INCREASED SENSITIVITY TO CGRP IS ASSOCIATED WITH CHRONIFICATION OF MIGRAINE AND POST-TRAUMATIC HEADACHE IN MALE AND FEMALE MICE

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by *Gege Guzman*, titled *Increased Sensitivity to CGRP is Associated with Chronification of Migraine and Post-Traumatic Headache in Male and Female Mice* and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.

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ABSTRACT

Migraine is one of the leading causes of disability affecting approximately 15% of the global population. Some individuals are more susceptible to the chronification of migraine with at least 2.5% of the patient population developing chronic migraine (CM) annually. Chronic headache disorders disproportionally affect women, with migraine occurring two to three times more often than in men and presenting with greater attack frequency and severity. These differences raise the possibility of sex-specific mechanisms contributing to migraine pathophysiology. The success of CGRP targeting therapies for both the acute and preventative treatment of migraine highlight CGRP's crucial role in migraine pathophysiology. Recent findings have demonstrated that CGRP produces female-selective pain, and additionally, acute migraine treatments targeting this peptide exhibit sex-preferential effectiveness that becomes comparable between sexes in chronic conditions. Men appear to experience less therapeutic benefit from CGRP targeting therapies for acute migraine treatment, despite these treatments providing significant relief for women. However, studies further exploring sexually dimorphic mechanisms and incorporating sex-based analysis remains limited, leaving the influence of biological sex on headache chronification largely unexplored. Preclinical studies further support the clinical observations of CGRP-targeting therapies for CM, demonstrating the essential contribution of CGRP for the chronification of migraine.

Interestingly, post-traumatic headache (PTH) from mild traumatic brain injury (mTBI) seems to share biologic features with migraine, however mechanisms are not fully understood. CGRP also appears to contribute to this headache condition, which shares migraine-like features and responds to migraine-specific therapeutics. Although concussive injuries are more frequently reported in men, women tend to experience worse outcomes and are more likely to develop persistent PTH, defined as injury-associated headaches lasting beyond three months. Differences in the physiology of the trigeminovascular system, specifically CGRP signaling within this system, may underlie susceptibility to headache chronification. This research aims to provide insight into the fundamental mechanisms of the sexually dimorphic nociceptive actions of CGRP and its contribution to migraine and migraine-like headaches as disorders evolve to chronic states.

BACKGROUND

OVERVIEW OF MIGRAINE AND BURDEN

Migraine is a highly prevalent, progressive neurological disorder that negatively impacts millions of men and women. It is one of the top leading causes of disability worldwide, and women are two to three times more likely to suffer from migraine (95, 108, 9). This primary headache disorder is characterized by its multiphasic and multisymptomatic nature requiring a complex treatment approach due to various triggers and heterogeneous symptoms (45, 22). This disorder causes an immense personal and economic burden for patients and their loved ones (9). According to The International Classification of Headache Disorders (ICHD-3), migraine headache typically features an intense, unilateral pulsing or throbbing and can be accompanied by nausea, photophobia, or phonophobia. Migraine encompasses several subtypes and may present with or without aura, characterized by visual and sensory symptoms that typically precede headache onset. Additionally, migraine can be classified by frequency, with chronic migraine defined as experiencing headache on 15 or more days per month for at least three months (ICHD-3). Approximately 2.5% of migraineurs will progress to chronic states annually (73). Access to effective diagnosis and treatment remains a significant challenge, often leading to mismanagement of the disorder and contributing to substantial personal and economic burdens for patients and their loved ones (9).

THE TRIGEMINAL SYSTEM IN MIGRAINE PATHOPHYSIOLOGY

Migraine involves complex interactions between peripheral and central neuronal and non-neuronal pathways, though the mechanisms are still not fully understood. The trigeminal nerve consists of three major branches, the ophthalmic (V1), maxillary (V2) and mandibular (V3) divisions, is responsible for both sensory and motor innervation to distinct regions of the face. Nerve fibers from the V1 branch innervate pain sensitive structures, such as the dura mater and intracranial vessels, that are important in headache pain sensation. Sensory information from these peripheral afferents converges at the trigeminal ganglion (TG), located bilaterally near the base of the skull, to then be relayed to second-order nuclei in the trigeminal nucleus caudalis (TNC), a region in the brainstem important for pain processing. Peripherally, vasoactive peptides and other pronociceptive neuropeptides, such as substance P, are co-released from trigeminal afferents that innervate the meningeal vasculature, particularly in the dura mater (119). The dura mater, the outermost of the three cranial meninges, is highly vascularized and innervated by C- and Aδ-fibers. These nerve fibers contain signaling molecules, such as substance P, CGRP, glutamate, neurokinin A/B, important for pain processes. Mechanical or chemical activation of these dural afferents elicits headache-like pain and has been directly implicated in migraine generation (44). This trigeminovascular system represents a key interface between peripheral input and central pain modulation, making it a critical target for both mechanistic investigation and therapeutic intervention in migraine. Migraine is characterized by reduced sensory thresholds resulting in a sensitized state vulnerable to triggers. Although the mechanisms underlying migraine are not fully clear, sensitization of the trigeminovascular system is a key driver of migraine pathophysiology.

CALCITONIN GENE-RELATED PEPTIDE (CGRP) IN MIGRAINE

Calcitonin gene-related peptide (CGRP) is an excitatory neuropeptide that regulates vascular function and modulates central pain transmission, thereby establishing its role as a key contributor to migraine pathophysiology. This neuropeptide is synthesized in peptidergic peripheral sensory neurons and in specific neurons within the central nervous system. Within the trigeminal system—particularly cranial nerve V (the trigeminal nerve) which innervates the face and head—CGRP plays a central role in cranial pain transmission. Noxious stimuli – such as mechanical, chemical, or electrical stimuli arising from tissue injury or inflammation – can activate dural afferents, triggering the release of CGRP and other inflammatory mediators from the peripheral and central terminals of unmyelinated C-fibers into innervated tissue and onto second-order neurons within the trigeminal nucleus caudalis (TNC) in the brainstem, where central pain processing is initiated (81, 37). Additionally, several studies provide evidence for local CGRP signaling within the trigeminal ganglion (TG), where it may act on neighboring neurons and glial cells expressing CGRP receptors (37, 65, 34, 113). Most TG neurons express either CGRP or its receptor components—but rarely both—suggesting a paracrine mechanism of action that may contribute to central sensitization (36, 37, 35). Intraganglionic neuronal-glial interactions may further facilitate this process, providing a probable mechanism for the recruitment and amplification of pain signaling leading to central sensitization. CGRP effects are mainly through the canonical G_s protein-coupled receptor complex comprising the calcitonin-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1), activating downstream cAMP, PKA, and MAPK pathways (97). CGRP acts at both peripheral and central sites, but mounting evidence supports a particularly important role for cranial peripheral signaling in migraine chronification (119, 82) supported by the efficacy of CGRP-targeting monoclonal antibodies that have limited blood-brain barrier permeability (35). This may involve a sustained activation of dural afferents, leading to lower sensory thresholds and increased vulnerability to triggers. Although the exact mechanisms remain unclear, CGRP has been shown to engage in autoregulatory loops via cAMP and MAPK pathways, which may establish a positive feedback cycle that promotes or sustains chronic migraine (97).

Preclinical studies further support the sexually dimorphic role of CGRP in meningeal nociception (11, 110, 69). Supradural application of CGRP produces migraine-like pain preferentially in female rodents (11), and sensitization of dural afferents has been linked to key events in migraine pathophysiology (67). This may reflect underlying differences in trigeminovascular signaling or hormone-modulated CGRP sensitivity, though further mechanistic work is needed.

Current Migraine Treatments & Limitations

Headache disorders often require specialized care for accurate diagnosis and effective management; however, most patients are treated by primary care physicians or other non-specialists, resulting in suboptimal care (9). Inadequate or ineffective care has been associated with a two-fold increased risk of developing new-onset CM (74, 32, 57), thereby placing individuals with frequent or chronic migraine at an even greater risk of worsening condition and chronification (17, 57, 79, 114, 58). Commonly, treatment aims to alleviate migraine symptoms with abortive medications or prevent a migraine attack with prophylactic treatments. Acute treatments approved for migraine include non-specific analgesics such as non-steroidal anti-inflammatories, as well as migraine specific agents like triptans, $5\text{-HT}_{1B/1D}$ receptor agonists, or gepants, small molecule CGRP receptor antagonists. However, these medications are often insufficient for effective migraine management, as acute medications frequently fail to provide adequate relief, leading to the overuse of analgesics and other acute headache medications (96, 20, 72). For example, triptans have been associated with high headache recurrence rates (42) and many migraine patients do not achieve satisfactory relief from this therapy (25, 96). Mismanagement or frequent use of these and other acute medications can cause medication overuse headache (MOH) (10, 105), a secondary headache disorder which further exacerbates pre-existing headache conditions (17, 72, 74, 32, 57, 58). Interestingly, analgesics present a seemingly exclusive risk for the development of MOH in patients with a pre-existing headache disorder, with migraineurs at particularly high risk (10, 17). Among available treatments, triptans are associated with one of the highest risks for MOH (10, 17), defined by their use on ≥10 days per month for >3 months with headache frequency exceeding 15 days per month. Established chronic headache conditions, such as CM, are more difficult to treat and respond less favorably to abortive medications compared to EM (56), highlighting the importance of preventative approaches and careful management to avoid complications such as MOH.

Most patients with MOH suffer from an underlying primary headache disorder, most commonly migraine, with frequent or chronic migraineurs at a heightened risk (17, 58). When MOH co-occurs with CM, it leads to an increase in headache frequency to nearly daily, accompanied by similar migraine-associated symptoms (32). This increased headache frequency is considered a form of CM in migraine patients (10) and presents an increased risk of EM transforming into CM, i.e., chronification (17, 72, 79, 32, 57). While MOH is reversible with the discontinuation of the offending medication, it can be challenging for clinicians to differentiate these conditions. Moreover, some patients are reluctant to reduce or stop the overused medication, and not all will revert from CM back to EM (87). Currently, MOH is mitigated by combining treatment with non-pharmacological interventions, such as trigger avoidance or behavioral methods, and prophylactic treatments (72, 87, 105). Preventative therapies aim to reduce headache frequency and minimize the risk of future attacks, thereby minimizing risk of chronification (73, 79). Approved options include topiramate, onabotulinumtoxinA (Botox), beta-blockers, antiepileptics, calcium channel blockers, and tricyclic anti-depressants, as well as migraine-specific agents such as atogepant and anti-CGRP monoclonal antibodies (68, 103).

