IDENTIFICATION OF FACTORS CONTRIBUTING TO THE INITIATION OF MIGRAINE: THE IMPACT OF SEX-, STRESS-, AND SYMPATHETIC-DEPENDENT CHANGES IN THE DURA

by

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Given evidence that the pain of migraine originates in the peripheral dural afferents, and that sterile inflammation contributes to the activation and sensitization of dural afferents, the question addressed in this dissertation was, how is inflammation in the dura initiated, which could then lead to the start of a migraine attack? Immune cells are one well-known primary source of inflammatory mediators and likely, at least in part, responsible for sterile dural inflammation. Therefore, changes in dural immune cells were characterized in association with three, key clinical features of migraine—that migraine is more common in women than in men, stress is the most common trigger for a migraine attack, and sympathetic dysregulation is observed in migraineurs. Dural immune cells were obtained for flow cytometry and fluorescence activated cell sorting from male or female, naïve or stressed, intact or with surgical denervation of sympathetic post-ganglionic neurons, adult Sprague Dawley rats. The total proportion of immune cells in the dura was identified for the first time, as well as the presence of lymphoid derived dural immune cells, even in naïve animals. Immune cell subtypes (macrophages in males, and T-cells in females) increased with a delay after stress, suggesting for the first time a possible role for T-cells in migraine. Furthermore, pro- (TNFα and IL-6) and anti- (IL-10 and POMC) inflammatory mediator mRNA expression was up- and down-regulated, respectively, in myeloid and lymphoid dural immune cells with a delay after stress, particularly in females, suggesting that a shift in the balance of pro- and anti-inflammatory mediators may also

contribute to the initiation of a migraine attack. This dissertation expands the current view of the dura, revealing it to be a much more immune rich and complex tissue than previously appreciated. Most importantly, this work underscores the possibility that it may not only be possible, but necessary to differentially treat migraine in men and women.

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PREFACE

This dissertation is dedicated in memory of Susan Carrie Stitt McIlvried. As both my mother and a migraineur, she taught me strength, passion, value, dedication, and courage—a foundation of tools I needed to make it through graduate school. I would also like to thank the rest of my incredibly supportive family, as well as my "statistically significant" other, Andrew Renckly ($p<0.001$ for the endless encouragement).

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1.0 INTRODUCTION

1.1 NEUROBIOLOGY OF MIGRAINE

Migraine is a common, debilitating pain disorder with high impact on both the patient and society. There is a clear sex difference in the manifestation of this disorder which has a prevalence of 6-8% in men and 15-25% in women. The first migraine attack commonly occurs at puberty, and frequency of attacks decreases in females after menopause (Goadsby, 2007). Consequently, this is a disorder that takes its toll over peak years in productivity. Migraine is ranked as one of the 20 most disabling diseases, according to the World Health Organization (Leonardi et al., 2005). The annual cost to employers is estimated between $12-17 billion, and yearly medical costs exceed $1 billion (Silberstein, 2004, Goldberg, 2005, Leonardi et al., 2005, Hawkins et al., 2007). Given that there is no known cure for migraine, and the available treatments are only effective in a fraction of patients (Goadsby, 2007), there is a significant need for the identification of novel therapeutic approaches for this disorder.

Criteria established by the International Headache Society in the International Classification of Headache Disorders (ICHD) are used to diagnose migraine. To meet the diagnosis of migraine, a patient must present with the occurrence of at least 5 headaches that fulfill two sets of criteria. First, the headaches must have at least two of the following: unilateral location, pulsating quality, moderate to severe pain intensity, and/or aggravation by movement.

Second, the headaches must be associated with at least one of the following: nausea/vomiting and/or photophobia/phonophobia (Lipton et al., 2004). In 20-30% of migraineurs, attacks are preceded by an aura, and diagnosed as "migraine with aura." The aura must be fully reversible, occur within 60 min of the start of the headache, and meet two additional sets of criteria. First, it must be associated with visual symptoms including positive features (scintillations—flickering) and/or negative features (scotoma—loss of vision), sensory symptoms including positive features (pins and needles) and/or negative features (numbness), or dysphasia (speech disturbance). Second, it must also be associated with development of aura symptoms over ≥5 min and/or each symptom lasting between 5 and 60 min (Lipton et al., 2004).

The most prominent feature of migraine is pain. Pain traditionally involves activation of specialized sensory afferents, colloquially referred to as nociceptors, that are responsive to noxious or potentially damaging stimuli. Nociceptors synapse centrally on the dorsal horn. Dorsal horn neurons in turn transmit this information to higher order brain structures to be interpreted as "pain." For migraine pain, this would involve activation of trigeminal (ophthalmic branch) afferents that innervate the meninges and associated blood vessels. Meningeal afferents have central projections to the trigeminocervical complex (TCC), consisting of C1 and C2 dorsal horns of the cervical spinal cord and the trigeminal nucleus caudalis (TNC). Neurons within TCC, in turn, project to ventroposteriomedial (VPM) and posterior (Po) thalamic nuclei, in addition to several brainstem nuclei as well as the hypothalamus (Pietrobon and Moskowitz, 2013). Dura-sensitive VPM neurons project mainly to cortical areas considered part of the pain matrix (i.e. somatosensory cortices and insular cortex), and are thought to play a role in the perception of the pain. Dura-sensitive Po neurons project to non-traditional "pain" cortical areas (i.e. auditory, visual, retrosplenial, parietal association cortices) and are thought to contribute to

other symptoms experienced during an attack such as altered limbic function, memory and cognitive performance (Noseda et al., 2011). Activation of the brainstem and hypothalamic nuclei are thought to contribute to various symptoms experienced during an attack, such as loss of appetite, nausea, sleepiness, irritability, fluid retention, and autonomic symptoms (Akerman et al., 2011). Additionally, the TCC receives descending projections from the cortex and particularly brainstem. Stimulation of the periaqueductal gray (PAG) or rostral ventral medulla (RVM) inhibits TCC responses to dural stimulation (Knight and Goadsby, 2001, Lambert et al., 2008, Akerman et al., 2011). The hypothalamus also has also been shown modulate TCC responses to dural stimulation (Bartsch et al., 2004, 2005, Charbit et al., 2009).

While there is still much to be resolved about this disorder, a topic of ongoing debate is the source of the headache pain. There are essentially two camps on this question, with one camp arguing in favor of the sensitization of afferents that innervate the meninges as the primary source of the pain, and the other arguing that the pain arises from within the central nervous system (CNS). Some of the most convincing evidence in support of the activation and sensitization of meningeal afferents comes from early studies of stimulation of the meninges in patients during brain surgery. Stimulation of the meninges and associated vasculature produced a sensation of pain that was qualitatively similar to that associated with a migraine (Ray and Wolff, 1940). Additionally, it has been reported over time that craniotomies and surgical disruption of the meninges performed for unrelated medical reasons (i.e., removal of neuromas) produced headaches for months in patients (4-84%, depending on the study), and even induced chronic headaches in some (Olesen et al., 2009).

Additional evidence in support of meningeal afferents as the source of migraine pain comes from evidence that migraine is associated with sterile inflammation of the dura. A

critical component of this inflammation is thought to be mediators released that are able to activate and sensitize dural afferents. Clinical data are consistent on the fact that migraine attacks are associated with increased levels of many inflammatory mediators in the plasma and CSF (Sarchielli et al., 2000, Perini et al., 2005, Waeber and Moskowitz, 2005, Fidan et al., 2006, Sarchielli et al., 2006). Non-steroidal anti-inflammatory drugs (NSAIDS) are also an effective acute treatment for migraine (Jakubowski et al., 2005). Preclinical evidence for the sterile inflammation hypothesis comes from the observation that the majority of dural afferents express the neuropeptides calcitonin-gene related peptide (CGRP) and substance P (SP) (Uddman et al., 1989, Messlinger et al., 1993, Shimizu et al., 2007). I have also shown that 66.0 ±6.0% of retrogradely labeled dural afferents express CGRP (McIlvried et al., 2010)(Apppendix). Peripheral release of these peptides produces local vasodilation, plasma extravasation, and degranulation of mast cells. These processes further release pro-inflammatory mediators and result in a pro-inflammatory feedback loop, known as sterile inflammation (Waeber and Moskowitz, 2005, Pietrobon and Moskowitz, 2013). Studies have also shown that application of pro-inflammatory mediators to dural afferents result in an increase in excitability (Strassman et al., 1996, Harriott and Gold, 2009). Evidence that this generic feedback loop may occur in the dura was provided by Levy and colleagues (2007) who demonstrated that mast cell degranulation in the dura is sufficient to drive dural afferent activity (Levy et al., 2007). Importantly, events associated with migraine, such as cortical spreading depression (CSD), the physiological phenomenon thought to underlie aura, are sufficient to drive mast cell degranulation (Karatas et al., 2013), and subsequently sensitization of dural afferents (Zhang et al., 2010). Similarly, triggers for migraine such as nitroglycerin (GTN) infusion, which induces a delayed migraine attack in migraineurs but not controls (Thomsen et al., 1994), also induced delayed mast cell

degranulation and inflammation in the rat dura (Reuter et al., 2001), as well as the sensitization and activation of dural afferents (Jones et al., 2001, Lambert et al., 2002).

CGRP has become a particularly important molecule in migraine because 1) CGRP is increased in plasma during attacks (Goadsby et al., 1990, Goadsby and Edvinsson, 1993), 2) infusion of CGRP produces a delayed migraine headache in migraineurs (Lassen et al., 2002), and 3) CGRP receptor antibodies have recently been identified as an effective treatment for migraine (Bigal et al., 2015). While the increase in CGRP levels observed during attacks could be due to central or peripheral sources, and CGRP receptors are present both centrally and peripherally (Lennerz et al., 2008), the successful use of CGRP to induce attacks, as well as antibodies to treat migraine, points to a peripheral site of action for at least the initiation of migraine pain, as neither peptide nor antibody should readily cross the blood brain barrier (Edvinsson et al., 2007, Asghar et al., 2012). Similarly, administration of other pro-inflammatory molecules that poorly cross the blood brain barrier such as histamine and PACAP are also able to induce migraine attacks (Lassen et al., 1995, Schytz et al., 2009).

Another line of evidence in support of the peripheral hypothesis comes from the changes observed following application of inflammatory soup to the dura. This is now widely used as a pre-clinical model of migraine because it results in periorbital mechanical allodynia (Edelmayer et al., 2009). Allodynia is a term used to describe the perception of pain in response to normally innocuous stimuli. Dynamic mechanical allodynia, which is pain associated with light brushing of the skin, is commonly associated with painful peripheral neuropathies such as diabetic or post-herpetic neuropathy. Because the stimulus intensities capable of evoking mechanical allodynia are well below those needed to activate even sensitized nociceptive afferents, this phenomena is thought to reflect changes in a neural circuit enabling low threshold

afferent input to the dorsal horn to gain access to nociceptive circuits in the presence of injury and/or following a burst of activity in nociceptive afferents (LaMotte et al., 1991). Mechanical allodynia is present in 63% (Lipton et al., 2008) to 79% (Burstein et al., 2000) of migraineurs, approximately an hour after the onset of pain. As is the case for neuropathic pain, the allodynia associated with migraine is thought to result from sensitization of second order TCC neurons that also receive input from facial cutaneous afferents (Dodick and Silberstein, 2006). Thus, the presence of mechanical allodynia following application of inflammatory mediators to the dura enables pre-clinical investigators to infer the presence of a headache.

Despite this evidence, there are still no direct data of a pathophysiology of the dura or an increase in meningeal afferent activity during a migraine. Many investigators cite this reason to support the hypothesis of a central origin of headache pain. Several different observations provide additional evidence in support of a central origin of the migraine attack. First, premonitory symptoms such as tiredness, yawning, mood change, and gastrointestinal symptoms have been described in 32.9% of patients for 9.42 hours preceding an attack (Kelman, 2004), suggesting activation of CNS pathways before an attack. Second, CSD is a propagating wave of excitation followed by a wave of depression that moves across the cortex at 3-5mm/min and has been correlated with the aura preceding attacks in 20-30% of migraineurs (Pietrobon and Striessnig, 2003). Again, that this is, by definition a CNS event that precedes an attack lends support to a central origin of pain. Lastly, the most compelling model to account for a central origin of migraine pain involves a “brainstem generator,” (i.e., in the PAG and/or RVM), that drives sensitization and/or activation of TCC neurons independent of aberrant peripheral input (Akerman et al., 2011). It has long been appreciated that the regulation of pain by the RVM is bi-directional, where descending facilitation results in an increase in the response to nociceptive

input at the level of the trigeminal and spinal dorsal horn (Gebhart, 2004). The possibility that such a process may be sufficient to account for migraine was supported by data from Lambert and colleagues who showed that a lidocaine block of the trigeminal ganglion (TG) did not prevent CSD evoked TCC activity (Lambert et al., 2011). Furthermore, imaging data indicate that activity in the brainstem correlates with the presence of pain during a migraine (Akerman et al., 2011).

However, there are problems with each of these lines of evidence. Most problematic is that while it is clear that there are changes in the CNS of migraineurs (premonitory symptoms or CSD), or at least in the processing of sensory information, it is not clear how any of these changes can lead to pain that is largely localized in the head (Olesen et al., 2009). Second, while recent evidence has confirmed that CSD can produce sensitization and activation of dural afferents (Zhang et al., 2010), there is compelling evidence against a necessarily causal link between aura and headache: 1) the aura can be aborted by dihydropyradines, but these compounds have no effect on the subsequent headache (Charles, 2010); 2) the headache can be aborted with a triptan or topiramate (Lampl et al., 2004, Wolthausen et al., 2009) but these drugs have no effect on the aura; 3) migrainuers with aura can have migraines without an aura as well as auras without migraine (Stewart et al., 1991, Charles, 2010); and 4) auras are only present in 20-30% of migraineurs (Rasmussen and Olesen, 1992). There is no evidence of "silent" auras (CSD wave across a cortically silent area) in migraine without aura patients.

Third, descending facilitation is distributed widely across the TCC and length of the spinal cord, and at least based on what we know about this process, cannot account for the specificity of head pain during an attack. And while widely championed by the proponents of the central theory of migraine, there were numerous technical problems with the Lambert study,

not the least of which was an incomplete and inadequately assessed block of the TG, but was covered in more detail by (Burstein et al., 2012). Consequently, conclusions drawn from this study should be made with caution. However, I acknowledge that it is possible that there is some resting level of activity in dural nociceptors that may be perceived as painful with changes in the CNS. Finally, if disinhibition of the TCC was the mechanism for the initiation of pain, allodynia should be present from the very beginning of an attack. Not only is there a delay between the start of the headache and the emergence of allodynia (Burstein et al., 2004), but allodynia does not occur in 21-37% of migraineurs (Burstein et al., 2000, Lipton et al., 2008). Thus, a key point to the peripheral hypothesis of migraine pain is that while the brainstem *modulates* the activity of afferent input *throughout* the body through descending inhibition/facilitation, there is no evidence that the brainstem *generates* the *focal* changes that could specifically cause a migraine (or pain in any other specific area of the body).

Due to the evidence outlined above in support of a peripheral origin of pain in migraine, and lack of evidence for a central origin, I chose to focus my dissertation research on mechanisms associated with migraine arising from the periphery. In particular, I chose to focus on the factors that would account for the release of sensitizing inflammatory mediators to initiate a migraine in the first place.

1.2 SEX DIFFERENCE AND GONADAL HORMONE REGULATION OF MIGRAINE

As mentioned above, there is a clear sex difference in the prevalence of migraine. Evidence in support of a link between gonadal hormones and migraine, particularly dynamic fluctuations in

estrogens, include the following: 1) the frequency and prevalence of migraine attacks increases with menarche and decreases with menopause (Neri et al., 1993, Brandes, 2006); 2) in about 50% of female migraineurs, attacks are reported more frequently around menstruation, just after estrogen levels drop in the cycle (Brandes, 2006); 3) estrogen treatment given just before menstruation to prevent the drop in estrogen associated with menstruation can prevent menstrual migraine (Martin and Behbehani, 2006); 4) in a study of 98 women undergoing *in vitro* fertilization where estrogen levels were suppressed prior to ovarian hyperstimulation, estrogen suppression was associated with a spike in migraine attacks in 82% of the women (Amir et al., 2005); and 5) when estrogen levels dramatically rise during pregnancy and do not drop, migraines improve in over 80% of migraineurs by the second trimester and 53% and 79% had complete remission during the second and third trimesters, respectively (Sances et al., 2003).

There are several lines of evidence suggesting that estrogen may influence nociceptive processing through an action on the primary afferent. First, estrogen receptors are present in both dorsal root ganglion (DRG) and TG neurons (Taleghany et al., 1999, Bereiter et al., 2005, Puri et al., 2006), providing a potential substrate for pain modulation in the peripheral nervous system. In DRG neurons, messenger RNA (mRNA) for estrogen receptor α (ERα) is localized almost exclusively in small diameter (putative nociceptive) neurons while estrogen receptor β (ERβ) is present in all sensory neurons (Taleghany et al., 1999). Second, Martin and colleagues found enhanced sensitization of TNC neurons during stages of the rat estrous cycle immediately following abrupt drops in estrogen (Martin et al., 2007). The excitability of primary afferents in the trigeminal system have been shown to be modulated by estrogen levels (Cairns et al., 2001), with evidence from our lab indicating that estrogen replacement in ovariectomized rats results in a significant increase in the excitability of afferents innervating the temporomandibular joint

(Flake et al., 2005). More relevant to migraine, our lab has also demonstrated that a significantly greater proportion of dural afferents from female rats are sensitized by inflammatory mediators than those from male rats (Scheff and Gold, 2011). Lastly, the threshold for CSD was 50% lower in female mice than males (Brennan et al., 2007). In a genetic model of familial hemiplegic migraine, R192Q knock-in mice had an increased susceptibility to CSD. This phenotype was stronger in females, abrogated by ovariectomy and restored by estrogen replacement (Eikermann-Haerter et al., 2009b). In contrast, orchiectomy increased CSD susceptibility in males and testosterone replacement restored the phenotype (Eikermann-Haerter et al., 2009a).

Due to evidence outlined above that pro-inflammatory processing as well as afferent activation is different in females compared to males, and that this is inherently important to the manifestation of this disorder, I chose to study mechanisms that may contribute to the initiation of migraine in both females and males.

1.3 STRESS AS A MIGRAINE TRIGGER

The physiological concept of "stress" has developed over the last century largely as a result of the pioneering work of Claude Bernard, Walter Cannon, and in particular Hans Selye. Organisms survive by maintaining a complex and dynamic state of internal balance, or homeostasis. Stressors are now largely viewed as perceived endogenous or exogenous challenges which may threaten homeostasis. The body reacts to stressors by activating the sympathoadrenal (SA) and hypothalamic-pituitary-adrenocortical (HPA) axes to generate a coordinated response to maintain homeostasis (stress response) (de Kloet et al., 2005, Ulrich-Lai

and Herman, 2009, Lucassen et al., 2014). The first phase of the stress response is often referred to as the "fight or flight" response, and involves the rapid activation two branches that make up the SA axis. One branch consists of sympathetic nervous system activation, where sympathetic post-ganglionic neurons (SPGNs) that innervate peripheral tissues release norepinephrine (NE) locally. The second branch consists of sympathetic activation of the adrenal medulla, and subsequent release of epinephrine and NE into the general circulation. Together, this release quickly elevates metabolic rate, blood pressure, respiration, and directs blood flow to vital tissues, such as the heart and skeletal muscles, that are critical for a fight or flight reaction (Ulrich-Lai and Herman, 2009, Lucassen et al., 2014). The second phase of the stress response involves activation of the HPA axis, which is triggered by corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus. CRH induces adrenocorticotropic hormone release from the pituitary, which in turn drives the release of glucocorticoid hormones (GCs) from the adrenal cortex. GCs (corticosterone in rodents and cortisol in humans) generally act more slowly as genomic transcriptional regulators, but can also act more quickly via membrane-bound receptors to coordinate tissue/cell functions that are geared towards coping with stress, recovery and adaptation, such as mobilizing substrates for energy metabolism. While these processes are complementary to that initiated with the activation of the SA axis, GCs also dampen primary SA axis stress and pro-inflammatory immune cell activation to prevent overshooting. Regulation of the HPA axis response occurs through GC-mediated negative feedback on the hypothalamus and pituitary (de Kloet et al., 2005, Ulrich-Lai and Herman, 2009, Lucassen et al., 2014).

Acute stress is analgesic in healthy animals and humans. The most common anecdotal evidence of this comes from soldiers wounded in battle and athletes injured in games, who report

less pain in the heat of the action. These anecdotes have been confirmed in the laboratory setting, where significant increases in both pain threshold and tolerance have been documented, and are mediated by activation of descending inhibition (Butler and Finn, 2009). While the pre-clinical literature on acute stress is extensive, there are two features of the stress response that may be of particular relevance of this dissertation. First, the nature of the response depends on the nature of the stressor. A dramatic example of this would be the set of parameters that produce opioid-dependent and opioid-independent stress-induced analgesia (Mogil et al., 1996), suggesting that there are not only quantitative differences in the response to stress, but qualitative differences in the neural circuitry engaged. Second, the response to stress is sexually dimorphic. For example, the parameters that produce an opioid-dependent stress-induced analgesia in male rodents, result in an opioid-independent stress-induced analgesia in females (Mogil et al., 1993).

While the response to acute stress is generally considered adaptive, the response to chronic or repeated, non-habituating stress is generally considered maladaptive. Chronic stress blunts the body's underlying acute responses to stress through down-regulation of GCs, and ultimately can lead to hyperalgesia. In animals, models of repeated cold stress, repeated swim stress and chronic restraint stress have been shown to produce mechanical and thermal hyperalgesia (Imbe et al., 2006, Jennings et al., 2014). Numerous possible mechanisms for the chronic stress-induced hyperalgesia have been identified, including alterations in the HPA axis reactivity, neurotransmitter release in the CNS as well as increased glutamate release from primary afferents in the spinal cord and dysregulation of the immune system in the PNS (Imbe et al., 2006, Jennings et al., 2014).

The most common trigger for migraine is stress. Approximately 76% of migraineurs report that their attacks have triggers, and 80% of these report stress as a common trigger

(Kelman, 2007) and 58% report it as their main trigger (Zivadinov et al., 2003). Stress also amplifies attack intensity (Chabriat et al., 1999) and duration (Wacogne et al., 2003). Furthermore, many of the other common triggers for migraine such as alterations in sleep/wake cycle, skipping meals, dehydration, caffeine or alcohol consumption, and hormonal variation across the menstrual cycle (Sauro and Becker, 2009) are all physiological stressors that can impact both the HPA and SA axes. In many pain syndromes, such as irritable bowel syndrome (Larauche, 2012, Larauche et al., 2012, Myers and Greenwood-Van Meerveld, 2012), interstitial cystitis (Theoharides et al., 1998), fibromyalgia (McEwen and Kalia, 2010) and rheumatoid arthritis (Wood, 2009), both acute and chronic stress exacerbate symptoms *during* the stress. In contrast, migraine has a unique temporal dynamic in response to stress. Attacks often occur during the relaxation phase after stress, up to 24 hours following the removal of stress. These observations lead to the "stress-relaxation model" of migraine, and such headaches have been called "let-down headaches" (Hering et al., 1992, Spierings et al., 1997, Torelli et al., 1999, Hashizume et al., 2008, Lipton et al., 2014). Both acute stressors, such as GTN, alcohol, or skipping meals, as well as chronic stressors such as an intense workweek, studying for an exam/presentation, or illness in the family, often trigger migraines, but with a delay after the termination of the stress (i.e., 4 hrs after GTN, "weekend migraines," or the evening/morning after an exam/presentation). While there is a clear link between the timing of stress and migraine, the exact relationship can become complicated in humans. For example, in one study 70% of migraineurs reported attacks after the resolution of stress, but 60% also reported attacks during stress (Hauge et al., 2010). However, among these patients, commonly reported stressors were "marital problems" and "economic problems"—stressors that do not create a clear resolution of stress and relaxation phase. Stressors that produce a large increase in stress and

clear relaxation following removal of the stressor are commonly reported to result in “let-down” migraines.

Due to the prevalence of stress as a trigger for migraine, I have chosen to study how stress may contribute to inflammatory processing in the dura and thus the initiation of an attack. Furthermore, due to the unique temporal dynamic between stress and the start of a migraine attack, I have studied in particular whether the delay following stress may provide insights regarding the mechanisms involved in attacks.

1.4 EVIDENCE OF A SPGN CONTRIBUTION TO MIGRAINE PAIN

There is evidence of sympathetic dysregulation in migraineurs, indicating alterations in the SA axis and specifically SPGNs. The majority of clinical data evaluating sympathetic function in migraineurs indicate sympathetic hypoactivity with secondary adrenergic receptor (AR) supersensitivity. A consistent finding is that migraineurs have significantly lower supine plasma NE levels between attacks than age and sex matched controls, pointing to sympathetic hypoactivity. In five published reports with a total of 113 migraineurs, NE levels were 61% of controls (Peroutka, 2004). NE and blood pressure changes remained impaired in migraineurs when challenged with a physiological sympathetic-activating task. For example, with orthostatic changes (standing up or head-up tilt) the resulting rapid increase in plasma NE levels remained significantly lower (59% of controls), and the decrease in both systolic and diastolic blood pressure was greater than controls in three published reports (Peroutka, 2004). Other sympathetic tests such as isometric exercise (sustained handgrip test), cold pressor test, and Valsalva maneuver were also impaired. The normal response to these tests—an increase in heart

rate and blood pressure—was significantly lower in migraineurs than in controls (Peroutka, 2004). Clinical studies have also recorded pupil size, which is under direct tonic control of both the sympathetic and parasympathetic post-ganglionic neurons, regulating pupil dilation and constriction, respectively. Significantly smaller pupils have been reported in 5-20% of migraineurs during interictal periods, again indicating SPGN hypoactivity (Herman, 1983, Drummond, 1991). Furthermore, an oral dose of fenfluramine, which releases NE stores from nerve terminals, resulted in decreased pupil dilation compared to controls, and pupils were still significantly smaller up to 8 hours after application, suggesting that migraineurs may have smaller NE stores (Fanciullacci, 1979). Guanethidine application, which disrupts NE vesicle release from SPGNs, resulted in a greater and longer lasting constriction of pupils in migraineurs than controls, suggesting that NE resynthesis may also be impaired (Fanciullacci, 1979).

Additional pharmacological tests and prophylactic treatments further point to AR supersensitivity, possibly as a compensatory change in receptors on SPGN targets in response to the sympathetic hypofunction. For example, the resulting increases in blood pressure and heart rate to an intravenous bolus injection of NE or phenylephrine (α_1-AR agonist) were significantly greater and longer lasting in migraineurs than controls (Gotoh et al., 1984, Boccuni et al., 1989). Also, during interictal periods, migraineurs had significantly increased pupil dilation following epinephrine or phenylephrine eye drops than controls (Fanciullacci, 1979, Fanciullacci et al., 1982, Gotoh et al., 1984, Drummond, 1991). Finally, one class of effective prophylactic treatments for migraine include drugs that act on the sympathetic system—in particular beta-adrenergic antagonists (β blockers) (Rapoport and Bigal, 2005). The efficacy of this treatment would support post-synaptic β-AR supersensitivity.

Several lines of preclinical evidence also pointed to the possibility of a link between SPGN and migraine. First, we (Harriott and Gold, 2008, McIlvried et al., 2010) and others (Keller et al., 1989) have described extensive innervation of the dura by SPGNs, which closely associate with dural afferents and vasculature as well as extravascular areas (Appendix A). In a mouse model of familial hemiplegic migraine, in which the wild-type P/Q type voltage-gated Ca^{2+} channel has been replaced with the R192Q mutant channel, I have found increased SPGN innervation of the dura (Appendix B). Additionally, our lab has previously shown that dural SPGNs contain the 5HT1D receptor, the target of triptans, which are the most effective acute treatment for migraine. Second, the possibility that SPGNs in the dura may *directly* influence dural afferent activity is suggested by the fact that SPGNs release a number of compounds that can activate and/or sensitize dural afferents. These include neurotransmitters (NE, NPY, ATP) subject to vesicular release and PGE2, synthesized and released on demand (Straub et al., 2006). Our lab has shown that application of PGE2 (either individually or in an inflammatory soup also containing histamine and bradykinin) to dissociated dural afferents results in an increase in excitability (Vaughn and Gold, 2010). Recently, NE application to dural afferents has also been shown to increase excitability (Wei et al., 2015). In dorsal root ganglion neurons, ATP has been well established to induce excitability (Burnstock, 2007, Dussor et al., 2008).

Third, the possibility that SPGNs in the dura may *indirectly* influence dural afferent activity is suggested by evidence that SPGNs can facilitate an inflammatory response. I have shown that dural SPGNs may regulate, at least in part, stress-induced mast cell degranulation and protein plasma extravasation in the dura (Appendix C), thereby contributing to an inflammatory response. Also, in an ex vivo dura preparation, Ebersberger and colleagues showed that NE can increase the release of PGE2 (Ebersberger et al., 2006). Further evidence of SPGN facilitation of

inflammation has been demonstrated in other tissues. For example, in the skin, the inflammatory mediator bradykinin (BK) induced SPGN dependent release of PGE2, resulting in mechanical hyperalgesia (Taiwo and Levine, 1988, Khasar et al., 1995). Also, in the knee joint, SPGN innervation was essential for the neurogenic inflammatory response (Green et al., 1999, Hucho and Levine, 2007).

Due to evidence outlined above that SPGNs are activated during stress and may directly or indirectly contribute to dural afferent activity, I chose to additionally study the impact to which SPGNs in particular regulate inflammatory responses in the dura, and thus migraine pain.

1.5 IMMUNE SYSTEM CONTRIBUTION TO MIGRAINE PAIN

One way sex, stress and SPGNs may impact migraine pain is through regulation of the immune system. Innate and adaptive immune responses require activation of immune cells (white blood cells, or leukocytes). Innate immunity, the first line of defense and non-specific response, largely involves myeloid-derived granulocytes, macrophages, mast cells, and dendritic cells which are recruited to sites of inflammation from the blood. Granulocytes, whose prominent granules give them characteristic staining patterns, include neurotrophils, eosinophils and basophils. In the acute phase of an inflammatory response, neutrophils are normally the earliest and most numerous cell type to infiltrate tissue. They are phagocytic, and release a variety of pro-inflammatory mediators, including ones that attract other immune cells such as macrophages. Eosinophils and basophils are thought to play more of a role in parasitic infections (Murphy, 2011). Macrophages are a key phagocytic cell. Their main function is to phagocytose foreign material, microbes, and other leukocytes to remove injured and dying tissue debris. They

also release a variety of pro-inflammatory mediators (Murphy, 2011). Mast cells also differentiate in tissue and mainly distribute near blood vessels. When stimulated, they degranulate and release pro-inflammatory mediators, contributing to inflammation and vascular permeability. They are classically known for their role in allergic reactions (Murphy, 2011). Dendritic cells, in an immature state, are both phagocytic and macropinocytic. However, when encountering a pathogen they are specialized to take up antigen, rapidly mature, and migrate to lymph nodes to display it for recognition by T lymphocytes, thereby activating the adaptive immune response (Murphy, 2011). Lastly, lymphoid-derived natural killer cells are also considered part of the innate immune system because they lack antigen receptors. These cells largely circulate in the blood, and recognize and kill abnormal cells such as tumor and virus-infected cells (Murphy, 2011).

