UNDERSTANDING THE PATHOPHYSIOLOGY OF MIGRAINE: ACTIVATION
AND SENSITIZATION OF DURAL AFFERENTS

by

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DEDICATION

For my dad and my grandparents
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LIST OF ABBREVIATIONS

5-HT - serotonin
\(\alpha,\beta\)-MeATP - \(\alpha,\beta\)-methylene ATP
AP - action potential
AMG – AMG-9810
ASICs - acid sensing ion channels
AOC - area over the time-effect curve
ANOVA - analysis of variance
BCA - bicinchoninic acid
CGRP - calcitonin gene-related peptide
CSD - cortical spreading depression
CRF - corticotrophin releasing factor
CSF - cerebrospinal fluid
CZP - Capsazepine
DMEM - Dulbecco's modified Eagle's medium
DMSO- dimethyl sulfoxide
DRG - dorsal root ganglion neurons
ERK - extracellular signal-regulated protein kinase
FG - Fluorogold
FHM - familial hemiplegic migraine
GMQ - 2-guanidine-4-methylquinazoline
LIST OF ABBREVIATIONS - CONTINUED

HBSS - hanks balanced-salt solution
IEM - inherited erythromelalgia
IL-6 - Interleukin-6
JAK - Janus kinase
L-15 – Liebovitz – 15
MAPK - Mitogen-Activated Protein Kinase
MMA - meningeal middle artery
NKA - neurokinin A
NSAIDs - nonsteroidal anti-inflammatory drugs
PACAP - pituitary adenylate cyclase-activating peptide
PAG - periaqueductal grey
PAR-2 - protease-activated receptor-2
PEPD - paroxysmal extreme pain disorder
RVM - rostral ventral medulla
SIF - Synthetic interstitial fluid
STAT - signal transducers and activators of transcription
TG - trigeminal ganglion neurons
TNF-α - tumor necrosis factor-α
TRPA 1 - transient receptor potential A1
TRPV1 - transient receptor potential vanilloid subfamily member 1
TRPV 4 - transient receptor potential vanilloid 4
LIST OF ABBREVIATIONS - CONTINUED

TTX - tetrodotoxin

VIP - vasoactive intestinal polypeptide
Migraine is one of the most common neurological disorders. The pathological conditions that initiate and sensitize afferent pain signaling are poorly understood. The goal of this study is to identify the ion channels and signaling proteins underlying activation and sensitization of meningeal nociceptors.

In trigeminal neurons retrogradely labeled from the cranial meninges, approximately 80% responded to a pH 6.0 application with a rapidly activating and desensitizing ASIC-like current. Pharmacological experiments and kinetics analysis demonstrated that dural afferent pH-sensitive currents were mediated via activation of ASIC3. In addition, applications of decreased pH solutions were able to excite these neurons and generate action potentials. In awake animals, application of decreased pH solutions to the dura produced dose-dependent facial and hindpaw allodynia, which was also mediated through activation of ASIC3. Accumulating evidence indicates that meningeal inflammation induced sensitization of dural afferents contributes to migraine headache. We have demonstrated here that in the presence of mast cell mediators, dural afferents showed a decreased pH threshold and increased activity in response to pH stimuli both in vivo and in vitro. These data provide a cellular mechanism by which decreased pH in the meninges directly excites afferent pain-sensing neurons potentially contributing to migraine headache. It also indicates that inflammatory events within the
meninges could sensitize afferent pain signaling and result in increased sensitivity of dural afferents.

Intracranial Interleukin-6 (IL-6) levels have been shown to be elevated during migraine attacks, suggesting that this cytokine may facilitate pain signaling from the meninges. Here we reported that in awake animals, direct application of IL-6 to the dura produced dose-dependent facial and hindpaw allodynia via activation of the ERK signaling pathway. IL-6 application was also able to increase neuronal excitability in a manner consistent with phosphorylation of Nav1.7. These data provide a cellular mechanism by which IL-6 in the meninges causes sensitization of dural afferents therefore contributing to the pathogenesis of migraine.

These findings are discussed in relation to how activation and sensitization of primary afferent neurons might initiate migraine pain signaling and how the research included in this dissertation relates to the development of new therapeutic strategies for migraine.
CHAPTER ONE:
INTRODUCTION AND BACKGROUND

1.1 Migraine

Epidemiology

Migraine is an extraordinarily common neurological disorder characterized by severe headache and associated manifestations, including nausea, vomiting and sensitivity to light and sound (Goadsby et al., 2002). It is recognized by the World Health Organization as a major cause of disability (2004). Disability caused by migraine is severe and produces significant negative impacts on patients’ performance at home, school or work in terms of impairment of normal daily functioning, absence and loss of productivity (Benemei et al., 2009). According to the American Migraine Prevalence and Prevention study, cumulative incidence was 43% in women and 18% in men (Stewart et al., 2008), which means half of the women will encounter migraine at some point in their lives. Given that prevalence of migraine peaks during the ages of 25 to 55 (Lipton et al., 1997), classic working years, the incidence imposes a huge burden on patients, their families, employers and society. Although migraine is among the most common health complaints, the problem gets surprisingly little attention from research, education and clinical resources, making migraine one of the most under-diagnosed, misdiagnosed, and poorly managed neurological conditions.
Symptoms and triggers

The characterization, classification and diagnosis of migraine are complex due to variable clinical symptoms between individuals and from one attack to another within a given individual. Based on the second edition of the International Classification of Headache Disorders, neurological and systemic symptoms that can occur before, during or after the headache phase, include mood change, neck stiffness, polyuria, yawning, fatigue, gastrointestinal disturbance, cognitive difficulties, and somatic sensory phenomena (2004). Five clinical phases can be identified in a migraine attack, including premonitory symptoms, aura, headache, resolution and recovery (Lane and Davies, 2006). However, during most migraine attacks, only one or two of these phases may be apparent. The premonitory symptoms, which begin hours to days before migraine attack, appear to be unique to the migraine phenotype. In an electronic diary study, most patients were able to predict the occurrence of their migraine up to days before headache based on their premonitory symptoms (Giffin et al., 2003), indicating that pathological process underlying migraine might occur well before the headache (Charles, 2009). The incidence of aura was reported by over one-third of migraineurs (Kelman, 2004). Aura is characterized by reversible focal neurological symptoms that usually precede or sometimes accompany the headache (2004). Cortical spreading depression (CSD), a slowly propagating wave of transient neuronal and glial depolarization has been recognized as the electrophysiologic substrate of migraine aura (Eikermann-Haerter and Ayata, 2010) since their travel velocities are similar. Headache is typically unilateral throbbing pain, which can last from 4 to 72 hrs and is usually worsened by sensory inputs,
including movements, light, odors and sound (Lane and Davies, 2006). Accompanied with headache, about 67% of the migraine patients developed cutaneous allodynia, defined as hypersensitivity of skin to innocuous mechanical or thermal stimuli which can extend to forearms (Burstein et al., 2000a). Sometimes the pain can be so severe that daily activities such as wearing jewelry and clothing, grooming can be irritating. It is not clear how a migraine attack terminates. Understanding the resolution process will help us develop better treatment strategies. Even after the migraine headache has resolved, many patients can experience hangover symptoms for several days.

The situation is further complicated by the variety of migraine triggers. Triggers are defined as particular agents or circumstances that will always induce migraine attacks (2004). In a large scale study, migraine was triggered in 75.9% of the patients (Kelman, 2007). Among all the triggers, stress/tension is the most frequently cited one in multiple studies (Martin, 2010). Menstruation is a major trigger for female sufferers (Martin, 2010), indicating the role of hormone change. Various sensory factors, including noise, odors, heat, head/neck movement, neck pain, coughing are able to initiate the attack, possibly due to increased sensory inputs (Martin, 2010). Other factors include sleep, exercise, smoking, hunger and certain foods. It is not clear how different triggers can initiate the activation processes under different circumstances that eventually lead to the migraine phenotype.

_Vascular theory_
For decades, researchers have been searching for the “ultimate” mechanism underlying migraine. Considering the number of migraine symptoms and triggers, it is unlikely that a single mechanism could explain migraine pathophysiology. Initially, migraine was thought to be a vascular disease, in which a theory which explained the pain of migraine to be due to dilation of cranial vessels (Goadsby, 2009). One obvious limitation of this theory is an explanation of the symptoms before, during and after the migraine attacks. Multiple imaging studies have observed the relationship between hypoperfusion and migraine headache phase (Olesen et al., 1990; Woods et al., 1994) to be inconsistent with vasodilation as a primary trigger for pain. Moreover, agents known to induce vasodilation, did not induce migraine headache (Rahmann et al., 2008). Therefore, now it is generally recognized that the vascular modulation of migraine is secondary to brain dysfunction.

Trigeminal vascular anatomy

Structures in the brain are largely insensate. Pain can be generated from large cerebral vessels, venous sinuses and the meninges since they are densely innervated by sensory fibers originating in the trigeminal ganglion ophthalmic branch or upper cervical dorsal roots (Goadsby et al., 2009). It is now generally recognized that the trigeminal sensory system is a key component in pain initiation and transmission in migraine. Trigeminal nerves are divided into 3 branches. The ophthalmic branch (V1) carries sensory information from the scalp, sinuses, parts of the meninges and the forehead including the upper eyelid and parts of the nose. The maxillary nerve (V2) and the
mandibular nerve (V3) cover the lower eyelid to lower lip, chin, jaw and parts of the meninges. The trigeminal nerves detect mechanical, chemical and temperature changes from intracranial and extracranial tissues, and send the information to the cell bodies located in trigeminal ganglion (primary afferent neurons). Primary afferent neurons mainly project to trigeminal nucleus caudalis and the first three cervical segments of the spinal dorsal horn as evidenced by c-Fos expression in these areas following meningeal stimulation (Hoskin et al., 1999; Strassman et al., 1994). Once sensory information reaches the trigeminal nucleus caudalis, it is relayed in a group of fibers to the thalamus through second-order neuronal connection. From the thalamus, the sensory information travels to higher brain areas. The decision as to whether the sensory information is perceived as painful or not depends not only on sensory input but also on integrated information from an extended brain network including the cingulate cortex, insulae, somatosensory cortex, amygdale and other areas (Goadsby et al., 2009). Apart from the ascending pain transmission pathway, several descending pain modulation pathways originating in brain stem are also involved in pain sensation. The nucleus raphe magnus is known to inhibit nociceptive responses by sending serotonergic projections to nucleus caudalis (Goadsby et al., 2009). The rostral ventromedial medulla (RVM) is another area of interest. It receives input from periaqueductal grey (PAG) and exerts bi-directional pain modulating actions under different physiological conditions (Porreca et al., 2002).

*Pathophysiology*
We do not completely understand the pathophysiology of pain in migraine. However, it is likely to be a combination of increased pain transmission in concert with abnormal endogenous pain modulation. Several hypotheses have been put forward falling into 3 categories: peripheral theories, central hypotheses and neuropeptide alteration. The peripheral theory proposes that activation and sensitization of primary afferent neurons by proinflammatory mediators result in enhanced sensory inputs to the spinal cord, which we will discuss later in this chapter. Here we are going to focus on the central mechanisms and neuropeptide alteration.

The central hypotheses maintain that abnormal neuronal activities in the central nervous system and brain stem are the underlying mechanisms of migraine. The presence of cutaneous allodynia in the upper limbs ipsilateral and contralateral to the headache site suggests the involvement of supraspinal neuronal sensitization (Burstein et al., 2000a; Burstein et al., 2000b), thus placing the pathophysiology of migraine within the central nervous system. Preclinical work also demonstrated that brain stem trigeminal neurons could be sensitized following dural chemical stimulation (Burstein et al., 1998), which further supports a possible role for central nervous system. Moreover, genetic studies have shown that mutations in calcium channels (van den Maagdenberg et al., 2004), Na+/K+ ATPase (Vanmolkot et al., 2006) and sodium channels (Dichgans et al., 2005) resulted in enhanced cerebral excitability and led to increased susceptibility to CSD and familial hemiplegic migraine (FHM).

Dysfunction of descending pain modulation pathways from the brain stem has also been suggested in migraine pathophysiology. One theory proposes that the
The immediate generator of migraine headache is located in the brain stem (Lambert and Zagami, 2009). It suggests that cortical activation induced by different triggers might converge on the PAG and inhibit the defective descending inhibitory pathway in migraineurs, thus releasing the ascending sensory inputs from inhibition leading to a migraine headache (Lambert and Zagami, 2009). Although not universally accepted, the idea is intriguing because it explains the multiple triggers and associated symptoms of migraine.

Along with the peripheral and central mechanisms, many agree with the role of neuropeptide alteration in migraine pathophysiology. Well-known contributors include calcitonin gene-related peptide (CGRP), substance P, neurokinin A and vasoactive intestinal polypeptide (VIP) etc (Samsam et al., 2007). Clinical studies demonstrated that during a migraine attack, levels of CGRP and neurokinin A (NKA) increased in human blood (Goadsby et al., 1988, 1990). The trigeminovascular theory proposes that following afferent activation, neuropeptides are released from nerve terminals (Moskowitz, 1993), located around blood vessels. These peptides can increase vascular permeability, dilate cerebral blood vessels and induce headache (Edvinsson et al., 1987; Holzer, 1998). Moreover, preclinical studies also demonstrated that neuropeptides could act on more than cerebral blood vessels. For example, they are able to induce peripheral and central sensitization by modulating ion channels and signaling cascades and enhance synthesis and release of other proinflammatory mediators by activating mast cells and endothelial cells.
However, none of these theories alone could explain all laboratory findings and clinical observations, suggesting that migraine is a disease that involves multifaceted molecular, cellular, neuroanatomic and neurochemical mechanisms.

*Current therapies*

Treatment strategies of migraine are very limited due to poor understanding of the disorder. Current approaches include non-pharmacologic interventions and pharmacologic therapies. Non-pharmacologic interventions focus on patient education about the disorder, treatment approaches and lifestyle changes, especially avoidance of migraine triggers. Pharmacologically, most drugs available only temporarily alleviate the symptoms but do not resolve the underlying causes. Drug treatments can be divided into two categories, prophylactic medications and abortive medications.

