MECHANISMS UNDERLYING MIGRAINE HEADACHE PATHOPHYSIOLOGY: NOVEL INSIGHTS FROM PRECLINICAL MODELS

by

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Dedicated to my family

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Migraine is a highly prevalent and complex disorder characterized by severe, unilateral, pulsating headaches associated with photophobia, phonophobia, nausea, and, in some cases, auras. Headaches are the most disabling component of the condition and, while treatments have improved over the last few decades, the complexity of migraine pathophysiology has made it extremely challenging to develop highly efficacious therapeutics. Patients are particularly susceptible to attacks following exposure to normally innocuous stimuli and mounting clinical and preclinical evidence suggests that this may be due to maladaptive sensitization of the trigeminal sensory system. Although it is widely accepted that the trigeminovascular system is responsible for the pain associated with migraine, the mechanisms by which dura-projecting trigeminal ganglia (TG) nociceptors become activated and sensitized remain poorly understood. In other preclinical pain models, reactive nitroxidative species such as nitric oxide (NO), but particularly peroxynitrite (PN), have been implicated in establishing long-lasting hypersensitivity and targeting these molecules has achieved antinociceptive efficacy. Despite NO donors being one of the most consistent triggers of headache, little is known about the role of nitroxidative species in migraine

mechanisms. Similarly, other mechanisms that have been shown to contribute to nociceptor activation and sensitization in preclinical pain models, such as translational dysregulation of mRNA, have not been studied in the context of migraine. Thus, the goal of our research was to utilize pharmacological techniques and transgenic animals in our novel preclinical migraine models to further understand the mechanisms that contribute to the development and persistence of migraine headache. The first part of our work highlights a novel, critical role for PN formation in mediating long-lasting hypersensitivity in preclinical models of migraine while the second part of our work defines MNK regulation of eIF4E phosphorylation as a key target for migraine therapeutics.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION TO HEADACHE AND THE MECHANISMS UNDERLYING ITS PATHOPHYSIOLOGY

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WHAT IS HEADACHE?

Headache is among the commonest afflictions in the world, affecting around 95% of the general population at least once in their lifetime. Headache disorders, characterized by recurrent headaches, are among the most common neurological disorders, with the World Health Organization estimating that global prevalence among adults is about 50%, with a female predominance. Headache disorders are not only painful, they are extremely disabling, collectively ranking as the third cause of disability in people under 50 years of age (migraine alone ranks first) [1]. Additionally, the economic burden of headache on society is estimated to be around $14 billion per year [2], with primary care providers seeing headache patients on a regular basis. Despite this, the underlying pathophysiology that causes headache is still poorly understood. Part of the complexity in understanding headache stems from the numerous ways in which it can present itself. Fortunately, the cranial nociceptive system is rather limited in terms of its structures and researching basic headache pharmacology has been a feasible goal.

The International Classification of Headache Disorders (ICHD), first published in 1988 and most recently updated in 2018 (ICHD-3), contains explicit criteria for determining headache type based on phenomenology [3]. Broadly, headache is divided into primary or idiopathic headache and secondary or symptomatic headache. Primary headaches generally have no known underlying cause while secondary headaches are the result of another condition causing tension on or inflammation of cranial nociceptive structures. Because the same nociceptive pathways are shared between primary and secondary headache disorders, primary headache has been the focus for understanding basic headache biology. The most common primary headaches include migraine, tension-type headache (TTH), and cluster headache (CH), while rarer ones include short-lasting

unilateral neuralgiform headache (SUNCT), thunderclap headache, etc. Common secondary headaches include those related to trauma, medication overuse (MOH), and vascular disease. Painful cranial neuropathies and other orofacial pain disorders that affect the cranial nociceptive system generally fall into their own category and include rare painful disorders such as trigeminal neuralgia and occipital neuralgia, among others.

HEADACHE VS. MIGRAINE

Much of our current understanding of headache pathophysiology comes from studying migraine, which is by far the most studied headache condition and for good reason, as it is the third most prevalent and seventh most disabling disorder worldwide [4]. However, migraine presents a phenotype that is unlike other primary headaches, including the development of hypersensitivity across four distinct phases- a prodrome phase consisting of irritability, depression, and fatigue; an aura phase (occurs in approximately 33% of migraineurs) which manifests sensory changes that are typically not present, such as intense visual cues, tingling sensations across the body, and other unusual sensory symptoms; an intense headache lasting 4-72 hours in most cases, but can last longer in certain phenotypes; and a postdrome phase marked by more fatigue, impairments in concentration, and depressed moods. The aura phase of migraine is thought to be induced by a physiological phenomenon called cortical spreading depression (CSD), defined as a propagation of cortical electrophysiological hyperactivity followed by a wave of inhibition [5]. This phenomenon is hypothesized to directly contribute to the experience of aura and is not thought to contribute to other headache disorders. The presence of migraine-specific pathologies may lead to the observations that many therapeutics that have efficacy in migraine are not useful in other types of

headache (e.g. NSAIDs have efficacy in migraine but not CH, SUNCT). Thus, in order to develop better and safer therapeutics, the mechanisms of other primary headache disorders and their distinctions from migraine must be better understood.

Table 1.1. Characteristics of primary headache disorders and their first-line treatments.

			← Trigeminal Autonomic Cephalalgias (TACs) →					
Name	Migraine	Tension-type headache	Cluster	Paroxysmal hemicrania	SUNCT	SUNA	Hemicrania continua	Trigeminal neuralgia
Location	Unilateral or bilateral	Bilateral	Unilateral orbital	V1, opthalmic	Unilateral orbital/ temporal	Unilateral orbital/ temporal	Unilateral	Unilateral V2/V3
Duration	4-72 h	30 min to 7 days	15-180 min	2-30 min	15 s to 4 min	15 s to 4 min	Persistent	1-120 s
Frequency	Episodic or chronic	Episodic or chronic	1-8/day	More than 5 daily for more than half the time	More than 1 daily for more than half the time	More than 1 daily for more than half the time	Continuous	Variable
Sex Ratio (M:F)	1:3	1:2	3:1	1:1	17:2	3:2	1:2	2:3
Associated Features	May include extreme sensitivity to light/sound (aura), nausea, vomiting, nasal congestion, insomnia, neck stiffness	Tenderness of scalp or neck, sensation of tightness across forehead, sides and/or back of head.	Lacrimation, hyperemia	Lacrimation, hyperemia	Lacrimation, hyperemia	Lacrimation, hyperemia, rhinorrhea, nasal congestion	Lacrimation, hyperemia, nasal congestion, ptosis, sweating eyelid edema	Aching/burning sensations in the face, spasms, other variable autonomic features
First-line treatment	Sumatriptan, other triptans	NSAIDs	Oxygen, verapamil, sumatriptan	Indomethacin	Lamotrigine, lidocaine	Lamotrigine, lidocaine	Indomethacin	Carbamezapine, oxcarbazepine

Trigeminal autonomic cephalalgias (TACs) are primary headache disorders characterized by similar autonomic symptoms and presentation of trigeminal pain (Table 1.1). TAC's usually share a common underlying pathophysiology involving the trigeminovascular system, the trigeminoparasympathetic reflex, and the brain centers controlling circadian rhythm [3]. The International Headache Society's classification of TACs includes cluster headache (CH) (the most common TAC), paroxysmal hemicrania (PH), and short-lasting, unilateral, neuralgiform headache with conjunctival injection and tearing (SUNCT). Although the underlying pathophysiology of

TACs is generally shared, there are some distinct differences between them. Diagnosis of TACs is currently based on three major features including trigeminal pain, changes in rhythmicity, and autonomic symptoms (such as lacrimation and rhinorrhea). Observations of trigeminal pain in TACs includes perivascular inflammation of the internal carotid artery, increased levels of various pro-inflammatory neuropeptides (which we will discuss in detail later), and sympathetic dysfunction (ptosis and miosis) [6]. In patients with chronic PH and CH, orbital vasculitis has been suggested to be one underlying pathology, indicating that vascular changes may also implicated in TACs [7]. Neuropathic mechanisms may also be involved in TACs, as roughly 10% of PH patients report subtle neck movement as a trigger while patients with CH and SUNCT report similar mechanical triggers, typically provoked by repetitive movements or increased pressure [6, 8]. These observations suggest that common neuropathic pain medications may also be effective in treating TACs. Additionally, disruptions in sleep associated with TACs suggests the involvement of central sites that mediate circadian rhythm and sleep cycles. Indeed, neuroimaging studies have identified structural and functional alterations in TACs, especially in CH [9, 10]. Furthermore, homeostatic functions may be altered in CH. The levels of various hormones such as orexin and melatonin have been shown to be altered in CH patients and could contribute to infrequent circadian cycles, suggesting that pharmacologically targeting hormonal pathways may be a useful therapeutic strategy for CH [11].

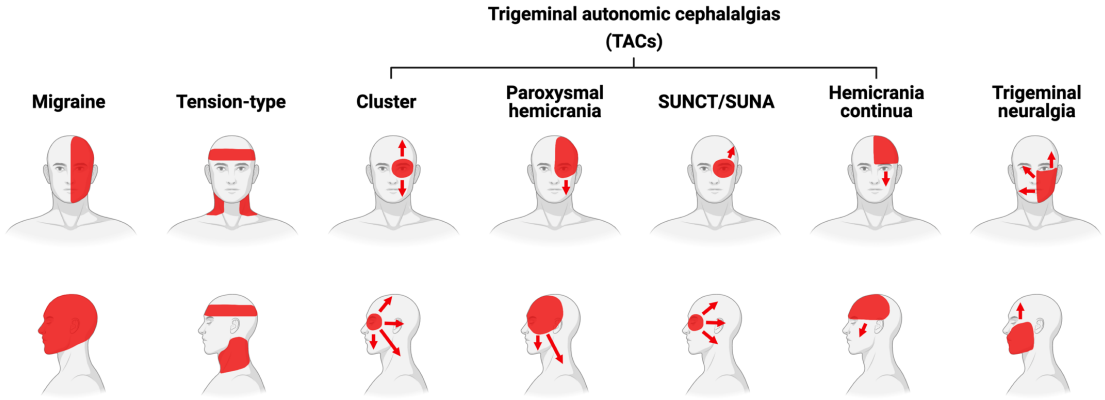

Figure 1.1. Comparison of the location of pain across primary headache disorders. The pain of migraine is generally unilateral and encompasses the entire side of the head affected, whereas the pain experienced in tension-type headache is reported to feel like a tight band of pressure around the head and extending into the neck. TACs are usually associated with unilateral pain in focused regions with the potential to spread to other areas depending on the headache type. Trigeminal neuralgia patients experience intense pain primarily in their lower face (trigeminal V2/V3 branches), but the pain can spread above the eye and into other regions of the face and forehead.

The primary distinction among TACs is in the lateralization, frequency, and duration of pain. While they share a unilateral element, the pain of CH is also known to affect the neck while PH can spread to other adjacent regions including the shoulders and arm (Figure 1.1) [6]. Additionally, the pain in CH has been known to switch sides between clusters and can be nonspecific with sudden jolts of intense pain. Perhaps not surprisingly, a recent survey of CH patients concluded that CH may be one of the most intensely painful human conditions [12]. In many cases, SUNCT is believed to be a variant of trigeminal neuralgia (TN) (discussed later) in that it shares similar pain unilaterality, cutaneous triggers, and frequency [13]. SUNCT pain is generally localized to ocular regions and attacks can occur in varying patterns involving single attacks and groups of stabbing attacks [3]. Additionally, although SUNCT and PH are short in duration (2-30 mins), they occur much more frequently (more than 8 attacks per day) than CH (2-8 attacks per day) which lasts

considerably longer (15-180 mins) [3]. Lastly, it should be noted that PH is primarily diagnosed by an absolute response to the indomethacin, which we will explore in further detail in the sections to follow [3].

Although hemicrania continua (HC) and short-lasting, unilateral, neuralgiform headache attacks with cranial autonomic features (SUNA) are not technically classified as TACs, increasing evidence has caused them to be considered as variants of TACs. HC, like PH, can be diagnosed based on patient response to indomethacin, in which 68% of reported cases have responded to the drug, suggesting a role for inflammation in HC [14]. Patients with HC generally experience daily and continuous attacks of moderate severity, with exacerbations disabling about 40% of patients [3]. HC can present in two forms- a remitting form characterized by headache that lasts for days followed by a pain-free period of about 2-15 days; and a continuous form. Triggers of HC include bending over, strong odors, and stress, similar to those reported in migraine, but are not always consistent in HC [6]. Additionally, functional imaging in HC patients has revealed activation of the posterior hypothalamus and dorsal rostral pons, both of which are considered to be markers of TACs and migraine-like symptoms, suggesting overlap of HC pathophysiology with that of other primary headache disorders [15, 16]. Likewise, in chronic HC attacks, this disorder is almost completely indistinguishable from migraine and even cases of HC with aura have been reported [14, 17]. These features, along with inconsistent autonomic symptoms, suggest that HC may in fact be a disorder that overlaps migraine and TACs; however, it is important to note that triptans lack efficacy in HC patients, therefore, distinguishing them from migraineurs [18]. Contrarily, the criteria distinguishing SUNA from SUNCT is minimal: attack duration is extended by up to 10 mins and can be accompanied by any type of autonomic symptom [3].

The final major primary headache disorder that we will cover here is tension-type headache (TTH). TTH is extremely common, occurring in about 30-78% of the general population and accounts for more missed work days than migraine [3]. TTH can be classified into three subtypes: infrequent episodic TTH (<1 day of headache per month), frequent episodic TTH (≥ 1-14 headache days per month), and chronic TTH (≥ 15 headache days per month). The most significant diagnostic feature of TTH is increased pericranial tenderness, which is exacerbated during an attack and increases in intensity with the frequency of attacks; however, the difficulty in diagnosing TTH stems from the overlap of TTH with mild migraine without aura, both of which are usually present in patients [3, 4]. Conversely, TTH is not usually associated with autonomic features normally observed in migraine patients and tends to be the most featureless primary headache disorder, mainly because many secondary headaches mimic the symptoms of TTH and, thus, must be excluded before a proper TTH diagnosis can be reached [19].

Other primary headache disorders such as primary cough headache, thunderclap headache, and nummular headache are far less common and generally consist of symptoms that mimic both primary and secondary headache. These headaches are usually categorized as headaches being attributed to physical exertion, physical stimuli, epicranial pain, and other miscellaneous signs [3]. Because these headaches generally present symptoms and pathological features that overlap with other primary and secondary headache disorders, their underlying pathophysiology is still poorly understood and treatment options are limited.

SECONDARY HEADACHE

As mentioned previously, secondary headaches arise from another underlying condition such as trauma, infection, vascular disease, tumors, medication use or withdrawal, etc. For example, medication overuse headache (MOH) arises from the tendency of some patients to overuse abortive or analgesic medications in the management of headache, particularly migraine, leading to the development of chronic daily refractory headaches that require immediate withdrawal from the acute drug and subsequent treatment with a preventive therapy. Conversely, post-traumatic headaches (PTH) are typically defined as arising within seven days of a traumatic head injury and are most commonly presented as being similar to either migraine headaches or tension-type headaches. PTH usually resolves within months of the injury, but in some cases can become persistent for years. Although the cause of secondary headache may not be known, the pain signaling systems overlap with those in primary headache and, thus, pharmacological targeting of these pathways is likely the best methods for understanding and treating secondary headache disorders. However, like primary headache, the identification of pharmacological targets for treating secondary headache will depend on developing a better understanding of the underlying pathology.

PAINFUL CRANIAL NEUROPATHIES AND OTHER OROFACIAL PAIN

Other rare types of headache and cranial pain can arise from irritation of or damage to the trigeminal nerve, which carries sensation from the face to the brain. Trigeminal neuralgia, for example, is similar to CH in that it is sometimes referred to as the most excruciating pain known to humanity and is commonly associated with compression of trigeminal nerve roots, causing the

nerve to misfire [20]. Still, other conditions such as multiple sclerosis can cause demyelination of the trigeminal nerve, leading to the development of trigeminal neuralgia. Painful neuralgia can also be limited to certain parts of the head and neck. For example, glossopharyngeal neuralgia can cause repeated episodes of severe pain in the tongue, throat, tonsils, and ear while occipital neuralgia starts with pain in the upper neck and spreads into the back of the head and upwards behind the ears. Interestingly, extremely rare conditions such as Tolosa-Hunt Syndrome can be characterized by severe periorbital headaches along with painful eye movements. Taken together, these painful neuropathies add to the complexity of trying to understand and treat headache disorders.

Despite the wide range of headache phenotypes among the general population, there is considerable overlap in terms of the pain expression systems in which they affect. Generally, headache biology and pharmacology can be best understood by studying the trigeminovascular system, cranial autonomic innervation, and pain modulation systems and by observing the relationships between peripheral and central pain expression systems. The following text will take an in-depth look at the current understandings of headache pharmacology and how this research has helped shape what we know about basic headache pathophysiology as well as how better therapeutics might be developed in order to treat these highly disabling and prevalent disorders.

PATHOPHYSIOLOGY OF HEADACHE

Although the origin of headache is still not truly known, the trigeminovascular system has been known to play a fundamental role in headache for decades, particularly in regards to peripheral sensitization [21-25] and neurogenic inflammation in the meninges (Figure 1.2) [26, 27]. This system also serves as the proposed location of action for many pharmaceutical agents, including

triptans, non-steroidal anti-inflammatory drugs (NSAIDS), ergots, and neuropeptide antagonists, all of which will be explored in further detail later [28-30].

Our current understanding of intracranial pain-sensitive structures is based on observations made during neurosurgical procedures in awake patients [31, 32], in human anatomical studies [33], and from experimental animal studies [22, 34-36]. In what is perhaps the earliest published study of pain-sensitive cranial structures, Ray and Wolff studied 30 patients undergoing awake craniotomies and found that mechanical or electrical stimulation of the dura mater and its immediate surrounding intracerebral vessels induced the perception of pain, providing the first evidence to implicate the dura mater in pain sensation of the head [31]. At the same time, Penfield and McNaughton made observations in both humans and monkeys that different regions of the dura mater are innervated by specific branches of the trigeminal nerve; namely, the ophthalmic (V1) division of the nerve innervates the posterior fossa and anterior base of the skull while the dura of the middle cerebral fossa and anterior falx cerebri are innervated by the maxillary (V2) and mandibular (V3) divisions [37]. Recently, a study involving patients undergoing awake craniotomies not only confirmed these early observations, but provided evidence that the pia mater and small cerebral vessels are also pain-sensitive structures that are innervated by V1 [32].

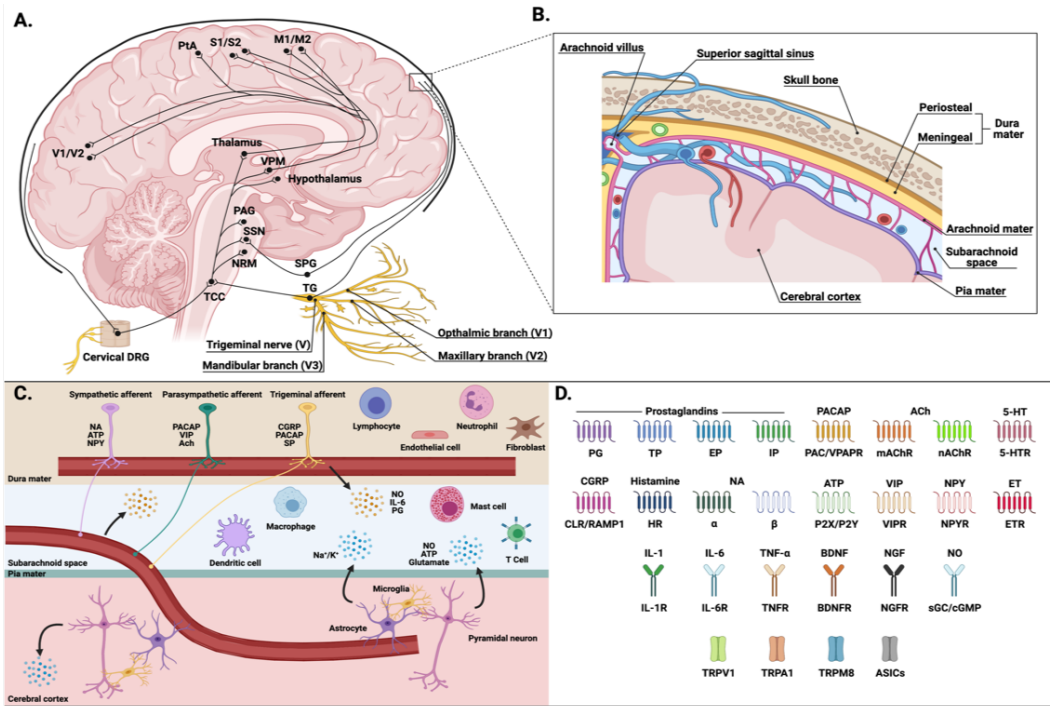

Figure 1.2. Overview of the central and peripheral pathways and signaling molecules involved in trigeminovascular signaling. (A) Peripheral projections from the TG and cervical DRG synapse in the TCC, the main relay center for trigeminal sensory information. The TCC sends projections centrally to various regions of the midbrain and into higher structures including the hypothalamus and the VPM of the thalamus, where they synapse onto third-order neurons that project into the cortex. Descending paraysympathetic and sympathetic projections carry sensory information into the periphery via the SPG and are thought to be responsible for the modulation of pain. (B) Schematic illustration of the meninges, which is innervated by axons from the TG, SPG, and cervical DRG. The meninges consists of the dura mater, which is comprised of periosteal and meningeal layers, the arachnoid mater, the subarachnoid space, and the pia mater, which separates the rest of the meninges from the cerebral cortex. Blood vessels, arteries, and lymphatic vessels can extend on either side of the skull and innervate the meningeal layers, ultimately protruding into the subarachnoid space and even the cerebral cortex, where they interact with many different cell types and signaling molecules. (C) Simplified overview of the interactions and cross-talk that can occur within the meninges and cortex. Axons from sympathetic, parasympathetic, or trigeminal neurons innervate meningeal vessels in the dura mater and can even branch down into the subarachnoid space and pia mater. Activation of these afferents can stimulate vasodilation of vessels as well as activation of various cell types, both of which can result in the release of many different pro-inflammatory mediators (yellow) into the subarachnoid space. These pro-inflammatory mediators can then activate and sensitize other cells, such as macrophages or mast cells, and can interact with the aforementioned sensory afferents that extend into this space. Further stimulation of vessels innervating the pia mater and cortex can result in activation of astrocytes, microglia, and neurons, all of which can release excitatory neurotransmitters (blue) and lead to further interaction with meningeal cells and other cortical neurons. (D) Representation of the ligands, receptors, and channels that have all been implicated in headache pathophysiology. The release

and/or expression of these molecules can all contribute to the cross-talk between different cell types and meningeal layers and may underlie the ways in which sensitization of the trigeminovascular system can occur. TG: trigeminal ganglia; SPG: sphenopalantine ganglia; DRG: dorsal root ganglia; TCC: trigeminocervical complex; NRM: nucleus raphe magnus; SSN: superior salivary nucleus; PAG: periaqueductal gray; VPM: ventral posterolateral nucleus of the thalamus; M1/M2: motor cortices; S1/S2 somatosensory cortices; PtA: parietal association area; V1/V2: visual cortices; CSF: cerebrospinal fluid; PACAP: pituitary adenylate cyclase-activating polypeptide; Ach: acetylcholine; 5-HT: 5-hydroxytyrosine; CGRP: calcitonin gene-related peptide; NA: noradrenaline; ATP: adenosine triphosphate; VIP: vasoactive intestinal peptide; NPY: neuropeptide Y; ET: endothelin; IL-1: interleukin-1; IL-6: interleukin-6; TNF-a: tumor necrosis factor; BDNF: brain-derived neurotrophic factor; NGF: nerve growth factor; NO: nitric oxide; sGC: soluble guanylyl cyclase; cGMP: cyclic guanosine monophosphate; TRP: transient receptor potential channel; ASICs: acid-sensing ion channels.

Still, nociceptive innervation of the meninges is not limited to inputs from the TG. Observations in cats found that projections from upper cervical dorsal root ganglia (DRG) contribute additional meningeal innervation [38]. This finding was recently expanded upon in rats to map out the possible origin of occipital headache and found that sensitization of C2 DRG neurons innervating the posterior dura results in hyper-responsiveness to stimulation of neck muscles, suggesting a difference in origin between occipital and frontal headache [39]. Critically, the dural axons of nociceptors have been characterized as having branches that extend into the pia mater, cross the arachnoid space, and innervate the periosteum and pericranial muscles, establishing a pathway of direct communication between extra- and intracranial structures that is capable of activating nociceptors on either side of the skull [40]. Such cross-cranial communication could underlie the pathophysiology of headaches that are triggered by cranial muscle tenderness and mild trauma to the skull.

The meninges is populated by small diameter unmyelinated C-fibers and thinly myelinated Aδ-fiber TG axons, both of which can be found in highest density in blood vessels. Additionally, large vessels such as the middle meningeal artery (MMA) serve a critical role in supplying the TG

and dura with numerous smaller vessels. Notably, electrical or mechanical stimulation of large meningeal blood vessels is associated with headache in humans, a phenomenon not observed upon stimulation of areas away from vessels [41]. Although early theories postulated that headache, particularly migraine, was caused by vasodilation of cerebral vessels, emerging clinical evidence suggests that vasodilation is merely one component involved in headache pathophysiology. Two recent clinical studies found that migraine pain was not accompanied by extracranial arterial dilation and that only a few intracranial vessels and the MMA exhibited slight changes in dilation, the latter of which was sustained in the late phase of migraine [42, 43]. These results suggest that vasodilation of the MMA might serve as an indicator of dural nociceptor activation. Importantly, these observations provide a rationale for why drugs that target dural nociceptor activation and vessel dilation have been effective in managing migraine and other headache disorders.