Adaptive immune responses involve activation of lymphoid derived B- and T-lymphocytes, which provide the long-term, specific immunity that can follow exposure to a pathogen. T-cells will recognize antigens expressed by antigen presenting cells (dendritic cells, B-cells, or host cells), and will mount the appropriate response. There are multiple subsets of T-cells, and the type of antigen expressed will determine the class of T cell activated and response generated. Cytotoxic T cells kill cells infected with viruses, where helper T cells induce a Th1 or Th2 type response. The Th2 response is characterized by the production of cytokines facilitating "cell-mediated immunity" (immune responses that do not involve antibodies, such as phagocytoic cells, natural killer cells, cytotoxic T-cells). The Th2 response supports antibody production (Murphy, 2011). More important for this dissertation is to note that processes that facilitate a shift from Th2 to Th1 are pro-inflammatory (Chapman et al., 2008). Regulatory T cells have been identified as negative regulators of adaptive immunity. They suppress aberrant

responses to self-antigens, and are more important in controlling autoimmune diseases. Lastly, when activated, B-cells differentiate into plasma cells that secrete antibodies to a specific antigen. These antibodies bind to the antigen, "flagging" them for phagocyotic cells. Some plasma cells become memory B cells, which survive to respond efficiently if the specific antigen is ever encountered again. Together the innate and adaptive immune systems provide an effective defense system (Murphy, 2011). While this brief overview does not begin to touch on the complexity of the immune system, it highlights some of the traditional types and roles of immune cells that are examined in this dissertation.

I hypothesized that immune cells may contribute to an inflammatory response in the dura, and therefore initiation of a migraine attack for multiple reasons. First, immune cells are a primary source of pro-inflammatory mediators. As noted previously, migraineurs have increased levels of pro-inflammatory mediators in their plasma and CSF during attacks, including the immune cell mediators IL-6, TNFα, and IL-1β (Perini et al., 2005, Fidan et al., 2006, Sarchielli et al., 2006). Second, stimuli that trigger migraines can activate immune cells. For example, stress [Appendix C and (Theoharides et al., 1995)], GTN (Reuter et al., 2001), and CSD (Karatas et al., 2013) can degranulate dural mast cells, and GTN can drive an upregulation of inducible NOS (iNOS) and IL-6 in dural macrophages (Reuter et al., 2001). Third, activation of immune cells can increase excitability of dural afferents. Degranulation of resident dural mast cells with the basic secretagogue agent compound 48/80 was sufficient drive dural afferent activation, which could be blocked with the mast cell stabilizer sodium cromoglycate (Levy et al., 2007). Fourth, immune cell pro-inflammatory mediators, such as IL-6 and TNFα can activate dural afferents. IL-6 has been shown to directly activate dissociated dural afferents (Yan et al., 2012), while TNFα mediated sensitization is indirect (Zhang et al., 2011). Zhang and colleagues

showed that TNFα application to the dura and consequent sensitization of dural afferents required activation of vascular endothelial cells and macrophages in the dura, and subsequent release of pro-inflammatory COX-derived prostanoids (Zhang et al., 2011).

I also chose to focus on immune cells in this dissertation due to evidence indicating that immune cells are regulated by factors that are also features of migraine: 1) the sex-difference and regulation by gonadal hormones, 2) attacks triggered by stress, 3) and sympathetic dysregulation. First, most major subtypes of immune cells have been shown to express estrogen (and other sex steroid hormone) receptors, and there are complex sex differences and hormone responses on immune cell number and activation (Oertelt-Prigione, 2012, Pennell et al., 2012, Yakimchuk et al., 2013). For example, females were reported to have more resident immune cells in the peritoneal cavity compared to males (Scotland et al., 2011). Also, in cycling females during the luteal phase when menstrual migraines occur, there were increased total monocyte (Mathur et al., 1979, Northern et al., 1994) and neutrophil (Jiemtaweeboon et al., 2011) numbers circulating in the blood. The low levels of estrogen during this phase stimulate increased TNFα release from monocytes (Brannstrom et al., 1999) and T cells (Correale et al., 1998). Low levels of estrogen are described to generally facilitate Th1 (pro-inflammatory) responses (Pernis, 2007, Oertelt-Prigione, 2012, Pennell et al., 2012). Ultimately, sex differences depend not only on genetic, hormonal and hormone receptor levels, but how immune cells are modulated by these factors during proliferation in the bone marrow and spleen, density and phenotype in the blood, trafficking to peripheral tissues, and ultimately their function within the tissue. In the dura specifically, it has been shown that mast cells express estrogen receptors (Rozniecki et al., 1999) and a combined estrogen and progesterone treatment following ovariectomy augmented mast cell degranulation (Boes and Levy, 2012). Also, dural mast cell density was increased in females,

which fluctuated with estrous cycle (highest at diestrus) and was dependent upon estrogen levels (Boes and Levy, 2012).

Second, it is well known that the immune system is dramatically influenced by stress. Stress can affect the immune system by multiple pathways—the HPA axis via CRH and GC receptors on immune cells and the SA axis via ARs on immune cells (see below). Lymph nodes are also directly innervated by SPGNs (Glaser and Kiecolt-Glaser, 2005, Chapman et al., 2008, Webster Marketon and Glaser, 2008, Rohleder, 2012, Reader et al., 2015). Generally, acute stress has been shown to be immune-enhancing while chronic stress is immunosuppressive. Chronic stress has been shown to delay wound healing and enhance the risk and prolong the duration of an infectious disease (Glaser and Kiecolt-Glaser, 2005, Chapman et al., 2008, Webster Marketon and Glaser, 2008, Rohleder, 2012, Reader et al., 2015). HPA-axis GCs down-regulate inflammation by binding to their cytoplasmic receptors and inducing translocation to the nucleus where the complex interferes with the DNA-binding activity of the major inflammatory transcription factor NF-κβ (McKay and Cidlowski, 1999). This mechanism has been therapeutically exploited for decades, as steroids are prescribed as potent anti-inflammatory drugs.

Third, immune cells also express ARs, which can regulate their proliferation, chemotaxis and activation in response to epinephrine and NE (Vizi, 1998, Elenkov et al., 2000). For example, β- and α-ARs can differentially suppress and facilitate, respectively, pro-inflammatory cytokine production in macrophages (Ignatowski et al., 1996, Szelenyi et al., 2000). Furthermore, there is evidence of time-dependent shifts in macrophages (Ignatowski et al., 1996) and T-cells (Sanders, 2012) and sexually dimorphic regulation of ARs in T-cells (Leposavic et al., 2008) and NK cells (Shakhar et al., 2000). Neutrophil number and chemokinesis is also

regulated by stress and β-ARs in a sexually dimorphic manner (de Coupade et al., 2004, Barker et al., 2005). Additionally, it has been hypothesized that whether the cell is already activated is important. Evidence suggests that AR signaling can suppress an inflammatory response in already activated immune cells, but can induce an inflammatory response in non-activated immune cells (Rohleder, 2012).

In this Introduction I have briefly reviewed the clinical features of migraine as well as some of the most relevant data regarding the neurobiology of migraine, at least in support of a peripheral origin of migraine pain. I have summarized evidence in support of the biological basis for the sex difference in the manifestation of migraine. I briefly summarized some of the critical features of a stress response, the link between stress and pain and the evidence in support of an important but unique link between stress and migraine. I also summarized evidence in support of a link between dural SPGNs and migraine. Finally, I again briefly described key features of the immune system, as well as evidence that dural immune cells are uniquely positioned to not only account for the key clinical features of migraine that I summarized, but to serve as the penultimate mediators of a migraine attack. This evidence served as the basis for the central hypothesis of my thesis research, which is that stress drives sex- and SPGN-dependent changes in the regulation of dural immune cells, setting the stage for the initiation of a migraine attack.

2.0 SEX-, STRESS-, AND SYMPATHETIC POST-GANGLIONIC-DEPENDENT CHANGES IN THE IDENTITY AND PROPORTIONS OF IMMUNE CELLS IN THE DURA

Due to compelling evidence in support of links between sex, stress, sympathetic post-ganglionic innervation, dural immune cells, and migraine, our aim was to characterize the impact of these factors on the type and proportion of immune cells in the dura. Dural immune cells were obtained from naïve or stressed adult male and female Sprague Dawley rats for flow cytometry. Rats with surgical denervation of sympathetic postganglionic neurons (SPGNx) of the dura were also studied. Immune cells comprise ~17% of all cells in the dura. These were comprised of: macrophages/granulocytes (Macs; 63.2% of immune cells), dendritic cells (0.88%), T-cells (4.51%), natural killer T-cells (0.51%), natural killer cells (3.08%), and B-cells (20.0%). There were significantly more Macs and fewer B-and natural killer T-cells in the dura of females compared to males. Macs and dendritic cells were significantly increased by stress in males, but not females. In contrast, T-cells were significantly increased in females with a 24 hr delay following stress. Lastly, Macs, dendritic cells, and T-cells, were significantly higher in SPGNx naïve males but not females. It may not only be possible, but necessary to use different strategies for the most effective treatment of migraine in men and women.

2.1 INTRODUCTION

Evidence suggests that a “sterile” inflammation of the dura is responsible for the activation and sensitization of dural afferents thought to be responsible for migraine pain (Waeber and Moskowitz, 2005, Olesen et al., 2009, Levy, 2012). However, the mechanisms underlying the initiation of the inflammation are poorly understood. One mechanism may be via dural immune cells. Consistent with this possibility, there is evidence that degranulation of resident mast cells is sufficient to drive the activation and sensitization of dural afferents (Levy et al., 2007) and that a migraine inducing stimulus, glyceryl trinitrate (GTN), can drive the degranulation of dural mast cells, and an upregulation of inducible NOS (iNOS) and IL-6 mRNA in dural macrophages (Reuter et al., 2001).

While the extent to which other immune cell types may contribute to migraine remains to be determined, the clinical manifestations of migraine suggest several additional reasons to focus on dural immune cells as an underlying contributor to this neurological disorder. First, the prevalence of migraine is roughly three times higher in women than in men (Pietrobon and Striessnig, 2003). That immune cells may contribute to this sex difference is suggested by the observations that there are sex differences in immune cell number, phenotype, and expression of hormonal receptors (Pennell et al., 2012, Sankaran-Walters et al., 2013, Yakimchuk et al., 2013). Second, roughly 80% of migraineurs report stress as a trigger for a migraine attack and 60% report it as their main trigger (Martin, 2010). Stress also amplifies attack intensity and duration (Sauro and Becker, 2009). The link between stress and migraine has an important and interesting temporal dynamic. Formally referred to as the “stress-relaxation model”, it has been proposed that a migraine attack occurs during the relaxation phase after stress (Sauro and Becker, 2009). And while there is evidence linking immune cells to the acute exacerbation of

pain observed in other pain syndromes (Theoharides et al., 1998, Wood, 2009, McEwen and Kalia, 2010, Larauche, 2012, Larauche et al., 2012), there is also evidence to suggest that time dependent changes in the regulation of immune cells could account for the delay in the migraine attack after the resolution of the stressor. For example, almost all immune cells express adrenergic receptors (AR), and β- and α-ARs differentially suppress and facilitate, respectively, pro-inflammatory cytokine production (Ignatowski et al., 1996, Szelenyi et al., 2000). Furthermore, there is evidence of time-dependent shifts (Ignatowski et al., 1996, Sanders, 2012) and sexually dimorphic regulation (Shakhar et al., 2000, de Coupade et al., 2004, Barker et al., 2005, Leposavic et al., 2008) of ARs on immune cells. Third, there is evidence of sympathetic dysregulation in migraineurs, which includes a) lower plasma norepinephrine levels between attacks than is observed in non-migraineurs; b) impaired sympathetic responses to challenge (i.e., pressor responses); and c) AR hypersensitivity, possibly secondary to the sympathetic hypofunction (Peroutka, 2004). There also is evidence that α-AR agonists and β-AR antagonists can decrease the frequency of migraine attacks (Goadsby and Sprenger, 2010). Finally, the link between these clinical features of migraine and immune cells is suggested by the observations that stress is associated with the activation of both the hypothalamic-pituitary-adrenal and the sympatho-adrenal axes, the dura is heavily innervated by sympathetic post-ganglionic neurons (SPGNs) (Keller et al., 1989, Harriott and Gold, 2008), and immune cells are under AR regulation.

Despite this compelling evidence in support of a link between dural immune cells and migraine, there have been no systematic analyses of the immune cells in the dura, let alone studies to address sex differences in these immune cells, and/or stress-induced changes in these immune cells that would be consistent with a role in migraine. To begin to address this issue, we

have performed a detailed characterization of the immune cells in the dura of male and female rats. The impact of stress was assessed by comparing immune cells in naïve rats to those immediately, or with a delay, following the termination of chronic stress. Finally, to determine whether local SPGN signaling may contribute to dural immune cell regulation, naïve and stressed rats were studied following SPGN denervation of the dura.

2.2 METHODS

2.2.1 Animals

Pathogen free adult (P50-70) male (200-300g) and female (150-250g) Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were used for all experiments. Rats were housed in pairs in microisolator cages [(Allentown Caging Equipment Co., Inc., Allentown, NJ), bedded with certified Aspen Coarse-grade Sani-chips® (P.J. Murphy Forest Products Corp, Montville, NJ)] in the University of Pittsburgh Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility on a 12:12 light:dark cycle at 72°F with food [ProLab® IsoPro® RMH 3000 5P76 irradiated (LabDiet, St. Louis, MO)] and water available ad lib unless indicated. Environmental enrichment was not provided, as it would counter-act the study of stress. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2.2 Chronic variable stress (CVS)

Animals were exposed to chronic variable stress (CVS), consisting of an ongoing series of "mild" stressors applied for variable durations over a period of 4 days (see experimental design in Figure 1A). The paradigm was adapted from unpredictable chronic mild stress models of depression (Grippo et al., 2004), and was compressed from 3 weeks to 4 days. This allowed us to model the length of stressors that typically trigger migraines (i.e., stress from a workweek or studying for an exam). The presentation of many different kinds of stressors for different lengths of time over the 4 days allowed us to mimic a more complex, non-habituating stress. Additionally, with stressors presented constantly (as opposed to "repeated" chronic stress models where one stressor is presented for a short period of time for multiple days), this design allowed us to model the constant stress that typically triggers a migraine, as well as create a clear "relaxation period" following the stress during which migraines typically occur.

After arrival, rats were housed in a "home" room for at least 6 days, and subsequently subjected to CVS in a separate, isolated housing room. Rats were single-housed, an average of 2 stressors were presented at any given time, and each stressor was presented twice over the 4 days. The schedule was semi-randomized to ensure that the same stressors were not always paired together or back-to-back. Nine different stressors were used and these included: food deprivation (12-16 hrs each), water deprivation (12-16 hrs each), overnight illumination (no 12hr dark cycle), cage tilt (18° angle, 8-16 hrs, once on each of the short and long cage axis), paired housing with a stranger (8-16 hrs each), damp bedding (12-16 hrs each), white noise (85dB of white noise, 3-5 hrs each), strobe light (1 Hz, 4-6 hrs each), and predator odor (two $5cm^2$ pieces of cloth from predator cages were hung in rat cages, 30 min ferret odor once and 1 hr cat odor

once). Rats with a delay period of "relaxation" after CVS were returned to the home housing room for that time.

It is important to note we are not proposing that CVS is a model of migraine, nor do we anticipate that this stress paradigm causes migraine in rats. Rather we have proposed to study changes in response to stress because we hypothesize that these changes may contribute to the initiation of a migraine attack in migraineurs.

2.2.3 Bilateral superior cervical ganglion sympathectomy (SPGNx)

One week prior to any experimentation, select animals underwent bilateral surgical removal of superior cervical ganglia (SCG), the cell bodies giving rise to sympathetic innervation of the dura (Figure 1A). Animals were deeply anesthetized with an intraperitoneal (IP) injection of anesthetic cocktail [55 mg/kg ketamine (Fort Dodge, Fort Dodge, IA), 5.5 mg/kg xylazine (LLOYD, Shenandoah, IA), and 1.1 mg/kg acepromazine (Butler Schein, Dublin, OH)], and monitored by respiratory rate and responses to toe pinch. SCG were accessed via a midline incision through the skin overlaying the trachea followed by blunt dissection of the muscles and connective tissue on either side of the trachea. Following excision of the ganglia and visual confirmation of hemostasis, the incision site was closed. Prior to recovery from anesthesia, rats received an intramuscular (IM) injection of buprenorphine (0.03 mg/kg; Reckitt Benckiser Pharmaceuticals, Richmond, VA). Sham animals underwent the same procedures, except the SCG were not removed. Animals were monitored daily following both surgical procedures.

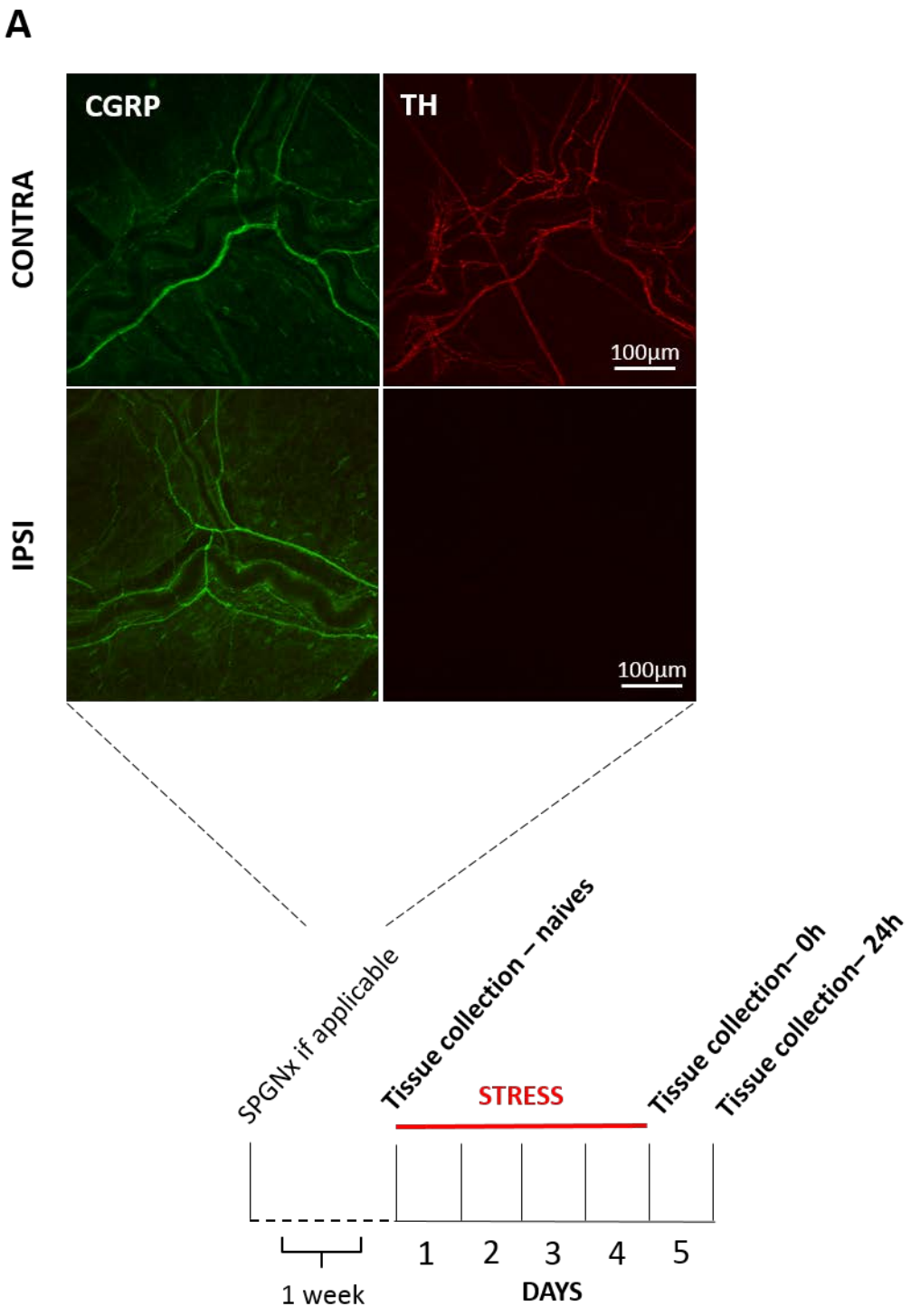
2.2.4 Isolation of dural cells

Tissue was collected from animals between 8-10am in the laboratory for CVS groups, and in a procedure room of the animal housing facility for naïve groups to minimize "transport stress." Prior to dural tissue collection, animals were deeply anesthetized with pentobarbital (60mg/kg IP; Sigma, St. Louis, MO; an anesthetic with minimal effect on vascular tone or adrenergic receptors, potential confounds for our studies) followed by a rapid transcardiac perfusion with 60mL cold 1x phosphate-buffered saline (PBS; pH 7.2). If appropriate, the uterus was also collected and weighed as an indirect measure of estrus cycle (Ji et al., 2008).

Following careful removal of occipital and parietal bones, dura extending from -4 to 15mm (caudal to rostral of convergence of sinuses) and ±11mm (lateral) was collected and placed in 5mL of Dulbecco's Modified Eagle Medium-F12+ [Advanced DMEM-F12 (Gibco, Grand Island, NY), to which 10 U/ml each of penicillin and streptomycin (Invitrogen, Carlsbad, CA), 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Calbiochem, San Diego, CA), and 2 mM L-glutamine (Invitrogen), were added] with 0.1% Collagenase P (Roche, Indianapolis, IN) in a sealed 60x15mm glass Petri dish and incubated for 1hr at 37°C in a shaking water bath. Dura were then mechanically dissociated in this solution with fire-polished Pasteur pipettes, centrifuged at 250g, resuspended in 5 mL fresh DMEM-F12+, centrifuged again, and resuspended in 1mL staining media [(SM); Ca^{2+}/Mg^{2+} free Dulbecco's PBS (Lonza, Walkersville, MD), 3% heat inactivated newborn calf serum, 1mM EDTA (ethylenediaminetetraacetic acid), 0.02% sodium azide]. The final cell suspension was filtered through 50µm mesh (Sefar, Heiden, Switzerland) and subsequently processed for flow cytometry or FACS. This protocol was adapted from (Borghesi et al., 2005, Steenhuis et al., 2008, Santos et al., 2014). Cell viability was uniformly >80%.

2.2.5 Flow cytometry and fluorescence activated cell sorting (FACS)

Flow cytometry and FACS, with the gating strategy illustrated in Figure 1B, was used to collect subpopulations of immune cells from each dissociated dura. All cell processing was performed on ice with SM. Initial cell counts were estimated with trypan blue staining. Cells were first blocked with 10% rat serum for 10min to prevent non-specific binding of antibodies, resuspended in SM, and then incubated in primary antibodies for 20min. They were then washed in SM 3 times prior to a final resuspension in 1µg/mL propidium iodide (PI; Molecular Probes, Eugene, OR) to exclude dead cells (viability), and immediately used for either flow cytometry or fluorescence activated cell sorting (FACS). All primary antibodies were mouse monoclonal raised against rat epitopes from BD Biosciences (San Diego, CA), used at the following dilutions: APC cy7 CD45 (clone OX-1) 1:100, PEcy7 CD11b/c (clone OX-42) 1:50, V450 CD11b (clone WT.5) 1:100, APC CD3 (clone 1F4) 1:50, PE CD161a (clone 10/78) 1:200, FITC CD45R (clone HIS24) 1:25. Specificity of the antibodies has been previously documented (Taieb et al., 2007, Hara et al., 2012, Ozaki et al., 2012, Osborn et al., 2013). Fluorescence minus one (FMO) antibody controls were used to identify negative staining in dural cells (Herzenberg et al., 2006). Flow cytometry was performed on a 5 laser, 18 detector LSR Fortessa (BD Biosciences) and FACS was performed on a 3 laser, 11 detector FACS Aria (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, San Carlos, CA).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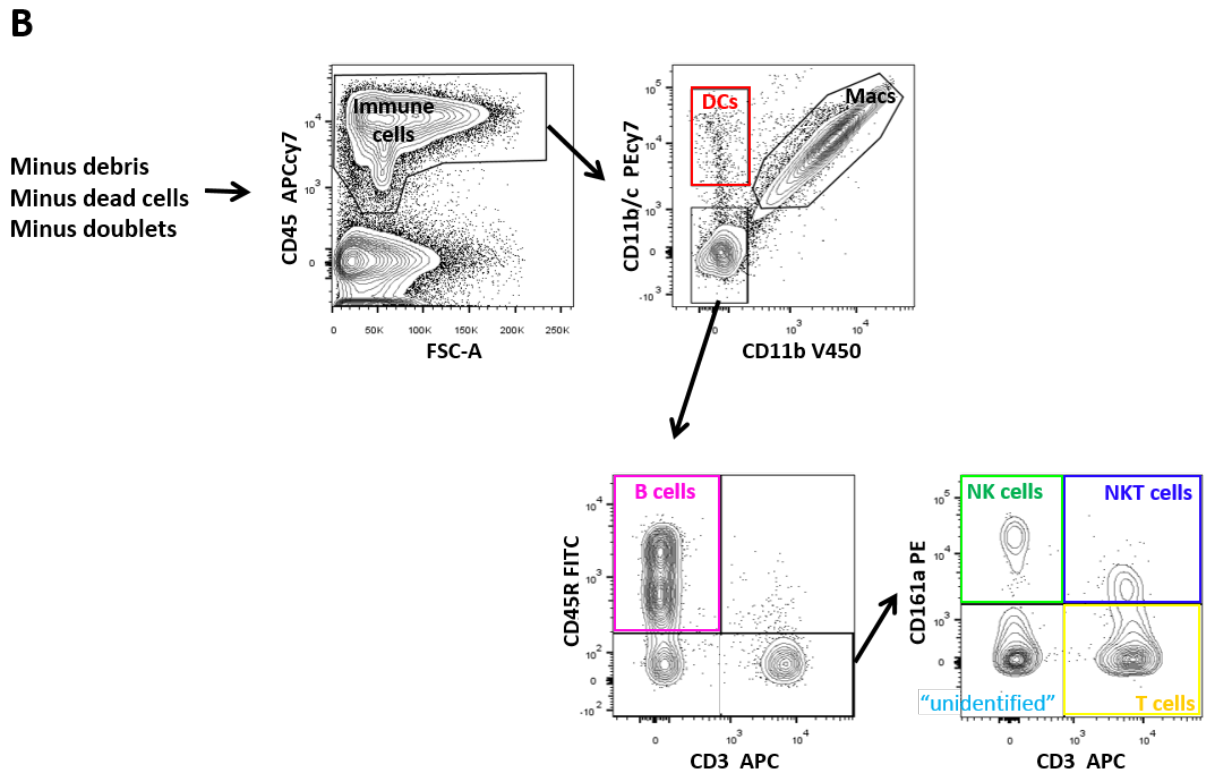


Figure 1. Experimental design.

(A) Intact and SPGNx, male and female rats were either considered "naïve" or exposed to constant, unpredictable, mild stressors over four days (chronic variable stress or CVS). Tissue was collected from "naïve" rats, rats immediately after the last day of stress ("CVS+0h") as a measure of the response to chronic stress, or rats after a 24 hr delay after stress ("CVS+24h") as a measure of the response to stress-relaxation. To test the impact of SPGN innervation, prior to stress superior cervical ganglia were surgically removed bilaterally (SPGNx) in appropriate groups. On the left a representative whole mount dura from an adult rat 7 days after a unilateral SPGNx is shown, probed with anti-calcitonin gene-related peptide (CGRP, green) or anti-tyrosine hydroxylase (TH, red) antibodies. Note the complete loss of TH-like immunoreactivity (a marker for sympathetic fibers) ipsilateral to the SPGNx, but no change in CGRP-like immunoreactivity (a marker for peptidergic afferents). (B) Gating strategy used for the isolation of immune cell subtypes from the dura. Data shown are from a naïve male rat. The day of the experiment, dural cells were dissociated and stained with antibodies specific to markers of immune cells and/or distinct immune

cell subtypes. The gating strategy illustrated was used for all flow and FACS experiments. CD45+ immune cells were first selected, and then sorted into CD11b+ Macrophages/granulocytes/mast cells (Macs) and CD11c+ dendritic cells (DCs). CD11b-/c- immune cells were further sorted into CD45R+ B-cells. CD11b-/c- and CD45R- immune cells were lastly sorted into either CD161a+ natural killer (NK) cells, CD3+ T-cells, and CD161a+/CD3+ NKT cells. A small subset of immune cells were left unstained by any of the immune subtype antibodies utilized in this strategy ("unidentified"). Viability was uniformly 80-85%, as determined by PI and trypan blue staining. This resulted in an average of 1.56 x 106 ± 0.18 x 106 live cells recovered per dura. Spleen cells were used for compensation controls (Borghesi et al., 2005, Santos et al., 2014). Images from FlowJo software, contour plots were used with 5% levels and outliers displayed.

2.2.6 Cell histology

Dissociated dural cells from 6 male rats were pooled and sorted via FACS into 1x Ca^{2+}/Mg^{2+} free PBS with 10% fetal bovine serum. Sorted populations showed purities >97% on reanalysis. Each sorted cell population (CD11b+, CD11c+, CD3+, CD161a+ and CD45R+; see Figure 1B) was resuspended into a volume of 50uL/50,000 cells and cytocentrifuged (Cytospin 3; Shandon Lipshaw, Pittsburgh, PA) onto slides at 800rpm for 3min. Slides were subsequently stained with the Diff Quik stain kit (Dade Behring, Newark, DE) or for 30sec with 1:1 of 1% toluidine blue (Sigma) in 70% EtOH : 1% NaCl pH 1.5. Five randomized fields per slide were photographed under brightfield with a Leica DM4000B upright microscope and DFC300FX camera (Leica, Wetzlar, Germany). Morphology and Diff quick staining was used to identify types of immune cells, and toluidine blue staining was used to identify mast cells. Each micrograph was scored by an investigator blinded to the test population.

2.2.7 Immunohistochemistry

Following deep anesthesia with anesthetic cocktail, rats were transcardially perfused with 200-250mL warm 1x PBS. Dura were dissected, fixed for 10min in ice cold acetone (or methanol for mouse anti-rat CD3 antibody) and processed as free-floating whole-mounts. Dura were incubated in blocking solution [10% normal donkey serum and 0.1% Triton X-100 in dilution buffer (1% BSA, 0.2% cold fish skin gelatin and 0.05% sodium azide in TBS)] for 1hr at room temperature, followed by the appropriate primary antibody in dilution buffer for 24 hrs at 4°C (or 48 hrs for FITC mouse anti-rat CD45R antibody). Primary antibodies were all raised in mouse

and included: ED-2 1:500 (Serotec), CD3 (clone G4.18) 1:25 (BD Biosciences), and CD161a (clone 10/78) 5µg/mL (BD Biosciences). Specificity of the antibodies has been previously documented (Brenan and Rees, 1997, Taieb et al., 2007, Zhang et al., 2009, Hara et al., 2012, Ozaki et al., 2012, Osborn et al., 2013). Antibodies were visualized with donkey anti-mouse secondary antibodies conjugated to cyanine 2 (Jackson ImmunoResearch, West Grove, PA, USA) at 1:200 in dilution buffer for 2 hours at room temperature.