Prophylactic medications are taken daily whether or not headache is present to decrease the severity and frequency of migraine attacks in patients who are un-responsive to abortive medications or experience migraine more than twice per month (Goadsby et al., 2002). Currently, major classes of migraine preventative drugs include the β-adrenergic blocker propranolol, the antidepressant amitriptyline and the antiepileptic drugs topiramate and valproic acid (Galletti et al., 2009). Emerging treatments include Ca\(^{2+}\)-channel blockers and botulinum toxin. Mechanisms of actions of prophylactic medications are not fully understood. Most likely, they exert their therapeutic effects through suppression of neuronal hyperexcitability in the central nervous system since most prophylactic medications are able to block CSD (Galletti et al., 2009). β-blockers
exhibit inhibitory effects in the ventroposteromedial nucleus (VPM) thalamic neurons in response to trigeminovascular input (Shields and Goadsby, 2005). They can also regulate the neuronal firing rate of PAG neurons (Xiao et al., 2008) and noradrenergic neurons in locus coeruleus (Hieble, 2000). The rationale for antidepressants use is based on the hypothesis that a low 5-HT levels facilitates trigeminovascular nociceptive activation pathways in migraine (Hamel, 2007). Antiepileptic drugs are thought to counteract hyperexcitability by modulation of voltage-gated ion channels, ligand-gated ion channels and long-term gene expression (Galletti et al., 2009). Botulinum toxin A was approved by F.D.A. in 2010 to prevent chronic migraine. It acts on primary peripheral neurons to reduce neurotransmitter release, thus inhibiting peripheral sensitization and subsequent central sensitization (Aoki, 2005).

Abortive medications are taken to treat the symptoms when the attacks arise. Available options include non-specific anti-pain compounds, such as NSAIDs (nonsteroidal anti-inflammatory drugs), opioids and more migraine-specific approaches, such as triptans, ergot derivatives and CGRP receptor antagonists. NSAIDs are used in migraine treatment for their anti-inflammatory effects, but they are the least effective among all options. Opioids such as morphine act on μ, δ or κ opioid receptors along the pain signaling pathway both in the peripheral and central nervous systems. Activation of opioid receptors, coupled with Gi/Go protein, initiates a large number of downstream intracellular events, including inhibition of adenyl cyclase activity, reduced opening of voltage-gated $\text{Ca}^{2+}$ channel and activation of G protein-activated inwardly rectifying $\text{K}^+$ channels (GIRKs), resulting in an decrease in overall neuronal excitability. Side effects
for all opioids include drowsiness, vomiting, nausea, constipation and respiratory depression. In addition, long term use of opioids can induce medication overuse headache (De Felice et al., 2011). Therefore, opioids should not be used as first-line therapy for migraine. Both ergot derivatives and triptans exert their therapeutic effects by activating 5-HT (serotonin) receptors. The better tolerated triptans have replaced ergot derivatives in most cases (Sprenger and Goadsby, 2009). Several mechanisms of action have been proposed for triptans, including cranial vasoconstriction (Humphrey et al., 1990), inhibition of trigeminal afferents activation (Hoskin et al., 1996) and disruption of communication between peripheral and central trigeminovascular neurons (Levy et al., 2004). CGRP, released at nerve terminals upon afferent activation, plays an important role in migraine pathogenesis. Infusion of CGRP can trigger a migraine attack and is also a potent vessel dilator (Doods et al., 2007). Thus, CGRP receptor antagonists provide another option for acute treatment of migraine.

Although many patients can be effectively treated, 35% of migraine patients are considered non-responders to current therapies (Lipton and Stewart, 1999). Even for responders, current medications provide limited benefits due to side effects and are sometimes contraindicated due to comorbidities (e.g. asthma). Since we do not understand the etiology of migraine, it is hard to predict which patients will respond to which treatment according to their symptoms or triggers. Trial and error is frustrating for both patients and physicians. Thus, a better understanding of the disease mechanism is crucial for developing specific and more effective treatments.
**Nociceptor activation and sensitization**

Our lab is particularly interested in where and how pain originates in migraine headache. Spontaneous onset of head pain is the most disabling symptom that prompts patients to seek medical care. The absence of any identifiable pathology in most patients leaves the genesis of migraine headache still unclear.

A large body of evidence supports the peripheral theory, which hypothesizes that migraine headache originates from activation of primary afferent neurons that innervate cranial tissues (Levy, 2010). This idea is based on the finding that direct stimulation of the meninges, particularly at vascular sites, led to headache mostly referred to similar cephalic locations as during migraine (Ray and Wolff, 1940). It is further supported by anatomical studies demonstrating that only the meninges and large cranial vessels, but not brain itself or its intrinsic blood vessels, are densely innervated by sensory fibers originating primarily from trigeminal ganglion (Mayberg et al., 1984; Strassman and Levy, 2006), indicating that the meninges and cranial vessels are the only possible sites of origination for migraine headache. It also suggests that primary afferent neurons have the potential to detect and transmit noxious stimuli of the cranial tissues. *In vivo* electrophysiological recording studies demonstrated the ability of primary afferent neurons to respond to mechanic stimuli, such as punctate probing and stroking (Strassman et al., 1996), thermal stimuli (Bove and Moskowitz, 1997) and chemical stimuli, such as KCl, capsaicin, acidic buffers and a mixture of inflammatory mediators (histamine, bradykinin, serotonin, and prostaglandin E2 at pH 5) (Bove and Moskowitz, 1997; Strassman et al., 1996).
Since there are no identifiable anatomic, biochemical or genetic changes in most migraine patients, the question is where do the noxious stimuli originate that can activate the primary afferent neurons. Preclinical and clinical studies suggest acute sterile neurogenic inflammation as a trigger. The notion is supported by clinical observations that intracranial and circulating levels of various inflammatory mediators are significantly higher during migraine attacks (Perini et al., 2005; Sarchielli et al., 2006; Sarchielli et al., 2001). Preclinical studies also identify dural mast cells as a potential source for proinflammatory mediators. In addition to sensory fibers, the meninges is also highly populated with mast cells (Dimlich et al., 1991; Strassman et al., 2004). These granulated cells reside within the dura and in close proximity to blood vessels and nerve terminals (Rozniecki et al., 1999), which make it possible that mast cell degranulation could activate the trigeminal nerve terminals and affect the neuronal excitability of primary afferent neurons.

Another endogenous process which has been proposed to promote meningeal inflammation is cortical spreading depression (CSD). In addition to causing aura, CSD has been proposed to trigger migraine headache. CSD is associated with a massive efflux of potassium (Mayevsky et al., 1974), hydrogen ions (Csiba et al., 1985) and neurotransmitters such as glutamate (Van Harreveld, 1959) into extracellular space, which can cause a multitude of changes in expressions of growth factors and proinflammatory mediators, such as tumor necrosis factor-α (TNF-α) and IL-1β (Jander et al., 2001). These mediators have the potential to activate or sensitize pial nociceptors. Following activation of the nociceptors, subsequent local release of neuropeptides from
pial nerve terminals may affect nearby blood vessels. Consistent with this hypothesis, preclinical studies have confirmed that CSD is able to induce a long lasting blood flow increase in the middle meningeal artery, cause plasma protein extravasation in dura mater, increase c-Fos staining in the ipsilateral trigeminal nucleus caudalis (Bolay et al., 2002) and cause delayed and long term activation of the meningeal nociceptors (Zhang et al., 2010).

Such proinflammatory mediators released from mast cell degranulation or a CSD event can not only activate the meningeal nociceptors but also promote sensitization of these neurons to mechanical or thermal stimuli. Once sensitized, these neurons show either decreased response threshold or increased responses to suprathreshold stimuli (Levy and Strassman, 2002), resulting in increased sensitivity to noxious or innocuous stimuli. These phenomena are consistent with the symptoms characteristic of migraine headache, for example, aggravation of pain during head movements and coughing.

Although it is well-known that meningeal nociceptors can be activated or sensitized by proinflammatory mediators (Harriott and Gold, 2009; Schepelmann et al., 1999; Strassman et al., 1996; Zhang et al., 2007) or following CSD events (Zhang et al., 2010) and mast cell degranulation (Levy et al., 2007), the mechanisms by which these events produce excitation of dural afferents are unknown. Multiple channels likely contribute to activation of dural afferents (as with any population of nociceptor). Without knowledge of which channels contribute and the mechanisms by which these channels are activated, it is difficult or impossible to predict which would be the most effective target for new migraine therapies.
1.2 Hypothesis and Organization of the Thesis

Overall Hypothesis: One fundamental question in migraine research that remains unanswered is what endogenous stimulus or stimuli activates pain signaling pathways and what mechanisms produce these responses. Understanding the headache initiation process is of critical importance in the development of new therapies for migraine. The primary goal of this dissertation is to address this issue by testing the hypothesis that inflammation and low pH within the meninges leads to activation and sensitization of dural afferents via specific receptors and signaling pathways. Once activated and sensitized, this increased afferent activity may drive the pain of migraine headache. The study proposed here will attempt to identify the ion channels, signaling molecules and cytokines mediating direct excitation of dural afferents. Future therapies directed toward blocking headache initiation by targeting these mechanisms may provide relief for the large numbers of migraine headache patients that are not adequately treated by currently available drugs.

Dural afferents will be identified by applying retrograde dye Fluorogold onto the dura. This method allows us to study a group of neurons relevant to migraine headache. Chapter three will characterize the properties and current kinetics of dural afferent pH-sensitive currents. The thresholds for pH-evoked currents and excitation will be determined. Following this, pH evoked behavioral responses will be assessed in an established migraine rat model. The ion channels underlying pH-evoked neuronal excitation and behavioral responses will be identified pharmacologically. Chapter four
will test the hypothesis that under sensitized conditions, dural afferents will exhibit a lowered threshold for pH evoked responses or increased responses to supra-threshold pH stimuli. The thresholds for pH evoked behavioral responses will also be determined and compared with or without coapplication of mast cell mediators. Chapter five will focus on evaluating mechanical allodynia induced by dural administration of Interleukin-6 (IL-6) and the underlying signaling pathway contributing to these effects. The effects of IL-6 on neuronal excitability and ion channel modulation will also be explored. This thesis aims to examine what dural afferents may be responding to in the extracellular environment and which receptors are used to produce these responses, thereby, providing new therapeutic targets for migraine.
CHAPTER TWO:
MATERIALS AND METHODS

2.1 Animals

Male Sprague-Dawley rats (150-300 g; Harlan, Indianapolis, IN) were maintained on a 12-hr light/dark cycle (lights on 7am / lights off 7pm) with food and water available ad libitum. All procedures were performed in accordance with the policies and recommendations of the International Association for the Study of Pain, the National Institutes of Health guidelines for the handling and use of laboratory animals and by the Animal Care and Use Committees of the University of Arizona. Rats weighing 150-200g were used in electrophysiology experiments. Rats weighing 250-275g were used in behavioral experiments.

2.2 Surgical Preparations

Tracer injection

Dural afferents were identified as previously described (De Felice et al., 2010) with several modifications. Male Sprague-Dawley Rats (150-175g) were used. Seven days prior to the sacrifice, animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg; Sigma-Aldrich). The top of the head was shaved using a rodent clipper (Oster Golden A5 w/size 50 blade) and cleaned with betadine and 70% ethanol. Rats were placed in a stereotaxic headholder (model 51600, Stoelting, Wood
Dale, IL), a 2 cm incision was made in the skin using a scalpel (#10 blade) and the underlying connective tissue and skin were retracted using hemostats to expose the skull. Under a dissecting microscope, as indicated in Fig.1, two holes (3 mm in diameter) were made in the skull using a Dremel Multipro 395 fitted with a dental drill bit (Stoelting) leaving a thin layer of bone at the bottom of the hole. Fine forceps were used to carefully remove the remaining bone and expose but not damage the dura. Fluorogold (5 μl/hole; 4% in synthetic interstitial fluid) was then applied onto the dura. A small piece of gelfoam was placed in the hole to increase the absorption of the dye and prevent spread of the tracer outside of the hole. Holes were covered with bone wax to prevent tracer spread and the incision was closed with sutures. Immediately postoperatively, animals received a single subcutaneous injection of gentamicin (8mg/kg) to minimize infection. Following recovery from anesthesia, animals were housed for 7 days to allow the retrograde transport of the dye from dura to the trigeminal ganglion neurons. Undamaged dura at the injection sites was evaluated at the time the animals were sacrificed and only data from animals with intact dura and no signs of damage were used for further analysis.

**Dura cannulation**

Dura cannulae were implanted as previously described (Edelmayer et al., 2009) with modifications. Male Sprague-Dawley Rats (250-275g) were used. Following skull exposure, the location of the bregma and midline bone sutures were identified, and a 1 mm hole (above the transverse sinus; 2 mm left of the sagittal suture and 2 mm anterior to the lambdoid suture) was made in the skull with a hand drill (DH-0 Pin Vise; Plastics
One, Roanoke, VA) to carefully expose the dura (Fig. 2). A guide cannula (22 GA, #C313G; Plastics One), designed to extend 0.5 mm from the pedestal was inserted into the hole and sealed into place with Superglue. The design of the cannula allows delivery of solutions to the underlying dural membrane without penetration or damage to the dura. Two additional 1 mm holes were made in the parietal bones to receive stainless-steel screws (#MPX-080-3F-1M, Small Parts Inc., Miami Lakes, FL). The screws were superficially placed in the skull to prevent damage to the underlying dural membrane and dental acrylic was used to secure the cannula and screws to the skull. After the dental acrylic dried (5 to 10 min), a dummy cannula (#C313DC, Plastics One Inc.) was inserted and secured to prevent contaminants from entering the guide cannula during the recovery period. Immediately postoperatively, animals received a single subcutaneous injection of gentamicin (8mg/kg) to minimize infection. Following recovery from anesthesia, rats were housed separately and allowed 6 to 8 days of recovery.

2.3 Behavioral Testing Protocols

Facial allodynia testing

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

*Hindpaw allodynia testing*

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

*Experimental testing protocols*

Baseline behavioral responses to probing of the face and hindpaws were obtained from all rats prior to drug administration. Rats then received experimental compounds via
the dura cannula, and behavioral responses were determined at 1 hr intervals for 5-6 hrs (Fig. 3)

*Calculation of facial and hindpaw tactile withdrawal thresholds*

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

*Injection Procedures & Reagents*

Animals were removed from the testing chamber and their dummy cannulas were removed. An injection cannula (28GA, #C313I, Plastics One Inc.) cut to fit the dura guide cannula, was connected to a 25 μl Hamilton Syringe (#1702SN) by Tygon tubing
(95607-14, Cole-Parmer, Vernon Hills, IL) and used to slowly inject 10 μl of the test compounds or vehicle onto the dura. Synthetic interstitial fluid (SIF) was used as vehicle. The SIF consisted (in mM) of 10 HEPES, 5 KCl, 1 MgCl₂, 5 CaCl₂, and 135 NaCl, pH 7.3 (adjusted with N-methyl glucamine), and was ~ 320 mosM (adjusted with sucrose). All testing compounds were dissolved or diluted in SIF solution at the indicated pH. Following injection, the dummy cannula was replaced and the animals were returned to their corresponding testing chambers.