Sex Differences in Migraine: Clinical Observations

Notably, treatments targeting CGRP signaling—through monoclonal antibodies (mAbs) or small molecule antagonists—have emerged as highly effective for both acute and preventative migraine treatment (40, 104, 103, 23). Consistent with this mechanistic consideration, elevated CGRP levels are consistently observed during migraine attacks (51) and are chronically elevated in patients with chronic migraine (CM) (21, 119). Additionally, triptans continue to be the most effective abortive therapy for relieving migraine symptoms (54), with clinical studies observing a strong correlation between their therapeutic benefit and a reduction in circulating CGRP levels (43). Inversely, intravenous CGRP administration induces delayed migraine headaches in migraineurs (66). Migraine disproportionally affects women in terms of prevalence, severity, and chronicity (75, 101). Interestingly, higher basal plasma levels of CGRP have been reported in women compared to men (115), a finding that reflects the higher prevalence of migraine and chronic headache in women (95, 108, 9) and highlights the relevance of CGRP signaling as a therapeutic target.

Sex differences are also evident in patients' responsiveness to anti-migraine therapies. Triptans, which act as a $5\text{-HT}_{1B/1D}$ receptor agonists, remain first-line abortive treatments for migraine. They alleviate pain by inhibiting central and peripheral of inflammatory peptide release from trigeminal nociceptors while also promoting cranial vasoconstriction (93, 28). However, clinical studies suggest that while initial headache relief is similar between sexes, women experience higher rates of recurrence (116). Triptan responsiveness may correlate with circulating CGRP levels (99), potentially reflecting individual variability in CGRP dynamics or receptor sensitivity. Additionally, anti-CGRP treatments demonstrate effective migraine prevention for most patients with CM, anti-CGRP therapeutics for acute treatment show limited benefit in men with EM (89). Our group recently conducted a meta-analysis evaluating the efficacy of anti-CGRP biologics in patients with episodic and chronic migraine, stratified by sex (89) and found that gepants show preferential effectiveness in women for the acute treatment of migraine, but benefits are not consistently observed in men. In contrast, both men and women respond favorably to preventative anti-CGRP treatments for CM. These observations suggest potential sex differences in CGRP-mediated pain mechanisms in migraine that may evolve as the condition transitions from episodic to more frequent or chronic states. The mechanisms underlying this discrepancy remain unclear but may involve sex-specific differences in CGRP signaling, receptor expression, or central sensitization.

Nitroglycerin as a Preclinical Model of Migraine

Nitroglycerin (NTG), a nitric oxide (NO) donor, triggers delayed migraine in migraineurs and headache in healthy individuals (111). The headache-inducing effects of NTG involves several mechanisms, including NO-mediated vasodilation, stimulation of dural nociceptive fibers, NO-driven CGRP release (15), and activation of inflammatory pathways (111). NTG releases NO that can then activate trigeminal nociceptive neurons, leading to the increased synthesis and release of CGRP. CGRP can in turn cause the release of NO from glial and endothelial cells in meningeal tissue, creating a reciprocal amplification loop of vasodilation and neurogenic inflammation leading to the sensitization of trigeminal system. NTG-evoked migraine is also correlated with increased plasma CGRP levels similarly observed in spontaneous migraine attacks in patients and in animal migraine models (46, 47) and can be attenuated by anti-migraine therapeutics (51). Preclinically, acute administration of NTG induces migraine-like phenotypes in rodents (111). Additionally, repeated NTG exposure in mice induces neuroplastic changes in the trigeminal system, resulting in latent sensitization that closely models chronic migraine states (90). Thus, as a NO-donor, NTG provides a reliable and widely used preclinical model that closely reflects migraine pathophysiology.

Triptan-Induced Medication Overuse Headache as a Preclinical Chronic Migraine Model

The overuse of triptans, is a common occurrence for chronic migraineurs and can lead to medication overuse headache (MOH), a risk factor for progression to CM (57). Possibly a shared mechanism with migraine, sensitization of trigeminal sensory pathways (98, 17), demonstrated through shared migraine features reported by patients (71) and preclinically state of latent sensitization to same challenges and increase in plasma CGRP, similar to patients experiencing spontaneous migraine (27). Preclinical evidence also suggests that triptan overuse induces sensitization of dural afferents, increasing susceptibility to migraine triggers and contributing to the development of medication overuse headache (MOH) (27). In rats, triptan exposure has been shown to modulate the expression of neuronal nitric oxide synthase (nNOS) in dural afferents, contributing to cutaneous allodynia and enhanced behavioral responses to common migraine triggers such as brainstem stimulation (BLS) and NO donors (27, 26). Consequently, this paradigm is widely used as a preclinical model of chronic migraine (27, 26).

Mild TBI-Induced Post-Traumatic Headache

Post-traumatic headache (PTH) is a common and often persistent sequela of mild traumatic brain injury (mTBI), with prevalence estimates ranging from 15% to over 50% (38, 30, 76). As defined by the IHCD-3, PTH occurs within seven days of injury and usually resolves by itself, however, when patients experience headaches continuing beyond three months post-injury, PTH is considered persistent (77). Although, female sex has been identified as risk factor (106), clinical data indicate that when prior headache history is accounted for, sex alone may not predict the risk of developing persistent PTH (76). Instead, a previous history of headaches appears to be the strongest risk factor for developing chronic PTH following mTBI (86, 117), though it is worth noting that persistent PTH is more common female PTH patients and they experience worse outcomes following mTBI (75, 14, 16, 107). Clinically, persistent PTH often mimics migraine in phenotype, including associated symptoms like photophobia, phonophobia, and nausea (78). Currently, there is no approved treatment for persistent PTH; management instead focuses on alleviating symptoms according to headache phenotype (52, 3, 4). However, these treatment approaches are often insufficient, with many patients remaining non-responsive or dissatisfied (4, 78), leaving a critical need to identify more targeted therapeutic strategies.

Preclinical Persistent Post-Traumatic Headache Model

Growing evidence points to CGRP signaling as a key contributor to PTH pathophysiology. PTH from mTBI seems to share biologic features with migraine, however mechanisms are not fully understood. Demonstrated in preclinical PTH models, injury-induced activation of the trigeminal system drives headache-like pain resulting from mTBI (85, 18). These models reveal CGRP-dependent mechanisms underlying headache-like pain behaviors and mTBI-induced latent sensitization (19, 85, 18), with some studies further noting sex differences in CGRP-driven responses (19). Most convincingly, CGRP infusion has been shown to induce migraine-like headaches in patients with persistent PTH, despite having no prior migraine history (5). Preclinical studies blocking CGRP signaling through pharmacological intervention have observed changes in headache-like pain behaviors in rodent models (18, 19, 60, 62, 84, 85).

RATIONALE AND SPECIFIC AIMS

Research Aim

The aim of this research was to investigate the role of CGRP sensitivity and possible sex differences in migraine chronification using preclinical models. We evaluated CGRP responsiveness in translationally relevant models that mimic frequent migraine-like attacks induced by repeated administration of nitroglycerin (NTG) or sumatriptan. To further assess whether enhanced CGRP sensitivity is a migraine-specific mechanism contributing to chronification, we compared these findings with a non-migraine chronic headache model—post-traumatic headache (PTH) following mild traumatic brain injury (mTBI).

Hypothesis

Previous preclinical studies have demonstrated a female-selective sensitivity to supradural administration of CGRP in producing migraine-like pain in rodents (11). Clinically, CGRP-targeted therapies appear more effective in women than in men with episodic migraine, suggesting underlying sex-dependent differences in CGRP signaling pathways. Yet, the reason why men become responsive to these therapeutics in chronic conditions remains to be explored. Additionally, increased frequency of migraine attacks may further promote CGRP sensitivity in females. We therefore investigated CGRP sexual dimorphism using animal models of chronic headache disorders.

Based on this evidence, the central hypotheses of this research were:

1. CGRP sensitivity increases with repeated migraine-like stimulation, contributing to the chronification of migraine in both sexes.
2. Females exhibit greater CGRP sensitization than males that might indicate a sex-specific vulnerability to chronic migraine.
3. Enhanced CGRP sensitivity is more prominent in other chronic headache disorders that share a migraine-like phenotype, supporting a shared mechanism of CGRP contributing to chronification.

These hypotheses were tested using behavioral and pharmacological analyses in validated rodent models of chronic migraine and PTH.

METHODS

ANIMALS

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Arizona and were performed in accordance with the ARRIVE guidelines, the ethical guidelines of the International Association for the Study of Pain regulations on animal welfare and the National Institutes of Health guidelines for the care and use of laboratory animals. A total of 136 male and female 8 - 12-week-old C57BL/6J mice (Jackson Laboratories) were used in these studies. Eight and four mice per group were used for behavior and IHC experiments, respectively. The estrous cycle of females was not monitored. Animals were housed 4 per cage under standard animal husbandry conditions of temperature-, humidity- and light cycle- controlled environment, with free access to food and water in the University of Arizona animal facility. Group size requirements to obtain significance at the α=0.05 and statistical power 0.9 were determined from previous experiments using power analysis. Mice were randomly divided into control and experimental groups. Experimenters were blinded to treatments when possible.

DRUGS

Nitroglycerin (NTG) was purchased from American Regent (Shirley, NY, USA), diluted in saline to 1 mg/mL, and administered intraperitoneally (i.p.) at 10 mg/kg. Sumatriptan was obtained from Abmole Bioscience (Houston, TX, USA), diluted in saline and administered i.p. at 10 mg/kg. Controls received saline at 10 mL/kg. CGRP (Bachem, Torrance, CA, USA) was dissolved in phosphate-buffered saline (PBS) and injected onto the dura mater (supradural) at 0.1 or 1 pg/5µL.

CUTANEOUS ALLODYNIA EVALUATION

Mice were placed in elevated individual Plexiglass chambers with mesh flooring and allowed to habituate for 3 days for 2 h each day before evaluations. Following a 2 h habituation period periorbital (cephalic) and hind paw (extracephalic) tactile frequency of response were measured by 10 applications of the 0.4 g (3.61N) von Frey filament to the periorbital region and 1g (4.08N) to the hind paw region (Stoelting, Wood Dale, IL, USA) with just enough pressure to cause the filament to display a slight arch. Swiping of the face (facial grooming) was considered a positive periorbital response. Sharp withdrawal, shaking and/or licking the paw were considered positive hind paw responses. Increased frequency of response was considered as a translationally relevant measure of cutaneous allodynia (CA). Frequency of response percentage was calculated as [(number of positive responses/10) × 100%] (60; 61).