To stain sensory and sympathetic fibers in the dura, rats were transcardially perfused with cold 1x PBS followed by cold 4% paraformaldehyde. Dura whole-mounts were placed in blocking solution (10% normal donkey serum and 0.3% Triton X-100 in PBS) for 30 minutes and incubated in primary antibodies of rabbit anti-CGRP (Peninsula/Bachem, San Carlos, CA; 1:500) and sheep anti-tyrosine hydroxylase (Millipore; 1:500) in blocking solution for 2 days at 4°C. Antibodies were visualized with donkey anti-sheep and donkey anti-rabbit secondary antibodies conjugated to cyanine 2 or 3 (Jackson) in blocking solution at 1:500 for 2 hours.

Omission of primary antibody was used as a control for non-specific binding of the secondary antibody. Dura were mounted on glass plus slides and coverslipped using Fluoromount-G (Southern Biotech). Slides were photographed under epifluorescence with a Fluoview FV1000 confocal microscope (Olympus) and processed for brightness and contrast with Adobe Photoshop (Adobe Systems, San Jose, CA).

2.2.8 Data analysis

We tested four related a priori hypotheses based on clinical features of migraine. The first hypothesis was that there are more immune cells in the dura of females than males. The second hypothesis was that stress increases the relative proportion of dural immune cells with a delay

following the cessation of stress that is greater in females than males. Thus, we examined the influence of both sex and stress on the proportion of immune cell subtypes in the dura by comparing naïve rats with rats exposed to 4 days of chronic variable stress (CVS), assessed either immediately following stress (CVS+0h) or with a 24 hr delay following stress (CVS+24h). The choice of a 24 hr delay was based on results from preliminary experiments also including a 12 hr delay group (N=4 for both male and female). No significant differences were detected between the 12 and 0 hr groups (data not shown), and thus the 12 hr delay group was not pursued further.

The third hypothesis was that the proportion of dural immune cells are dependent on SPGN innervation, and more pronounced in females than males. To test this hypothesis, we assessed changes in the proportion of dural immune cells in rats surgically sympathectomized (SPGNx) by removal of the superior cervical ganglion bilaterally. Lastly, given that stress drives activity in the SPGN, our fourth hypothesis was that the stress-induced increase in dural immune cells in females is dependent on the presence of SPGN innervation.

To address all four a priori hypotheses, rats were semi-randomized to groups in a 2x3x3 design (Figure 1A) with groups defined by sex (male/female), stress (naïve/CVS+0h/CVS+24h), and SPGN innervation (intact/SPGNx/sham). Rats were semi-randomized because they were assigned to groups by cages so that both rats in a cage were in the same group. As it was only possible to process tissue from four rats per day, tissue from two groups were processed on any given day. The order in which rats from any particular group were processed was also randomized. The power analysis (G-power software, Softpedia) used to determine group size was based on preliminary analysis of CD11b+/c- cells from naïve males and females. A group

size of six would enable us to detect a medium effect size of both sex and stress with a power of 0.8 and alpha at 0.05.

Immune cells from each animal were quantified with flow cytometry, and analyzed as a percent of total live cells so that changes in one subtype of immune cells could be determined independently of changes in another cell type. Thus figures 4-7 are presented as a percent of total live cells, and normalized to the respective male naïve group for comparison.

Data were analyzed with t-tests for sex effects (male vs. female), 2-way ANOVAs for sex and stress effects (naïve, 0, 12 or 24 hrs following CVS) as well as sex and SPGN effects (intact vs. SPGNx) in naïve animals, and 3-way ANOVAs for sex, stress, and SPGNs effects with $p<0.05$ considered significant. The Tukey HSD test was used for post-hoc comparisons if significant interactions were detected. An ANCOVA was used to assess the effect of estrus cycle with uterus weight as a rough measure of cycle stage.

2.3 RESULTS

This study was completed in two parts. First we used flow cytometry to identify immune cell types present in the dura, and validated these data with complementary morphology following single cell sorting and IHC of intact dura. Second, we used flow cytometry to determine the impact of sex, stress and SPGN innervation on the relative proportions of dural immune cell subtypes. Any data related to this paper can be accessed by sending a request to the corresponding author.

2.3.1 Immune cell types present in the dura

Flow cytometry, with the gating strategy illustrated in Figure 1B, was used to characterize immune cell types. Within CD45+ leukocytes, six types of immune cells were detected (Figure 2) as assessed using antibodies previously validated in the rat (Taieb et al., 2007, Hara et al., 2012, Ozaki et al., 2012, Osborn et al., 2013). In the naïve male dura, 63.2 ± 0.9% of immune cells in the dura were CD11b+ macrophages/monocytes, granulocytes and mast cells ("Macs"); 0.9 ± 0.2% were CD11b/c+/CD11b- dendritic cells ("DCs"); 20.0 ± 0.1% were CD45R+ B-cells; 3.1 ± 0.6% were CD161a+ natural killer ("NK") cells; 4.5 ± 0.4% were CD3+ T-cells; and 0.5 ± 0.1% were CD161a+/CD3+ natural killer T ("NKT") cells. An average of 7.8 ± 0.3% of immune cells were not specifically identified in our panel ("unidentified"). The major subpopulations of immune cells were validated with morphological and histological analysis. Diff quik staining (Figure 2, insets) confirmed that Macs consisted of 89.1% macrophages/monocytes with a characteristic and pronounced granularity, 5.1% neutrophils, 3.6% mast cells (identified with toluidine blue stain), and 2% contamination by lymphocytes. The B-cell population consisted of 93.0% small lymphocytes (≤10μm) and 5.1% large lymphocytes (>10μm), both of which were non-granular, with <2% contamination by macrophages/monocytes. The NK cell population consisted of 51.3% large and 48.7% small lymphocytes, of low granularity. The T-cell population consisted of 79.7% small and 20.3% large non-granular lymphocytes.

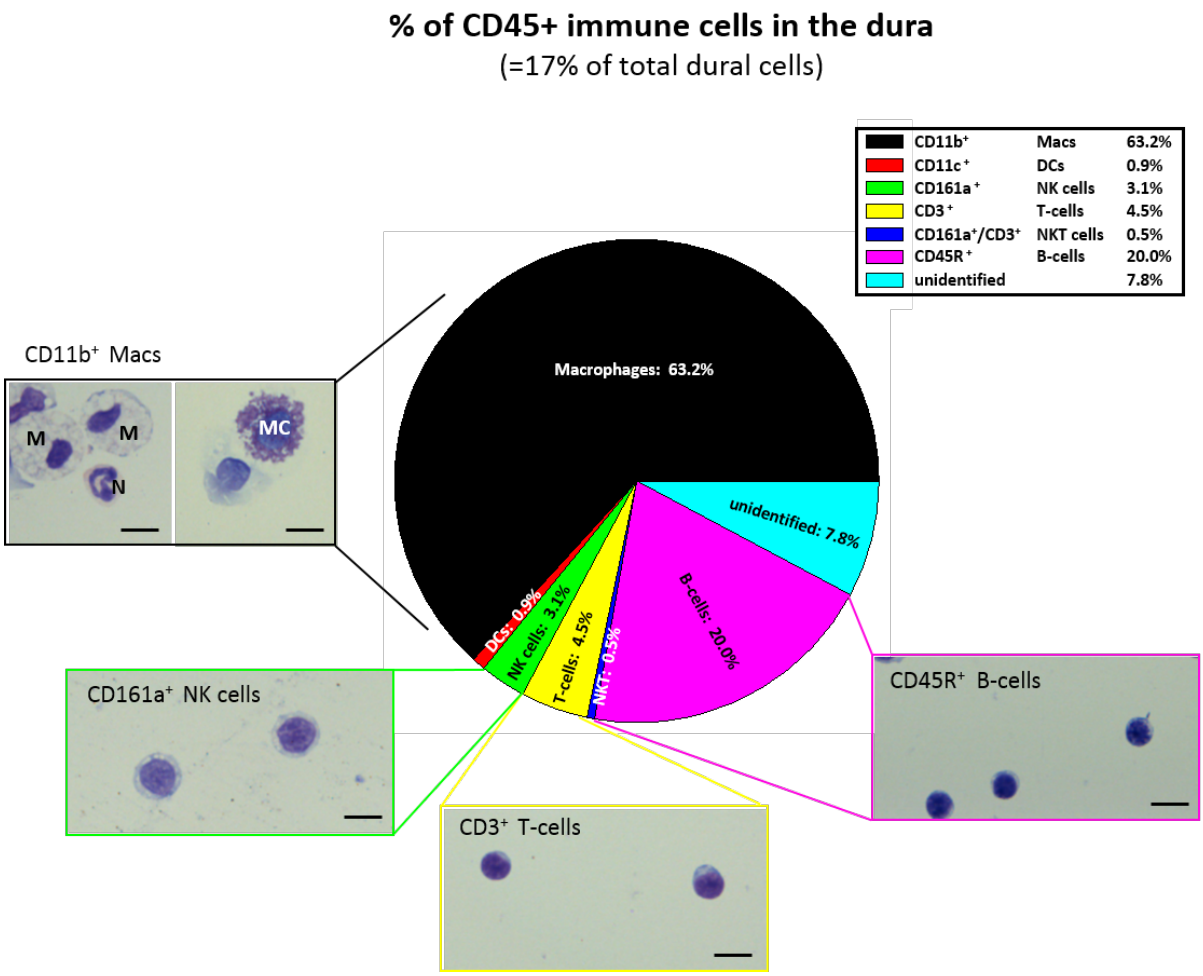


Figure 2. Proportion of immune cell subtypes in the naïve male dura as determined with flow cytometry using the gating strategy shown in Figure 1B.

Of the live cells recovered from the dura, an average of 16.9 ± 0.90% were CD45+, and thus determined to be immune cells. The pie chart and legend show the relative proportion of the 6 immune cell subtypes identified in the dura. The insets show FACS sorted, cytocentrifuged preparations of immune cell subtypes, subsequently stained with Diff quik. Macs consisted of 89.1% macrophages/monocytes (M), 5.1% neutrophils (N), 3.6% mast cells (MC; identified with toluidine blue stain), and 2.2% lymphocytes. The NK cell population consisted of 51.3% large lymphocytes (>10µm) and 48.7% small lymphocytes (≤10µm). The T-cell population consisted of 79.7% small lymphocytes and 20.3% large lymphocytes. Lastly, the B-cell population consisted of 93.0% small lymphocytes, 5.1% lymphocytes, and 1.9% macrophages/monocytes. Scale bars 10µm.

To exclude the possibility that immune cell estimates were confounded by incomplete perfusion of the dural vasculature, as well as to determine the distribution of major immune cell populations detected within the dura, Macs, NK, and T-cells were assessed in situ with ED2-, CD161a-, and CD3-like immunoreactivity (-LI), respectively. There was a high density of ED2-LI cells throughout the dura, both surrounding dural vasculature (Figure 3A, top panel and insert) as well as in "extravascular" areas (Figure 3A, bottom panel). A much lower density of CD161a-LI and CD3-LI cells were present surrounding the vasculature (Figure 3B and C, top panels and insets) as well as in "extravascular" areas (Figure 3B and C, bottom panels). Together, these observations suggest that there is a large and diverse immune cell population in the dura, raising the question as to how these populations vary in association with migraine-relevant conditions.

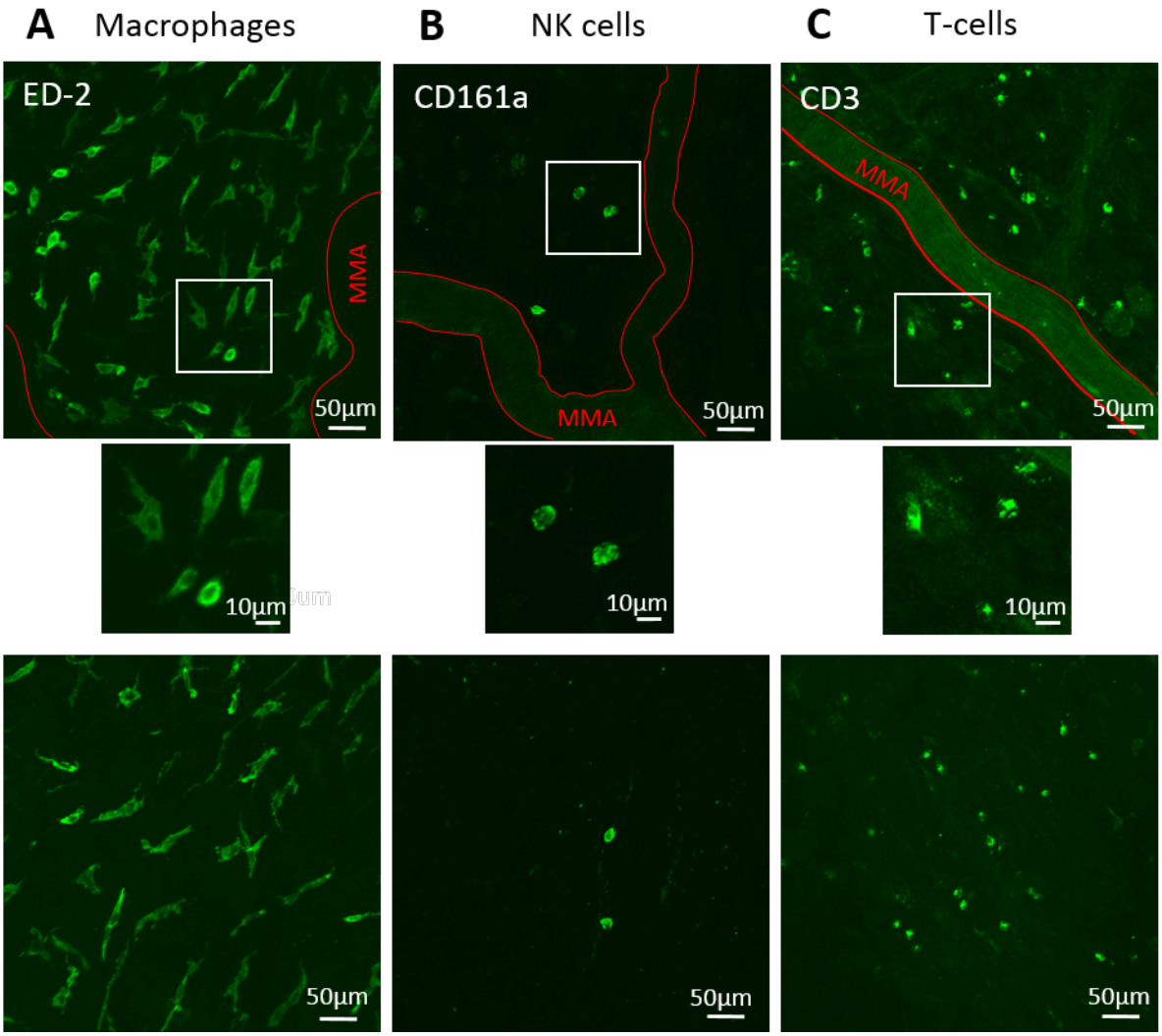


Figure 3. Distribution of immune cells in the naïve male dura.

Macrophage, NK cell and T cell distributions were examined in situ with immunohistochemistry for ED2- (**A**), CD161a- (**B**), and CD3- (**C**) like immunoreactivity (-LI), respectively. Immune cells were observed in high density throughout the dura, both surrounding dural vasculature (top panels and inserts) as well as in "extravascular" areas (bottom panels). Smaller numbers of CD161a-LI and CD3-LI cells were observed.

2.3.2 Sex, stress, and sympathetic innervation influence dural immune cell proportions

2.3.2.1 Immune cells in the dura

While there was no detectable difference between naïve male (n=7) and female (n=6) rats in the proportion of total immune cells (CD45+) in the dura (Figure 4A, "Intact, naïve" groups), there was a significant ($p<0.05$) interaction between sex and stress ("Intact" groups). This was due to a stress-induced increase in dural immune cells present in males, but not females, that was significantly greater ($p<0.05$; n=6) immediately after stress (0 hr) and maintained ($p<0.05$; n=8) for 24 hr after stress.

There was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve animals on the proportion of immune cells in the dura ("Intact, naive" and "SPGNx, naive" groups). This was due to the significant ($p<0.05$) decrease in immune cells with SPGNx in naïve females (n=6), and increase with SPGNx in naïve males (n=6). Folding all additional groups into the analysis revealed a significant ($p<0.05$) interaction between sex, stress, and SPGN innervation on dural immune cells. Importantly, some SPGNx effects were also seen in sham operated rats (Figure 4B).

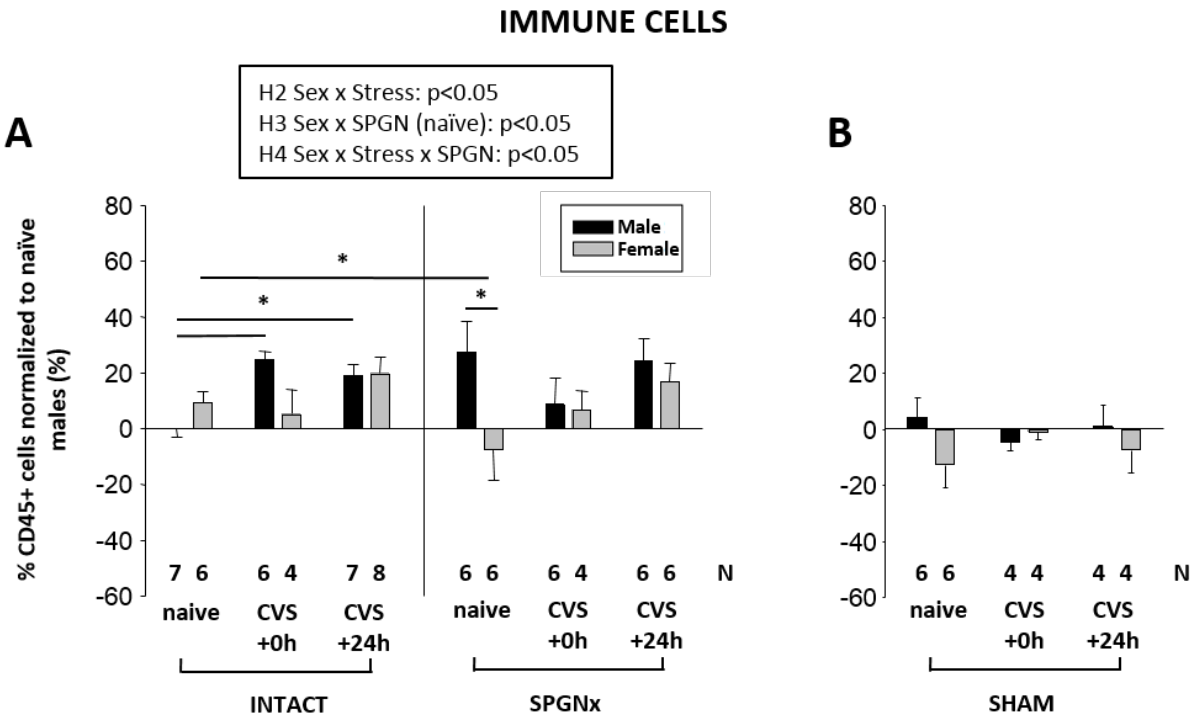


Figure 4. Sex-, stress- and SPGN-dependent changes in the percent of CD45+ immune cells in the dura (relative to the total number of live cells recovered).

Data are presented as a percent of naïve males to facilitate comparisons between groups. (**A**) There was a significant interaction between sex and stress on the proportion of live immune cells in the dura, $p<0.05$ ("intact" groups). Also, there was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve animals on the proportion of immune cells in the dura ("Intact, naive" and "SPGNx, naive" groups). Lastly, there was an interaction between sex, stress, and SPGN innervation ($p<0.05$) on the proportion of immune cells in the dura. (**B**) Similar to male SPGNx groups, male sham operated rats also showed the loss of a stress-induced increase in immune cells. Number of animals per group (N) included above x-axis group labels. (CVS=chronic variable stress; SPGNx= surgical removal of sympathetic post ganglionic neurons; H1=a priori hypothesis 1: sex difference in "Intact, naïve" groups, analyzed with a t-test; H2=a priori hypothesis 2: sex x stress comparison in "Intact" groups, analyzed with a 2-way ANOVA; H3=a priori hypothesis 3: sex x SPGNx in "naïve" groups (ie "Intact" vs "SPGNx" naïve), analyzed with a 2-way ANOVA; H4=a priori hypothesis 4: sex x stress x SPGNx interaction between all groups, analyzed with a 3-way ANOVA). * is $p < 0.05$.

2.3.2.2 Myeloid derived dural immune cell subtypes

Macrophages/monocytes, granulocytes, mast cells: The proportion of Macs was significantly ($p<0.05$) higher in the dura from intact naïve females than intact naïve males (Figure 5A). There was also a significant ($p<0.05$) interaction between sex and stress on the proportion of Macs in the dura. This was due to a stress-induced increase in Macs present in males, but not females, that was significantly greater immediately after stress ($p<0.05$) and maintained with a 24 hr delay after stress ($p<0.01$), compared to naïves. Additionally, there was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve rats on the proportion of Macs in the dura. The increase in Macs in naïve SPGNx males was significant ($p<0.05$), where there was only a trend ($p=0.056$) toward a decrease in Macs in naïve SPGNx females. Of note, while the trend in the decrease in naïve SPGNx females was observed in the sham surgery group, the significant increase Macs in naïve SPGNx males was not (Figure 5B). Lastly, there was a significant ($p<0.05$) interaction between sex, stress, and SPGN innervation on Macs in the dura (Figure 5A), although some SPGNx effects were also seen in sham operated rats (Figure 5B).

Dendritic cells: There was a significant ($p<0.05$) interaction between sex and stress on the proportion of dural DC cells (Figure 5C). This was due to a stress-induced increase in DCs present in males, but not females, which was significant ($p<0.001$) immediately after stress. There was also a significantly ($p<0.05$) higher proportion of DCs in naïve males than females following SPGNx. Lastly, there was a significant ($p<0.05$) interaction between sex, stress and SPGNx on the proportion of dural DCs. The stress-induced increase in DCs in SPGNx females was significant ($p<0.001$) immediately after stress. Interestingly, in sham rats the stress induced increase in DCs was only significant with a 24hr delay following stress (Figure 5D).

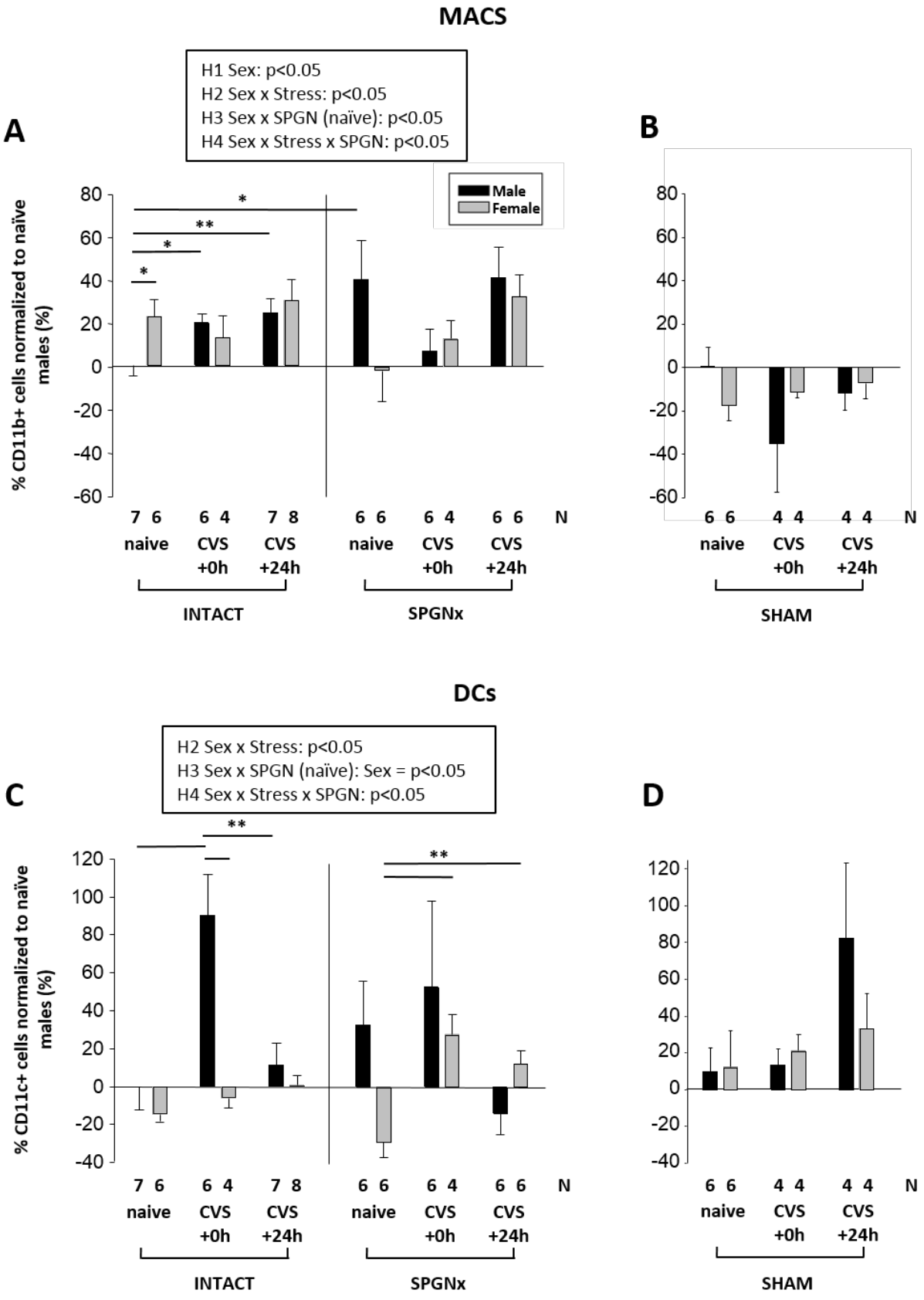

MACS
H1 Sex: p<0.05
H2 Sex x Stress: p<0.05
H3 Sex x SPGN (naïve): p<0.05
H4 Sex x Stress x SPGN: p<0.05
A
B
Male
Female
% CD11b+ cells normalized to naïve males (%)
7 6 6 4 7 8 6 6 6 4 6 6 N
naive CVS +0h CVS +24h naive CVS +0h CVS +24h
INTACT
SPGNx
6 6 4 4 4 4 N
naive CVS +0h CVS +24h
SHAM
DCs
H2 Sex x Stress: p<0.05
H3 Sex x SPGN (naïve): Sex = p<0.05
H4 Sex x Stress x SPGN: p<0.05
C
D
% CD11c+ cells normalized to naïve males (%)
INTACT
SPGNx
SHAM

Figure 5. Sex-, stress- and SPGN-dependent changes in the percent of myeloid derived immune cells dissociated from the dura (relative to the total number of live cells from each dura).

Data are presented as a percent of naïve males for comparison. (**A**) The proportion of Macs was significantly ($p<0.05$) higher in the dura from intact naïve females than males. There was a significant ($p<0.05$) interaction between sex and stress on the proportion of Macs in the dura, ("Intact" groups). There was also a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve rats on the proportion of Macs in the dura ("Intact, naive" and "SPGNx, naive" groups). Lastly, there was an interaction between sex, stress, and SPGN innervation ($p<0.05$). (**B**) Similar to male SPGNx groups, male sham operated rats also showed the loss of a stress-induced increase in Macs. (**C**) There was a significant ($p<0.05$) interaction between sex and stress on the proportion of dural DC cells ("Intact" groups). Additionally, there was a significant ($p<0.05$) interaction between sex, stress, and SPGNx. (**D**) Interestingly, in sham rats the stress induced increase in DCs was only seen with a delay following stress. Groups, group sizes and a priori hypotheses (H1-4) tested were the same as those in Figure 4. * is $p < 0.05$ and ** is $p < 0.01$.

2.3.2.3 Lymphoid derived dural immune cell subtypes

T-cells: There was a significant ($p<0.05$) interaction between sex and stress (Figure 6A) on the proportion of dural T-cells. This was due to a stress-induced increase in T cells present in females, but not males, that was significantly greater 24 hrs after stress than immediately after stress ($p<0.05$) or naïves ($p<0.01$). Additionally, there was a significant main effect ($p<0.05$) of SPGN innervation. This was due to an increase in the proportion of T cells in the dura from SPGNx than intact rats. Lastly, the sex and stress interaction and SPGN main effect persisted with all groups folded into the analysis. Some SPGNx effects were again seen in sham operated rats (Figure 6B).

NKT cells: The variability was large for the minor subgroup of T cells, NKT cells, likely due to the overall low number of NKT cells in the dura. Nevertheless, there was a significant ($p<0.05$) main effect of sex on the proportion of NKT cells in intact groups (Figure 6C). This was due to a smaller proportion of NKT cells in females than males. This overall pattern was maintained in SPGNx rats and as a result, SPGN innervation had no significant effect on NKT cells. However, some SPGNx effects were also seen in sham operated rats (Figure 6D).

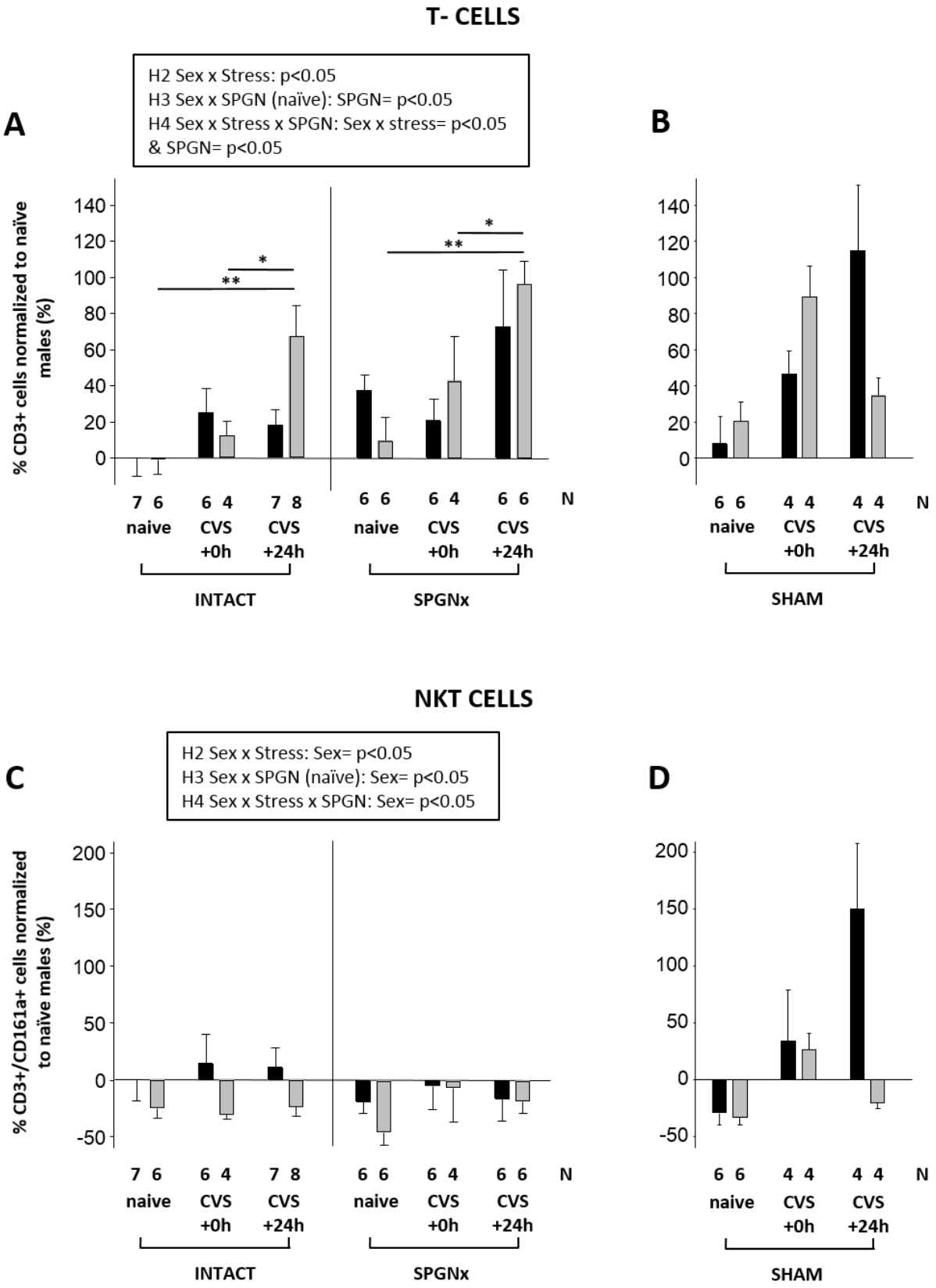
Natural killer cells: The variability was considerably larger for NK cells than any other immune cell type studied. Nevertheless, we were still able to detect a significant ($p<0.05$) sex by stress interaction when all groups were folded into the analysis (Figure 6E). This was due to a significant ($p<0.001$) increase in NK cells immediately after stress in females. Neither SPGNx, nor sham surgery (Figure 6F), appeared to affect NK cells.