MECHANISMS OF PERIPHERAL SENSITIZATION IN HEADACHE

Activation of meningeal afferents results in the release of pro-inflammatory cytokines, growth factors, excitatory neurotransmitters, and neuropeptides that can directly contribute to the sensitization of TG nociceptors and their targets in the TNC [23, 25, 26, 44-46]. Neuropeptides such as calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) have been highly implicated in headache pathophysiology, especially in migraine, CH, and PH [47-49]. CGRP is perhaps the most potent vasodilator known and is thought to contribute to the release of other inflammatory mediators via vasodilation of cranial and cerebral vessels [50]. Additionally, CGRP seems to contribute to both peripheral and central sensitization, making it a very attractive therapeutic target, which we will explore further in section 3.3.

Neurogenic inflammation in the meninges has been attributed to numerous factors that are released upon activation of dural afferents, including maladaptive activation of transient-receptor potential (TRP) channels, acid-sensing ion channels (ASICS), and excitatory channels as well as degranulation of mast cells and activation of dural fibroblasts [27, 51-55]. Inflammatory mediators such as nerve growth factor (NGF), interleukin-1beta (IL-1β), and interleukin-6 (IL-6) have been shown to sensitize dural afferents and targeting these molecules has achieved some degree of therapeutic relief in preclinical models [24, 56, 57]. Norepinephrine, a well-studied stress hormone and neurotransmitter, increases action potential firing of dural afferents and stimulates the release of IL-6 from dural fibroblasts, suggesting a potential mechanism for stress-induced headache [58]. Additionally, nitric oxide (NO), a vasoactive and pronociceptive molecule that has been highly implicated in migraine headache, is released from perivascular nerves fibers during CSD and is thought to contribute to IL-6 expression, mast cell degranulation, and plasma protein extravasation (PPE) [59-61]. Interestingly, NO donors, such as glyceryl trinitrate, are commonly used as experimental headache triggers and NO donor administration is known to only cause headache and no other types of pain [62, 63]. Thus, targeting NO production as a therapeutic strategy has recently gained clinical interest.

Perhaps the most direct consequence of dural afferent activation is the sensitization of TG nociceptors. Sensitization of TG nociceptors is known to be induced by strong nociceptive inputs which can lead to the development of enlarged receptive fields, increased responsiveness to afferent stimulation, reduced activation thresholds, or impairment of inhibition of these neurons [64-67]. Stimulation of the MMA, has been shown to activate trigeminal nociceptors in the brain stem [68]. Furthermore, repeated or intense noxious stimulation has been shown to cause maladaptive

changes in synaptic plasticity within nociceptive circuits and leading to peripheral and central sensitization [69]. Based on this, dural stimulation has served as an important method for modeling headache in rodents, in which development of cutaneous mechanical hypersensitivity and sensitization occur following administration of a noxious stimulus [70, 71]. Utilizing these rodent models has led to the identification of multiple potential drug targets for managing headache, primarily through modulating mechanisms that contribute to TG nociceptor activation and sensitization.

THE TRIGEMINOCERVICAL COMPLEX: THE RELAY CENTER OF TRIGEMINAL PAIN

Sensitization is not limited to peripheral nociceptors, as higher-ordered neurons in the brain stem, thalamus, and cerebral cortex are also capable of being sensitized and are thought to play a critical role in headache pathophysiology [72]. TG neurons projecting to the meninges also project to second-order neurons in the trigeminal nucleus caudalis (TNC) of the trieminocervical complex (TCC), where they converge on neurons that receive additional input from periorbital skin and pericranial muscles [73, 74]. The TCC receives sensory input both from trigeminal nociceptors and higher-ordered brain structures, including the periaqueductal gray (PAG) [75], nucleus raphe magnus (NRM) [76], posterior hypothalamus [77], thalamus [78], rostral ventromedial medulla [79], and cortices [80]. Because of its integration of trigeminal and cortical pathways, the TCC is a key relay center in the processing of nociceptive information from the head. Preclinical studies have shown that electrical, mechanical, or chemical stimulation of dural afferents causes neuronal activation and sensitization in the TCC [81, 82]. Notably, there are a variety of currently available therapeutics that affect, either directly or indirectly, neurons in the TCC. For example, specific triptans, NSAIDs, anti-epileptics,

NO synthase inhibitors, and CGRP antagonists have all been demonstrated to have effects on TCC neurons that are activated by dural stimulation [83]. Additionally, synaptic transmission between TG and TNC neurons within the brainstem has been shown to be a primary target of triptans and calcitonin gene-related peptide (CGRP) antagonists [84, 85]. Based on this, it is hypothesized that maladaptive activation of the TCC is a primary mechanism for central sensitization in headache pathology. Unfortunately, the inability of some of these therapeutics to inhibit or reverse central sensitization points to the need for further testing of these compounds directly on the TCC as well as other higher brain centers, as they can also modulate inputs into the TCC.

The PAG is thought to be a central region in a powerful descending antinociceptive neural network [86-88]. Despite this, studies of the PAG in headache have produced conflicting results. For example, an early clinical study found correlations between electrode stimulation of the PAG during surgery and the development of headache in otherwise headache-free patients, suggesting that activation of the PAG may produce headache-like pain [89]. Conversely, early rodent studies found that deep brain stimulation of the PAG resulted in analgesic effects and stimulation of the PAG has been used to relieve severe pain, including headache, although not without controversy [86, 88, 90]. In cats, stimulation of the PAG was found to inhibit the excitation of TNC neurons in response to stimulation of the face or the superior sagittal sinus [91, 92]. Likewise, a recent study in rats demonstrated that excitation of PAG and NRM nuclei inhibits the responses of dural-projecting TCC neurons, suggesting that impaired modulation of these regions may underlie headache pathophysiology [93]. Furthermore, functional studies of the brains of migraine patients have revealed impairments in brainstem structures, such as the PAG, NRM, and substantia nigra [94, 95]. In patients with MOH, in which dysfunction of the PAG is thought to be a critical underlying

factor, the volume of the PAG is significantly increased and high concentrations of iron have been identified [96, 97]. Given the PAG is an important site of action of many compounds with analgesic properties (e.g. opioids and cannabinoids), better understand of how this region contributes to headache may lead to more optimal use of current therapeutics or may identify novel targets.

The role of central structures in headache pathophysiology cannot be understated. In rats, fibers carrying trigeminal nociceptive information from the caudal medulla and upper cervical spinal cord project directly to the hypothalamus, thalamus, and basal ganglia and possibly mediate autonomic, affective, and homeostatic functions [98-100]. The paraventricular nucleus (PVN) of the hypothalamus directly modulates meningeal-evoked trigeminovascular activity via trigeminal neurons in the medulla, further implicating trigeminohypothalamic signaling in headache [101]. Additionally, chemical stimulation of the dura is capable of sensitizing thalamic sensory neurons and thalamic activation in response to stimulation of skin on the hand has been observed in patients undergoing a migraine attack, both of which suggest that thalamic sensitization may underlie widespread allodynia in migraine patients [102].

Critically, these areas also project to higher cortical regions which have been shown to directly and indirectly modulate trigeminovascular pain [80]. In cats, stimulation of dural vessels excites the somatosensory cortex (SI) via activation of the ventroposteromedial thalamus, which itself is capable of receiving antidromic excitatory inputs from the SI [103]. Similarly, descending cortical projections from SI and insular (Ins) cortices to the TCC have been identified and are believed to differentially modulate meningeal nociceptive inputs to the TCC [104]. Perhaps one of the most important implications of cortical structures has been their role in the manifestation of CSD [105, 106]. CSD is known to initiate waves of membrane depolarization within the cortex, which

is associated with a large efflux of potassium, influx of sodium and calcium, shifts in pH, glutamate release, and neuronal swelling [107-109]. In rodents, stimulation of the NRM was found to suppress trigeminal neuron activity caused by mechanical stimulation of the dura, an effect that was inhibited by multiple waves of CSD propagation, suggesting cortico-NRM regulation of trigeminal excitation [76]. Based on these observations, cortical regions that receive direct or indirect trigeminal input are thought to play a critical role in the regulation of trigeminovascular pain and CSD events and underscore the therapeutic potential in pharmacologically targeting these pathways.

The complex anatomy of the trigeminovascular system underscores the complexity in understanding headache pathophysiology. Pathways conveying head pain clearly involve both peripheral and central components as well as a number of complex interactions between those components. Experimental and clinical studies have revealed the ability of these mechanisms to process incoming nociceptive information and either relay it to other regions or, ultimately, activate antinociceptive pathways. Consequently, dysfunction of one or more of these mechanisms may result in amplification of trigeminal pain and possibly underlies the pathophysiology of the most severe headache disorders.

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CHAPTER 2
CURRENT AND NOVEL THERAPEUTIC TARGETS IN HEADACHE

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SEROTONERGIC (5-HT) AGONISTS

5-hydroxytrptamine (5-HT) agonists, namely the triptans, have traditionally been the first-in-line treatment option for those suffering from migraine headaches. The discovery of this class of drugs was based on early observations that serotonin and ergotamine, acting primarily via 5-HT receptors, were effective in aborting migraine, but caused adverse effects in vascular and pulmonary systems due to their vasoconstrictive properties [1]. This launched the search for compounds with vasoconstrictive properties that could selectively modulate serotonin. More than a decade later, the discovery that 5-HT_{1B} receptors were largely localized to cranial, rather than peripheral, blood vessels led to the development of the highly selective 5-HT_{1B} agonist sumatriptan by Patrick Humphrey and colleagues [2]. Triptans are usually recommended as abortive therapeutics meant to be taken at the onset of a migraine headache since there is little to no evidence of efficacy when taken preceding aura [3]. Unfortunately, triptans have major limitations: not all patients respond, not all patients tolerate the medicines, and they are not recommended for patients with cardiovascular or cerebrovascular diseases due to their vasoconstrictive activity [4]. The most effective triptans are those specific to both the 5-HT_{1B} receptors, which are expressed on meningeal smooth muscle cells and cause vasoconstriction when activated, and 5-HT_{1D} receptors, expressed on peripheral and central trigeminal nerve fibers and contributing to neuropeptide release [5]. Thus, the goal in developing 5-$HT_{1B/1D}$ specific agonists was to induce vasoconstriction of meningeal vasculature while simultaneously inhibiting neuropeptide release from trigeminal nerve terminals.

As mentioned earlier, sumatriptan was the first selective 5-$HT_{1B/1D}$ agonist, having high affinity for both receptors as well as low affinity for 5-HT_{1F}, and was specifically designed for the acute treatment of migraine headache. Based on the success of this drug in migraine, several other

triptans were developed to have greater bioavailability, longer plasma half-life, faster absorption, and increased lipophilicity, allowing for greater brain penetration [6]. These include naratriptan (GlaxoSmithKline), zolmitriptan (Zeneca), rizatriptan (Merck & Co), eletriptan (Pfizer), frovatriptan (Vanguard Medica), almotriptan (Almirall/Pharmacia & Upjohn), and avitriptan (Bristol-Meyers Squibb). The choice of which triptan to use depends on several factors including the route of administration, side effects, cost, and headache duration and symptomology. Generally, standard doses of triptans relieve headache pain within 2 hours in 42-76% of patients, with around 18-50% of patients experiencing sustained relief during that time [7]. At 24 hours, standard dose triptans provide relief in about 29-50% of patients, with 18-33% experiencing sustained relief. Specifically, efficacy data have shown support for eletriptan as offering the most consistent efficacy in migraine patients at 2 hours and 24 hours when taken orally, despite frovatriptan having a significantly longer half-life [8]. Conversely, when administered subcutaneously, sumatriptan generally offers the highest efficacy and the fastest onset of relief, providing relief to 70% of patients within just one hour.

In an effort to move away from the vasoconstrictive properties of classical serotonin receptor agonists, a new class of selective $5HT_{1F}$ agonists termed the "ditans" have recently gained momentum as novel therapeutics for headache management. Recently, a phase 3 clinical study of Lasmitidan demonstrated efficacy in resolving migraine symptoms, in which 30-40% of patients were reported to be headache free at 2 hours following administration of the drug [9]. Lasmiditan works by binding $5HT_{1F}$ receptors, which effectively reduces the production of CGRP and glutamate and without inducing vasoconstriction, making it safer compared to its predecessors [10]. Lasmitidan is also readily able to cross the BBB and has been shown to inhibit CGRP release from

peripheral and central trigeminal nerve terminals [11]. Notably, several other large clinical studies have demonstrated Lasmitidan as being an overall safe and efficacious therapeutic for migraineurs; however, future aftermarket analyses will need to be conducted in order to verify its long-term safety in the population and further research is needed to support its use in other types of headache disorders. Lasmitidan is currently available under its brand name Reyvow (Eli Lilly) as an acute treatment for migraine with or without aura.

Although the most important site of triptan action remains unknown, there are several sites within the trigeminovascular system that are targeted by the pharmacological actions of triptans, including vasoconstriction of meningeal and cerebral blood vessels, inhibition of neuropeptide release from trigeminal nerves, central modulation of pain transmission in the TCC and thalamus, and inhibition of CGRP release from TG neurons [12-15]. Of course, the involvement of triptans in central pain processing is dependent on their ability to cross the blood-brain barrier (BBB), with some being more lipophilic than others. Evidence from early studies demonstrated the ability of cells in the TCC to be inhibited by eletriptan [16], naratriptan [17], rizatriptan [18], and zolmitriptan [19]. Interestingly, there is still debate about the role of sumatriptan in the CNS, given its poor ability to cross the BBB. Early studies demonstrated that sumatriptan does not inhibit TCC activity unless the BBB is disrupted, possibly underscoring the reason for sumatriptan's lack of efficacy when administered during migraine aura [20-22]. Conversely, preclinical studies in rats have demonstrated sumatriptan's ability to block induction of central sensitization following administration of an inflammatory soup on the dura [23]. Whole-cell patch recordings from rat brain slices have demonstrated sumatriptan's ability to inhibit membrane excitability and synaptic transmission in the PAG [24]. Additionally, a recent PET investigation in six migraine patients revealed the ability

of subcutaneous sumatriptan to normalize attack-related increases in brain serotonin synthesis, suggesting penetration of the CNS by sumatriptan [25]. Taken together, these findings emphasize a need for further research into triptan pharmacology, as understanding the exact mechanisms and sites of action of these drugs could lead to better recommendations in the clinic.

Triptans are also one of the preferred first-line abortive strategies for cluster headache (CH). Clinical assessment found that 75% of CH patients that received a 6 mg subcutaneous dose of sumatriptan obtained pain relief within 15 min [26, 27]. Other studies have demonstrated efficacy of intranasal and oral zolmitriptan in the treatment of CH, although these are usually recommended for people who cannot tolerate subcutaneous or intranasal sumatriptan [28]. Still, a major drawback of triptans lies in their ability to cause MOH, which is likely attributed to impaired modulation of 5-HT following frequent use of abortive medications [29]. Notably, chronic triptan use has been found to increase the expression of pro-nociceptive 5HT-2A receptor binding sites and decrease production of 5-HT in the CNS, resulting in neuronal hyperexcitability, enhanced CSD, and trigeminal nociception [30, 31]. Thus, while triptans may be considered an effective first-line treatment for migraine headache and, in some cases, cluster headache, caution should be used when using them consistently.

The ergot alkaloids, consisting of ergotamine and its derivative, dihydroergotamine (DHE) are similarly used in the acute treatment of moderate to severe migraine and cluster headache; however, their efficacy suffers from poor bioavailability. The activity of DHE and other ergots does not directly correlate with their plasma concentrations and their exact mechanism of action is still currently unknown [6]. DHE is not selective for serotonin receptors, as it exhibits affinity for adrenergic and dopamine receptors as well [32]. Furthermore, DHE does maintain a relatively long

half-life and early studies indicate that DHE has a lower headache recurrence rate than sumatriptan [33]. DHE is also a potent agonist of 5-$HT_{1B/1D}$ and induces vasoconstriction similar to that of triptans. A summary of recent clinical findings indicate that certain formulation and administration approaches can improve the efficacy of DHE in migraine headache [34]. For example, a phase 1 study of the intranasal DHE powder, STS101, demonstrated rapid absorption and improved consistency of response compared with Migranal, an intranasal DHE spray, suggesting that this non-injected, acute treatment for migraine could have significantly improved efficacy in patients [35]. The pharmacokinetics of STS101 also suggest that this formulation does not cause nausea, unlike IV administered DHE, making it more attractive for migraineurs. Another phase 1 study investigated the pharmacokinetics of INP104, a DHE intranasal spray administered via a Precision Olfactory Delivery device, in the treatment of episodic migraine and found that this formulation and method of delivery provided up to four times the plasma concentration of DHE compared to Migranal, suggesting improved bioavailability as well as a favorable tolerability profile [36]. Thus, improved DHE formulations and delivery methods may be key to improving the efficacy of ergots in the treatment of migraine; however, further studies will need to address not only how these compounds perform in later clinical trials of migraine, but also in other forms of headache. As it stands, the limitations of ergots as well as the superior efficacy of triptans still make them less recommendable and generally not preferred in most cases [37].

TARGETING NEUROGENIC INFLAMMATION IN HEADACHE

Neurogenic inflammation in the meninges underlies one mechanism of peripheral and central sensitization in headache disorders. Stimulation of dural afferents by various triggers

causes the release of neuropeptides, such as CGRP, which then activate smooth muscle cells, mast cells, and platelets, resulting in vasodilation, plasma-protein extravasation, and the release of inflammatory mediators [38]. This process can lead to further activation of trigeminal afferents and central structures, causing a prolonged pain state and the development of peripheral and central sensitization [39, 40]. Indeed, pharmacologically targeting this process of neuro-inflammation has been widely effective in treating in headache patients, with non-steroidal anti-inflammatory drugs (NSAIDs) being used as both acute and prophylactic drugs in episodic migraine and episodic TTH.

NSAIDS

NSAIDs work by inhibiting cyclooxygenase (COX), preventing the synthesis of prostaglandins and prostanoids and, ultimately inhibiting the inflammatory response. COX exists as two structurally similar isoforms, COX-1 and COX-2, both of which are constitutively expressed at stable levels under normal physiological conditions, but increase in expression in response to pro-inflammatory cytokines [41, 42]. NSAIDs can be differentiated based on their effects on each isoform: nonspecific COX inhibitors, which inhibit both COX-1 and COX-2 (most NSAIDs, ibuprofen, meclofenamate); selective COX-1 inhibitors (indomethacin, piroxicam, sulindac); selective COX-2 inhibitors, which inhibit COX-2 in therapeutic doses and inhibit COX-1 in higher doses (meloxicam, diclofenac, nimesulid, etodolac); specific COX-1 inhibitors, which do not inhibit COX-2 (only low dose aspirin); specific COX-2 inhibitors, which do not inhibit COX-1 (celecoxib, rofecoxib, valdecoxib, etoricoxib, parecoxib, acetaminophen). In general, specific COX-2 inhibitors are preferred, as they are reported to have lower gastric side effects, such as gastrointestinal bleeds and ulcers [6, 43].

NSAIDs are the mainstay treatment option for aborting mild, episodic classifications of TTH, tension-like post-traumatic headache (PTH), paroxysmal hemicrania (PH), hemicrania continua (HC), and most secondary headaches. Interestingly, patients with PH or HC have an absolute response to indomethacin, which is often also used as a diagnostic criteria for these two conditions [44, 45]. Ibuprofen, aspirin, and naproxen are commonly used where NSAIDs are effective, but can lead to gastric issues and may also have a role in the development of MOH, though the latter requires further study. Conversely, NSAIDs are not usually effective in CH or short-lasting uniform neuralgiform headache (SUNCT) and are generally not considered as a prophylactic treatment option for chronic headache, underscoring the complexity of headache and the numerous mechanisms that are likely contribute to its pathophysiology.

GLUCOCORTICOIDS

Corticosteroids mainly work through binding to glucocorticoid receptors (GR) and repressing the transcription of many genes encoding pro-inflammatory cytokines and chemokines as well as key enzymes involved in the development and maintenance of inflammatory responses [46]. Specifically, the activity of NF-κB and AP-1, both of which are important inflammatory transcriptional regulators, is significantly repressed by GR agonists [47, 48]. Intravenous administration of the corticosteroid dexamethone has been recommended for shortening migraine attacks [49]. Additionally, prednisone has shown efficacy in treating episodic CH patients, possibly through reducing CGRP levels and increasing melatonin levels, altering trigeminal activation and improving hypothalamic function [50-52]. In some situations, prednisone is also recommended for treating MOH by reducing the duration of symptomology [53, 54]. Unfortunately, corticosteroids

themselves can induce headache attacks when taken frequently and generally do not offer the highest efficacy in most primary headache disorders.

NITRIC OXIDE SYNTHASE/NITRIC OXIDE INHIBITION

Administration of a NO donor has been one of the most consistent triggers of vascular headache in humans, with approximately 75% of human migraine patients developing an attack within six hours of NO donor administration [55, 56]. NO is a gaseous signaling molecule that is present in most tissues throughout the body. It is formed by oxidation of L-arginine into NO and L-citrulline, a process that is catalyzed by three different isoforms of nitric oxide synthase (NOS), which include endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) [57]. eNOS can be found in the endothelial cells of dural arteries, whereas nNOS is expressed throughout numerous cerebral tissues as well as peripheral arteries, both of which indicate potential vasodilatory effects. NOS inhibition has been shown to lower the activity of dural-projecting spinal TG neurons in rats as well as lower c-fos expression in the TCC following stimulation of the superior sagittal sinus [58, 59]. Additionally, in rats, administration of glyceryl trinitrate (GTN) led to decreased cortical superoxide concentrations, an effect that was reversed by sumatriptan [60]. Infusion of GTN has been found to induce immediate, violent headaches in chronic TTH patients, an effect that is also observed in CH patients during a cluster period [61-63]. In MOH, abnormal activation of Nav1.9 channels by NO was shown to evoke CGRP release, causing vasodilation of arterial vessels and degranulation of mast cells, suggesting that pharmacological targeting of NO may prove useful treating MOH [64].

Despite the overwhelming evidence for a role of NO in headache pathophysiology, there are currently no clinically approved drugs that target NO for headache management. Past clinical studies have demonstrated the efficacy of non-selective NOS inhibitors, such as L-NMMA, in reducing migraine and TTH pain, but have incurred concerns due to their adverse effects on blood pressure [65-67]. Recent efforts have focused on the development of selective NOS inhibitors [68]. Unfortunately, two clinical trials of the selective iNOS inhibitor, GW274150, have failed in achieving efficacy in the abortive or preventive treatment of migraine headache and clinical trials of selective eNOS inhibition have not yet been conducted, particularly due to concerns over the safety of targeting vascular endothelial function and possible cardiovascular issues [68-70]. Conversely, although clinical evidence involving selective nNOS inhibition is limited, several nNOS inhibitors have been developed. The compound NXN-323 (developed by NeurAxon Inc.) was found to be more effective when co-administered with sumatriptan due to the observation that triptan administration increases nNOS expression in dural afferents, suggesting that co-administration of a triptan and a selective nNOS inhibitor may provide greater therapeutic relief [71]. Interestingly, NXN-188, which is selective for both nNOS antagonism and 5-$HT1_{B/D}$ agonism has demonstrated the ability to inhibit CGRP release in preclinical migraine models and has already been utilized in two clinical trials with positive outcomes, suggesting that a combination of nNOS inhibition and activation of 5-$HT1_{B/D}$ may prove to be an effective target for the treatment of migraine [71-73]. Fortunately, NXN-188, along with another selective nNOS antagonist, NXN-462, are listed in phase II and phase III development for the treatment of migraine.

Alternatively, targeting the downstream effects of NO may provide better and safer therapeutic relief. This could include inhibition of cyclic guanylate phosphate (cGMP), which is

involved in NO signaling cascades and has been shown to provoke migraine headaches in patients, or peroxynitrite (PN), which is formed downstream of NO. Specifically, PN is known to activate protein kinase C, p38, NF-xB, nitration of NMDA channels, glutamate transporters, and glutamine synthase, and increase the expression of COX enzymes, all of which can contribute to pain [74, 75]. PN has also been shown to promote DRG hyperexcitability in neuropathic pain and a wide variety of PN scavengers (molecules that react stochiometrically with PN have demonstrated efficacy in preclinical neuropathic and inflammatory pain models [74-77]. However, the role of PN and other mechanisms downstream of NO in headache require much more extensive research before the development and clinical testing of such compounds can occur.

Overall, despite a clear role in headache, there are still no effective therapeutics that target NO in the management of headache. Those that do exist, namely NOS inhibitors, are limited in their ability to reduce pain and are likely to incur adverse effects pertaining to blood pressure. Thus, further research into mechanisms downstream of NO is critical for the discovery, development, and testing of future NO-modulating drugs.

CALCITONIN GENE-RELATED PEPTIDE (CGRP) RECEPTOR ANTAGONISTS

As mentioned earlier, CGRP, a 37 amino acid neuropeptide, has been highly implicated in migraine and CH pathophysiology. CGRP can be found in TG neurons innervating major cerebral and meningeal blood vessels and acts as a potent vasodilator, mast cell degranulator, and inflammatory mediator [78-80]. In animal models of inflammation, CGRP levels have been shown to be elevated in the sagittal sinus following chemical or electrical stimulation of the TG nerve, an effect that is blocked by DHE and sumatriptan [81, 82]. Similarly, CGRP levels are elevated in the

serum of human migraine, CH, and PH patients, an effect that is reduced by subcutaneous administration of sumatriptan [83-86]. Thus, the release of CGRP and other neuropeptides from trigeminal afferents is thought to mediate sterile inflammation within the meninges, ultimately contributing to peripheral and central sensitization in headache patients.

