THE ROLE OF RELEVANT PEPTIDES ON THE SEX-SPECIFIC NATURE OF MIGRAINE

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Migraine is considered to be both one of the most disabling and common disorders worldwide yet, migraine is both more common and disabling among women. While this disorder effects 2-3 times as many women as men across the globe the reasons for this disparity are not known. These data present new insights regarding how calcitonin gene-related peptide interacts with the predominantly female hormone prolactin to create female-specific hypersensitivity in rodent models of migraine. This sensitivity to dural CGRP is also contingent on the presence of ovarian secreted hormones, although it is not yet clear which are responsible for mediating this effect. While CGRP is likely in part responsible for the prevalence of migraine in women, in this dissertation we present evidence that dural amylin leads to hypersensitivity in males, but not in females. As such amylin may mediate migraine in males; however, ovariectomized females demonstrate more robust responses to dural amylin, than their male counterparts. This suggests that ovarian derived hormones may block responses to dural amylin. The data covered in this project may provide valuable information for the development of sex-specific migraine therapeutics.

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CHAPTER 1

THE ROLE OF SEX-SPECIFIC HORMONES AND MIGRAINE RELEVANT PEPTIDES IN MIGRAINE

Migraine is a complex neurological disorder the symptoms of which are variable among patients, but commonly consist of unilateral throbbing pain, photophobia, phonophobia, cutaneous hypersensitivity in the face and body, nausea, and fatigue [40; 42; 142; 175; 202]. Due to the nature of these symptoms migraine is considered to be the 2nd most disabling disease world-wide [62]. Migraine symptoms can surface prior to the headache phase of migraine during what is referred to as the prodrome, or premonitory, phase of migraine. During the prodrome patients may feel fatigue, nausea, photophobia or phonophobia. Migraine is a highly multifactorial disorder and what may trigger an attack in one migraineur will not necessarily evoke a migraine in another patient [6; 142]. Commonly reported triggers include consumption of alcohol, alterations in sleep wake patterns and stress. The most commonly self-reported trigger for migraine attacks is stress [142]. While certain studies suggest that women more commonly perceive stress as a trigger for their migraine attacks when compared with men, [126] whether or not there are differences in stress induced migraine experienced by men and women remain unclear.

The circuitry of migraine is complex, but migraine related pain is thought to originate from activation and sensitization of dural afferents typically via inflammatory or mechanical stimulation [158; 253]. these signals are received the trigeminal ganglia (TG) [41]. These are then sent to higher order neurons in the trigeminal nucleus caudalis (TNC), followed by processing in the

cortex to allow for the perception of pain. It is thought that inflammatory cytokines likely play a role in meningeal afferent stimulation [41; 103].

Migraine is one of the top 6 most common disorders across the globe in both men and women [71]. Yet migraine is primarily experienced by women, with 2-3 times as many women suffering from migraine as men. In childhood there is not an obvious sex discrimination in the incidence of migraine [27]. This disparity typically begins around the onset of puberty and is lost following menopausal years [43; 230; 231]. Among women aged 15-49 migraine is considered to be the leading cause of disability [230]. Reasons for the sexually dimorphic nature of migraine are not clear, although here we present data offering new insights into how various migraine relevant peptides may differentially mediate migraine between the sexes. Given the onset of migraine during puberty and that a large portion of women report that their migraines coincide with changes in the menstrual cycle or occur with menstruation, there is likely a hormonal component [166]. There is long held evidence that sexually specific hormones may protect against or mediate migraine attacks.

The predominantly male hormone testosterone has long been thought to serve a protective role in inflammatory and chronic pain conditions. Testosterone treatment in men decreases the presence of the proinflammatory cytokines tumor necrosis factor α (TNFα), interleukin (IL)-1β, and IL-6 [168; 170; 187] both of which are upregulated in during migraine[91; 214; 270]. Studies have demonstrated that men with chronic migraine have significantly lower testosterone levels than healthy controls [221]. Testosterone treatment in men with cluster headache resolved headaches and in some patients lead to remission [232]. Additionally, testosterone not only

contributes to antinociception in males, but has similar effects in females. For example, women with chronic migraine that received continuous testosterone via subcutaneous implant reported decreased severity and frequency of migraines [102].

As previously stated, the literature establishes the role of testosterone as being widely protective against pain and inflammation. However, the role of estrogen remains unclear. E2 was shown to increase facial receptive fields of trigeminal ganglia (TG) neurons in rats as well as decrease the thresholds for activation these fields, making the argument that E2 may contribute to female-specific pain states mediated by the TGs, such as migraine [77]. Within the last year, estrogen receptors (ERs) were found to be more abundant in female than male rats in the TG [262]. These data, combined with data showing ER agonists lead to blood vessel dilation in the dura [262], have led to speculation that E2 may contribute to predominantly female pain disorders such as migraine and temporomandibular joint syndrome. In contrast, many data have been published that suggest estrogen plays a protective role against inflammation and pain disorders. Estrogen has been shown to attenuate lymphocyte extravasation following exposure to TNFα, as well as reduce the trafficking of immune cells in response to inflammation [169]. Moreover, the estrogen steroid hormone 17β-E2 serves a protective role at the blood-brain barrier, helping to maintain tight junctions between endothelial cells [149; 262]. Decreases in E2 following menopause correlate with increases in the proinflammatory cytokines IL-6, IL-1β, and TNFα [201]. Ultimately, E2 levels may be responsible for these conflicting reports, as low levels of estrogen have been shown to increase inflammatory mediators, but estrogen replacement can lead to a reduction in these inflammatory cytokines. In agreement with this statement, certain migraine attacks are evoked by

estrogen withdrawal [1; 167]; however, the use of estrogen-containing contraceptives has been noted to induce migraine attacks [4; 107; 173]. These data indicate that fluctuations in E2 levels may aid in the development of pain states in women, whereas stability of E2 levels may serve a protective role against nociceptive signaling (see Fig. 2).

Prolactin (PRL) is a female-specific hormone that is also thought to contribute to migraine. Women that have previously not experienced migraines have reported onset of migraines following development of PRL secreting tumors called microprolactinomas [31]. In these patients increased PRL levels are associated with migraine attacks [31]. Furthermore bromocriptine, which prevents secretion of PRL from the pituitary, has been shown to treat migraine [106; 121]. A cross-sectional study found that women with migraine had no significant difference in levels of estrogen, luteinizing hormone, or follicle stimulating hormone, but women with migraine did have significantly higher levels of PRL than their healthy counterparts [194]. Additionally, PRL sensitizes the pain signaling channels TRPV1, TRPM8, and TRPA1 within the dorsal root ganglia of female, but not male mice [199]. PRL additionally mediates other pain states including heat induced hyperalgesia [197] and chronic pain [195] a female-specific manner.

Interestingly, PRL also acts as an inflammatory cytokine [29; 127]. PRL belongs to the Interleukin-6 (IL-6) family cytokines, which includes IL-6, IL-11, IL-27, Cardiotrophin-1, Cardiotrophin-like cytokine, and oncostatin M [208]. The cytokines in this family share the receptor subunit glycoprotein 130 kDa activation of which is mediated via janus kinases or JAKS which dimerize with signal transducer and activator of transcription (STATs) [154; 209]. The IL-6 family cytokines signal through JAK1/STAT3 [222; 257]. Among these cytokines IL-6 has

additionally been heavily implicated in migraine. IL-6 is upregulated in the serum and blood of migraine patients during an attack [91; 214; 270]. PRL has may also aid in the production of IL-6 induced peripheral allodynia [195], intrathecal administration of a PRL receptor antagonist (ΔPRL) blocks responses to IL-6 in female mice. Furthermore, co-injection of PRL with IL-6 increases hindpaw hypersensitivity in female mice when compared with those that received IL-6 alone [74; 186; 258]. IL-6 also has been shown to cause facial hypersensitivity following direct application onto the dura of both mice and rats [37; 39]. Due to the likely contribution of IL-6 to the migraine pathology, and prolactin in the migraine pathology we have chosen to explore the potential differences in IL-6 induced hypersensitivity in male and female rats.

These hormones and cytokines, while involved in the migraine pathology, likely are not a sole cause of migraine rather it is probable that they interact with one of, or a series of, peptides that have been implicated in the migraine pathology. observed to lead to migraine attacks. Among these are poly adenylate cyclase activating peptide (PACAP), calcitonin gene-related peptide (CGRP), and the CGRP relative amylin. PACAP is a potent vasodilator found in the TG and TNC [24; 87; 250]. Administration of the PACAP isoform PACAP-38 has been shown to induce migraine attacks in migraineurs [218]. Plasma levels of PACAP-38 are also increased during migraine attacks [249]. PACAP is heavily involved in regulation of the hypothalamic-pituitary-adrenal (HPA) axis and the stress response [122] and is likely involved in stress induced-migraine.

Calcitonin gene-related peptide (CGRP) has long been associated with migraine. CGRP is widely expressed in dural afferent fibers and the TG [88; 131]. CGRP is upregulated in the blood, saliva, and cerebrospinal fluid of patients during an attack [14; 23]. Additionally, intravenous

administration of CGRP triggers migraine attacks in migraineurs [27]. Intravenous CGRP also leads to cranial vasodilation [9]. Furthermore, CGRP antagonists and monoclonal antibodies against both CGRP and the CGRP receptor have been proven effective in reducing migraine frequency and severity [12; 19].

While the role of PACAP and CGRP in migraine have been heavily explored for a long duration of time, amylin has more recently been implicated in the migraine pathology. Similar to CGRP and PACAP the amylin analog pramlintide has been shown to induce migraine in migraine patients [101]. Amylin and the amylin receptor components calcitonin receptor (CTR), and receptor activity modifying protein 1, 2, and 3 (RAMP1, RAMP2, and RAMP3) have all been found within the TGs [84; 260]. Amylin and CGRP share affinity for the canonical CGRP receptor which is comprised of calcitonin receptor like receptor (CLR) and RAMP1 [124]. Therefore, it is possible that they would exert similar effects within the trigeminal ganglia and potentially result in similar behavior responses in migraine models.

Here we have chosen to explore the role of the outlined migraine relevant peptides and ovarian derived hormones, with an emphasis on the role of PRL, in the sexual dichotomy of migraine. We examine the role of these peptides in a previously established migraine models [39; 81]. In addition to these models, which involve direct dural stimulation, we utilize a model of stress induced migraine that we have developed here.

REFERENCES

[1] Headache Classification Committee of the International Headache Society (IHS) The International Classification of Headache Disorders, 3rd edition. Cephalalgia 2018;38(1):1-211.

[2] Allais G, Chiarle G, Sinigaglia S, Airola G, Schiapparelli P, Benedetto C. Estrogen, migraine, and vascular risk. Neurol Sci 2018;39(Suppl 1):11-20.

[3] Andress-Rothrock D, King W, Rothrock J. An analysis of migraine triggers in a clinic-based population. Headache 2010;50(8):1366-1370.

[4] Asghar MS, Hansen AE, Amin FM, van der Geest RJ, Koning P, Larsson HB, Olesen J, Ashina M. Evidence for a vascular factor in migraine. Ann Neurol 2011;69(4):635-645.

[5] Bhatt DK, Gupta S, Olesen J, Jansen-Olesen I. PACAP-38 infusion causes sustained vasodilation of the middle meningeal artery in the rat: possible involvement of mast cells. Cephalalgia 2014;34(11):877-886.

[6] Borba VV, Zandman-Goddard G, Shoenfeld Y. Prolactin and autoimmunity: The hormone as an inflammatory cytokine. Best Pract Res Clin Endocrinol Metab 2019;33(6):101324.

[7] Bosco D, Belfiore A, Fava A, De Rose M, Plastino M, Ceccotti C, Mungari P, Iannacchero R, Lavano A. Relationship between high prolactin levels and migraine attacks in patients with microprolactinoma. J Headache Pain 2008;9(2):103-107.

[8] Burgos-Vega CC, Quigley LD, Avona A, Price T, Dussor G. Dural stimulation in rats causes brain-derived neurotrophic factor-dependent priming to subthreshold stimuli including a migraine trigger. Pain 2016;157(12):2722-2730.

[9] Burgos-Vega CC, Quigley LD, Trevisan Dos Santos G, Yan F, Asiedu M, Jacobs B, Motina M, Safdar N, Yousuf H, Avona A, Price TJ, Dussor G. Non-invasive dural stimulation in mice: A novel preclinical model of migraine. Cephalalgia 2019;39(1):123-134.

[10] Burstein R, Cutrer MF, Yarnitsky D. The development of cutaneous allodynia during a migraine attack clinical evidence for the sequential recruitment of spinal and supraspinal nociceptive neurons in migraine. Brain 2000;123 (Pt 8):1703-1709.

[11] Burstein R, Yamamura H, Malick A, Strassman AM. Chemical stimulation of the intracranial dura induces enhanced responses to facial stimulation in brain stem trigeminal neurons. J Neurophysiol 1998;79(2):964-982.

[12] Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ, Bajwa ZH. An association between migraine and cutaneous allodynia. Ann Neurol 2000;47(5):614-624.

[13] Buse DC, Loder EW, Gorman JA, Stewart WF, Reed ML, Fanning KM, Serrano D, Lipton RB. Sex differences in the prevalence, symptoms, and associated features of migraine, probable migraine and other severe headache: results of the American Migraine Prevalence and Prevention (AMPP) Study. Headache 2013;53(8):1278-1299.

[14] DALYs GBD, Collaborators H. Global, regional, and national disability-adjusted life-years (DALYs) for 333 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 2017;390(10100):1260-1344.

[15] Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2018;392(10159):1789-1858.

[16] Dohler KD, Wuttke W. Changes with age in levels of serum gonadotropins, prolactin and gonadal steroids in prepubertal male and female rats. Endocrinology 1975;97(4):898-907.

[17] Duarte DB, Vasko MR, Fehrenbacher JC. Models of Inflammation: Carrageenan Air Pouch. Curr Protoc Pharmacol 2016;72:5 6 1-5 6 9.

[18] Edelmayer RM, Vanderah TW, Majuta L, Zhang ET, Fioravanti B, De Felice M, Chichorro JG, Ossipov MH, King T, Lai J, Kori SH, Nelsen AC, Cannon KE, Heinricher MM, Porreca F. Medullary pain facilitating neurons mediate allodynia in headache-related pain. Ann Neurol 2009;65(2):184-193.

[19] Edvinsson L, Grell AS, Warfvinge K. Expression of the CGRP Family of Neuropeptides and their Receptors in the Trigeminal Ganglion. J Mol Neurosci 2020;70(6):930-944.

[20] Eftekhari S, Salvatore CA, Gaspar RC, Roberts R, O'Malley S, Zeng Z, Edvinsson L. Localization of CGRP receptor components, CGRP, and receptor binding sites in human and rhesus cerebellar cortex. Cerebellum 2013;12(6):937-949.

[21] Eftekhari S, Warfvinge K, Blixt FW, Edvinsson L. Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. J Pain 2013;14(11):1289-1303.

[22] Fidan I, Yuksel S, Ymir T, Irkec C, Aksakal FN. The importance of cytokines, chemokines and nitric oxide in pathophysiology of migraine. J Neuroimmunol 2006;171(1-2):184-188.

[23] Ghanizada H, Al-Karagholi MA, Walker CS, Arngrim N, Rees T, Petersen J, Siow A, Morch-Rasmussen M, Tan S, O'Carroll SJ, Harris P, Skovgaard LT, Jorgensen NR, Brimble M, Waite JS, Rea BJ, Sowers LP, Russo AF, Hay DL, Ashina M. Amylin Analog Pramlintide Induces Migraine-like Attacks in Patients. Ann Neurol 2021;89(6):1157-1171.

[24] Glaser R, Dimitrakakis C, Trimble N, Martin V. Testosterone pellet implants and migraine headaches: a pilot study. Maturitas 2012;71(4):385-388.

[25] Goadsby PJ, Holland PR, Martins-Oliveira M, Hoffmann J, Schankin C, Akerman S. Pathophysiology of Migraine: A Disorder of Sensory Processing. Physiol Rev 2017;97(2):553-622.

[26] Gordon ML, Lipton RB, Brown SL, van Praag HM. The neuroendocrine challenge paradigm in headache research. Cephalalgia 1995;15(4):292-296.

[27] Granella F, Sances G, Zanferrari C, Costa A, Martignoni E, Manzoni GC. Migraine without aura and reproductive life events: a clinical epidemiological study in 1300 women. Headache 1993;33(7):385-389.

[28] Hartman N, Voron SC, Hershman JM. Resolution of migraine following bromocriptine treatment of a prolactinoma (pituitary microadenoma). Headache 1995;35(7):430-431.

[29] Hashimoto H, Shintani N, Tanida M, Hayata A, Hashimoto R, Baba A. PACAP is implicated in the stress axes. Curr Pharm Des 2011;17(10):985-989.

[30] Hay DL, Garelja ML, Poyner DR, Walker CS. Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. Br J Pharmacol 2018;175(1):3-17.

[31] Hedborg K, Anderberg UM, Muhr C. Stress in migraine: personality-dependent vulnerability, life events, and gender are of significance. Ups J Med Sci 2011;116(3):187-199.

[32] Hinterberger-Fischer M. [Prolactin as pro-inflammatory cytokine--considerations on consolidated immunotherapy after high dosage therapy]. Acta Med Austriaca Suppl 2000;52:16-20.

[33] Iyengar S, Johnson KW, Ossipov MH, Aurora SK. CGRP and the Trigeminal System in Migraine. Headache 2019;59(5):659-681.

[34] Kelman L. The triggers or precipitants of the acute migraine attack. Cephalalgia 2007;27(5):394-402.

[35] Kuruca SE, Karadenizli S, Akgun-Dar K, Kapucu A, Kaptan Z, Uzum G. The effects of 17beta-estradiol on blood brain barrier integrity in the absence of the estrogen receptor alpha; an in-vitro model. Acta Histochem 2017;119(6):638-647.

[36] Leaman DW, Pisharody S, Flickinger TW, Commane MA, Schlessinger J, Kerr IM, Levy DE, Stark GR. Roles of JAKs in activation of STATs and stimulation of c-fos gene expression by epidermal growth factor. Mol Cell Biol 1996;16(1):369-375.

[37] Levy D, Strassman AM. Mechanical response properties of A and C primary afferent neurons innervating the rat intracranial dura. J Neurophysiol 2002;88(6):3021-3031.

[38] Maasumi K, Tepper SJ, Kriegler JS. Menstrual Migraine and Treatment Options: Review. Headache 2017;57(2):194-208.

[39] MacGregor EA, Frith A, Ellis J, Aspinall L, Hackshaw A. Incidence of migraine relative to menstrual cycle phases of rising and falling estrogen. Neurology 2006;67(12):2154-2158.

[40] Maggio M, Basaria S, Ble A, Lauretani F, Bandinelli S, Ceda GP, Valenti G, Ling SM, Ferrucci L. Correlation between testosterone and the inflammatory marker soluble interleukin-6 receptor in older men. J Clin Endocrinol Metab 2006;91(1):345-347.

[41] Maggioli E, McArthur S, Mauro C, Kieswich J, Kusters DHM, Reutelingsperger CPM, Yaqoob M, Solito E. Estrogen protects the blood-brain barrier from inflammation-induced disruption and increased lymphocyte trafficking. Brain Behav Immun 2016;51:212-222.

[42] Malkin CJ, Morris PD, Pugh PJ, English KM, Channer KS. Effect of testosterone therapy on QT dispersion in men with heart failure. Am J Cardiol 2003;92(10):1241-1243.

[43] Massiou H, MacGregor EA. Evolution and treatment of migraine with oral contraceptives. Cephalalgia 2000;20(3):170-174.

[44] Mathew NT, Kailasam J, Seifert T. Clinical recognition of allodynia in migraine. Neurology 2004;63(5):848-852.

[45] Neill JD. Effect of "stress" on serum prolactin and luteinizing hormone levels during the estrous cycle of the rat. Endocrinology 1970;87(6):1192-1197.

[46] Norata GD, Tibolla G, Seccomandi PM, Poletti A, Catapano AL. Dihydrotestosterone decreases tumor necrosis factor-alpha and lipopolysaccharide-induced inflammatory response in human endothelial cells. J Clin Endocrinol Metab 2006;91(2):546-554.

[47] Parashar R, Bhalla P, Rai NK, Pakhare A, Babbar R. Migraine: is it related to hormonal disturbances or stress? Int J Womens Health 2014;6:921-925.

[48] Patil M, Belugin S, Mecklenburg J, Wangzhou A, Paige C, Barba-Escobedo PA, Boyd JT, Goffin V, Grattan D, Boehm U, Dussor G, Price TJ, Akopian AN. Prolactin Regulates Pain Responses via a Female-Selective Nociceptor-Specific Mechanism. iScience 2019;20:449-465.

[49] Patil MJ, Green DP, Henry MA, Akopian AN. Sex-dependent roles of prolactin and prolactin receptor in postoperative pain and hyperalgesia in mice. Neuroscience 2013;253:132-141.

[50] Patil MJ, Ruparel SB, Henry MA, Akopian AN. Prolactin regulates TRPV1, TRPA1, and TRPM8 in sensory neurons in a sex-dependent manner: Contribution of prolactin receptor to inflammatory pain. Am J Physiol Endocrinol Metab 2013;305(9):E1154-1164.

[51] Pfeilschifter J, Koditz R, Pfohl M, Schatz H. Changes in proinflammatory cytokine activity after menopause. Endocr Rev 2002;23(1):90-119.

[52] Plesh O, Adams SH, Gansky SA. Self-reported comorbid pains in severe headaches or migraines in a US national sample. Headache 2012;52(6):946-956.

[53] Rose-John S. Interleukin-6 Family Cytokines. Cold Spring Harb Perspect Biol 2018;10(2).

[54] Rose-John S, Scheller J, Schaper F. "Family reunion"--A structured view on the composition of the receptor complexes of interleukin-6-type and interleukin-12-type cytokines. Cytokine Growth Factor Rev 2015;26(5):471-474.

[55] Sarchielli P, Alberti A, Baldi A, Coppola F, Rossi C, Pierguidi L, Floridi A, Calabresi P. Proinflammatory cytokines, adhesion molecules, and lymphocyte integrin expression in

the internal jugular blood of migraine patients without aura assessed ictally. Headache 2006;46(2):200-207.

[56] Schytz HW, Birk S, Wienecke T, Kruuse C, Olesen J, Ashina M. PACAP38 induces migraine-like attacks in patients with migraine without aura. Brain 2009;132(Pt 1):16-25.

[57] Shields LBE, Seifert T, Shelton BJ, Plato BM. Testosterone levels in men with chronic migraine. Neurol Int 2019;11(2):8079.

[58] Shien K, Papadimitrakopoulou VA, Ruder D, Behrens C, Shen L, Kalhor N, Song J, Lee JJ, Wang J, Tang X, Herbst RS, Toyooka S, Girard L, Minna JD, Kurie JM, Wistuba, II, Izzo JG. JAK1/STAT3 Activation through a Proinflammatory Cytokine Pathway Leads to Resistance to Molecularly Targeted Therapy in Non-Small Cell Lung Cancer. Mol Cancer Ther 2017;16(10):2234-2245.

[59] Steiner TJ, Stovner LJ, Vos T, Jensen R, Katsarava Z. Migraine is first cause of disability in under 50s: will health politicians now take notice? J Headache Pain 2018;19(1):17.

[60] Stewart WF, Shechter A, Rasmussen BK. Migraine prevalence. A review of population-based studies. Neurology 1994;44(6 Suppl 4):S17-23.

[61] Stillman MJ. Testosterone replacement therapy for treatment refractory cluster headache. Headache 2006;46(6):925-933.

[62] Tuka B, Helyes Z, Markovics A, Bagoly T, Szolcsanyi J, Szabo N, Toth E, Kincses ZT, Vecsei L, Tajti J. Alterations in PACAP-38-like immunoreactivity in the plasma during ictal and interictal periods of migraine patients. Cephalalgia 2013;33(13):1085-1095.

[63] Uddman R, Tajti J, Hou M, Sundler F, Edvinsson L. Neuropeptide expression in the human trigeminal nucleus caudalis and in the cervical spinal cord C1 and C2. Cephalalgia 2002;22(2):112-116.

[64] Vaughn AH, Gold MS. Ionic mechanisms underlying inflammatory mediator-induced sensitization of dural afferents. J Neurosci 2010;30(23):7878-7888.

[65] Villarino AV, Kanno Y, O'Shea JJ. Mechanisms and consequences of Jak-STAT signaling in the immune system. Nat Immunol 2017;18(4):374-384.

[66] Voogt JL, Chen CL, Meites J. Serum and pituitary prolactin levels before, during, and after puberty in female rats. Am J Physiol 1970;218(2):396-399.

[67] Walker CS, Hay DL. CGRP in the trigeminovascular system: a role for CGRP, adrenomedullin and amylin receptors? Br J Pharmacol 2013;170(7):1293-1307.

[68] Warfvinge K, Krause DN, Maddahi A, Edvinsson JCA, Edvinsson L, Haanes KA. Estrogen receptors alpha, beta and GPER in the CNS and trigeminal system - molecular and functional aspects. J Headache Pain 2020;21(1):131.

[69] Yucel M, Kotan D, Gurol Ciftci G, Ciftci IH, Cikriklar HI. Serum levels of endocan, claudin-5 and cytokines in migraine. Eur Rev Med Pharmacol Sci 2016;20(5):930-936.

CHAPTER 2

DURAL CALCITONIN GENE-RELATED PEPTIDE PRODUCES FEMALE-SPECIFIC RESPONSES IN RODENT MIGRAINE MODELS

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ABSTRACT

Migraine is the second leading cause for disability worldwide and the most common neurological disorder. It is also three times more common in women; reasons for this sex difference are not known. Using preclinical behavioral models of migraine, we show that application of calcitonin gene-related peptide (CGRP) to the rat dura mater produces cutaneous periorbital hypersensitivity. Surprisingly, this response was observed only in females; dural CGRP at doses from 1 pg to 3.8 μg produce no responses in males. In females, dural CGRP causes priming to a pH 7.0 solution after animals recover from the initial CGRP-induced allodynia. Dural application of interleukin-6 causes acute responses in males and females but only causes priming to subthreshold dural CGRP (0.1 pg) in females. Intracisternal application of BDNF also causes similar acute hypersensitivity responses in males and females but only priming to subthreshold dural CGRP (0.1 pg) in females. Females were additionally primed to a subthreshold dose of the NO-donor sodium nitroprusside (0.1 mg/kg) following dural CGRP. Finally, the sexually dimorphic responses to dural CGRP were not specific to rats as similar female-specific hypersensitivity responses were seen in mice, where increased grimace responses were also observed. These data are the first to demonstrate that CGRP-induced headache-like behavioral responses at doses up to 3.8 μg are female-specific both acutely and following central and peripheral priming. These data further implicate dural CGRP signaling in the pathophysiology of migraine and propose a model where dural CGRP-based mechanisms contribute to the sexual disparity of this female-biased disorder.

Significance statement

Calcitonin gene-related peptide (CGRP) has long been implicated in the pathophysiology of migraine, and CGRP-based therapeutics are efficacious for the treatment of migraine in humans. However, the location of action for CGRP in migraine remains unclear. We show here that application of CGRP to the cranial meninges causes behavioral responses consistent with headache in preclinical rodent models. Surprisingly, however, these responses are only observed in females. Acute responses to meningeal CGRP are female-specific and sensitization to CGRP after two distinct stimuli are also female-specific. These data implicate the dura mater as a primary location of action for CGRP in migraine and suggest that female-specific mechanisms downstream of CGRP receptor activation contribute to the higher prevalence of migraine in women.

INTRODUCTION

Migraine is a complex neurological disorder that is characterized by throbbing head pain, increased sensitivity to light, sound, and touch, as well as nausea and vomiting. Globally ranked as the second highest cause of disability (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017), migraine is 2–3 times more common in women than in men. Further, migraine is the most common cause of disability in women ages 15–49 [230]. Little is known as to why sex differences exist in migraine prevalence. In females, the incidence of migraine rises following the onset of puberty and decreases after menopause, suggesting an

influence of hormones on the pathology [43; 230; 231]. However, hormones may be only one of many contributing factors leading to the higher prevalence in women, and their downstream mechanisms may promote sex differences in the actions of numerous signaling pathways.

Calcitonin gene-related peptide (CGRP) has been implicated in the pathology of migraine for several decades. CGRP is elevated in venous blood, saliva, and CSF of migraine patients during an attack [82; 135]. Attacks can be triggered in migraine patients by intravenous CGRP [152] and can be treated by inhibitors of CGRP signaling [241]. Recent studies have further confirmed a role for CGRP because monoclonal antibodies recognizing the peptide or its receptor are efficacious at reducing the frequency of both episodic and chronic migraine in humans [72; 73; 117]. Importantly, because CGRP administration likely triggers attacks without crossing the blood–brain barrier, and because only a small fraction of therapeutic monoclonal antibodies reaches the brain, these data argue strongly for a role of peripheral CGRP in migraine [132]. Although the exact location of peripheral CGRP action in migraine remains unclear, nociceptive signaling from the dura mater is considered a necessary event in the headache phase of attacks. CGRP has dilatory actions on the vasculature in human dura when given intravenously, including dilation restricted to the headache side during attacks [9; 10]. Similar dilation of vessels in human dura on the headache side has also recently been shown during spontaneous migraine [143]. These data suggest that CGRP in the dura mater, likely released by nociceptive nerve endings, is one key site of peptide action during migraine attacks.

Despite the differences in migraine prevalence between sexes and the clear role of CGRP in the disorder, surprisingly few studies have examined whether CGRP has sex-specific actions. While it has been shown that estrogen is capable of regulating the release of CGRP [203; 236], studies investigating the connection of CGRP to migraine have been almost entirely conducted in males. In prior studies that have administered CGRP to both males and females, none has compared responses to dural application of CGRP; this is surprising considering the role afferent nociceptive signaling from the meninges is thought to play in the pathology of the headache phase of migraine.

Here, we initially aimed to address whether dural application of CGRP caused behavioral responses in awake animals using preclinical migraine models. While our data support prior studies showing no effect of dural CGRP in males [157], we unexpectedly found a robust action of dural CGRP in females. This sexually dimorphic effect of CGRP occurs in both rats and mice and is present in both naive animals as well as after priming.

MATERIALS AND METHODS

Animals

In this study, 12- to 14-week-old ~260–300 g female and ~300–350 g male Sprague Dawley rats (Taconic) and 6- to 8-week-old female and male ICR mice (Envigo) were used for experiments. Animals were housed on a 12 h light/dark cycle with access to food and water *ad libitum*. Animals were housed in the facility for at least 72 h before handling and habituation of animals to testing rooms. In all experiments, investigators were blinded to treatment groups. All procedures were conducted with prior approval of the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

Rat cannula implantation and drug delivery

Dural injections in rats were administered via cannula at a total of 10 µl injections of CGRP, interleukin-6 (IL-6), or synthetic interstitial fluid (SIF) as vehicle (see **Table 2.1**). Animals were anesthetized initially at 5% isoflurane via a nose cone; once animals no longer demonstrated a paw pinch reflex, isoflurane was lowered to 2.5%–3.5% for the entirety of the surgery. The scalp was incised longitudinally and retracted from the midline to expose the skull. Using a pin vise (Grainger Industries) set to a length of 1 mm, a 1 mm burr hole was created using a stereotaxic frame at the target coordinates to sit above the middle meningeal artery (8 mm anteroposterior, −2 mm mediolateral, 1 mm dorsoventral) to puncture the skull while leaving the dura intact. A guide cannula (Plastics One C313G/SPC gauge 22) was implanted into the burr hole using a stereotaxic frame and sealed using Vetbond (3M). Two screws were inserted above the guide cannula on both sides of midline below bregma. Perm reline repair resin (Coltene) was used to anchor the cannula to the screws and skull. To prevent clogging, a dummy cannula (Plastics One 313DC-SPC 0.014–0.36 mm fit 1 mm) was inserted into the guide cannula. After surgery,

animals were given 8 mg/kg gentamicin and 0.25 mg meloxicam to prevent infection and for pain management, respectively. Animals were returned to their home cage and allowed to recover for 7 d.

Mouse dural injections

Mouse dural injections were performed as previously described [39]. Mice were anesthetized under isoflurane for <2 min <2.5%–3% isoflurane via a nose cone. While anesthetized, treatments were injected in a volume of 5 μl via a modified internal-cannula (Invivo1, part #8IC313ISPCXC, Internal Cannula, standard, 28 gauge, fit to 0.5 mm). The inner projection of the cannula was used to inject through the soft tissue at the intersection of the lambdoidal and sagittal sutures. The length of the projection was adjusted, using calipers, to be from 0.5 to 0.65 mm based on the animal weight (30–35 g) as to not puncture the dura.

Rat intracisternal injections

Intracisternal injections were administered in a volume of 10 μl at a rate of 1 μl/s and performed in rats as previously described [52]. A 25 gauge, 1.5 inch needle was contorted ∼7 mm from the tip at a 45° angle with the bevel facing outwards. The needle was attached to a 25 gauge Hamilton syringe. Animals were anesthetized for <2 min <2.5%-3% isoflurane via a nose cone. The head of the animal was tilted forward at an ∼120° angle between body to allow access to the cisterna magna. The needle was positioned above C1 and inserted through the cisterna magna along the midline.

Intraplantar injections

Rats were anesthetized initially at 5% isoflurane via a nose cone; once animals no longer exhibited a pinch reflex, isoflurane was lowered to 2.5%–3%, during which time animals were injected. These animals received a volume of 50 µl into the left hindpaw via injection with a 30 gauge, 0.5 inch needle attached to a Hamilton syringe. Animals were kept under isoflurane for <2 min.

von Frey testing Rats

Animals were conditioned for 5 continuous minutes by handling, 24 h before habituation. Rats were habituated to testing chambers and testing room by being placed in testing chambers for 2 h/d for 3 d before the first day of testing. Food was placed in the chamber of each rat for the duration of the testing day. Mechanical sensitivity baselines were assessed before drug administration. Only animals that met a facial baseline threshold of 8 g (rats) or 0.6 g (mice) were included in the study. These animals were then randomly allocated to experimental groups and remained in the study until completion of the experiment. Immediately after baseline, animals were injected; and 1 h after injection, mechanical sensitivity thresholds were assessed both on the periorbital region and hindpaw for a maximum of 5 s, or shorter if the investigator observed a response. von Frey filament thresholds were determined by the Dixon "up-and-down" method. Testing began with the 1 g filament on the face and 2 g on the hindpaw and increased weight to a maximum of 8 g on the face and 15 g on the hindpaw. For acute pain, animals were tested every other hour until a maximum of 5 h after injection was reached. Animals were then tested once every day from the time of injection until animals returned to baseline. Once animals returned to baseline, a normally subthreshold stimulus was administered to examine the ability of

the initial stimulus to cause priming. The same testing times were maintained following priming stimuli. All investigators were blinded to experimental conditions.

Mice

Mice were conditioned for 5 continuous minutes by handling, 24 h before habituation. Mice were habituated to paper cups (Choice 4 oz paper cups: 6.5 cm top diameter, 4.5 cm bottom diameter, 72.5 cm length) while in testing chambers [39]. Each mouse typically used their same assigned paper cup for the remainder of the experiment. Animals were given food while in testing chambers to allow for testing as previously described [39]. Filament thresholds were determined using the Dixon "up-and-down" method. Testing in mice began with 0.07 g on the face and increase weight to a maximum of 0.6 g on the face. Mice maintained the same testing timeline as rats. All investigators were blinded to experimental conditions.

Grimace

Discomfort following treatment was recorded in male and female mice in five characterized areas (orbital, nose, cheek, ears, and whiskers) on a 3 point scale (0 = not present; 1 = moderate; and 2 = obviously present) as previously characterized [151]. Grimace was conducted on mice before von Frey testing for all time points. All investigators were blinded to experimental conditions.

Drugs

α-Rat CGRP (Bachem) stock solution was prepared in ddH_2O (1 mg/ml) and diluted in SIF consisting of 135 mm NaCl, 5 mm KCl, 10 mm HEPES, 2 mm $CaCl_2$, 10 mm glucose, 1 mm $MgCl_2$ (pH 7.4, 310 mOsm). Rat recombinant IL-6 (R&D Systems) stock solution (10

μg/ml) was prepared in sterile 0.1% BSA and diluted to 1 ng/ml in SIF. Human recombinant BDNF (R&D Systems) stock solution was made in sterile PBS containing 1% BSA. BDNF was dissolved into aCSF containing 126 mm NaCl, 2 mm KCl, 1.25 mm KH_2PO_4, 2 mm $CaCl_2$, 2 mm $MgSO_4$, and 11 mm glucose. Sodium nitroprusside (SNP; Sigma-Aldrich) was dissolved in sterile PBS. Details on drugs used in these studies can be found in **Table 1**.

Table 2.1:

Drugs administered in these experiments, sources, doses/volumes, and routes of administration

Drug	**Source**	**Dose**	**Administration Route**
α-Rat CGRP	Bachem	3.8 μg/10 μl; 3.8 ng/10 μl; 1 pg/10 μl; 0.1 pg/10 μl	Dural; intraplantar
Rat recombinant IL-6	R&D Systems	0.1 ng/10 μl	Dural
Human recombinant BDNF	R&D Systems	1 pg/10 μl	Intracisternal
SNP	Sigma-Aldrich	0.1 mg/kg	Intraperitoneal

Experimental design and statistical analysis

Data are presented as mean ± SEM. Data were analyzed among groups at each time point via two-way ANOVA and followed by Bonferroni post test when appropriate. Data analysis was performed using Prism (GraphPad Software). Significance was set at $p < 0.05$ for all analyses. Power analysis was performed using G power for comparison of the means between groups using expected effect sizes based on pilot studies as well as previously published results in the case of dural IL-6 and cisternal BDNF [37]. For dural CGRP experiments in mice, the sample

size was determined based on pilot studies with rats. In the case of CGRP administered in the hindpaw of animals, we were unsure of the effect size, so a minimum sample size of 9 was used. All sample sizes reflect or surpass the suggested sample sizes for all experiments, with a minimum of 6 animals in each group for rat experiments and a minimum of 4 for mice experiments.

RESULTS

Dural CGRP induces mechanical sensitivity in female, but not male, rodents

Previous studies demonstrated that 100 μm CGRP applied to the dura does not sensitize or excite meningeal nociceptors in anesthetized rats [157]. One important caveat to this study is that only males were used to record nociceptor activity. Because migraine is more common in females, we set out to establish whether CGRP could trigger migraine-like pain in female rats using the same concentration of CGRP from prior studies, 100 μm. Dural application of 100 μm (3.8 μg) CGRP elicited significantly lower facial withdrawal thresholds at 3, 5, and 24 h after injection in females, but not males (**Fig. 1***A*). The vehicle administration had no significant effect in either male or female rats.

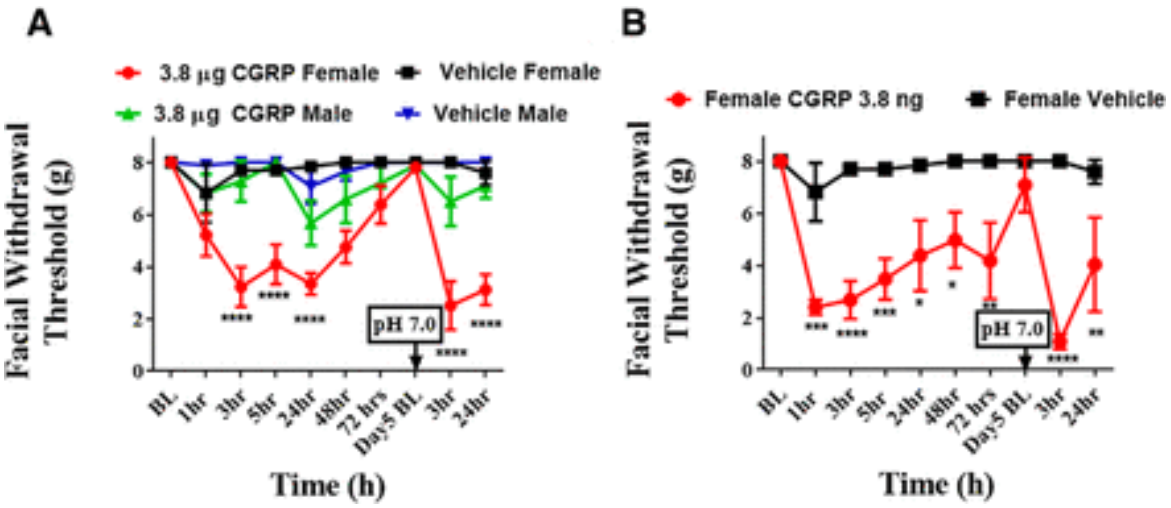

Figure 2.1: Dural administration of CGRP produces facial hypersensitivity and priming in female, but not in male, rats. Baseline facial withdrawal thresholds were determined before dural stimulation with 100 µm CGRP (***A***) (n = 10 females, n = 7 males), 0.1 µm CGRP (n = 4 females) (*B*), or vehicle (n = 5 females, n = 7 males). A dural injection of pH 7.0 SIF was given in all groups at day 5 following either dural CGRP or dural vehicle. Two-way ANOVA followed by Bonferroni *post hoc* test indicated significant differences in females treated with 100 µm CGRP ($F_{(27,250)}$ = 2.591, $p < 0.0001$) and in females treated with 0.1 µm CGRP ($F_{(27,250)}$ = 2.591, ****$p < 0.0001$). These data are represented as means ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$. We have previously shown that "priming" of the dural afferent system with IL-6 can induce sensitivity to subthreshold stimulation with decreased pH (7.0) after the animals returned to baseline [37]. Here we show that, 5 d after injection of CGRP, all animals returned to baseline withdrawal threshold. Rats were then injected with 10 µl of SIF at pH 7.0 onto the dura. Three hours following injection of pH 7.0 SIF, only females presented with significant allodynia, and this effect persisted for at least 24 h (Fig. 1*A*).

While female rats responded to 3.8 μg CGRP, it was important to determine whether this high dose was necessary for responses in females or whether they respond to lower doses. We thus started a series of experiments where CGRP dose was lowered 10-fold in each case. The data for doses of CGRP between 3.8 μg and 3.8 ng are not shown. Figure 1*B* shows the results of application of the thousand-fold lower dose of 3.8 ng CGRP on the dura of cannulated female rats. Even at this low CGRP dose, significant decreases in withdrawal thresholds at 1, 3, 5, 24, 48, and 72 h after injection were observed in females. Additionally, to examine whether this lower dose of dural CGRP could also prime animals to respond to a normally non-noxious stimulus, animals were given dural SIF at pH 7.0 after they returned to baseline withdrawal thresholds, which was 5 d following dural CGRP. Animals that received CGRP were primed to dural pH 7.0 as they experienced significantly reduced mechanical withdrawal thresholds 3 and 24 h after pH 7.0 injection (Fig. 1*B*). As with the high dose in Figure 1*A*, males did not respond to any lower doses of CGRP (data not shown).

The initial use of the 3.8 μg value as a dose was based on the original publication using dural application of 100 μm CGRP (Levy et al., 2005). We next switched to more standard dose units to determine the threshold for responses to dural CGRP in females. Following dural administration of 1 pg, but not 0.1 pg CGRP, female rats showed significantly lower facial mechanical thresholds at 1, 3, and 5 h thresholds compared with rats that were administered ($F_{(8,85)} = 11.07$, $p < 0.0001$; Fig. 2). Because 0.1 pg CGRP failed to produce a hypersensitivity response in these animals, we used this dose as a subthreshold CGRP stimulus in subsequent experiments.

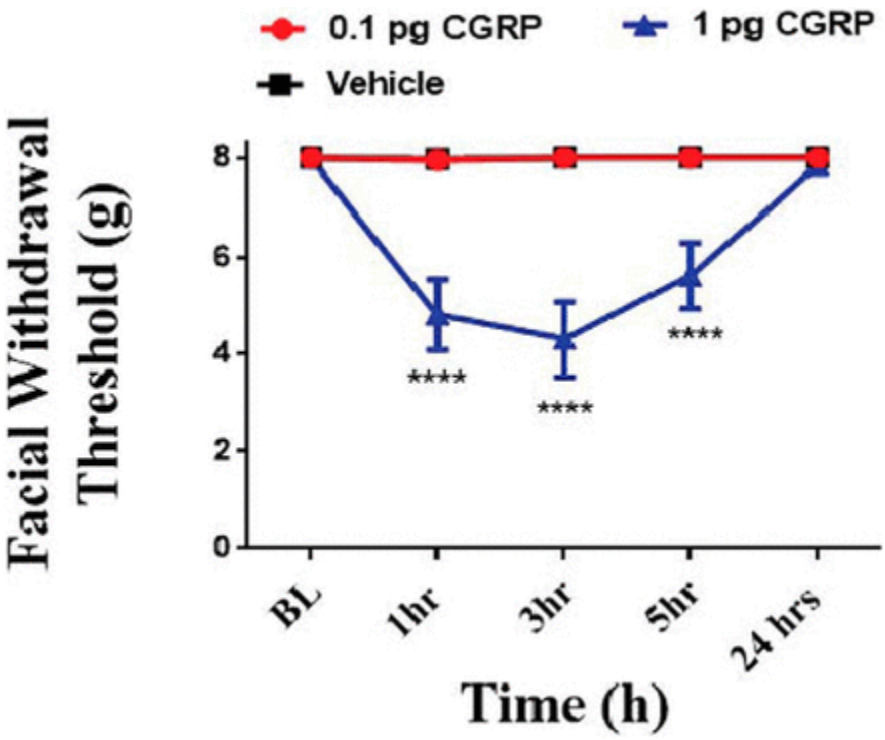

Figure 2.2: Dural application of CGRP produces facial hypersensitivity in female rats at doses as low as 1 pg. Facial withdrawal thresholds were measured before and following dural stimulation with 0.1 pg CGRP ($n = 6$), 1 pg CGRP ($n = 6$), or vehicle pH 7.4 SIF ($n = 8$). Two-way ANOVA followed by Bonferroni *post hoc* test revealed significant differences in the 1 pg CGRP-treated group ($F_{(8,85)} = 11.07$; ****$p < 0.0001$). These data are represented as means ± SEM.