B- cells: The relative proportion of B-cells was significantly ($p<0.05$) lower in the dura from intact naïve females than males (Figure 6G). This sex difference persisted as a main effect

($p<0.05$) after folding in stress groups to the analysis. Additionally, when folding in all groups there was a significant ($p<0.05$) main effect of SPGN innervation. The decrease in the proportion of B-cells across all SPGNx groups was not detected in the sham surgery groups (Figure 6H).

2.3.2.4 "Unidentified" dural immune cell subtypes

When all groups were folded into the analysis, the proportion of "unidentified" immune cells in females was significantly ($p<0.05$) greater than in males (Figure 7A). No significant influence of sham surgery was detected on "unidentified" cells (Figure 7B).


T- CELLS
H2 Sex x Stress: p<0.05
H3 Sex x SPGN (naïve): SPGN= p<0.05
H4 Sex x Stress x SPGN: Sex x stress= p<0.05
& SPGN= p<0.05
A
B
% CD3+ cells normalized to naïve males (%)
140
120
100
80
60
40
20
0
*
**
*
**
7 6
naive
6 4
CVS
+0h
7 8
CVS
+24h
6 6
naive
6 4
CVS
+0h
6 6
CVS
+24h
N
INTACT
SPGNx
6 6
naive
4 4
CVS
+0h
4 4
CVS
+24h
N
SHAM
NKT CELLS
H2 Sex x Stress: Sex= p<0.05
H3 Sex x SPGN (naïve): Sex= p<0.05
H4 Sex x Stress x SPGN: Sex= p<0.05
C
D
% CD3+/CD161a+ cells normalized to naïve males (%)
200
150
100
50
0
-50
7 6
naive
6 4
CVS
+0h
7 8
CVS
+24h
6 6
naive
6 4
CVS
+0h
6 6
CVS
+24h
N
INTACT
SPGNx
6 6
naive
4 4
CVS
+0h
4 4
CVS
+24h
N
SHAM

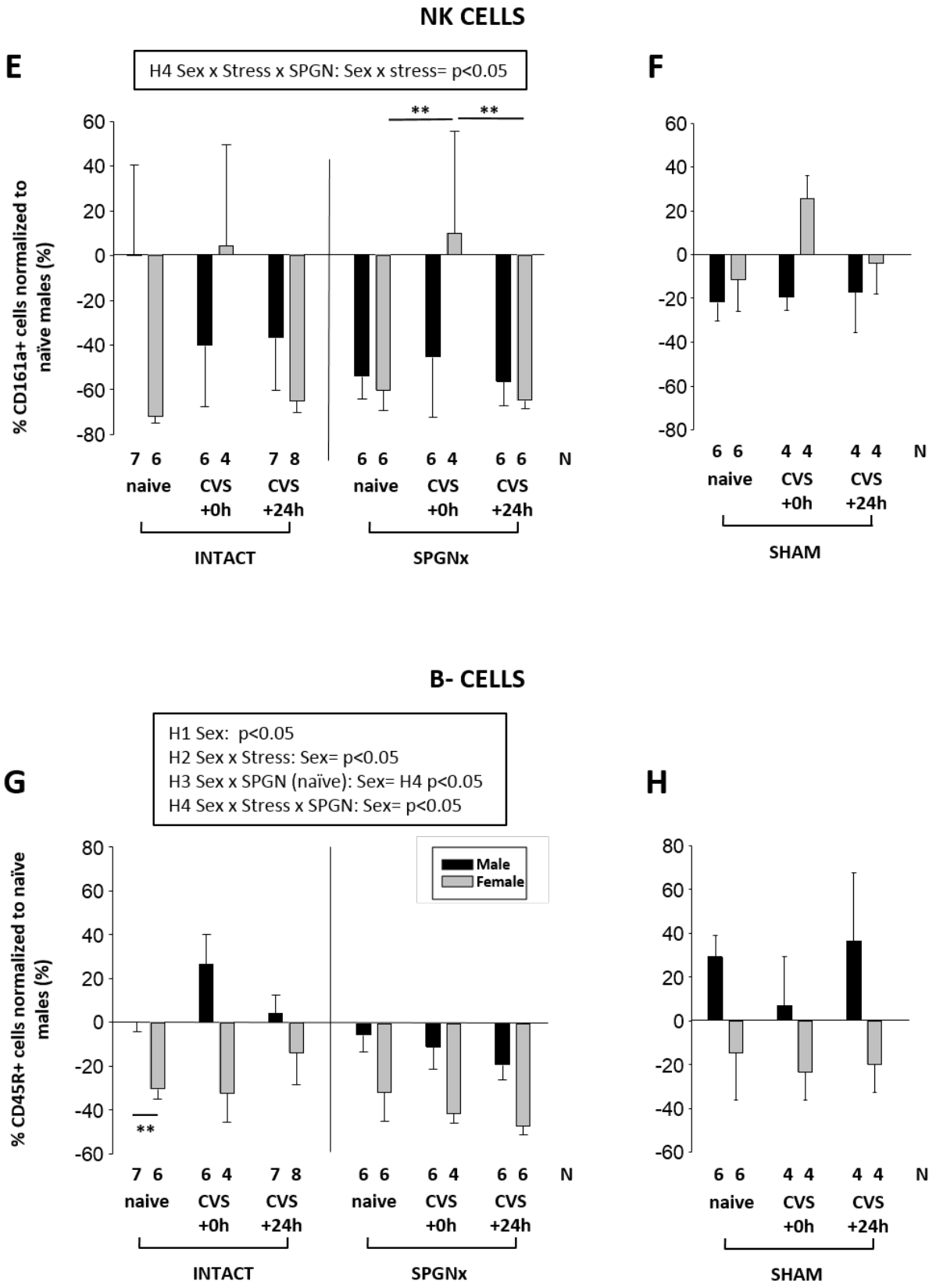

NK CELLS
E
H4 Sex x Stress x SPGN: Sex x stress= p<0.05
F
% CD161a+ cells normalized to naïve males (%)
**
**
7 6 naive
6 4 CVS +0h
7 8 CVS +24h
6 6 naive
6 4 CVS +0h
6 6 CVS +24h
N
INTACT
SPGNx
6 6 naive
4 4 CVS +0h
4 4 CVS +24h
N
SHAM
B- CELLS
G
H1 Sex: p<0.05
H2 Sex x Stress: Sex= p<0.05
H3 Sex x SPGN (naïve): Sex= H4 p<0.05
H4 Sex x Stress x SPGN: Sex= p<0.05
H
% CD45R+ cells normalized to naïve males (%)
Male
Female
**
7 6 naive
6 4 CVS +0h
7 8 CVS +24h
6 6 naive
6 4 CVS +0h
6 6 CVS +24h
N
INTACT
SPGNx
6 6 naive
4 4 CVS +0h
4 4 CVS +24h
N
SHAM

Figure 6. Sex-, stress- and SPGN-dependent changes in the percent of lymphoid derived immune cells dissociated from the dura (relative to the total number of live cells from each dura).

Data are presented as a percent of naïve males for comparison. (**A**) There was a significant ($p<0.05$) interaction between sex and stress on the relative proportion of T-cells ("Intact" groups). Additionally, there was a significant main effect ($p<0.05$) of SPGN innervation in naïve rats. Lastly, the sex and stress interaction and SPGN main effect persisted with all groups folded into the analysis. (**B**) Comparable stress-induced changes in T-cells were observed in sham surgery groups as in the SPGNx groups. (**C**) There was a main effect of sex ($p<0.05$) on the proportion of NKT cells. (**D**) Sham operated groups showed a stress effect in males, which may have been masked by SPGNx. (**E**) There was a significant ($p<0.05$) sex and stress interaction when all groups were folded into the analysis of NK cells. (**F**) Sham surgery did not appear to affect NK cells. (**G**) The proportion of B-cells was significantly ($p<0.001$) lower in the dura from intact naïve females than males, and this sex difference persisted as a main effect ($p<0.05$) after folding in stress groups to the analysis ("Intact" groups). Additionally, when all groups were folded in, there was a significant ($p<0.05$) main effect of SPGN innervation. (**H**) Sham surgery did not appear to affect B-cells. Groups, group sizes and a priori hypotheses (H1-4) tested were the same as those in Figure 4. * is $p < 0.05$ and ** is $p < 0.01$.

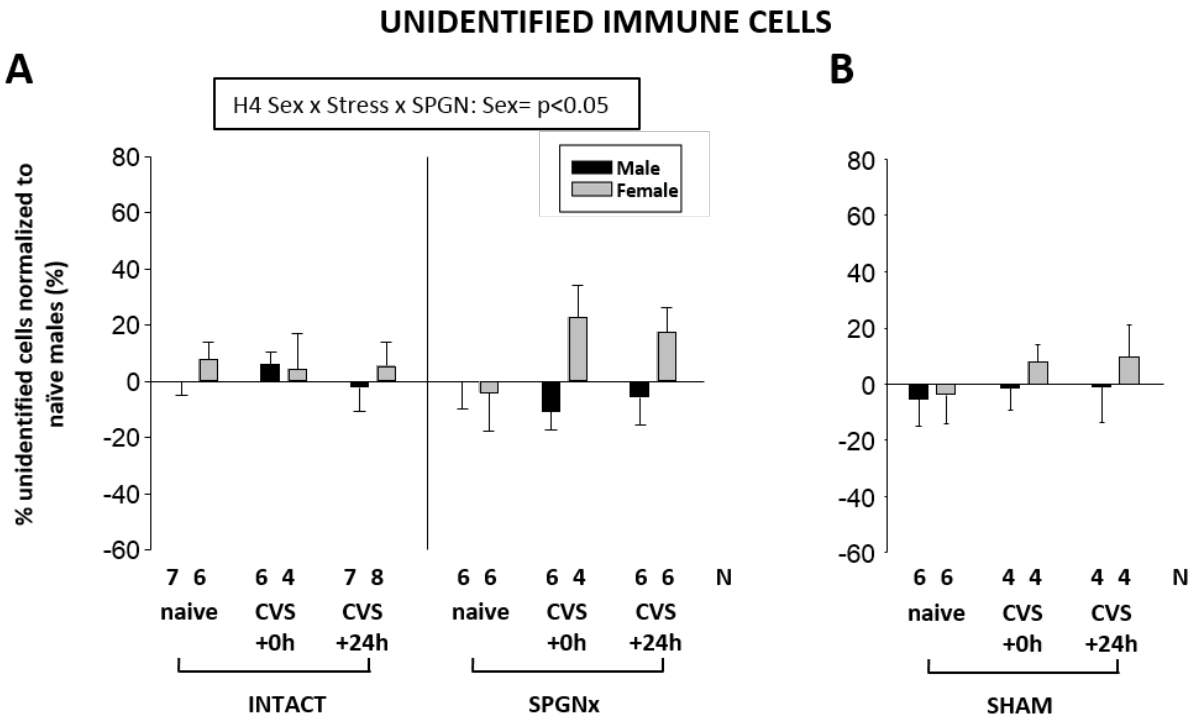


Figure 7. Sex-, stress- and SPGN-dependent changes in the percent of unidentified immune cells dissociated from the dura (relative to the total number of live cells from each dura).

Data are presented as a percent of naïve males for comparison. (**A**) With all groups folded into the analysis, there was a main effect of sex ($p<0.05$) on the proportion of "unidentified" immune cells. (**B**) Sham surgery did not appear to affect "unidentified" cells. Groups, group sizes and a priori hypotheses (H1-4) tested were the same as those in Figure 4.

2.4 DISCUSSION

The purposes of this study were twofold: 1) to characterize the identity and proportion of immune cells present in the dura; and 2) to determine how the proportion of immune cells in the dura was influenced by sex, stress and/or SPGN innervation. Using a combination of flow-cytometry, immunohistochemistry and FACS of rat dura, we found that ~17% of the total cells in the dura were immune cells, composed of at least 6 subtypes: myeloid derived Macs and DCs, and lymphoid derived T-, NKT, NK, and B-cells, identified by lineage specific cell surface markers, morphology, and histology. These 6 immune cell types accounted for ~92% of the total dural immune cells. The relative proportion of these immune cell types was influenced sex, stress, and SPGN innervation. There were significantly more myeloid derived Macs and less lymphoid derived B-and NKT cells in the dura of females compared to males. There was a significant influence of stress on the proportion of myeloid derived Macs and DCs in males but not females: both immune subtypes were increased by stress in males, and this increase was maintained for at least 24 hrs following stress for the Macs. In contrast, lymphoid derived T-cells were significantly increased in females with a 24 hr delay following stress.

Previous data indicate that the dura is enriched with resident myeloid derived mast cells (Dimlich et al., 1991, Rozniecki et al., 1999, Strassman et al., 2004, Levy et al., 2007) and macrophages (McMenamin, 1999, Reuter et al., 2001, McMenamin et al., 2003, Zhang et al., 2009). Our observations extend these previous results in three important ways. First, we quantified the relative proportion of resident immune cells in the dura. Second we described the

impact of sex, stress and SPGN innervation on the proportion of these cells. And third, our results clearly indicate that in addition to the classical resident immune cells, a wide array of immune cells traditionally described as "recruited" are also present. Recruitment versus local proliferation may be distinguished in future flow cytometry experiments with lineage markers. Previously, lymphoid-derived immune cells have only been described in the dura under pathological conditions such as meningitis (Rossi et al., 2004, Nakamura et al., 2007, Tokushige et al., 2012) or tumors (Fang et al., 2013). Because we minimized vascular contamination of immune cells by removing blood prior to tissue collection and demonstrated the presence of NK and T-cells in situ with IHC, our results strongly argue against the possibility that the presence of lymphoid derived immune cells in the dura was an artifact.

The localization of both lymphoid- and myeloid-derived immune cells near vasculature and large nerve bundles (not shown) may enable these cells to play a role in initiation, as well as resolution of migraine pain. We suggest that this is true for all the immune cell types detected, despite the relatively small numbers in which some types were present. Indeed, mast cell degranulation drives dural afferent activity (Levy et al., 2007), despite the fact that this subpopulation of immune cells appears to represent only ~2.4% of immune cells in the dura as estimated by the results of our cytological analysis and toluidine blue staining (Fig 5).

The available evidence suggests that the relative distribution of immune cell types not only varies between tissue types, but even within a tissue type. For example, differences in the number and/or proportion of immune cell types in ear, footpad, back skin and tail have been described (Tong et al., 2015). Similarly, there are differences in the proportion of immune cells types in the ascending and descending colon (Braak et al., 2012). Although Macs are consistently the most abundant resident immune cell type in peripheral tissue, the relative ratios

of T-cells:Macs and Mast cells:Macs appear to be larger in colon (Braak et al., 2012)>skin (Tong et al., 2015)>dura (our study). These differences are likely to reflect the unique environmental demands on various tissues, but may also contribute to the differences in the relative contribution of immune cells to chronic pain disorders.

Clearly, dura is not the only tissue in which immune cells have either been implicated in pathophysiology or in which immune cells have been shown to be regulated by sex and/or stress. For example, irritable bowel syndrome (IBS) and interstitial cystitis are both more prevalent in women than in men and the pain of both of these syndromes are exacerbated by stress. In both cases, immune cell activation has been implicated in the stress-induced increase in pain. Immediately following stress we detected increases in myeloid derived immune cells (Macs, DCs), although the prevailing theory of stress effects on the immune systems is that acute stress (minutes) activates the immune system whereas chronic stress (weeks to months) is immunosuppressive (Dhabhar, 2009, Hall et al., 2012). More recent evidence suggests that the immune system can be locally depressed in the skin during any length of a stressor and is activated following stress cessation (Neeman et al., 2012). However, due to the relatively tight temporal link between stress and other pain disorders, changes in immune cells over time following the termination of a stressor, have not been described in detail in these other tissue types.

While performed as a control, the results from the sham surgery groups were interesting in several respects. Our data showed differential immune responses to sham surgery. There are clear examples of a stressor sensitizing or priming the immune system response to a subsequent stress (Frank et al., 2012, Weber et al., 2013), and this may explain the enhanced responses in most of the immune cell subtypes with sham surgery. However surgery, as a stressor, is well

documented to result in immunosuppression for less than one week (Cristaldi et al., 1997, Leaver et al., 2000, Hogan et al., 2011, Marik and Flemmer, 2012). That any effect would last for 12 days in the sham groups was unexpected. Ultimately, the relative impact of SPGN innervation on stress-induced changes in dural immune cells was confounded by the influence of surgery, per se, as suggested by the results obtained with the sham surgery group. However, there were still cases of marked differences between SPGNx and sham surgery groups, implicating a role of SPGN in the regulation of dural immune cells. This was most clearly evident in the proportion of Macs, DCs, and T-cells, which were significantly higher in SPGNx naïve males but not females.

Our observations raise several new areas of investigation concerning the mechanisms underlying the impact of sex, stress and SPGN innervation on immune cells in the dura. Given evidence of gonadal hormone regulation of immune cells (Oertelt-Prigione, 2012, Pennell et al., 2012, Yakimchuk et al., 2013), it will be important to determine the extent to which differential regulation of immune cells in the dura is dependent on gonadal hormones. To begin to address this issue, we analyzed uterus weights as an indirect measure of cycling in the rats (Ji et al., 2008). Although a subtype of migraine is correlated with the menstrual cycle in humans, we did not find correlations between immune cell subtypes and uterus weights of rats, suggesting that hormone fluctuations associated with the estrus cycle in female rats was not likely responsible for the sex differences described observed in the dura (data not shown). Additionally, with evidence of adrenergic regulation of immune cells as well, it will be important to determine if norepinephrine is the mediator primarily responsible for the impact of SPGN innervation on immune cells. However, given our evidence of several immune cell types influenced by stress

but not SPGN innervation or in which there was an interaction between stress and SPGN innervation, additional mediators are likely to contribute to the regulation of dural immune cells. While the full implications of the dynamic regulation of dural immune cells will ultimately require functional analysis, the changes observed in this study have at least two important implications. First, the observation that there is a sex difference in the stress-induced increase in dural immune cells suggests that it may not only be possible, but necessary to use different strategies for the most effective treatment of migraine in men and women. Second, our observations provide the first evidence implicating T-cells in migraine pathogenesis, where the changes observed may not only account for the sex difference in the manifestation of migraine, but the delay in the initiation of a migraine attack following a period of stress.

3.0 SEX-, STRESS-, AND SYMPATHETIC POST-GANGLIONIC NEURON-DEPENDENT CHANGES IN THE EXPRESSION OF PRO- AND ANTI-INFLAMMATORY MEDIATORS IN RAT DURAL IMMUNE CELLS

Migraine attacks are associated with sterile inflammation of the dura. Immune cells are a primary source of inflammatory mediators, and we therefore sought to further explore the link between dural immune cells and migraine. Based on the observations that migraine is more common in women than in men, stress is the most common trigger for a migraine attack, and sympathetic post-ganglionic innervation of the dura enables local control of dural immune cells, we hypothesized that stress shifts the balance of inflammatory mediator expression in dural immune cells toward those that trigger a migraine attack, where these changes are larger in females and dependent, at least in part, on sympathetic post-ganglionic innervation of the dura. Our objective was to test this hypothesis. Dura were obtained from naïve or stressed, intact or surgically sympathectomized, adult male and female rats. Dura were assessed immediately or 24 hrs after termination of four continuous days of unpredictable, mild stressors. Following enzymatic digestion of each dura, myeloid and lymphoid derived dural immune cells were isolated by fluorescence activated cell sorting for semi-quantitative polymerase chain reaction analysis. In myeloid derived dural immune cells there was an increase in pro-inflammatory mediator mRNA following stress, particularly in females, which remained elevated with a 24 hr delay after stress. There was a stress-induced decrease in anti-inflammatory mediator mRNA

immediately after stress in females, but not males. The stress-induced changes were attenuated in sympathectomized females. In lymphoid derived dural immune cells, there was a persistent increase in pro-inflammatory mediator mRNA following stress, particularly in females. A stress-induced increase in anti-inflammatory mediator mRNA was also observed in both males and females, and was further attenuated in sympathectomized females. Consistent with our hypothesis, there is a stress-induced shift in the balance of pro- and anti-inflammatory mediator expression in dural immune cells that is more pronounced in females, and is dependent, at least in part, on sympathetic post-ganglionic innervation in females. This shift in the balance of inflammatory mediator expression may not only play an important role in triggering migraine attacks, but suggests it may be possible, if not necessary to employ different strategies to most effectively treat migraine in men and women.

3.1 INTRODUCTION

While still an area of active debate, there continues to be compelling evidence in support of sterile inflammation of the dura as an underlying mechanism of a migraine attack (Waeber and Moskowitz, 2005). Pro-inflammatory mediators are increased in plasma and cerebrospinal fluid during migraine attacks (Sarchielli et al., 2000, Perini et al., 2005, Fidan et al., 2006, Sarchielli et al., 2006), and application of these mediators can activate and/or sensitize dural afferents in rats (Strassman et al., 1996, Harriott and Gold, 2009, Zhang et al., 2011, Yan et al., 2012). Additionally, stimuli such as glyceryl trinitrate, which can evoke a delayed migraine in migraineurs but not controls (Thomsen et al., 1994), are associated with an increase in the expression and release of pro-inflammatory mediators in the dura (Reuter et al., 2001). Immune

cells are one primary source of pro- and anti-inflammatory mediators in the dura, and in the present study we therefore began to explore immune cell regulation of inflammation in the dura and thus migraine pain.

To assess the link between immune cells and migraine, we focused on three clinical features of migraine. The first is the sex difference in the prevalence of migraine, which is present in women at a rate roughly three times that seen in men (Pietrobon and Moskowitz, 2013). The second is stress. Over 80% of migraineurs report that their attacks are commonly triggered by stress, and 60% report it as their main trigger (Martin, 2010) Importantly, the stress-exacerbation of migraine has a unique temporal pattern, where attacks often occur during the relaxation phase after stress (Sauro and Becker, 2009). The third feature is sympathetic dysregulation, where sympathetic hypofunction is often observed in migraineurs (Peroutka, 2004), and α-adrenergic agonists and β-adrenergic antagonists can decrease the frequency of migraine attacks (Goadsby and Sprenger, 2010). Further evidence suggesting sympathetic post-ganglionic neurons (SPGNs) may contribute to the link between immune cells and migraine includes: 1) the dura is richly innervated by SPGNs (Keller et al., 1989, Harriott and Gold, 2008), enabling local control of resident immune cells, 2) SPGN fibers are activated by stress, and 3) the 5-HT1D receptor, a primary target for triptans, is present on these neurons (Harriott and Gold, 2008). Each of these features may influence immune cells either directly or indirectly (Ignatowski et al., 1996, Shakhar et al., 2000, Szelenyi et al., 2000, de Coupade et al., 2004, Barker et al., 2005, de Coupade et al., 2007, Leposavic et al., 2008, Pennell et al., 2012, Sanders, 2012, Yakimchuk et al., 2013).

Thus, we hypothesized that stress shifts the balance of inflammatory mediator expression in dural immune cells toward those that trigger a migraine attack, where these changes are larger

in females and dependent, at least in part, on SPGN innervation of the dura. To begin to test this hypothesis, we analyzed the mRNA expression of pro- and anti-inflammatory mediators in immune cells in the dura of male and female rats. The impact of stress was assessed by comparing immune cells in naïve rats to those immediately, or with a delay, following the termination of chronic stress. Finally, to determine whether local SPGN signaling may contribute to dural immune cell regulation, naïve and stressed rats were studied following SPGN denervation of the dura.

3.2 METHODS

3.2.1 Animals

Adult male (200-300g; n=35) and female (150-250g; n=40) Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were used for all experiments. Animals were housed in the University of Pittsburgh Association for the Assessment and Accreditation of Laboratory Animal Care approved animal facility two per cage on a 12:12 light:dark cycle with food and water ad lib unless indicated. Procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2.2 Chronic variable stress (CVS)

Animals were exposed to chronic variable stress (CVS), consisting of an ongoing series of "mild" stressors applied for variable durations over a period of 4 days. The paradigm was adapted from unpredictable chronic mild stress models of depression (Grippo et al., 2004), and was compressed from 3 weeks to 4 days. This allowed us to model the length of stressors that typically trigger migraines (i.e., stress from a workweek or studying for an exam). The presentation of many different kinds of stressors for different lengths of time over the 4 days allowed us to mimic a more complex, non-habituating stress. Additionally, with stressors presented constantly (as opposed to "repeated" chronic stress models where one stressor is presented for a short period of time for multiple days), this design allowed us to model the constant stress that typically triggers a migraine, as well as create a clear "relaxation period" following the stress during which migraines typically occur.

After arrival, rats were housed in a "home" room for at least 6 days, and subsequently subjected to CVS in a separate, isolated housing room. Rats were single-housed, an average of 2 stressors were presented at any given time, and each stressor was presented twice over the 4 days. The schedule was semi-randomized to ensure that the same stressors were not always paired together or back-to-back. Nine different stressors were used and these included: food deprivation (12-16 hrs each), water deprivation (12-16 hrs each), overnight illumination (no 12hr dark cycle), cage tilt (18° angle, 8-16 hrs, once on each of the short and long cage axis), paired housing with a stranger (8-16 hrs each), damp bedding (12-16 hrs each), white noise (85dB of white noise, 3-5 hrs each), strobe light (1 Hz, 4-6 hrs each), and predator odor (two $5cm^2$ pieces of cloth from predator cages were hung in rat cages, 30 min ferret odor once and 1 hr cat odor

once). Rats with a delay period of "relaxation" after CVS were returned to the home housing room for that time.

It is important to note we are not proposing that CVS is a model of migraine, nor do we anticipate that this stress paradigm causes migraine in rats. Rather we have proposed to study changes in response to stress because we hypothesize that these changes may contribute to the initiation of a migraine attack in migraineurs.

3.2.3 Bilateral superior cervical ganglion sympathectomy (SPGNx)

One week prior to any experimentation, a group of animals underwent bilateral surgical removal of superior cervical ganglia, the cell bodies giving rise to sympathetic innervation of the dura. Animals were deeply anesthetized with an intraperitoneal injection of anesthetic cocktail [55 mg/kg ketamine (Fort Dodge, Fort Dodge, IA), 5.5 mg/kg xylazine (LLOYD, Shenandoah, IA), and 1.1 mg/kg acepromazine (Butler Schein, Dublin, OH)]. Superior cervical ganglia were accessed via a midline incision through the skin overlaying the trachea followed by blunt dissection of the muscles and connective tissue on either side of the trachea. Following excision of the ganglia and visual confirmation of hemostasis, the incision site was closed. Prior to recovery from anesthesia, rats received an intramuscular injection of buprenorphine (0.03 mg/kg; Reckitt Benckiser Pharmaceuticals, Richmond, VA).

3.2.4 Isolation of dural cells

Prior to dural tissue collection, animals were deeply anesthetized with an intraperitoneal injection pentobarbital (60-70 mg/kg; Vortech Pharmaceuticals, Dearborn, Michigan, USA)

followed by a rapid transcardiac perfusion with 60mL cold 1x phosphate-buffered saline (PBS; pH 7.2).

Following careful removal of occipital and parietal bones, dura extending from -4 to 15mm (caudal to rostral of the convergence of the sinuses) and ±11mm (lateral) was collected and placed in 5mL of Dulbecco's Modified Eagle Medium-F12+ [Advanced DMEM-F12 (Gibco, Grand Island, NY), to which 10 U/mL each of penicillin and streptomycin (Invitrogen, Carlsbad, CA), 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Calbiochem, San Diego, CA), and 2 mM L-glutamine (Invitrogen), were added] with 0.1% Collagenase P (Roche, Indianapolis, IN) in a sealed 60x15mm glass Petri dish and incubated for 1hr at 37°C in a shaking water bath. Dura were then mechanically dissociated in this solution with fire-polished Pasteur pipettes, centrifuged at 250g, resuspended in 5 mL fresh DMEM-F12+, centrifuged again, and resuspended in 1mL staining media [(SM); Ca^{2+}/Mg^{2+} free Dulbecco's PBS (Lonza, Walkersville, MD), 3% heat inactivated newborn calf serum, 1mM EDTA (ethylenediaminetetraacetic acid), 0.02% sodium azide]. The final cell suspension was filtered through 50µm mesh (Sefar, Heiden, Switzerland) and subsequently processed. This protocol was adapted from (Borghesi et al., 2005, Steenhuis et al., 2008, Santos et al., 2014). Cell viability was uniformly >80%.

3.2.5 Fluorescence activated cell sorting (FACS)

FACS, with the gating strategy illustrated in Figure 8, was used to collect myeloid and lymphoid populations of immune cells from each dissociated dura. Cells were first blocked with 10% rat serum for 10min to minimize non-specific binding of antibodies, resuspended in SM, and then incubated in primary antibodies for 20min. They were then washed in SM 3 times prior to a

final resuspension in 1µg/mL propidium iodide (PI; Molecular Probes, Eugene, OR) to exclude dead cells (viability), and immediately sorted. Post sort purity was >97%. Both primary antibodies were mouse monoclonal raised against rat epitopes from BD Biosciences (San Diego, CA) and were used at the following dilutions: APC cy7 CD45 (clone OX-1) 1:100 and PEcy7 CD11b/c (clone OX-42) 1:50. Myeloid cells were defined as $CD45^{+}CD11b^{+}CD11c^{+}$. Lymphoid cells were defined as $CD45^{+}CD11b^{-}CD11c^{-}$. Specificity of the antibodies has been previously documented (Ozaki et al., 2012). Fluorescence minus one antibody controls were used to identify negative staining in dural cells(Herzenberg et al., 2006). FACS was performed on a 3 laser, 11 detector FACS Aria (BD Biosciences). Samples were sorted into buffer for qPCR and immediately processed. An average of 132,974 ± 3675 myeloid cells and 45,394 ± 1520 lymphoid cells were sorted per dura.

