MENINGEAL AFFERENT SIGNALING AND THE PATHOPHYSIOLOGY OF MIGRAINE

by

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Migraine is the most common neurological disorder. Attacks are complex and consist of multiple phases but are most commonly characterized by intense, unilateral, throbbing headache. The pathophysiology contributing to migraine is poorly understood and the disorder is not well managed with currently available therapeutics, often rendering patients disabled during attacks. The mechanisms most likely to contribute to the pain phase of migraine require activation of trigeminal afferent signaling from the cranial meninges and subsequent relay of nociceptive information into the central nervous system in a region of the dorsal brainstem known as the trigeminal nucleus caudalis (TNC). A three-fold higher prevalence of migraine in females implies sex-dependent mechanisms are involved in migraine. Yet, sex-dependent mechanisms of migraine remain unknown and have yet to be investigated thoroughly. Events leading to activation of meningeal afferents are unclear but nerve endings within this tissue are mechanosensitive and also express a variety of ion channels including acid-sensing (ASIC) and transient receptor-potential (TRP) channels. These properties may provide clues into the pathophysiology of migraine by suggesting

that decreased extracellular pH and environmental irritant exposure in the meninges contributes to headache. Neuroplasticity is also likely to play a role in migraine given that attacks are triggered by routine events that are typically non-noxious in healthy patients and clear evidence of sensitization occurs during an attack. Where and how plasticity develops is also not clear but may include events directly on the afferents and/or within the TNC. Among the mediators potentially contributing to plasticity, calcitonin gene-related peptide (CGRP) has received the most attention within the migraine field but other mechanisms may also contribute. Ultimately, greater understanding of the molecules and mechanisms contributing to migraine will undoubtedly lead to better therapeutics and relief for the large numbers of patients across the globe who suffer from this highly disabling neurological disorder.

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INTRODUCTION

Migraine is a subtype of headache characterized by repetitive episodes of intense unilateral throbbing head pain lasting many hours to days. However, it is a multiphasic disorder comprised of complex symptomology is highly variable among patients. These complex features of migraine differentiate it from other types of chronic pain and include the presence of premonitory symptoms, an aura, and during the headache phase, severe nausea, vomiting, and hypersensitivity to light and sound. Although migraine is episodic in nature, together these collections of symptoms cause an entire attack to last close to a week, and given the severity, migraine has a significant negative impact on a patient's quality of life. The World Health Organization's Global Burden of Disease Study conducted in 2010 found migraine headache to be the third most prevalent disease across the globe, preceded only by dental caries and tension type headache[1]. Given the current understanding of migraine as a pathological condition of the nervous system, migraine is thus the most common neurological disorder with an estimated 33% of women and 10% of men affected by the condition[1-3]. Despite the prevalence, the basic physiology and underlying contributing factors to the development of migraine headache is still poorly understood.

Features of Migraine

Migraines are comprised of 4 phases; premonitory phase (also commonly referred to as the prodrome), aura, headache, and postdrome[2,3] (Figure 1.1). Studies have estimated that the number of patients with migraine that experience premonitory symptoms may range from as low 37% to as high as 80%[4,5]. The premonitory phase occurs hours to days before the actual

headache and can be a reliable predictor of an upcoming migraine for some patients[4]. This phase consists of symptoms that range in severity such as excessive yawning, food cravings, mood changes, fatigue, sore neck, and confusion among others[4-6]. Following the premonitory phase is the aura phase that takes place up to an hour prior to the headache phase, but is only experienced by 15 to 30% of migraineurs. Aura involves sensory disturbances, often visual, with moving and intensifying regions of flashing lights or scintillations accompanied by partial vision loss or scotomas (areas of decreased acuity in the visual field). Scotomas are reported as typically originating in the center of a patients visual field and progressing towards the edges[7]. Aura may present other somatosensory symptoms that can affect normal motor function, perception of language, or even production of discernible language. The next phase is headache which must, by definition, be a minimum of 4 hours but can last up to 72 hours without medical intervention[7-9]. It is described as moderate to severe throbbing pain, initially localized to one side of the head[7]. The headache can be aggravated by repositioning of the head, by normal physical activity, or by changes in intracranial pressure e.g. coughing or sneezing. Common symptoms that accompany the throbbing pain include sensitivity to light, sounds, and smells also commonly referred to as photophobia, phonophobia, and osmophobia respectively, although the first two are more correctly described by the terms photo- and phonoallodynia as these stimuli cause pain. Additionally nausea and vomiting are common during the headache phase. After the headache has resolved it can often be followed by a postdrome phase where the patient still does not feel recovered or entirely back to normal. The postdrome can typically last from 18-24 hours and often consists of exhaustion, irritability, and an inability to concentrate [7].

Migraine currently lacks a known cause or source that can be identified as the driver of

the disorder. Nonetheless, individual patients may be susceptible to distinct stimuli that are capable of evoking a migraine. These stimuli, referred to as triggers (Figure 1.1), can vary between individuals and yet they share the commonality of eliciting a migraine attack. However, it should be noted that these triggers do not exhibit a noxious effect in non-migraine sufferers and therefore are not the true source of the condition. Sensitivity to triggers in migraine patients suggests the presence of maladaptive plasticity in the nervous system (discussed below) that contributes to the development of attacks in response to stimuli that are innocuous in normal individuals.

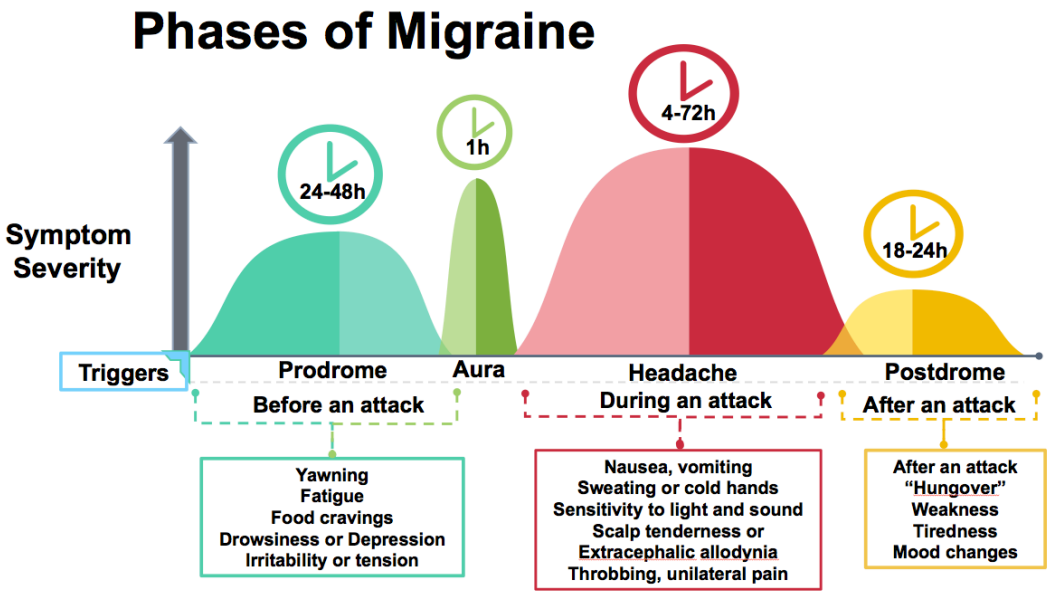

Figure 1.1. Timeline of migraine attacks.

Migraine typically consists of four phases although not all patients experience all phases. Attacks often begin with triggers, which can be routine events such as stress or fatigue. The first phase, prodrome, lasts between 24 and 48 h and consists of premonitory symptoms such as yawning or irritability. The next phase to develop is aura, which affects less than 30% of migraine patients for about one hour and consists of sensory disturbances, mostly visual. The most debilitating phase of a migraine attack is the headache phase. This uni- lateral and throbbing head pain can last between 4 and 72 h and is often accompanied by photo- and phonoallodynia as well as nausea and vomiting. The fourth phase of a migraine attack is the postdrome. Symptoms related

to the postdrome phase often vary; however, patients commonly report being exhausted and irritable. Together, all four phases of a migraine attack can last between 3 and 5 days.

One extremely common trigger in those susceptible to migraine is stress. In fact, studies demonstrate that anywhere from 59% to over 80% of patients indicate that stress is their primary trigger for migraine [10-12], thus making stress the most commonly reported trigger. The effect of stress exposure may be cumulative. Stress appears to have a greater influence with 2 consecutive days of moderate or greater stress in a row. On the other hand, low stress followed by a day with moderate or greater stress on the subsequent day is associated with less risk [9]. Conversely this study also found that if stress is moderate or greater on the first day, but low on the following day the migraine risk is still increased, thus suggesting a complex relationship between stress and migraine. Recent reports also show that the peak susceptibility to migraine is in the 18-24 hours after a stressful event[13], further supporting the complex nature of the contribution of stress to migraine. It is hypothesized that stress itself may not be the trigger, however it may evoke changes in normal behaviors such as sleep or food intake that can aggravate the condition[14]. Patients able to identify their triggers can benefit and potentially reduce migraine attacks by minimizing their encounters with that particular stimulus (or stimuli)[15]. Other common triggers include foods (and alcohol), environmental irritants (cigarette smoke, odors), exercise, changes in the environment (barometric pressure), too much/lack of sleep, hormone fluctuations, sensory stimuli (such as intense light), and skipping meals. Notably, drugs such as nitric oxide donors (e.g. nitroglycerin) are capable of eliciting headaches reliably amongst migraineurs[16], an important observation since NO donors are often used to experimentally trigger attacks in clinical studies.

Current Migraine Treatments

Migraine is an extremely disabling disorder[1], a fact made more troubling by the lack of efficacy of currently available therapeutics. Treatment typically falls in one of two categories, abortive and prophylactic. Abortive agents are taken at the onset of headache or during aura with the goal being termination or at least a decrease in intensity of the building attack. Prophylactic therapies are given daily to patients who have frequent migraines (e.g. >5 per month) with the goal of decreasing the frequency of attacks. The most commonly prescribed abortive therapies are the triptans and nonsteroidal anti-inflammatory drugs or (NSAIDs)[17,18]. Triptans are a family of $5HT_{1b}$ and $5HT_{1d}$ agonists that include members such as sumatriptan, zolmitriptan, rizatriptan, eletriptan, and naritriptan. Triptans alone account for up to 80% of medications prescribed for migraine[2,19,20]. Despite the high propensity of their use triptans are not always effective. For example, the percentage of patients that report being completely pain free 2 hours after dosing ranges from 12% (frovatriptan 2.5 mg) to 40% (rizatriptan 10 mg)[21]. Further, triptans possess vasoconstrictive properties and are therefore contraindicated in patients at risk for cardiovascular complications or abnormal blood pressure[22]. Another issue with triptan use (this can also occur with many migraine medications) is a clinical concern that triptan overuse can lead to phenomenon known as medication-overuse headache (MOH)[2,23,24]. MOH is characterized by worsened or more frequent headache caused by regular triptan intake that resolves after removal of the offending medication. Thus, acute treatments such as triptans are not recommended for daily use and cannot be used as prophylactic agents[18]. Opioids coupled with NSAIDs or acetaminophen, as well as barbiturates are also used by migraine patients, even though they too can induce MOH[25,26].

As mentioned above, prophylactic migraine treatment is given on a daily basis in order to attempt to prevent the onset of migraine attacks or decrease frequency. Commonly used prophylactic agents are antiepileptics such as topiramate, gabapentin, or valproate, beta blockers such as propranolol, or antidepressants such as amitriptyline. Use of propranolol, valproate, and topiramate for example can result in a 50% reduction in migraine frequency in 40–50% of patients [21]. However, prophylactic treatment is generally not well tolerated by patients (with the exception of propranolol) due to numerous adverse side affects. These side effects can include significant decreases in cognitive function, nausea, vomiting, weight gain, and even withdrawal symptoms upon discontinued use of the medication[18,19,27]. Taking into account the statistics noted above for both types of agents (<40% of patients pain free with triptans and 50% of patients achieving 50% frequency reduction with prophylactics), it is clear that many patients are unable to achieve relief. Thus, there remains unmet need to develop novel treatment strategies treatments for migraine patients.

Anatomy and Physiology of the Cranial Meninges

The meninges consist of three layers that envelop the brain; the dura mater, the arachnoid and the pia mater. Unlike the meningeal layers beneath it, the pachymeninges or dura is made up of functionally distinct laminae [28], making it the thickest meningeal layer. While the maturing dura is well attached to the cranial sutures, as grows it becomes thicker, tougher, and upon fusion separates from the suture junction [29].

The outermost endosteal lamina (or endosteum) is a tough inelastic structure firmly attached to the inner surface of the cranium to form the periosteal lining. Despite containing relatively few cells attached by gap junctions; flattened fibroblasts are fortified by a dense matrix

of extracellular collagen fibrils interwoven with micro-fibrils [30]. This matrix also provides the structural support for the meningeal circulation and afferent innervation. Blood vessels and nerves can be interwoven within dural fibrils, giving the appearance continuity of the respective adventitia and epineuria with the dural surface [28].

The inner meningeal layer of the dura is affixed to the endosteal layer. However, these larger fibroblasts are devoid of extracellular connective collagen fibers and gap junctions. The meningeal layer can be further divided into the inner and the border cell layer. Border layer cells are flattened cells overlying one another to form thin sheets that range in thickness- dependent on location-facing the arachnoid side[30].

Although, the dura is strong its fibrous composition renders it incapable of encapsulating CSF within the skull. However, the numerous tight cell junctions between fibroblasts lacking extracellular space and collagen make the arachnoid layer equipped to serve as foremost barrier; impermeable to fluids and ions[30,31]. Fibroblasts form arachnoid trabeculae in order to attach to the inner surface of the arachnoid layer, span the subarachnoid space, surround subarachnoid space vessels and attach to pial lined surface of the brain. CSF flows through the narrow arachnoid and pia into the subarachnoid space that expands to create arachnoid granulations in the dural border layer, as well as the cisternal spaces found at the skull base.

Finally cells that resemble those of the arachnoid trabecular cells; with elongated overlaying flat processes form the innermost pia. Desmosomes and gap junctions link superimposed fibroblasts onto adjacent cells to produce a smooth and thin covering of the gyri and sulci.

Vasculature of the Cranial Meninges

As mentioned above, the outer endosteal dural lamina serves at the substrate for the vasculature of the meninges. The internal carotid and vertebral arteries provide small anterior and posterior branches, however the arterial workhorse is the middle meningeal artery (MMA). The MMA ascends from the maxillary artery branch of the external carotid artery. It then divides into three major branches; the anterior (bregmatic), middle (obelic), and posterior (lambdoidal)[28]. Venous drainage of the dura occurs either through satellite veins (running along meningeal arterial trunks) or at the dural venous sinuses[32].

Trigeminal Nerve Anatomy

The trigeminal nerve is the fifth and largest cranial nerve; exiting the brainstem at the level of the pons as a single nerve root, passing through the trigeminal ganglion and continues distally from the ganglion as separate nerve branches [33,34]. There are three major branches that emerge from the ganglion into V1, V2, and V3 subdivisions. Each branch innervates a distinct dermatome. The first division, V1 is also referred to as the ophthalmic nerve, and it innervates the nose, areas above the eye, and the scalp. The second division is V2, or the maxillary nerve, and it innervates areas below the eye and above the mouth as well as the mandible. Lastly, V3 is the mandibular nerve that innervates the mouth and lower portions of the jaw and face. Most relevant to migraine is the V1 branch as this is the branch that provides the majority of the innervation of the meninges (dura and blood vessels). These trigeminal afferents send afferent input from the head into the brainstem (at the pons) and they descend into the medulla where they synapse on second order neurons and interneurons within a region of the dorsal medulla known as the spinal trigeminal nucleus or trigeminal nucleus caudalis (TNC) (Figure 1.2). The

nucleus caudalis lies below the obex of the brainstem and is the most caudal part of the trigeminal nucleus[35]. Multiple projections into the caudalis may share the same interneuron, allowing crosstalk between afferents and providing the basis for referred pain seen in the trigeminal system[36]. The axons of second order neurons form the trigeminothalamic pathway then decussate and ascend to the ventroposteromedial nucleus of the thalamus. From the thalamus connections are made to cortical sites.

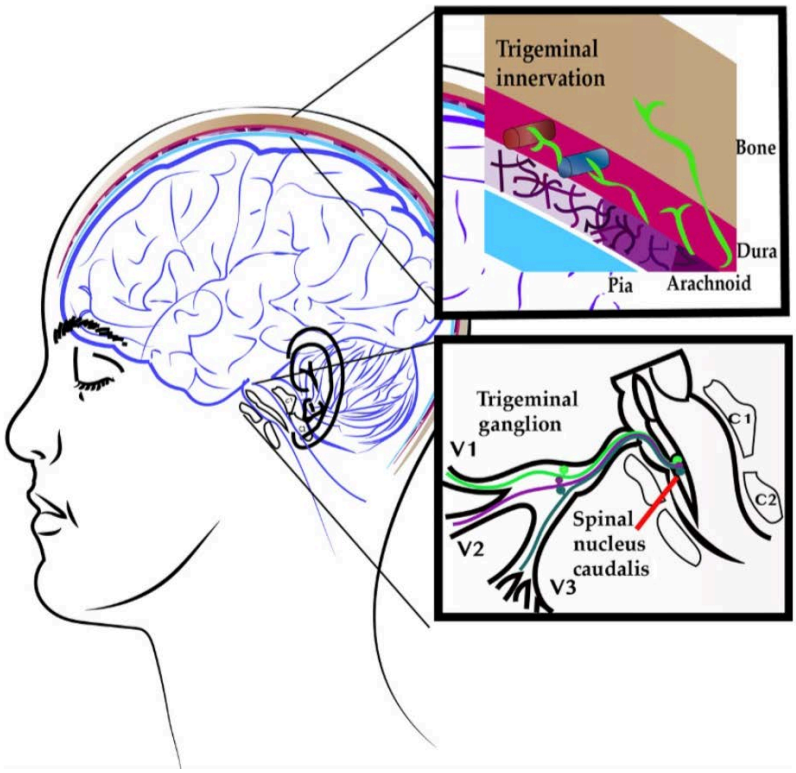

Figure 1.2. Anatomy of the meningeal afferent system.

Illustration of the meningeal layer and its relationship to the overlying bone and brain below. The dura is highly innervated by sensory afferents projecting from the trigeminal ganglion. Three major branches emerge from the ganglion into V1, V2, and V3 subdivisions. Each branch innervates a distinct dermatome of the head with V1 providing innervation to the meninges. Trigeminal afferents send input into the brainstem (at the pons) and they descend into the medulla where they synapse on second-order neurons and interneurons within a region of the dorsal medulla known as the spinal trigeminal nucleus or trigeminal nucleus caudalis.

Afferent Innervation of the Meninges

The cerebral dura mater is innervated predominantly by afferents that accompany the dural vasculature, whose cell bodies reside in the ipsilateral trigeminal ganglion. Comparatively, the dural vasculature contains much less sympathetic and parasympathetic innervation; arising from the ipsilateral cervical sympathetic ganglia and sphenopalatine ganglion respectively. Afferents dedicated to sensations of pain and temperature form the spinotrigeminal pathway and end in the spinal trigeminal nucleus[37]. Recent studies have shown meningeal nerve fibers of all origins capable of traversing through periosteal, diploe (the osseous part of the skull), endosteum, dura, arachnoid and pia at multiple sites in humans and rodents [38,39]. Human surgical procedures stimulating dural branches of the trigeminal nerve have been shown to activate the trigemino-cardiac reflex, causing autonomic slowing of the heartrate, hypotension, apnea, and gastric hypermobility[40,41]. This brain stem reflex is generated by reticular formations that connect efferent pathways in the spinal motor nucleus of the vagus nerve[41,42]. Experimental models have shown stimulation of the trigeminal nucleus caudalis can cause the trigemino-cardiac reflex in animals[42]. Ongoing meningeal afferent activity in migraine patients may intiate this reflex in migraine patients, potentially contributing to symptoms autonomic dysfunction seen in the condition.

Migraine Pathophysiology

Although the brain lacks sensory innervation, which renders it incapable of sensing noxious stimuli, studies dating as far back as 1940 have shown that sensory afferents that innervate the cranial meninges, particularly the dura mater, are in fact mechanically (and chemically) sensitive [43-49]. The earliest of these studies showed that in response to stimulation of

the meninges, conscious humans report pain and no other sensation, indicating that the meninges are innervated purely by nociceptors. However, the events within the meninges that occur during migraine are not yet known. Migraine has been extensively hypothesized to arise from vascular swelling in the brain and meninges (supported by the triggering ability of vasodilators such as NO donors) that produced the distinct throbbing characteristic of the pain, thus many antimigraine treatments have possessed vasoconstrictive properties [50-52]. Together, the mechanical sensitivity of dural afferents and the alleged contribution of vasodilation has been a leading hypothesis in the field for decades. However, it is becoming increasingly evident that vasodilation of dural vessels during migraine is a secondary effect of the disorder rather than the clinical cause[52,53]. Several recent imaging studies in humans, the most recent examining natural (i.e. non experimentally triggered) migraines, found no extracranial dilation of vessels and only slight intracranial dilation[54-56]. The recent study also showed efficacy for headache by the vasoconstrictor sumatriptan, even in the absence of any vasodilation present. These studies cast serious doubt on vasodilation being the primary mediator of migraine. Further evidence supporting this concept is that functional imaging studies have concluded that migraine headache appears to arise within the brain and does not require vasodilation in order to occur[54,57-61]. In fact, the hypothalamus is now regularly cited as a potential central origin of migraine attacks[62] and activation of the hypothalamus is known to occur in the premonitory phase of migraine[63]. In relation to pain, the hypothalamus is also known to modulate afferent trafficking in the TNC and can enhance afferent input from the meninges[64,65]. Although attacks are potentially driven centrally, the pain phase of migraine still requires activation of peripheral afferents and these afferents can be modulated by central outputs. Importantly, activation of afferents need not be in

response to vasodilation. Thus, migraine is now becoming increasingly accepted as a neurological disorder where maladaptive responses (plasticity) of dural afferents innervating the vasculature or the dura itself, possibly modulated by descending pathways from the brain, can provide the basis of the headache[7,9,66].

Attention is now focused on identifying mechanisms of activation and plasticity of dural afferents that may contribute to the pain of migraine. The primary goal of this dissertation is to investigate direct and indirect mechanisms of meningeal afferent activation, sensitization, and sex specific pharmacology in preclinical migraine models. Together, the studies presented here provide new mechanistic insight into the intrinsic neuroplastic properties of the cranial meninges.

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CHAPTER 2

MENINGEAL TRPM8 ACTIVATION CAUSES CUTANEOUS FACIAL AND HINDPAW ALLODYNIA IN A PRECLINICAL RODENT MODEL OF HEADACHE

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Abstract

Migraine headache is a neurological disorder affecting millions worldwide. However, little is known about the mechanisms contributing to migraine. Recent genome wide association studies have found single nucleotide polymorphisms (SNPs) in the gene encoding transient receptor potential channel M8 (TRPM8). TRPM8 is generally known as a cold receptor but it has been implicated in pain signaling and may play a role in migraine pain. In order to investigate whether TRPM8 may contribute to the pain of migraine, the TRPM8 activator icilin was applied to the dura mater using a rat behavioral model of headache. Cutaneous allodynia was measured for five hours using Von Frey filaments. Dural application of icilin produced cutaneous facial and hind paw allodynia that was attenuated by systemic pretreatment with the TRPM8-selective antagonist AMG1161 (10 mg/kg p.o.). Further, the anti-migraine agent sumatriptan (0.6 mg/kg s.c.) or the non-selective NOS inhibitor L-NAME (20 mg/kg i.p.) also attenuated allodynia when given as a pretreatment. These data indicate that TRPM8 activation in the meninges produces behaviors in rats that are consistent with migraine and that are sensitive to pharmacological mechanisms known to have efficacy for migraine in humans. The findings suggest that activation of meningeal TRPM8 may contribute to the pain of migraine.

Introduction

Migraine is a common, often debilitating, neurological disorder that can last from hours to several days. The recent Global Burden of Disease Study found migraine headache to be the third most prevalent disease on the planet, following dental caries and

tension type headache [1]. Despite the magnitude and impact of migraine, the mechanisms that lead to this disorder remain unclear. Trigeminal nociceptors innervate the meninges and are sensitive to both chemical and mechanical stimulation[1,2]. These nociceptors may play a role in the development of migraine pain, but the mechanisms by which they are activated remain to be fully elucidated.

The transient receptor potential or TRP family of non-selective cation channels are involved in several physiological and pathological processes[3]. TRP channels are sensitive to both thermal and chemical stimuli and have been proposed to act in mammals as thermosensors as well as detectors of endogenous inflammatory states and external irritants [4,5]. In support of this hypothesis, pharmacological as well as genetic evidence has clearly implicated certain TRP channels in the detection or transduction of sensory stimuli. There are three super-families of TRP channels, TRPV, TRPC and TRPM[6], as well as several other smaller TRP families, some with only a single member (e.g. TRPA). The TRPM subfamily has eight members: TRPM1–M8. The TRPM8 channel was formerly known as the cold and menthol receptor (CMR1) due to its responsiveness to cold temperature as well as to cooling agents such as menthol[7-9]. TRPM8 is activated by noxious and non-noxious cold ranging from ~28°C, down to 8°C[10]. TRPM8 can also be activated by cooling agents such as icilin (as well as menthol mentioned above), behaving like many ligand-gated channels in response to these agents [11]. Transcripts of TRPM8 are found in a subset (<15%) of small diameter sensory neurons [7,9]. As alluded to above, TRPM8 expression in the trigeminal and dorsal root ganglia is thought to confer innocuous cold sensitivity to the somatosensory system [12,13], particularly in sensory

neurons innervating cutaneous tissues. However, TRPM8 is also expressed in neurons innervating deep tissues such as the bladder and colon[14,15]. As these neurons are not exposed to cold temperatures, cold may not be an activating stimulus in these tissues and the channel may respond to other endogenous activators. [16,17] Possible endogenous activators/sensitizers in deep-tissue afferents include stimuli such as lysophospholipids, cyclopentenone prostaglandins, and phosphatidylinositol biphosphate among others.[18] Thus, TRPM8 may be a sensor of a variety of internal and external stimuli.

One of the most consistent genetic findings in migraine patients comes from several recent genome wide association studies (GWAS) that revealed single nucleotide polymorphisms (SNPs) in and around the TRPM8 locus [19-21]. The mutations can lie in coding regions of the gene, but are often within the 5' untranslated region (5'UTR). It remains unclear how these mutations influence TRPM8, e.g. by altering channel expression or function, but these mutations have been verified across several populations of migaineurs[22-25] and suggest that TRPM8 may play a role in migraine. The purpose of this study was to investigate whether activation of TRPM8 in the meninges produces behaviors in rodents consistent with migraine pain using a preclinical model of headache.

Methods

Subjects

Adult male Sprague-Dawley rats (250–300g, Harlan) were maintained in a temperature-controlled room on a 12-hr light/dark cycle with food and water ad libitum. All procedures were performed in accordance with the policies of the IASP as well as the

NIH guidelines for use of laboratory animals. All procedures were approved by the IACUC of the University of Arizona.

Surgeries

Dura cannulation surgeries were performed on rats (250-300) grams as previously described [26,27]. Briefly, animals were anesthetized and an incision exposing the skull was made to the top of the skull. Once the skull was exposed, a 1-mm hole was made in the skull to expose the dura (1mm left of midline, 1mm anterior to bregma). A 1mm guide cannula (Plastics One) was then inserted into the hole and secured with Vetbond™ (3M™). Two screws (Small Parts) were placed rostral to the cannula on either side of the skull. Dental acrylic was used to adhere the cannula and screws to the skull. A dummy cannula (Plastics One) was placed into the cannula to ensure patency. Postoperatively, animals received gentamicin (8 mg/kg) to minimize infection. Rats were housed individually and given 6–8 days for recovery prior to behavioral testing.

Testing

The animals were allowed to habituate in the testing chambers for one hour prior to baseline. Animal weights were recorded and oral gavage was given post baseline. At 30 minutes post oral gavage, dura injections were given. Testing of both facial and hind paw allodynia was conducted every hour for 5 hours using calibrated Von Frey filaments. Upon completion of allodynia testing, all animals' cannulas patency was verified by ink injection into the cannula.

Solution Preparation

The TRPM8 antagonist AMG1161, previously published as Compound 45[28],(10 mg/mL) was dissolved in 2.5% methylcellulose diluted from 5% stock and was kept at room temperature prior to oral gavage. Icilin (Cayman Chemical) was prepared at a concentration of 1 nmol in Polyethylene glycol- 300 (PEG 300). 10 μL of the 1 nmol solution was injected at approximately 2 μL per second. Sumatriptan succinate (Amgen) was dissolved into saline and a dose of 0.6 mg/kg was administered via sub-cutaneous injection, as previously described [27,29]. The non-selective NOS inhibitor L-NG-Nitroarginine Methyl Ester (L-NAME) (Cayman Chemical) was given at 20 mg/kg via intraperitoneal injection.