2.4 Cell Culture

Seven days following Fluorogold application, trigeminal ganglia were removed, enzymatically treated and mechanically dissociated. Rats were anesthetized with isoflurane (Phoenix Pharmaceuticals) and sacrificed by decapitation. The trigeminal ganglion were removed and placed in ice-cold Hanks balanced-salt solution (divalent free). Ganglia were chopped with scissors and incubated for 25 mins in 20 U/ml Papain (Worthington) followed by 25 mins in 3 mg/ml Collagenase TypeII (Worthington). Ganglia were then triturated through fire-polished pasteur pipettes and plated on poly-D-lysine (Becton Dickinson) and laminin (Sigma)-coated plates. After several hours at room temperature to allow adhesion, cells were cultured in a room-temperature, humidified chamber in Liebovitz L-15 medium supplemented with 10% FBS, 10 mM glucose, 10 mM HEPES and 50 U/ml penicillin/streptomycin. Cells were used within 24 h post plating.
2.5 Electrophysiology

Recording solutions

Pipette solution contained (in mM) 140 KCl, 11 EGTA, 2 MgCl₂, 10 NaCl, 10 HEPES, 2 MgATP, and 0.3 Na₂GTP, 1CaCl₂ at pH 7.3 (adjusted with N-methyl glucamine), and was ~ 320 mosM. For recording acid sensing ion channels (ASICs), the external solution contained (in mM) 135 NaCl, 2 CaCl₂, 1 MgCl₂, 5 KCl, 10 Glucose, 5 HEPES, and 5 MES, pH 7.4 (adjusted with N-methyl glucamine), and was ~ 320 mosM. 5 HEPES/5 MES (4-morpholineethanesulfonic acid) buffer was used to prepare extracellular solutions with pHs ranging from 6.0 to 7.4. For recording IL-6 induced excitation, 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. The pipette solution was the same as above.

Solutions were rapidly changed during recordings using gravity-fed flow pipes positioned near the cell and controlled by computer driven solenoid valves. The solution exchange time was ~ 20 ms. No currents were observed when solutions were switched from pH 7.4 to pH 7.4 using our drug application system.

Whole-cell patch clamp 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 5 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 60–80%. 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).

Recording protocols

ASIC currents were evoked under voltage clamp by a 5 to 120 second step from pH 7.4 to the indicated pH. Effects of decreased pH on membrane excitability were recorded in the current-clamp configuration by a 5 second step from pH 7.4 to indicated pH.

Current clamp configuration was used to determine the current threshold, i.e. the minimum current required to initiate an action potential. Action potentials were elicited by injecting rectangular current steps (25 nA, = 10 pA). To mimic the slow depolarization, action potentials were elicited under current clamp by 1 second ramp current injection ranging from 0.1 to 0.7 nA in 0.2 nA increments from resting membrane potential.

2.6 Western Blotting

Rats were anesthetized with isoflurane (Phoenix Pharmaceuticals) and sacrificed by decapitation. The meninges was then carefully dissected out and transferred in lysis
buffer (50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA at pH 7.4) containing protease and phosphatase inhibitor mixtures (Sigma) on ice. The meninges was sonicated and spun down at at 14,000 RCF for 20 min at 4°C. Supernatants were then transferred and used for protein assay and western blotting. Protein concentration was determined by the (bicinchoninic acid) BCA protein assay (Thermo Scientific) with bovine serum albumin as the standard. Thirty micrograms of protein per well were loaded and separated by standard 10% SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and then blocked with 5% dry milk for 3 h at room temperature. The blots were incubated with ASIC3 antibody (1:1000, Neuromics) overnight at 4°C. After washing, blots were incubated in a horseradish peroxidase-conjugated secondary antibody (1:10,000; goat anti-guinea pig IgG) for 1 hr at room temperature. Signal was detected by ECL on chemiluminescent films.

2.7 Compounds

Fluorogold was purchased from Fluorochrome, LLC. and dissolved in synthetic interstitial fluid (pH 7.4, 310 Osm) to 4%. 2-guanidine-4-methylquinazoline (GMQ, Sigma) used bath solutions as a vehicle.

Amiloride was purchased from Sigma and dissolved in DMSO to 100 mM as a stock solution and diluted to the designated concentration in bath solution for patch clamp experiments. Capsazepine was from Ascent Scientific and AMG-9810 was from Tocris. Capsazepine and AMG-9810 were dissolved in DMSO to 10 mM and diluted in bath solution to the indicated concentration for patch clamp experiments. Final DMSO
concentration never exceeded 0.1% for patch clamp experiments. For behavioral experiments, stock amiloride solutions (1 M in DMSO) and stock AMG-9810 solutions (100 mM in DMSO) were prepared and diluted in SIF to the final concentration of 10 mM and 1 mM at the indicated pH, respectively. rAPETx2 (Alomone Labs) was dissolved in pH 6 SIF solutions to a final concentration of 20 µM.

Stock mast cell mediators were composed of 100 mM Histamine in distilled water (Sigma), 100 mM serotonin in distilled water (Sigma), 10 mM AC55541 in DMSO (Tocris) and 13.8 mM Iloprost in 0.5% in methyl acetate (Caymen Chemical). Stock mast cell mediators were diluted to the desired concentrations in bath solutions (electrophysiology experiments) or SIFs at indicated pH (behavioral experiments).

Rat recombinant IL-6 (rIL-6) was from R&D Systems. Stock rIL-6 (10 µg/ml) was prepared in sterile 0.1% BSA in PBS and diluted to final concentrations of 50 ng/ml in bath solution (electrophysiology experiments) and 100 ng/ml in SIF (behavioral testing), respectively. U0126 was from Tocris Biosciences. Stock U0126 (10 mM) was prepared in DMSO and added to the culture media and the recording chamber to produce a final concentration of 10 μM in 0.1% DMSO for patch experiment. For the behavioral experiments, stock U0126 solutions (100 mM in DMSO) were prepared and diluted in SIF to the final concentration of 1 mM. Vehicle control was SIF with 1% DMSO for behavioral experiments.

2.8 Data Analysis

Patch clamp
All data are presented as means ± SEM unless otherwise noted. Statistical evaluation was performed by linear regression analysis for the comparison of difference in the mean numbers of action potentials among groups. Comparison among several groups for time-to-first spike was performed by two-factor analysis of variance (ANOVA). Differences among means of current thresholds for each group were determined by analysis of variance followed by Dunnett's post hoc test.

*Behavioral testing*

All data are presented as means ± SEM unless otherwise noted. Behavioral studies among groups and across time were analyzed by two-factor ANOVA. Data were converted to area over the time-effect curve (AOC) and normalized as a percentage of the pH 5 or pH 6-treated group to allow for multiple comparisons. Significant differences between groups were assessed by one-way ANOVA with Dunnett’s multiple comparison post hoc.
Figure 2.1 Location of tracer injection. The location of the bregma and midline bone sutures were identified, and as indicated by arrow and black circle, two 3mm holes were drilled for the dye injection. Rostal = toward the nose. Caudal = toward the tail.
Figure 2.2 Location of dura cannulation. The location of the bregma and midline bone sutures were identified, and as indicated by arrow and black circle, and a 1 mm hole (above the transverse sinus; 2 mm left of the sagittal suture and 2 mm anterior to the lambdoid suture) was made in the skull with a hand drill (DH-0 Pin Vise; Plastics One, Roanoke, VA) to carefully expose the dura. A guide cannula (22 GA, #C313G; Plastics One), designed to extend 0.5 mm from the pedestal to avoid irritation of the dural tissue, was inserted into the hole and sealed into place with glue. The design of the cannula allows delivery of solutions to the underlying dural membrane without penetration or damage to the dura. Two additional 1 mm holes were made in the parietal bones to receive stainless-steel screws (#MPX-080-3F-1M, Small Parts Inc., Miami Lakes, FL).
The screws were superficially placed in the skull to prevent damage to the underlying dural membrane.
Figure 2.3 Experimental testing protocol. Animals were allowed to recover 6-8 days following dura cannulation surgery. Baseline behavioral responses to probing of the face and hindpaws were obtained from all rats prior to drug administration. Rats then received test compounds via the dura cannula, and behavioral responses were determined at 1 hr intervals for 5-6 hrs.
CHAPTER THREE:

DURAL AFFERENTS EXPRESS ACID-SENSING ION CHANNELS: A ROLE FOR DECREASED MENINGEAL pH IN MIGRAINE HEADACHE

3.1 Introduction

While multiple hypotheses regarding the pathophysiology of migraine headache have been proposed, the exact nature of the triggering process itself is largely unknown (Levy, 2009). Activation of trigeminal neurons innervating the intracranial meninges and their related large blood vessels is likely required to generate the headache experienced during a migraine attack. However, the cellular mechanisms that initiate pain signaling in these neurons are unknown as most of the mediators do not directly open ion channels to produce firing of action potentials.

Acid sensing ion channels (ASICs) belong to the ENaC/DEG (epithelial amiloride-sensitive Na+ channel and degenerin) family of ion channels. ASICs share overall structural similarity with other members of this family, characterized by two hydrophobic transmembrane regions flanking a large extracellular domain (Lingueglia, 2007). ASICs are neuronal voltage-insensitive cationic channels activated by increases in the concentration of extracellular protons (Wemmie et al., 2006). The ASIC family consists of 4 members, ASIC1 through ASIC4, with several splice variants (Lingueglia, 2007). Functional ASIC channels are assembled as heteromeric or homomultimeric channels. Different subunit composition of these channels gives rise to different
pharmacology, current kinetics and sensitivity to pH change (Lingueglia, 2007). Among all subunits, ASIC3 and ASIC1a exhibit extreme sensitivity to pH change, with half maximum activation pH ranging from pH 6.2 to pH 6.8 (Benson et al., 2002; Lingueglia, 2007; Yagi et al., 2006). Conversely, ASIC4 channels are pH-insensitive and ASIC2 channels respond to strong acidification with activation thresholds close to pH 6.0 (Baron et al., 2002; Lingueglia, 2007). Activation of ASIC channels by extracellular protons triggers transient rapid activating and desensitizing inward currents, which lasts from hundreds of milliseconds to several seconds (Li and Xu, 2011). ASICs are widely expressed in the nervous system. ASIC1a and ASIC2 subunits are expressed in the central nervous system (Baron et al., 2008; Price et al., 1996; Waldmann et al., 1996; Wu et al., 2004), while almost all ASIC subunits are displayed in peripheral sensory neurons (Mamet et al., 2002; Voilley et al., 2001; Waldmann et al., 1997).

Among all subunits, the ASIC3 subunit is of particular interest for several reasons. ASIC3 exhibits greater pH sensitivity than other ASIC subunits. Except for transient peak current, ASIC3 is also able to generate a non-desensitizing current in response to a physiologically relevant pH (Yagi et al., 2006), making it suitable to detect prolonged and slow acidification. ASIC3 is highly expressed in sensory neurons and largely restricted to the periphery (Lingueglia, 2007; Waldmann et al., 1997), which is consistent with its role in detecting pH change in peripheral tissues. Peripheral sensory neurons expressing ASIC3 innervate visceral organs including the colon and heart as well as skeletal muscles (Jones et al., 2005; Molliver et al., 2005; Page et al., 2005; Sutherland et al., 2001). Tissue acidosis has been associated with a variety of pathological conditions in these
tissues, including inflammation, ischemia, muscle incision model and tumors, therefore ASIC3 channels have been proposed to modulate associated painful conditions, including angina, postoperative pain, various GI disorders and muscle pain (Deval et al., 2011; Jones et al., 2005; Page et al., 2005; Sluka et al., 2007; Sutherland et al., 2001; Yagi et al., 2006). With respect to migraine, ASICs on dural afferents have been proposed as a sensor of decreased extracellular pH within the dura (Burstein, 2001). However, this has never been determined experimentally. The aim of this study was to investigate the effects of decreased extracellular pH on dural afferents, including the mechanism by which these neurons may respond to decreased pH within the dura. Additionally, these studies examined the ability of decreased meningeal pH to produce migraine-related behavior in awake animals.

3.2 Results

Verification of retrograde labeling

Patch clamp electrophysiology was performed on rat trigeminal ganglion neurons (TG) in culture from rat in which Fluorogold was previously applied onto the dura. Retrogradely-labeled cells were selected for recording. Several important controls were performed for this technique to confirm that cells selected were dural afferents. 1) Incomplete holes were made in the skull with a thin layer of bone left intact at the bottom to guarantee that Fluorogold did not penetrate the skull and contact the dura. Fluorogold thus remained in the hole and could label tissues other than dura. The hole was sealed with gel foam and bone wax. Fluorogold-positive cells were rarely observed in trigeminal
cultures from these animals. 2) Since the UV light required to excite Fluorogold may damage cells, we studied cells from naïve animals and labeled cells from Fluorogold-labeled animals that had ASIC-like currents before and after 10 min of UV light exposure (passed through the Fluorogold filter cube) and have seen no significant differences in the properties of these cells following UV light exposure. Importantly, under typical experimental conditions the normal UV exposure during the process of locating Fluorogold-positive neurons was less than 1 min. 3) We compared our preliminary patch-clamp results to data we obtained using DiI as a retrograde tracer (this tracer was used in (Harriott and Gold, 2009)) and have observed no significant differences. 4) we observed identical properties, including rapidly activating and rapidly desensitizing ASIC-like currents with amplitudes above 20 nA as well as action potential firing upon decreased pH application, in a fraction of randomly-selected trigeminal neurons taken from unlabeled animals (the innervation target of these neurons is unknown), indicating that the properties that we observed in retrogradely-labeled cells did not occur due to the labeling process. Thus, Fluorogold-positive neurons were used for this study since we believe that these cells are dural afferents and that they are not significantly altered by the retrograde labeling.

**Dural afferent respond to multiple mediators**

To explore what substances dural afferents might respond to in extracellular space, we applied α,β-methylene ATP (α,β-MeATP) and pH 6.0 solutions. Among 160 dural afferents from 11 rats, 80% exhibited ASIC-like pH 6-evoked currents. (Fig. 3.1).
Additionally, α,β-MeATP evoked currents in 75% of dural afferents (Fig. 3.1), indicating the presence of P2X channels. It suggested that changes in extracellular ATP and pH may be present during migraine. Here we focus on dural afferent ASIC-like pH sensitive currents in this thesis.