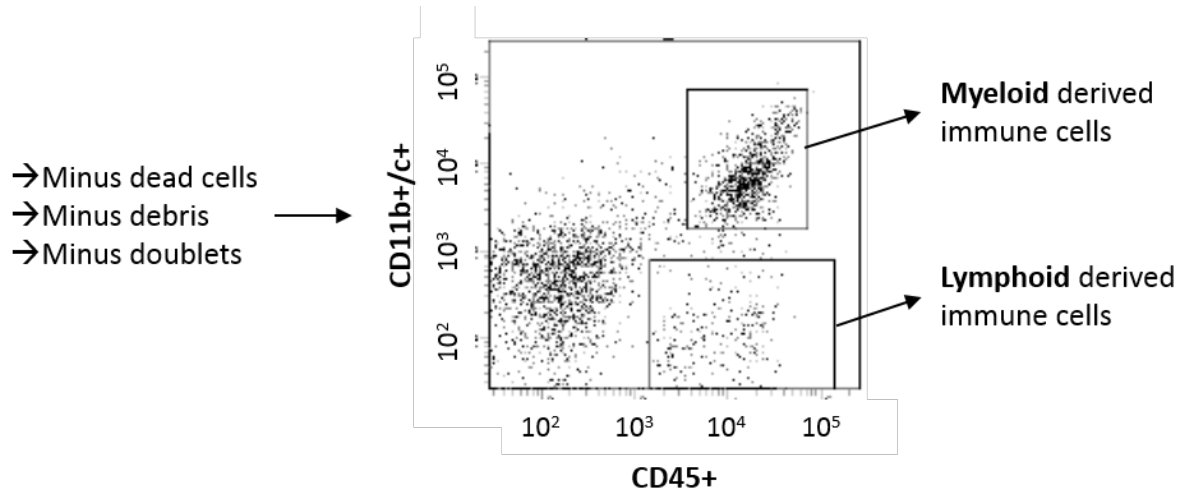


Figure 8. Gating strategy example used for the isolation of myeloid and lymphoid immune cell subtypes from the dura.

Data are from a male naïve rat. The day of the experiment, dural cells were dissociated and stained with antibodies specific to markers of immune cells (CD45+) and of myeloid derived immune cells (CD11b/c+). Non-myeloid derived immune cells (CD45+/CD11b/c-) were considered lymphoid derived immune cells. Viability was uniformly 80-85%, as determined by PI and trypan blue staining. Spleen cells were used for compensation controls. This resulted in an average of 132,974 ±3675 (n=75) myeloid cells and 45,394 ±1520 (n=75) lymphoid cells sorted per dura. Image from Diva software.

3.2.6 Real time polymerase chain reaction (qPCR)

RNA extraction was performed according to RNeasy® Micro Kit (Qiagen, Hilden, Germany) specifications. Briefly, cells were sorted directly into 350µL of RLT buffer with 10µL/mL β-mercaptoethanol. Samples were homogenized by passing the lysate 6 times through a 1mL syringe with a 27G needle followed by 30s of vortexing. One volume of 70% ethanol was added and the sample spun through a column. The column was washed, treated with DNase 1 for 15min, washed with buffer followed by 80% ethanol, and RNA eluted from the column in 20µL RNase-free water followed by first stand cDNA synthesis by random-hexamer/oligo-d(T)-primed, Superscript III (Invitrogen)-mediated reverse transcription. Gene expression of pro- and anti-inflammatory mediators was measured by semi-quantitative, real time polymerase chain reaction (qPCR) on a CFX Connect real time cycler (Bio-Rad, Hercules, CA, USA). The targets and sequences of the primer pairs are listed in Table 1. The gene expression of each inflammatory molecule was quantified by linear regression of individual amplification curves using LinReg PCR (Ruijter et al., 2009), and normalized to the quantity of internal reference standard gene transcript Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, or β2 microglobulin (β2M). Normalization to each internal reference produced very similar results. For example, the expression ratio of β-actin to GAPDH in myeloid derived immune cells from naïve intact and stressed (0 hr and 24 hr) males were 0.863 ± 0.002, 0.869 ± 0.005 and 0.861 ± 0.003, respectively, and 0.873 ± 0.003, 0.875 ± 0.004 and 0.873± 0.005, respectively for the same groups of females. Similarly, the expression ratio of β2M to GAPDH was 1.000 ± 0.004, 1.001 ± 0.004 and 1.001 ± 0.002 in the three groups of intact males and 0.994 ± 0.002, 0.994 ± 0.004 and 0.989 ± 0.004 in the intact groups of females. Comparable ratios were observed in the

SPGNx groups and for lymphoid derived cells as well. Because of the small but consistent difference between males and females with respect to the relative expression of all internal reference targets, we did not assess the presence of sex differences in the expression of inflammatory mediators. Rather, we only assessed the impact of sex on the stress and/or SPGNx-induced changes in inflammatory mediator expression. Data are displayed relative to GAPDH.

The pro- and anti-inflammatory mediators studied were chosen based on established links to migraine. Tumor necrosis factor alpha (TNFα), interleukin 1β (IL-1β), and interleukin 6 (IL-6) are potent pro-inflammatory mediators increased in the plasma of migraineurs during an attack (Perini et al., 2005, Fidan et al., 2006, Sarchielli et al., 2006). TNFα (Zhang et al., 2011) and IL-6 (Yan et al., 2012) have been shown to sensitize dural afferents. Additional pro-inflammatory mediators included inducible nitric oxide synthase (iNOS) and cyclooxygenase 1 and 2 (COX1 and COX2). Glyceryl trinitrate, a nitric oxide (NO) donor, is a well-described and reliable trigger for delayed migraine attacks (Thomsen et al., 1994). Additionally, NO metabolites are increased in the plasma during attacks (Sarchielli et al., 2000, Fidan et al., 2006), and there is an increase in NO production in monocytes from the blood of migraine patients (Sarchielli et al., 2006). A marker for NO production, iNOS, was also increased in dural macrophages stimulated with nitroglycerin (Reuter et al., 2001). The pro-inflammatory actions of prostaglandins, primary COX metabolites, have been well described and are upregulated in the plasma of migraineurs during an attack (Sarchielli et al., 2000). Non-steroidal anti-inflammatory drugs, which inhibit the activity of COXs, remain one of the most effective abortive treatments for migraine (Jakubowski et al., 2005).

The anti-inflammatory mediators studied included: interleukin 10 (IL-10), interleukin 4 (IL-4), and pro-opiomelanocortin (POMC). IL-10 and IL-4 are potent anti-inflammatory cytokines released from immune cells, and are upregulated in the plasma of migraineurs during an attack (Munno et al., 1998, Munno et al., 2001, Perini et al., 2005). β-endorphin, an endogenous opioid, is decreased in the plasma of migraineurs during an attack (Leone et al., 1992, Misra et al., 2013). POMC, the precursor peptide for the production of β-endorphin, is produced in immune cells and endogenous opioids can be released from immune cells (Rittner et al., 2005, Rittner and Stein, 2005). Lastly, in a transgenic knock-in mouse model of familial hemiplegic migraine type 1, the trigeminal ganglia expressed increased numbers of activated monocytes and mRNA levels of IL-1β, IL-6, IL-10, and TNFα (Franceschini et al., 2013).

Table 1. Primer sequences (5'→3')

	Forward	Reverse	Accession #	Size (bp)
TNFα	**AGAAACACACGAGACGCTGA**	**ACTCAGGCATCGACATTCCG**	**NM_012675.3**	**119**
IL-1β	**ACAAGGAGAGACAAGCAACGAC**	**TCTTCTTTGGGTATTGTTTGGG**	**NM_031512.2**	**140**
IL-6	**ATCTGCTCTGGTCTTCTGGA**	**TTGCTCTGAATGACTCTGGC**	**NM_012589.2**	**98**
iNOS	**GACCAGAAACTGTCTCACCTG**	**CGAACATCGAACGTCTCACA**	**NM_012611.3**	**137**
COX1	**TGACTATCTGACGGGTGACT**	**TTGCTGGACATTGGGTTCTT**	**NM_017043.4**	**156**
COX2	**ATGCTACCATCTGGCTTCGG**	**TGGAACAGTCGCTCGTCATC**	**NM_017232.3**	**88**
IL-4	**CTTACGGCAACAAGGAACAC**	**TTCTTCAAGCACGGAGGTAC**	**NM_201270.1**	**104**
IL-10	**GCATAGAAGCCTACGTGACA**	**TTGGAGAGAGGTACAAACGAG**	**NM_012854.2**	**151**
POMC	**GAACGCCATCATCAAGAACG**	**CTCTAAGAGGCTGGAGGTCA**	**NM_139326.2**	**114**
GAPDH	**CAGCAACTCCCATTCTTCCA**	**GTGGTCCAGGGTTTCTTACT**	**NM_017008.4**	**163**
β-actin	**GCAGGAGTACGATGAGTCCG**	**ACGCAGCTCAGTAACAGTCC**	**NM_031144.3**	**74**
β2M	**TTGTGGCTGGAGGTTTAGTC**	**GCTGCCTTTATACCAACCCT**	**NM_012512.2**	**130**

3.2.7 Data Analysis

We tested three related a priori hypotheses based on clinical features of migraine. The first hypothesis was that stress increases the relative expression of pro- vs. anti-inflammatory mediators in dural immune cells with a delay following the cessation of stress, based on the stress relaxation model of migraine (Sauro and Becker, 2009), and that the stress-induced increase is greater in females than males, based on the higher prevalence of migraine in women than in men (Pietrobon and Moskowitz, 2013). Thus, we examined the influence of both sex and stress on the expression of pro- and anti-inflammatory mediators in myeloid and lymphoid dural immune cells by comparing naïve rats with rats exposed to 4 days of stress (CVS), assessed either immediately following stress (CVS+0h) or with a 24 hr delay following stress (CVS+24h). The choice of a 24 hr delay was based on results from preliminary experiments also including 6 and 12 hr delay groups (N=4 each, female). No significant differences were detected between the 6 and 0 hour groups (data not shown). Larger changes in the expression of both pro- and anti-inflammatory mediators were observed with a 24 hr delay compared to 12 hrs (data not shown), and thus the 6 and 12 hr delay groups were not pursued further. The second hypothesis was that the relative expression of pro- and anti-inflammatory mediators in dural immune cells is dependent on the presence of SPGN innervation, and is more pronounced in females than males, based on evidence of both extensive SPGN innervation of the dura (Keller et al., 1989, Harriott and Gold, 2008) and adrenergic regulation of immune cells (Ignatowski et al., 1996, Szelenyi et al., 2000, Sanders, 2012). To test this hypothesis, we assessed changes in the expression of pro- and anti-inflammatory mediators in dural immune cells from groups of rats surgically sympathectomized (SPGNx) by removal of the superior cervical ganglion bilaterally. Lastly, given that stress drives activity in the SPGN, our third a priori hypothesis was that the stress-

induced increase in the expression of pro- vs. anti-inflammatory mediators in dural immune cells in females is dependent on the presence of SPGN innervation.

The Pfaffl method was used to assess changes in relative expression (Pfaffl, 2001). Groups were each set to the respective male or female naïve group as the "control" in the Pfaffl calculations to account for the slight sex difference in internal comparators. To better visualize the impact of sex, stress and SPGNx on the pattern of gene expression, data were then normalized to the male naïve group for comparison in figures.

Data were analyzed with 2-way analysis of variance (ANOVA) for sex and stress effects (naïve vs. 0, 6, 12 or 24 hrs following CVS), as well as sex and SPGN effects (intact vs. SPGNx) in naïve animals, and 3-way ANOVAs for sex, stress, and SPGNs effects with $p<0.05$ considered significant. The Tukey HSD method was used for post-hoc comparisons if significant interactions were detected.

3.3 RESULTS

Myeloid and lymphoid derived immune cells interact to mediate the innate and adaptive responses of the immune system. We examined the influence of sex, stress and sympathetic innervation on the expression of pro- and anti-inflammatory mediators in these two general populations of immune cells in the dura.

3.3.1 Myeloid derived dural immune cells: pro-inflammatory mediators

TNFα: There was a significant ($p<0.05$) interaction between sex and stress on the relative expression of TNFα in myeloid derived dural immune cells in intact groups (Figure 9A). A significant ($p<0.05$) stress-induced increase in TNFα was present in females, but not males. Post-hoc analysis indicated that the stress-induced increase in TNFα in females was associated with a trend towards an increase immediately after stress ($p=0.057$; $n=7$) as well as with a 24 hr delay following stress ($p=0.054$; $n=6$), compared to naïve ($n=9$). Also, TNFα in females was significantly higher than males ($n=6$) both immediately following stress ($p<0.05$) and with a 24 hr delay ($p<0.01$). Folding all additional groups into the analysis revealed a significant ($p<0.05$) interaction between sex, stress, and SPGN innervation on TNFα expression. Post-hoc analysis indicated that there was a loss of the stress-induced increase in TNFα in females with SPGNx in addition to the emergence of a stress-induced increase in TNFα in males with SPGNx that was significant ($p<0.05$) immediately after stress ($n=5$), and persisted for 24 hrs following stress ($n=6$).

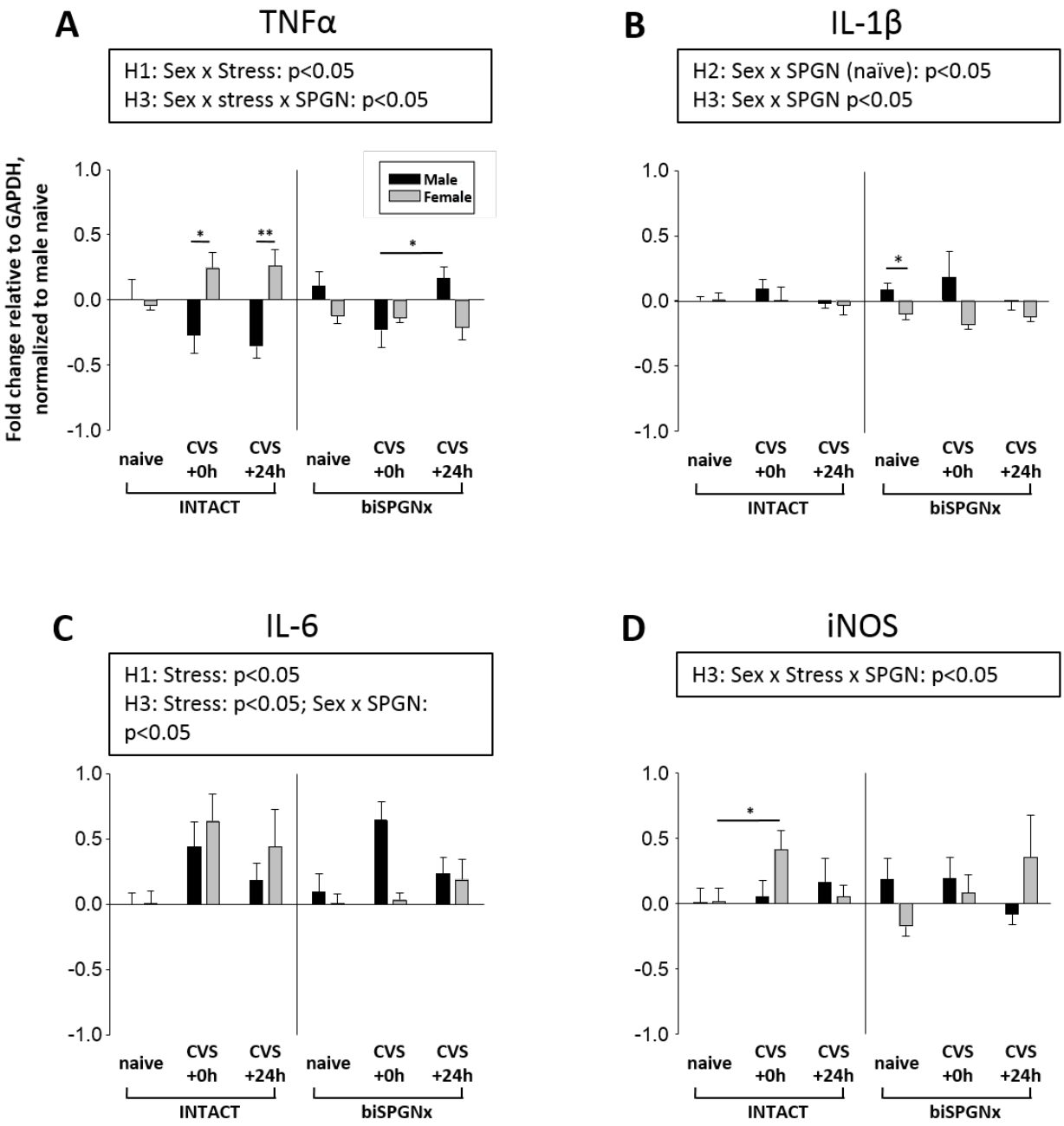
IL-1β: While there was no significant sex or stress effects in intact animals, there was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve animals on the expression of IL-1β in myeloid derived dural immune cells (Figure 9B). Post-hoc analysis indicated that there was a small but significant ($p<0.05$) increase in IL-1β with SPGNx in naïve males compared to females. Folding in all additional groups revealed a significant ($p<0.05$) overall interaction between sex and SPGN innervation. Post-hoc analysis indicated that there was significantly ($p<0.001$) more IL-1β in SPGNx males compared to females, which was not present in intact animals. In females, there also was significantly ($p<0.05$) less IL-1β in SPGNx animals compared to intact.

IL-6: There was a significant ($p<0.05$) main effect of stress on the relative expression of IL-6 in myeloid derived cells in intact groups (Figure 9C). Post-hoc analysis indicated that the increase in IL-6 immediately after stress was significant ($p<0.01$). Folding in all groups revealed a significant ($p<0.05$) interaction between sex and SPGN innervation. Post-hoc analysis indicated that the reduced IL-6 in SPGNx females was significant ($p<0.05$) compared to intact females.

iNOS: There was a significant ($p<0.05$) interaction between sex, stress, and SPGNs on the relative expression of iNOS in myeloid cells (Figure 9D). Post-hoc analysis indicated that the increase immediately after stress in intact females was significant ($p<0.05$), but not in SPGNx females.

COX1: It was only with all groups folded into the analysis that a significant ($p<0.05$) main effect for stress emerged with respect to the expression of COX1 in myeloid cells (Figure 9E). Post-hoc analysis indicated that the increase in COX1 with a 24 hr delay after stress, but not immediately after stress, was significant ($p<0.01$).

COX2: No significant sex, stress, or SPGN effects were present in COX2 expression in myeloid cells (Figure 9F).


A
TNFα
H1: Sex x Stress: p<0.05
H3: Sex x stress x SPGN: p<0.05
Male
Female
Fold change relative to GAPDH, normalized to male naive
1.0
0.5
0.0
-0.5
-1.0
*
**
*
naive
CVS +0h
CVS +24h
naive
CVS +0h
CVS +24h
INTACT
biSPGNx
B
IL-1β
H2: Sex x SPGN (naïve): p<0.05
H3: Sex x SPGN p<0.05
*
naive
CVS +0h
CVS +24h
naive
CVS +0h
CVS +24h
INTACT
biSPGNx
C
IL-6
H1: Stress: p<0.05
H3: Stress: p<0.05; Sex x SPGN: p<0.05
naive
CVS +0h
CVS +24h
naive
CVS +0h
CVS +24h
INTACT
biSPGNx
D
iNOS
H3: Sex x Stress x SPGN: p<0.05
*
naive
CVS +0h
CVS +24h
naive
CVS +0h
CVS +24h
INTACT
biSPGNx

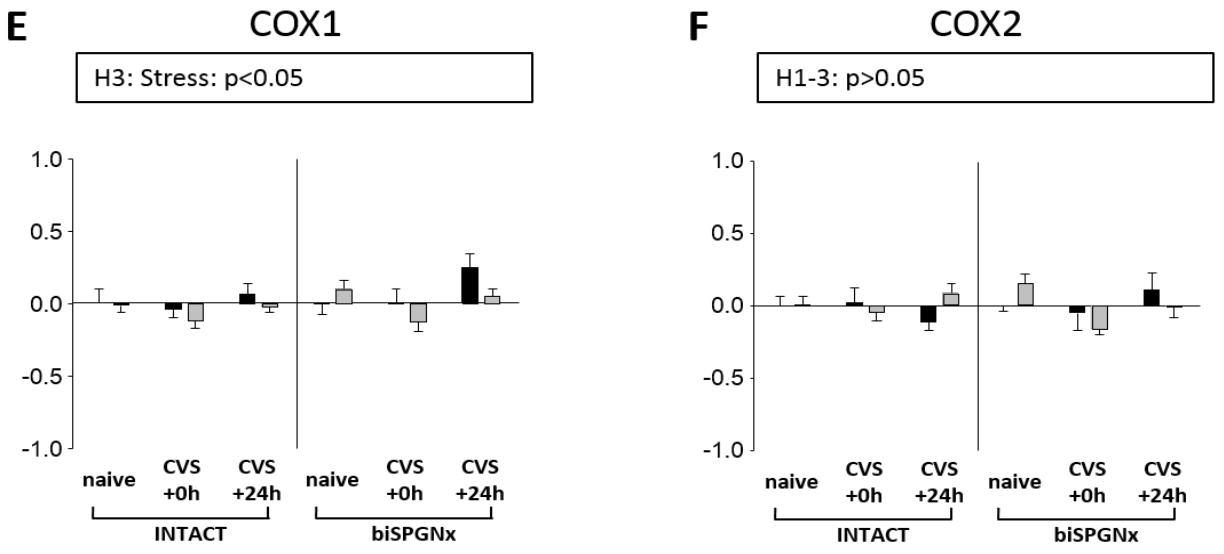


Figure 9. Sex-, stress-, and SPGN- dependent changes in pro-inflammatory mediators in dural myeloid cells.

(**A**) There was a significant ($p<0.05$) interaction between sex and stress on the relative expression of TNFα in myeloid derived dural immune cells ("Intact" groups). Folding in all groups revealed a significant ($p<0.05$) interaction between sex, stress, and SPGN innervation on TNFα expression. (**B**) There was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve animals on the expression of IL-1β ("Intact" vs "SPGNx" naïve groups). Folding in all additional groups revealed a significant ($p<0.05$) overall interaction between sex and SPGN innervation. (**C**) There was a significant ($p<0.05$) main effect of stress on the relative expression of IL-6 ("Intact" groups). Folding in all groups revealed a significant ($p<0.05$) interaction between sex and SPGN innervation. (**D**) There was a significant ($p<0.05$) interaction between sex, stress, and SPGNs on the relative expression of iNOS. (**E**) It was only with all groups folded into the analysis that a significant ($p<0.05$) main effect for stress emerged with respect to the expression of COX1. (**F**) No significant effects were present in COX2 expression. N: naïve (M=6; F=9), CVS+0h (M=6; F= 7), CVS+24h (M=6; F= 6), SPGNx naïve (M=6; F=6), SPGNx CVS+0h (M=5; F=6), SPGNx CVS+24h (M=6; F=6). (CVS=chronic variable stress; SPGNx= surgical removal of sympathetic post ganglionic neurons; H1=a priori hypothesis 1: sex x stress comparison in "Intact" groups, analyzed with a 2-way ANOVA; H2=a priori hypothesis 2: sex x SPGNx in "naïve" groups (ie "Intact" vs "SPGNx" naïve), analyzed with a 2-way ANOVA; H3=a priori hypothesis 3: sex x stress x SPGNx interaction between all groups, analyzed with a 3-way ANOVA) * is $p < 0.05$, and ** is $p < 0.01$.

3.3.2 Myeloid derived dural immune cells: anti-inflammatory mediators

IL-10: There was a significant ($p<0.05$) interaction between sex and stress on the relative expression of IL-10 in myeloid derived dural immune cells in intact groups (Figure 10A). Post-hoc analysis indicated that in females, it was only the stress-induced decrease in IL-10 immediately after stress that was significant ($p<0.01$), while in males, it was only the stress-induced decrease 24 hrs after stress that was significant ($p<0.01$). Folding in all additional groups revealed a significant ($p<0.05$) interaction between sex, stress, and SPGNs. Post-hoc analysis indicated that the stress-induced decrease in IL-10 in intact males was not significant ($p=0.53$) in SPGNx males.

IL-4: IL-4 expression was below the level of detection in myeloid derived immune cells.

POMC: There was a clear sex difference in the expression of POMC, which was below the level of detection in myeloid cells harvested from any groups of females (Figure 10B). With all groups folded into the analysis, there was also a significant ($p<0.05$) interaction between sex and stress. Post-hoc analysis indicated that while small, the stress-induced decrease in males immediately after stress was significant ($p<0.05$).

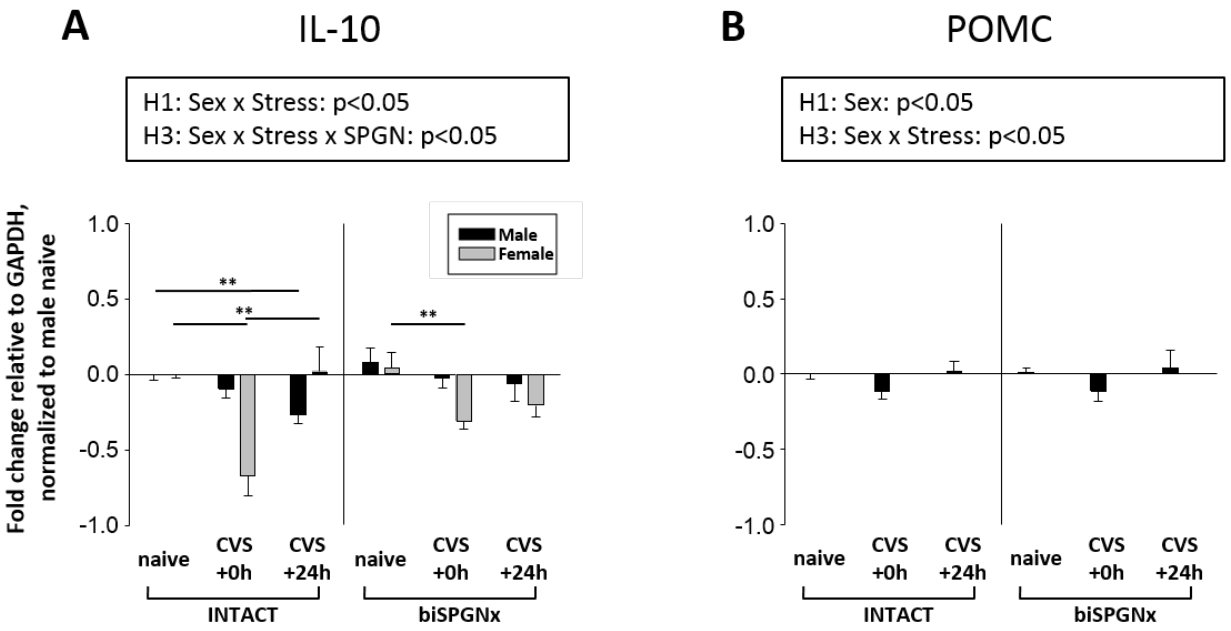


Figure 10. Sex-, stress-, and SPGN- dependent changes in anti-inflammatory mediators in dural myeloid cells.

(**A**) There was a significant ($p<0.05$) interaction between sex and stress on relative expression of IL-10 in myeloid derived dural immune cells ("Intact" groups). Folding in all additional groups revealed a significant ($p<0.05$) interaction between sex, stress, and SPGN. (**B**) There was a clear sex difference in the expression of POMC, which was below the level of detection in myeloid cells harvested from any groups of females. With all groups folded into the analysis, there was also a significant ($p<0.05$) interaction between sex and stress. Group sizes and a priori hypotheses tested were the same as those in Figure 9. ** is $p < 0.01$.

3.3.3 Lymphoid derived dural immune cells: pro-inflammatory mediators

TNFα: There was a significant ($p<0.05$) interaction between sex and stress on the relative expression of TNFα in lymphoid derived dural immune cells in intact groups (Figure 11A). TNFα was significantly higher in females than males immediately following stress ($p<0.05$). Folding all additional groups into the analysis revealed a significant ($p<0.05$) interaction between sex and SPGN innervation. Post-hoc analysis indicated that while the difference between intact females compared to males was significant ($p<0.01$), TNFα levels in SPGNx females and males were comparable. Also, in males, there was significantly ($p<0.05$) more TNFα in the SPGNx group compared to the intact group.

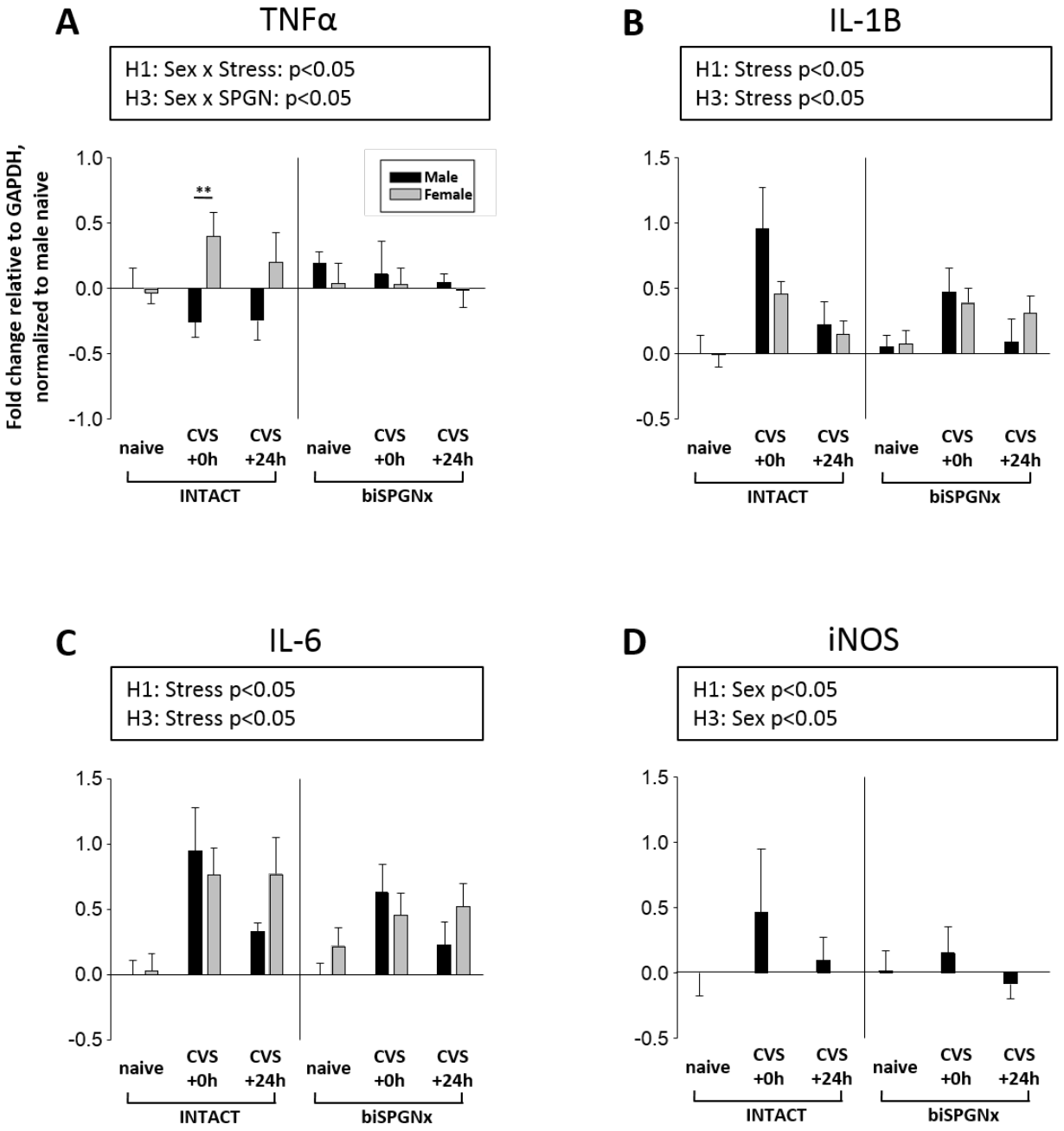
IL-1β: There was a significant ($p<0.05$) main effect of stress on the relative expression of IL-1β in lymphoid derived dural immune cells in intact animals, which persisted when SPGNx groups were folded into the analysis (Figure 11B). Post-hoc analysis indicated that the level of IL-1β immediately after stress was significantly greater than that in naïve ($p<0.01$) and 24 hrs after stress ($p<0.01$).