In Vitro TRPM8 Functional Assay

Recombinant rat TRPM8 plasmid DNA was stably transfected into Chinese hamster ovary (CHO) cell lines using a tetracycline-inducible T-REx™ expression plasmid from Invitrogen, Inc. (Carlsbad, CA). To enable a luminescence readout based on intracellular calcium increase[30], the cell lines were co-transfected with a pcDNA3.1 plasmid containing jellyfish aequorin cDNA. The cells were maintained in Ham's F-12 nutrient media containing tetracycline-free fetal bovine serum, glutamine-penicillin-streptomycin, genetecin, blasticidin-S-HCl, and zeocin. Twenty four hours before assay, the cells were induced with 0.5 μg/mL tetracycline in Ham's F-12 for TRPM8 expression and plated at a density of 3.0×10^4 per well, in 96-well black plates with clear bottoms and grown at 37°C in a humidified atmosphere of 5% CO_2. On the day of assay, culture media was removed and cells were incubated for 2 hrs at 37°C with assay buffer (Ham's

F-12 containing 30 mM HEPES) containing 15 µM Coelenterazine (stock prepared in ethanol). Stock solution of AMG1161 was prepared in 100% DMSO and diluted to required final concentrations (0.2 nM to 20 µM) in assay buffer, limiting final concentration of DMSO to < 0.5%. TRPM8 antagonist AMG1161 or a positive control (AMG0762) was added 2.5 min prior to the addition of agonist (1 µM icilin) or 1 min prior to the addition of cold buffer (10°C) [31,32]. Luminescence was measured on a charge-coupled device camera-based FLASH-luminometer built by Amgen, Inc. A cooling device attached to the FLASH luminometer was used for cold (10°C) activation of TRPM8. A TRPM8 antagonist control (AMG0762) at a final concentration of 1 µM was considered zero percent control for cold activation. Compound activity was calculated using GraphPad Prism, version 5.03 (GraphPad Software Inc, San Diego, CA) or Genedata Screener (San Francisco, CA).

Data analysis

All data are graphed as means ± SEM. Allodynia studies were analyzed among groups and across time by 2-factor analysis of variance (ANOVA) for treatment and time. Data were also converted to area over the time-effect curve to allow for analysis of multiple treatment groups and analyzed with a 1-factor ANOVA and Bonferroni posttest. Statistics were calculated using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Significance was set at $P < .05$ for all data analysis.

Results

Dural application of 1 nmol icilin produced robust facial (Figure 2.1A) and hindpaw (Figure 2.1B) allodynia that peaked 3-4 hours later. This allodynia was dose dependent as

shown in the time courses and by area-over-curve plots in Figure 2.1C and 2.1D. Allodynia was not observed in response to dural application of vehicle (PEG 300). Animals treated with 1nmol icilin displayed facial withdrawal thresholds significantly different from controls from 2 hours- 5 hours, thresholds were trending towards baseline at 5 hours, and had completely returned to baseline by 24 hours. Animals treated with 100 pmol or 10 pmol icilin did not produce facial or hindpaw responses significantly different than controls.

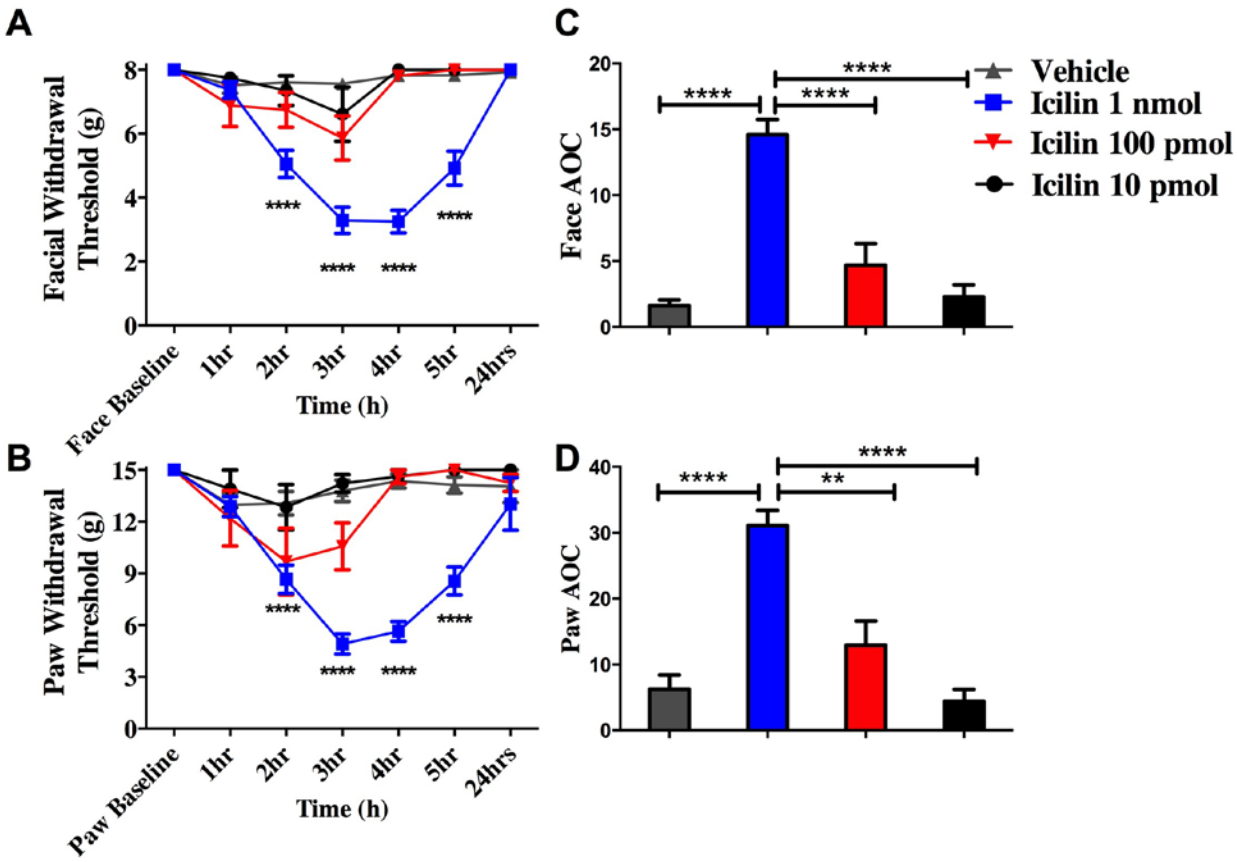

Figure 2.1. Activation of meningeal TRPM8 produces headache-related behaviors.

Dural application of 1 nmol icilin induced cutaneous facial and hindpaw allodynia. Withdrawal thresholds to tactile stimuli applied to the face (a) and the hind paws (b) were measured in rats prior to and after dural application of 1 nmol icilin (N = 39 at time points 1–5 hours, N = 8 at 24 hours), 100 pmol icilin (N = 8 at all timepoints), 10 pmol icilin (N = 8 at all timepoints) vehicle (PEG300) (N = 29 at time points 1–5 hour, N = 8 at 24 hours) For both facial and hind-paw responses, two-factor ANOVA indicated a significant effect of both treatment and time of both

the face and hind paws. This figure comprises all data run in this manuscript with these stimuli (icilin or vehicle). Withdrawal thresholds to tactile stimuli measured for five hours and data were converted to area over the time-effect curve (AOC) for face (c) and hind paw (d). A one-factor ANOVA with Bonferroni's post test revealed significantly more allodynia with 1 nmol icilin injection compared to both vehicle, 100 pmol icilin and 10 pmol icilin in both the face and hind paws. Facial: treatment $F(3, 508) = 26.38$, $P < 0.0001$, time $F(6, 508) = 9.764$, $P < 0.0001$; Hind paw: time $F(6, 508) = 9.543$, $P < 0.0001$, treatment $F(3, 508) = 31.13$, $P < 0.0001$.

Although icilin is commonly used as an activator of TRPM8, blockade of this channel with a selective antagonist would further support the conclusion that headache-like responses following dural icilin are mediated by TRPM8. First, an in vitro calcium influx assay was used to examine blockade of TRPM8 by the antagonist AMG1161. In cultured CHO cells stably transfected with the rat TRPM8 channel, AMG 1161 (Figure 2.2) displayed concentration-dependent inhibition of TRPM8 activation by icilin and cold with IC_{50} values of 23 ± 0.9 nM (n=6 independent experiments with 3 replicates for each concentration) and 11 ± 0.4 nM (n=2 independent experiments with 3 replicates for each concentration), respectively. In order to address selectivity, similar studies were performed against related TRP channels including TRPV1 (activated with capsaicin), TRPV3 (activated with 2-APB), TRPV4 (activated with 4-alpha PDD), TRPC5 (activated with cold temperature), and TRPA1 (activated with AITC). Corresponding IC_{50} values were >10 μm, >10 μm, >20 μm, >40 μm, and >40 μm, respectively (data not shown). These data indicate that AMG1161 has functional selectivity over similarly related TRP channels.

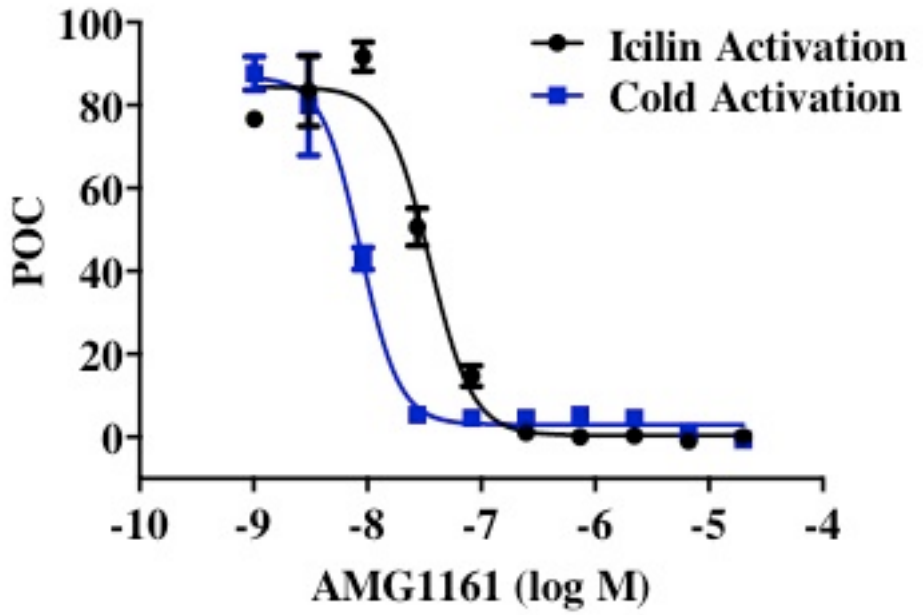

Figure 2.2. AMG1161 is a TRPM8 antagonist.

AMG 1161 showed a concentration-dependent inhibition of TRPM8 activation by icilin and cold in cultured CHO cells stably transfected with rat TRPM8 channel. IC_{50} values were 23 ± 0.9 nM (*n* = 6 independent experiments with three replicates for each concentration) and 11 ± 0.4 nM (*n* = 2 independent experiments with three replicates for each concentration) against icilin and cold, respectively and Y-axis is percent of control (POC).

In order to determine whether icilin produces its behavioral effects via activation of TRPM8, AMG1161 was given to rats prior to dural stimulation. Oral pretreatment with AMG1161 (10 mg/kg) 30 minutes prior to application of 1 nmol icilin on the dura prevented the reduction in facial and paw withdrawal thresholds due to icilin administration. There was a significant decrease in facial and hindpaw allodynia at the 3 hour and 4 hour time points compared to icilin alone and also for facial allodynia at the 5 hour time point (hindpaw allodynia was not significant at 5 hours) (Figure 2.3A and 2.3B). Oral pretreatment with vehicle or

AMG1161 had no effect on animals given vehicle on the dura (Figure 2.3A and 2.3B) indicating that AMG1161 alone had no effect on withdrawal thresholds.

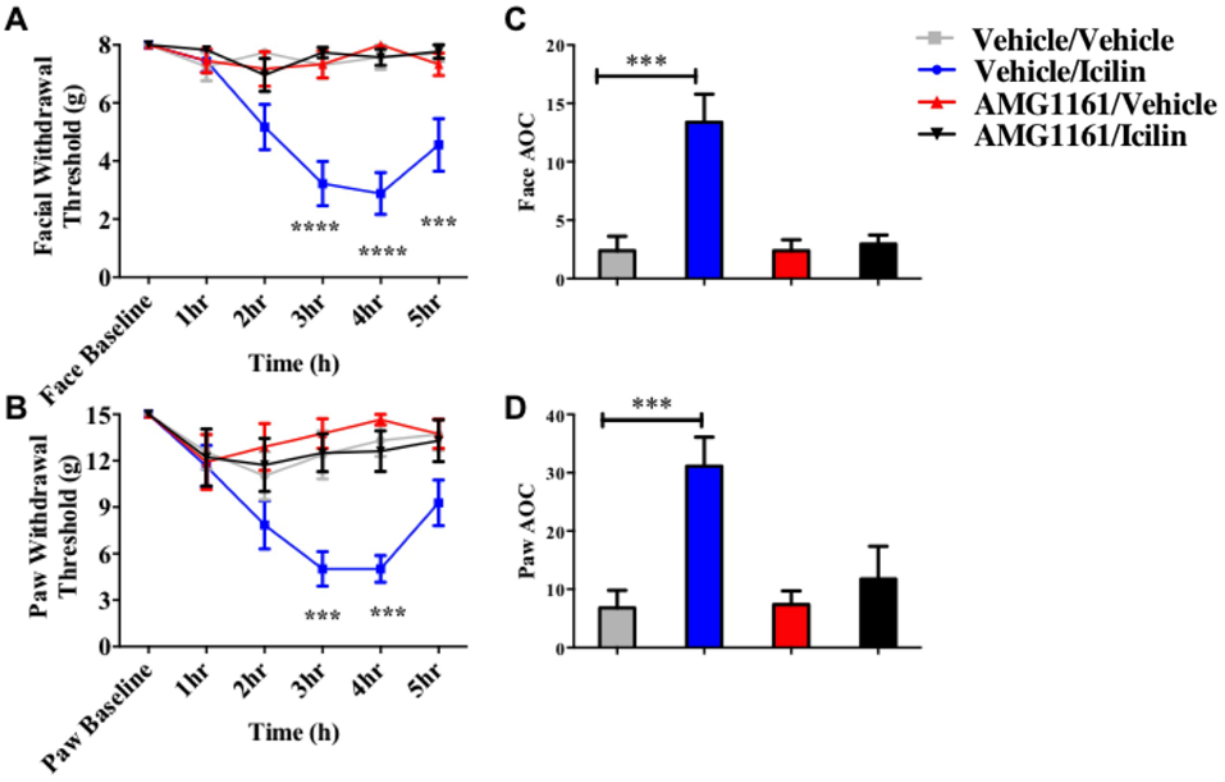

Figure 2.3. Headache-like responses due to dural icilin are prevented by a systemic TRPM8 antagonist.

Pretreatment with AMG1161 (10 mg/kg) 30 min prior to application of 1 nmol icilin attenuated cutaneous allodynia. Withdrawal thresholds to tactile stimuli applied to the face (a) and the hind paws (b) were measured in rats prior with AMG1161 or vehicle then given dural application of 1 nmol icilin. AMG1161/vehicle (N = 9), AMG1161/icilin (N = 9), vehicle/vehicle (N = 11) or vehicle/icilin (PEG300) (N = 14) (for both facial and hind-paw responses, two-factor ANOVA indicated a significant effect of both treatment and time of both the face and hind paws). Facial: treatment $F(3, 234) = 31.07$, $P < 0.0001$, time $F(5, 234) = 5.621$, $P < 0.0001$; Hind paw: time $F(5, 138) = 8.023$, $F(5, 234) = 6.168$, $P < 0.0001$, treatment $F(3, 234) = 17.63$, $P < 0.0001$.

Sumatriptan (as well as other triptans) is considered to be a standard first-line abortive agent in the treatment of human migraine patients but is not given for other forms of pain[33].

Prior animal studies have used sumatriptan as a probe to determine whether behavioral responses are headache-like[27,29]. Thus, efficacy of sumatriptan against icilin-induced behavioral responses was examined to determine whether these behaviors are also consistent with headache. Simultaneous treatment of rats with sumatriptan (0.6 mg/kg, s.c.) and dural icilin led to partial attenuation of the decrease in both facial and paw withdrawal thresholds observed with icilin alone (Figure 2.4). Sumatriptan treatment did not cause any changes in withdrawal thresholds in animals given vehicle (PEG-300) onto the dura (Figure 2.4).

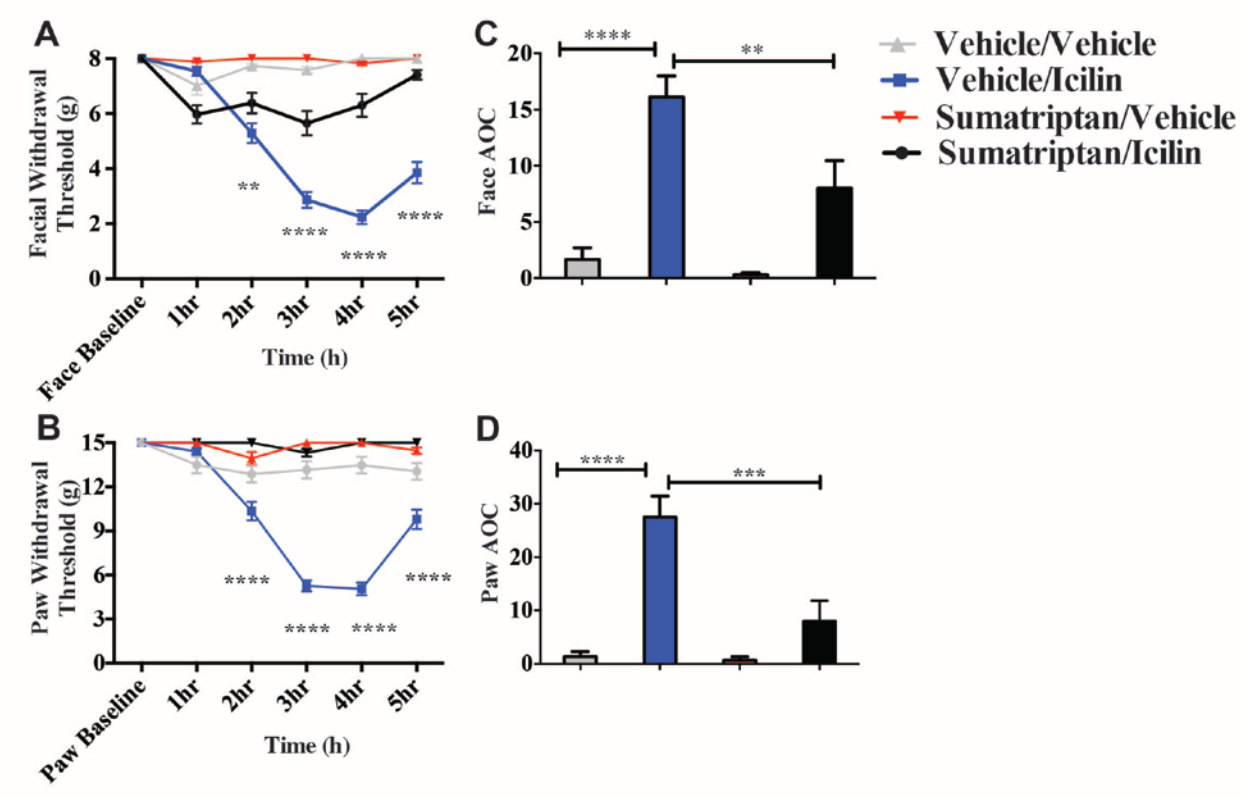

Figure 2.4. Headache-like responses following dural icilin are sensitive to the migraine abortive agent sumatriptan.

Dural application of 1 nmol icilin induced cutaneous allodynia that is prevented by simultaneous treatment of rats with sumatriptan (0.6 mg/kg, s.c.). Withdrawal thresholds to tactile stimuli applied to the face (a) and the hind paws (b) were measured in rats prior to and after dural application of sumatriptan/vehicle (N = 7), sumatriptan/icilin (*N* = 7), vehicle/vehicle (*N* = 6) or

vehicle/icilin (PEG300) (N = 8) Withdrawal thresholds to tactile stimuli measured for 5 hours and data were converted to area over the time-effect curve (AOC) for face (c) and hind paw (d). A one-factor ANOVA with Bonferroni's post test revealed significantly more allodynia with vehicle injection followed by icilin compared to sumatriptan followed by icilin in both the face and hind paws. Facial: treatment $F(15, 144) = 27.38$, $P < 0.0001$, time $F(5, 144) = 37.30$, $P < 0.0001$; Hind paw: time $F(15, 144) = 29.90$, $P < 0.0001$, treatment $F(5, 144) = 41.71$, $P < 0.0001$.

NOS inhibitors have also been found to be efficacious in humans with migraine as pharmacological blockade of NOS with the non-selective inhibitor L-NMMA significantly reduced migraine pain in a small clinical trial[34,35]. Similar to the experiments described above with sumatriptan, we also assessed whether the icilin-induced behaviors in this preclinical model were sensitive to NOS inhibitors. Animals were pretreated with another non-selective NOS inhibitor L-NAME (20 mg/kg, s.c.) or vehicle 15 minutes prior to icilin administration (Figure 2.5 A&B). Animals that received L-NAME/icilin displayed significantly higher thresholds (i.e., reduced allodynia) both in the face and hindpaw compared to those of the vehicle/icilin group. No significant differences were found between the L-NAME/icilin group and vehicle groups. Together with the efficacy of sumatriptan, these data indicate that the behavioral response in rats due to dural TRPM8 activation is consistent with headache.

Discussion

Migraine is a debilitating disorder that affects a population of otherwise healthy individuals and for which current treatments are often ineffective. Despite the prevalence of migraine, the underlying pathophysiology is not well understood and thus identification of novel targets for migraine therapies is challenging. Although pain signaling from the meninges has been proposed to play a role in the development of the pain of migraine, little is known about the mechanisms by which afferents are activated

during a migraine event. We and others have described numerous mechanisms capable of initiating nociceptive signaling from the meninges in animals [26,27] [36] but human data supporting a role for many of these mechanisms is largely absent. In contrast, human data implicating TRPM8 in migraine has been reported in several recent GWAS of migraine patients[21-25,37] but preclinical data supporting a role for TRPM8 in migraine does not yet exist. Using a preclinical model of headache, we now demonstrate that stimulation of the dura with the TRPM8 agonist icilin evokes cutaneous allodynia, a common feature of migraine.

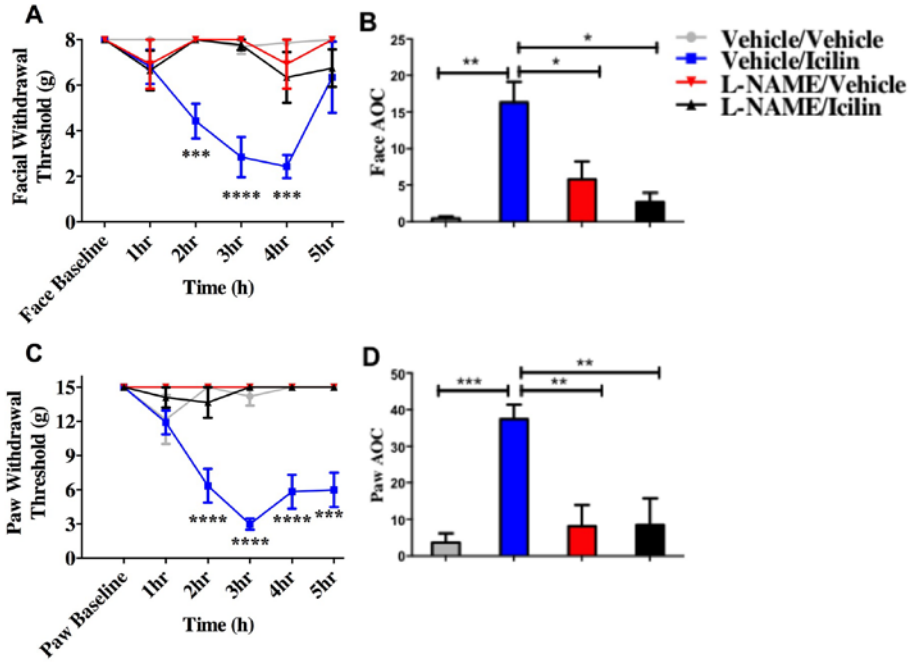

Figure 2.5. Headache-like responses following dural icilin are sensitive to NOS inhibition.

Administration of L-NAME (20 mg/kg) i.p.15 min prior to dural icilin (1 nmol) prevented cutaneous allodynia. Withdrawal thresholds to tactile stimuli applied to the face (a) and the hind paws (b) were measured in rats L-NAME/vehicle (*N* = 7), L-NAME/icilin (*N* = 9),

vehicle/vehicle (N = 4) or vehicle/icilin (PEG300) (N = 4). Withdrawal thresholds to tactile stimuli measured for five hours and data were converted to area over the time-effect curve (AOC) for face (c) and hind paw (d). A one-factor ANOVA with Bonferroni's post test revealed significantly more allodynia with vehicle pretreatment followed by icilin compared to L-NAME followed by icilin in both the face and hind paws. Facial: treatment $F(5, 120) = 3.107$, $P = 0.0113$, time $F(3, 120) = 16.85$, $P < 0.0001$; Hind paw: time $F(15, 78) = 5.172$, $P < 0.0001$, treatment $F(5, 120) = 4.008$, $P = 0.0021$.

Previous preclinical work has shown cutaneous allodynia after dural afferent activation with numerous compounds[38-40] and thus these data are consistent with other pro-nociceptive stimuli applied to the dura. An interesting observation from these studies is the development of mechanical allodynia following TRPM8 activation, a type of hypersensitivity not typically observed after activation of this channel (cold allodynia is usually the phenotype associated with TRPM8). However, the current studies are not testing the primary site of activation (i.e., the dura mater) and are dependent on referred allodynia of the facial and hindpaw regions. It is thus not known whether thermal thresholds are altered in the dura mater where TRPM8 is activated. Allodynia was nonetheless attenuated by pretreatment with the TRPM8 antagonist AMG1161, indicating this affect was due to specific activation of TRPM8 in the dura. Additionally, both sumatriptan and L-NAME attenuated the cutaneous facial and hindpaw allodynia due to dural application of icilin. This is presumably due to attenuation of afferent input from the meninges and inhibition of subsequent central sensitization necessary to establish referred facial and hindpaw allodynia[27], although our data do not prove this mechanism. The ability of sumatriptan and L-NAME to attenuate icilin-induced allodynia in this model suggests that the behavioral responses due to activation of TRPM8 within the dura are consistent with headache. Triptans are one of the most common abortive agents for

migraine and NOS inhibitors have also been shown to be efficacious in inhibiting migraine pain in humans[34,35]. Taken together, these data suggest that activation of TRPM8 on meningeal afferents contributes to headache.

Although several GWASs now exist implicating SNPs in TRPM8 in migraine, the actual effects of the mutations on channel expression and function are not yet known. Consequently, it is difficult to determine whether patients with these mutations have increased or decreased TRPM8 function. Activation of TRPM8 on sensory neurons can initiate afferent signaling but interestingly in the case of this channel, the sensation that is ultimately perceived may be pro-nociceptive or anti-nociceptive possibly depending on the context. In animals, TRPM8 is essential for both neural and behavioral responses to noxious cold, as well as cold mimetics.[41] And deletion or antagonism of TRPM8 reduces inflammation-induced cold hypersensitivity [42-44] indicating that this channel is necessary for cold allodynia in pain states. But TRPM8 can also produce analgesia in states of inflammation [8,45] and menthol is widely used as a topical analgesic. Ultimately, it may depend on whether TRPM8 is activated alone or in the presence of other stimuli. In support of this concept, it was recently shown that while activation of TRPM8 alone is nociceptive, TRPM8 activation can decrease nociception due to stimulation of other TRP channels.[46] Our data demonstrate that activation of TRPM8 alone in the meninges is pro-nociceptive but whether TRPM8 is activated alone during migraine in humans is not clear. Future studies will ideally determine how migraine-associated TRPM8 SNPs impact channel function. These studies will also shed light on whether patients are more likely to have increased or decreased channel expression and function and will help

further uncover how TRPM8 may contribute to migraine (i.e., in a protective or causative).

It should be noted that prior studies on TRPM8 expression in meningeal afferents found little to no channel expression on these neurons[47]. This is seemingly at odds with our current findings that activation of TRPM8 in the dura is pro-nociceptive. Differences in species could account for the discrepancy in findings as our studies were performed in rats while the prior work was in mice and expression of TRPM8 on dural afferents may be species dependent. Additionally, other studies using mice have found TRPM8 expression in the meninges but found that the expression is restricted to specific regions[48]. TRPM8 may thus be expressed in the meninges in mice, but not equally across the tissue. Another possibility is that TRPM8 is not expressed on meningeal afferents but is found on other cell types in the dura. Mast cells have been implicated in migraine [49] and TRPM8 is expressed on this cell type[50]. Additionally, we recently reported that dural fibroblasts may also contribute to headache pathology[51] and TRPM8 is expressed on several types of fibroblasts[52]. Thus, activation of the channel on non-neuronal cells could indirectly initiate afferent signaling. Future work is necessary to more conclusively determine the expression pattern of the channel in the meninges and how its activation may ultimately contribute to headache.

These studies provide the first preclinical evidence that TRPM8 can play a role in headache disorders such as migraine and they provide a potential mechanism for how the channel may contribute to migraine pain i.e. activation within the meninges and subsequent initiation of afferent signaling. Although it still remains to be determined

how TRPM8 is impacted by the mutations in human migraine patients, these data suggest that meningeal TRPM8 is capable of contributing to headache. Importantly, these findings imply that TRPM8 antagonists may have efficacy as novel migraine therapeutics.