MODELS OF CHRONIC MIGRAINE

CHRONIC NTG MODEL

To model CM-like pain, mice were administered NTG (10 mg/kg, i.p.) every other day for 9 days (five treatments in total), with treatments performed at the same time each day (11:00 a.m.), immediately after the behavior evaluation according to the previously reported protocol (70, 90).

MEDICATION OVERUSE HEADACHE (MOH) MODEL

To mimic MOH, mice were administered sumatriptan (10 mg/kg, i.p.) once daily for 9 consecutive days, with treatments performed at the same time each day (11:00 a.m.), immediately after the behavior evaluation as reported previously (49).

Post-Traumatic Headache (PTH) model

PTH was modeled using a translationally relevant closed mild traumatic head injury model previously reported (53, 85). Under light anesthetic with 5% isoflurane for 15 seconds and 2% isoflurane for 30 seconds, mice were positioned unrestrained in a crucifix position and ventral side down on tissue paper elevated above a plexiglass apparatus with a soft sponge floor. Placement of a 100 g weighted drop was standardized using a metal guide tube and released from 94 cm height above the mouse's skull and between the ears. Mice that receive mTBI undergo a single concussive impact with rotational and linear head forces, reproducing clinically relevant mechanisms of mTBI. Sham animals were similarly anesthetized but do not receive a weighted impact or rotational movement. All animals woke up and regained their righting reflex within 5 minutes. Animals were returned to their home cages and monitored for behavioral abnormalities indicative of potential neurological complications. The chronic headache state induced by this model is referred to as persistent post-traumatic headache (PTH). It is characterized by the return of transient tactile thresholds to baseline levels, typically around 14 days post-induction.

Supradural injection

Injectors were modified from commercially available cannulas (Plastic One/Invivo1, part #C313I/SPC) by inserting a stopper to establish a length of 0.65-0.7 mm to maintain dura mater integrity. The injectors were connected to Tygon tubing (Cole Parmer Co. Vernon Hills, Illinois, USA), which was then attached to a 25 μL Hamilton syringe (Hamilton, Reno, Nevada, USA). Under brief isoflurane anesthesia (5% isoflurane for induction and 2% for maintenance), the injector was inserted through the sagittal and lambdoid suture junction for supradural delivery (61).

Immunohistochemistry

Tissue Preparation

Mice were transcardially perfused with 4% paraformaldehyde (PFA). Following perfusion, skulls with attached dura mater and TGs were harvested and post-fixed in 4% PFA for 4 hours at room temperature. TGs were subsequently cryoprotected in a graded sucrose series (15% followed by 30% in PBS) and stored at 4°C until sectioning. TG tissues were embedded in optimal cutting temperature (OCT) compound, sectioned at 10 μm using a cryostat and mounted on slides. Following post-fixation, dura mater tissue was carefully dissected from the skull and stored at −20°C in anti-freeze until staining.

Trigeminal Ganglia (TG) Staining

TG sections containing cell bodies in the V1 region were selected and rehydrated in PBS. Antigen retrieval was performed using a citrate-based solution (1X, Cat #C9999, Sigma-Aldrich) at 90°C for 10 minutes. Sections were blocked and permeabilized for 1 hour at room temperature in PBS containing 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 to minimize non-specific binding.

Slides were incubated overnight at 4°C with goat anti-CGRP primary antibody (1:200; ab36001, Abcam). The following day, after PBS washes, sections were incubated with donkey anti-goat Alexa Fluor 488 secondary antibody (1:600, Cat# 705-546-147, Jackson ImmunoResearch) and anti-NeuN Alexa Fluor 555 conjugated antibody (1:100, MAB377A5, Millipore Sigma) for 2 hours at room temperature. Sections were then counterstained with DAPI and mounted using Prolong Glass Antifade Mountant (Cat# P36980, Invitrogen).

Dura Mater Staining

Following post-fixation, dura mater tissue was carefully dissected from the skull and stored at −20°C until staining. For staining, free-floating samples underwent antigen retrieval as above, followed by blocking and permeabilization for 1 hour in PBS containing 0.1% BSA and 0.3% Triton X-100.

Tissues were incubated overnight at 4°C with the following primary antibodies: goat anti-CGRP (1:200; ab36001, Abcam) and mouse anti-β-Tubulin III (Tubb3) (1:200, T8578, Millipore). After PBS washes, tissues were incubated with donkey anti-goat Alexa Fluor 488 (1:600, Cat# 705-546-147, Jackson ImmunoResearch) and donkey anti-mouse Cy3 (1:600, Cat# 705-546-147, Jackson ImmunoResearch) secondary antibodies for 1 hour at room temperature. Samples were counterstained with DAPI and mounted on slides using Prolong Gold Antifade Mountant (Cat# P36934, Invitrogen).

Confocal Microscopy and Image Analysis

The ophthalmic branch (V1) of the TG was selected for analysis due to its key role in innervating the cranial dura mater (24). The V1 was identified in longitudinal sections using an Echo Revolve microscope. Confocal z-stacks of TGV1 and dura mater were acquired using an Olympus Fluoview FV1200 confocal microscope with a 20×/0.8 objective. Imaging parameters and software settings were kept consistent across all samples.

Neuronal counts were conducted using IMARIS surface detection software. NeuN-positive ($NeuN^+$) and CGRP-positive ($CGRP^+$) cells were quantified using an automatic thresholding based on IMARIS-generated statistics, followed by manual refinement. Data were collected from n = 4 mice per group, with 2-4 replicates per animal.

Dural innervation density was quantified using FIJI (ImageJ). $CGRP^+$ and $Tubb3^+$ fibers were measured using Otsu thresholding to calculate positive area per mm^2. Measurements were taken from both vascular and avascular regions of the dura in each mouse (n=4 mice per group).

Low magnification overviews images (Fig. 3 and 6) were acquired using an Echo Revolve microscope with a 4×/0.13 objective and stitched together using FIJI. All maximum intensity projections in figures underwent background subtraction based on the mean intensity of all image pixels.

Experimental design

TG and dura mater were collected from naïve female and male mice one week following the animal arrival at the University of Arizona animal care facility to assess the number of CGRP-positive cell bodies and CGRP-positive fibers using immunohistochemistry.

CGRP was administered at 1 pg/5µL onto the dura mater of a different cohort of naïve female and male mice immediately after collecting baseline tactile frequency of response. Von Frey testing was performed at 10, 20, 40, 60, 120 and 180 min after supradural injections.

The effect of supradural administration of subthreshold CGRP on inducing CA was assessed using a dose of 0.1 pg/5µL for females and 1 pg/5µL for males. Subthreshold doses of CGRP were previously reported for both female and male mice (11, 70). Tactile frequency of response was collected before (baseline) and at 10, 20, 40, 60, 120 and 180 min after supradural CGRP injection. On the following day, post-CGRP time course, tactile frequency of response was collected (baseline) and CM-like, MOH-like and persistent PTH-like behaviors were induced by repeated administration of NTG or sumatriptan or induction of mTBI, respectively. Control mice received either saline or sham procedure. CA was evaluated on indicated days post-NTG, -sumatriptan onset administration, or post-mTBI induction. On days 18 post-NTG, or 21 post-sumatriptan, or 14 post-mTBI, tactile frequency of response was collected as a baseline, and animals received the same previous supradural subthreshold dose of CGRP, i.e., 1 pg for males and 0.1 pg for females, followed by CA evaluation at 10, 20, 40, 60, 120 and 180 min after CGRP injection. TG and dura mater were collected the following day to assess the number of CGRP-positive cell bodies using immunohistochemistry.

The timeline of each experimental procedure is represented in Figure 1.

STATISTICAL ANALYSIS

Sample size was determined using the GPower 3.1 software, with an established significance level of $p < 0.05$. Data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software). Time course experiments for sensory thresholds were analyzed using two-way analysis of variance (ANOVA) with repeated measurements followed by Tukeys's multiple comparisons test. An unpaired two-tailed Student t-test was used to analyze immunohistochemistry data. Statistically significant differences were considered when $p < 0.05$. The statistical analysis, numbers of animals used (n), p values, and F ratios are reported in Table 1.

RESULTS

SEXUALLY DIMORPHIC EFFECT OF SUPRADURAL CGRP IN ELICITING HEADACHE-LIKE PAIN BEHAVIOR

Administration of CGRP at 1 pg/5 µL onto the dura mater elicited robust headache-like behaviors in female, but not male, mice. In female mice, supradural CGRP administration significantly increased cutaneous allodynia (CA) as evidenced by heightened response to tactile stimulation in both the periorbital (Fig. 2a) and hind paw regions (Fig. 2b) compared to vehicle-treated controls (Fig. 2a, 2b). In contrast, male mice did not exhibit a significant increase in tactile sensitivity following CGRP administration (Fig. 2c, 2d). These findings confirm a sexually dimorphic response to CGRP as previously reported, with females showing increased susceptibility to CGRP-induced headache-like pain and highlight the dose of 1 pg/5 µL as a subthreshold dose of CGRP in males.

INCREASED NUMBER OF CGRP-POSITIVE TGV1 NEURONS IN NAÏVE FEMALE, COMPARED TO MALE, MICE BUT SIMILAR CGRP-POSITIVE FIBERS IN THE DURA MATER

Immunohistochemical analysis of longitudinal sections of the ophthalmic branch (V1) of the trigeminal ganglion (TGV1) revealed an increased number of CGRP-immunoreactive neurons in naïve female mice compared to males. Sections were stained with anti-CGRP (green), pan-neuronal anti-NeuN (magenta), and counterstained with DAPI (blue). Quantitative analysis showed 42.4% of NeuN-positive neurons were CGRP-positive in female TGV1 (Fig. 3a), whereas 28.8% were CGRP-positive in males (Fig. 3b), indicating a sex-dependent difference in CGRP expression within the TG (Fig. 3e).