Small truncated fragments of CGRP peptide, such as $CGRP_{8-37}$, were the first molecules used to target CGRP receptors, which are composed of the calcitonin-like (CL) receptor and the receptor activity modifying protein type 1 (RAMP1) [87]. $CGRP_{8-37}$ consists of the first seven amino acids of the normal peptide and, although it has demonstrated efficacy in preclinical models of inflammation, it has ultimately failed in the clinic due to its short half-life and poor potency. Furthermore, other shortened versions of the CGRP peptide have also proven to be ineffective in clinical trials [88].

Small non-peptidergic CGRP antagonists, termed "gepants", target CGRP ligands and have demonstrated clinical efficacy in the acute treatment and prevention of migraine. These drugs have been shown to inhibit the vascular release of CGRP in preclinical models [89, 90]. Currently, rimegepant (Biohaven) and ubrogepant (Allergan) are the only two FDA approved gepants for use in the abortive treatment of migraine. Other gepants, such as telcagepant and olcegepant, demonstrated efficacy early on as prophylactic migraine drugs; however, both were abandoned for use in humans after further trials found elevated levels of liver enzymes in patients who received either drug [91, 92]. Unfortunately, so far there have been no clinical studies investigating the efficacy of gepants in CH, despite the overwhelming evidence that implicates CGRP in CH. Thus, further testing must be performed with these molecules as to determine their efficacy and safety in CH patients.

Currently, there are dozens of clinical trials involving monoclonal CGRP antibodies (Mabs), which target CGRP receptors and offer the greatest potency in humans. Because Mabs are impermeable to the BBB, their mechanism of action likely occurs in the TG, inhibiting CGRP release and ultimately preventing the downstream nociceptive pathways it activates. To date, there are four Mabs that have been clinically approved, four of which must be injected and are long-lasting (typically one to three months), making them remarkably effective for prophylactic use, and include eptinezumab (Alder), erenumab (Amgen/Novartis), Fremanzuman (Teva), galcanezumab (Lilly). All of these demonstrate similar safety profiles, although further testing is still needed to determine long-term effects. Interestingly, recent clinical studies have demonstrated that galcanezumab, but not fremanezumab, is effective in treating episodic CH; however, neither seem to be effective in managing chronic CH [93-95]. Furthermore, no studies to date have examined the use of Mabs in PH or other primary headache disorders.

The above evidence strongly implicates a role for CGRP in the underlying pathophysiology of migraine. CGRP is expressed throughout the central and peripheral nervous systems and mounting preclinical and clinical data support a role for its effects on vasodilation, PPE, and regulating inflammation. Although clinical studies have demonstrated efficacy of gepants and Mabs in the management of migraine, there is still little evidence for their use in CH or PH, despite the observation that CGRP levels are elevated during attacks in both conditions. Thus, it is critical to continue testing these molecules in the management of CH and other primary headache disorders where CGRP may be implicated.

MODULATING EXCITATORY AND ION CHANNEL FUNCTIONS

Although clinical evidence has suggested that increased neuronal hyperexcitability exists in migraine patients, this phenomenon has not been well examined in other primary headache disorders [96]. Various regulators of excitatory neurotransmission, such as glutamatergic signaling through N-Methyl-D-aspartate receptors (NMDARs), have been implicated in the generation and propagation of CSD and neuronal plasticity [97-99]. Likewise, glutamatergic release from presynaptic P/Q type calcium channels and voltage gated sodium channels may also play a role in the transmission of nociception throughout the TNC and, to some extent, have been implicated in familial hemiplegic migraine [100, 101]. Interestingly, the L-type calcium channel blocker, verapamil, has been highly effective in preventing CH in patients, although its mechanism of action is still poorly understood [50]. Flunarizine, a calcium entry blocker, is considered the most potent calcium blocker antagonist for the prophylactic treatment of migraine [102].

Perhaps the most effective drugs in modulating excitability are anti-epileptics, which have long been used to prevent migraine headache [96]. These drugs modulate different aspects of neuronal excitability through modulating sodium channels and altering glutamatergic or calcinergic signaling and their accompanying downstream pathways in the TNC, ultimately suppressing CSD and nociceptive transmission [6, 96, 102-104]. Sodium valproate, topiramate, and gabapentin are among the most commonly used drugs in this class for the prevention of migraine. Topiramate is also the recommended go-to treatment for nummular and daily persistent headaches, demonstrating remarkable efficacy in both conditions [105]. Additionally, gabapentin has demonstrated efficacy in a small clinical study of SUNCT patients and lamotrigine has been approved as a first-line treatment for SUNCT [106]. Unfortunately, evidence for the use of anti-

epileptics in the treatment of other primary headache disorders is lacking and these drugs are limited by their low adherence in chronic migraine patients as well as the wide array of side effects they can cause, likely due to their overall impedance on excitatory mechanisms in both the CNS and PNS [107].

OTHER CHANNEL TARGETS IN HEADACHE

Potassium channels have also been implicated in migraine pathophysiology, less so in other headache conditions. They are likely involved in the antinociceptive actions of several drugs, including agonists of G-protein coupled receptors and opioid receptors, NSAIDs, TCAs, potassium channel openers such as levcromakalim, and have been demonstrated to modulate the neuronal excitability of trigeminal neurons [108, 109]. The antinociceptive actions of opening potassium channel blockers may be mediated by G protein-coupled receptors such as $GABA_B$ receptors, effectively reducing neurotransmission in the trigeminovascular system [110]. Conversely, the K_{ATP} channel opener levcromakalim has been shown to dilate the MMA and subsequently cause migraine attacks [111, 112]. Thus, the therapeutic potential of targeting potassium channels may involve targeting specific potassium channel subtypes over others in order to effectively mediate TG excitability. Other channels regulating neuronal excitability, including transient receptor potential (TRP) channels and purinergic (P2X) receptors are also potential therapeutic targets in headache pathophysiology. Activation of these channels promotes excitation of nociceptive fibers, CGRP release, and sensitization of trigeminal afferents [113-115]. Unfortunately, despite showing efficacy in preclinical migraine models, clinical trials involving TRPV1 antagonists have so far failed to

demonstrate therapeutic relief [116, 117]. Still, preclinical and clinical evidence supports the therapeutic potential in targeting both of these channels [118-120].

Taken together, the above evidence strongly supports a role for various excitatory mechanisms in the pathophysiology of headache. Although clinical evidence of targeting these mechanisms is still lacking in most, if not all primary headache disorders, there is sufficient rationale for the development of specific antagonists of excitatory neurotransmission in the treatment of headache. So far, the most effective and currently available drugs to target these mechanisms are the anti-epileptics, which are both limited in their efficacy and capable of incurring substantial adverse effects. Novel drugs should be specific to their target and limited in their ability to cause off-target effects.

NORADRENERGIC ANTAGONISTS AND ANTI-DEPRESSANTS

Noradrenaline mediates a diverse range of responses through alpha or beta-adrenoreceptors located on both neuronal and nonneuronal cells and have been shown to contribute to vasodilation when activated [121-123]. Currently, non-selective beta-adrenergic antagonists, such as propranolol, are a first-line treatment in preventing high-frequency episodic migraine as well as benign exertional headaches [102, 105]. Other beta-adrenergic drugs, such as metoprolol, atenolol, and alprenolol are non-selective but also show efficacy as prophylactic migraine medications [124]. These drugs, with the exception of atenolol, are easily able to cross the BBB and likely mediate hypothalamic function, influencing cortical excitability and reducing CSD [104, 125]. Beta-blockers have also been effective at reducing TTH, though usually exerts maximum efficacy when combined with a (TCA) anti-depressant, such as amitriptyline [105].

TRICYCLIC ANTI-DEPRESSANTS

Substantial clinical evidence supports the use of TCAs in the treatment of headache. Recent meta-analyses of these compounds in adults have found TCAs to be modestly effective in reducing chronic TTH and preventing migraine attacks, being more effective than SSRIs, but incurring greater adverse effects [126, 127]. TCAs work by blocking the reuptake of norepinephrine and serotonin in presynaptic terminals, leading to an increased concentration of these neurotransmitters in the synaptic cleft, and additionally these drugs can block voltage-gated sodium channels. Their efficacy in the treatment of depression is likely due to restoring balanced levels of norepinephrine and serotonin, as well as subsequent regulation of receptors for these transmitters. Amitriptyline, imipramine, clomipramine, desipramine, nortriptyline, and maprotiline are the most well-studied TCAs; however, only amitriptyline has shown efficacy in preventing migraine and managing TTH [128, 129]. Additionally, TCAs can also impose a wide range of adverse side effects, making them unsuitable for use in most patients.

SELECTIVE SEROTONIN/NORADRENALINE REUPTAKE INHIBITORS

Selective serotonin reuptake inhibitors (SSRIs) and selective noradrenaline reuptake inhibitors (SNRIs) have also been considered in the management of TTH and migraine. The SSRI, fluoxetine, produces antinociceptive effects via central opioid pathways [130]. Likewise, duloxetine is a selective noradrenaline/serotonin reuptake inhibitor that is capable of producing antinociceptive effects in rats and may be a suitable in attenuating headache pathophysiology [131]. Unfortunately, the use of SSRIs and SNRIs in treating headache has been debated, with most patients not responding to these drugs or incurring relapse of headache [132]. Additionally, they are

generally less efficacious than TCAs [133, 134]. Despite this, venlafaxine and mirtazapine are both recommended as second choice treatments for TTH in patients who do not respond to or are unable to take TCAs [129].

OTHER PHARMACOLOGICAL TARGETS

For patients who are not responsive or who are unable to tolerate the previously described treatments, there exist a few other options for managing headache disorders. For example, inhalation of 100% oxygen is the preferred acute treatment for CH, having better tolerability and greater efficacy over subcutaneous sumatriptan [50]. Oxygen consumption can reduce levels of CGRP in the blood and is thought to cause vasoconstriction of blood vessels and overall inhibition of the trigeminal system. Additionally, hyperoxia has been shown to inhibit PPE in dural vessels caused by stimulation of the TG in rats [135]. Thus, oxygen is likely effective in managing trigeminal hyperexcitability and possibly even neurogenic inflammation.

Administration of 100% carbon dioxide (CO2) has also gained attention as a treatment option for headache, as CO2 has been shown to block CGRP release in cultured rat TG neurons and inhibit overall trigeminal nerve activity [136]. Likewise, CO2 may mediate trigeminal antihyperalgesic effects via TRPV1 and ASIC receptor modulation [137]. In contrast to oxygen therapy, CO2 treatment involves non-inhaled intranasal delivery and should ideally cause no change in arterial CO2 levels. Whether or not treatment with CO2 is as efficacious as treatment with oxygen remains to be observed. A phase II clinical trial conducted in 2005 by Spierings and colleagues found that 30% of 67 migraine patients experienced pain relief within 2 hours of intranasal CO2, providing the first clinical evidence of the benefits of CO2 as a migraine therapy.

Currently, further clinical trials are being conducted to determine the efficacy and safety of using intranasal CO2 in the treatment of migraine and CH.

Botulinum neurotoxin type A (BoNT) has been FDA approved for the treatment of chronic migraine and has been increasingly utilized in TTH, PTH, and other primary headache disorders in recent years [138-140]. BoNT blocks the presynaptic release of acetylcholine at neuromuscular junctions via cleavage of SNAP25, although its mechanism of action in headache is likely due to its ability to block the release of CGRP, glutamate, and substance P from TG neurons and upper cervical neurons of the DRG, resulting in attenuation of peripheral and central sensitization [141, 142]. So far, BoNT seems to be a promising therapeutic for managing primary headache; however, further research into the safety and efficacy of this drug are necessary.

Lastly, gap-junction inhibitors have proven to be effective treatment options for headache. Communication between neurons and glia across gap junctions is known to contribute to peripheral sensitization of TG nerves and possibly even CSD [143, 144]. Tonabersat, which inhibits gap junction communication between neurons and glia, has been shown to inhibit inflammation and CSD, making it an ideal target in headache pathophysiology [145, 146]. Although initial clinical studies found that Tonabersat failed to demonstrate a greater reduction in migraine headache than placebo, the drug was well-tolerated and demonstrated efficacy in preventing aura, consistent with its known actions on CSD [147]. Thus, further studies evaluating the efficacy and safety of Tonabersat should be performed to determine whether this drug is a viable treatment to prevent migraine and other types of headache.

NON-PHARMACOLOGICAL TARGETS

While treatments with various medications may be the most effective in managing headache disorders, non-pharmacological approaches are always recommended when possible. Although options are limited in this category, behavioral treatments such as cognitive-behavioral therapy, relaxation, biofeedback, as well as acupuncture have all demonstrated some level of effectiveness in managing headache. These treatments are typically most effective in managing TTH, daily persistent headaches, and stress-induced headaches. Non-invasive neuromodulation using transcutaneous cranial nerve stimulation, vagus nerve stimulation, and transcranial magnetic stimulation as well as invasive approaches such as occipital nerve stimulation have all demonstrated some level of efficacy in relieving certain characterizations of headache [148-150]. Although these techniques are not effective in all patients, they certainly present viable options for the non-pharmacological management of headache and are generally recommended when reasonable.

NOVEL TARGETS FOR TREATING HEADACHE

In addition to the headache therapies covered in this chapter, new molecular insights and drug targets are constantly being explored in an effort to develop more effective and long-term treatments for the many presentations of headache. For example, pituitary adenylate cyclase activating polypeptide (PACAP) has recently gained traction as a novel headache target. PACAP is expressed in human TCC neurons and infusion of PACAP-38 into migraineurs triggers migraine headaches [151, 152]. Pre-clinically, stimulation of the superior sagittal sinus has been shown to elevate PACAP levels in the cranial blood of cats, an observation that was consistent with increased

PACAP levels in the external jugular vein of migraineurs during a headache [151]. These observations have made PACAP and its receptors an increasingly attractive target in the headache field. The monoclonal PACAP antibody, ALD1910, antagonized PACAP signaling via PACAP-1 (PAC-1) receptor, VIP1, and VIP2 and has demonstrated efficacy in a rat model of neurogenic vasodilation and parasympathetic lacrimation, further indicating the therapeutic potential of targeting this polypeptide [153]. Contrarily, the PAC-1 receptor antagonist AMG 301, was recently found to be ineffective in a phase 2 clinical study for migraine prevention, underscoring the need for further research into whether targeting PACAP or its receptors can actually produce robust therapeutic results [154].

Another class of compounds with implications for headache management are the delta-opioid receptor (DOR) agonists, which differ both mechanistically and clinically from mu opioids receptor agonists. DOR agonists have been shown to regulate cutaneous mechanosensory neuronal input at presynaptic junctions in the spinal dorsal horn, suggesting a mechanism for how these drugs might regulate nociception [155]. Additionally, DOR agonists are capable of attenuating CGRP release and inhibiting CGRP receptor-mediate pronociceptive signaling and may explain how these compounds inhibit migraine-associated pain [156]. The most notable of these compounds, SNC80, is a selectively potent DOR agonist with considerable anti-nociceptive effects in various preclinical models [157, 158]. Recently, SNC80 was shown to be effective at reducing peripheral and cephalic allodynia in preclinical models of chronic migraine, PTH, MOH, and even OIH, providing substantial basis for further investigation of the drug as a headache therapy [159]. Additionally, SNC80 was found to reduce the number of CSD events as well as hyperalgesia in an NTG model of migraine, suggesting an effect of DOR agonists on multiple mechanisms that may contribute to

migraine-like symptoms [160]. Despite these benefits, SNC80 has been associated with producing convulsions [161]; therefore, other DOR agonists with non-convulsant properties, such as KNT-127, which has demonstrated efficacy in reducing CSD and attenuating NTG-induced allodynia in mice, may present better alternatives for developing drugs in this class [162]. Notably, a phase 1 study has already been completed for TRV250, a DOR agonist with a preferential selectivity for G-protein signaling and non-convulsant properties that has demonstrated efficacy in attenuating NTG-induced allodynia in rodents [163]. In this study, the compound was found to have good overall oral bioavailability and tolerability in healthy adults, suggesting the need for further research into this compound as a novel headache treatment.

Compounds targeting acid-sensing ion channels (ASICs) have also demonstrated therapeutic potential. Decreased extracellular pH and subsequent activation of ASICs is thought to contribute to several key processes underlying migraine pathophysiology [164]. Perhaps the best indication of a role for ASICs was the observation that amiloride, an epithelial sodium channel blocker, acts via ASICs to inhibit CSD and trigeminal activation in *in vivo* migraine models [165]. In the same study, amiloride demonstrated strong clinical efficacy in reducing aura and headache symptoms in 4 of 7 patients with intractable aura. Furthermore, a recent study found that blocking ASIC3 leads to inhibition of durovascular and nitric oxide-mediated trigeminal pain, suggesting therapeutic potential in blocking specific ASIC channels [166].

Other recent advances in headache management include developing more potent drugs for targets that already have established therapeutic benefits. For example, the drug NOX-L41, a CGRP-neutralizing Spiegelmer, has been shown to inhibit PPE in a rat model of electrically evoked meningeal PPE and offers hope in developing a more potent class of CGRP-neutralizing

compounds [167]. Additionally, researchers have been working to improve drug delivery systems in order to enhance the precision and therapeutic benefit of currently available drugs. Solid lipid nanoparticles, for example, have been shown to increase the uptake of triptans by the brain, encouraging the use of these delivery systems for other drugs with poor CNS penetration [168-171]. Still, most of these new developments have been focused towards the migraine field and must therefore be thoroughly researched to determine their potential in other headache disorders.

THE UNMET NEED FOR BETTER MIGRAINE THERAPEUTICS

While decades of research have shed light on some of the mechanisms involved in headache pathophysiology as well as enabled the development of potent therapies, there is still an enormous gap in our understanding of this common and widespread disorder. The complexity of headache pathophysiology is marked both by the number of distinct presentations that occur across individuals as well as by the differences in the efficacy of first-line treatments. The discovery of the triptans, especially sumatriptan, led to the first robust therapeutics for headache management and remain first-line treatment strategies to this day, despite their ability to cause MOH and other side effects. Decades later, advancements in our understanding of CGRP signaling and its contributions to migraine paved the way for the development of CGRP Mabs, offering a novel solution to managing headache with minimal side effects. Unfortunately, CGRP Mabs, effective as they are, still do not provide a permanent solution to treating headache, as they are not effective in all patients or all headache subtypes. Perhaps the most important point to make in this chapter is the fact that many of the currently available therapeutics have only been approved for use in migraine or have only been minimally researched for other headache disorders. Although migraine

is the most prevalent of these, there is no shortage of individuals who suffer from other headache subtypes and treatment options for these patients are lacking. Future research should aim to identify and elucidate the mechanisms that underlie other types of headache in an effort to better understand why some therapies only work for certain types and not others. Additionally, filling in these gaps in our understanding of headache should encourage the identification of novel targets for advanced drug development, while simultaneously highlighting the structural, functional, and molecular differences among headache subtypes. In conclusion, the future of headache therapeutics is bright, but there is still much work to do and insight to gain in order to meet the demand of the many patients who suffer from this disorder.

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CHAPTER 3

THE ROLE OF REACTIVE NITROXIDATIVE SPECIES IN PAIN PROCESSING: IMPLICATIONS FOR MIGRAINE PATHOPHYSIOLOGY

NO DONORS ARE CONSISTENT EXPERIMENTAL TRIGGERS OF MIGRAINE HEADACHE

The ability of NO donors to produce long-lasting headaches has been well known for more than a century and, in recent decades, it has been well established that approximately 75% of human migraineurs develop an attack within six hours of intravenous NO donor administration [1, 2]. Additionally, several clinical phenomena have demonstrated a potential role for NO in migraine, including elevated concentrations of the metabolite in venous outflow from the head both during and in-between migraine attacks compared to healthy controls. Interestingly, inhibition of NO production has been shown to have efficacy during migraine attacks, indicating NO to not only be important for initiating, but also for maintaining ongoing attacks [3]. Similar to spontaneous migraine attacks, NO-induced headaches can be associated with elevated levels of calcitonin gene-related peptide in plasma, an effect capable of being mitigated by treatment with sumatriptan [4, 5]. Exposure to these metabolites in migraine patients consistently leads to long-lasting acute headaches and can trigger premonitory symptoms similar to those experienced during naturally-occurring migraines [6]. Likewise, migraineurs exhibit significantly enhanced hypersensitivity in response to nitroglycerin (NTG) compared to healthy controls [7]. Collectively, these observations suggest that migraine patients are sensitized to mechanisms by which NO donors trigger migraine. Given that NO has a half-life of five seconds, it is more likely that migraines are not due to NO itself, but to mechanisms downstream of NO such as activation of guanylyl cyclases, nitrosylation

of proteins, and production of PN [8-11]. Although NO is a vasodilator, there is little evidence suggesting that vasodilation occurs during spontaneous migraine in humans, therefore, challenging the potential of dilation as a mechanism [12]. Despite the overwhelming evidence for a role of NO in migraine, few mechanistic studies have actually been attempted.

The most widely accepted mechanism for the pain of migraine is activation of afferent trigeminal nociceptors innervating the cranial meninges [13, 14]. These neurons project into the TNC, where they synapse onto second-order neurons projecting further into other brain regions [15]. Pre-clinical studies have shown that administration of NO can activate and sensitize this system. NO donors cause calcitonin gene-related peptide (CGRP) release and increased meningeal blood flow in ex vivo preparations of dura mater [16], increased CGRP release from TG neurons in vitro [17], sensitization of mechanical responses to dural stimulation in vivo [18], increased spontaneous activity in TG neurons in vivo [19, 20], and NOS inhibitors can decrease spontaneous activity of trigeminal afferents in vivo [21]. Furthermore, NTG has been shown to increase levels of interleukin-1B and interleukin-6 in the meninges and CSF for up to 6 hours [22]. Systemic injection of a high dose (10 mg/kg) of NTG induces mechanical hypersensitivity lasting for several hours, an effect that is blocked by sumatriptan [23, 24]. Repeated administration of high dose (10 mg/kg) NTG increases multiple behavioral measures including cutaneous hypersensitivity and photosensitivity, both of which are mitigated by sumatriptan, topiramate, and propranolol [25-27]. Interestingly, lower doses (1 mg/kg) of NO donors cause no behavioral responses [28]. While it is clear that high doses of NO donors produce behavioral responses in all animals, it is critical to understand why lower doses of NO donors have the ability to cause attacks in human migraineurs compared to controls; however, to date there have only been a few studies addressing this phenomenon. For example,

continuous administration of sumatriptan for 7 days causes sensitization to a low dose (3 mg/kg) of the NO donor sodium nitroprusside (SNP) 14 days following discontinuation of the drug [29, 30]. In preclinical models of post-traumatic headache, rats and mice are primed to low-doses (100 μg/kg) of the NO donor glyceryl trinitrate, an effect that is resolved upon treatment with sumatriptan, an anti-CGRP monoclonal antibody, or topiramate [31-33]. Additionally, our lab has recently published two studies implicating low-dose NO donors in preclinical migraine. We have previously shown that direct dural stimulation with the pro-inflammatory cytokine interleukin-6 (IL-6) or intracisternal administration of brain-derived neurotrophic factor primes rats to sub-threshold doses of SNP (3 mg/kg) after resolution of acute hypersensitivity [34]. Similarly, following repeated restraint stress, mice become primed to sub-threshold doses of SNP (0.1 mg/kg) [35]. These studies suggest that plasticity within the dural afferent system can sensitize animals to NO and demonstrate that responses to NO donors in rodent models of headache mimic those observed in human migraine studies.

PEROXYNITRITE FORMATION CONTRIBUTES TO HYPERSENSITIVITY IN PRECLINICAL PAIN MODELS

It is possible that NO may not directly contribute to migraine and, while non-selective inhibition of NOS has been efficacious in clinical studies, the therapeutic potential of NOS inhibition remains limited due to complications with blood pressure in patients. Despite this, there are several mechanisms activated downstream of NO that have been proposed to have a role in migraine pain. One of these pathways involve the formation of PN. Few studies have addressed PN in migraine, despite its ability to activate and sensitize sensory neurons in preclinical models

of pain (ref). PN causes activation of protein kinase C, p38, NF-kB, inhibition of MnSOD, nitration of NMDA channels and glutamate transporters, and increased expression of inflammatory mediators [36, 37]. Additionally, PN promotes dorsal root ganglia (DRG) and dorsal horn hyper-excitability in neuropathic pain [38] and a wide variety of PN-modulating compounds have demonstrated efficacy in preclinical models of neuropathic and inflammatory pain [36, 39]. Although clearly relevant to pain, to date there have been no studies investigating the relationship between PN and migraine, despite migraine being the only type of pain directly triggered by PN-forming substances [40]. In addition to preclinical evidence, numerous clinical studies have demonstrated a potential role for PN in migraine. Decreased SOD activity and elevated levels of NO, both of which are necessary for PN formation, have been shown to occur during migraine attacks [41]. Furthermore, in a clinical study involving migraine patients, administration of l-arginine led to significantly higher levels of PN in platelet counts between migraine attacks compared to healthy controls [42], suggesting that PN may play a role in establishing a hypersensitive state in migraineurs. Collectively, these studies support the hypothesis that formation of PN contributes to migraine; however, studies using specific tools to address this question have not yet been performed.

Based on these observations, our lab wanted to further investigate mechanisms of PN formation and the overall contribution of this nitroxidative molecule in migraine pathophysiology.

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CHAPTER 4

PEROXYNITRITE MEDIATES STRESS-INDUCED HYPERSENSITIVITY AND PRIMING TO A NITRIC-OXIDE DONOR IN A PRECLINICAL MODEL OF MIGRAINE

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INTRODUCTION

Migraine headache is an extremely complex and disabling disorder that affects more than one billion people worldwide [1]. Thought to result from abnormal activation and sensitization of the trigeminovascular system, migraine attacks are known to be triggered by various stimuli that are typically non-noxious in healthy individuals [2]. Although current therapeutics are efficacious in some patients, the larger migraine population is still burdened by issues of low drug efficacy and high rates of headache relapse following treatment [3]. Because of this, the need to better understand migraine pathophysiology to improve drug development cannot be over-stated.