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CHAPTER 3

CENTRAL BDNF PRIMES THE DURAL AFFERENT SYSTEM TO SUBTHRESHOLD STIMULI INCLUDING A COMMON MIGRAINE TRIGGER

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Abstract

Migraine is an episodic disorder where, between attacks, patients are otherwise normal but are sensitized to a variety of non-noxious events known as triggers. The purpose of these studies was to investigate mechanisms that may lead to sensitivity to subthreshold events. A rat behavioral model of migraine was used to test whether stimulation of the dura mater with interleukin-6 (IL-6) can prime the afferent system to later dural stimulation and to a nitric oxide donor. Animals were tested for cutaneous facial and hindpaw allodynia. Intracisternal injections of either brain-derived neurotrophic factor (BDNF) or BDNF sequestering agents were given to investigate a role for this neuropeptide in priming at central sites. Dural IL-6 leads to cutaneous allodynia and following resolution of this response animals are primed to dural stimulation with pH 6.8 or pH 7.0 and to a systemic NO donor. Systemic or intracisternal delivery of BDNF signaling inhibitors attenuated this primed response. Intracisternal administration of BDNF without prior dural stimulation also produced cutaneous allodynia. Following resolution of BDNF-induced allodynia, animals were primed to dural pH 6.8/7.0 and an NO donor. These data indicate that afferent input from the meninges primes the dural nociceptive system to later subthreshold events and that the primed state is dependent on central BDNF. This primed state mimics the interictal period of migraine where attacks can be triggered by non-noxious events. Understanding mechanisms by which BDNF-induced plasticity occurs within the nervous system may lead to development of new therapeutic targets for migraine.

Introduction

Migraine headache is an episodic pain condition that is the 3rd most common disease worldwide [1]. Despite this prevalence, the pathophysiology contributing to

migraine is poorly understood. Biomarkers for migraine do not yet exist and few changes can be found in patients that potentially explain the disorder. Although structural changes in the brain have been identified [2], it is not clear whether these changes contribute to migraine or whether they are a consequence of migraine. Migraine is often triggered by certain stimuli such as stress, skipping meals, foods/scents, excess/inadequate sleep, and changes in hormone levels [3]. However, these same stimuli are innocuous in non-migraineurs. This suggests that changes in the nervous system of migraine patients enhances their susceptibility to triggers of migraine. The location(s) and mechanism(s) of these changes are not well understood.

The frequency of migraine attacks is variable and can increase over time. Episodic migraine (defined as 0-14 migraine days/month) can progress to chronic migraine (15 or more days/month) at a rate of 2.5% per year [4], although a transition from chronic to episodic migraine can also occur [5], and higher frequency is associated with higher risk of progression [6]. Another risk factor for progression is adequacy of treatment of acute migraines. When maximum treatment efficacy of acute attacks was achieved, the progression to chronic migraine was 1.9% while the progression increased to 2.7%, 4.4%, and 6.8% with moderate, poor, and very poor treatment efficacy, respectively [7]. These data indicate that migraine attacks and the severity of those attacks can increase the likelihood of future migraines. Although mechanisms contributing to progression of migraine are not known, plasticity within the nervous system due to events occurring during attacks may play a role [8].

Several animal behavioral models have been developed that allow investigation of potential mechanisms contributing to headache disorders. Acute stimulation of the dura mater of rats produces behavioral responses consistent with headache [9]. Repetitive stimulation of the dura mater promotes increased responses over time and animals eventually transition into a state of chronic cutaneous allodynia [10] that is dependent on neuronal sensitization in the trigeminal nucleus caudalis (TNC) [11]. Repetitive administration of sumatriptan to rats [12] or nitric oxide donors to mice [13] also increases responses to these stimuli over time and sensitizes these animals to migraine triggers, even well after drug administration has ceased. Although these behavioral models cause sensitization to migraine triggers and mimic migraine progression, the underlying mechanisms have not been fully uncovered.

Previously, we showed that interleukin-6 (IL-6) administration to the rat dura produced cutaneous allodynia for at least 24 hours [14]. IL-6 administration to other tissues produces a state of hyperalgesic priming where, after resolution of IL-6 allodynia, animals display enhanced responses to non-noxious or mildly noxious stimuli for extended periods of time [15]. The purpose of the current studies was to determine whether meningeal IL-6 administration primes the dural afferent system to later subthreshold stimuli and to investigate the potential mechanisms by which this state of sensitization is mediated.

Methods

Animals

Adult male and female Sprague-Dawley rats (250–300g, Harlan) were kept in a temperature-controlled room on 12-hr light/dark cycle with food and water ad libitum.

All procedures were performed in accordance with the policies of the IASP and the NIH guidelines for use of laboratory animals. The Institutional Animal Care and Use Committee (IACUC) of both the University of Arizona and The University of Texas at Dallas approved all procedures.

Surgeries

As previously described [4,5], dura cannulation surgeries were performed on rats (250-300) grams. Animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg) and an incision exposing the skull was made to the top of the skull. Once the skull was exposed, a 1-mm hole was made in the skull to expose the dura (1mm left of midline, 1mm anterior to lambda). A 1mm guide cannula (Plastics One) was then inserted into the hole and secured with Vetbond™ (3M™). Two screws (Small Parts) were placed rostral to the cannula biparietal to sagittal suture and posterior to bregma. Dental acrylic was used to secure the cannula to both the screws and the skull. A dummy cannula (Plastics One) was placed into the cannula to ensure patency. Animals received gentamicin (8 mg/kg) to minimize infection following surgery. Rats were housed separately and allowed 6–8 days for recovery prior to behavioral testing.

Behavioral Testing

The animals were habituated in the testing chambers for a minimum of one hour prior to baseline. Animal weights were recorded and dural injections were given post baseline. Testing of both facial and hind paw allodynia was conducted every hour for 5 hours using calibrated Von Frey filaments thresholds were determined by the “up-down” method[6]. The Von Frey filaments were applied to the peri-orbital region of the face or to

the plantar surface of the hind paw perpendicularly until the entire force was applied, and held for approximately 5 seconds or until animals withdrew. Maximum filaments used were 8 g for the peri-orbital region and 15 g for the hindpaw. Upon completion of allodynia testing, all animals' cannula patency was verified by ink injection into the cannula and dissection post mortem. The experimenter collecting measurements was blinded to the experimental conditions.

Intracisternal Administration

Curved 25 gauge needles 1.5 inches long (BD Needles) were bent at approximately 40° to no more than 45° at a distance 7 mm from the bevel of the tip. These needles were then used, 1 per animal, while attached to a 25 μL micro-syringe (Hamilton Syringes). Intracisternal injections were performed under light isoflurane anesthesia administered via nose cone from a vaporizer. Animals were anesthetized for <2 min. Animal heads were tilted forward to allow for exposure of the cisterna magna in order to perform injections. The curved needle was then positioned at the back of the neck just below the occiput and above the C1 vertebrae. The needle was inserted through the cisterna magna at the midline. Central placement of the needle was verified by drawing cerebrospinal fluid back into the needle prior to infusion of a given solution. Injections of 10 μL volume are made over a 10 sec period after which the needle is removed. Animals recover from anesthesia for 5 min before being returned to cages or testing chambers[16].

Rotarod

Animals were tested for motor impairments following intracisternal injection by their ability to balance and walk on a slowly rotating rod (Rotamex 4/8, 6.5 cm diameter for rats, respectively; rate of rotation, 10 revolutions per min, Columbus Instruments, Columbus, Ohio). Animals were trained for three consecutive days prior to challenge with a maximal cut-off time of 180 seconds [8]. Training consisted of a habituation to the rod while stationary for 180 seconds, as well as while rotating for a maximum of 180 seconds. Animals unable to stay on the rod for the full training period were excluded. On the fourth day the length of time that an animals were able to stay on the rotating rod during a 10 minute period was recorded as a baseline. Compounds were then administered via intracisternal injections and rats were assessed for time spent on the rod at peak behavioral response times (for allodynia) for the compound (3 hours post injection). Vehicle-treated rats were also tested for time spent on the rod.

Solution Preparation

Rat recombinant IL-6 (R&D Systems) stock solution (10 μg/ml) was prepared in sterile 0.1% bovine serum albumin (BSA) and diluted to final concentrations of 10 ng/ml in synthetic interstitial fluid (SIF) (pH 7.4, 310 Osmolality). Human recombinant BDNF and TrkB/Fc (R&D Systems) were made into stock solutions (50ng/ml & 25ug/ml respectively). BDNF stock solution was made in sterile phosphate buffered saline (PBS) containing 0.1% BSA and TrkB/Fc stock solution was made in sterile PBS. Both BDNF and TrkB/Fc were dissolved in artificial cerebrospinal fluid (aCSF) for intracisternal administration. Vehicle control was aCSF. ANA-12 (Maybridge) was diluted to final

concentration of .5 mg/kg in 10% polyethyl glycol 300 (PEG 300) and administered via intraperitoneal injection. APETx2 (Alomone Labs) was diluted in SIF solution at pH 6.8 to a final concentration of 20 μM/mL.

Data analysis

All behavioral data are graphed as means ± SEM. Statistical evaluations of allodynia studies were conducted using GraphPad Prism Version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Data was analyzed among groups and across time by one- or two-way analysis of variance (ANOVA) for treatment and time followed by Bonferroni post-test where appropriate. Data were also converted to area over the time-effect curve to allow for analysis of multiple treatment groups and analyzed with a 1-factor ANOVA and Bonferroni post-test. In experiments where differences in allodynia at the 72-hour time point and 3 hours following this time point are being compared, t-tests were used. Significance was set at $P < 0.05$ for all data analysis.

Results

We have shown previously that application of IL-6 to the rat dura mater produced cutaneous facial and hindpaw allodynia that remained significantly different from baseline at 24 hours post injection [14]. IL-6 was applied in the current studies to determine the duration of the allodynic response. Dural application of IL-6 produced both facial and hindpaw allodynia that resolved by 48 hours post application (Figure 3.1) and remained at baseline at 72 hours.

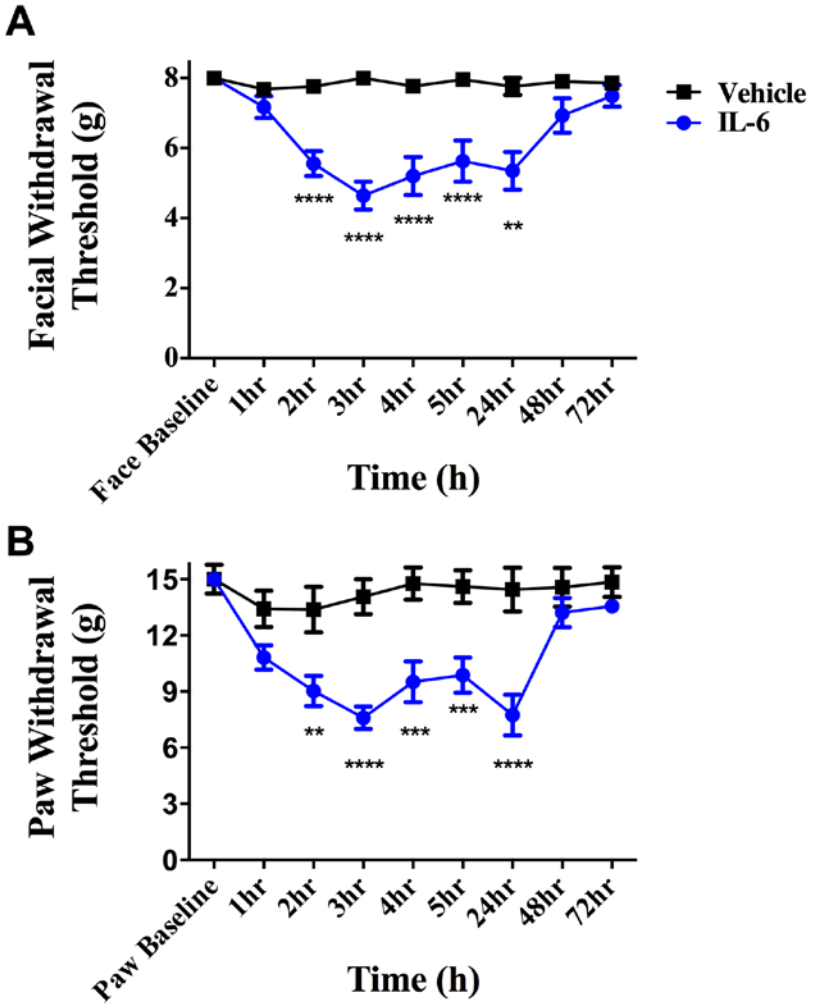

Figure 3.1. Experimental design and met Dural application of IL-6 produces headache-related behaviors.

Withdrawal thresholds to tactile stimuli applied to the face (A) and hindpaws (B) were measured in animals prior to and after dural application of .1 ng IL-6 (n = 39) or Vehicle(pH 7.4) (n = 28). Administration of IL-6 produced significant allodynia that lasted 24 hours in both face and hindpaw. Two-factor analysis of variance (ANOVA) indicated a significant effect of both treatment and time of both the face and hindpaws. Significant differences among means for each group were determined by analysis of variance followed by Bonferroni post hoc test. (A) Facial: time $F(8, 418) = 5.029$, $P < 0.0001$, treatment $F(1, 418) = 49.79$, $P < 0.0001$; (B) Hindpaw: time $F(8, 347) = 7.818$, $P < 0.0001$, treatment $F(1, 347) = 81.33$, $P < 0.0001$.

At the 72-hour time point, when animals had remained at baseline for 24 hours, they were tested for responses to pH 6.8 and pH 7.0 application to the dura mater. Previously, pH 6.8 was shown to not produce significant facial and hindpaw allodynia [17] indicating that this pH value is subthreshold in normal animals. At 72 hours following IL-6, stimulation of the dura with the normally subthreshold pH values of pH 6.8 or pH 7.0 produced robust facial and hindpaw allodynia at 3 hours post administration (Figure 3.2). Only animals that had previously received IL-6, but not vehicle-treated animals (vehicle is pH 7.4 SIF), responded to the subsequent low pH stimulus. Higher pH values (pH 7.2) did not produce significant facial allodynia in either IL-6 or vehicle-treated rats (Figure 3.2C). In order to determine whether acid-sensing ion channels (ASICs) contribute to the behavioral response to low pH after sensitization (as we have shown previously in the absence of sensitization [17-18]), the ASIC3 blocker APETx2 was given with pH 6.8 at the 72-hour time point following IL-6. In the presence of APETx2, pH 6.8 did not produce significant facial allodynia indicating that ASICs still detect the decrease in pH even after sensitization with IL-6. Additionally, stimulation of the dura with pH 7.4 at the 72-hour time point following IL-6 did not produce facial allodynia, indicating that the animals are not simply responding to the pressure of the injection but that they are responding to decreased pH.

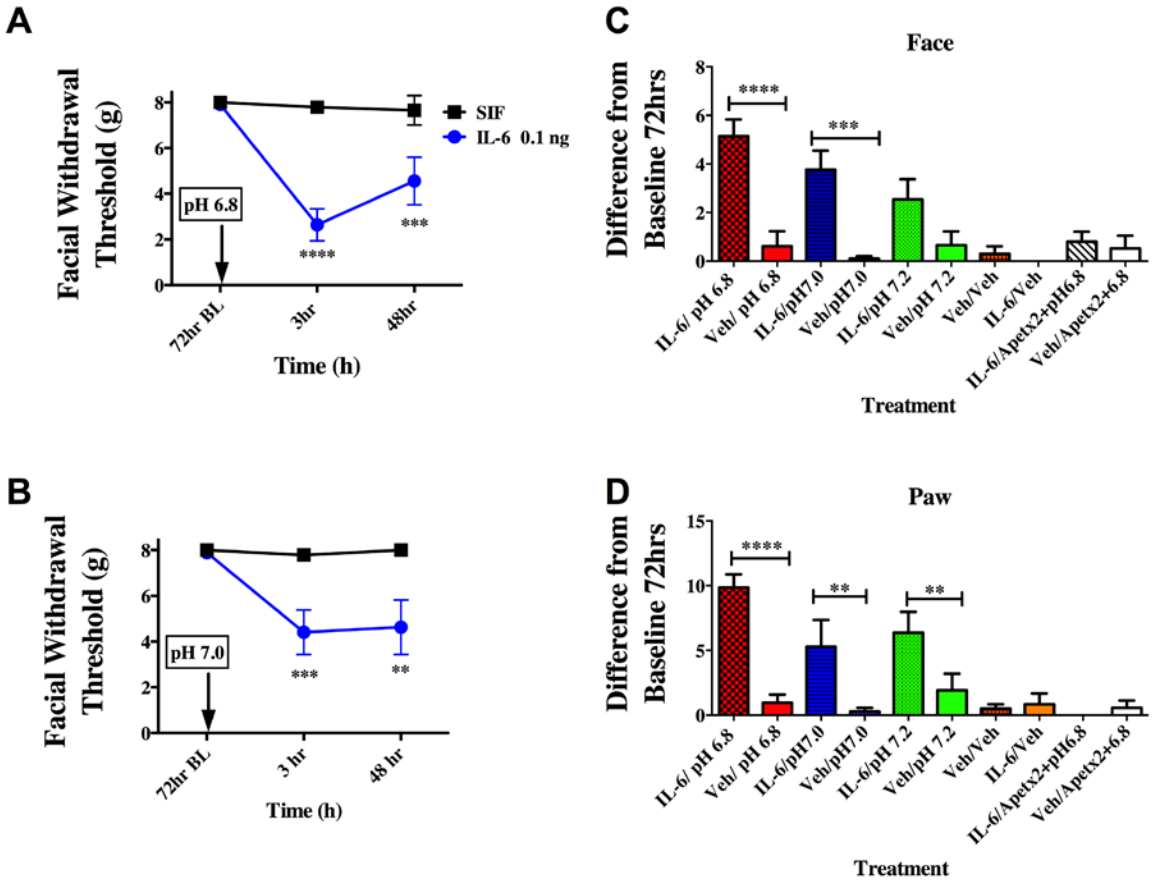

Figure 3.2. Dural application of IL-6 primes animals to sub-threshold stimuli.

Animals that received a prior application of IL-6 exhibit cutaneous allodynia when dural pH 6.8 (A, n=9 for all groups) or pH 7.0 (B, n=10 for all groups) is applied after the resolution of allodynia at 72 hours. Withdrawal thresholds to tactile stimuli applied to the face (A & B) were measured in animals after application of dural pH 6.8 or pH 7.0 72 hours post IL-6. Significant differences among means for each group were determined by analysis of variance followed by Bonferroni post hoc test. Increasing the pH value to pH 7.2 did not produce significant facial allodynia in either IL-6 or vehicle-treated rats (C). ASIC3 blocker APETx2 was able to attenuate facial (C) and hindpaw (D) allodynia when administered with pH 6.8 at 72 hours post IL-6. Stimulation of the dura with Vehicle at 72 hours post IL-6 did not produce facial allodynia (C). Significant (**p < 0.01) differences among change of means at 72 hours for each group were determined using a t-test (C & D). (A) Facial: time $F(2, 36) = 26.53$, $P < 0.0001$, treatment $F(1, 87) = 0.0046$, $P = 0.0001$; (B) Facial: time $F(2, 36) = 5.112$, $P = 0.0111$, treatment $F(1, 36) = 19.70$, $P < 0.0001$.

In order to determine whether IL-6 primes the dural afferent system only to subthreshold pH stimulation or whether they are sensitive to other stimuli, primed rats were given sodium nitroprusside (SNP, 3 mg/kg) at 72 hours following IL-6. NO donors are consistent migraine triggers in humans susceptible to migraine, but they do not trigger attacks in non-migraine patients [19], suggesting sensitivity to these stimuli contributes to the pathophysiology of the disorder. This dose of SNP was chosen as it has been shown to be below threshold in normal rats [12]. In rats treated with dural IL-6, systemic SNP produced allodynia when given 72-hours later while animals treated with dural vehicle did not respond to SNP (Figure 3.3). These data demonstrate that dural stimulation with IL-6 produces changes in the nervous system that prime animals to normally subthreshold stimuli including common triggers of migraine.

Prior studies have shown that IL-6 injection into the mouse hindpaw primes animals to a subsequent injection of subthreshold prostaglandin E_2 (PGE_2) and that the primed state is dependent on brain-derived neurotrophic factor (BDNF) in the spinal cord [15a]. In order to test whether priming in the context of headache is dependent on BDNF, rats were treated systemically with ANA-12, previously characterized as a selective and CNS-penetrant antagonist of the BDNF receptor TrkB [20]. Rats were given i.p. injections of ANA-12 (0.5 mg/kg) 24 hours following IL-6 administration on the dura (Figure 3.4). Systemic administration of ANA-12 blocked both the facial and hindpaw response to dural pH 7.0 at the 72-hour time point. These data implicate BDNF in the development and/or maintenance of the primed state but do not provide information regarding the location of BDNF signaling necessary for priming.

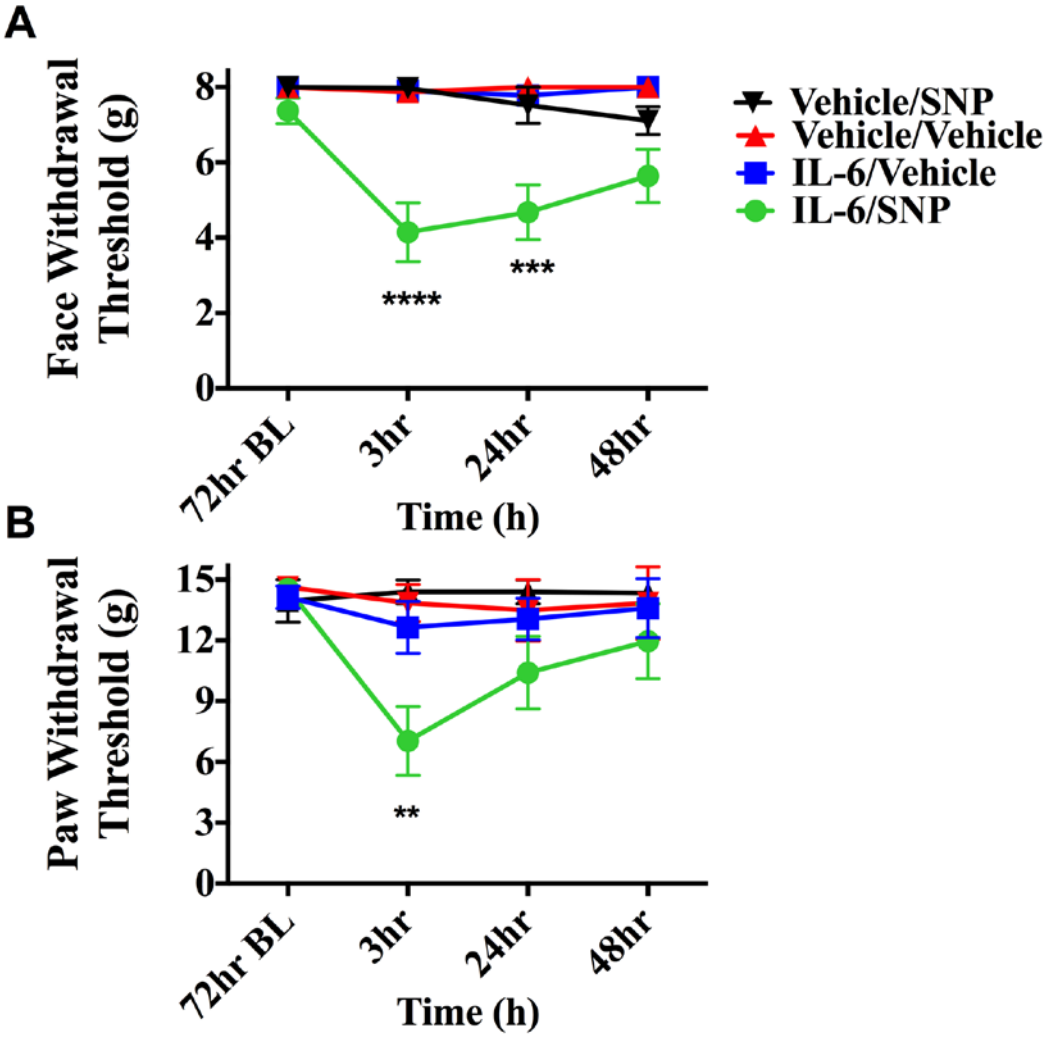

Figure 3.3. Dural application of IL-6 primes animals to systemic NO donors.

Animals treated with meningeal IL-6 (n=8 IL-6/SNP 3mg/kg , n=7 IL-6/Vehicle) respond to systemic SNP; producing both facial (A) and hindpaw (B) allodynia at 72 hours post IL-6 while animals treated with Vehicle (n=5 for all groups)did not respond to SNP. Withdrawal thresholds to tactile stimuli applied to the face (A) and the hindpaw (B) after systemic SNP given 72 hours post IL-6. Significant differences among means for each group were determined by analysis of variance followed by Bonferroni post hoc test. Facial: time $F(3, 88) = 1.578$, $P = 0.2004$, treatment $F(3, 88) = 9.588$, $P < 0.0001$; Hind paw: time $F(3, 80) = 1.172$, $P = 0.3256$ treatment $F(3, 80) = 20.31$, $P < 0.0001$.

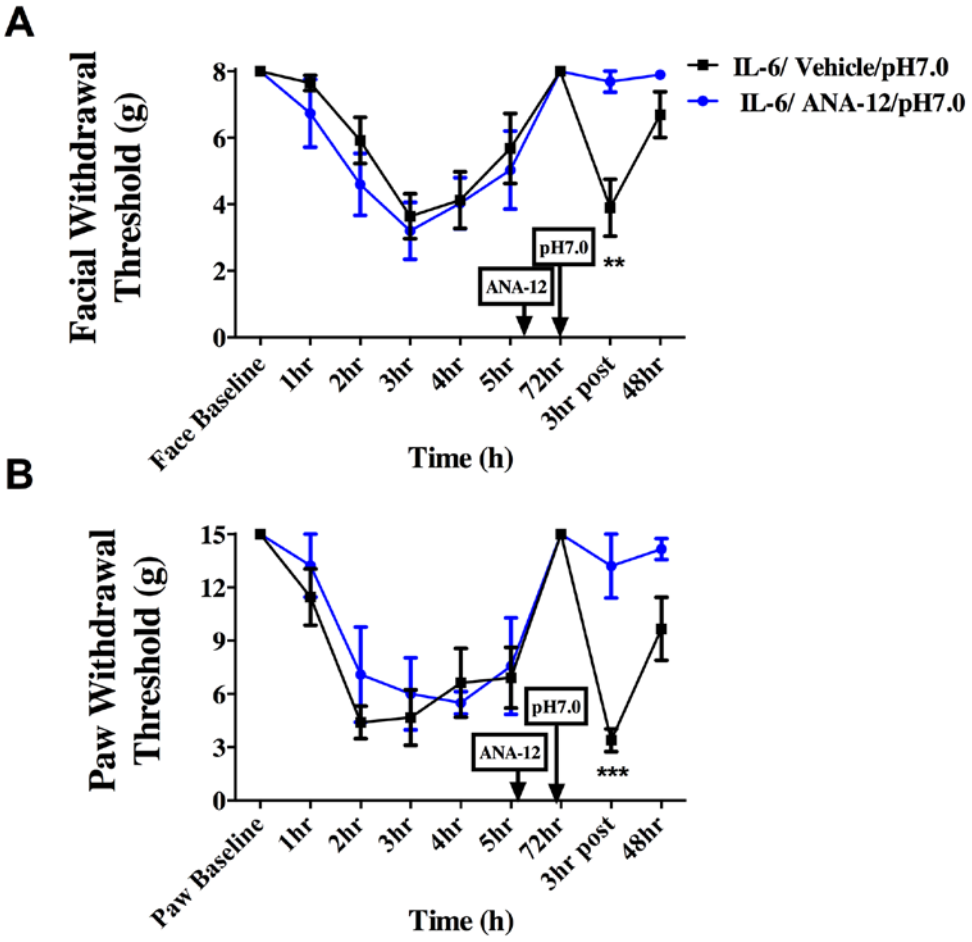

Figure 3.4. Priming following dural IL-6 stimulation is dependent on BDNF signaling in the brainstem.