In contrast, analysis of dural innervation revealed no sex difference in CGRP-positive fiber density. Free floating dura mater samples were immunostained with anti-CGRP (green) and the neuronal marker βIII-tubulin (Tubb3) (magenta) to visualize overall innervation. The percentage of CGRP-positive fibers, normalized to total Tubb3-positive fibers, did not differ between female (Fig. 3c) and male (Fig. 3d) mice, suggesting comparable CGRP-positive fiber distribution in both vascular and avascular regions of the dura mater (Fig. 3f).

Together, these findings suggest that while CGRP expression in the trigeminal ganglion is sexually dimorphic, peripheral CGRP fiber density in the dura mater is similar between sexes (Fig. 3).

INCREASED CGRP SENSITIVITY FOLLOWING INDUCTION OF CM WITH REPEATED ADMINISTRATION OF NTG

Administration of a subthreshold dose of CGRP at 0.1 pg onto the dura mater of female mice did not alter tactile sensory responses in the periorbital (Fig. 4a) or hind paw (Fig. 4b) regions. In male mice, a dose ten times higher (1 pg) applied supradurally also failed to induce CA, as measured by tactile threshold testing in the same regions (Fig. 4c, 4d). These findings confirm that the doses of 0.1 and 1 pg/5 µL of CGRP are insufficient to elicit headache-like pain behaviors in naïve female and male animals.

In contrast, repeated systemic administration of NTG induced a robust CA phenotype in both female and male mice. Sensitivity to tactile stimulation in the periorbital and hind paw regions significantly increased by day 5 of the NTG treatment regimen and persisted until day 17, at which point thresholds returned to baseline (Fig. 4e, 4f, 4g, 4h).

Following recovery, on day 18, the same previously subthreshold doses of CGRP (0.1 pg in females, 1 pg in males) now induced significant CA in both sexes (Fig. 4i, 4j, 4k, 4l). No changes in tactile thresholds were observed in vehicle-control animals at any point (Fig. 4).

These results indicate that repeated NTG administration primes the trigeminovascular system enhancing behavioral sensitivity to previously ineffective doses of CGRP, and support a model in which migraine chronification is associated with peripheral CGRP sensitization.

Repeated NTG Treatment Increases CGRP Expression in Trigeminal Neurons but Not in Dural Fibers

Immunohistochemical analysis of TG tissue revealed increased CGRP immunoreactivity in the V1 region of the TG in both female and male mice following repeated NTG treatment. Enhanced CGRP-positive staining in neuronal cell bodies was observed in NTG-treated animals compared to vehicle-treated controls (Fig. 5a, 5c). Quantification confirmed a significant increase in the percentage of CGRP-positive neurons in both sexes after repeated NTG administration (Fig. 5b, 5d), indicating upregulation of CGRP expression within the TG.

In contrast, no significant differences were observed in the percentage of CGRP-positive fibers within the dura mater between NTG- and vehicle-treated animals in either male (Fig. 6d) or female (Fig. 6b) mice. This suggests that repeated NTG exposure selectively enhances CGRP expression in trigeminal cell bodies without altering the density of CGRP-containing fibers in the dura.

These findings support the hypothesis that chronic migraine-like conditions upregulate CGRP signaling at the level of the trigeminal ganglion, potentially contributing to enhanced sensitivity to peripheral CGRP in sensitized animals.

Sumatriptan-induced MOH Enhances CGRP Sensitivity

Initial testing confirmed sex-dependent subthreshold doses of CGRP did not alter tactile sensory responses in the periorbital and hind paw regions of either female (Fig. 7a, 8b) or male (Fig. 7b, 8b) as in the previous experiment (Fig. 4).

Following this confirmation, mice underwent a repeated sumatriptan treatment protocol to induce a model of medication overuse headache (MOH). Repeated administration of sumatriptan resulted in the development of persistent CA in both the periorbital and hind paw regions of female (Fig. 7c, 8c) and male (Fig. 7d, 8d) mice. CA onset began by day 5 and resolved by day 20, with tactile thresholds returning to baseline levels (Fig. 7, 8).

On day 21, after resolution of sumatriptan-induced allodynia, supradural administration of the previously subthreshold dose of CGRP now elicited significant CA in both male (Fig. 7f, 8f) and female (Fig. 7e, 8e) animals. Conversely, no changes in tactile sensitivity were observed in vehicle-treated control animals. These findings indicate that repeated sumatriptan exposure induces a sensitized state that enhances nociceptive behavioral responses to CGRP, consistent with a role for peripheral CGRP sensitization in MOH pathophysiology.

mTBI-Induced PTH Increases Sensitivity to Supradural CGRP

Animals subjected to mTBI developed transient periorbital and hind paw CA (Fig. 9) consistent with the development of acute PTH. mTBI-induced CA was resolved by day 5 in male mice (Fig. 9c, 9d) and by day 7 in female mice (Fig. 9a, 9b), while no changes in tactile sensitivity were observed in sham animals.

On day 13 post-injury, when tactile thresholds were back to baseline levels, administration of sex-specific subthreshold doses of CGRP (0.1 and 1 pg/5 μL for females and males, respectively) onto the dura mater elicited robust CA in both male (Fig. 9g, 9h) and female (Fig. 9e, 9f) mice, with no response observed in controls (Fig. 9). These results indicate that mTBI induces a sensitized trigeminovascular state that enhances susceptibility to non-noxious doses of CGRP, supporting the involvement of CGRP in the pathogenesis of PTH persistence.

DISCUSSION AND CONCLUSIONS

This study aimed to investigate the sexually dimorphic contribution of CGRP to migraine as attacks become more frequent, and to contrast these findings to a non-migraine chronic headache model, specifically persistent PTH induced by mTBI. We observed increased sensitivity to CGRP in repeated NTG and sumatriptan exposure and PTH induced by mTBI in both male and female mice. This led to the conclusion that increased frequency of migraine attacks is associated with increased sensitivity to CGRP and with higher increased expression of CGRP in TGV1 in both male and female animals. In addition, this increased sensitivity to CGRP is also associated with the persistence of PTH.

Sex differences underlying sensitivity to supradural CGRP were observed in naïve male and female mice. Higher density of CGRP-expressing neurons was observed in the TG of naïve female compared to male. These results support previous findings of female-preferential nociceptive effects of supradural CGRP seen in other preclinical rodent models (11, 94, 84) and align with other evidence of sex differences in migraine pain mechanisms observed in non-human primate models and post-mortem human tissue (104, 49, 59, 63, 80, 88, 92, 102, 109, 112, 118, 115). Despite this enhanced response in females, CGRP can elicit nociceptive behaviors in male mice, an effect largely overlooked in previous studies (88, 11, 12). In the current study, we demonstrated nociceptive effects of CGRP in male mice at a dose 10 times higher than that required for female mice. Female sensitivity to CGRP is further reflected clinically in the higher prevalence of migraine among women and by the preferential efficacy of CGRP-targeting therapies in the acute treatment of EM (95, 108, 9, 89). Moreover, female sex hormones have been shown to have great influence on the regulation of CGRP, further underscoring its sexually dimorphic effects (91, 29, 41, 12, 49, 115). Interestingly, all chronic headache models tested in the current study produced heightened sensitivity to CGRP in both sexes, suggesting that increased CGRP responsiveness may contribute to greater susceptibility to subsequent attacks. Notably, females exhibited hypersensitivity to CGRP's nociceptive effects compared with males, potentially contributing to the well-established association of female sex and an increased risk of developing chronic migraine, MOH, and persistent PTH – all of which CGRP-targeting therapies have demonstrated notable efficacy in managing (95, 108, 9, 48, 100, 85, 62, 6). Although there were no observed sex differences in CGRP-positive dural sensory innervation, area-based measurements should be noted as limitations. Differences in smaller microvascular structures within the dural stroma could still provide meaningful insight into peripheral mechanisms contributing to sex-specific CGRP sensitivity, such as changes in receptor expression.

Repeated NTG administration modeling frequent headache-like attacks sensitized both male and female mice to CGRP. As a nitric oxide (NO) donor, NTG can induce migraine-like headaches in migraineurs, including headache pain and other migraine symptoms that can be relieved with anti-migraine treatments (55, 50). Like spontaneous migraine headaches, NTG-induced attacks correlate with increased plasma levels of CGRP in patients and similarly in animal models (51). Vasodilation produced from NO can activate nociceptive trigeminal afferents, and repeated exposure leads to sensitization of the trigeminal system (2, 31, 39). NTG-triggered headaches have also been linked to central actions through activation of pain centers in brain imaging studies (111, 13, 1). Therefore, repeated NTG-induced attacks serve as a translationally relevant model of CM. Previous preclinical studies have linked NTG exposure to increased CGRP promoter activity (15), which aligns with our current findings showing an increased number of CGRP-positive neurons in the TGs of both male and female mice sensitized to CGRP following NTG treatment. Increased CGRP expression correlates with nociceptive behavior evoked by subthreshold CGRP dose. Additionally, frequent exposure to sumatriptan resulted in MOH-like pain and plasticity changes that promote headache through increased CGRP sensitivity in both male and female mice. Similarly, sumatriptan-induced MOH has been linked to increased neuronal nitric oxide synthase in rodent TGs (26) as well as increased CGRP expression in TG cell bodies retrogradely labeled from the dura in male rats (27). Through possible compensatory mechanisms, frequent sumatriptan use may be upregulating NO mechanisms influencing CGRP signaling therefore developing a CM-like condition.

Notably, repeated use of gepants (i.e. ubrogepant) does not induce a state of latent sensitization like other acute migraine medications (i.e. triptans, NSAIDs, oipioids) (83) and blocking CGRP signaling with small molecule antagonists or anti-CGRP mAbs blocks chronification in preclinical models (83, 85, 64) and proves to be effective prophylactic therapy in chronic migraine patients with co-occurring MOH (87, 100). Peripheral CGRP signaling may engage a cascade of events that sensitizes the trigeminal system, possibly through inflammatory mechanisms or sensitization of nociceptive fibers, to amplify pain signaling (45). However, as the frequency of migraine increases and develops into chronic conditions, other CGRP-independent mechanisms or parallel contributions of other neuropeptide or neurotransmitter systems may be recruited resulting in anti-CGRP therapies becoming less effective. Such effects have been observed in randomized controlled trials evaluating the efficacy of preventative CGRP-targeting therapies in CM compared to EM in patients (33).