One of the most consistent triggers of migraine headache is administration of or exposure to a nitric oxide (NO) donor, in which around 75% of migraineurs experience an attack within six hours of NO donor administration [4, 5]. While most individuals experience short, mild headaches upon first exposure to an NO donor, they also typically develop a tolerance upon repeated exposure [6]. Furthermore, even at sub-threshold doses, NO donors trigger longer, more intense headaches in migraineurs [5]. Interestingly, studies have shown that these compounds trigger premonitory symptoms that are commonly experienced during naturally-occurring migraines [7, 8]. Notably, these effects are not present in healthy controls; however, high enough doses of the NO donor glyceryl trinitrate (GTN or NTG) have been shown to induce migraine even in healthy individuals [9, 10]. Despite these observations, targeting NO as a therapeutic strategy has achieved mixed results [11]. For example, inhibiting neuronal NOS (nNOS) has proven to be effective in preclinical studies of trigeminal nociceptive activation [12, 13] and pharmacological blockade of NOS with the non-selective inhibitor L-NMMA has demonstrated efficacy in reducing migraine pain [14, 15]; however, despite being mechanistically important observations, non-selective NOS inhibition is not an

attractive therapeutic strategy due to cardiovascular issues likely caused by actions on endothelial NOS (eNOS) [16]. Additionally, selective inhibition of inducible NOS (iNOS) was ineffective in treating human migraineurs [17]. Thus, targeting the downstream effects of NO may prove to be a more therapeutically viable strategy moving forward.

Indeed, preclinical studies have implicated a role for several pathways downstream of NO in the development of migraine, including stimulation of the NO-selective receptor soluble guanylyl cyclase (sGC) and its downstream activation of cyclic guanosine monophosphate (cGMP) [18-20]; degranulation of meningeal mast cells [21]; release of the migraine-implicated neuropeptide calcitonin gene-related peptide (CGRP)[22]; induction of meningeal inflammation[23]; and phosphorylation of extracellular signal-related kinase (ERK)[24]. Although NO is also known to cause vasodilation [25, 26], the effects of NO donors on migraine are much longer-lasting than the short-lived release and dissociation of NO, which is typically less than 10 minutes [27], suggesting that the effects of NO donors on migraine mechanisms are likely due to downstream pathways that are activated by these compounds [28-32]. Additionally, while vasodilation was once thought to be the cause of migraine headaches, minimal vasodilation is actually observed in spontaneous migraine patients, thus challenging vasodilation as an actual mechanism for migraine [32].

NO reacts with superoxide (O_2^-; SO) to produce peroxynitrite ($ONOO^-$; PN), a reactive nitro-oxidative molecule that has been implicated in nociceptive processing in various pain models [33]. PN, like other reactive nitro-oxidative species (RNOS), has been shown to mediate the development and maintenance of painful states and has an active role in sensitizing neurons and disrupting homeostasis through the activation of protein kinase C, p38, NF-kB, nitration of NMDA channels and glutamate transporters, and increasing expression of inflammatory mediators [31, 33].

Additionally, PN promotes dorsal root ganglia (DRG) and dorsal horn hyper-excitability in neuropathic pain [34] and a wide variety of PN-modulating compounds (PNMCs) have demonstrated efficacy in preclinical models of neuropathic and inflammatory pain [33, 35]. Endogenous PN induces oxidative damage through disrupting mitochondria respiration via inhibition of the mitochondrial electron transport chain (mETC), ATPases, aconitase, and manganese superoxide dismutase (MnSOD) and increasing calcium release [36-38]. Furthermore, a recent study found that administration of a PNMC, but not a NOS inhibitor, prevented nociceptive responses caused by dural injection of CGRP in male rats, providing the first direct evidence for a role of PN in migraine pathophysiology [39]. Clinical studies have also generated support for a role of PN in migraine. A 2015 meta-analysis of over 1000 migraine patients and controls found significantly decreased concentrations of superoxide dismutase (SOD) (indicating increased SO levels) and increased levels of NO during migraine attacks, both of which are necessary for PN formation [40]. In another clinical study, administration of l-arginine led to increased levels of PN in the platelets of migraineurs during headache-free periods, an effect that is thought to be regulated by NO pathways [41-43]. Despite these observations, at the time of this writing, there are no other published reports regarding the potential role of PN formation in headache.

Our lab has previously demonstrated that rodents are capable of being sensitized to normally non-noxious doses of dural pH 7.0 or an NO donor following dural stimulation or repeated restraint stress (stress is the number one reported trigger of migraine in humans [44]), respectively [45, 46]. Based on this as well as the previous findings mentioned above, we sought to explore whether PNMCs, which accelerate the breakdown of PN, had any effect on nociceptive responses following dural stimulation or repeated restraint stress.

METHODS

Experimental Animals

Unless otherwise indicated, all behavioral experiments presented in this paper used female and male ICR (CD-1) mice aged 6-8 weeks (~25-30 g) which were outbred and purchased from Envigo. All experiments were performed between the hours of 9:00 AM and 5:00 PM. All mice were housed in groups of four animals per cage on a 12-hour light-dark cycle and had access to food and water *ad libitum*. Upon arrival to the animal care facility, animals were allowed a minimum of 72 hours to acclimate to their new environment before being handled for experiments. All procedures were conducted with prior approval of the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

Drugs and compounds

For dural injections, human recombinant interleukin-6 (IL-6) protein (R&D Systems) stock solution (100 mg/mL) was prepared in sterile 0.1% BSA and diluted to 1 ng/mL in synthetic interstitial fluid (SIF) consisting of 135mM NaCl, 5mM KCl, 10mM HEPES, 2mM CaCl2, 10mM glucose, 1mM MgCl2 (pH 7.4, 310 mOsm). The NO donor sodium nitroprusside (SNP) (Sigma-Aldrich) was prepared in sterile phosphate buffered saline (PBS) at the time of use and was kept away from light. To assess the role of PN in our models, a 150 µL IP injection of 30 mg/kg IP of the PN scavenger Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBap) or the PN decomposition catalyst (PNDC) Fe(III)5,10,15,20-tetrakis(N-methylpyridinium-4-yl) porphyrin (FeTMPyP) was administered following repeated stress or before hyperalgesic priming. This dose was based on previous studies showing that PNMCs achieve efficacy in behavioral pain and trigeminal

nociception assays when administered IP [12, 34]. To test for the presence of hyperalgesic priming, mice were given a 150 µL IP injection of 0.1 mg/kg of SNP or a 5 µL dural injection of SIF pH 7.0 solution.

Mouse dural injections

Mouse dural injections were performed as previously described [47]. Mice were anesthetized under isoflurane for <2 min with <2.5–3% isoflurane via a chamber and given a 5 µL injection via a modified internal cannula (Invivo1, part #8IC313ISPCXC, Internal Cannula, standard, 28 gauge, fit to 0.5 mm). The inner projection of the cannula was used to inject through the soft tissue at the intersection of the lambdoidal and sagittal sutures. Using a caliper, the length of the projection was adjusted to be from 0.6 to 0.7mm based on animal weight (25–30 g) in order to avoid puncturing the dura mater. Control mice received a 5 µL dural injection of SIF (pH 7.4, 310 mOsm). Upon completion of injections, mice were placed back into their respective cups in the testing chamber for 1 hr before testing.

Repeated restraint stress

Mice were stressed as previously described [46]. Mice were stressed between the hours of 10:00 AM to 12.00 PM for 2 h per day for three consecutive days. Mice were placed right-side up into tail vein injection tubes (Stolting #51338) with the nose through the provided breathing hole. The slotted tail piece was tightened so as to prevent the mouse from rotating in the tube, but loose enough to allow the animal to breathe. Mice were restrained at a level that allowed for adequate respiration and care was taken to avoid any trauma caused by the restraint tube. Control mice were

placed into a separate room and deprived of food and water for the same 2-h interval for three consecutive days. Animals subjected to stress were housed separately from control mice in order to avoid potential transfer of the stress phenotype.

Measuring mechanical hypersensitivity and grimace

Mice were handled and conditioned for a single 5-minute session, approximately 24 h before habituation. Mice were habituated to paper cups (Choice 4 oz paper cups: 6.5 cm top diameter, 4.5 cm bottom diameter, 72.5 cm length) while in testing chambers for 2 h per day and for at least 2 days before measuring a baseline. Each mouse typically used their same assigned paper cup for the remainder of the experiment. Animals were given food while in testing chambers. Grimace measurements were recorded for each animal in 10-minute increments using an Apple iPhone 11 Pro video camera and analyzed as previously described [45, 48]. Analysis of 5 characterized pain behaviors (orbital tightening, nose bulging, cheek bulging, flattening of whiskers, and flattening of the ears) were scored on a scale from 0 to 2 (0 = not present, 1 = somewhat present, 2 = clearly present). Following grimace measurements, von Frey testing of the periorbital region of the face was used to measure mechanical hypersensitivity of the face as previously described [47, 49]. Filament thresholds were determined using the Dixon "up-and-down" method. Testing in mice began with 0.07 g on the face and increased in weight to a maximum of 0.6 g on the face. The testing timelines for dural injection experiments and stress experiments were conducted as previously described [46, 47]. In both experimental paradigms, once the mice returned to baseline, a sub-threshold dose of compound was administered either onto the dura (pH 7.0) or intraperitoneally (sodium nitroprusside) to test for hyperalgesic priming. Responses were defined as a mouse

removing/swiping the filament away from its face upon brief application of the filament. All animals were randomly allocated to experimental groups by drawing for groups. All experimenters were blinded to animal treatments.

Statistical Analysis

All data presented here are shown as mean ± SEM. Female and male mice were used in almost all experiments to determine any sex differences. Behavioral data were analyzed for multiple comparisons at each time point via two-way ANOVA followed by Bonferroni's *post-hoc* analysis. F-values for these analyses are presented in (**Table 1**). Significance was set at $p < 0.05$ for all statistical comparisons. Each experiment was independently replicated at least twice. All behavioral graphs were analyzed using a two-way ANOVA with multiple comparisons between groups. Data analysis was performed using Prism version 9.2 for Mac OS X. All investigators were blinded to treatment during testing and scoring.

RESULTS

PN mediates NO donor-induced hypersensitivity following repeated stress

Although the exact mechanism of how NO donors induce sensitization in migraine or other types of pain is still not understood, targeting the downstream formation the reactive nitro-oxidative molecule PN has been gaining interest based on its observed role in mediating painful states [33, 35, 39, 50]. Previously, our laboratory published that repeated restraint stress is capable of cephalically sensitizing mice to sub-threshold, typically non-noxious doses of the NO donor SNP [46]. Based on this, we wanted to test whether modulating PN prior to NO donor administration was capable of

preventing hyperalgesic priming following repeated stress. Following baseline measurements, female and male mice were subjected to repeated restraint stress and measured for facial pain via grimace and von Frey. Upon returning to baseline thresholds, mice were administered 30 mg/kg of the PN scavenger MnTBap, the decomposition catalyst FeTMPyP, or vehicle approximately 30 mins prior to an IP administration of 0.1 mg/kg SNP and again tested for facial hypersensitivity (Figure 4.1A).

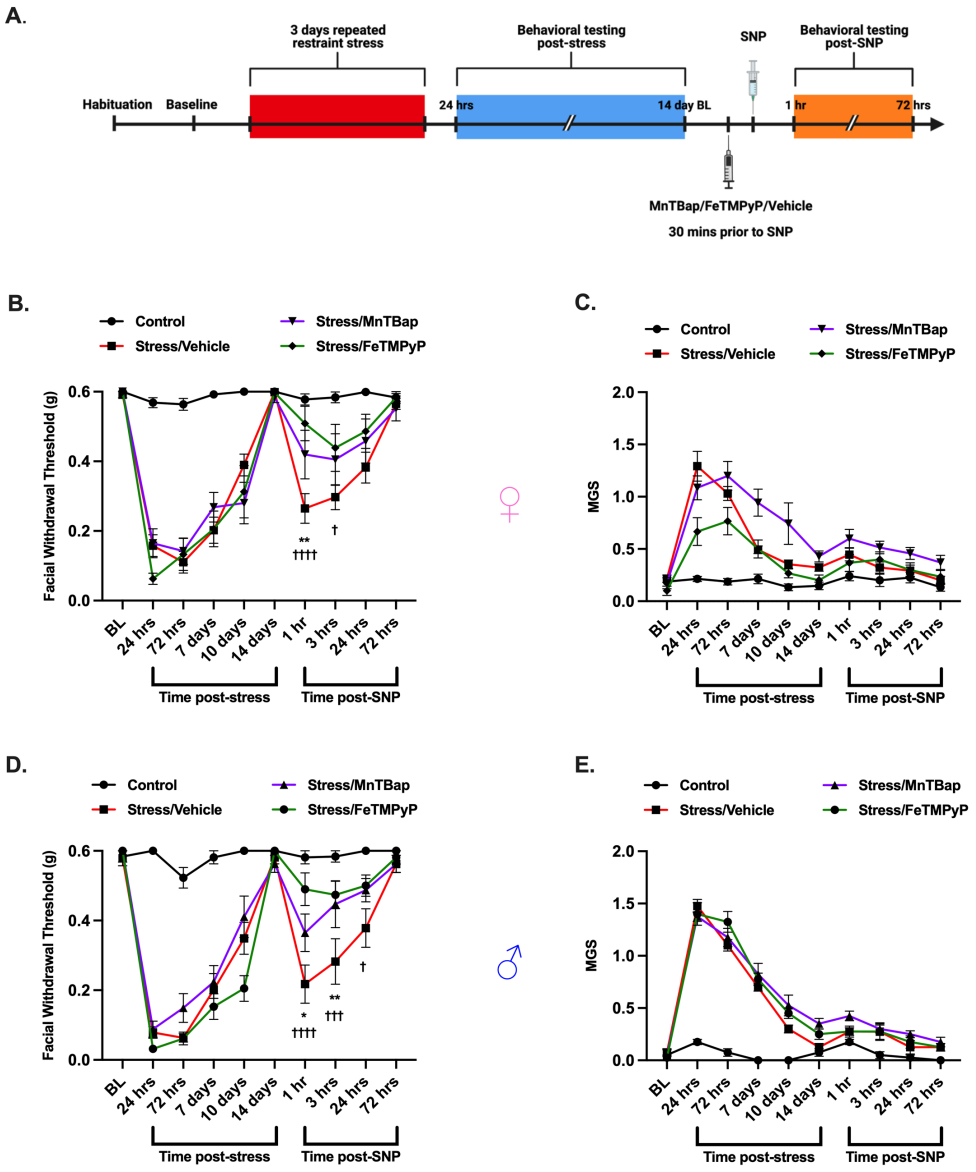

Figure 4.1. Peroxynitrite mediates NO donor-induced mechanical hypersensitivity in stress-primed mice. A schematic of the stress paradigm used is shown in (A). Mice were subjected to repeated restraint stress or control conditions and tested for facial allodynia via von Frey assessment and mean grimace scores (MGS). Upon returning to baseline thresholds 14 days after stress, mice received a 30 mg/kg IP injection of either a PN scavenger (MnTBap), PN decomposition catalyst (FeTMPyP), or vehicle (PBS) 30 mins prior to injection of the NO donor SNP (0.1 mg/kg, IP) and were again tested for facial allodynia. MnTBap and FeTMPyP both significantly attenuated facial hypersensitivity caused by SNP in stress-primed female (B) and male (D) mice. No differences in grimace scoring were found in either sex (C and E). All control groups received vehicle prior to SNP. Two-way ANOVA followed by Bonferroni *post-hoc* analysis revealed significant differences in the priming phase between stressed mice that received vehicle prior to SNP and stressed mice that received

MnTBap (denoted by *) or FeTMPyP (denoted by †) prior to SNP. n≥6 for all groups in A and C; n=8 for all groups in D and E. Data are represented as mean ± SEM. See Table 1 for F-values. *†p<0.05, **p<0.01, ***†††p<0.001, ****††††p<0.0001.

Stress induced acute facial hypersensitivity and noxious grimace responses in both females (Figure 4.1B and C) and males (Figure 4.1D and E) that lasted for roughly 14 days. Interestingly, stress-primed mice that received MnTBap or FeTMPyP, but not vehicle, prior to SNP exhibited significantly reduced facial withdrawal thresholds, suggesting a role for PN formation in mediating NO donor-induced priming following stress. Grimace measurements were insignificant in the priming phase of these experiments, consistent with what we have previously reported [46].

Modulating PN does not prevent dural IL-6 -induced hyperalgesic priming

Although the bulk of this report is focused on the role of PN formation in stress and NO donor-induced hypersensitivity, several reports have suggested a role for PN in the development of inflammatory hyperalgesia [34, 51, 52]. Additionally, there have been mixed reports about the role of PN in regulating inflammatory gene expression, with some showing that NO and PN induce gene expression of pro-inflammatory interleukin receptors, such as IL-6, while others have concluded that PN can actually decrease inflammatory gene expression depending on the nature of the RNOS pathway that is activated [53, 54]. Our lab has previously shown that dural IL-6 is capable of inducing facial hypersensitivity in mice and sensitizing them to dural pH 7.0, which is typically non-noxious in healthy control mice [47]. Based on the previous reports mentioned above as well as the notion that NO is capable of modulating acid-sensing ion channels (ASICs) [55], we wanted to test whether modulating PN prior to dural pH 7.0 would attenuate the facial hypersensitivity caused by dural IL-6. Female and male mice were administered a non-invasive 5 µL injection of IL-6 onto

their dura mater and tested for facial hypersensitivity and grimacing. Upon resolution of acute hyperalgesia, mice were given an IP injection of 30 mg/kg MnTBap, FeTMPyP, or vehicle followed by a 5 μL dural injection of pH 7.0 solution approximately 30 mins later (Figure 4.2A). Although grimace responses were slightly altered in the early time points following dural pH 7.0, we found that neither MnTBap or FeTMPyP were able to attenuate the facial hypersensitivity caused by dural pH 7.0 in either sex (Figure 4.2B-E), suggesting that PN formation may not be critical to the development of facial priming in this model.

Temporal effects of modulating PN following repeated stress

After observing that modulation of PN was capable of preventing NO donor-induced hypersensitivity in stress-primed mice, we wanted to expand on our understanding of the role of PN, if any, in the acute stress response. As mentioned earlier, stress is the number one reported trigger of migraine in human patients. Preclinical migraine studies have used different variations of stress to induce migraine-like phenotypes in rodents, including bright light, unpredictable sounds, wet bedding, predator exposure, restraint, or a combination of stressors. Acute or chronic stress has been shown to increase NOS expression in the dura mater of rats as well as increase rat tail-flick responses to high doses (10 mg/kg) of NTG [56, 57]. Based on this, we wanted to explore PN formation plays a role in stress-induced acute facial hypersensitivity. Following the third day of stress, we gave mice a single dose of FeTMPyP or MnTBap (30 mg/kg, IP) 1-hr after removing them from the restraint tubes and tested them for acute facial hypersensitivity and grimace.

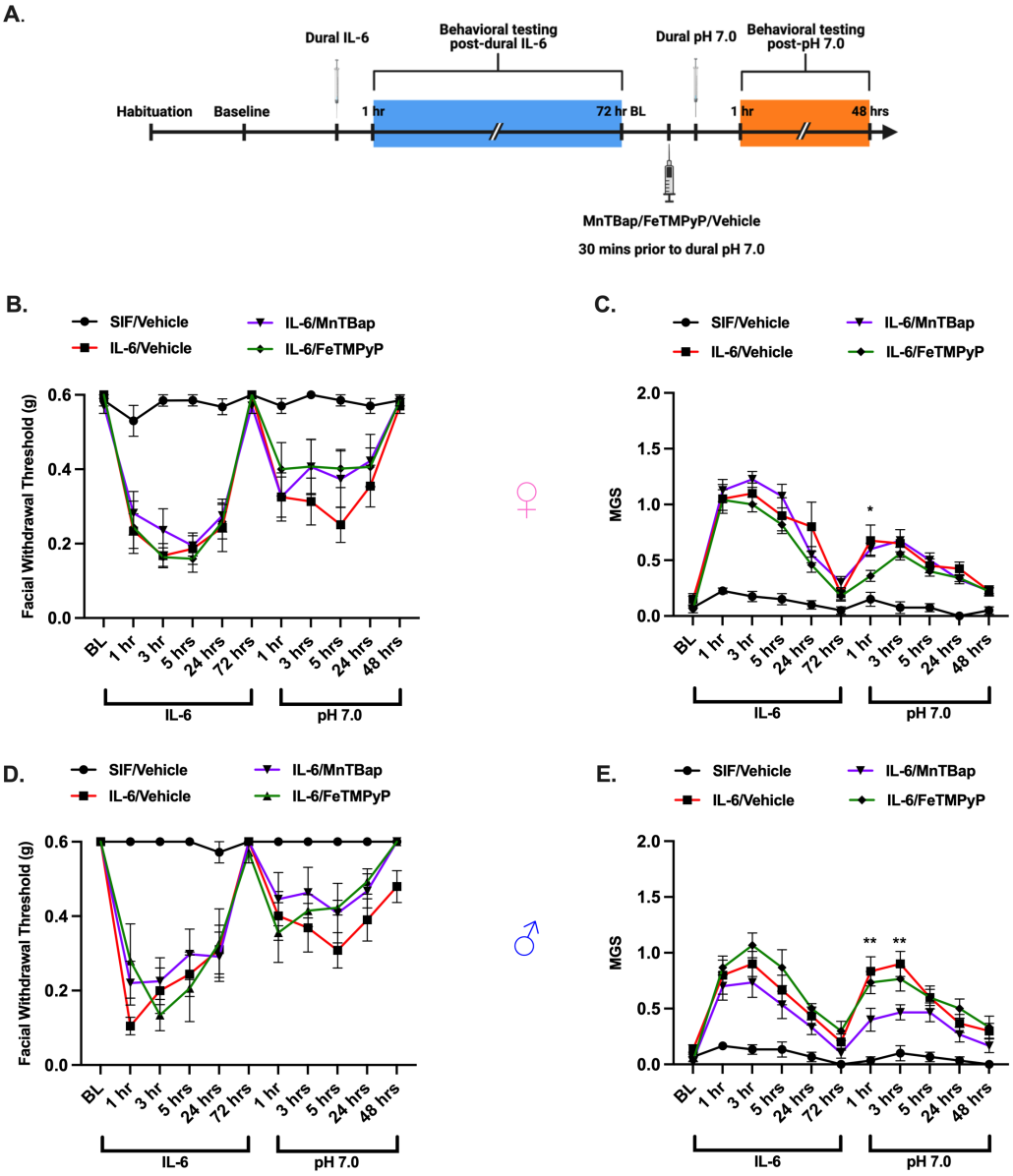

Figure 4.2. Modulating PN does not attenuate facial priming to dural pH 7.0. Dural injections and behavioral testing timelines are presented in (A). Female (B-C) and male (D-E) mice received a 5 µL dural injection of vehicle (SIF) or IL-6 (0.1 ng) to induce acute periorbital hypersensitivity and grimacing that lasted out to 72 hrs. After the pain resolved, mice were given a 30 mg/kg IP injection of either MnTBap, FeTMPyP, or vehicle (SIF) 30 mins prior to a second 5 µL dural injection of a SIF pH 7.0 solution to check for the presence of hyperalgesic priming. Mice that received a PN-modulating compound did not exhibit significant differences in nociceptive thresholds from those that received vehicle after IL-6; however, a two-way ANOVA with Bonferroni *post-hoc* analysis of the priming phase revealed significantly lower grimace scores between the group that received MnTBap (denoted by *) and the IL-6/vehicle group within the first three hours following dural pH 7.0. All control mice

received pH 7.0 solution in the priming phase. n≥8 for all groups in A and B; n=6 for all groups in D and E. Data are represented as mean ± SEM. See Table 1 for F-values. *p<0.05, **p<0.01.

Upon returning to baseline thresholds, we administered SNP (0.1 mg/kg, IP) to check for hyperalgesic priming (Figure 4.3A). Interestingly, stressed female (Figure 4.3B-C) and male (Figure 4.3D-E) mice that received a PNMC exhibited significantly reduced facial withdrawal thresholds and grimace responses and did not prime to low-dose SNP compared to those that received vehicle, suggesting a critical role for PN formation in mediating the acute stress response and development of a primed state in mice. Because no sex differences were observed up to this point and, in order to reduce animal use, we chose to only use female mice moving forward.

While undoubtedly an interesting observation, we wanted to know whether this therapeutic effect of PNMCs was temporally dependent on when the compound was given following stress. Thus, we gave female mice a single dose of FeTMPyP (30 mg/kg, IP) at 24-hrs following stress (approximately 1-hr prior to our first behavioral time point) (Figure 4.4A) and checked for priming to SNP (0.1 mg/kg, IP). Contrary to the effects of FeTMPyP at 1-hr post-stress, we found no significant differences in acute facial hypersensitivity or grimacing between the treated group and the stress/vehicle group (Figure 4.4B-C). Additionally, FeTMPyP at 24-hrs post-stress did not prevent priming to low-dose SNP. These data suggest that the effects of PNMC administration on the acute stress response and development of priming are temporally dependent and that PN formation may mediate the initial onset of acute hypersensitivity.

Lastly, we wanted to determine the effects of repeated dosing with a PNMC on the acute stress response and priming to SNP. Thus, following the third day of stress, female mice were given a 30 mg/kg IP injection of FeTMPyP or vehicle at approximately 1, 24, 48, and 72-hrs

following stress and tested for facial allodynia (Figure 4.5A). Similar to the effects of single dosing at 1-hr post-stress, this repeated dose regimen was found to significantly attenuate facial hypersensitivity (Figure 4.5B), grimacing behaviors (Figure 4.5C), and priming to SNP compared to stressed mice that received vehicle. Importantly, no adverse effects or reactions were observed in these studies. Taken together, these data suggest that PNMCs have therapeutic efficacy in a clinically-relevant rodent model of migraine, an effect which is temporally dependent, and implicate PN in the development and maintenance of stress-induced hypersensitivity.