IL-6: Like IL-1β, there was a significant ($p<0.05$) main effect of stress on the relative expression of IL-6 in lymphoid derived dural immune in intact animals, which persisted when SPGNx groups were folded into the analysis (Figure 11C). Post-hoc analysis indicated that both the increase in IL-6 immediately after stress ($p<0.01$) and with a 24 hr delay after stress ($p<0.01$) were significant relative to naïves.

iNOS: There was a sex difference in the expression of iNOS, which was below the level of detection in lymphoid cells from all female groups (Figure 11D). Within males, no significant influence of stress or SPGNx was detected.

COX1: There was a significant ($p<0.05$) main effect of stress on the relative expression of COX1 in lymphoid cells (Figure 11E). Post-hoc analysis indicated that the increase of COX1 immediately following stress was significant ($p<0.01$). This stress effect persisted when SPGNx groups were folded into the analysis. A significant ($p<0.05$) influence of sex also emerged in the analysis of all groups. There was a significantly ($p<0.01$) higher level of COX1 expression in females than males.

COX2: There was a significant ($p<0.05$) main effect of stress on the relative expression of COX2 in lymphoid derived dural immune cells in intact animals, and this difference persisted when SPGNx groups were folded into the analysis (Figure 11F). Post-hoc analysis indicated that only the increase in COX2 immediately after stress was significantly ($p<0.01$) different than naïves.


A
TNFα
H1: Sex x Stress: p<0.05
H3: Sex x SPGN: p<0.05
Fold change relative to GAPDH, normalized to male naive
Male
Female
**
naive
CVS +0h
CVS +24h
INTACT
biSPGNx
B
IL-1B
H1: Stress p<0.05
H3: Stress p<0.05
C
IL-6
H1: Stress p<0.05
H3: Stress p<0.05
D
iNOS
H1: Sex p<0.05
H3: Sex p<0.05

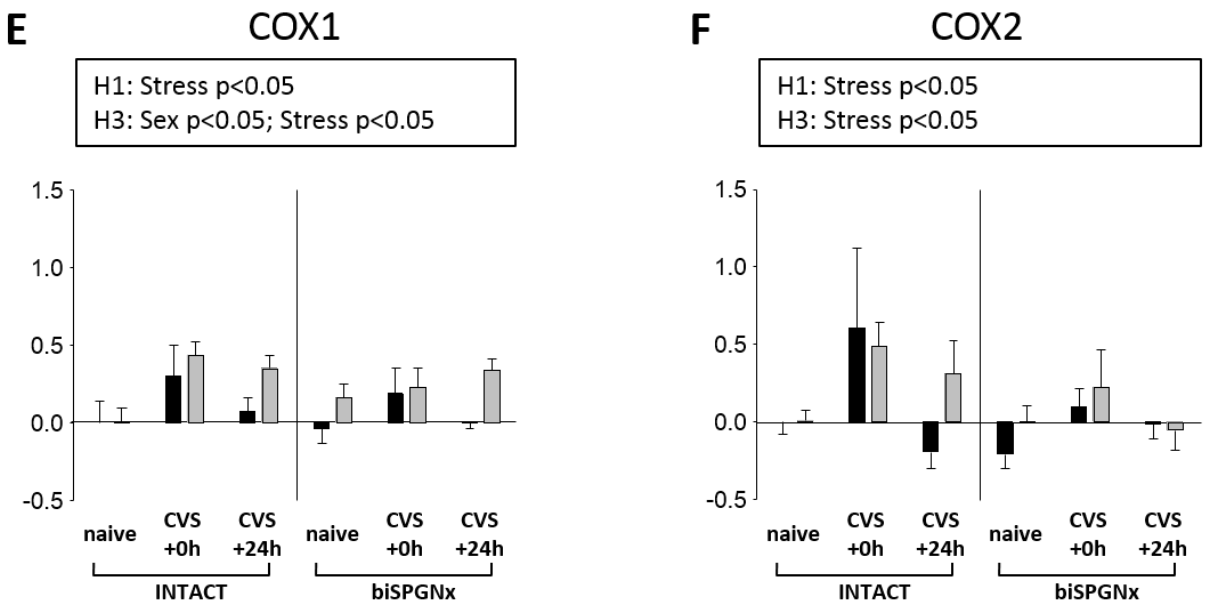


Figure 11. Sex-, stress-, and SPGN- dependent changes in pro-inflammatory mediators in dural lymphoid cells.

(**A**) There was a significant ($p<0.05$) interaction between sex and stress on the relative expression of TNFα in lymphoid derived dural immune cells ("Intact" groups). Folding all additional groups into the analysis revealed a significant ($p<0.05$) interaction between sex and SPGN innervation on TNFα expression. (**B**) There was a significant ($p<0.05$) main effect of stress on the relative expression of IL-1β. (**C**) There was also a significant ($p<0.05$) main effect of stress on the relative expression of IL-6. (**D**) There was sex difference in the expression of iNOS, which was below the level of detection in all female groups. (**E**) There was a significant ($p<0.05$) main effect of stress on the relative expression of COX1 in lymphoid cells. A significant ($p<0.05$) main effect of sex also emerged in the analysis of all groups. (**F**) There was a significant ($p<0.05$) main effect of stress on the relative expression of COX2. Group sizes and a priori hypotheses tested were the same as those in Figure 9. ** is $p < 0.01$.

3.3.4 Lymphoid derived dural immune cells: anti-inflammatory mediators

IL-10: In intact animals, there were significant ($p<0.05$) main effects for sex and stress on the relative expression of IL-10 in lymphoid derived dural immune cells (Figure 12A). There was significantly ($p<0.05$) more IL-10 expression in intact males than females. Also, the increase in IL-10 immediately after stress was significant ($p<0.01$) compared to naives. When the SPGNx groups were folded into the analysis, the increase in IL-10 with a 24 hr delay after stress was also significant ($p<0.01$).

IL-4: In intact animals, there was a significant ($p<0.05$) main effect of sex on the relative expression of IL-4 in lymphoid derived dural immune cells (Figure 12B). Post-hoc analysis indicated a significantly ($p<0.05$) higher level of IL-4 expression in females than males. Additionally, there was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve animals. Post-hoc analysis indicated that IL-4 expression in SPGNx females was significantly ($p<0.01$) greater than in intact females, while no difference between SPGNx and intact males was detected. The level of IL-4 in SPGNx females was also significantly ($p<0.01$) larger than in SPGNx males. Lastly, with all groups folded into the analysis, there was a significant ($p<0.05$) interaction between sex, stress, and SPGN innervation. However, post-hoc analysis indicated that the trend toward a stress-induced *increase* in IL-4 in intact female animals was not significant ($p=0.094$), nor was the trend towards a stress-induced *decrease* in SPGNx females ($p=0.061$).

POMC: POMC was below the level of detection in lymphoid derived immune cells from all groups.

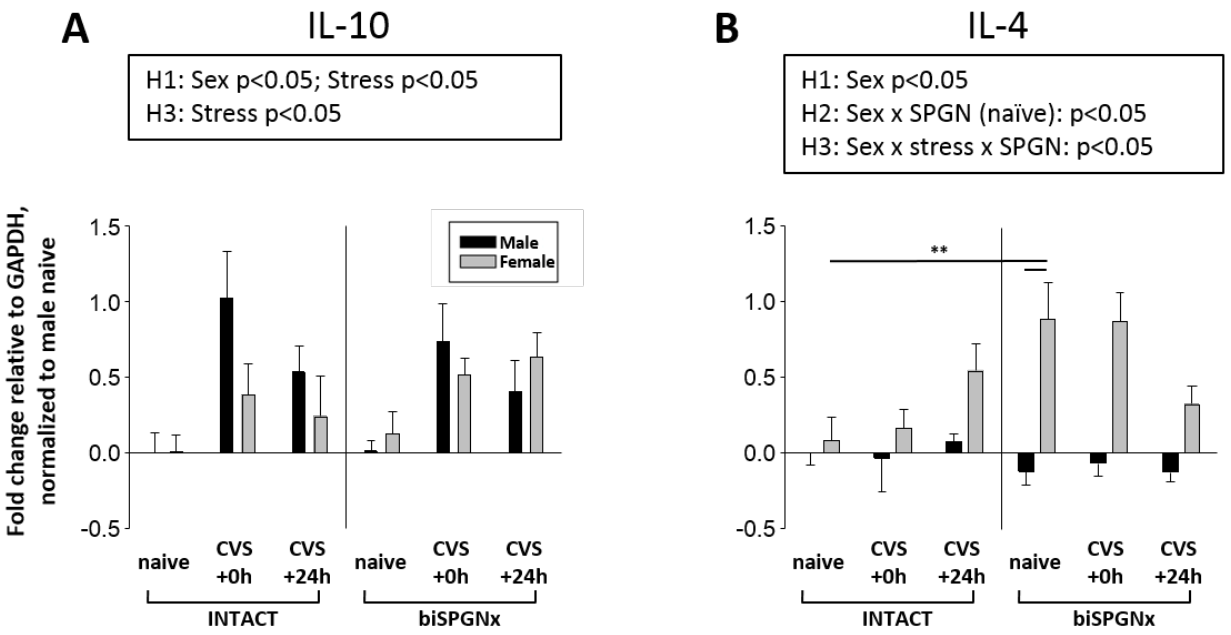


Figure 12. Sex-, stress-, and SPGN- dependent changes in anti-inflammatory mediators in dural lymphoid cells.

(**A**) There were significant ($p<0.05$) main effects for sex and stress on the relative expression of IL-10 in lymphoid derived dural immune cells ("Intact" groups). The significant stress effect persisted when the SPGNx groups were folded into the analysis. (**B**) There was a significant ($p<0.05$) main effect of sex on the relative expression of IL-4 ("Intact" groups). Additionally, there was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve animals. Lastly, with all groups folded into the analysis, there was a significant ($p<0.05$) interaction between sex, stress, and SPGN innervation. Group sizes and a priori hypotheses tested were the same as those in Figure 9. ** is $p < 0.01$.

3.4 DISCUSSION

The purpose of the present study was to begin to test the hypothesis that migraine attacks are due, at least in part, to a stress-induced shift in the balance of pro- and anti-inflammatory mediator expression in dural immune cells toward those that activate and sensitize dural afferents, where these changes are larger in females and dependent on sympathetic post-ganglionic innervation of the dura. We therefore analyzed of the impact of sex, stress and SPGN innervation on the expression of pro- and anti-inflammatory mediators in dural immune cells. The most robust changes observed in myeloid derived cells (macrophages, granulocytes, and dendritic cells) were stress-induced increases in IL-6 in both males and females, and even more strikingly, stress-induced increases in TNFα and iNOS that were selectively observed in females. Moreover, in females these changes appeared to depend, at least in part, on the presence of cephalic SPGN innervation, as the stress-induced increases in TNFα, IL-6 and iNOS in intact females were all attenuated in SPGNx females. Additionally, in females there was a stress-induced decrease in expression of the anti-inflammatory mediator IL-10, at least acutely, and POMC expression was undetectable. In lymphoid derived dural immune cells (B-, T-, and natural killer cells), there were stress-induced increases in IL-1β, IL-6, COX1 and COX2 in both males and females, but a stress-induced increase in TNFα that was only present in females. In contrast to the changes observed in myeloid-derived immune cells, only the stress-induced increase in TNFα in females appeared to depend on SPGN innervation, as the stress-induced increase in TNFα was attenuated in SPGNx rats. Finally, there were sex, stress and SPGNx effects on the expression of IL-10 and IL-4, with stress-induced increases in IL-10 that were

greater in males than females and stress-induced increases in IL-4 in females that appeared to be dependent on SPGN innervation. The overall trend of a stress-induced increase in the expression of pro-inflammatory mediators and decrease in the expression of anti-inflammatory mediators is consistent with a role for dural immune cells in the initiation of a migraine attack.

These results raise two important implications for the treatment of migraine. First, observations that 1) there is a sex difference in the stress-induced increase in pro-inflammatory, and decrease in anti-inflammatory mediators in dural immune cells, 2) differential anti-inflammatory mediators are engaged between males and females, 3) some mediators such as POMC and iNOS are only expressed in males, and 4) SPGN innervation influenced stress-induced increases in pro-inflammatory mediators in females but not males, suggests that it may not only be possible, but necessary to use different strategies for the most effective treatment of migraine in men and women. For example, opioids in immune cells have been shown to regulate persistent inflammation and neuropathic pain (Rittner et al., 2005, Rittner and Stein, 2005), and while it may be possible to therapeutically access such an opioid-dependent mechanism in males, it may not be possible to do so in females. Interestingly, this sex difference appears to be tissue specific given that in contrast to the dura, levels of iNOS and POMC in myeloid derived immune cells from the spleen were comparable in males and females (data not shown). Second, the timing of the stress-induced changes in the expression of several mediators including TNFα, IL-6, and IL-10 appeared to be particularly relevant to migraine attacks in females, given the persistence of the upregulation of the pro-inflammatory mediators and the transient decrease in the expression of the anti-inflammatory mediator. Strategies aimed toward downregulating TNFα and IL-6, and upregulating IL-10 and POMC signaling may prove to be viable treatment options for migraine.

Acute stress is classically thought to exacerbate immune responses while chronic stress induces immunosuppression (Dhabhar, 2009, Hall et al., 2012). Yet in other female-prevalent, stress-exacerbated inflammatory disorders there has also been clear evidence of an upregulation of immune cell cytokine release (Dinan et al., 2006, Dinan et al., 2008, Corcoran et al., 2013, Jiang et al., 2013, Logadottir et al., 2014, Schrepf et al., 2014). However, migraine attacks uniquely occur with a delay after stress (Sauro and Becker, 2009), and the temporal dynamics of stress-immune interactions with a delay have not been well described in the context of other tissues or disorders, nor in the context of migraine. Due to the potential importance of these temporal dynamics and the fact that the timing of changes in gene expression do no always correlate with changes in protein levels, it will ultimately be necessary to determine the impact of stress on inflammatory mediator protein levels in dural immune cells.

Interestingly, the stress-induced increases in pro-inflammatory mediators were SPGN-dependent in females but not males. We suggest that this difference may be due to the impact of gonadal hormones, in particular estrogens, on the regulation of immune cell adrenergic receptors (ARs) and consequently adrenergic regulation of immune cell properties. Both ERα and ERβ estrogen receptors have been described in most immune cells (Pennell et al., 2012, Yakimchuk et al., 2013). Estrogens have been shown to influence the expression of both α- and β-AR in immune cells (Shakhar et al., 2000, de Coupade et al., 2007, Leposavic et al., 2008). For example, β ARs are increased on neutrophils in females (de Coupade et al., 2004), and β blockers *increased* LPS-induced neutrophil recruitment in females and *attenuated* recruitment in males (Barker et al., 2005). Thus, the results of these different patterns of regulation are sex differences in the adrenergic regulation of chemotaxis and more relevantly, gene expression (Ignatowski et al., 1996, Szelenyi et al., 2000, Sanders, 2012).

In summary, we have demonstrated a stress-induced increase in the expression of pro-inflammatory mediators and decrease in the expression of anti-inflammatory mediators in females, which is consistent with a role for dural immune cells in the initiation of a migraine attack. Our results suggest specific mediators such as IL-6, TNFa, IL-10 and POMC could be viable therapeutic targets, and that it may be necessary to employ different treatment strategies in men and women. Understanding how migraine triggers initiate an inflammatory response in the dura will help identify underlying mechanisms of the disorder.

4.0 DISCUSSION

Given evidence that the pain of migraine originates in the peripheral dural afferents, and that sterile inflammation contributes to the activation and sensitization of dural afferents, the question I wanted to address coming into this field was, how is inflammation in the dura initiated, which could then lead to the start of a migraine attack? The idea was that if we could start to identify these mechanisms, we may be able to develop better prophylactic treatments for migraine. Immune cells are a well-known primary source of inflammatory mediators and were a likely source of the mediators responsible for sterile dural inflammation. To me, the most logical and relevant way to approach this question, was to characterize changes in immune cells in association with three, key clinical features of migraine (sex, stress, and SPGNs). The strategy was that any changes in immune cells/mediators occurring in females, with a delay after stress, and which were dependent on SPGN innervation, would be targets that could contribute to the initiation of a sterile inflammatory response in the dura.

4.1 IDENTIFICATION AND CHARACTERIZATION OF IMMUNE CELLS IN THE DURA

When I started this dissertation, the current view of the immune competency of the dura was that there were mast cells (Theoharides et al., 1995, Rozniecki et al., 1999, Levy et al., 2007) and

macrophages (Reuter et al., 2001), although dendritic cells have been identified as well (McMenamin et al., 2003). However, it was not known if other types of immune cells were present in this tissue. I needed to address this issue first, to begin to examine the possibility of immune-mediated inflammation in the dura. In the migraine field, the standard technique for identifying immune cells has been immunohistochemistry (IHC). Due to limitations of IHC in terms of the number of markers that can be studied in the same tissue, the limited quantification it affords, and the difficulty in performing any secondary analysis on the labeled tissue, I chose to pursue flow cytometry and FACS for the analysis of dural immune cells. While novel to the field of migraine, these powerful techniques address many limitations of IHC and maximize the amount of data that can be collected from each animal. This approach allowed me to identify 6 different phenotypic subtypes of immune cells in the dura simultaneously, accounting for ~92% of the immune cells present. While I complemented initial experiments with IHC, flow cytometry therefore eliminated the need to stain 2-3 dura with 6 different antibodies to identify each immune cell subtype with IHC, cutting the animals needed for these studies at least in half. Additionally, in rat, many of the immune cell antibodies for IHC are not even available and/or do not work in the dura, making the IHC approach even more difficult. Furthermore, because a large number of cells are analyzed (I used 200,000 events per dura) one cell at a time, it is easy to quantify changes in cell number as well as fluorescence intensity with a very high level of precision. Finally, with the ability to isolate cell types with FACS, it was possible to use quantitative PCR to assess changes in expression of 9 different mediators in subpopulations of immune cells. This enabled me to ascribe changes to a cell type with considerably more confidence than that afforded by more traditional approaches involving Western blot or PCR analysis of whole dura (Reuter et al., 2001).

While the numerous advantages of these approaches vastly outweighed any limitations, I acknowledge there were trade-offs to take into consideration. First, due to the need for dissociation, flow cytometry and FACS did not allow me to identify changes in the distribution patterns of immune cells throughout the dura. This highlights the need to use multiple approaches to complement results. Second, the impact of a dissociation process, which introduces both enzymatic and mechanical stimuli, as well as a time delay between tissue collection and analysis, is not fully known. However, these variables were controlled for as much as possible. I performed initial control experiments to demonstrate that the process of mechanical and chemical dissociation, per se, was not activating the immune cells (macrophages did not express CD80 or CD86, and after plating, released TNFα in response to LPS stimulation, data not shown). Also, all animals were treated with the same protocol and data were expressed as a percent change from naïve. Third, the dura was dissected from the top of the cortex. It is very likely that pieces of the pia and even some arachnoid were not fully recovered. This may be an issue because there is evidence suggesting that the distribution of immune cells varies among the different layers of the meninges. For example, dendritic cells are located more predominantly in the arachnoid and pia while macrophages are located more predominantly in the dura (McMenamin et al., 2003), so I may have underestimated the dendritic cell population in these experiments. Lastly, more than ~10,000 sorted cells were required to reliably and accurately recover enough RNA for qPCR. Due to the number of cells present in any given dura, I was unable to obtain enough of each subtype of immune cells from a single animal to enable expression analysis in each immune cell subtype. Nevertheless, I was able to characterize changes in immune cells more broadly separated by lineage into myeloid and lymphoid derived.

4.2 STUDYING SEX DIFFERENCES

The large sex difference in migraine (3:1 females: males) has been generally disregarded in basic science, as most studies have only been conducted in males. It was important to me that if I was going to study a disorder that occurs most often in women, that I should study it in females as well as males. Inflammatory processing in the dura can be very different in females (see Introduction). Only studying males will most certainly not help explain why women are more vulnerable to migraine, and may preclude the identification of mechanisms uniquely responsible for migraine in women as well.

While the analysis of sex differences was a central focus of my thesis, aside from an indirect measure of estrus cycle afforded by the analysis of uterine weights (which did not significantly correlate with results), I did not even begin to analyze the basis for the sex differences observed. The first question to address this basis would be whether the magnitude or even presence of sex differences change across the estrous cycle. Any changes would then be used to implicate gonadal hormones on the differences observed. However I chose not to assess the impact of estrus cycle on dural immune cells for multiple reasons. First, when studying sex differences it is important to initially determine if there are sex differences present before proceeding with additional experiments to address the basis of the differences observed. This was the purpose of the experiments in this dissertation. Second, while the timing of the onset of migraine with menarch and resolution with menopause (Neri et al., 1993, Brandes, 2006) suggests a role for an activational influence of gonadal hormones in migraine, there are pre-clinical data to suggest that an organizational influence of gonadal hormones is critical for the manifestation of a sex difference in SPGN-dependent inflammation (Green et al., 2001, Green and Levine, 2005). Third, using a rat for the determination of an activational influence of

hormones is problematic for several reasons. Rat estrus cycles differ substantially from human (Becker et al., 2005). This raises concerns about how well information from rat cycling could be extrapolated to changes that happen in humans. Fourth, use of daily vaginal smears for staging rats in their estrous cycle is problematic in the context of a study on stress. Stress is known to disrupt cycling, performing the smears is a stressor itself, and there is no appropriate control for this procedure to apply to males (Greenspan et al., 2007). The alternative approach of gonadectomy with hormone replacement is a potentially viable alternative. However, removal of the gonads results in the loss of more than just estrogens (or testosterone), and as a result, negative results will only enable ruling out a necessary role of the hormone manipulated. Nevertheless, having observed a clear sex difference in a number of endpoints, it will be important to determine the basis for this sex difference in futures studies. Such a determination would require a systematic approach to manipulating gonadal hormones in both males and females (Greenspan et al., 2007).

4.3 USING CHRONIC VARIABLE STRESS AS AN APPROACH TO IDENTIFY HOW INFLAMMATION IS INITIATED IN THE DURA

Three general approaches have been used to study the effects of inflammation in the dura in the context of migraine. The first has been to use exogenous application of inflammatory mediators to the dura or dissociated cells (Strassman et al., 1996, Oshinsky and Gomonchareonsiri, 2007, Edelmayer et al., 2009, Harriott and Gold, 2009). This approach has provided compelling support for the suggestion that dural inflammation is critically involved in the initiation of a migraine attack as well as the development of chronic migraine (Oshinsky and

Gomonchareonsiri, 2007), however does not address the origin of the inflammation. The second approach has been to employ a non-physiological stimulus [i.e., compound 48/80 (Levy et al., 2007)] capable of directly activating immune cells. While results obtained with this approach confirm that activation of immune cells (although not necessarily dural with the systemic administration of such compounds) can activate dural afferents, it is still necessary to confirm that such activation occurs in the context of migraine. The third has been to use stimuli that are associated with and/or can trigger a migraine attack [i.e., CSD (Karatas et al., 2013), GTN (Reuter et al., 2001), and stress (Theoharides et al., 1995)]. This third approach was the most appealing to me, particularly when based on a physiological trigger such as stress. Strikingly, I have only been able to find a single study on the effects of stress on inflammation in the dura. Theoharides and colleagues reported mast cell degranulation in the dura in response to acute restraint stress (Theoharides et al., 1995).

What was also appealing to me about the study of stress in the context of migraine, was that the temporal delay between stress and a migraine attack uniquely differentiates migraine from all other pain syndromes and disorders. However, despite some very well characterized models of stress in the literature (Imbe et al., 2006), to maximally exploit the unique temporal dynamic of migraine, it became clear to me that I needed to develop my own model of stress, which I referred to as chronic variable stress (CVS). The problem with most established models is that they are associated with relatively short exposures to the stressor (i.e., 30 minutes in the repeated sound stress model), once a day over several days. The result is repeated periods of stress and relaxation. The repeated presentation of the stress clearly has a profound effect (Strausbaugh et al., 2003), but produced highly variable results for me (data not shown). Repeated exposure to short durations of stress is also very different than the time course of stress

that is often an effective trigger for a migraine attack in humans. Ongoing stress over several days is often sufficient to precipitate an attack after the stress has resolved. Furthermore, repetition can often result in habituation. By designing my stress paradigm with many different, randomized stressors applied continuously, I was able to model the constant stress that typically triggers a migraine, as well as create a clear relaxation period following the stress during which migraines often occur.

While I was able to demonstrate marked changes in body weight in response to CVS (data not shown), in addition to the changes in dural immune cells, it will ultimately be necessary for me to confirm that the model is both stressful and non-habituating with more traditional endpoints such as plasma CORT, ACTH or NE levels. It was not possible to collect these data on the animals in which dural immune cells were studied, because plasma CORT levels rise relatively rapidly (minutes) to even handling and movement, and are confounded by anesthetics (Vahl et al., 2005). It will therefore be necessary to collect unanesthetized core blood from additional animals to address this issue, as chronic catheterization and habituation to handling for blood collection are both potential confounds with the model employed.

4.4 EXAMINING THE IMPACT OF SPGN INNERVATION

Lastly, while parasympathetic innervation of the dura has been studied due to its contribution to vasodilation and release of pro-inflammatory mediators (Bolay et al., 2002, Yarnitsky et al., 2003), SPGN innervation and mediators (i.e. NE) have been generally ignored, despite evidence of sympathetic dysregulation in migraineurs (Peroutka, 2004), stress (and thus sympathetic activation) as a common trigger for migraine, and evidence of SPGN contribution to

inflammatory pain in the periphery (Green et al., 1999, Hucho and Levine, 2007). Only two studies have addressed NE signaling in the dura. Ebersberger and colleagues demonstrated NE-induced PGE2 release (Ebersberger et al., 2006), and recently Wei and colleagues have shown that NE increases dural afferent excitability and fibroblast activity (Wei et al., 2015).

I chose to study the effects of SPGN innervation with a bilateral surgical removal of the superior cervical ganglion (SCG), eliminating all cephalic SPGN innervation (SPGNx). A bilateral SPGNx was conducted because preliminary experiments (data not shown) using a unilateral SPGNx and comparing intact vs. SPGNx hemi-dura revealed partial effects on the intact hemi-dura compared to fully intact animals. While the SPGN innervation of the dura from each SCG stops at midline, immune cells may move and released mediators can diffuse across the midline. To avoid this problem, bilateral SPGNx were therefore used. However I observed sham surgery effects on immune cell proportions, even though surgeries were performed 12 days before experiments were performed, incision sites had completely healed, there were no signs of ptosis, and the effects of surgery as a stressor have only been reported to last for up to a couple days (Hogan et al., 2011, Marik and Flemmer, 2012). While surgeries were performed carefully, the nearby vagus nerve could have been damaged, resulting in dysregulation of the cardiac response to stress. Ultimately, additionally experiments will need to be performed to determine the basis for the sham effects. First, sham surgeries can be performed at even earlier time points. Also, examination of cardiac responses and vagus nerve recordings can be obtained following sham surgeries to confirm or reject the hypothesis of vagus damage. Another approach to eliminate SPGN activity could include a systemic guanethidine injection (Chen et al., 2010), however this would unnecessarily remove all SPGN innervation in the rats. Any attempt to create a localized pharmacological manipulation has multiple caveats: 1) this would require a

craniotomy for cannula, which is extremely invasive and is known to disrupt resident immune cells (Levy et al., 2007), 2) the drug may not diffuse throughout the entire dura from the point of the cannula, confounding results, 3) depending on the kinetics of the drug, would require many infusions to last through a 5 day experimental design, and 4) again depending on the drug, would likely only produce a partial block of SPGN activity.

4.5 FUTURE DIRECTIONS

Immune cell mediated inflammation is an incredibly complex processes. We are only just scratching the surface with these initial studies, and more questions have been raised than answered by this dissertation. In light of the approaches I used, discussed above, there are multiple directions to take this research in the future. First, in order to probe for multiple pro- and anti-inflammatory mediators in immune cells, I assessed changes in mRNA. Naturally the next steps will be to confirm protein changes and release of the targets identified with mRNA. Also, determining changes in activation patterns in subtypes of immune cells (i.e., M1 vs M2 in macrophages and Th1 vs Th2 in T cells) will give a larger picture of the immune contribution to an inflammatory response. Classically activated M1 macrophages have been implicated in initiating and sustaining inflammation in chronic inflammation, while M2 macrophages are associated with the resolution of inflammation (Martinez et al., 2009). This response mirrors the Th1-Th2 polarization of T-cells, where Th1 cells secrete pro-inflammatory cytokines and Th2 cells secrete anti-inflammatory cytokines (Chapman et al., 2008).

A particularly interesting question about stress in the context of migraine is, what are the possible mechanisms for the unique timing of stress-triggered migraines? This is an important

observation that will help discern mechanisms that may be causal to migraine versus general stress mechanisms that are unrelated to migraine. It also creates a unique window for treatment. One mechanism that could account for the timing would be if pro- and anti-inflammatory pathways activated by stress "turn off" at different rates. That is, while both pathways may balance out during stress, if anti-inflammatory mechanisms resolve more quickly than pro-inflammatory mechanisms following termination of stress there would be a vulnerable window for an inflammatory response in the dura after stress. Although there were mixed effects on the timing of anti-inflammatory mediator expression in immune cells, there were clear examples of pro-inflammatory mediator expression (TNFα, IL-6) that were upregulated with stress, and remained upregulated with a delay after stress, in females. An analogous mechanism that may account for the delay in the initiation of a migraine attack would be the timing of stress-induced changes in receptor density and/or sensitivity, where anti-inflammatory signaling is blunted following chronic stress and pro-inflammatory signaling is enhanced. For example, traditionally in both humans and animal models, acute stress causes an increase in immune responses while chronic stress suppresses it (decreased trafficking, activity, and production of cytokines that translate to decreases in wound healing, responses to vaccines and autoimmune diseases) (Padgett and Glaser, 2003). However in this study and in studies on other stress exacerbated pain disorders, an increase in the pro-inflammatory immune response was found following chronic stress. This is thought to be due to GR desensitization, and loss of CORT-mediated anti-inflammatory signaling. Future studies should be directed to examining these possible mechanisms for the unique timing of stress-triggered migraines.

To determine how stress was effecting the changes in dural immune cell number and/or expression of mediators, additional experiments will need to be conducted that include

manipulations at different levels of the stress axes (i.e., adrenalectomy, hypophysectomy) and of specific mediators (i.e., CRH antibody, CORT given to naïve animals, AR antagonist/agonists). Barker and colleagues have shown that the sexually dimorphic, stress-induced changes in neutrophil recruitment are dependent on an intact SA axis, as demonstrated with adrenalectomy and AR antagonist/agonists (Barker et al., 2005). I have provided evidence to suggest that some stress-induced effects on dural immune cells were SPGN dependent, but some were SPGN independent and would require these additional experiments.