Post-traumatic headache, a common and debilitating symptom following concussive head injury, involves CGRP-dependent mechanisms that contribute to its migraine-like features (76, 8, 18, 19, 85, 7). Preclinical studies have shown immediate and sustained treatment with anti-CGRP therapeutics can block the development of persistent PTH in rodents but are insufficient as acute treatment once established (85, 19). Furthermore, a recent open-label trial showed that the CGRP receptor antagonist erenumab significantly reduced headache frequency in patients with migraine-type persistent PTH (6), and additional migraine-specific acute and prophylactic treatments have likewise demonstrated partial benefit in persistent PTH (78) but remain largely insufficient (4). This study further supports an enhanced role for CGRP in persistent PTH following mTBI, underscoring its potential as promising therapeutic target. We observed an increase in CGRP sensitivity in both male and female mice, indicating that mTBI induces a sensitized state in which subthreshold CGRP, administered onto the dura mater, was sufficient to elicit headache-like behaviors. Many persistent PTH patients experience migraine-like symptoms during injury-associated headaches that are associated with the consequential sensitization of the trigeminal system (4). Furthermore, a prior history of migraine and female sex are among the biggest risk factors for PTH persisting months after initial injury (107, 78). Given these overlaps and the demonstrated efficacy of CGRP-targeted therapies in reducing and preventing headaches in women and chronic migraine patients, such therapies may offer a promising option for PTH – particularly in the absence of established treatment guidelines or approved treatments.

Together, these findings suggest that CGRP assumes an increasingly prominent role in pain signaling through dural afferents as migraine and PTH transition to a chronic, sensitized state compared with a non-sensitized state. This shift may explain why females, who can be considered inherently more susceptible to CGRP-mediated mechanisms, show early responsiveness to CGRP-targeted therapies, whereas males demonstrate comparable therapeutic benefit once a chronic or sensitized state is established (89). In these studies, supradural application of subthreshold CGRP was sufficient to evoke headache-like pain behavior only in mice induced with chronic migraine or PTH, indicating that heightened sensitivity in these chronic headache conditions correlates with greater headache frequency. Migraine, a lifelong disorder, is characterized by reduced sensory thresholds resulting in a sensitized state susceptible to headache triggers (45, 22). Repeated attacks progressively lower the activation threshold of meningeal nociceptors, increasing reactivity to normally innocuous stimuli, including CGRP signaling. This compounding effect may promote and sustain a state of central sensitization that is hyperresponsive to CGRP's nociceptive effects, thereby amplifying pain processing so that normally innocuous stimuli can trigger migraine attacks. The findings of this study highlight the therapeutic potential of anti-CGRP interventions as a first-line or preventative strategy, particularly for individuals who may be susceptible to the effects of CGRP.

FIGURES

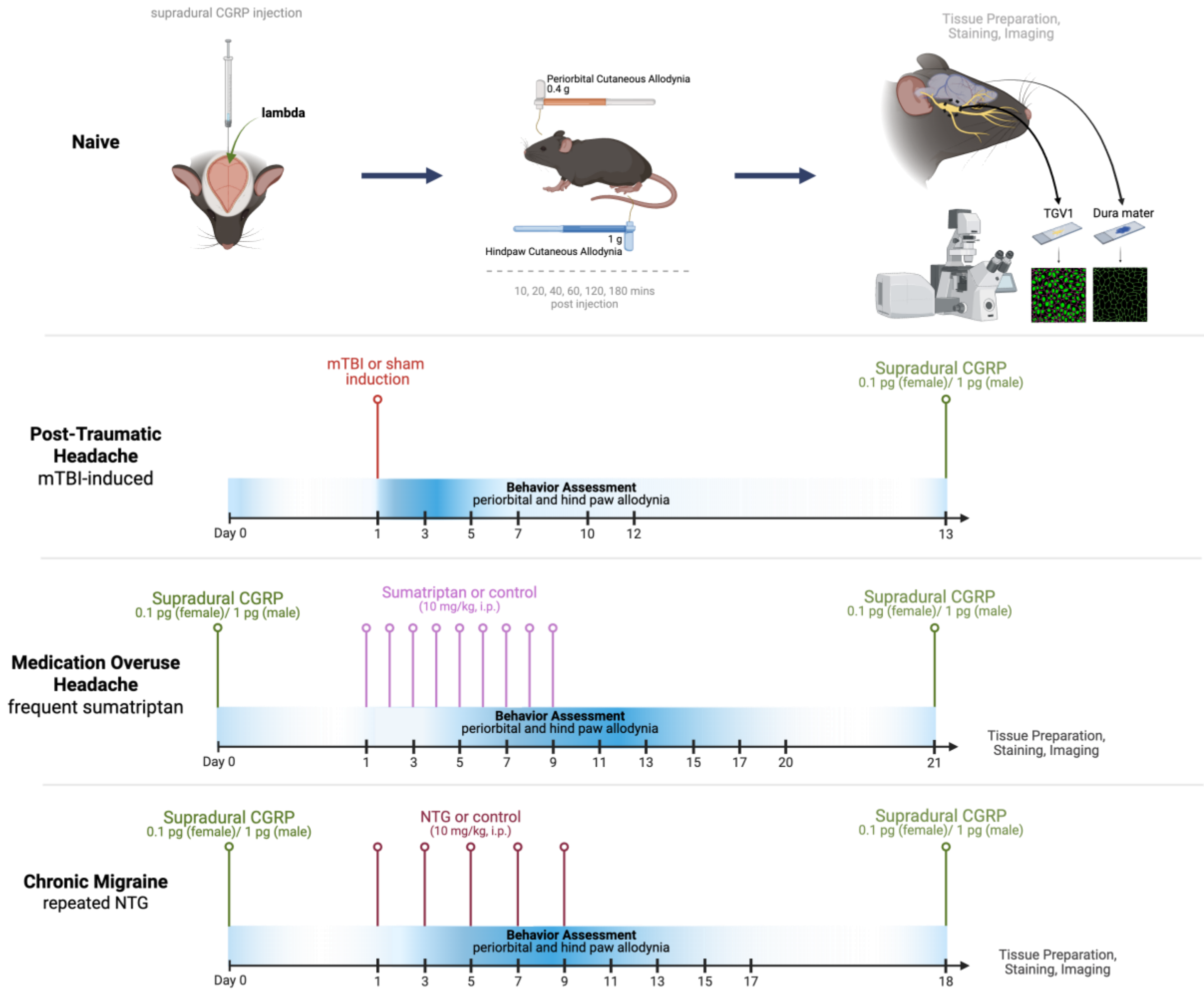

Figure 1. Timeline of experimental procedures. CGRP = calcitonin gene-related peptide; NTG = nitroglycerin; MOH = medication overuse headache; PTH = post-traumatic headache; TGV1 = ophthalmic V1 region of the trigeminal ganglion. Created with Biorender.

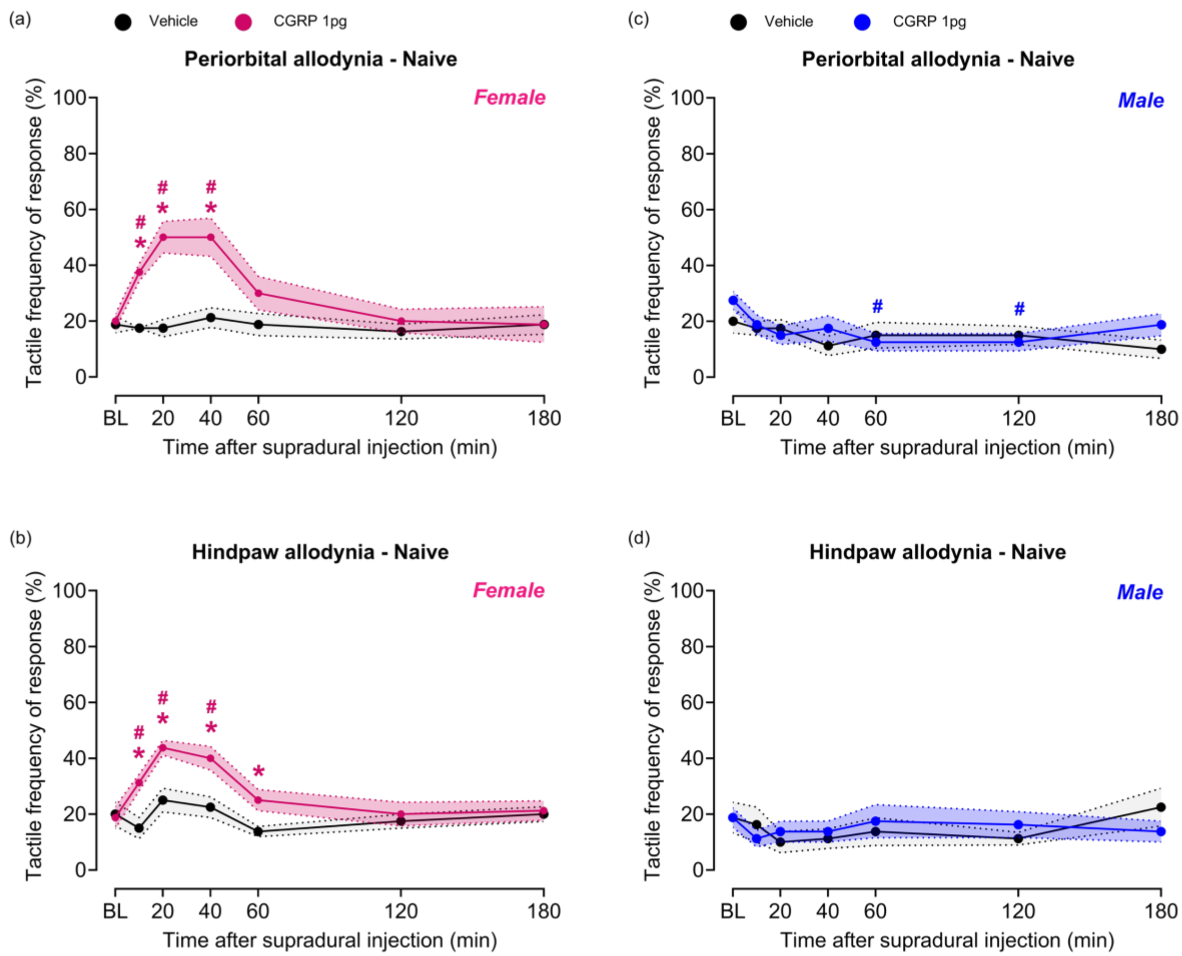

Figure 2. Female, but not male, naïve mice display migraine-like pain behavior in response to 1 pg of CGRP administered onto the dura mater. Periorbital tactile frequency responses were collected at a baseline (BL) and 10, 20, 40, 60, 120, and 180 min after supradural injection of CGRP at a dose of 1 pg in (a, b) female and (c, d) male naïve mice. Data are presented as mean ± SEM and were analyzed using two-way ANOVA followed by Tukey's multiple comparison test for (n = 8 per group), with * representing $p < 0.05$ for comparisons between groups and # representing $p < 0.05$ for comparisons to baseline within the CGRP-treated group. Details of the statistical analysis can be found in Table 1.

