminal ganglion that innervate cerebral vasculature. Previous work has shown that CGRP levels are increased in animal models of inflammatory pain and in the external jugular vein of humans during migraine attacks. The CGRP receptor is comprised of three proteins: a G–protein coupled receptor called calcitonin-like receptor (CLR), a receptor activity-modifying protein (RAMP1), and a coupling protein, receptor component protein (RCP). Thus, the availability and sensitivity of this receptor is subject to regulation at numerous levels.

The objectives of this study were to develop a preclinical behavioral model of chronic migraine, to test sensory and motor behaviors relevant to International Headache Society diagnostic criteria, to examine whether there are sex differences in these behaviors, and to assess whether alterations in the expression pattern of genes encoding CGRP and its receptor components are

associated with sex differences or changes in pain-related behaviors.

Male and female Sprague-Dawley rats were implanted with a dural cannula placed over the occipital cortex. Groups of rats were treated with 10 or 20 microliter volumes of an inflammatory soup containing 1 mM each of histamine, serotonin, and bradykinin, as well as 0.1 mM prostaglandin E_2 (pH 5.5). A control group received sterile phosphate-buffered saline (pH 7.4) alone. Baseline behavioral testing was conducted on all eight groups of animals prior to surgery and seven days later. The inflammatory soup or control solution was administered supradurally 3 times/week for a total of eight applications. Locomotor activity was assessed using force plate actimetry during and following application of the inflammatory soup or vehicle. Total RNA was isolated from ipsilateral trigeminal ganglia and ipsilateral medulla. Real-time polymerase chain reaction was used to quantify the expression of amplified constructs using gene specific primers for CGRP, RAMP1, CLR, and RCP.

The results reveal pronounced sex differences in behavior following application of the inflammatory soup. Female rats displayed dose-dependent migraine-related behaviors and a longer duration of these effect in measurements of distance traveled, bouts of low mobility, and spatial confinement. Both males and females experienced allodynia following exposure to the inflammatory mixture. Moreover, females displayed a higher baseline gene expression of CGRP and lower baseline gene expression of RAMP1, CLR, and RCP in the medulla than male animals. In addition to these baseline differences, gene expression of CLR and RCP was induced in the medulla of female rats but

not in males. No sex difference in CGRP gene expression was noted in the trigeminal ganglia, although females do have a lower baseline expression of CGRP receptors, RAMP1, CLR, and RCP than males. It was also found that in the trigeminal ganglia RAMP1, CLR and RCP are inducible, especially in males, and that at least a portion of these responses are the result of volume effects associated with application to the dura of the inflammatory soup or vehicle.

These findings indicate significant sex dependent changes in rat locomotor activity and CGRP-related gene expression in the brainstem and trigeminal ganglia associated with the application to the dura of an inflammatory soup. As the behavioral endpoints utilized in this study correspond to clinical signs considered by the International Headache Society as diagnostic for migraine, these data confirm that CGRP and its receptors are involved in migraine pathophysiology and reveal for the first time that alterations in the response to this peptide may be related to the increased prevalence of migraine in females. Such findings could be of value in devising new therapeutic strategies for the treatment of the debilitating condition.

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List of Abbreviations

5-HT	serotonin
AM	adrenomedullin
AMY	amylin
ASIC	acid sensitive ion channel
Ca^{++}	calcium
CALCA	CGRP/calcitonin gene
cAMP	cyclic adenosine monophosphate
cDNA	complimentary deoxyribonucleic acid
CFA	Complete Freund's Adjuvant
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CLR	calcitonin-like receptor
CT	calcitonin
DHE	dihydroergotamine
DRG	dorsal root ganglia
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FHM	Familial Hemiplegic Migraine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GTN	glyceryl trinitrate

HIV	human immunodeficiency virus
HLH	helix-loop-helix
IHS	International Headache Society
IP_3	inositol 1,4,5-triphosphate
IS	inflammatory soup
K_{ATP}	ATP-senstive potassium channels
KCl	potassium chloride
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
Na_v	voltage-gated sodium channel
NEP	neutral endopeptidase
NGF	nerve growth factor
NK-1	neurokinin-1
NO	nitric oxide
NSAID	non-steroidal antinflammatory drug
OB2	octamer-binding motif
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
qRT-PCR	quantitative real-time polymerase chain reaction

RAMP	receptor activity modifying protein
RCP	receptor component protein
SSS	superior sagittal sinus
TGF-β	tumor growth factor β
TRPV	transient receptor potential vanilloid
USF	upstream stimulating factor
USV	ultrasonic vocalization
VIP	vasoactive intestinal peptide
$\Delta\Delta C_t$	double-delta threshold cycle

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I. General Introduction

Historical context for migraine

The earliest evidence for migraine come from skulls exhumed from the Andean region of South America. The skulls show early Peruvian societies practiced neurosurgery over 8000 years ago. While the exact reasons for this neurosurgery are not known, it is likely that some of these operations were aimed at relieving headache (Marino and Gonzales-Portillo, 2000). These societies also practiced intentional cranial deformation to modify the growth axis in infancy perhaps to differentiate different social classes (Schijman, 2005). It is possible that increased intracranial pressure in regions of the cerebral cortex necessitated trephination for relief of pressure and headache symptoms (Gerszten et al., 1998). We know trephination was recommended as treatment for migraine as late as the 17th century by the famous British physician William Harvey (Rapoport and Edmeads, 2000).

Migraine is mentioned in the oldest extant medical text, the Ebers Papyrus, which was written in the mid 15th century (Garcia-Albea, 1999). In this text migraine is described as being caused by “pain-matter demons”, with magical and surgical techniques being the prescription for punishing and protecting against this demon power; the suggested remedy: “for pains in one side of the head. Skull of catfish; were heated until they turned to ashes and boiled with oil; the head is rubbed therewith for four days” (Karenberg and Leitz, 2001). Catfish was thought to represent the demon sphere and burning it may have been intended to destroy its power. Other techniques used involved binding various combinations of vegetable, animal, and mineral materials directly to the

patient's head. It is possible that the act of binding may have offered some relief by compressing the cranial blood vessels of the external carotid system (Rapoport and Edmeads, 2000). Greek and Roman cures for headache employed similar prescriptions (Karenberg and Leitz, 2001), with head binding being one of the standard treatments for migraine well into the 19th century (Rapoport and Edmeads, 2000).

Hippocrates (circa 400 BCE) was one of the first to report vomiting and visual aura, two hallmark symptoms for the diagnosis of migraine today. He described a young patient who experienced a bright light in his right eye and whose symptoms were relieved through vomiting. Hippocrates was among the first to understand that migraine was caused by a physical imbalance in the body and not by some supernatural force. He mentions that migraines are caused by the pathologic rise of vapors from the liver to the head. He also identified migraine as benign and advocated as treatment cessation of activities which appeared to trigger the headache pain (Rapoport and Edmeads, 2000).

Galen (200 CE) was able to glean a great deal of medical and anatomical information from animal dissections and trauma surgeries on gladiators. He generally followed Hippocratic theory on the origin of migraine and is believed to have coined the term that lead to the word "migraine". His term for the disorder was the ancient Greek word "hemicrania" which became the Old English term "migrem" which was ultimately transformed by the French to "migraine" (Rapoport and Edmeads, 2000; Waeber and Moskowitz, 2003).

It wasn't until the late 17th century that Dr. Thomas Willis advanced the

understanding of migraine beyond the knowledge imparted by the Greeks with his publication of “The London Practice of Physick.” He states in this tome that headache pain must be associated with the “parts of the Head that are most nervous, that is, the nerves themselves.” The meninges were considered “nervous”, while he noticed that the cerebral cortex, cerebellum, and brainstem “want sensible fibers” and remained pain-free when distended (Rapoport and Edmeads, 2000). Later he noted that the cerebral and meningeal vasculature was heavily innervated and hypothesized that migraine is caused by cranial vasodilation. This speculation led to the vascular theory of migraine.

Modern diagnosis

Migraine is considered a syndrome, reflecting the current lack of understanding about the underlying pathology of this condition. Currently, migraine is characterized as a collection of idiopathic symptoms that, taken together, are labeled migraineous. At present the symptomatic diagnosis of migraine is made on the basis of International Headache Society (IHS) criteria (IHS, 2004).

Migraine without aura

Menstrual migraines are classified within the grouping of migraine without aura because they are not typically accompanied by it. The IHS diagnosis for migraine without aura is defined as follows:

At least five headache attacks lasting 4 - 72 hours (untreated or unsuccessfully treated), and has at least two of the four following characteristics:

1. Unilateral location

2. Pulsating quality
3. Moderate or severe intensity (inhibits or prohibits daily activities)
4. Aggravated by walking stairs or similar routine physical activity

During headache at least one of the two following symptoms must occur:

1. Phonophobia and photophobia
2. Nausea and/or vomiting

Migraine with aura

Classical migraine has the additional symptom of aura. According to the IHS, a migraine with aura must meet the following diagnostic criteria:

At least two attacks fulfilling at least three of the following:

1. One or more fully reversible aura symptoms indicating focal cerebral cortical and/or brain stem functions
2. At least one aura symptom develops gradually over more than four minutes, or two or more symptoms occur in succession
3. No aura symptom lasts more than 60 minutes; if more than one aura symptom is present, accepted duration is proportionally increased
4. Headache follows aura with free interval of at least 60 minutes (it may also simultaneously begin with the aura)

At least one of the following aura features establishes a diagnosis of migraine with typical aura:

1. Homonymous visual disturbance
2. Unilateral paresthesias and/or numbness
3. Unilateral weakness

4. Aphasia or unclassifiable speech difficulty

Although the temporal progression of migraine is not consistent across patients, it can be divided into several stages. The first stage, the prodrome or premonitory phase, can last many hours and consists of feelings of uneasiness, heightened perception, and fluid retention. In the classical description of migraine the prodrome is followed by a visual aura. Some believe that the aura can also be manifested in the sensory perceptions if the disturbance is located in the sensory cortex. The aura may last for up to 30 minutes. At the conclusion of the aura the migraineur enters the headache phase which may be accompanied by nausea, vomiting, pallor, photophobia, and phonophobia. This phase can persist for hours or days and is most commonly resolved by sleep. After resolution, migraineurs enter the postdrome phase, which is often referred to as the postdromal hangover. During this phase migraineurs may feel tired, have limited food tolerance, and experience diuresis (Blau, 1992).

Epidemiology of migraine

Migraine has a strong genetic component. Several studies place the heritability of migraine at up to 50% (Kors et al., 2004; Pietrobon, 2005). Interestingly, migraines with aura seem to show a higher genetic component than those without aura. One rare, but well studied, inherited form of migraine is Familial Hemiplegic Migraine (FHM). This condition shows an autosomal dominant inheritance pattern and is associated with genes CACNA1A and ATP1A2 in FHM type 1 and FHM type 2, respectively. The CACNA1A gene encodes a subunit of a calcium channel while ATP1A2 encodes for a subunit of a

Na^+/K^+ ion pump (Kors et al., 2004).

The prevalence of migraine, which is higher in women than men, is approximately 13% of the population according to studies performed in the United States and Europe (Linde, 2006). The 1-year prevalence of migraine among men is 6–9% , whereas it is 15–17% among women (Linde, 2006). The sex disparity of prevalence begins at puberty, increases during the reproductive years, and becomes less pronounced after menopause (Stewart et al., 2000a). Migraine incidence also decreases during pregnancy especially in the third trimester (Chen and Leviton, 1994). The dynamic nature of menstrual migraine may provide important insights into the pathophysiology of this condition in general.

Migraine pathophysiology

Our understanding of migraine remains rudimentary, with considerable disagreement among scientists about the etiology of this disorder. Some describe migraine pain as unusual and unlike other types of pain. According to the generally accepted chronic pain classification system, pain is either nociceptive or neuropathic (Merskey, 1986). Nociceptive pain is caused by the stimulation of nociceptors by noxious stimuli, and is generally classified as thermal, mechanical, or chemical. Stimulation of these pain pathways is thought to be the part of a protective healing process in response to tissue damage. Neuropathic pain is associated with damage to the nerve itself. This damage may result from, for example, trauma, infection, or ischemia. Contrary to the cancer literature, where inflammatory pain is classified as neuropathic pain (Berger et

al., 2006), efforts are being made to tailor the pain classification system to divide nociceptive pain into either inflammatory or dysmodulatory pain (Jensen, 2006). In this system inflammatory pain is considered a response to tissue injury and subsequent neurogenic inflammation. Signs of inflammation are present in this form of pain. Others would prefer to categorize migraine pain as dysfunctional or dysmodulatory pain (Figure 1). This classification is quite controversial. In dysfunctional pain there are no signs of tissue injury (inflammatory changes) or of nerve injury (neuropathic changes). Rather, it is hypothesized that due to a poorly understood innocuous event, nociceptive neurons undergo a change and transmit signals abnormally. As the stimulus is not, in itself, potentially damaging to tissue, it cannot be considered inflammatory and is not neuropathic because there is no damage to the nerve itself (Dodick and Silberstein, 2006).

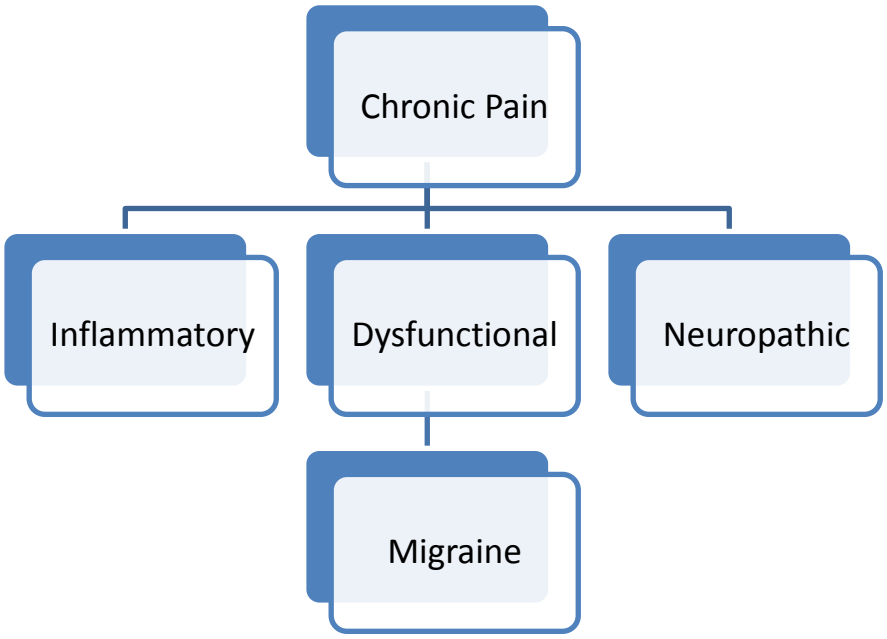

Figure 1. Migraine pain classification

Trigeminal system

It is accepted that the neurons at the center of migraine pain pathophysiology are located in the trigeminal ganglion, alternately referred to as the semilunar or gasserian ganglion. This ganglion houses the cell bodies of the pseudounipolar neurons of the sensory tract of the trigeminal nerve. The fifth cranial nerve, the trigeminal nerve has both motor and sensory components. The sensory component of the trigeminal is the exclusive focus of the work presented in this report. The sensory component is responsible for transmitting sensory information to the central nervous system. The myelinated fibers carry information about touch and position while the unmyelinated fibers, termed nociceptors, carry information about pain and temperature. Nociceptors are thought to be the fibers involved in migraine pain because they transmit the type of dull aching pain associated with this condition. The trigeminal neuron transmits sensory information from the face, forehead, and scalp. It is comprised of 3 divisions: the first or ophthalmic division, the second or maxillary division, and the third or mandibular division. The ophthalmic division innervates the supraorbital regions, the forehead, and scalp, in addition to the meningeal and cranial vasculature. The maxillary division innervates the lower eyelid, the cheek, and upper lip, whereas the mandibular division innervates the jaw and temporal region on the side of the head (Olesen et al., 2006).

Four regions of the trigeminal vascular system are thought to be involved in migraine. One is the cerebral and cranial vasculature where vasodilation is blocked by the serotonin receptor 1B and 1D (5-HT_{1B}, 5-HT_{1D}) agonists, the

antimigraine triptans (Perren et al., 1989; Nilsson et al., 1999), and the calcitonin gene-related peptide (CGRP) antagonist, olecegepant (Edvinsson et al., 2002). A second is the dura mater and resident inflammatory mast cells which are activated by CGRP and subsequently release inflammatory agents and cytokines that produce neurogenic inflammation (Theoharides et al., 2005). The third is the trigeminal ganglion, which express $5HT_{1B}$ and $5\text{-}HT_{1D}$ and CGRP receptors (Hou et al., 2001) and where CGRP is believed to have an autocrine or paracrine action in increasing its own production and in inducing the release of nitric oxide and inflammatory cytokines (Li et al., 2008). Another region of the trigeminal vascular system thought to be involved in migraine is the second-order sensory neurons with their cell bodies located in the spinal trigeminal nucleus of the brainstem that express post-synaptic CGRP receptors that can be blocked by olecegepant (Storer et al., 2004; Levy et al., 2005).

Nociceptor function

Trigeminal nociceptors forward sensory information to the secondary neurons in the spinal trigeminal nucleus where it is then transmitted for processing in the thalamus and cerebral cortex. These nociceptors are capable of both orthodromic transmission and antidromic signaling to peripheral nerve terminals. The antidromic signaling is thought to be key in the migraine process. At the peripheral terminal, these neurons release neuropeptides such as CGRP and substance P (Moskowitz et al., 1979). The neuropeptides then induce neurogenic inflammation by inducing mast cell degranulation by binding to receptors on these cells. The mast cell release of histamine, serotonin, and

prostaglandins leads to further inflammation and plasma extravasation. It is hypothesized that these chemical substances cause the afferent nociceptors to depolarize in response to normally innocuous stimuli, sending a pain signal to the spinal trigeminal nucleus and on to the thalamus and cerebral cortex. If the signal is sufficiently strong, it can initiate another round of antidromic signaling and neuropeptide release, further fueling the vicious cycle of pain and inflammation (Moskowitz and Macfarlane, 1993).

The manner in which the trigeminal neuron is able to affect cranial blood vessels was defined further by anatomical studies showing the meninges and dural sinus of both humans and laboratory animals are innervated by all three branches of the trigeminal nerve (Andres et al., 1987; Moskowitz and Macfarlane, 1993). These nociceptors are thought to be components of the major pathway for transmitting head pain. Interestingly, nonvascular areas of the dura are known to be insensitive to pain (Ray and Wolff, 1940; Davis and Dostrovsky, 1986). It has only relatively recently that a direct response to noxious stimuli was measured in neurons of the cerebral cortex (Hutchison et al., 1999).

Sensitization

With respect to pain, sensitization refers to the process whereby the threshold for neuronal activation is lowered such that a normally innocuous stimulus is capable of activating a response. Sensitization is further defined on the basis of its location, with peripheral sensitization involving first order afferents. This ability of noxious stimuli to produce long lasting changes in thresholds is observed in primary afferents (Perl, 1976; Perl et al., 1976). Central

sensitization is that which occurs in the brainstem or higher order neurons. Central sensitization was first investigated in decerebrate animals where contralateral increases in the excitability of spinal cord neurons was observed in response to thermal injury (Woolf, 1983).

With regard to migraine pain, Strassman and others proposed that trigeminal nerve sensitization plays a role in mediating the symptoms of this condition. This was demonstrated by the discovery that application of inflammatory chemical stimuli to the dura produces depolarization and sensitization to mechanical stimulation of afferents that innervate the dural venous sinuses. It was hypothesized that once sensitized, these afferents trigger vascular changes associated with migraine pain even when they encounter normally innocuous mechanical stimuli (Strassman et al., 1996). Since this report, electrophysiologists have used a preparation, termed “Inflammatory Soup” (IS), which is composed of some of the substances released following mast cell degranulation, such as 5-HT, histamine, prostaglandin E_2 (PGE_2), and bradykinin (Strassman et al., 1996) to study the sensitization of primary and secondary trigeminal nociceptors. Over the past ten years, many have successfully used IS to study central and peripheral sensitization in rodents (Burstein and Jakubowski, 2004; Oshinsky and Luo, 2006; Jakubowski et al., 2007; Oshinsky and Gomonchareonsiri, 2007).

While mechanisms of peripheral sensitization have been intensively studied in spinal sensory neurons (Kessler et al., 1992; Steen et al., 1992; Aley et al., 2001; Amaya et al., 2006), less attention has been paid in this regard to

trigeminal neurons. Moreover, no studies have attempted to relate migraine-like behaviors to increases in CGRP or its receptors in trigeminal neurons. Although it is now well-known that central sensitization in the dorsal horn of the spinal cord is accompanied by increases in substance P and neurokinin-1 (NK-1) receptors (McCarson and Krause, 1994; Krause et al., 1995; McCarson and Krause, 1996; Allen et al., 2003; Seybold et al., 2003), virtually nothing is known about possible changes in CGRP receptor expression or function in the spinal trigeminal nucleus caudalis, even though such alterations could underlie a central sensitization of the CGRP system associated with migraine.

Calcitonin gene-related peptide and migraine

Sensitization of sensory neurons is associated with increases in expression of CGRP, a pro-nociceptive neuropeptide (Nahin and Byers, 1994). A splice variant of the calcitonin/CGRP gene (CALCA) (Amara et al., 1982; Rosenfeld et al., 1984; Amara et al., 1985), CGRP is released from nerve terminals in association with inflammation and inflammatory pain (Devor, 1991b; Ohtori et al., 2001; Ohtori et al., 2003; Adwanikar et al., 2007; Benemei et al., 2007; Tzabazis et al., 2007). Also, CGRP is a potent dilator of meningeal blood vessels (Gupta et al., 2006). Other evidence pointing to a possible involvement of this peptide in migraine is the fact that CGRP levels increase in the trigeminal ganglion following injections of irritants into peripheral targets, and that this increase is associated with pain severity (Ambalavanar et al., 2006b). Moreover, up-regulation of CGRP occurs within a few hours after application of an inflammatory stimulus (Ambalavanar et al., 2006b), and CGRP levels continue to

increase over weeks during chronic inflammation (Ambalavanar et al., 2006b).

While it has been reported that circulating CGRP levels are increased in external jugular venous blood during a migraine attack (Goadsby et al., 1990), others have not been able to reproduce this finding (Tvedskov et al., 2005). This has led to continued debate about the circulating levels of CGRP during migraine and its involvement in this condition (Tfelt-Hansen and Le, 2009). Intravenous administration of CGRP to migraineurs without aura elicits migraine-like symptoms (Lassen et al., 2002). Although CGRP application to the dura mater does not sensitize dural nociceptors (Levy et al., 2005), transcranial electrical stimulation causes nerve terminal release of CGRP and meningeal artery and arteriole dilation in a rat model of migraine (Petersen et al., 2004). However, the physiological stimulus responsible for the release of CGRP from peripheral nerve terminals remains unknown.