*Extreme pH sensitivity of dural afferents*

Examples of currents evoked by a 5s step from pH 7.4 to pH 6.0 in a representative dural afferent are shown in Fig. 3.2. Dural afferent pH 6.0-evoked currents ranged from 0.07 to 63 nA (Fig. 3.2A). Approximately 50% of the dural afferents could generate currents with amplitudes well above 20 nA. Densities of pH 6.0-evoked currents in dural afferents were determined with some cells demonstrating up to 1058 pA/pF (Fig. 3.2B), suggesting a high density of channel expression, thus making it possible that dural afferents might respond to more physiologically relevant pH.

Most of the dural afferents exhibiting pH 6.0-evoked currents also exhibit pH 6.8-evoked currents, ranging from 0.03 to 6.6 nA (Fig. 3.3A). To calculate the percentage of neurons responding with inward currents to pH 6.9, 7.0 and 7.1, the minimum amplitude for response was set at 20 pA and neurons generating ASIC-like currents larger than 20 pA were counted as positive. In response to pH 6.9, 7.0 and 7.1, 73%, 56% and 30% of dural afferents exhibited currents, respectively (Fig 3.3B).

*pH-evoked currents in dural afferents exhibit variable kinetics*
The pH-evoked currents in dural afferents were fast inactivating suggesting the presence of ASIC1 or ASIC3-containing subtypes. To attempt to determine which subtype mediates these pH-evoked currents, the time constant for recovery from desensitization at pH 6.0 was measured. The decay time constant varied in dural afferents (Fig. 3.4A) and single exponentials fit to the data showed time constants ranging from 142 to 3102 ms (Fig. 3.4B). However, 75% of the dural afferents (n = 128) had decay time constant of 0 - 500 ms (Fig. 3.4B). Further analysis of the currents with a decay time constant less than 500 ms showed that 18.75 % and 44.53% of the dural afferents displayed a decay time constant between 100 – 200 ms and 200 – 300 ms, respectively (Fig. 3.4B). Prior work has shown that homomeric ASIC3 decay time constants average 320 ± 70 ms, which suggests the presence of ASIC3 homomers in dural afferents. Several lines of evidence indicated that pH evoked currents with decay time constants less than 300 ms were mediated by ASIC3 heteromers. The decay time constants for ASIC1a/ASIC3, ASIC1b/ASIC3, ASIC2a/ASIC3 and ASIC2b/ASIC3 average 160 ± 30, 230 ± 10, 190 ± 20 and 230 ± 20 ms, respectively (Hesselager et al., 2004). The mean decay time constant for wild type dorsal root ganglion neurons (DRG) pH-evoked current was shorter than that for homomeric ASIC3 transfected in COS-7 cells and was mimicked by coexpression of ASIC3 with other ASIC subunits (Benson et al., 2002). Additionally, 25% of dural afferents had time constants that suggested the presence of other ASIC subunits as they were longer than those shown previously for channels containing ASIC3. Taken together, the extreme sensitivity of dural afferents to changes in pH along with the desensitization time constants suggests that pH-evoked currents in
Dural afferents are most likely mediated by ASICs but the exact makeup of these subtypes is yet to be determined.

**pH-evoked currents in dural afferents are blocked by the ASIC antagonist amiloride**

To further determine whether dural afferent pH-evoked currents were mediated by ASICs, the effects of amiloride, a non-specific blocker of the ENaC/DEG channels (Kellenberger et al., 2002), were determined. Current amplitude in the presence of the antagonists was normalized to the average current amplitude in response to the preceding control pH applications. As shown in Fig. 3.5, 1 mM amiloride reversibly blocked the pH 6.0-evoked current (Fig. 3.5A and C; average blockade of 92% at 1 mM and 58% at 10 µM), while 10 µM capsazepine or 10 µM AMG-9810 produced virtually no effect (Fig. 3.5B and C; average blockade of 3.05% and 1.26%, respectively). Previous studies have shown that 10 µM AMG-9810 fully blocks proton-induced TRPV1 activation (Gavva et al., 2005) thus the lack of effect of either capsazepine or AMG-9810 indicates that TRPV1 channels do not contribute to the pH-evoked currents in dural afferents at these proton concentrations.

Amiloride block of ASIC currents is less evident at higher pH (Yagi et al., 2006). The mechanism by which this occurs is not clear but demonstrates an unusual pharmacology between amiloride and ASICs that appears to depend on the pH used as a stimulus i.e. amiloride concentrations that block ASIC currents at pH 6.0 are not necessarily the same as those that block currents at pH 7.0. Current evoked by pH 6.9 application was partially blocked by 1 mM amiloride (53% block compared to 92% block
at pH 6.0 shown above), but not by 10 µM capsazepine or 10 µM AMG-9810 (Fig. 3.6A and B). These data indicate that pH-evoked currents in dural afferents at these higher pH values were also generated by ASICs but not TRPV1. In transfected cells, ASIC3 is known to generate a “window current” due to a window of overlap between the activation and inactivation curves around neutral pH. This property allows the channel to generate sustained depolarizing currents for at least 20 mins. Previously, amiloride has been shown to enhance the window current in cells transfected specifically with ASIC3 subunits (Yagi et al., 2006). Similar effects were observed here (Fig. 3.6C) since the pH 7.0-evoked sustained currents were increased by 1 mM amiloride.

**pH-evoked responses are mediated through activation of ASIC3 subunit in vitro**

To further confirm the contribution of the ASIC3 subunit in pH evoked currents, we tested whether pH responsive dural afferents also responded to a specific ASIC3 agonist in vitro. Recently, a non-proton ligand was identified for ASIC3 channels (Yu et al.). At physiological pH (7.4), 2-guanidine-4-methylquinazoline (GMQ) caused persistent activation of the ASIC3, but not ASIC1 or ASIC2 channels. Thus, this ligand is selective for ASIC3 containing channels and can be used as a tool to probe the contribution of ASIC3 to pH-sensitive currents in dural afferents. As we have discussed above, decay time constants below 500 ms suggested the presence of an ASIC3 subunit. Here we showed that fifteen pH-responsive dural afferents with decay time constants below 500 ms also exhibited currents in response to 3 mM GMQ application, which ranged from 100 pA to 1400 pA (Fig. 3.7). On the other hand, a decay time constant
above 500 ms excluded the presence of ASIC3 (Hesselager et al., 2004). One dural afferent with a decay time constant of 2400 ms and six pH-insensitive neurons did not respond to a 3 mM GMQ application at the cutoff amplitude (50 pA), which was consistent with the fact that GMQ selectively activated the ASIC3 subunit. Given the fact that a decay time constant of pH 6.0-evoked currents in dural afferents were mostly within the range of ASIC3-containing subtypes, pH-sensitive dural afferents also exhibited GMQ currents, which were presumably mediated through an ASIC3 subunit and amiloride could increase the dural afferent pH 7.0-evoked window current, the subtypes responsible for these currents in dural afferents most likely contain the ASIC3 subunit.

**pH-evoked firing of action potentials in dural afferents**

Current clamp recordings were performed to determine the effects of decreased pH on membrane excitability. Responses of cells to solutions of different pH were initially performed under voltage-clamp conditions to determine current amplitudes (Fig. 3.3). Subsequently, responses to different pH applications were recorded in the current-clamp configuration. The percentage of dural afferents firing action potentials at different pH values was plotted in Fig. 3.8B. In 53% of the dural afferents, a short burst of action potentials was rapidly evoked by a 5s application of pH 6.8 (Fig. 3.8A). In 30% of the dural afferents, pH 6.9 evoked either a single action potential or a burst of action potentials. Remarkably, pH 7.0 also evoked action potentials in 7% of the dural afferents. No action potentials were observed in response to application of pH 7.1 solutions.
However, this pH was often able to evoke small membrane depolarizations in dural afferents (Fig. 3.8B).

**pH-evoked sustained current in dual afferents**

In retrogradely-labeled dural afferents which exhibited pH 7.0-evoked currents, 80% (n = 32) exhibited sustained currents during prolonged application with no sign of development of complete desensitization by the end of application (a sustained pH 7.0-evoked current is shown in a representative dural afferent in Fig. 3.9). This 60 sec sustained current (Fig. 3.9A) was able to evoke a sustained 60 sec membrane depolarization (Fig. 3.9B), which was consistent among the cells tested in both voltage and current clamp with prolonged application of pH 7.0 solutions. Given that these window currents are able to produce sustained membrane depolarization and that window currents can presumably last for the duration of exposure of the cell to moderate pH values, these properties may be important in prolonged afferent signaling during migraine headache in the presence of decreased dural pH.

**Cutaneous allodynia following acidic stimulation of the dura via activation of ASIC3**

As we have discussed in chapter one, 79% migraine patients developed cutaneous allodynia during their migraine attack. Testing for allodynia has been used as a strategy in humans for migraine diagnosis. It has also been used in preclinical works to evaluate the efficacy of abortive migraine treatments (Edelmayer et al., 2009). Morphine, sumatriptan and CGRP antagonist were able to abolish allodynia in a rat migraine model
(Edelmayer et al., 2009) which parallels the clinical pharmacology of pain associated with migraine headache. It has been hypothesized that the development of cutaneous allodynia is correlated with a series of pathological changes along the trigeminovascular pain pathway, from activation of nociceptors to sensitization of neurons in central nervous system (Burstein et al., 2000a). Therefore, allodynia testing can be utilized to study the pain initiation and transmission processes of migraine.

Application of pH 5.0 SIF solution to the dura produced significant (p < 0.0001) time dependent and reversible reductions in withdrawal thresholds to tactile stimuli applied to the face or the hind-paws (Fig. 3.10A and B) compared with pH 7.4 SIF application. Maximal effects occurred 2 hours after pH 5.0 application, and facial and hind-paw responses approached baseline by 5 hrs after pH 5.0 application (Fig. 3.10 A and B). To explore the ion channels underlying pH 5.0-induced cutaneous allodynia, western blotting was performed using an antibody which recognizes the ASIC3 protein and showed that ASIC3 protein was expressed in the dura (Fig. 3.11A), consistent with its role in detecting dural pH change. Coapplication of amiloride (100 nmol) with pH 5.0 prevented facial and hind-paw cutaneous allodynia (Fig. 3.11B). In contrast, AMG-9810 (10 nmol) application did not prevent pH 5.0-induced facial and hind-paw allodynia (Fig. 3.11B). To test that the AMG-9810 dose used here was sufficient to block TRPV1 activity, we examined facial and hind-paw allodynia produced following application of 0.01 nmol capsaicin to the dura. AMG-9810 significantly blocked the capsaicin-induced decrease in facial and hind-paw withdrawal threshold (p < 0.01, Fig 3.12A and B)
indicating that this dose is sufficient to block any TRPV1-mediated contribution to the behavior shown in Fig. 3.11B.

To further explore whether moderate dural pH change could evoke mechanical allodynia, we applied pH 6.0 SIF solutions onto the dura. Consistent with our previous findings with pH 5.0, pH 6.0 SIF dural application also produced significant (p < 0.0001) time dependent and reversible reductions in withdrawal thresholds to tactile stimuli applied to the face or the hind-paws (Fig. 3.13A and B) compared with pH 7.4 SIF application. pH 6.0 evoked cutaneous allodynia was also significantly (***p < 0.01, *p < 0.01) blocked by coapplication with 100 nmol amiloride, a non-specific ASIC antagonist, but not with 10 nmol AMG-9810, a TRPV1 antagonist, suggesting that the responses were also mediated through activation of ASICs (Fig. 3.14A). Identifying the ASIC subtype which was responsible for the pH evoked mechanical allodynia would help us better understand the dural afferent pain signaling and develop new targets for treatment.

Based on our electrophysiology results, we hypothesized that dural afferent pH evoked responses were mediated through ASIC3 subtype containing channels. Here we tested the effects of pharmacological inhibition of pH evoked mechanical allodynia by a selective ASIC3 inhibitory peptide APETx2. APETx2, a sea anemone peptide, blocked ASIC3 homomeric and heteromeric channels both in transfected cells and rat primary sensory neuron culture (S. Diochot et al., 2004). It also blocked ASIC3 evoked sustained window current in DRG neurons (Deval et al., 2011). Coapplication of APETx2 (10 µl, 20 µM) with pH 6.0 solution significantly blocked the development of tactile allodynia of the face.
and the hindpaws (Fig. 3.14B), indicating that pH-evoked migraine-related pain behaviors were mediated by ASIC3 subtype containing channels.

3.3 Discussion

*Decreased extracellular pH activates dural afferents in vitro and in vivo*

The studies described here demonstrated that even small decreases in extracellular pH were able to directly excite primary dural-afferent neurons via the opening of ASICs. This is the first study providing experimental evidence that ASICs are important in dural afferent signaling and these findings further suggest that decreased pH within the dura is an initiating factor in the pathophysiology of migraine headache.

Whole-cell recording experiments performed here showed that changes in pH from 7.4 to pH 6.8, 6.9 or 7.0 alone were sufficient to directly excite many dural afferents and to produce sustained membrane depolarization in others. Thus, even small changes in pH due to the release of the acidic mast-cell granular contents could lead to activation of dural afferents via opening of ASICs. Further acidification as well as sensitization of dural afferents by other mast-cell derived substances (Levy et al., 2007) could enhance this activation leading to increased pH-induced excitation of dural afferents. The studies described here also showed that dural afferents were able to generate window currents at pH 7.0. These window currents have been shown to last for at least 20 min at pH 7.0 (Yagi et al., 2006) and would presumably last as long as the pH stimulus was present. Although the pH that might be achieved within the dura prior to or during migraine headache is not known, pH 7.0 is not far from normal physiological pH. Furthermore,
sustained currents and membrane depolarizations were observed at pH 7.1. Although these currents/depolarizations are not able to evoke action potentials under the normal, non-sensitized recording conditions used here, other pathological events may also occur during migraine attack (e.g. mast-cell degranulation). This may convert sustained pH-induced depolarization to sustained firing of action potentials in sensitized neurons. Thus, persistent activity through ASICs may contribute to the sustained activation of dural afferents leading to the development of migraine headache.

These studies also demonstrated that application of varying pH to the dura produced dose-dependent mechanical allodynia with thresholds at pH 6.4 and pH 6.6 for facial and hindpaw, respectively, which is consistent with the observation that the majority of migraine patients experience cutaneous allodynia during the headache phase (Burstein et al., 2000b). Thus, this behavioral response represents migraine-related behavior in rats following exposure of the dura to a decrease in pH. It is not clear how quickly this solution is buffered and what pH is ultimately present at the nerve endings embedded within the dura. Therefore, the pH that could produce allodynia might be higher than what was reported here. Furthermore, these results showed that despite the transient ASIC-mediated activity observed in vitro, a single application of a decreased pH solution to the dura was sufficient to produce prolonged allodynia. The exact mechanisms leading to prolonged ASIC-mediated behavior are not yet clear but will be discussed in this chapter.