TrkB-Fc was given i.c. 24 hours after dural application of IL-6. Only animals given i.c. vehicle (males n=12 A & females n=7 B) respond to dural pH 7.0 at 72 hours post IL6. Males treated with IL-6/i.c vehicle/ dural pH 7.0 showed significant facial and hindpaw responses at 3 hours and 24 hours. However, females treated with IL-6/i.c vehicle/dural pH 7.0 had significant facial responses at 3 hours and hindpaw responses at 3 hours and 24 hours. Both male(n=8 A) and female (n=7 B) animals treated with i.c. TrkB-Fc after IL-6 failed to display allodynia 3 hours post administration of pH 7.0 on to the dura. Significant differences among means for each group were determined one-way ANOVA followed by Bonferroni post hoc test. Males facial: time $F(3, 72) = 11.74$, $P < 0.0001$, treatment $F(1,72) = 10.29$, $P < 0.0001$; Hind paw: time $F(3, 64) = 1.944$, $P = 0.134$, treatment $F(3, 64) = 13.21$, $P < 0.0001$. Females Facial: time $F(3, 64) = 20.92$, $P < 0.0001$, treatment $F(3, 72) = 1.109$, $P = 0.0013$; Hind paw: time $F(1, 72) = 6.152$, $P = 0.0012$, treatment $F(3, 64) = 24.51$, $P < 0.0001$

In order to test whether BDNF signaling at central terminals of dural afferents in the TNC contributes to priming in this headache behavioral model, the BDNF sequestering agent TrkB-Fc was given via intracisternal injection to decrease extracellular levels of BDNF in the TNC. When TrkB-Fc was given 24 hours following dural IL-6, rats did not respond to dural pH 7.0 at the 72-hour time point (Figure 3.5). Animals treated with i.c. vehicle after IL-6 displayed allodynia 3 hours after dural pH 7.0 administration, similar to data shown in Figure 3.2. This effect occurred regardless of the sex of the animal as both males and females were tested. These data demonstrate a role for central BNDF release in the maintenance of the primed state. If central BDNF is released following dural IL-6, and BDNF release contributes to the sensitivity to subsequent exposure to dural pH 7.0, then direct central administration of BDNF should recapitulate some or all of the features present in the primed state. As shown in Figure 3.6, i.c. administration of 1 or 10 picograms (pg) of BDNF produced cutaneous facial allodynia that in the case of 1 pg is resolved by 72 hours. Since 1 pg i.c. BDNF produced a time course of allodynia similar to dural IL-6, this dose was chosen for future studies.

To determine whether i.c. administration of BDNF is capable of producing priming in the absence of dural stimulation, pH 7.0 was applied to the dura at the 72-hour time point after i.c. BDNF (1 pg). In animals treated with i.c. BDNF, pH 7.0 application to the dura 72 hours later produced facial allodynia while dural vehicle given to i.c. BDNF animals produced no response at this time point (Figure 3.7). Prior administration of i.c. vehicle did not prime rats as neither pH 7.0 nor vehicle on the dura produced allodynia at 72 hours. Additionally, i.c. BDNF-treated animals were tested for responses to systemic NO donor administration after resolution of allodynia due to BDNF (Figure 3.8).

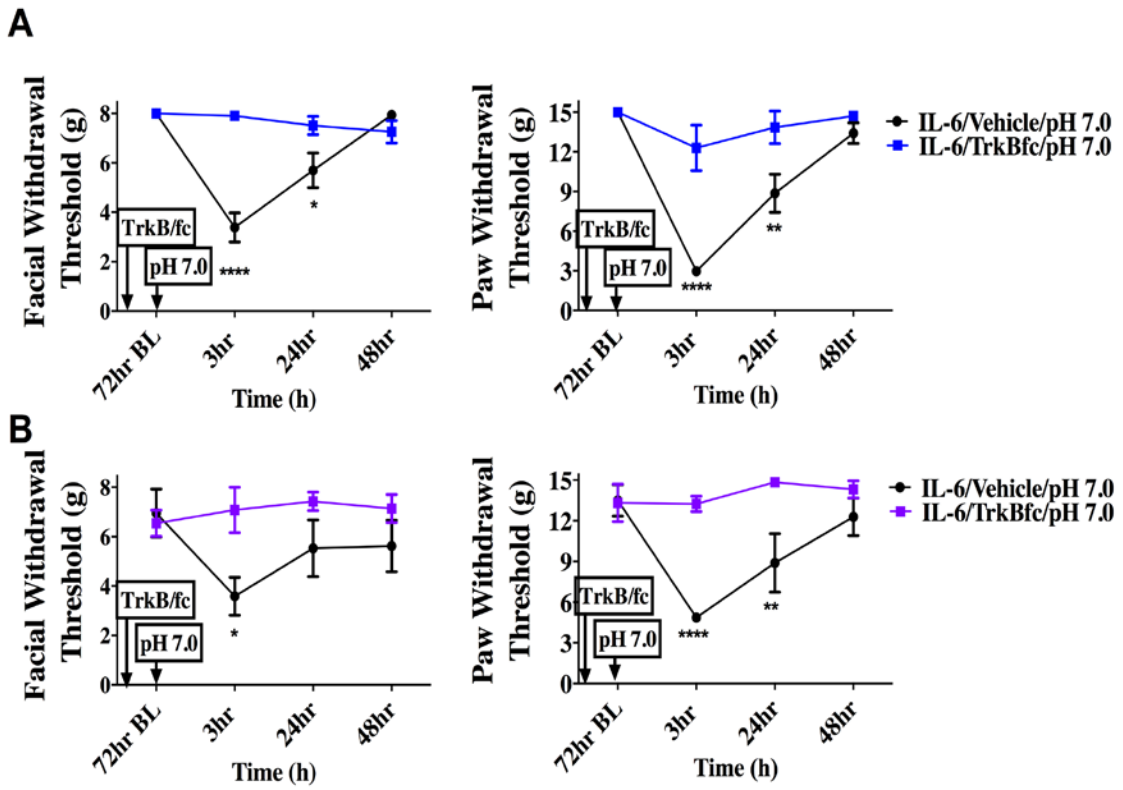

Figure 3.5. Priming following dural IL-6 stimulation is dependent on BDNF signaling in the brainstem. TrkB-Fc was given i.c. 24 hours after dural application of IL-6.

Only animals given i.c. vehicle (males n=12 A & females n=7 B) respond to dural pH 7.0 at 72 hours post IL6. Males treated with IL-6/i.c vehicle/ dural pH 7.0 showed significant facial and hindpaw responses at 3 hours and 24 hours. However, females treated with IL-6/i.c vehicle/dural pH 7.0 had significant facial responses at 3 hours and hindpaw responses at 3 hours and 24 hours. Both male(n=8 A) and female (n=7 B) animals treated with i.c. TrkB-Fc after IL-6 failed to display allodynia 3 hours post administration of pH 7.0 on to the dura. Significant differences among means for each group were determined one-way ANOVA followed by Bonferroni post hoc test. Males facial: time $F(3, 72) = 11.74$, $P < 0.0001$, treatment $F(1,72) = 10.29$, $P < 0.0001$; Hind paw: time $F(3, 64) = 1.944$, $P = 0.134$, treatment $F(3, 64) = 13.21$, $P < 0.0001$. Females Facial: time $F(3, 64) = 20.92$, $P < 0.0001$, treatment $F(3, 72) = 1.109$, $P = 0.0013$; Hind paw: time $F(1, 72) = 6.152$, $P = 0.0012$, treatment $F(3, 64) = 24.51$, $P < 0.0001$

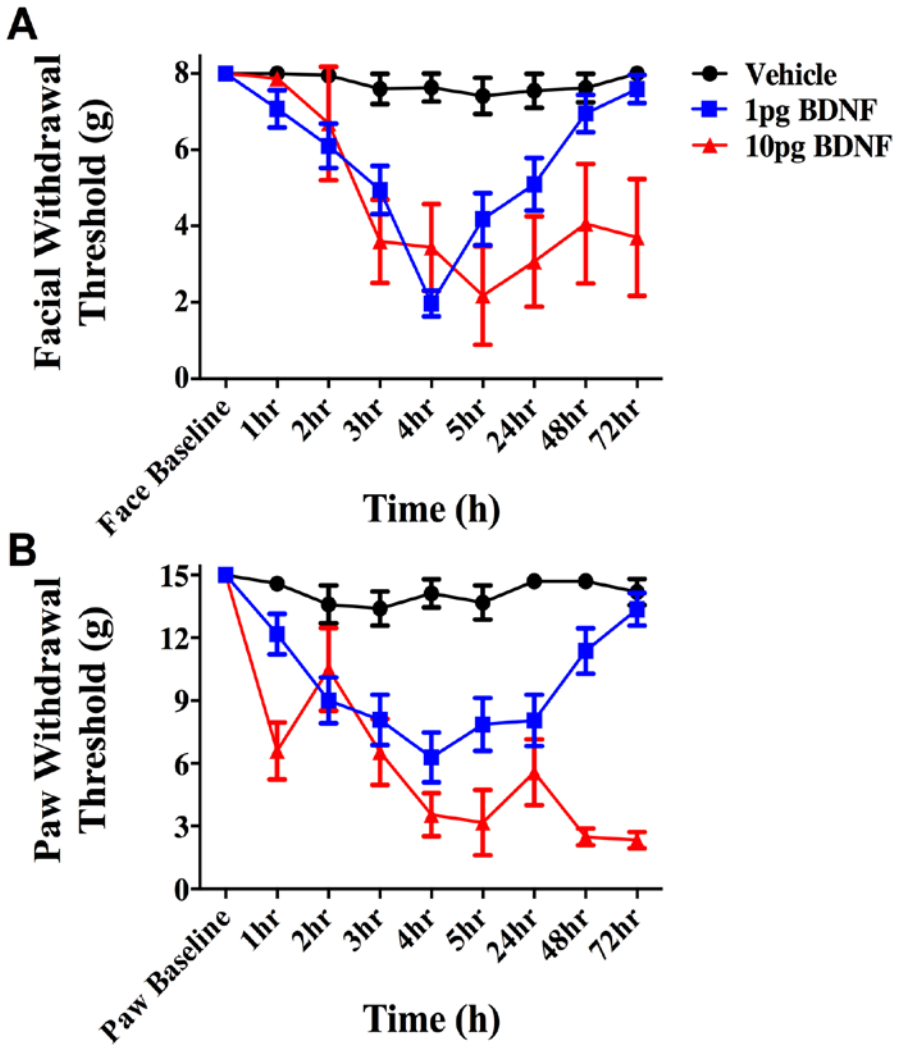

Figure 3.6. Intracisternal BDNF produces cutaneous allodynia.

Administration of 1 or 10 picograms (pg) of BDNF i.c produces cutaneous facial (A) and hindpaw (B) allodynia. Allodynia induced by BDNF at 1 pg (n=20) resolved within 72 hours, whereas that induced by 10pg (n=6) of BDNF persisted beyond 72 hours. Vehicle (n=5) i.c. showed no significant results at any time point. Facial: time $F(8, 252) = 5.85$, $P < 0.0001$, treatment $F(2,252) = 31.02$, $P < 0.0001$; Hind paw: time $F(3, 64) = 4.937$ $P < 0.0001$, treatment $F(3, 64) = 60.07$, $P < 0.0001$

At the 72-hour time point after i.c. BDNF, only BDNF-treated rats developed facial allodynia when given i.p SNP. Transient allodynia was observed in i.c. BDNF animals given vehicle at 72 hours but i.c. vehicle–treated animals did not respond to later administration of SNP or vehicle. Finally, in order to examine whether BDNF priming lasts beyond the 72-hour time point, animals were given i.c. BDNF or vehicle followed by systemic (i.p.) SNP at day 16 following BDNF (Figure 3.8). BDNF-treated rats developed allodynia to SNP at this late time point while there was no response to vehicle in these rats, indicating that BDNF priming lasts for weeks. Together, these data show that similar to stimulation of the dura with IL-6, direct central administration of BDNF is capable of producing priming to subsequent subthreshold stimuli including common migraine triggers.

Discussion

Migraine in its most common form is an episodic disorder where, between attacks, patients are otherwise normal. However, during interictal periods, migraine patients likely carry underlying neuroplasticity that causes susceptibility to mild triggering events. The findings from these studies show that afferent input from the meninges, such as that occuring during the headache phase of migraine, can prime the nociceptive system to future subthreshold stimuli. Importantly, this primed state is present in rats that display no outward signs of hypersensitivity (e.g. no cutaneous allodynia). Despite an otherwise normal phenotype, exposure to stimuli such as moderate changes in dural pH or systemic delivery of low doses of NO donors can provoke headache-related behavior. These studies also show that this primed state is dependent on central release of BDNF within the brainstem. Although migraine is a complex neurological disorder with contributions from throughout the nervous system, the findings presented here

implicate BDNF-dependent mechanisms within the TNC as a contributing factor to the sensitivity to events that are subthreshold in non-migraineurs.

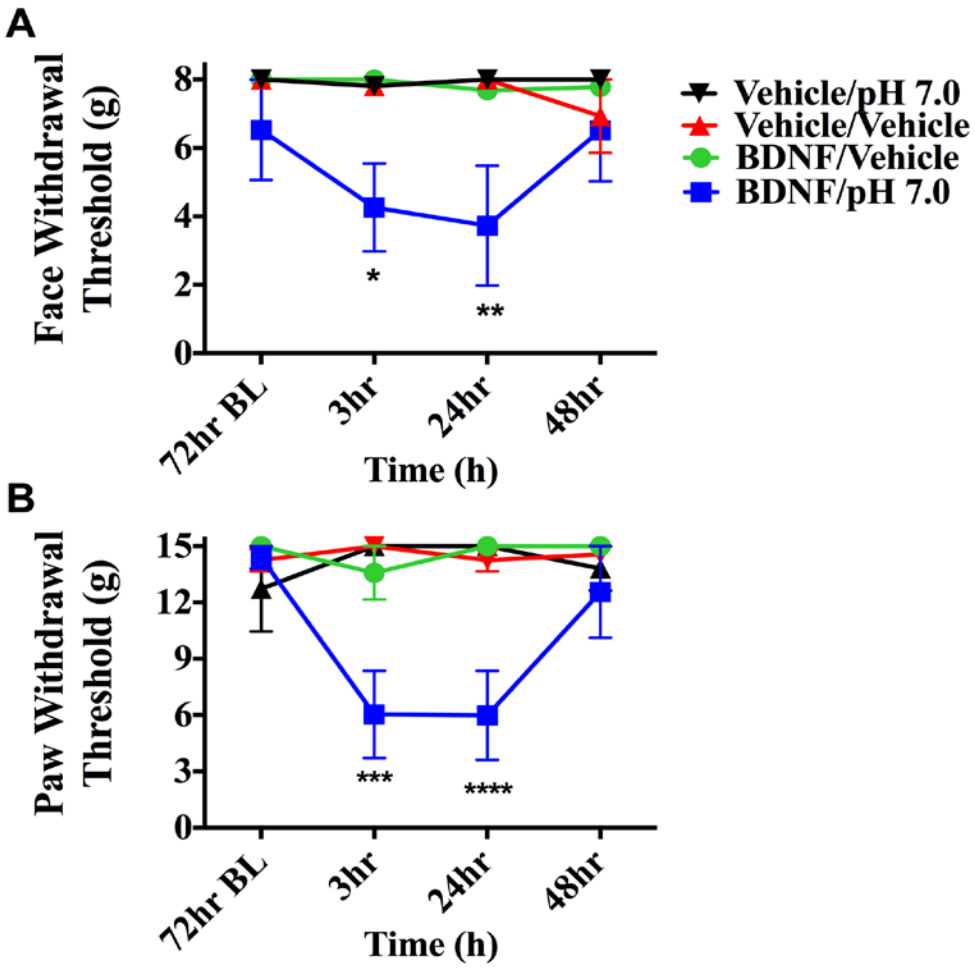

Figure 3.7. Intracisternal BDNF primes animals to sub-threshold dural pH stimulation.

Animals treated with i.c. BDNF followed by pH 7.0(n=5 for all groups) application to the dura 72 hours later displayed facial allodynia (A &B) while BDNF-treated animals given i.c dural vehicle showed no response at this time point. Significance was determined by one-way ANOVA analysis if the differences of the means for each group, (****$p < 0.0001$, *$p < 0.01$) . Facial: time F (3, 64) = 0.7616, P <=0.5198, treatment F (3, 64) = 10.29, P < 0.0001; Hind paw: time F (3, 64) = 1.944, P =0.134, treatment F (3, 64) = 13.21, P < 0.0001

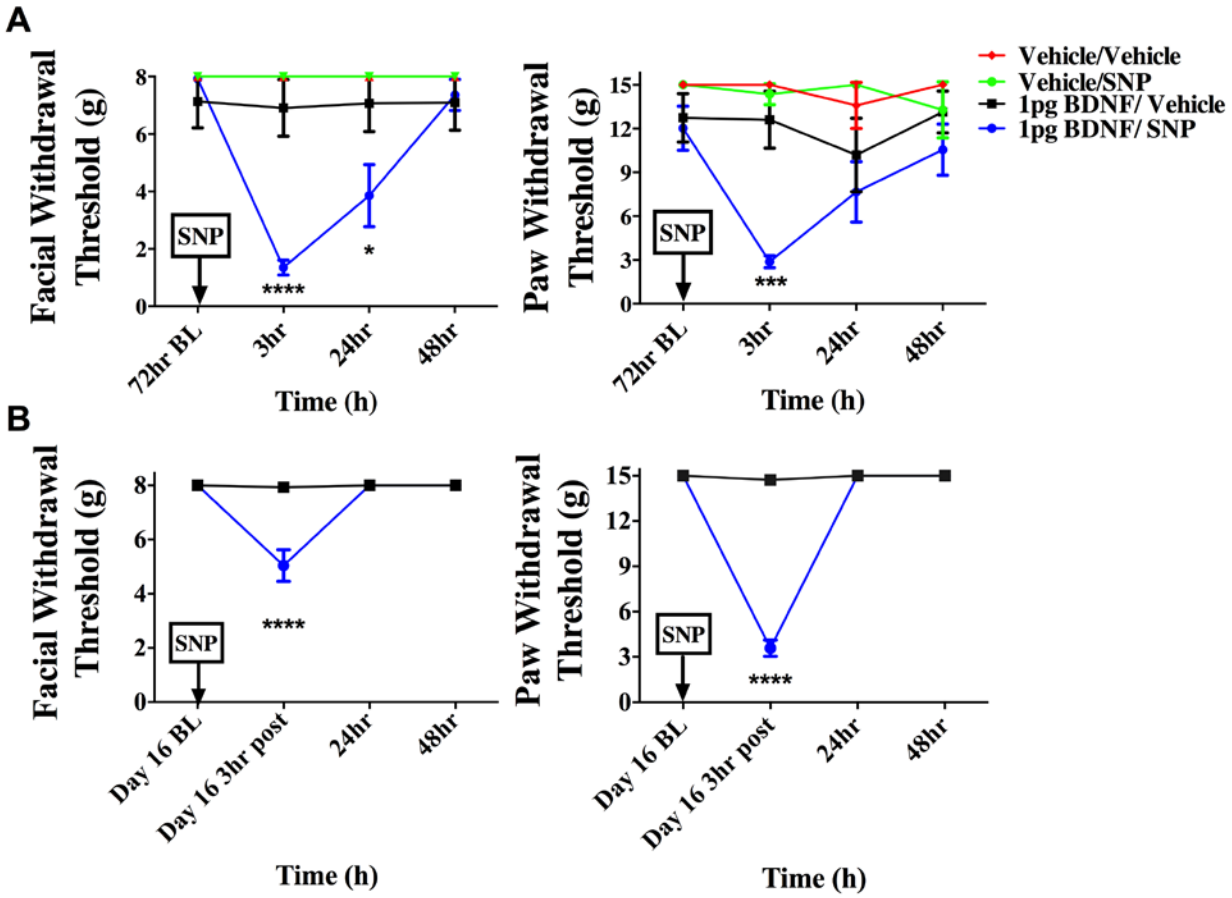

Figure 3.8. Priming following intracisternal BDNF lasts for at least several weeks.

BDNF-treated animals developed facial allodynia when given i.p SNP at 72 hours (A) (BDNF/SNP n=10, BDNF/vehicle n=8, Vehicle/Vehicle n=5, Vehicle/SNP n=5) or day 16 (B) (n=8 for all groups) post BDNF treatment. A significant attenuation of withdrawal thresholds to tactile stimuli applied to the face were observed at both 3 hours and 24 hours, whereas hindpaw responses were only significant 3 hours after 72 hour SNP administration. However, on day 16 a significant attenuation of withdrawal thresholds was observed 3 hours after SNP administration for facial and hindpaw responses. Allodynia was not observed in i.c. BDNF-treated animals given vehicle at 72 hours or day 16(. Significant (****$p < 0.0001$) differences among means for each group were determined one-way ANOVA followed by Bonferroni post hoc test. (A) Facial: time $F(3, 64) = 8.347$, $P =< 0.0001$, treatment $F(1, 64) = 12.30$, $P =0.0008$; Hind paw: time $F(3, 96) = 1.789$, $P =0.1544$, treatment $F(3, 96) = 11.55$, $P < 0.0001$(B)Facial: time $F(3, 56 = 26.59$, $P < 0.0001$, treatment $F(1,56) = 24.15$, $P < 0.0001$; Hind paw: time $F(8, 406) = 8.027$, $P < 0.0001$, treatment $F(1, 406) = 87.69$, $P < 0.0001$

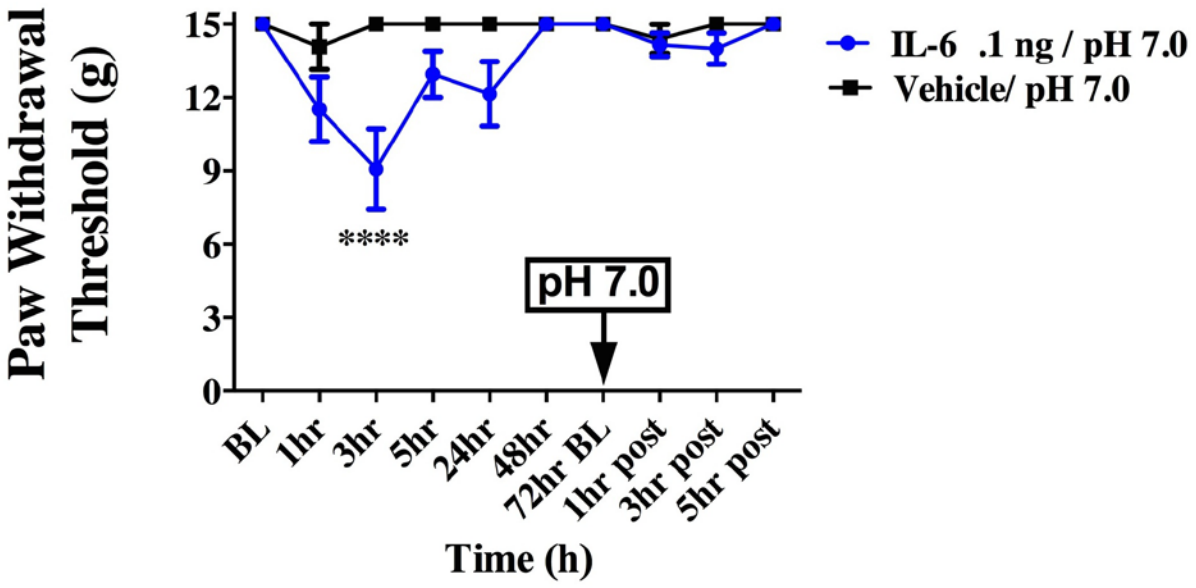

Figure 3.9. Hindpaw application of IL-6 fails to prime animals to sub-threshold stimuli administered in the hindpaw.

Animals that received a prior application of IL-6 do not exhibit cutaneous allodynia when pH 7.0 is applied to the hindpaw (n=5 for all groups) after the resolution of allodynia at 72 hours. Two-factor analysis of variance (ANOVA) indicated a significant effect of both treatment and time of the hindpaws. Significant differences among means for each group were determined by analysis of variance followed by Bonferroni post hoc test. Hind paw: time $F(9, 90) = 4.11$, $P=0.0002$, treatment $F(1, 90) = 20.47$, $P < 0.0001$

The presence of a primed state in otherwise normal rats where typically subthreshold events cause headache-related behaviors mimics the phenotype of common episodic migraine. Our findings show that this state can develop following a single exposure of the dura to IL-6 or a single injection of i.c. BDNF. This suggests that a primed state in episodic migraine patients may be present after a minimal number of prior events. While previous studies have shown that animals transition into a state of persistent allodynia following repetitive exposure to dural inflammatory mediators [10], to repetitive/continuous exposure to triptans [12], or to repetitive NO

donor administration [13], these models more closely mimic chronic migraine where headaches and allodynia are present most of the time [6a]. Our study demonstrates that the neuroplasticity contributing to these chronic states may develop early and with repetitive exposure (as in other studies) it may allow the transition from episodic to chronic migraine. Identification of plasticity mechanisms present during the episodic phase may allow these mechanisms to be targeted early, before the disorder progresses to a chronic state.

These studies demonstrate a role for central BDNF in priming of the dural afferent system, consistent with a pain-promoting role for this neuropeptide [21]. This conclusion stems from several experimental findings. First, we show that the initiation and/or maintenance of priming requires BDNF signaling since priming was not present in IL-6 treated rats that were given the TrkB receptor antagonist ANA-12 24 hours after IL-6. Second, the maintenance of priming (in both males and females) requires central BDNF since sequestration of BDNF via i.c. injection of TrkB-Fc 24 hours following dural IL-6 can reverse the primed state. Third, we show that direct central administration of BDNF can both produce cutaneous allodynia and induce priming following resolution of allodynia. These actions of BDNF are consistent with a similar role for this neuropeptide in priming in the spinal system [15a]. Our findings suggest that input from the meninges through dural afferents promotes the release of BDNF and that this release contributes to neuroplasticity that underlies the primed state. Other studies have shown that dural stimulation leads to sensitization within the TNC (for example see [22]), and our current data suggest that BDNF may play a role in these findings. Additionally, BDNF signaling may contribute to the sensitized state in models of chronic migraine (e.g. due to repeated inflammatory mediator application to the dura or repetitive triptan/NO donor administration).

Repetitive stimulation of the dura with inflammatory mediators was recently shown to both sensitize TNC neurons and to impair diffuse noxious inhibitory control (DNIC, a form of central descending modulation) in the TNC [11]. Chronic morphine administration, using a protocol similar to chronic triptan delivery published in [12], also sensitized dural input into the TNC and impaired DNIC [23]. Although BDNF was not examined in these DNIC studies, chronic morphine exposure is known to increase spinal BDNF [24] providing a possible mechanistic link between BDNF and the loss of TNC DNIC. Our current work suggests that BDNF may be a unifying factor in the generation of plasticity in the TNC that contributes to migraine.

An important observation from these studies is that central administration of BDNF, without any prior stimulation of the dura, promotes headache-like behavior following subsequent stimulation of the dura with pH 7.0. Dural stimulation with IL-6 produced similar priming to pH 7.0 and while we have shown previously that IL-6 can sensitize dural afferents [14], central BDNF administration is not likely to sensitize nerve endings in the dura mater. Thus, this central mechanism can exist independent of any hypersensitivity present in peripheral terminals before or during headache. Central sensitization in the TNC may enhance transmission of background levels of input from the meninges. Additionally, hypersensitivity in dural nerve endings would likely further enhance afferent trafficking. A variety of activating/sensitizing mechanisms within the dura have been proposed to contribute to migraine [8, 25] and these may occur in addition to central sensitization creating efficient afferent signaling in response to otherwise non-noxious events.

BDNF can be released from both primary afferents [26] and microglia [27]. Calcitonin gene-related peptide (CGRP), which is closely linked to migraine [28], can promote the release of BDNF

from trigeminal neurons [29] and this may be one downstream mechanism by which administration of CGRP triggers migraine. Levels of serum BDNF have been found to be elevated in migraine patients during attacks compared to between attacks [30] although they were unchanged in one study [31], further implicating BDNF in migraine in humans. However, recent work in several mouse pain models found that release of BDNF from spinal microglia does not contribute to pain in female mice[32]. Our data show that sequestration of central BDNF reverses priming in both males and females. This may indicate that the source of BDNF in this primed state is not microglia but that BDNF released from afferents contributes to priming. Future work is necessary to identify the source of BDNF for priming, potentially using tools such as mice where BDNF is deleted from primary afferents [26a] or deleted from microglia [33].

Although our findings suggest that targeting BDNF/TrkB may have potential for the treatment of migraine, BDNF is thought to contribute to a variety of beneficial neurological processes and decreased BDNF may contribute to several disorders. Among these, BDNF (or altered BDNF levels) has been proposed to contribute to learning/memory, major depressive disorder, anxiety, and addiction [34]. Thus, a more likely therapeutic strategy will be to identify downstream mechanisms by which BDNF and TrkB signaling contribute to priming of the dural nociceptive system. BDNF increases the excitability and signaling of dorsal root ganglia and postsynaptic dorsal horn neurons via altered Cl^- gradients and increased glutamate (AMPA/NMDA), acid-sensing ion channel (ASIC1a), and voltage-gated Na^+ currents [21, 35] and it can also regulate the synthesis and activity of atypical protein kinase C (PKC) [15a]. In the trigeminal system, BDNF application increased expression of the ATP-gated ion channel

P2X3 [36] and decreased multiple subtypes of voltage-gated K^+ currents in trigeminal ganglion neurons projecting to the trigeminal nucleus interpolaris/caudalis transition zone [37], a region known to contribute to the development of persistent orofacial pain [38]. It was also recently shown that activation of PKCγ-expressing interneurons in the medullary dorsal horn contributes to pain states in the trigeminal system [39], and this mechanism may be engaged downstream of BDNF/TrkB signaling. The above mechanisms may contribute to the primed state and it will be important to examine differences between BDNF actions here and those in central circuits contributing to processes such as memory as these differences could be exploited for novel migraine therapeutics.