Calcitonin superfamily of peptides

Because the CGRP receptor is a heterotrimer that includes components that are also part of receptors for other members of the calcitonin family, it is useful to understand related peptides and receptor components. The calcitonin family of peptides includes 6 members: calcitonin (CT), adrenomedullin (AM), adrenomedullin-2 (AM_2), amylin (AMY), and CGRP, with α-CGRP and β-CGRP isoforms (Juaneda et al., 2000; Poyner et al., 2002). Calcitonin is a 32 amino acid peptide produced in the thyroid (parafollicular C-cells) that regulates calcium (Ca^{++}) homeostasis throughout the body by inhibiting intestinal absorption and osteoclast activity (Copp and Cheney, 1962; Nicholson et al., 1986). It also has a

role in satiety (Erdogan et al., 2006). Adrenomedullin (AM) is a 52 amino acid peptide in its active form. Expressed in several tissues, it was first isolated from a pheochromocytoma cell line (Kitamura et al., 1998). A potent vasodilator, AM is also thought to be involved in angiogenesis and to impart resistance to oxidative stress (Kim et al.). Adrenomedullin-2, a 47 amino acid peptide expressed in the pituitary and the gastrointestinal tract, displays diverse cardiovascular and gastrointestinal effects (Roh et al., 2004). It may play a role in angiogenesis (Smith et al., 2009), as well as in placental development and blood pressure reduction in pregnancy (Chauhan et al., 2009). Amylin is a 37 amino acid peptide produced in the β-cells of the pancreas. Released in a 1:100 ratio with insulin, AMY acts synergistically with insulin to maintain glycemic control. Along with other members of the calcitonin family, AMY also has a role in bone metabolism (Pittner et al., 1994).

The secondary structures of these peptides are similar, with a single disulfide bond forming a six amino acid ring structure near the N-terminus. The bodies of these peptides are all comprised of an α-helix with potential amphiphilic character. In all cases, the C-termini are amidated (Poyner et al., 2002).

CGRP structure

There are two forms of CGRP; α-CGRP and β-CGRP. Although these peptides have similar biological activities, they differ with regard to their sites of action and chemical structure. The α-CGRP is a 37 amino acid splice variant of the CALCA gene, which contains 6 exons. In neurons, CGRP messenger ribonucleic acid (mRNA) is generated by joining exons 1 through 3 to exons 5

and 6. Alternatively, in thyroid C-cells, calcitonin mRNA is produced by joining exons 1through 3 to 4 (Zhou et al., 2007). While CGRP, in general, is distributed predominantly in the central and peripheral nervous systems (Amara et al., 1982; Poyner et al., 2002), human β-CGRP is more highly localized in the enteric nervous system. Human β-CGRP differs by 3 amino acids from human α-CGRP, while the rat analogs differ by only one amino acid (Mulderry et al., 1988). Interestingly, β-CGRP is encoded by its own gene with high homology to the calcitonin gene (Poyner et al., 2002). CGRP shares 50% sequence homology with adrenomedullin and some homology with other members of the calcitonin superfamily (Arulmani et al., 2004a). Because the N-terminal loop of calcitonin is required for receptor activation and signal transduction (Conner et al., 2002), one of the first high affinity CGRP receptor antagonists, $CGRP_{8-37}$, was produced by creating a peptide lacking the first seven amino acids of the parent compound (Chiba et al., 1989). The amphiphilic α-helix spanning the middle of the peptide is critical for receptor binding, with its deletion resulting in a 100-fold decline in receptor affinity (Conner et al., 2002). The amidated C-terminal is required for maintaining the peptide in the conformation necessary for receptor binding (Conner et al., 2002).

With regard to migraine pathophysiology, α-CGRP is 3 to 6 times more concentrated in the sensory nervous systems than β-CGRP (Mulderry et al., 1988). In addition,, α-CGRP is the major form of this peptide found in the trigeminal ganglion (Amara et al., 1985) and cerebral artery vasodilation is attributed primarily to the actions of this isoform (Jansen-Olesen et al., 1996). In

the remainder of this report, use of the term CGRP refers solely to α-CGRP.

Cranial location and vascular effects of CGRP

There is widespread distribution of CGRP positive innervation of cerebral vascular structures and CGRP is extensively expressed in nociceptors. The first cerebrovascular immuno-positive fibers to be discovered were thought to be of trigeminal origin (Uddman et al., 1985). Further work showed the venous structures of the dura are more extensively innervated by CGRP positive fibers than arterial structures. The superior sagittal sinus (SSS) is the most densely innervated of these cerebral vascular structures. In addition to the perivascular innervations, some CGRP positive fibers also traverse through the dura obliquely from posterolateral to anteromedial. These fibers follow a long straight path and are associated primarily with dura mater and not the vasculature. Some end in bulbous terminals within the dura and others pass through the dura to form arterial associations or to connect to the nerve plexus of the SSS. These dural fibers are in close relation to dural mast cells (Keller and Marfurt, 1991). Information is lacking about the specific receptor field sizes of the trigeminal neurons innervating the dura. Nevertheless, using retrograde tracers, the dural fibers were found to originate from the sensory neurons of the trigeminal ganglion, with a few originating in the internal carotid and dorsal root ganglia (DRG) of the cervical spinal segment (Keller and Marfurt, 1991).

Other locations and biological functions of CGRP

Immunoreactive CGRP is found in many regions of the brain. A high density of CGRP positive neurons terminate in the olfactory bulb and are present

in the auditory processing regions of the lateral olivochochlear system (Van Rossum et al., 1997; Maison et al., 2003).

Other roles for CGRP possibly include regulation of cholinergic function in olfaction and auditory processing (Maison, Adams et al. 2003). This peptide is also expressed in the posterior thalamus and is thought to be involved in acquisition, consolidation, and retrieval of passively learned information, especially that associated with acoustic stimuli (Van Rossum et al., 1997). Shown to be involved in feeding and satiety centers (Van Rossum et al., 1997), CGRP is present in many parts of the gustatory pathway, including taste bud fiber endings and the nucleus of the solitary tract. It is present in structures involved in autonomic functioning via the vagus and glossopharyngial nerves, and it has been localized in brain regions involved in vision, growth hormone secretion, and thermoregulation (Van Rossum et al., 1997).

It has been proposed that CGRP has a role in the formation, maintenance, and functioning of the neuromuscular junction. It is thought to act in concert with acetylcholine to enhance the rate of acetylcholine receptor aggregation and to potentiate the acetylcholine response in developing myotubes (Buffelli et al., 2001). With CGRP receptors widely distributed in the cardiovascular system and abundantly expressed in conducting system of the heart (Chang et al., 2001), CGRP has an ionotrophic effect on heart (Saetrum Opgaard et al., 2000) and may have a role in the treatment of congestive heart failure where it has been shown to lower blood pressure and increase cardiac output without increasing heart rate (Van Rossum et al., 1997). It dilates the renal vasculature and, in

isolated kidney, CGRP increases glomerular filtration rate and sodium excretion (Kurtz et al., 1989). This peptide also increases renin production and sodium excretion, but not glomerular filtration rate in healthy human volunteers (Kurtz et al., 1988; Gnaedinger et al., 1989).

Transcriptional activation of CGRP gene expression

Work on transcriptional activators of CGRP has been performed using cultured thyroid C-cells and trigeminal ganglion cells. In these cells, expression of the CGRP gene is controlled by a cell-specific, 18 base pair distal enhancer with several helix-loop-helix (HLH) binding domains (Lanigan and Russo, 1997). Enhancer activation requires an HLH protein heterodimer comprised of upstream stimulating factor (USF) 1 and 2 and a cell-specific octamer-binding motif, OB2 (Lanigan and Russo, 1997). The USF proteins are regulated by the mitogen activated protein kinase (MAPK) p38 -mediated phosphorylation (Galibert et al., 2001), with phosphorylation of the specific MAPK extracellular signal regulated kinase (ERK) suggested as a possibility as well (Kutz et al., 2006). It has been shown that the CGRP enhancer is stimulated by the MAPK pathway (Durham and Russo, 2003), and that USF proteins are activators of the CGRP promoter in cultured trigeminal ganglion neurons (Park and Russo, 2008). The CGRP promoter contains an element that is cyclic adenosine monophosphate (cAMP) and Ras responsive (deBustros et al., 1986).

Splice determinants of CGRP gene expression

In neurons, CGRP mRNA is generated by alternate processing of the CALCA gene. This processing is subject to many complex RNA regulatory

elements and protein factors. Most of these regulators affect the calcitonin-specific inclusion of exon 4. These include an exonic enhancer element on exon 4 (Tran et al., 2003), and an intronic enhancer element located downstream of exon 4 (Lou et al., 1994). In HeLa cells, Fox1 and Fox2 proteins are key silencers of exon 4 expression via the UGCAUG element (Zhou et al., 2007). Fox1 and Fox2 and are expressed exclusively in neurons in muscle, heart, and brain (Underwood et al., 2005). Although not the only factors involved, Fox proteins appear to be critical for determining the splice fate of CGRP pre-mRNA.

Regulation of CGRP expression and release

Following an inflammatory insult, nociceptors increase expression of CGRP (Devor, 1991a; Ambalavanar et al., 2006a) and medium-sized neurons with myelinated axons also begin to express this peptide (Neumann et al., 1996). By this process, nociceptors increase their signal strength, and many neurons that previously carried only light touch information begin to signal nociception. *In vitro* studies revealed that trigeminal CGRP release is stimulated by potassium chloride (KCl), a model of neuronal depolarization (Durham and Russo, 1999), capsaisin, through activation of vanilloid receptors (Caterina et al., 1997; Durham and Russo, 1999), and by an application of IS, a model neurogenic inflammation (Strassman et al., 1996; Durham and Russo, 1999). Studies indicate that prolonged elevation of CGRP is facilitated by the nerve growth factor (NGF) and MAPK pathways (Durham and Russo, 2003). Sequences near the CGRP gene promoter are responsive to various signal transduction pathways, including those induced by cAMP (deBustros et al., 1986; Monla et al., 1995), NGF (Watson and

Latchman, 1995), and activated Ras (Thiagalingam et al., 1996). Additionally, because tumor growth factor β (TGF-β) activates USF it has the potential to promote CGRP expression (Riccio et al., 1992). Moreover, as CGRP receptor activation auto-activates the CGRP promoter through a protein kinase A (PKA) dependent mechanism, trigeminal CGRP system could create a self-reinforcing, feed-forward amplification of CGRP expression (Zhang et al., 2007b). Release of CGRP is inhibited by sumatriptan through a prolonged increase in intracellular Ca^{++} levels (Durham and Russo, 1999), and estrogen increases CGRP mRNA and protein expression in the DRG of female rats (Sarajari and Oblinger, 2010). Our study focuses on changes in gene expression but those studies described above highlight the numerous other important mechanisms that are involved in CGRP release and signaling.

Catabolism of CGRP

The actions of CGRP are terminated through cleavage of the peptide by ubiquitously expressed neutral endopeptidase (NEP). This 100 kDa type II transmembrane glycoprotein cleaves a variety of neuropeptides, including CGRP and substance P (Erdos and Skidgel, 1988). This enzyme is widely distributed throughout the body where it is, mostly, membrane bound although a soluble form has been identified in blood and urine (Erdos and Skidgel, 1988). The NEP located in the lung appears to be a major inactivator of substance P, displaying 88-fold lower activity against CGRP (Katayama et al., 1991). NEP is involved in the catabolism of opioid peptides and neutrophil NEP is activated by morphine (Wang and Hung, 2003), an effect that may contribute to withdrawal headaches.

CGRP receptor

CGRP receptors are formed of three components including a G-protein coupled calcitonin-like receptor (CLR), the receptor activity-modifying protein (RAMP1) and the receptor component protein (RCP), a coupling protein (Arulmani et al., 2004a). The CGRP receptors are divided into two classes, CGRP1 and CGRP2, based on the association of RAMP1 with CLR, and in part on their sensitivity to the antagonist $CGRP_{8-37}$ (Dennis et al., 1989; Quirion et al., 1992). The RAMP1 amplifies the CGRP signal and contributes to sensitization of CGRP receptors (Zhang et al., 2007b).

Calcitonin receptor-like receptor

The rat and human calcitonin receptor-like receptors (CLRs) were first identified in 1993 (Chang et al., 1993), two years after the CT receptor complimentary deoxyribonucleic acid (cDNA) sequence was determined (Lin et al., 1991). The receptor is a seven transmembrane domain G-protein coupled receptor (GPCR) of the family B or type II variety (Sexton, 1999). The CLR is a 58 kDa immature protein and 66 kDa protein when glycosylated to its mature form (Poyner et al., 2002). Originally considered an orphan receptor, CLR was found in transfected HEK cells to produce cAMP accumulation in response to CGRP (Aiyar et al., 1996). This work also indicated that the receptor functions through a $G_{s\alpha}$ protein to stimulate adenylyl cyclase. Gene cloning studies subsequently identified an accessory protein, RAMP1, was required for CGRP activation (McLatchie et al., 1998).

Receptor activity modifying proteins

The family of receptor activity modifying proteins (RAMPs), RAMP1, RAMP2, and RAMP3, act as pharmacological switches, imparting ligand specificity (Table 1) when they associate with CPCR (Poyner et al., 2002). These single transmembrane domain proteins, which are composed of 148-175 amino acids, help determine the selectivity of the calcitonin family receptors. The RAMPs possess a large N-terminal extracellular domain and a 9 amino acid C-terminal intracellular domain (Fitzsimmons et al., 2003). They enable intracellular translocation and glycosylation of CLR protein and its insertion into the plasma membrane (McLatchie et al., 1998; Foord and Marshall, 1999). When coupled to CLR, RAMP1, a 17 kDA protein, produces the CGRP receptor (Banerjee et al., 2006).

In contrast to RAMP1, RAMPs 2 and 3 impart preferential receptor specificity for AM (McLatchie et al., 1998). These receptors are termed AM_1 and AM_2, respectively (Poyner et al., 2002). Because the AM_2 site is activated by CGRP at pharmacological concentrations, as with many of the CT family receptors it is somewhat promiscuous. The antagonist $CGRP_{8-37}$ blocks the AM_2 receptor, revealing some pharmacologic characteristics of the previously termed $CGRP_2$ while the RAMP/CLR receptor imparts the pharmacological characteristics of the previously termed $CGRP_1$ (Hay et al., 2003). While the coupling of RAMP with CT produces some specificity for amylin, RAMP1/CT (AMY_1) is potently activated by α-CGRP while the RAMP3/CT (AMY_3) receptor subtype is activated by CGRP similarly to the AM_2 receptor (Hay et al., 2005). It

has been concluded that the CGRP1 receptor corresponds to the CLR/RAMP1 complex, and CGRP2 likely corresponds to the AMY_1, and less so to the AMY_3 and AM_2 receptors. For this reason, it is recommended the term CGRP2 not be used any longer (Hay et al., 2008).

G-protein Coupled Receptor	RAMP isoform	Resultant receptor
Calcitonin receptor-like (CLR)	RAMP1	CGRP
	RAMP2	adrenomedullin (AM) receptor, designated AM_1
	RAMP3	dual CGRP/AM receptor, designated AM_2
Calcitonin receptor (CT)	None	Calcitonin receptor (CT)
	RAMP1	amylin receptor AMY_1
	RAMP2	amylin receptor AMY_2
	RAMP3	amylin receptor AMY_3

Table 1. CGRP receptor classification

CGRP-receptor component protein

The CGRP-receptor component protein (RCP) is an important accessory protein expressed in CGRP responsive tissues that directly interacts with CLR (Evans et al., 2000). The RCP is an intracellular, membrane-associated protein (Evans et al., 2000) composed of 146 amino acids with a molecular weight of 17 kDa (Luebke et al., 1996; Naghashpour et al., 1997). In NIH3T3 cells expressing RCP antisense there is a dramatic reduction in CGRP- and AM-stimulated cAMP signal responses, although there is no reduction in CGRP binding or receptor density. This indicates that RCP is not, as previously thought, involved as a chaperone protein for trafficking CLR to the cell membrane (Evans et al., 2000). The RCP is thought to work with CLR in coupling the receptor to downstream signaling pathways including the cAMP and the protein kinase A pathway (Prado

et al., 2002). It has been proposed that RCP is also involved in transcription based on the function of its yeast homologue, the C17 subunit of yeast RNA polymerase III (Siaut et al., 2003).

Signal transduction mechanisms

In general, CGRP receptors signal through $G_{\alpha s}$ to activate adenylyl cyclase and produce cAMP (Wimalawansa, 1996; Poyner et al., 2002). There is evidence, however, suggesting that CGRP receptors can also signal through $G_{\alpha q/11}$ to stimulate phospolipase C (PLC) and thereby activate inositol 1,4,5-triphosphate (IP_3) formation and release of Ca^{++} from the endoplasmic reticulum (Drissi et al., 1998). Changes in G-protein coupling is a potential mechanism to central sensitization (Winter and McCarson, 2005).

The MAPK pathway is also activated through stimulation of the CGRP receptor (Schaeffer et al., 2003; Vause and Durham, 2009). In smooth muscle, CGRP increases the activity of ERK1/2 and p38 MAPK (Schaeffer et al., 2003) and in DRG neurons ERK1/2 is activated in response to CGRP (Anderson and Seybold, 2004). Stimulation of these pathways is known to increase expression of inflammatory mediators (Kaminska, 2005) and to play a role in initiating and perpetuating peripheral sensitization (Ji, 2004).

The vasodilatory effects of CGRP are mediated through both endothelial-dependent and -independent mechanisms (Wimalawansa, 1996). With respect to the endothelial-dependent mechanism, activated CGRP receptors in endothelial cells activate endothelial nitric oxide synthase (eNOS). This stimulates the production of nitric oxide (NO) which, after diffusion to nearby smooth muscle,

increases cyclic guanosine monophosphate (cGMP) which causes relaxation of smooth muscle (Muff et al., 2001; de Hoon et al., 2003). The endothelial-independent mechanism is the process thought to be responsible for vasodilation in human cranial arteries. In this process, CGRP reaches the perivascular smooth muscle by diffusion or release from local nerve terminals. It binds to its receptor and activates adenylyl cyclase, increasing intracellular cAMP, leading to vasodilation. In a separate endothelial-independent mechanism, CGRP is able to phosphorylate and open ATP-senstive potassium channels (K_{ATP}) channels, thereby hyperpolarizing the cell and causing vascular relaxation (Wimalawansa, 1996; Van Rossum et al., 1997; Gozalov et al., 2008).

Regulation of CGRP receptor expression

Very little is known about what influences the expression of CGRP receptor components. While it has been reported that *in vitro* stimulation of CGRP receptors with 1 μM CGRP does not change expression of CLR, RAMP1, RAMP2 or RAMP3 mRNA in osteoclasts (Granholm et al., 2008), *in vivo* data are lacking and other cell systems remain to be examined.

CGRP receptor distribution

CGRP receptors are found in a variety of tissues including those in the central and peripheral nervous systems (Arulmani et al., 2004a), as well as in the gastrointestinal, cardiovascular, respiratory, endocrine, and musculoskeletal systems (Hagner et al., 2002; Rossi et al., 2003). There are high levels of CGRP receptor expression in heart and blood vessel tissue (Wimalawansa, 1996), with the highest density of receptors located in the atria (Chang et al., 2001). These

receptors have also be detected in coronary arteries, veins, arterioles, heart valves, and endocardium (Wimalawansa, 1996), helping to explain the chronotropic and ionotropic effects of this peptide (Saetrum Opgaard et al., 2000; Poyner et al., 2002). In DRG neurons, CGRP receptors are co-expressed with receptors for other neurotransmitters, such as substance P, vasoactive intestinal peptide (VIP), neuropeptide Y, and norepinephrine (Van Rossum et al., 1997), and in autonomic neural tissue they are co-expressed with tachykinins and substance P (Ursell et al., 1991). Co-localization of CGRP and CGRP receptor components in primary sensory neurons and motor neurons suggests this peptide has autocrine or paracrine functions in these systems (Ma et al., 2003).

CGRP receptor expression in migraine relevant tissues

The relevance of these proteins to migraine is indicated by the fact that CLR, RAMP1, and RCP are expressed in human cerebral blood vessels (Moreno et al., 2002), dura mater, dural mast cells, trigeminal ganglion and presynaptic nerve terminals in the spinal trigeminal nucleus (Lennerz et al., 2008). They are also found in peripheral and central nervous system neurons and glial cells (Levy et al., 2004), Schwann cells (Lennerz et al., 2008) and trigeminal ganglia glial cells (Li et al., 2008). It is notable, however, that CGRP receptors do not appear to be present on trigeminal nerve endings (Lennerz et al., 2008).

Basis for sex differences in migraine

Migraines are 3 times more prevalent in women and the severity of migraine pain often varies with the menstrual cycle, peaking around the time of menstruation (Welch, 1997; Stewart et al., 2000b). An associated trigeminal pain

condition, temporomandibular disorder, has also been shown to vary with the menstrual cycle, indicating higher levels of trigeminal pain when estrogen drops to its lowest levels in the perimenstrual periods (LeResche et al., 2003). Moreover, it is notable that CGRP receptor protein expression and the effects of CGRP on myometrial contraction change with the estrous cycle in mice (Naghashpour and Dahl, 2000). Receptive field sizes of neurons in the spinal trigeminal nucleus are larger when estrogen levels are high (Bereiter and Barker, 1975). Estrogen increases excitability of trigeminal nociceptors (Flake et al., 2005) as well as ERK activation and orofacial allodynia in a model of inflammatory pain (Liverman et al., 2009a). These studies indicate that estrogen regulates the excitability of trigeminal neurons and increases responses to inflammation. However, there have been few studies addressing the sex differences in peripheral or central sensitization in migraine models.

Published studies in the Berman laboratory have demonstrated that estrogen amplifies the behavioral response to inflammatory pain in a temporomandibular model. These investigators have also found that two types of estrogen receptors are present in likely nociceptors, and that estrogen stimulates the intracellular MAPK signaling pathway ERK, which is also activated during sensitization in chronic pain conditions (Puri et al., 2005; Puri et al., 2006).

Migraine pharmacotherapy

Because of the data indicating that migraine results from a dysfunction of the vascular system, pharmacologic therapy has focused on the use of vasoactive mechanisms. Ergotamine, a vasoconstrictor, is an early example of

this approach. However, because of its diverse pharmacodynamic actions that contribute to adverse effects, ergotamine is not an ideal therapy for the relief of migraine symptoms (Tfelt-Hansen, Saxena et al., 2000; Tfelt-Hansen and Koehler, 2008).

Analgesics

Many studies have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) and opioids are effective in the acute management of migraine. Efficacy has been demonstrated for acetylsalicylic acid in doses up to 1000 mg (Tfelt-Hansen and Olesen, 1984), ibuprofen at 200-800 mg (Diener et al., 2004), diclofenac at 50-100 mg (Linde, 2006), and acetaminophen at 1000 mg (Peatfield et al., 1983). In addition, opioids such as propoxyphene (Hakkarainen et al., 1978) and codeine (Somerville, 1976) are known to be effective treatments as well. Therefore, analgesics are considered first line drugs for the acute treatment of migraine and are often administered in combination with antiemetics such as metoclopramide or domperidone to treat the nausea that may accompany this condition. The antiemetics may also reverse some of the gastric stasis that results from sympathetic activation due to migraine and thereby aid in drug absorption (Welch, 1993).