*pH evoked responses are mediated through activation of ASIC3 in vitro and in vivo*
Protons can activate both ASICs and the transient receptor potential vanilloid subfamily member 1 (TRPV1). TRPV1, which is highly expressed on primary afferent neurons, is activated directly by capsaicin, low pH and noxious temperatures (above 43 °C) (Caterina and Julius, 2001). TRPV1 is presumably expressed on dural afferents since these neurons have been shown to respond to capsaicin (Bove and Moskowitz, 1997). In contrast to the rapid activating and inactivating pH sensitive currents observed here in dural afferents, pH evoked TRV1 current appeared to be a slow desensitizing current (Neelands et al., 2005). Thus, based on the current kinetics, TRPV1 is less likely to play a role in sensing pH changes under current experimental settings. However, several lines of evidence support a role for ASICs at the pH values used in this study. The decay time constants of dural afferent pH evoked current were within the range of ASICs. The pH-evoked currents were not blocked by TRPV1 antagonists, capsazepine or AMG-9810 but were blocked by the ASIC antagonist, amiloride. The behavioral responses to pH 5.0 and pH 6.0 application in the present study were blocked by the amiloride but not by AMG-9810 indicating that ASICs mediated these effects regardless of the final pH at the nerve endings. Although we did not further block pH responses by AMG-9810 at any pH above 6, it is unlikely that TRPV1 would play a role since at pH above 6, less than 10% of TRPV1 channels are able to open and generate currents (Neelands et al., 2005).

Targeting an ion channel subtype is a common strategy in developing new therapeutics. Among the ASIC subtypes, ASIC3-containing channels are the most suitable candidates for detecting pH change within the dura and initiating the afferent signaling because of its extreme sensitivity to pH change compared with other ASIC
subtypes and high expression level in sensory neurons. It is important to fully determine the role of ASIC3 as drugs targeting ASIC3 would not have CNS side effects since ASIC3 is restricted to peripheral sensory neurons. Combined with our previous data, several lines of evidence supported the role of the ASIC3 subunit. We were testing the effects of SIF solutions with pH values above 6 \textit{in vitro} and \textit{in vivo}. This experimental setting minimized the contributions of ASIC4, which is pH insensitive and ASIC2, which has a half maximum activating pH between 4 and 5 (Lingueglia, 2007). The decay time constants of most dural afferent pH-evoked current were below 500 ms, which were within the range of ASIC3-containing channels, while ASIC1 had much longer decay time constant, longer than 1 second (Benson et al., 2002). Most dural afferents also exhibited sustained currents at moderate pHs which were exclusive to ASIC3-containing channel activation (Yagi et al., 2006; Yan et al., 2010). Amiloride enhanced a dural afferent pH 7.0-evoked sustained current. While the mechanism by which amiloride increases sustained currents at this pH is unknown, this effect is observed only in ASIC3-containing channels previously (Yagi et al., 2006). Most pH sensitive dural afferents, but not pH insensitive neurons could be activated by a specific ASIC3 agonist, GMQ (Yu et al.). The selective ASIC3 antagonist, APETx2 could block pH-evoked allodynia in awake animals. Taken together, these data support the conclusion that dural afferent pH responses are mediated through ASIC3-containing channels \textit{in vitro} and \textit{in vivo}.

\textit{Sensation of meningeal pH change}
The expression of ASIC channels on dural afferents would allow these neurons to immediately respond to changes in pH within the dura thus initiating afferent signaling. However, the source of change in pH within the dura is not known. Cortical-spreading depression (CSD), defined as a spreading wave of cortical excitation followed by depression of neuronal activity, has been linked to migraine, especially to migraine aura (Bolay et al., 2002). CSD has been shown to be accompanied by dural ischemia (Lambert and Michalicek, 1994) which could produce a drop in dural pH. Given the fact that dural afferents express a high density of ASIC channels and ASICs are extremely sensitive to pH change, even small decreases in pH resulting from dural ischemia can activate ASIC channels and initiate signaling. Alternatively, mast cells are located in close proximity to nerve endings. Prior work has shown that the intragranular pH of isolated mast cells was 5.55 ± 0.06 (De Young et al., 1987), which makes it possible that mast cell degranulation could acidify the environment surrounding sensory nerve endings.

The pH of different cellular compartments, bodily fluids, and organs is usually tightly regulated. Consequently, noxious afferent signaling is a strategy utilized by many systems to signal changes in pH to avoid tissue damage. Thus, it is not surprising that the expression of ASICs and the ability to generate large pH-induced inward currents is not unique to neurons innervating the dura. For example, a recent study of trigeminal ganglion neurons innervating the masseter muscle showed that 64% of these neurons displayed robust ASIC-like current at pH 6.8 (average amplitude 4.9 ± 0.5 nA) and the average amplitude of pH 6.0-evoked currents in these neurons was almost 12 nA (Connor et al., 2005). Although our study is the first demonstration of ASIC-dependent signaling
from the dura, our findings are in line with pH-induced activation of afferents innervating other tissues. These studies highlight the importance of signaling changes in pH throughout the body but unlike other tissues, the mechanisms leading to pH changes within the meninges have yet to be fully determined.

**Conclusion**

In conclusion, this study identified the ASIC3 subunit as a key element in mediating pH evoked dural afferent activation and migraine-related pain behavior. These data reveal a cellular mechanism by which decreased pH in the meninges directly excites afferent pain-sensing neurons through activation of ASIC3 channels, potentially contributing to migraine headache initiation.
Figure 3.1 Dural afferents responded to multiple mediators. 75% of dural afferents responded to α,β-MeATP, while 80% exhibited ASIC-like pH 6-evoked currents.
Figure 3.2 Dural afferents exhibited huge ASIC currents. (A) Dural afferents exhibited currents well above 20 nA in response to pH 6.0 application. (B) Current density (pA/pF) of pH 6.0 evoked currents in dural afferents (n = 30).
Figure 3.3 The action of modest pH stimuli on dural afferents. (A) Recordings from a dural afferent in response to a 5 second pH step from 7.4 to 6.0, 6.8, 6.9, 7.0 and 7.1, respectively. (B) Percentage of dural afferents responding to step from pH 7.4 to pH 6.0, 6.8, 6.9, 7.0 and 7.1, respectively (n = 30). Cutoff was set at 20 pA.
Figure 3.4 Dural afferents pH-evoked currents exhibited variable kinetics. (A) Examples of acid evoked currents from two representative dural afferents in response to a 5 second pH step from 7.4 to 6.0. ASIC3 like (left) and ASIC1a like (right) B) Histograms showing the distribution of desensitization time constants (s) (n = 128).
Figure 3.5 Amiloride blockade of pH 6.0 evoked currents in dural afferents. (A–C) pH was stepped from 7.4 to 6.0 for 1 second or 5 second every 20 s. pH 6.0 evoked current in a representative dural afferent was reversibly blocked by 1 mM amiloride (A) but not 10 µM capsazepine (B) or 10 µM AMG-9810 (C). (D) The current amplitude
after drug treatment was normalized to the average current amplitude in response to the preceding control pH applications. Blockade of pH 6 evoked peak current amplitude by amiloride, capsazepine or AMG-9810 was averaged (mean ± SEM). pH 6.0 evoked current was blocked by 10 µM and 1 mM amiloride (n = 9 and 10, respectively), but not 10 µM capsazepine (n = 10) or AMG-9810 (n = 13).
Figure 3.6 Amiloride exhibited a paradoxical effect on higher pH. (A) The pH 6.9 evoked current in a representative dural afferent is blocked by 1 mM amiloride. (B) The percentage of pH 6.9 evoked peak current amplitude blocked by 1 mM amiloride (n = 9), 10 µM capsazepine (n = 5) and 10 µM AMG-9810 (n = 14), respectively. (C) 1 mM amiloride enhanced sustained current evoked by a pH step from 7.4 to pH 7.0.
Figure 3.7 pH-evoked responses were mediated through ASIC3 subunits *in vitro*. GMQ evoked current in pH-sensitive dural afferents. Representative traces illustrating acid- (pH 6) and GMQ-induced currents in a dural afferent.
Figure 3.8 pH evoked depolarization and firing of action potentials in dural afferents. (A) pH evoked depolarization and firing of action potential recorded in a representative dural afferent by a 5 second step from pH 7.4 to the indicated pH. The four traces are on the same vertical scales. (B) Percentage of dural afferents exhibiting firing of action potentials in response to the pH indicated.
Figure 3.9 Small pH changes evoked sustained current in dural afferents. (A) The sustained current was undiminished throughout a 60 second stimulus to pH 7.0 (beginning and ending pH is 7.4) in a representative cell. (B) In turn, a 60 second stimulus to pH 7.0 evoked an undiminished depolarization in the same cell.
Figure 3.10 Application of a pH 5.0 SIF solution to the dura elicited cutaneous allodynia. Withdrawal thresholds to tactile stimuli applied to the face (A) and the hind-paws (B) were measured in rats before and immediately after dural application of pH 5.0 SIF (n = 18) or pH 7.4 SIF (n = 8). For both facial and hind-paw responses, two-factor analysis of variance indicated that response thresholds of pH 5.0 SIF-treated rats were significantly (p < 0.0001) less than those of pH 7.4 SIF-treated rats.
Figure 3.11 pH 5.0-induced cutaneous allodynia was mediated by activation of ASICS. (A) Western blot analysis indicated that ASIC3 protein was expressed in dura. (B) Application of pH 5.0 SIF was given alone or with either amiloride (gray bars, 100 nmol, n = 10) or AMG-9810 (black bars, 10 nmol, n = 9). Vehicle control was pH 5.0 SIF containing 1% DMSO (white bars). Significant (*p < 0.05) differences among means for each group were determined by analysis of variance followed by Dunnett’s post hoc test. Coapplication of amiloride significantly abolished behavioral signs of tactile alldynia of the face and hind-paw (*p < 0.05). Cotreatment with AMG-9810 failed to prevent development of behavioral signs of tactile alldynia of the face or hind-paw.
Figure 3.12 AMG-9810 blocked capsaicin-induced cutaneous allodynia. Withdrawal thresholds to tactile stimuli applied to the face A) and the hind-paw B) were measured in rats before and immediately after dural application of 0.01 nmol capsaicin (n = 8), synthetic interstitial fluid (SIF) (n = 8) or 0.01 nmol capsaicin + 10 nmol AMG9810 (n= 9). For both facial and hind-paw responses, two-factor analysis of variance indicated that response thresholds of capsaicin -treated rats were significantly (p < 0.0001) less than those of SIF-treated rats. C) Data were converted to area over the time-effect curve and normalized as a percentage of the capsaicin – treated group to allow for multiple comparisons. Significant (p < 0.05) differences among means for each group were determined by student’s t-test. Coapplication of AMG-9810 (black bars) significantly abolished behavioral signs of tactile allodynia of the face and hind-paws evoked by capsaicin (white bars) (p < 0.01).
Figure 3.13 Application of pH 6.0 SIF solution to the dura elicited cutaneous allodynia. Withdrawal thresholds to tactile stimuli applied to the face (A) and the hind-paws (B) were measured in rats before and immediately after dural application of pH 6.0 SIF (n = 11) or pH 7.4 SIF (n = 13). For both facial and hind-paw responses, two-factor analysis of variance indicated that response thresholds of pH 6.0 SIF-treated rats were significantly (p < 0.0001) less than those of pH 7.4 SIF treated.
Figure 3.14 pH 6.0-induced cutaneous allodynia was mediated by activation of ASIC3. (A) Application of pH 6.0 solution was given with either vehicle (1% DMSO, white bar, n = 13), amiloride (100 nmol, grey bar, n = 12) or AMG-9810 (10 nmol, black bar, n = 10). Withdrawal thresholds to tactile stimuli were measured for 5 hrs and data were converted to area over the time-effect curve and normalized as a percentage of the pH6.0-treated group. Coapplication of amiloride significantly abolished behavioral signs of tactile allodynia of the face and hind-paw (*p < 0.05, **p < 0.01). Cotreatment with AMG-9810 failed to prevent development of behavioral signs of tactile allodynia of the
face or hind-paw. (B) Application of pH 6.0 solution was given alone (white bar, n = 13) or with the selective ASIC3 antagonist APETx2 (10 μl, 20 μM, grey bar, n = 13). Coapplication of APETx2 (10 μl, 20 μM) significantly abolished behavioral signs of tactile allodynia of the face and hind-paw (*p < 0.05, **p < 0.01).
CHAPTER FOUR:  
SENSITIZATION OF PH-EVOKED RESPONSES BY MAST CELL 
MEDIATORS  

4.1 Introduction

In recent years, accumulating evidence has suggested the involvement of neurogenic inflammation in migraine pathophysiology. The hypothesis is supported by the established efficacy of NSAIDs in migraine therapy as well as increased intracranial levels of inflammatory mediators during migraine attacks in humans (Waeber and Moskowitz, 2005). As discussed in chapter one, dural mast cells have been suggested to be the endogenous source for inflammatory mediators since activation of these granulated cells can potentially activate and sensitize nearby nerve terminals (Dimlich et al., 1991; Strassman et al., 2004). Mast cells have established roles in modulating a variety of inflammatory conditions such as asthma, arthritis and coronary artery disease (Theoharides and Kalogeromitros, 2006). Several lines of clinical evidence have pointed to its role in migraine. Epidemiological studies show that migraine is associated with higher than expected incidences of mast cell-related disorders, such as asthma, rhinitis, and interstitial cystitis (Low and Merikangas, 2003). Cohort studies conducted at Mayo Clinic (Rochester, MN) showed a high prevalence of headache in patients with mastocytosis, a disorder characterized by an increased number of tissue mast cells (Smith et al.). Moreover, compound 48/80, a potent mast cell degranulator was found to trigger
migraine-like headache in humans following injection into cranial circulation (Sicuteri, 1963). Dural mast cell activation following trigeminal stimulation can be blocked by sumatriptan, a widely used migraine abortive medication.