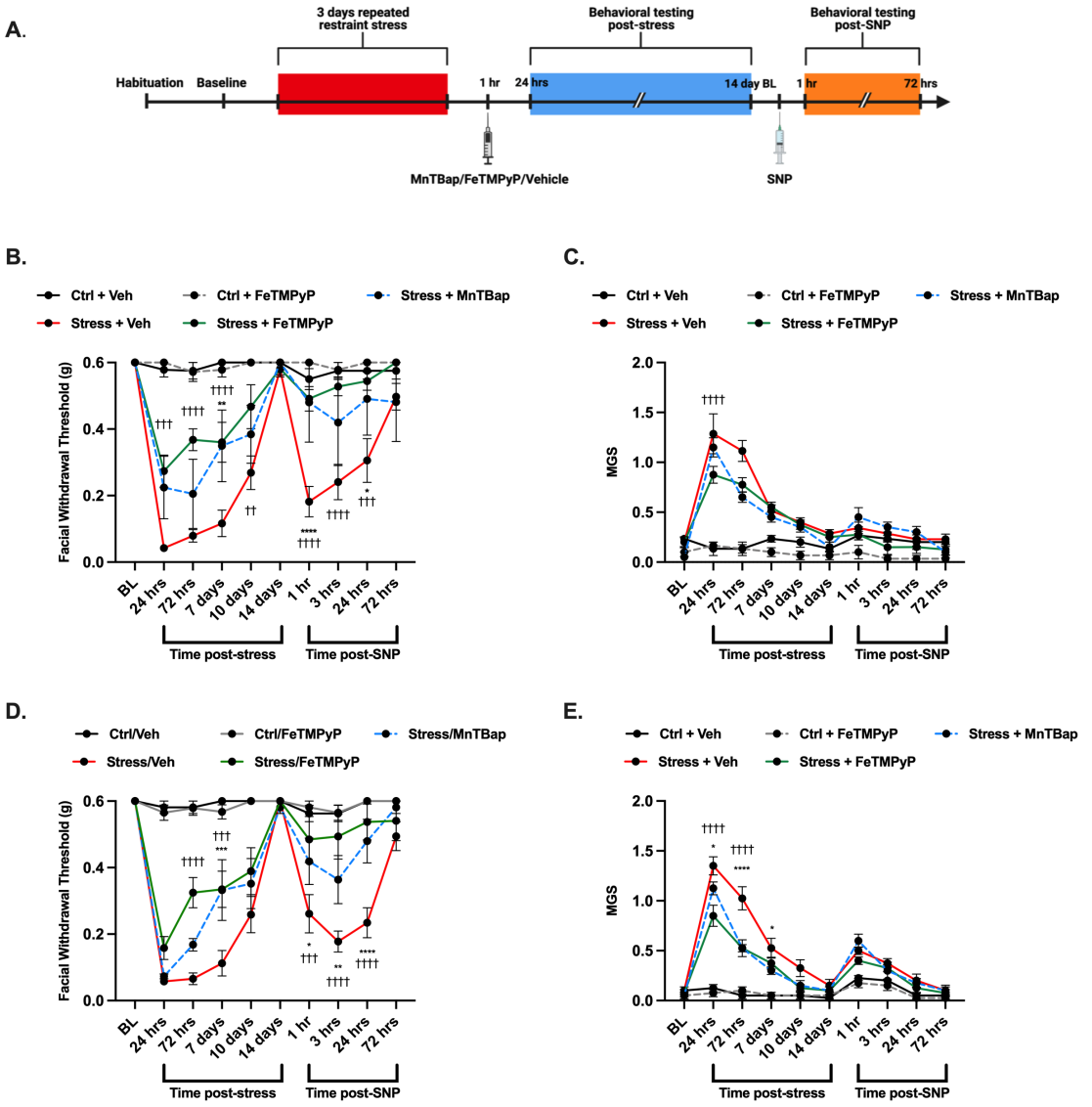

Figure 4.3. Administration of a PNMC at 1 hr following stress results in attenuation of acute facial hypersensitivity and prevents priming to an NO donor. Following repeated stress, mice were administered FeTMPyP or MnTBap (30 mg/kg, IP) 1 hr post-stress and tested for facial hypersensitivity, grimacing, and priming to low-dose SNP (0.1 mg/kg, IP) (A). Compared to stressed mice that received vehicle, stressed mice that received a PNMC were found to have significant attenuation of acute allodynia and grimace scores and did not prime to SNP in both females (B-C) and males (D-E). * denotes significance between stressed mice that received MnTBap and those that received vehicle. † denotes significance between Stress/FeTMPyP and Stress/Vehicle groups. All control groups received vehicle and were administered SNP prior to the priming phase. (n≥4 in B and C; n=8 in D and E). Data are represented as mean ± SEM. See Table 1 for F-values. *†p<0.05, **††p<0.01, ***†††p<0.001, ****††††p<0.0001.

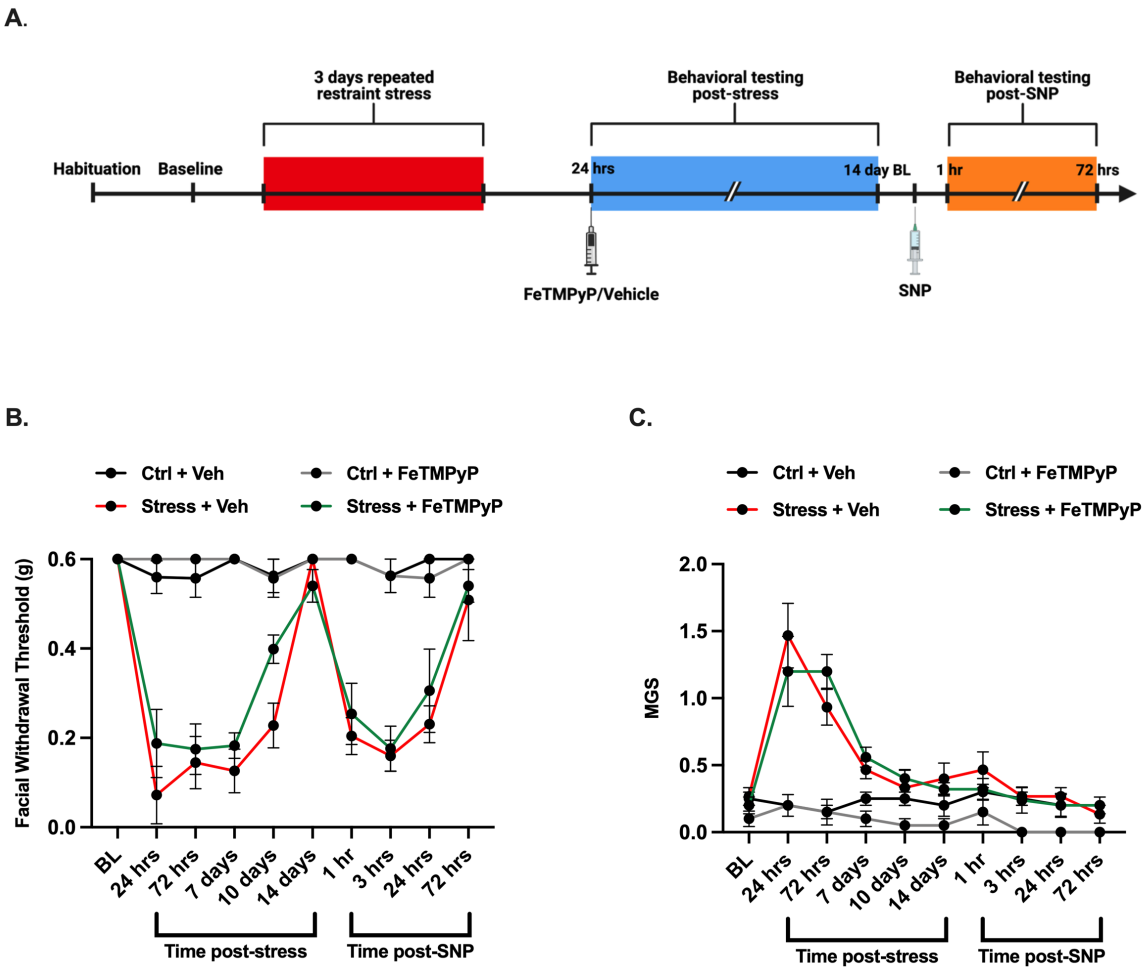

Figure 4.4. Administration of a PNMC 24 hrs following repeated stress does not block facial allodynia. Stress paradigm and dosing regimen are shown in (A). Following stress, female ICR mice exhibited robust facial hypersensitivity (B) and grimacing (C) and were primed to low-dose SNP (0.1 mg/kg, i.p.). A two-way ANOVA with Bonferroni *post-hoc* analysis revealed no significant differences in acute hypersensivitiy or priming in stressed mice that received a PNMC at 24 hrs following stress compared to stressed mice that received vehicle. (n= 3-5 for all groups). Data are represented as mean ± SEM. See Table 1 for F-values.

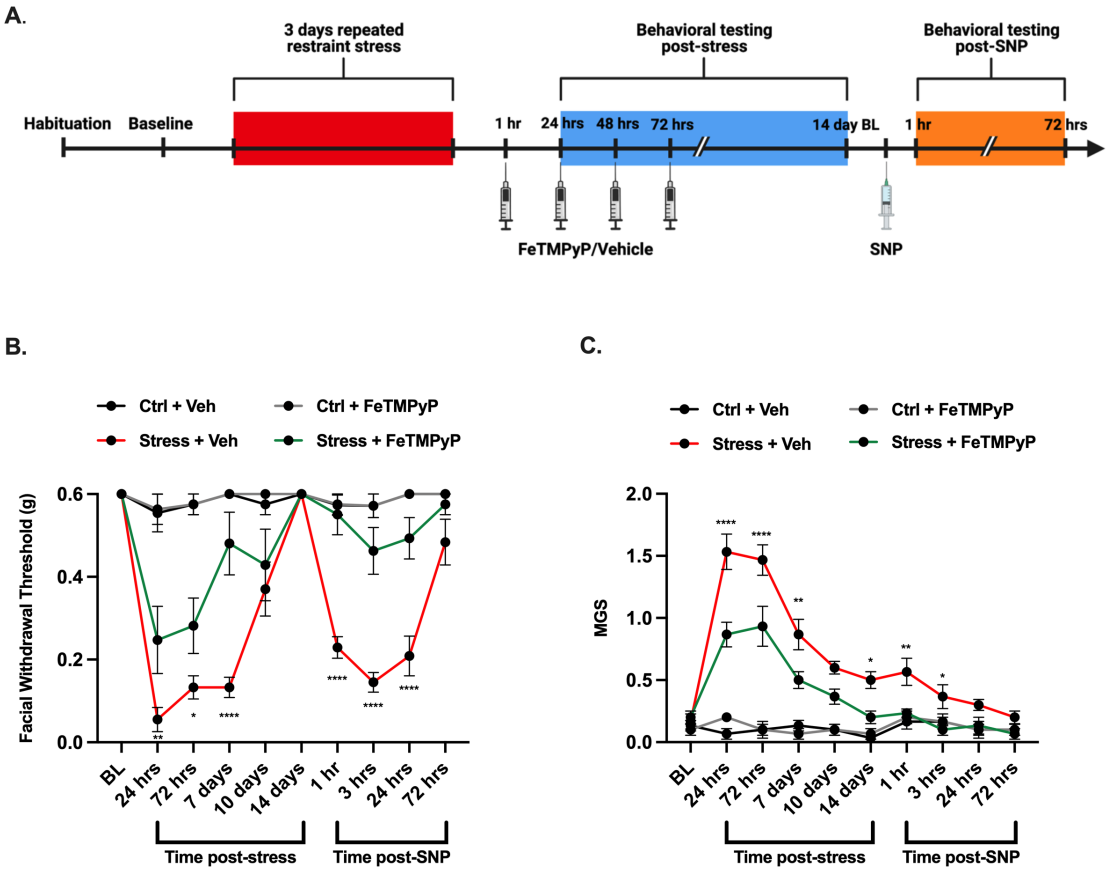

Figure 4.5. Multiple dosing with a PNMC attenuates stress-induced hypersensitivity and priming to an NO donor. Stress paradigm and dosing regimen are shown in (A). Following 3 days of repeated stress, female ICR mice were administered FeTMPyP (30 mg/kg, IP) or vehicle at 1, 24, 48, and 72 hrs post-stress and tested for acute facial hypersensitivity (B) and grimacing (C). Upon returning to baseline thresholds, mice were checked for priming to low-dose SNP (0.1 mg/kg, IP). Stress induced acute mechanical hypersensitivity and grimace responses in mice that received multiple injections of vehicle; however, these effects were attenuated by multiple injections of FeTMPyP, determined by a two-way ANOVA with Bonferroni *post-hoc* analysis. * denotes significance between stressed mice that received FeTMPyP and those that received vehicle. All control groups received vehicle and were administered SNP before the priming phase; (n=6 for all groups). Data are represented as mean ± SEM. See Table 1 for F-values. *p<0.05, **p<0.01, ****p<0.0001.

DISCUSSION

Here, we report a novel role for PN in the development and maintenance of stress-induced facial hypersensitivity and priming to a low-dose NO donor, a clinically relevant observation given

the extreme sensitivity of migraine patients to both stress (the most common trigger) and NO producing agents [44]. Our data support previous findings which underscore the role of PN in nociceptive processing [31, 33] and, specifically, migraine pain [39]. Perhaps of greatest significance, these data encourage a new appreciation for endogenous PN as a nitro-oxidative mediator of stress-induced facial allodynia and priming. Repeated daily dosing with a PNMC beginning 1-hr after repeated restraint stress was able to attenuate acute facial hypersensitivity. Interestingly, a single dose of a PNMC at 1-hr post-stress resulted in a reduction in pain thresholds; however, this therapeutic effect was not achieved when administered as a single dose 24-hrs post-stress, suggesting that the effects of these PNMCs on acute stress may be temporally sensitive. Critically, these data implicate endogenous PN in the mechanisms responsible for the onset of hypersensitivity. Furthermore, administering a PNMC 30 mins prior to administration of SNP in stress-primed mice proved efficacious in reducing nociceptive responses, suggesting that PN mediates at least some of the effects of NO-donor-induced hypersensitivity. Together, these data strongly implicate PN in migraine pathophysiology and underscore the therapeutic potential in targeting this molecule.

Our data also highlight potential differences in nociceptive sensitization between models of stress-induced headache and headache produced by dural stimulation. Stimulation of the dura mater with pro-inflammatory mediators sensitizes meningeal afferents to pH 7.0 [47], an effect that is mediated by ASICS, which have been highly implicated in migraine pathophysiology and suggested as novel therapeutic targets for migraine [58-61]. Of particular relevance, NO has been shown to modulate ASIC channels in DRG neurons, an effect that is reversed upon treatment with oxidative reducing agents, but not inhibitors cGMP inhibitor, suggesting a role for RNOS in the

modulation ASICS during inflammation [55]. Additionally, inhibiting ASIC3 prevents durovascular and NO-mediated trigeminal pain in rats [62]. Based on these observations, we were interested in determining whether PN plays a role in the development of priming to dural pH 7.0. Contrary to our findings in the stress model, we found no significant effect of either PNMC in preventing hypersensitivity to dural pH 7.0, suggesting that sensitization of ASICS may not involve PN formation. Conversely, another possible explanation for the lack of efficacy of PNMCs in this model could be due to issues in pharmacokinetics of these compounds. Specifically, MnTBap and FeTMPyP are both large metalloporphyrins that poorly penetrate the blood-brain barrier [63]. Given the fact that IL-6 and pH 7.0 were administered locally, these specific PMNCs may not be able to access the pharmacologically relevant site(s) of action in this model, which are not entirely known. Contrarily, a recent study showed that the same dose and route of injection (30 mg/kg, IP) of another metalloporphyrin inhibits spontaneous firing in trigeminocervical neurons following dural activation [39], thereby suggesting that PN may differentially contribute to trigeminal hypersensitivity depending on the activating stimulus. Nonetheless, further testing with more pharmacologically potent PNMCs is necessary to determine the extent to which PN contributes to trigeminal nociceptive sensitization.

Currently, there is no clear mechanism for how endogenous PN may contribute to stress-induced hyperalgesia or priming to NO donors. Stress is a highly variable trigger among migraine patients as the type, intensity, and frequency of stress can all have differential impacts between individuals, making it a particularly complex model to study even in rodents. Preclinically, activation of meningeal afferents provides a more direct and observable model of facial allodynia, given the cross-talk between the dura and TG; however, both stress and NO donor administration

affect the entire body and, undoubtedly, numerous tissues, cellular populations, and mechanisms. Similarly, PN contributes to pain and sensitizes neurons via several distinct mechanisms: it directly causes lipid peroxidation; it can induce chronic inflammation via activation of NF-kB and activator protein-1 and the subsequent release of numerous pro-inflammatory cytokines and chemokines; it inhibits MnSOD and the mETC and increases mitochondrial calcium release, all of which contribute to mitochondrial dysfunction; it causes endothelial dysfunction by reducing the amount of NO available for stimulation of G-protein coupled receptors and, thus, vasodilation; lastly, it binds to and modulates all types of biomolecules and biological targets, including proteins, lipids, thiols, and DNA [36, 37, 64]. Clearly, pinpointing which of these mechanisms, if any, are affected by PN in the context of migraine presents a significant challenge.

Critically, PN plays an essential role in mediating nitro-oxidative stress (also known as nitro-oxidative damage, a term which we will use interchangeably in order to avoid confusion with perceived stress). Nitro-oxidative stress is defined as an imbalance between the production of RNOS (NO, O_2^-, $ONOO^-$) and antioxidant defenses. Although RNOS such as NO and SO have important physiological roles in maintaining homeostasis, these mechanisms can become maladaptively altered under intense levels of stress, also defined as allostatic load [65, 66]. Indeed, noxious psychological and physical stress experienced by humans has been linked to oxidative damage and inflammation caused by imbalances in RNOS [67, 68]. This damage is thought to underlie many different diseases and pain states, including migraine [66, 69]. Notably, deficits in bioenergetics are observed in migraine patients and mitochondrial dysfunction is a prominent theory behind the cause of cortical spreading depression and other hallmarks of migraine [70-72]. Some indications suggest that migraineurs may be more susceptible to an attack during the period *following* stress,

also known as the "let-down" phase [73]. This is supported by clinical observations which conclude that the greatest susceptibility to a stress-related attack is typically 6 to 18 hours following resolution of stress [74, 75]. Based on this, it has been suggested that symptoms such as fatigue, which can be caused by stress, may reflect premonitory symptoms associated and could be indicative of an oncoming attack [76]. One of the most important consequences of nitro-oxidative stress is the disruption of proper mitochondrial function, ultimately culminating in impaired bioenergetics and homeostasis [37, 50]. As mentioned earlier, PN nitrates and subsequently inhibits MnSOD, the essential antioxidant enzyme that detoxifies free radical species, and shuts down mitochondrial respiration via inhibiting the mETC and enzymes that are essential for mitochondrial function [36, 37]. Additionally, PN can increase mitochondrial calcium release, providing a potential mechanism for how PN might sensitize nociceptors [38]. Preclinical models have also generated support for mitochondrial dysfunction as a critical contributor to migraine pathophysiology and treatment with MnTBAP has shown efficacy in reducing oxidative damage in other preclinical pain models, further implicating PN-mediated oxidative damage as a potential mechanism in neuronal sensitization [77-79]. Together, these observations create a strong argument for PN-mediated oxidative damage and mitochondrial dysfunction as possible mechanisms that underlie the development of trigeminal hypersensitivity following stress or NO donor administration.

The above data highlight a novel role for PN in mediating the development of long-lasting stress-induced facial hypersensitivity and priming to an NO donor and further implicate it as a therapeutically attractive target for migraine. Future studies should focus on determining the extent to which PN contributes to nociception in this disorder, as well as establish relevant mechanisms of action. Currently, more potent and efficacious variations of PNMCs are being developed and

phase II trials have begun for chemotherapy-induced peripheral neuropathy as well as surgical, osteoarthritic, and diabetic pain [80]. Based on their preclinical efficacy across different models, these compounds may attenuate toxic PN-based modifications via several different mechanisms, including inflammation and nitro-oxidative stress. Additionally, although we did not observe any sex differences in our experiments, future studies should take care to address the potential for sex-specific effects regarding PN-mediated nociception, as migraine affects women disproportionately to men. Given the important roles of NO and SO in maintaining normal physiological function, targeting the overproduction of PN presents a unique approach to resolving nitro-oxidative stress and other consequences of RNOS formation that may play a critical role in migraine pathophysiology.

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CHAPTER 5

REGULATION OF MITOCHONDRIAL FUNCTION BY PEROXYNITRITE IN PRECLINICAL MODELS OF MIGRAINE

PEROXYNITRITE INTERACTIONS IN MITOCHONDRIA BIOENERGETICS

Beyond testing the behavioral effects of PN in migraine, it is important to understand *where* and *how* PN might be contributing to this underlying pathology. Mt are the key loci for the formation of intracellular PN and its reactions and has been recognized as a primary pathway in mediating the pathological effects of both NO and PN. PN is formed both intra- and extramitochondrially and is able to rapidly undergo reactions with mt membrane proteins [1]. Interestingly, one of the primary indicators of mt disease is episodic head pain and emerging evidence has supported a role for mt dysfunction in migraine pathophysiology. The idea that mt dysfunction may contribute to migraine was first hypothesized in 1988, when a study of nine migraine patients found ragged red fibers (RRF) deficient in cytochrome c oxidase (COX) as well as decreased respiratory chain enzymes in muscle tissues, indicating altered mt function [2]. Mt play a central role in several cellular functions, including production of reactive oxygen species (ROS), regulation of apoptosis, maintaining Ca^{2+} concentration, and, ultimately, regulating cellular energy homeostasis [3]. Mt also function in controlling vascular tone by stabilizing concentrations of Ca^{2+} and ROS. Because migraine is associated with deficits in energy homeostasis, dysregulation of vasodilation, and neuronal sensitization, it is likely that mt dysfunction contributes to some of the underlying pathophysiology of this disorder.

NO binds directly with COX, which can sequester NO and ultimately prevent vasodilation [4]; however, an overload of NO activity and, subsequently, increased PN formation can disrupt

these maintenance processes [5]. NO has also been shown to inhibit mt nicotinamide adenine dinucleotide phosphate (NADH) reductase activity through PN formation, which can lead to intravascular platelet dysfunction and dysregulation of vasodilation and vasoconstriction [6]. Importantly, impairments in both NADH dehydrogenase and COX have been found in migraineurs with and without aura [7]. PN can also interact with and oxidize or nitrate critical mt membrane proteins, such as adenine nucleotide translocase and voltage-dependent anion channels, which can lead to activation of the pyridine nucleotide-dependent Ca^{2+} release pathway and opening of the permeability transition pore (PTP), ultimately resulting in disruption of Ca^{2+} homeostasis and cell death [8-11]. Impairment of Ca^{2+} homeostasis has been linked to cortical spreading depression (CSD), in which increases in Ca^{2+} concentration within astrocytes causes vasoconstriction during CSD [12, 13]. Because mt are the primary organelles that sequester Ca^{2+}, mt dysfunction that results in an imbalance of Ca^{2+} may contribute to downstream processes that result in neuronal sensitization, further increasing susceptibility to migraine [14].

MITOCHONDRIA DYSFUNCTION IN MIGRAINE PATHOPHYSIOLOGY

Impairments in mt respiratory chain function and mETC enzymes have also been observed in migraineurs and suggest depletions in cellular energy [2, 15]. Energy depletion due to mt dysfunction can impair astrocytic function and further increase neuronal susceptibility to CSD [16]. PN has been shown to interact with and inactivate complexes I, II, and V of the mETC and is believed to be the primary mechanism by which NO disrupts mt function [1, 17]. Other markers of mt metabolic dysfunction, such as lactic and pyruvate acid, are also increased in migraineurs, suggesting dysregulation of metabolic processes and an increased vulnerability to oxidative stress

[18]. These findings are further supported by preclinical evidence; functional analyses using rat migraine models have identified decreases in spare respiratory capacity in the TNC as well as impairment of mt biogenesis in the TG, strongly suggesting altered mt function [19, 20]. Furthermore, depletions in levels of N-acetyl aspartate, an important marker of neuronal integrity that is produced exclusively in neuronal mt, have been observed in the serum of migraine patients compared with healthy controls, suggesting the presence of mt-mediated neuronal dysfunction [21, 22].

Therapeutically, the actions of various drugs that are commonly used to treat migraine have been found to have beneficial effects on mt function. Valproate, an anti-epileptic drug used in the treatment of migraine, has been shown to attenuate nitroglycerin-induced TG activation by preserving mt energy metabolism and biogenesis in a rat model of migraine [23]. Similarly, topiramate, another anti-epileptic drug used to treat migraine, has been shown to protect against mt membrane depolarization caused by high Ca^{2+} concentrations, ultimately increased mt survival [24].

Taken together, the above evidence strongly suggests a role for mt dysfunction in migraine pathophysiology likely caused, in part, by PN formation. The ability of PN to directly and indirectly interact with several key mt membrane proteins and mETC components resulting in dysregulation of Ca^{2+} and energy homeostasis, enhanced generation of ROS, and initiation of apoptosis provides strong rationale for its role in mt dysfunction. Furthermore, the ability of NO to trigger attacks in migraineurs along with the identification of mt abnormalities in many patients suggests that one mechanism underlying the effects of NO in migraine could very well be mediated by PN-induced impairments in mt function. Despite these observations, no study to date has

directly investigated the role of PN-induced mt dysfunction in the acute or primed phases of migraine.