Ergot alkaloids

Targeted therapies for treating migraine were not available until the 20th century. In 1906, Dale showed that an extract of ergotamine tartrate inhibits the pressor effect of sympathetic stimulation, and, in 1918, Stoll isolated and purified the active alkaloid. At the time migraine was thought to be caused by an increase

in sympathetic activity. As ergotamine was shown to have sympatholytic effects, it was a logical choice as a possible therapy for this condition and, in 1925, Rothlin proposed that it be used to treat migraine (Rothlin, 1955; Stoll, 1955). His recommendation appeared strengthened by positive results of a clinical trial conducted that year (Tfelt-Hansen and Koehler, 2008).

In 1938, Wolff and Graham proposed that the effectiveness of ergotamine in treating migraine is because the drug constricts cranial vasculature. This group reported that the temporal artery pulse pressure amplitude was decreased with administration of ergotamine (Graham and Wolff, 1938; Wolff, 1955; Tfelt-Hansen and Koehler, 2008). This study provided the first direct evidence in support of the vascular theory of migraine and led to the hypothesis that the condition is the result of cranial vasodilation. This finding led to the refinement of the treatment options for migraine. Synthesized in 1943, dihydroergotamine (DHE) was predicted to be more effective than ergotamine because it is more selective for α-adernergoceptors. While clinical trials revealed that DHE is slightly less potent than ergotamine in the acute treatment of migraine, it was found it could also be used prophylactically to reduce the number and severity of migraine attacks (Rothlin, 1955; Tfelt-Hansen and Koehler, 2008).

Although the ergot alkaloids were the recommended class of drugs for acute therapy, there are few placebo controlled, randomized clinical trials attesting to their efficacy. It is now appreciated that the ergot alkaloids probably act primarily as 5-HT_1 receptor agonists in treating migraine, although with less selectivity than the triptans. The ergots induce both venous and arterial

constriction. Regardless of the route of administration, ergotamine tartrate is effective in less than 50% of migraine patients (Welch, 1993). When given parenterally, even when together with an antiemetic, DHE is less efficacious than sumatriptan but as effective as opioids (Colman et al., 2005). Both ergotamine and DHE treatment are associated with numerous adverse effects, including drug-overuse headaches that occur quickly and often at low doses (Linde, 2006).

Triptans

More selective 5-HT receptor agonists for the treatment of migraine headache, the triptans, were developed in the late 1980's. The first member of this class developed for human use was sumatriptan (Feniuk et al., 1991; Humphrey et al., 1991). The triptans are thought to be superior to the ergot alkaloids in the treatment of migraine symptoms because of their greater selectivity for cerebral vascular 5-HT_{1B} receptors and for the 5-HT_{1D} receptors in trigeminal neurons. At therapeutic doses the triptans cause constriction of the cerebral vasculature, and in addition, they inhibit trigeminal function peripherally by reducing CGRP release and centrally by blocking action potentials in the spinal trigeminal nucleus (Tfelt-Hansen et al., 2000).

Triptans, the best studied migraine drugs, are selective for the $5HT_{1D}$ receptor subtype located on the peripheral terminal of trigeminal neurons of vascular and meningeal structures and the $5HT_{1B}$ receptor subtype found on the smooth muscle and endothelium of cerebral arteries that mediate vasoconstriction (Nilsson et al., 1999). The first drug of this class, Introduced for clinical use in 1991, sumatriptan has been extensively utilized for the treatment of

migraine since then. Comparative trials of sumatriptan versus other triptans such as rizatriptan, zolmitriptan, and naratriptan have shown small, but statistically significant, differences in efficacy. The clinical relevance of these differences is still being debated (Saxena and Tfelt-Hansen, 2000). Suggested doses of sumatriptan are 6 mg and 100 mg for subcutaneous and oral administration, respectively. Triptans are slightly more efficacious than most analgesic/anti-emetic combinations and are effective in 60% of patients whose migraines are refractory to NSAIDs. The triptans are less effective when administered during the aura phase of the migraine, with administration during the headache phase only recommended (Linde, 2006). Due to its short elimination half-life (2 hours), headache can return within 24 hours but is typically relieved by a second dose of sumatriptan (Welch, 1993). Because triptans are vasoconstrictors, they are contraindicated in those with significant cardiovascular disease, unmanaged hypertension, and for certain types of migraine, such as hemiplegic and basilar-type migraine (Dodick et al., 2004). Vasocontractile responses to triptans correlates with plasma concentrations in cerebral, but not coronary (Edvinsson et al., 2005). Each one of these approaches has had limited to moderate effects in treatment of migraine. The NSAIDs are less effective than other available therapies. Ergot alkaloids and triptans while more effective cannot be prescribed in patients with hypertension and cardiovascular risk factors for stroke. This has led to development of a new class of drugs, the CGRP antagonists.

CGRP antagonists

The newest class of antimigraine drugs is the CGRP receptor antagonists.

The first of these, olcegepant (Figure 2), was developed by Boehringer Ingelheim (Doods, 2001). In animal models and isolated human cerebral arteries, olcegepant blocks the cerebral vascular effects of CGRP and trigeminal stimulation (Doods et al., 2000; Kapoor et al., 2003a; Kapoor et al., 2003b; Petersen et al., 2004), while having no effect in the absence of CGRP exposure (Kapoor et al., 2003b; Arulmani et al., 2004b). Initial clinical trials revealed an acceptable safety profile (Petersen et al., 2005a) and efficacy in preventing CGRP induced headache and vascular changes in healthy volunteers (Petersen et al., 2005b). A phase II clinical trial demonstrated the antimigraine effectiveness of olcegepant following intravenous administration, with the optimal dose being 2.5mg (Olesen et al., 2004).

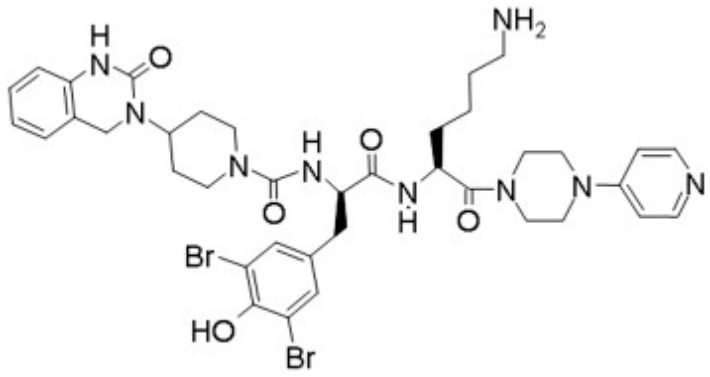

Figure 2. Olcegepant

Because olcegepant is not orally active, efforts were undertaken to develop CGRP antagonists that are bioavailable by this route (Williams et al., 2006). These resulted in the identification of telcagepant (Figure 3) (Paone et al., 2007). *In vitro* testing revealed that telcagepant binds with high affinity to CGRP receptors, is a competitive antagonist of CGRP-induced cAMP accumulation in

transfected cells expressing human CGRP receptor, has high selectivity for CLR/RAMP1 receptors versus other calcitonin family receptors, and displays a 1500-fold higher affinity for human CGRP receptors over dog and rat receptors (Salvatore et al., 2008). Telcagepant inhibits endogenous CGRP-induced and transient receptor potential vanilloid (TRPV) 1 receptor facilitated cutaneous vasodilation in the rhesus forearm in a concentration-dependent manner (Salvatore et al., 2008). A phase II, dose-ranging study showed that 300 mg of telcagepant displays an efficacy similar to a 10 mg dose of rizatriptan (Ho et al., 2008b). Approximately 69% of both groups reported pain relief 2 hours following treatment. The most common adverse events reported following administration of telcagepant were nausea, dizziness, and somnolence (Ho et al., 2008b). In a phase III trial of telcagepant, a 300 mg dose of telcagepant is as effective for the acute treatment of migraine as the most effective (5 mg) dose of zolmitriptan and more effective than placebo (Ho et al., 2008a). In this study there were fewer side effects reported by those taking telcagepant than by those treated with zolmitriptan. Importantly, it does not appear that CGRP antagonists have direct vasoconstrictor properties (Ho et al., 2008a), as suggested by a study showing that treatment with 500 mg of telcagepant does not block the vasodilatory effects of sublingual glyceryl trinitrate (GTN) in healthy male volunteers (Van Der Schueren et al., 2009). This suggests that telcagepant can be employed for the treatment of migraine in those with high cardiac risk profiles.

It was recently reported that liver transaminases are elevated following administration of telcagepant (Tepper and Cleves, 2009). Although the dosing

schedule used in this study differs from that required for acute treatment of migraine, the results have slowed the clinical development of this compound as its potential toxicity is reassessed (Merck, 2009).

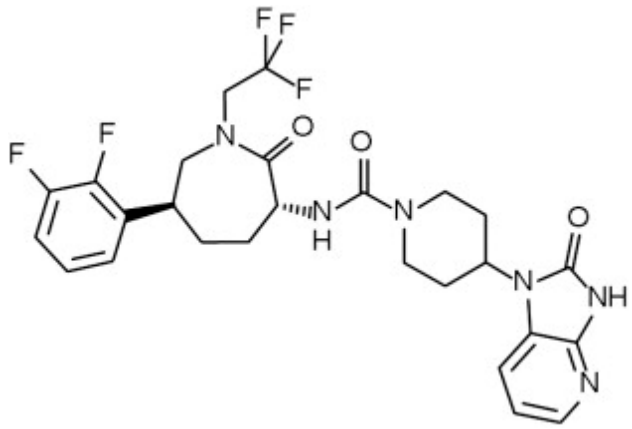

Figure 3. Telcagepant

MK-3207

Other CGRP receptor antagonists, such as MK-3207 (Figure 4) have been developed that display a higher affinity than telcagepant for the CGRP receptor (Salvatore et al., 2010). For MK-3207, the K_i for inhibition of ^{125}I-hCGRP binding to membranes taken from stably transfected HEK293 cells expressing CLR/RAMP1 receptors is 21 pM in comparison to 770 pM for telcagepant, and the MK-3207 IC_{50} for inhibiting CGRP-induced cAMP formation is 0.12 nM as compared to 2.2 nM for telcagepant (Salvatore et al., 2010). This agent also demonstrates good oral bioavalability in rhesus monkeys, dogs, and rodents. In a capsaicin-induced dermal vasodilation model in rhesus monkeys, MK-3207 is reported to be approximately 100-fold more potent than telcagepant (Salvatore et al., 2010). Currently MK-3207 is in phase II clinical trials. Another compound,

MK-8825, displays a similar preclinical profile to MK-3207 (Bell et al., 2010).

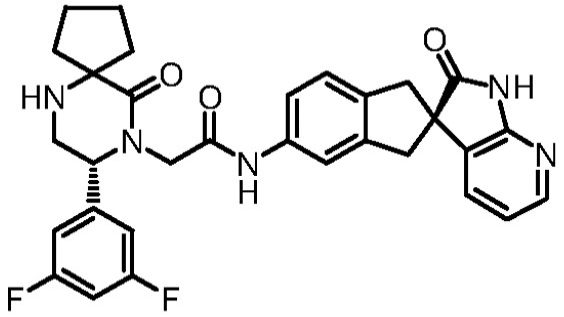

Figure 4. MK-3207

Prophylactic therapy

Prophylactic therapy is employed for only the most severe cases of migraine. Many of the agents used for this purpose have potentially serious adverse effects and can interact with other drugs. Currently, the drugs of choice for prophylactic use are the beta-adrenoceptor antagonists propranolol and metoprolol, the calcium channel blocker flunarizine, and the anticonvulsants valproic acid and topiramate (Linde, 2006). Whereas, estrogen is indicated for prophylactic use in menstrual migraine. In some cases non-pharmacological approaches, such as relaxation and meditation, have also proven effective as prophylactic treatments for migraine (Linde, 2006).

Overall, treatment of migraine even today is empirically driven, reflecting the lack of understanding about the underlying pathophysiology. In the coming years it is anticipated that new findings about the underlying physiological mechanisms of this disorder will lead to the design of more effective episodic and

prophylactic therapies and a more rational approach to it treatment. The present study was undertaken to obtain some of the data needed to attain these objectives.

Models of migraine pain

The IHS-2 criteria for migraine include at least 5 headache attacks lasting 4-72 hours that have at least two of the following characteristics: unilateral, pulsating, moderate or severe pain intensity aggravated by routine physical activity, and at least one of these characteristics: nausea and/or vomiting, photophobia and phonophobia. Allodynia occurs when normally innocuous stimuli are able to induce pain, and hyperalgesia is an increased sensitivity to a stimulus that was previously painful. The presence of allodynia is associated with more severe headaches (Burstein et al., 2004), with many migraineurs experiencing thermal and mechanical allodynia in regions innervated by the trigeminal nerve (Burstein et al., 2000b; Goadsby, 2005; LoPinto et al., 2006). While many of these IHS targeted migraine behaviors can be examined in rodents most animal models of trigeminal pain were designed to study temporomandibular disorder. Some have used neuropathic pain models to examine conditions associated with trigeminal pain. Nerve ligation, a method for inducing neuropathic pain, has been employed in studying rodent behavior in the presence of pain. For example, an increased mechanical sensitivity has been quantified after ligation of the infraorbital nerve (Vos et al., 1994).

Common approaches for the study of temporomandibular disorder involve inducing nociceptive pain by injection of irritants into the areas innervated by the

third branch of the trigeminal nerve followed by measurement of various behavioral endpoints. Examples of this approach include injection of Complete Freund's Adjuvant (CFA) into the temporomandibular joint or masseter muscle to cause facial allodynia (Ambalavanar et al., 2006b) and altered feeding behavior (Kerins, 2005). Formalin has been injected into the same regions followed by an analysis of facial grooming (Clavelou et al., 1995), hyperalgesia, and allodynia (Imbe et al., 2001). Grooming has also been measured after injection of capsaicin into the vibrissa (Pelissier et al., 2002). A newer approach, and one that appears more relevant for the study of migraine, is production of allodynia by application of an IS to the dura of conscious rats 3 times/week for 4 weeks (Oshinsky and Gomonchareonsiri, 2007).

Behavioral assays

The difficulty of creating a migraine behavioral model was aptly summarized by Harold Wolff in 1938:

> *Pain is certainly the commonest complaint with which the physician must deal and headache probably the commonest pain. Pain must be studied in conscious human beings. Since it is a sensory experience it must be reported by man. Thus, when the effects of noxious stimulation are studied in animals by observation of skeletal muscle responses, these cannot be considered as responses to pain because animals cannot give subjective report of their sensation. In animals, one studies not pain, but reaction to noxious stimulation* (Graham and Wolff, 1938).

Studies of migraine pain have, for the most part, employed single unit

recording or c-fos immunohistochemistry in the spinal trigeminal nucleus as surrogate measures of trigeminal pain (Bergerot et al., 2006). Because in the present study, behavioral assays are used as measures of trigeminal pain, it is important to review the behavioral measures used in the past for such studies...

Behavioral assays: trigeminal pain models

For years, behavioral measures of trigeminal pain have included facial allodynia and hyperalgesia, both thermal and mechanical, facial grooming, and altered feeding behavior. In the studies described here we used several of the behavioral assays described below.

Mechanical facial allodynia and hyperalgesia

Testing mechanically for facial allodynia and hyperalgesia is one of the most common behavioral tests used in models of trigeminal pain. In these assays, rats are habituated to stand still either by restraining them by hand, restraining them in an enclosure, or using operant conditioning to drink sweetened water to ensure immobilization (Ambalavanar et al., 2006b; Oshinsky and Gomonchareonsiri, 2007; Liverman et al., 2009a). Testing of the three different branches of the trigeminal innervation most frequently involves mechanically stimulating the masseter, whiskerpad, and periorbital regions of the face with standard force von Frey filaments. A variable force transducer can be coupled to the filament to measure allodynia. This model has shown that multiple infusions of IS on the dura induce an interictal allodynia that lasts for longer than 3 weeks after the final infusion. In this model, increased allodynia and increased levels of extracellular glutamate in brain are elicited in response to GTN infusion

are further potentiated by IS-induced chronic trigeminal hypersensitivity (Oshinsky and Gomonchareonsiri, 2007). Another study showed that facial allodynia peaks 3 hours following application of IS to the dura and is reversed by administration of sumatriptan, naproxen, or the CGPR antagonist $CGRP_{8-37}$, but not affected administration of a neurokinin-1 (NK-1) antagonist (Edelmayer et al., 2009). Similarly, induction of mechanical facial allodynia is observed following application of either human immunodeficiency virus (HIV) glycoprotein gp120 or an IS (Wieseler et al., 2009). Another model utilizes a focused air current directed at the animal's head to elicit ultrasonic vocalization (USV). Rats receiving intracerebroventricular lipopolysaccharide displayed an increase in USV in comparison to naïve animals. The relevance of this model to migraine is indicated by the fact that the increased USV is abolished by administration of morphine, ketorolac, zolmiptriptan, sumatriptan, dihydorergotamine, or $CGRP_{8-37}$ (Martino and Perkins, 2008).

Thermal facial allodynia and hyperalgesia

Others have measured thermal hyperalgesia in the trigeminal territories of rats. The regions to be thermally stimulated are shaved to ensure contact between the thermal source and the skin. Similar to mechanical testing, the rat is trained to hold its head still by drinking water allowing the skin to come into contact with the thermal source. As the rat accesses the reward, the temperature of the thermal stimulus is increased. The sensitivity of the animal is measured by recording the temperature at which the rat withdraws its head from the water source and thermal stimulus (Neubert et al., 2005).

Hind paw mechanical hyperalgesia models

In order to measure hind paw mechanical hyperalgesia, the rodent is placed on a mesh floor and a von Frey filament or similar object is used to prod the foot pad from underneath. The normal response to this stimulus is a nocifensive withdrawal reflex and a relatively quick return of the foot to the floor. Increased sensitivity to this mechanical stimulus is indicated by a more pronounced foot withdrawal and a longer period of withdrawal. In some cases a hyperalgesic animal will attend to the stimulated foot by licking it. The withdrawal time can be measured with a stopwatch. This procedure, combined with a measure of mechanical allodynia, are together referred to as the up-down method (Chaplan et al., 1994).

For the up-down method, mechanical allodynia is tested using von Frey filaments. These filaments are of differing diameters, each of which is designed to exert a standardized force when pressed against the skin to the point of bending. The force required to elicit a response is then recorded as the threshold for mechanical allodynia (Chaplan et al., 1994). This procedure has been used to demonstrate that hind paw allodynia peaks at 3 hours following application of IS to the dura and, as with mechanical facial allodynia, this mechanical hind paw allodynia is reversed by administration of sumatriptan, naproxen, or the CGRP receptor antagonist, $CGRP_{8-37}$, but not affected by administration of the NK-1 receptor antagonist (Edelmayer et al., 2009).

The Randall-Selitto method is another common procedure for measuring mechanical sensitivity. In this assay the rodent is restrained and a special

apparatus is used to move a screw against a specific location on the footpad. The pressure is slowly increased, with that required to stimulate a foot withdrawal or vocalization recorded as the pain threshold (Randall and Selitto, 1957).

Facial grooming

Facial grooming is also used to assess trigeminal nerve sensitization. After injection of capsaicin into the whisker pad, the rat is observed and the pain score measured by the number of seconds spent grooming the injection site with the ipsilateral fore- or hind paw within a 42 minute period (Clavelou et al., 1995; Pelissier et al., 2002)

Altered feeding behavior

Other behavioral measurements of pain include changes in meal duration, meal size, and number of meals (Kerins et al., 2004; Kerins et al., 2005).Notably, meal duration appears to be the best indicator of temporomandibular pain, being significantly increased for 24 to 48 hours after CFA injection into the temporomandibular joint (Kerins et al., 2005).

Photophobia

Few have attempted to test for photophobia. In principle, rats and mice are normally photophobic because they avoid well illuminated open spaces at all times. There is a report of a photophobic test using a strain of mice that displays photophobicity when placed in a divided light/dark enclosure (Thiels, 2008). A study of transgenic mice that over-express human RAMP1 protein in peripheral and central nervous tissues revealed enhanced light-aversive behavior, as

defined by an increased time spent in the dark chamber of a 1000 lux light-dark box. The light-aversive behavior was exacerbated by the intracerebroventricular administration of CGRP, and blocked by co-administration of olcegepant, a CGRP receptor antagonist (Recober et al.; Recober et al., 2009).

Behavioral assays in other pain models

Behavior has been an endpoint in a variety of animal pain models. It is possible that some of these might be appropriate for establishing preclinical models of migraine pain.

Thermal sensory models

An approach for measuring thermal hyperalgesia of the footpad is to place a light below a glass bottomed enclosure. The light is positioned below the resting footpad of an unrestrained rat and then turned on. As the light source begins to heat the glass and the footpad, the latency for withdrawal of the foot is measured by a sensor coupled to a timer. This assay displays better sensitivity in a carrageenan-induced inflammatory model then the Randall-Selitto mechanical sensitivity assay (Hargreaves et al., 1988).

Cold allodynia can also be used to study a decrease in pain threshold. In one assay procedure, the animal is placed on a cold metal plate at 4° C for 20 minutes. Allodynia is quantified by counting the number of nocifensive withdrawal reflexes observed when the affected foot pad touches the floor. This value is compared with the number of nocifensive withdrawals recorded when the animal is placed on a metal floor warmed to 30° C (Choi et al., 1994).

Another cold approach uses acetone applied to the footpad. This is

typically performed with rats and utilizes on the wire mesh floor used for mechanical testing. As it evaporates, the acetone causes a cooling sensation within 1-2 seconds. Normal animals ignore this sensation, but sensitized subjects display strong nocifensive withdrawal responses that can be readily quantified by counting.

Actimetry

Actimetry is a method for measuring gross animal behavior. For the test, the animal is placed in an enclosed area where high sensitivity force transducers are coupled to the four corners of a rigid, low-mass floor to monitor and track locomotor activity. Software processes the force-location data and provides a detailed analysis of locomotor behavior. Additional analysis can be employed to yield information about ambulation, tremors, and certain physiological parameters. Actimeter measurements have been used to describe animal behavior following manipulation of brain dopaminergic systems (Bonatz et al., 1987) and to measure pharmacologically-induced behavioral changes (Kuczenski and Segal, 1999; Chen et al., 2003; Fowler et al., 2003; Fowler et al., 2007) and aging (Stanford et al., 2002).

II. Statement of Purpose

Migraine is among the most common neurological diseases, occurring in 12% of the population (Stovner et al., 2007; Bigal and Lipton, 2009) and there is evidence that the condition is under-diagnosed (Buse et al., 2009). The true societal impact is compounded by the debilitation and losses in productivity that accompany the disorder (Buse et al., 2009). It is 3 times more prevalent in females than males (Bigal and Lipton, 2009). The pathophysiology of migraine is poorly understood, in part because of the lack of preclinical models of headache. In addition, the reason for the female predominance is not understood (Rasmussen, 1993; Bigal and Lipton, 2009). Because there is no objective test for the condition, migraine is diagnosed using clinical criteria established by the IHS. These are based on presenting behavioral signs and symptoms, including duration and intensity of pain, pulsating and unilateral nature of pain, and avoidance of routine physical activity.