Together, the studies presented here provide new mechanistic insight into neuroplasticity that can cause hypersensitivity to otherwise non-noxious events, a hallmark of migraine. They indicate that afferent input from the meninges, which occurs during headache, primes the dural nociceptive system using central release of BDNF. This suggests that headache events themselves contribute to future headaches. Our data demonstrate that a single round of meningeal input (from dural IL-6) or a single administration of BDNF intracisternally can induce neuroplasticity leading to priming. Although other preclinical migraine models use repetitive stimulation to produce sensitization [10, 12-13], BDNF in the TNC may provide a common link between these models as it is a critical mediator of plasticity throughout the nervous system including plasticity related to pain [21]. Assessment of a role for BDNF in the variety of stimuli that contribute to migraine, and identification of downstream events following activation of the BDNF receptor TrkB, could lead to novel therapeutics targeting the neuroplasticity present in migraine patients.

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CHAPTER 4

NON-INVASIVE DURAL STIMULATION IN MICE PRODUCES CUTANEOUS FACIAL ALLODYNIA

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Abstract

Migraines are a collection of abnormal neurological symptoms accompanied with unilateral throbbing pain in the absence of injury. The World Health Organization found migraine to be the 8th most burdensome disease on the planet in the 2012 Global Burden of Disease study. Targeted therapies and interventions are needed to understand the underlying pathophysiology of the condition in order to develop new therapeutics. The purpose of these studies was to develop a mouse model utilizing supradural injections, as an alternative to cannulation surgery in mice. Mouse trigeminal neurons retrogradely labeled from the dura were found to generate pH-evoked currents using whole-cell patch-clamp electrophysiology, similar to prior findings in rats. Behavioral responses to stimuli known to induce headache behavior in rats such as low pH, interleukin-6 (IL-6), and mustard oil (AITC) were shown to produce similar allodynia in this model. In these experiments we found that pH 6 (but not pH 6.8 or 7.0), IL-6 and 10% mustard oil produces time-dependent facial and hindpaw cutaneous allodynia, similar to that observed in rats. Further, exposure of mouse dura to IL-6 can sensitize responses to subsequent stimulation with a normally subthreshold pH stimulus given after the resolution of IL-6 induced allodynia. Finally, we show that repetitive stress may serve as a more naturalistic method to induce priming of the meninges.

Introduction

Migraine headaches are long lasting, persistent, and recurrent episodes of aberrant neurological symptoms that cause disruption or impairment of normal sensory, social, and occupational functions[1-3]. Marked by debilitating unilateral throbbing head pain, over 36 million Americans migraines suffer from the third most prevalent disease in the world[4]. Despite the high

prevalence the aetiology of migraine headaches is enigmatic. Atypical from other chronic conditions the underlying pathology for progression of the disease remains unidentified[5]. It is currently thought that migraine pain is driven by the activation of nociceptors within the meninges[6-8]. Previous work in rat models of migraine pain have shown that administration of various stimuli into the cisterna magna[9,10] or onto the dura produces headache behaviors (cutaneous allodynia) [6,11-13].

Current techniques allowing access to the dura in rats involve multiple drawbacks. First, a craniotomy must be performed which is an invasive procedure and can potentially compromise the underlying tissue[14]. The procedure requires a lengthy recovery period or anesthetization of the surrounding tissue[15,16] following the procedure that may interfere with dural stimulation results. Existing non-invasive migraine models e.g. using systemic administration of NO donors lack direct activation of menengial nociceptors[17-20], thus relying on non-direct mechanisms of afferent activation. Development of a novel technique to circumvent these limitations would allow for more direct evaluation of dural stimulation.

The purpose of these studies was to develop a novel approach for acute stimulation of the mouse dura. Notably, this model bypasses the need for a surgery and a recovery period. This technique utilizes the natural separation between cranial sutures, prior to fusion, as the injection point. The projection of the injector is a fixed length to prevent damaging the underlying tissues. Thus, the ability of stimuli to produce headache behavior without a prior surgery can be assessed. Using non- invasive supradural injections in mice we have characterized responses to stimuli previously shown to induce headache behavior in rats (mustard oil, low pH, and (IL-6), and to compare allodynia to data published from rats that had surgery. We investigated if several

of these initial stimuli could produce priming in mice[21]. We have utilized stress, the most reported migraine trigger [22,23], as a naturalistic stimulus of dural afferent system activation. Lastly, we investigated if dural IL-6 or stress could produce sensitization of dural afferent system.

Methods

Animals

Male and Female adult ICR (>8 weeks) mice were maintained in a temperature-controlled room on a 12-hour light/dark cycle with food and water *ad libitum*. All procedures were performed in accordance with the policies of the IASP as well as the NIH guidelines for use of laboratory animals. All procedures were conducted with prior approval of the IACUC at The University of Texas at Dallas.

Injections

Supradural injections are given ~8.0 mm posterior to the bregma on the junction of the saggital and lamboidal suture junction. All mice used were under 30 grams, but differences in mouse age and body weight may require adjustments of the injection coordinates and/or injector length. The head of the mouse is shaved and then stabilized in a stereotaxic frame. Using a .5mm injector the cranial bone sutures bregma and lambda are identified via topographical features of the cranial plates. The junction of the saggital and lamboidal sutures are felt with the injector and verified by re- positioning the injector along the skull. The injector was placed between the junction of the saggital and lamboidal sutures to allow for injection. Injections are performed under light isoflurane anesthesia administered via nose cone from a vaporizer. Mice were under isofluorane anesthesia for less than 2 minutes and injections were administered onto the dura of

the animals. The animals were then returned to the testing boxes. Fluoro-Gold was injected onto mice dura 7-10 days before prior to dissection of the trigeminal ganglia.

Solution Preparation

Allyl isothiocyanate (AITC; Mustard oil or MO; Sigma) 10% was prepared in mineral oil. MO was prepared at a concentration known to induce dural nociceptor activation in anesthetized animals[6] and in our behavioral studies[24]. The vehicle injection was 100% mineral oil (Min oil). Rat recombinant interleukin-6 (IL-6) (R&D Systems) stock solution (10 μg/ml) was prepared in sterile 0.1% bovine serum albumin (BSA) and diluted to final concentrations of 10 ng/ml in synthetic interstitial fluid (SIF) (pH 7.4, 310 Osmolality). SIF was used at pH 7.0. Fluorogold (Fluorochrome, LLC) was dissolved in synthetic-interstitial fluid (pH 7.4, 310 Osm) to 4%.

Testing

Male and Female adult ICR mice were habituated to tapered single poly-coated paper cylindrical facial testing chambers (2.5” top diameter, 1.75” bottom diameter) while contained in clear acrylic compartments (2” Length x 3” Width x 9” Height) for three days prior to baseline. The use of this larger compartment lets mice freely access and explore the facial testing chamber without physical restraint but they are unable to move about the acrylic compartment. Animals were not deprived of food or water; they were given a food pellet and Hydrogel® (Clear H2O).

Prior to injection, baseline facial and hindpaw thresholds were recorded for each animal. Using a 0.5mm injector, an injection was made between the sutures of the skull (see injections). The animals were then returned to the testing boxes. Facial and hindpaw thresholds were measured at 1, 3, 5, and 24 hours post-injection using the Von Frey up-down method of

testing. The Von Frey filaments were applied to the peri-orbital region of the face or to the plantar surface of the hind paw perpendicularly until the entire force was applied and held for approximately 3 seconds or until animals withdrew. Maximum filaments used were 0.6 g for the peri-orbital region and 2 g for the hindpaw. For biphasic behavioral experiments, facial and hindpaw withdraw thresholds were measured until mice recovered then the second stimulus was given as described

Stress

Animals were habituated (explained above) to facial testing chambers. The next three consecutive days animals were stressed in tail vein injector tubes (Stoelting, 15-30 gm) for 2 hours each day. Weights were recorded on all habituation and stress days. Animals were placed in stress chambers at the same time on consecutive stress days and stress was always conducted before 12 pm. Controls stress animals remained in their home cages deprived of food and water for the same two-hour span in a separate room. Testing for facial and hind-paw allodynia was conducted at the following time points post stress: 24 hours, 72 hours, 7 days, 10 days, 14 days.

Trigeminal Neuron Culture

The left and right trigeminal ganglia were dissected out and was enzymatically treated with Papain and Collagenase Type II. The tissue was triturated 4-5 times, plated onto Poly-D-Lysine and laminin coated 35mm Petri dishes, flooded with a L-15 solution 2-3 hours post-plating, and kept at room temperature. Cells were used for patch-clamp experiments within 24 hours of plating.

Whole Cell Patch-clamp Electrophysiology

A MultiClamp 700B patch-clamp amplifier with pClamp 10 acquisition software were used on isolated rat trigeminal ganglia neurons. Pipettes were pulled and polished to 1.5-3 MΩs resistance. During the recording, series resistance was usually less than 7 MΩs, with 80% compensation. Solenoid valves attached to gravity-fed flow tubes were used to perfuse extracellular solution over the cells during recording Whole cell patch-clamp experiments were performed on isolated rat TG using a MultiClamp 700B (Axon Instruments) patch-clamp amplifier and pClamp 10 acquisition software (Axon Instruments). Recordings were sampled at 2 kHz and filtered at 1 kHz (Digidata 1322A, Axon Instruments). Pipettes (OD: 1.5 mm, ID: 0.86 mm, Sutter Instrument) were pulled using a P-97 puller (Sutter Instrument) and heat polished to 2.5 – 4 MΩ resistance using a microforge (MF-83, Narishige). Series resistance was typically < 7 MΩ and was compensated 80%. All recordings were performed at room temperature. A Nikon TE2000-S Microscope equipped with a mercury arc lamp (X-Cite® 120) was used to identify FG-labeled dural afferents. Data were analyzed using Clampfit 10 (Molecular Devices) and Origin 8 (OriginLab). Cell sizes were not significantly different among groups. Labeled neurons fall in a range of capacitance values from 15 to 80 pF (average of 57.47 +/− 5.47). Pipette solution contained (in mM) 140 KCl, 11 EGTA, 2 MgCl2, 10 NaCl, 10 HEPES, 1CaCl2 pH 7.3 (adjusted with N-methyl glucamine), and was ~ 320 mosM. External solution contained (in mM) 135 NaCl, 2 CaCl2, 1 MgCl2, 5 KCl, 10 Glucose, 10 HEPES, pH 7.4 (adjusted with N-methyl glucamine), and was ~ 320 mosM. Solution exchange time was around 20 ms.

Results

To verify that supradural injections effectively access dural afferents, we applied a retrograde dye to label cell bodies in the trigeminal ganglion. As expected, flourogold injections resulted in labeling in TG cell bodies. Conducting these experiments in mice without the need for craniotomy to apply the tracer allowed us to rule outdetermine whether ASIC responses of dural afferents we previously reported [25,26](either their presence or magnitude) were due to surgery in rats. Dural afferents from mice were tested with stimulation from an acidic pH, identical to the experiments conducted previously in rats[25]. Mouse trigeminal ganglion neurons retrogradely labeled from the dura had a 65% response rate to acidic pH 6.0 with a current generation (Figure 4.1). This is similar to an 80% response rate previously observed in rat dural afferents[25]. Currents ranged from 314 pA to 1123 pA. Examples of typical currents generated from mouse dural afferents upon pH 6.0 application are shown in Figure 4.1A and 4.1B. Cell sizes ranged from 22.7 to 62.02 pF. As seen in Figure 4.1C, pA/pF distribution ranged from ~0 – 30, with one outlier current at 64.2 pA/pF. Cell traces were typical of ASIC3 homomers and heterotetramers, and also ASIC1 homomeric channels[27].

To further validate the ASIC3 responses in dural afferents APETx2, a known inhibitor of ASIC3 heteromers and homomers, was applied after currents were generated with pH 6.0. Channel inhibition was observed in a range between 48% to 94% inhibition, with an average of 77.868% (n = 5). Current profiles with fast inactivation times were characteristics of ASIC3 homomeric or heteromeric channels. APETx2 inhibition of pH-evoked current was also shown to be reversible (Figure 4.2C).

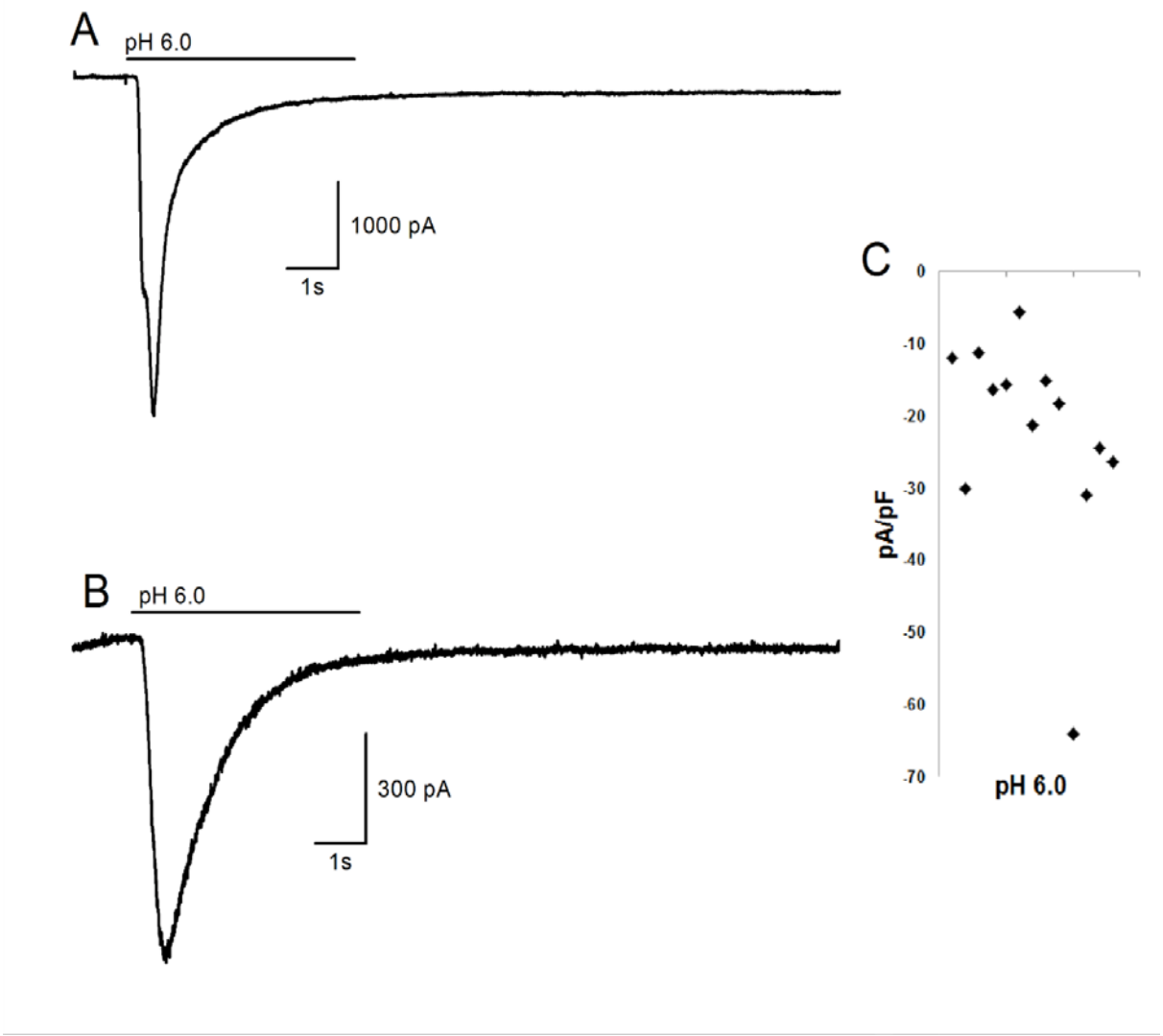

Figure 4.1. Dural afferent recordings.

Recordings from mouse dural afferents in response to a 5s pH step from 7.4 to 6.0 are characteristic of ASIC3 currents in A, B. A) Current size of 887 pA. Shape characteristics of ASIC1A. B) Current size of 3982 pA. Shape characteristics of ASIC3. C) Current density (pA/pF) of pH 6.0 evoked currents in mouse dural afferents (n =13)

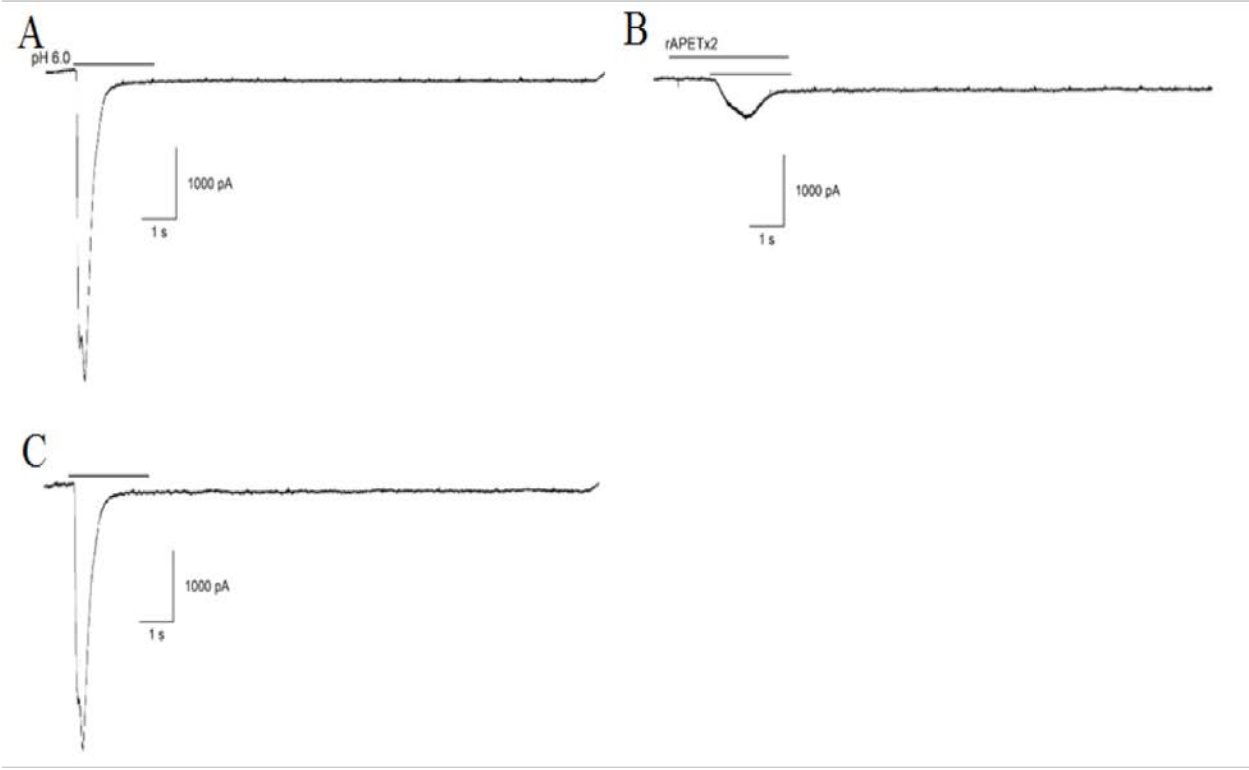

Figure 4.2. APETx2 block of pH 6.0 evoked currents in dural afferents of rat TG.

Applied solution was perfused for 5s over 20 s total. **A**) ASIC current response from pH 6.0 alone (3758pA) **B**) Block of ASIC current with 200 pMolAPETx2 in pH (587pA) at roughly 85% 6.0 **C**) Regeneration of ASIC

Behavioral responses following supradural injection in mice were evaluated for comparison to results seen in rats. In the first series of experiments, pH 6, 6.8, and 7.0 SIF were compared with SIF at normal pH 7.4. This was done to characterize dural responses to a range of pH in mice. Dural application of pH 6, but not pH 6.8, 7.0, and 7.4 (vehicle) produce allodynia in male mice. Likewise dural application of pH 6 but not pH 7.4 (vehicle) produces allodynia in female mice.

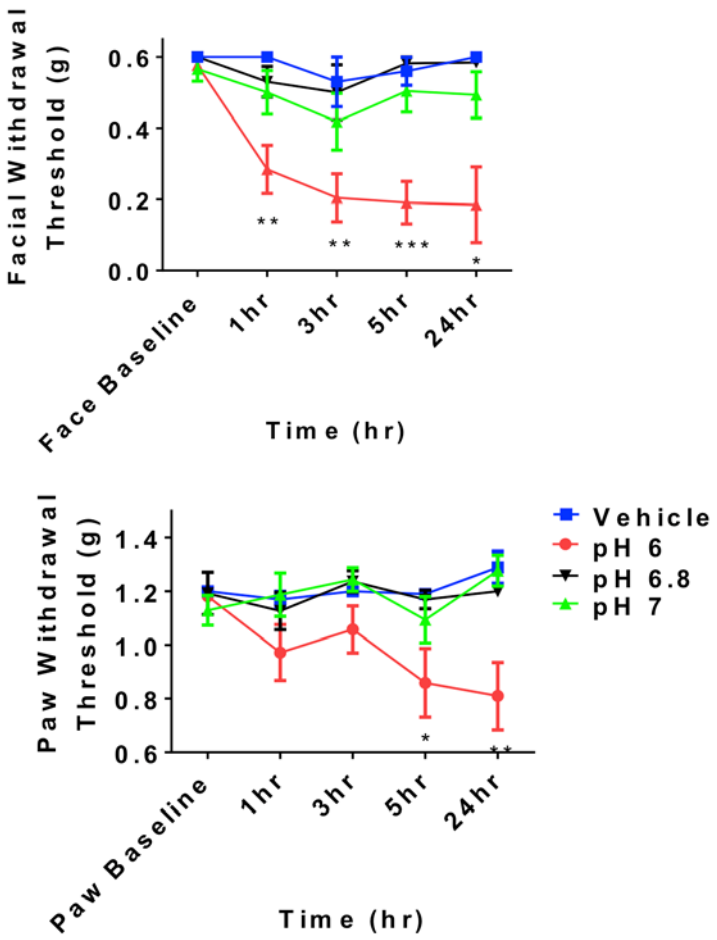

Figure 4.3. Dural application of pH 6, 6.8, and 7.0, but not pH 7.4 (vehicle) produces allodynia in male mice.

We have also shown previously that application of IL-6 to the rat dura mater via a cannula produced acute cutaneous facial and hindpaw allodynia that remained significantly different from baseline at 24 hours post injection[28]. IL-6 was applied to the mouse dura in the current studies to characterize the allodynic response in mice. Dural application of IL-6 produced both facial and hindpaw allodynia that resolved by 48 hours post application in both males and females (Figure 4.2) and remained at baseline at 72 hours. Next we investigated the effects of application of 10% AITC. Dural application of 10% AITC produced allodynia in both male and female mice. Both vehicle and null injections failed to produce significant changes in

threshold, indicating that the allodynia is due to the injection of specific stimuli ().Null injections were prepped with 5ul MO where the injector was advanced through the skull suture, but the MO was not delivered onto the dura. Null injections were able to verify that responses were due to administration of noxious stimuli rather than an artifact of the injection.

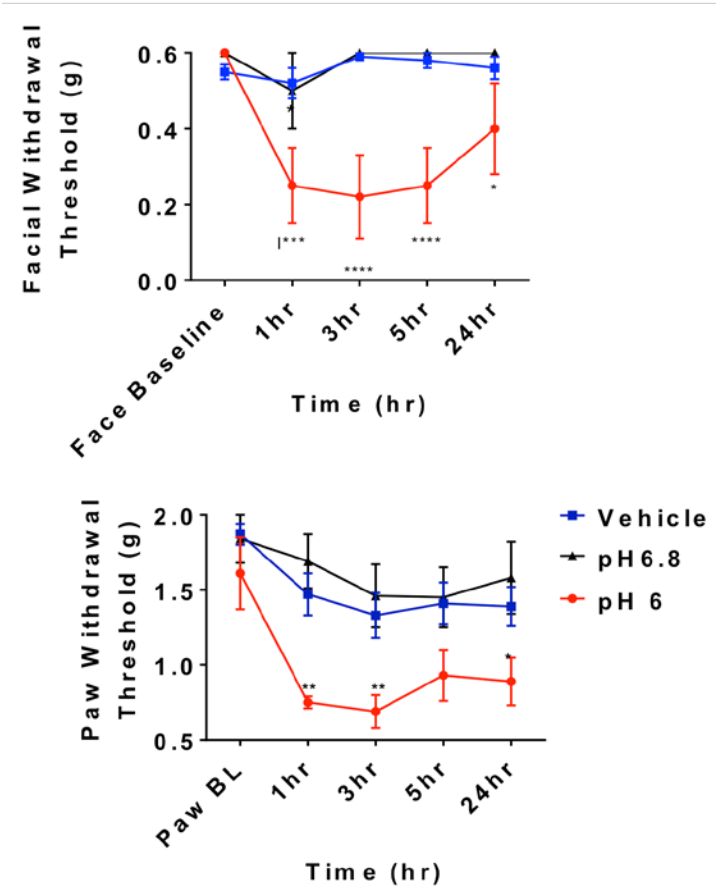

Figure 4.4. Dural application of pH 6 but not pH 7.4 (vehicle) produces allodynia in female mice.

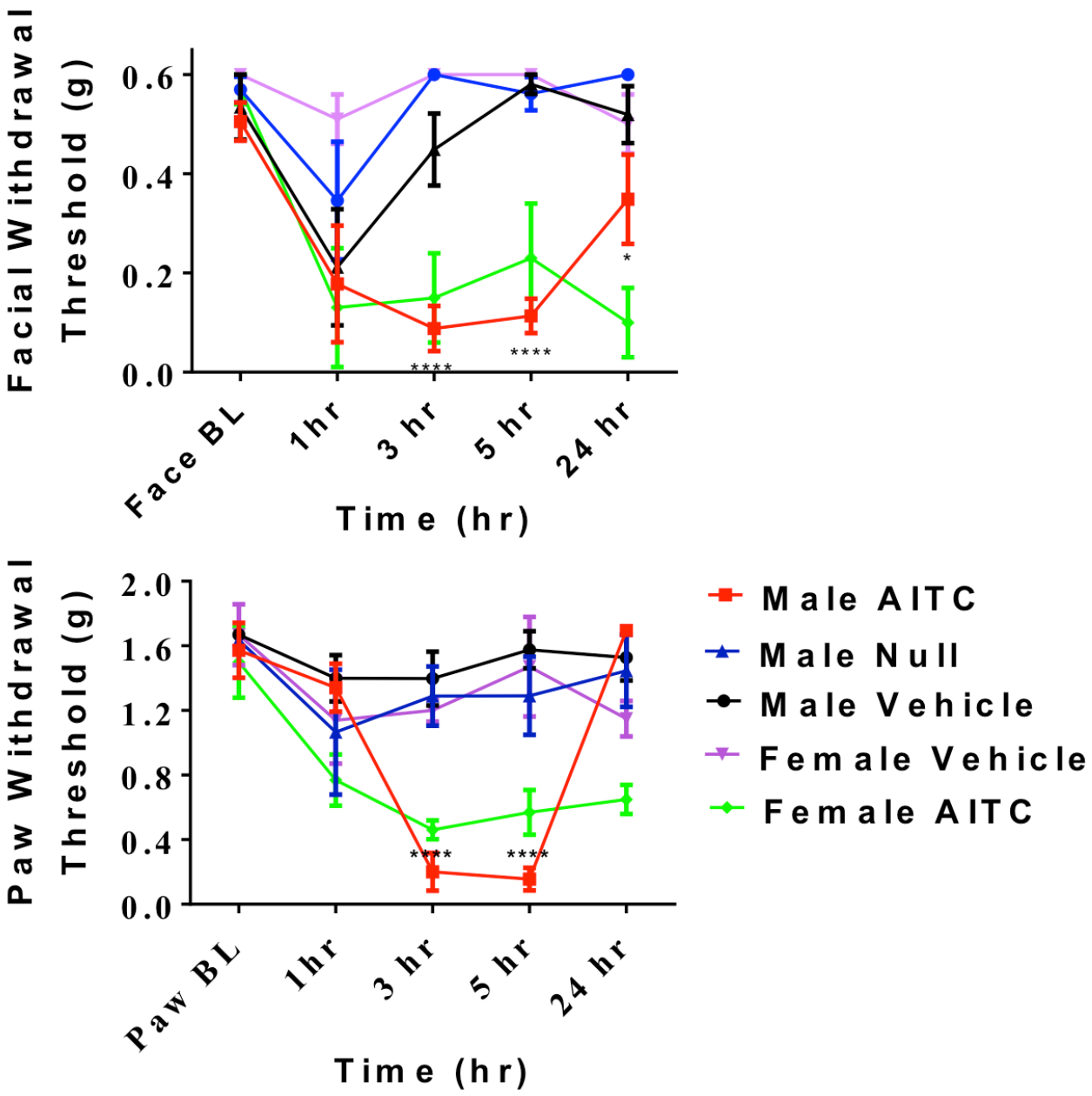

Figure 4.5. Dural application of 10% Mustard oil (AITC), but not vehicle or a null injection, produces allodynia in both male and female mice

Recently we showed that dural application of IL-6 sensitizes rats to normally non-noxious pH[9]. To investigate if mice were capable of responding to sub-threshold stimuli after priming we gave IL-6 to female and male mice and a subsequent application of pH 7.0. Only animals whose initial stimulus was IL-6 were primed and thus responded to supradural application of pH 7.0. These data are consistent with those shown previously in rats demonstrating that IL-6 induced priming is indeed due to dural IL-6 and not influenced by the craniotomy in rats.