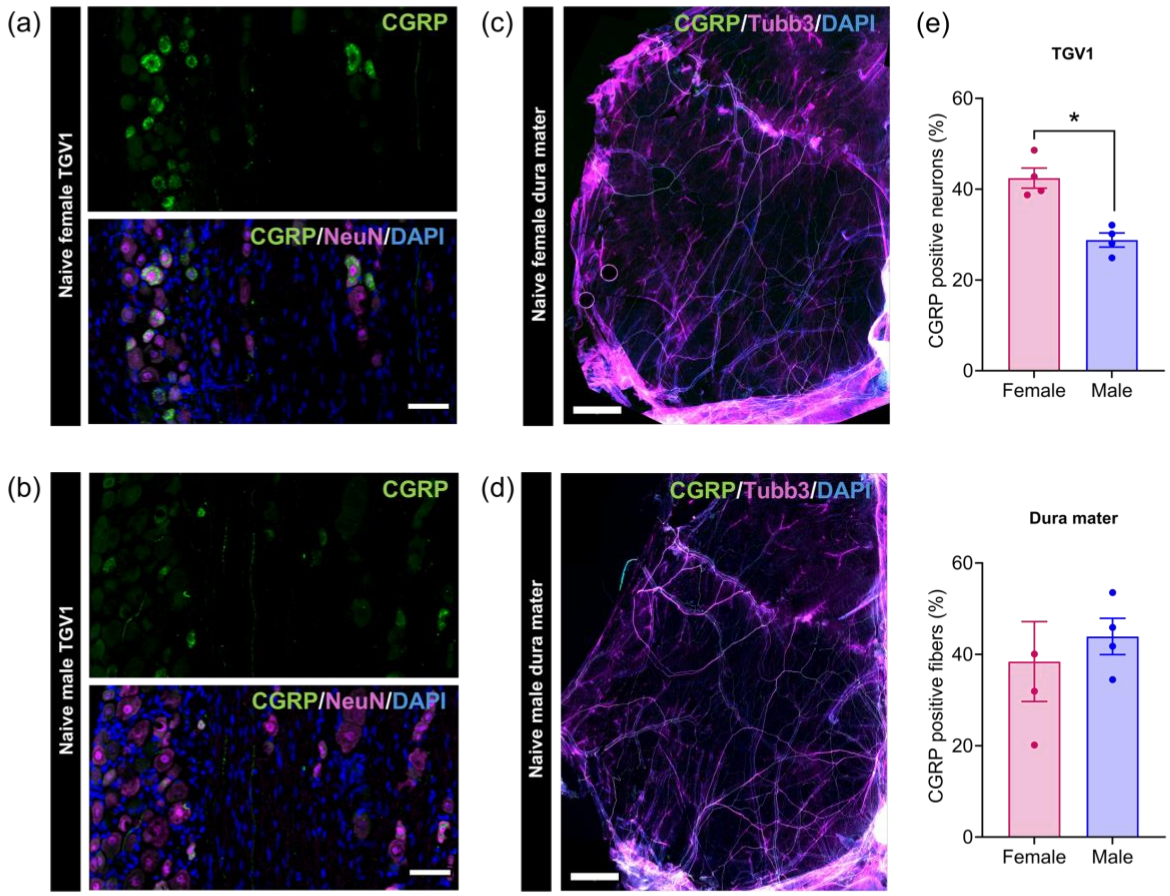

Figure 3. Female mice exhibit higher number of CGRP-positive neurons in the TGV1. There are no observed differences in CGRP-positive neuronal fiber density in the dura mater between the sexes. Representative photomicrographs of sections of naïve (c) female and (d) male mouse dura mater immunostained with antibodies targeting CGRP (green), Tubb3 (magenta) and DAPI (blue). Scale bar: 1 mm. Quantification of CGRP positive neurons in (e) the TGV1 and (f) neuronal fibers in the dura mater of female and male naïve mice. Data are presented as mean ± SEM and were analyzed using (e and f) unpaired two-tailed Student's t-test ($n = 4$ per group with 2-4 replicates per mouse), with * representing $p < 0.05$ for comparisons between groups. Data values for individual mice are shown as small symbols in (g and h). Details of the statistical analysis can be found in Table 1.

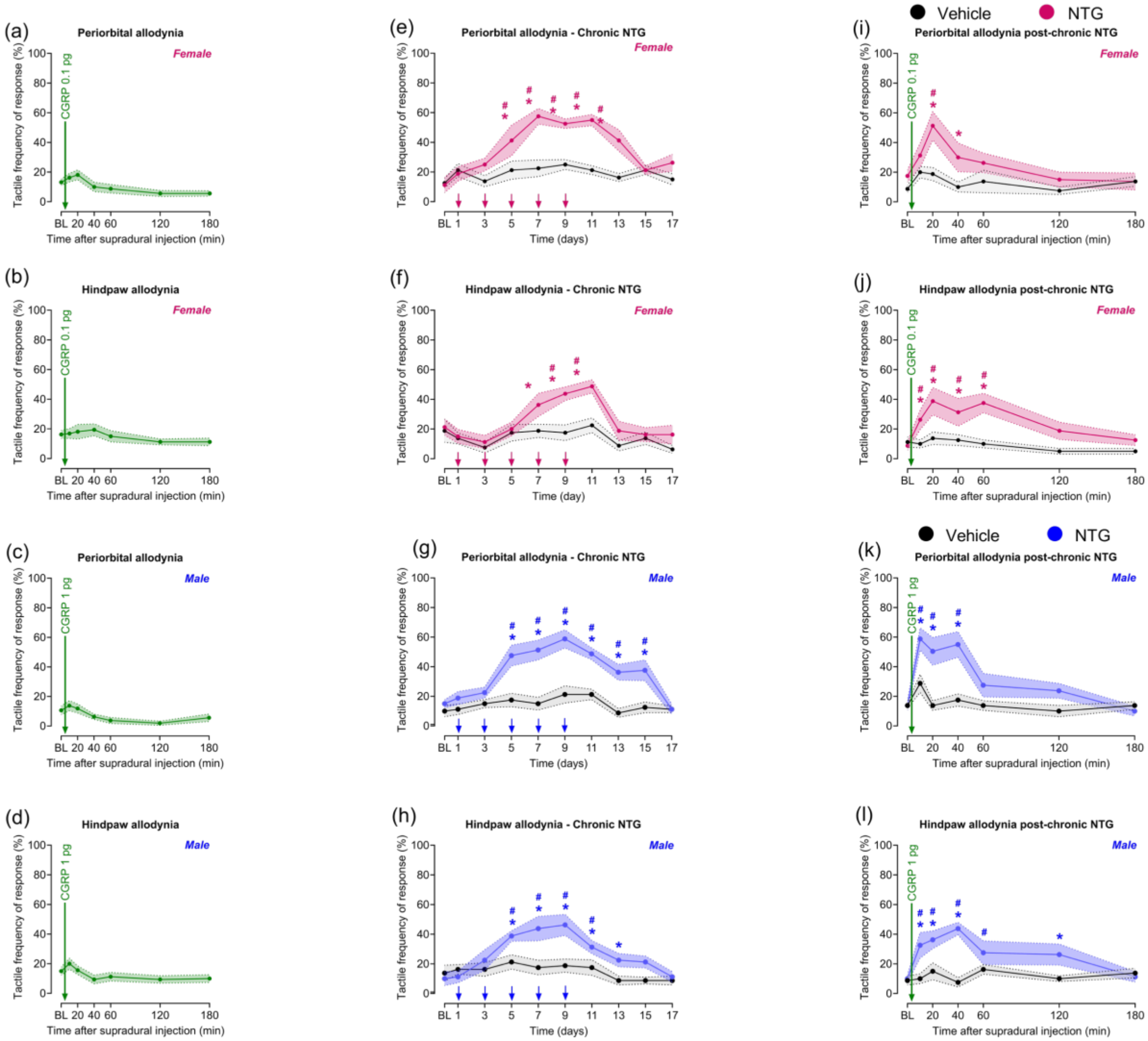

Figure 4. Repeated NTG increases CGRP sensitivity to promote chronic migraine (CM)-like pain. Periorbital tactile frequency responses were collected at baseline (BL) and at indicated times after supradural injection of CGRP at a subthreshold dose of (a) 0.1 pg for females and (c) 1 pg for males. On the following day, BL periorbital tactile frequency responses were collected, and CM-like pain was induced in (e) female and (g) male mice by repeated administration of NTG (10mg/kg, i.p.) every other day for 9 days (5 treatments in total), as indicated by arrows. Control mice received saline. Periorbital tactile frequency responses were measured on days 1, 3, 5, 7, 9, 11, 13, 15 and 17 post-NTG onset. On treatment days, CA was evaluated before NTG administration. On day 18 post-NTG onset (i) female and (k) male mice received a supradural injection of the respective subthreshold dose of CGRP, followed by periorbital tactile frequency response measurements. Data are presented as mean ± SEM and were analyzed using two-way repeated measures ANOVA followed by Tukey's multiple comparison test (n = 8 per group), with * representing $p < 0.05$ for comparisons between groups, and # representing $p < 0.05$ for comparisons to BL within the NTG-treated group. Details of the statistical analysis can be found in Table 1.