Previous attempts to model and study migraine have employed electrophysiological techniques, yielding important information about the neuronal sensitization that appears to underlie the disorder (Burstein et al., 1998; Jakubowski et al., 2007). Others have focused on studying related trigeminal pain conditions such as temporomandibular disorder, which have symptoms that are well suited for study in animal behavioral tests (Clavelou et al., 1995; Kerins et al., 2004; Ambalavanar et al., 2006b; Takeda et al., 2007; Liverman et al., 2009a). However, none of these approaches include the behaviors specifically associated with the IHS criteria for diagnosing migraine in humans.

There is strong evidence that CGRP and its receptors are functionally

important in migraine pathophysiology. Because CGRP is a vasoactive neuropeptide, it could contribute to the vasodilatory component of migraine (McCulloch et al., 1986). Moreover, it has been shown that CGRP levels in plasma are increased following activation of the trigeminal system and CGRP is present in nociceptors including those in the trigeminal ganglion that innervate cerebral vasculature (Goadsby et al., 1988; Moskowitz and Macfarlane, 1993; Ambalavanar et al., 2006a). ***The overall hypothesis of this study is that dural inflammation will induce allodynia, reduced locomotor activity, and increase CGRP and CGRP receptor gene expression in the trigeminal nervous system in male and female rats. Changes in behavior and gene expression will be more pronounced in females.***

The following dissertation is organized in a format based on prepared publications such that each specific aim contains an abstract preceding each report of results and discussion. In some cases there is repetition of background information first presented in the general introduction section within the aims section. This organization enables each aim to be read and understood individually.

Based on previous findings, this dissertation project has the following aims:

1. ***To test the hypothesis that locomotor activity and mechanical facial allodynia are modulated in a sex-dependent manner in response to inflammation of the dura mater in a rat model of migraine.***

Earlier studies indicate that application of IS to the dura mater causes mechanical sensitization of the trigeminal nervous system (Burstein and

Jakubowski, 2004; Jakubowski et al., 2007) and mechanical periorbital allodynia. Mechanical periorbital allodynia was demonstrated acutely following a single application (Edelmayer et al., 2009; Wieseler et al., 2009) and interictally following chronic application of IS (Oshinsky and Gomonchareonsiri, 2007). In the present study, a new behavioral assay is developed that measures changes in routine activity, one of the IHS criteria for migraine, using actimetry to measure distance traveled and bouts of low mobility during application of IS to freely-moving rats. An established procedure for measuring periorbital allodynia is coupled with the locomotor assay to obtain data from both male and female rats receiving two different volumes of IS or vehicle. The data are analyzed to investigate whether there are sex differences in any IS-induced changes in locomotor activity and mechanical orofacial allodynia.

2. ***To test the hypothesis that expression of genes encoding for calcitonin gene-related peptide and related receptor components are modulated in a sex-dependent manner in response to inflammation of dura mater in a rat model of migraine.***

To address this issue, sex-dependent variations in gene expression of CGRP and its associated receptors, CLR, RAMP1, and RCP in the trigeminal pathway are examined following application of an IS to the rat dura.

Others have shown that intravenous administration of CGRP to migraineurs without aura elicit migraine-like symptoms as defined by IHS criteria (Lassen et al., 2002). However, no one has examined whether there are sex-related differences in gene expression of CGRP and CGRP receptor or how expression of these genes is modulated in a model of neurogenic inflammation. In this study the gene expression of CGRP and CGRP receptors is evaluated using quantitative real-time polymerase chain reaction and an attempt made to determine if there are sex-related variations in baseline gene expression and induction.

III. Materials and Methods

Common methods

Animals

Animal care and use procedures were approved by and conducted according to University of Kansas Medical Center Institutional Animal Care and Use Committee and Institute of Laboratory Animal Research guidelines. Ten week old, sexually mature Sprague-Dawley rats were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). The animals were housed in the Kansas University Medical Center Laboratory Animal Resources Facility in plastic cages and provided water and food *ad libitum*. The animals were all subject to a 12 hour light-dark cycle. The rats were divided into groups based on sex, application of IS or control vehicle, and volume of application. All behavioral experiments were conducted between 9:00 and 14:00 h in a dedicated, temperature-controlled room.

Cannula implantation

The cannula for dural delivery of the IS or vehicle was a Plastics One (Roanoke, VA) product made of biocompatible material implanted into the skull under isoflurane anesthesia (3.5% isoflurane for induction and 2.7% for maintenance in 4% oxygen). During surgery, sterile ophthalmic ointment was used to protect the rat's eyes and absence of a corneal reflex confirmed the level of anesthesia. The animal was placed in a stereotactic apparatus, immobilized at the external auditory meati and incisors, and the cannula placed on the right side 5 mm lateral to midline and halfway between the bregma and lambda over the

occipital lobe to model unilateral migraine. The dura mater was exposed by first thinning a 1 mm diameter region of the skull using a burr drill to remove the outer layer, taking care to irrigate the skull with sterile saline to prevent heating. A McCall currette was used to carefully remove the inner layer of bone while leaving the underlying dura intact. Sterile petrolatum was placed over the intact dura to prevent dural fixation to the cannula and calvarium. Single rostral and caudal drill holes were placed 3 mm from the center of the cannula, and stainless steel screws attached to the skull to provide anchors for the dental cement (Ortho-Jet, Lang Dental Mfg. Co., Inc., Wheeling IL) used to provide stability to the cannula. An internal obturator was placed within the cannula to maintain patency. The scalp was folded over the base of the cannula and sutured using #4 sterile silk. Following recovery from the isoflurane anesthesia, the animal was administered buprinorphine (0.05 mg/kg in 1 mL normal saline) by intraperitoneal injection for analgesia, and transferred to a clean cage with an external heat source. Breathing and movement were observed until the animal was fully awake and mobile. The rat was then returned to its home cage. Following implantation, the cannula and obturator cap projected less than one centimeter from the right side top of the head. Closure of the skin around the cannula was confirmed prior to behavioral testing. A preliminary study showed no behavioral differences between rats with cannulas implanted and non-surgical control animals (data not shown). In the rare case (2/43) where animals became ill following surgery, they were removed from the study. All animals were monitored regularly for health status by veterinary staff at the animal resources facility.

Treatment

The vehicle control groups received regular application to the dura of 10 or 20 µL of pyrogen-free phosphate buffered saline (PBS) adjusted to a pH of 7.4. Groups selected for the inflammatory stimulus received regular application to the dura of 10 or 20 µL of IS consisting of 1 mM histamine, 1 mM 5-HT, 1 mM bradykinin, and 0.1 mM PGE_2, in PBS at a pH of 5.5 (Table 2). For application to the dura of IS or vehicle, the obturator cap of the cannula was removed and an internal delivery cannula inserted and connected to PE50 tubing attached to an infusion pump (Cole Parmer) that delivered vehicle or IS at a rate of 2 or 4 µL/min over 5 min (Burstein and Jakubowski, 2004; Oshinsky and Gomonchareonsiri, 2007). Following application of IS or vehicle and locomotor testing, the internal delivery cannula was removed and replaced with the obturator cap.

Treatment Group	Sex	Treatment	Volume	Behavioral Studies	Molecular Studies
Naïve control	Male	-	-	-	mRNA
	Female	-	-	-	mRNA
Vehicle control	Male	PBS	10 µL	Locomotor, Allodynia	mRNA
			20 µL	Locomotor, Allodynia	mRNA
	Female	PBS	10 µL	Locomotor, Allodynia	mRNA
			20 µL	Locomotor, Allodynia, hind paw testing	mRNA
Inflammatory group	Male	IS	10 µL	Locomotor, Allodynia	mRNA
			20 µL	Locomotor, Allodynia	mRNA
	Female	IS	10 µL	Locomotor, Allodynia,	mRNA
			20 µL	Locomotor, Allodynia, hind paw testing	mRNA

Table 2. Treatment groups

Timeline

Prior to surgery the rats were trained for 10 days in the allodynia task, and presurgical baseline behavioral responses measured for all tasks one day prior to surgery. Rats were allowed to recover for 7 days following surgery before resuming behavioral testing, a time when preliminary studies revealed the behavioral responses had returned to presurgical baseline values. Postsurgical behavioral measurements were obtained two days before delivering the first application of vehicle or IS.

Behavioral testing was performed between 0 and 30 minutes following

application of IS to the dura, when cutaneous allodynia responses are most pronounced (Oshinsky and Gomonchareonsiri, 2007). Because the temporal relationship between inflammation of the dura and onset of headache is not well understood, animals were tested during application of IS and during a second period 5 minutes later to determine the duration of the effects. The first five minutes (during IS application) was termed the “onset phase”, while the 10-15 minute time period after IS application was termed the “persistence phase”. Behavioral data were collected after application of IS on every 3rd day for a 7 total applications. A final behavioral data point was taken 48 hours after the last application of IS. Locomotor and facial allodynia results were recorded for comparison with other studies, most of which focused on the measurement of interictal time points (Oshinsky and Gomonchareonsiri, 2007; Edelmayer et al., 2009). The post-surgical baseline interictal data were subtracted from the Day 8 interictal results to calculate changes occurring over the 2.5 weeks of the experiment. For all behavioral testing, rats were coded and experimenters blind to treatment.

Aim 1 methods

Mechanical allodynia test

An operant conditioning paradigm was used to train rats to hold their heads in a fixed position while testing their withdrawal responses to mechanical stimulation of the face. For this task, rats were placed in a start box and conditioned to traverse a plastic tube to drink an aqueous solution of 0.1 M sucrose. The device is positioned so the rat places its head in a fixed, repeatable

position allowing von Frey monofilaments to be applied to specific locations, such as the masseter and periorbital region. While performing this task, rats consumed less than 2 mL of the sucrose solution, amounting to less than 10% of their daily water consumption. The amount of fluid required to alter sensory thresholds is over 1000 times this volume (Kanarek et al., 2001). The rats were not restrained in any way. Threshold withdrawal was assessed using a 4 g von Frey monofilament to test for mechanical allodynia on both the ipsilateral periorbital region and a 4 g von Frey monofilament on the ipsilateral masseter region. Previous studies using a 4 g von Frey monofilament demonstrated this procedure is sensitive enough to detect changes in withdrawal responses due to inflammation or estrogen status (Liverman et al., 2009a; Liverman et al., 2009b).

Open-field locomotor activity

General open-field locomotor activity was measured using an actimeter arena (BASi, San Diego CA). The apparatus utilizes high sensitivity force transducers coupled to the four corners of a rigid, low-mass floor to monitor and track the subject's locomotor activity. Software processes the force-location data and provides detailed analysis of locomotor behaviors including distance traveled, bouts of low mobility, spatial confinement, and stereotypy. These position and force measurements are captured by the actimeter hardware and processed by accompanying software according to formulas developed by Dr. Stephen Fowler at the University of Kansas (Fowler et al., 2001). Briefly, distance traveled is calculated by summing distance over a set time period (5 minutes) using the center of force reported according to the sampling rate (100 Hz). Bouts

of low mobility are scored when the center of force defined did not move beyond a radius of 15 mm within a 10 second period. The spatial confinement score was designed to compare a test rat to a theoretical rat that visits every sector of the arena in a given time period. This value is essentially the standard deviation of the rat's movement from an evenly distributed theoretical value. If the center of force does not move beyond a single sector, a maximum spatial confinement score of 100 is recorded. The aggregate score is the sum of individual scores over 5 minutes. Focused energy is calculated by computing the weighted average of the force variance over a 5 minute period. Taken together, these measures provide quantitative assessments of several aspects of routine animal behavior.

Hind paw testing

Mechanical withdrawal thresholds were measured using an electronic von Frey apparatus (Model 2390; IITC Inc., Woodland Hills, CA). The animals were brought to the testing room and allowed to acclimate for at least 30 minutes prior to initiating the test. Following this acclimation period, the rats were placed in individual Plexiglas chambers located atop a steel mesh table and allowed to the environment for 15 minutes. After acclimation, the electronic von Frey filament was applied perpendicular to the surface of the hind paw. Mechanical pressure from the filament was applied until the animal exhibited a withdrawal behavior, including one of the following: movement of the paw away from the stimulus, shaking of the paw, and biting or licking of the paw. The absence of all of these withdrawal behaviors is defined as no response. The force transducer attached

to the filament automatically records the maximum force that applied to the paw. A measurement was taken three times at 2 to 5 minute intervals. The results were averaged to determine the mechanical withdrawal threshold to the nearest gram. All mechanical threshold testing was performed 2 hours following application to the dura of IS or vehicle. A total of 6 testing sessions were performed during the pre-surgical baseline, post-surgical baseline, and treatment days 1, 2, 3, and 6.

Statistical analysis

A two-way repeated-measures ANOVA with a Holm-Sidak post hoc comparison and confidence intervals was used to compare groups. The data were examined for non-normality and equal variance. Differences between groups are considered significant with a two-sided alpha level of 0.05. SigmaStat 3.5 (Systat Software Inc., Chicago, IL) was used for data analyses.

Aim 2 methods

Control animals

In addition to the vehicle control and IS groups animals mentioned in general methods this aim used male (n = 6) and female (n = 6) naïve control animals for baseline gene expression comparisons.

Animal sacrifice

Animals were sacrificed after undergoing behavioral testing, approximately 20 minutes after receiving the final administration to the dura of vehicle or inflammatory soup. Prior to dissection of tissue samples, all instruments and

surfaces were thoroughly cleaned with RNaseZap (Applied Biosystems/Ambion, Austin, TX). The ipsilateral and contralateral dura, medulla and trigeminal ganglia were dissected and isolated under sterile conditions. All tissue samples were immersed immediately in RNALater (Applied Biosystems/Ambion, Austin, TX) and placed into 4° C refrigerator to preserve the mRNA for isolation.

Quantitative real-time polymerase chain reaction (qRT-PCR)

An RNA analysis was used to measure CGRP receptor (RAMP-1, CLR and RCP) gene expression in dura, trigeminal ganglia, and medulla. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and was treated with DNaseI to remove any contaminating DNA. The cDNA was synthesized using 10 µg total RNA from each sample and random hexamers in a Taqman reverse transcription reaction (Applied Biosystems, Foster City, CA, USA). Expression of CGRP, RAMP1, CLR, and RCP mRNA was analyzed by real-time PCR using SYBR Green dye. The cDNA and gene-specific primers (Table 3) were added to SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaqDNA polymerase, dNTPs mixture, dUTP, and optimal buffer components; Applied Biosystems, Foster City, CA, USA) and subjected to PCR amplification.

Primer	Sequence	Amplicon Size	Gene ID
GAPDH	F –ATGACATCAAGAAGGTGGTG R -CATACCAGGAAATGAGCTTC	177bp	NM_017008
CGRP	F –CTGTCACTGCCCAGAAGAGATC R -CAAAGTTGTCCTTCACCACACC	101bp	
RAMP1	F –ACTGGGGAAAGACCATAGGGAG R -AGTCATGAGCAGTGTGACCGT	230bp	NM_031645
CLR	F –AACAACAGCACGCATGAGAA R -ACCCCCAGCCAAGAAAATAA	403bp	NM_012717
RCP	F –GCGAACGCTGCCCTGCTCAGTA R -CGCGTCATTGCTAGTGCTTTTG	405bp	NM_053670

Table 3. qRT-PCR primers

The PCR reactions were conducted in triplicate and the amplified transcripts quantified with the comparative double-delta threshold cycle (ΔΔCt) method using expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The resulting amplicon product was visualized on agarose gel to check size (and reaction specificity) and to confirm results. A published method for statistical analysis of real-time qRT-PCR data was employed (Pfaffl et al., 2002).

Statistical analysis

The rats were randomly assigned to the experimental and control groups. A student's t-test was used to compare groups. The data were examined for evidence of non-normality and equal variance. Differences between groups are considered significant with a two-sided alpha level of 0.05. SigmaStat 3.5 (Systat Software Inc., Chicago, IL) was used for data analyses.

IV. Sex Differences in Allodynia and Motor Behaviors in a Rodent Model of Migraine

Hypothesis

Locomotor activity and mechanical facial allodynia are modulated in a sex-dependent manner in response to inflammation of the dura mater in a rat model of migraine.

Abstract

The objectives of this study were to develop a preclinical behavioral model of chronic migraine for the purpose of testing sensory and motor behaviors relevant to International Headache Society (IHS) diagnostic criteria, and to determine whether there are sex differences in pain-related behaviors between male and female rats.

Most previous animal studies of migraine employed electrophysiological techniques to assess neuronal sensitization, but in the present study changes in routine physical activity and mechanical sensitivity of facial regions were measured in response to inflammatory soup (IS) induced dural inflammation.

Application to the dura of IS or control vehicle was performed 3 times/week for a total of 8 applications. Locomotor activity was assessed using force plate actimetry during and following application of the IS or vehicle, and periorbital and masseter sensory testing was performed 20 min post-application to measure allodynia.

These measurements revealed pronounced sex differences with regard to these measures. Thus, although both males and females showed behavioral effects following IS application, females were affected at the lower dose and had a longer duration of response with regard to distance traveled, bouts of low

mobility, and spatial confinement. Moreover, females showed an increase in behavioral changes with increased dose of IS. Both males and females develop allodynia and in females distance traveled correlated with allodynia severity.

This study demonstrates sex-related changes in locomotor activity associated with application of IS to the dura and the potential value of using International Headache Society diagnostic criteria to guide development of rodent models of migraine.

Results

Behavioral responses during onset of application of IS

Locomotor activity measurements during application of the IS were designed to measure changes in routine activity at the onset of the headache (onset phase). Measurements of total distance traveled provided spatial quantification of these changes, i.e. a quantitative measure of ambulation (Figure 5A). Male 10 µL groups showed no difference in the distance traveled in the 5 minutes during application while the female group receiving 10 µL IS traveled less distance than control animals during the infusion ($P < .001$). Both the male and female 20 µL IS groups traveled less distance than vehicle controls in the same period (male 20 µL $P < .05$, female 20 µL $P < .01$). A dose effect was noted when comparing the female 10 µL and female 20 µL groups. The female 20 µL group that received IS were less active than female 10 µL group that received IS as measured by total distance traveled ($P < .05$). These data demonstrate that females are sensitive to the effects of IS in a dose-dependent manner and respond to the IS at a lower dose than males.

Quantification of bouts of low mobility assessed the temporal component of inactivity in response to IS treatment, i.e. time spent remaining inactive. Male 10 µL IS groups showed no change in time spent inactive when compared to control animals during the IS infusion (Figure 5B), whereas the female 10 µL IS group spent more time inactive than did their vehicle control cohorts ($P < .01$). There was no significant difference between the male 20 µL group and female 20 µL IS groups and their control cohorts. A sex difference was noted with respect to bouts of low mobility. Thus, at 10 µL doses of IS, females showed more bouts of low mobility as compared with vehicle controls than males, indicating that IS causes greater inactivity in females than males.

Spatial confinement measurements quantify the area explored; i.e., with a high spatial confinement measure, animals confined their movement to their immediate vicinity and did not explore the whole arena. Normally, rats spend much of their time exploring a new environment and relatively little time remaining in a confined area. Thus, an increase in the spatial confinement score represents a change in routine activity. While the males 10 µL IS group were not found to differ from their vehicle control cohorts, the female 10 µL IS group displayed a higher spatial confinement score than controls (Figure 5C). That is, the females explored less area than the controls ($P < .001$). Male 20 µL and female 20 µL IS groups explored less area than did 20 µL vehicle control animals ($P < .05$ and $P < .01$ respectively). Only the male 20 µL, but both the female 10 µL and 20 µL IS groups displayed higher confinement scores than their respective controls, indicating a sex-related difference in sensitivity to IS with

regard to the behavioral endpoint.

Focused energy was used to quantify the amount of restless activity that occurs while the animal is stationary (Figure 5D). Male and female groups receiving 10 µL IS were not different from controls with regard to this measure. In contrast, male and female groups receiving 20 µL IS displayed a lower focused energy score than did vehicle control animals ($P < .005$ and $P < .05$ respectively). It is noteworthy that the control rats increased their focused energy scores over time, while the IS rats remained relatively immobile.

Behavioral response following application of IS

Locomotor activity measurements in the period starting 10 minutes after the infusion of IS or vehicle were designed to measure the persistence of changes in routine activity. In this period, the male 10 µL IS group showed no change in distance travelled when compared with vehicle controls (Figure 6A), while the female 10 µL IS group traveled less distance than vehicle controls in the period following infusion ($P < .001$). Neither the male 20 µL nor the female 20 µL IS groups showed any statistical difference in distance traveled as compared to the vehicle treated groups. Thus, the female 10 µL group showed the most persistent changes in distance traveled following IS treatment.

Vehicle and IS treatment in the male 10 µL group produced no statistical difference in bouts of low mobility during the persistence phase (Figure 6B). On the other hand, the female 10 µL IS group spent less time exploring the environment as compared to vehicle control animals ($P < .001$). Male 20 µL and female 20 µL IS groups showed no statistical difference in bouts of low mobility

during this period as compared to controls. As with the earlier behavioral tests, the female 10 µL IS group showed the most persistent effects.

The male 10 µL IS group showed no changes in the spatial confinement metric when compared to vehicle controls (Figure 6C), while the female 10 µL IS group explored less area than controls ($P < .0005$). Male 20 µL and female 20 µL IS groups showed no changes in the area they explored as compared to vehicle controls. Thus, the female 10 µL IS animals was the only group showing persistent effects with respect to this measure.

The male 10 µL IS group was no different with regard to its focused energy score as compared to vehicle control animals (Figure 6D), whereas, in contrast, the female 10 µL IS group displayed lower focused energy score ($P < .005$). Both the male 20 µL and female 20 µL IS groups receiving showed higher focused energy scores than control animals ($P < .05$ and $P < .05$ respectively). In this case, both 20 µL groups displayed persistent changes in focused energy with the 10 µL females showing changes in focused energy in the persistence phase but not in the onset phase.

Facial allodynia following IS treatment

Mechanical testing of the periorbital region measured sensitization of the first division of the trigeminal nerve (Figure 7A). The male 10 µL IS group showed significant allodynia of the periorbital region only on testing day 5 ($P < .05$) as compared to controls, with the male 20 µL IS group showing significant periorbital allodynia when compared with vehicle cohorts on testing days 3, 4, 5 and 7 ($P < .05$). The female 10 µL IS group showed significant periorbital allodynia when

compared with their vehicle cohort on days 4, 5, 6 and 7 ($P < .05$). Thus, for the 10 µL IS groups, females showed more allodynia than males, but in the 20 µL groups, males showed more allodynia than females.

Mechanical testing of the masseter region measured sensitization of the third division (mandibular branch) of the trigeminal nerve (Figure 7B). The male 10 µL IS group showed no allodynia during mechanical stimulation of the masseter region in comparison with vehicle controls, although significant allodynia was noted in the male 20 µL IS group on testing days 1, 3, 4, and 5 ($P < .05$). In contrast, the female 10 µL IS group experienced allodynia during mechanical stimulation of the masseter region only on testing days 5 and 7 ($P < .05$). Thus, the male 20 µL IS group showed the greatest allodynia in the masseter region under these conditions.