Induction of female-specific priming to subthreshold dural CGRP

We have previously reported that dural injection of IL-6 can establish sustained hyperalgesic priming [37]. We thus asked whether IL-6 (0.1 ng) can sensitize the dura to a subthreshold dose of CGRP (0.1 pg) and whether this response is also female-specific. In both male (**Fig. 3***B*) and female rats (**Fig. 3***A*), IL-6 induced significant allodynia from 1 to 24 h after administration and

animals returned to baseline at 72 h after injection, following which, either 0.1 pg CGRP or vehicle SIF was applied to the dura. Females showed priming to this subthreshold dose, as they exhibited significantly reduced facial withdrawal thresholds 1 and 3 h after injection ($F_{(9,176)}$ = 7.065, $p < 0.0001$; **Fig. 3***A*). Males experienced no hypersensitivity to dural CGRP at any time point despite their initial response to IL-6 (**Fig. 3***B*).

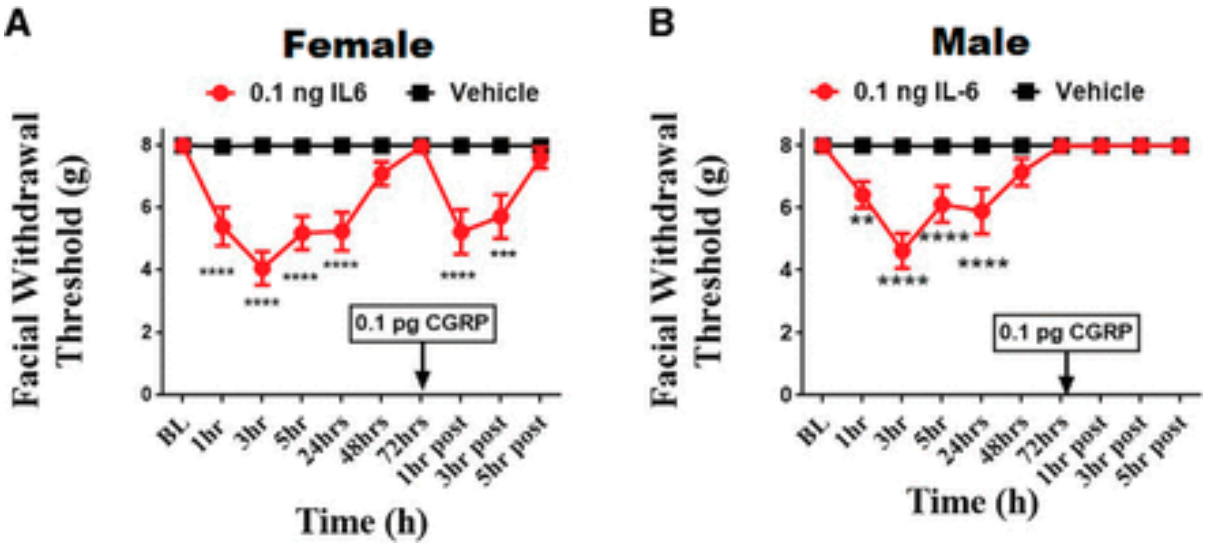

Figure 2.3: Dural application of IL-6 induces priming to dural CGRP in females, but not in males. Facial withdrawal thresholds were measured in female (***A***) and male (***B***) rats before and after administration of either 0.1 ng dural IL-6 (n = 10 females, n = 11 males) or vehicle pH 7.4 SIF (n = 9 females, n = 11 males). At 72 h after dural IL-6, all animals received a subthreshold dose of dural CGRP (0.1 pg). Two-way ANOVA indicated a significant effect of CGRP in the IL-6-treated group of females. The significant differences of the means for each group were

determined by ANOVA followed by Bonferroni *post hoc* test ($F_{(9,176)} = 7.065$; $^{**}p < .01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). These data are represented as means ± SEM.

To determine whether initial dural stimulation was required to produce priming to subthreshold CGRP, rats were given 1 pg intracisternal BDNF, a stimulus that we have shown previously leads to priming to dural pH 7.0 and a systemic NO donor [37]. Following intracisternal BDNF, both male and female rats displayed significant facial and allodynia out to 24 h. Once these animals returned to baseline withdrawal thresholds, at 72 h after injection, they were given either subthreshold 0.1 pg dural CGRP or vehicle. Females exhibited significant facial allodynia 1 h after CGRP injection compared with controls ($F_{(9,100)} = 7.467$, $p < 0.0001$; Fig. 4*A*). Males did not respond to CGRP at any time point following dural injection (Fig. 4*B*). These data demonstrate that, like dural IL-6, males respond to intracisternal BDNF but they do not prime to dural CGRP. Together, these experiments show that only females respond to suprathreshold CGRP doses and only females prime to CGRP following dural IL-6 or intracisternal BDNF.

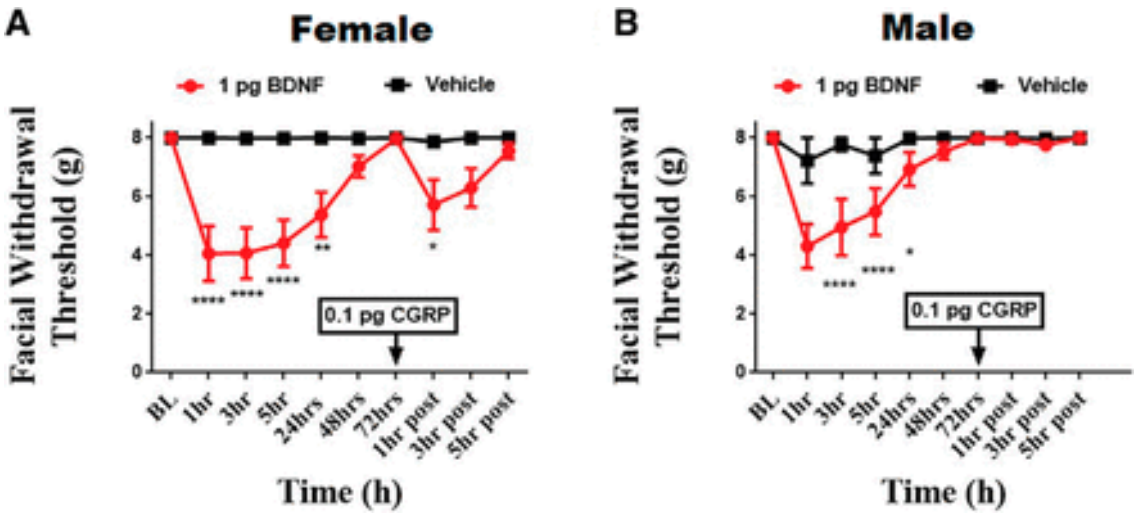

Figure 2.4: Intracisternal administration of BDNF induces priming to dural CGRP in females, but not males. Facial withdrawal thresholds were measured in female (*A*) and male (*B*) rats before and after either cisternal injection of 1 pg BDNF (n = 6 females, n = 6 males) or vehicle (aCSF, pH 7.4) (n = 6 females, n = 6 males). At 72 h after dural IL-6, when animals had baselined, 0.1 pg CGRP was administered to the dura. Two-way ANOVA indicated a significant effect of CGRP in the BDNF-treated group of females. The significant differences of the means for each group were determined by ANOVA followed by Bonferroni *post hoc* test ($F_{(9,100)}$ = 7.467). These data are represented as means ± SEM; *$p < .05$, **$p < .01$, ****$p < 0.0001$).

Female-specific responses to dural CGRP in mice

To determine whether sex-dependent hypersensitivity following dural CGRP is a rat-specific effect, we administered CGRP to the dura of both male and female mice. Similar to what was seen with rats, female mice exhibited a reduced mechanical threshold following 1 pg dural CGRP ($F_{(20,121)}$ = 4.466, $p < 0.0001$). Female mice that received 1 pg dural CGRP exhibited

persistent facial hypersensitivity that did not return to baseline until ~14 d after injection (Fig. 5*A*), much longer than rat behavior observed in Figure 1. Additionally, male mice also exhibited a marked hypersensitivity response from vehicle injection, but only at 1 h after injection. By 3 h following injection, male mice were no longer hypersensitive (Fig. 5*A*).

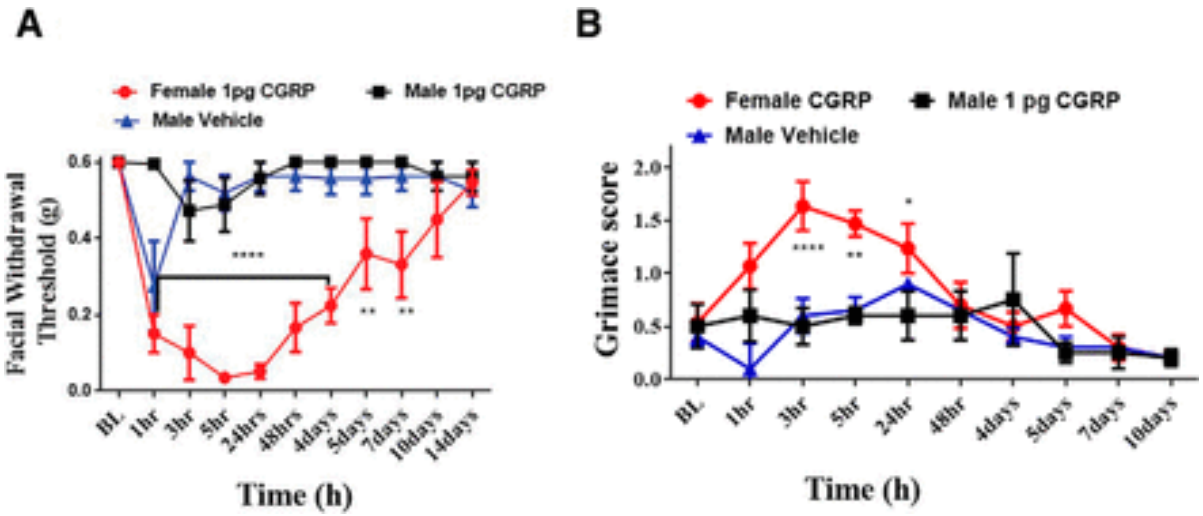

Figure 2.5: Dural application of CGRP in mice causes hypersensitivity (*A*) and grimace (*B*) in females, but not males. For all animals, von Frey baselines as well as Grimace Score baselines were recorded following dural injection of CGRP ($n = 6$ females, $n = 4$ males) or vehicle ($n = 4$ males); then ANOVA with Bonferroni *post hoc* test showed significant differences in withdrawal thresholds female-treated group ($F_{(20,121)} = 4.466$; $^{**}p < 0.01$, $^{****}p < 0.0001$) as well as significant grimacing ($F_{(16,99)} = 2.033$; $^{*}p < .05$, $^{**}p < 0.01$, $^{****}p < 0.0001$).

It has been shown recently that systemic administration of CGRP causes facial grimace responses suggestive of spontaneous pain [204]. To examine whether the hypersensitivity

responses following dural CGRP in mice are also accompanied by spontaneous pain behavior, we quantified facial grimace responses using the mouse grimace scale. Female mice that received dural CGRP had significant grimace scores out to 24 h after injection ($F_{(16,99)}$ = 2.033, p = 0.0178), whereas males that received dural CGRP or vehicle did not show grimace responses at any time point (Fig. **5***B*). These data show that, although male mice respond to dural CGRP, their response is minimal compared with the prolonged hypersensitivity seen in females. Additionally, dural CGRP produces behavioral responses suggestive of spontaneous pain, similar to that seen previously with systemic CGRP.

Dural CGRP primes females to subthreshold SNP

Acute administration of nitric oxide donors has been previously shown to induce migraine in humans and produce migraine-like behaviors in rodents [189; 190]. It has also been shown that nitric oxide applied to the dura causes CGRP release [234]. We have also shown previously that dural IL-6 or intracisternal BDNF primes rats to subthreshold doses of NO donors [37]. We next determined whether dural CGRP could prime rats to a subthreshold systemic dose of the NO donor SNP. Prior studies have reported that doses up to 3 mg/kg SNP are subthreshold on otherwise naive animals [64]. Consistent with the findings in Figure 2, female rats that were administered 1 pg dural CGRP exhibited a reduction in withdrawal threshold in the facial region. Approximately 24 h following CGRP, female withdrawal thresholds returned to baseline (Fig. 6). Once animals returned to baseline, a dose of 0.1 mg/kg SNP was given via intraperitoneal injection. One hour after injection, females that received dural CGRP demonstrated significant

facial allodynia compared with controls ($F_{(6,98)} = 9.171$, $p < 0.0001$), showing that dural CGRP causes priming to NO donors in female rats. Because males do not show acute responses to dural CGRP, they were not tested for priming to SNP.

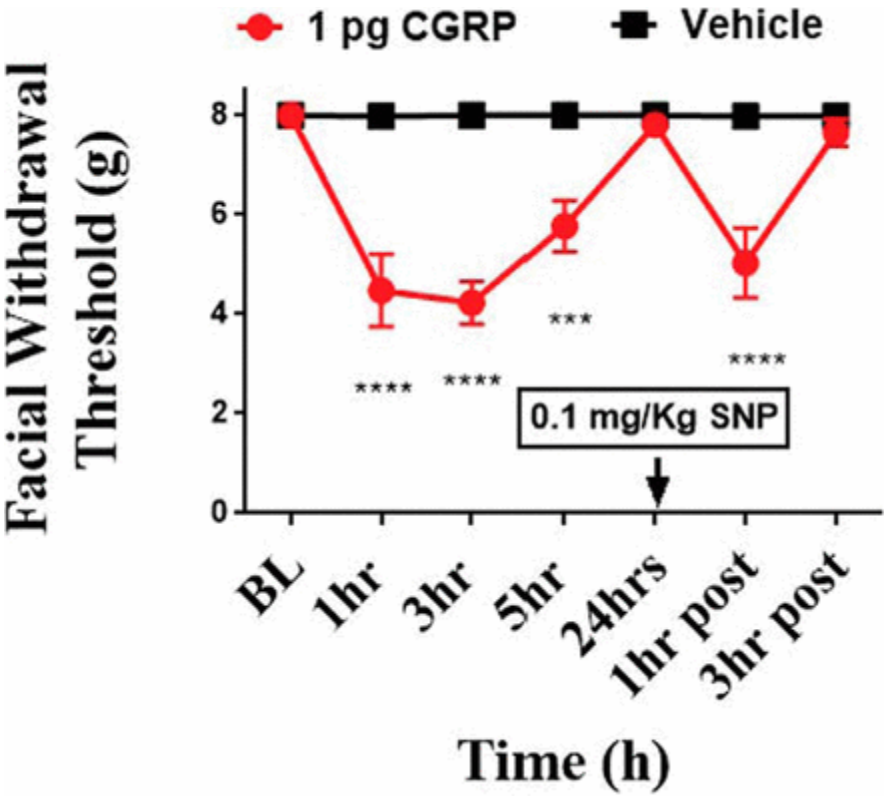

Figure 2.6: Dural application of CGRP primes female rats to a subthreshold nitric oxide donor. Facial withdrawal thresholds were measured in females before and after either 1 pg CGRP (n = 9) or vehicle pH 7.4 SIF (n = 7) applied to the dura. At 24 h following dural CGRP or dural vehicle, all rats were given 0.1 mg/kg SNP (i.p.). Two-way ANOVA indicated significant effects of SNP in the CGRP-treated group with no response to SNP in the vehicle-treated group.

Significant differences in the means of each group were determined by ANOVA followed by Bonferroni post hoc test ($F_{(6,98)} = 9.171$). These data are represented as means ± SEM; ***$p <$ .001, ****$p < .0001$).

Intraplantar CGRP produces hindpaw allodynia in female rats

To examine whether the specificity of CGRP to induce pain in females was specific to dural administration, low-dose (1 pg) CGRP was administered into the hindpaw of otherwise naive male and female rats. At 1 and 3 h following injection of CGRP, females exhibited significant hindpaw allodynia compared with controls ($F_{(4,80)} = 5.103$, $p = 0.0010$; Fig. **7***D*). Males that received CGRP exhibited no significant hindpaw allodynia at any time point (Fig. **7***B*). Neither males nor females experienced significant facial allodynia at any time point following hindpaw injections (Fig. **7***A*,*C*). This suggests that the sex specificity of pain responses following low-dose local injections of CGRP may not be specific to the dura.

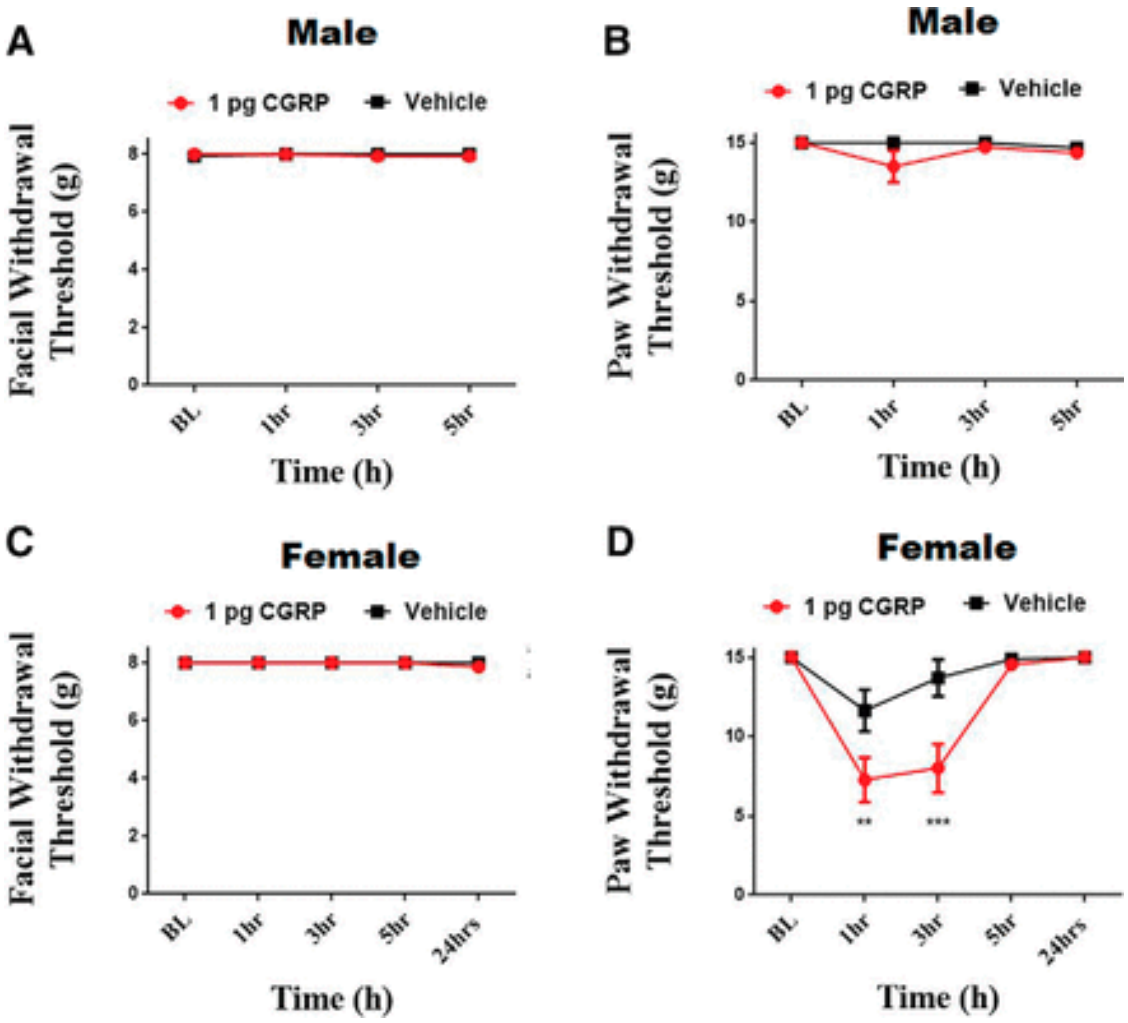

Figure 2.7: Intraplantar administration of CGRP results in hypersensitivity of the hindpaw in females, but not males. Facial and paw withdrawal thresholds for males (***A***, ***C***) and females (***B***, ***D***) were tested before and following injection of 1 pg CGRP (n = 9 females, n = 10 males) or vehicle (1× PBS, pH 7.4) (n = 9 females, n = 10 males). Two-way ANOVA followed by Bonferroni *post hoc* test indicated significant hindpaw allodynia in the hindpaws of CGRP-treated females ($F_{(4,80)} = 5.103$; $^{**}p < 0.01$, $^{***}p < 0.001$). No significant facial hypersensitivity

was observed following intraplantar CGRP administration. These data are represented as means ± SEM to the end of this paragraph.

DISCUSSION

The contribution of CGRP to migraine has been investigated for >30 years. However, a role for peripheral CGRP has only recently been clearly demonstrated by the efficacy of predominantly peripherally restricted peptide or receptor monoclonal antibodies in human migraine patients. The location of peripheral CGRP that contributes to migraine attacks is not fully known, but given that afferent signaling from the meninges is necessary for the headache phase, and CGRP dilates meningeal vessels including on the headache side during attacks [9; 10] meningeal actions of CGRP are one plausible site. While the mechanisms leading to the higher prevalence of migraine in females are likely complex and not mediated by a single factor, the data shown here suggest that CGRP-based signaling from the meninges may contribute to the female-biased nature of this disorder.

Here we show that CGRP administered at doses as low as 1 pg onto the dura of rats and mice produces facial and hindpaw allodynia in females but fails to produce allodynia in males at doses as high as 3.8 μg. At doses of 3.8 μg and 3.8 ng, female rats were also primed to dural pH of 7.0 and SNP, the latter a migraine trigger in humans. This implicates dural CGRP not only in acute nociceptive responses but in the sensitization of females to normally non-noxious triggers of migraine, such as nitric oxide donors. Similar findings in 2 outbred species suggest that these female-specific responses to dural CGRP are not a peculiarity of mice and are not an artifact of genetic manipulation, increasing confidence that these data may translate to other species. The

acute responses of both males and females to dural IL-6 show that overall noxious responses to dural stimulation occur in males (see also [39]), but that female-specific responses only occur with certain stimuli. We have also shown previously that priming to dural pH 7.0 occurs in both males and females when the primed state is initiated by IL-6 [37], demonstrating that priming following dural stimulation is not female-specific. Further, we have shown that the priming to dural pH 7.0 induced by intracisternal BDNF occurs in males [37], and priming in the DRG/spinal dorsal horn system to BDNF also occurs in male and female rats [183]. Thus, centrally induced priming is also not exclusive to females. Together, these data show that the actions of dural CGRP, and not dural signaling or priming in general, are female-specific.

The anatomical and physiological mechanisms explaining the female-specific responses to dural CGRP are not clear. Our data showing responses to hindpaw CGRP injection only in female rats suggest that these mechanisms may be a feature of peripheral tissue and not unique to the dura. It was shown recently that CGRP administered into the peritoneal cavity does not result in differential photosensitivity and grimace between male and female mice [172], that subcutaneous CGRP given into the periorbital skin results in facial allodynia in male mice [66], and intracisternal CGRP caused behavioral signs of headache in male rats [58]. While these studies may seem at odds with the data shown in the current work, differential doses used in these experiments likely explain the responses in males. The most closely related study based on injection location/type is that of [66], which gave up to 15 nmol (s.c.) under the periorbital skin, where we gave up to 3.8 μg (which is 1 nmol, a dose that showed a small but nonsignificant response in males; Fig. 1*A*) onto the dura. In other experiments, we gave 0.1 and 1 pg (which is

0.03 and 0.3 fmol) onto the dura. In the hindpaw experiments, we gave 1 pg, whereas other studies that found that responses in males gave intraplantar doses between 1 and 10 μg [26; 220; 264]. Females may thus respond selectively at low doses of CGRP, whereas males respond at much higher doses. Additionally, the administration route/location of CGRP may cause differential effects (i.e., systemic or into the CSF vs locally onto the dura). CGRP signaling mechanisms may only be sexually dimorphic in certain locations, and the location of action of CGRP-mediated behavioral responses was not determined in those prior studies (e.g., when given intraperitoneally or intracisternally). Nonetheless, CGRP may have sex-specific actions at nerve endings in peripheral tissue. Within the dura of rats, CGRP is expressed in C-fibers, whereas its receptor is expressed in Aδ fibers; and these fibers have been reported to run in parallel with one another [88; 155]. Because this study was only conducted on males, it is difficult to determine whether the presence of CGRP or the receptors would show a different expression pattern between the sexes. There have been reported differences in gene expression of CGRP as well as its receptor components. Naive female rats have significantly lower levels of RAMP1, CLR, and RCP mRNA in the trigeminal ganglion compared with male counterparts, but there is no difference in CGRP mRNA [236]. Whether or not these differences in transcript levels in the trigeminal ganglia lead to differential protein expression on nerve endings in the dura is not clear.

The female-specific actions of CGRP may be mediated in part by blood vessels where CGRP is one of the most potent endogenous vasodilators. CGRP-expressing fibers are found near both human and rat dural vessels, and CLR and RAMP1 are expressed on smooth muscle cells of

these vessels [155]. Thus, CGRP can act on smooth muscle cells, causing vasodilation as well as other dilation-independent events that may ultimately lead to afferent nociceptive signaling [133]. Unfortunately, the previously mentioned study of dural CGRP/receptor expression was conducted on male rats only, and the sample size from human tissue was too small (1 female and 2 males) to address sex differences in receptor expression patterns. While sex differences in the expression of CGRP receptors on blood vessels remain largely unexplored, sex-specific hormones have been shown to modulate responses within the vasculature to CGRP. When stimulated with a bolus injection of CGRP, pregnant and ovariectomized rats receiving hormone replacement of 17β-estradiol and progesterone experience larger decreases in total vascular resistance compared with ovariectomized rats not receiving hormone replacement [98]. It has also been shown that pregnant rats and hormone-treated ovariectomized rats experience larger decreases in mean arterial blood pressure in response to CGRP compared with both nonpregnant and ovariectomized rats [97]. Further, 17β-estradiol can potentiate the dilating effects of CGRP on isolated vessels of ovariectomized rats compared with ovariectomized females with no hormone replacement [112]. These data demonstrate that female sex hormones can cause increases in vasodilation in response to CGRP.

One additional possibility explaining the sex difference in responses is that dural CGRP may cause hypersensitivity via the degranulation of mast cells native to the meninges. Degranulation of mast cells within the dura has been shown to result in excitation of meningeal nociceptors, activation of the spinal trigeminal nucleus [156], and headache behavioral responses in mice [123]. The ability of CGRP to degranulate mast cells may differ between males and females. In

male rats, the CGRP receptor components CLR and RAMP1 have been located on mast cells [88]; however, the population of these receptor components and the potential differences in their expression on mast cells have yet to be explored in female rats. The presence of other functional CGRP receptor components, such as CTR on dural mast cells, and the possible differences in distribution of these components on mast cells are largely unexplored in both sexes. Mast cells also express receptors for estrogen as well as receptors for progesterone, both of which have been shown to degranulate mast cells [136; 184; 275]. The potential interaction of estrogen and progesterone in CGRP-mediated mast cell degranulation are also unexplored. In humans, it has been demonstrated that mast cell tryptase was colocalized with CLR, but not RAMP1 [88]. However, this finding was derived from 2 males and 1 female, too small of a sample size to be conclusive for sex differences. The interactions of female hormones and CGRP on mast cell degranulation requires further study before it is clear whether this mechanism contributes to the female-specific response to dural (or hindpaw) CGRP.

There are several technical limitations to the findings in this study. While cutaneous hypersensitivity is present in a large percentage of migraine patients during the headache phase of attacks [42; 160], it is not headache. Consequently, our findings using facial von Frey testing could potentially be limited to a female-specific role of dural CGRP in the cutaneous facial hypersensitivity that is present along with migraine attacks. However, the presence of increased facial grimace responses in females, but not males, in response to dural CGRP (**Fig. 5**) adds an important nonevoked component to the study and demonstrates that this female-specific effect exists beyond von Frey responses. Another technical limitation of the study relates to blinding of

the experimenters to the sex of the rodents. It is difficult, if not impossible, to blind those conducting behavioral responses in rodents to whether the rodent is male or female. While all other aspects of the behavioral assays are blinded, including the treatment groups, we cannot completely eliminate the impact that knowledge of the sex of the animal may have on the experimental outcomes. Multiple investigators have nonetheless observed essentially identical responses (i.e., female-specific behaviors with dural CGRP) in experiments conducted at different times in the laboratory.

The findings shown here may raise questions regarding the efficacy of CGRP/CGRP receptor monoclonal antibodies for migraine in males. Patient demographics from published clinical trials on erenumab, fremanezumab, galcanezumab, and eptinezumab all show patient populations that are >80% female, so it may not be clear whether these therapeutics are efficacious in a smaller percentage of males or whether higher doses may be required to achieve the same efficacy in males [72; 206; 210; 252]. Alternatively, because we show that substantially lower doses of CGRP cause behavioral responses in females, the minimum concentration of CGRP necessary to contribute to migraine may be much lower in females, which may also be a contributing factor to why females are more susceptible to attacks. However, once CGRP reaches the minimum concentrations to cause effects (which again, is lower in females), the antibodies could then work equally in males and females because in either case they are simply lowering CGRP concentrations or blocking receptor signaling. Efficacy in males may also depend on the site of action of these antibodies in humans and whether they act in a location that displays sexually dimorphic CGRP signaling. If their primary site of action is not in a location where CGRP has a

female-specific effect, the antibodies will likely work equally in males and females. In any case, when considering that females make up the majority of the clinical population of migraine sufferers [72; 117; 181; 254], the higher percentage of females in both the clinical trials and the general patient population fortunately aligns with any female-specific efficacy of these therapeutics. While reasons for the sexually dimorphic response to dural CGRP shown here are still unknown, these findings demonstrate the need for sex-specific migraine therapeutics, as mechanisms contributing to migraine in males and females likely differ. And although CGRP plays a clear role in migraine, this does not imply that migraine is exclusively a CGRP-based disorder; other peptides, such as pituitary adenylate-cyclase activating polypeptide, may also contribute [211], and there may be similar sex-specific actions of this peptide to consider. These findings also highlight the need for determining whether new therapeutics have sex-specific mechanisms of action, as selecting the proper sex in clinical trials may be key in demonstrating efficacy.

REFERENCES

[1] Asghar MS, Hansen AE, Amin FM, van der Geest RJ, Koning P, Larsson HB, Olesen J, Ashina M. Evidence for a vascular factor in migraine. Ann Neurol 2011;69(4):635-645.

[2] Asghar MS, Hansen AE, Kapijimpanga T, van der Geest RJ, van der Koning P, Larsson HB, Olesen J, Ashina M. Dilation by CGRP of middle meningeal artery and reversal by sumatriptan in normal volunteers. Neurology 2010;75(17):1520-1526.

[3] Bileviciute I, Stenfors C, Theodorsson E, Lundeberg T. Unilateral injection of calcitonin gene-related peptide (CGRP) induces bilateral oedema formation and release of CGRP-like immunoreactivity in the rat hindpaw. Br J Pharmacol 1998;125(6):1304-1312.

[4] Burgos-Vega CC, Quigley LD, Avona A, Price T, Dussor G. Dural stimulation in rats causes brain-derived neurotrophic factor-dependent priming to subthreshold stimuli including a migraine trigger. Pain 2016;157(12):2722-2730.

[5] Burgos-Vega CC, Quigley LD, Trevisan Dos Santos G, Yan F, Asiedu M, Jacobs B, Motina M, Safdar N, Yousuf H, Avona A, Price TJ, Dussor G. Non-invasive dural stimulation in mice: A novel preclinical model of migraine. Cephalalgia 2019;39(1):123-134.

[6] Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ, Bajwa ZH. An association between migraine and cutaneous allodynia. Ann Neurol 2000;47(5):614-624.

[7] Buse DC, Loder EW, Gorman JA, Stewart WF, Reed ML, Fanning KM, Serrano D, Lipton RB. Sex differences in the prevalence, symptoms, and associated features of migraine, probable migraine and other severe headache: results of the American Migraine Prevalence and Prevention (AMPP) Study. Headache 2013;53(8):1278-1299.

[8] Chen Y, Imai H, Ito A, Saito N. Novel modified method for injection into the cerebrospinal fluid via the cerebellomedullary cistern in mice. Acta Neurobiol Exp (Wars) 2013;73(2):304-311.

[9] Cornelison LE, Hawkins JL, Durham PL. Elevated levels of calcitonin gene-related peptide in upper spinal cord promotes sensitization of primary trigeminal nociceptive neurons. Neuroscience 2016;339:491-501.

[10] De Felice M, Ossipov MH, Wang R, Lai J, Chichorro J, Meng I, Dodick DW, Vanderah TW, Dussor G, Porreca F. Triptan-induced latent sensitization: a possible basis for medication overuse headache. Ann Neurol 2010;67(3):325-337.

[11] De Logu F, Landini L, Janal MN, Li Puma S, De Cesaris F, Geppetti P, Nassini R. Migraine-provoking substances evoke periorbital allodynia in mice. J Headache Pain 2019;20(1):18.

[12] Dodick DW, Goadsby PJ, Silberstein SD, Lipton RB, Olesen J, Ashina M, Wilks K, Kudrow D, Kroll R, Kohrman B, Bargar R, Hirman J, Smith J, investigators ALDs. Safety and efficacy of ALD403, an antibody to calcitonin gene-related peptide, for the prevention of frequent episodic migraine: a randomised, double-blind, placebo-controlled, exploratory phase 2 trial. Lancet Neurol 2014;13(11):1100-1107.

[13] Dodick DW, Silberstein SD, Bigal ME, Yeung PP, Goadsby PJ, Blankenbiller T, Grozinski-Wolff M, Yang R, Ma Y, Aycardi E. Effect of Fremanezumab Compared With Placebo for Prevention of Episodic Migraine: A Randomized Clinical Trial. JAMA 2018;319(19):1999-2008.

[14] Edvinsson L, Goadsby PJ. Neuropeptides in migraine and cluster headache. Cephalalgia 1994;14(5):320-327.

[15] Eftekhari S, Warfvinge K, Blixt FW, Edvinsson L. Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. J Pain 2013;14(11):1289-1303.

[16] Gangula PR, Zhao H, Supowit S, Wimalawansa S, DiPette D, Yallampalli C. Pregnancy and steroid hormones enhance the vasodilation responses to CGRP in rats. Am J Physiol 1999;276(1):H284-288.

[17] Gangula PR, Zhao H, Wimalawansa SJ, Supowit SC, DiPette DJ, Yallampalli C. Pregnancy and steroid hormones enhance the systemic and regional hemodynamic effects of calcitonin gene-related peptide in rats. Biol Reprod 2001;64(6):1776-1783.

[18] Gupta S, Mehrotra S, Villalon C, De Vries R, Garrelds I, Saxena P, Vandenbrink AM. Effects of female sex hormones on responses to CGRP, acetylcholine, and 5-HT in rat isolated arteries. Headache 2007;47(4):564-575.

[19] Halker Singh RB, Aycardi E, Bigal ME, Loupe PS, McDonald M, Dodick DW. Sustained reductions in migraine days, moderate-to-severe headache days and days with acute medication use for HFEM and CM patients taking fremanezumab: Post-hoc analyses from phase 2 trials. Cephalalgia 2019;39(1):52-60.

[20] Hassler SN, Ahmad FB, Burgos-Vega CC, Boitano S, Vagner J, Price TJ, Dussor G. Protease activated receptor 2 (PAR2) activation causes migraine-like pain behaviors in mice. Cephalalgia 2019;39(1):111-122.

[21] Iyengar S, Ossipov MH, Johnson KW. The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. Pain 2017;158(4):543-559.

[22] Jacobs B, Dussor G. Neurovascular contributions to migraine: Moving beyond vasodilation. Neuroscience 2016;338:130-144.

[23] Jang MU, Park JW, Kho HS, Chung SC, Chung JW. Plasma and saliva levels of nerve growth factor and neuropeptides in chronic migraine patients. Oral Dis 2011;17(2):187-193.

[24] Jensen F, Woudwyk M, Teles A, Woidacki K, Taran F, Costa S, Malfertheiner SF, Zenclussen AC. Estradiol and progesterone regulate the migration of mast cells from the periphery to the uterus and induce their maturation and degranulation. PLoS One 2010;5(12):e14409.

[25] Khan S, Amin FM, Christensen CE, Ghanizada H, Younis S, Olinger ACR, de Koning PJH, Larsson HBW, Ashina M. Meningeal contribution to migraine pain: a magnetic resonance angiography study. Brain 2019;142(1):93-102.

[26] Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS. Coding of facial expressions of pain in the laboratory mouse. Nat Methods 2010;7(6):447-449.

[27] Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. Cephalalgia 2002;22(1):54-61.

[28] Lennerz JK, Ruhle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF, Messlinger K. Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. J Comp Neurol 2008;507(3):1277-1299.

[29] Levy D, Burstein R, Kainz V, Jakubowski M, Strassman AM. Mast cell degranulation activates a pain pathway underlying migraine headache. Pain 2007;130(1-2):166-176.

[30] Levy D, Burstein R, Strassman AM. Calcitonin gene-related peptide does not excite or sensitize meningeal nociceptors: implications for the pathophysiology of migraine. Ann Neurol 2005;58(5):698-705.

[31] Lipton RB, Bigal ME, Ashina S, Burstein R, Silberstein S, Reed ML, Serrano D, Stewart WF, American Migraine Prevalence Prevention Advisory G. Cutaneous allodynia in the migraine population. Ann Neurol 2008;63(2):148-158.

[32] Mason BN, Kaiser EA, Kuburas A, Loomis MM, Latham JA, Garcia-Martinez LF, Russo AF. Induction of Migraine-Like Photophobic Behavior in Mice by Both Peripheral and Central CGRP Mechanisms. J Neurosci 2017;37(1):204-216.

[33] Monteith D, Collins EC, Vandermeulen C, Van Hecken A, Raddad E, Scherer JC, Grayzel D, Schuetz TJ, de Hoon J. Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of the CGRP Binding Monoclonal Antibody LY2951742 (Galcanezumab) in Healthy Volunteers. Front Pharmacol 2017;8:740.

[34] Moy JK, Kuhn JL, Szabo-Pardi TA, Pradhan G, Price TJ. eIF4E phosphorylation regulates ongoing pain, independently of inflammation, and hyperalgesic priming in the mouse CFA model. Neurobiol Pain 2018;4:45-50.

[35] Narita S, Goldblum RM, Watson CS, Brooks EG, Estes DM, Curran EM, Midoro-Horiuti T. Environmental estrogens induce mast cell degranulation and enhance IgE-mediated release of allergic mediators. Environ Health Perspect 2007;115(1):48-52.

[36] Olesen J, Iversen HK, Thomsen LL. Nitric oxide supersensitivity: a possible molecular mechanism of migraine pain. Neuroreport 1993;4(8):1027-1030.

[37] Olesen J, Jansen-Olesen I. Nitric oxide mechanisms in migraine. Pathol Biol (Paris) 2000;48(7):648-657.

[38] Pota V, Quagliariello V, Armenia E, Aurilio C, Passavanti MB, Sansone P, Iannotti M, Catauro M, Coaccioli S, Barbarisi M, Pace MC. CGRP and Visceral Pain: The Role of Sex Hormones in In Vitro Experiment. J Cell Biochem 2017;118(3):510-517.

[39] Rea BJ, Wattiez AS, Waite JS, Castonguay WC, Schmidt CM, Fairbanks AM, Robertson BR, Brown CJ, Mason BN, Moldovan-Loomis MC, Garcia-Martinez LF, Poolman P, Ledolter J, Kardon RH, Sowers LP, Russo AF. Peripherally administered calcitonin gene-related peptide induces spontaneous pain in mice: implications for migraine. Pain 2018;159(11):2306-2317.

[40] Reuter U, Goadsby PJ, Lanteri-Minet M, Wen S, Hours-Zesiger P, Ferrari MD, Klatt J. Efficacy and tolerability of erenumab in patients with episodic migraine in whom two-to-four previous preventive treatments were unsuccessful: a randomised, double-blind, placebo-controlled, phase 3b study. Lancet 2018;392(10161):2280-2287.

[41] Rosen N, Pearlman E, Ruff D, Day K, Jim Nagy A. 100% Response Rate to Galcanezumab in Patients With Episodic Migraine: A Post Hoc Analysis of the Results From Phase 3,

Randomized, Double-Blind, Placebo-Controlled EVOLVE-1 and EVOLVE-2 Studies. Headache 2018;58(9):1347-1357.

[42] Rubio-Beltran E, Correnti E, Deen M, Kamm K, Kelderman T, Papetti L, Vigneri S, MaassenVanDenBrink A, Edvinsson L, European Headache Federation School of Advanced S. PACAP38 and PAC1 receptor blockade: a new target for headache? J Headache Pain 2018;19(1):64.

[43] Shi X, Wang L, Li X, Sahbaie P, Kingery WS, Clark JD. Neuropeptides contribute to peripheral nociceptive sensitization by regulating interleukin-1beta production in keratinocytes. Anesth Analg 2011;113(1):175-183.

[44] Steiner TJ, Stovner LJ, Vos T, Jensen R, Katsarava Z. Migraine is first cause of disability in under 50s: will health politicians now take notice? J Headache Pain 2018;19(1):17.

[45] Stewart WF, Shechter A, Rasmussen BK. Migraine prevalence. A review of population-based studies. Neurology 1994;44(6 Suppl 4):S17-23.

[46] Strecker T, Dux M, Messlinger K. Nitric oxide releases calcitonin-gene-related peptide from rat dura mater encephali promoting increases in meningeal blood flow. J Vasc Res 2002;39(6):489-496.

[47] Stucky NL, Gregory E, Winter MK, He YY, Hamilton ES, McCarson KE, Berman NE. Sex differences in behavior and expression of CGRP-related genes in a rodent model of chronic migraine. Headache 2011;51(5):674-692.

[48] Tepper SJ. History and Review of anti-Calcitonin Gene-Related Peptide (CGRP) Therapies: From Translational Research to Treatment. Headache 2018;58 Suppl 3:238-275.

[49] VanderPluym J, Dodick DW, Lipton RB, Ma Y, Loupe PS, Bigal ME. Fremanezumab for preventive treatment of migraine: Functional status on headache-free days. Neurology 2018;91(12):e1152-e1165.

[50] Vermeersch S, Benschop RJ, Van Hecken A, Monteith D, Wroblewski VJ, Grayzel D, de Hoon J, Collins EC. Translational Pharmacodynamics of Calcitonin Gene-Related Peptide Monoclonal Antibody LY2951742 in a Capsaicin-Induced Dermal Blood Flow Model. J Pharmacol Exp Ther 2015;354(3):350-357.

[51] White S, Marquez de Prado B, Russo AF, Hammond DL. Heat hyperalgesia and mechanical hypersensitivity induced by calcitonin gene-related peptide in a mouse model of neurofibromatosis. PLoS One 2014;9(9):e106767.

[52] Zierau O, Zenclussen AC, Jensen F. Role of female sex hormones, estradiol and progesterone, in mast cell behavior. Front Immunol 2012;3:169.

CHAPTER 3

MENINGEAL CGRP-PROLACTIN INTERACTION EVOKES FEMALE-SPECIFRIC MIGRIANE BEHAVIOR

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ABSTRACT

Objective: Migraine is three times more common in women. CGRP plays a critical role in migraine pathology and causes female-specific behavioral responses upon meningeal application. These effects are likely mediated through interactions of CGRP with signaling systems specific to females. Prolactin (PRL) levels have been correlated with migraine attacks. Here, we explore a potential interaction between CGRP and PRL in the meninges.

Methods: Prolactin, CGRP, and receptor antagonists CGRP8-37 or Δ1-9-G129R-hPRL were administered onto the dura of rodents followed by behavioral testing. Immunohistochemistry was used to examine PRL, CGRP and Prolactin receptor (Prlr) expression within the dura. Electrophysiology on cultured and back-labeled trigeminal ganglia (TG) neurons was used to assess PRL-induced excitability. Finally, the effects of PRL on evoked CGRP release from ex vivo dura were measured.

Results: We found that dural PRL produced sustained and long-lasting migraine-like behavior in cycling and ovariectomized female, but not male rodents. Prlr was expressed on dural afferent nerves in females with little-to-no presence in males. Consistent with this, PRL increased excitability only in female TG neurons innervating the dura and selectively sensitized CGRP release from female ex vivo dura. We demonstrate crosstalk between PRL and CGRP systems as CGRP8-37 decreases migraine-like responses to dural PRL. Reciprocally, Δ1-9-G129R-hPRL

attenuates dural CGRP-induced migraine behaviors. Similarly, Prlr deletion from sensory neurons significantly reduced migraine-like responses to dural CGRP.

Interpretation: This CGRP-PRL interaction in the meninges is a mechanism by which these peptides could produce female-selective responses and increase the prevalence of migraine in women.

Abbreviations: acid-sensing ion channels (ASICs), Calcitonin Gene-Related Peptide (CGRP), Cluster of differentiation 31 (CD31), Conditional Knockout (CKO), Dorsal Root Ganglia (DRG), Hypothalamic-Pituitary-Ovarian axis (HPO-axis), Prolactin (PRL), Prolactin Receptor (Prlr), Δ1-9-G129R-hPRL (ΔPRL), Tyrosine Hydroxylase (TH), Trigeminal Ganglia (TG).

INTRODUCTION

Migraine is one of the most sexually dimorphic neurological disorders with prevalence in women at 2-3 times more than men. Among women ages 15-49, migraine is ranked as the most common cause of disability [207; 230]. While little is known about the underlying causes for the higher prevalence of migraine in females, the largest differences between sexes occurs after the onset of puberty and before menopause, many women have increased susceptibility to attacks during specific days of the cycle, and the frequency/severity of attacks can change across pregnancy, all

of which strongly suggest that sex-specific hormone levels influence the pathology [43; 230; 231].

Among the hormones potentially mediating the increased migraine prevalence in women, the pituitary-derived hormone prolactin (PRL) is among the least studied. PRL release from the pituitary of humans and rodents is regulated by gonadal hormones, but also by trauma and stress [20]. Although migraine patients do not have higher basal serum PRL levels compared to controls [110], PRL has been shown to rise during migraine attacks in patients with microprolactinoma; importantly, PRL levels do not rise during tension-type-headaches [32]. Further, some cases of migraine can be treated by bromocriptine, which blocks the release of PRL from the pituitary [105; 121], a treatment typically given for hyperprolactinemia. It has been reported that women who have never experienced migraine, began reporting migraine attacks that correlate with being diagnosed with microprolactinomas [31]. Moreover, patients with a history of migraine were found to develop chronic migraine that was correlated with increasing PRL levels [50]. This suggests that the elevated PRL levels in hyperprolactinemic patients increases the susceptibility to migraine.