According to the neurogenic inflammation theory of migraine, following activation of primary afferent neurons, vasoactive neuropeptides released from peripheral terminals could trigger a cascade of events, including vasodilatation and plasma protein extravasation (Waeber and Moskowitz, 2005). Preclinical studies have shown that mast cells act on multiple sites in this process. First, mast cell degranulation activated primary afferent neurons and initiated the signaling process (Levy et al., 2007). Mediators released following mast cell activation promoted the sensitization of dural afferents to mechanical stimuli (Zhang and Levy, 2008; Zhang et al., 2007). It has also been shown that plasma extravasation within the dura following trigeminal ganglion stimulation was dependent on mast cell activation (Dimitriadou et al., 1992). Furthermore, neuropeptides such as CGRP and substance P released from primary afferents could trigger release of mast cell mediators (Ali et al., 1986; Piotrowski and Foreman, 1986), which sustain the activation and sensitization of dural afferents, resulting in persistent throbbing headache and mechanical allodynia.

We have showed in the previous chapter that decreased meningeal pH could promote activation of dural afferents. However, the pH drop might not be the solo pathological change during migraine attacks. Under sensitized conditions, allodynia might be produced at a higher pH. Since activation of dural mast cells could promote
sensitization of dural afferents, it would be interesting to determine whether this event alters the threshold for pH-evoked responses in vitro and in vivo.

4.2 Results

Dural application of pH solutions dose-dependently elicit cutaneous alldynia

Here we determined the pH threshold for evoking facial and hindpaw mechanical allodynia following application of SIF solutions at different pH values. Application of pH 6.4 SIF solutions to the dura produced significant (*p < 0.05) time dependent and reversible reductions in both facial and hindpaw withdrawal thresholds compared with SIF pH 7.4 application (Fig. 4.1B). The effect peaked at 2 hrs and returned to baseline values 5 hrs after pH 6.4 applications (Fig. 4.1A). pH 6.6 dural application caused a significant (*p < 0.05) decrease in hindpaw withdrawal threshold, but not facial withdrawal threshold (Fig. 4.1B). Tactile alldynia was not observed following pH 6.8 application (p > 0.05) (Fig. 4.1B). Therefore, we determined that pH dose-dependently caused facial and hindpaw alldynia with thresholds at pH 6.4 and pH 6.6 for the face and hindpaw, respectively.

Sensitization of pH-evoked responses by mast cell mediators in vitro and in vivo

Fig. 4.1 indicated that dural afferents exhibited extreme sensitivity to pH changes within the dura, which made it possible that mast-cell derived substances (Zhang et al., 2007) could enhance pH-induced tactile alldynia. Among the list of mast cell mediators, many can sensitize dural afferents resulting in increased excitability. In anesthetized
animals, *in vivo* electrophysiological single-unit recordings have showed that mast cell mediators, including histamine, serotonin, prostacyclin sensitized the meningeal nociceptors (Zhang et al., 2007). Along with these mediators, tryptase was the most abundant secretory granule-derived serine proteinase contained in mast cells (Vanderslice et al., 1990). Tryptase has been shown to act on dural afferents through cleavage and activation of the protease-activated receptor-2 (PAR2) receptor (Zhang and Levy, 2008).

In this study we used a combination of well known mast cell mediators including histamine, serotonin, AC55541 (PAR2 agonist) and iloprost (prostacyclin analogue).

First we identified a combination of sub-threshold mast cell mediators (10 μl, 200 μM Histamine 200 μM, Serotonin 10 μM, AC55541 and 20 μM Iloprost), which did not produce allodynia when it was injected alone (black bar in Fig. 4.2A and B, n = 9). Coapplication of sub-threshold mast cell mediators and pH 6.6 significantly (**p < 0.01, red bar in Fig. 4.2A, n = 12) decreased facial withdrawal threshold compared with pH 7.4 SIF application (white bar in Fig 4.2A, n = 11), whereas pH 6.6 by itself did not (red bar, in Fig. 4.2A, n = 13). On the other hand, the pH threshold for evoking hindpaw allodynia shifted from pH 6.6 (Fig. 4.2B) to pH 6.8 following coapplication with sub-threshold mast cell mediators (red bar in Fig. 4.2B, n = 12).

Since coapplication with mast cell mediators shifted pH thresholds to a higher pH *in vivo*, we would expect that ability of dural afferents to generate pH-induced action potential firing would increase following application of mast cell mediators *in vitro*. The percentage of dural afferents firing action potentials was calculated before and after 5 mins application of mast cell mediators (100 μM Histamine, 10 μM Serotonin, 10 μM
AC55541, 1 μM Iloprost). The percentage of neurons firing action potentials was increased at each pH tested (Fig. 4.3). Number of spikes generated was counted from the same dural afferent before and after application of mast cell mediators. Dural afferents exhibited significantly more spikes following application of mast cell mediators (Fig. 4.4, paired t-test, **p < 0.01 for pH 6.9, *p < 0.05 for pH 6.8 and pH 7.0). In two representative dural afferents, application of mast cell mediators led to the transient and persistent firing of action potentials at pH 7.0 and 6.9, respectively (Fig. 4.5). Enhanced pH-induced excitation was likely mediated through sensitization of dural afferents instead of modulation of ASIC current amplitudes since there was no significant change between ASIC current amplitudes before (-9350 ± 3146 pA, n = 9) and after (-10245 ± 3204 pA, n = 9) application of mast cell mediators (p > 0.05, Fig 4.6). However, resting membrane potentials were significantly (*p < 0.05, paired t-test) depolarized following application of mast cell mediators (-63.13 ± 1.485 mV, n = 44) compared with baseline levels (-69.31 ± 1.255 mV, n = 44). Taken together, dural afferents showed an increased sensitivity to pH changes within the dura following sensitization induced by application of mast cell mediators.

4.3 Discussion

Sensitization of dural afferents by mast cell mediators facilitate pH-evoked responses

Given the clear temporal dissociation between the short duration of action potential firing and the time course of migraine headache, the behavioral response (as well as the time course of migraine headache), sensitization of these responses leading to
prolonged excitation may better explain how ASIC-dependent signaling could mediate many hours of pain. Since mast cells are potential sources for endogenous inflammatory mediators, here we determined the pH thresholds in the presence of classic mast cell mediators. Following application of mast cells mediators, we showed depolarization of membrane potential, an indication of dural afferent sensitization. Under sensitized conditions, the percentage of dural afferents firing action potentials increased for each pH tested, indicating increased sensitivity to pH change. On the other hand, dural afferents exhibited persistent firing following sensitization, which might contribute to sustained activation of dural afferents, leading to the development of migraine headache which can last from hours to days. In awake animals, we also observed increased sensitivity to meningeal pH change following sensitization induced by dural application of mast cell mediators. These experiments highlighted the pathophysiologic effect of inflammatory events happening within the dura and how it might alter the threshold of dural afferents to respond to other stimuli.

Although we did not observe any changes in ASIC currents following application of mast cell mediators, we cannot rule out the possibility that due to limitations of our experimental setting, we were unable to capture the modulation of ASIC currents by mast cell mediators. Proinflammatory mediators including NGF, serotonin, interleukin-1, and bradykinin have been shown to increase ASIC current amplitude by enhancing ASIC3 encoding gene expression (Mamet et al., 2002). In our study, the huge variability in ASIC current amplitudes between dural afferents might prevent the detection of a statistically
significant change in peak amplitudes. It still remains possible that ASIC expression is upregulated in migraine patients.

Conclusion

These findings provide a cellular mechanism by which inflammatory events within the dura result in increased sensitivity of dural afferents to other stimuli. Increased sensitivity of dural afferents following sensitization is likely to be an important contributor to prolonged activation underlying migraine pathophysiology. Drugs targeting at stabilizing mast cells might provide a new therapeutic target for migraine treatments.
Figure 4.1 Application of pH solution to the dura dose dependently elicited cutaneous allodynia. (A) Withdrawal thresholds to tactile stimuli applied to the face and the hind-paws were measured in rats before and immediately after dural application of pH 6.4, pH 6.6 pH 6.8 and pH 7.4 solutions. (B) Data were converted to area over the time-effect curve. pH dose-dependently decreased the withdrawal threshold both in the face and the hindpaws. Significant (*p < 0.05) differences among means for each group were determined by analysis of variance followed by Dunnett's post hoc test.
Figure 4.2 Dural afferents exhibited enhanced withdrawal responses to pH changes following coapplication with sub-threshold mast cell mediators. Sub-threshold mast cell mediators (M) were composed of 200 μM Histamine 200 μM, Serotonin 10 μM, AC55541 and 20 μM Iloprost in 10 μl. Application of sub-threshold mast cell mediators (black bar, n = 9) by itself did not cause significant changes in facial and hindpaw withdrawal thresholds compared with SIF administration (white bar, n = 11). Significant (**p < 0.01) differences among means for each group were determined by analysis of variance followed by Dunnett’s post hoc test. (A) Coapplication of sub-threshold mast cell mediators and pH 6.6 solution caused a significant decrease in facial withdrawal threshold (red bar, n = 12) compared with pH 7.4 application, whereas application of pH 6.6 solution by itself did not (red bar, n = 13). (B) Coapplication of sub-threshold mast cell mediators and pH 6.8 solution caused a significant decrease in hindpaw withdrawal threshold (blue bar, n = 14) compared with pH 7.4 application, whereas application of pH 6.8 solution by itself did not (blue bar, n = 14).
Figure 4.3 Dural afferents exhibited enhanced sensitivity to pH-induced action potential firing following application of mast cell mediators. Mast cell mediators were composed of 100 μM Histamine, 10 μM Serotonin, 10 μM AC55541, 1 μM Iloprost. The percentage of neurons firing action potentials was calculated before and after a 5 minute application of mast cell mediators. At each pH tested, the percentage of neurons firing action potentials increased following application of mast cell mediators.
Figure 4.4 Dural afferents exhibited enhanced excitability to pH-induced action potential firing following application of mast cell mediators. Number of spikes was counted from the same dural afferent before and after a 5 minute application of mast cell mediators. Application of mast cell mediators significantly increased number of spikes for each pH tested (paired t-test, **p < 0.01 for pH 6.9, *p < 0.05 for pH 6.8 and pH 7.0)
Figure 4.5 Application of mast cell mediators led to the transient (B) and persistent (A) firing of action potentials at pH 7.0 (B) and 6.9 (A) in two representative dural afferents.
Figure 4.6 Effects of acute application of mast cell mediators on dural afferent pH 6 evoked currents. Current amplitudes were recorded in the same dural afferent before (white bar, n = 9) and after (grey bar, n = 9) a 5 minute application of mast cell mediators, 100 μM Histamine, 10 μM Serotonin, 10 μM AC55541, 1 μM Iloprost. There was no significant difference in peak current amplitudes (p > 0.05) before and after acute application of mast cell mediators.
CHAPTER FIVE:
SENSITIZATION OF DURAL AFFERENTS UNDERLIES MIGRAINE-RELATED PAIN BEHAVIOR FOLLOWING MENINGEAL APPLICATION OF INTERLEUKIN-6 (IL-6)

5.1 Introduction

As suggested by neurogenic inflammation theory, inflammatory substances play important roles in modulation of migraine by interfering with nociceptive transmission. Interleukin-6 (IL-6) is a proinflammatory cytokine with an established role in induction and maintenance of a variety of inflammatory pain conditions, including skin incision, carrageenan injection and pancreatitis-induced pain (Clark et al., 2007; De Jongh et al., 2003; Vardanyan et al., ; Xu et al., 1997). The expression levels of IL-6 and its specific receptor IL-6R (gp80) increased under inflammatory conditions increases in IL-6 paralleling pain intensity over time (Arruda et al., 1998; Bao et al., 2001; Kurek et al., 1996). Preclinical studies showed that local injection of IL-6 into hindpaw or muscle was able to induce mechanical hyperalgesia (Dina et al., 2008). Moreover, for human rheumatoid arthritis, neutralizing IL-6 using a monoclonal antibody is effective in ameliorating symptoms of inflammation as well as the associated pain (Nishimoto et al., 2009; Smolen et al., 2008).

With migraine, IL-6 levels were found to be elevated during migraine attacks (Fidan et al., 2006; Sarchielli et al., 2006). Following nitroglycerin infusion, a well-
known migraine trigger, IL-6 expression increased in dura mater and IL-6 levels were elevated in cerebrospinal fluid (CSF) (Reuter et al., 2001), indicating its role in mediating delayed inflammation. IL-6 was strongly correlated with stress, a reliable migraine trigger. Stress has been shown to be capable of evoke IL-6 release in a mast cell dependent manner (Huang et al., 2003). Thus, accumulating evidence points to IL-6 as a contributing factor in many inflammatory conditions, possibly including migraine.

However, the contributions of IL-6 to this process and the mechanisms by which this may occur have not yet been explored. IL-6’s pain promoting actions are thought to be mediated by a direct action on nociceptors because sensory neuron specific knockout of the IL-6 co-receptor reduced nociceptive sensitization (Andratsch et al., 2009). Sensitization of meningeal nociceptors leads to afferent signaling is thought to contribute to the headache that occurs during migraine. Following acute IL-6 application, trigeminal ganglion neurons display phosphorylation of extracellular signal-regulated protein kinase (ERK), a neuronal activation marker (Melemedjian et al., 2010), indicating that these neurons respond to IL-6 through the Mitogen- Activated Protein Kinase (MAPK) signaling pathway. It is interesting to note that activation of the ERK1/2 MAPK pathway has been implicated in modulating various pain conditions via transcriptional, translational or post-translational regulation (Ji et al., 2009; Karim et al., 2001; Melemedjian et al., 2010). Recent work has identified the voltage-gated sodium channel Nav1.7 as a novel downstream post-translational target for MAPK. Nav1.7 is a threshold sodium channel expressed on small and medium DRG neurons (Rush et al., 2007) and
inhibition of ERK1/2 decreased neuronal excitability by inhibiting Nav1.7 phosphorylation and altering its gating properties (Stamboulian et al., 2010).

Taken together, these studies led us to propose that increased levels of IL-6 in the meninges produce migraine-related pain behavior and this hypothesis was addressed using a preclinical model of headache. Further, we examined whether dural afferent excitability was increased following IL-6 exposure and whether this increased excitability is consistent with sodium channel phosphorylation.