Interictal behavioral response (48 hours)

Locomotor activity measurements 48 hours after the infusion of vehicle or IS were designed to measure longer-term changes in routine activity over the course of the 2.5 week experiment. The male and female 10 µL IS groups displayed no statistically significant difference in distance traveled in the interictal period over the course of the experiment as compared to the 10 µL vehicle controls (Figure 8A). The male 20 µL IS group showed a decrease in distance traveled in the interictal period as compared to the vehicle controls ($P < .05$) over the course of the experiment. This contrasts with the female 20 µL IS subjects who showed difference in distance traveled as compared to controls. Thus, the 20 µL male IS group displayed more significant interictal changes the females in

distance traveled over the 2.5 week experiment.

The male 10 µL IS group showed no statistical difference in changes in bouts of low mobility with respect to controls (Figure 8B), whereas the female 10 µL IS group displayed a statistically significant increase in the number of bouts of low mobility when compared to controls ($P < .05$). Neither the male nor female 20 µL IS groups demonstrated any statistical differences as measured by bouts of low mobility during the interictal period (Figure 8B). Thus, the female 10 µL IS group showed the most significant interictal changes in bouts of low mobility.

Neither the male nor the female 10 µL or 20 µL IS groups showed any difference in spatial confinement when compared to vehicle control animals (Figure 8C).The male and female 10µL IS groups did not show any difference in the focused energy score when compared to vehicle control animals (Figure 8D), although differences were noted compared to controls in both the male and female 20 µL IS groups (both $P < .05$).

Interictal facial allodynia (48 hours)

Mechanical testing of the periorbital region measured sensitization of the first division (ophthalmic branch) of the trigeminal nerve during the interictal time period (48 hours post application of IS). Neither the male nor female 10 and 20 µL IS groups showed any significant allodynia in this facial region (Figure 9A). Mechanical testing of the masseter region measured sensitization of the third division (mandibular branch) of the trigeminal nerve (Figure 9B). None of the treatment groups displayed any allodynia in the masseter region under these conditions.

Correlation of locomotor and allodynia data

In female subjects, onset phase distance traveled is highly correlated with interictal periorbital mechanical facial allodynia (Figure 10). Following a general linear fit, a goodness of fit analysis was performed using an ANOVA F-test. The correlation is statistically significant with a $P = .0003$. There was no statistical correlation when comparing onset phase distance traveled and interictal periorbital mechanical facial allodynia.

Hind paw testing

There was no significant difference in hind paw withdrawal thresholds between the groups receiving IS and vehicle, suggesting no allodynia in this extremity (Figure 11).

Figures

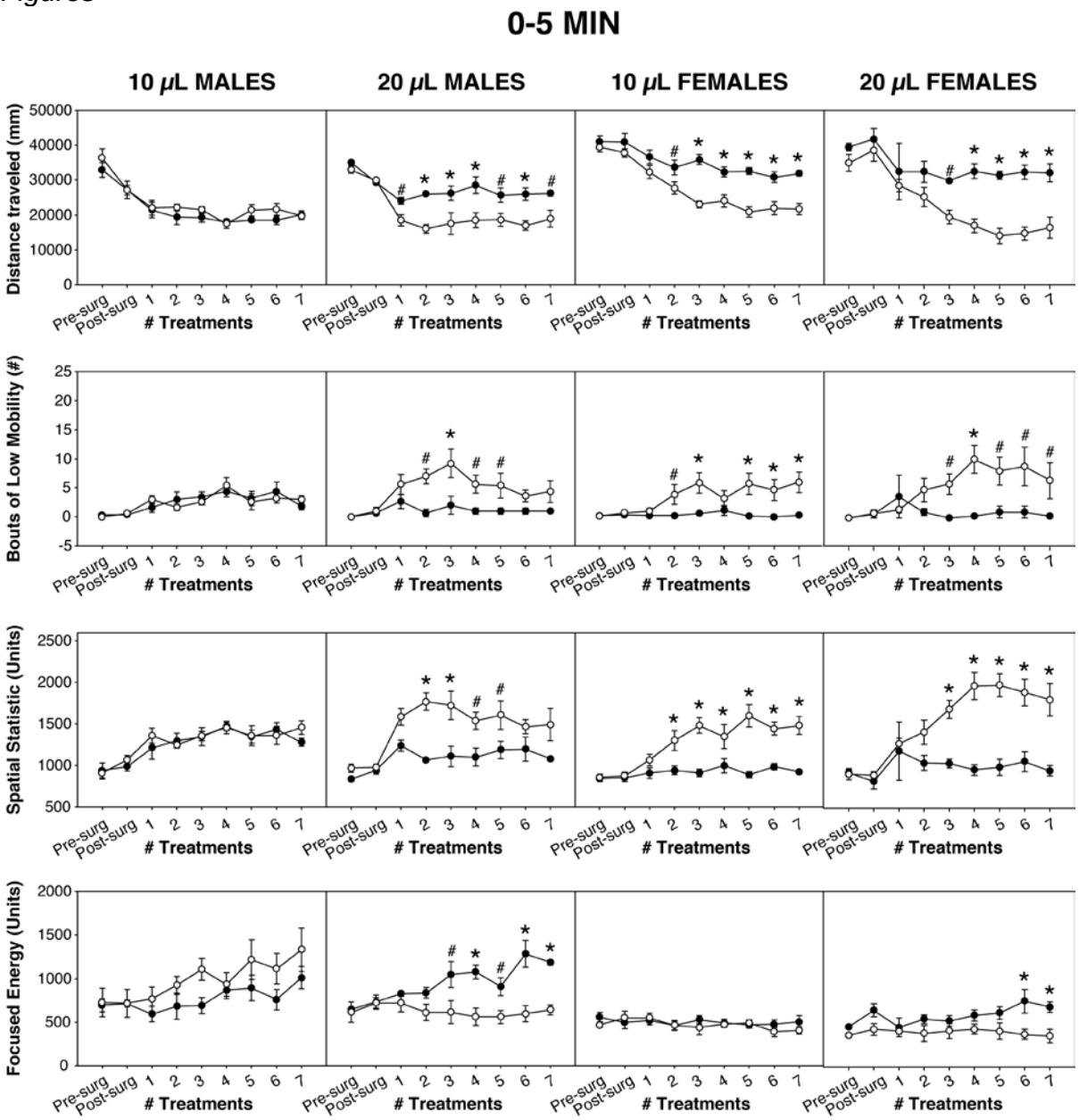

Figure 5. Inflammatory soup decreases locomotor activity in onset phase.

A) Distance traveled measurement: millimeters of distance traveled between t = 0 and t = 5 minutes. **B**) Bouts of low mobility: number of ten-second periods of inactivity between t = 0 and t = 5 minutes. **C**) Spatial confinement score: unit measure with inverse relation to area explored between t = 0 and t = 5

minutes. **D**) Focused energy: unit measure of activity while animal remains stationary between t = 0 and t = 5 minutes. X-axes show number of treatments. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 (closed circles) or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 (open circles) was delivered to the surface of intact dura via cannula. Male 10 µL vehicle group, n = 5; male 10 µL IS group, n = 5; female 10 µL vehicle group, n = 9; female 10 µL IS group, n = 7; male 20 µL vehicle group, n = 3; male 20 µL IS group, n = 5; female 20 µL vehicle group, n = 3; female 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistical significance with $P < .05$, * indicates $P < .005$.

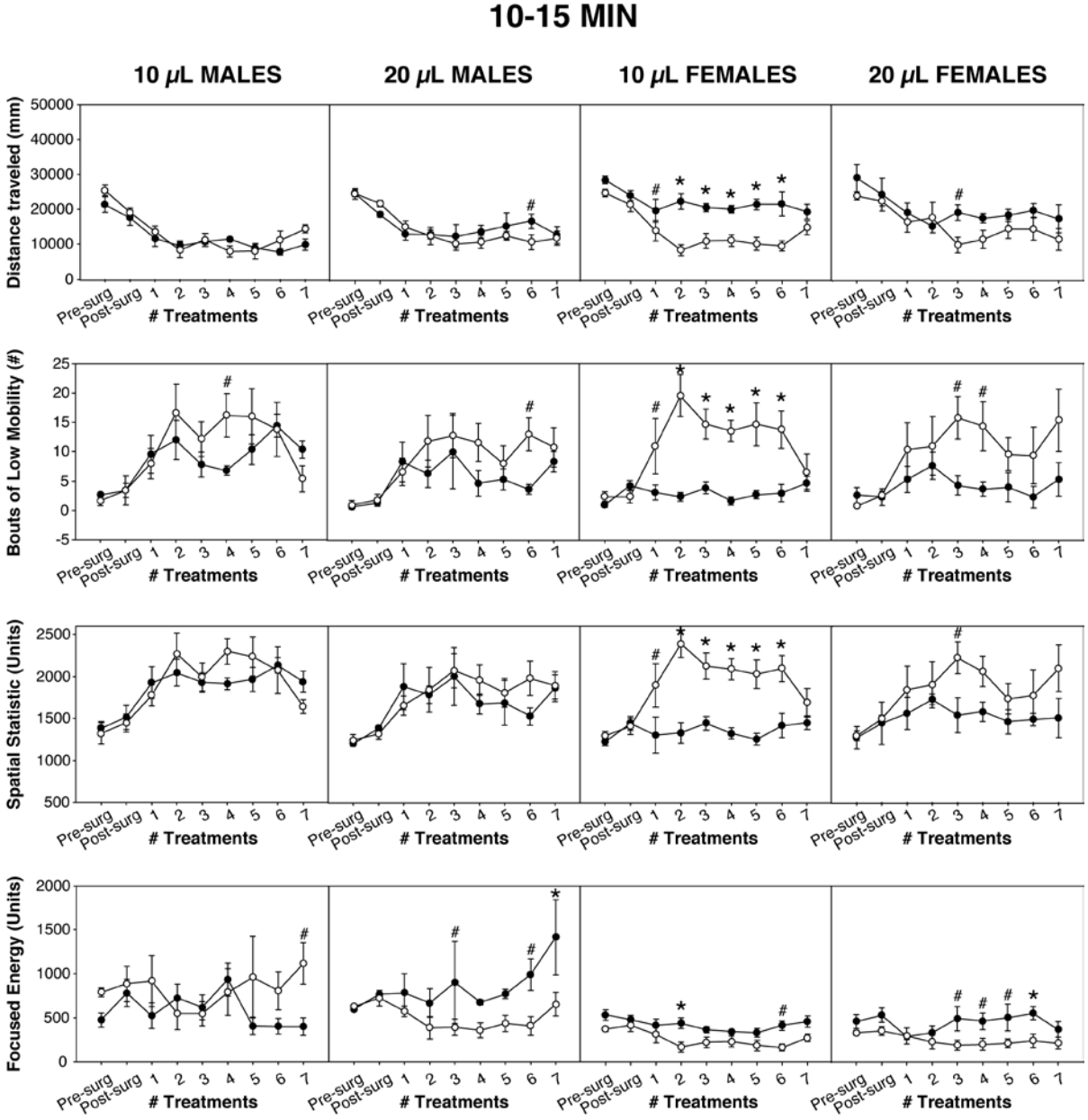

Figure 6. Inflammatory soup decreases locomotor activity in persistence phase.

A) Distance traveled measurement: millimeters of distance traveled between t = 10 and t = 15 minutes. **B**) Bouts of low mobility: number of ten-second periods of inactivity between t = 10 and t = 15 minutes. **C**) Spatial confinement score: unit measure with inverse relation to area explored between t = 10 and t = 15 minutes. **D**) Focused energy: unit measure of activity while

animal remains stationary between t = 10 and t = 15 minutes. X-axes show number of treatments. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 (closed circles) or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 (open circles) was delivered to the surface of intact dura via cannula. Male 10 µL vehicle group, n = 5; male 10 µL IS group, n = 5; female 10 µL vehicle group, n = 9; female 10 µL IS group, n = 7; male 20 µL vehicle group, n = 3; male 20 µL IS group, n = 5; female 20 µL vehicle group, n = 3, female 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistical significance with $P < .05$, * indicates $P < .005$.

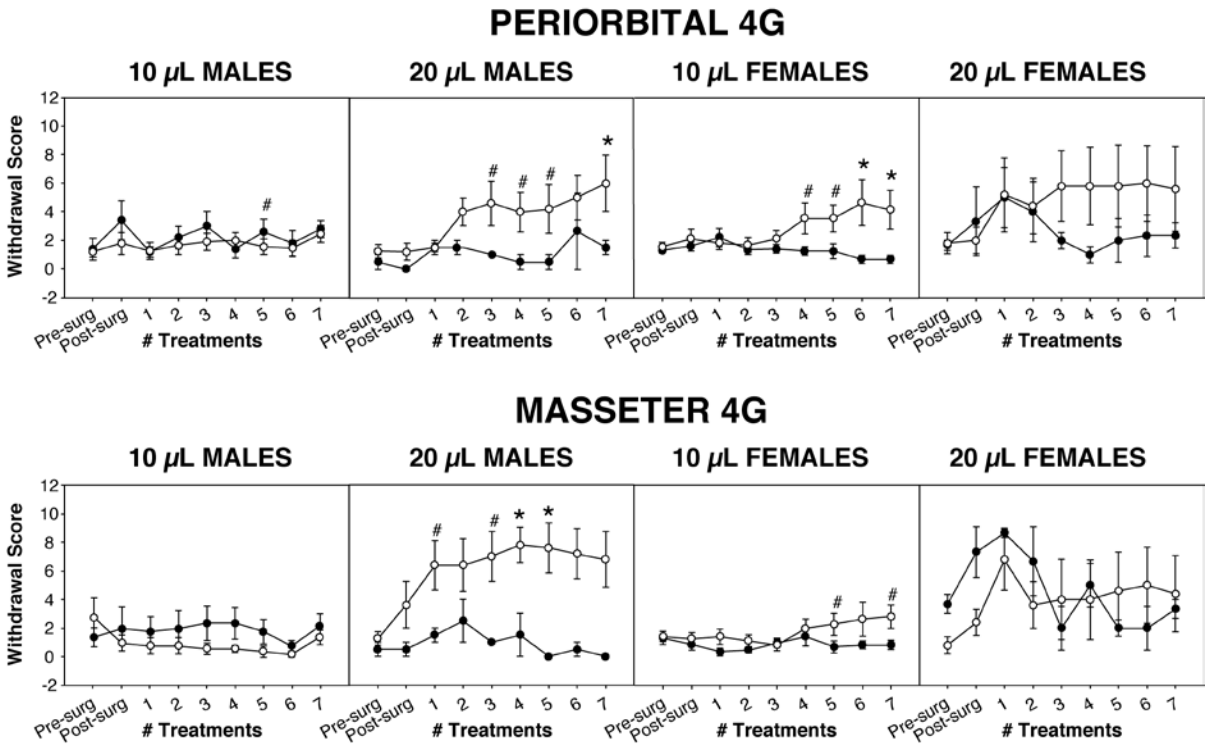

Figure 7. Inflammatory soup induces facial allodynia.

A) Primary division allodynia: Withdrawal scores in response to monofilament (4g) stimulation of the periorbital region. **B**) Secondary division allodynia. Withdrawal scores in response to monofilament (4g) stimulation of the masseter. Over the course of testing the withdrawal scale measures the maximum withdrawal as a score of 15 and no response as a score of 0. X-axis shows number of treatments. For each animal, 10 μL or 20 μL phosphate-buffered saline (PBS) pH 7.4 (closed circles) or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 (open circles) was delivered to the surface of intact dura via cannula. Male 10 μL vehicle group, n = 5; male 10 μL IS group, n = 5; female 10 μL vehicle group, n = 9; female 10 μL IS group, n = 7; male 20 μL vehicle group, n = 3; male 20 μL IS group, n = 5; female 20 μL vehicle group, n = 3, female 20 μL IS group, n = 5. Standard error

is shown in error bars. # indicates a statistical significance with $P < .05$, * indicates $P < .005$.

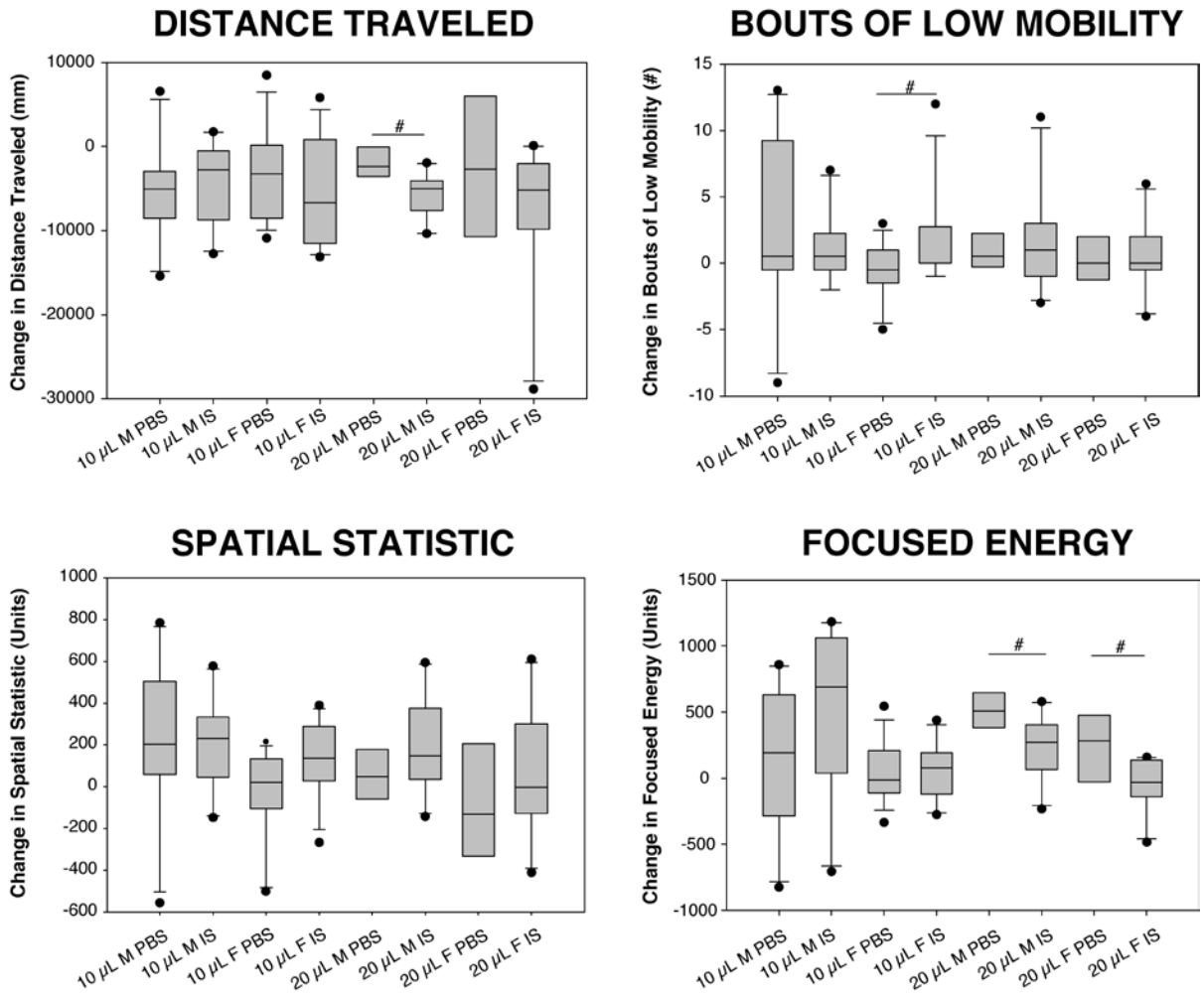

Figure 8. Inflammatory soup decreases routine activity interictally.

A) Distance traveled measurement: difference in millimeters of distance traveled between post-surgical baseline and treatment day 8. **B**) Bouts of low mobility: difference in number of ten second periods of inactivity between post-surgical baseline and treatment day 8. **C**) Change in spatial confinement score between post-surgical baseline and treatment day 8. **D**) Change in focused energy score between post-surgical baseline and treatment day 8. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 (closed circles) or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2

in PBS) pH 5.5 (open circles) was delivered to the surface of intact dura via cannula. Male 10 µL vehicle group, n = 5; male 10 µL IS group, n = 5; female 10 µL vehicle group, n = 9; female 10 µL IS group, n = 7; male 20 µL vehicle group, n = 3; male 20 µL IS group, n = 5; female 20 µL vehicle group, n = 3, female 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistical significance with $P < .05$, * indicates $P < .005$. Data are shown as a box plot with a line within the box representing the 50th percentile or median, while the boundary of the box closest to zero indicates the 25th percentile and the boundary of the box farthest from zero indicates the 75th percentile. The whiskers mark the 10th and 90th percentiles. Outlying data points are shown.

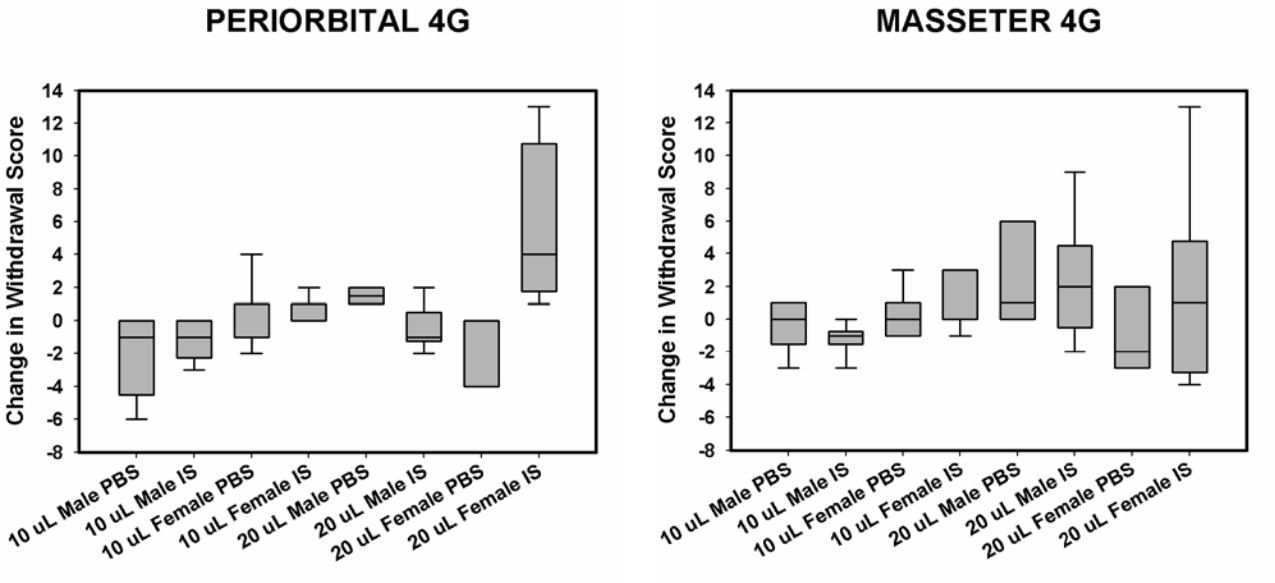

Figure 9. Inflammatory soup does not illicit interictal allodynia.