EVIDENCE FOR PN-MEDIATED MITOCHONDRIAL DYSFUNCTION IN PRECLINICAL MIGRAINE MODELS

Our lab has begun investigating the complex relationship between PN mitochondrial function in our preclinical migraine models. Following repeated restraint stress, we harvested and cultured mouse TGs from male and female ICR mice and allowed them to grow overnight. The following day, we processed these cultures using the Mito Stress Test Kit (Agilent) and measured oxygen consumption rates (OCR) using an Agilent Seahorse XFp apparatus. From the OCR rates, parameters of mt function, including basal respiration, maximal respiration, spare reserve capacity, proton leak, ATP production, and coupling efficiency were calculated as described in the Agilent Seahorse XF Cell Mito Stress Test Report Generator manual. Approximately 24 hrs following day 3 of repeated restraint stress, basal respiration levels are increased in stressed male, but not female, mice compared with controls; however, maximal respiration levels are significantly upregulated in both sexes, suggesting that female and male mice exhibit differences in respiration outputs shortly after repeated stress (Figure 5.1). Conversely, at 14 days following repeated stress, when animals typically return to baseline nociceptive thresholds, we found significant increases in spare respiratory capacity (a measure of the cell's ability to adapt to environmental stressors), ATP production, and proton leak compared to controls in female mice, but not in males (Figure 5.2).

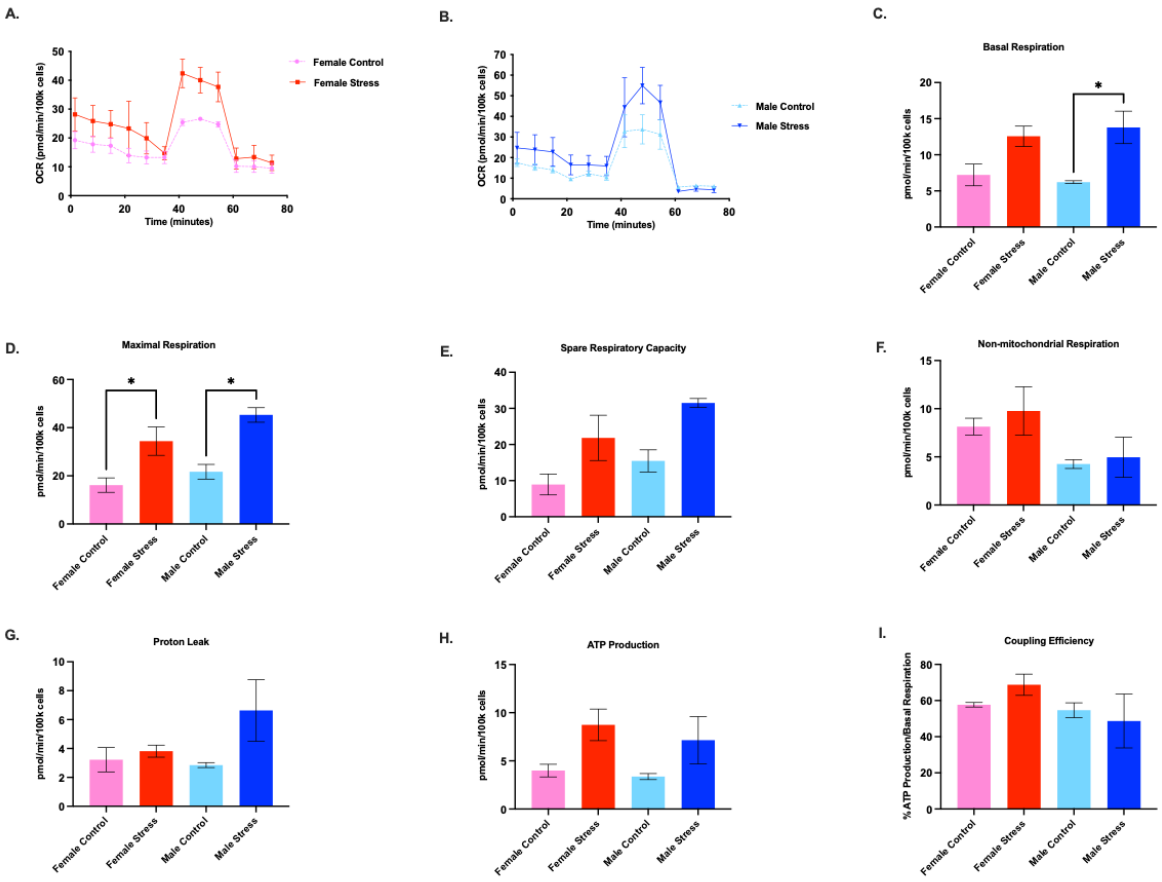

Figure 5.1. Mitochondrial respiration is increased in the TGs of male and female mice 24 hrs following repeated restraint stress. Oxygen consumption rates (OCRs) were measured for females (A) and males (B) from which rates of basal respiration (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial respiration (F), proton leakage (G), ATP production (H), and ATP coupling efficiency (I) were calculated. Male mice exhibit increased basal respiration levels while both sexes were found to have increased maximal respiration, indicating that stress increases respiration rates in TG mitochondria.

Most importantly, these data seem to suggest that mt undergo significant changes within the period of hypersensitivity following stress and may serve as a potential biological basis for the development of hyperalgesic priming. While an important output of mt respiration is to produce ATP, abnormal production of ATP has also been shown to be indicative of abnormal Ca^{2+} signaling, which can directly contribute to neuronal hyperexcitability [25]. Additionally, it

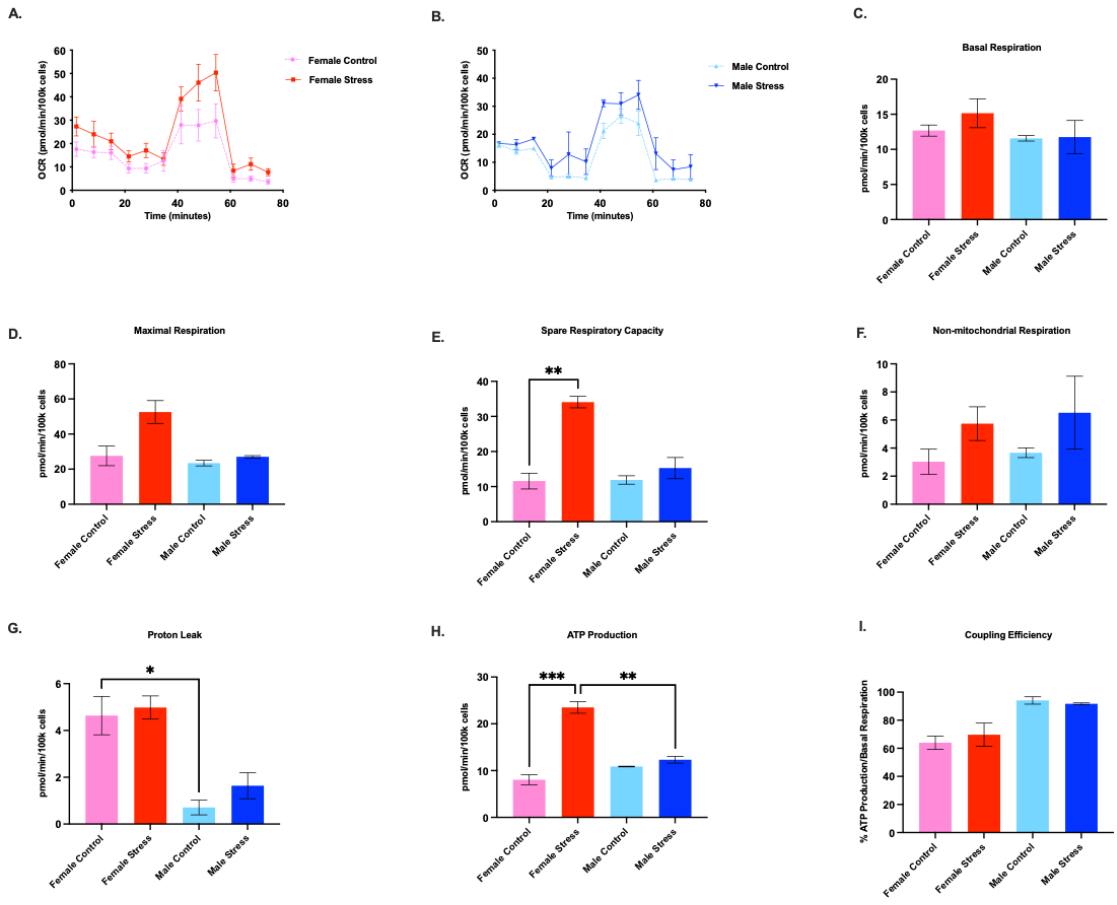

Figure 5.2. 14 days following repeated stress, spare respiratory capacity and ATP production are increased in female mice. Oxygen consumption rates (OCRs) were measured for females (A) and males (B) from which rates of basal respiration (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial respiration (F), proton leakage (G), ATP production (H), and ATP coupling efficiency (I) were calculated. Female mice exhibited increased mitochondrial spare respiratory and ATP production in their TGs, an effect that was not observed in males, suggesting a potential sex difference in the long-term effects of stress on mitochondrial function.

can be speculated that an increase in spare respiratory capacity, or the adaptive ability of mt, is not necessarily a positive effect, as mt are crucial to maintaining homeostasis and even minute disruptions in mt respiration can be detrimental to the cell. Furthermore, proton leak has been shown to regulate mt RNOS generation in models of inflammation, suggesting that an increase in proton leak may be indicative of mt dysfunction [26]. It should also be noted that although we have

not observed sex differences in nociceptive behavior following stress, the mechanisms underlying those behaviors may very well be sex-specific and should not be ruled out.

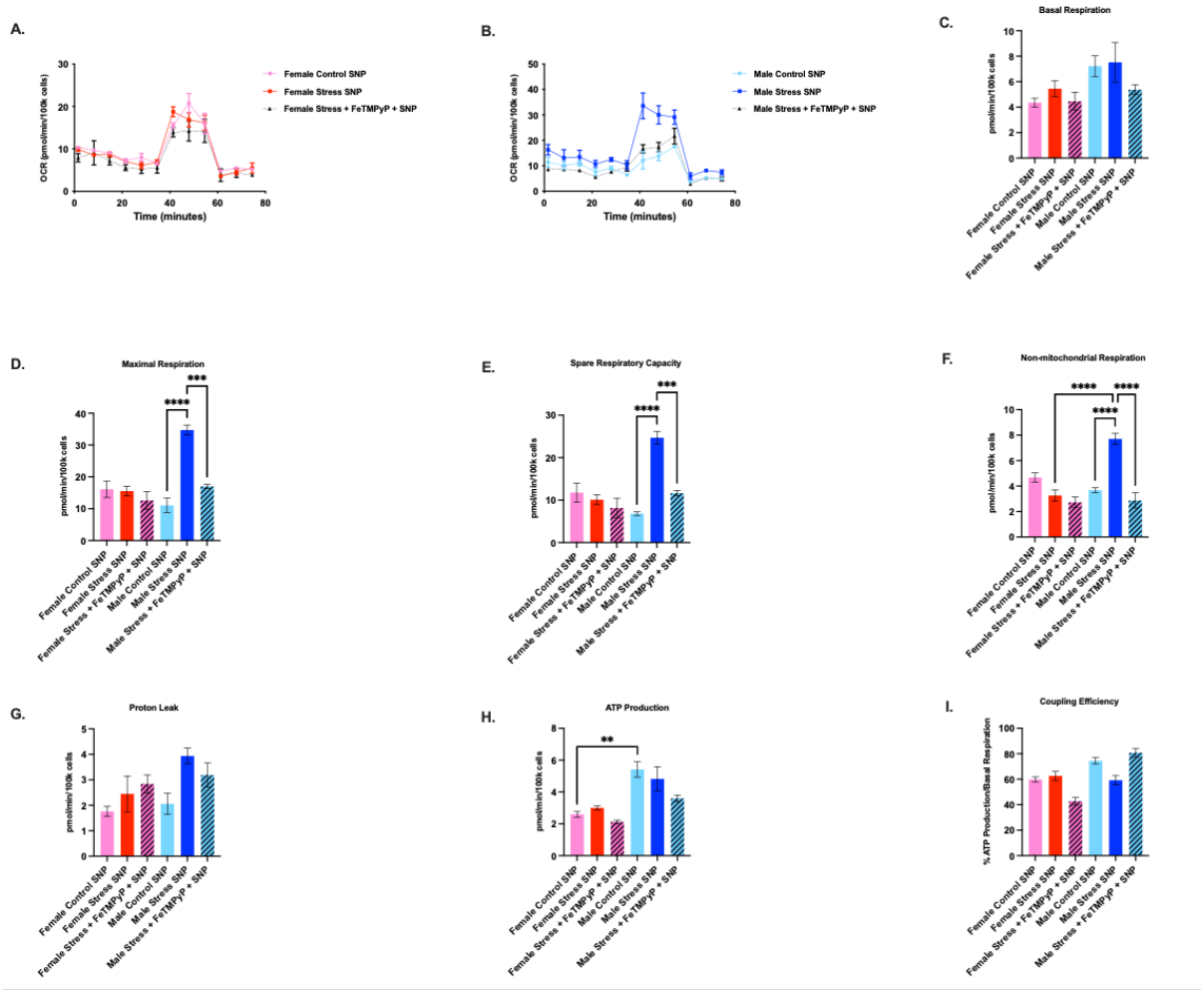

Figure 5.3. Administration of low-dose SNP induces robust changes in mitochondrial function in the TGs of male, but not female mice, an effect that is attenuated by pre-treatment with FeTMPyP. 14 days following repeated stress, mice were administered either FeTMPyP or vehicle and primed with low-dose SNP. Mitochondrial Oxygen consumption rates (OCRs) were measured for females (A) and males (B) from which rates of basal respiration (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial respiration (F), proton leakage (G), ATP production (H), and ATP coupling efficiency (I) were calculated. Interestingly, stress-primed male mice exhibited robust increases in maximal respiration, spare respiratory capacity, and non-mitochondrial function in response low-dose SNP (0.1 mg/kg, IP). Notably, these changes

were attenuated by pre-treatment with FeTMPyP (30 mg/kg, IP). No changes were observed in female mice.

To parallel our behavior experiments with stress, we also measured OCR rates in mice that were administered either vehicle or FeTMPyP (30 mg/kg, IP) 30 mins prior to SNP (0.1 mg/kg, IP) at day 14 of stress. Contrary to what we observed in earlier studies, we found that maximal respiration, spare respiratory capacity, and non-mitochondrial respiration are all increased in male, but not female, mice following SNP and, critically, this effect is attenuated by pre-treatment with FeTMPyP (Figure 5.3). While it is difficult to draw firm conclusions from these data without additional studies, these observations suggest that NO donors more readily impact mt function in stress-primed male mice than in females, an effect that seems to be mediated by PN formation, given the efficacy of FeTMPyP in correcting the resulting dysfunction. Likewise, the increase in spare respiratory capacity in females at day 14 following stress may provide a basis for protecting mt from the effects of NO donors at this stage, although this is only speculative.

Taken together, these preliminary, yet critical data provide insight into how PN-mediated mt dysfunction may contribute to long-lasting hypersensitivity in preclinical models of migraine. Future studies investigating these effects should take care to study changes in other critical parameters of mt function, including Ca^{2+} signaling, antioxidant levels, and levels of relevant enzymes, such as NADH.

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CHAPTER 6

TRANSLATION DYSREGULATION IN PERIPHERAL SENSORY NEURONS

Although acute pain is critical to species survival, chronic pain is extremely disabling. Chronic pain is thought to be caused by maladaptive changes in nociceptor plasticity and is associated with persistent peripheral and central sensitization, in which nociceptors become hyperexcitable and exhibit ectopic activity, resulting in mechanical hypersensitivity, thermal hypersensitivity, and spontaneous pain. Although pain transmission is mediated by both central and peripheral mechanisms, targeting peripheral sensory neurons as a therapeutic strategy has gained increasing interest due to the potential to minimize off-target effects that typically occur when targeting the brain directly. Here, we will briefly review the mechanisms by which dorsal root ganglia (DRG) and trigeminal ganglia (TG) nociceptor signaling may contribute to chronic pain conditions.

PAIN NEUROTRANSMISSION AND PERIPHERAL SENSITIZATION

The sensation of pain is critical to species survival, as it warns an individual of potential dangers resulting in damage to the body and allows for protection during the healing process. The transmission of noxious information is mediated by a class of sensory neurons called nociceptors which transmit pain signals to the spinal cord and up through the spinothalamic and spinoreticulothalamic tracts, ultimately projecting to the brainstem, thalamus, and higher cortical regions where sensory information is processed. Importantly, this process involves three key steps [1, 2]. First, tissue damage results in the local release of several pro-inflammatory endogenous compounds which bind to their respective membrane receptors in peripheral sensory axons, effectively transducing the noxious stimulus into a chemical signal. Next, this chemical signal is

converted into an electrical signal that results in propagation of an action potential and transmission of nociceptive information to first-order DRG or TG neurons. Lastly, sustained activation and modulation of primary afferent input leads to continuous transmission of pain signals to higher ordered neurons and brain regions, ultimately culminating in persistent pain. Thus, sensitization of sensory neurons serves a critical role in alerting individuals to additional harm, allowing for adequate recovery from the initial noxious stimulus.

Despite its role in acute pain, sensitization in the absence of tissue damage is no longer beneficial and is thought to underlie chronic pain. Peripheral sensitization in nociceptors is caused by robust changes in signaling cascades and gene expression, leading to decreased action potential thresholds and, thus, hyperexcitability of the cell. Following injury, the release of various pro-inflammatory cytokines and neuropeptides, such as IL-6 and CGRP, results in the activation of G-protein coupled receptors that work downstream to stimulate transcription and translation of new proteins [3]. Indeed, *de novo* protein synthesis of voltage-gated ion channels and other proteins within the nociceptor cell body and its peripheral axon induce maladaptive changes in nociceptor plasticity and lead to neuronal hyperexcitability [4].

SENSITIZATION IN MIGRAINE PATHOPHYSIOLOGY

One of the hallmarks of migraine patients is their susceptibility to normally innocuous stimuli, suggesting underlying sensitization of the trigeminovascular system. As mentioned earlier, priming of the trigeminovascular system is associated with maladaptive neuroplasticity. Trigeminal activation releases a multitude of excitatory neurotransmitters such as CGRP, Substance P and neurokinin A and also causes vasodilation of blood vessels and release of pro-

inflammatory mediators, all of which can contribute to dural afferent plasticity [5-8]. Repetitive stimulation of dural afferents not only contributes to peripheral sensitization but also to central sensitization at synapses in the TNC, which has been shown in humans to account for facial allodynia [9, 10]. Second-order neurons in the TNC also receive inputs from fibers innervating the face and studies have shown that when inflammatory mediators such as mustard oil, capsaicin or an inflammatory soup are put directly onto the dura, the receptive fields of these neurons expand and there is a development of facial input from these neurons in response to tactile stimulation [11]. This resulting second-order neuronal plasticity might indicate a change in the molecular machinery at these synapses, such as an up-regulation of NMDA and AMPA receptors, which have been highly implicated in other forms of plasticity, such as learning and memory [12, 13].

Two molecules that have been shown to contribute to changes in neuroplasticity are CGRP and BDNF. CGRP can act at both peripheral and central sites and, in trigeminal neurons, facilitates the release of BDNF, which is thought to be involved in the maintenance phase of long-term potentiation (LTP), a process that has long been shown to contribute to plasticity at central synapses [14]. BDNF facilitates transmission through NMDA receptors and NMDA-dependent LTP [15]. In preclinical models of migraine, dural stimulation of meningeal afferents caused BDNF-dependent priming to innocuous stimuli [16]. Furthermore, in the spinal cord, pain plasticity is blocked and reversed with a local injection of the BDNF scavenger TrkB/Fc [17].

Thus, the above evidence strongly suggests a role for maladaptive plasticity in migraine headache, likely occurring at trigeminal synapses and regulated by *de* novo protein synthesis.

REGULATION OF NASCENT PROTEIN SYNTHESIS VIA EIF4E

Translation control of gene expression is a critical process for the regulation of plasticity in the nervous system and numerous lines of evidence have implicated the dysregulation of this process in chronic pain plasticity [18]. In dorsal root ganglia (DRG) and trigeminal ganglia (TG) sensory neurons, pain-inducing ligands bind to their receptors and activate mechanistic target of rapamycin (mTOR) and extracellular signal-regulated kinase (ERK) pathways, both of which converge on the 5' cap of mRNAs to initiate protein synthesis via eukaryotic translation initiation factor (eIF) 4F complex formation [19, 20]. The eIF4F complex is comprised of the scaffolding protein eIF4G, the RNA helicase eIF4A, and the 5' cap-binding protein eIF4E. mTOR controls the rate of translation via phosphorylation of 4E-binding protein (4E-BP) and p70S6 kinase (S6K1/2) [21]. 4E-BPs are small translational inhibitors that suppress the assembly of eIF4F through inhibiting the binding of eIF4E and eIF4G. Phosphorylation of 4E-BP leads to dissociation of this molecule from eIF4E, thus, increasing the amount of free eIF4E and promoting eIF4F complex formation. Conversely, ERK-dependent activation of mitogen-activated protein kinase interacting kinases (MNKS) 1/2, which directly phosphorylate eIF4E at serine 209, and, ultimately, promote translation initiation [22, 23]. Furthermore, activation of p38, another important kinase in the pain pathway, can also promote eIF4E-dependent translation via phosphorylating MNK1 [24]. Importantly, eIF4E regulates the translation of a distinct subset of "eIF4E-sensitive" mRNAs which encode proteins that regulate synaptic function and plasticity in neurons, such as brain-derived neurotrophic factor (BDNF) [17, 18, 25].

TRANSLATION DYSREGULATION IN PERSISTENT PAIN

Activation of peripheral sensory fibers following nerve injury or inflammation results in the release of numerous pro-inflammatory molecules, including interleukin-6 (IL-6) and nerve growth factor (NGF), which induce protein synthesis via ERK/mTOR activation [19]. In the spinal cord and DRG, there is overwhelming evidence for the contribution of ERK and mTOR pathways to nociceptive basal hypersensitivity as well as modulation of many channel subtypes, including Kv4.2, Nav1.7, and Cav2.2, all of which contribute to the generation and amplification of pain transmission [4, 18, 26-30]. Additionally, eIF4E phosphorylation has been shown to contribute to injury-induced nociceptive plasticity and the development of sensitization in preclinical models of pain [31, 32].

Inhibition of mTOR using rapamycin attenuates tactile allodynia in neuropathic mice, but also induces a negative feedback loop in which ERK activation is enhanced, resulting in the promotion of pain [33]. Conversely, phosphorylation of AMP-activated protein kinase (AMPK) suppresses both ERK and mTOR activity and activators of AMPK have been shown to attenuate nociceptive responses in inflammatory and neuropathic pain models [34-36]. Furthermore, targeting MNK 1 and 2 has also achieved therapeutic efficacy in preclinical pain models. Genetic inhibition of eIF4E via mutation of its phosphorylation site at Ser 209 reduces inflammatory pain and spontaneous neuronal firing following treatment with IL-6, an effect also observed after pharmacological inhibition of MNK by cercosporamide and eFT508 [31, 37, 38]. Thus, targeting eIF4E-mediated translation via activation of AMPK and targeting the MNK-eIF4E axis offer promising therapeutic strategies moving forward.

In Chapter 7, we will provide evidence of a novel role for eIF4E-mediated translation in migraine pathophysiology.

OTHER MECHANISMS OF TRANSLATION CONTROL IN PAIN

Another major mechanism of translation regulation occurs via phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) [39]. eIF2α integrates pathways involved in cellular stress responses, also known as the integrated stress response (ISR), and regulates mRNA translation via binding guanosine 5'-triphosphate (GTP), the initiator RNA, and the small ribosomal unit to form the translation pre-initiation complex. Phosphorylation of eIF2α converts it from a substrate to an inhibitor of eIF2β and ultimately coincides in the suppression of general translation. Conversely, although phosphorylation of eIF2α decreases general translation, it also promotes translation of mRNAs with upstream open reading frames (uORFs) in their 5' UTRs, some of which encode transcription factors which activate gene expression programs involved in adaptation and cell survival. Interestingly, eIF2α phosphorylation is increased in the DRG in models of inflammation, nerve injury, and diabetes [18]. Additionally, preclinical evidence suggests that eIF2α phosphorylation promotes thermal, but not mechanical hypersensitivity [40]. Despite these observations, the extent to which this mode of translation contributes to long-lasting hypersensitivity is still unknown.

Furthermore, mRNA translation is regulated by several additional mechanisms, including internal ribosome entry sites (IRES), which mediate cap-independent translation initiation as well as mechanisms that regulate the length of the poly(A) tail, which protects mRNA from degradation and stimulates mRNA translation via promoting mRNA circularization [41, 42]. Although

physiologically important, the role of these other translation control pathways in pain contexts is not yet clear.

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CHAPTER 7

DE NOVO PROTEIN SYNTHESIS IS NECESSARY FOR PRIMING IN PRECLINICAL MODELS OF MIGRAINE

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ABSTRACT

Background: Migraine attacks are often triggered by normally innocuous stimuli, suggesting that sensitization within the nervous system is present. One mechanism that may contribute to neuronal sensitization in this context is translation regulation of new protein synthesis. The goal of this study was to determine whether protein synthesis contributes to behavioral responses and priming in preclinical models of migraine.

Methods: Mice received a dural injection of interleukin-6 in the absence or presence of the protein synthesis inhibitor anisomycin or the translation initiation inhibitor 4EGI-1 and were tested for facial hypersensitivity. Upon returning to baseline, mice were given a second, non-noxious dural injection of pH 7.0 to test for priming. Additionally, *eIF4E*^{S209A} mice lacking phosphorylation of mRNA cap-binding protein eIF4E received dural interleukin-6 or were subjected to repeated restraint stress and then tested for facial hypersensitivity. After returning to baseline, mice were given either dural pH 7.0 or a systemic sub-threshold dose of the nitric oxide donor sodium nitroprusside and tested for priming.