Calcitonin gene-related peptide (CGRP) signaling plays a critical role in migraine pathology since plasma levels rise during attacks, intravenous administration can trigger attacks, and there are now multiple FDA-approved therapeutics that target CGRP or its receptor [83]. Despite the established role for CGRP in migraine, the locations and mechanisms that mediate its actions remain unclear. We showed previously that dural application of CGRP caused female-specific behavioral responses in a preclinical migraine model [12], but the signaling system mediated the

sexual dimorphism was not identified. The purpose of the present study was to 1) evaluate whether dural PRL signaling, particularly in sensory afferents, exhibits sexually-dimorphic effects that may contribute to sex/gender differences in migraine; and 2) determine whether there is potential crosstalk between PRL and CGRP signaling within the meninges that can explain the female-selective effects of CGRP in this tissue.

METHODS

Animals:

In this study, 12-to 14-week-old 260-300 g female and 300-350 g male Sprague Dawley rats (Taconic; Rensselaer, NY), 6- to 8-week old female and male ICR mice (Envigo; Indianapolis, IN), 6-8 week-old ovariectomized CD-1 mice (Charles River; Houston, TX; ovariectomy surgeries were performed by Charles River before shipping) and 6- to 8-week-old Nav1.8$^{cre/-}$/ Prlr$^{fl/fl}$ mice on C57/BL6 background were used for behavioral experiments. The Prlr$^{fl/fl}$ line was generated by inserting lox sites for deletion of the 4th exon and causing a mRNA-eliminating frame shift as previously described [35]. Ex-vivo experiments were conducted on 8-12-week-old female and male C57/BL6 mice (Jackson Laboratory; Bar Harbor, ME). Primary cultures from Prlr$^{cre/-}$/Rosa26$^{LSL\text{-}tdTomato/+}$ animals were used for electrophysiology experiments. Additionally, these animals were used in immunohistochemistry experiments. Estrous phases were determined by vaginal gavage as described previously[45]. Animals were housed with access to food and water *ad libitum* on a 12 h light/dark cycle. Animals were allowed to acclimate to the facility for 72 hours prior to handling and habituation.

Rat Cannula implantation and drug delivery

All rat dural injections were administered at a total volume of 10 µl via an implanted cannula. For the cannula implantation surgery, rats were initially anesthetized at 5% isoflurane via a nose cone; once animals no longer demonstrated a paw-pinch reflex, isoflurane was lowered to 2-3.5% for the entirety of the surgery. A longitudinal incision was made through the scalp down the midline to expose the skull. Two screws were inserted below bregma on either side of midline. Using a pin vise (Grainger Industries; Lake Forest, IL) set to a length of 1 mm to leave the dura intact. A 1 mm burr hole was created using a stereotaxic frame at the coordinates to sit above the middle meningeal artery (8 mm anteroposterior, -2 mm mediolateral, 1 mm dorsoventral). A guide cannula (P1 Technologies; Roanoke, VA; C313G/SPC gauge 22) was then inserted into the burr hole and sealed using Vetbond (3M). Hygenic orthodontic resin (Coltene) was used to anchor the cannula to the screw and skull. After the resin hardened a dummy cannula (P1 Technologies 313DCSPC 0.014–0.36 mm fit 1 mm) was inserted into the guide cannula to prevent clogging. Following surgery, animals were given 8 mg/kg gentamicin and 0.25 mg meloxicam to prevent infection and for pain management, respectively. Animals were returned to their home cage and allowed to recover for approximately 7 days.

Mouse dural injections

Mouse dural injections were performed according to previously published methods [38]. Briefly, mice were anesthetized under isoflurane for <2 minutes and injected with a modified internal-cannula (Invivo1, part #8IC313ISPCXC, Internal Cannula, standard, 28 gauge). The inner

portion of the cannula was adjusted with calipers to extend from 0.5 to 0.65 mm in length. This inner portion was used to inject a volume of 5 µl through the soft tissue at the intersection of the lambdoidal and sagittal sutures.

Mouse Von Frey testing

Mice were handled for a single 5-min session at 24-hours prior to habituation to the behavior chambers. During each session of habituation animals were placed in 4 oz paper cups (Choice) for 2 hours a day for 3 consecutive days as previously described [38]. Habituation was done in the rooms where all further behavioral testing occurred to acclimate animals to the room and light conditions. Von Frey testing of the periorbital skin [38] was used to assess baseline values following habituation prior to stress as well as mechanical hypersensitivity that resulted from drug treatments. Food was placed in the chamber for each animal for each day of testing. Baselined animals were defined as animals that exhibited a withdrawal threshold approximately 0.5 g - 0.6 g. Filaments greater than 0.6 g were not used. This was set as the maximum gram weight in our initial development of this preclinical model [38].The majority of mice showed facial responses above the 0.6 g threshold. Mice with a baseline threshold lower than 0.5 g at the end of 4 days were excluded from experiments. Mechanical thresholds were determined by applying von Frey filaments to the periorbital region of the face (the midline of the forehead at the level of the eyes) in an ascending/descending manner starting from the 0.07 g filament. Briefly, if an animal did not respond, increasing filament forces were applied until the 0.6 g filament was reached or until a response was observed. If the animal responded to a specific

filament, decreasing filament forces were applied until the 0.008 g filament was reached or until there were no responses. A response was defined as a mouse actually removing/swiping the filament away from its face during application. All animals were numbered and randomly allocated to experimental groups by drawing from pre-labeled paper slips. All experimenters were blinded to the treatment groups until the end of each experiment.

Rat Von Frey testing

Rats were handled for a single 5-min session at 24-hours prior to habituation to the behavior chambers. Rats were subject to 2-hour sessions of habituation a day for 3 days. During each session of habituation animals were placed in testing chambers in the rooms where all further behavioral testing occurred to acclimate animals to the room and light conditions. Baselined rats were required to have a periorbital von Frey threshold of 8 g, animals were not tested above 8 g for comparison with previous experiments[12; 37]. If a rat did not meet this threshold by end of habituation they were not included in experiments. Rats withdrawal thresholds were assessed in the same ascending/descending manner as mice; however, testing began at 1 g with a maximum of 8 g, and minimum of 0.4 g.

Primary TG neuron Cultures

Trigeminal ganglia (TG) neurons that innervate the dura mater were identified by dural injection (5 μl) of 1% WGA-488 (Applied Biosystems; Foster City, CA) 48 hours prior to vaginal smear/TG neuronal culture generation. Vaginal smears were performed on mice from separate

cages. Only mice in the estrous phase of the reproductive cycle were used for TG neuronal culture preparation and subsequent patch clamp recording. WT or reporter mice, including $Prlr^{cre/-}/Rosa26^{LSL\text{-}tdTomato/+}$, were used for patch clamp recording. For TG dissection, mice were deeply anaesthetized with isoflurane (0.3 ml in 1 liter administered for 60-90 sec) and sacrificed by cervical dislocation. The V1 area of the TG was quickly removed, and neurons were dissociated by treatment with a 1 mg/ml collagenase-dispase (Roche, Indianapolis, IN) solution. Cells were maintained in DMEM supplemented with 2% fetal bovine serum (FBS), 2mM L-glutamine, 100U/ml penicillin and 100 μg/ml streptomycin and no NGF. Experiments were performed within 6-24 hr after TG neuron plating.

Dural CGRP Release Assay

Mustard oil (MO: Fluka, St. Louis, MO) stock (100%) was diluted in Hank's buffer (HBSS) to 0.01% (1 mM). HBSS was pH adjusted to 6.9 using HEPES (20 mM). The entire mouse dura from 2 separate mice were combined and submerged into a single well and treated as an N of 1. Dura remained submerged for the entirety of release experiments. Experiments were carried out at 37ºC. Dura tissues were washed once with HBSS and then soaked in HBSS for 30 min to equilibrate, the supernatant was collected for measurement of baseline CGRP release after 15 min in HBSS. Tissues were then exposed for 3 min to MO, pH 6.9 HBSS, MO+PRL or pH 6.9 HBSS+PRL (PRL was used at 1 μg/ml), then each solution was replaced with HBSS. Tissues were maintained for additional 15 min in HBSS. The total evoked CGRP release was measured by pooling the 3 min treatment exposure sample with a 15 min vehicle post-exposure sample.

Dura biopsies were only used once and only exposed to one sequence of treatments. The CGRP radioimmunoassay was conducted as previously described[212] with a primary antibody against CGRP (final dilution, 1:1x10^6; kindly donated by Dr. Michael J. Iadarola (NIDCR/NIH, Bethesda, MD). CGRP release data were normalized by the weight of fresh dura biopsies, to avoid compromising tissue as it dries. Data are presented as percent release above baseline.

Electrophysiology

Recordings were made in whole-cell current clamp configuration at 22-24ºC. Data were acquired and analyzed using an Axopatch 200B amplifier and pCLAMP 10.6 software (Molecular Devices, Sunnyvale, CA). Recorded data were filtered at 5 kHz and sampled at 20 kHz. Borosilicate pipettes (Sutter, Novato, CA) were polished to resistances of 2-3 MΩ. Access resistance (Rs) was compensated (40-80%) to maintain resistance <6-8 MΩ. Data were rejected when Rs changed >20% during recording, leak currents were >100pA, or input resistance was <300 MΩ. Standard external solution (SES) contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 HEPES, pH 7.4. The standard pipette (internal) solution (SIS) contained (in mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 D-glucose, 10 HEPES, pH 7.3, 2.5 ATP and 0.2 GTP. Drugs were applied by a fast, pressure-driven and computer controlled 4-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA) with quartz application pipettes.

Small (<30 pF) WGA^+/Prlr-cre^+ TG neurons from $Prlr^{cre/-}$/$Rosa26^{LSL\text{-}tDTomato/+}$ reporter mice were randomly selected for recording (Fig. 5A). To characterize modulation of these TG neurons

excitation by vehicle (control) or PRL (200 ng/ml), the following sequence of recording protocols were applied: (1) a single AP in current clamp configuration was generated with a 0.5ms and 1nA current step to define the type of sensory neurons[198] (Fig. 5B); (2) a linear ramp from 0 to 0.2 nA for 1 sec was applied to generate a control AP train (Fig. 5C); (3) the patched neuron was treated for 2-5 min with vehicle or PRL; and then (4) the ramp as in step 2 was re-applied (Fig. 5C). Data was accumulated from 4 independent mouse TG neuronal cultures for each sex. Each culture was generated from one male or estrous female mouse. Changes in neuronal excitability were calculated by dividing AP frequency generated by a current ramp after vehicle or drug-treatment to AP frequency produced by the ramp before treatment. Excitability was determined to be regulated by PRL when the PRL treatment produced statistically significant increase in AP frequency than vehicle-treatment (i.e. control).

Immunohistochemistry (IHC)

Dura mater from perfusion-fixed female and male WT and $Prlr^{cre/-}$/$Rosa26^{LSL\text{-}tDTomato/+}$ mice were fixed again with 4% paraformaldehyde and cryoprotected with 30% sucrose in phosphate buffer. Anti-Prlr rabbit polyclonal (NSJ Bioreagents; San Diego, CA; catalogue R31199; 1:200) ([44];[196]); anti-CGRP rabbit polyclonal antibodies (Sigma; C8198; 1:300) ([196];[198]); anti-PRL rabbit polyclonal antibodies (Bioss, Boston, MA; cat: BS-23763R; 1:200); and anti-CD31 rat monoclonal (Clone: MEC 13.3) antibodies (BD Pharmingen; cat: 553370; 1:400) were used for IHC. Sections were incubated with species appropriate Alexa Fluor secondary antibodies (1:200; Molecular Probes, Eugene, OR). Images were acquired using a Nikon Eclipse 90i

microscope (Melville, NY, USA) equipped with a C1si laser scanning confocal imaging system. Images were processed with NIS-elements software (Nikon Instruments, Melville, NY). Control IHC was performed on tissue sections processed as described but either lacking primary antibodies or lacking primary and secondary antibodies. IHC images were obtained from 3-5 independent tissue sections or whole tissues from 3-4 animals. Z-stack images were used for presentation and analysis.

Drugs

See table 1 for information on Drugs sources, doses, and administration routes.

Statistics

Data are presented as mean SEM. Data were analyzed among groups at each time point via two-way ANOVA and followed by Bonferroni post-test where appropriate. Prism (Graph-Pad Software) was also used for data analysis. Significance was set to $p < 0.05$ for all analyses. Experimenters were blinded to treatment groups. Allocation of animals to treatment groups was randomized by a "blinder" that drew animal numbers from a bag of paper slips.

Study approval

All procedures were conducted with prior approval of the Institutional Animal Care and Use Committee at the University of Texas at Dallas and the University of Texas Health Science Center at San Antonio.

RESULTS

Prolactin causes female-specific migraine-like behavioral responses in rodents.

In order to investigate whether PRL signaling within the meninges causes differential behavioral responses in females and males, we used a preclinical migraine model in which stimuli are applied to the dura and cutaneous periorbital hypersensitivity and facial grimace are measured [38]. Initially, 5 μg of PRL was applied to the dura of both female and male wild-type ICR mice. Significant facial hypersensitivity was observed in both female (Fig. 1A) and male (Fig. 1D) mice in response to PRL. However, this effect was prolonged in the female mice, lasting out to at least 72 hours following application of PRL. In contrast, we observed a transient allodynia in males with significant facial hypersensitivity only at 3 hours following PRL application. Next, since 5 μg PRL likely causes tissue concentrations that are higher than those found under physiological and pathological conditions [78; 197], we applied a lower dose of PRL (0.5 μg) to the dura to determine whether this dose caused responses only in females. This lower PRL dose caused facial hypersensitivity in females (Fig. 1B), lasting out to at least 7 days, while no effect was observed in males (Fig. 1E). Additionally, only female mice exhibited grimace behaviors in response to this low dose PRL when compared with their respected controls (Fig. 1C). These data demonstrate that dural application of PRL causes migraine-like responses that are female-specific. However, high, likely non-physiological dosages of PRL can cause transient and smaller-magnitude periorbital allodynia in males.

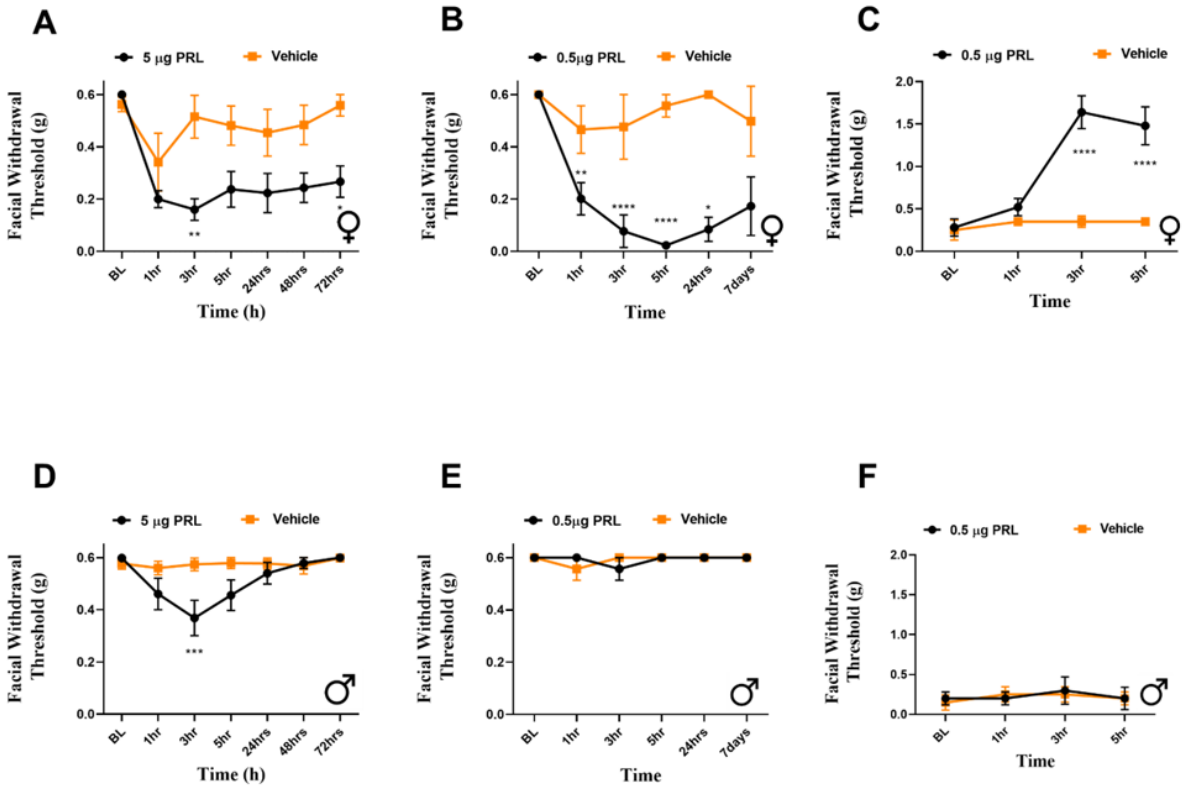

Figure 3.1: Dural prolactin induces greater behavioral responses in female mice. Male and female mice had mechanical withdrawal thresholds assessed prior to dural injection of either 5 µg or 0.5 µg PRL. Following 5 µg PRL, both female (A) (n = 7 PRL, 6 vehicle) and male (D) (n = 7 PRL, 7 vehicle) mice exhibited facial hypersensitivity. Only females exhibited a significant hypersensitivity at low dose PRL (B) (n = 5 PRL, 4 vehicle). Animals that received 0.5 µg of PRL were additionally assessed for grimace prior to facial testing at each time point (C). Female mice that received this dose of PRL experienced significant grimace in comparison with respective controls, while male mice (n = 4 PRL, 4 vehicle) exhibited no significant grimace. Two-way ANOVA followed by Bonferroni multiple comparison analysis indicated significant

differences between females that received PRL when compared with those that received vehicle. Data are represented as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. See Table S1 for additional results of analysis.

To rule out the possibility of a species-specific effect, PRL was also applied to the dura of Sprague-Dawley female and male rats. Unlike in mice, even the higher dose of PRL (5 μg) applied to the dura caused significant facial hypersensitivity only in females (Fig. 2A, 2C). These responses were significant at 1 hour and 3 hours post-injection, and animals returned to baseline by 5 hours. The lower dose of PRL (0.5 μg; Fig. 2B, 2D) was also female-specific, with hypersensitivity observed at 1 hour and 3 hours following administration, and animals returned to baseline by 5 hours. These findings demonstrate that dural application of PRL causes female-specific behavioral responses in both mice and rats.

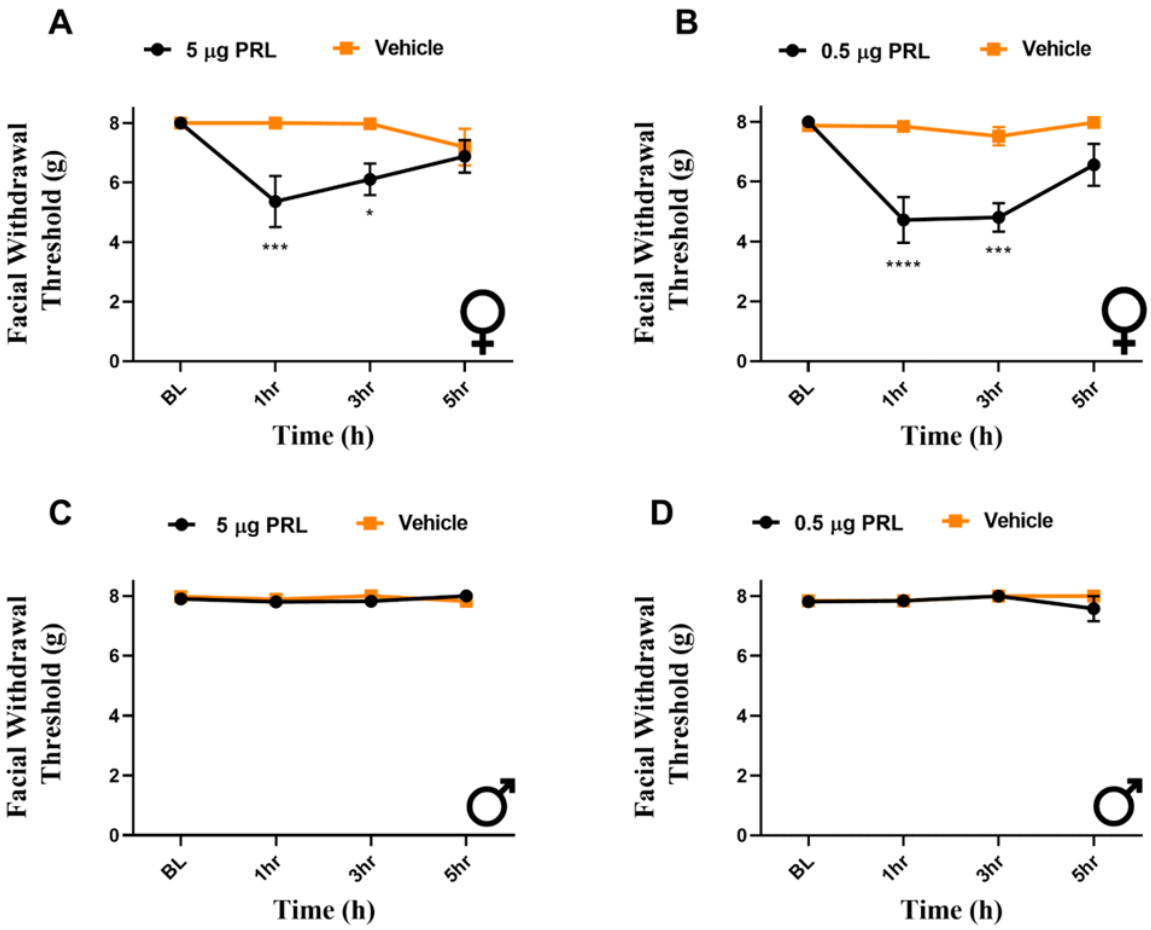

Figure 3.2: Dural prolactin causes female-specific behavioral responses in rats**.** Facial withdrawal thresholds were measured in female and male rats prior to and following dural injection of either 5 µg (A, C) or 0.5 µg dural PRL (B, D). Two-way ANOVA followed by Bonferroni multiple comparison analysis indicated significant differences between females that received 5 µg PRL (n = 8 PRL)when compared with those that received vehicle (n = 9). No significant responses were seen in males at this dose (n=8PRL, 7 vehicle).At the 0.5 µg PRL dose, female mice that received PRL (n = 8) demonstrated significant hypersensitivity when

compared with controls (n = 7).No significant effect was found in males (n = 5 PRL, 5 vehicle). Data are represented as means ± SEM. *p < 0.05,***p < 0.001, ****p < 0.0001. See Table S1 for additional results of analysis.

Ovariectomy does not prevent responses to dural PRL

Given the role for ovarian-derived estrogen and progesterone in prolactin release and Prlr regulation, we asked whether disruption of the hypothalamic-pituitary-ovarian (HPO) axis would decrease the behavioral responses in females to levels similar to those observed in males. To address this, ovariectomized females were purchased 2 weeks following ovariectomy and received injection of either 0.5 μg PRL or vehicle onto the dura 3 weeks following surgery (Fig. 3A, 3B). Ovariectomized females that received PRL demonstrated significant responses at 3, 24, and 48-hours post injection compared with those that received vehicle. PRL-injected OVX females returned to baseline by 72 hours, in contrast to intact females (Fig. 1B). These data suggest that while there may be subtle differences in regulation of Prlr expression or signaling in the dura by HPO axis, PRL-induced migraine-like pain is largely independent of regulation by ovarian-derived hormones.

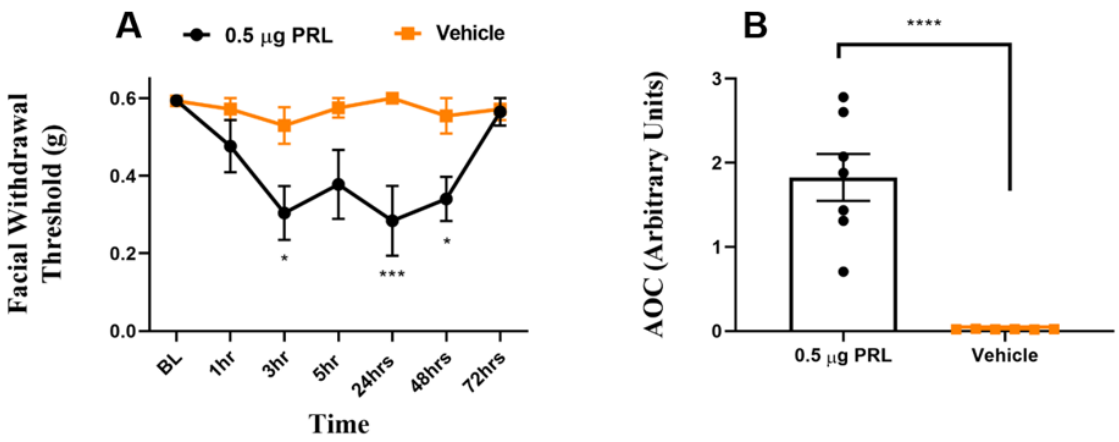

Figure 3.3: Dural PRL induces behavioral responses in ovariectomized female mice. Ovariectomized female mice received dural injection of either 0.5 µg PRL (n = 8) or vehicle (n = 8), 3 weeks after surgery. Data are additionally represented as area over the curve (B). Two-way ANOVA followed by Bonferroni multiple comparison analysis indicated significant differences between OVX females that received PRL and those that received vehicle. Data are represented as means ± SEM. *p < 0.05, **p < 0.01,,***p < 0.001, ****p < 0.0001. See Table 1 for additional analyses.

Expression of Prlr is higher in female dura mater.

Given the ability of PRL to induce female-selective facial hypersensitivity, we investigated whether these sexually dimorphic effects could be due to differential expression of (Prlr) within this tissue. We took advantage of a mouse line where tdTomato is present in cells that express Prlr ($Prlr^{cre/-}$/$Rosa26^{LSL\text{-}tdTomato/+}$). In 5-week-old females (i.e. pre-puberty), Prlr expression appeared to be restricted to non-neuronal cells (Fig. 4A). However, in 10-week-old

reproductively mature females, Prlr expression was clearly evident on both resident dural cells as well as neuronal fibers that innervate the dura mater (Fig. 4B). In 10-week-old males, tdTomato was observed almost entirely in non-neuronal cells, with only sparse expression on nerve fibers (Fig. 4C). Next, we identified Prlr+ cell types. Co-labeling for endothelial cells in blood vessels (CD31) and sensory fibers (CGRP) in female dura showed that Prlr is co-localized on both structures (Fig. 4D-4E'), as previously reported [195; 207]. Using immunohistochemistry for Prlr (instead of genetically-driven tdTomato expression), labeling was similarly found on both blood vessels and dural nerve fibers (Fig. 4F, 4F'). These PRL-expressing fibers were found to run alongside CGRP expressing fibers (Fig. 4G, 4G') and fibers expressing Tyrosine hydroxylase (TH) (Fig. 4I, 4I'). Labeling associated with blood vessels was not different between female and male dura while neuronal labeling appeared less extensive. These data suggest that PRL-induced female-selective migraine behavior is likely due to differential expression of Prlr on CGRP+ nerve fibers in female and male dura.

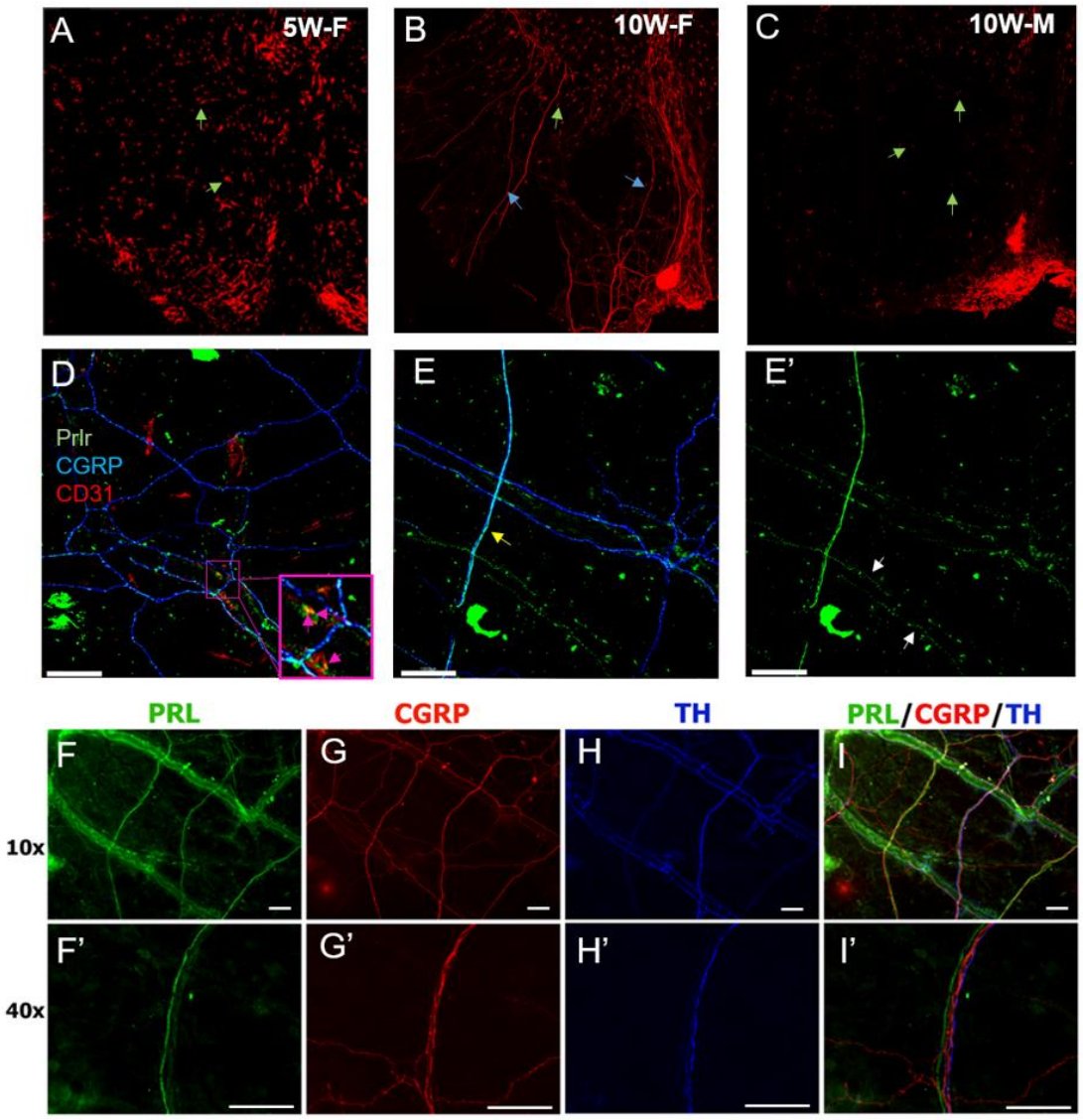

Figure 3.4: Expression of Prolactin and prolactin receptor in mouse dura. A 3 × 3 mm square area of dura mater from 5-week-old female (A), 10-week-old female (B) and 10-week-old male (C) Prlrcre/-/TdT mice. Green arrows mark Prlr-cre+non-neuronal cells. Blue arrows mark Prlr-cre+dural afferent fibers. Objective (10×, scale bars represent 120 μm). Dura mater from 10-

week-old female Prlrcre/-/TdT mice labeled with anti-CGRP and anti-CD31 antibodies (D). Pink arrows show dural blood vessel coexpressing Prlr-cre+(Green) and CD31+ (Red) cells. Objective (20×, scale bars = 120 μm). Prlr-cre+dural afferent fibers express CGRP (Blue) and are indicated with yellow arrows (E). White arrows mark Prlr-cre+ expression on non CGRP expressing dural fibers (E'). Objective (20×). PRL (F, F'), CGRP (G, G'), and TH (H, H0) expression in female mouse dura. Overlap of (F–H) shown in (I). For 40× images scale bars represent 100 μm.

PRL has female-specific effects on dural afferent excitability and CGRP release

Since the Prlr appeared to be more highly expressed on nerve endings in female dura, we tested whether there are selective actions of PRL on dural afferents using both *in vitro* patch-clamp electrophysiology of TG neurons innervating dura and evoked CGRP release from *ex vivo* dura tissue. First, TG neurons innervating the dura were identified by retrograde labeling using application of the tracer wheat-germ agglutinin 488 (WGA-488) to the dura. TG were dissected from females in estrous and male $Prlr^{cre/-}/Rosa26^{LSL\text{-}tdTomato/+}$ mice within 48 hours after application of WGA-488 to the dura and neurons were cultured. Dural afferents were selected for recording in the cultures based on the presence of WGA-488, indicating retrograde tracing from the dura, and Prlr expression was determined by the presence of tdTomato (Fig. 5A). Importantly, among the 40 culture dishes used from female mice in these experiments, there were 541 neurons that expressed both Prlr and WGA-488, and a majority of them were small-sized (<35□m) neurons. Among 44 dishes from male mice, there were only 140 neurons expressing both Prlr and WGA-488, and approximately 90% of them were medium-sized

(>35□m) neurons. The predominant expression of Prlr in small-sized sensory neurons in females and medium-sized sensory neurons in males is consistent with that reported for dorsal root ganglia (DRG) [196]. A typical action potential (AP) shape from a small sized (<30 pF) female WGA-488^{+}/tdTomato^{+} neuron is shown in Fig. 4B, similar to those shown previously [176; 198]. Dural afferents were stimulated with 1 sec ramps from 0 to 200 pA. When PRL (200 ng/mL) was applied to these neurons after the first ramp protocol, the number of AP's fired on a subsequent ramp protocol was significantly greater in female but not male neurons (Fig. 5C,5D). These data show that PRL application to dural afferent cell bodies selectively sensitizes female neurons.

Next, we used freshly dissected dura from the mouse skull to measure CGRP release, which presumably originated from the sensory nerve endings innervating the dura. As mustard oil (MO) is known to stimulate sensory neurons and cause release of CGRP [108] and also cause migraine-like behaviors when applied to the dura [38; 79], we used this stimulus (0.01%) to determine whether PRL application to the dura would potentiate this response. Female dura treated with PRL (1 μg/mL) prior to MO exhibited an approximately 200% increase in CGRP release compared to females treated with MO and vehicle (Fig. 5E; MO and vehicle causes a 200% increase in CGRP release over baseline). There was no significant difference in release of CGRP in males treated with vehicle or PRL, despite similar increase to females in CGRP release over baseline with MO alone. We also tested a pH 6.9-buffered solution, which activates acid-sensing ion channels (ASICs), a family of receptors that we have previously shown to play a role in afferent signaling from the dura [37; 263; 268]. Unlike MO, the pH 6.9 solution caused little

release of CGRP over baseline on its own (Fig. 5F). However, the application of PRL (1 µg/mL) along with pH 6.9 led to a 200% increase in CGRP release over baseline, and at least 5-fold potentiation of responses. As with MO, the potentiating effect of PRL only occurred in females. These female-specific effects of PRL on dural afferents *in vitro* and on CGRP release from dura are consistent with the female-specific behavioral responses of dural PRL in Figs. 1 and 2, as well as higher Prlr expression on sensory afferents in females shown in Fig.4.

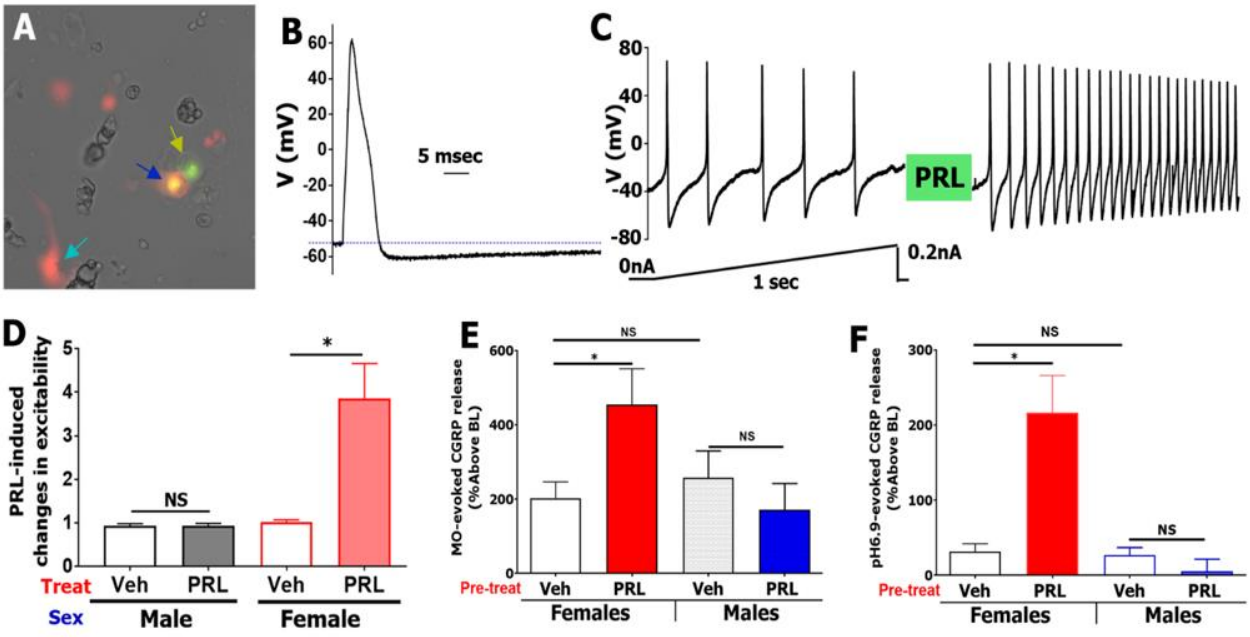

Figure 3.5: PRL selectively sensitizes Prlr-positive TG neurons innervating the dura of female mice. Prlr-cre+/WGA-488+ back traced neurons from dura TG neuron (blue arrow) were selected for recording. Yellow arrow shows Prlr-cre-/WGA-488+ TG neuron. Sapphire arrow shows Prlr-cre+ non-neuronal cell. (A) Action potential (AP) from small-sized (25 pF) selected Prlr-cre +/WGA-488+ TG neuron. Stimulus waveform is 1,000 pA, 0.5 ms. (B) Train of APs in a selected Prlr-cre+/WGA-488+ TG neuron (same neurons as panels A and B) was stimulated with

current ramp protocol shown below trace. The neuron was treated with exogenous PRL (200 ng/ml) for 5 min, and the same current ramp protocol was applied. Ratio of post-PRL AP frequency to before-PRL AP frequency reflects changes in excitability. (C) PRL-induced changes in excitability of Prlr-cre+/WGA-488+ TG neuron from females and males. Control is vehicle treatment between two current ramps. n = 7–14 (D) PRL (1 μg/ml)-induced sensitization of MO (0.01%)-evoked CGRP release from female and male dura biopsies. n = 4–6. (E) PRL (1 μg/ml)-induced sensitization of pH 6.9-evoked CGRP release from female and male dura biopsies. n = 4 (F) Statistics are 2-way ANOVA with variables as sex and treatment (NS, non-significant; *$p < 0.05$).

Loss of Prlr on sensory neurons or block of meningeal Prlr attenuates migraine-like behavioral responses to dural CGRP

The female-specific nature of the behaviors caused by dural application of PRL (Figs. 1, 2) are remarkably similar to the female-specific responses to dural application of CGRP we showed previously [12]. This led us to ask whether there might be a relationship between the signaling mechanisms engaged downstream of dural Prlr and CGRP receptor activation. We thus took advantage of a conditional knockout (CKO) mouse where the Prlr is selectively deleted from Nav1.8-positive sensory neurons, using a Nav1.8-cre line crossed to a mouse line a floxed Prlr ($Prlr^{fl/fl}$). We have previously characterized this mouse, showing the loss of Prlr expression in $Nav1.8^{+}$ dorsal root ganglion neurons [195]. Female Prlr CKO mice and their control littermates ($Prlr^{fl/fl}$ only) were administered 1 pg dural CGRP, a dose we showed previously to have female-specific effects in mice [12]. While responses to dural CGRP in Prlr CKO mice were similar to

their wild type counterparts at 1 hour, the Prlr CKO mice showed significantly attenuated periorbital withdrawal thresholds at hours 3 and 5 post-injection as well as 24 hours post injection (Fig. 6A). These values are additionally represented as AOC (Fig. 6B). In control mice, migraine-like responses to dural CGRP returned to baseline at 4 days post-injection. In contrast, in the Prlr CKO mice, return to baseline occurred significantly faster at 24 hours post-injection. These critical findings suggest that the mechanism by which dural CGRP causes hypersensitivity is partially mediated via Prlr signaling in sensory neurons.

To determine whether a similar Prlr-dependent mechanism of action of dural CGRP exists in rats, we tested whether the Prlr antagonist Δ1-9-G129R-hPRL (modified human PRL molecule that sterically inhibits Prlr activity in humans and rodents [23], hereafter referred to as ΔPRL) blocked the response to CGRP. In our previous study, dural administration of CGRP produced female-specific facial hypersensitivity in rats as well as mice [12]. Here, female rats received either 1 pg CGRP or co-administration of 1 pg CGRP + 5 μg ΔPRL, and migraine-like behaviors were tested as in Fig. 2. Female rats responded to dural CGRP as shown previously, but these responses were significantly blocked at the 3-hour time point by ΔPRL (Fig. 6C). While we cannot precisely identify the location of the Prlr's sites of actions in this experiment, the findings are nonetheless consistent with mice where Prlr was deleted from Nav1.8-expressing neurons. This suggests that one of the locations of Prlr in these rat experiments is on sensory fibers.

Given the data described above showing that Prlr contributes to the response to dural CGRP, we next asked whether the reverse might also be true, i.e. whether the CGRP receptor contributes to responses to dural PRL. Female rats received either 0.5 μg dural PRL or a co-injection of 0.5 μg

PRL and 100 ng of the CGRP receptor antagonist $CGRP_{8-37.}$ Co-administration of PRL and $CGRP_{8-37}$ significantly attenuated the response to dural PRL at the 3-hour timepoint following injection (Fig. 6E). These data demonstrate that not only does Prlr contribute to the CGRP response but the CGRP receptor also contributes to the PRL response. Thus, there is crosstalk between these two signaling systems in the mechanisms that are used to produce female-specific responses from the dura. Moreover, this crosstalk involves Prlr+ sensory neurons.

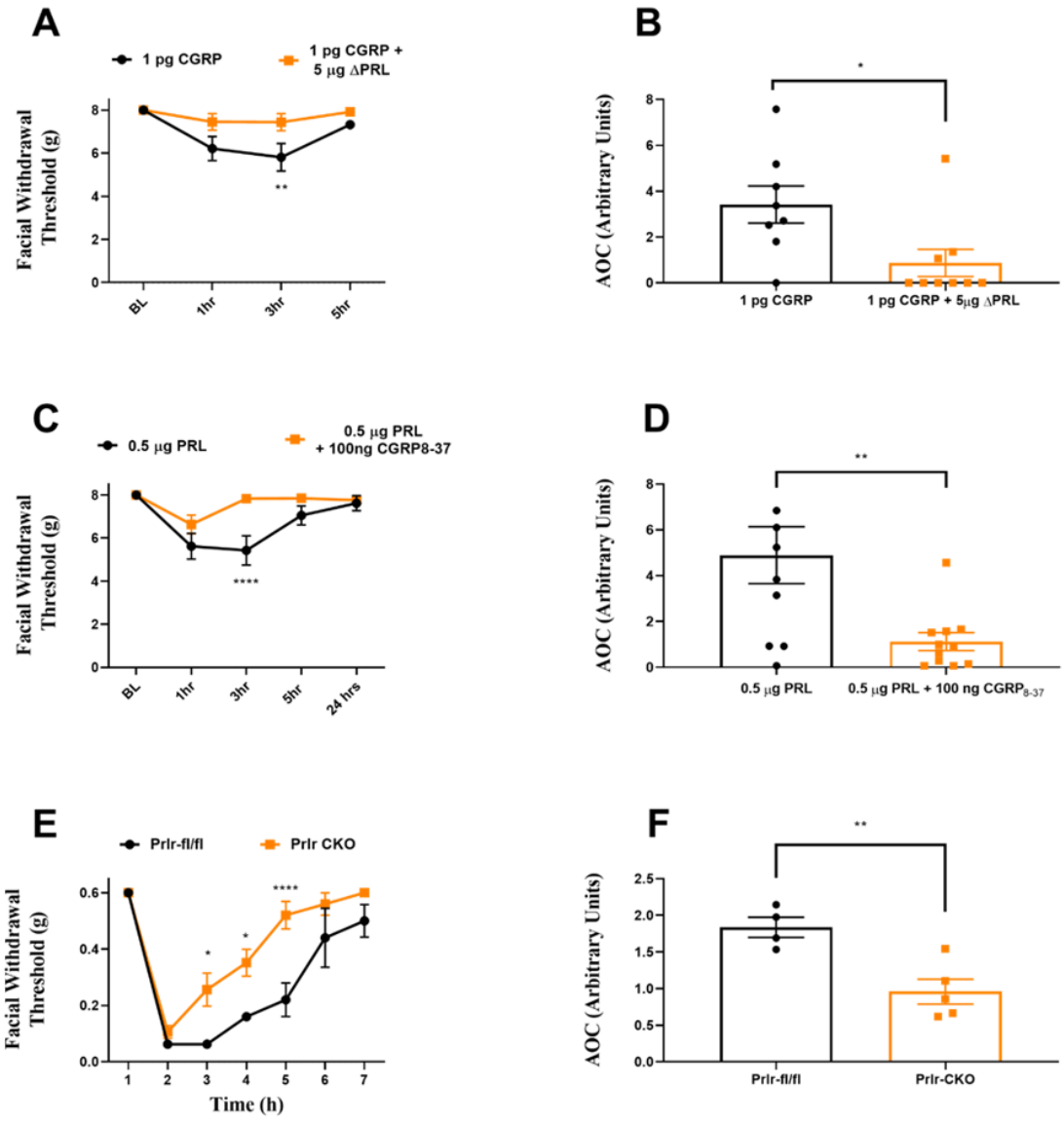

Figure 3.6: Crosstalk between the CGRP and PRL signaling systems within the dura. Female prolactin receptor CKO mice (n = 5) and their control littermates (n = 4) had mechanical withdrawal thresholds tested prior to and following injection with CGRP (A, B). Female rats had

baseline thresholds assessed and received dural injection of 1 pg CGRP (C, D) (n = 9) or 1 pg of CGRP co-injected with 5 μg of delta PRL (n = 9). Co-injection with delta prolactin significantly attenuated CGRP induced responses, as indicated via two-way ANOVA followed by Bonferroni post-hoc analysis. A separate cohort of female rats received injection of either 0.5 μg dural PRL (n = 10) or co-injection of 0.5 μg PRL with 100 ng CGRP8-37 (E, F) (n = 11). Co-injection with CGRP8-37 significantly reduced behavioral responses to dural PRL. Data are represented as means ± SEM. **p < 0.01,****p < 0.0001. See Table S1 for additional results of analysis.