A) Primary division allodynia: Withdrawal scores in response to monofilament (4g) stimulation of the periorbital region. **B**) Secondary division allodynia. Withdrawal scores in response to monofilament (4g) stimulation of the masseter. Change calculated as difference between post-surgical baseline and treatment day 8. Over the course of testing the withdrawal scale measures the maximum withdrawal as a score of 15 and no response as a score of 0. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 (closed circles) or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 (open circles) was delivered to the surface of intact dura via cannula. Male 10 µL vehicle group, n = 5; male 10 µL IS group, n = 5; female 10 µL vehicle group, n = 9; female 10 µL IS group, n = 7; male 20 µL vehicle group, n = 3; male 20 µL IS group, n = 5; female 20 µL vehicle group, n = 3, female 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistical significance with $P < .05$, * indicates $P < .005$. Data are shown as a box plot with

a line within the box representing the 50th percentile or median, while the boundary of the box closest to zero indicates the 25th percentile and the boundary of the box farthest from zero indicates the 75th percentile. The whiskers mark the 10th and 90th percentiles. Outlying data points are shown.

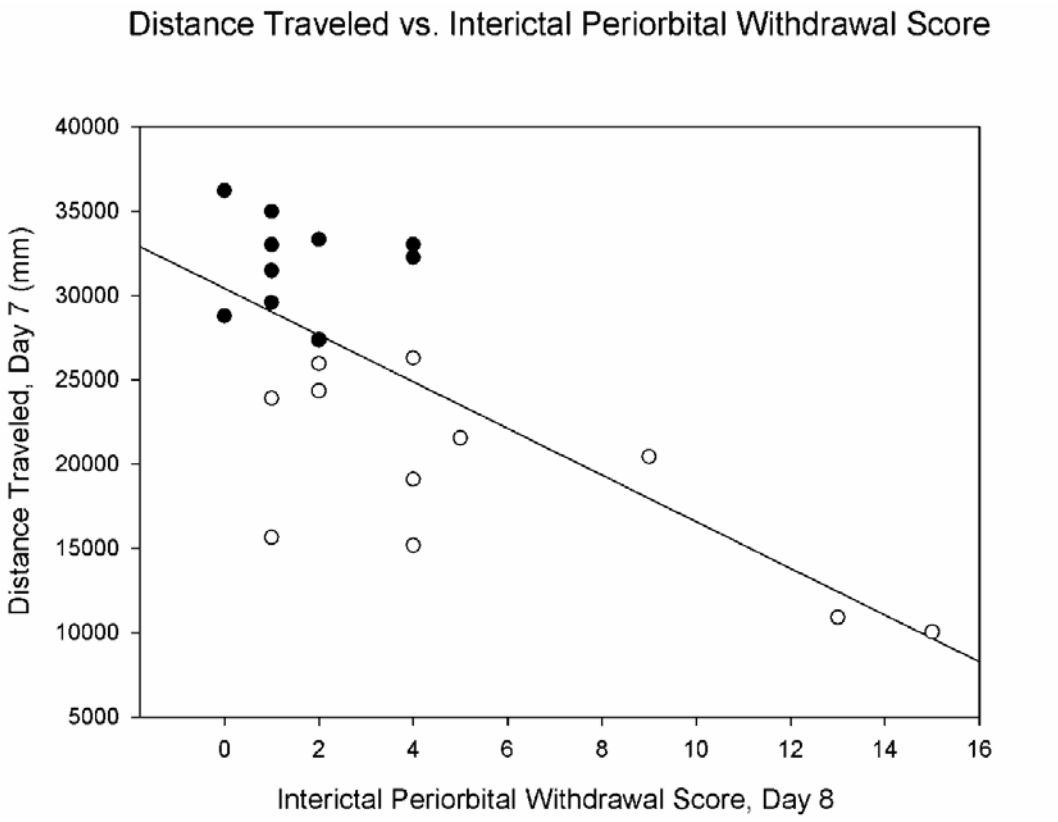

Figure 10. In female subjects, onset phase distance traveled is highly correlated with interictal periorbital mechanical facial allodynia.

X-axis shows interictal periorbital withdrawal score. Withdrawal scores in response to monofilament (4g) stimulation of the periorbital region during interictal period on Day 8. Y-axis shows distance traveled. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 (closed circles) or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 (open circles) was delivered to the surface of intact dura via cannula. Female 10 µL vehicle group, n = 5; female 10 µL IS group, n = 6; female 20 µL vehicle group, n = 3, female 20 µL IS group, n = 5. The data are shown with a general linear regression with a goodness of fit analysis performed using ANOVA F-test. The correlation is statistically significant with a $P = .0003$.

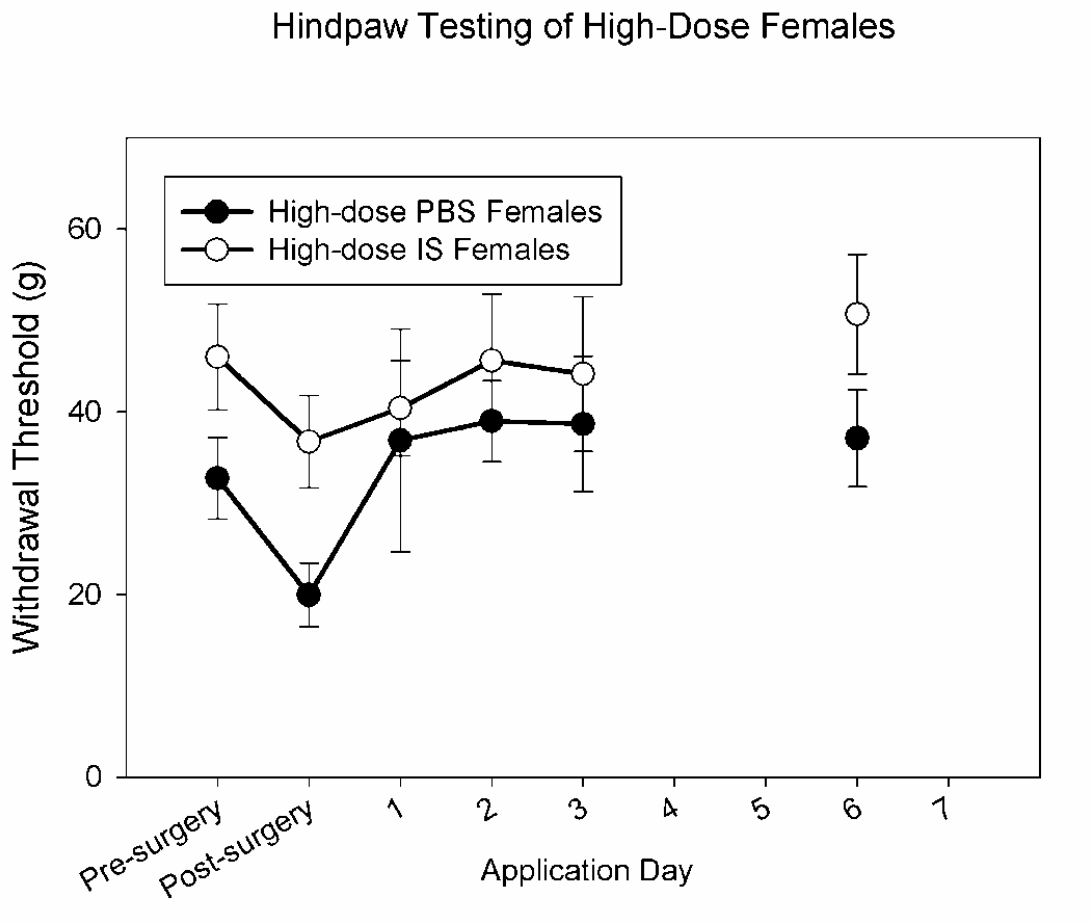

Figure 11. Inflammatory soup does not illicit ipsilateral hind paw allodynia.

Withdrawal threshold values recorded in response stimulation of the hind paw by electronic von Frey apparatus. X-axis shows number of applications. Y-axis shows withdrawal threshold in grams. For each animal, 20 µL phosphate-buffered saline (PBS) pH 7.4 (closed circles) or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 (open circles) was delivered to the surface of intact dura via cannula. Withdrawal testing was performed 2 hours after application. Female 20 µL vehicle group, n = 3, female 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistical significance with $P < .05$, * indicates $P < .005$.

Discussion

This is the first study to demonstrate that changes in behavioral activity occur during dural inflammation in a preclinical model of migraine. Using actimetry, it was found that animals receiving IS display a decrease activity similar to that observed during migraine attacks in humans (Kelman, 2006; Martins et al., 2006). These findings provide face validity for this animal model as useful tool for studying the behavioral and physiological changes associated with migraine.

Previous studies demonstrated that IS sensitizes the trigeminal system (Kessler et al., 1992; Steen et al., 1992; Knyihar-Csillik et al., 2000; Ter Horst et al., 2001; Burstein and Jakubowski, 2004; Jakubowski et al., 2007; Zhang et al., 2007a). Clinically, a change in routine activity is used as a key diagnostic criterion for migraine (IHS, 2004). Because changes in daily activity are associated with decreased quality of life, a goal of therapies is to maintain normal levels of activity. However, previous preclinical models of migraine have not measured this parameter. Furthermore, avoidance of routine activity is one of the most commonly reported migraine symptoms, with over half of migraineurs surveyed requiring bed rest to manage their migraine (Brandes, 2002). Another study found that each year there are 2.7 million days of work for men and 18.8 million missed days for women in the United States because of migraine symptoms (Stang and Osterhaus, 1993). Avoidance of routine activity is a criteria for migraine diagnosis but there is no validated questionnaire/instrument for measuring this change. This data would be helpful to be able to correlating the

extent of headache severity with activity.

In the present study, primary division facial mechanical allodynia was measured in the periorbital region (i.e. the same sensory dermatome as dura) and secondary allodynia was measured in the masseter region (different trigeminal dermatome from dura) as a measure of sensitization of the trigeminal pain pathway. Previous behavioral work assessed mechanical facial allodynia hours to days after application of IS, but did not thoroughly examined changes in activity within minutes of administration of the IS (Oshinsky and Gomonchareonsiri, 2007; Edelmayer et al., 2009; Wieseler et al., 2009). Periorbital region cutaneous allodynia, as measured by increased withdrawal scores, was significantly increased in females receiving inflammatory soup application, while males had significant sensitivity increases in response to stimulation of the masseter. Previous studies have shown allodynia of the periorbital region, but those studies did not test other regions of the face or compare males to females (Oshinsky and Gomonchareonsiri, 2007).

A dose-response relationship was found with regard to the IS in locomotor measurements, including changes in distance traveled and spatial confinement in females (Figure 5A and 5C). Others have shown dose responsive decreases in mechanically stimulated firing thresholds of C-unit and A-delta neurons in the dura in response to 5-HT, histamine, and PGI_2 (Zhang et al., 2007a). In the present study, although the molar equivalents (10 and 20 pmol equivalents for 5-HT and histamine) are at the upper range of those investigated by Zhang et al. the same trend is observed, suggesting that the behavioral effects result from the

IS constituents acting on dural C and A-delta fibers.

The results of this study demonstrate that males and females show differences in their response to IS application to the dura. A 10 µL application of IS decreased activity in female but not male rats, as measured by decreased distance traveled, increased bouts of low mobility, and increased spatial confinement. This finding is of particular interest in that migraine, like a number of pain conditions, is more prevalent in females than males (Greenspan et al., 2007; Bigal and Lipton, 2009). An explanation for this may be provided by the finding that regulation of nociceptive sensitization in the trigeminal ganglia is strongly linked to estrogen (Flake et al., 2005; Puri et al., 2005; Liverman et al., 2009a; Liverman et al., 2009b). In addition, the frequency and propagation speeds of cortical spreading depression, a condition thought to be linked to migraine with aura, is increased in cycling females as compared to males and ovariectomized females in a mouse model of familial hemiplegic migraine type 1 (Eikermann-Haerter et al., 2009). It is unknown whether estrogen alters the dose-response characteristics of dural nociceptors.

Data from the current work indicates that alteration of locomotor activity was not as robust when measured interictally as it is when measured immediately after administration of the IS. Changes that generally correlated with both acute changes (e.g. distance traveled was reduced in groups that received IS versus vehicle controls) and with differences in sex and dose differences (e.g. females showed changes at lower volumes of IS applied) were apparent. The general trend observed was that groups treated with IS tended to ambulate less

and display less head and body movement even while standing still. In this animal model of chronic migraine, some effects of chronic IS application are apparent as long as 48 hours following application of the soup (Figure 8).

Another model of chronic migraine revealed sustained interictal periorbital allodynia 48 hours after IS application (Oshinsky and Gomonchareonsiri, 2007). In the present study, the female 20μL IS group displayed the most pronounced periorbital allodynia (Figure 7), although it was not statistically significant when compared with changes noted in the control vehicle group. Studies of migraineurs that suffer 1-6 migraine attacks per month show significantly lower pain thresholds acutely when compared with interictal values (Burstein et al., 2000a; Burstein et al., 2000b).

None of the groups examined in this work showed interictal cutaneous peri-masseter allodynia, indicating that allodynia in this region, innervated by a separate, third division of the trigeminal nerve, may present acutely and subside interictally. Again, this finding is consistent with clinical research showing that facial mechanical pain thresholds decrease 63% acutely (4 hours following onset of headache) as compared to interictal values (Burstein et al., 2000a).

In female subjects, the distance traveled is highly correlated with interictal periorbital mechanical facial allodynia. Although interictal allodynia did not develop following chronic application of IS in any of the groups studied as compared to vehicle control, it was highly correlated with the decrease in routine activity noted during the onset phase (Figure 10). This connects the new assay measuring reductions in distance traveled to previous studies using facial

allodynia as a migrainous end point.

In contrast with other reports, no increase in hind paw mechanical sensitivity was observed following application to the dura of inflammatory soup (Edelmayer et al., 2009; Wieseler et al., 2009). There are several possible explanations for this difference. One is that females were tested in the present work, whereas in previous studies only males were examined. Secondly, the IS application in the current work was unilateral, as opposed to the bilateral administration employed by some others (Wieseler et al., 2009). Moreover, hind paw allodynia in a model of unilateral dural inflammation described in a previous report was in response to twice the concentration of IS used in the present study (Edelmayer et al., 2009), and the IS was administered repeatedly over two weeks in the current work, whereas others have administered it only once or twice before measuring a response (Edelmayer et al., 2009; Wieseler et al.).

Others have shown the magnitude of force required to elicit hind paw withdrawal responses is considerably different from those reported in the present study. The work reported herein shows baseline withdrawal thresholds of approximately 40 g, which is consistent with forces required for hind paw withdrawal reported by others (Hernstadt et al., 2009; Ito et al., 2009). Other studies using measurements of hind paw withdrawal in models of dural inflammation have reported baseline force withdrawal thresholds ranging from 15 g (Edelmayer et al., 2009) to 3.16 g (Wieseler et al., 2009) of force. A response to a force in the range of 3 g is similar to the withdrawal threshold seen following formalin or CFA injection (Intondi et al., 2008; Hernstadt et al., 2009).

Several clinical studies have identified extracephalic allodynia in migraineurs (Tfelt-Hansen et al., 1981; Burstein et al., 2000b; Ashkenazi et al., 2007; Guy et al., 2009; Kalita et al., 2009). These studies often tested the forearm region. While a population-based study report that 63% of migraineurs experience allodynia during their migraine attacks (Lipton et al., 2008) this dropped to 8% to 49% when considering only those presenting with extracephalic allodynia (Guy et al., 2009; Kalita et al., 2009). Most extracephalic allodynia is thermal, not mechanical (Burstein et al., 2000b; Guy et al., 2009). One group reported that extracephalic allodynia correlates with more severe and less frequent headache episodes than cephalic allodynia alone (Guy et al., 2009). Extracephalic allodynia has also been reported in fibromyalgia and has been shown to be mostly thermal (Smith et al., 2008). Migraine is co-morbid with fibromyalgia in 22-36% of patients (Ifergane et al., 2006; de Tommaso et al., 2009). Indeed, there is speculation that migraine and fibromyalgia share pathophysiology and are components of the same disorder (Centonze et al., 2004; Ifergane et al., 2006).

It is impossible fully to explain all of the differences between the present findings and earlier reports. Nonetheless, the present findings indicate there may be sex related differences in extracephalic allodynia or, perhaps, subtle effects related to method or chronicity of application to the dura that can influence the appearance of extracephalic allodynia.

In conclusion, the results of this study demonstrate significant decreases in locomotor activity and development of mechanical allodynia in both male and

female rats in response to application to the dura of IS in a model of chronic migraine. The changes in locomotor activity occur at lower doses of IS, are more persistent, and are dose-related in females but not males. While the differences in the overall pattern of allodynia and changes in locomotion noted in this study are complex, they suggest that females are more sensitive to dural inflammation and the effects of dural inflammation may be longer lasting in females. This model provides a new tool to further investigate the reason for sex differences in migraine and a more refined analysis of the locomotor and pain-related behaviors that are evoked by dural inflammation in a freely-moving subject.

V. Changes in Gene Expression of CGRP and Receptor Components in a Rodent Model of Migraine

Hypothesis

Expression of genes encoding for CGRP and CGRP-related receptor components will be modulated in a sex-dependent manner in response to inflammation of dura mater in a rat model of migraine.

Abstract

The objectives of this study were to determine the differences in the pain-evoked expression pattern of genes encoding CGRP and components of its receptor in regions of the trigeminal pathway (trigeminal ganglion and medulla) following application to the dura of inflammatory soup (IS), to study sex differences in expression of these genes, and to attempt to correlate the migraine-like behavioral changes in a preclinical rat model of migraine to any changes in gene expression.

The vasoactive substance, calcitonin gene related peptide (CGRP) is thought to be an important mediator of migraine. The CGRP receptor consists of a G–protein coupled calcitonin-like receptor (CLR), a receptor activity-modifying protein (RAMP1), and a receptor component protein (RCP). Following chronic IS application to model migraine, real-time polymerase chain reaction was used to quantify the expression of mRNA for CGRP, RAMP1, CLR, and RCP.

In the medulla, females displayed higher baseline gene expression of CGRP and lower baseline expression of RAMP1, CLR, and RCP than males, with CLR and RCP being induced in all of the experimental conditions. In the trigeminal ganglia, females displayed lower baseline expression of RAMP1, CLR, and RCP than males. It was also found that females have a greater range of

induction in gene expression than males. In males, RCP gene expression was increased in the trigeminal ganglia of animals displaying migraine-like behaviors. These findings provide new evidence that changes in the expression of CGRP-related genes may underlie the differences in prevalence of migraine in men and women.

Results

This portion of the study focuses on CGRP and related receptors and how they are affected by dural inflammation. Given the data suggesting a relationship between CGRP and the symptoms of migraine, these experiments were performed in an attempt to determine whether sex-related changes in the CGRP system my underlie the gender differences in the prevalence of this disorder. To this end, measurements were made in the changes in expression of qRT-PCR of CGRP, RAMP1, CLR and RCP in a new animal model of migraine headache.

Sex differences

Sex-related differences in mRNA expression in the trigeminal ganglia (Figure 12) noted in the present study include significantly lower expression of mRNA in females than in males for RAMP1, CLR, and RCP ($P = .008$, $P < .001$, and $P = .003$, respectively) genes. In the medulla (Figure 13), sex-related differences were found in the significantly lower expression of mRNA in female subjects as compared to males for RAMP1, CLR, and RCP ($P = .007$, $P = .002$, and $P = .001$, respectively) genes, and a higher expression of CGRP in the medulla of female subjects ($P = .041$).

Induction in gene expression in trigeminal ganglia in male rats

Gene expression of CGRP (Figure 14) is decreased in all experimental groups (male 10 µL vehicle, P = .008; male 10 µL IS, P = .008; male 20 µL vehicle, P = .024, and male 20 µL IS, P = .017) and gene expression of RAMP1 (Figure 15) is decreased in the male 10 µL vehicle and male 10 µL IS groups (P < .001 and P = .011, respectively) as compared to controls. As for CLR expression (Figure 16), it is decreased in the male 10 µL vehicle and male 10 µL IS groups (P = .001 and P < .001) and increased in the male 20 µL vehicle group (P = .024) and male 20 µL IS group (P = .007) relative to the control subjects. Gene expression of RCP (Figure 17) is decreased in the male 10 µL vehicle (P = .004) and increased in the male 20 µL vehicle group (P = .029) and the male 20 µL IS group (P = .004) as compared to controls.

Induction in gene expression in medulla in male rats

The expression of the CGRP gene (Figure 18) is significantly lower in male subjects receiving 20 µL vehicle and 20 µL IS (P = .026 and P = .007), and RAMP1 expression (Figure 19) is significantly lower in males receiving 20 µL of IS compared with control male subjects (P = .002). It was also found that CLR expression (Figure 20) is lower in male 10 µL vehicle and 10 µL IS-treated groups than in controls (P = .026 and P = .004, respectively). Gene expression of RCP (Figure 21) is unchanged in all groups when compared to control subjects.

Induction in gene expression in trigeminal ganglia in female rats

There is no statistical difference in gene expression for CGRP or RAMP1 among any of the groups compared to the female controls (Figure 14, Figure 15,

respectively). However, CLR expression (Figure 16) is higher in both the female 20 µL vehicle group ($P < .001$) and the female 20 µL IS group ($P = .004$) as compared to controls, and gene expression of RCP (Figure 17) is higher in all groups as compared to control subjects (female 10 µL vehicle, $P = .03$; female 10 µL IS, $P = .015$; female 20 µL vehicle, $P = .048$, and female 20 µL IS, $P = .004$).

Induction in gene expression in medulla in female rats

In comparison to female control subjects, CGRP gene expression (Figure 18) is significantly lower in female subjects receiving 20 µL IS ($P = .042$) and RAMP1 expression (Figure 19) is significantly higher in females receiving 10 µL of IS as compared about female control subjects ($P = .020$). In contrast, CLR expression (Figure 20) is higher in all groups, including vehicle treated groups, than in the untreated control group (female 10 µL vehicle, $P = .004$; female 10 µL IS, $P = .002$; female 20 µL vehicle, $P = .024$, and female 20 µL IS, $P = .004$). Gene expression of RCP (Figure 21) is also higher in all groups when compared to control subjects (female 10 µL vehicle, $P = .03$; female 10 µL IS, $P = .003$; female 20 µL vehicle, $P = .024$, and female 20 µL IS, $P < .001$).

Gene induction in males displaying migraine-like behaviors

Statistically significant behavioral changes noted with these animals (Stucky, 2010) were compared to the significant changes in detected in gene expression under the various treatment conditions. The results indicate that males show significantly decreased expression of RAMP1 in the medulla in the group receiving 20 µL IS (Figure 19), which also displayed migraine-like behaviors when compared to controls (Stucky, 2010). Likewise, gene expression

of RCP is significantly increased in trigeminal ganglia of the group receiving 20 µL IS (Figure 17), which is also a group that displayed migraine-like behaviors (Stucky, 2010).

Gene induction in females displaying migraine-like behaviors

Females receiving 20 µL IS show a decrease in the expression of CGRP in the medulla (Figure 18) and those receiving 10 µL IS display an increased expression of RAMP1 (Figure 19). These groups displayed migraine like behaviors when compared to control subjects (Stucky, 2010).