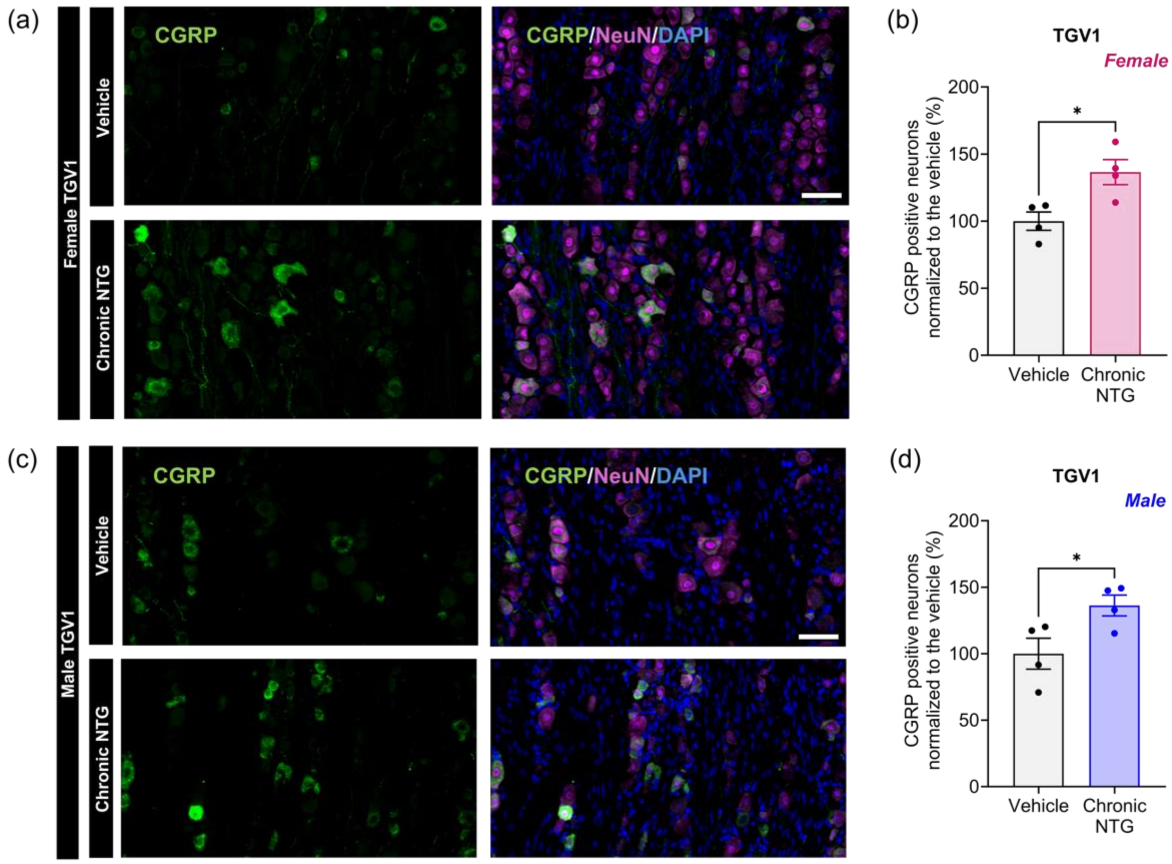

Figure 5. Repeated NTG increased the number of CGRP-positive cell bodies in the TGV1 of female and male mice. Representative photomicrographs of longitudinal sections of repeated NTG- or vehicle-treated (a) female and (c) male mouse TGV1 immunostained for anti-CGRP (green), NeuN (magenta), and DAPI (blue). Scale bar: 50 µm. Quantification of CGRP positive neurons in the TGV1 of (b) female and (d) male mice. Data are presented as mean ± SEM and were analyzed using (b and d) an unpaired two-tailed Student's t-test ($n = 4$ per group with 2-4 replicates per mouse), with * representing $p < 0.05$ for comparisons between groups. Data values for individual mice are shown as small symbols in (b and d). Details of the statistical analysis can be found in Table 1.

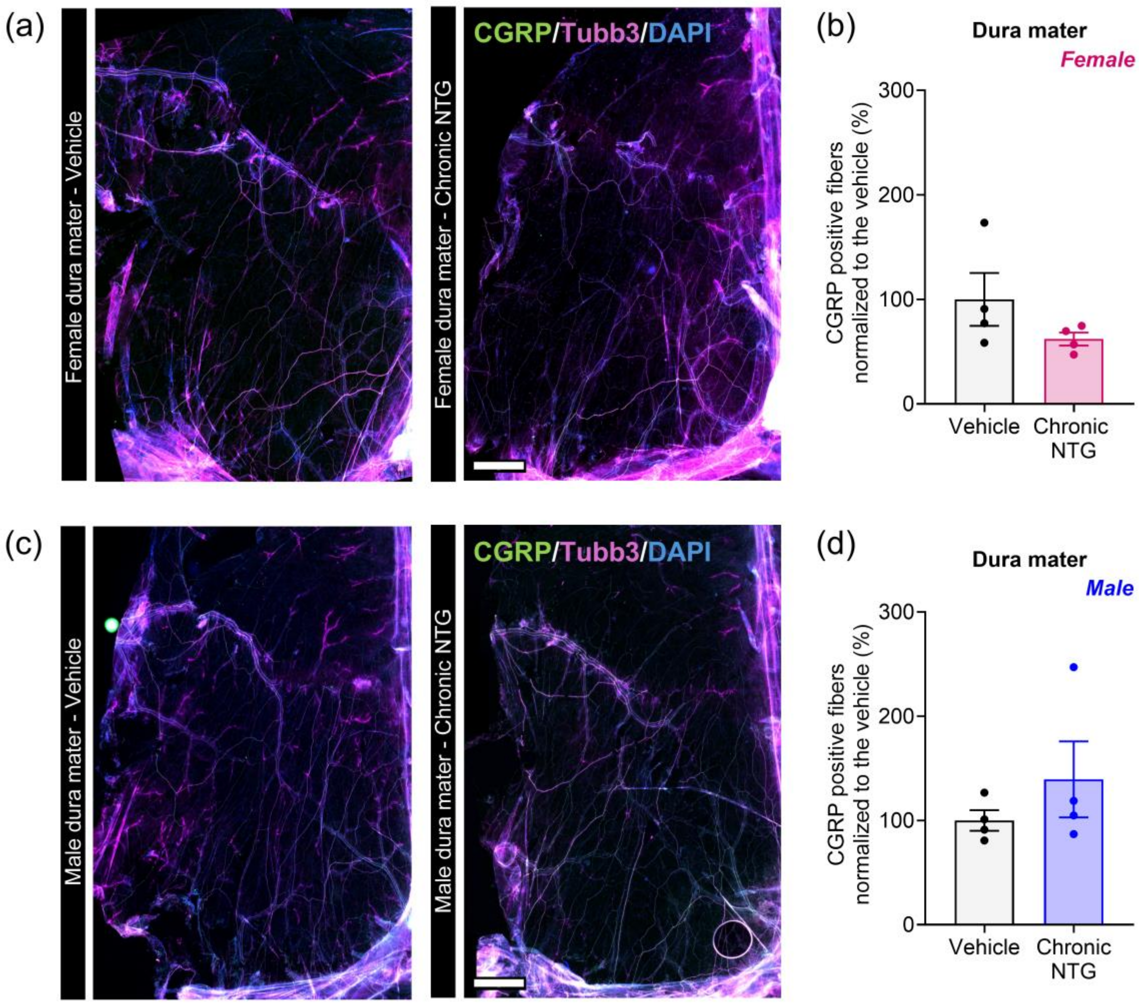

Figure 6. Repeated NTG did not modify the proportion of CGRP-positive neuronal fibers in the dura mater of female and male mice. Representative confocal photomicrographs of dura mater sections of (a) female and (c) male mouse treated with repeated NTG- or vehicle immunostained with antibodies against CGRP (green), Tubb3 (magenta) and DAPI (blue). Scale bar: 1 mm. Quantification of CGRP positive fibers as percent of Tubb3 fibers in the dura mater of repeated NTG- or vehicle-treated (b) female and (d) male mice. Data are presented as mean ± SEM and were analyzed using (b and d) an unpaired two-tailed Student's t-test (n = 4 per group with 2-4 replicates per mouse). Data values for individual mice are shown as small symbols in (b and d). Details of the statistical analysis can be found in Table 1.