Additionally, it will be important to distinguish the impact of activity in the SPGN from local, activity independent processes. Levine and colleagues have demonstrated that NE-induced release of PGE2 from the SPGN can occur independent of activity (Gonzales et al., 1991, Sherbourne et al., 1992). One approach to address this issue would be to stimulate the SPGNs by implanting an electrode in the SCG. I performed initial experiments of this nature (data not shown). Chronically instrumented animals would be needed, however, to address this issue.

In future experiments it will also be essential to put these results in the context of a model of migraine, such as GTN infusion. The prediction is that if immune cell mediated inflammation is relevant, I should be able to see differences, particularly in the targets I identified in these studies, with a delay after GTN infusion. Furthermore, I should be able to see differences in the response to GTN with a delay after stress. Additionally, demonstrating an impact on dural afferent signaling, second-order neurons in the TNC, as well as changes in "migraine behavior", such as periorbital allodynia and photophobia, will support the relevance of my results to a physiological, behaviorally meaningful outcome.

While my data have broad implications for the understanding of migraine, it would be significant to confirm that the primary observations made in this rat model are relevant to

humans. Species differences notwithstanding, one of the biggest limitations to the use of the rat model is that the rats are not migraineurs. Thus, it is possible that changes I observed are normal physiological responses to stress that are in fact protective against migraine. This raises the question of whether migraineurs are always migraineurs or if anyone can become a migraineur. Polymorphisms and genetic models of migraine (familial hemiplegic migraine) (Eising et al., 2013) support the idea that migraine may be a unique phenomenon for some people. However, evidence that PFO closures can abolish migraines (Ailani, 2014), and PTSD with or without a mild TBI (Theeler et al., 2013) can evoke migraines, suggest that migraine may be on a continuum for others. Oshinsky and colleagues have made the first steps to address these difficult issues. First, through identification of a rat with spontaneous periorbital allodynia and subsequent breeding, they have developed a genetic model of spontaneous migraine (Oshinsky et al., 2012). Alternatively, they addressed the question, can you make a rat a migraineur if you "push" the system hard enough? They modeled recurrent headache by applying repeated infusions of inflammatory soup over the dura through a cannula. This produced long-lasting periorbital allodynia and pronounced responses to GTN (Oshinsky and Gomonchareonsiri, 2007). However, an important parallel line of investigation that I (and others) am pursuing will be to compare dura tissue from migraineurs and non-migraineurs.

4.6 SUMMARY AND CONCLUSIONS

The strategy and tools utilized in this dissertation allowed me to make a number of novel observations about dural immune cells. I was the first to identify the total proportion of immune cells in the dura, and also the first to identify T-, B-, NK- and NKT cells in the dura—immune

cells other than mast cells and macrophages. I found significant proportions of these lymphoid derived immune cells in the dura—immune cells generally considered a recruited, adaptive response—even in naïve animals. These observations should change the way those working in the field will think about the immune competency of the dura. Additionally, I was able to identify different immune cell subtypes (macrophages in males, and T-cells in females), which increased with a delay after stress, suggesting for the first time a possible role for T-cells in migraine. I was also able to identify pro- (TNFa and IL-6) and anti- (IL-10 and POMC) inflammatory mediators, up- and down-regulated, respectively, with a delay after stress, particularly in females, suggesting that a shift in the balance of pro- and anti-inflammatory mediators may also contribute to the initiation of a migraine attack. Minimally, I have been able to expand our view of the dura, revealing it to be a much more immune rich and complex tissue than previously appreciated. Most importantly, I believe my results underscore the possibility that it may not only be possible, but necessary to differentially treat migraine in men and women.

APPENDIX A

DISTRIBUTION OF ARTEMIN AND GFRA3 LABELED NERVE FIBERS IN THE DURA MATER OF RAT

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(*Headache* 2010;50:442-450)

Objective.—We examined the distribution of artemin and its receptor, glial cell line-derived neurotrophic factor family receptor α3 (GFRα3), in the dura mater of rats.

Background.—Artemin, a member of the glial cell line-derived neurotrophic factor family, is a vasculature-derived growth factor shown to regulate migration of sympathetic neuroblasts and targeting of sympathetic innervation. The artemin receptor,GFRα3, is present in both sympathetic efferents and a subpopulation of nociceptive afferents. Recent evidence has shown that artemin may contribute to inflammatory hyperalgesia. The extent to which artemin is present in the dural vasculature and its relationship to GFRα3 containing fibers have yet to be investigated.

Methods.—We used retrograde labeling, double and triple labeling with immunohistochemistry on the dura mater and trigeminal ganglia of female Sprague-Dawley rats.

Results.—Artemin-like immunoreactivity (-LI) was detected in the smooth muscle of dural vasculature. GFRα3-LI was present in nerve fibers that closely associated with tyrosine hydroxylase or calcitonin gene-related peptide (CGRP). CGRP-LI and transient receptor potential ion channel 1 (TRPV1)-LI were present in all GFRα3-positive dural afferents, which constituted 22% of the total population of dural afferents.

Conclusions.—These anatomical results support the hypothesis that artemin contributes to dural afferent activity, and possibly migraine pain, through modulation of both primary afferent and sympathetic systems.

Key words: peptidergic afferent, sympathetic postganglionic neuron, sympathetically mediated pain

Abbreviations: Artn artemin, CGRP calcitonin gene-related peptide, DiI 1,1_-dioctadecyl-3,3,3_,3_-tetramethylindocarbocyanine perchloride, DRG dorsal root ganglia, GDNF glial cell line-derived neurotrophic factor, GFRα3 GDNF family receptor a3, -LI -like immunoreactivity, MMA middle meningeal artery, SMA smooth muscle actin, SPGN sympathetic postganglionic neuron, TG trigeminal ganglia, TH tyrosine hydroxylase, TRPV1 transient receptor potential ion channel 1

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A.1 INTRODUCTION

Artemin (Artn) is a member of the glial cell line derived neurotrophic factor (GDNF) family of proteins that signals through the receptor complex of ret and its specific GDNF family receptor (GFR)α3.[1] Artn is derived from vascular smooth muscle cells and regulates the development of sympathetic postganglionic neurons (SPGN), in particular SPGN innervations of the vasculature.[2] Recent evidence suggests that both Artn and GFRα3 expression persists into adulthood in vascular smooth muscle and SPGNs,[3] respectively.

The GFRα3 is also expressed in a subset of primary afferent neurons that appear to function as nociceptors and contribute to inflammatory hyperalgesia. GFRα3-labeled neurons in the dorsal root ganglion (DRG) and trigeminal ganglion (TG) have a small to medium-cell body size, and express the proinflammatory neuropeptide calcitonin gene-related peptide (CGRP) and the transient receptor potential ion channel 1 (TRPV1).[4] TRPV1 is activated by heat, protons, and endogenous lipid metabolites, and is critical for the manifestation of inflammatory thermal hyperalgesia.[5] Artn application significantly potentiates TRPV1 signaling in small diameter DRG neurons.[6] Additionally, peripheral inflammation is associated with an increase in Artn expression and injection of Artn causes thermal hyperalgesia.[6,7]

Vasodilation of the meningeal vessels is due in part to CGRP release and is considered an important component of the neurogenic inflammation thought to underlie migraine. Vasodilation, plasma extravasation, and mast cell degranulation contribute to the release of proinflammatory substances into the meninges, which can activate meningeal primary afferents ultimately resulting in migraine pain.[8] The role of vasodilation in migraine is supported by observations that headaches can be induced by vasodilatory, nitric oxide-generating agents such as nitroglycerin,[9] and relieved with vasoconstricting agents such as ergots.[10] Artn expression in vasculature and its influence on sensory nociception and sympathetic development led us to hypothesize that Artn may be contributing to migraine pain through both primary afferent and sympathetic systems. The goal of this study was to determine if the underlying anatomy supports this hypothesis.

A.2 METHODS

Animals.—Female adult Sprague-Dawley rats (150-250 g, aged 8-12 weeks; Harlan, Indianapolis, IN, USA) were used for all experiments. Procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh, and performed in accordance with National Institutes of Health guidelines for the use of laboratory animals.

Tissue Preparation.—Animals were deeply anesthetized with an intraperitoneal injection of anesthetic cocktail (55 mg/kg ketamine, 5.5 mg/kg xylazine, and 1.1 mg/kg acepromazine), and transcardially perfused with cold 1x phosphate-buffered saline (PBS; pH 7.2) followed by cold 4% paraformaldehyde. TG, superior cervical ganglia (SCG), and dura were collected and postfixed in 4% paraformaldehyde for 1 hour. Dural membranes were processed for immunohistochemistry as free-floating whole-mounts, while TG and SCG were equilibrated in 30% sucrose, frozen in OCT (Tissue Tek, Torrance, CA, USA), sectioned at 16 mm and thaw-mounted on SuperFrost plus slides (Fisher Scientific). One dura was embedded in paraffin and 30 mm cross sections collected and processed for immunohistochemistry. Slides were heated to 50°C on a hotplate, soaked in CitriSolv (Fisher Scientific) for 10 minutes and rehydrated through a series of decreasing ethanol concentrations. Antigen retrieval was obtained by boiling slides for 10 minutes in 0.01 M sodium citrate, 0.05%Tween 20,pH 6.0. After cooling, slides were washed in PBS and processed for immunohistochemistry.

Immunolabeling.—Dura whole-mounts were placed in blocking solution (10% normal donkey serum and 0.3% Triton X-100 in PBS) for 30 minutes and incubated in primary antibodies in blocking solution for 2 days at 4°C. TG sections on slides were blocked in 10% normal donkey serum and 0.03% Triton X-100 in PBS, and incubated in primary antibodies overnight at room temperature. The following antibodies were used: goat anti-GFRα3 (R&D

Systems; 1:500), goat anti-mouse Artn (R&D Systems; 1:40), rabbit anti-CGRP (Sigma; 1:500) and rabbit anti-tyrosine hydroxylase (Chemicon; 1:500), and rabbit anti-TRPV1 (Alomone Labs: 1:1000). The specificity of the Artn and GFRα3 antibodies has been previously documented (manufacturer's information[11,12]). Antibodies were visualized with donkey anti-goat and donkey anti-rabbit secondary antibodies conjugated to cyanine 2 or 3 (Jackson ImmunoResearch, West Grove, PA, USA) in blocking solution at 1:500 for 2 hours. Dura were mounted on glass plus slides and coverslipped using Fluoromount-G (Southern Biotech). Slides were photographed under epifluorescence with a Leica DM4000B upright or confocal microscope (Leica, Wetzlar, Germany). Images were captured using a Leica DFC300FX camera and processed for brightness and contrast with Adobe Photoshop (Adobe Systems, San Jose, CA).

Retrograde Labeling.—TG and SCG neurons innervating the dura were retrogradely labeled as described previously.[13] Briefly, a 3 x 3 mm craniotomy was made over the superior sagittal sinus, leaving the dura intact. A single drop of 1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine perchloride (DiI; 170 mg/mL in dimethylsulphoxide diluted 1:10 in saline), or true blue chloride powder (Invitrogen; 1 mg) was applied to the exposed dura. The area was covered by dental dam and an acrylic cap. Postoperatively, animals received intramuscular injections of penicillin G (100,000 U/kg) and buprenorphine (0.03 mg/kg). Animals were sacrificed and ganglia extracted for immunohistochemistry 10-14 days following labeling.

Data Analysis.—Staining was considered positive when the immunofluorescence was clearly greater than the signal obtained with no primary antibody. Omission of primary antibody was used as a control for non-specific binding of the secondary antibody. The percentage of GFRα3-positive dural afferents was determined by ~50 DiI-labeled neurons in each of 4 rats (8

total ganglia) that exhibited GFRα3- labeling, and ~100 true blue-labeled neurons in each of 3 rats (6 ganglia).

A.3 RESULTS

Artn is Located in Smooth Muscle Cells of Dural Blood Vessels.—Co-localization of Artn-like immunoreactivity (-LI) and smooth muscle actin (SMA), a marker for smooth muscle cells, was assessed using double-label immunohistochemistry on paraffin embedded cross sections of the dura. Artn-LI was evident surrounding blood vessels and co-localized with SMA (Fig. 1). These results indicate that Artn is expressed in smooth muscle cells of the dural vasculature.

GFRα3-LI is Present in Neuronal Fibers of the Dura Mater.—The distribution of GFRα3 throughout the dura mater was also assessed in a whole-mount preparation using immunohistochemistry. GFRα3-LI was present in a network of nerve fibers, in large bundles of axons as well as fine fibers (Fig. 2). Many of these fibers followed the vasculature, with the greatest density concentrated around the middle meningeal artery (MMA). Many fibers also followed the superior and transverse sinuses (not shown). However, some fibers did not follow the vasculature or sinuses, and were observed running perpendicular to the MMA (Fig. 2) and other vessels. GFRα3-LI was not detected in mast cells or the vasculature.

GFRα3-LI is Present in Sympathetic Postganglionic Efferents.—Given the developmental link between Artn and sympathetic innervation of the vasculature[2] as well as evidence of GFRα3 expression in adult SCG[3] we sought to determine whether dural fibers with GFRα3-LI were SPGNs. Although a small subpopulation of TG neurons also display tyrosine hydroxylase reactivity (TH)-LI, dural afferents do not.[13] Therefore, TH was used as a marker for

SPGN fibers in the dura. TH-LI was present in an extensive network of fibers throughout the dura mater, which closely followed the vasculature (Fig. 2A). Close association of GFRα3-LI and TH-LI in fibers throughout the dura was extensive. Interestingly, while most double-labeled fibers closely followed the dural vasculature, some did not.

GFRα3-LI, TRPV1-LI, and CGRP-LI are Present in Peptidergic Neuronal Afferents.—As mentioned above, GFRα3 is present in a subpopulation of peptidergic DRG neurons. To determine whether the same is true in the trigeminal system, we first assessed whether the receptor was co-localized with CGRP in the dura. Of note, while CGRP is also present in a subset of SPGNs in some species, it is not detectable in the SCG of rat.[13] As illustrated in Figure 2B, the close association of GFRα3-LI and CGRP-LI was evident in a subpopulation of fibers. To confirm that at least some of the GFRα3-LI dural fibers were sensory afferents, TG neurons innervating the dura mater were back-labeled with DiI or true blue, and subsequently probed for the presence of GFRα3-LI. Approximately 50 DiI-labeled neurons from each of 4 rats and 100 true blue-labeled neurons from each of 3 rats were assessed. To avoid double-counting, we only counted cells in non-adjacent sections when a nucleus was present. While it is possible that true blue and DiI labeled different subpopulations of dural afferents, there was no statistically significant difference (P = .10) in the proportion of dural afferents with GFRα3-LI labeled with each tracer – 25.9 ±3.9% (mean ± SEM) vs 17.7 ± 1.4% for DiI and true blue, respectively. Therefore, data obtained with the 2 tracers were pooled as 22.4 ± 3.0% of dural afferents with GFRα3-LI. The TRPV1-LI (Fig. 3) and CGRP-LI (Fig. 4) were seen in 43.2 ± 1.7% and 66.0 ±6.0% of dural afferents, respectively. Twenty-six percent (25.5 ± 2.7%) of dural afferents that were labeled with TRPV1 did not show GFRα3-LI, and 47.6 ±3.6% labeled

with CGRP did not show GFRα3-LI. All GFRα3-LI dural afferents were TRPV1- and CGRP positive. These data are summarized in charts shown in Figure 5.

A.4 DISCUSSION

The main findings of this study are: (1) Artn-LI was detected in the smooth muscle of adult rat dura mater vasculature; (2) expression of the Artn receptor GFRα3 was detected in afferents that innervate the dura; (3) there was extensive close association of GFRα3-LI and TH-LI in dural fibers; (4) GFRα3 was present in a minority of dural afferents, but all of these afferents were both TRPV1- and CGRP-positive.

While this is the first demonstration of Artn in the dural vasculature, this observation is consistent with other studies reporting Artn expression in vascular structures. For example, Damon et al demonstrated the presence of Artn mRNA and protein in carotid, femoral, and tail arteries of neonatal and adult rats, as well as in cultured vascular smooth muscle cells. In neonatal lacZ Artn+/- mice, Honma et al also found lacZ-LI co-localization with SMA-LI in the superior mesenteric artery. This evidence, in combination with previous data supporting the specificity of the antibody used,[11] supports our conclusion that Artn-LI co-localization with SMA-LI in the dural vasculature reflects Artn expression in the smooth muscle.

We suggest that the majority of GFRα3 expression in the dura is present in fibers of SPGNs. This is supported by the comparable labeling patterns of GFRα3-LI and TH-LI in dural fibers (Fig. 2A), and the absence of TH staining in dural afferents.[13] Additional evidence comes from data linking Artn/GFRα3 signaling to proper SPGN innervation of the vasculature during development,[2] and that the receptor is expressed in the SCG in adults.[3] Furthermore, the more

limited overlap of GFRα3-LI and CGRP-LI in dural fibers (Fig. 2B,C), in combination with the observation that only 28% of dural afferents with CGRP-LI were double labeled with GFRα3 (Figs. 4 and 5), is also consistent with the suggestion that a minority of GFRα3-LI in the dura was associated with dural afferents. However, because it was not possible to assess GRFα3 levels in SCG, and therefore quantify the extent of co-localization of this receptor in dural efferents, and because staining patterns at the cell body may not be the same as those in the periphery, conclusions about the extent of co-localization in dural fibers are made cautiously.

Interestingly, while only present in a minority of dural afferents, the subpopulation of GFRα3- containing afferents may play a particularly important role in nociceptive processing in the dura. Artn potentiates TRPV1 signaling in DRG neurons, a change that appears to contribute to thermal hyperalgesia associated with inflammation of the hindpaw.[6,11] Furthermore, activation of TRPV1, a calcium permeable ion channel, has been shown to promote the release of CGRP from a number of tissues[14-16] and contribute to vasodilatation, neurogenic inflammation, and mast cell activation.[17,18] Our anatomical results raise the possibility that similar processes occur in the dura mater, underscored by observations that GFRα3 appears to be present in similar subpopulations of nociceptive afferents in the TG and DRG.[4]

The anatomical results of the present study suggest a model in which Artn contributes to migraine pain indirectly, via the SPGN as well as directly via the sensitization and/or activation of dural afferents. In such a model, triggers for migraine such as stress, exercise, and nitric oxide which are associated with alterations in cerebrovascular tone would result in the release of Artn from vascular smooth muscle. The release of Artn secondary to the modulation of vascular tone would account for the mounting evidence against the vascular hypothesis of migraine that is based on a direct link between the cerebrovasculature and migraine pain. While the impact of

Artn on SPGN properties in the adult has yet to be described, the results of the present study highlight the proximity of ligand and receptor. Importantly, there is increasing evidence implicating a critical role for the SPGN in neurogenic inflammation and inflammatory hyperalgesia.[19,20] Additional evidence in support of a link between the SPGN and migraine comes from the recent observations that (1) norepinephrine, a neurotransmitter released by sympathetic efferents, increases basal levels of prostaglandin E2 from a rat dural preparation;[21] (2) the serotonin 1D receptor, a primary target for the antimigraine triptans, is present in a subpopulation of SPGNs.[13] Evidence in support of the link between Artn/GFRα3 signaling in the primary afferent and migraine pain comes from our evidence of the co-localization of GRFa3 with TRPV1 and CGRP in combination with evidence for (1) Artn-induced sensitization of TRPV1;[6,22] (2) TRPV1-mediated release of CGRP (discussed above); (3) a critical role for CGRP in migraine pain;[23-25] (4) the role for dural afferents in migraine pain.[26]While further studies are needed to confirm the details of this model, it does raise the intriguing possibility that Artn/GFRα3 signaling may serve as a novel target for the treatment of migraine.

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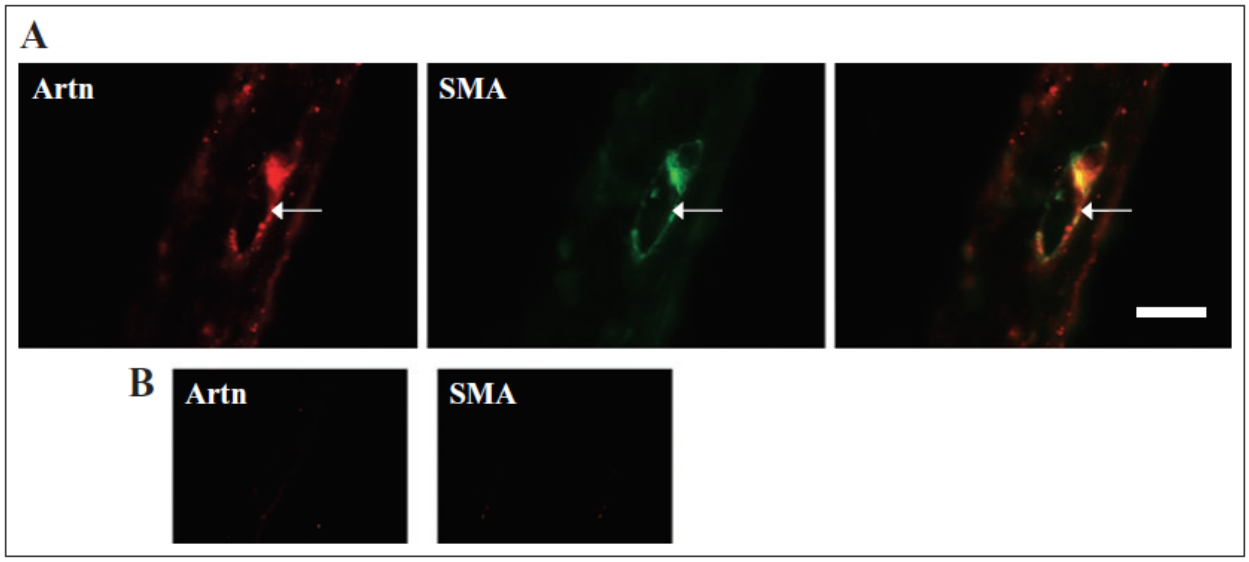
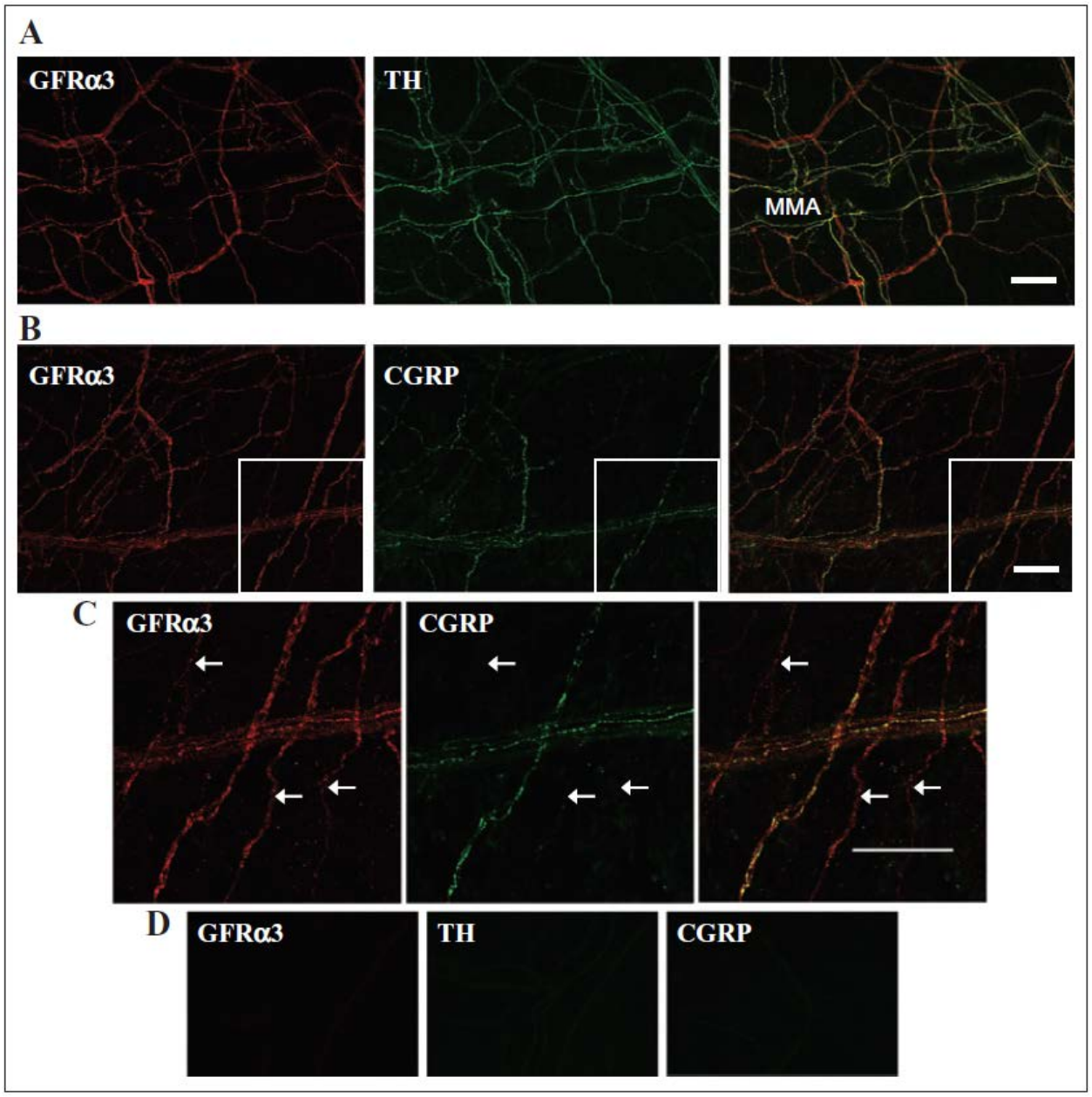


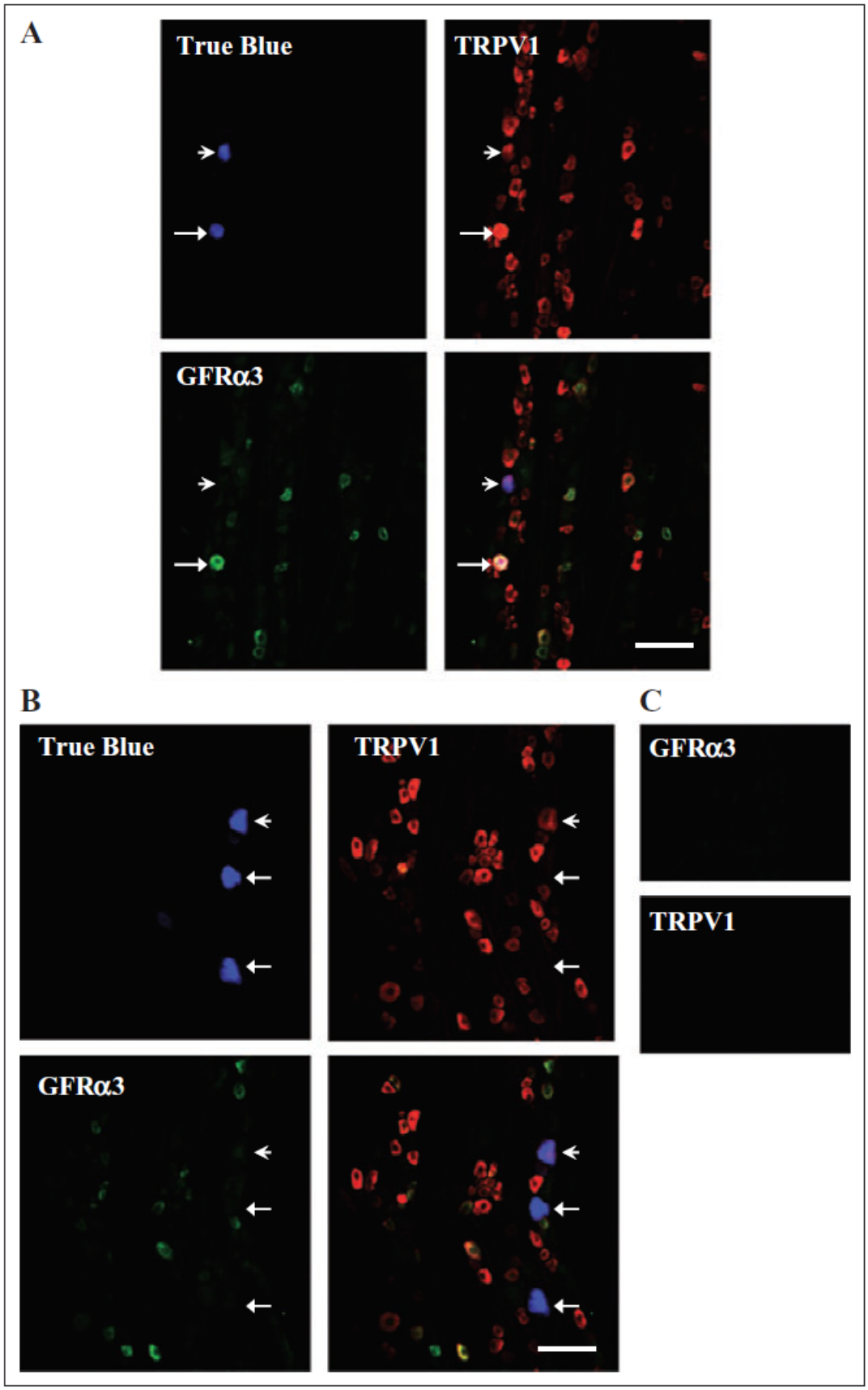
Appendix A Figure 1.

(A) A cross section of the rat dura mater was immunolabeled for artemin (Artn, left panel) and smooth muscle actin (SMA, middle panel). The merged image (right panel) illustrates co-localization. (B) Controls in which the primary antibody was omitted show lack of reactivity. Scale bar, 100µm.



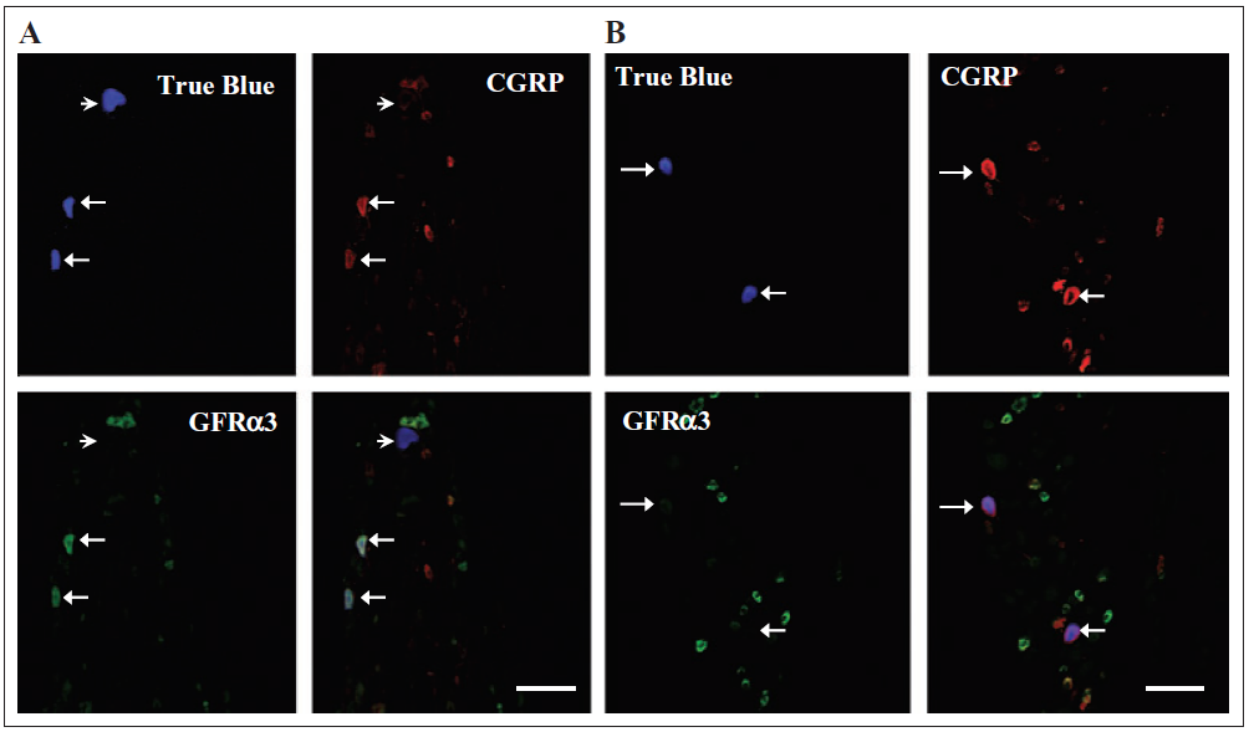
Appendix A Figure 2.