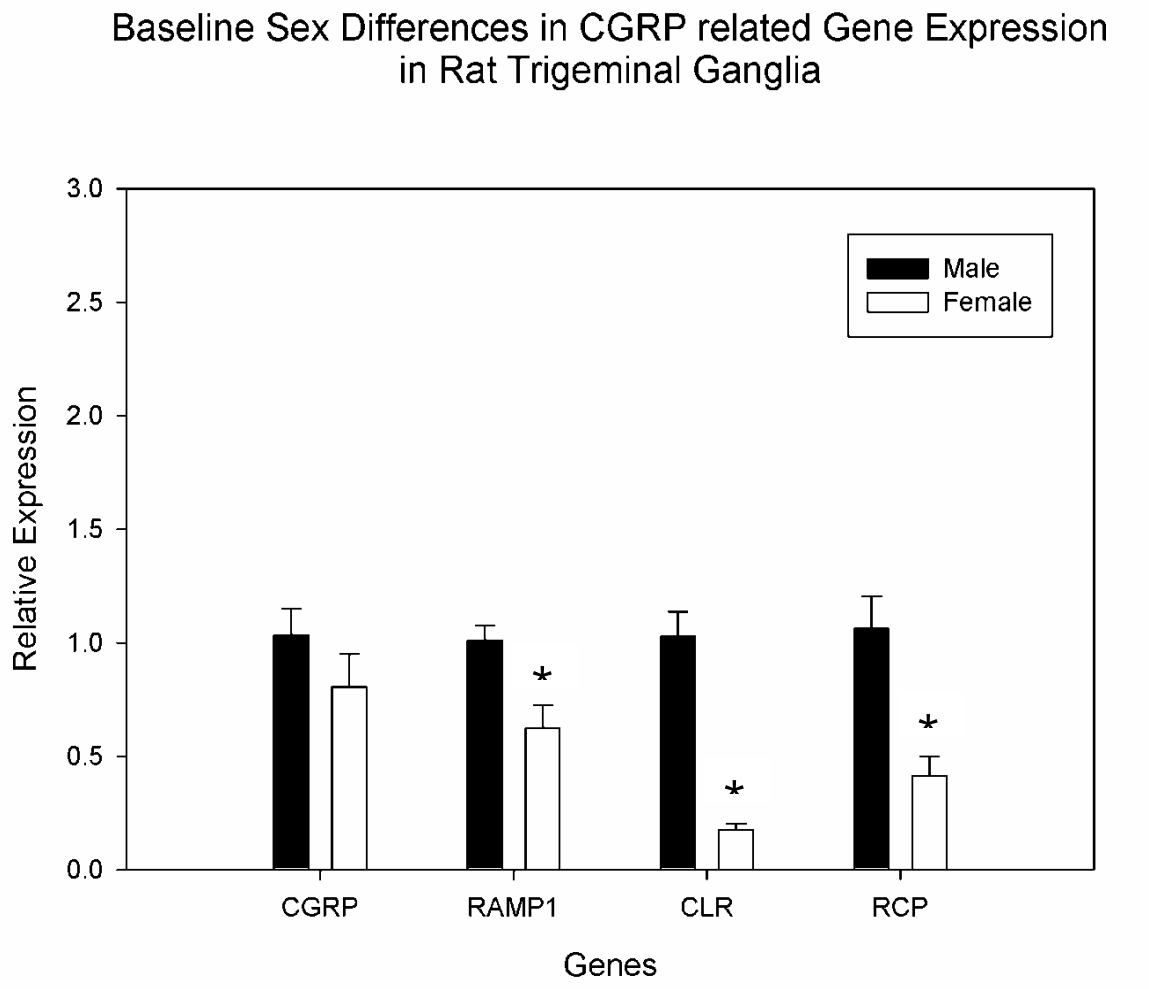

Figure 12. Baseline sex differences in CGRP-related gene expression in rat trigeminal ganglia

Relative expression of genes of interest in the trigeminal ganglia as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. X-axis shows gene of interest. Y-axis shows relative expression. Animals are naïve control male or naïve control female Sprague-Dawley rats. Male group, n = 6 (black bars), female group, n = 6 (white bars). Standard error is shown in error bars. # indicates a statistically significant difference from male control with $P < .05$, * indicates $P < .005$.

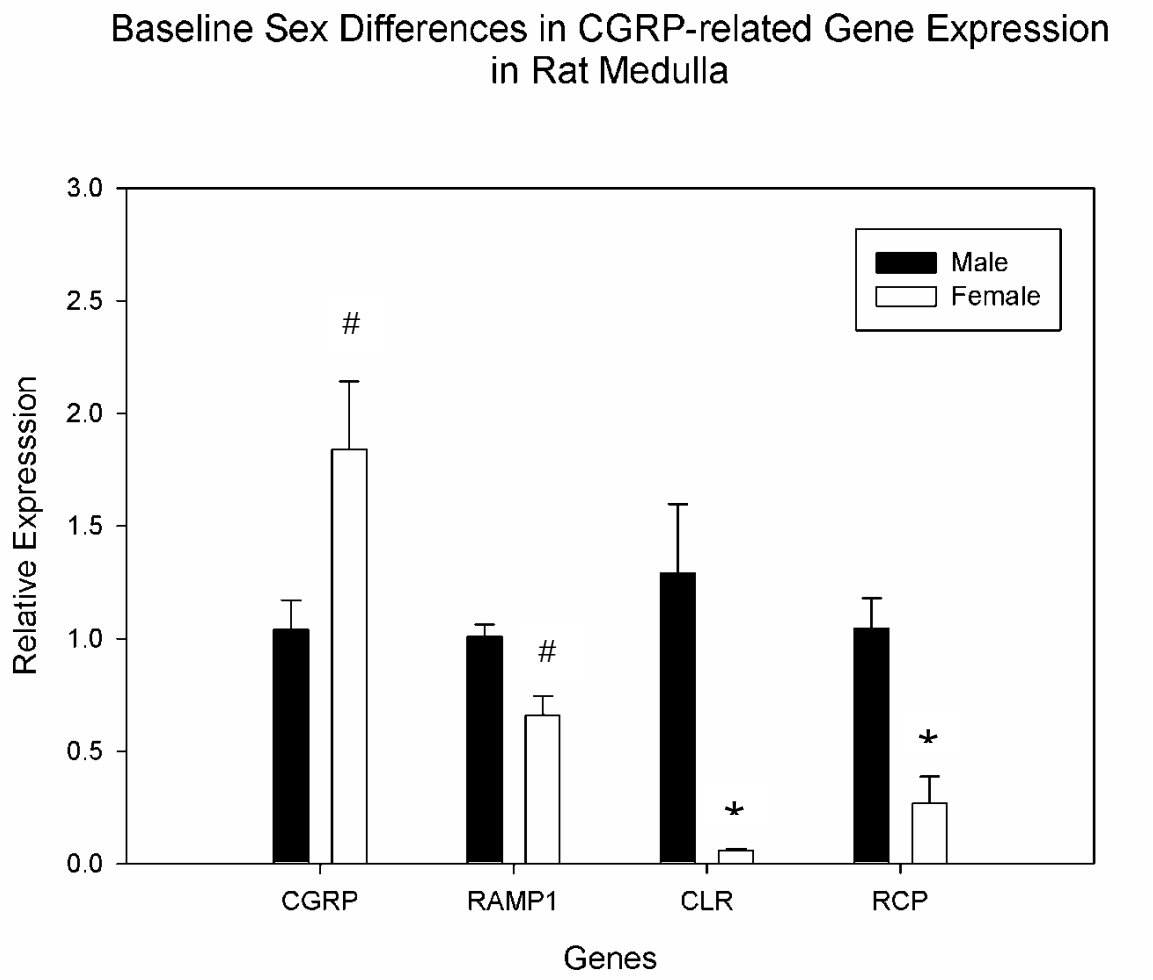

Figure 13. Baseline sex differences in CGRP-related gene expression in rat medulla

Relative expression of genes of interest in the medulla as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. X-axis shows gene of interest. Y-axis shows relative expression. Animals are naïve control male or naïve control female Sprague-Dawley rats. Male group, n = 6 (black bars), female group, n = 6 (white bars). Standard error is shown in error bars. # indicates a statistically significant difference from male control with $P < .05$, * indicates $P < .005$.

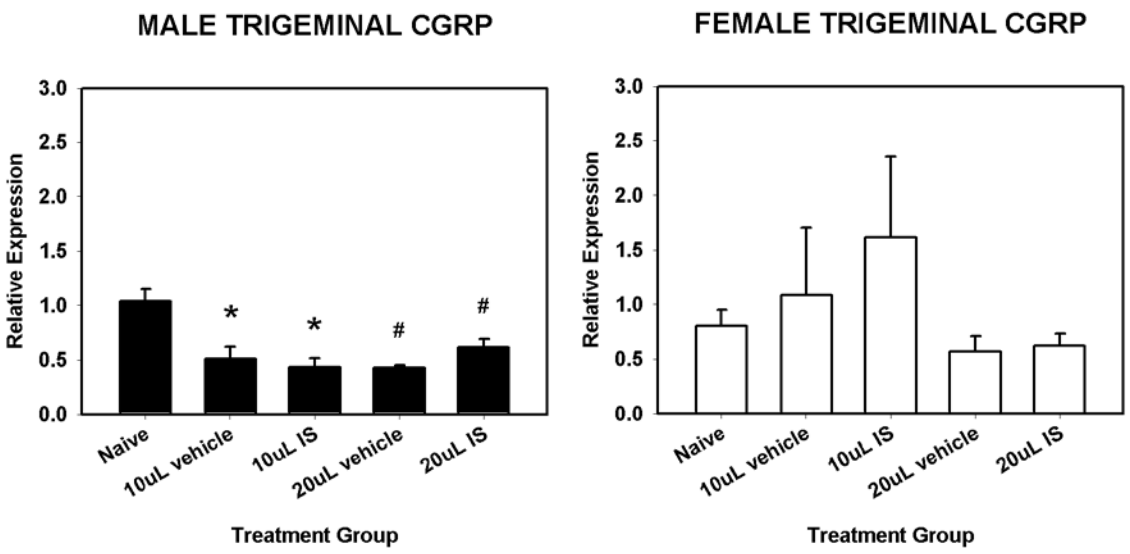

Figure 14. Sex differences in induction of gene expression of CGRP in rat trigeminal ganglia

Relative expression of CGRP in the trigeminal ganglia as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. Average naïve male expression used as subject control. X-axis shows treatment groups. Y-axis shows relative expression. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 was chronically delivered to the surface of intact dura via cannula for 8 applications. Male (black bars) naïve group, n = 6; 10µL vehicle group, n = 5; 10 µL IS group, n = 5; 20 µL vehicle group, n = 3; 20 µL IS group, n = 5; Female (white bars) naïve group, n = 6; 10 µL vehicle group, n = 9; 10 µL IS group, n = 7; 20 µL vehicle group, n = 3, 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistically significant difference from the sex matched naïve control with $P < .05$, * indicates $P < .005$.

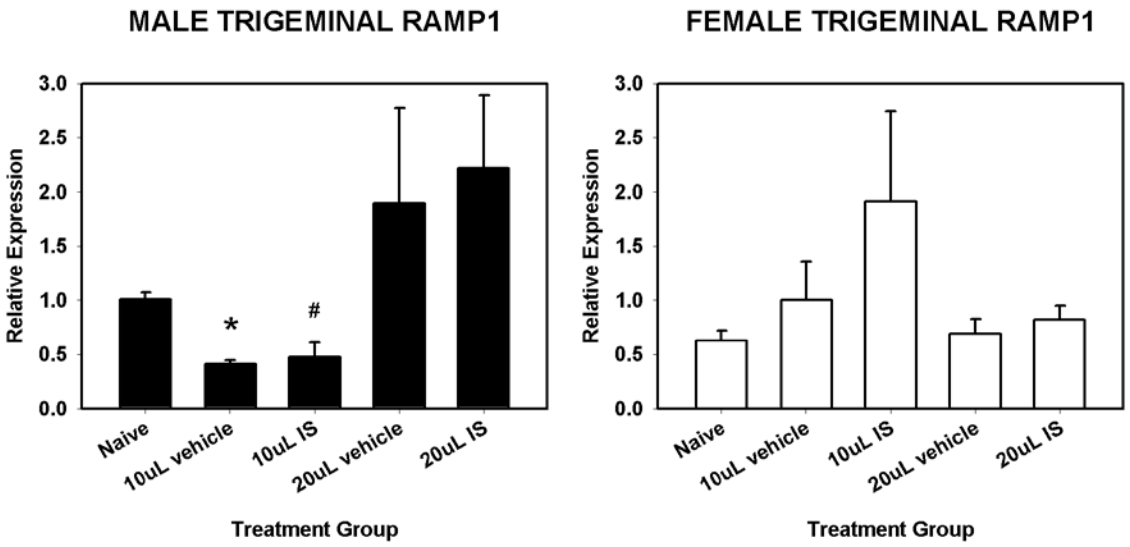

Figure 15. Sex differences in induction of gene expression of RAMP1 in rat trigeminal ganglia

Relative expression of RAMP1 in the trigeminal ganglia as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. Average naïve male expression used as subject control. X-axis shows treatment groups. Y-axis shows relative expression. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 was chronically delivered to the surface of intact dura via cannula for 8 applications. Male (black bars) naïve group, n = 6; 10µL vehicle group, n = 5; 10 µL IS group, n = 5; 20 µL vehicle group, n = 3; 20 µL IS group, n = 5; Female (white bars) naïve group, n = 6; 10 µL vehicle group, n = 9; 10 µL IS group, n = 7; 20 µL vehicle group, n = 3, 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistically significant difference from the sex matched naïve control with $P < .05$, * indicates $P < .005$.

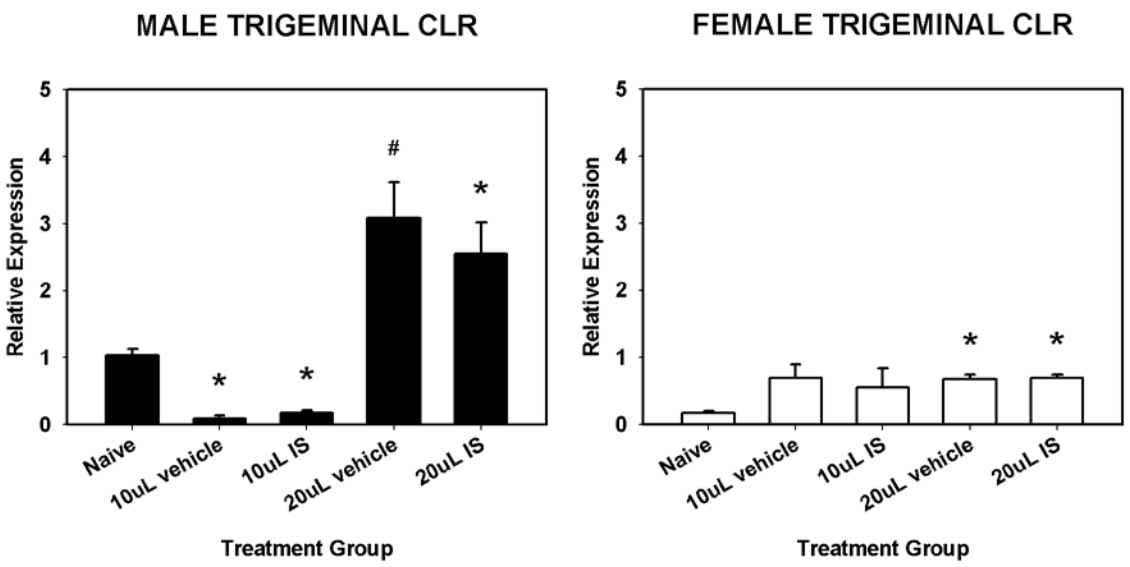

Figure 16. Sex differences in induction of gene expression of CLR in rat trigeminal ganglia

Relative expression of CLR in the trigeminal ganglia as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. Average naïve male expression used as subject control. X-axis shows treatment groups. Y-axis shows relative expression. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 was chronically delivered to the surface of intact dura via cannula for 8 applications. Male (black bars) naïve group, n = 6; 10 µL vehicle group, n = 5; 10 µL IS group, n = 5; 20 µL vehicle group, n = 3; 20 µL IS group, n = 5; Female (white bars) naïve group, n = 6; 10 µL vehicle group, n = 9; 10 µL IS group, n = 7; 20 µL vehicle group, n = 3, 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistically significant difference from the sex matched naïve control with $P < .05$, * indicates $P < .005$.

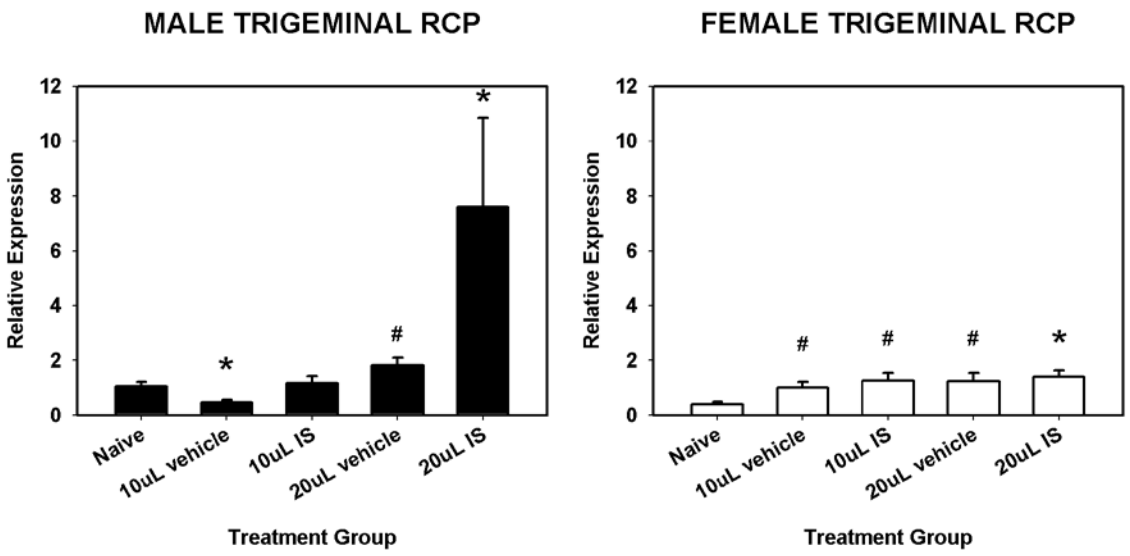

Figure 17. Sex differences in induction of gene expression of RCP in rat trigeminal ganglia

Relative expression of RCP in the trigeminal ganglia as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. Average naïve male expression used as subject control. X-axis shows treatment groups. Y-axis shows relative expression. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 was chronically delivered to the surface of intact dura via cannula for 8 applications. Male (black bars) naïve group, n = 6; 10 µL vehicle group, n = 5; 10 µL IS group, n = 5; 20 µL vehicle group, n = 3; 20 µL IS group, n = 5; Female (white bars) naïve group, n = 6; 10 µL vehicle group, n = 9; 10 µL IS group, n = 7; 20 µL vehicle group, n = 3, 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistically significant difference from the sex matched naïve control with $P < .05$, * indicates $P < .005$.

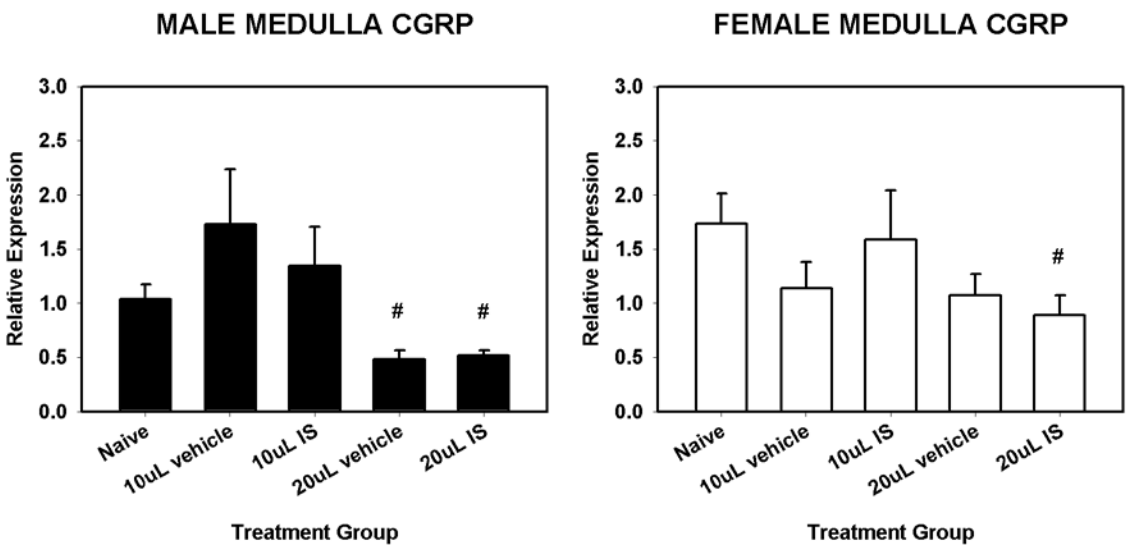

Figure 18. Sex differences in induction of gene expression of CGRP in rat medulla

Relative expression of CGRP in the medulla as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. Average naïve male expression used as subject control. X-axis shows treatment groups. Y-axis shows relative expression. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 was chronically delivered to the surface of intact dura via cannula for 8 applications. Male (black bars) naïve group, n = 6; 10 µL vehicle group, n = 5; 10 µL IS group, n = 5; 20 µL vehicle group, n = 3; 20 µL IS group, n = 5; Female (white bars) naïve group, n = 6; 10 µL vehicle group, n = 9; 10 µL IS group, n = 7; 20 µL vehicle group, n = 3, 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistically significant difference from the sex matched naïve control with $P < .05$, * indicates $P < .005$.