PRL and Prlr expression on multiple cell types within the dura

The effects observed in the Prlr CKO mice (Fig. 6A) as well as with ΔPRL (Fig. 6C) following dural CGRP suggest that endogenous PRL is involved in these behavioral responses which may be produced locally within the dura from extra-pituitary cells [21; 75; 171]. To address this, dura from 10-week-old female mice were extracted and used for IHC. We observed PRL immunoreactivity in immune cells as marked by CD11b (Fig. 7A-C; Blue arrows) indicating a potential role for PRL release from immune-like cell types of the dura. Ten-week-old males had similar expression patterns (Fig. 7D-F), suggesting that differences in PRL signaling within the dura are likely due to differential receptor expression patterns (Fig. 4A-C) and not due to differential expression of the ligand.

To address whether other cell types within the dura may contribute to local PRL-dependent signaling following dural CGRP, we examined Prlr expression in relation to the immune cell marker CD45 in control females (Fig. 7G-I) and Prlr CKO females (Fig. 7J-L). These data show

expression of Prlr on CD45-positive cells, and as expected, there was no influence of Prlr CKO on expression of Prlr within the CD45 cell population. This suggests that the responses to dural CGRP in the Prlr CKO mice shown in Figure 6 may involve Prlr activation of CD45-positive immune cells as part of this local dural signaling mechanism.

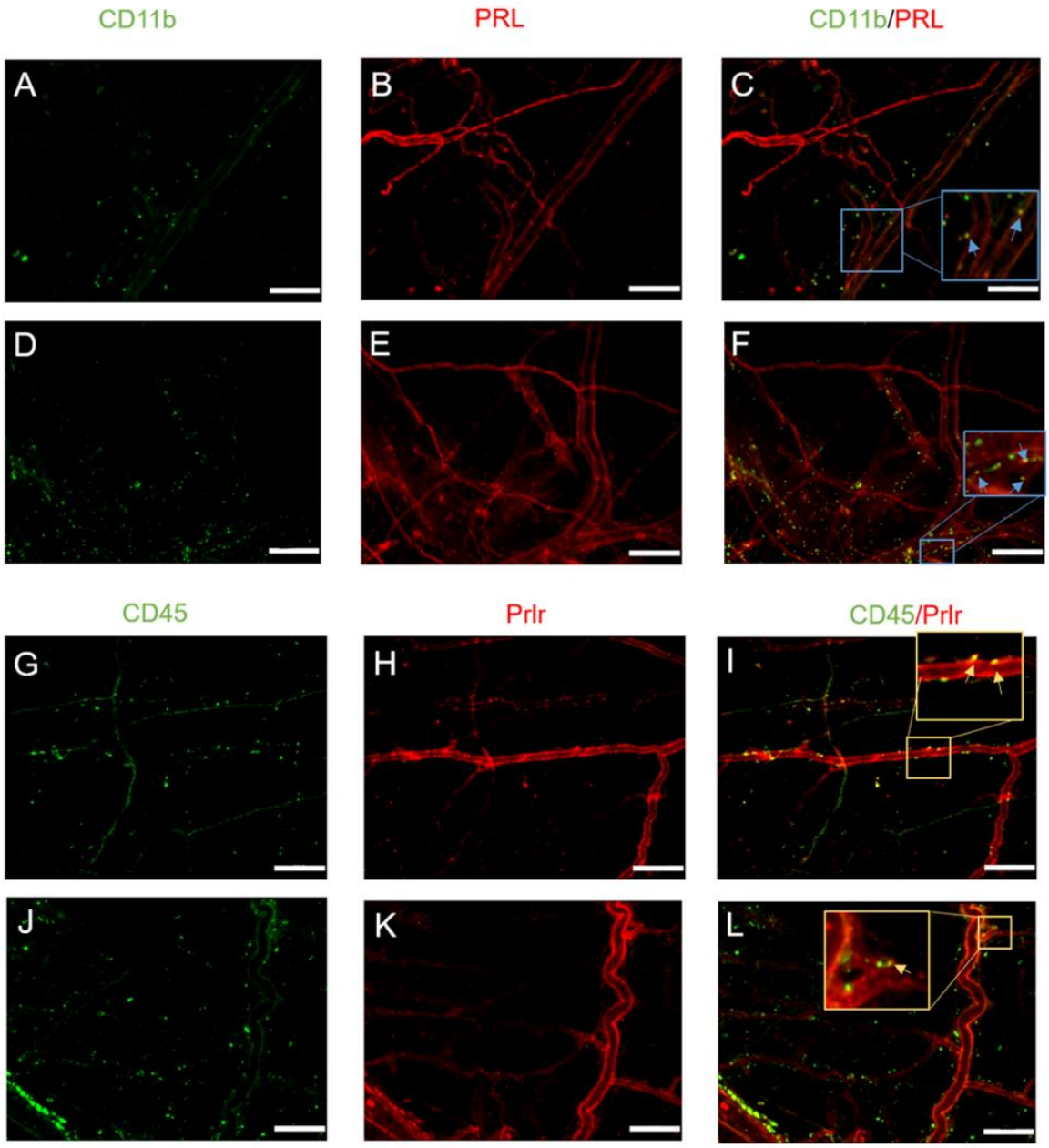

Figure 3.7: Expression of prolactin and prolactin receptor in non-neuronal cells in the dura. Dura mater was removed from 3–5-month-old female (A–C) and male (D–F) mice. These dura were processed and stained for PRL expression and co-stained with CD11b. Images were taken at 20×.

Scale bars represent 100 µm. Blood vessels are included as a potential source of prolactin release. Orange arrow heads indicate the middle meningeal artery (MMA) which served as a biological marker to ensure images were taken from the same region of animals. CD11b expression (A, D), PRL expression (B, E) and the overlay is shown in (C, F). Inserts in (C, F) highlight overlap of PRL and CD11b. Expression of Prlr in intact females. Prolactin receptor expression (H) was assessed in 3–5-month-*old female mice, dura was co-stained for CD45 (G) and overlap shown in (I). Dura of littermate Prlr conditional knockout animals, that have Prlr deleted from Nav1.8 sensory neurons, were also stained to assess the presence of Prlr (J–L). Inserts in (I, L) highlight overlap of Prlr and CD45.

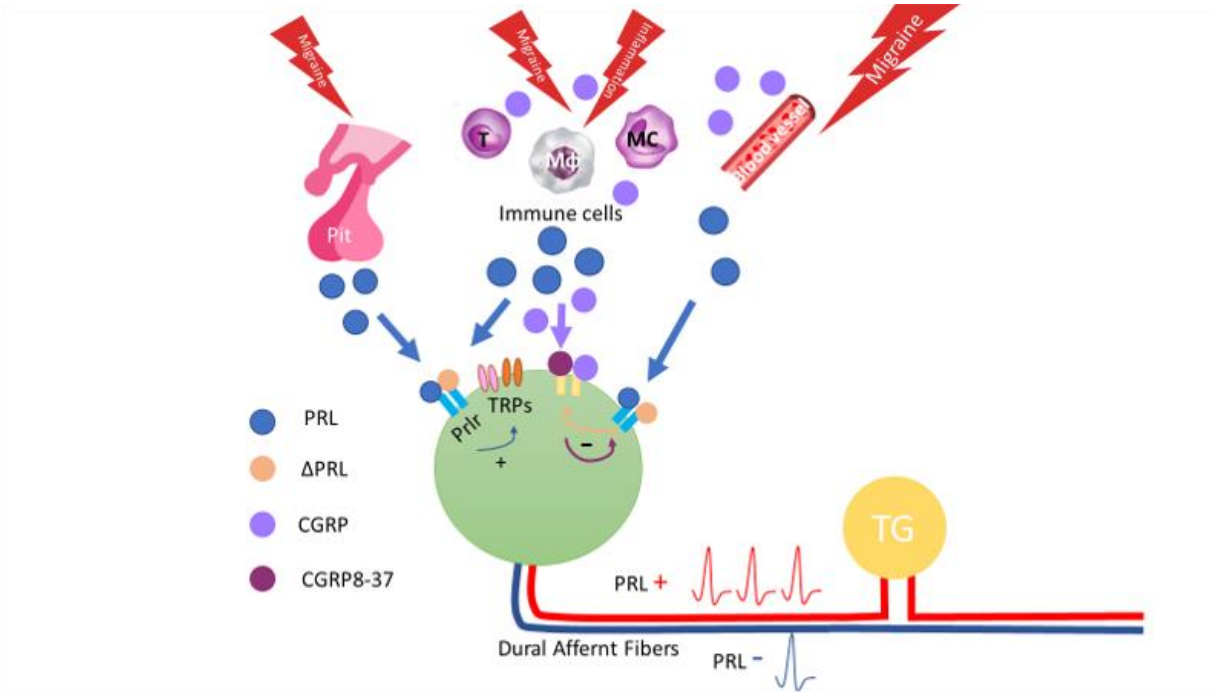

Figure 3.8: Hypothesized intercellular interactions within the dura**.** The pituitary (Pit),

endothelial cells on blood vessels, immune cells (macrophages (Mφ), mast cells (MC), T cells (T)), and sensory neurons can all serve as potential PRL release sites during migraine. PRL can sensitize dural afferents in a paracrine manner via TRP and other channels. CGRP released from dural afferents may interact with immune cells and blood vessels leading to further PRL release. CGRP and PRL receptor antagonists mitigate intercellular signaling, where CGRP8-37 may block release of PRL from dural cells and nerve fibers (indicated via purple arrow), and ΔPRL may block additional sensitization of dural afferents (yellow arrow). These combined actions lead to sensitized afferent signaling from the dura (represented by PRL+).

DISCUSSION

While the mechanisms responsible for the increased prevalence of migraine in females are likely to be complex, the data shown here are the first to describe a female-specific signaling circuit within the meninges that may be an important component in the headache phase of attacks. This circuit is based on an interaction between PRL and CGRP, the latter a critical contributor to migraine, but one whose mechanisms in the disorder remain unclear. We show that exogenous application of PRL onto the dura of both rats and mice produces female-specific responses at the 0.5 μg dose. At the 5 μg dose, male mice demonstrated short lasting allodynia. The lower potency and reduced response in males are likely due to low neuronal expression of Prlr within the dura (Fig. 4). Surprisingly, this same high dose (5 μg) of PRL in females (Fig.1A) resulted in

less robust allodynia than the lower 0.5 μg dose (Fig. 1B). This observation may be due to the higher concentrations of PRL causing Prlr internalization in females, as shown previously with Prlr on pancreatic β islet cells in rat [34]. Regardless, both doses of dural PRL demonstrated facial hypersensitivity that was clearly more robust in females.

These female-specific responses are likely due to higher Prlr expression on sensory nerve endings in adult female dura compared to males (Fig.4 A-C). Consistent with the behavioral data and female-specific expression of Prlr on nerve endings in the dura, we found a sensitizing effect of PRL on isolated female dura that led to increased CGRP release, while no effect was seen on male dura. This was similar in TG neurons, as electrophysiology on cultured TG cells back-labeled from the dura revealed that PRL only induced hyperexcitability in neurons from female mice. This may explain how PRL is able to potentiate CGRP release from female but not male dura. Regardless of the source of endogenous PRL release, whether from the pituitary or cells native to the dura, the pro-nociceptive actions of PRL are likely to be limited to females due to the mechanisms shown here. We also show bi-directional communication between the PRL and CGRP signaling systems as interventions targeted toward each receptor block responses to the opposing peptide. These data suggest that both PRL and CGRP play a more significant role in migraine in females and that these signaling systems are dependent on each other for their actions in the meninges.

These experiments also show that responses to dural PRL are maintained in ovariectomized females. This is not surprising, as ovariectomy only transiently alters endogenous PRL levels.

Prolactin mRNA decreases following ovariectomy in female rats for the initial 2 weeks following surgery but returns to those of intact controls by week 3 and 4 [2]. Additionally, ovariectomy does not alter serum PRL in female rats at various stages of the reproductive cycle[239]. In women that have undergone total abdominal hysterectomy with bilateral salpingo-oophorectomy, PRL levels returned to pre-surgery baseline levels by 6 weeks post-surgery [8] and remain non-significantly altered one year after surgery [49]. These data suggest continued Prlr expression after ovariectomy given the lack of changes in circulating levels of PRL caused by this surgery.

Our data are consistent with prior studies in models of postoperative and inflammatory pain, where PRL has been shown to contribute to nociception in a female-specific manner [196]. These data support previous findings showing PRL increased TRPV1-, TRPM8-, and TRPA1-mediated responses in sensory neurons from DRG in female mice, but not males [199]. Moreover, female Prlr CKO mice, showed reduced behavioral responses to CFA, whereas males did not show significant deficits [199]. While these findings were in the spinal system, PRL has been shown to sensitize TG neurons and increase capsaicin-evoked CGRP release from TG neurons [69]. In the current study, Prlr expression was not seen on dural fibers of 5-week female mice. During human adolescence the mean serum PRL levels in males and females are similar while adult PRL levels are significantly increased in females but not in males [114], which aligns with the findings here of differential receptor expression across age. In the approximately 10-week old animals used here, PRL causes a more robust allodynic response in females and induces excitability of TG neurons back-labeled from female dura. Thus, our data may fit with a

general pattern for a role of PRL in nociceptive signaling in adult females but not males and may offer insight to the emergence of migraine following the onset of puberty [89; 159].

Importantly, this work demonstrates the apparent crosstalk between PRL and CGRP signaling in the meninges. Given the similar pattern of female-specific behavioral responses between PRL shown here and that of CGRP (shown previously [12]), it may not be surprising that the signaling systems interact. Prlr is known to be expressed on CGRP-positive sensory fibers [195] and activation of these fibers by PRL (pituitary and/or extra-pituitary) likely promotes the release of CGRP (as shown in Fig. 5). It is well known that CGRP is a potent vasodilator and its receptors are located on cells of the vasculature. CGRP may then act on these nearby blood vessels as well as immune cells [11; 16], or other nerve fibers [88; 155] to promote a positive-feedback loop that potentiates local inflammatory or sensitizing conditions. Additionally, the CGRP receptor is expressed on dural mast cells [155] and elevated CGRP may lead to mast cell degranulation resulting in increased PRL within the dura. Blocking CGRP receptors in the presence of PRL stimulation would disrupt this loop (Fig. 6E).

CGRP application to the dura may act on blood vessels, immune cells, and/or other nerve fibers, causing PRL release from these structures ([75; 104; 174; 195]; Fig. 7) since PRL can also be supplied by sources outside of the pituitary (i.e. extra-pituitary PRL) to act via paracrine or autocrine mechanisms [21]. Moreover, residential dural cells express mRNA for PRL [55] and are immunoreactive for PRL protein expression. Endothelial and immune cells express Prlr and are capable of releasing PRL [93; 269]. Here we confirm that PRL is expressed on CD11b

expressing cells within the dura of female and male mice; CD11b cell types are likely to be myeloid cells (macrophages, granulocytes, and mast cells) (Fig.7A-F), which are the predominant type of immune cells in dura [177]. Additionally, we show that Prlr is expressed on CD45 expressing cells (Fig. 7G-L). CD45 staining may further indicate mast cells, which have been found to present similarly in shape at dural blood vessels[7; 188]. PRL may then have one of two actions: 1) on Prlr expressed on nerve fibers, sensitizing afferent nociceptive signaling from the dura; 2) on Prlr expressed on immune cells in a cytokine-like manner causing further release of PRL and recruitment of more immune cells [33; 68]. Blocking Prlr may similarly disrupt this feedback loop (Fig. 8). While there is some debate about the role of PRL in inflammation, PRL levels have been shown to increase as a result of peripheral inflammation [219] and PRL can also induce inflammation [266], which may contribute to this potential feedback loop. Here we show that female mice that have Prlr deleted from Nav1.8-expressing fibers within the dura exhibited attenuated facial allodynia in response to previously reported effective doses of dural CGRP[12] (Fig. 6A). This is consistent with findings that CGRP co-injected with the Prlr antagonist Δ1-9-G129R-hPRL blocked behavioral responses (Fig. 6C). In either case, the mechanisms are female-specific, given the dimorphic expression/function of Prlr on dural afferents, as well as potential unidentified dimorphisms in CGRP receptor expression/function. While there could be dimorphic expression of PRL in cells native to the dura, we did not observe obvious differences in nerve fibers, blood vessels, or non-neuronal cells. This does not rule out the potential that PRL-expressing cell types are differentially recruited to the dura under specific conditions, but there does not appear to be baseline

differences in PRL expression within the dura. These data show an interaction between the downstream mechanisms of PRL and CGRP within the dura that may have an important role in promoting meningeal afferent nociceptive signaling in females.

Finally, the data presented here demonstrate involvement of Prlr on sensory neurons in CGPR-PRL signaling within the dura. Moreover, our data suggest that communication between these signaling pathways could be an important female-selective mechanism contributing to the increased prevalence of migraine in females. Accordingly, targeting PRL signaling, whether through suppression of pituitary and/or extra-pituitary PRL release or development of a Prlr antagonist, could be novel therapeutic approach for migraine. Our data suggest that peripherally-restricted Prlr antagonists could be an option for the effects of PRL on the headache phase of attacks. While these therapeutic approaches may not be completely devoid of adverse effects, pharmacological reduction of pituitary PRL release is chronically prescribed to thousands of patients with hyperprolactinemia with minimal ill effects [145] and would likely only be contraindicated in women who are pregnant or nursing, both conditions where extensive drug restrictions are common. Targeting PRL signaling for migraine could ultimately represent one the first sex/gender-specific therapeutics for a neurological disorder and may pave the way for future approaches that would be designed to have efficacy only in females or males.

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AUTHOR CONTRIBUTIONS

A.A, C.B.V, T.J.P, A.N.A, G.D contributed to design of experiments

A.A, C.B.V, A.H.H, S.N.B, J.M, V.G, N.W conducted experiments

A.A, B.N.M, A.H.H, S.N.B, J.M, V.G, N.W contributed to data analysis

A.A, B.N.M, A.N.A, G.D contributed to writing the manuscript.

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REFERENCES

[1] Abbot SD, Docherty K, Clayton RN. Regulation of LH subunit mRNA levels by gonadal hormones in female rats. J Mol Endocrinol 1988;1(1):49-60.

[2] Arac A, Grimbaldeston MA, Galli SJ, Bliss TM, Steinberg GK. Meningeal Mast Cells as Key Effectors of Stroke Pathology. Front Cell Neurosci 2019;13:126.

[3] Aravantinos D, Tzingounis V. [Changes in plasma prolactin levels after gynecologic surgery]. J Gynecol Obstet Biol Reprod (Paris) 1983;12(5):461-463.

[4] Assas BM, Pennock JI, Miyan JA. Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. Front Neurosci 2014;8:23.

[5] Avona A, Burgos-Vega C, Burton MD, Akopian AN, Price TJ, Dussor G. Dural Calcitonin Gene-Related Peptide Produces Female-Specific Responses in Rodent Migraine Models. J Neurosci 2019;39(22):4323-4331.

[6] Baliu-Pique M, Jusek G, Holzmann B. Neuroimmunological communication via CGRP promotes the development of a regulatory phenotype in TLR4-stimulated macrophages. Eur J Immunol 2014;44(12):3708-3716.

[7] Ben-Jonathan N, LaPensee CR, LaPensee EW. What can we learn from rodents about prolactin in humans? Endocrine Reviews 2008;29(1):1-41.

[8] Ben-Jonathan N, Mershon JL, Allen DL, Steinmetz RW. Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. Endocr Rev 1996;17(6):639-669.

[9] Bernichtein S, Kayser C, Dillner K, Moulin S, Kopchick JJ, Martial JA, Norstedt G, Isaksson O, Kelly PA, Goffin V. Development of pure prolactin receptor antagonists. J Biol Chem 2003;278(38):35988-35999.

[10] Bosco D, Belfiore A, Fava A, De Rose M, Plastino M, Ceccotti C, Mungari P, Iannacchero R, Lavano A. Relationship between high prolactin levels and migraine attacks in patients with microprolactinoma. The journal of headache and pain 2008;9(2):103-107.

[11] Bosco D, Belfiore A, Fava A, De Rose M, Plastino M, Ceccotti C, Mungari P, Iannacchero R, Lavano A. Relationship between high prolactin levels and migraine attacks in patients with microprolactinoma. J Headache Pain 2008;9(2):103-107.

[12] Brand JM, Frohn C, Cziupka K, Brockmann C, Kirchner H, Luhm J. Prolactin triggers pro-inflammatory immune responses in peripheral immune cells. Eur Cytokine Netw 2004;15(2):99-104.

[13] Brelje TC, Stout LE, Bhagroo NV, Sorenson RL. Distinctive roles for prolactin and growth hormone in the activation of signal transducer and activator of transcription 5 in pancreatic islets of langerhans. Endocrinology 2004;145(9):4162-4175.

[14] Brown RS, Kokay IC, Phillipps HR, Yip SH, Gustafson P, Wyatt A, Larsen CM, Knowles P, Ladyman SR, LeTissier P, Grattan DR. Conditional Deletion of the Prolactin Receptor Reveals Functional Subpopulations of Dopamine Neurons in the Arcuate Nucleus of the Hypothalamus. J Neurosci 2016;36(35):9173-9185.

[15] Burgos-Vega CC, Quigley LD, Avona A, Price T, Dussor G. Dural stimulation in rats causes brain-derived neurotrophic factor-dependent priming to subthreshold stimuli including a migraine trigger. Pain 2016;157(12):2722-2730.

[16] Burgos-Vega CC, Quigley LD, Trevisan Dos Santos G, Yan F, Asiedu M, Jacobs B, Motina M, Safdar N, Yousuf H, Avona A, Price TJ, Dussor G. Non-invasive dural stimulation in mice: A novel preclinical model of migraine. Cephalalgia 2018:333102418779557.

[17] Buse DC, Loder EW, Gorman JA, Stewart WF, Reed ML, Fanning KM, Serrano D, Lipton RB. Sex differences in the prevalence, symptoms, and associated features of migraine, probable migraine and other severe headache: results of the American Migraine Prevalence and Prevention (AMPP) Study. Headache 2013;53(8):1278-1299.

[18] Buteau H, Pezet A, Ferrag F, Perrot-Applanat M, Kelly PA, Edery M. N-glycosylation of the prolactin receptor is not required for activation of gene transcription but is crucial for its cell surface targeting. Mol Endocrinol 1998;12(4):544-555.

[19] Caligioni CS. Assessing reproductive status/stages in mice. Curr Protoc Neurosci 2009;Appendix 4:Appendix 4I.

[20] Castelo-Branco C, Martinez de Osaba MJ, Vanrezc JA, Fortuny A, Gonzalez-Merlo J. Effects of oophorectomy and hormone replacement therapy on pituitary-gonadal function. Maturitas 1993;17(2):101-111.

[21] Cavestro C, Rosatello A, Marino MP, Micca G, Asteggiano G. High prolactin levels as a worsening factor for migraine. J Headache Pain 2006;7(2):83-89.

[22] Clapp C, Lopez-Gomez FJ, Nava G, Corbacho A, Torner L, Macotela Y, Duenas Z, Ochoa A, Noris G, Acosta E, Garay E, Martinez de la Escalera G. Expression of prolactin mRNA and of prolactin-like proteins in endothelial cells: evidence for autocrine effects. J Endocrinol 1998;158(1):137-144.

[23] Dill R, Walker AM. Role of Prolactin in Promotion of Immune Cell Migration into the Mammary Gland. J Mammary Gland Biol Neoplasia 2017;22(1):13-26.

[24] Diogenes A, Patwardhan AM, Jeske NA, Ruparel NB, Goffin V, Akopian AN, Hargreaves KM. Prolactin modulates TRPV1 in female rat trigeminal sensory neurons. J Neurosci 2006;26(31):8126-8136.

[25] Donoso AO, Banzan AM. Release of prolactin and LH and histamine-containing cells in brain. J Neural Transm 1979;44(4):327-332.

[26] Dutia R, Kim AJ, Mosharov E, Savontaus E, Chua SC, Jr., Wardlaw SL. Regulation of prolactin in mice with altered hypothalamic melanocortin activity. Peptides 2012;37(1):6-12.

[27] Edelmayer RM, Le LN, Yan J, Wei X, Nassini R, Materazzi S, Preti D, Appendino G, Geppetti P, Dodick DW, Vanderah TW, Porreca F, Dussor G. Activation of TRPA1 on dural afferents: a potential mechanism of headache pain. Pain 2012;153(9):1949-1958.

[28] Edvinsson L, Goadsby PJ. Discovery of CGRP in relation to migraine. Cephalalgia 2019;39(3):331-332.

[29] Eftekhari S, Warfvinge K, Blixt FW, Edvinsson L. Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. J Pain 2013;14(11):1289-1303.

[30] Ferrari MD, Klever RR, Terwindt GM, Ayata C, van den Maagdenberg AM. Migraine pathophysiology: lessons from mouse models and human genetics. Lancet Neurol 2015;14(1):65-80.

[31] Galsgaard ED, Rasmussen BB, Folkesson CG, Rasmussen LM, Berchtold MW, Christensen L, Panina S. Re-evaluation of the prolactin receptor expression in human breast cancer. J Endocrinol 2009;201(1):115-128.

[32] Gonzalez C, Rosas-Hernandez H, Jurado-Manzano B, Ramirez-Lee MA, Salazar-Garcia S, Martinez-Cuevas PP, Velarde-Salcedo AJ, Morales-Loredo H, Espinosa-Tanguma R, Ali SF, Rubio R. The prolactin family hormones regulate vascular tone through NO and prostacyclin production in isolated rat aortic rings. Acta Pharmacol Sin 2015;36(5):572-586.

[33] Gordon ML, Lipton RB, Brown SL, van Praag HM. The neuroendocrine challenge paradigm in headache research. Cephalalgia : an international journal of headache 1995;15(4):292-296.

[34] Grant AD, Pinter E, Salmon AM, Brain SD. An examination of neurogenic mechanisms involved in mustard oil-induced inflammation in the mouse. Eur J Pharmacol 2005;507(1-3):273-280.

[35] Guldiken S, Guldiken B, Demir M, Kabayel L, Ozkan H, Turgut N, Hunkar R, Kat S. Soluble CD40 ligand and prolactin levels in migraine patients during interictal period. J Headache Pain 2011;12(3):355-360.

[36] Guyda HJ, Friesen HG. Serum prolactin levels in humans from birth to adult life. Pediatr Res 1973;7(5):534-540.

[37] Hartman N, Voron SC, Hershman JM. Resolution of migraine following bromocriptine treatment of a prolactinoma (pituitary microadenoma). Headache 1995;35(7):430-431.

[38] Koivisto MB, Eschricht F, Urhausen C, Hoppen HO, Beyerbach M, Oei CHY, Gunzel-Apel AR. Effects of Short-term Hyper- and Hypoprolactinaemia on Hormones of the Pituitary, Gonad and -Thyroid Axis and on Semen Quality in Male Beagles. Reprod Domest Anim 2009;44:320-325.

[39] Lennerz JK, Ruhle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF, Messlinger K. Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. J Comp Neurol 2008;507(3):1277-1299.

[40] Lipton RB, Bigal ME. Migraine: epidemiology, impact, and risk factors for progression. Headache 2005;45 Suppl 1:S3-S13.

[41] Marano RJ, Ben-Jonathan N. Minireview: Extrapituitary prolactin: an update on the distribution, regulation, and functions. Mol Endocrinol 2014;28(5):622-633.

[42] Matera L, Muccioli G, Cesano A, Bellussi G, Genazzani E. Prolactin receptors on large granular lymphocytes: dual regulation by cyclosporin A. Brain Behav Immun 1988;2(1):1-10.

[43] McCarthy PW, Lawson SN. Differing action potential shapes in rat dorsal root ganglion neurones related to their substance P and calcitonin gene-related peptide immunoreactivity. J Comp Neurol 1997;388(4):541-549.

[44] McIlvried LA, Cruz JA, Borghesi LA, Gold MS. Sex-, stress-, and sympathetic post-ganglionic-dependent changes in identity and proportions of immune cells in the dura. Cephalalgia 2017;37(1):36-48.

[45] Nurkhametova D, Kudryavtsev I, Guselnikova V, Serebryakova M, Giniatullina RR, Wojciechowski S, Tore F, Rizvanov A, Koistinaho J, Malm T, Giniatullin R. Activation of P2X7 Receptors in Peritoneal and Meningeal Mast Cells Detected by Uptake of Organic Dyes: Possible Purinergic Triggers of Neuroinflammation in Meninges. Front Cell Neurosci 2019;13:45.

[46] Patil M, Belugin S, Mecklenburg J, Wangzhou A, Paige C, Barba-Escobedo PA, Boyd JT, Goffin V, Grattan D, Boehm U, Dussor G, Price TJ, Akopian AN. Prolactin Regulates Pain Responses via a Female-Selective Nociceptor-Specific Mechanism. iScience 2019;20:449-465.

[47] Patil M, Hovhannisyan AH, Wangzhou A, Mecklenburg J, Koek W, Goffin V, Grattan D, Boehm U, Dussor G, Price TJ, Akopian AN. Prolactin receptor expression in mouse dorsal root ganglia neuronal subtypes is sex-dependent. J Neuroendocrinol 2019;31(8):e12759.

[48] Patil MJ, Green DP, Henry MA, Akopian AN. Sex-dependent roles of prolactin and prolactin receptor in postoperative pain and hyperalgesia in mice. Neuroscience 2013;253:132-141.

[49] Patil MJ, Hovhannisyan AH, Akopian AN. Characteristics of sensory neuronal groups in CGRP-cre-ER reporter mice: Comparison to Nav1.8-cre, TRPV1-cre and TRPV1-GFP mouse lines. PLoS One 2018;13(6):e0198601.

[50] Patil MJ, Ruparel SB, Henry MA, Akopian AN. Prolactin regulates TRPV1, TRPA1, and TRPM8 in sensory neurons in a sex-dependent manner: Contribution of prolactin receptor to inflammatory pain. Am J Physiol Endocrinol Metab 2013;305(9):E1154-1164.

[51] Reuwer AQ, Nowak-Sliwinska P, Mans LA, van der Loos CM, von der Thusen JH, Twickler MT, Spek CA, Goffin V, Griffioen AW, Borensztajn KS. Functional consequences of prolactin signalling in endothelial cells: a potential link with angiogenesis in pathophysiology? J Cell Mol Med 2012;16(9):2035-2048.

[52] Ruparel NB, Patwardhan AM, Akopian AN, Hargreaves KM. Homologous and heterologous desensitization of capsaicin and mustard oil responses utilize different cellular pathways in nociceptors. Pain 2008;135(3):271-279.

[53] Scotland PE, Patil M, Belugin S, Henry MA, Goffin V, Hargreaves KM, Akopian AN. Endogenous prolactin generated during peripheral inflammation contributes to thermal hyperalgesia. Eur J Neurosci 2011;34(5):745-754.

[54] Steiner TJ, Stovner LJ, Vos T, Jensen R, Katsarava Z. Migraine is first cause of disability in under 50s: will health politicians now take notice? J Headache Pain 2018;19(1):17.

[55] Stewart WF, Shechter A, Rasmussen BK. Migraine prevalence. A review of population-based studies. Neurology 1994;44(6 Suppl 4):S17-23.

[56] Szawka RE, Rodovalho GV, Helena CV, Franci CR, Anselmo-Franci JA. Prolactin secretory surge during estrus coincides with increased dopamine activity in the hypothalamus and preoptic area and is not altered by ovariectomy on proestrus. Brain Res Bull 2007;73(1-3):127-134.

[57] Wemmie JA, Price MP, Welsh MJ. Acid-sensing ion channels: advances, questions and therapeutic opportunities. Trends Neurosci 2006;29(10):578-586.

[58] Wu W, Sun M, Zhang HP, Chen T, Wu R, Liu C, Yang G, Geng XR, Feng BS, Liu Z, Liu Z, Yang PC. Prolactin mediates psychological stress-induced dysfunction of regulatory T cells to facilitate intestinal inflammation. Gut 2014;63(12):1883-1892.

[59] Yan J, Wei X, Bischoff C, Edelmayer RM, Dussor G. pH-evoked dural afferent signaling is mediated by ASIC3 and is sensitized by mast cell mediators. Headache 2013;53(8):1250-1261.

[60] Yang X, Meyer K, Friedl A. STAT5 and prolactin participate in a positive autocrine feedback loop that promotes angiogenesis. J Biol Chem 2013;288(29):21184-21196.

CHAPTER 4

REPETITIVE STRESS IN MICE CAUSES MIGRAINE-LIKE BEHAVIORS AND CGRP-DEPENDENT HYPERALGESIC PRIMING TO A MIGRAINE TRIGGER

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ABSTRACT:

Migraine is one of the most disabling disorders worldwide but the underlying mechanisms are poorly understood. Stress is consistently reported as a common trigger of migraine attacks. Here we show that repeated stress in mice causes migraine-like behaviors that are responsive to a migraine therapeutic. Adult female and male mice were exposed to 2 hours of restraint stress for 3 consecutive days, after which they demonstrated facial mechanical hypersensitivity and facial grimace responses that were resolved by 14d-post stress. Hypersensitivity or grimace was not observed in either control animals or those stressed for only 1 day. Following return to baseline, the NO-donor sodium nitroprusside (SNP; 0.1 mg/kg) elicited mechanical hypersensitivity in stressed but not in control animals, demonstrating the presence of hyperalgesic priming. This suggests the presence of a migraine-like state, since NO-donors are reliable triggers of attacks in migraine patients but not controls. The stress paradigm also caused priming responses to supradural pH 7.0 treatment. The presence of this primed state after stress is not permanent as it was no longer present at 35 days post-stress. Finally, mice received either the CGRP monoclonal antibody ALD405 (10 mg/kg) 24 hours prior to SNP or a co-injection of sumatriptan (0.6 mg/kg). ALD405, but not sumatriptan, blocked the facial hypersensitivity due to SNP. Altogether, this stress paradigm in mice and the subsequent primed state caused by stress, allow further preclinical investigation of mechanisms contributing to migraine, particularly those caused by common triggers of attacks.

INTRODUCTION:

Migraine is among the top 10 most common diseases and is the second-most disabling disease world-wide [70]. Migraine is characterized by unilateral throbbing pain, cutaneous allodynia, and is often accompanied by nausea and sensitivity to light and sound. Attacks are often caused by triggers that differ among migraineurs and include hormonal changes, changes in sleep-wake patterns, skipping meals, and consumption of alcohol and certain foods [142]. These common stimuli are not unique to migraine patients thus the susceptibility to attacks following exposure to these triggers suggests maladaptive changes have occurred within migraine-related nociceptive pathways. The mechanisms by which these changes happen and the location(s) in which they are present remain poorly understood. While the sensitivity to, and presence of, these triggers differs among migraine patients, the most commonly reported trigger for migraine is stress [142]. Not only can stress serve as a trigger for migraine, but stress has been reported to increase the duration of headache [259] and may even play a role in the development of chronic headache disorders [215]. Migraineurs that experience stress as a trigger often do not have a migraine attack while the stress is ongoing, but rather once the stressful event has passed [228; 229]. The greatest susceptibility to attacks is in the 6 to 18 hours following resolution of stress [161]. Additionally, multiple days of intense stress are more likely to trigger attacks than a single day[161]. Despite the close correlation between stress and migraine, mechanistic links between the two remain

poorly understood. Development of better preclinical models to study this connection could lead to better understanding of how stress contributes to migraine.

One of the most widely utilized methods of inducing stress in preclinical models is repeated restraint stress. Such stress paradigms can be mimicked through physical restraint which typically consists of confining rodents to a small space allowing minimal movement for variable durations of time. Restraint stress in rats has been shown to induce thermal and mechanical allodynia in the hindpaw [17]. It has also been shown to cause hyperalgesia in response to administration of the nitric oxide (NO) donor nitroglycerin in the tail-flick test [59] and to increase responses in the temporomandibular joint formalin test [95]. In the work described here, we attempted to further explore the ability of repeated stress to produce migraine-related behaviors by combining a repeated restraint-stress paradigm with previously developed models to assess cutaneous facial hypersensitivity. Our previously published data showed that application of noxious stimuli to the meninges is capable of causing acute behavioral responses consistent with headache, but also causing priming to normally non-noxious dural stimuli as well as to systemic administration of the NO-donor sodium nitroprusside (SNP) [37]. Here we tested whether single or repeated stress paradigms were able to cause hypersensitivity similar to that seen with direct dural stimulation and whether repeated restraint stress also causes priming to subsequent dural stimulation or to a subthreshold dose of SNP. Additionally, we tested the efficacy of two commonly-used migraine therapeutics, sumatriptan and an anti-CGRP monoclonal antibody, in blocking the priming

response in previously stressed animals. These data suggest that repeated stress in rodents can be used as a model to recapitulate migraine-like hypersensitivity.

METHODS:

Animals:

Female and Male ICR mice ages 6-8 weeks (Envigo; Livermore, CA) were used for all experiments. Animal weights varied between 19 g up to a maximum of 30 g. Animals were housed on a 12-hour light-dark cycle and had access to food and water *ad libitum*. Prior to being handled for experiments, animals were allowed a minimum of 72 hours in the animal facility. In all experiments, investigators were blinded to treatment groups. All procedures were conducted with prior approval of the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

Measurement of facial mechanical hypersensitivity:

All animals were handled for a minimum of 5 minutes, 24 hours prior to habituation, as well as each day prior to habituation. During each session of habituation animals were placed in 4 oz paper

cups (Choice) for 2 hours a day for 3 consecutive days as previously described [38]. Habituation was done in the rooms where all further behavioral testing occurred to acclimate animals to the room and light conditions. von Frey testing of the periorbital skin [38] was used to assess baseline values following habituation prior to stress as well as mechanical hypersensitivity that resulted from restraint stress and drug treatments. von Frey thresholds were not measured on the days that animals were subject to restraint stress. Testing began 24 hours after the third day of restraint unless otherwise noted. Mechanical thresholds were determined by the Dixon "up-and-down" method. von Frey filaments were applied to the periorbital region for a maximum of five seconds, or less if a response was observed. Testing began with the 0.07 g filament on the face and progressed to a maximum of 0.6 g. Only animals that met a baseline value of 0.6 g were included in the study. Following baseline, animals were numbered and randomly allocated to experimental groups by drawing from pre-labeled paper slips.

Repetitive stress paradigm:

Animals were subjected to restraint stress 24 hours following baseline. Sensory threshold values were determined with von Frey filaments. Animals were placed in cylindrical tail access rodent restrainers designed for animals 15-30 g (Stoelting 51338). Animals were placed in these restraint devices so that their tail was threaded through the moveable disk and their faces project out of the hole in the acrylic front face of the tube. Animals were introduced to the tube by placing the restrainer in front of the animals on the first day of stress and guiding them into the restrainer with the animal facing the acrylic front. Once the animal was in position the tail was threaded through

the moveable disk, the disk was moved toward the animal and tightened to ensure that the animal was incapable of movement. Care was taken to avoid any trauma to the mice due to injuries from moving the disk or from threading the tail. Mice were also restrained at a level that still allowed normal respiration. Animals were placed in the restraint tube so that the moveable disk faced upward and an opening on the tube was on the bottom. The animals were restrained for 2 hours a day for 3 consecutive days. Restraint stress started no earlier than 9:30 am and stress ended prior to 12:00pm in all cases to account for natural rising in corticosterone levels that have been shown occur in rodents in the afternoon starting at 1:00 pm [60]. Sham animals were left in their home cages without access to water or food to ensure that water/food deprivation alone did not contribute to the stress responses. Sham animals were kept in a separate room from stressed animals for the duration of stress. Once animals were in restraint devices, checks were made every 15-20 minutes to ensure that animals had not altered their position; if an animal altered their position, they were readjusted by the experimenter by loosening the movable disk without completely removing the animal from the restrainer.

Animal weight was taken into consideration to ensure that all animals were restrained equivalently. Animals above 34 g were not used for stress due to the maximum weight of the restrainer and animals that weighed under 22 g had a custom 3D printed 1 mm thick plastic insert fit into the restrainer. Animals weighing 18 g or less were not used for restraint stress as in pilot studies, animals at this weight were capable of exiting through the hole in the acrylic front of the restrainer.

Animals subjected to stress were not co-housed with sham animals to avoid the transfer of a stressed phenotype between mice.

Measurement of grimacing pain behavior: Grimace was performed according to previously published methods [12; 38; 151] prior to von Frey testing for all time points. Assessment of 5 characterized pain behaviors on portions of the face (orbital tightening, nose bulging, cheek bulging, flattening of ears, and flattening of whiskers). These behaviors were scored on a scale of 0-2 (0 = not present, 1 = somewhat present, 2 = clearly present).

Dural Injections of reagents/drugs: For experiments where dural stimulation was applied, dural injections were performed according to previously published methods [38]. Briefly, animals were anesthetized for < 2 minutes under isoflurane using a nose cone. While under anesthesia, stimuli were administered onto the dura through the junction of the lambdoid and sagittal sutures via a modified internal cannula (Invivo1, part #8IC313ISPCXC, Internal Cannula, Standard, 28 gauge, fit to 0.5 mm) in a volume of 5 µl. Following injection, animals were placed back in their respective cups in the testing chamber for 1 hour prior to von Frey testing.

Drugs: Drugs applied via dural injection were made in synthetic interstitial fluid (SIF) consisting of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl2, 10 mM glucose, 1 mM MgCl2 (pH 7.4, 310 mOsm). Solutions at pH 7.0 were made using SIF and adjusting pH with HCl. Sodium Nitroprusside (Sigma Aldrich) and sumatriptan (Sigma Aldrich) were dissolved in sterile 1 X PBS. Drug concentrations and administration routes are reported in *Table 4.1*.

Table 4.1: Drug information

Drugs	**Source**	**Doses**	**Administration**
SNP	Sigma-Aldrich	0.1 mg/kg	Intraperitoneal
Sumatriptan	Bachem	0.6 mg/kg	Intraperitoneal
ALD405	Alder Biopharmaceuticals	10 mg/kg	Intraperitoneal
Isotype control	Alder Biopharmaceuticals	10 mg/kg	Intraperitoneal

Statistics:

All data here are shown as mean ± SEM. These data were analyzed among groups for each time point via two-way ANOVA and then followed by Bonferroni post hoc analysis. Data was analyzed using Prism 8.0 (Graph-Pad Software). Significance was set at $p < .05$ for all analyses. The Resulting F and P values are reported in *Table 4.2*.

RESULTS

Repeated stress using restraint causes facial hypersensitivity and priming to an NO donor in mice

Prior studies using restraint as a model of stress show wide variations in protocols for mice with some using 30 minutes for 1 day [163] while others use 1 hour a day for 2 weeks [48], 2 hours a day for 3 days [144] or 5 hours a day for 14 days [227]. Due to this variation between stress protocols, we first aimed to determine the amount of stress required to produce facial hypersensitivity in mice. Because the durations of a single session of stress are varied, we decided

to use 2-hour sessions as an intermediate duration of each stress session. Because consecutive days of stress are more likely to trigger attacks in migraine patients than is a single day, we decided to test this stress across a 3 day paradigm [144; 161]. Males and females were restrained for 2 hours for 3 consecutive days, and von Frey testing was then conducted 24 hours after the end of the final stress session. The 24-hour time point was chosen as the earliest von Frey test after stress in order to avoid the potential of additional stress from behavioral testing as well as stress-induced acute analgesia, both of which have been documented immediately following restraint in rats [94]. Three consecutive days of stress produced significant facial hypersensitivity in both males (Fig. 1a) and females (Fig. 1b) that lasted from 24 hours following stress out to between 7 and 10 days in both males and females. Mice returned to baseline withdrawal thresholds typically by 14 days following the last session of stress. In a few instances, animals did not return to baseline until longer than 14 days, with a few animals even remaining hypersensitive out to 21 days after stress (data not shown as these animals were not used for the figures presented in this manuscript). In order to measure spontaneous non-evoked pain in these animals, we assessed grimace scores in female mice following stress. Females showed significant grimacing that lasted from 24 hours post stress to 7 days' post stress (Fig. 1c).

Prior studies have shown that in contrast to acute restraint, repeated restraint stress in rats is capable of inducing hypersensitivity to nitroglycerin in the tail-flick test [59]. In order to determine whether these repeated stress exposures caused priming to the NO-donor, animals were injected with the normally subthreshold dose of 0.1 mg/kg SNP following a return to baseline. Both male and female

mice that received SNP had significantly reduced facial withdrawal thresholds at 1 and 3 hours following SNP injection. Female mice were also observed for facial grimace responses following SNP injection. While females that received the NO donor showed significantly reduced withdrawal thresholds, these animals showed no significant grimacing following SNP (Fig. 1c).

To ensure that stress responses to this paradigm were maintained across strains, mice with a mixed background of both C57BL6 and 129J were exposed to stress (Supplementary fig. 1). Both male and female mice off this background responded to initial stress from 24 hours following stress out to 14 days following stress. Additionally, male and female mice were primed to respond to 0.1 mg/kg SNP. These data demonstrate that this stress paradigm is not specific to a single mouse strain.