Results: Dural injection of interleukin-6 in the presence of anisomycin or 4EGI-1 or in *eIF4E*^{S209A} mice resulted in the partial attenuation of acute facial hypersensitivity and complete block of hyperalgesic priming. Additionally, hyperalgesic priming following repeated restraint stress was blocked in *eIF4E*^{S209A} mice.

Conclusions: These studies show that *de novo* protein synthesis regulated by activity-dependent translation is critical to the development of priming in two preclinical models of migraine. This suggests that targeting the regulation of protein synthesis may be a novel approach for new migraine treatment strategies.

INTRODUCTION

A distinct characteristic of migraine patients is their enhanced sensitivity to innocuous stimuli, which can trigger and exacerbate a migraine headache [1, 2]. In preclinical models of migraine, repetitive stimulation of dural afferents not only contributes to peripheral plasticity, but also to central plasticity at synapses in the trigeminal nucleus caudalis (TNC), which is thought to account for the cutaneous facial allodynia present in humans during attacks [3, 4]. Activation of meningeal afferents results in the release of numerous pro-inflammatory cytokines, growth factors, excitatory neurotransmitters, and neuropeptides from primary sensory neurons, degranulated mast cells, and dural fibroblasts [5-8]. Many of these endogenous factors, including IL-6 and calcitonin gene-related peptide (CGRP), directly contribute to the sensitization of primary sensory neurons as well as their downstream targets in the TNC [6, 8-13]. Based on this, mechanisms underlying peripheral and central sensitization have been proposed to contribute to these symptoms.

Repeated or intense noxious stimulation can cause maladaptive changes in synaptic plasticity within nociceptive circuits, leading to peripheral and central sensitization [14]. Nascent protein synthesis in response to noxious stimuli can induce long-term changes in nociceptor activity and gene expression that can lead to the development of chronic pain [15-19]. Additionally, regulation of nascent protein synthesis via activity-dependent translation has been shown to be a highly critical molecular event for neuroplasticity and plays a key role in changing nociceptor functionality [17, 20-23]. Activity-dependent translation can be induced by various endogenous compounds and membrane receptors and is regulated via the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) and the extracellular-signal-regulated kinase (ERK) pathways which converge on eukaryotic initiation factor 4E (eIF4E) of the eIF4F translation pre-initiation

complex. Interestingly, in the dorsal root ganglion and dorsal horn of the spinal cord, these pathways are robustly activated following peripheral nerve inflammation and nerve injury and have been demonstrated as being essential for the persistence of chronic pain. For example, inhibition of mTORC1, activity-dependent translation, or general translation by local administration of rapamycin, 4EGI-1, or anisomycin, respectively, reduces mechanical hypersensitivity and injury-induced changes in nociceptor excitability, further implicating mechanisms of translation in the maintenance of pain [22, 24].

Despite the overwhelming evidence for a role of translational regulation in chronic pain, no studies to date have examined this mechanism in the development and persistence of migraine headache. Here, using a preclinical model of dural stimulation and a model of repeated stress-induced hypersensitivity, we demonstrate a critical role for *de novo* protein synthesis in the behavioral responses and priming in these models.

MATERIALS AND METHODS

Experimental animals. Male and female *eIF4E*^{S209A} mice on a C57BL/6 background were generated in the Sonenberg laboratory at McGill University as previously described [25] and bred at The University of Texas at Dallas to generate experimental animals. These animals were genotyped using DNA from tail clips taken at the time of weaning and were backcrossed to C57BL/6 background for at least 10 generations before experiments. In experiments involving transgenic mice, the control mice used were wild-type (WT) mice generated in the UT Dallas breeding colony from crossings of heterozygous *eIF4E*^{S209A} mice with WT C57BL/6 mice (Envigo). Male and female ICR (CD-1) mice were outbred and purchased from Envigo. All

behavior experiments were performed using mice aged 6-8 weeks (~25-30 g) at the start of the experiment. All mice were housed on 12 h light-/dark cycles with lights on at 7:00 A.M. All mice were housed in groups of 4 animals per cage and had food and water available *ad libitum.* All behavioral experiments were performed between the hours of 9:00 A.M. and 5:00 P.M. Mice were randomized to groups from multiple cages and investigators were blinded to treatment groups in all experiments. All animal procedures were approved by the Institutional Animal Care and Use Committees at The University of Texas at Dallas and were performed in accordance with the ARRIVE guidelines as well as the policies of the International Association for the Study of Pain and the National Institutes of Health guidelines for animal research.

Drugs and antibodies. Human recombinant IL-6 protein (R&D Systems) stock solution (100 µg/mL) was prepared in sterile 0.1% BSA and diluted to 1 ng/µL in synthetic interstitial fluid (SIF) consisting of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl2, 10 mM glucose, 1 mM MgCl2 (pH 7.4, 310 mOsm). Anisomycin (Tocris) stock solution (135 µg/µL) and 4EGI-1 (Enzo) stock solution (20 µg/µL) were both prepared in sterile 0.1 % BSA and diluted to 5 µg/µL in SIF. Sodium nitroprusside (SNP) (Sigma-Aldrich) was prepared in sterile phosphate buffered saline (PBS) at the time of use and was kept away from light. For dural injections, mice received 5 µL injections of either IL-6, anisomycin, 4EGI-1, SIF, or a combination of IL-6 and anisomycin or IL-6 and 4EGI-1 for acute testing. For testing the ability of mice to prime to the initial stimulus in these dural injection experiments, 5 µL of SIF pH 7.0 were administered onto the dura. In repeated restraint stress experiments, which used SNP to test priming, a subthreshold dose of 0.1 mg/kg of SNP was administered intraperitoneally as a 150 µL injection. For western blotting experiments,

p-eIF4E (Cell Signaling #9741S) and total-eIF4E (Cell Signaling #9742S) antibodies were used for primary incubation.

Mouse dural injections. Mouse dural injections were performed as previously described [13]. Mice were anesthetized under isoflurane for <2 min with <2.5%–3% isoflurane via a chamber. While anesthetized, treatments were injected in a volume of 5 μL via a modified internal cannula (Invivo1, part #8IC313ISPCXC, Internal Cannula, standard, 28 gauge, fit to 0.5 mm). The inner projection of the cannula was used to inject through the soft tissue at the intersection of the lambdoidal and sagittal sutures. The length of the projection was adjusted, using calipers, to be from 0.5 to 0.7 mm based on the animal weight (25–30 g) so as to not puncture the dura. Control mice received a 5 uL dural injection of SIF (pH 7.4, 310 mOsm).

Repeated restraint stress. Mice were stressed as previously described [26]. Mice were placed right-side up into tail vein injection tubes (Stolting #51338) with the nose through the provided breathing hole and the tail through the slotted tail piece. The slotted tail piece was tightened so as to prevent the mouse from rotating in the tube, but loose enough to allow the animal to breathe. Mice were stressed between the hours of 10am-12pm for two hours per day for three consecutive days. Control mice were placed into a separate room and deprived of food and water for the same two-hour interval for three consecutive days.

Von Frey testing. Mice were conditioned for 5 continuous minutes by handling, 24 h before habituation. Mice were habituated to paper cups (Choice 4 oz paper cups: 6.5 cm top diameter, 4.5

cm bottom diameter, 72.5 cm length) while in testing chambers for two hours per day and for at least two days before measuring a baseline [13]. Each mouse typically used their same assigned paper cup for the remainder of the experiment. Animals were given food while in testing chambers to allow for testing as previously described. Filament thresholds were determined using the Dixon "up-and-down" method. Testing in mice began with 0.07 g on the face and increased in weight to a maximum of 0.6 g on the face. The testing timelines for dural injection experiments and stress experiments were conducted as previously described in [13] and [26], respectively. In both experimental paradigms, once the mice returned to baseline, a sub-threshold dose of compound was administered either onto the dura (pH 7.0) or intraperitoneally (sodium nitroprusside). Mice were then tested for the ability of the initial stimulus to cause priming to the sub-threshold stimulus. All investigators were blinded to experimental conditions.

Western blotting. Female mice were used for all western blotting experiments and were killed by decapitation following anesthesia with tissues being flash frozen on dry ice. Frozen tissues were homogenized using a pestle in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, and 1% Triton X-100) containing protease and phosphatase inhibitors (Sigma-Aldrich) and then sonicated for 10 s. TG and dura tissues were harvested following three days of repeated restraint stress at the time-points provided in the Results section. To clear debris, samples were centrifuged at 14,000 rpm for 15 min at 4°C. 15 μg of protein was loaded into each well and separated by a 10% SDS-PAGE gel. Proteins were transferred to a 0.45 PVDF membrane (Millipore) at 30 V overnight at 4°C. Membranes were then blocked with 5% nonfat dry milk in 1x Tris buffer solution containing Tween 20 (TTBS) for 2 h. Membranes were washed in 1x TTBS

three times for 5 min each then incubated with primary antibody overnight at 4°C. The following day, membranes were washed three times in 1x TTBS for 5 min each then incubated with the goat anti-rabbit secondary antibody (Jackson Immunoresearch) at room temperature for 1 h. Membranes were then washed with 1x TTBS six times for 5 min each. Signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Bands were visualized with a Bio-Rad ChemiDoc Touch and over-saturated pixels were excluded from the final analysis. Blots were first probed for phosphorylated eIF4E (peIF4E) (Cell Signaling; 1:3000), then stripped and re-probed for total eIF4E (teIF4E) (Cell Signaling; 1:3000). Equal loading was verified using GAPDH (Cell Signaling; 1:5000) as a control. For quantitative analysis, peIF4E was normalized to teIF4E. Analysis was performed using Image Lab version 6.0.1.

Experimental design and statistical analysis. We used only female mice in the dural IL-6 experiments involving ICR mice given the higher frequency of migraine among women. The rationale for this was based on pilot studies in which co-injection of dural IL-6 with either anisomycin or 4EGI-1 revealed no sex differences in these animals. Additionally, previously reported findings from our laboratory revealed similar effects in males when IL-6 is co-injected with anisomycin or 4EGI-1 into the hindpaw [27]. Since there have been no comparable studies in *eIF4E*^{S209A} mice, we used both females and males to explore the possibility of a sex difference in this genotype with these stimuli. All behavioral data are represented as individual data points with means (lines). Western blot data are represented as means ± SEM. Behavioral data were analyzed for multiple comparisons at each time point via two-way ANOVA and Bonferroni's *post-hoc* test. F-values for each analysis are presented (Table 7.1). Student's *unpaired two-tailed t-test* was used

for individual mean comparisons when appropriate. Data analysis was performed using Prism version 8.3 for Mac OS X. Significance was set at $p < 0.05$ for all analyses. Power analysis was performed using G power for comparison of the means between groups using expected effect sizes based on pilot studies and previously published data in other models [24]. All investigators were blinded to genotype and treatment during testing and scoring. Each experiment was independently replicated twice.

Table 7.1. F-values obtained from Two-way ANOVA analysis comparing mean effects within rows are presented for each figure.

Figure	Interaction	Row Factor	Column Factor
1	F (30, 385) = 3.569	F (10, 385) = 17.81	F (3, 385) = 98.19
2	F (30, 308) = 2.948	F (10, 308) = 9.244	F (3, 308) = 36.81
3a	F (30, 275) = 3.402	F (10, 275) = 10.43	F (3, 275) = 66.87
3b	F (30, 286) = 2.219	F (10, 286) = 8.967	F (3, 286) = 47.16
4a	F (24, 189) = 4.444	F (8, 189) = 14.01	F (3, 189) = 41.06
4b	F (24, 180) = 11.43	F (8, 180) = 32.85	F (3, 180) = 80.98

RESULTS

Priming induced by dural IL-6 is blocked by general protein synthesis inhibition

Previously, our laboratory has demonstrated the ability of dural IL-6 to sensitize mice to cutaneous mechanical stimulation following a sub-threshold stimulus in a model of hyperalgesic priming [13]. Given recent data supporting a role for general protein synthesis in the development of hyperalgesia via DRG and spinal pathways [28, 29] , and given the potential greater dependence on

translation regulation signaling for sensitization in TG versus DRG neurons [30] we tested the hypothesis that protein synthesis is necessary for facial sensitization in this dural stimulation priming model. We first administered either 0.1 ng of IL-6, 25 µg of anisomycin (a general protein synthesis inhibitor), or a co-injection of both onto the dura of female mice to induce mechanical facial allodynia that persisted for more than 24 h and resolved by 72 h (Fig. 7.1). Upon returning to baseline, we administered a second stimulus, SIF (pH 7.0), onto the dura to reveal the presence of hyperalgesic priming from the initial IL-6 stimulus. Although there were only minor differences in acute mechanical hypersensitivity, mice that were initially administered only IL-6 exhibited robust facial hypersensitivity when exposed to low pH. This hypersensitivity persisted for more than 5 h. Conversely, mice that received a co-injection of both IL-6 and anisomycin did not respond to the low pH, suggesting that general protein synthesis is required for the generation of a primed state in mice.

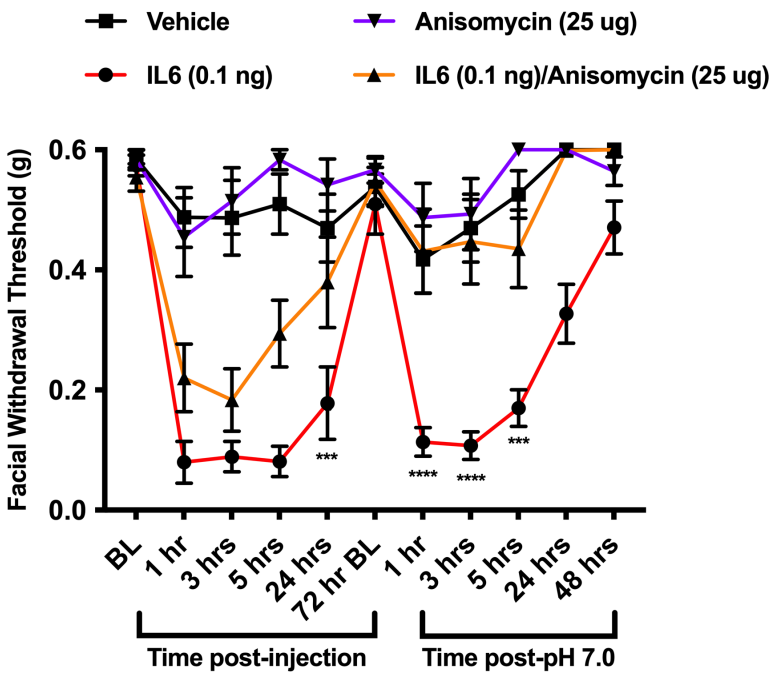

Figure 7.1. Dural co-injection of IL-6 with the general protein synthesis inhibitor anisomycin blocks hyperalgesic priming to dural pH 7.0 in female ICR mice (n=10 for all groups). Analysis of groups was performed using two-way ANOVA followed by Bonferroni *post hoc* test (See Table 1 for F-values). *p < .05, **p < .01, ***p < .001, ****p < .0001.

Inhibiting cap-dependent translation prevents long-lasting facial hypersensitivity

Previous reports have demonstrated that cap-dependent translation induced by IL-6 is dependent on the binding of eIF4E/eIF4G to induce eIF4F complex formation and interruption of this binding via stabilizing 4E-BP1 to eIF4E prevents the priming induced by pronociceptive mediators [27]. Translation control by 4E-BP1 has been shown to regulate mechanical hypersensitivity and genetic loss of 4E-BP1 increases excitatory synaptic transmission in the spinal cord, thereby enhancing mechanical nociception [31]. To gain a better understanding of the molecular mechanisms underlying the priming induced by IL-6, we sought a similar approach to investigate the role of eIF4E phosphorylation and subsequent cap-dependent translation. Similar to the

experiments in Fig. 7.1, we administered 0.1 ng of IL-6, 25 µg of 4EGI-1, a compound that mimics the activity of 4E-BP1, or a co-injection of both onto the dura of female mice (Fig. 7.2). The resulting mechanical allodynia induced by IL-6 persisted for more than 24 h and resolved after 72 h. Upon returning to baseline, mice were exposed to a dural injection of low pH. Mice previously treated with IL-6 alone demonstrated cutaneous mechanical hypersensitivity. As with anisomycin, acute facial hypersensitivity was partially attenuated and the response to low pH was robustly blocked in mice that received a co-injection of IL-6 and 4EGI-1. Thus, assembly of the eIF4F complex locally in the dura appears to be critical to the development of long-lasting mechanical hypersensitivity following repeated stress.

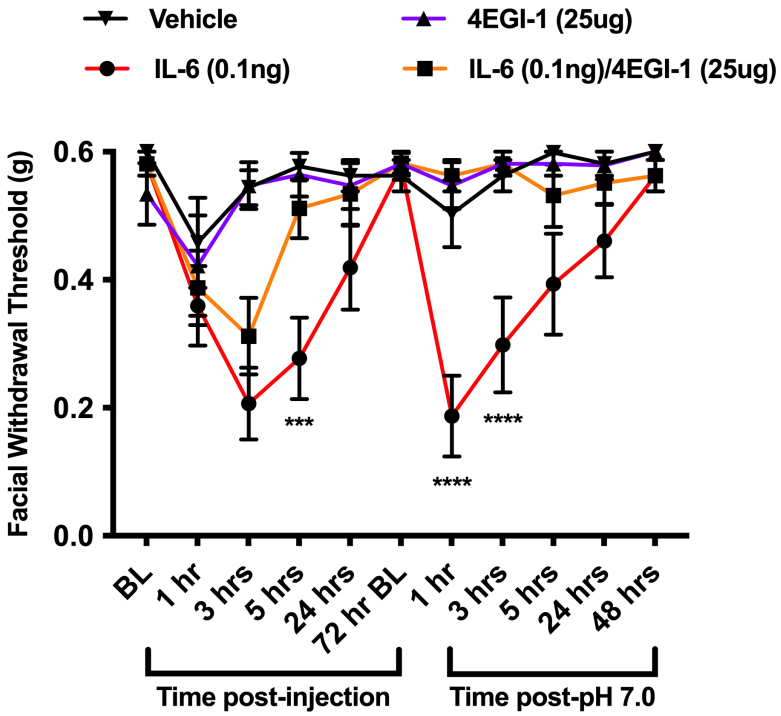

Figure 7.2. IL-6-induced priming to pH 7.0 is blocked by co-treatment with 4EGI-1 in female ICR mice (n=8 for all groups). Analysis of groups was performed using two-way ANOVA followed by Bonferroni *post hoc* test (See Table 1 for F-values). *p < .05, **p < .01, ***p < .001, ****p < .0001.

eIF4E phosphorylation is necessary for priming following repeated stress

Previous studies have shown that *eIF4E*^{S209A} mice, which lack phosphorylation of eIF4E at Ser209, exhibit reduced nociceptor sensitization and hyperalgesic priming in response to pro-nociceptive and inflammatory factors, including NGF and IL-6 [24, 32]. To test the hypothesis that eIF4E phosphorylation is important for priming following dural stimulation or repeated stress, we utilized these same *eIF4E*^{S209A} in these models. As previously described, we once again administered IL-6 onto the dura of female and male *eIF4E*^{S209A} mice, followed by dural pH 7.0 (Fig. 7.3). Unlike WT mice, acute mechanical hypersensitivity was partially attenuated and priming to dural pH 7.0 was blocked in both sexes of *eIF4E*^{S209A} mice, supporting our hypothesis that eIF4E phosphorylation is critical to establishing long-lasting hypersensitivity induced by dural IL-6.

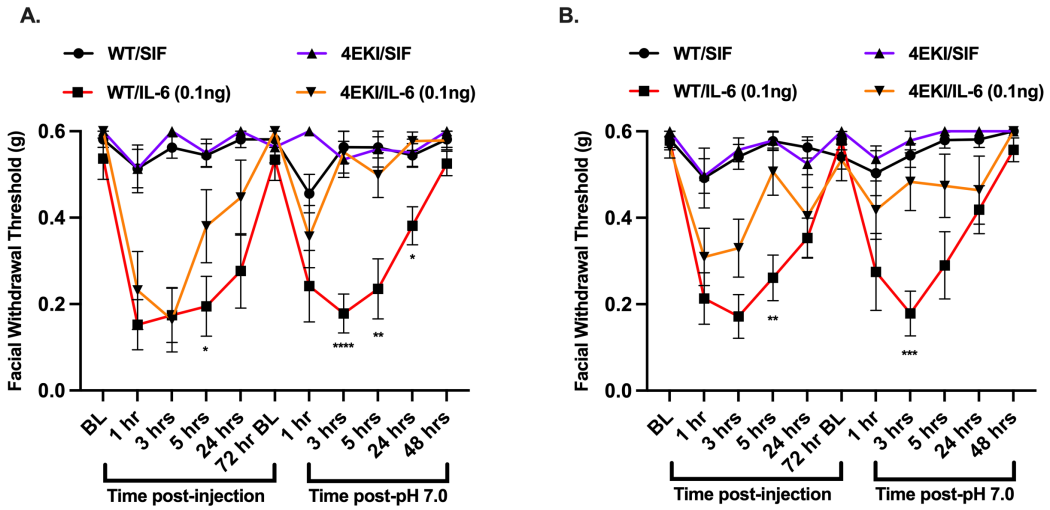

Figure 7.3. Female (a) and male (b) *eIF4E*^{S209A} mice have decreased mechanical hypersensitivity to dural IL-6 and do not prime to dural pH 7.0. For **(a)** n=8 for WT groups; n=7 for 4EKI/SIF and n=8 for 4EKI/IL-6. For **(b)** n=8 for WT/SIF and 4EKI/IL-6; n=7 for WT/IL-6 and 4EKI/SIF. Analysis of groups was performed using two-way ANOVA followed by Bonferroni *post hoc* test (See Table 1 for F-values). *p < .05, **p < .01, ***p < .001, ****p < .0001.

We recently showed that mice exposed to repeated restraint stress become primed to sub-threshold doses of the nitric oxide-donor sodium nitroprusside (SNP). Stress is the number one reported trigger of migraine among humans [33] and NO donors are among the most reliable experimental triggers of migraine attacks [34, 35]. We subjected both WT and *eIF4E*^{S209A} mice to repeated restraint stress as previously described. Following their return to baseline nociceptive thresholds, we administered a 0.1 mg/kg IP injection of SNP (Fig 7.4). Interestingly, priming to SNP was completely blocked in stressed *eIF4E*^{S209A} mice compared to a robust increase in mechanical hypersensitivity in stressed WT mice. This identifies a key role for eIF4E phosphorylation in the transition from acute to long-lasting hypersensitivity and suggests eIF4E phosphorylation as a potential mechanism underlying neuronal plasticity in migraine.

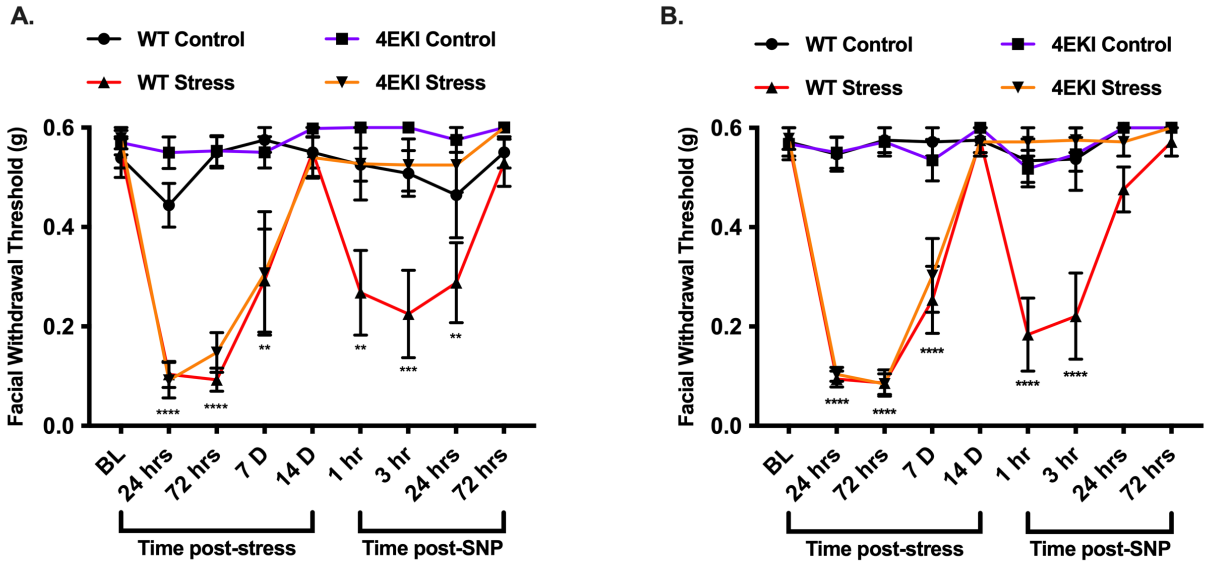

Figure 7.4. Female (a) and male (b) *eIF4E*^{S209A} mice exhibit acute mechanical hypersensitivity similar to WT mice following repeated restraint stress, but fail to prime to a sub-threshold dose of the nitric oxide donor SNP (n≥6). For **(a)**, n=6 for all groups except 4EKI/Stress, which n=7. For **(b)** n=6 for all groups. Analysis of groups was performed using two-way ANOVA followed by Bonferroni *post hoc* test (See Table 1 for F-values). *p < .05, **p < .01, ***p < .001, ****p < .0001.