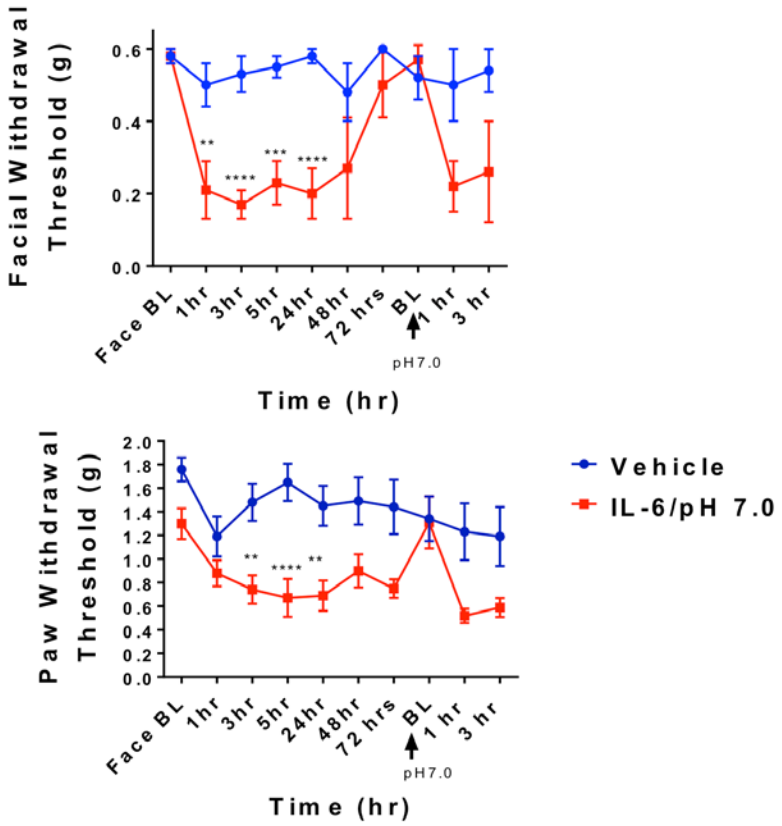

Figure 4.6. Dural application of IL-6 but not pH 7.4 produces allodynia in male mice

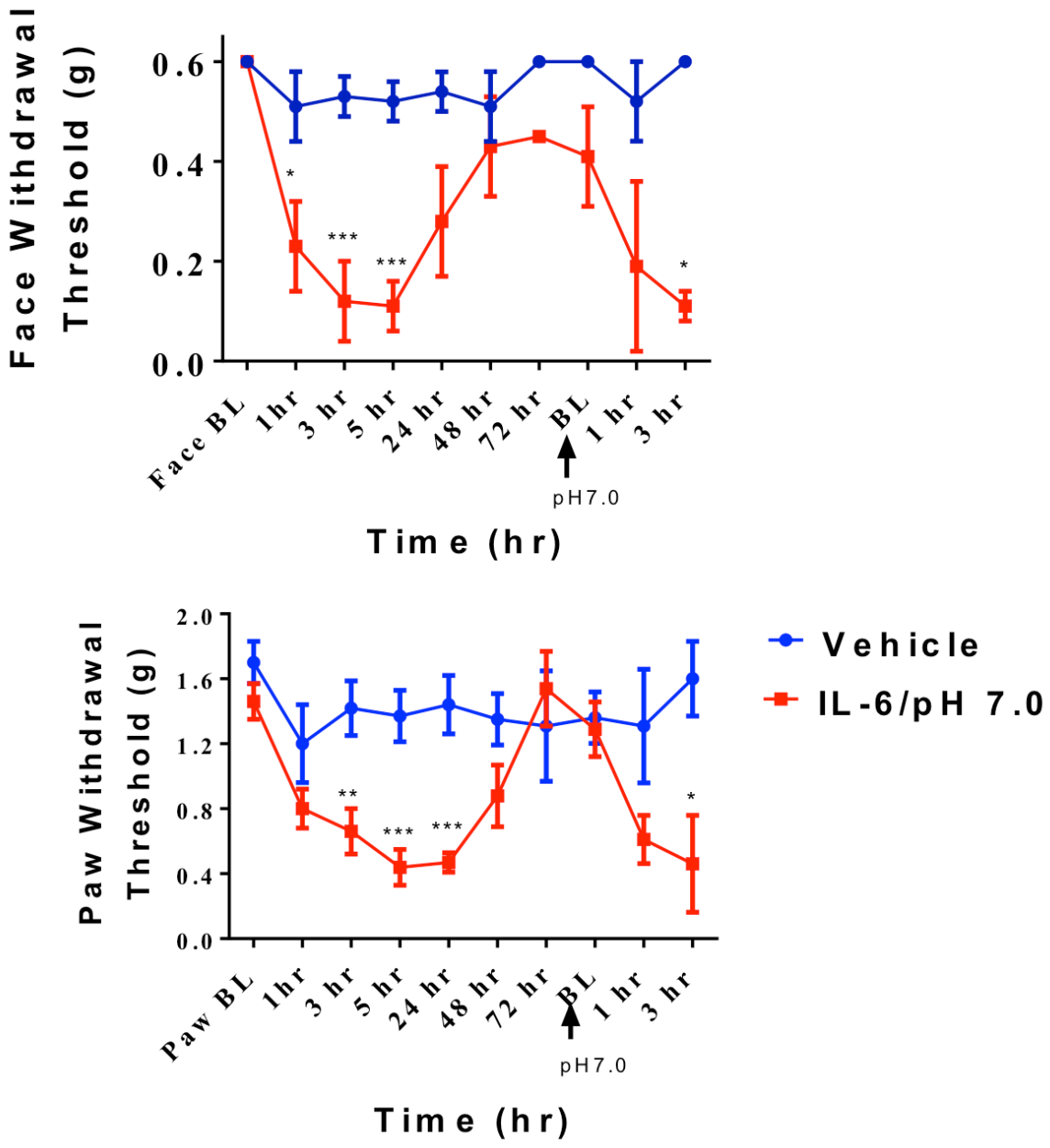

Figure 4.7. Dural application of IL-6 sensitizes female mice to subsequent application of pH 7.0

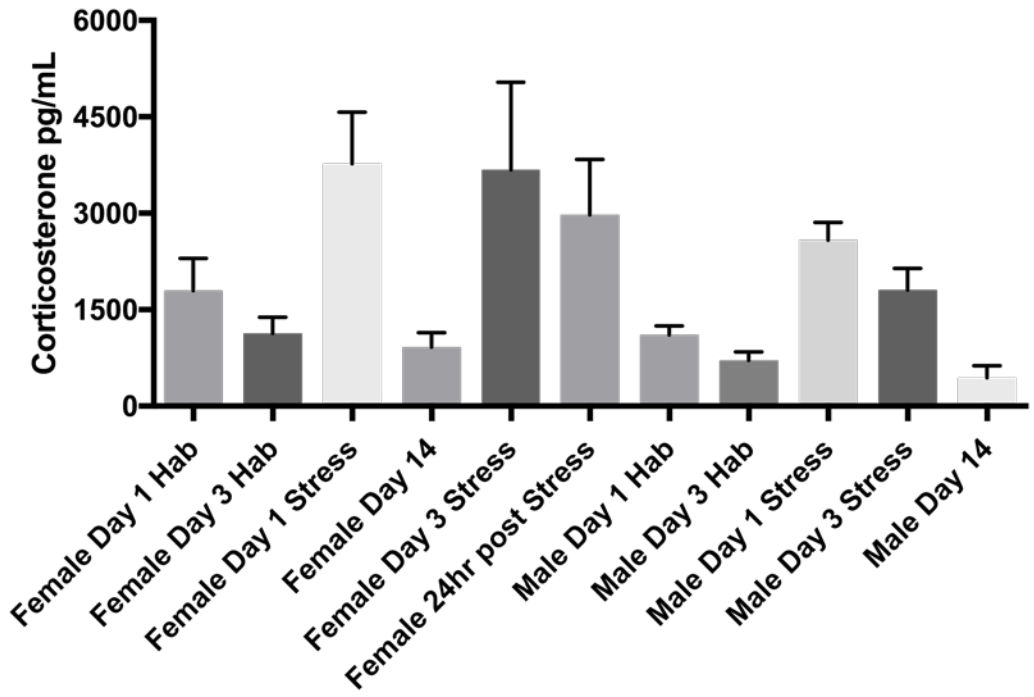

Figure 4.8. Serum corticosterone levels are increased with restraint stress.

Animals show increased serum corticosterone on both day 1 and day 3 of stress. Males produce significantly less serum corticosterone in response to restraint on stress days in comparison to females.

How stress triggers in migraine is unclear; however studies show the highest susceptibility to migraine is in the 18-24 hours after a stressful event[29]. Here three consecutive days of restraint stress elevates corticosterone levels. Stress also produced both facial and hindpaw allodynia 24hrs post stress, in male and female mice that resolved by 14 days(). Subsequent administration of pH 7.0 onto the dura produced significant facial allodynia only in stress animals. Nitric oxide (NO) donors are consistent migraine triggers in humans susceptible to migraine, but they do not trigger attacks in non-migraine patients [29]. Following recovery from stress, at 14 days, a single administration of the nitric oxide donor sodium nitroprusside (SNP, .1mg/kg) produced widespread cutaneous allodynia at 3hrs in females.

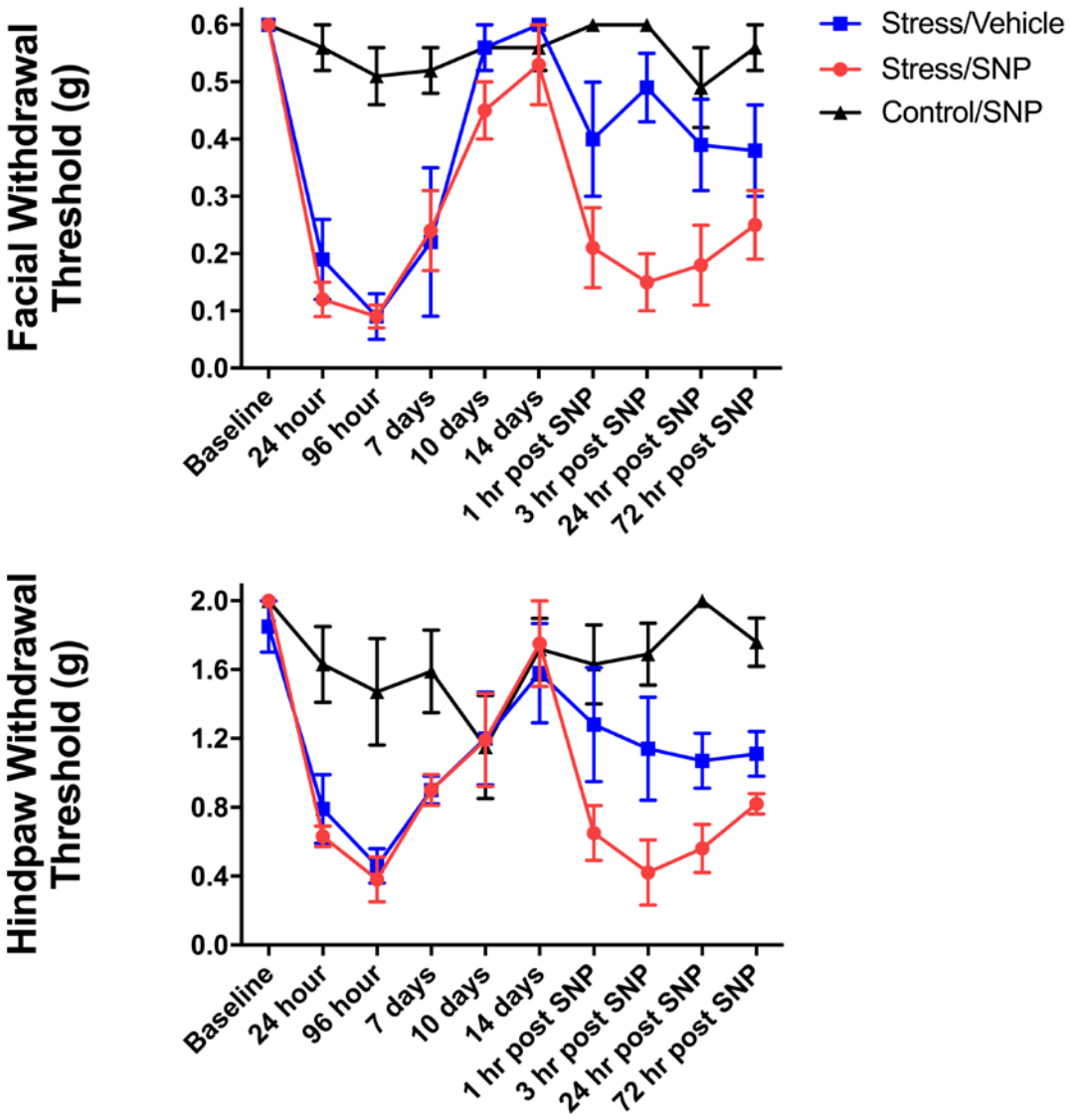

Figure 4.9. Restraint stress primes females to NO donor

Restraint stress produces both hindpaw and facial allodynia at 24hours that lasts for up to 10days. Upon recovery at 14days, subsequent systemic injection with .1mg/kg SNP causes significant drops in facial and hindpaw mechanical thresholds.

Discussion

Migraines occur in the absence of apparent injury; non-invasive preclinical models may further investigation into activation, sensitization, and plasticity of the present dural afferent system of patients. Understanding these mechanisms may uncover the underlying pathophysiology ultimately contributing generation of migraine headaches. Supportive of our previous work, we demonstrate here that mice exhibit pH-evoked dural afferent activation both in vitro and in vivo. Additionally, the effects of supradural injections of pH 6.0, AITC, and IL-6 mirrored those seen in rats, by producing widespread cutaneous allodynia in both male and female mice (Figure 4.9). We also demonstrate that mouse dural following the recovery from IL-6 dural afferents respond to pH 7.0, paralleling recent findings in rats[9].

Anywhere from 59% to over 80% of patients state that stress is their primary trigger for migraine[23,30,31]. Although, stress alone may not directly promote migraine it may disrupt normal behaviors, like sleep quality or food intake, which collectively trigger the condition[31]. Animal studies have found acute stress to be analgesic [32], whereas many chronic stress models have been shown to promote allodynia [33]. Incorporation of stress into this model allows the development of a sensitized state in these animals using a more naturalistic stimulus. Stress, as expected elevated corticosterone levels. Also, it is important to note that three days of repetitive restraint stress produced widespread cutaneous allodynia initially in this model (Figure 4.4). It has been hypothesized that repeated exposure to stressful conditions can result in dysregulation of normal adaptive responses by negatively influencing biological consequences or allostatic load[34]. Future studies could investigate if other known triggers (i.e., bright light) can develop noxious responses

following chronic exposure, mechanism by which stress sensitizes to migraine triggers, and whether sensitivity due to stress is unique to the trigeminovascular system.

NO donors are consistent migraine triggers in humans susceptible to migraine, but do not trigger attacks in healthy controls [29], suggesting that the sensitivity to a single NO donor challenge is a hallmark of migraine. Thus far studies in mice have used repetitive administration of high-dose NO donors to model sensitization seen in the condition[18]. These studies demonstrate sensitivity to a single low-dose NO donor injection, which is more representative of what is seen in humans.

These findings indicate that dural stimulation in mice produces migraine-like responses similar to those seen in rat models of migraine[9,25,28]. This suggests that mice are a suitable species for behavioral testing of headache following non-invasive stimulation of the dura mater. Additionally, adaptation of these studies to mice allows for the use of genetically-modified animals to better understand the underlying mechanisms of dural afferent activation/sensitization and ultimately migraine. For example, genetic tools such as ASIC knockout mice can allow testing of a role for these channels in migraine behavioral assays providing complementary support to the use of pharmacological agents. Additionally, dural stimulation can now be applied to familial-hemiplegic migraine (FHM) knockin models to address how these mutants differ in their responses to dural stimulation. Finally, optogenetic/chemogenetic tools can be used to dissect the contribution of various cell types (e.g. fibroblasts) within the dura to nociceptive signaling.

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CHAPTER 5

MENINGEAL APPLICATION OF PROLACTIN AND CGRP PRODUCES FEMALE SPECIFIC MIGRAINE-RELATED BEHAVIOR IN RODENTS

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Abstract

Migraine is the third most prevalent disease worldwide and is three times more prevalent among females with no currently known pathophysiology supporting the sexual dichotomy. Migraine has a significant negative impact on a patient's quality of life and current treatments only provide relief for 50% of patients. Studies have shown that migraine patients have elevated serum levels of both calcitonin gene related peptide (CGRP) and prolactin (PRL). Although these factors are capable of being modulated by female hormones, it is unknown how CGRP and PRL may mediate sex differences in migraine pain. The purpose of these studies was to determine whether nociceptive input from the dura mater, considered an essential event for migraine pain, may be modulated by PRL or CGRP and whether these stimuli produce sexually dimorphic headache behavior in rodents. Dural application of 0.5 μg of PRL caused robust facial and hindpaw hypersensitivity as well as increased grimacing for at least 7 days in females but not males. Following return to baseline, animals were sensitized to dural stimulation with a pH 7.0 solution, a typically subthreshold stimulus. Further, we show that stress, a widely-reported migraine trigger, can sensitize animals to dural pH 7.0 and a systemic nitric oxide donor in both males and females. However, stress increases serum PRL only in females. Finally, application of 1.9 pg CGRP to the dura produces cutaneous facial and hindpaw allodynia only in females, and this behavior was blocked by a PRL receptor antagonist. Together, these data support the hypothesis that PRL and CGRP can promote female-specific headache behavior, they may contribute to the sex differences observed in migraine, and they may be targets for sex-specific migraine therapeutics.

Introduction

Migraine headache is characterized by recurring episodes of disabling unilateral head pain**.** Migraine affects 3 times more females than males, it is the 8th most prevalent disease in the world[1,2]. Given the severity, migraine has a significant negative impact on a patient's quality of life and current treatments only provide relief for 50% of patients. Migraine involves the progression of aberrant neurological symptoms- in addition to disabling pain- patients experience a spectrum of abnormal sensory disturbances that can cause an attack to last almost a week. Migraine can be triggered by certain stimuli that are innocuous in non-migraineurs, such as stress, dietary changes, smells, physical activity, as well as hormone changes[3-7]. Maladaptive plasticity in the nervous system likely contributes to migraine pathology by increasing susceptibility to triggers, which are typically non-noxious. The maladaptive plasticity is likely caused, in part, by activation of the meningeal afferents innervating the dura. Participation of dural primary sensory neurons in headache mechanisms is further supported by the observation that pathophysiological events may sensitize meningeal sensory nerves.

Current therapies only provide for less than 50% of migraine patients. Many analgesics for pain management demonstrate marked sex-based differences for efficacy, safety profile and abuse/addiction potential[8-10]. Given that migraine is times more prevalent in women than in men; identification of sex-dependent mechanisms contributing to the pathophysiology of migraine is critical for more development of targeted headache therapies**.** However, further work is needed to better understand the role of female hormones in pain processes, particularly those most closely related to migraine.

Despite the higher prevalence in females, sex-dependent mechanisms of migraine have yet to be elucidated. However, studies have shown that migraine patients have elevated serum levels of calcitonin gene related peptide (CGRP) and prolactin (PRL). Both CGRP and PRL are capable of being modulated by female hormones [11-17], but it is unknown how these factors may mediate sex differences in migraine pain and whether they contribute to plasticity in the dural afferent system.

In view of the direct implication of CGRP, and PRL and their ability to contribute to neuroplasticity these neuropeptides may contribute in a sex-dependent manner to activation and sensitization of the dural afferent system. The purpose of these studies was to investigate the contribution of PRL and CGRP to migraine-related behavior induced by stimulation of the dura in females and males. We will also investigate if CGRP or PRL contribute to stress induced priming in mice.

Methods

Animals

Male and Female adult ICR (>8 weeks) mice were maintained in a temperature-controlled room on a 12-hour light/dark cycle with food and water *ad libitum*. All procedures were performed in accordance with the policies of the IASP as well as the NIH guidelines for use of laboratory animals. All procedures were conducted with prior approval of the IACUC at The University of Texas at Dallas.

Adult male and female Sprague-Dawley rats (250–300g, Harlan) were kept in a temperature-controlled room on 12-hr light/dark cycle with food and water ad libitum. All procedures were performed in accordance with the policies of the IASP and the NIH guidelines

for use of laboratory animals. The Institutional Animal Care and Use Committee (IACUC) of The University of Texas at Dallas approved all procedures.

Surgeries

As previously described[18], dura cannulation surgeries were performed on rats (250-300) grams. Animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg) and an incision exposing the skull was made to the top of the skull. Once the skull was exposed, a 1-mm hole was made in the skull to expose the dura (1mm left of midline, 1mm anterior to lambda). A 1mm guide cannula (Plastics One) was then inserted into the hole and secured with Vetbond™ (3M™). Two screws (Small Parts) were placed rostral to the cannula biparietal to sagittal suture and posterior to bregma. Dental acrylic was used to secure the cannula to both the screws and the skull. A dummy cannula (Plastics One) was placed into the cannula to ensure patency. Animals received gentamicin (8 mg/kg) to minimize infection following surgery. Rats were housed separately and allowed 6–8 days for recovery prior to behavioral testing.

Injections

Supradural injections in mice were given ~8.0 mm posterior to the bregma on the junction of the saggital and lamboidal suture junction. All mice used were under 30 grams. The head of the mouse was shaved and stabilized in a stereotaxic frame. An internal cannula was altered to adjust the length of the injector (Plastics One, part #C313I/SPC) by moving the outer plastic pedestal manually down the length of the injector in order to shorten the projection. A digital caliper was used measure the injector projection length. The modified injectors are then attached to a 10µl glass syringe cemented needle (Hamilton Company, 700 series) via tygon

tubing (Cole-Palmer, Item # EW-96460-16).Using a .5mm injector the cranial bone sutures bregma and lambda were identified via topographical features of the cranial plates. The junction of the saggital and lamboidal sutures were felt with the injector and verified by re- positioning the injector along the skull. The injector was placed between the junction of the saggital and lamboidal sutures to allow for injection. Injections are performed under light isoflurane anesthesia administered via nose cone from a vaporizer. Mice were under isofluorane anesthesia for less than 2 minutes and injections were administered onto the dura of the animals. The animals were then returned to the testing boxes.

Internal cannula with 11mm projection were used (Plastics One, part #C313I/SPC) to deliver injections in rats that had undergon cannulation surgery. They internal cannula are attached to a 10µl glass syringe cemented needle (Hamilton Company, 700 series) via tygon tubing (Cole-Palmer, Item # EW-96460-16. Animals were injected awake and then returned to the testing boxes.

Solution Preparation

Allyl isothiocyanate (AITC; Mustard oil or MO; Sigma) 10% was prepared in mineral oil. MO was prepared at a concentration known to induce dural nociceptor activation in anesthetized animals[19] and in our behavioral studies[20]. The vehicle injection was 100% mineral oil (Min oil). Prolactin and Prolactin receptor antagonist (ΔPRL) was made in synthetic interstitial fluid (SIF) (pH 7.4, 310 Osmolality). SIF was used at pH 7.0. and pH 7.4. Fluorogold (Fluorochrome, LLC) was dissolved in synthetic-interstitial fluid (pH 7.4, 310 Osm) to 4%.

Grimace Scale

Coding of facial expressions was conducted using a 3-point scale (0=not present, 1=moderately present & 2=obviously present) [21]. Observers were blinded in order to prevent bias when attempting to score facial expressions. Average grimace score was determined and analyzed as described below.

Mouse Behavioral Testing

Male and Female adult ICR mice were habituated to tapered single poly-coated paper cylindrical facial testing chambers (2.5" top diameter, 1.75" bottom diameter) while contained in clear acrylic compartments (2" Length x 3" Width x 9" Height) for three days prior to baseline. The use of this larger compartment lets mice freely access and explore the facial testing chamber without physical restraint but they are unable to move about the acrylic compartment. Animals were not deprived of food or water; they were given a food pellet and Hydrogel® (Clear H2O).

Prior to injection, baseline facial and hindpaw thresholds were recorded for each animal. Using a 0.5mm injector, an injection was made between the sutures of the skull (see injections). The animals were then returned to the testing boxes. Facial and hindpaw thresholds were measured at 1, 3, 5, and 24 hours post-injection using the Von Frey up-down method of testing. The Von Frey filaments were applied to the peri-orbital region of the face or to the plantar surface of the hind paw perpendicularly until the entire force was applied and held for approximately 3 seconds or until animals withdrew. Maximum filaments used were 0.6 g for the peri-orbital region and 2 g for the hindpaw. In priming experiments facial and hindpaw withdraw thresholds were measured until mice recovered then a subsequent stimulus was given as described.

Rat Behavioral Testing

The animals were habituated in the testing chambers for a minimum of one hour prior to baseline. Animal weights were recorded and dural injections were given post baseline. Testing of both facial and hind paw allodynia was conducted every hour for 5 hours using calibrated Von Frey filaments thresholds were determined by the "up-down" method[22]. The Von Frey filaments were applied to the peri-orbital region of the face or to the plantar surface of the hind paw perpendicularly until the entire force was applied, and held for approximately 5 seconds or until animals withdrew. Maximum filaments used were 8 g for the peri-orbital region and 15 g for the hindpaw. Upon completion of allodynia testing, all animals' cannula patency was verified by ink injection into the cannula and dissection post mortem. The experimenter collecting measurements was blinded to the experimental conditions

Stress

Animals were habituated to facial testing chambers. The next three consecutive days animals were stressed in tail vein injector tubes (Stoelting, 15-30 gm) for 2 hours each day. Weights were recorded on all habituation and stress days. Animals were placed in stress chambers at the same time on consecutive stress days and stress was always conducted before 12 pm. Controls stress animals remained in their home cages deprived of food and water for the same two-hour span in a separate room. Testing for facial and hind-paw allodynia was conducted at the following time points post stress: 24 hours, 72 hours, 7 days, 10 days, 14 days.

Enzyme-linked immunoassay (ELISA)

Blood was collected using submandibular puncture[23]. Blood was were centrifuged at 14,000 rpm for 15 min at 4 °C in EDTA coated tubes. Supernant was collected and stored at -20

°C. The following kits were used according to the manufacturer's protocol: Corticosterone (Bicin Choninic Acid; Pierce, Rockford, IL); CGRP ELISA Kit (Cayman Chemicals, Ann Arbor, MI, cat# 589001) was used to measure serum CGRP levels. Each sample was run in duplicate and data were reported as pg/uL respectively.

Data analysis

All behavioral data are graphed as means ± SEM. Statistical evaluations of allodynia studies were conducted using GraphPad Prism Version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Data was analyzed among groups and across time by one- or two-way analysis of variance (ANOVA) for treatment and time followed by Bonferroni post-test where appropriate.

Results

Dural application of 0.5 µg of PRL caused significant grimacing at 3 hours and 5 hours in females but not males (Figure 5.1). Application of 0.5 µg of PRL to the dura elicited robust facial and hindpaw hypersensitivity (Figure 5.2), for at least 7 days in females but not males..

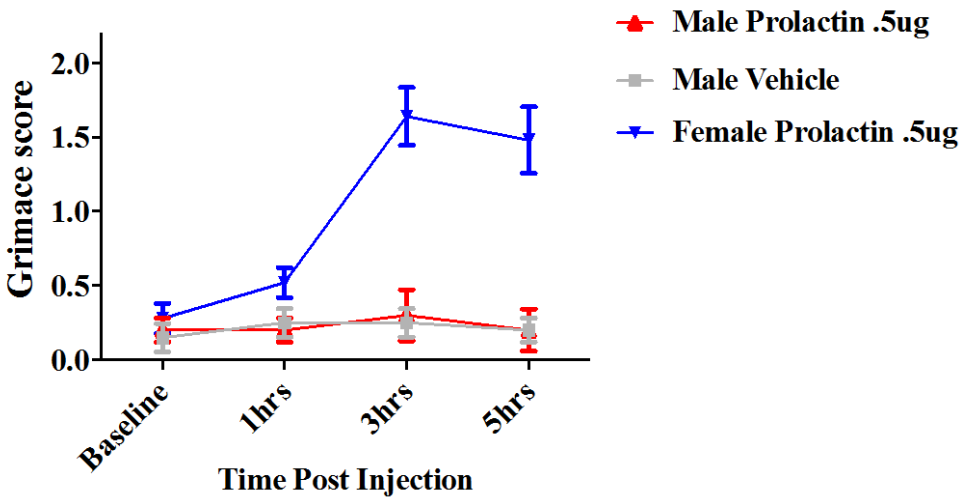

Figure 5.1. Prolactin produces robust grimacing in females

Following return to baseline from PRL, at 72 hours, females were sensitized to dural stimulation with a pH 7.0 solution, a typically subthreshold stimulus (Figure 5.3).

Restraint stress causes allodynia 24 hours post-stress, that is resolved by 14 days post stress. Stress sensitizes animals to dural pH 7.0 and to a systemic nitric oxide donor in females (Figure 5.4). Serum PRL was measured 10 min following stress and 10 min following habituation to the facial testing chambers. Serum PRL was increased only in females on day 1 and day 3 of stress (Figure 5.5).

Application of 1.9 pg CGRP to the dura produced cutaneous facial and hindpaw allodynia selectively in females, and this behavior was blocked by a PRL receptor antagonist (Figure 5.6).