As previously mentioned, stress across consecutive days is more likely to cause migraine in migraine patients when compared with 1 day of stress [161]. In order to explore the potential differences in repeated sessions of stress in this model male and female mice underwent 1 session of 2-hour restraint stress. One 2-hour session of restraint stress failed to produce significantly reduced facial withdrawal thresholds in both male (Fig. 2a) or female mice (Fig 2b). Mice were tested out to 5 days following stress to ensure that delayed hypersensitivity did not occur. While the stress produced no acute facial hypersensitivity, our prior work demonstrated that a single stimulus (dural stimulation with IL-6 or CGRP) can cause priming to subthreshold doses of a common migraine trigger i.e. an NO donor [12; 37] so it was also important to test for potential priming following a single stress exposure. Prior work using the tail-flick test in rats found that 1

session of acute stress caused stress-induced analgesia and resulted in no response the NO-donor nitroglycerin [59]. We thus tested whether acute stress would cause priming to a subthreshold dose of the NO-donor sodium nitroprusside (SNP). Following the fifth day of testing, neither males nor females that received 0.1 mg/kg SNP showed facial hypersensitivity. These data demonstrate that a single session of stress is not sufficient to cause acute hypersensitivity nor is it sufficient to induce priming to SNP. The 3-day stress paradigm was used for all subsequent experiments

The previous experiments show that 3 days of restraint stress cause priming to SNP when given relatively shortly after mice return to baseline. In order to determine whether stress-induced priming is extinguished over time, animals underwent the 3-day stress protocol and were tested with von Frey filaments until they returned to baseline at approximately 14 days after stress. Following a return to baseline, animals were then tested once weekly until they reached 35 days post stress (i.e. 35 days following the last session of restraint). Animals were then injected with 0.1 mg/kg SNP at this late time point. Neither males (Fig. 3a) nor females (Fig. 3b) that received SNP at 35-days post stress showed reduced facial withdrawal thresholds when compared with controls. These data indicate that while repeated stress causes priming to SNP, the primed state is not permanent.

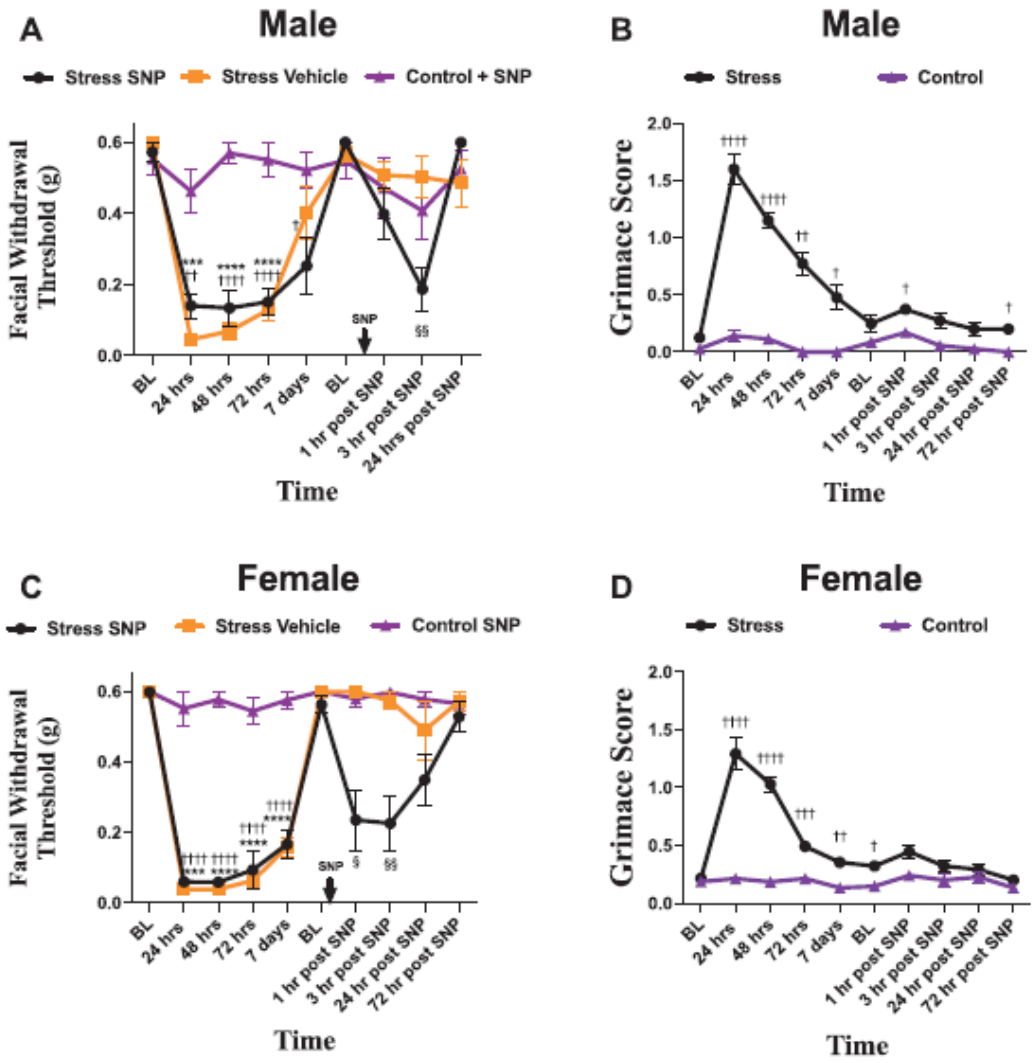

Figure 4.1: The repeated stress paradigm primes male and female mice to subthreshold doses of an NO donor. Facial withdrawal thresholds were measured in male (A) and female (C) mice after repeated restraint stress. Upon returning to baseline thresholds at 14 days after the final day of stress, stressed mice were administered either 0.1 mg/kg SNP (n 5 8 males, n 5 8 females) or vehicle (n 5 10 males, n 5 6 females). All control mice were given SNP (n 5 8 males, n 5 7 females).

†Statistical significance between stressed mice that received SNP and control mice; *Statistical significance between stressed mice that received vehicle and control mice. §Significance between stressed mice that received SNP and stressed mice that received vehicle. In a separate cohort of mice, grimace responses (B and D) were measured for stressed (n515) and control (n513)mice after acute stress and administration of 0.1 mg/kg SNP. Two-way RMANOVA followed by Bonferroni multiple comparison analysis indicated significant differences between stressed and control mice after SNP in both males and females. Data are represented as mean 6 SEM. †,§P , 0.05, ††,§§P , 0.01, †††,***P , 0.001, ††††,****P , 0.0001. See Table 4.2 for additional results of analysis. ANOVA, analysis of variance; RM, repeated-measures; SNP, sodium nitroprusside.

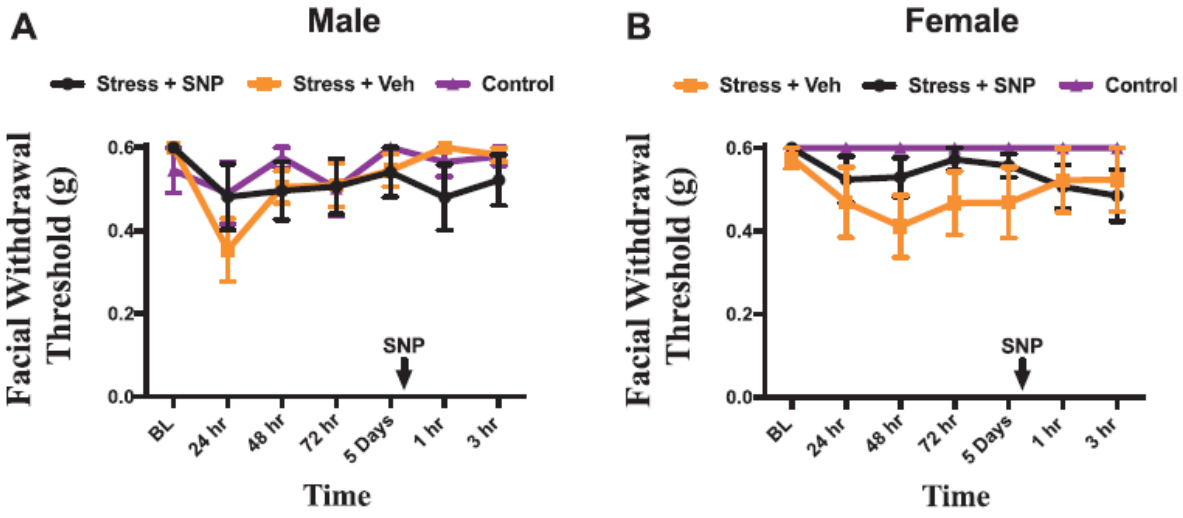

Figure 4.2: Single-restraint stress does not produce facial allodynia or priming to SNP. (A) Facial withdrawal thresholds of male mice after a single session of stress and administration of either

SNP (n510) or vehicle (n57) after 5 days of testing or naive mice (n59). (B) Facial withdrawal thresholds of female mice after a single session of stress and administration of either SNP (n 5 7) or vehicle (n 5 6) after 5 days of testing or naive mice (n 5 6). Two-way RM with Bonferroni multiple comparison analysis indicates there was no statistical difference among control mice and all cohorts that were stressed in both the acute phase and the priming phase (mean 6 SEM). See Table 4.2 for additional statistical analysis. SNP, sodium nitroprusside.

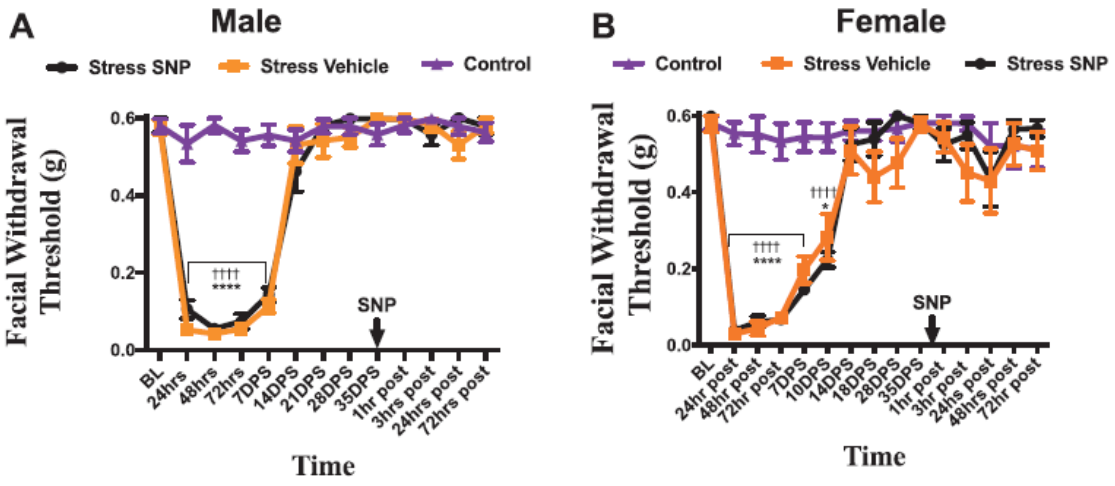

Figure 4.3: Repeated stress induces transient priming to SNP in male and female mice**.** Facial withdrawal thresholds were measured in (A) male and (B) female mice after acute stress (n 5 8 male, 8 female). After the final day of stress, animals were tested out to 35 days and received either 0.1 mg/kg SNP (n 5 8 males, n 5 9 females) or vehicle (n59males, n58 females). All controls received SNP. †Statistical significance between stressed mice that will receive SNP and control mice. *Statistical significance between stressed mice that will receive vehicle and control mice. §Statistical significance between stressed mice that received vehicle and stressed mice that

received SNP. Two-way RM ANOVA with Bonferroni multiple comparison analysis indicated a significant difference between stressed and control mice in the acute phase, but revealed no significant differences after administration of SNP. Data are represented as mean6SEM. *P,0.05, ††††,****P, 0.0001. See Table 4.2 for additional results of analysis. SNP, sodium nitroprusside.

Efficacy of human migraine therapeutics against priming to SNP following stress

CGRP has been strongly implicated in the pathology of migraine, and the use of monoclonal antibodies against CGRP and the CGRP receptor have now been demonstrated to be efficacious in the preventive-treatment of migraine [72; 73; 205; 210]. To determine the possible role for CGRP in stress-induced hypersensitivity, both male and female animals underwent the 3-day restraint stress protocol and were tested until they returned to baseline withdrawal thresholds. At 24 hours following return to baseline, animals received 10 mg/kg i.p. of ALD405, a monoclonal antibody against CGRP, or an isotype control. At 24 hours after dosing of the antibody, 0.1 mg/kg SNP was given systemically as described above. ALD405 significantly blocked the effects of SNP in females (Fig. 4a) from 1-72 hours following SNP injection. Whereas males (Fig. 4a) that received ALD405 only experienced decreased mechanical sensitivity at 48 hours following SNP administration. Females that received the active form of the antibody showed no significant differences from controls in response to SNP. These findings show that a monoclonal antibody against CGRP is capable of blocking the primed response to SNP in a sex-specific manner. This suggests that not only does CGRP have a prominent role in a stress induced priming to NO donors, but that this role may be sexually dimorphic.

Sumatriptan, as well as other triptans, are among the most commonly used acute migraine therapeutics. It was thus important to determine whether this stress priming model is sensitive to the effects of sumatriptan. To test this question, we examined the efficacy of sumatriptan against the hypersensitivity caused by SNP in stress-primed mice. Female mice underwent the restraint stress protocol and were allowed to return to baseline as described for the prior experiments. At 24-hours following return to baseline, mice received either vehicle (1X PBS), 0.1 mg/kg SNP, 0.6 mg/kg sumatriptan (the standard dose used throughout preclinical migraine studies [36; 63; 172; 185]), or a co-injection of SNP and sumatriptan (Fig. 5). Animals that received only sumatriptan were not significantly different from baseline at any time point. As in prior experiments, animals that received SNP showed significantly reduced withdrawal thresholds when compared with controls out to 24 hours. Animals that received a co-injection of SNP and sumatriptan experienced significantly reduced withdrawal thresholds when compared with controls and animals that received sumatriptan alone out to 3 hours following injection; these animals showed no significant differences from those that received only SNP at any time point. These data demonstrate that repeated stress leads to a state of priming where sumatriptan lacks efficacy against subsequent exposure to an NO donor.

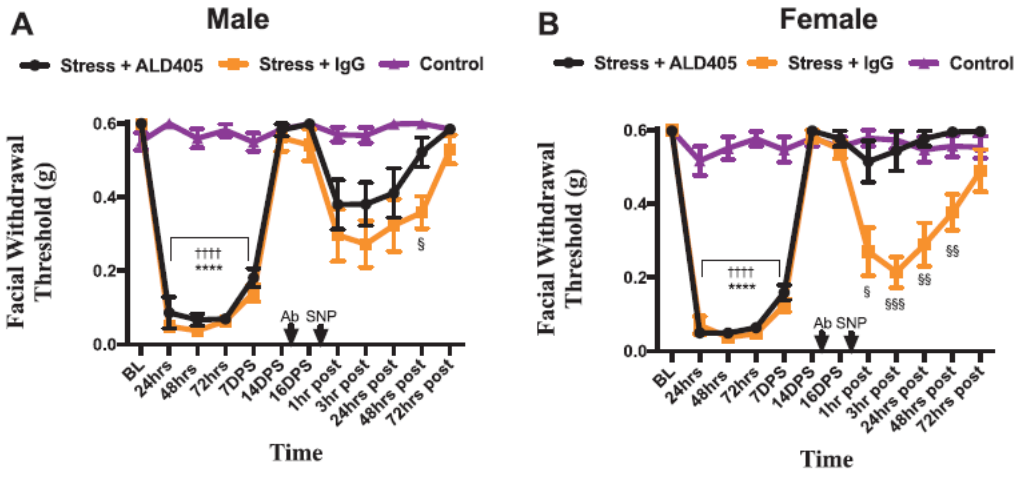

Figure 4.4: Effects of CGRP monoclonal antibodies in repeated stress induced priming to SNP in mice. Facial withdrawal thresholds were measured in male (A) and female (B)mice after acute stress (n510 males, n57 females). 15 days after the final day of stress, animals received either the anti-CGRP antibody ALD405 (n5 10 males, n 5 8 females) or an isotype control (n 5 9 males, n 5 9 females). 24 hours after administration of antibody, all animals received 0.1 mg/kg SNP. In the acute phase, † denotes statistical significance between controlmice and stressedmice that will receive ALD405 and SNP. *Statistical significance between control mice and stressed mice that will receive isotype control Immunoglobulin G. In the priming phase, § denotes statistical significance between stressed mice that received ALD405 and those that received the isotype control before SNP. Two-way ANOVA followed by Bonferroni multiple comparison analysis revealed significant differences in the priming phase between female stressed mice that received ALD405 and those that received the isotype. Statistical significance between these groups was observed in males at 48 hours after SNP. Data are represented as mean6SEM. §P,0.05,§§P,0.01,

§§§P,0.001, ††††,****P, 0.0001. See Table 2 for additional results of analysis. CGRP, calcitonin gene-related peptide; SNP, sodium nitroprusside.

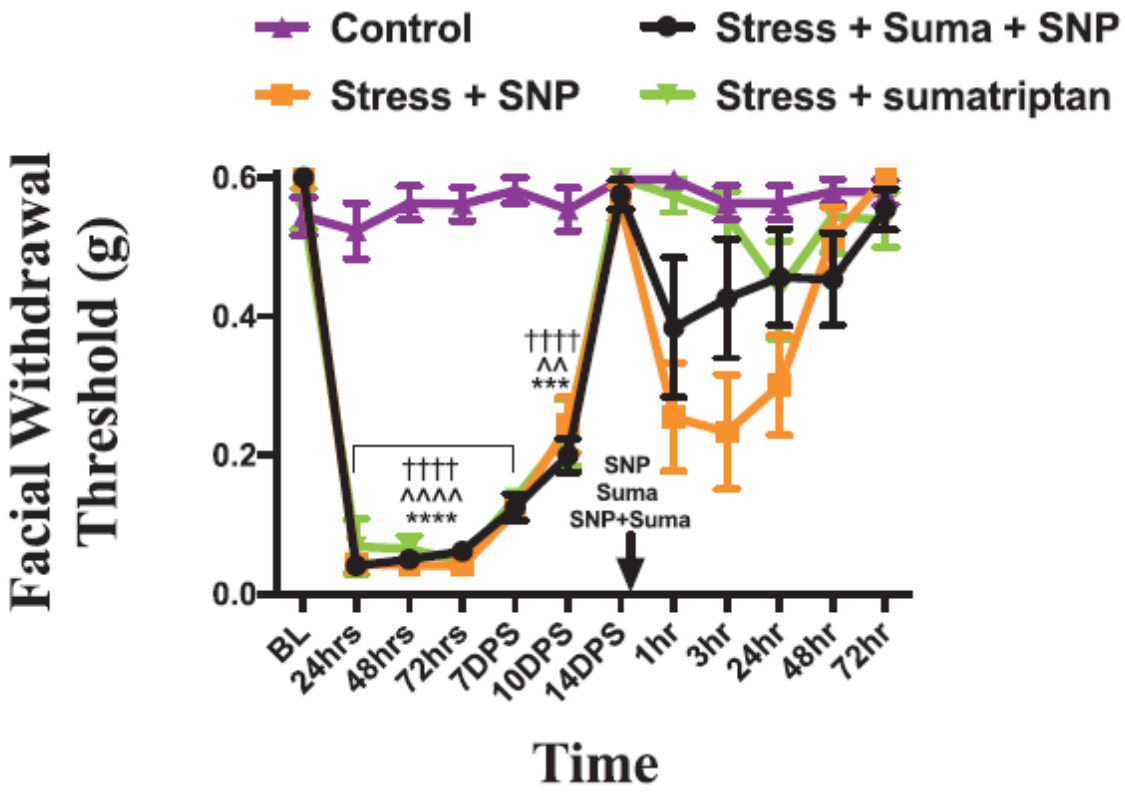

Figure 4.5: Sumatriptan does not block SNP responses in stress-primed mice. Facial withdrawal thresholds of female mice either naive (n 5 8) or after acute stress and administration of either SNP (0.1 mg/kg; n58), suma (0.6 mg/kg; n 5 7), or a coadministration of suma and SNP (n 5 7). In the acute phase, * denotes significance between control mice and stressed mice that will receive SNP; ^significance between control mice and stressed mice that will receive suma; †significance between control mice and stressed mice that will receive suma and SNP. For 24-hour-7DPS in the acute phase, the statistical significance is the same for each time point. Two-

way RM with Bonferroni multiple comparison analysis indicates a statistical difference among control mice and all cohorts that were stressed. In the priming phase, there was no statistical difference detected between stressed mice that received SNP and those that received suma and SNP (mean 6 SEM, ^^P , 0.01, ***P , 0.001, ****,^^^^, ††††P , 0.0001). See Table 2 for additional statistical analysis. SNP, sodium nitroprusside.

Repeated stress primes mice to dural stimulation with a subthreshold pH

These studies demonstrate that repeated stress primes mice to subthreshold doses of NO-donors. However, it is not clear from these studies whether stress and NO donors have sensitizing actions within the meninges as both stimuli are non-specific to any location within the body. We thus investigated whether repeated restraint stress could prime animals to a direct dural stimulus. Female mice were subject to the repeated stress protocol and allowed to return to baseline withdrawal thresholds as in the prior experiments. At 24 hours after return to baseline, mice received dural injections of either physiological pH of 7.4 or a slightly decreased, but normally non-noxious pH 7.0, the latter a stimulus that we have previously shown animals become primed to by prior dural application of IL-6 or CGRP[12; 37]. Stressed females that received dural pH 7.0 experienced significant allodynia at 1 hour, 3hours, and 24 hours post injection when compared with stressed animals that received pH 7.4 and control non-stressed animals that received either dural pH 7.0 and pH 7.4 (Fig. 6a). In addition, these animals showed significant grimacing in response to stress out to 7 days (Fig. 6b), however none of the animals showed any significant

grimace in response to any secondary stimulus. These data demonstrate that repeated stress primes mice to both systemic migraine triggers as well as direct stimuli applied to the dura.

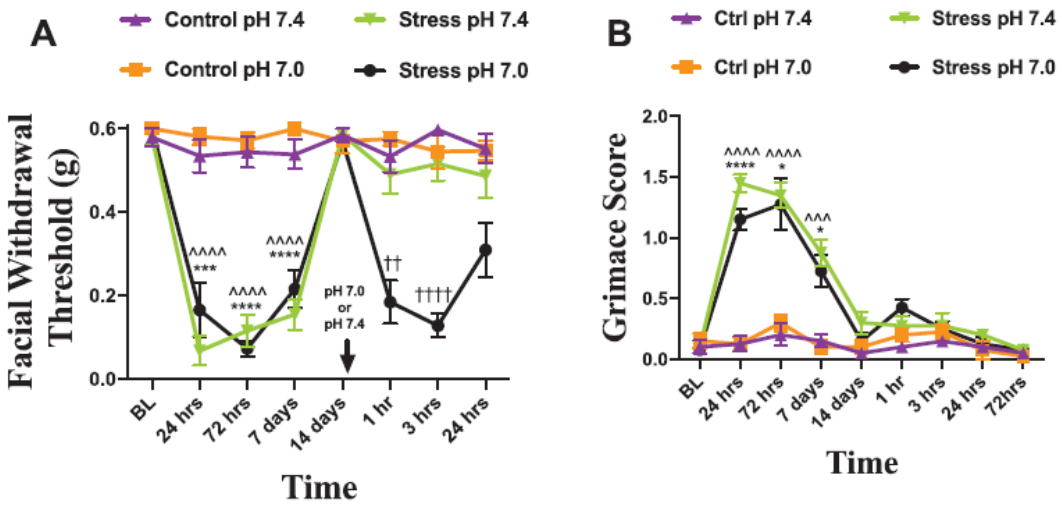

Figure 4.6: Repeated stress primes female mice to decreased dural pH**.** (A) Facial withdrawal thresholds of female mice after acute stress and priming to dural pH 7.0 (n512) or 7.4 (n512) and controlmice given pH 7.0 (n512) and pH 7.4 (n511). (B)Grimace scores of mice that were first stressed and then administered either pH 7.0 (n 5 8) or pH 7.4 (n 5 8) or mice that were naive and then administered either pH 7.0 (n 5 8) or pH 7.4 (n 5 8). For the acute phase, *significance between stressed and control mice that will receive dural pH 7.0; ^significance between stressed and control mice that will receive dural pH 7.4. For the priming phase; †significance between stressed mice that received pH 7.0 and stressed mice that received pH 7.4. Dural pH stimuli are given at the time point indicated by the arrows in (A); before dural pH injections, animals were

only exposed to either stress or control. Two-way ANOVA RM with Bonferroni multiple comparison analysis indicate a significant difference between stressed and control mice and stressed mice that received pH 7.0 compared to stressed mice that received pH 7.4 (mean 6 SEM, *P , 0.05, ††P , 0.01, ***, ^^^P , 0.001, ****,^^^^,††††P , 0.0001). See Table 2 for additional statistical analysis.

DISCUSSION:

The mechanisms by which stress contributes to migraine are poorly understood. Better understanding of these mechanisms may lead to insights for new therapeutic targets as well as other potential approaches. Here we show that repeated stress using restraint results in significant facial hypersensitivity in both male and female mice, starting approximately 24 hours after the end of stress (although this was the earliest time point tested here). Repeated stress also causes grimace in females and primes mice to subthreshold doses of the nitric oxide donor SNP as well as subthreshold dural stimulation with pH 7.0. This stress paradigm induces a time window in which animals are primed to subthreshold dose of SNP; mice remained primed for 2 weeks following stress but not at 5 weeks post-stress. Responses to subthreshold doses of SNP in primed animals were blocked by a CGRP monoclonal antibody, implicating CGRP in the mechanisms that contribute to low-dose NO donors responses in the primed state. This was not the case when sumatriptan was used, demonstrating that priming due to stress may lead to a "triptan-unresponsive state." Together, these data support the use of a stress priming model to help further study the mechanisms by which stress contributes to migraine. Importantly, this model can be induced

without significant tissue injury and the presence of a primed state can be determined without tissue injury (e.g. by using SNP injections).

Calcitonin gene-related peptide (CGRP) has long been implicated in the pathology of migraine as it is upregulated in the saliva, venous blood, and CSF of migraine patients during an attack [82; 135]. Additionally, intravenous administration of CGRP to migraine patients triggers migraine attacks [152]. The observation that the response to SNP following repeated stress is blocked by a CGRP monoclonal antibody (although to a greater extent in females; see additional discussion below) suggests that exposure to a subthreshold dose of SNP in the primed state due to stress is similar to migraine attacks. However, sumatriptan had no effect on SNP-donor induced responses in primed mice. This is in contrast to humans, where sumatriptan reduces nitroglycerin-induced headaches [130]. In rodent studies, both sumatriptan and the monoclonal antibody ALD405 were shown to reduce pain from repeated glyceryl trinitrate administration [53]. In other rodent studies, sumatriptan was not efficacious in blocking NO donor induced facial hypersensitivity when given prior to repeated NO-donor administration across 5 days [61]. Neither of these studies used stress however. The inability of sumatriptan to prevent NO-donor mediated hypersensitivity following repeated stress may be able to provide insight into mechanisms that are present in the approximately 40% of migraine patients that take oral triptans and do not experience relief [90] and also how stress may contribute to triptan response/non-response. Further, this model may be helpful in identifying mechanisms by which NO donors contribute to migraine since only primed animals respond to low-dose SNP (thus mimicking the human observation that NO donors do not

trigger attacks in controls). Because the increased sensitivity to SNP is not permanent i.e. it was not present 5 weeks following the end of the stress paradigm, this model may be valuable for identifying mechanisms by which sensitivity to the effects of stress are extinguished.

Migraine affects women disproportionately to men; mechanisms underlying this dimorphism are not known. We have recently shown that female mice are more sensitive to the pain-promoting effects of CGRP in the meninges than are males [12]. Consistent with the prior finding of female-specific CGRP effects, ALD405 was more effective in blocking responses to SNP following stress in females than in males. While anti-CGRP therapeutics are effective in many migraine patients, both female and male, factors that predict efficacy are not currently known. Additionally, mechanisms by which stress and NO donors contribute to migraine are poorly understood. While far more investigation is needed, our work is a substantial advance because it suggests that stress combined with NO donors may act through CGRP-dependent mechanisms in females but not in males. The model system that we have developed, and similar model systems that can be developed in the future, may start to draw mechanistic connections between psycho-social migraine findings and specific molecular mechanisms that can predict who will respond to certain types of therapeutics, like CGRP-targeting drugs or triptans.

How and where CGRP contributes to migraine pathology is still a matter of debate but the repeated stress-induced priming model may be helpful in addressing this question. It is possible that repeated stress causes plasticity in the hypothalamic-pituitary-adrenal (HPA) axis, altering the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the

hypothalamus and adrenocorticotropic hormone (ACTH) from the pituitary in response to internal and external challenges. When administered to the PVN and in the lateral ventricle, CGRP increases ACTH and plasma CRH [67; 146]. CGRP has also been shown to increase ACTH from cultured pituitary cells [129]. An increased role for CGRP in these processes following stress may occur. Additionally, repeated stress has been shown to increase vascular permeability and plasma extravasation within the dura [138]. Plasma extravasation typically requires substance P and CGRP [96; 164]. Repeated stress may change the role of CGRP within the meninges, particularly how it contributes to the response to NO donors. While some debate exists about whether these antibodies act in the periphery or in the central nervous system, due to the size of these antibodies they are unlikely to cross the blood brain barrier, and may act on mechanisms that occur outside of the BBB. The repeated stress priming model is an additional preclinical tool with which to test these types of CGRP-dependent mechanisms that may contribute to migraine.

The hypersensitivity of the dura to pH 7.0 following repeated stress may be in part caused by mast cell (MC) degranulation. Acute restraint stress in mice and rats has been shown to activate dural mast cells in a CRH dependent fashion [138; 244]. Mast cell degranulation is thought to contribute to migraine by causing excitation of dural nociceptors [156]. In addition, mast cell degranulation has been shown to increase sensitivity to decreased dural pH via activation of ASICs [268]. Stress not only leads to mast cell degranulation but increases the presence of pro-inflammatory agents within dural immune cells, [177] likely potentiating the effects of inflammation within the dura. CGRP contributes to release of inflammatory mediators from mast cells within the dura [65].

Additionally, mast cells express the CRH1 receptor and are likely to be responsive to both CGRP and CRH [47]. While sumatriptan has been reported to attenuate mast cell degranulation-evoked activity in the trigeminal nucleus caudalis [156] it did not have efficacy in the repeated stress priming model to SNP. This may be because the initial stress-induced mast cell degranulation may have already increased the sensitivity of dural nociceptors, and the timing of sumatriptan administration (given well after stress) may not be capable of reversing this sensitivity. Stress has also been shown to increase innervation of peripheral tissues by sympathetic fibers [226] and post-ganglionic sympathetic neurons can alter the immune cell populations in the dura in response to chronic stress [177]. It is thus possible that changes in sympathetic innervation of the dura could result from restraint stress, and that this restructuring ultimately contributes to increases in immune response and consequently hypersensitivity of dural nociceptors. This model may lend itself to further exploration of the role of sympathetic dural innervation, immune cells, and the link between migraine and stress.

The data presented here support the use of repeated stress-induced priming as a new preclinical model of migraine in which to further investigate the mechanisms of the disorder and their regulation by stress. It may also be a valuable tool to investigate efficacy of novel therapeutics, especially those that are likely to act within mechanisms or pathways regulated by stress. Finally, it offers the opportunity to study conditions related to migraine in the absence of tissue injury. While several preclinical behavioral models of migraine currently exist, it is unlikely that any single model will accurately capture the complexity that is human migraine. This model may be

an important addition to the current list of preclinical tools, and may be able to capture aspects of the disorder not currently represented in the currently-available models.

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STATISTICAL TABLES

Table 4.2:

Figure	Analysis	Statistics
Fig. 1A BL-BL	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(10, 115)}$=12.04, $p<0.0001$ $F_{(3.008, 69.19)}$=50.31, $p<0.0001$ $F_{(2, 23)}$=28.16, $p<0.0001$

Fig. 1A SNP 1- 24hr	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(4, 46)}$=3.862, $p=0.0087$ $F_{(1.854, 42.63)}$=6.911, $p=0.0031$ $F_{(2, 23)}$=1.685, $p=0.2075$
Fig. 1B BL-BL	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(10, 89)}$=36.19, $p<0.0001$ $F_{(2.822, 50.23)}$=180.0, $p<0.0001$ $F_{(2, 18)}$=149.7, $p<0.0001$
Fig. 1B SNP 1- 24hr	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(6, 54)}$=3.952, $p=0.0024$ $F_{(1.799, 32.37)}$=2.446, $p=0.1074$ $F_{(2, 18)}$=14.38, $p=0.0002$
Fig. 1C	Two-way repeated measure ANOVA Interaction Factor	 $F_{(5, 130)}$=35.53, $p<0.0001$

BL-BL	Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	$F_{(2.574, 66.93)}$=42.04, $p<0.0001$ $F_{(1, 26)}$=124.6, $p<0.0001$
Fig. 1C SNP 1-24hr	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(3, 78)}$=1.571, $p=0.2032$ $F_{(2.6, 67.60)}$=7.452, p=0.0004 $F_{(1, 26)}$=4.448, $p=0.0447$
Fig. 2A	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(12, 138)}$=1.608, $p=0.0961$ $F_{(3.257, 74.91)}$=4.811, $p=0.0032$ $F_{(2, 23)}$=0.1607, $p=0.8525$
Fig. 2B	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor	 $F_{(12, 108)}$=0.7945, $p=0.6652$ $F_{(3.763, 67.74)}$=0.9845, $p=0.4186$ $F_{(2, 18)}$=2.249, $p=0.1343$

	Bonferroni's multiple comparisons between treatments	
Fig. 3A BL-35D	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(16, 176)}$=33.03, $p<0.0001$ $F_{(4.232, 93.11)}$=144.2, $p<0.0001$ $F_{(2, 22)}$=170.6, $p<0.0001$
Fig. 3A SNP1-72hr	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(6, 66)}$=1.526, $p=0.1834$ $F_{(2.334, 51.35)}$=0.7346, $p=0.5043$ $F_{(2, 22)}$=0.2865, $p=0.7537$
Fig. 3B BL-35D	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments	 $F_{(18, 198)}$=16.84, $p<0.0001$ $F_{(4.475, 98.44)}$=76.59, $p<0.0001$ $F_{(2, 22)}$=78.94, $p<0.0001$

REFERENCES

[1] Avona A, Burgos-Vega C, Burton MD, Akopian AN, Price TJ, Dussor G. Dural Calcitonin Gene-Related Peptide Produces Female-Specific Responses in Rodent Migraine Models. J Neurosci 2019;39(22):4323-4331.

[2] Bardin L, Malfetes N, Newman-Tancredi A, Depoortere R. Chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rat: Relevance to human stress-associated painful pathologies. Behav Brain Res 2009;205(2):360-366.

[3] Burgos-Vega CC, Ahn DD, Bischoff C, Wang W, Horne D, Wang J, Gavva N, Dussor G. Meningeal transient receptor potential channel M8 activation causes cutaneous facial and hindpaw allodynia in a preclinical rodent model of headache. Cephalalgia 2016;36(2):185-193.

[4] Burgos-Vega CC, Quigley LD, Avona A, Price T, Dussor G. Dural stimulation in rats causes brain-derived neurotrophic factor-dependent priming to subthreshold stimuli including a migraine trigger. Pain 2016;157(12):2722-2730.

[5] Burgos-Vega CC, Quigley LD, Trevisan Dos Santos G, Yan F, Asiedu M, Jacobs B, Motina M, Safdar N, Yousuf H, Avona A, Price TJ, Dussor G. Non-invasive dural stimulation in mice: A novel preclinical model of migraine. Cephalalgia 2018:333102418779557.

[6] Cao J, Papadopoulou N, Kempuraj D, Boucher WS, Sugimoto K, Cetrulo CL, Theoharides TC. Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. J Immunol 2005;174(12):7665-7675.

[7] Carneiro de Oliveira PE, Zaniboni CR, Carmona IM, Fonseca AR, Canto-de-Souza A. Preliminary behavioral assessment of cagemates living with conspecifics submitted to chronic restraint stress in mice. Neurosci Lett 2017;657:204-210.

[8] Christensen SL, Petersen S, Kristensen DM, Olesen J, Munro G. Targeting CGRP via receptor antagonism and antibody neutralisation in two distinct rodent models of migraine-like pain. Cephalalgia 2019:333102419861726.

[9] Costa A, Smeraldi A, Tassorelli C, Greco R, Nappi G. Effects of acute and chronic restraint stress on nitroglycerin-induced hyperalgesia in rats. Neurosci Lett 2005;383(1-2):7-11.

[10] D'Agostino J, Vaeth GF, Henning SJ. Diurnal rhythm of total and free concentrations of serum corticosterone in the rat. Acta Endocrinol (Copenh) 1982;100(1):85-90.

[11] Dallel R, Descheemaeker A, Luccarini P. Recurrent administration of the nitric oxide donor, isosorbide dinitrate, induces a persistent cephalic cutaneous hypersensitivity: A model for migraine progression. Cephalalgia 2018;38(4):776-785.

[12] De Felice M, Ossipov MH, Wang R, Dussor G, Lai J, Meng ID, Chichorro J, Andrews JS, Rakhit S, Maddaford S, Dodick D, Porreca F. Triptan-induced enhancement of neuronal nitric oxide synthase in trigeminal ganglion dural afferents underlies increased responsiveness to potential migraine triggers. Brain 2010;133(Pt 8):2475-2488.

[13] De Jonge F, De Laet A, Van Nassauw L, Brown JK, Miller HR, van Bogaert PP, Timmermans JP, Kroese AB. In vitro activation of murine DRG neurons by CGRP-mediated mucosal mast cell degranulation. Am J Physiol Gastrointest Liver Physiol 2004;287(1):G178-191.

[14] Dhillo WS, Small CJ, Jethwa PH, Russell SH, Gardiner JV, Bewick GA, Seth A, Murphy KG, Ghatei MA, Bloom SR. Paraventricular nucleus administration of calcitonin gene-related peptide inhibits food intake and stimulates the hypothalamo-pituitary-adrenal axis. Endocrinology 2003;144(4):1420-1425.

[15] Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 2017;390(10100):1211-1259.

[16] Dodick DW, Goadsby PJ, Silberstein SD, Lipton RB, Olesen J, Ashina M, Wilks K, Kudrow D, Kroll R, Kohrman B, Bargar R, Hirman J, Smith J, investigators ALDs. Safety and efficacy of ALD403, an antibody to calcitonin gene-related peptide, for the prevention of frequent episodic migraine: a randomised, double-blind, placebo-controlled, exploratory phase 2 trial. Lancet Neurol 2014;13(11):1100-1107.

[17] Dodick DW, Silberstein SD, Bigal ME, Yeung PP, Goadsby PJ, Blankenbiller T, Grozinski-Wolff M, Yang R, Ma Y, Aycardi E. Effect of Fremanezumab Compared With Placebo for Prevention of Episodic Migraine: A Randomized Clinical Trial. JAMA 2018;319(19):1999-2008.

[18] Edvinsson L, Goadsby PJ. Neuropeptides in migraine and cluster headache. Cephalalgia 1994;14(5):320-327.

[19] Ferrari MD, Roon KI, Lipton RB, Goadsby PJ. Oral triptans (serotonin 5-HT(1B/1D) agonists) in acute migraine treatment: a meta-analysis of 53 trials. Lancet 2001;358(9294):1668-1675.

[20] Gamaro GD, Xavier MH, Denardin JD, Pilger JA, Ely DR, Ferreira MB, Dalmaz C. The effects of acute and repeated restraint stress on the nociceptive response in rats. Physiol Behav 1998;63(4):693-697.

[21] Gameiro GH, Gameiro PH, Andrade Ada S, Pereira LF, Arthuri MT, Marcondes FK, Veiga MC. Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress. Physiol Behav 2006;87(4):643-649.

[22] Gamse R, Holzer P, Lembeck F. Decrease of substance P in primary afferent neurones and impairment of neurogenic plasma extravasation by capsaicin. Br J Pharmacol 1980;68(2):207-213.

[23] Iino K, Oki Y, Tominaga T, Iwabuchi M, Ozawa M, Watanabe F, Yoshimi T. Stimulatory effect of calcitonin gene-related peptide on adrenocorticotropin release from rat anterior pituitary cells. J Neuroendocrinol 1998;10(5):325-329.

[24] Iversen HK, Olesen J. Headache induced by a nitric oxide donor (nitroglycerin) responds to sumatriptan. A human model for development of migraine drugs. Cephalalgia 1996;16(6):412-418.

[25] Jang MU, Park JW, Kho HS, Chung SC, Chung JW. Plasma and saliva levels of nerve growth factor and neuropeptides in chronic migraine patients. Oral Dis 2011;17(2):187-193.

[26] Kandere-Grzybowska K, Gheorghe D, Priller J, Esposito P, Huang M, Gerard N, Theoharides TC. Stress-induced dura vascular permeability does not develop in mast cell-deficient and neurokinin-1 receptor knockout mice. Brain Res 2003;980(2):213-220.

[27] Kelman L. The triggers or precipitants of the acute migraine attack. Cephalalgia 2007;27(5):394-402.

[28] Koh SJ, Kim JW, Kim BG, Lee KL, Kim JS. Restraint stress induces and exacerbates intestinal inflammation in interleukin-10 deficient mice. World J Gastroenterol 2015;21(28):8580-8587.

[29] Kovacs A, Biro E, Szeleczky I, Telegdy G. Role of endogenous CRF in the mediation of neuroendocrine and behavioral responses to calcitonin gene-related peptide in rats. Neuroendocrinology 1995;62(4):418-424.

[30] Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS. Coding of facial expressions of pain in the laboratory mouse. Nat Methods 2010;7(6):447-449.

[31] Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, Olesen J. CGRP may play a causative role in migraine. Cephalalgia 2002;22(1):54-61.

[32] Levy D, Burstein R, Kainz V, Jakubowski M, Strassman AM. Mast cell degranulation activates a pain pathway underlying migraine headache. Pain 2007;130(1-2):166-176.

[33] Lipton RB, Buse DC, Hall CB, Tennen H, Defreitas TA, Borkowski TM, Grosberg BM, Haut SR. Reduction in perceived stress as a migraine trigger: testing the "let-down headache" hypothesis. Neurology 2014;82(16):1395-1401.

[34] Long CC, Sadler KE, Kolber BJ. Hormonal and molecular effects of restraint stress on formalin-induced pain-like behavior in male and female mice. Physiol Behav 2016;165:278-285.

[35] Louis SM, Jamieson A, Russell NJ, Dockray GJ. The role of substance P and calcitonin gene-related peptide in neurogenic plasma extravasation and vasodilatation in the rat. Neuroscience 1989;32(3):581-586.

[36] Mason BN, Kaiser EA, Kuburas A, Loomis MM, Latham JA, Garcia-Martinez LF, Russo AF. Induction of Migraine-Like Photophobic Behavior in Mice by Both Peripheral and Central CGRP Mechanisms. J Neurosci 2017;37(1):204-216.

[37] McIlvried LA, Cruz JA, Borghesi LA, Gold MS. Sex-, stress-, and sympathetic post-ganglionic-dependent changes in identity and proportions of immune cells in the dura. Cephalalgia 2017;37(1):36-48.

[38] Nation KM, Dodick DW, Navratilova E, Porreca F. Sustained exposure to acute migraine medications combined with repeated noxious stimulation dysregulates descending pain modulatory circuits: Relevance to medication overuse headache. Cephalalgia 2019;39(5):617-625.

[39] Reuter U, Goadsby PJ, Lanteri-Minet M, Wen S, Hours-Zesiger P, Ferrari MD, Klatt J. Efficacy and tolerability of erenumab in patients with episodic migraine in whom two-to-four previous preventive treatments were unsuccessful: a randomised, double-blind, placebo-controlled, phase 3b study. Lancet 2018.

[40] Rosen N, Pearlman E, Ruff D, Day K, Jim Nagy A. 100% Response Rate to Galcanezumab in Patients With Episodic Migraine: A Post Hoc Analysis of the Results From Phase 3, Randomized, Double-Blind, Placebo-Controlled EVOLVE-1 and EVOLVE-2 Studies. Headache 2018;58(9):1347-1357.

[41] Scher AI, Stewart WF, Buse D, Krantz DS, Lipton RB. Major life changes before and after the onset of chronic daily headache: a population-based study. Cephalalgia 2008;28(8):868-876.

[42] Sloan EK, Capitanio JP, Tarara RP, Mendoza SP, Mason WA, Cole SW. Social stress enhances sympathetic innervation of primate lymph nodes: mechanisms and implications for viral pathogenesis. J Neurosci 2007;27(33):8857-8865.

[43] Son SW, Lee JS, Kim HG, Kim DW, Ahn YC, Son CG. Testosterone depletion increases the susceptibility of brain tissue to oxidative damage in a restraint stress mouse model. J Neurochem 2016;136(1):106-117.

[44] Sorbi MJ, Maassen GH, Spierings EL. A time series analysis of daily hassles and mood changes in the 3 days before the migraine attack. Behav Med 1996;22(3):103-113.

[45] Spierings EL, Sorbi M, Maassen GH, Honkoop PC. Psychophysical precedents of migraine in relation to the time of onset of the headache: the migraine time line. Headache 1997;37(4):217-220.

[46] Theoharides TC, Spanos C, Pang X, Alferes L, Ligris K, Letourneau R, Rozniecki JJ, Webster E, Chrousos GP. Stress-induced intracranial mast cell degranulation: a corticotropin-releasing hormone-mediated effect. Endocrinology 1995;136(12):5745-5750.

[47] Wacogne C, Lacoste JP, Guillibert E, Hugues FC, Le Jeunne C. Stress, anxiety, depression and migraine. Cephalalgia 2003;23(6):451-455.

[48] Yan J, Wei X, Bischoff C, Edelmayer RM, Dussor G. pH-evoked dural afferent signaling is mediated by ASIC3 and is sensitized by mast cell mediators. Headache 2013;53(8):1250-1261.