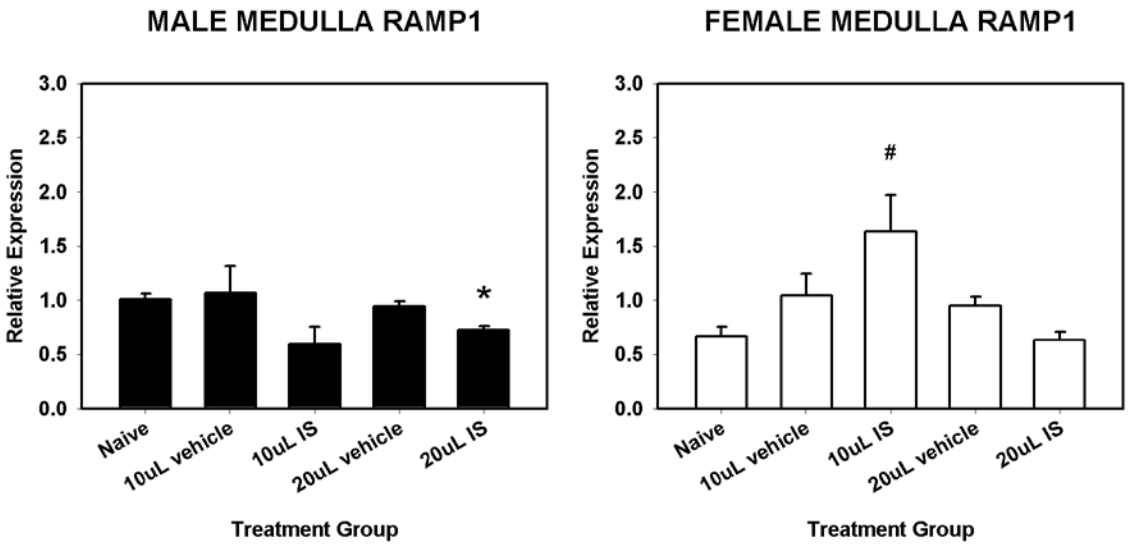

Figure 19. Sex differences in induction of gene expression of RAMP1 in rat medulla

Relative expression of RAMP1 in the medulla as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. Average naïve male expression used as subject control. X-axis shows treatment groups. Y-axis shows relative expression. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 was chronically delivered to the surface of intact dura via cannula for 8 applications. Male (black bars) naïve group, n = 6; 10 µL vehicle group, n = 5; 10 µL IS group, n = 5; 20 µL vehicle group, n = 3; 20 µL IS group, n = 5; Female (white bars) naïve group, n = 6; 10 µL vehicle group, n = 9; 10 µL IS group, n = 7; 20 µL vehicle group, n = 3, 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistically significant difference from the sex matched naïve control with $P < .05$, * indicates $P < .005$.

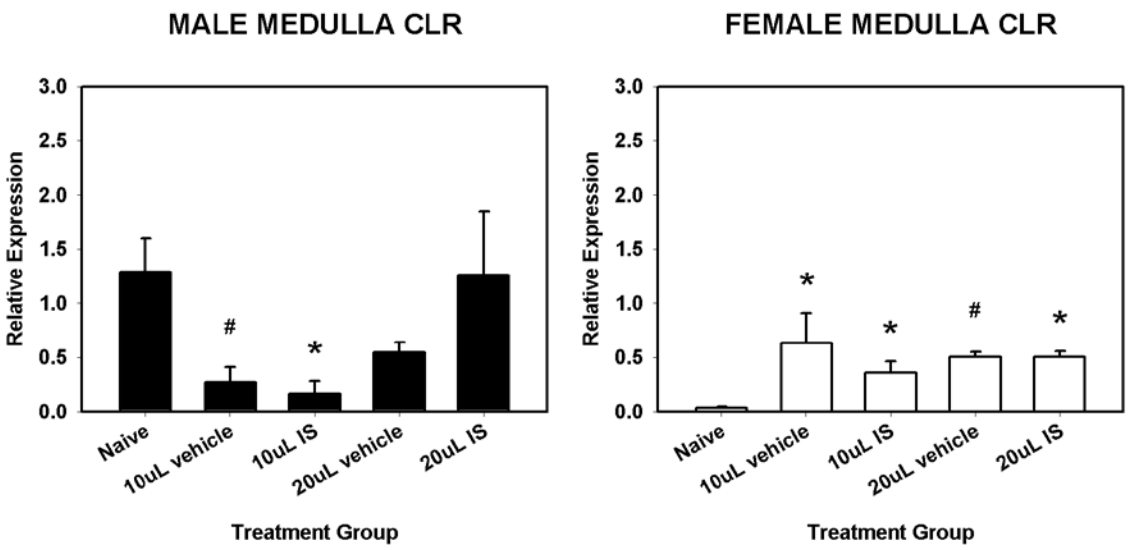

Figure 20. Sex differences in induction of gene expression of CLR in rat medulla

Relative expression of CLR in the medulla as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. Average naïve male expression used as subject control. X-axis shows treatment groups. Y-axis shows relative expression. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 was chronically delivered to the surface of intact dura via cannula for 8 applications. Male (black bars) naïve group, n = 6; 10 µL vehicle group, n = 5; 10 µL IS group, n = 5; 20 µL vehicle group, n = 3; 20 µL IS group, n = 5; Female (white bars) naïve group, n = 6; 10 µL vehicle group, n = 9; 10 µL IS group, n = 7; 20 µL vehicle group, n = 3, 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistically significant difference from the sex matched naïve control with $P < .05$, * indicates $P < .005$.

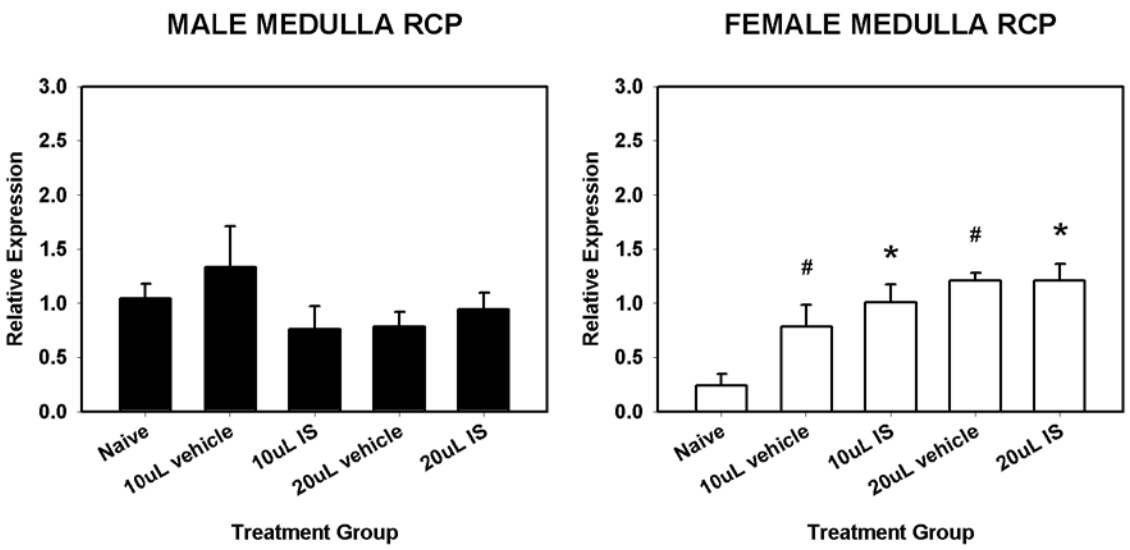

Figure 21. Sex differences in induction of gene expression of RCP in rat medulla

Relative expression of RCP in the medulla as measured by qRT-PCR using $\Delta\Delta C_t$ analysis to calculate relative expression. GAPDH was used as internal control. Average naïve male expression used as subject control. X-axis shows treatment groups. Y-axis shows relative expression. For each animal, 10 µL or 20 µL phosphate-buffered saline (PBS) pH 7.4 or inflammatory soup (1 mM histamine, serotonin, bradykinin and 0.1 mM PGE_2 in PBS) pH 5.5 was chronically delivered to the surface of intact dura via cannula for 8 applications. Male (black bars) naïve group, n = 6; 10 µL vehicle group, n = 5; 10 µL IS group, n = 5; 20 µL vehicle group, n = 3; 20 µL IS group, n = 5; Female (white bars) naïve group, n = 6; 10 µL vehicle group, n = 9; 10 µL IS group, n = 7; 20 µL vehicle group, n = 3, 20 µL IS group, n = 5. Standard error is shown in error bars. # indicates a statistically significant difference from the sex matched naïve control with $P < .05$, * indicates $P < .005$.

Discussion

Studies by others have localized the protein expression of CGRP, RAMP1 and CLR in the trigeminal pathway in male rats (Lennerz et al., 2008). In the trigeminal ganglia, CLR and RAMP1 are found on Schwann and satellite cells. The CLR, RAMP1 and CGRP are co-localized on trigeminal neurons and their central projections. In the spinal trigeminal nucleus of the medulla, because CLR, RAMP1 and CGRP are found on glomerular-shaped structures and not central glia or neuron cell bodies, it was concluded that primary afferent nerve endings are being labeled (Lennerz et al., 2008). In the present study, gene expression was evident in the medulla indicating that either mRNA was transported to the primary afferent fiber endings or that CGRP and its receptors are expressed in resident neurons or glia.

The results of the current work reveal that mRNA for CGRP, RAMP1, CLR and RCP is expressed in both medulla and trigeminal ganglia, and there are significant sex differences in gene expression of CGRP and CGRP receptors, including sex-dependent changes in expression of these genes in response to the chronic application of IS to the dura. A concurrent study (Stucky, 2010) demonstrated sex-related IS-induced migraine-like behavioral changes in these same test subjects.

It was found in this study that females have higher baseline gene expression of CGRP in the medulla than males (Figure 13), and that the expression of CGRP in the medulla declines following chronic application of IS to the dura (Figure 18). Decreased expression of CGRP in the medulla also occurs

in males, although this appears to be due primarily to the volume of fluid placed on the dura, suggesting a volume effect in these subjects. Changes in CGRP protein levels have be influenced by female sex hormones (Lanlua et al., 2002; Pardutz et al., 2002; Yallampalli et al., 2002), which may account for the sex differences in gene expression observed in the present work. Little is known about gene expression of CGRP in the medulla or dorsal horn. Thus, these findings indicate that CGRP gene expression in the medulla is differentially regulated between sexes in a model of chronic migraine.

In the medulla, females have lower baseline gene expression of RAMP1, CLR, and RCP than males (Figure 13), and CLR and RCP are induced by application of IS or vehicle to the dura in all of the experimental conditions (Figure 20 and Figure 21). The RAMP1 mRNA is induced by repeated application of 10 µL IS in females, whereas in males there appears to be a small, but statistically significant, IS-induced reduction in gene expression of RAMP1 (Figure 19). Although RAMP1 and CLR are reported to be induced in reproductive tissues by ovarian steroids (Dong et al., 2003), little is known about their induction in the trigeminal system. As the female 10 µL IS group showed significant allodynia and the 10 µL IS male group did not, it is possible that the induction of RAMP1 in the brainstem may contribute to central sensitization.

An increase in gene expression of CLR and RCP was noted in this study across all experimental female groups. A possible explanation for this is that a baseline inflammatory response to the cannula implantation surgery is sufficient to induce expression of CLR and RCP in the female medulla. Indeed, the work of

others has shown that crainiectomy or parietal bone scoring alone is sufficient to produce mast cell degranulation, local dural histamine release, and edema, which might mimic in some ways the inflammation induced in the present study (Stokely and Orr, 2008).

No baseline sex differences were observed in gene expression of CGRP in the trigeminal ganglia in the current study, although others have found more trigeminal CGRP-positive neurons in male rats as compared to females (Ambalavanar et al., 2003). In the present study, gene expression of CGRP in the trigeminal ganglia was decreased in male experimental groups but not in females following repeated application of IS to the dura. This effect was present across all experimental groups. While others have reported increases in CGRP gene expression from 30 minutes to 24 hours following a single injection of CFA in the masseter of male rats (Ambalavanar et al., 2006a), this is an acute response as opposed to chronic nature of the present work.

CGRP levels in blood plasma and DRGs are increased during pregnancy and in ovariectomized animals receiving sex hormones (Lanlua et al., 2002). Estrogen has been shown to increase activation of intracellular signaling pathways in the trigeminal ganglia (Liverman et al., 2009a), pathways known to be involved in activating CGRP transcriptional enhancers (Durham and Russo, 2003). This may be, in part, responsible for why in the present study there are reductions in gene expression of CGRP in trigeminal ganglia in response to repeated IS application in males but not females.

The present data indicate that females have lower baseline expression of

CGRP receptors RAMP1, CLR, and RCP in the trigeminal ganglia than males. Expression of RAMP1 in the trigeminal ganglia is decreased in males receiving 10 µL applications of either IS or vehicle. Indeed, it was found that gene expression is subject to a volume effect in males, with reduction of RAMP1 expression with a 10 µL volume, and an increase at 20 µL. Gene expression of CLR follows the same pattern as RAMP1. A small but significant volume effect on gene expression is also noted in females.

The RCP protein is induced in males receiving 20µL applications of either IS or vehicle, being greatest in those repeatedly exposed to 20µL of inflammatory soup, a condition which also induces migraineous behaviors (Stucky, 2010). As for females, RCP gene expression is induced in all experimental groups. This could be in response to surgery, as previously described for RCP and CLR in the medulla. In the trigeminal ganglia, RAMP1, CLR and RCP are inducible especially in males and volume effects are present. This suggests a regulatory role for mechanically or osmotically active sensory channels in this region. Acid sensitive ion channel (ASIC) family channels, such as TRPV4 or voltage-gated sodium channels (Na_v) family channels, such as Na_v 1.8, are possible candidates for this role. For example, TRPV4 channels are known to be present in trigeminal ganglia, and their activation has been shown to increase the excitability of nociceptors through a protein kinase C (PKC)-dependent mechanism (Chen et al., 2009a). The ion channels, such as Na_v 1.7, Na_v 1.3 and Na_v 1.8, are reported to increase excitability of the trigeminal nociceptors in much the same way (Chen et al., 2009b). It would be interesting to know whether TRPV4 or Na_v 1.8 are

activated in response to osmotic or mechanical changes in neurons co-expressing RCP or CLR and whether there is a correlation in the expression of these genes.

In the present study, females displayed a greater dynamic range of induction in gene expression than males. Sex differences in baseline values of gene expression appear to prime females for a greater percentage change in CGRP receptor gene expression. For example, female CLR gene expression is increased 12.6-fold when comparing the naïve control group to the 20 µL IS group, while expression is increased only 2.2-fold in males.

The present findings also suggest greater induction in the trigeminal ganglia of males and more induction in the medulla in females in response to exposure to the IS. This suggests that migraine pathophysiology may occur more centrally in females, and that central sensitization may be more likely in females than males. This gene expression data correlates with the finding in a related study that there is more allodynia in females and it occurs in response to smaller volumes of IS than in males (Stucky, 2010).

Taken together with the behavioral study, the results of these experiments support the possibility that sex differences in CGRP and CGRP receptor expression may be responsible for the increased prevalence of migraine in females. These data indicate that gene expression changes in CGRP and CGRP receptors are differentially regulated in a model of chronic dural inflammation, demonstrating further the complexity of migraine pathophysiology.

VI. General Summary, Conclusions, and Future Directions

Summary of findings

A major aim of this dissertation project was to establish a model of migraine headache and use it to identify sex differences in behavior. The existing model was extended to encompass an IHS criterion for headache diagnosis in humans, namely, reduction in routine activity. Decreased locomotor activity and increased allodynia were noted in both sexes at a dose of 20 µL of IS to the dura, supporting the validity of the technique as a putative model for migraine.

Using assays for mechanical facial allodynia and locomotor activity together allowed for assessment of the behavioral differences between males and females in their reactions to dural inflammation. The results revealed that both locomotor activity and mechanical facial allodynia are modulated in sex-dependent manners in response to repeated applications of IS. In addition to higher dose treatment, females also showed effects at a dose of 10 µL IS, in accord with the fact that women are more likely to experience migraine than men. Females also showed allodynia in regions innervated by the ophthalmic division which is the same division of the trigeminal ganglia that innervates the site of dural inflammation. Males on the other hand demonstrated more allodynia in the peri-masseter region, innervated by the mandibular division of the trigeminal ganglia.

Once the model was established and sex-differences in behavioral changes were identified, evaluation of baseline sex-dependent variations in gene expression in the trigeminal pathway of CGRP and its receptor components was

performed to investigate whether expression of these genes changes in response to application of the IS. Significant findings showed lower CGRP receptor and higher ligand gene expression levels in females at baseline and greater percentage induction of receptors in females. More central changes were seen in females while the largest expression changes happened peripherally in males, and volume effects were present in both groups. Before addressing the implications of this work a discussion of limitations of the study is necessary.

Limitations

There are important behavioral correlates of migraine that this study did not attempt to measure. The two most prominent are photophobia and phonophobia. Both are included in the IHS diagnostic criteria and are adaptable to a rodent model. One laboratory has created mouse model of photophobia (Recober et al., 2009) and in models of acoustic startle have been used to study anxiety and stress (Davis et al., 1997; Walker et al., 2009) and could be adapted to use in a migraine model.

The CGRP-related gene expression is one possible regulatory mechanism of sensitization, but there are many others. Migraine pathophysiology may be regulated at many points including gene expression, post-transcriptional processing, protein expression, ligand or receptor trafficking, ligand binding, and receptor signaling. This study investigated baseline expression and IS-induced changes at the mRNA level but did not thoroughly look at changes in protein. An attempt was made to perform protein quantification using western blot analysis but the available antibodies were found to be non-specific and showed multiple

bands that did not correspond to any published isoforms. Further functional studies could be performed to look at ligand binding and receptor signaling. It is feasible to look at G-protein signaling using GTPγs (Winter and McCarson, 2005) and preliminary studies have been performed with tissue from this study. With these limitations in mind, the model can be extended to new areas of research.

Future directions

Using with the behavioral data and tissue that has been collected in this study it is possible to use additional qRT-PCR primers to perform additional analysis of other genes that may be involved in migraine. As mentioned previously genes that are involved in osmotic or mechanical signaling such as Nav 1.8, and TRPV4 may be induced by application of IS to the dura. Actimetry and qRT-PCR are both highly quantitative and data intensive methods and this study has produced a voluminous dataset of greater than 6,000 data points. A systems biology approach is well suited to analyze the data from this study. For example, principal component analysis could be used for data reduction to isolate the genes that change most independently from those that are highly interdependent. In this study linear regression analysis was used to determine correlations between genes and behavior, but it is likely that interactions are more complex than can be modeled using this technique. Further modeling can be performed to search for interactions that may be revealed by more sophisticated techniques. The many signaling motifs possible in the trigeminal system are likely to produce complex interactions that will be suitable for study with these strategies.

The finding that application volume influenced gene expression was unexpected and indicates that application volume could significantly influence trigeminal activation. One strategy to investigate this phenomenon would be to serially increase volumes of vehicle (the present study investigated 2 different volumes) and measure behavioral effects. A second strategy would be to isolate volume effects from the IS effects by applying a constant volume of increasingly concentrated IS and measure behavioral changes. Certainly, CGRP related gene expression changes should be measured in these same groups.

To further investigate the role of neurogenic inflammation and dural mast cells in migraine, the present model can be used with agents that eliminate the influence of resident dural mast cells through stabilization or depletion using pretreatment of the dura with the mast cell stabilizer sodium dicromoglycate (Markowitz et al., 1989) or with the mast cell depleting agent 48/80 (Marotta et al., 2009).

One of the most exciting future directions is to move beyond neurogenic inflammation and employ the model to study behavioral correlates and molecular changes in other processes involved in migraine pathogenesis and related disorders. Cortical spreading depression can be induced by applying KCl directly to the dura in rats (Colombo et al., 1973) and there is still considerable debate about how this biological effect and is involved in migraine (Pietrobon, 2005). The model could be adapted to study menstrual migraine by manipulating estrogen levels in rats through ovariectomy and estrogen replacement. Additionally, this behavioral model could be used to identify behavioral and molecular changes

associated with medication overuse of headache, a condition associated with a number of drugs used to treat migraine (Tepper and Tepper, 2010). In this condition, migraineurs find that increasing pharmacological treatment paradoxically increases the frequency of migraine attacks, and the migraine episodes become more resistant to therapy. This is most often encountered with opioid drugs (Bigal et al., 2004; Tepper and Tepper, 2010). Unfortunately, the incidence of mild traumatic brain injury is increasing in the U.S. population as soldiers return from serving in Iraq and Afghanistan (Afari et al., 2009). Development of a model of trauma induced headache would be especially relevant and can be investigated in this model by performing a mild traumatic brain injury prior to initiating neurogenic inflammation. Using the techniques in the current study each of these studies could be accomplished in a straightforward manner.

In the future, the model will be useful to investigate new drugs and therapies. CGRP antagonists are currently under development for use in migraine and new more effective therapies are needed for of migraine sufferers that have increased cardiovascular risk profiles or are not helped by existing therapies. The locomotor component of the behavioral model is highly quantitative and measures activity, which is an important functional outcome. This characteristic of the preclinical model may allow early detection of suboptimal therapies that alleviate pain but produce adverse effects such as somnolence.

Conclusion

A major contribution from the present project was development of behavioral assays for a model of migraine that appear to be more robust than others. The measurements are highly quantitative and less susceptible to experimenter bias than previous measures of allodynia. By incorporating the actimeter and the ability to precisely quantify the level of ambulation and force variance using metrics such as distance traveled and bouts of low mobility this study allowed for an assessment in rats that is analogous to those clinical signs and symptoms used to diagnose humans as migraineurs. This strengthens the value of this preclinical model considerably. Reductions in locomotor activity were present in the model developed in the present study and that this reduction correlates with periorbital mechanical allodynia in females make for a significantly stronger preclinical model for investigating migraine pathophysiology and for testing therapeutic interventions.

The allodynia behavioral task presented in the present study has advantages over other models of facial mechanical testing in migraine (Oshinsky and Gomonchareonsiri, 2007; Edelmayer et al., 2009; Wieseler et al., 2009) because it does not require restraint of the rats and therefore the results are less confounded by the significant analgesic effects which have been reported in response to restraint (Botelho et al., 2010).

Evidence of increased expression of RCP in the trigeminal ganglia of males and of RCP in the medulla of females displaying migraine-like behaviors links allodynia and decreased motor activity to increases in gene expression, and

indicates trigeminal sensitization may involve CGRP signaling, which is a mechanism that is a major target of drug development.

This study identified major sex differences that are significant and have never been looked at before in migraine model, in both aspects of behavioral and molecular changes. The present study with this model has shown significant sex differences in susceptibility to migraine, with females displaying migraine-related behavioral responses at lower doses of the inflammatory soup than males, and with females displaying a greater persistence of effect than males. As the effects in female were dose-related, it appears they represent pharmacologically and physiologically relevant effects of the inflammatory agents contained in the IS.

An important element of these findings is that females have a greater dynamic range of induction in gene expression. It appears that sex differences in baseline values of gene expression prime females for a greater percentage change in CGRP receptor gene expression than is the case for males. This change could be responsible for the greater susceptibility of females to migraine.

Previous work in other organ systems revealed that gene and protein expression of RAMP1 and CLR are highly inducible in females, and that 17β-estradiol inhibits expression, while progesterone stimulates expression of these proteins in the rat placenta during pregnancy (Dong et al., 2003). These hormone-related RAMP1 and CLR expression changes have also been observed in the rat mesenteric artery (Yallampalli et al., 2004). As it is also believed that CGRP plays an essential role in maintaining normal blood pressures in response to the increase in blood volume that occurs during pregnancy (Yallampalli et al.,

2004). Perhaps it is necessary that the CGRP system be highly adaptable in females and a possible adverse effect of this adaptability may be increased susceptibility to trigeminal activation and migraine.

While the mechanisms underlying migraine remain poorly understood, for the first time these results provide evidence that sex differences in the regulation of the expression of CGRP and CGRP receptors in migraine correlate with changes in migraine-like behaviors and may be responsible for the increased prevalence of migraine in females. As gene expression changes in CGRP and CGRP receptors are differentially regulated in this model of migraine, the results provide further evidence for their potential involvement in modulating pain and the symptoms of migraine headache.

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