5.2 Results

Cutaneous allodynia following IL-6 administration to the dura

A preclinical in vivo migraine model was used to evaluate the effect of meningeal IL-6 application on mechanical withdrawal thresholds both to the face and hindpaws (Edelmayer et al., 2009). Application of 1 ng IL-6 in SIF solution to the dura produced significant (p < 0.0001) time-dependent reductions in withdrawal thresholds to tactile stimuli applied to the face or the hind-paws compared with SIF application alone (Fig. 5.1A). Maximal effects occurred 2 hours after IL-6 application with facial and hind-paw remaining sensitive for at least 24 hrs (Fig. 5.1A). IL-6 dose-dependently reduced the withdrawal thresholds compared with vehicle control as 1 ng IL-6 produced greater allodynia than that observed at 0.1 ng (Fig. 5.1B). Coapplication of the MEK inhibitor, U0126 (10 nmol) with 1 ng IL-6 prevented facial and hind-paw cutaneous allodynia compared to vehicle control (Fig. 5.2) indicating that IL-6 produces allodynia following dural application via activation of the MAP kinase (ERK) signaling pathway.
Activation of the ERK pathway mediates IL-6-induced hyperexcitability of dural afferents

Retrograde labeled cells *in vitro* were selected for patch clamp experiments. Current clamp configuration was used to determine the current threshold, i.e. the minimum current required to initiate an action potential. Action potentials were elicited by injecting rectangular current steps (25 ms, $\Delta = 10$ pA). Current threshold was significantly decreased for dural afferents pretreated with IL-6 for 15 mins (217.4 ± 17.98 nA, n = 31) compared with dural afferents treated with vehicle (319.3 ± 25.14 nA, n = 30, **$p < 0.01$ vs vehicle) (Fig. 5.3 A and B). Resting membrane potential was significantly hyperpolarized following pretreatment with the MEK inhibitor U0126 pretreatment (-67.24 ± 1.474 mV) compared with IL-6 treatment alone (-62.40 ± 0.7737 mV *$p < 0.05$), consistent with phosphorylation of the sodium channel Nav1.7 downstream of the ERK signaling pathway (Stamboulian et al., 2010). Pretreatment with the MEK inhibitor U0126 for 10 mins reversed the IL-6-induced changes in current threshold (374.6 ± 52.45 nA, n = 13, ##$p < 0.01$ vs IL-6 alone), again indicating that IL-6 acts through the MAP kinase pathway.

Nav1.7 is known to generate current in response to slow ramp depolarization due to its slow inactivation kinetics, hence a ramp stimulus protocol was used to preferentially elicit activity of Nav1.7 (Cummins et al., 1998). Slow ramp currents from 0.1 to 0.7 nA with $\Delta = 0.2$ nA were injected over 1 second (Fig. 5.4A) to mimic slow depolarization. Cutoff was set at 2 nA and cells that did not fire action potentials at 2 nA were excluded from the study. Dural afferents acutely treated with 50 ng/ml IL-6 for 10
min showed a significant increase in the number of spikes and a decrease in the latency to the first AP spike (Fig. 5.4A and B), consistent with increased Nav1.7 activity. Pretreatment with 10 μM U0126 for 10 min significantly reversed the IL-6-induced increase in excitability (Fig. 5.4 A and B) indicating that, similar to IL-6 induced allodynia and changes in current threshold, these changes are due to activation of ERK signaling.

5.3 Discussion

Sensitization of dural afferents by IL-6 via activation of the ERK signaling pathway

With respect to migraine, IL-6 level was found to be elevated during migraine attacks (Fidan et al., 2006; Sarchielli et al., 2006). IL-6 can be released from activated human mast cells (Grabbe et al., 1994; Kruger-Krasagakes et al., 1996) and dural macrophages (Reuter et al., 2001). In addition to immune cells, most nucleated cells have been shown to be able to synthesize IL-6 in vitro (De Jongh et al., 2003), which implies the possibility of de novo synthesis by dural afferents following activation. Regardless of the source, studies here demonstrated that direct meningeal application of exogenous IL-6 caused migraine-like pain behaviors in rats, indicating its ability to sensitize dural afferents. However, the downstream signaling pathway underlying IL-6 induced mechanical allodynia is not clear.

IL-6 first binds specifically to its α-receptor subunit IL-6R and this complex efficient recruit the signaling receptor subunit gp130, which initiates the signaling cascades (Heinrich et al., 2003). Downstream signaling cascades include activation of
JAK (Janus kinase) tyrosine kinase family members, leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family (Heinrich et al., 2003). Another major signal transduction pathway involves activation of the MAPK (mitogen-activated protein kinase) cascade (Heinrich et al., 2003). It is interesting to note that these intracellular signal proteins downstream of IL-6 are also involved in promoting sensitization of nociceptors (Ji et al., 2009; Obreja et al., 2005). Recent studies demonstrated that in addition to regulating immune responses, IL-6’s pain promoting actions could be mediated by direct action on nociceptors (Andratsch et al., 2009). IL-6 has the ability to sensitize TRPV1 channels in sensory neurons via a gp130/Jak/PKC-dependent mechanism (Andratsch et al., 2009; Obreja et al., 2005). IL-6 could also act on sensory neurons to enhance translation through activation of the MAPK pathway (Melemedjian et al., 2010), contributing to nociceptive plasticity. Conditional knockout of gp130 on sensory neurons alleviated heat hyperalgesia in vivo in models of pathological pain with an inflammatory background (Andratsch et al., 2009), all of which suggest sensory neurons as the site of actions of IL-6. Here we demonstrated that IL-6 evoked mechanical allodynia was also mediated through activation of the MAPK pathway, providing another piece of evidence that activation of the MAPK pathway is able to induce and maintain pain hypersensitivity. Therefore, drugs targeting at IL-6, its coreceptors or downstream signaling proteins might provide new therapeutic targets for migraine.
Sensitization of sodium channel Nav1.7 via activation of the ERK pathway in dural afferents

Human genetic studies have demonstrated an important role for the sodium channel Nav1.7 in pain (Dib-Hajj et al., 2007). Gain-of-function mutations of Nav1.7 lead to DRG neuron hyperexcitability and are associated with several extreme pain conditions in humans, including inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD), whereas loss-of-function mutations of Nav1.7 are associated with congenital insensitivity to pain (Dib-Hajj et al., 2007). Preclinical work has also indicated an important role for Nav1.7 in mediating inflammatory pain as supported by the evidence that formalin-induced mechanical allodynia and thermal hyperalgesia were abrogated in Nav1.7 knockout mice (Nassar et al., 2004). Hence, preclinical and clinical studies have created a compelling rationale for targeting Nav1.7 in pain modulation.

Nav1.7 is characterized by slow closed-state inactivation, which allows it to generate currents in response to ramp stimuli (Cummins et al., 1998). The present work showed that IL-6 application increased the number of spikes and decreases the latency to the first action potential (AP) in response to ramp stimuli protocols, which were consistent with the hyperexcitability induced by Nav1.7 phosphorylation (Stamboulian et al., 2010). Moreover, this IL-6-induced hyperexcitability was mediated through ERK signaling, which was similar to prior work showing that inhibition of ERK1/2 decreases excitability in DRG neurons (Stamboulian et al., 2010). Although we cannot rule out the possibility that modulation of other channels contributes to these effects at this point, the
findings reported here support the hypothesis that IL-6 produces sodium channel-dependent hyperexcitability and migraine-related behavior through activation of the ERK pathway. Further studies need to be conducted to examine the effect of IL-6 on Nav1.7 current amplitude and current kinetics.

**Conclusion**

This study provides direct evidence that IL-6 can sensitize dural afferents in a manner consistent with sodium channel phosphorylation and that it produces prolonged migraine-related pain behavior through activation of the ERK pathway. These findings suggest that drugs targeting IL-6 and its signaling pathway may have efficacy in the treatment of migraine headache.
Figure 5.1 Application of IL-6 to the dura elicited cutaneous allodynia. (A) Withdrawal thresholds to tactile stimuli applied to the face and the hind-paws were measured in rats before and immediately after dural application of 1 ng IL-6 (n = 16) or SIF (n = 17). For both facial and hind-paw responses, two-factor analysis of variance indicated that response thresholds of IL-6-treated rats were significantly (p < 0.0001) less than those of SIF-treated rats. (B) Rats received dural administration of SIF (white bar, n = 17), 0.1 ng IL-6 (gray bar, n = 22) or 1 ng IL-6 (black bar, n = 16). Withdrawal thresholds to tactile stimuli were measured for 5 hrs and data were converted to area over the time-effect curve. IL-6 dose-dependently decreased the withdrawal threshold both in
the face and in the paw. Significant (*p < 0.05, **p < 0.01) differences among means for each group were determined by analysis of variance followed by Dunnett's post hoc test.
Figure 5.2 IL-6-induced cutaneous allodynia was mediated via activation of the MEK/ERK pathway. Application of 1 ng IL-6 was given with vehicle (white bars, 1% DMSO, n = 12) or with U0126 (black bars, 1 nmol, n = 12). Withdrawal thresholds to tactile stimuli were measured for 5 hrs and data were converted to area over the time-effect curve and normalized as a percentage of the IL-6 – treated group. Coapplication of U0126 significantly abolished behavioral signs of tactile allosthynia of the face and hind-paw (*p < 0.05, **p < 0.01).
Figure 5.3 IL-6 treatment significantly increased the dural afferent excitability, which was blocked by pretreatment with U0216. (A) Current threshold was measured in dural afferents treated with vehicle (white bar, n = 30), 50 ng/ml IL-6 (black bar, n = 32) or 50 ng/ml IL-6 + 10 µM U0126 (gray bar, n = 13). Significant (**p < 0.01) differences among means for each group were determined by analysis of variance followed by Dunnett's post hoc test. Current threshold was significantly lowered after IL-
6 treatment (**p < 0.01). Pretreatment with U0126 for 10 mins significantly reversed the hyperexcitability induced by IL-6 (##p < 0.01). (B) Action potentials were elicited by 25ms step current injection from resting membrane potential. Horizontal line and arrow indicate 0 and -70mV membrane potential, respectively. Current threshold for action potentials in the representative dural afferents treated with vehicle, 50 ng/ml IL-6 or 50 ng/ml IL-6 + 10 μM U0126.
Figure 5.4 IL-6 promoted ERK-dependent hyperexcitability of dural afferents in response to ramp current stimuli. (A) Action potentials were elicited by 1 second ramp current injection ranging from 0.1 to 0.7 nA in 0.2 nA increments from resting membrane potential. Dural afferents treated with IL-6 show increased numbers of action potentials and shorter time-to-first AP peak compared with vehicle-treated dural afferents. IL-6-induced hyperexcitability was blocked by pretreatment with 10 μM U0126. (B) Difference in the mean numbers of action potentials among groups was analyzed by comparing the slopes and intercepts generated from linear regression. Comparison among several groups for time-to-first spike was performed by two-factor analysis of variance. Dural afferents treated with 50 ng/ml IL-6 (red square, n = 16) showed a significant (p < 0.05) increase in number of action potentials and a significant decrease in time-to-first peak compared with vehicle-treated dural afferents (black circle, n = 12). Pretreatment with 10 μM U0126 (blue triangle, n = 13) for 10 mins significantly reversed IL-6-induced hyperexcitability.
CHAPTER SIX:  
DISCUSSION

Migraine is estimated to affect 10% of people worldwide (Robbins and Lipton, 2010). Despite recent advances in drug development, there is a huge unmet need for better medications. Modern drug discovery relies on identification of therapeutic targets and subsequent testing of drug candidates (Ohlstein et al., 2000), which requires design of in vitro and in vivo assays that reflect disease-relevant biological processes (Drews, 2000). Development of new therapies for migraine has previously been hampered by a lack of understanding of the disease mechanism and predictive animal models. Over the past few decades, great progress has been made in migraine research: the neurobiology of trigeminovascular system has been elucidated (Goadsby, 2007; Moskowitz and Macfarlane, 1993); several signaling molecules have been implicated (Lassen et al., 2002; Olesen et al., 1994) and animal models have been developed (Edelmayer et al., 2009; Strassman et al., 1996). However, the origin of headache pain is still not fully understood. Understanding the mechanisms that directly activate primary afferent neurons innervating the cranial meninges is important in understanding the events that initiate migraine headache. In this work, we describe the evidence suggesting that peripheral activation of meningeal nociceptors is sufficient to initiate pain signaling process and produce behavioral responses. In particular, we focus on identifying ion channels, pro-inflammatory mediators and signaling molecules underlying activation and sensitization
of primary afferent neurons, which will hopefully provide novel therapeutic targets for migraine drug development. This discussion will address the data presented in the previous chapters in relation to our current understanding of the initiation process of migraine.

6.1 Sites of nociception in migraine

Several nociceptive sites have been implicated in migraine pathophysiology, including extracranial and intracranial tissues. Extracranial pain-sensitive tissues include skin, muscles, arteries, periosteum and calvarial bones. Skin is less likely to play a role in migraine since nociception from skin is often perceived as superficial and is precisely located, whereas sensory inputs from deeper tissues feel as if they originate from inside of the head (Olesen et al., 2009). Anatomic studies showed that meningeal nerves infiltrated the periosteum through the calvarial sutures (Kosaras et al., 2009), suggesting the possibility of transmitting painful impulses from these extracranial tissues.

Intracranial pain sensitive tissues include the cranial vessels and the meninges since they are densely innervated by nerve fibers (Goadsby et al., 2009) and stimulation of large cranial vessels and the meninges evoked headache in similar cephalic locations as migraine (Ray and Wolff, 1940). The pulsating nature of the headache in 80% of migraine patients suggests the involvement of cranial arteries in migraine pathophysiology (Ray and Wolff, 1940).

Within the meninges, the dura mater has been extensively studied in relation to migraine pain, whereas the role of the pia mater still remains unknown since it is
inaccessible with conventional craniotomy. Although preclinical studies have linked the dura mater with migraine pain, there is still no convincing evidence of any structural or biochemical changes of the human dura mater during migraine attacks. Thus, only hypothetical mechanisms of nociception from the meninges can be proposed here. First, like cranial vessels, dilation of the meningeal middle artery (MMA) has been proposed. Immunohistochemical analysis has shown a bundle of axons running in parallel with the MMA (Strassman et al., 2004), indicating potential neurovascular cross-talk. CGRP or histamine released from activated primary afferent neurons nearby or degranulated mast cells have been shown to be potent vasodilators (Brain et al., 1985). Therefore, pulsation of a dilated MMA could in turn activate primary afferent fibers which are close to the MMA and result in pulsating headache. In addition to sensory fibers, dura mater is also densely innervated by sympathetic fibers (Keller et al., 1989), which suggests the possibility that aberrant release of norepinephrine from these fibers could promote sensitization of neighbouring sensory fibers. Last but not least, the dura mater is also highly populated with mast cells, macrophages and fibroblasts, among which mast cells have drawn much attention from migraine researchers. The role of mast cells in migraine pathophysiology will be discussed later in this chapter. To further understand the role of the dura mater in initiating migraine headache, neuroimaging studies aimed at identifying structural and biochemical changes during migraine attacks must be conducted. And it is important to understand how migraine triggers could cause these changes in the meninges.