CHAPTER 5

INTERLEUKIN-6 INDUCES SPATIALLY DEPENDENT WHOLE-BODY HYPERSENSITVITY IN RATS: IMPLICATIONS FOR EXTRACEPHALIC HYPERSENSITIVITY IN MIGRAINE.

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ABSTRACT

Background: Migraine is a complex neurological disorder that is characterized by throbbing head pain, increased sensitivity to light, sound, and touch, as well as nausea and fatigue. It is one of the most common and most disabling disorders globally but mechanisms causing migraine are poorly understood. While head pain is a typical feature of attacks, they also often present with cutaneous hypersensitivity in the rest of the body. In contrast, primary pain conditions in the lower parts of the body are less commonly associated with cephalic hypersensitivity. Previous studies indicate that application of stimuli to the meninges of rodents causes cutaneous facial as well as hindpaw hypersensitivity. In the present study, we asked whether widespread hypersensitivity is a unique feature of dural stimulation or whether body-wide responses occur similarly when the same stimulus is given in other locations.

Methods: Rats were given the same dose of IL-6 either via dural, intraplantar, subcutaneous, intramuscular, intracisternal, or intrathecal injection. Cutaneous facial and hindpaw allodynia was assessed using Von Frey following injection into each location.

Results: Hindpaw allodynia was observed following dural and intraplantar injection of IL-6 in both males and females. Hindpaw allodynia was only observed in females following intracisternal and intrathecal IL-6 injections. In contrast, facial allodynia was only observed in either sex following dural and intracisternal injections, which would activate meningeal afferents and the trigeminal nucleus caudalis (TNC), respectively.

Conclusions: Here we show that while stimulation of upper body regions with IL-6 including the meninges and brainstem can cause widespread hypersensitivity spreading to the paws, similar stimulation of the lower body does not cause the spread of hypersensitivity into the head. These data are consistent with the observations that whole body hypersensitivity is specific to conditions such as migraine where pain is present in the head and they may provide insight into co-morbid pain states associated with migraine.

Abbreviations: Interleukin-6 (IL-6), Trigeminal Ganglia (TG), Trigeminal Nucleus Caudalis (TNC), Toll-like Receptor 4 (TLR4), Calcitonin Gene-Related Peptide (CGRP), Δ1-9-G129R-hPRL (ΔPRL), Prolactin (PRL), Cortical Spreading Depression (CSD).

BACKGROUND

Migraine is among the top 6 most common disorders globally in both men and women [71] and is the 2nd-most disabling disease world-wide [70]. Despite the prevalence of migraine, little is known about the pathophysiology. Migraine is characterized by throbbing head pain, cutaneous allodynia, nausea, and sensitivity to light and sounds. These symptoms vary among migraine patients, as do the triggers that spawn these attacks. Common self-reported triggers include stress, hormonal changes, changes in sleep-wake patterns, skipping meals, and consumption of alcohol and certain foods [142].

Migraineurs often report increased sensory sensitivity and cutaneous allodynia in extracephalic regions during migraine attacks [40; 175; 202; 247]. This is in contrast to other pain conditions in the lower body which are not typically associated with hypersensitivity in the head. This suggests that migraine, and the concomitant activation of meningeal afferents that causes the headache phase, may have a distinctive circuitry that leads to widespread hypersensitivity. Numerous prior studies using rodent migraine models have shown a remarkably consistent finding that stimulation of the dura mater causes cutaneous hypersensitivity of both the facial skin as well as that of the hindpaw [37; 80; 192; 265; 267]. These prior studies thus show that headache-inducing conditions lead to widespread hypersensitivity in rodents as in humans. While it is not generally reported that facial hypersensitivity exists in models of pain in the lower spinal system, these studies typically do not test for the presence of cephalic responses. The purpose of the present work was to test, using the same stimulus applied to multiple locations throughout the rat, whether dural stimulation is unique in its ability to cause body-wide hypersensitivity.

While migraine pathology remains poorly understood, inflammation is thought to be involved. Debate exists about the type, sites, and role of inflammation in migraine [85], but it is thought that peripheral inflammatory stimuli cause activation and hyper-excitability of meningeal afferents. These signals are received by the trigeminal ganglia, which sends these signals to higher order neurons in the trigeminal nucleus caudalis (TNC) [41]. From the TNC, these signals are then processed by the cortex for the perception of head pain [103]. One particular inflammatory mediator that has been implicated in migraine is interleukin-6 (IL-6). IL-6 is a pro-

inflammatory cytokine that is upregulated in the serum and blood of migraine patients during an attack [91; 214; 270]. We have shown previously that application of IL-6 on to the dura of female and male rats as well as mice results in cutaneous facial allodynia [12; 37; 39]. Importantly, these studies also showed that IL-6 applied to the rodent dura causes facial and hindpaw allodynia [37; 267]. Additionally, this same dose of IL-6 given onto the dura is capable of sensitizing male and female rats to respond to subthreshold doses of migraine relevant triggers such as lowered dural pH and dural calcitonin gene-related peptide (CGRP) [12; 37]. Furthermore, we have shown that IL-6 applied to the dura of male rats causes facial and hindpaw allodynia [37; 267]. Based on the link between IL-6 and migraine and headache-relevant behavioral responses in preclinical models with dural IL-6, we chose this stimulus as the probe to test whether activation of meningeal afferent neurons can cause differential spread of hypersensitivity compared to the same stimulus given elsewhere.

METHODS:

Animals:

In this study, 12-14 week-old approx. 260-300g female and approx. 300-350g male Sprague-Dawley rats (Taconic; Rensselaer, NY) were used for all experiments. Animals were housed on a 12-hour light/dark cycle with access to food and water *ad libitum*. Animals were housed in the facility for at least 72 hours prior to handling and habituation of animals to testing rooms. All

procedures were conducted with prior approval of the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

Rat cannula implantation and drug delivery:

Dural injections in rats were administered via cannula at a total of 10 µl injections. Cannulation surgeries were performed according to previously published methods [12; 13; 81] in which animals were anesthetized initially at 5% isoflurane via a nose cone; once animals no longer demonstrated a paw pinch reflex, isoflurane was lowered to 2.5-3.5% for the entirety of the surgery. The scalp was incised longitudinally and retracted from the midline to expose the skull. Using a pin vise (Grainger Industries) set to a length of 1 mm, a 1 mm burr hole was created using a stereotaxic frame at the target coordinates to sit above the middle meningeal artery (8mm AP, -2mm ML, 1mm DV) to puncture the skull while leaving the dura intact. A guide cannula (Plastics One C313G/SPC gauge 22) was implanted into the burr hole using a stereotaxic frame and sealed using Vetbond (Vetbond). Two screws were inserted above the guide cannula on both sides of midline below bregma. Perm reline repair resin (Coltene, Altstätten, Switzerland) was used to anchor the cannula to the screws and skull. To prevent clogging, a dummy cannula (Plastics One 313DC-SPC 0.014-0.36mm fit 1 mm) was inserted into the guide cannula. Post-surgery, animals were given subcutaneous 8 mg/kg gentamicin diluted in sterile saline and 0.25 mg meloxicam self-administered via Mouse MD's™ (MD275-0125) bacon flavored tablets, to

prevent infection and for pain management, respectively. Animals were returned to their home cage and allowed to recover for 7 days.

Intraplantar injections:

Intraplantar injections were administered according to previously published methods [12]. Briefly, rats were anesthetized initially at 5% isoflurane via a nose cone, once animals no longer exhibited a pinch reflex, isoflurane was lowered to 2.5-3%, during which time animals were injected. These animals received a volume of 50 µl into the left hindpaw via injection with a 30-gauge 0.5 inch needle attached to a Hamilton syringe. Animals were kept under isoflurane for <2 minutes. Following intraplantar injection the left paw was subject to von Frey.

Subcutaneous injections: Subcutaneous scalp injections were performed while animals were anesthetized under a nose cone under 2.5-3% isoflurane. Injections were administered where a dural cannula would otherwise be implanted in a volume of 10 µl via a 30-gauge 0.5 inch needle. In all cases animals were kept under anesthesia for less than 2 minutes.

Gastrocnemius injections: Injections into the gastrocnemius muscle were performed in a volume of 10 µl administered into the left gastrocnemius muscle while the animals were anesthetized with 2.5-3% isoflurane administered via a nosecone. Subsequently the left hindpaw was tested to see if any hypersensitivity had developed as a result of injection.

Intracisternal Injections:

Intracisternal injections were administered in a volume of 10 µl at a rate of 1 µl/sec and performed as previously described [12; 37; 52]. A 25-gauge 1.5 inch needle was contorted approximately 7 mm from the tip at a 45° angle with the bevel facing outwards. The needle was attached to a 27-gauge Hamilton syringe. Animals were anesthetized for < 2 minutes under 2.5-3% isoflurane via a nose cone. The head of the animal was tilted forward at approximately a 120° angle to allow access to the cisterna magna. The needle was positioned above C1 and inserted through the cisterna magna along the midline.

Intrathecal injections

Intrathecal injections were performed according to previously published methods [179]. Injections of either 10 µl IL-6 or vehicle were administered into the L5-L6 intervertebral space while animals were under 2.5-3% isoflurane administered via a nose cone. Injections were administered using a 30-gauge 0.5 inch needle.

Von Frey testing

Rats were allowed to acclimate to testing room, chambers, and light conditions for 2 hours a day for 3 days prior to facial testing. Rats were handled for a single 5-min session at 24-hours prior to habituation to the behavior chambers. Only rats that met a baseline of 8 g facial withdrawal threshold and 15 g hindpaw withdrawal threshold were included in the study. Following establishment of baseline, animals were given their respective injections. Facial withdrawal thresholds were determined by applying von Frey filaments to the periorbital region of the face (the midline of the forehead at the level of the eyes) in an ascending/descending manner starting from the 1 g filament. Briefly, if an animal did not respond, increasing filament forces were applied until the 8 g filament was reached or until a response was observed. If the animal responded to a specific filament, decreasing filament forces were applied until the 0.4 g filament was reached or until there were no responses observed. If no responses are observed, i.e facial baseline is reached, the filaments tested are 1 g, 2 g, 4 g, 6 g, 8 g. Hindpaw withdrawal thresholds were determined via the same paradigm with a maximum of beginning with the 2 g

filament and with a maximum of 15 g and a minimum of 0.6 g if an animal reaches a baseline withdrawal threshold on the paw the filaments tested are 2 g, 4 g, 6 g, 8 g, 15 g. Following central administration of IL-6 both the left and right hindpaws were tested; the paw showing greater hypersensitivity was then tested to be included in the data set.

Drugs:

Rat recombinant IL-6 (R&D systems, cat: 506-RL-050/CF) was diluted to a concentration of 0.1 ng for all experiments. For dural, hindpaw, and gastrocnemius muscle injections IL-6 was diluted in synthetic interstitial fluid (SIF) comprised of 135 mM NaCl, 5 mM KCl, 10mM HEPES, 2mMCaCl2, 10mM glucose, and 1mM MgCl2 (pH 7.4, 310 mOsm). For intracisternal injections IL-6 was diluted in artificial cerebrospinal fluid (aCSF) which was comprised of 125mM NaCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 2.5mM KCl, 1mM $MgCL_2$, 2mM $CaCl_2$, and 10mM D-glucose (pH 7.4). For intrathecal injection IL-6 was diluted in 0.9% saline. Diluents were used as respective vehicles for all experiments.

Experimental design and statistical analysis:

In all experiments, investigators were blinded to which animals received drug in all experiments. Allocation of animals to treatment groups was randomized via the “blinder” who chose animal

identification numbers from a bag of pre-labeled paper slips. Data here are presented as mean ± SEM. Data were analyzed at each time point via two-way ANOVA and followed by Bonferroni post-hoc assessment where appropriate. Prism (GraphPad) was used for all data analyses. Significance was set to $p < 0.05$ for all analyses.

RESULTS:

Dural IL-6 causes facial and hindpaw sensitivity in female rats.

Previously we found that application of IL-6 onto the dura of male rats not only resulted in facial hypersensitivity, but hindpaw sensitivity [37]. While we have additionally reported that dural IL-6 leads to facial allodynia in females [37], we did not test the ability of this stimulus to cause whole body allodynia in females. Given that cutaneous allodynia is more common in female migraineurs [25; 160] we aimed to test whether female rats would experience hindpaw allodynia similarly to male rats. Here we confirm that female rats that receive dural IL-6 demonstrate facial allodynia out to 72 hours (Fig. 1A), and additionally experience significant hindpaw sensitivity, persisting for 24 hours following injection (Fig. 1B).

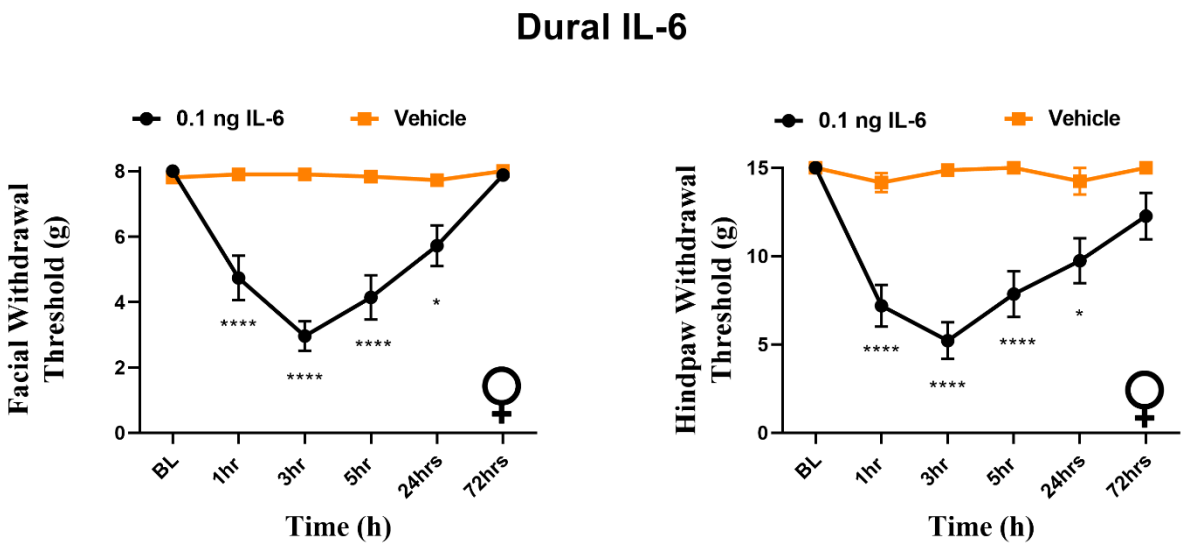

Figure 5.1: Dural IL-6 produces facial and hindpaw hypersensitivity in females. Female rats had baseline withdrawal thresholds established prior to receiving administration of 0.1 ng IL-6 onto the dura. Dural IL-6 (n=10) elicited significant effect of treatment on facial (A) $F(1, 66) = 31.58$, $p<0.0001$) and hindpaw (B) ($F(1, 66) = 43.66$, $p<0.0001$) hypersensitivity when compared with animals that received vehicle (n=4). *p<0.05, ****p<0.0001.

Intraplantar administration of IL-6 results in hindpaw, but not facial, allodynia.

Our data here and in our prior publications indicates that dural stimulation with IL-6 causes referred hypersensitivity to the hindpaw. Previously we have reported that intraplantar IL-6 leads to sensitivity in the hindpaw [37]. We next sought to test whether intraplantar IL-6 causes periorbital hypersensitivity like that observed following dural IL-6. Both sexes were tested since females and males exhibit differential responses to migraine-relevant peptides in multiple

peripheral tissues including the hindpaw [12; 195]. Both female and male rats received 0.1 ng IL-6 into the left hindpaw and were subject to periorbital and hindpaw von Frey testing. Females demonstrated acute hindpaw allodynia at 1- and 3-hours following injection (Fig. 2), consistent with hindpaw responses shown previously with intraplantar IL-6 in males [37]. In contrast, neither females nor males exhibited any facial hypersensitivity responses at any time point following intraplantar IL-6 (Fig. 3). These data demonstrate that while dural IL-6 causes referred hypersensitivity to the hindpaw, intraplantar IL-6 does not cause referred hypersensitivity to the facial skin.

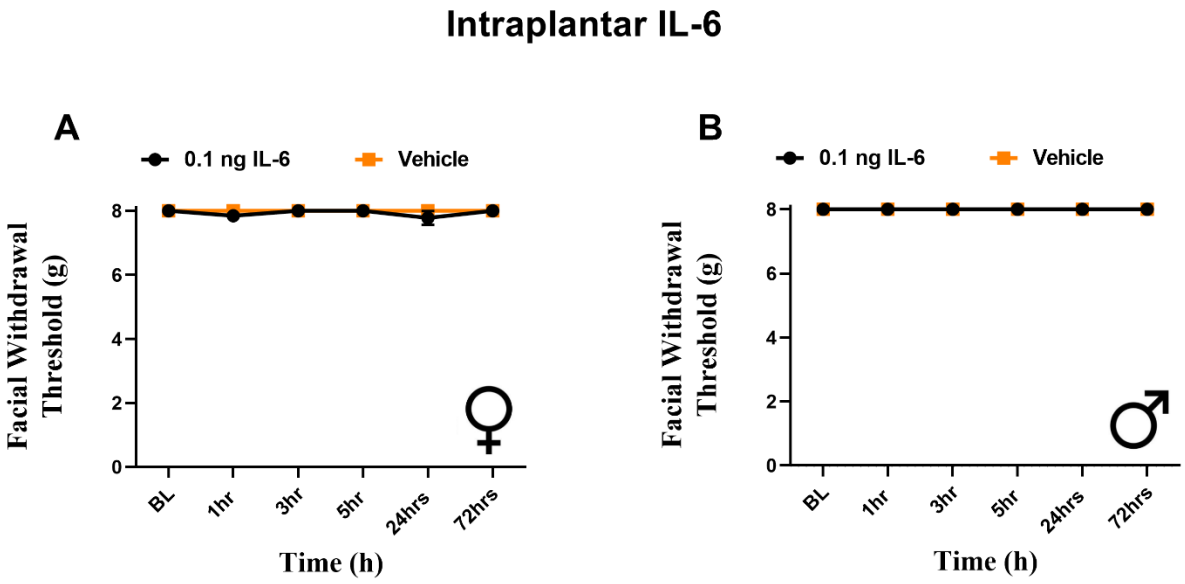

Figure 5.2: Intraplantar IL-6 produces hindpaw hypersensitivity in females. Female rats from the cohort in figure 2 also had baseline hindpaw withdrawal thresholds established prior to receiving intraplantar injection of 0.1 ng IL-6. IL-6 (n=6) elicited significant effect of treatment on

hindpaw hypersensitivity ($F(5, 60) = 2.512$, $p=0.0394$) when compared with animals that received vehicle (n=6). *$p<0.05$, ***$p<0.001$.

Subcutaneous IL-6 in the scalp does not elicit facial or hindpaw hypersensitivity.

Prior studies have found that migraine-relevant peptides injected into the periorbital region of the face of rats results in facial allodynia [66]. This is likely due to activation of fibers from the trigeminal ganglia that innervate the peri-orbital region of the face i.e. direct activation and sensitization of nerve endings near the site of von Frey testing. In contrast, for dural stimulation to cause periorbital hypersensitivity, central sensitization leading to referred responses from the dura to the facial skin must be present. This led us to question whether the periorbital hypersensitivity that results from 0.1 ng dural IL-6 would be observed if this stimulus was applied subcutaneously to the scalp where the dural cannula would otherwise be implanted. Thus, we administered subcutaneous IL-6 to the rostral part of the scalp in rats that were otherwise naïve, i.e., they had no dural cannula. Despite the noted projection of dural afferents to the extracranial periosteum [217], neither females nor males exhibited any periorbital (Fig. 4A, C) or hindpaw (Fig. 4B, D) hypersensitivity at any time point following subcutaneous IL-6. These data show that the referred periorbital hypersensitivity that develops following activation of dural afferents is not a general response to IL-6 injected anywhere in the head.

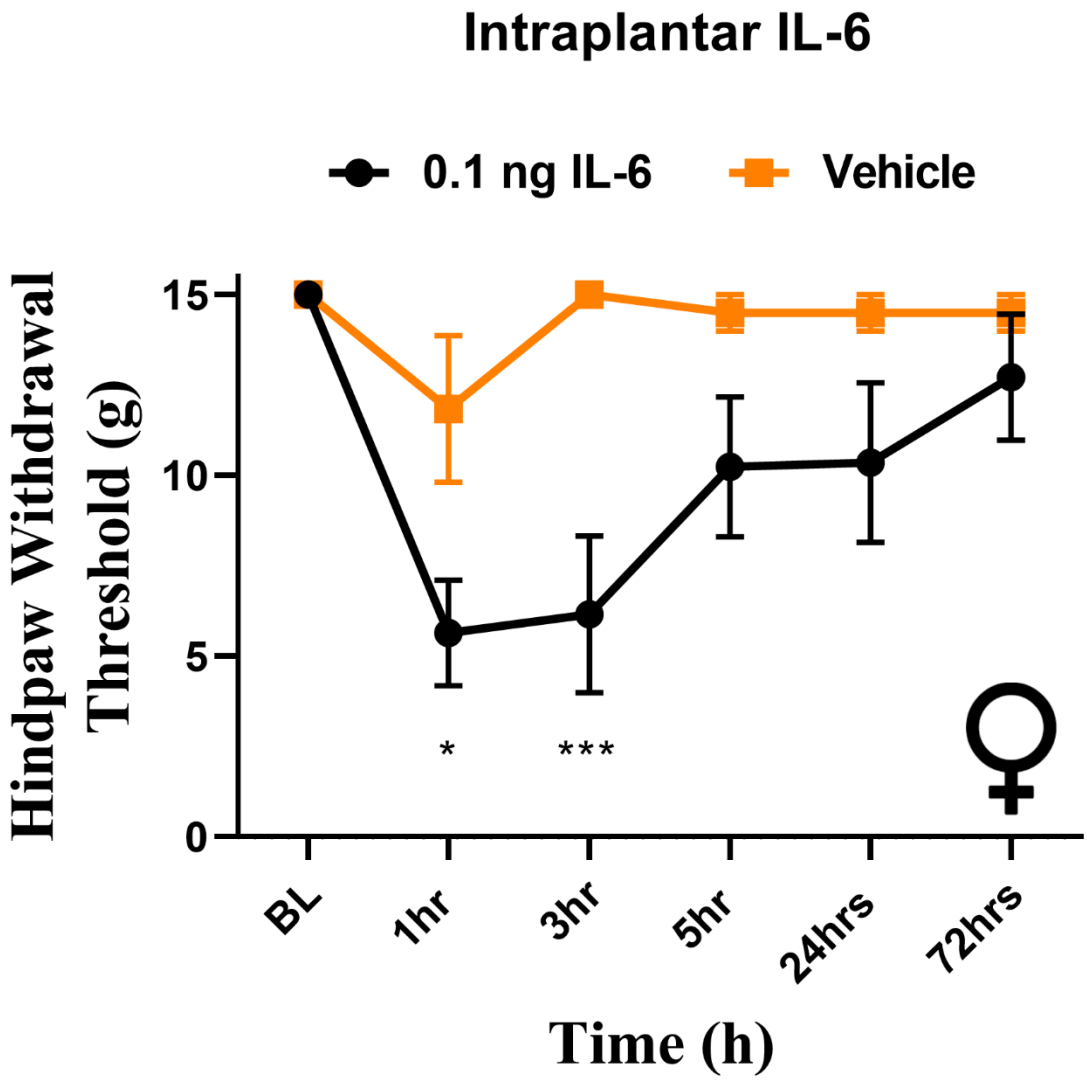

Figure 5.3: Intraplantar administration of IL-6 produces no facial responses in female or male rats. Female and male rats had facial hindpaw withdrawal thresholds assessed prior to and following intraplantar injection of 0.1 ng IL-6 (6 females, 6 males) or vehicle (6 females, 6 males). Two-way ANOVA followed by Bonferroni posthoc analysis revealed no significant

facial responses in female (A) ($F(1, 40) = 1.844$, $p=0.1821$) or male (B) (no variation among groups) rats.

Injection of IL-6 into the gastrocnemius muscle fails to produce facial or hindpaw hypersensitivity in male or female rats.

Peripheral cutaneous injections into the scalp produced no facial or hindpaw hypersensitivity, suggesting that whole body responses are not simply caused by general activation of the afferents within trigeminal system. One alternate possibility is that widespread referred responses are caused by activation of deep-tissue afferents but not cutaneous afferents. This is a well-known phenomenon observed with gastrointestinal or cardiac pain that is commonly referred to the surface of the abdomen. It is possible that hindpaw and scalp injections of IL-6 do not cause widespread referred hypersensitivity since they are subcutaneous while stimulation of the dura mater is more similar to activation of deep-tissue afferents. To address this possibility, we administered 0.1 ng IL-6 into the gastrocnemius muscle. We observed no significant reductions in facial or hindpaw withdrawal response to this intramuscular injection in either female (Fig. 5A, B) or male rats (Fig. 5C, D). This indicates that the referred hypersensitivity of the facial and hindpaw skin following dural stimulation with IL-6 is not a generalized response to activation of non-cutaneous afferents.

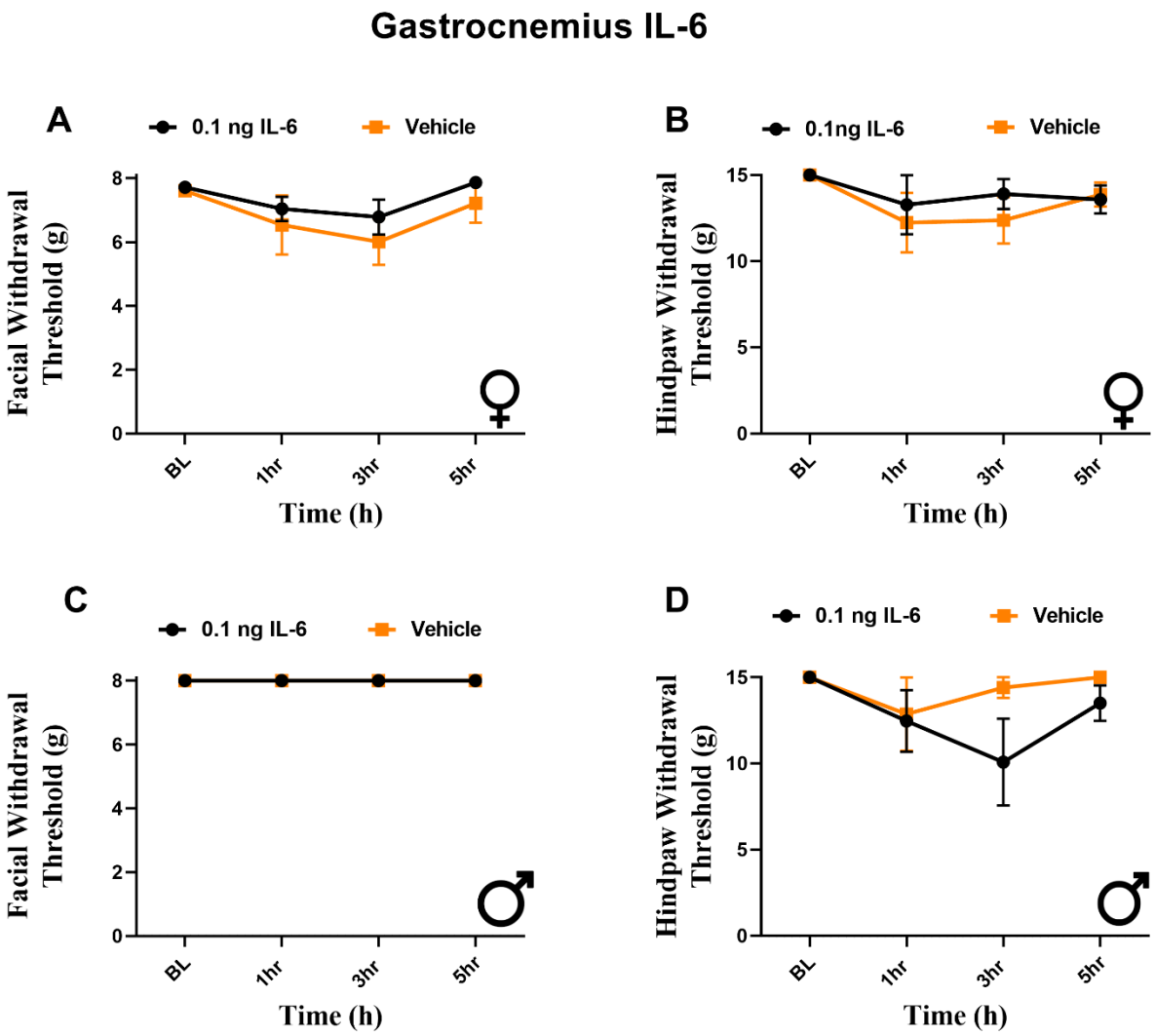

Figure 5.4: Subcutaneous injection of IL-6 in the scalp produces no significant facial or hindpaw responses in male and female rats**.** Rats had facial and hindpaw withdrawal thresholds assessed to establish baseline withdrawal thresholds, as well as following administration of 0.1 ng IL-6 (6 females, 8 males) or vehicle (7 females, 6 males) subcutaneously in the scalp. Two-way ANOVA followed by Bonferroni posthoc analysis revealed no significant facial or hindpaw

responses in female (A, B) ($F(1, 44) = 0.2761$, $p=0.6019$) ($F(1, 44) = 1.502$, $p=0.2269$) or male (C,D) (no variation among groups) ($F(1, 48) = 1.371$, $p=0.2473$) rats.

Intracisternal IL-6 produces facial and hindpaw hypersensitivity in rats.

It has been shown previously that dural application of inflammatory soup (IS) can lead to sensitization of neurons within the TNC [18; 41; 191]. This sensitization that develops has been suggested to be the underlying mechanism of referred facial hypersensitivity following dural stimulation. We thus aimed to determine whether direct stimulation of the central terminals of trigeminal afferents or of second-order neurons within the TNC with IL-6 would produce whole body hypersensitivity. To test this, rats received 0.1 ng IL-6 into the cisterna magna. Female rats exhibited facial (Fig. 6A) and hindpaw (Fig. 6B) hypersensitivity at 3 hours following injection. Male rats exhibited no hindpaw hypersensitivity (Fig. 6D) but demonstrated significant facial allodynia from 1 to 5 hours following injection (Fig. 6C). These data show that activation of circuits within the brainstem by IL-6 is capable of causing referred facial hypersensitivity, and also referred paw hypersensitivity in females, but that only dural stimulation causes referred paw hypersensitivity in males.

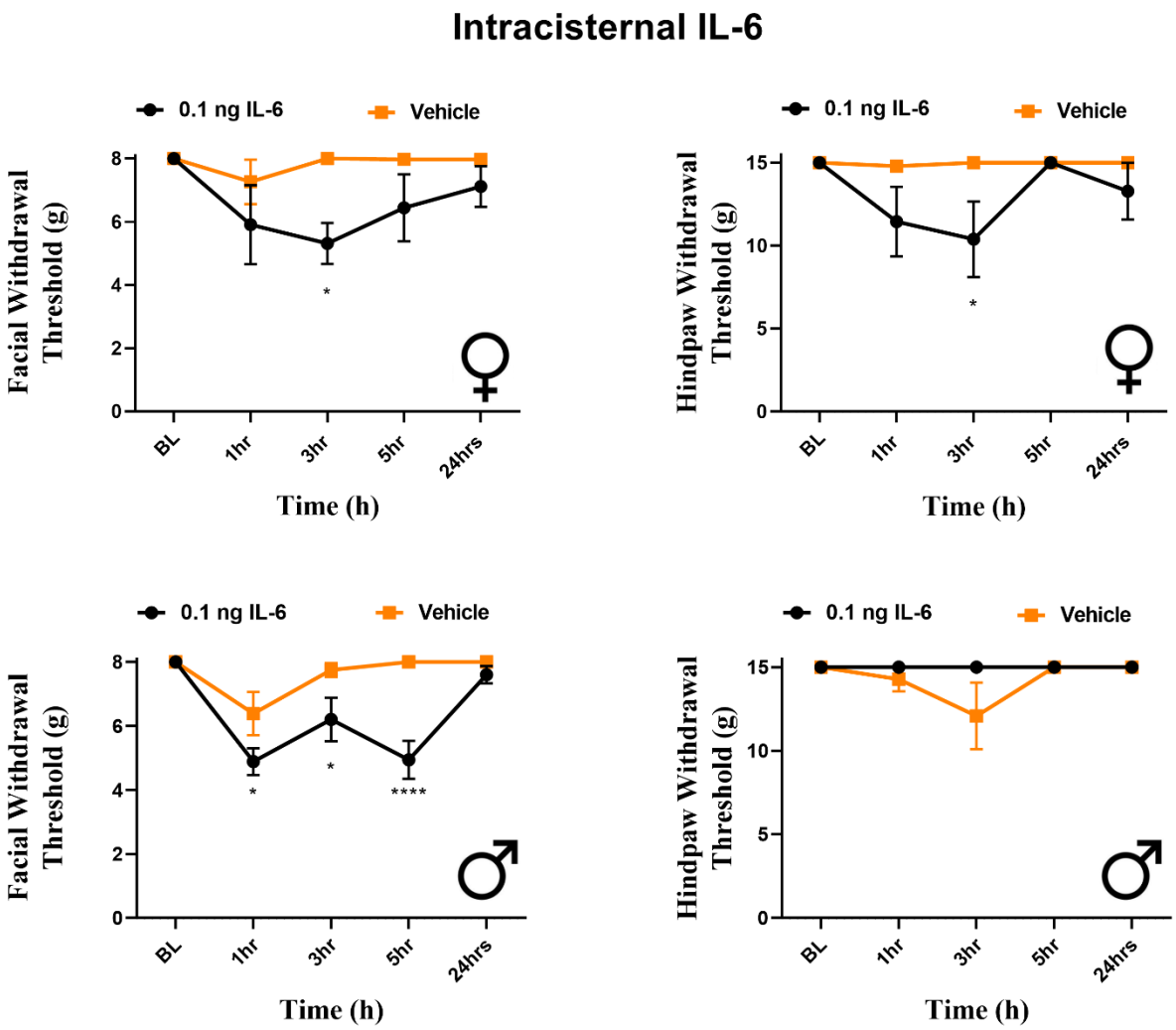

Figure 5.5: Intracisternal IL-6 produces facial hypersensitivity in both sexes, but differential hindpaw responses. Female (A, B) and male (C, D) rats had baseline withdrawal thresholds of the face and hindpaw determined prior to intracisternal injection of IL-6 (4 females, 6 males) or vehicle (5 females, 7 males). Both females and males demonstrated significant effects of treatment on facial responses ($F(1, 35) = 12.24$, $p=0.0013$) ($F(1, 55) = 27.98$, $p<.0001$);

however, only females presented with hindpaw responses. (F (1, 35) = 9.640, p=0.0038)

*p<0.05, ****p<0.0001.

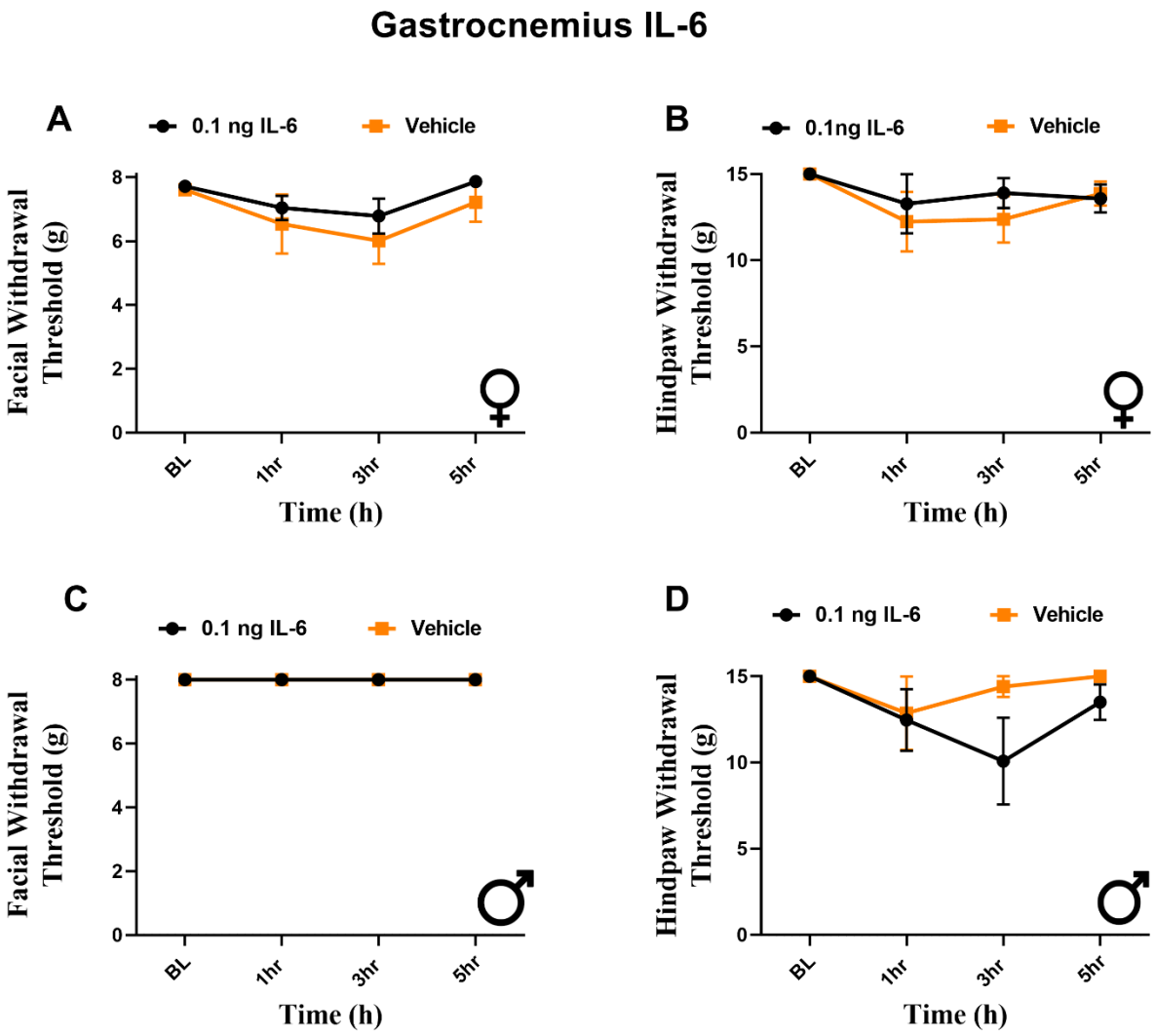

Figure 5.6: Gastrocnemius injection of IL-6 produces no significant facial or hindpaw responses in male and female rats**.** Rats had facial and hindpaw withdrawal thresholds assessed prior to and following administration of 0.1 ng IL-6 (6 females, 6 males) or vehicle (6 females, 5 males) into

the gastrocnemius muscle. Two-way ANOVA followed by Bonferroni posthoc analysis revealed no significant effect of treatment on facial or hindpaw responses in female (A, B) ($F(1, 40) = 1.844$, $p=0.1821$) ($F(3, 36) = 0.2904$, $p=0.8320$) or male (C, D) (no variation among groups) ($F(1, 36) = 2.289$, $p=0.1390$) rats.

Intrathecal IL-6 causes hindpaw hypersensitivity in females.

The findings showing that intracisternal injection of IL-6 leads to whole body hypersensitivity, at least in females, led us to question whether central sensitization in general leads to widespread hypersensitivity, or whether this is specific to the activation of the trigeminal pathways. To address this question, we administered 0.1 ng IL-6 intrathecally in both male and female rats. IL-6 produced significant acute hindpaw hypersensitivity in females 1 hour following injection (Fig. 7B). No hindpaw hypersensitivity was observed in males at any time point (Fig. 7D). Importantly, no facial hypersensitivity responses were observed in either sex at any time point (Fig. 7A, C). These findings show that referred hypersensitivity to the periorbital region is not a general feature of injections of IL-6 into the spinal canal but that it is unique to activation of meningeal afferents or the TNC.

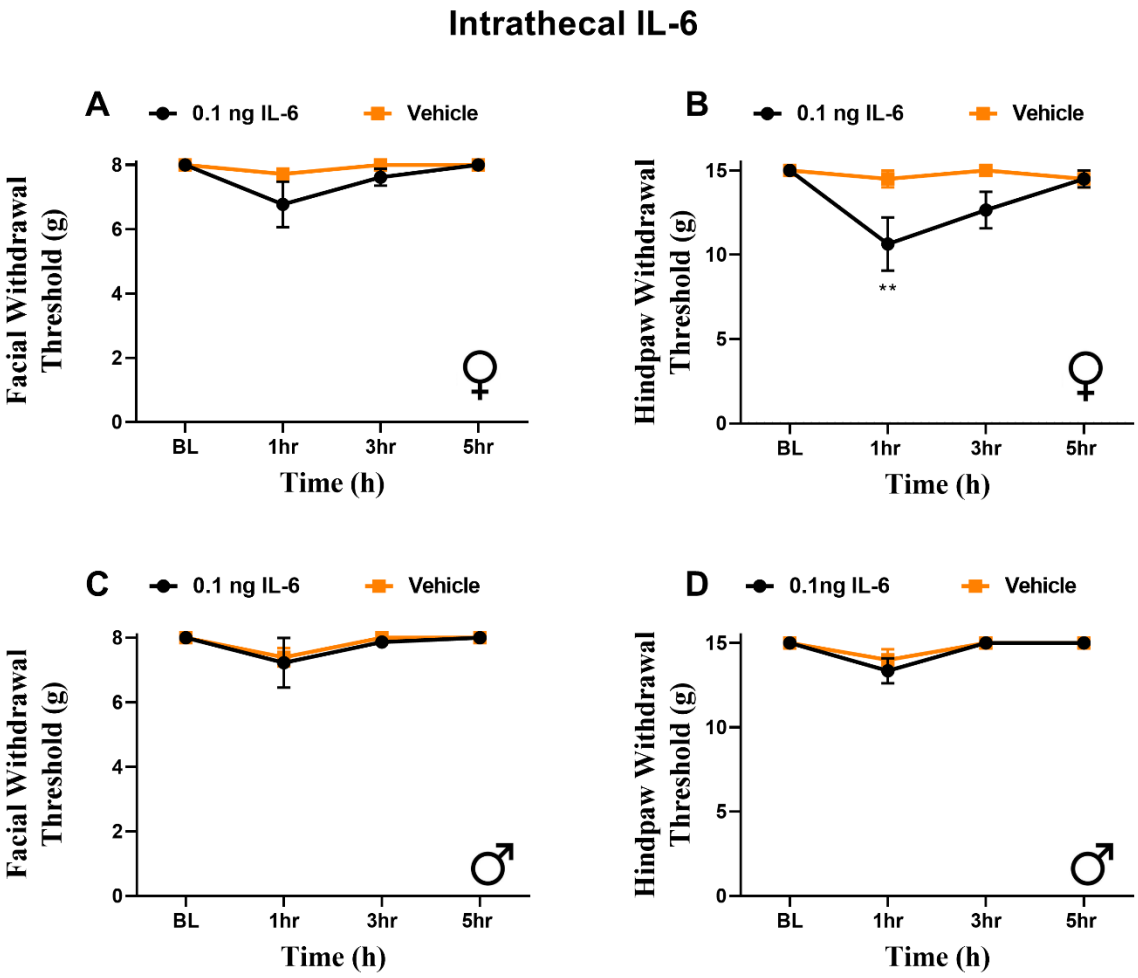

Figure 5.7: Intrathecal IL-6 produces hindpaw responses in female, but not male rats. Rats had periorbital and hindpaw withdrawal thresholds assessed prior to and following intrathecal administration of 0.1 ng IL-6 (6 females, 6 males) or vehicle (6 females, 6 males). Two-way ANOVA followed by Bonferroni posthoc analysis revealed no significant effect of treatment on facial responses in female (A, B) ($F(1, 40) = 2.924$, $p=0.0950$) or male (C, D) rats ($F(1, 40) = 1.280$, $p=0.7223$). Female rats demonstrated significant hindpaw allodynia. ($F(1, 40) = 8.700$, $p=0.0053$) $^{**}p<0.01$.

DISCUSSION:

While many migraine patients report cutaneous hypersensitivity in cephalic and in one or multiple additional extracephalic sites during attack [40; 175; 247], it is less common that patients with pain states in the lower part of the body experience cephalic hypersensitivity. Little is known about how or why this occurs. Here we examined whether a similar effect occurs in rats where provoking migraine-like conditions causes body wide hypersensitivity and whether these widespread responses are absent when the same stimulus is given into locations that would cause other types of pain. We used the pro-inflammatory cytokine IL-6, which is upregulated during migraine attacks, but is a stimulus that is also implicated in pain states in other body regions [274]. IL-6 administered directly onto the dura of female rats results in facial and hindpaw hypersensitivity similarly to our previous reports in male rats [37]. In contrast, intraplantar IL-6 at the same dose only produces hindpaw responses in either females or males. Subcutaneous IL-6 onto the scalp produced no facial or hindpaw allodynia in either sex. We also show that IL-6 administered intracisternally produces facial hypersensitivity in both sexes, but only leads to whole body responses in females. Finally, when IL-6 was administered intrathecally, neither males nor females exhibit facial hypersensitivity, but females demonstrated significant decreases in hindpaw withdrawal thresholds.

Together, these data show that the only locations where administration of IL-6 leads to periorbital hypersensitivity is onto the dura or via intracisternal injection, the latter of which is capable of activating central trigeminal pathways for all innervation targets. These are also the

only stimulus locations where hypersensitivity is referred to distant locations across the body. This finding suggests the presence of unique pathways through the trigeminal system that are capable of establishing widespread behavioral responses and that similar pathways do not exist within the lower spinal system. These data may help to offer additional insight into the mechanistic differences by which meningeal afferents and other sensory inputs engage central circuits to cause pain and also may aid in the understanding of how and why migraine is such a debilitating disorder.