Double-label immunohistochemistry of whole-mount, adult rat dura mater with anti-glial cell line-derived neurotrophic factor family receptor α3 (GFRα3) and (A) anti-tyrosine hydroxylase (TH) or (B,C) anti-calcitonin gene-related peptide (CGRP), showed GFRα3-like immunoreactivity (-LI) in sympathetic efferents and peptidergic sensory afferents, respectively. (C) Enlarged portion of boxed area in B showing GFRα3-LI fibers that do not show CGRP-LI. (D) Controls in which the primary antibody was omitted show lack of reactivity. Scale bars, 100 μm.


A
True Blue
TRPV1
GFRα3
B
True Blue
TRPV1
GFRα3
C
GFRα3
TRPV1

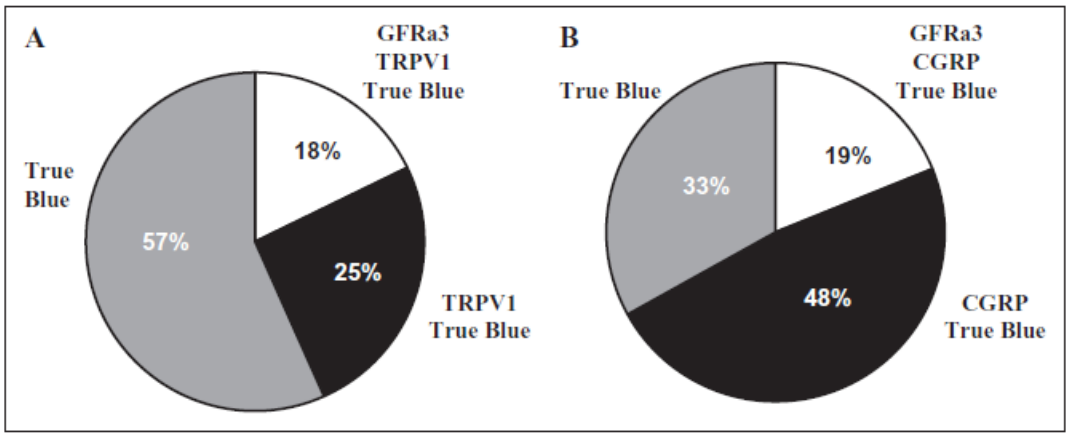
Appendix A Figure 3.

Glial cell line-derived neurotrophic factor family receptor α3 (GFRα3)-like immunoreactivity (-LI) and transient receptor potential ion channel 1 (TRPV1)-LI were seen in 17.7 ± 1.4% and 43.2 ±1.7%, respectively, of dural true blue back-labeled neurons in adult rat trigeminal ganglia (TG). All GFRα3-LI neurons were also TRPV1-LI. Example of dural afferent that was (A) positively labeled for both GFRα3 and TRPV1 (arrow), labeled for only TRPV1 (arrowhead), and (B) negatively labeled (arrows). (C) Controls in which the primary antibody was omitted show lack of reactivity. Scale bar, 100 µm.



Appendix A Figure 4.

Glial cell line-derived neurotrophic factor family receptor α3 (GFRα3)-like immunoreactivity (-LI) and calcitonin generelated peptide (CGRP)-LI were seen in 18.6 ± 2.6% and 66.0 ± 6.0%, respectively, of dural true blue back-labeled neurons in adult rat trigeminal ganglia (TG). All GFRα3-LI neurons were also CGRP-LI. Example of dural afferents that were (A) positively labeled for both GFRα3 and CGRP (arrows), negatively labeled (arrowhead) and (B) labeled for only CGRP (arrows). Scale bar, 100 µm.



Appendix A Figure 5.

Summary of glial cell line-derived neurotrophic factor family receptor α3 (GFRα3), transient receptor potential ion channel 1 (TRPV1), and calcitonin gene-related peptide (CGRP) in dural afferents. (A) TRPV1-like immunoreactivity (-LI) was seen in 43.2 ± 1.7% of dural afferents, and 17.7 ± 1.4% were labeled for both TRPV1 and GFRα3. (B) CGRP-LI was seen in 66.0 ± 6.0% of dural afferents, and 18.6 ± 2.6% were labeled for both CGRP and GFRα3. All GFRα3-positive dural afferents were CGRP- and TRPV1-positive.

APPENDIX B

INCREASED SYMPATHETIC INNERVATION IN A MOUSE MODEL OF FAMILIAL HEMIPLEGIC MIGRAINE

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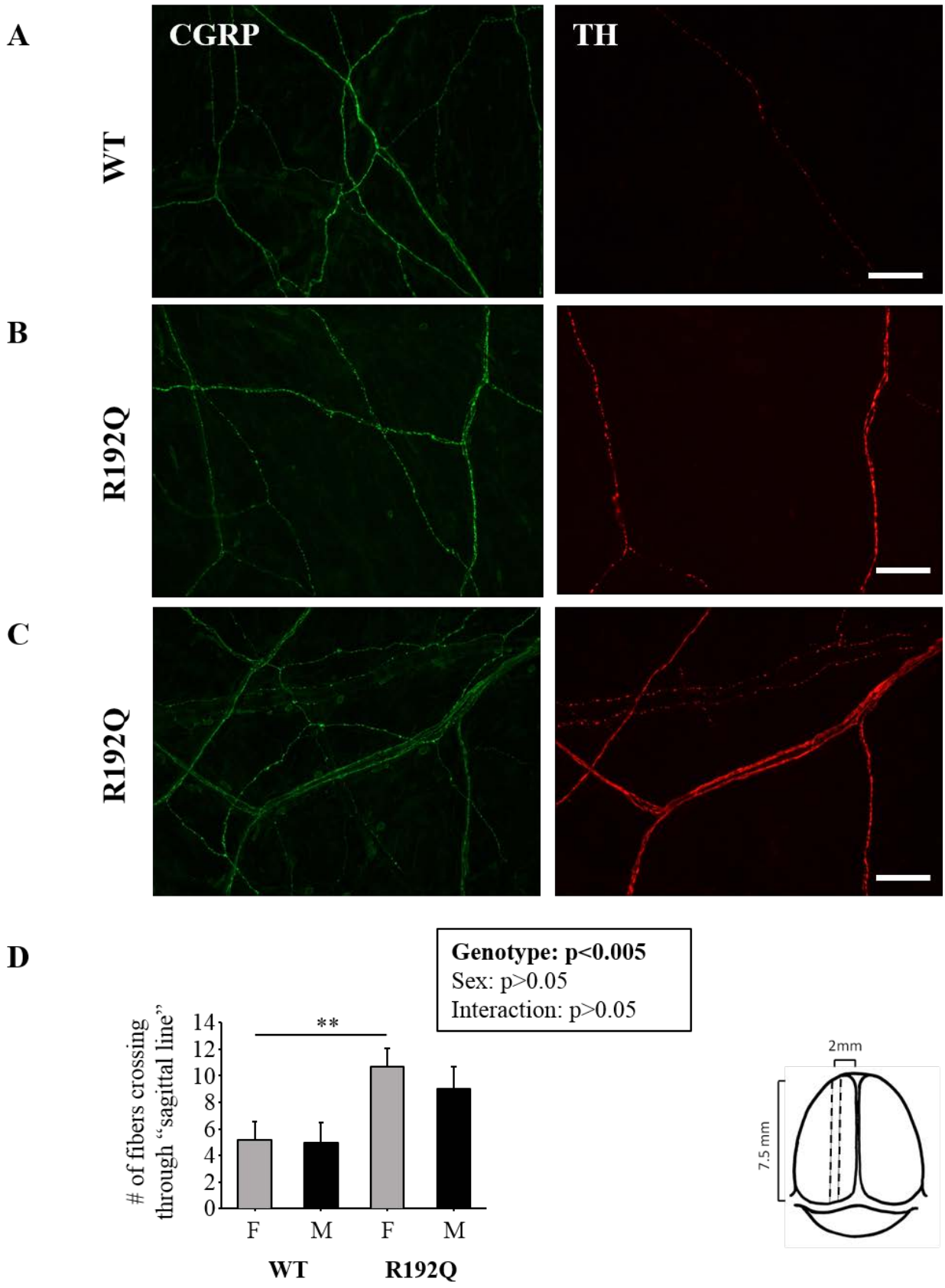
Introduction—Mechanisms underlying migraine pain have yet to be fully elucidated. Stress is the most commonly reported trigger for migraine and involves activation of the sympathetic nervous system. More specifically, alterations have been observed in sympathetic post-ganglionic neuron (SPGN) activity and excitability of nociceptive afferents in the dura mater have been implicated in the pathophysiology of migraine. Therefore, we have begun to test whether alterations are present in the sympathetic innervation of the dura of transgenic knock-in mice that harbor an R192Q missense mutation (i.e. R192Q KI mice) in the $\alpha 1$ subunit of voltage-gated Ca^{2+} channels. In patients, this mutation causes familial hemiplegic migraine (FHM) type 1, a monogenic type of migraine with aura associated with long-lasting hemiparesis.

Methods—Afferent and sympathetic efferent innervation of the dura from male and female R192Q KI and wild-type mice was assessed with Western blotting and immunohistochemistry.

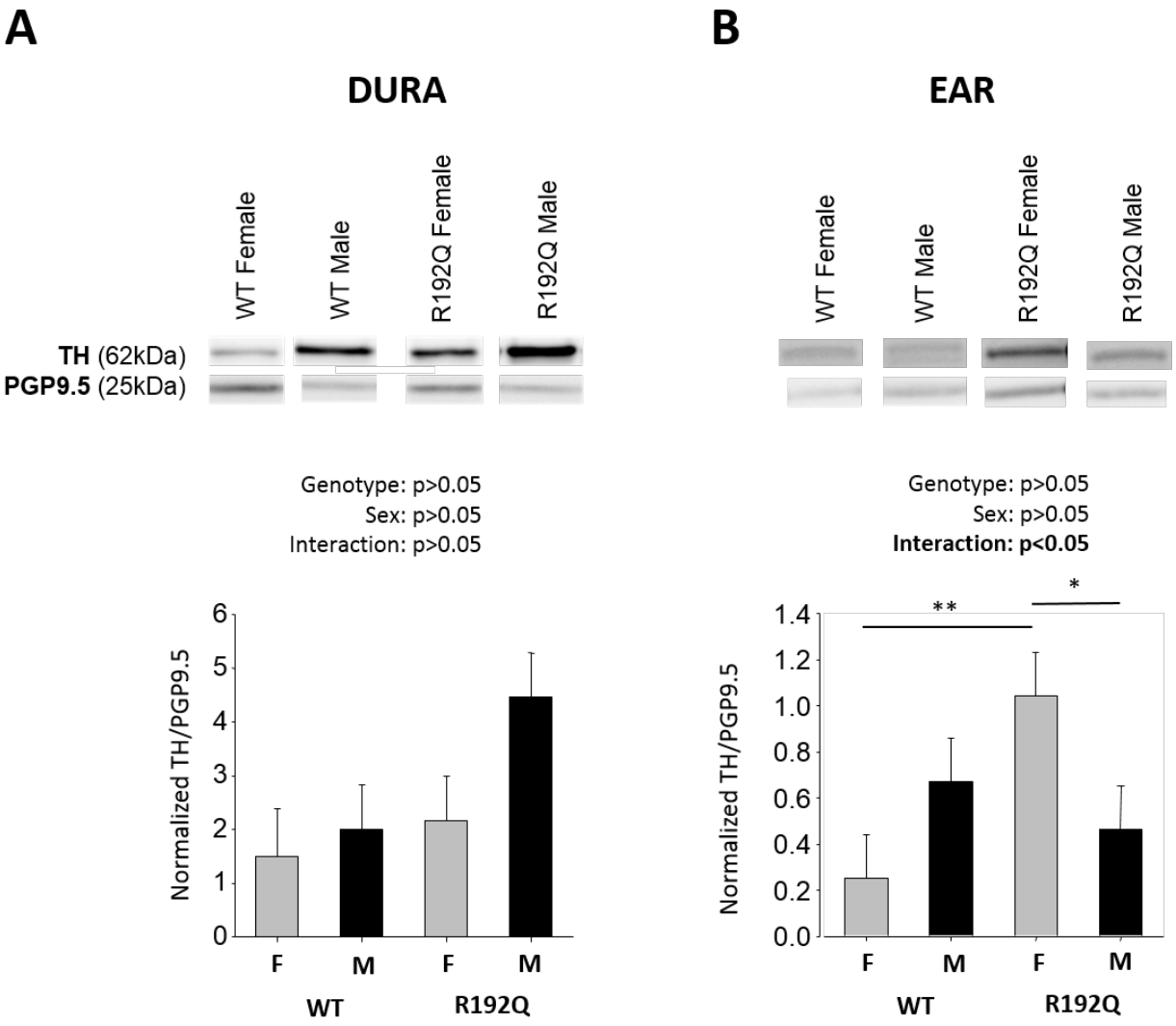
Results—The R192Q mutation had no significant influence on total dural tyrosine hydroxylase-like immunoreactive (TH-LI) protein, a marker of sympathetic innervation. However, the mutation was associated with significantly more TH-LI vasculature-independent fibers innervating the dura ($p<0.01$), particularly proximal to the transverse sinus. There was also significantly more total TH-LI protein in female mutant non-migraine associated tissue (ear), than that in wild-type females or mutant males.

Conclusions—Hyper-sympathetic innervation, particularly of the dura, was observed in a genetic model of migraine. This may provide an anatomical substrate for the link between stress and a migraine attacks.


A
CGRP
TH
WT
B
R192Q
C
R192Q
D
Genotype: p<0.005
Sex: p>0.05
Interaction: p>0.05
# of fibers crossing through "sagittal line"
**
14
12
10
8
6
4
2
0
F
M
F
M
WT
R192Q
2mm
7.5 mm

Appendix B Figure 1. Increased TH-LI vasculature-independent fibers in dura of R192Q mutant mice.

Whole mounts of adult (**A**) wild-type (WT) female and (**B**) R192Q mutant male and (**C**) female mouse dura mater were probed with anti-CGRP (green) or anti-TH (red) antibodies. (**D**) Quantification of TH-LI fibers showed a main effect of genotype. Significantly more vasculature-independent fibers were seen in R192Q mice than WT ($p<0.005$). Specifically, more fibers were found in female R192Q mice than WT ($p<0.05$). Quantification was performed by counting TH-LI fibers that passed through an area parallel but 2mm lateral to the sagittal sinus (**D**, right). Density of TH-LI vasculature independent fibers in R102Q mutant mice was greatest proximal to the transverse sinus. N=6-8 per group. Scale bars 100 μm.



Appendix B Figure 2. Increased TH-LI innervation in female R192Q mutant mice ear tissue.

Top: Western blots of adult female and male, wild-type (WT) and R192Q mutant mouse dura (**A**) and ear (**B**) tissue probed for TH, a marker of sympathetic innervation, and PGP9.5, a marker of total innervation. Bottom: (**A**) Quantification of western blots of dural tissue showed no significant effects of genotype or sex ($p>0.05$). (Female WT, n=6; other groups n=7.) (**B**) Western blots of a non-migraine associated tissue, ear, showed significantly more TH in female mutant mice than males ($p<0.05$) or WT females ($p<0.01$). (All groups n=7.)

APPENDIX C

STRESS INDUCED MAST CELL DEGRANULATION AND PLASMA EXTRAVASATION IN THE DURA MATER OF RATS IS REGULATED BY SYMPATHETIC POSTGANGLIONIC NEURONS

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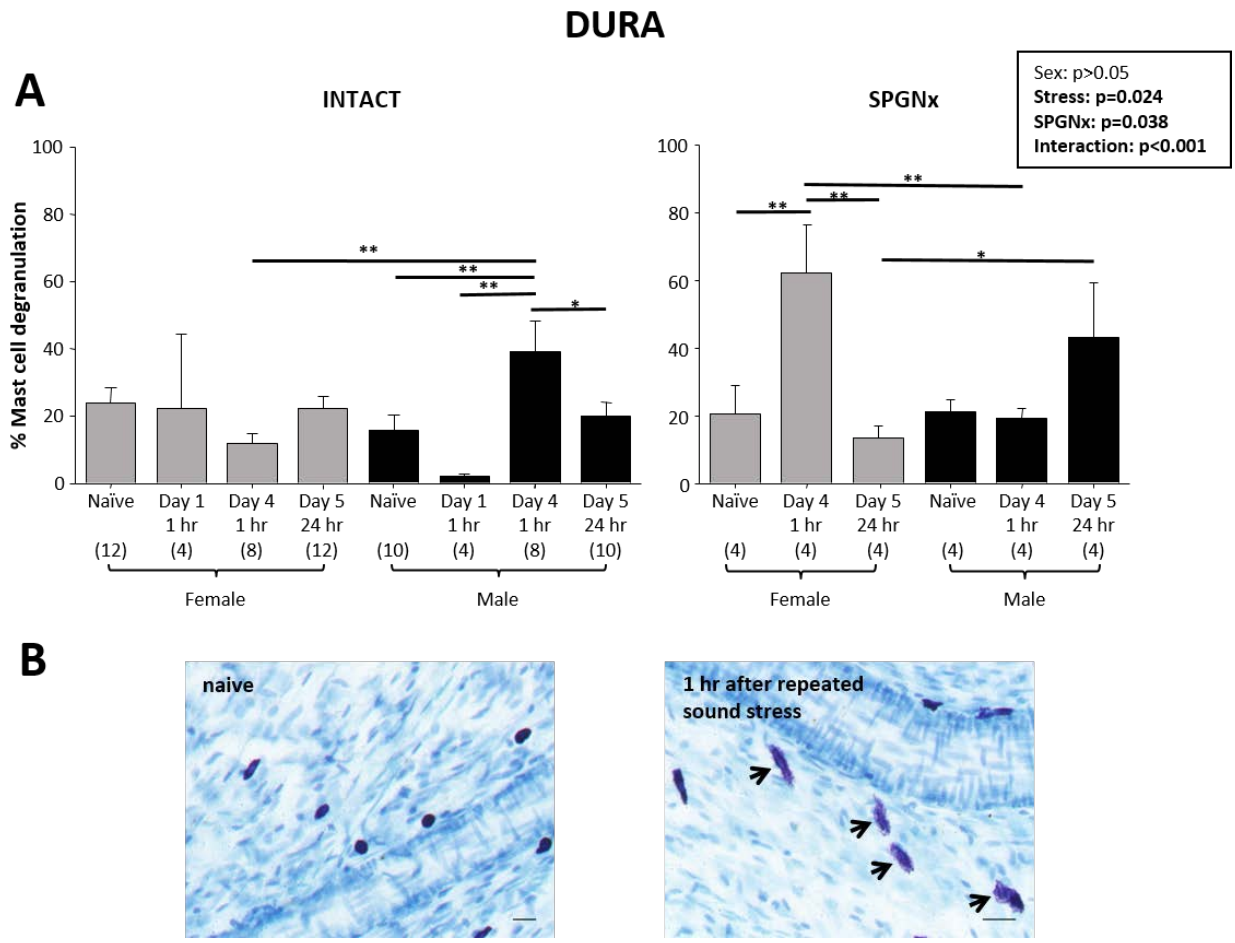
Introduction—Migraine is a debilitating disorder characterized by long lasting attacks of severe head pain, with a high prevalence in females (3:1). Evidence suggests that inflammatory mediators from the dural vasculature and/or dural mast cells contribute to the activation of dural afferents during a migraine. Evidence also suggests that migraine attacks are triggered and that stress is the most commonly reported trigger for migraine. The aim of this investigation was to determine whether there is a link between stress and changes in dural mast cells and plasma extravasation (PE).

Methods— Adult male and female Sprague Dawley rats were used in all experiments. Rats were stressed with a nonhabituating sound stress paradigm consisting of exposures to 5-10 second 11-19 kHz tones at 105 dB, played randomly once per minute for 30 minutes. Rats were exposed to the sound stress on days 1, 3 and 4 to create a non-habituating stress (Strausbaugh et

al., 2003). Groups consisted of “naïve”, “Day 1-1hr” (immediately after the first day of stress), “Day 4-1hr” (immediately after the last day of stress), and “Day 5- 24hrs” (24 hr delay after the last day of stress). Furthermore, to determine whether sympathetic postganglionic neurons (SPGN) in the dura contribute to the link between stress and changes in the dura, additional groups were studied in which superior cervical ganglia were surgically removed (SPGNx) prior to any experimental manipulation. Mast cells were visualized in fixed tissue stained with toluidine blue. PE was quantified with a ten minute exposure to Evans blue (50mg/kg IV), subsequent extraction from tissue in DMSO and assessment by spectrophotometry at 620 nm. Analysis was performed as 2 or 3-way ANOVAs, as appropriate.

Results— Mast cell degranulation was significantly increased immediately following stress, with sex-dependent regulation by SPGNs. There was no change in the density of dural mast cells. PE in the dura, ear, and viscera was significantly increased immediately following stress in both males and females, which recovered by 24hrs. These effects were SPGN dependent.

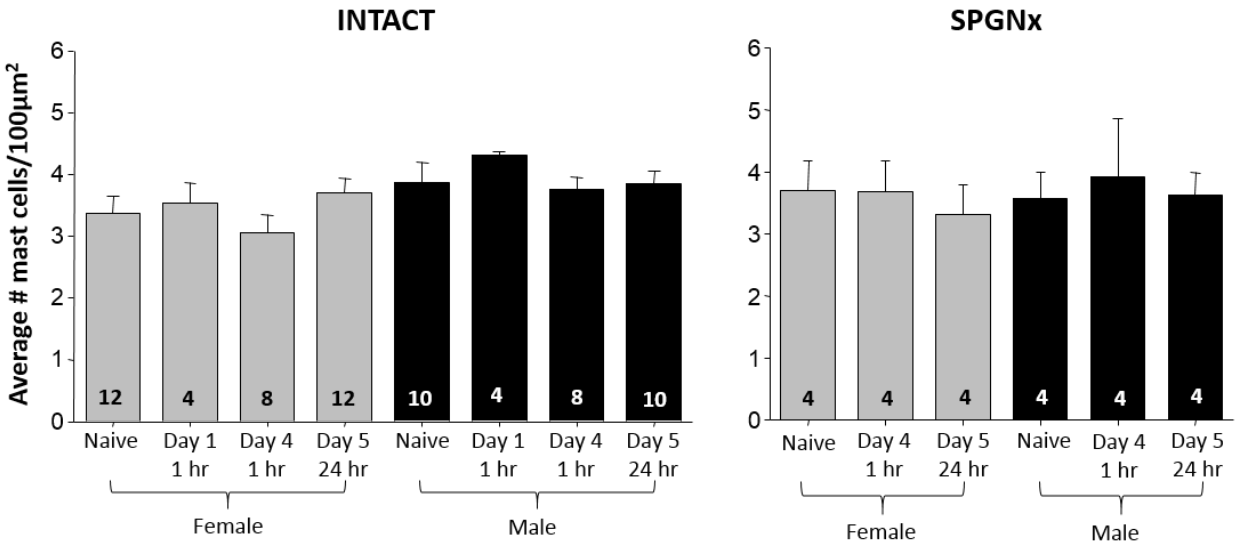
Conclusions— There is a complex interaction between the influences of sex, stress and sympathetic innervation on the regulation of mast cell degranulation and plasma extravasation in the dura. SPGNs differentially stabilize dural mast cells in males and females during repeated non-habituating stress. An impaired sympathetic system, as observed in migraineurs, could result in dysregulation of dural mast cells and a lower threshold for degranulation following a stressful trigger, providing a mechanism for subsequent sensitization of dural afferents. However, an increase in neither mast cell degranulation nor plasma extravasation *per se*, can account for the delay between the end of stress and the initiation of a migraine attack.



Appendix C Figure 1. Stress-induced, sex-dependent changes in dural mast cell degranulation are regulated by SPGNs.

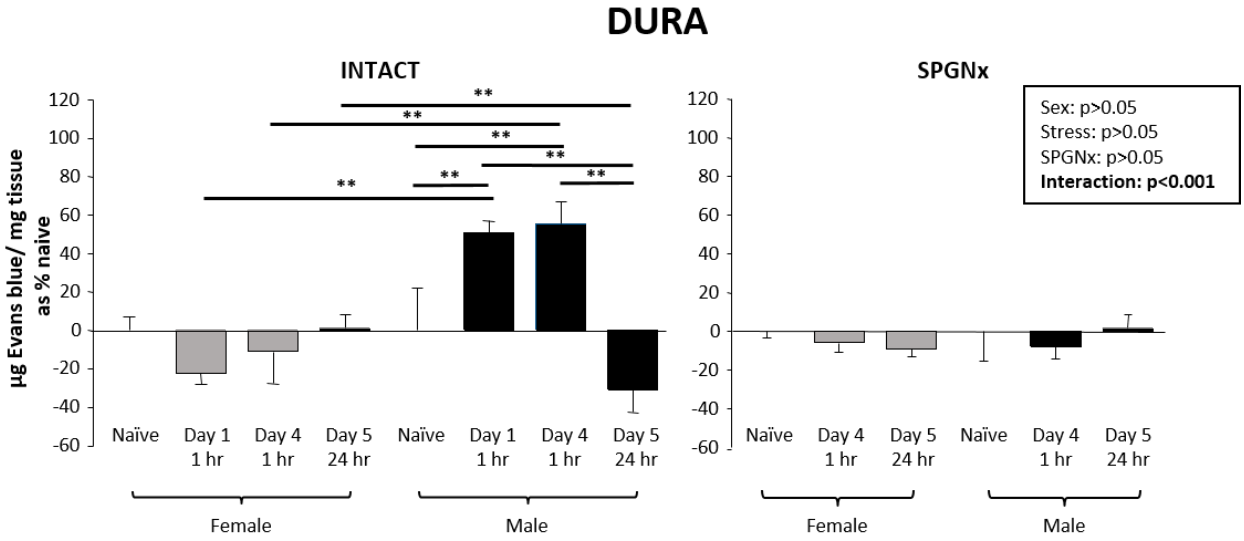
There was no significant effect of sex on mast cell degranulation in intact rats. However, there was a significant effect of sound stress on mast cell degranulation, due to the stress-induced changes in degranulation in males. There was a significant effect of SPGNx on mast cell degranulation due to increases in degranulation associated with stress in both males and females. Finally, there was a significant interaction between sex, stress and SPGNx due to the differential pattern of degranulation observed in response to stress in intact and SPGNx rats. Numbers in parenthesis are the number of animals per group. SPGNx=bilateral surgical removal of superior cervical ganglia, resulting in loss of cephalic SPGN innervation; Naive=not exposed to stress paradigm; Day 1-1hr= immediately after the first day of stress; Day 4-1hr-immediately after the last day of stress; Day 5-24hrs=a 24hr delay after the last day of stress. (**B**) Representative whole mounts of toluidine blue stained dura from a naïve male (left) and

"Day4-1hr" stressed male (right). Note the absence of degranulated mast cells in the naïve dura and robust signs of degranulation in the stressed dura (arrows). Scale bar 10 μM for both.



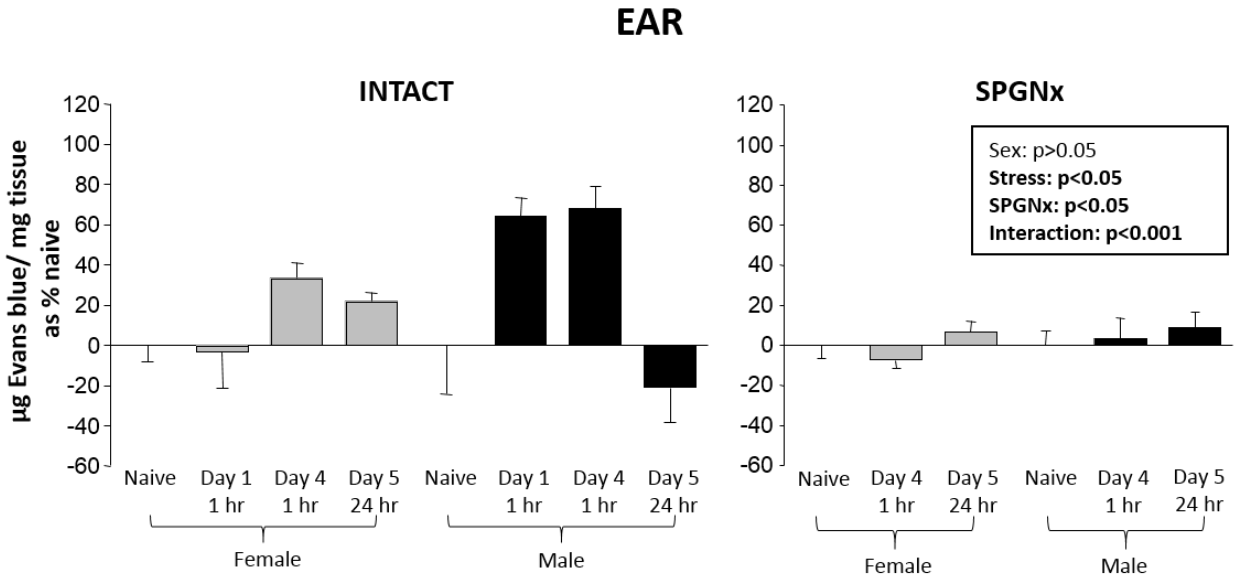
Appendix C Figure 2. No change in density of dural mast cells.

The density of dural mast cells did not change with sex, stress, or SPGNx, suggesting that mast cell recruitment is not a mechanism of stress-induced migraine.



Appendix C Figure 3. Stress-induced, sex-dependent changes in dural plasma extravasation are SPGN dependent.

In intact males, but not females, stress produced a significant increase in dural plasma extravasation, which recovered by 24hrs after 4 days of repeated sound stress. Effects were SPGN dependent. Sound stress and groups were the same as in Appendix C Figure 1. N=4 for all groups.



Appendix C Figure 4. Stress-induced increases in plasma extravasation in the ear is SPGN dependent.

In intact animals, sound stress produced a significant increase in PE in the ear, a non-migraine associated tissue, which in males recovered by 24 hrs. These effects were SPGN dependent. Sound stress and groups were the same as in Appendix C Figure 1. N=4 for all groups.

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