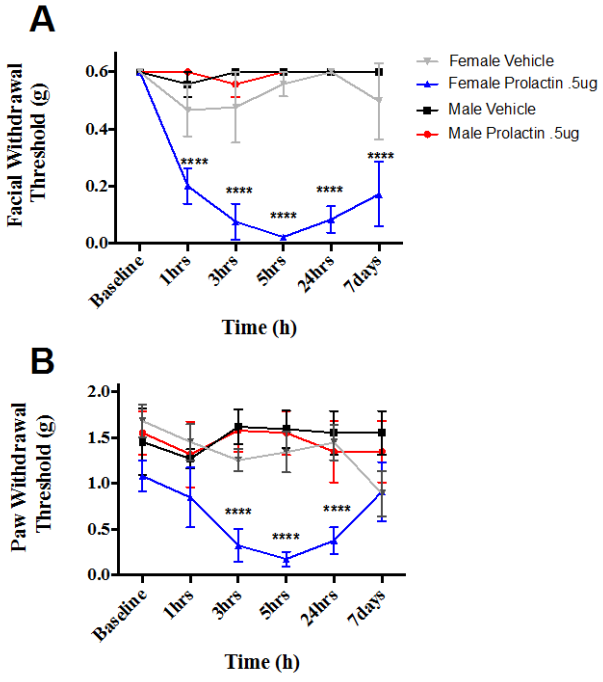

Figure 5.2. Prolactin produces facial and hindpaw allodynia selectively in females

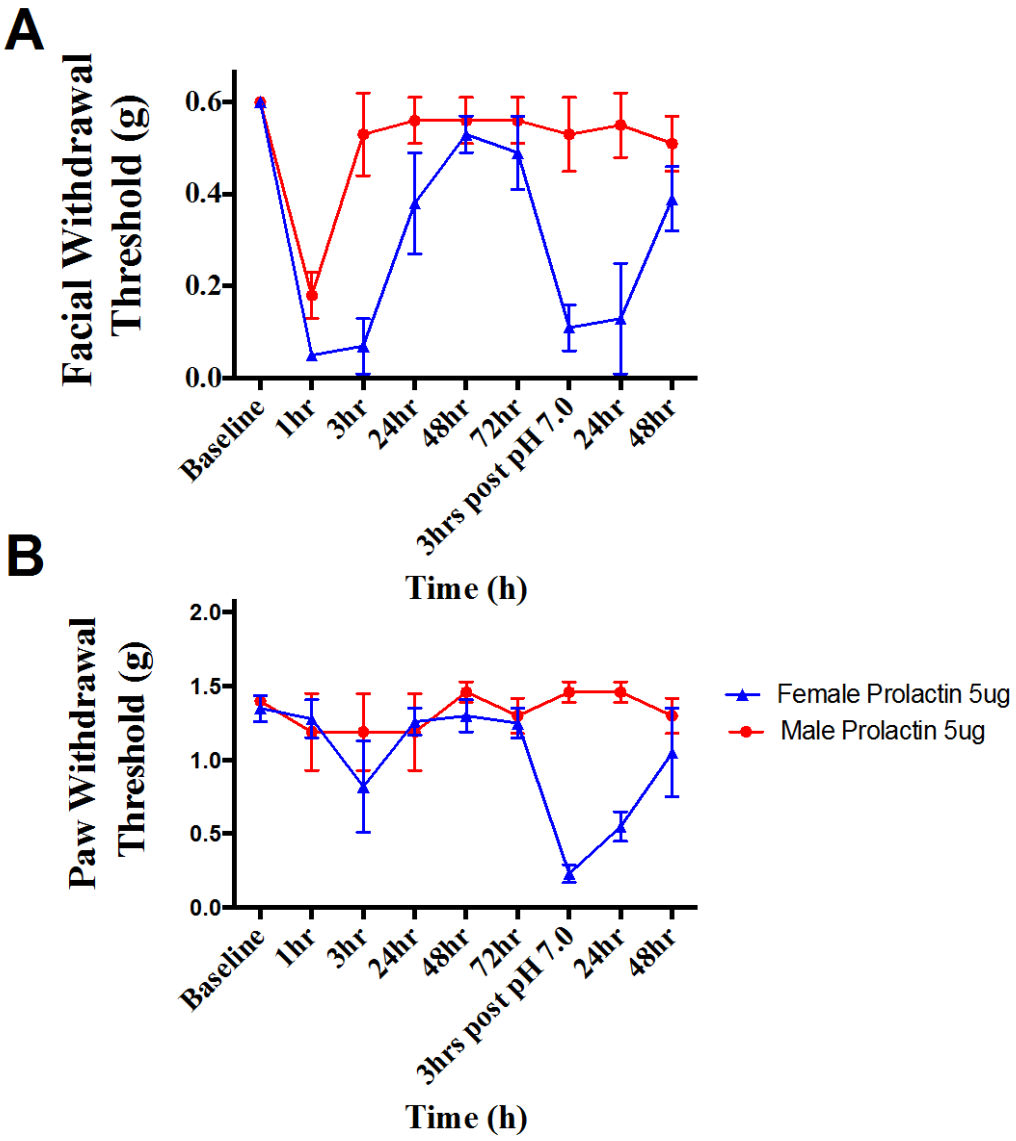

Figure 5.3. Prolactin sensitizes females to dural pH 7.0

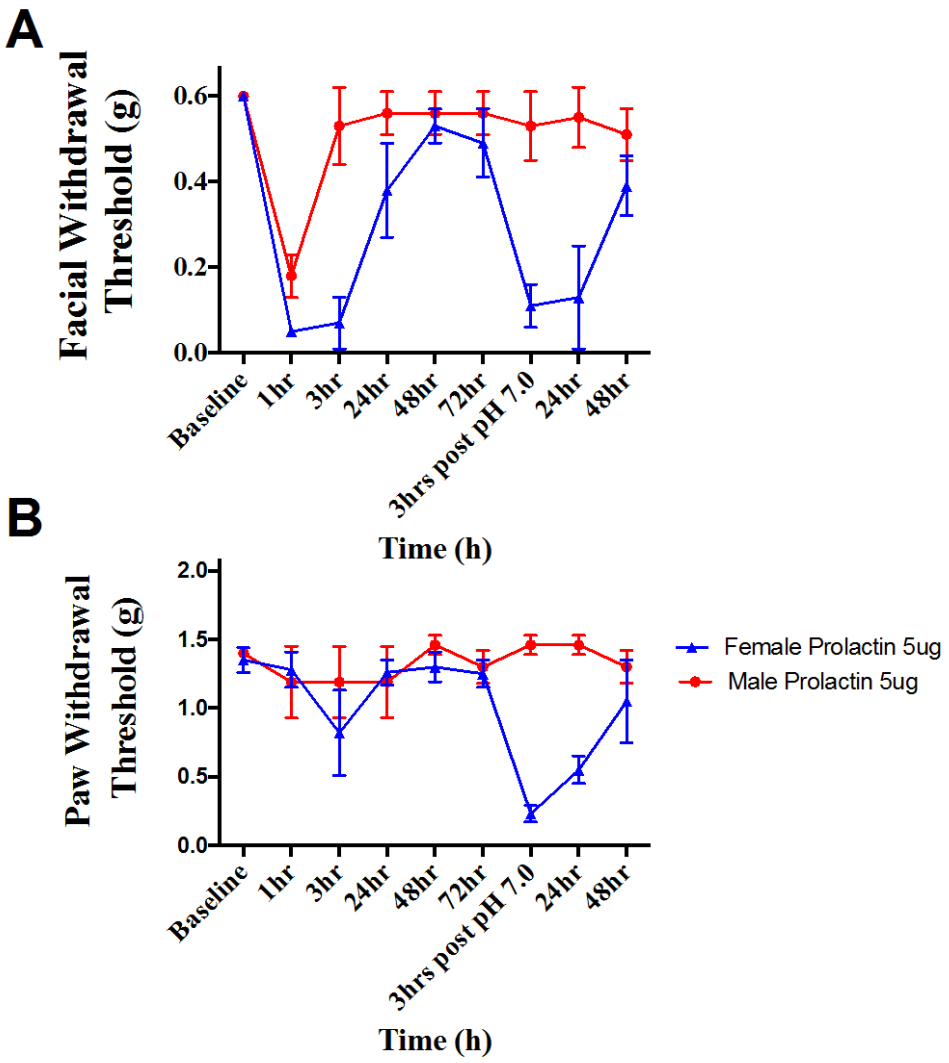

Figure 5.4. Stress sensitizes females to systemic NO donor (SNP) on day14

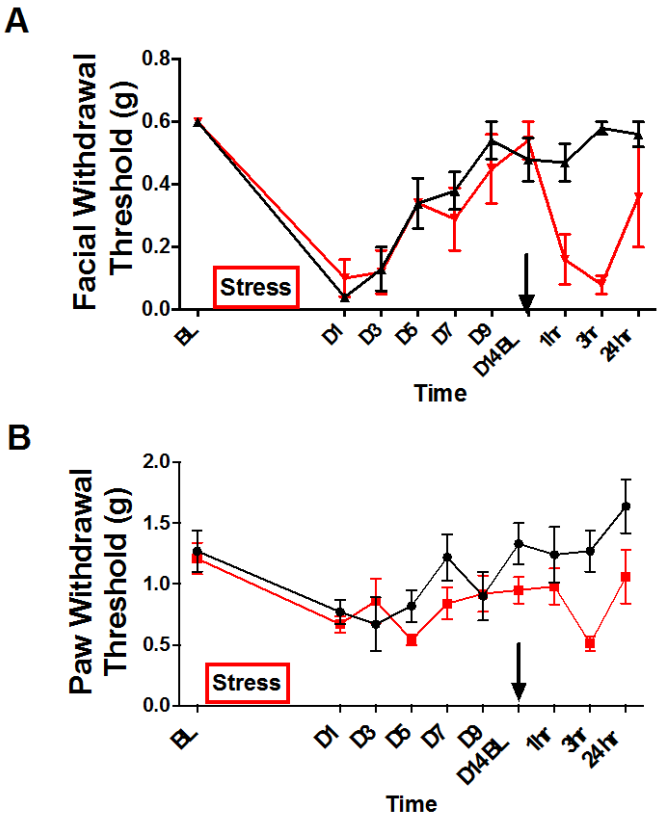

Figure 5.5. Stress sensitizes females to dural afferent to pH 7.0 on day 14

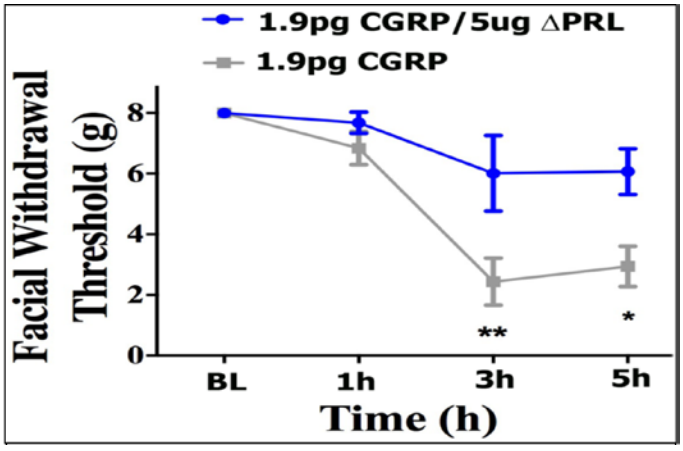

Figure 5.6. Responses to dural CGRP are attenuated with a PRL antagonist

Discussion

Migraine headache causes severe uni-lateral head pain and is the 8th most disabling condition worldwide. The severity of headache attacks between women and men are similar, but the frequency of migraine is significantly higher in women[24-26]. Migraine affects as many as three times as many women as it does men in the adult population in the United States (~18% vs. ~ 6%) [2]. However, this marked gender disparity is not present in adolescents. Prior to puberty the incidence of migraine is 4% for both males and females [5-7,26]. The trigeminal afferent system in humans may be influenced by hormonal changes that occur in the transition from adolescence, which could underlie sexually dimorphic prevalence seen in adults. Interestingly, the trigeminal afferent system of rats has shown differences in sensitization between adolescent and adult rats[27]. Other preclinical studies indicate that the trigeminal vascular system can be modulated by sex hormones at both expression and functional levels[28,29]. For example, receptive field sizes of neurons in the spinal trigeminal nucleus of rats are larger when estrogen levels are high[11]. Acute application of estradiol to trigeminal neurons in vitro increased functional activity of pro-inflammatory bradykinin receptors[30]. Mechanisms mediating the sexual dichotomous presentation of migraine have yet to be elucidated, despite substantial evidence for the involvement of sex hormones.

Estradiol regulates serum PRL levels throughout the estrous cycle[31,32] and levels are altered by psychological or physical stress[33]. The traditional role for hypothalamic PRL involved its role in the initiation of milk production throughout lactation[32]. However, elevation of serum PRL is *strongly correlated* to migraine attacks [34,35] but the mechanisms by which PRL may

contribute to migraine pain are not known. It has been reported that PRL can be synthesized in extra-pituary sites and act both as a circulating hormone and a cytokine[36]. Yet, the function of PRL in afferents innervating the dura/meninges of females and males has not been studied. Despite the clear effects of estrogen on mediators implicated in migraine, the precise actions of sex hormones on the trigeminal afferent system is not yet clear.

Estrogen is known to regulate serotonin and calcitonin gene-related peptide (CGRP) [37,38] with most studies showing that CGRP, CGRP receptor, serotonin (5HT), and 5HT receptor expression are positively modulated by estrogen[17]. 5HT and CGRP have been strongly implicated in migraine pathophysiology [39-41] and drugs targeting both transmitter systems are efficacious for migraine. However, the contribution of estrogen to modulation of trigeminal CGRP and the 5HT1b/1d receptors (those activated by triptans) have not shown consistent effects[42-44]. Recently a study found that intra-dermal sumatriptan induced dose-dependent decreases mechanical thresholds in females, but elicited no such effects males[45]. Previous studies found CGRP administered onto the dura of male rats was unable to sensitize dural afferents[46]. Our results indicate that while males fail to respond to high doses of CGRP, females are sensitive to CGRP. These results may implicate that sex-dependent Prlr activity in dural afferent peripheral terminals could differentially impact neuronal CGRP release in females and males. Females innately have more Prlr activity, and this may positively modulate CGRP release leading to increased vasodilation and neurogenic inflammation in the dura, that may contribute to the development of migraine. Here we demonstrate sex dependent trigeminal sensitivity by prolactin and CGRP. These studies provide additional evidence that hormones influence pain processing in the craniofacial region. In the future the efficacy of antibodies to Prlr should be

investigated in the context of migraine disorders; both in preclinical models and eventually humans. Ultimately, further studies must be conducted to eludicate the role of Prlr and CGRP in the sexually dimorphic clinical presentation of migraine.

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CHAPTER 6

CONCLUSIONS

Potential Mechanisms of Dural Afferent Activation

Dural afferents are capable of sensing noxious stimuli generated within the dura and the pain that arises during migraine is likely to involve the activation of these primary afferent nociceptors[1,2]. However, the events that lead to the activation and/or sensitization of these neurons are not fully known[3-5]. The dura is often overlooked as just one of the three meningeal layers, however the dura mater regulates events in the overlying skull during development. Ultimately the dura will control the size and shape of the cranial bones, along with suture patency, and the proper maturation of the these bones and sutures is dependent on interactions with the dura mater[6,7]. Whether there is any interaction between the dura and the skull that contributes to migraine is not known but, interestingly, some afferents that innervate the dura send branches that penetrate into the skull, particularly at sutures[8-10]. There are thus sensory afferents that innervate both tissues suggesting importance of both in signaling from the dura.

Within the dura mater are fibroblasts, which generate the fibrous tissue and accompanying elastic fibers. These cells have recently been shown to play a potential role in headache as they release factors that both sensitize dural afferents and cause headache behaviors in rodents when applied to the dura[11]. Among the factors released by these cells is the pro-inflammatory cytokine interleukin-6 (IL-6). The dura also contains other non-neuronal cell types such as mast cells and macrophages. There are numerous arteries within the dura mater, the most prominent being the middle meningeal artery

(MMA) and the branches originating from this artery. Mast cells are known to aggregate around blood vessels within the dura and may play potential role in migraine pathophysiology[12,13]. Mast cells have also been found in direct contact with afferents within the dura, which could allow for potential modulation[12-14]. The degranulation of meningeal mast cells has been shown to activate and sensitize dural afferents and this is due, at least in part, to activation of the protease-activated receptor 2 (PAR_2) by mast-cell derived tryptase [14-17]. Mast cell degranulation can occur as a result of factors known to be associated with migraine in humans including; the release of the stress hormone corticotropin releasing factor (CRF), CGRP release, nitroglycerin infusion, and also increases in estrogen [15,17-19]. Degranulation of mast cells could result in the release of factors such as histamine, serotonin, and protons capable of contributing to sensitization of dural afferents. Among this list of chemical mediators, many can sensitize dural afferents resulting in increased excitability. Application of a cocktail of inflammatory mediators (IM) that included histamine, serotonin, bradykinin, and PGE2 all at pH 5.0 cause widespread cutaneous allodynia in rodents. [20,21] This has been shown recently following application of bradykinin, PGE_2, and histamine leading to sensitization of ionic currents that contribute to the increased excitability of dural afferents[11,22].

Ion Channels and Dural Afferent Activation

Acid Sensing Ion Channels or ASICS are a family of 4 ion channels consisting of, ASIC1-ASIC4, with several splice variants[23-25]. ASIC1-3 are sensitive to various ranges of pH from approximately 4.5 to just above 7.0. ASIC4 is not a pH sensitive channel. Although ASICs are abundant throughout the nervous system, including expression

primary sensory neurons, ASIC3 is largely restricted to the periphery [25,26]. ASICs on dural afferents may act as an indicator of changes in extracellular pH. However, a change in extracellular pH within the dura remains to be demonstrated. Cortical spreading depression (CSD), which may contribute to migraine headache through activation of the trigeminovascular system, [27,28] was found to be associated with a decrease in dural blood flow [29,30] as well as cortical hypoxia [28,30,31], events that may decrease the pH of the cortex and/or meninges.

ASICs may play a role in dural afferent signaling and as a result, in the pain of migraine. Decreased pH has been shown to activate dural afferents in several studies using *in vivo* electrophysiological recording techniques. These studies found the following: pH 4.7 activated 75% of rat dural afferents 8, pH 5.0 activated 37% in guinea pigs [32], and pH 6.1 activated 64% in rats [33]. Although these studies show pH sensitivity of dural afferents, they did not identify mechanisms. Electrical stimulation of the dura activates afferent signaling that is blocked by the ASIC blocker amiloride[34] . And pH 5.4 to 5.9 promotes calcitonin gene-related peptide (CGRP) release from dura and trigeminal ganglia that is sensitive to ASIC blockers [35,36].

However, one of the best examples supporting a role for ASICs in migraine is the finding of ASIC currents in 80% of dural afferents at pH 6.0 and 50% at pH 7.0 [37] that were blocked by amiloride or activated by an ASIC3-specific agonist [37]. When low pH (from 5.0 to 6.4) was applied to the rat dura, animals displayed headache-related behaviors that were blocked by ASIC antagonists. These findings provide some of the most direct evidence that ASICs contribute to processes related to migraine.

Recently, it was also demonstrated that CSDs are sensitive to ASIC blockers [34] implicating these channels in migraine-related pathophysiology. Also included in this study was an exciting clinical trial on 7 difficult to treat migraine patients. When given the ASIC blocker amiloride, 4 out of 7 patients achieved significant relief of both headache and aura symptoms with minimal side effects. These studies suggest that targeting ASICs in humans may be an effective strategy for novel migraine therapeutics.

Mast cell degranulation (discussed above) may decrease meningeal pH as the contents of mast cell granules are approximately pH 5.5 [38]. This may lead to pH-dependent activation of dural afferents and these nerve endings may also be sensitized by additional factors released from mast cells. Supporting this idea is the observation that the number of dural afferents that fire action potentials is doubled at pH 7.0 in the presence of mast cell mediators (40% vs. 20% in controls) [37]. This study also showed that higher pH values applied to the dura can produce headache behaviors in the presence of mast cell mediators than under control conditions. Together, mast cell degranulation may provide both a drop in pH and a sensitizing stimulus enhancing the afferent input mediated by dural afferents. These studies argue for further investigation of ASICs for a role in migraine and ultimately as novel therapeutic targets.

TRP channels are a family of membrane ion channels known to be responsive to stimuli such as temperature, changes in extracellular osmolarity, pH, and an extensive list of natural products [39-43]. There are 6 subfamilies of TRP channels, denoted with a letter corresponding to the primary amino acid sequences; TRPA, TRPC, TRPM, TRPP, TRPL, & TRPMV [44-46]. TRP channels are non-selective ion channels that aid in depolarization as

well as activation of second messenger signaling pathways via influx of Na^+ and Ca^{++} [47]. They are proposed to contribute to many types of sensory input in varying tissues from cutaneous to visceral and have been implicated in many pain conditions [40,48] [49,50].

Relevant to migraine, the TRP channel most implicated in headache pathophysiology is TRPA1. TRPA1 is activated by a diverse array of substances including the environmental irritants acrolein[51,52], chlorine[53,54], formaldehyde[55,56], and cigarette smoke[57,58], natural products like cinnamaldehyde[22,59], isothiocyanates[60], and allicin [61] and endogenous substances like 4-hydroxynonenal[62,63], nitro-oleic acid[64], and reactive prostaglandins[65]. Many of these are linked to migraine, commonly as triggers[66-69].

Preclinical studies continue to build a case that TRPA1 contributes to the pathophysiology of headache. TRPA1 is expressed on [70] and generates currents [71] in dural afferents in rodents. When TRPA1 agonists, including acrolein, are introduced into the nasal cavity, there is an increase in meningeal blood flow that is blocked by either a CGRP or a TRPA1 antagonist. This study implicates TRPA1 in headaches due to inhaled environmental irritants[72]. More direct evidence comes from studies showing that dural application of mustard oil produces headache behaviors (both cutaneous facial hypersensitivity and a decrease in exploratory activity) that are blocked by an oral TRPA1 antagonist.

Interestingly, a tree native to northern California and southern Oregon known as the “headache tree” *(U. californica)* can trigger cluster headache-like attacks in individual susceptible to headaches [69]. A primary component of the extracts of this tree is

umbellulone, a TRPA1 activator [69]. Dural application of umbellulone leads to headache behaviors identical to those described above with mustard oil [71]. How umbellulone gains access to the meninges to produce headache is not clear. It may be via the nasal route since preclinical studies found that intranasal delivery can produce meningeal vasodilation in a TRPA1-dependent mechanism [52]. Together, the studies described here provide an intriguing case for a contribution of TRPA1 to headache and like ASICs, argue for the development of TRPA1 therapeutics to test in migraine patients.

As described above, dural afferents are mechanically sensitive but they are also sensitive to changes in extracellular osmolarity [73] suggesting that they express the mechano- and osmo-sensitive channel TRPV4 [74-77]. Prior studies have shown TRPV4 mRNA in trigeminal ganglion neurons [78] and the channel appears to be functional, at least *in vitro* [79,80]. Deletion of TRPV4 in mice results in a loss of the ability to detect both osmotic and pressure stimuli . Additionally,[81,82] TRPV4 can be sensitized by pro-inflammatory mediators and its mechanosensitivity may be enhanced under inflammatory conditions as administration of inflammatory mediators to the hindpaw leads to osmotic and mechanical sensitivity that is lost in TRPV4 null mice [83].

Recently, TRPV4 currents were found on dural afferents *in vitro* in response to hypotonic stimulation and the TRPV4 activator 4αPDD [84]. Using the behavioral model described above, application of TRPV4 activators to the dura of rats caused headache behaviors [84], indicating that these channels in the dura can contribute functionally to headache. Changes in osmolarity, at least to the degree required to activate TRPV4, do not likely contribute to migraine. But as mentioned previously, changes in intracranial

pressure (head movements, coughing etc) can worsen headache in migraine patients [2] and this may be mediated by the mechanically-sensitive TRPV4. More work is still necessary to determine whether TRPV4 contributes to headache in humans and whether targeting this channel may have efficacy for migraine.

The TRPM subfamily contains eight members: TRPM1–M8. The TRPM8 channel is sensitive to both cold temperature as well as chemical cooling agents such as menthol[85-87]. TRPM8 is activated by both noxious and non-noxious temperatures from ~ 28°C to 8°C[88]. TRPM8 transcripts are expressed in a small percentage (<15%) of small diameter sensory neurons [85,87]. TRPM8 expression within the nervous system is thought to confer innocuous cold sensitivity to the somatosensory system [89,90], especially in sensory neurons innervating cutaneous tissues. TRPM8 is not isolated to the cutaneous afferents and has been found in neurons innervating deep tissues such as the bladder and colon[91,92]. As these neurons are not directly exposed to external cold stimuli, cold may not be an exclusive activator in these tissues supporting the hypothesis that the channel may respond to endogenous activators. [93,94] Possible endogenous activators/sensitizers in visceral tissues include lysophospholipids, cyclopentenone prostaglandins, and phosphatidylinositol biphosphate among others. [42] Ultimately, TRPM8 may be able to sense various internal and external stimuli. Additionally, recent genome wide association studies (GWAS) have consistently revealed single nucleotide polymorphisms (SNPs) in and around the TRPM8 locus in several populations of migaineurs [95-97] further strengthening the hypothesis of possible TRP channel involvement in migraine. It is not currently known how these mutations impact channel function but these data nonetheless

provide exciting support for a potential role of this channel in the pathophysiology of migraine.

Dural Afferent Input may lead to Neuroplasticity

Although the events that actually initiate a migraine attack remain unknown, the ultimate activation of the trigeminovascular system is considered to be essential[98,99] . Trigeminovascular activation may provoke release of multiple excitatory neurotransmitters including substance P, neurokinin A, and CGRP from dural afferent terminals resulting in neurogenic vasodilation of dural blood vessels, release of proinflammatory mediators, degranulation of mast cells and plasma protein extravasation [100-102]. Activation of these primary nociceptors innervating the meninges may also play a role in maladaptive changes in central circuits that contribute to migraine. However, how these nociceptors might contribute to central plasticity is not well understood. Dural afferents project into the trigeminal nucleus caudalis (TNC) and activation, or repetitive activation of these neurons may contribute to plasticity in the central circuitry in the TNC that contributes to the pathophysiology of migraine (Figure 2). Preclinical studies have demonstrated widespread cutaneous allodynia following dural afferent stimulation with a variety of compounds[37,71,103], an effect that implicates plasticity at central sites following stimulation of the dura. Migraineurs also experience hypersensitivity that can diverge from the initial or primary site of the spontaneous head pain over the course of an attack [104,105]. For example, patients commonly initially present with facial allodynia that over time expands to extracephalic regions as well [2], again strongly implicating central plasticity during migraine attacks. The mechanisms that contribute to the plasticity likely

required for these observed changes have yet to be fully elucidated. Elevated cytokines, neuropeptides, and growth factors such IL-6, CGRP, and brain-derived neurotrophic factor (BDNF) have been shown in patients during migraine attacks compared to migraine free periods[57,106,107], and these mediators, among others, may contribute to the development of neuronal plasticity.

Neuroplasticity and Migraine

As discussed above, there are a number of proposed mechanisms that may contribute to the pathophysiology of migraine, one of which involves "neuroplasticity" in the peripheral and central nervous systems. There are two different kinds of neuroplasticity, one that is beneficial, contributing processes within the brain that lead to learning and memory, and one that is harmful, contributing to pathological pain or migraine. The underlying mechanisms mediating these forms of plasticity are thought to be similar. Plasticity in the nervous system manifests as a change in the response of a neuron or circuit to a stimulus such that after plasticity develops, a given stimulus produces a different response than before the onset of plasticity. In the case of pain, this can present as an increase in sensitivity of primary sensory neurons or a painful response in an individual to a normally non-noxious stimulus. For example, cutaneous allodynia or pain in response to touching of the skin is thought to be mediated by plasticity within the nociceptive system. Migraine is another example of maladaptive plasticity in the nervous system, especially since it seems to play no beneficial role in warning affected patients of ongoing tissue damage. Migraine is a chronic, episodic, pain disorder in which there is no identifiable injury or pathology. Consequently, the changes within the body that lead to

migraine are likely subtle. This strongly suggests maladaptive plasticity to relatively normal events. Additionally, the rate at which migraine episodes occur often increases. Approximately 14% of patients who develop episodic migraines will transition to chronic migraines (15 or more headache days per month), suggesting a pathophysiology based on plasticity [108-110]. These data suggest that the presence of migraine may contribute to further plasticity that perpetuates the disorder.

In general, neuroplasticity can occur throughout the central and peripheral nervous systems at many sites within the neuron including at synapses, near the cell soma (e.g. at the axon initial segment), or in axons. Relevant to the pain of migraine, the peripheral neurons, where plasticity is likely to occur are meningeal afferents (innervating the dura and pia) and the central endings of these neurons at synapses in the TNC(6.1 & 6.2). Importantly, prior studies implicate both of these anatomical locations as potential sites of plasticity contributing to migraine.