The data presented here show a robust ability of dural stimulation with IL-6 to cause both facial and hindpaw hypersensitivity, demonstrating a role for meningeal inputs in these responses. While this may also result from the ability of dural stimuli to penetrate the CNS [272] and lead to activation of central pain targets, IL-6 is a much larger peptide than the small molecules that were observed to have these properties and is unlikely to migrate to the CNS. Additionally, our previous work showed that dural application of pH 6.0 caused body-wide hypersensitivity [39] and it is unlikely that the H^+ concentration rises enough throughout the CNS to cause this response. This suggests that dural IL-6 likely exerts the effects observed here via activation of dural afferents near the injection site. However, intracisternal injection of IL-6 should activate these dural inputs in addition to many, if not all, other trigeminal inputs to the TNC. Thus, it is surprising that intracisternal IL-6 did not cause hindpaw hypersensitivity in males. These data may be due to the release of additional inflammatory mediators or other signaling molecules downstream of dural IL-6 that are not present following intracisternal IL-6, ultimately adding to

the stimulus intensity of the former. For example, there may be release of factors such as histamine, serotonin, proteases, and other sensitizing agents from mast cells [57; 243]. Given that mast cells are more abundant in the dura mater and leptomeninges [271], the release of additional inflammatory mediators from these cells may lead to increased engagement of trigeminal pathways in response to dural IL-6. This may also explain why the facial hypersensitivity responses of males and females to intracisternal IL-6 are less robust and of shorter duration than those resulting from dural application.

We also surprisingly observed that while subcutaneous IL-6 on the scalp failed to produce hindpaw hypersensitivity, consistent with IL-6 injections into numerous other locations, it was also unable to cause hypersensitivity of the relatively nearby periorbital skin. This is despite the fact that the scalp IL-6 injection should activate or sensitize dural fibers in the periosteum that pass through the calvarial sutures [216]. Activation of these fibers has previously been demonstrated with stimuli such as KCl and inflammatory soup [273]. In contrast, capsaicin and low pH 5.0 failed to activate these afferents reliably or robustly [273]. It is thus possible that not all stimuli are able to activate these fibers and IL-6 may be among those that cause no responses. Additionally, the location of subcutaneous scalp injection may not effectively target these periosteal trigeminal afferents directly.

While many of the responses to IL-6 shown here were not sexually dimorphic, there were several notable exceptions. We show that intrathecal administration of IL-6 only leads to hindpaw responses in female rats (Fig. 6). Similarly, IL-6 applied into the cisterna magna resulted in facial

hypersensitivity in both females and males, potentially via activation of pial afferents within the subarachnoid space [19; 120], but only led to hindpaw hypersensitivity in females (Fig. 5). Prior studies show that estrogen can lead to inhibition of IL-6 production and release [46; 140], but whether this leads to changes in effects of exogenous IL-6 is not clear. Recently, a potential role for spinal prolactin (PRL) has been implicated in the production of IL-6 induced hindpaw allodynia [195], as intrathecal administration of a prolactin receptor antagonist (ΔPRL) prevents responses to IL-6 in female mice. Similarly, co-injection of PRL with IL-6 increases hindpaw hypersensitivity in female mice. The increased endogenous levels of PRL in females rats compared to their male counterparts [74; 186; 258] may explain the ability of intracisternal and intrathecal IL-6 to produce hindpaw responses in females, but not in males at this dose, as it may be a more intense stimulus in females. These findings demonstrate a clear role for the trigeminal system in *producing* whole body hypersensitivity; central administration of IL-6 to the TNC leads to whole body allodynia, while central administration that does not activate the trigeminal system is incapable of eliciting a facial response.

There are several potential limitations to this study. The only stimulus tested was IL-6 and other migraine-relevant stimuli may lead to differential responses across the body; in particular, other stimuli applied to the lower body may lead to facial hypersensitivity. However, we have previously demonstrated that hindpaw injection of CGRP leads to hindpaw allodynia in female rats, but does not result in any facial hypersensitivity [12]. Next, while we did not observe

widespread hypersensitivity when IL-6 was injected subcutaneously into the scalp, there may have been different findings with IL-6 injected into other trigeminal targets such as the temporomandibular joint. Stimulation of other such tissues may be more effective at referring hypersensitivity to the facial skin. Similarly, we used the gastrocnemius muscle as a representative deep tissue, but injection of IL-6 may lead to more robust and widespread referred pain from other deep tissues such as visceral organs. While colonic inflammation has been shown to induce periorbital hypersensitivity in rodents, this model required administration of dextran sodium sulfate into the drinking water for 7 days and the location of action leading to periorbital hypersensitivity is not clear [134]. Additionally, as a result of the circulation of CSF we cannot accurately state the concentration of IL-6 at the cisterna magna or intrathecally. Furthermore, given the rate and direction of CSF [139] it is possible that intracisternal IL-6 diffuses to lower targets aiding in the development of hindpaw sensitivity. Finally, IL-6 administration at this dose leads to transient behavioral responses that may better reflect signaling pathways associated with acute pain states. Testing chronic pain models in other body locations may lead to more widespread hypersensitivity, including into the facial region.

Overall, these data show the unique ability of dural and intracisternal stimulation to produce robust whole-body hypersensitivity and are consistent with the building list of studies supporting differential connections of trigeminal and dural pathways with central circuits relevant for pain. These studies raise the possibility that afferent input from the head may more effectively engage

central pain and affective circuits as an enhanced protective mechanism given the importance of the brain and other sensory structures within the head. Similarly, hypotheses have been proposed that migraine evolved as a defense mechanism, and to lead to detection of potentially harmful events such as the ingestion of toxins, lack of sleep, or hunger [162]. They further support the notion that all forms of pain have evolved to contribute to the survival of an organism [261]. This idea also offers context to the common symptoms, and diagnostic criteria for migraine, photophobia and phonophobia where the increased sensitivity to lights and sounds would signal for the animals to remain in a covered location, to become less susceptible to predators [1]. In the case of rodents, they often remain immobile to heal and recover, therefore the hindpaw allodynia we observe here in response to dural and intracisternal stimulation may serve to prevent the animal from moving. This is consistent with findings that cortical spreading depression (CSD) leads to significant freezing behavior in rats [92]. Furthermore, this freezing behavior is attenuated by CGRP inhibitors [92].

CONCLUSIONS:

The findings of this study show that activation of trigeminal afferents, and in particular dural afferents, are uniquely able to generate widespread cutaneous hypersensitivity in both female and male rats. Mechanisms underlying these effects may contribute to the distinct collection of symptoms present in pain states such as migraine where sensory symptoms spread beyond the

cephalic region. Better understanding of these mechanisms may lead to novel therapeutic approaches that are differentially effective for migraine compared to pain in the rest of the body.

DECLARATIONS:

Ethics approval and consent to participate: All procedures were conducted with prior approval of the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

Consent for publication: Not applicable.

Availability of data and materials: Raw data are available upon request via contacting the corresponding author: Gregory.Dussor1@utdallas.edu/

Competing interests: Not applicable.

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Authors Contributions: Design of work: AA, TJP, GD,

Acquisition and analysis of work: AA

Interpretation of data: AA, GD

Draft of the manuscript: AA, GD

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REFERENCES

[1] Headache Classification Committee of the International Headache Society (IHS) The International Classification of Headache Disorders, 3rd edition. Cephalalgia 2018;38(1):1-211.

[2] Avona A, Burgos-Vega C, Burton MD, Akopian AN, Price TJ, Dussor G. Dural Calcitonin Gene-Related Peptide Produces Female-Specific Responses in Rodent Migraine Models. J Neurosci 2019;39(22):4323-4331.

[3] Avona A, Mason BN, Burgos-Vega C, Hovhannisyan AH, Belugin SN, Mecklenburg J, Goffin V, Wajahat N, Price TJ, Akopian AN, Dussor G. Meningeal CGRP-Prolactin interaction evokes female-specific migraine behavior. Ann Neurol 2021.

[4] Bartsch T, Goadsby PJ. Increased responses in trigeminocervical nociceptive neurons to cervical input after stimulation of the dura mater. Brain 2003;126(Pt 8):1801-1813.

[5] Bedussi B, van Lier MG, Bartstra JW, de Vos J, Siebes M, VanBavel E, Bakker EN. Clearance from the mouse brain by convection of interstitial fluid towards the ventricular system. Fluids Barriers CNS 2015;12:23.

[6] Bigal ME, Ashina S, Burstein R, Reed ML, Buse D, Serrano D, Lipton RB, Group A. Prevalence and characteristics of allodynia in headache sufferers: a population study. Neurology 2008;70(17):1525-1533.

[7] Burgos-Vega CC, Quigley LD, Avona A, Price T, Dussor G. Dural stimulation in rats causes brain-derived neurotrophic factor-dependent priming to subthreshold stimuli including a migraine trigger. Pain 2016;157(12):2722-2730.

[8] Burgos-Vega CC, Quigley LD, Trevisan Dos Santos G, Yan F, Asiedu M, Jacobs B, Motina M, Safdar N, Yousuf H, Avona A, Price TJ, Dussor G. Non-invasive dural stimulation in mice: A novel preclinical model of migraine. Cephalalgia 2019;39(1):123-134.

[9] Burstein R, Cutrer MF, Yarnitsky D. The development of cutaneous allodynia during a migraine attack clinical evidence for the sequential recruitment of spinal and supraspinal nociceptive neurons in migraine. Brain 2000;123 (Pt 8):1703-1709.

[10] Burstein R, Yamamura H, Malick A, Strassman AM. Chemical stimulation of the intracranial dura induces enhanced responses to facial stimulation in brain stem trigeminal neurons. J Neurophysiol 1998;79(2):964-982.

[11] Canellada A, Alvarez I, Berod L, Gentile T. Estrogen and progesterone regulate the IL-6 signal transduction pathway in antibody secreting cells. J Steroid Biochem Mol Biol 2008;111(3-5):255-261.

[12] Chen Y, Imai H, Ito A, Saito N. Novel modified method for injection into the cerebrospinal fluid via the cerebellomedullary cistern in mice. Acta Neurobiol Exp (Wars) 2013;73(2):304-311.

[13] Conti P, Kempuraj D, Di Gioacchino M, Boucher W, Letourneau R, Kandere K, Barbacane RC, Reale M, Felaco M, Frydas S, Theoharides TC. Interleukin-6 and mast cells. Allergy Asthma Proc 2002;23(5):331-335.

[14] De Logu F, Landini L, Janal MN, Li Puma S, De Cesaris F, Geppetti P, Nassini R. Migraine-provoking substances evoke periorbital allodynia in mice. J Headache Pain 2019;20(1):18.

[15] Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet 2017;390(10100):1211-1259.

[16] Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2018;392(10159):1789-1858.

[17] Dohler KD, Wuttke W. Changes with age in levels of serum gonadotropins, prolactin and gonadal steroids in prepubertal male and female rats. Endocrinology 1975;97(4):898-907.

[18] Edelmayer RM, Ossipov MH, Porreca F. An experimental model of headache-related pain. Methods Mol Biol 2012;851:109-120.

[19] Edelmayer RM, Vanderah TW, Majuta L, Zhang ET, Fioravanti B, De Felice M, Chichorro JG, Ossipov MH, King T, Lai J, Kori SH, Nelsen AC, Cannon KE, Heinricher MM, Porreca F. Medullary pain facilitating neurons mediate allodynia in headache-related pain. Ann Neurol 2009;65(2):184-193.

[20] Edvinsson L, Haanes KA, Warfvinge K. Does inflammation have a role in migraine? Nat Rev Neurol 2019;15(8):483-490.

[21] Fidan I, Yuksel S, Ymir T, Irkec C, Aksakal FN. The importance of cytokines, chemokines and nitric oxide in pathophysiology of migraine. J Neuroimmunol 2006;171(1-2):184-188.

[22] Filiz A, Tepe N, Eftekhari S, Boran HE, Dilekoz E, Edvinsson L, Bolay H. CGRP receptor antagonist MK-8825 attenuates cortical spreading depression induced pain behavior. Cephalalgia 2019;39(3):354-365.

[23] Goadsby PJ, Holland PR, Martins-Oliveira M, Hoffmann J, Schankin C, Akerman S. Pathophysiology of Migraine: A Disorder of Sensory Processing. Physiol Rev 2017;97(2):553-622.

[24] Hannocks MJ, Pizzo ME, Huppert J, Deshpande T, Abbott NJ, Thorne RG, Sorokin L. Molecular characterization of perivascular drainage pathways in the murine brain. J Cereb Blood Flow Metab 2018;38(4):669-686.

[25] Jain P, Materazzi S, De Logu F, Rossi Degl'Innocenti D, Fusi C, Li Puma S, Marone IM, Coppi E, Holzer P, Geppetti P, Nassini R. Transient receptor potential ankyrin 1 contributes to somatic pain hypersensitivity in experimental colitis. Sci Rep 2020;10(1):8632.

[26] Karimy JK, Kahle KT, Kurland DB, Yu E, Gerzanich V, Simard JM. A novel method to study cerebrospinal fluid dynamics in rats. J Neurosci Methods 2015;241:78-84.

[27] Kassem M, Harris SA, Spelsberg TC, Riggs BL. Estrogen inhibits interleukin-6 production and gene expression in a human osteoblastic cell line with high levels of estrogen receptors. J Bone Miner Res 1996;11(2):193-199.

[28] Kelman L. The triggers or precipitants of the acute migraine attack. Cephalalgia 2007;27(5):394-402.

[29] Lipton RB, Bigal ME, Ashina S, Burstein R, Silberstein S, Reed ML, Serrano D, Stewart WF, American Migraine Prevalence Prevention Advisory G. Cutaneous allodynia in the migraine population. Ann Neurol 2008;63(2):148-158.

[30] Loder E. What is the evolutionary advantage of migraine? Cephalalgia 2002;22(8):624-632.

[31] Mathew NT, Kailasam J, Seifert T. Clinical recognition of allodynia in migraine. Neurology 2004;63(5):848-852.

[32] Mestre C, Pelissier T, Fialip J, Wilcox G, Eschalier A. A method to perform direct transcutaneous intrathecal injection in rats. J Pharmacol Toxicol Methods 1994;32(4):197-200.

[33] Neill JD. Effect of "stress" on serum prolactin and luteinizing hormone levels during the estrous cycle of the rat. Endocrinology 1970;87(6):1192-1197.

[34] Oshinsky ML. Sensitization and ongoing activation in the trigeminal nucleus caudalis. Pain 2014;155(7):1181-1182.

[35] Oshinsky ML, Gomonchareonsiri S. Episodic dural stimulation in awake rats: a model for recurrent headache. Headache 2007;47(7):1026-1036.

[36] Patil M, Belugin S, Mecklenburg J, Wangzhou A, Paige C, Barba-Escobedo PA, Boyd JT, Goffin V, Grattan D, Boehm U, Dussor G, Price TJ, Akopian AN. Prolactin Regulates Pain Responses via a Female-Selective Nociceptor-Specific Mechanism. iScience 2019;20:449-465.

[37] Plesh O, Adams SH, Gansky SA. Self-reported comorbid pains in severe headaches or migraines in a US national sample. Headache 2012;52(6):946-956.

[38] Sarchielli P, Alberti A, Baldi A, Coppola F, Rossi C, Pierguidi L, Floridi A, Calabresi P. Proinflammatory cytokines, adhesion molecules, and lymphocyte integrin expression in the internal jugular blood of migraine patients without aura assessed ictally. Headache 2006;46(2):200-207.

[39] Schueler M, Messlinger K, Dux M, Neuhuber WL, De R. Extracranial projections of meningeal afferents and their impact on meningeal nociception and headache. Pain 2013;154(9):1622-1631.

[40] Schueler M, Neuhuber WL, De Col R, Messlinger K. Innervation of rat and human dura mater and pericranial tissues in the parieto-temporal region by meningeal afferents. Headache 2014;54(6):996-1009.

[41] Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, Asadi S, Vasiadi M, Weng Z, Miniati A, Kalogeromitros D. Mast cells and inflammation. Biochim Biophys Acta 2012;1822(1):21-33.

[42] Tietjen GE, Brandes JL, Peterlin BL, Eloff A, Dafer RM, Stein MR, Drexler E, Martin VT, Hutchinson S, Aurora SK, Recober A, Herial NA, Utley C, White L, Khuder SA. Allodynia in migraine: association with comorbid pain conditions. Headache 2009;49(9):1333-1344.

[43] Voogt JL, Chen CL, Meites J. Serum and pituitary prolactin levels before, during, and after puberty in female rats. Am J Physiol 1970;218(2):396-399.

[44] Walters ET, Williams ACC. Evolution of mechanisms and behaviour important for pain. Philos Trans R Soc Lond B Biol Sci 2019;374(1785):20190275.

[45] Wieseler J, Ellis A, Sprunger D, Brown K, McFadden A, Mahoney J, Rezvani N, Maier SF, Watkins LR. A novel method for modeling facial allodynia associated with migraine in awake and freely moving rats. J Neurosci Methods 2010;185(2):236-245.

[46] Yan J, Melemedjian OK, Price TJ, Dussor G. Sensitization of dural afferents underlies migraine-related behavior following meningeal application of interleukin-6 (IL-6). Mol Pain 2012;8:6.

[47] Yucel M, Kotan D, Gurol Ciftci G, Ciftci IH, Cikriklar HI. Serum levels of endocan, claudin-5 and cytokines in migraine. Eur Rev Med Pharmacol Sci 2016;20(5):930-936.
[48] Zappulla JP, Arock M, Mars LT, Liblau RS. Mast cells: new targets for multiple sclerosis therapy? J Neuroimmunol 2002;131(1-2):5-20.

[49] Zhao J, Bree D, Harrington MG, Strassman AM, Levy D. Cranial dural permeability of inflammatory nociceptive mediators: Potential implications for animal models of migraine. Cephalalgia 2017;37(11):1017-1025.

[50] Zhao J, Levy D. The sensory innervation of the calvarial periosteum is nociceptive and contributes to headache-like behavior. Pain 2014;155(7):1392-1400.

[51] Zhou YQ, Liu Z, Liu ZH, Chen SP, Li M, Shahveranov A, Ye DW, Tian YK. Interleukin-6: an emerging regulator of pathological pain. J Neuroinflammation 2016;13(1):141.

CHAPTER 6

DURAL AMYLIN AND CGRP LEAD TO DIFFERENTIAL SEX-SPECIFIC RESPONSES CONTINGENT ON OVARIAN HORMONES IN RODENT MIGRAINE MODELS

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ABSTRACT

Migraine affects approximately 303 million people worldwide, in addition to being common migraine is also considered to be one of the most disabling disorders. Migraine is considered to be more disabling and common in women. The reasons for the disparity are not known, however previous studies have demonstrated that the migraine relevant peptide CGRP applied onto the dura of female rodents causes facial hypersensitivity yet caused no facial responses in male rodents. Recently the calcitonin family peptide amylin has been implicated in migraine as amylin and the receptor components for amylin have been found within the trigeminal ganglia. Additionally, administration of the amylin analog pramlintide induces migraine in migraine patients. Here we report that despite similarities in receptors and signaling mechanisms dural amylin causes facial hypersensitivity and grimacing in male but not female mice. Ovariectomized mice demonstrate robust hypersensitivity in response to a previously non-noxious dose of dural amylin yet demonstrate no hypersensitivity to a previously noxious dose of CGRP. This suggests that ovarian hormones play a role in the sex-specific responses to dural administration of these peptides. Ovariectomized animals also demonstrated increased glucose which may be indicative of alterations to amylin signaling. These sex biased responses are not only specific to the dura but extend to other peripheral tissues intraplantar administration of amylin results in hindpaw allodynia of male, but not female mice. Finally, despite similarities in responses and signaling with PACAP, dural PACAP results in similar hypersensitivity in male

and female mice. These data suggest that there are specific mechanisms by which calcitonin family peptides lead to the sexual dichotomy of migraine.

INTRODUCTION

Migraine is considered to be one of the most common and disabling disorders across the globe. Although migraine is the 2nd most disabling disorder over all [71] it is considered to be even more debilitating in women [255] , among women ages 15-49 migraine is actually the leading cause of disability [230]. Calcitonin gene-related peptide (CGRP) has long been thought to contribute to migraine. Studies indicate that CGRP is elevated in the blood, saliva, and cerebrospinal fluid of migraine patients when they experience an attack [86]. Additionally, intravenous administration of CGRP will elicit a migraine attack in migraineurs [256]. Recent work has demonstrated that CGRP may lead to female-specific migraine like-behavior in rodent models of migraine, contingent on the presence of the predominantly female hormone prolactin [12; 13]. There has also been increasing evidence that the calcitonin family peptide amylin is involved in migraine. Expression of amylin and the components of the amylin receptors AMY1 calcitonin receptor (CTR) and receptor activity modifying proteins (in this case RAMP1), AMY2 (CTR and RAMP2), and AMY3 (CTR and RAMP3) have all been found within the trigeminal ganglion (TG) [84; 260]. Additionally, clinical trials have demonstrated the ability of the amylin analogue pramlintide to induce migraine in patients without aura [101].

Amylin and CGRP are both comprised of 37 amino acids, 16 of which are shared. Furthermore, both the canonical CGRP receptor and the AMY1 receptor include RAMP1 as a

receptor component. [124] Amylin and CGRP share equal affinity for the AMY1 receptor [124]. Although in the canonical CGRP receptor RAMP1 is coupled with calcitonin-like receptor (CLR) in place of CTR, both CGRP and amylin signal through G-protein coupled receptors leading to increases in cAMP [153; 182]. Despite these similarities, little is known about how amylin is involved in the migraine pathology. Amylin is co-secreted with insulin and is heavily involved in regulation of glucose levels. Additionally, CGRP and amylin inhibit insulin secretion [118; 200; 240], this is particularly intriguing because hyperinsulinism, hypoglycemia, and hyperglycemia are all associated with migraine [22; 28; 51; 109; 115].

Given this potential role for metabolic instability in migraine, we additionally sought to explore the role of PACAP-38. PACAP-38 has been implicated in the migraine pathology. Similarly, to amylin and CGRP PACAP-38 is capable of inducing migraines in migraine patients [218]. PACAP and CGRP both cause similar light aversive behaviors in mice [148]. One receptor for PACAP-38, the VPAC1 receptor also associates with RAMP1 [54]. PACAP-38 additionally signals via cAMP, however, one striking difference is that while amylin and CGRP lead to inhibition of insulin [223], PACAP-38 is insulinotropic [30]. Given these similarities in signaling and function this study aims to explore the potential role for amylin and PACAP-38 in previously established rodent migraine models, as well as to determine whether these peptides contribute to the sexually dimorphic nature of migraine, in the nature of the results we have previously seen with dural application of CGRP [12].

METHODS:

Dural injection

Dural injections were preformed according to previously published methods [39]. Animals were briefly placed under anesthesia using 1.5% isoflurane administered via a nose cone for less than 2 minutes. While anesthetized, drugs were administered onto the dura via a modified internal cannula internal-cannula (Invivo1, part #8IC313ISPCXC, Internal Cannula, standard, 28 gauge, fit to 0.5 mm). The inner projection length was modified to be approximately 0.6mm in length. This inner portion of the projection was inserted through the soft tissue at the junction of the lambdoidal and sagittal sutures. All dural injections were administered in a volume of 5 µl.

Hindpaw injection

Intraplantar injections were administered into the hindpaw of mice in a volume of 5 µl according to previously published methods. Intraplantar injections were preformed while animals were anesthetized in the same manner as animals that received dural injections. Animals were anesthetized under 1.5% isoflurane administered via a nose cone for less than 2 minutes, during which time amylin was injected with a 30-gauge needle attached to a Hamilton 700 microliter syringe (Hamilton™ 80401). All injections were preformed into the animals left hindpaw.

Facial von Frey

Prior to and following dural injections animals were subject to facial von Frey. Animals were all handled for a single 5-minute session 24 hours prior to habituation to testing chambers and room conditions. Each habituation session was carried out in rooms where all further testing would be conducted. A habituation session consists of placing animals in 4oz paper cups (Choice) within an individual testing chamber for 2 hours a day as previously described [39]. Filaments are then applied to the periorbital region of the face via the Dixon up-down method. Beginning with 0.07 g each filament is applied for a maximum of 5 seconds or until the animal responds. A response is counted if the animal swipes at the filament. If the mouse does not respond to the tested filament, filaments of increasing gram weights are tested. In order these filaments would be 0.07 g, 0.2 g, 0.4 g, and a maximum of 0.6 g. If an animal responds to the tested filament, the filament of the next lower gram weight is applied. The descending order of filaments is 0.07 g, 0.02 g, and 0.008 g which is the minimum.

Hindpaw von Frey

Animals that received intraplantar injections of amylin subsequently had mechanical withdrawal thresholds assessed with von Frey filaments prior to and following injection. Only the hindpaws that were injected were tested. An initial gram weight of 0.6 g was applied to the center of the paw away from the digits. If an animal did not respond this gram weight was increased to 1.0 g, 1.4 g, and finally 2.0g. If the animal does not respond to 2.0 g the animal is considered to have reached a baseline hindpaw withdrawal threshold. All animals are required to meet this baseline

prior to inclusion in the study and injection. If an animal responds to the initial filament the gram weight is decreased to 0.4 g, 0.2 g, and finally 0.07 g which is considered to be the minimum.

Grimace scoring

Grimace behaviors were assessed according to previously published methods [151]. Animals were observed for grimacing behaviors prior to establishing von Frey withdrawal thresholds at each time point. The grimace score averages 5 characteristic pain-like behaviors that are scored as either 0 (not present), 1 (somewhat present), 2 (clearly present). These behaviors are comprised of orbital tightening, nose bulging, cheek bulging, flattening of the ears, and flattening of the whiskers. All characteristics are weighted the same in the average.

Glucose Measurement

Glucose was measured using a glucometer all glucose levels were measured while animals were fasting for 12 hours. All animals were fasted overnight (during dark cycle). Blood was taken from tail tips of each animal. And measured on the glucometer. Results are reported as mg/dl.

Drugs

Please refer to table 6.1 for all information regarding drug sources, doses, and administration routes.

Table 6.1: Drug information

Drug	Source	Doses	Administration route
Amylin	Bachem	0.1 ng, 0.1 μg	Dural, Intraplantar
CGRP	Bachem	1 pg	Dural
PACAP-38	Bachem	10 ng, 0.1 μg	Dural

Statistics

All data shown here are represented as SEM. All Data were analyzed using Prism (Graph-Pad Software) All behavioral data were analyzed among groups at each time point via a two-way ANOVA and followed by Bonferroni post-hoc test where it was appropriate. Differences in blood glucose level were assessed via one-way ANOVA. Significance was set to $p < 0.05$ for all analyses. For all experiments testers were blinded to treatment group. Prior to all experiments animals were allocated to groups via random selection of pre-labeled paper slips.

RESULTS

Dural amylin leads to facial hypersensitivity in male, but not in female mice.

Male mice that received 0.1 μg amylin onto the dura exhibited significant facial hypersensitivity acutely when compared with control animals that received vehicle at 1- and 3-hours following injection (Fig. 1A). A separate cohort of male mice that received a lower dose of 0.1 ng amylin onto the dura failed to exhibit any significant hypersensitivity when compared with controls (Fig.

1C). These data suggest that high concentrations of amylin may lead to migraine like behaviors in male mice. Female mice that received dural administration of 0.1 µg amylin showed no significant facial hypersensitivity when compared with animals that received dural injection of vehicle either acutely, at 1-, 3-, or 5-hours following injection, or sustained out to 72 hours following injection (Fig. 1B). Female mice that received 0.1 ng also did not demonstrate any significant hypersensitivity when compared with controls (Fig. 1D). This suggests that the ability of dural amylin to induce migraine-like behavior in mice is specific to males.

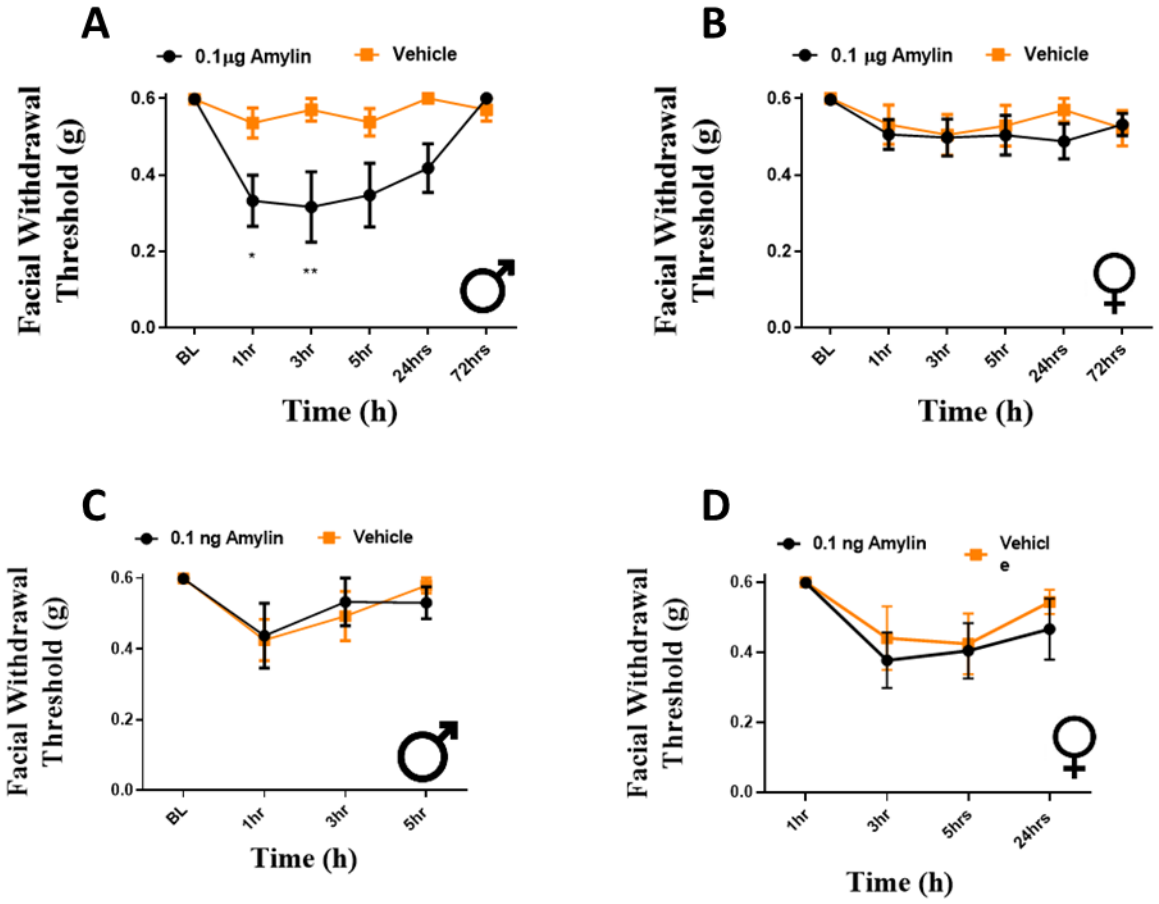

Figure 6.1: Dural amylin leads to facial hypersensitivity in male, but not in female mice.

Dural amylin was administered to both male and female mice following baseline facial withdrawal assessment. Amylin administered at 0.1 µg resulted in significant facial hypersensitivity in male mice when compared with controls (n = 6 amylin, 5 vehicle) no significant facial hypersensitivity was observed in females at this dose (n= 12 amylin, 9 vehicle). When 0.1 ng amylin was given on to the dura of male (n = 6 amylin, 6 vehicle) and female (n = 9 amylin, 5 vehicle) no significant hypersensitivity was observed. *p< 0.05, ** p<0.01.

Dural amylin produces grimacing behaviors in male mice, but not in female mice.

Given the finding that amylin results in facial hypersensitivity in male mice we aimed to determine whether this sex-specific effect was true of additional pain behaviors. Cohorts of animals that were separate from those subjected to von Frey received either 0.1 µg, 0.1 ng, or vehicle SIF, and were then observed for grimace every other hour beginning at 1 hour post injection. Male mice that received 0.1 µg of amylin exhibited significant grimace when compared with males that received either vehicle or 0.1 ng at 1 hour following injection (Fig. 2A). No significant grimace was observed in females that received either 0.1 µg or 0.1 ng amylin at any time point (Fig. 2B).

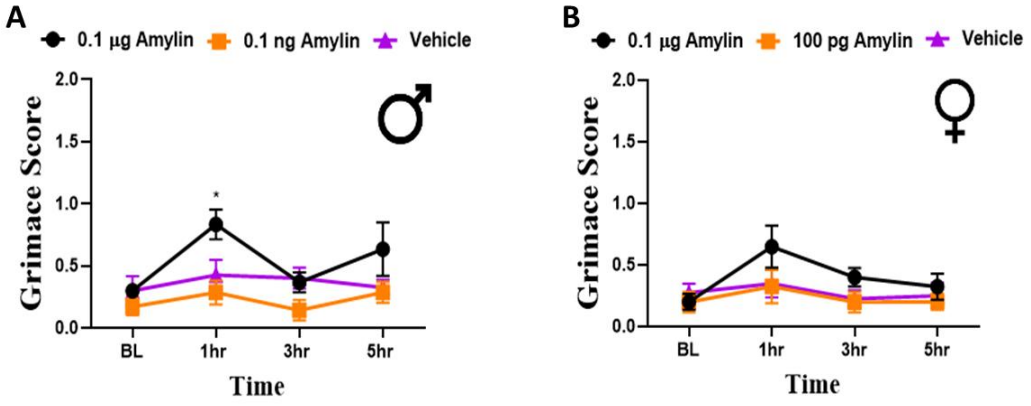

Figure 6.2: Dural amylin produces grimacing behaviors in male mice, but not in female mice.

Male and female mice were observed for grimacing behaviors prior to and following dural injection of either 0.1 μg amylin, 0.1 ng amylin, or vehicle. Two-way ANOVA followed by Bonferroni post hoc analysis indicated significant grimace caused by Dural 0.1 μg amylin caused in male mice at 1 hour following injection when compared with males that received vehicle. No significant responses were observed in males that received 0.1 ng amylin or vehicle. No significant grimace was observed in any group of females at any time tested. *$p < 0.05$.

Dural amylin produces robust facial hypersensitivity in ovariectomized mice.

In order to examine the potential role for ovarian secreting hormones in the observed sexually dimorphic responses to dural amylin, female mice that had undergone ovariectomy received 0.1 μg of amylin onto the dura. In contrast to intact females those that had ovariectomy exhibited significant and robust allodynia in response to amylin at 1 and 3 hours post injection (Fig. 3A). Ovariectomized animals also showed significant acute grimace at 3 and 5 hours following dural administration of amylin (Fig. 3B). This suggests that ovarian hormones may in some part suppress hypersensitivity caused by dural amylin. Interestingly, ovariectomized mice that received dural injection of 1 pg CGRP, previously reported to cause robust female-specific facial hypersensitivity [12], demonstrated no significant hypersensitivity at any time point when compared with controls. These animals were observed for grimace behaviors prior to von Frey testing at each time point. OVX mice that received amylin demonstrated significant grimace behaviors at 1- and 3-hours following injection. Although we have previously reported that female mice that receive dural 1 pg CGRP exhibit significant grimace behavior at 3, 5, and 24 hours [12], ovariectomized females that received this same dose of CGRP demonstrated no significant grimace behaviors when compared with controls.

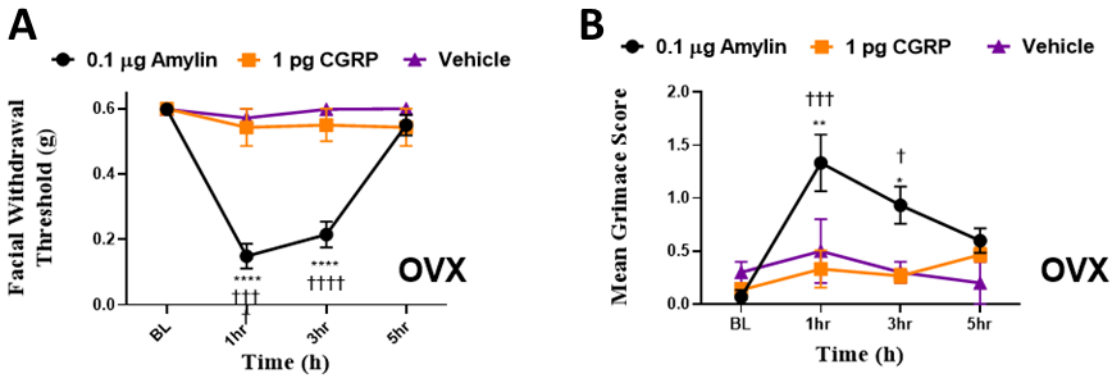

Figure 6.3: Dural amylin produces robust facial hypersensitivity in ovariectomized mice.

Female mice that had been ovariectomized had periorbital mechanical withdrawal thresholds assessed prior to and following dural application of either 0.1 µg amylin (n= 6), 1 pg CGRP (n=3), or vehicle (n=6). Grimace was assessed in a separate co-hort of ovariectomized mice. Two-way ANOVA indicated significant effect of dural amylin on both mechanical withdrawal thresholds and grimacing behaviors. Asterix denote significance between animals that received amylin and vehicle, dagger marks denote significance between amylin and CGRP groups. * p< 0.05, ** p<0.01, ***p< 0.001, ****p<0.0001.

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Fasting blood glucose levels are increased in ovariectomized females.

Given the ability of amylin to decrease blood glucose levels, and the stark behavioral differences we observed between male, intact female, and OVX females, we sought to measure blood glucose levels [180; 193]. Average male blood glucose levels were 50.25 mg/dl, intact females averaged 56.63 mg/dl, and ovariectomized females had an average blood glucose level of 74.50 mg/dl (Fig. 4A). The differences in blood glucose levels between males and intact females were not found to be significant (p = 0.6128); however, ovariectomized females had significantly higher blood glucose levels when compared with both intact female (p = 0.0047) and male mice

($p = 0.0026$). This may reflect a decrease in amylin production. In support of this OVX females had significantly increased weight when compared with intact age-matched controls (Fig. 4B).

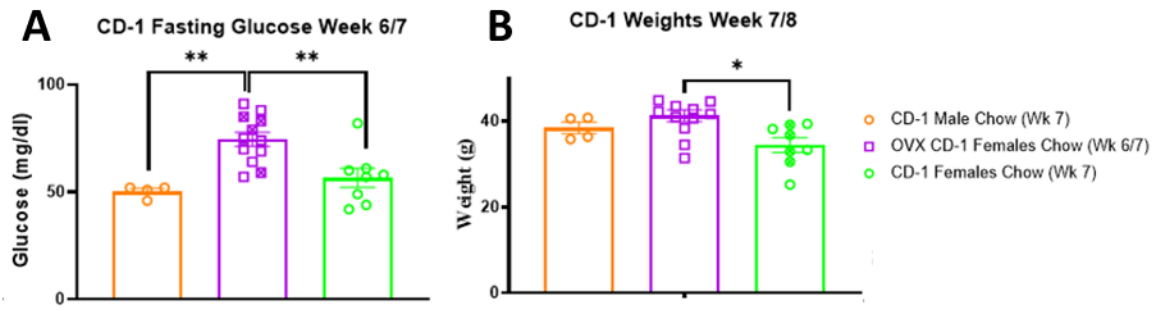

Figure 6.4: Fasting blood glucose levels are increased in ovariectomized females.

Blood glucose levels were measured from fasted animals. One-way ANOVA of blood glucose levels followed by Tukey post hoc analysis indicated that female OVX animals had significantly increased blood glucose levels when compared with intact female and male mice. There were significant differences in weight of OVX and intact females. * $p < 0.05$, ** $p < 0.01$.

Intraplantar amylin leads to male specific hypersensitivity.

Given previous findings showing that CGRP exerts female-specific hypersensitivity when administered peripherally both onto the dura and into the hindpaw of rodents [12], we aimed to see whether the sex-specific effects of amylin in males were restricted to the dura or maintained throughout multiple peripheral tissues. Injection of 0.1 µg amylin into the hindpaw of male mice caused significant allodynia at 1 hour when compared with controls (Fig. 5A). Injection of the same dose of amylin into the hindpaw of female mice resulted in no significant responses between animals that received vehicle or amylin at any time point tested (Fig. 5B).

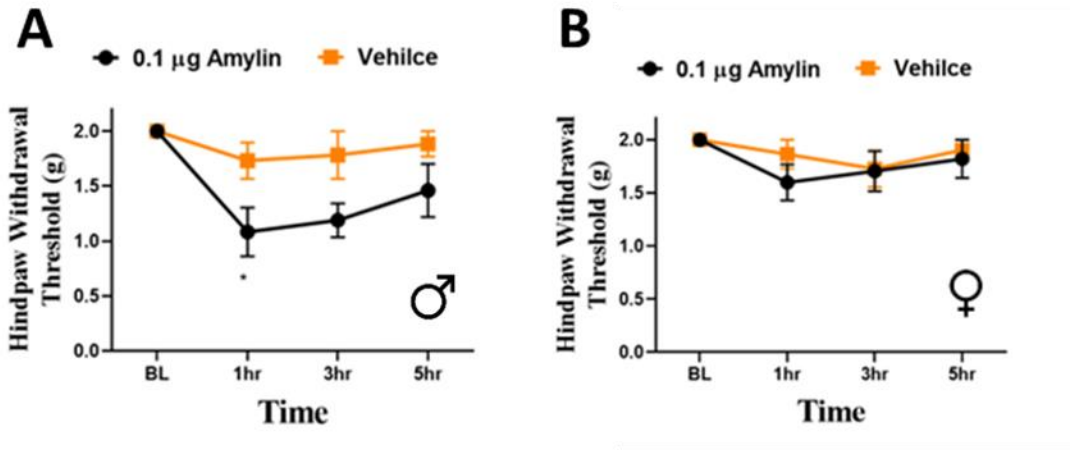

Figure 6.5: Intraplantar amylin produces hypersensitivity in the male hindpaw.
Female and male mice had hindpaw withdrawal thresholds assessed before and after intraplantar injection of 0.1 µg amylin. Two-way ANOVA and Bonferroni post hoc analysis revealed significant effect of treatment in male (n = 5 amylin, 6 vehicle) mice at one hour following injection. No significant effect of treatment was found in female mice (n = 6 amylin, 6 vehicle). * $p < 0.05$.

Dural PACAP-38 leads to facial allodynia in both male and female mice.

Due to the association of amylin with diabetes and the co-secretion of amylin with insulin, we decided to examine potential sex-specific effects of the insulinotropic migraine related peptide PACAP-38. Dural administration of 0.1 µg PACAP-38 resulted in robust facial hypersensitivity that lasted from 1 hour out to 72 hours following injection in both male (Fig. 6A) and female mice (Fig. 6B).

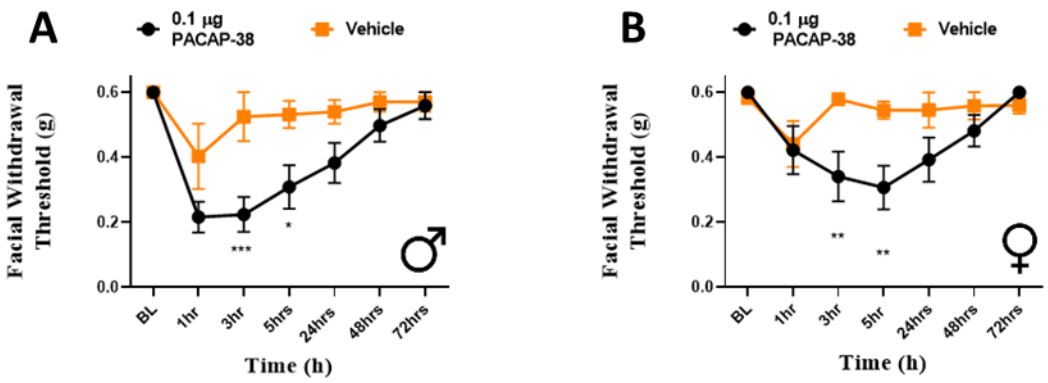

Figure 6.6: Dural PACAP-38 leads to facial allodynia in both male and female mice.
Male and female mice had facial withdrawal thresholds assessed prior to and following 0.1 µg of amylin (n = 8 females, 6 males) or vehicle (n = 8 females, 5 males). Two-way ANOVA followed by Bonferroni post hoc analysis indicated significant effect of treatment in both male and female mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

DISCUSSION

Here we show that dural administration of 0.1 µg amylin results in facial hypersensitivity and grimace in male, but not female mice. Dural 0.1 ng amylin results in no significant facial hypersensitivity in male or female mice. However, this same dose of amylin applied onto the dura of ovariectomized mice leads to robust facial hypersensitivity. A dose of CGRP that was previously reported to be noxious when applied to the dura of intact female mice, results in no facial hypersensitivity when administered to ovariectomized mice. Additionally, ovariectomized females have significantly higher blood glucose levels when compared with intact females and males. Intraplantar injection of 0.1 µg amylin causes hypersensitivity in the hindpaw of male mice, but not in females. Finally, despite similarities in amylin and PACAP-38 dural

administration of 0.1 μg PACAP-38 resulted in significant facial hypersensitivity of similar time and magnitude in both male and female mice.

Given similarities in function and signaling between CGRP and amylin, the finding that dural amylin causes facial allodynia in male but not in female mice may seem at odds with previously reported findings that CGRP produces robust migraine-like behavior in female but not male mice [12]. However, it is not certain that male mice would not exhibit sensitivity to higher doses of dural CGRP, as CGRP injected subcutaneously into the periorbital region of the face produces facial hypersensitivity in male mice [66]. Nevertheless, despite shared affinity for the AMY1 receptor [124; 125] CGRP and amylin have clear opposing sex-dependent effects when applied to the dura at the tested doses. This suggests that these nociceptive effects of amylin and CGRP may not be dependent on activation of the AMY1 receptor, but rather CGRP induced effects may be mediated by activation of the CGRP receptor. In regard to amylin specific effects, these may be mediated by AMY2 (CTR + RAMP2), and/or AMY3 (CTR + RAMP3) receptors which are solely activated by amylin [124]. Alternatively, these effects may still be mediated in part by the AMY1 receptor, but because the functions of CLR and CTR are dependent on the expression of, and coupling with, certain RAMPs [178] the sexually dimorphic effects of dural amylin and dural CGRP could result from differential expression of CTR, CLR, and RAMPs within the dura between male and female mice. While this has not been yet explored in the dura or TG, female rats express higher levels of RCP in the trigeminal nucleus caudalis (TNC) than their male counter parts [137]. RCP is required for the CGRP receptor but not the amylin receptors, to couple with Gs [224]. When taking this into consideration with our observations

here these data may indicate that there are more CGRP receptors in the female dura are more abundant when compared with males, rather there may also be increased AMY1 receptor in the dura of males. In order to make this claim, future studies examining the potential difference in sex-dependent expression of amylin and CGRP receptors throughout the dura and trigeminal ganglia are warranted.