6.2 Mast cells as a potential endogenous source for inflammatory mediators
Migraine occurs more frequently in patients with allergy and asthma, which implies involvement of brain mast cells (Theoharides et al., 2005). Recent studies support the theory that episodes of local sterile meningeal inflammation contribute to migraine headache pathogenesis (Levy, 2009) and dural mast cells are hypothesized to be the endogenous source for these inflammatory mediators. Mast cells are known to reside mostly within the dura compared to other meningeal layers (Dimlich et al., 1991; Strassman et al., 2004; Theoharides et al., 2005) and they have been demonstrated to be in direct contact with afferent endings within the dura (Rozniecki et al., 1999). Upon activation, mast cells can release a host of cytokines and vasoactive and proinflammatory mediators (Theoharides and Kalogeromitros, 2006) and induce de novo synthesis of cytokines, chemokines, and eicosanoids (Rivera and Gilfillan, 2006; Theoharides and Kalogeromitros, 2006). Mediators secreted following mast cell activation are able to activate and sensitize dural afferents (Zhang et al., 2007). Therefore, it is possible that dural mast cell and nerve terminals could be assembled in functional microdomains, whereby activated sensory fibers may release neuropeptides and evoke exocytosis of adjacent mast cells, which, in turn, can modulate the functions of nerve fibers.

Several migraine precipitants have been shown to activate mast cells and promote the release of mediators, such as the neuropeptides CGRP, substance P and pituitary adenylate cyclase-activating peptide (PACAP) (Ottosson and Edvinsson, 1997; Seebeck et al., 1998). Levels of stress are strongly correlated with the incidence of migraine (Wacogne et al., 2003) and acute stress could induce increased vascular permeability in a mast cell dependent manner, possibly via the release of corticotropin releasing factor.
CRF) (Chrousos, 1995; Esposito et al., 2001; Rozniecki et al., 1999). Fluctuations of estrogen levels, believed to play a role in menstrual migraine turned out to be a modulator for mast cells (Rozniecki et al., 1999). Moreover, infusion of nitroglycerin, a reliable migraine trigger, was also able to trigger dural mast cell degranulation (Reuter et al., 2001). Therefore, dural mast cells could serve both as “sensor” and “effector” cells to participate in detecting changes in the meninges induced by migraine triggers and promoting development of neurogenic inflammation.

Regarding the observation that mast cells are found throughout the body, in respiratory tract, skin and digestive systems, one might ask what is special about the dural mast cell or, in other words, why do migraine triggers cause activation of dural mast cells and the headache phenotype in the absence of systemic symptoms. We are certainly far from understanding how migraine triggers initiate pain signaling and there is even less knowledge about dural mast cells, allowing only hypothetical ideas to be listed to answer the above questions. Based on current knowledge, the following differences might be functionally important.

1. The expression of surface receptors on mast cells can be different from one tissue to another, which would result in a different response to the same stimuli in different tissues. In addition to IgE and IgG receptors, mast cells can also express chemokine receptors, estrogen receptors and receptors for neuropeptides, such as CGRP receptor and Neurokinin 1 (NK1) receptor (Juremalm and Nilsson, 2005; Rozniecki et al., 1999; van der Kleij et al., 2003). This idea is supported by studies showing that CGRP selectively evoked release of histamine in dural mast cells, but not peritoneal mast cells
(Ottosson and Edvinsson, 1997). CGRP-induced dural mast cell exocytosis was blocked by a CGRP receptor antagonist (Ottosson and Edvinsson, 1997), suggesting that it is a receptor-mediated effect. In fact, dural mast cells were more sensitive to CGRP than substance P (Ottosson and Edvinsson, 1997; Reynier-Rebuffel et al., 1994), whereas skin mast cells showed opposite sensitivity (Lowman et al., 1988). This is also consistent with clinical studies showing that a CGRP receptor antagonist was effective in alleviating migraine headache (Olesen et al., 2004), whereas substance P antagonists were not (Diener, 2003). However, there is no direct evidence of these possibilities since detailed immunohistochemical analysis of receptor expression profiles for dural mast cells are not available.

2. In addition to degranulation, brain mast cells could undergo progressive and time-dependent ultrastructural changes indicative of secretion in the absence of degranulation (Dimitriadou et al., 1991), a phenomenon occurring within 1 min following trigeminal sensory fiber stimulation, whereas degranulation happens later (Dimitriadou et al., 1991). This type of activation is possibly associated with the ability to release some mediators selectively (Theoharides et al., 1982). In fact, mast cells are rarely seen to degranulate in inflammatory disease (Theoharides and Kalogeromitros, 2006). Selective secretion of mediators in the absence of degranulation might be the only way that mast cells could regulate inflammatory process without causing systemic allergic reactions (Theoharides and Kalogeromitros, 2006). Given the fact that mast cells often “wrapped” around blood vessels (Rozniecki et al., 1999), it is possible that an increase of
inflammatory mediators within the microdomain is sufficient to evoke vascular responses following this type of activation.

3. Cortical spreading depression has been shown to activate primary afferent neurons (Zhang et al., 2010). Stimulated primary afferent neurons were able to degranulate or activate dural mast cells (Dimitriadou et al., 1991; Folgueras et al., 2009), which makes it possible that CSD could promote the release of inflammatory mediators from mast cells.

4. Finally, in inflammatory skin conditions, increased nerve-mast cell contacts and increased numbers of mast cells were observed (Harvima et al., 1990; Naukkarinen et al., 1996; Sugiura et al., 1992), which might also apply to migraine pathophysiology.

Taken together, these studies suggest the involvement of dural mast cells in migraine pathophysiology and provide a hypothesis for how neurogenic inflammation might initiate and sustain pain signaling. Detailed morphological, biochemical and functional analysis of dural mast cell under normal and pathological conditions should be conducted to better understand the meningeal inflammation process.

6.3 Nav1.7 as a potential integrator for amplifying generator potentials in dural afferents

Recent studies have identified Nav1.7 as a key contributor in regulating nociceptive neuronal excitability. Nav1.7 is found to be highly expressed in DRG neurons, their nerve endings and sympathetic ganglion neurons, but at negligible levels in spinal cord, brain, skeletal muscle and cardiac muscle (Sangameswaran et al., 1997;
Toledo-Aral et al., 1997). The expression pattern of Nav1.7 makes it an attractive drug target since inhibition of Nav1.7 is less likely to induce side effects in the central nervous system and cardiovascular systems. Within DRG, Nav1.7 was expressed in a higher proportion of nociceptive than low threshold mechanoreceptive neurons (Djouhri et al., 2003), consistent with its role in nociception.

Nav1.7 produces a fast activating and inactivating current which is sensitive to tetrodotoxin (TTX-S) (Sangameswaran et al., 1997). In addition to contributing to all-or-none action potentials like other sodium channel isoforms (Renganathan et al., 2001), Nav1.7 is able to generate graded responses in response to small, slow depolarizations due to its distinctive slow development of closed-state inactivation (Cummins et al., 1998). Therefore, Nav1.7 is able to amplify small depolarizations, such as generator potentials and promote depolarization of membrane potentials (Dib-Hajj et al., 2007). Depolarization of the membrane potential induced by activation of Nav1.7 brings the neuron close to the activation threshold of Nav1.8, which is responsible for evoking all-or-none action potentials in sensory neurons (Renganathan et al., 2001). Hence, regarding migraine, Nav1.7 is a suitable candidate to amplify generator potentials caused by stimulating channels on the meninges.

Dural afferents express a variety of ligand-gated ion channels, including ASICs, P2X, TRPV1, TRPV4, TRPA1, all of which have the potential to produce generator currents in response to external environmental changes in the meninges. Generator currents converge to activate Nav1.7 and lead to a depolarizing generator potential. Under inflammatory conditions, mRNA and protein levels of Nav1.7 increase, which
parallels the increase in TTX-S currents (Black et al., 2004). Our studies shown here indicated that activation of the ERK signaling pathway downstream of pro-inflammatory mediators could potentially cause a hyperpolarizing shift of the Nav1.7 activation curve and allowing the channel to open with smaller depolarizations. A recent computer simulation study indicates that at potentials more negative than -50 mV, increasing Nav1.7 expression reduces the current required to evoke an action potential (Choi and Waxman, 2011). In another words, increasing Nav1.7 expression or enhancing Nav1.7 activity could enhance neuronal excitability. Therefore, in theory, enhanced Nav1.7 activity or increased Nav1.7 current following meningeal inflammation has the potential to facilitate the summation of generator potentials, induce supra-threshold depolarization which would otherwise be sub-threshold, evoke action potentials and initiate subsequent afferent signaling. These studies indicate that Nav1.7 might provide another therapeutic target for migraine.

6.4 Activation of dural afferents as a headache generator

There has been a long-term debate about the origin of headache in migraine; whether it is due to activation of peripheral nociceptors or dysfunction of the brain stem. Studies from our laboratory have shown that dural afferents could generate currents in response to decreased pH solutions, hypotonic solutions, capsaicin and mustard oil via activation of ASICs, TRPV4, TRPV1 and TRPA1, respectively. Consistent with \textit{in vitro} findings, meningeal application of these solutions were able to elicit cutaneous allodynia. Moreover, blockade of peripheral inputs with coapplication of antagonists for ASIC,
TRPV4, TRPV1 and TRA1 abrogate the behavioral responses, supporting the hypothesis that activation of peripheral nociceptors is the driver for migraine headache.

In addition to initiating afferent signaling, our data also imply that activation of nociceptors by itself is sufficient to induce central sensitization. Applications of these solutions created a similar behavioral response pattern, similar to what has already been published following meningeal application of inflammatory mediators (2mM histamine, serotonin, bradykinin and 0.2mM PGE2 in 10mM Hepes buffer, pH 5.0) (Edelmayer et al., 2009). Facial and hindpaw allodynia peaked 2 hours following meningeal injection, consistent with the time course for the development of cutaneous allodynia seen in migraine patients (Burstein et al., 2000a). The development of facial and hindpaw allodynia suggests that regardless of the initial triggers, peripheral inputs could promote the development of central sensitization, which is consistent with what has been reported from other groups. Dural application of inflammatory mediators has been shown to promote sensitization of both brain stem trigeminal neurons (Burstein et al., 1998) and ON cells in the RVM (Edelmayer et al., 2009).

In line with this theory, drugs targeted at inhibiting peripheral inputs must be administered as early as possible before the establishment of central sensitization to abort headache and prevent the development of cutaneous allodynia. This was confirmed by the study showing that pretreatment or early post-treatment with sumatriptan was able to block the development of inflammatory mediator-induced alldoynia (Edelmayer et al., 2009). However, efficacy was lost after central sensitization was established (Edelmayer et al., 2009). Although the site of action for sumatriptan is not clear, it has been proposed...
to induce cranial vasoconstriction (Humphrey et al., 1990), inhibit peripheral neuronal activation (Moskowitz and Cutrer, 1993) and disrupt transmission between peripheral and central trigeminal neurons (Levy et al., 2004), all of which contribute to reduce peripheral input. Clinical studies also showed the same phenomena for migraine patients, since administration of almotriptan within 1 hr of pain onset when the pain intensity was still mild could significant decrease the headache duration (Valade, 2009). Since we are proposing potential drug targets aimed at blocking activation and sensitization of peripheral nociceptors in this thesis, these compounds must also be administered as early as possible. Given the fact that most migraine patients could predict their headache based on premonitory symptoms and aura, patients should be educated about the importance of taking these medications at the onset of migraine.

6.5 Conclusion

Activation of primary afferent neurons is critical for initiating migraine headache. To this end, the present study explored the ion channels responsible for excitation of meningeal nociceptors. Here we proposed that ion channels expressed on dural afferents are responsible for sensing external environmental changes in the meninges following CSD, ischemic or inflammatory events. The present study supported this hypothesis demonstrating that activation of these channels, such as TRPV1 and ASICs was able to excite dural afferents and produce migraine-related pain behavior.

Sensitization of primary afferent neurons makes this group of neurons more sensitive and more excitable to external environmental changes as evidenced by that in
the presence of mast cell mediators, dural afferents exhibited increased sensitivity to pH change in vitro and in vivo. IL-6 and its downstream activation of the ERK signaling pathway robustly sensitized meningeal nociceptors potentially through phosphorylating the sodium channel Nav1.7. In my opinion, Nav1.7 is a very promising and exciting drug target for pain since it integrates generator currents temporally and spatially and brings the neurons close to the activation threshold for action potentials. Moreover, Nav1.7 itself can be modulated by inflammatory mediators which provides amplification of generator potentials and a further depolarization shift of membrane potential. In theory, Nav1.7 plays a very important role in modulating excitability of meningeal nociceptors since electrical changes of dural afferents following meningeal external environmental changes eventually converge to Nav1.7.

**Future project:** Several questions have remained unanswered from analysis of the present work. With regard to the sources of pH drop and IL-6, immunohistochemical analysis of the localizations of mast cells, nerve fibers, ASICs and gp130 in dura whole mount would be critical to determine anatomically whether mast cell degranulation could activate and sensitize dural afferents by causing pH drop or releasing IL-6. Furthermore, functionally it would be of interest to know whether mast cell degranulation could evoke cutaneous allodynia and to elucidate the mechanism by which receptors or ion channels mediate mast cell degranulation-evoked responses. Compound 48/80, which is used to promote mast cell degranulation, will be applied to the dura and facial and hindpaw allodynia will be measured. Together, these studies may provide direct evidence that endogenous events happening in the meninges have the potential to activate and sensitize
dural afferents directly. Finally, it would be worth studying the association between ERK and Nav1.7 and the modulation of Nav1.7 kinetics following IL-6 application. These data will further establish the role for Nav1.7 in migraine pathophysiology.

**Significance:** current therapies for the treatment of migraine have been restricted to triptans, tricyclic antidepressants and anticonvulsants or combinations with NSAIDs. However, such compounds provide limited benefits to migraine patients. While the molecular mechanisms for the initiation of migraine headache remain unknown, the present study provides evidence that peripheral sensory inputs from the meninges have the potential to induce the development of cutaneous allodynia, a sign of central sensitization. Specifically, these studies maybe one of the first to demonstrate the role of ASICs, IL-6 and Nav1.7 in activation and sensitization of dural afferents, providing novel drug targets for future migraine research. These data also validate the therapeutic rationale that migraine must be treated as early as possible.
REFERENCES