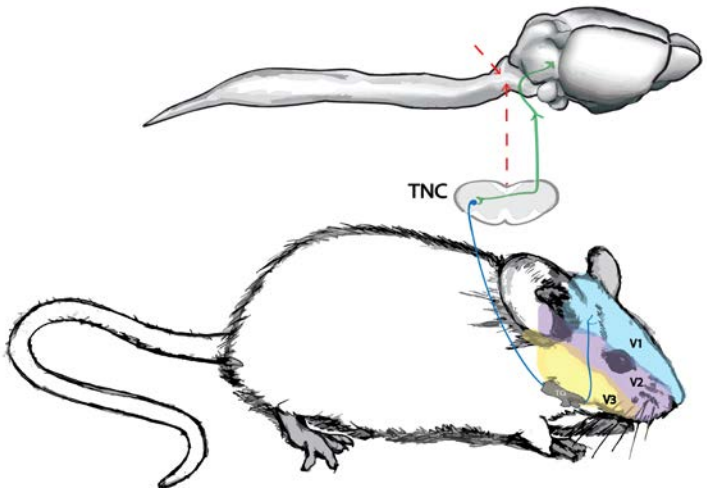

Figure 6.1. Anatomical organization of trigeminal dermatomes in mouse.

The dura mater is almost entirely innervated by pain-sensing fibers, Aδ and C [89], generally considered to be responsible for migraine pain [4,99,111]. As mentioned above, the pain of migraine is commonly described as unilateral, spontaneous, and throbbing in nature and it is also well documented that changes in intracranial pressure such as head movements or coughing, can exacerbate headache pain. This clinical picture fits with the preclinical observation that meningeal afferents are responsive to mechanical stimulation [99,112,113]. However, it is not clear whether baseline mechanical sensitivity contributes to the pain of migraine or whether plasticity in these fibers plays a greater role. Plasticity in meningeal afferents is supported by the increase in their mechanical sensitivity after application of noxious stimulants [114]. After sensitization, these neurons may respond to stimuli that normally do not initiate activity. They may then generate afferent input in response to normal events such as coughing, which under normal circumstances does not cause headaches. These neurons may also begin to respond to normal arterial pulsations following sensitization that contributes to the throbbing nature of migraine pain.

Headache-producing events within the meninges can lead not only to activation/sensitization of primary afferents but also to sensitization of the central terminals of these neurons in the TNC [115]. The second-order neurons in the TNC, where meningeal afferents synapse, receive input from all primary trigeminal neurons innervating tissues throughout the head (Figure 6.2). The second-order neurons that receive input from the dura can also receive input from the facial skin, particularly after sensitization when the receptive fields of these neruons expand. It has been shown that after applying an inflammatory soup to the dura, there is a development of cutaneous

sensitivity in the periorbital region [115]. Chemical stimulation of dural afferents is shown to expand cutaneous trigeminal and cervical receptive fields in the TNC [116], lowering the threshold for pain sensitivity in the skin of the face and cervical regions. When recording excitability from second order neurons in the TNC that receive input from the dural meninges, there is a development of facial input from these neurons (in response to tactile stimulation) after applying chemical mediators such as capsaicin, inflammatory soup, or mustard oil to the dura [32,71,73,84]. This demonstrates that peripheral activation of afferents in the dura leads to plasticity at second order neurons in the TNC such that input from the facial skin is now unmasked. These mechanisms described above may explain the cutaneous cephalic allodynia observed in the majority of migraine patients during the headache phase [99].

While the exact mechanism of how these plastic changes happen is unknown, it is clear that there is a change in the mechanical threshold of peripheral afferents and also in the receptive fields of second-order neurons in the TNC receiving input from the dura. One potential explanation for these events is that activation of dural afferents could cause the release of neurotransmitters that can lead to a maladaptive state. This may be due to the release of substance P and CGRP from afferent endings in the dura, promoting the degranulation of mast cells, and sensitizing afferents to mechanical and other stimuli. Central release of transmitters in the TNC may also lead to loss of inhibitory networks such that afferent input from distant sites (such as facial skin), which would normally not gain access to second-order neurons with dural input, begin to activate these pathways leading to facial allodynia along with headache. The candidate transmitters leading to

plasticity in migraine are not fully known, but among them CGRP has received the most attention.

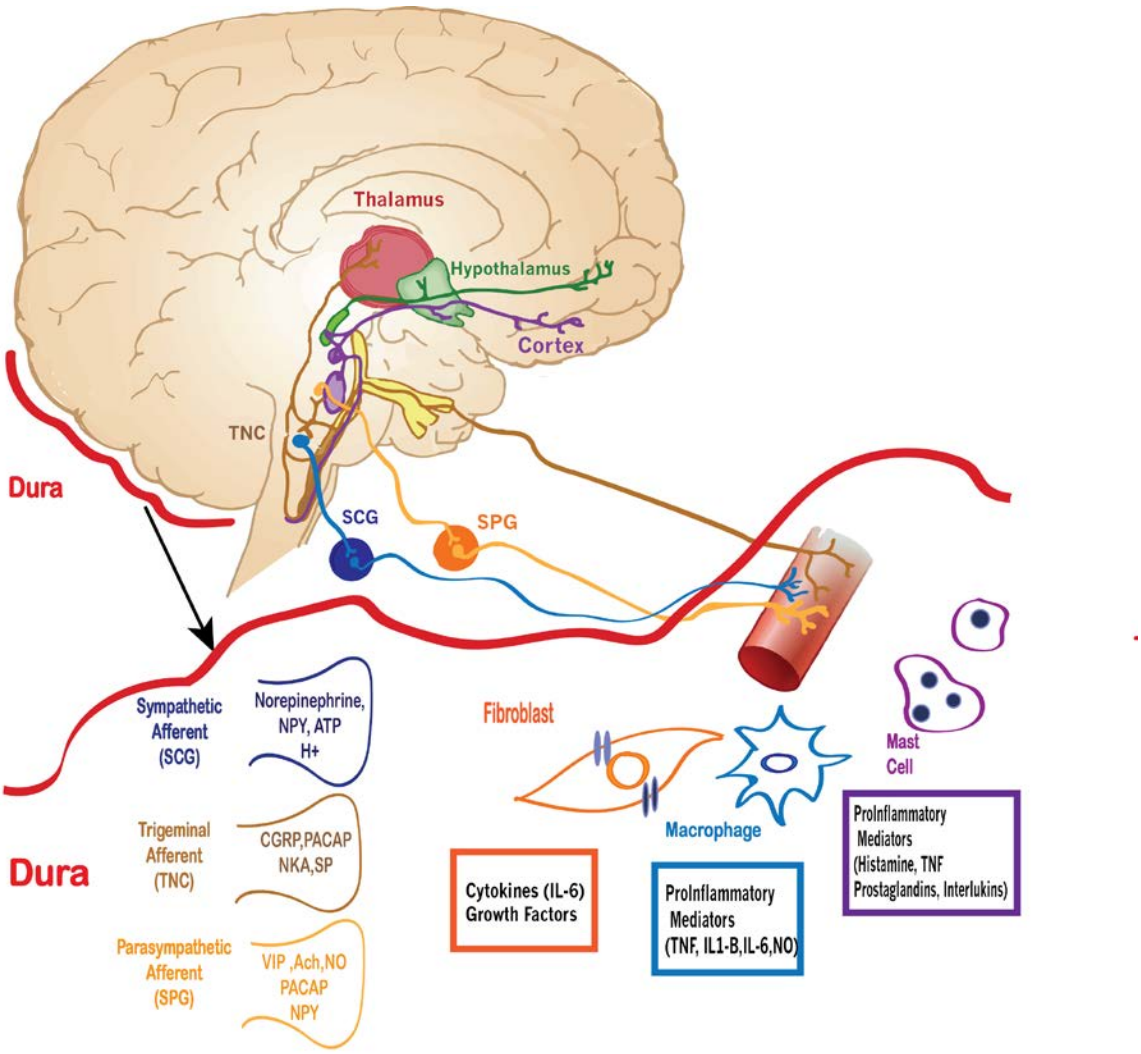

Figure 6.2. Schematic of afferent innervation and resident cells within the dura mater.

Cell bodies of cerebrovascular and dural nociceptors reside in the trigeminal ganglion (TG), sphenopalatine ganglion (SPG), and superior cervical ganglion (SCG). Second-order neurons are found at the level of the brainstem within trigeminal nucleus caudalis (TNC). The close relationship of afferents with both the vasculature and resident cells in the dura allows for possible direct and indirect mechanisms of nociceptor activation-that may ultimately lead to migraine.

CGRP and Migraine Plasticity

Numerous studies show that CGRP plays a critical role in migraine headache. CGRP is a neuropeptide produced by peripheral neurons commonly affiliated with nociceptive signals entering the spinal cord due to its release from C-fibers at this site [117-119]. The role of CGRP in migraine is still evolving, but many advances have been made recently. As described above, it was previously hypothesized that the cause of migraine pain was due to vasodilation of blood vessels innervating the cerebrum [62 100] and CGRP released from trigeminal sensory neurons caused this dilation [6]. While it is no longer thought that vasodilation is the cause of migraine pain, CGRP is proven to play a key role in the pathophysiology of migraine [6]. In migraine patients, levels of blood CGRP are elevated during migraine attacks [102], and in animals, dural activation precipitates the release of CGRP [1]. It has also been shown that intravenous delivery of CGRP triggers migraine headaches in migraineurs, however, no headaches were observed in healthy individuals [3,5]. These studies have lead to a number of studies looking at the different therapies for migraine patients by blocking the effects of CGRP [6]. While it is now widely accepted that CGRP plays a role in migraine, its mechanism is still unknown. The question remains as to how and where CGRP is acting to contribute to migraine pain.

There are two main sites where CGRP can act to contribute to the mechanisms of migraine: at the level of meninges where it could be released from the peripheral terminals of nociceptors, or at the spinal trigeminal nucleus where it could be released from the central terminals of nociceptors. Blocking CGRP receptors via intravenous delivery of an antagonist, olcegepant, led to a decrease in markers of afferent activity in

the trigeminal ganglion [12]. CGRP receptor antagonists at the central site in the TNC were also able to block markers of afferent activation in second-order neurons [12]. However, dural application of the CGRP antagonist failed to be effective in preclinical models [15]. These studies add to the uncertainty of where CGRP antagonists act in migraine. CGRP can trigger the release of inflammatory agents [15 20 23] and these agents may participate in the activation/sensitization of dural afferents. The lack of efficacy of dural CGRP antagonists is thus not easy to explain. However, recently published clinical data demonstrate efficacy of a systemically-delivered antibody to CGRP in migraine[120]. Given the unlikely access of antibodies to central sites, these data support a peripheral role for CGRP in migraine.

CGRP has been shown to contribute to synaptic plasticity at both peripheral and central sites. In the periphery, CGRP released presynaptically (at the neuromuscular junction) produces an augmented response to acetylcholine in the postsynaptic terminal through protein kinase A (PKA) mechanisms [26], possibly altering the development of new synapses. CGRP release has been shown to be increased in the spinal cord 8 days after applying inflammatory mediators at peripheral sites [27], suggesting that upregulation of CGRP release from the central terminals of primary afferents in the spinal cord contributes to pain signaling induced by peripheral inflammation. Moreover, a CGRP antagonist was able to reverse synaptic plasticity in dorsal horn synapses in arthritic rats but had no effect on synaptic responses in normal rats [29]. This same study showed a greater enhancement of post-synaptic response by CGRP in neurons from arthritic rats compared to control rats. These effects were found to be post- rather than pre-synaptic.

Similarly, CGRP antagonists acting at postsynaptic sites have shown to be effective in inhibiting pain-plasticity in the amygdala [31]. These studies establish a role for CGRP in plasticity in peripheral and central synapses, but a role for CGRP-induced plasticity in migraine has not yet been established with these types of studies.

BDNF and Migraine

Long-term potentiation (LTP) is an example of neuroplasticity, where the persistent activation of a synapse leads to long-lasting changes in transmission between two neurons. While LTP has long been implicated in learning and the formation of memories at central synapses in the cortex and hippocampus, LTP can play an important role in the dorsal horn of the spinal cord leading to pain plasticity [39]. LTP is separated into two phases: an early and late or maintenance phase. While early phase LTP is known to require the contribution of protein kinases to regulate AMPA receptors [44], the mechanisms involved in the maintenance of LTP has different requirements. The maintenance phase of LTP is thought to involve a member of the neurotrophin family of growth factors: BDNF [116].

BDNF is widely accepted to play a role in the development of LTP in the hippocampus [48] and in LTP-like processes in the spinal dorsal horn [49]. This links BDNF to pain plasticity through memory-like mechanisms. BDNF is released from the central terminals of nociceptive afferent neurons to act on its postsynaptic receptor, tyrosine receptor kinase B (trkB) in the spinal cord [51] [53]. Thus, the activity of BDNF on the spinal cord may be crucial for the development of pain plasticity. This hypothesis is supported by several findings. Application of BDNF to spinal cord slices potentiated synaptic transmission in an NMDA-dependent manner[121]. BDNF is also able to increase functional connectivity of sensory afferents with motor neurons, indicating a role for

BDNF in plasticity in the circuitry mediating pain reflexes[122]. When BDNF is genetically deleted from nociceptors, a variety of acute and prolonged pain behaviors are significantly attenuated[123], implicating a role for the release of BDNF in pain signaling. Further, pain plasticity fails to develop and is also reversed in the presence of an injection of a BDNF scavenger, TrkB/fc, into the spinal cord [55].

While the majority of BDNF related pain plasticity is focused on the dorsal root ganglion and the spinal cord, this concept may be similar in the trigeminal system and the TNC for the pain of migraine. In fact, similarly to CGRP, BDNF levels are increased during migraine attacks in episodic migraineurs [57]. Additionally, CGRP facilitates the release of BDNF from trigeminal neurons [59], suggesting an interaction between the two peptides where release of CGRP during migraine can enhance the release of BDNF. These studies together suggest that BDNF is a contributor to the plastic changes involved in the transition to chronic migraine.

PRL and Migraine

Another candidate mediator for the control of pain by gonadal hormones is the pituitary hormone prolactin (PRL). Estradiol regulates serum PRL levels throughout the estrous cycle[124,125] and levels are altered by psychological or physical stress[126]. Elevation of serum PRL is *strongly correlated* to migraine attacks [127,128] but the mechanisms by which PRL may contribute to migraine pain are not known. It is also not clear whether *modulation is sex-dependent*. The function of PRL in afferents innervating the dura/meninges of females and males has not been studied.

Stress

As previously stated. Stress is the most commonly reported migraine trigger. In fact,

studies demonstrate that anywhere from 59% to over 80% of patients indicate that stress is their primary trigger for migraine[129-131]. Studies show the highest susceptibility to migraine is in the 18-24 hours after a stressful event[132], supporting the complex nature of the contribution of stress to migraine. Stress itself may not be a direct trigger of migraine, rather it may evoke changes in normal behaviors such as sleep or food intake that can aggravate the condition[131]. Acute stress has been shown suppress pain behaviors[133], whereas chronic models of restraint stress have been shown to promote allodynia [134]. Sex hormones may also play a role in stress related behaviors; one study found chronic restraint stress reduced formalin responses only in males[135]. These studies suggest stress may be able to modulate pain, and this remained to be studied in preclinical models of migraine. Here we demonstrate that repetitive mild restraint can induce allodynia, increases in sex linked hormones and meningeal afferent plasticity.

Conclusion

Migraine is the most common neurological disorder and is one of the most disabling disorders worldwide. Surprisingly, the pathophysiological mechanisms contributing to this highly prevalent disorder remain poorly understood. One primary result of the poor understanding of the condition is slow development of novel therapeutics. The gold standard in the treatment of migraine, triptans, were developed on an outdated hypothesis for the mechanism contributing to migraine, vasodilation. Although they have efficacy in some patients, the percentage of patients that achieve complete relief is quite low. In those that do achieve relief with triptans, concerns over MOH with repetitive use can limit the utility of these drugs. Prophylactic agents can have efficacy in the reduction of migraine frequency in some patients, but side effects are

often intolerable. As a result, patients must often choose between frequent migraines and extreme side effects of medications. The lack of adequate treatments for migraine creates a significant negative impact on the quality of life for patients.

Better understanding of the pathophysiology of migraine will undoubtedly lead to more efficacious therapeutics. What was once considered a vascular disorder is now considered a pathology of the nervous system involving the brain and peripheral nociceptive system. While there is much that is still unknown, progress is being made towards uncovering mechanisms that may contribute to migraine, particularly in relation to the meningeal afferent system. Traditionally, the meninges were thought to serve several key protective and homeostatic functions; physical protection of the brain, regulation of hydrostatic pressure in the CNS, and maintenance the blood–brain barrier[136]. However, the meninges have are of clinical significance to migraine because they are the primary intracranial sensory environment. The dura mater was believed to be a metabolically inert and protective fibrous sheath, but is now well defined as home to the neuronal innervation of the meninges. It has been known for decades that stimulation of the dura causes pain in humans, that meningeal afferents mediate this pain, and that these neurons are mechanically sensitive. Unlike sensory innervation in the periphery or cutaneous tissues the dense sensory network of the dura does not relay exteroceptive signals. More recent work has expanded on the understanding of these neurons now implicating a number of processes in their activation. These processes include mast cell degranulation and activation of several families of ion channels such as ASICs and TRPs.

With the discovery of these mechanisms comes potential for development of novel therapeutics based on these targets.

There is also a likely role for neuroplasticity in migraine that needs further exploration. Although, plasticity has been demonstrated, for example, in meningeal afferents with enhanced mechanosensitivity and in the TNC with the expansion of receptive fields in second-order neurons. The molecules and mechanisms that mediate plasticity are largely unexplored in this context. The dura may be capable of augmenting nociceptive responses based on both previous neuronal activation and modulatory mechanisms mediating transduction of noxious stimuli; thus ultimately altering the set point of initiation for the transmission of electrical signals to the CNS that would be perceived as painful through the modification of release of neurotransmitters and neuropeptides at central and peripheral terminals.

CGRP has long been implicated in migraine and it can potentially contribute to plasticity in the meninges, via pro-inflammatory mechanisms, and in the TNC where it acts as a transmitter across the first synapse. It is now clear from late-phase clinical trials that blocking CGRP receptors has efficacy for migraine but it is still not clear how and where CGRP contributes to migraine. Understanding even the location of action of these drugs could help provide insights into critical regions of the nervous system necessary for migraine but this awaits the results of future studies. Additional mechanisms of plasticity in the TNC could involve LTP-like mechanisms, as these have been shown to contribute to pain plasticity in the spinal cord. As with afferents, discovery of mechanisms contributing to plasticity in the TNC may lead to novel therapeutics. Mechanisms have

yet to be determined but may involve BDNF, a plasticity-related neuropeptide that is elevated in migraine patients. Similarly, the potential actions of sex hormones on the trigeminal afferent system is not yet clear. Although, these studies provide evidence of hormones influencing pain processing in the craniofacial region further work is needed to better understand the role of female hormones in pain processes, particularly those most closely related to migraine.

Ultimately, more research is clearly necessary to further uncover the pathophysiology of migraine. Given that migraine is the most common neurological disorder, one that has a significant negative impact on the lives of patients, and one that is poorly treated by current therapeutics, it is difficult to provide a more compelling rationale for the need for research to better understand this complex disorder.

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BIOGRAPHICAL SKETCH

Carolina Burgos-Vega grew up in Tacoma, Washington. She did her undergraduate work at the University of New Mexico and graduated in 2011 with a Bachelor's of Science in Biology and Genetics. During her undergraduate program she worked in multiple research labs investigating genetic resistance in plants. She participated in the Minority Access to Research Program that enabled her to be exposed to a research environment. This program led to her interest in joining the PhD program at the University of Arizona in 2011. Her PhD work is focused on uncovering the underlying mechanisms of the trigeminal system that contribute to migraine headache.

CURRICULUM VITAE

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EDUCATION:

2011-Current	Ph.D. Neuroscience	The University of Texas at Dallas, Dallas, TX
2007-2011	B.S. Genetics and Biotechnology	New Mexico State University, Las Cruces, NM
2007-2011	B.S. Biology	New Mexico State University, Las Cruces, NM
2005-2007	Biology Major	University of Puerto Rico, Cayey, PR

RESEARCH EXPERIENCE:

2012-Present *(Dr Gregory Dussor)*

PhD Dissertation Work:

Investigated mechanisms of sensitization in the trigeminal afferent system.
Conducted studies designed to investigate sex differences in rodent migraine models.
Developed strong in vivo techniques (including aseptic surgery, multiple routes of drug administration, and tissue collection from rodents).
Development, characterization, and implementation of several novel models of migraine in mice.
Designed and tested operant box system to assess photosensitivity and cognitive function in rodent migraine models.
Active participant in collaborative research environment executing experiments in a team and independently.

2011-2012 *(Dr Frank Porreca)*

PhD Rotation:

Screened compounds for synergistic effects when paired with mu/delta opioid agonists in neuropathic pain.
Conducted conditioned place preference (CPP) experiments.
Performed stereotaxic surgeries; (RVM, ACC, & NAc).

2010-2011 *(Dr Tamara King)*

Undergraduate Research Internship:

Investigated the role of the P2X4 receptor in thermal and spontaneous pain.
Became proficient in lumbar puncture injection, spinal nerve ligation surgeries and in intrathecal catheterization (> 95 success rate).

2009-2011

Undergraduate Thesis:

(Dr Champa Gopalan) Isolated and analyzed both 3' and 5' components of a gene of interest to understand regulatory components.
Generated vectors with target genes, and screened for proper integration.
Utilized genetic engineering techniques to modify genes in *Capsicum Annum* in order to select for herbicide resistance

PROFICIENCIES AND SKILLS:

Animal models	Migraine models (surgical & naturalistic); Neuropathic pain models; Spinal Nerve (L5/L6) Ligation; CCI. Inflammatory pain; capsaicin; carrageennan; CFA
Behavioral Assays	Hargreaves; Von Frey; and grimace
Imaging	Confocal imaging; tissue processing & embedding; immunohistochemistry; immunofluorescence; faxitron imaging
Molecular and Cellular Biochemistry	Protein extraction; gel electrophoresis, Western blot, Enzyme-linked immunosorbent assays; PCR; cell culture
Surgical Techniques	Dural cannulations; spared nerve injury; Brennan model; sciatic nerve ligation; chronic constriction injury; intrathecal catheter implantation; cranial window placement; stereotaxic surgeries; fluorogold back-labeling surgery
Management	Interviewing, training, and supervising students/laboratory assistants (> +10); organize project teams; student mentoring (high school - graduate level); Write new Standard Operating Procedures; file amendments to protocols; ordering supplies/reagents; budgeting; maintaining clean/safe lab environment
Pharmacological Techniques	Design/execute experiments and evaluate data. Drug calculations, dose- and time- response curves, agonist and antagonist studies, multiple routes of administration, statistics and plotting
Communication	Fully Bi-lingual (Spanish & English) Able to translate and report scientific findings at student seminars, professional conferences and in peer- reviewed journals

RESEARCH INTERESTS:

Migraine; sexually dimorphic disease presentation; pain chronification; pain plasticity; neuropharmacology

AWARDS AND RECOGNITIONS:

February 2017	Travel Scholarship	American Pain Society, for presentation at the annual APS meeting to be held in Pittsburgh, Pennsylvania, May 2017
December 2017	Grant	Hamilton Company; Hamilton product grant for innovators dedicated to teaching through experimentation
October 2015 their	*Scholarship*	Pleneurethics Society; support for students achieving educational goals *scholarship*
August 2012 related	Award	NINDS Supplement to Promote Diversity in Health- research supplement to 5R01NS072204; The role of ASICs in migraine pathophysiology
August 2011	Award	National Institutes of Health Initiative for Maximizing Student Diversity (IMSD) Graduate Study Program
June 2010 **Disparities**	Summer Research	UA Graduate College Consortium **Minority Health** **Program, University of Arizona**
Jan 2010	*Scholarship*	First recipient of Mr.Ananda & Dr.Uday Segupta University Memorial Scholarship for Genetics Students, New Mexico State University
Jan 2009 University	*Award*	Undergraduate Student Training in Academic Research (U*STAR) Awards (T34) *Scholar*, New Mexico State
May 2006	Deans List	College of Science, University of Puerto Rico
June 2005	Scholarship	Hispanic Leadership Award, Centro Latino
May 2005 Experience)	Internship	Project H.O.P.E. (Health Occupations Preparatory Washington State Health Department

PUBLICATIONS:

Burgos-Vega CC, Quigley LD, Trevisan G., Motina M., Saafar N., Price T., and Dussor G. Non-invasive dural stimulation in mice produces cutaneous facial allodynia. *In preparation*

Moy, JK. Khoutorsky, A. Asiedu, MN. Black, BJ. Kuhn, JL. Barragán-Iglesias, P. Megat, S. Burton, MD. **Burgos-Vega, CC**. Melemedjian, OK. Boitano, S. Vagner, J. Gkogkas, CG. Pancrazio, JJ. Mogil, JS. Dussor, G. Sonenberg, N. Price, TJ. eIF4E phosphorylation regulates behavioral pain plasticity and sensory neuron excitability through sodium current density. The Journal of Neuroscience. *In revision.*

Burgos-Vega CC, Quigley LD, Avona A, Price T, Dussor G. Dural stimulation in rats causes brain-derived neurotrophic factor-dependent priming to subthreshold stimuli including a migraine

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Burgos-Vega CC, Moy J, Dussor G. Meningeal afferent signaling and the pathophysiology of migraine. Prog Mol Biol Transl Sci. 2015;131:537-64 Review. PubMed PMID: 25744685.

Burgos-Vega CC, Ahn DD, Bischoff C, Wang W, Horne D, Wang J, Gavva N, **Dussor G** (2015) Meningeal transient receptor potential channel M8 activation causes cutaneous facial and hindpaw allodynia in a preclinical rodent model of headache. Cephalalgia. May 5. Pii: 0333102415584313. PMC4635063

Tillu DV, Hassler SN, **Burgos-Vega CC**, Quinn TL, Sorge RE, Dussor G, Boitano S, Vagner J, Price TJ. Protease-activated receptor 2 activation is sufficient to induce the transition to a chronic pain state. Pain. 2015 May;156(5):859-67. PubMed PMID: 25734998; PubMed Central PMCID: PMC4589228

RESEARCH PRESENTATIONS:

Burgos-Vega C.C., Avona A., Price T., and Dussor G. IL-6 induces spatially-dependent referred allodynia in a rat model of migraine. *American Pain Society*; Austin, TX, May 2016

Burgos-Vega C.C., Quigley L., Trevisan G., Price T., and Dussor G. Meningeal application of low pH, IL-6 and allyl isothiocyanate produces migraine-related behavior in mice. *American Pain Society;* Austin, TX, May 2016

Burgos-Vega C.C., Quigley L., Dahn D., Price T., and Dussor G. Brainstem BDNF contributes to hyperalgesic priming in both males and females in a rat model of migraine. *American Pain Society;* Austin, TX, May 2016

Burgos-Vega C.C., Quigley L., Trevisan G., Price T., and Dussor G. Meningeal application of low pH, IL-6 and allyl isothiocyanate produces migraine-related behavior in mice. *Society for Neuroscience*; Chicago, IL, October 2015 (poster)

Avona A., **Burgos-Vega C.C**, Quigley L., Trevisan G., Price T., and Dussor G. IL-6 Induces spatially dependent hyperalgesic priming in a rat model of migraine. Texas Pain Consortium Conference; Dallas, TX, August 2015 (poster)

Quigley L., **Burgos-Vega C.C.**, Trevisan G., Price T., and Dussor G. Development and characterization of a mouse model of migraine. Texas Pain Consortium Conference; Dallas, TX, August 2015 (poster)

Burgos-Vega C.C., Ahn D., Wei X., Price T., and Dussor G. Brainstem BDNF contributes to hyperalgesic priming in a rat model of migraine. *15th World Congress: International Association for the Study of Pain;* Buenos Aires Argentina, October 2014 (poster)

Burgos-Vega C.C., Ahn D., Wei X., Price T., and Dussor G. Brainstem BDNF contributes to hyperalgesic priming in a rat model of migraine. *Society for Neuroscience;* Washington, DC, November 2014 (poster)

Burgos-Vega C.C., King T, and Porreca F. Inhibitors of Evoked and Spontaneous Components of Neuropathic Pain. *The University of Arizona Summer Research Institute Annual Research Conference;* Tucson, AZ, August 2010 (poster & talk)

Burgos-Vega C.C. and C. Sengupta-Gopalan. Glyphosate resistance in chile (Capsicum Annuum). *Undergraduate Research and Creative Arts Symposium*; NMSU, Las Cruces, NM, April 2010 (poster)

TEACHING EXPERIENCE

Fall 2014- Spring 2016	**Graduate Teaching Assistant**, The University of Texas at Dallas Directed the course with approval from supervising instructor. Implemented weekly review sessions for students. Designed exams and quizzes for classroom assessment. Provided weekly lectures, oversaw experiments, gave constructive feedback on essays and processed grades.
Fall 2006-Fall 2007	**Conversational English Tutor**, The University of Puerto Rico Prepared individualized lesson plans to accelerate improvement of communication skills of each student. Prepared weekly assessments on the progress of each student

PEER REVIEWING EXPERIENCE;

Ad hoc Reviewer, Journal of Pain (2014-)
Ad hoc Reviewer, British Journal of Pharmacology (2014-)
Ad hoc Reviewer, European Journal of Pain (2013-)
Ad hoc Reviewer, Brain (2013-)
Ad hoc Reviewer, Journal of Neuroscience (2013-)
Ad hoc Reviewer, Cephalgia (2012-)
Ad hoc Reviewer, Headache (2012-)

PROFESSIONAL ORGANIZATIONS;

Society for Neuroscience (SfN)
American Pain Society (APS)
International Association for the Study of Pain (IASP)