Phosphorylation of eIF4E is differentially regulated after repeated restraint stress

In an effort to determine the time points at which eIF4E phosphorylation is altered in the stress paradigm, we examined protein lysates from the dura and TG of WT C57BL/6 mice at multiple time points after day 3 of the stress protocol (Fig 7.5). Our data indicate an almost 50% increase in the expression level of p-eIF4E in the TG 1 h after day 3 of stress, an effect that was diminished by 3 h. In the dura, the effects were completely opposite, with an almost 50% decrease in p-eIF4E expression levels 1 h after stress, but an increase of over 75% in expression levels by 3 h. Phosphorylation levels of eIF4E were decreased 24 h after day 3 of stress in both tissues. Although the implications of these data remain unclear, they indicate that eIF4E phosphorylation-mediated events occur earlier in the TG than they do in the dura in response to stress, providing evidence that the temporal components of this key biochemical event are dynamically regulated.

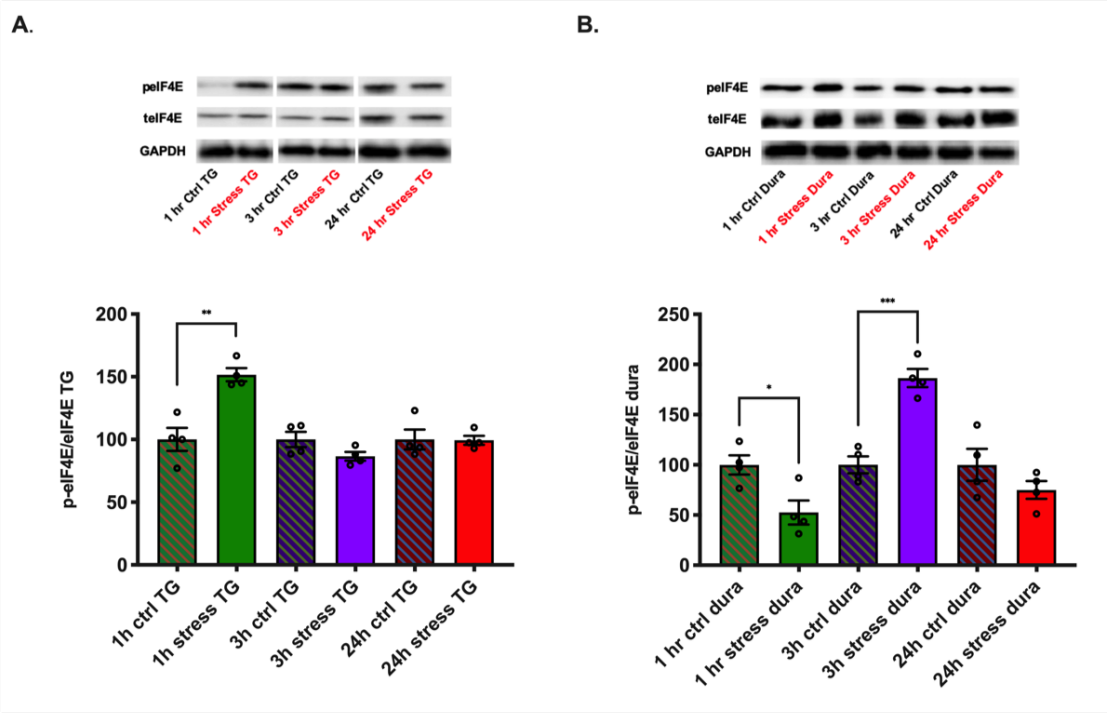

Figure 7.5. Compared to controls, phosphorylation of eIF4E is robustly increased in the TG of WT C57BL/6 mice at 1 h following repeated restraint stress, an effect that is diminished by 3 h (a). In contrast, decreases in eIF4E phosphorylation initially occur in the dura before robustly increasing to peak levels by 3 h, indicating that eIF4E is differentially regulated in the TG and dura following stress (n=4 mice pooled for each time point) (b). GAPDH was used as a loading control. Significance between treatments was determined via student's unpaired two-tailed t-test. Data are represented as means ± SEM. *p < .05, **p < .01, ***p < .001.

DISCUSSION

The susceptibility of migraine patients to attacks following exposure to normally innocuous triggers strongly implicates sensitization of the trigeminovascular system, leading to lower activation thresholds for nociception to occur. Robust changes in gene expression regulated by activity-dependent translation are key to nociceptor plasticity and phenotypic alterations and are thought to underlie neuronal sensitization [29]. IL-6-induced phosphorylation of ERK has been shown to modulate the sodium channel Nav1.7, resulting in prolonged migraine-related pain [36] and

activation of ERK/MAPK pathways can ultimately lead to changes in transient receptor potential (TRP) channel function, a family of proteins which have been implicated in migraine pathophysiology [37-39]. Co-application of dural IL-6 and anisomycin partially attenuated the resulting acute facial hypersensitivity and completely blocked the development of a primed state in mice, suggesting that protein synthesis may be critical for the development of long-lasting mechanical hypersensitivity. The comparable results between local administration of anisomycin and 4EGI-1 suggest that the newly synthesized proteins required to establish priming are regulated by cap-dependent translation. In accordance with this, our results from injecting IL-6 onto the dura of *eIF4E*^{S209A} mice suggest that eIF4E phosphorylation is key for long-lasting facial mechanical hypersensitivity. Thus, similar to reports in other preclinical models of pain [24], activity-dependent translation appears to be critical for the sensitization of trigeminal nociceptors by pronociceptive factors.

Headaches can be triggered in migraine patients by a wide range of noxious and innocuous stimuli, making it difficult to parse out which mechanisms may be most relevant for trigeminal activation and sensitization. Since the most common and frequent trigger of migraine is stress [33], use of this trigger is one of the more clinically relevant models for mechanistic investigation. Utilizing our novel repeated stress model with *eIF4E*^{S209A} mice allowed us to gauge the role of eIF4E phosphorylation in a much more robust model of hypersensitivity that was not isolated to local cephalic regions, but rather the entire body. In *eIF4E*^{S209A} mice, the loss of NO-donor induced priming normally observed after stress highlights a critical role for eIF4E phosphorylation in the development of long-lasting mechanical hypersensitivity. Additionally, in WT mice, phosphorylation of eIF4E in the dura and TG was differentially expressed across multiple time

points following stress, with an increase in p-eIF4E occurring 1 h after stress in the TG and being downregulated by 3 h while the opposite effect was observed in the dura. While the significance of these data remains unclear, our data indicate that robust changes in eIF4E phosphorylation occur after repeated stress and that a genetic loss of this phosphorylation prevents the development of hyperalgesic priming. Additionally, the experiments in this study are helping to establish the sample sizes needed for significance based on effect sizes in the stress model.

Targeting local translation in DRG sensory axons has been proposed as a potential treatment for many types of inflammatory and neuropathic pain [23]; however, whether the translation of mRNAs that contribute to priming of dural afferents occurs locally in meningeal sensory axons or at distal sites in the TG remains unclear. A previous study from our lab found that while local co-injection of IL-6 with anisomycin into the hindpaw prevented acute mechanical hypersensitivity, co-injection with a transcription inhibitor had no effect, suggesting that local translation contributing to acute pain in the hindpaw is dependent on pre-existing pools of mRNA [27]. Given the similarities between the DRG and TG, it is plausible to suggest that the translation events regulating acute and long-lasting hypersensitivity caused by dural IL-6 are dependent on local pools of mRNA as well; however, one key difference between these studies is that attenuation of dural IL-6-induced facial allodynia begins at later time points in the acute phase. One potential explanation for this can be attributed to the location of testing following injection. For example, other studies tested the hindpaw in the same location that the injection was given; here, we inject directly onto the dura, but test the periorbital region of the face. The delay in attenuation of acute hypersensitivity might indicate that nascent protein synthesis in response to dural stimulation occurs some distance away from the initial injection site. Additionally, if these translation events

are indeed occurring locally in meningeal sensory axons, then changes in synaptic plasticity in the TG and possibly even the ophthalmic nerve (innervating the periorbital region) may be dynamically and temporally regulated following injection.

Further studies will be necessary to determine both the location and identity of the mRNAs that are translated to mediate long-lasting sensitization of the trigeminal pathway. Recently, studies have demonstrated robust increases in the expression levels of mRNAs that modulate acid-sensing ion channels (ASICs) [40] and TRP channels [41] in the TNC in response to noxious odors and repeated dural stimulation, respectively. Although it is currently unknown which eIF4E-dependent mRNAs are most critical in these models, eIF4E phosphorylation has been shown to regulate the translation of brain-derived neurotropic factor (BDNF) mRNA in mouse DRG [32]. BDNF is a key player in the maintenance of long-term potentiation (LTP) [42-44] and has been implicated in maintaining persistent pain states [45, 46]. Additionally, multiple lines of evidence suggest a role for BDNF in headache [47-50]. In support of these claims, our lab has recently demonstrated that afferent input from the meninges is capable of producing BDNF-dependent priming of the trigeminovascular system [51].

Although the exact mechanism is still unclear, our findings are the first to demonstrate that *de novo* protein synthesis regulated by activity-dependent translation is critical to the development of cutaneous facial hypersensitivity following dural stimulation or repeated stress. Further exploration of how these translation pathways as well as other modes of translation contribute to trigeminal sensitization may provide additional insight into how to develop more efficient therapies for migraine.

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CHAPTER 8

DISCUSSION AND CONCLUSIONS

Although emerging migraine therapeutics, such as the CGRP antagonists, offer hope for patients in need, there still exists a large gap in relief among the migraine population. Namely, while some treatments offer short-term relief for many patients, this relief typically does not last long after discontinuation of treatment. While relief, in general, is a great milestone, it cannot be denied that novel targets and more efficacious treatments must be uncovered and developed, respectively, in order to provide long-lasting, if not permanent, relief from migraine headaches, even after discontinuation of treatment. Undoubtedly, this has not been a trivial pursuit, but identifying novel mechanisms of migraine pathophysiology may provide the first stepping stones in developing better therapeutics. The above evidence implicates two novel mechanisms in migraine headache pathology: the formation of peroxynitrite and eIF4E-mediated translation dysregulation. Critically, we have shown that targeting these mechanisms in preclinical models provides therapeutic relief, thus, warranting further investigation of these mechanisms.

TARGETING PEROXYNITRITE FORMATION IN MIGRAINE

The most significant contributions of PN to migraine pathology uncovered in our studies include its role in mediating both acute facial hypersensitivity as well as hyperalgesic priming to low-dose NO donors following repeated restraint stress. Importantly, these findings are of high clinical significance, as stress is the most common trigger of migraine in humans while NO donors are very consistent experimental triggers. Notably, blocking PN formation following repeated stress or administration of low-dose NO donor significantly attenuates facial nociceptive

thresholds, suggesting therapeutic value in targeting this process. While these observations are important and may aid in steering novel drug development, further studies investigating how PN contributes to these painful phenotypes on a biological level must be conducted.

Due to its very short half-life at physiological pH (~5 seconds), the biological impact of PN on the cell is likely maintained through other various mechanisms that PN acts on [1]. As mentioned earlier, PN is capable of modulating various ion channels and proteins via nitrotyrosination of protein residues [2]. Additionally, PN is capable of increasing Ca^{2+} release from both mitochondrial and non-mitochondrial sources and can directly modulate mitochondrial respiration [3-5]. As these processes contribute both to biological homeostasis as well as neuronal excitability, investigation of the impact of PN on these mechanisms in a migraine headache context is required to truly parse out the role of this nitroxidative molecule in this disease. To that end, our work also provides a framework for the potential role of PN-mediated mitochondrial dysfunction as one mechanism in which PN may contribute to a migraine-like phenotype.

TARGETING TRANSLATION DYSREGULATION IN MIGRAINE

As described above, our data also implicate translational dysregulation of *de novo* protein synthesis in the development and persistence of migraine headache. Specifically, regulation of mRNA translation by eIF4E appears to be a critical mechanism underlying hyperalgesic priming, as mice lacking the phosphorylation site in eIF4E are not sensitized to the same innocuous triggers that induce facial hypersensitivity in stress-primed mice and mice primed with dural IL-6. Additionally, phosphorylation of eIF4E is necessary for the establishment of priming in other pain models [6, 7]. Indeed, targeting this pathway has achieved therapeutic relief in other disease states,

such as cancer and fragile X syndrome, in which translation of eIF4E-sensitive mRNAs is upregulated [8, 9].

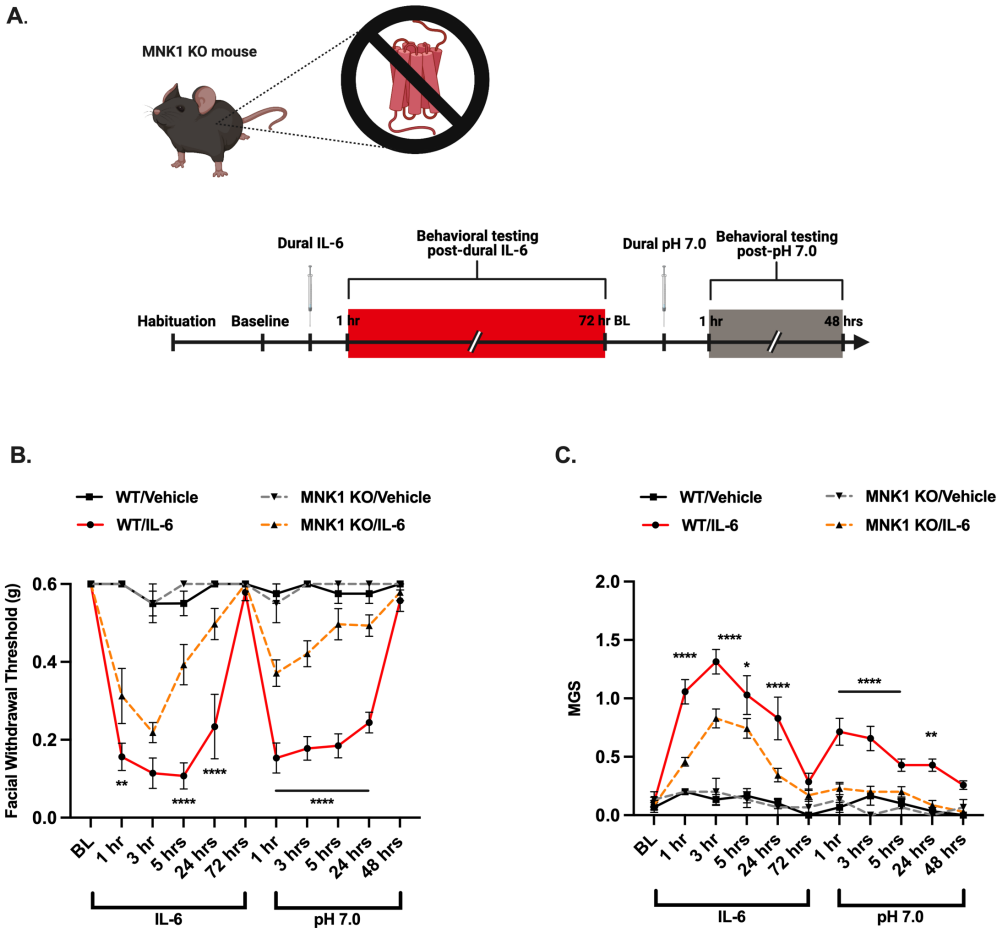

Figure 8.1. Genetic inhibition of MNK partially attenuates facial hypersensitivity and hyperalgesic priming caused by dural IL-6. (A) Male and female wild-type (WT) C57/BL6 and MNK1 KO mice (which lack the essential translation-initiating kinase, mitogen-activated protein kinase-interacting kinase 1 [MNK1]) were given a 5 µL injection of the pro-inflammatory cytokine interleukin-6 (IL-6) (0.1 ng) and tested for facial mechanical hypersensitivity and grimacing. 72 hours following dural IL-6, mice returned to baseline nociceptive thresholds, upon which they were administered a second 5 µL dural injection of synthetic interstitial fluid (SIF) solution (pH=7) to check for the presence of hyperalgesic priming. Control mice received dural SIF at physiological pH. (B) Dural IL-6 induced acute periorbital hypersensitivity and hyperalgesic priming to dural pH 7.0 in WT mice; however, MNK1 KO mice exhibited significantly reduced von Frey thresholds following dural IL-6 and were not primed. (C) A similar effect was observed in grimace measurements, in which MNK1 KO mice had significantly lower mean grimace scores (MGS) compared to WT mice following

both dural IL-6 and pH 7.0. No sex differences were observed. * denotes significance between MNK1 KO/IL-6 and WT/IL-6 groups. For all groups, n= 3-7. Data are represented as mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001.

The phosphorylation of eIF4E by MNK is essential to initiate translation in the cell [10]. In further support of these findings, we have generated preliminary evidence that confirms the importance of the MNK-eIF4E signaling axis in the development of long-lasting facial hypersensitivity (Figure 8.1). Interestingly, compared to WTs, mice lacking MNK1 exhibit significantly reduced acute facial hypersensitivity and grimace scores following application of dural IL-6. These mice also do not prime to dural pH 7.0, suggesting that phosphorylation of eIF4E by MNK is a critical event for the development of persistent facial hypersensitivity in this model. Notably, these findings corroborate our earlier evidence showing that pharmacological inhibition of MNK attenuates priming to dural pH 7.0. Taken together, our studies reveal eIF4E-mediated mRNA translation as a novel mechanism underlying migraine pathophysiology and suggest therapeutic potential in targeting this process. Future work should focus on identifying which eIF4E-sensitive mRNAs are translated in response to nociceptive stimuli, including those that directly contribute to maintaining neuronal hyperexcitability. Identification and targeting of these mRNAs could potentially pave the way for highly-specific drug targeting in painful conditions.

CONCLUSIONS

Migraine sufferers face the burden of living with one of the most debilitating diseases in the world and with few effective treatments to seek relief from. In this dissertation, we have revealed two novel mechanisms that may contribute to migraine pathophysiology and warrant further investigation. Critically, we strongly propose that pharmacologically targeting PN formation or

eIF4E phosphorylation may have therapeutic potential in migraine headache. Future studies should also consider how eIF4E phosphorylation might influence PN-mediated processes, as no ties between these mechanisms have been clearly established. Additionally, because both of these processes play important biological roles under normal physiological conditions, we propose that targeting these processes in the periphery may provide the greatest therapeutic advantage while limiting off-target effects. Collectively, these studies have advanced our understanding of migraine pathophysiology, in hopes of improving the way we treat this disease moving forward.

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BIOGRAPHICAL SKETCH

Jacob Edward Lackovic was born in Jacksonville, Arkansas to Ken and Sharon Lackovic. He graduated from North Little Rock High School in 2010 and went on to pursue a Bachelor of Science degree in Biology and Biochemistry with a minor in General Business at the University of Arkansas, upon which he completed in 2014. As an undergraduate, Jacob performed basic scientific research in the lab of Dr. Paul Adams, where he gained technical training in basic laboratory skills and was part of a research project focused on the biochemical characterization of proteins. Currently, he is enrolled in the Cognition and Neuroscience PhD program at The University of Texas at Dallas, where he has spent the last several years researching mechanisms that underlie migraine pathophysiology in the laboratory of Dr. Gregory Dussor.

Jacob's dissertation research implicated two novel biological mechanisms, mRNA translation dysregulation and peroxynitrite formation, in the development and persistence of migraine headache in rodents. This discovery parallels findings in other chronic pain models and suggests that targeting these mechanisms may have significant therapeutic potential, which was an important end-goal of this work, considering that migraine patients still suffer from a lack of effective therapeutics. He hopes that his research will lead to the identification of novel drug targets and the development of better therapeutics for this highly debilitating disease.

Upon completion of his PhD, Jacob plans to pursue independent research opportunities with the goal of improving the drug identification, development, and approval process in an effort to reduce both the number of failed drugs in clinical trials as well as the amount of time it takes to move from target selection to FDA approval. He has a strong passion for pain research, advanced

scientific technologies, collaboration, and scientific entrepreneurship and hopes to make a lasting impact in these areas.

CURRICULUM VITAE

Education and Training

08/2015-present	PhD, Cognition and Neuroscience University of Texas at Dallas (Mentor: Gregory Dussor) *Dissertation Title: Mechanisms underlying migraine headache pathophysiology: Novel insights from preclinical models*
08/2010-12/2014	BS, Biology and Biochemistry / Minor in Business University of Arkansas-Fayetteville (Mentor: Paul Adams) *Thesis Title: Biochemical characterization of intersectin-1*

Research Experience

Research Associate, Pain Neurobiology Research Group, Center for Advanced Pain Studies, University of Texas at Dallas

I currently perform basic science research under the mentorship of Dr. Gregory Dussor for the following projects:

- Characterizing novel pre-clinical models of migraine headache
- Investigating the contributions of activity-dependent translation to the development and persistence of migraine headache (first author publication)
- Examining the role of peroxynitrite formation in the development and persistence of migraine headache (first author publication)
- Examining mechanisms underlying dural nociceptor sensitization

Research Associate, Learning and Memory Lab, University of Texas at Dallas

Performed basic research in the laboratory of Dr. Jonathan Ploski on the mechanisms underlying memory reconsolidation:

- Developed adeno-associated viruses and lenti viruses for the delivery of pharmacological compounds into the baso-lateral amygdala and hippocampus of mice and rats
- Performed cannulation surgeries on mice and rats for delivery of the aforementioned compounds
- Examined rodent behavior from a pharmacological perspective while utilizing Pavlovian Fear Conditioning
- Memory learning, consolidation, reconsolidation, and extinction behavior paradigms
- Constructed, created, and purified DNA plasmids to use in virus development

Research Assistant, Protein Biochemistry Research Lab, University of Arkansas-Fayetteville

Performed basic science research under the mentorship of Dr. Paul Adams:

- Characterized the structure and functional relationships of intersectin-1
- Growth and harvest of bacterial cultures for overexpression of intersectin-1 protein
- Trained in protein purification methods
- Trained in mass spectrometry and nuclear magnetic resonance spectrometry procedures and data analysis
- Presented this work at the 2014 IdeA Network of Biomedical Research conference

Publications

Peer-reviewed scientific papers

1. Mason BN, Avona A, **Lackovic J**, Dussor G. Dural Stimulation and Periorbital von Frey Testing in Mice As a Preclinical Model of Headache. J Vis Exp. 2021 Jul 29;(173). doi: 10.3791/62867. PMID: 34398161.
2. **Lackovic J**, Price TJ, Dussor G. *De novo* protein synthesis is necessary for priming in preclinical models of migraine. Cephalalgia. 2021 Feb;41(2):237-246. doi: 10.1177/0333102420970514. Nov 17. PMID: 33200943.
3. Avona A, Mason BN, **Lackovic J**, Wajahat N, Motina M, Quigley L, Burgos-Vega C, Moldovan Loomis C, Garcia-Martinez LF, Akopian AN, Price TJ, Dussor G. Repetitive stress in mice causes migraine-like behaviors and calcitonin gene-related peptide-dependent hyperalgesic priming to a migraine trigger. Pain. 2020 Nov;161(11):2539-2550. doi: 10.1097/j.pain.0000000000001953. PMID: 32541386; PMCID: PMC7572536.

Book chapters

1. **Lackovic, J**. and G. Dussor (2021). Headache. Reference Module in Biomedical Sciences, Elsevier.

In preparation/review

1. **Lackovic J**, Dussor G. Peroxynitrite mediates stress-induced hypersensitivity and priming to a nitric oxide-donor in preclinical models of migraine. (manuscript in progress)

Selected Presentations

- *The Effects Of Nitric Oxide On Migraine Headache Are Mediated By Peroxynitrite Formation.* **Gulf Coast Consortia, 2021. Virtual conference. [#38]** *Poster and data blitz*
- *The Effects Of Nitric Oxide On Migraine Headache Are Mediated By Peroxynitrite Formation.* **American Gasotransmitter Symposium, 2020. Virtual conference.** *Poster*
- *Examining the role of peroxynitrite in preclinical models of migraine headache.* **UT Dallas 2021.** *Presentation*

- *De novo protein synthesis is necessary for priming in preclinical models of migraine*. **International Headache Conference, 2019. Dublin, IE. [#IHC-PO-325]** *Poster*
- *De novo protein synthesis is necessary for priming in preclinical models of migraine*. **Society for Neuroscience, 2018. San Diego, CA. [#058.11/FF11]** *Poster*
- *De novo protein synthesis is necessary for priming in preclinical models of migraine.* **UT Dallas 2018.** *Presentation*
- *The molecular basis of the induction of memory reconsolidation*. **UT Dallas 2016.** *Presentation*
- *Characterizing the structure and functional relationships of intersectin-1*. **IDeA Networks of Biomedical Research Excellence, 2014. Fayetteville, AR.** *Poster*

Professional Organizations

2019-2020 International Headache Society

2018-2019 Society for Neuroscience

2014-2015 American Chemical Society

Patents

2021 **Lackovic J**, Dussor G. *Regulation of eIF4E activity for migraine therapy.* U.S. Patent No. 17/495,902

Awards and Other Honors

2018-2019	Brain and Behavioral Sciences Travel Award
2014	INBRE Summer Undergraduate Mentored Research Program Scholar
2010-2012	Fulbright College of Arts and Sciences Honors Student
2010-2014	Arkansas Academic Challenge Scholarship

Teaching and Training

Courses taught/assisted teaching:

2018 (Fall)	Neuroscience Laboratory Methods
2018 (Summer)	Introduction to Neuroscience
2018 (Spring)	Introduction to Neuroscience
2017 (Fall)	Neuroscience Laboratory Methods
2017 (Summer)	Cellular Neuroscience
2017 (Spring)	Genes, Brain, & Behavior

2016 (Fall) Neuroscience Laboratory Methods
2016 (Summer) Introduction to Neuroscience
2016 (Spring) Neuroscience Laboratory Methods
2015 (Fall) Neuroscience Laboratory Methods

Mentored undergraduate pre-med and/or honors students (22 total)

Helped review manuscripts for peer-review publication (12 total)