Here we present data showing that ovariectomy results in robust facial hypersensitivity in response to dural amylin. This suggests that ovarian derived hormones may protect against nociception caused by amylin. This is interesting when comparing the finding that a previously noxious dose of dural CGRP [12] administered to OVX mice no longer causes facial hypersensitivity, this may indicate that ovarian hormones are required to elicit CGRP dependent migraine. Although we have shown that the facial hypersensitivity caused by dural CGRP is contingent on prolactin, this is not likely to be ovarian produced prolactin, as serum prolactin levels have not been found to be altered following ovariectomy in rats [13; 239]. Furthermore, we have previously noted that ovariectomy in mice does not prevent responses to dural prolactin [13]. The ablation of the CGRP response in OVX mice may instead reflect conflicting reports regarding the possible contribution of estrogen to migraine. Increases and decreases in estrogen have both been reported to cause migraine in women [1; 4; 107; 167; 173]. While the interactions between estrogen and CGRP are likely to be complex there is substantial evidence that estrogen and CGRP interact with one another [150]. Estradiol leads to increased CGRP released from perivascular nerves within the dura, although it does not alter dural vasodilation in response to treatment with CGRP [113]. Women with low estrogen levels have increased dermal

blood-flow to the forehead resulting from capsaicin [128]. This capsaicin induced dermal blood flow to this region is thought to be mediated via CGRP [225]. In female rats baseline CGRP release from TGs is higher in pro-estrous (higher estrogen) when compared with the estrous phase of the reproductive cycle (low estrogen) [262], conversely ovariectomy increases CGRP mRNA in the rat TG [3]. The increase in CGRP following ovariectomy could ultimately lead to decreased CGRP sensitivity including the reduced sensitivity to CGRP we observe here. As repeated CGRP stimulation leads to desensitization of CGRP evoking stimuli [111].

The hypersensitivity that occurs following dural stimulation with amylin in OVX, but not intact females would suggest that ovarian secreted hormones reduce the response to amylin. While potential interactions between estrogen and amylin have not been deeply explored in the context of the migraine pathology, estradiol attenuated amylin induced weight loss in OVX rats [165; 248]. The data here showing that ovariectomy results in facial hypersensitivity to dural amylin may indicate estrogen further prevents amylin induced behaviors, including pain. However, further studies are required to ascertain the potential interactions between amylin and estrogen within the TG or dura.

While hormone induced alterations of calcitonin family receptors have not been explored in the meninges, they have been studied in the uterine wall of rodents, wherein treatment with exogenous progesterone has been found to increase CLR, RAMP1, in both intact and OVX rats [245; 246]. This presents a possibility that the loss of ovarian derived progesterone in OVX animals could lead to decreases in CGRP receptor components within the meninges, and thereby lead to decreased sensitivity to dural CGRP. In OVX animals progesterone treatment does not

alter the presence of RAMP2 and RAMP3 in intact females, nor does it alter the presence of RAMP3 in OVX animals [245; 246]. Therefore, this could be specific to loss of CGRP sensitivity.

As previously mentioned, fluctuations in insulin and glucose levels may also contribute to migraine. Both amylin and CGRP inhibit further insulin release. Amylin has demonstrated sex-dependent effects on glucose levels. Amylin deficient male mice have increased insulin responses and faster blood glucose elimination in response to glucose administration when compared with WT controls. However, responses to glucose treatment were no different in female amylin deficient mice and their sex matched WT controls [100]. This suggests that there is a sex-dependent role for amylin in insulin secretion and blood glucose elimination. More recently, amylin has been found to have differential sex-specific effects on food intake in RAMP1 and RAMP3 KO mice[56]. Because hyperglycemia, hypoglycemia, and hyperinsulinemia have all been noted to trigger migraine in migraineurs [51; 100; 109]. Here we show that ovariectomy leads to increased fasting blood glucose levels which may indicate altered insulin and amylin levels that may also impact hypersensitivity to amylin and CGRP.

The finding that intraplantar amylin leads to hypersensitivity in the hindpaw of male mice, but not in female mice, suggests that there are body wide sex-dependent differences to peripheral amylin. This agrees with our previous findings that CGRP applied both onto the dura and into the hindpaw of mice resulted in female-specific hypersensitivity [12]. While these data signify that peripheral administration of CGRP and amylin lead to female and male specific hypersensitivity respectively. Subcutaneous injection of amylin into the periorbital region has

failed to induce facial hypersensitivity in male mice and administration of CGRP into the same region has resulted in facial hypersensitivity in male mice [66]. This disparity likely results from the route of administration. In conjunction, we have previously observed that subcutaneous injection of the migraine associated substance IL-6 also does not result in facial hypersensitivity while injection of the same dose of IL-6 applied to the dura results in robust facial hypersensitivity [15].

Although PACAP-38 has many similarities with both CGRP and amylin dural PACAP-38 resulted in no sex-specific effect. Rather both female and male mice demonstrated reduced facial withdrawal thresholds of similar magnitude and time course. This is not unusual as decreased dural pH, IL-6, inflammatory soup, and mustard oil have all produced similar behavioral responses in male and female mice [39]. This may further indicate that activation of certain CGRP and amylin receptors at the doses tested here are required to elicit the observed sexually dimorphic effects.

Together these data not only have implications for sexually dimorphic causes of migraine pain but may offer insights for the development of sex-specific therapeutics. Small molecule CGRP receptor antagonists including Rimegepant, Telcagepant, and Olcegepant while all more selective for CGRP activity at the CGRP receptor, also have some efficacy at blocking both CGRP activity at the AMY1 receptor and amylin activity at the AMY1 receptor [99]. The data here would suggest that because CGRP and amylin may differentially contribute to migraine in males and females there may be altered efficacy of these therapeutics in men and women with migraine based on their receptor selectivity. These data also advocate for further exploration of

the efficacy of monoclonal antibodies against the CGRP receptor in both men and women. Lastly, these data propose that amylin receptor antagonists could potentially be developed to serve as migraine therapeutics particularly in male migraineurs.

REFERENCES

[1] Headache Classification Committee of the International Headache Society (IHS) The International Classification of Headache Disorders, 3rd edition. Cephalalgia 2018;38(1):1-211.

[2] Aggarwal M, Puri V, Puri S. Effects of estrogen on the serotonergic system and calcitonin gene-related peptide in trigeminal ganglia of rats. Ann Neurosci 2012;19(4):151-157.

[3] Allais G, Chiarle G, Sinigaglia S, Airola G, Schiapparelli P, Benedetto C. Estrogen, migraine, and vascular risk. Neurol Sci 2018;39(Suppl 1):11-20.

[4] Avona A, Burgos-Vega C, Burton MD, Akopian AN, Price TJ, Dussor G. Dural Calcitonin Gene-Related Peptide Produces Female-Specific Responses in Rodent Migraine Models. J Neurosci 2019;39(22):4323-4331.

[5] Avona A, Mason BN, Burgos-Vega C, Hovhannisyan AH, Belugin SN, Mecklenburg J, Goffin V, Wajahat N, Price TJ, Akopian AN, Dussor G. Meningeal CGRP-Prolactin interaction evokes female-specific migraine behavior. Ann Neurol 2021.

[6] Avona A, Price TJ, Dussor G. Interleukin-6 induces spatially dependent whole-body hypersensitivity in rats: implications for extracephalic hypersensitivity in migraine. J Headache Pain 2021;22(1):70.

[7] Bernecker C, Pailer S, Kieslinger P, Horejsi R, Moller R, Lechner A, Wallner-Blazek M, Weiss S, Fazekas F, Truschnig-Wilders M, Gruber HJ. GLP-2 and leptin are associated with hyperinsulinemia in non-obese female migraineurs. Cephalalgia 2010;30(11):1366-1374.

[8] Blau JN, Pyke DA. Effect of diabetes on migraine. Lancet 1970;2(7666):241-243.

[9] Borboni P, Porzio O, Pierucci D, Cicconi S, Magnaterra R, Federici M, Sesti G, Lauro D, D'Agata V, Cavallaro S, Marlier LN. Molecular and functional characterization of pituitary adenylate cyclase-activating polypeptide (PACAP-38)/vasoactive intestinal polypeptide receptors in pancreatic beta-cells and effects of PACAP-38 on components of the insulin secretory system. Endocrinology 1999;140(12):5530-5537.

[10] Burgos-Vega CC, Quigley LD, Trevisan Dos Santos G, Yan F, Asiedu M, Jacobs B, Motina M, Safdar N, Yousuf H, Avona A, Price TJ, Dussor G. Non-invasive dural stimulation in mice: A novel preclinical model of migraine. Cephalalgia 2019;39(1):123-134
.

[11] Cavestro C, Rosatello A, Micca G, Ravotto M, Marino MP, Asteggiano G, Beghi E. Insulin metabolism is altered in migraineurs: a new pathogenic mechanism for migraine? Headache 2007;47(10):1436-1442.

[12] Christopoulos A, Christopoulos G, Morfis M, Udawela M, Laburthe M, Couvineau A, Kuwasako K, Tilakaratne N, Sexton PM. Novel receptor partners and function of receptor activity-modifying proteins. J Biol Chem 2003;278(5):3293-3297.

[13] Coester B, Pence SW, Arrigoni S, Boyle CN, Le Foll C, Lutz TA. RAMP1 and RAMP3 Differentially Control Amylin's Effects on Food Intake, Glucose and Energy Balance in Male and Female Mice. Neuroscience 2020;447:74-93.

[14] De Logu F, Landini L, Janal MN, Li Puma S, De Cesaris F, Geppetti P, Nassini R. Migraine-provoking substances evoke periorbital allodynia in mice. J Headache Pain 2019;20(1):18.

[15] Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2018;392(10159):1789-1858.

[16] Edvinsson L, Grell AS, Warfvinge K. Expression of the CGRP Family of Neuropeptides and their Receptors in the Trigeminal Ganglion. J Mol Neurosci 2020;70(6):930-944.

[17] Edvinsson L, Haanes KA, Warfvinge K, Krause DN. CGRP as the target of new migraine therapies - successful translation from bench to clinic. Nat Rev Neurol 2018;14(6):338-350.

[18] Garelja ML, Walker CS, Hay DL. CGRP receptor antagonists for migraine. Are they also AMY1 receptor antagonists? Br J Pharmacol 2021.

[19] Gebre-Medhin S, Mulder H, Pekny M, Westermark G, Tornell J, Westermark P, Sundler F, Ahren B, Betsholtz C. Increased insulin secretion and glucose tolerance in mice lacking islet amyloid polypeptide (amylin). Biochem Biophys Res Commun 1998;250(2):271-277.

[20] Ghanizada H, Al-Karagholi MA, Walker CS, Arngrim N, Rees T, Petersen J, Siow A, Morch-Rasmussen M, Tan S, O'Carroll SJ, Harris P, Skovgaard LT, Jorgensen NR, Brimble M, Waite JS, Rea BJ, Sowers LP, Russo AF, Hay DL, Ashina M. Amylin Analog Pramlintide Induces Migraine-like Attacks in Patients. Ann Neurol 2021;89(6):1157-1171.

[21] Granella F, Sances G, Zanferrari C, Costa A, Martignoni E, Manzoni GC. Migraine without aura and reproductive life events: a clinical epidemiological study in 1300 women. Headache 1993;33(7):385-389.

[22] Gruber HJ, Bernecker C, Pailer S, Fauler G, Horejsi R, Moller R, Lechner A, Fazekas F, Truschnig-Wilders M. Hyperinsulinaemia in migraineurs is associated with nitric oxide stress. Cephalalgia 2010;30(5):593-598.

[23] Gupta S, Amrutkar DV, Mataji A, Salmasi H, Hay-Schmidt A, Sheykhzade M, Messlinger K, Olesen J, Jansen-Olesen I. Evidence for CGRP re-uptake in rat dura mater encephali. Br J Pharmacol 2010;161(8):1885-1898.

[24] Gupta S, Villalon CM, Mehrotra S, de Vries R, Garrelds IM, Saxena PR, MaassenVanDenbrink A. Female sex hormones and rat dural vasodilatation to CGRP, periarterial electrical stimulation and capsaicin. Headache 2007;47(2):225-235.

[25] Haghighi FS, Rahmanian M, Namiranian N, Arzaghi SM, Dehghan F, Chavoshzade F, Sepehri F. Migraine and type 2 diabetes; is there any association? J Diabetes Metab Disord 2015;15(1):37.

[26] Halloran J, Lalande A, Zang M, Chodavarapu H, Riera CE. Monoclonal therapy against calcitonin gene-related peptide lowers hyperglycemia and adiposity in type 2 diabetes mouse models. Metabol Open 2020;8:100060.

[27] Hay DL, Garelja ML, Poyner DR, Walker CS. Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. Br J Pharmacol 2018;175(1):3-17.

[28] Hay DL, Walker CS. CGRP and its receptors. Headache 2017;57(4):625-636.

[29] Ibrahimi K DA, Villalón CM, van den Meiracker AH, MaassenVanDenBrink A. Influence of varying estrogen levels on trigeminal CGRP release in healthy women. The Journal of Headache and Pain 2013;14:123.

[30] Ji Y, Rizk A, Voulalas P, Aljohani H, Akerman S, Dussor G, Keller A, Masri R. Sex differences in the expression of calcitonin gene-related peptide receptor components in the spinal trigeminal nucleus. Neurobiol Pain 2019;6:100031.

[31] Kuburas A, Mason BN, Hing B, Wattiez AS, Reis AS, Sowers LP, Moldovan Loomis C, Garcia-Martinez LF, Russo AF. PACAP Induces Light Aversion in Mice by an Inheritable Mechanism Independent of CGRP. J Neurosci 2021;41(21):4697-4715.

[32] Labastida-Ramirez A, Rubio-Beltran E, Villalon CM, MaassenVanDenBrink A. Gender aspects of CGRP in migraine. Cephalalgia 2019;39(3):435-444.

[33] Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS. Coding of facial expressions of pain in the laboratory mouse. Nat Methods 2010;7(6):447-449.

[34] Laufer R, Changeux JP. Calcitonin gene-related peptide elevates cyclic AMP levels in chick skeletal muscle: possible neurotrophic role for a coexisting neuronal messenger. EMBO J 1987;6(4):901-906.

[35] Lutz TA. Amylin may offer (more) help to treat postmenopausal obesity. Endocrinology 2011;152(1):1-3.

[36] MacGregor EA, Frith A, Ellis J, Aspinall L, Hackshaw A. Incidence of migraine relative to menstrual cycle phases of rising and falling estrogen. Neurology 2006;67(12):2154-2158.

[37] Massiou H, MacGregor EA. Evolution and treatment of migraine with oral contraceptives. Cephalalgia 2000;20(3):170-174.

[38] McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 1998;393(6683):333-339.

[39] Mietlicki-Baase EG. Amylin-mediated control of glycemia, energy balance, and cognition. Physiol Behav 2016;162:130-140.

[40] Morfis M, Tilakaratne N, Furness SG, Christopoulos G, Werry TD, Christopoulos A, Sexton PM. Receptor activity-modifying proteins differentially modulate the G protein-coupling efficiency of amylin receptors. Endocrinology 2008;149(11):5423-5431.

[41] Otto-Buczkowska E, Mazur-Dworzecka U, Dworzecki T. [Role of amylin in glucose homeostasis and its perspective use in diabetes management]. Przegl Lek 2008;65(3):135-139.

[42] Pettersson M, Ahren B, Bottcher G, Sundler F. Calcitonin gene-related peptide: occurrence in pancreatic islets in the mouse and the rat and inhibition of insulin secretion in the mouse. Endocrinology 1986;119(2):865-869.

[43] Schytz HW, Birk S, Wienecke T, Kruuse C, Olesen J, Ashina M. PACAP38 induces migraine-like attacks in patients with migraine without aura. Brain 2009;132(Pt 1):16-25.

[44] Silvestre RA, Salas M, Garcia-Hermida O, Fontela T, Degano P, Marco J. Amylin (islet amyloid polypeptide) inhibition of insulin release in the perfused rat pancreas: implication of the adenylate cyclase/cAMP system. Regul Pept 1994;50(2):193-199.

[45] Simms J, Routledge S, Uddin R, Poyner D. The Structure of the CGRP and Related Receptors. Handb Exp Pharmacol 2019;255:23-36.

[46] Sinclair SR, Kane SA, Van der Schueren BJ, Xiao A, Willson KJ, Boyle J, de Lepeleire I, Xu Y, Hickey L, Denney WS, Li CC, Palcza J, Vanmolkot FH, Depre M, Van Hecken A, Murphy MG, Ho TW, de Hoon JN. Inhibition of capsaicin-induced increase in dermal blood flow by the oral CGRP receptor antagonist, telcagepant (MK-0974). Br J Clin Pharmacol 2010;69(1):15-22.

[47] Steiner TJ, Stovner LJ, Vos T, Jensen R, Katsarava Z. Migraine is first cause of disability in under 50s: will health politicians now take notice? J Headache Pain 2018;19(1):17.

[48] Szawka RE, Rodovalho GV, Helena CV, Franci CR, Anselmo-Franci JA. Prolactin secretory surge during estrus coincides with increased dopamine activity in the hypothalamus and preoptic area and is not altered by ovariectomy on proestrus. Brain Res Bull 2007;73(1-3):127-134.

[49] Tanaka H, Kashiwagi R, Koizumi T. Inhibition of calcitonin gene-related peptide (CGRP) has the potential to extend first-phase insulin secretion. Exp Clin Endocrinol Diabetes 2013;121(5):280-285.

[50] Thota C, Gangula PR, Dong YL, Yallampalli C. Changes in the expression of calcitonin receptor-like receptor, receptor activity-modifying protein (RAMP) 1, RAMP2, and RAMP3 in rat uterus during pregnancy, labor, and by steroid hormone treatments. Biol Reprod 2003;69(4):1432-1437.

[51] Thota C, Yallampalli C. Progesterone upregulates calcitonin gene-related peptide and adrenomedullin receptor components and cyclic adenosine 3'5'-monophosphate generation in Eker rat uterine smooth muscle cell line. Biol Reprod 2005;72(2):416-422.

[52] Trevaskis JL, Turek VF, Wittmer C, Griffin PS, Wilson JK, Reynolds JM, Zhao Y, Mack CM, Parkes DG, Roth JD. Enhanced amylin-mediated body weight loss in estradiol-deficient diet-induced obese rats. Endocrinology 2010;151(12):5657-5668.

[53] Vetvik KG, MacGregor EA. Sex differences in the epidemiology, clinical features, and pathophysiology of migraine. Lancet Neurol 2017;16(1):76-87.

[54] Villalon CM, Olesen J. The role of CGRP in the pathophysiology of migraine and efficacy of CGRP receptor antagonists as acute antimigraine drugs. Pharmacol Ther 2009;124(3):309-323.

[55] Walker CS, Hay DL. CGRP in the trigeminovascular system: a role for CGRP, adrenomedullin and amylin receptors? Br J Pharmacol 2013;170(7):1293-1307.

[56] Warfvinge K, Krause DN, Maddahi A, Edvinsson JCA, Edvinsson L, Haanes KA. Estrogen receptors alpha, beta and GPER in the CNS and trigeminal system - molecular and functional aspects. J Headache Pain 2020;21(1):131.

CHAPTER 7

DIFFERENTIAL SEX-SPECIFIC RESPONSES IN MIGRAINE ARE LIKELY MEDIATED BY HORMONES AND CALCITONIN FAMILY-PEPTIDES

The data shown here demonstrate that CGRP is likely involved in female-specific migraine related pain. Females are more sensitive to dural CGRP than their male counterparts. This is not to say that CGRP is not involved in migraine attacks in males, but that smaller amounts of CGRP may be required to elicit migraine in females. The reasons for this seem to be contingent on the presence of the predominantly female hormone prolactin. As we show here in chapter 3 the prolactin receptor antagonist Δ1-9-G129R-hPRL blocks the hypersensitivity that results from application of dural CGRP in females, and the CGRP antagonist $CGRP_{8-37}$ prevents dural prolactin induced hypersensitivity [7]. This is likely carried our via cross talk between PRL and CGRP expressing fibers. This may be facilitated by prlr present on CGRP expressing fibers within the dura. Given that CGRP is a potent vasodilator CGRP application to the dura may act on blood vessels, immune cells[5; 9], and/or other nerve fibers [18; 30], causing PRL release from these structures [17; 19; 33; 36]. Moreover, residential dural cells are immunoreactive for PRL protein expression and express mRNA for PRL [13]. In chapter 3 we confirm that PRL is expressed on CD11b expressing cell types (Fig. 3.7) which are likely to be macrophages, granulocytes, and mast cells, as myeloid cells are the the predominant type of immune cells in dura [34]. The finding that Prlr is expressed on CD45 expressing cells (Fig. 3.7) may additionally indicate mast cells, which have been found to present at dural blood vessels in a fashion similar

to what we observe here [3; 35]. PRL may then act on on Prlr expressed on immune cells in a cytokine-like manner causing further release of PRL and recruitment of more immune cells [10; 16].

Mast cell degranulation via CGRP-linked prolactin release presents another possible explanation for CGRP sensitivity in female mice, particularly in regards to the finding that CGRP seems to be involved in the hypersensitivity following stress-induced migraine in females, but not in males presented in chapter 4 [8]. Although male and female mice present with similar hypersensitivity following the stress response and subsequent sensitivity to the NO donor SNP, the female response to SNP induced by stress is blocked by ALD403, a monoclonal antibody against CGRP, whereas the male response was not blocked by the same dose of this antibody (Fig. 4.4). This suggest that the stress induced priming is dependent on CGRP in female but not in male mice. This may have larger implications for CGRP induced mast cell degranulation and subsequent prolactin release in the female stress response. Mast cell degranulation is thought to contribute to migraine by causing excitation of dural nociceptors [31]. Both CGRP and PACAP have been shown to contribute to release of inflammatory mediators from mast cells within the dura [14] and mast cell degranulation leads to increased plasma levels of prolactin [17]. Interestingly, amylin has been observed to suppress mast cell degranulation [47]. Given that in the data shown in chapter 6 dural amylin leads to male-specific migraine like behavior, while CGRP and prolactin lead to female-specific migraine-like behavior. This could in part be due to a sex-specific mechanism for increased hypersensitivity due to prolactin released via CGRP-induced mast cell degranulation.

Additionally, PRL, PACAP, and CGRP all heavily contribute to the stress response, and as such may contribute to the observed female specific CGRP dependent priming that follows the stress response. Acute restraint stress in mice and rats has been shown to de-granulate dural mast cells in a CRH dependent fashion [26; 44]. Additionally, mast cells express the CRH1 receptor and may not only be responsive to CGRP and PACAP, but to neuropeptide induced increases in CRH [11]. PACAP is thought to modulate the hypothalamic pituitary adrenal (HPA) axis via increasing the release of ACTH from the pituitary as well by increasing CRH release from the hypothalamus during immobilization [39]. Furthermore, rats that undergo chronic variant stress (CVS) show increase in PACAP and the PAC(1) receptor in the bed nucleus of the stria terminalis, signaling from which is thought to aid in the emotional response in the emotional response to stress [21]. PACAP is thought to modulate the hypothalamic pituitary adrenal (HPA) axis via increasing the release of ACTH from the pituitary as well by increasing CRH release from the hypothalamus during immobilization [39]. CGRP administered to the paraventricular nucleus of the hypothalamus and in the lateral ventricle, increases ACTH and plasma CRH [15; 28]. CGRP has also been shown to increase ACTH from cultured pituitary cells [25] In preclinical models, different stressors have been shown to increase plasma levels of prolactin [27; 43]. PRL is also thought to modulate the activity of the HPA axis by increasing the release of ATCH from the pituitary. Because Prolactin, PACAP, and CGRP all serve regulatory functions within the HPA-axis, specifically leading to increases in ACTH and CRH, it is possible that increases in sensitivity to and presence of these peptides could lead to maladaptive responses

or dysregulation of the HPA-axis and the subsequent sex-specific CGRP dependent priming we observe here.

While this is likely to occur during the stress response, the data we have indicating stark sex-differences following direct dural stimulation, in particular those between amylin and CGRP, may not directly involve activation of the HPA axis. We report that dural amylin leads to facial hypersensitivity in male, but not female mice and dural CGRP leads to hypersensitivity in female, but not male rodents. These disparities may result from differential expression of the calcitonin family peptides CGRP and amylin, and/or their receptors within the dura or other migraine relevant structures. These structures include the TG and TNC. As covered in chapter 6 CGRP and amylin share affinity for the AMY1 receptor which is comprised of calcitonin receptor (CTR) and receptor activity modifying protein 1 (RAMP1) [23] but both amylin and CGRP also signal through additional receptors. CGRP signals through the CGRP receptor, which also includes RAMP1, but is coupled with calcitonin receptor like receptor (CLR) [24]. Amylin signals through the AMY2 receptor (CTR and RAMP2) as well as AMY3 (CTR and RAMP3) [23]. There has yet to be a comprehensive study of potential differences in receptor expression of these 4 receptors in either the meninges, trigeminal ganglia, or TNC between males in females. Therefore, it is possible that the differences in sensitivity we observe in males and females to dural amylin and CGRP, respectively, are the result of differential receptor expression. This may be via increased CGRP receptor expression in females when compared with males, and increased AMY2 or AMY3 expression in males.

In Chapter 6 we noted that ovariectomized (OVX) females no longer demonstrated sensitivity to a previously noxious dose of CGRP [6]. We also noted that OVX mice demonstrated robust hypersensitivity to a dose of dural amylin that was found to be non-noxious in intact females. This finding suggests that ovarian derived hormones are likely partially responsible for these responses. Despite our finding that prolactin interacts with CGRP to produce female-specific sensitivity to both CGRP, the source of this prolactin is not likely to be the ovaries as PRL ovariectomy does not alter responses to dural PRL in females, and ovariectomy only transiently alters PRL levels in both women and rodents [1; 4; 12; 42]. This would suggest that either estrogen or progesterone are involved in the dichotomy of female responses to calcitonin family peptides. Namely that both, or one, of these hormones leads to increased sensitivity to CGRP and reduced sensitivity to amylin. It is important to note it is also possible that these hormones have differential effects i.e. one prevents sensitivity to amylin and the other may increase CGRP sensitivity. This may be mediated by hormonal regulation of receptor presence as has been reported in response to exogenous application of these hormones in the uterine wall of rodents [45; 46]; however, additional studies are required to explore the role of these hormones further. These additional studies would also help to provide insight that may develop sex-specific migraine therapeutics. As well as offer explanations for the efficacy of either estrogen or progesterone containing oral contraceptives for the treatment of migraine. As both oral contraceptives have been reported to trigger and alleviate migraine in women [2; 32; 37].

While this project explores sexual differences in migraine, an important caveat of this body of work is that pre-clinical models cannot account for gender differences. While there is strongly evidenced sex differences in migraine there are very few studies that examine potential differences in how migraine is differential among gender. An increasing body of evidence has shown that there are differences in anatomical and functional aspects of the brain that are aligned with gender rather than sex [20; 29; 40; 41]. While little of this data has shown that there are differences in gender on pain conditions. A study conducted by Strath et. Al examined evoked pain in individuals living with HIV. This study concluded that both trans and cis gender women experienced no significant differences in their responses to heat pulses or mechanical sensitivity; however, both groups of women presented with significantly greater pain responses following heat pulse in comparison with men. Correspondingly transgender women had significantly greater pain responses to mechanical stimuli than men [38]. For these reasons sex-specific findings may not correlate with gender differences and the role of gender in migraine should be considered in future studies.

Nevertheless, the data shown here offer insight for the development of sex-specific therapeutics for migraine. Specifically, that amylin receptor antagonists, while typically not sensitive enough to distinguish receptor subtype [22], could be used in the treatment of male migraineurs. While, as further discussed in chapter 3, peripherally restricted prolactin receptor antagonists may serve as effective therapeutics in female migraineurs. These data also imply that there may be differential efficacy in existing therapeutics for migraine such as small molecule CGRP receptor antagonists and monoclonal antibodies against CGRP and/or the CGRP receptor

among men and women. The insights provided by these data will ultimately be informative for development of future therapeutics and will help to clarify the sexually dimorphic nature of the migraine pathology.

REFERENCES

[1] Abbot SD, Docherty K, Clayton RN. Regulation of LH subunit mRNA levels by gonadal hormones in female rats. J Mol Endocrinol 1988;1(1):49-60.

[2] Allais G, Gabellari IC, Airola G, Borgogno P, Schiapparelli P, Benedetto C. Headache induced by the use of combined oral contraceptives. Neurol Sci 2009;30 Suppl 1:S15-17.

[3] Arac A, Grimbaldeston MA, Galli SJ, Bliss TM, Steinberg GK. Meningeal Mast Cells as Key Effectors of Stroke Pathology. Front Cell Neurosci 2019;13:126.

[4] Aravantinos D, Tzingounis V. [Changes in plasma prolactin levels after gynecologic surgery]. J Gynecol Obstet Biol Reprod (Paris) 1983;12(5):461-463.

[5] Assas BM, Pennock JI, Miyan JA. Calcitonin gene-related peptide is a key neurotransmitter in the neuro-immune axis. Front Neurosci 2014;8:23.

[6] Avona A, Burgos-Vega C, Burton MD, Akopian AN, Price TJ, Dussor G. Dural Calcitonin Gene-Related Peptide Produces Female-Specific Responses in Rodent Migraine Models. J Neurosci 2019;39(22):4323-4331.

[7] Avona A, Mason BN, Burgos-Vega C, Hovhannisyan AH, Belugin SN, Mecklenburg J, Goffin V, Wajahat N, Price TJ, Akopian AN, Dussor G. Meningeal CGRP-Prolactin interaction evokes female-specific migraine behavior. Ann Neurol 2021.

[8] Avona A, Mason BN, Lackovic J, Wajahat N, Motina M, Quigley L, Burgos-Vega C, Moldovan Loomis C, Garcia-Martinez LF, Akopian AN, Price TJ, Dussor G. Repetitive stress in mice causes migraine-like behaviors and calcitonin gene-related peptide-dependent hyperalgesic priming to a migraine trigger. Pain 2020;161(11):2539-2550.

[9] Baliu-Pique M, Jusek G, Holzmann B. Neuroimmunological communication via CGRP promotes the development of a regulatory phenotype in TLR4-stimulated macrophages. Eur J Immunol 2014;44(12):3708-3716.

[10] Brand JM, Frohn C, Cziupka K, Brockmann C, Kirchner H, Luhm J. Prolactin triggers pro-inflammatory immune responses in peripheral immune cells. Eur Cytokine Netw 2004;15(2):99-104.

[11] Cao J, Papadopoulou N, Kempuraj D, Boucher WS, Sugimoto K, Cetrulo CL, Theoharides TC. Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. J Immunol 2005;174(12):7665-7675.

[12] Castelo-Branco C, Martinez de Osaba MJ, Vanrezc JA, Fortuny A, Gonzalez-Merlo J. Effects of oophorectomy and hormone replacement therapy on pituitary-gonadal function. Maturitas 1993;17(2):101-111.

[13] Clapp C, Lopez-Gomez FJ, Nava G, Corbacho A, Torner L, Macotela Y, Duenas Z, Ochoa A, Noris G, Acosta E, Garay E, Martinez de la Escalera G. Expression of prolactin mRNA and of prolactin-like proteins in endothelial cells: evidence for autocrine effects. J Endocrinol 1998;158(1):137-144.

[14] De Jonge F, De Laet A, Van Nassauw L, Brown JK, Miller HR, van Bogaert PP, Timmermans JP, Kroese AB. In vitro activation of murine DRG neurons by CGRP-mediated mucosal mast cell degranulation. Am J Physiol Gastrointest Liver Physiol 2004;287(1):G178-191.

[15] Dhillo WS, Small CJ, Jethwa PH, Russell SH, Gardiner JV, Bewick GA, Seth A, Murphy KG, Ghatei MA, Bloom SR. Paraventricular nucleus administration of calcitonin gene-related peptide inhibits food intake and stimulates the hypothalamo-pituitary-adrenal axis. Endocrinology 2003;144(4):1420-1425.

[16] Dill R, Walker AM. Role of Prolactin in Promotion of Immune Cell Migration into the Mammary Gland. J Mammary Gland Biol Neoplasia 2017;22(1):13-26.

[17] Donoso AO, Zarate MB. Release of prolactin and luteinizing hormone by histamine agonists in ovariectomized, steroid-treated rats under ether anesthesia. Exp Brain Res 1983;52(2):277-280.

[18] Eftekhari S, Warfvinge K, Blixt FW, Edvinsson L. Differentiation of nerve fibers storing CGRP and CGRP receptors in the peripheral trigeminovascular system. J Pain 2013;14(11):1289-1303.

[19] Gonzalez C, Rosas-Hernandez H, Jurado-Manzano B, Ramirez-Lee MA, Salazar-Garcia S, Martinez-Cuevas PP, Velarde-Salcedo AJ, Morales-Loredo H, Espinosa-Tanguma R, Ali SF, Rubio R. The prolactin family hormones regulate vascular tone through NO and prostacyclin production in isolated rat aortic rings. Acta Pharmacol Sin 2015;36(5):572-586.

[20] Hahn A, Kranz GS, Kublbock M, Kaufmann U, Ganger S, Hummer A, Seiger R, Spies M, Winkler D, Kasper S, Windischberger C, Swaab DF, Lanzenberger R. Structural Connectivity Networks of Transgender People. Cereb Cortex 2015;25(10):3527-3534.

[21] Hammack SE, Cheung J, Rhodes KM, Schutz KC, Falls WA, Braas KM, May V. Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): roles for PACAP in anxiety-like behavior. Psychoneuroendocrinology 2009;34(6):833-843.

[22] Hay DL, Chen S, Lutz TA, Parkes DG, Roth JD. Amylin: Pharmacology, Physiology, and Clinical Potential. Pharmacol Rev 2015;67(3):564-600.

[23] Hay DL, Garelja ML, Poyner DR, Walker CS. Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. Br J Pharmacol 2018;175(1):3-17.

[24] Hay DL, Walker CS. CGRP and its receptors. Headache 2017;57(4):625-636.

[25] Iino K, Oki Y, Tominaga T, Iwabuchi M, Ozawa M, Watanabe F, Yoshimi T. Stimulatory effect of calcitonin gene-related peptide on adrenocorticotropin release from rat anterior pituitary cells. J Neuroendocrinol 1998;10(5):325-329.

[26] Kandere-Grzybowska K, Gheorghe D, Priller J, Esposito P, Huang M, Gerard N, Theoharides TC. Stress-induced dura vascular permeability does not develop in mast cell-deficient and neurokinin-1 receptor knockout mice. Brain Res 2003;980(2):213-220.

[27] Kehoe L, Janik J, Callahan P. Effects of immobilization stress on tuberoinfundibular dopaminergic (TIDA) neuronal activity and prolactin levels in lactating and non-lactating female rats. Life Sci 1992;50(1):55-63.

[28] Kovacs A, Biro E, Szeleczky I, Telegdy G. Role of endogenous CRF in the mediation of neuroendocrine and behavioral responses to calcitonin gene-related peptide in rats. Neuroendocrinology 1995;62(4):418-424.

[29] Kruijver FP, Zhou JN, Pool CW, Hofman MA, Gooren LJ, Swaab DF. Male-to-female transsexuals have female neuron numbers in a limbic nucleus. J Clin Endocrinol Metab 2000;85(5):2034-2041.

[30] Lennerz JK, Ruhle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF, Messlinger K. Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. J Comp Neurol 2008;507(3):1277-1299.

[31] Levy D, Burstein R, Kainz V, Jakubowski M, Strassman AM. Mast cell degranulation activates a pain pathway underlying migraine headache. Pain 2007;130(1-2):166-176.

[32] Massiou H, MacGregor EA. Evolution and treatment of migraine with oral contraceptives. Cephalalgia 2000;20(3):170-174.

[33] Matera L, Muccioli G, Cesano A, Bellussi G, Genazzani E. Prolactin receptors on large granular lymphocytes: dual regulation by cyclosporin A. Brain Behav Immun 1988;2(1):1-10.

[34] McIlvried LA, Cruz JA, Borghesi LA, Gold MS. Sex-, stress-, and sympathetic post-ganglionic-dependent changes in identity and proportions of immune cells in the dura. Cephalalgia 2017;37(1):36-48.

[35] Nurkhametova D, Kudryavtsev I, Guselnikova V, Serebryakova M, Giniatullina RR, Wojciechowski S, Tore F, Rizvanov A, Koistinaho J, Malm T, Giniatullin R. Activation of P2X7 Receptors in Peritoneal and Meningeal Mast Cells Detected by Uptake of Organic Dyes: Possible Purinergic Triggers of Neuroinflammation in Meninges. Front Cell Neurosci 2019;13:45.

[36] Patil M, Belugin S, Mecklenburg J, Wangzhou A, Paige C, Barba-Escobedo PA, Boyd JT, Goffin V, Grattan D, Boehm U, Dussor G, Price TJ, Akopian AN. Prolactin Regulates Pain Responses via a Female-Selective Nociceptor-Specific Mechanism. iScience 2019;20:449-465.

[37] Ryan RE. A controlled study of the effect of oral contraceptives on migraine. Headache 1978;17(6):250-252.

[38] Strath LJ, Sorge RE, Owens MA, Gonzalez CE, Okunbor JI, White DM, Merlin JS, Goodin BR. Sex and Gender are Not the Same: Why Identity Is Important for People Living with HIV and Chronic Pain. J Pain Res 2020;13:829-835.

[39] Stroth N, Eiden LE. Stress hormone synthesis in mouse hypothalamus and adrenal gland triggered by restraint is dependent on pituitary adenylate cyclase-activating polypeptide signaling. Neuroscience 2010;165(4):1025-1030.

[40] Swaab DF. Sexual differentiation of the human brain: relevance for gender identity, transsexualism and sexual orientation. Gynecol Endocrinol 2004;19(6):301-312.

[41] Swaab DF. Sexual differentiation of the brain and behavior. Best Pract Res Clin Endocrinol Metab 2007;21(3):431-444.

[42] Szawka RE, Rodovalho GV, Helena CV, Franci CR, Anselmo-Franci JA. Prolactin secretory surge during estrus coincides with increased dopamine activity in the hypothalamus and preoptic area and is not altered by ovariectomy on proestrus. Brain Res Bull 2007;73(1-3):127-134.

[43] Terkel J, Blake CA, Sawyer CH. Serum prolactin levels in lactating rats after suckling or exposure to ether. Endocrinology 1972;91(1):49-53.

[44] Theoharides TC, Spanos C, Pang X, Alferes L, Ligris K, Letourneau R, Rozniecki JJ, Webster E, Chrousos GP. Stress-induced intracranial mast cell degranulation: a corticotropin-releasing hormone-mediated effect. Endocrinology 1995;136(12):5745-5750.

[45] Thota C, Gangula PR, Dong YL, Yallampalli C. Changes in the expression of calcitonin receptor-like receptor, receptor activity-modifying protein (RAMP) 1, RAMP2, and RAMP3 in rat uterus during pregnancy, labor, and by steroid hormone treatments. Biol Reprod 2003;69(4):1432-1437.

[46] Thota C, Yallampalli C. Progesterone upregulates calcitonin gene-related peptide and adrenomedullin receptor components and cyclic adenosine 3'5'-monophosphate generation in Eker rat uterine smooth muscle cell line. Biol Reprod 2005;72(2):416-422

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[47] Umarova BA, Smirnova EA, Luk'yantseva GV, Kopylova GN, Samonina GE, German SV, Zhuikova SE. Effect of amylin on mast cell secretion as a possible mechanism increasing gastric mucosa resistance. Bull Exp Biol Med 2001;132(4):929-931.

BIOGRAPHICAL SKETCH

Amanda Avona is currently a student in the cognition and neuroscience PhD program at The University of Texas at Dallas. Her interest in neuroscience and training has helped her to develop a set of skills that have prepared her to continue her scientific interests in an industry position. She performed her undergraduate degree at The University of Texas at Dallas where she began her research career as a volunteer in the Dussor lab. This initial experience allowed her to learn the basics of experimental design in behavioral models of migraine. Following completion of her bachelor's degree in Neuroscience, she continued her master's degree at The University of Texas at Dallas in applied cognition and neuroscience and continued developing her skills in the Dussor lab. During her master's degree she also performed two short internships at the University of Marburg, the first of which was in the Weihe lab where she worked on molecular techniques that included PCR, in situ hybridization, plasma ligation, and bacterial transformation. The second internship in the Decher lab gave her the foundations of electrophysiology via training in two microelectrode voltage clamp techniques. Following these internships Amanda continued her scientific training in the Dussor lab where she has primarily worked on projects further examining potential reasons for the prevalence of migraine in women with a particular focus on calcitonin family peptides and prolactin. During this time Amanda has authored 4 papers and co-authored an additional 4.

CURRICULUM VITAE

EDUCATION

EDUCATION

2018 – present	PhD in Systems Neuroscience University of Texas at Dallas Mentor: Gregory Dussor, PhD Field of Study: Neuroscience
2016 – 2018	Master of Science University of Texas at Dallas Mentor: Gregory Dussor, PhD Field of Study: Applied Cognition and Neuroscience
2007 – 2011	Bachelor of Science University of Texas at Dallas Mentor: Gregory Dussor, PhD Field of Study: Neuroscience

EMPLOYMENT

2019 – present	Research Assistant University of Texas at Dallas Systems Neuroscience
2018 – 2019	Teaching Assistant University of Texas at Dallas
2015-2016	Undergraduate Researcher University of Texas at Dallas Mentors: Gregory Dussor, PhD Field of Study: Neuroscience

ACADEMIC AND PROFESSIONAL RECOGNITION

2019 Journal of Neuroscience Spotlight
University of Texas at Dallas- Bio and Behavioral Research Award
Nature reviews Neurology

PUBLICATIONS

1. Avona A, Price TJ, Dussor G. Interleukin-6 induces spatially dependent whole-body hypersensitivity in rats: implications for extracephalic hypersensitivity in migraine. J Headache Pain. 2021 Jul 13;22(1):70. doi: 10.1186/s10194-021-01286-8. PMID: 34256692; PMCID: PMC8278737.

2. Mason BN, **Avona A**, Lackovic J, Dussor G. Dural Stimulation and Periorbital von Frey Testing in Mice As a Preclinical Model of Headache. J Vis Exp. 2021 Jul 29;(173). doi: 10.3791/62867. PMID: 34398161.

3. Lenert ME, **Avona A**, Garner KM, Barron LR, Burton MD. Sensory Neurons, Neuroimmunity, and Pain Modulation by Sex Hormones. Endocrinology. 2021 Aug 1;162(8):bqab109. doi: 10.1210/endocr/bqab109. PMID: 34049389; PMCID: PMC8237991.

4 Avona A, Mason BN, Burgos-Vega C, Hovhannisyan AH, Belugin SN, Mecklenburg J, Goffin V, Wajahat N, Price TJ, Akopian AN, Dussor G. Meningeal CGRP-Prolactin Interaction Evokes Female-Specific Migraine Behavior. Ann Neurol. 2021 Jun;89(6):1129-1144. doi: 10.1002/ana.26070. Epub 2021 Apr 22. PMID: 33749851; PMCID: PMC8195469.

5. Avona A, Mason BN, Lackovic J, Wajahat N, Motina M, Quigley L, Burgos-Vega C, Moldovan Loomis C, Garcia-Martinez LF, Akopian AN, Price TJ, Dussor G. Repetitive stress in mice causes migraine-like behaviors and calcitonin gene-related peptide-dependent hyperalgesic priming to a migraine trigger. Pain. 2020 Nov;161(11):2539-2550. doi: 10.1097/j.pain.0000000000001953. PMID: 32541386; PMCID: PMC7572536.

6. Avona A, Burgos-Vega C, Burton MD, Akopian AN, Price TJ, Dussor G. Dural Calcitonin Gene-Related Peptide Produces Female-Specific Responses in Rodent Migraine Models. J Neurosci. 2019 May 29;39(22):4323-4331. doi: 10.1523/JNEUROSCI.0364-19.2019. Epub 2019 Apr 8. PMID: 30962278; PMCID: PMC6538861.

7. Burgos-Vega CC, Quigley LD, Trevisan Dos Santos G, Yan F, Asiedu M, Jacobs B, Motina M, Safdar N, Yousuf H, **Avona A**, Price TJ, Dussor G. Non-invasive dural stimulation in mice: A novel preclinical model of migraine. Cephalalgia. 2019 Jan;39(1):123-134. doi: 10.1177/0333102418779557. Epub 2018 May 31. PMID: 29848109; PMCID: PMC6499065.

8. Burgos-Vega CC, Quigley LD, **Avona A**, Price T, Dussor G. Dural stimulation in rats causes brain-derived neurotrophic factor-dependent priming to subthreshold stimuli including a migraine trigger. Pain. 2016 Dec;157(12):2722-2730. doi: 10.1097/j.pain.0000000000000692. PMID: 27841839; PMCID: PMC5315498.

In preparation

1. Avona A, Mason BN, Price TJ, Dussor G. Dural amylin and CGRP lead to differential sex-specific responses contingent on ovarian hormones in rodent migraine models.

PRESENTATIONS

1. Dural Calcitonin Gene-Related Peptide produces female specific responses in rodent migraine models. August 2019. European pain school. *Talk*

2. CGRP and prolactin signaling in the meninges produces female-specific migraine-related behavior in rodents. August 2018. International association for the study of pain. *Poster*

3. CGRP produces female-specific migraine behavior in rat. 2017. *Poster*

4. Prolactin signaling in the meninges produces female-specific migraine-related behavior in rats. American Pain Society 2019. *Poster*

5. IL-6 induces spatially dependent hyperalgesic priming in a rat model of migraine. Texas Pain Research Consortium, 2015. *Post*