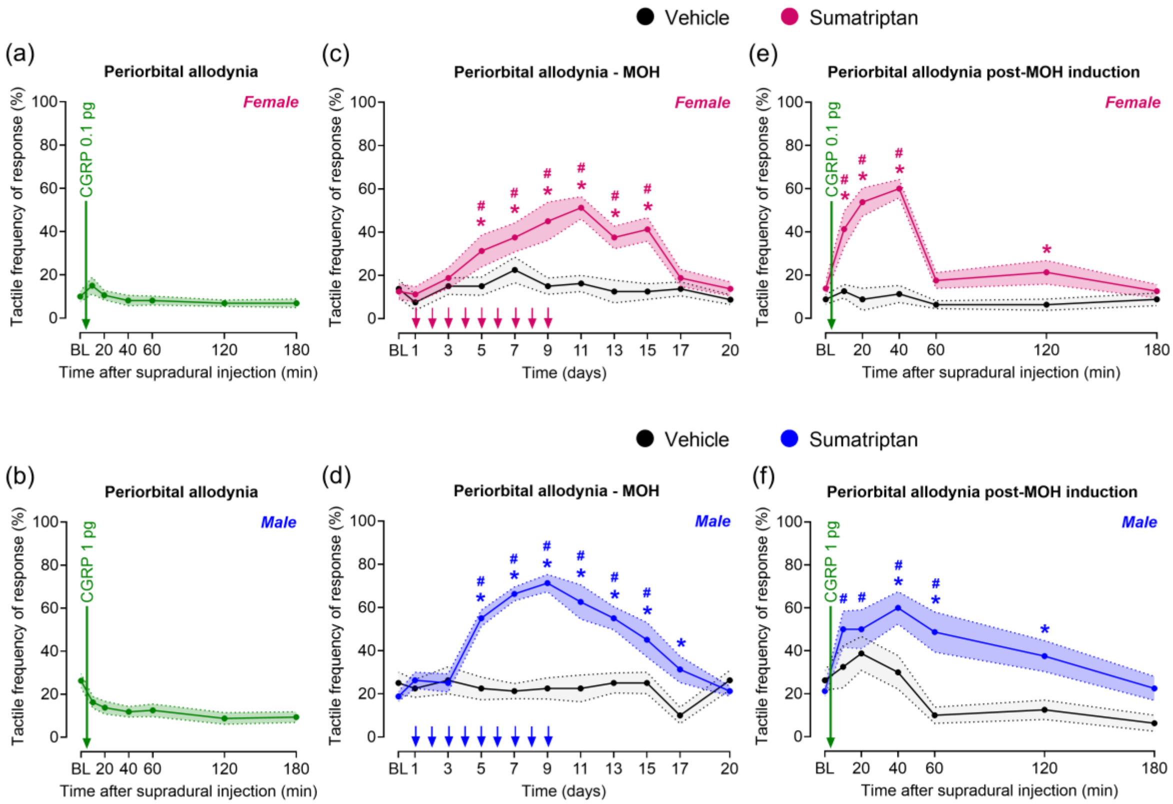

Figure 7. Repeated sumatriptan-induced MOH enhances CGRP sensitivity to promote migraine-like pain behavior. Periorbital tactile frequency responses were collected at baseline (BL) and after supradural injection of CGRP at a subthreshold dose of (a) 0.1 pg for females and (b) 1 pg for males. On the following day, BL periorbital tactile frequency responses were collected, and CM-like pain was induced in (c) female and (d) male mice by repeated administration of sumatriptan (10 mg/kg, i.p.) for 9 consecutive days, as indicated by arrows. Control mice received saline. Periorbital tactile frequency responses were measured on indicated days post-sumatriptan onset. On treatment days, CA was evaluated before sumatriptan administration. On day 21 post-sumatriptan onset, (e) female and (f) male mice received a supradural injection of the subthreshold dose of CGRP, followed by periorbital tactile frequency response measurements. Data are presented as mean ± SEM and were analyzed using (c-f) two-way repeated measures ANOVA followed by Tukey's multiple comparison test (n = 8 per group), with * representing $p < 0.05$ for comparisons between groups and # representing $p < 0.05$ for comparisons to baseline within the sumatriptan-treated group. Details of the statistical analysis can be found in Table 1.

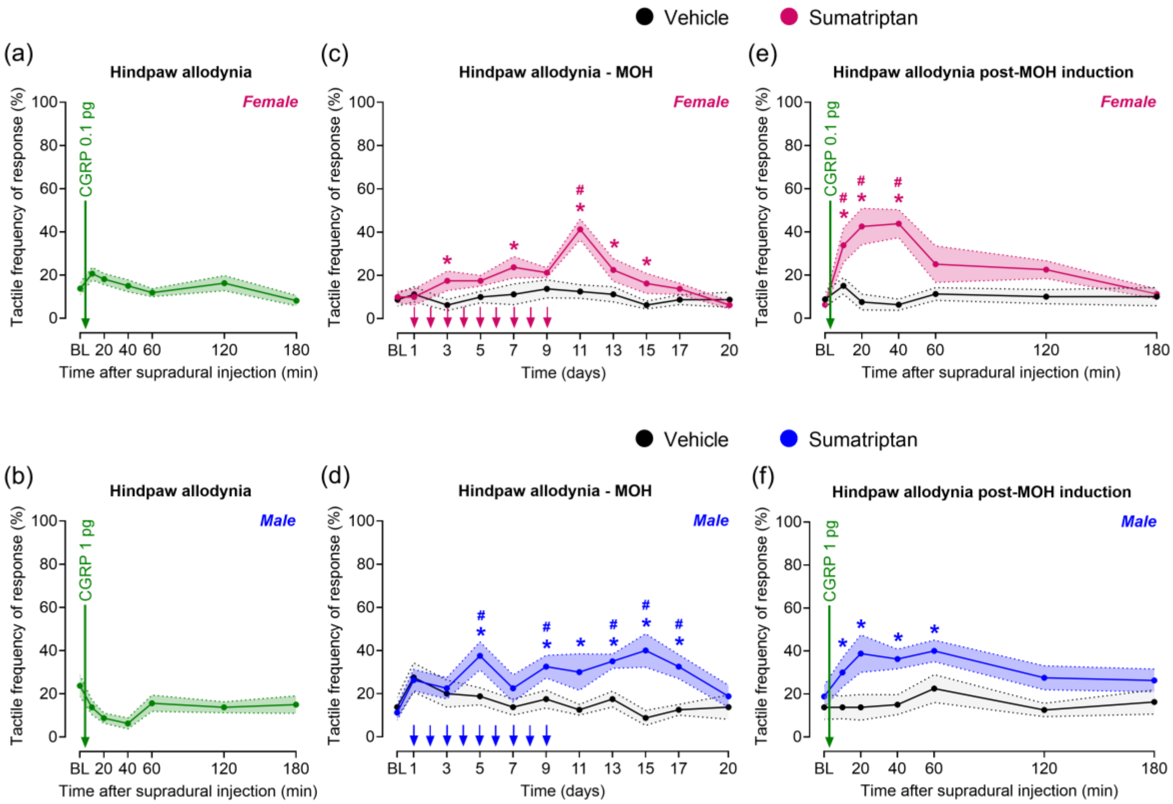

Figure 8. Repeated sumatriptan-induced MOH increases CGRP sensitivity to promote extracephalic CA. Hindpaw tactile frequency responses were collected at baseline (BL) and at 10, 20, 40, 60, 120, and 180 min after supradural injection of CGRP at a subthreshold dose of (a) 0.1 pg for female and (b) 1 pg for male mice. On the following day, post-CGRP time course, BL hindpaw tactile frequency responses were collected, and CM-like pain was induced in (c) female and (d) male mice by repeated administration of sumatriptan (10 mg/kg, i.p.) for 9 consecutive days, as indicated by arrows. Control mice received saline. Hindpaw tactile frequency responses were measured on days 1, 3, 5, 7, 9, 11, 13, 15, 17, and 20 post-sumatriptan onset. On treatment days, CA was evaluated before sumatriptan administration. On day 21 post-sumatriptan onset, hindpaw tactile frequency responses were collected as BL, and (e) female and (f) male mice received a supradural injection of the subthreshold dose of CGRP, followed by hindpaw tactile frequency response measurements. Data are presented as mean ± SEM and were analyzed using two-way repeated measures ANOVA followed by Tukey's multiple comparison test (n = 8 per group), with * representing $p < 0.05$ for comparisons between groups, and # representing $p < 0.05$ for comparisons to baseline within the sumatriptan-treated group. Details of the statistical analysis can be found in Online Supplementary Table 1.

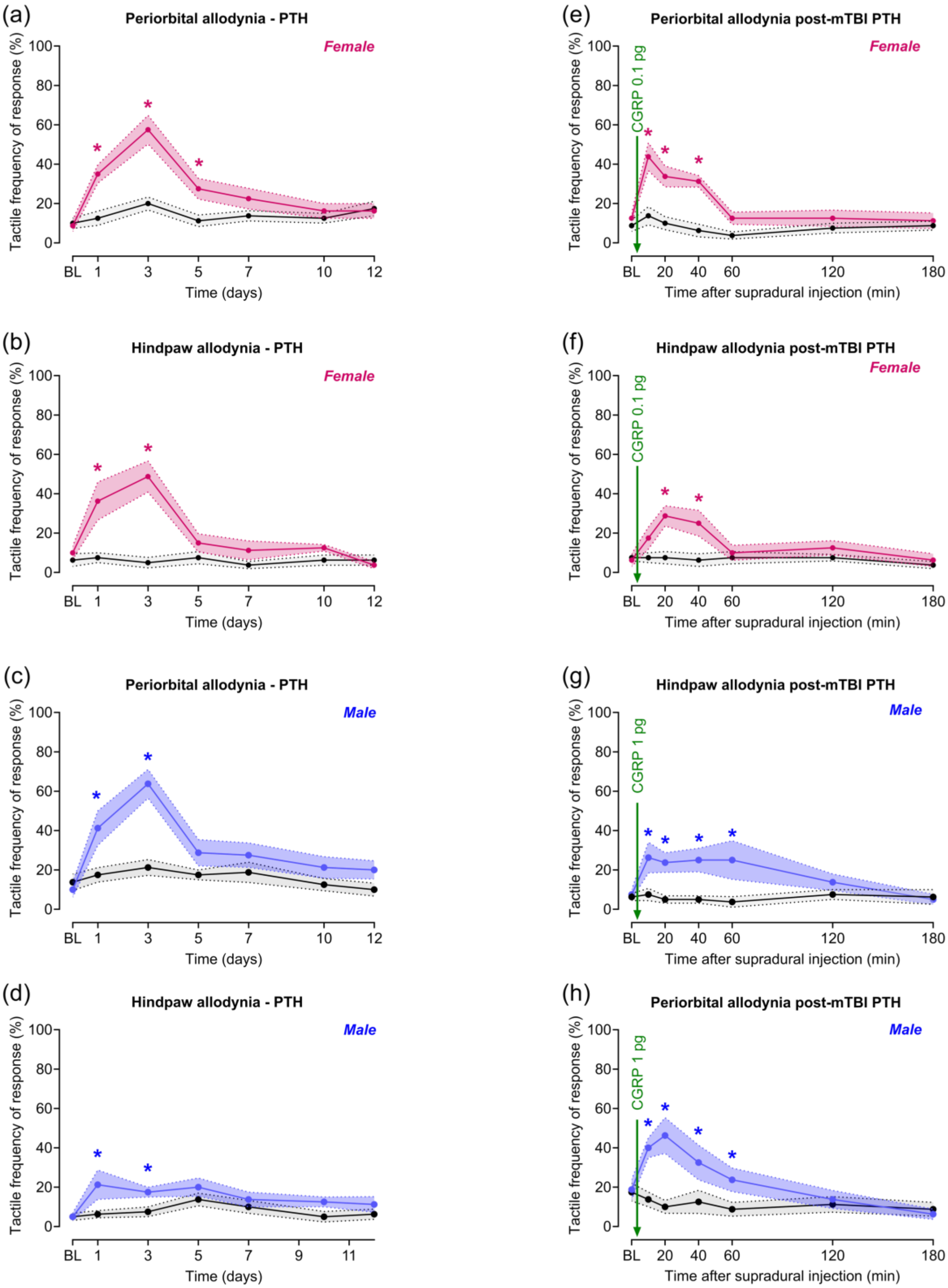
(a)
Periorbital allodynia - PTH
Female
Tactile frequency of response (%)
Time (days)
(b)
Hindpaw allodynia - PTH
Female
Tactile frequency of response (%)
Time (days)
(c)
Periorbital allodynia - PTH
Male
Tactile frequency of response (%)
Time (days)
(d)
Hindpaw allodynia - PTH
Male
Tactile frequency of response (%)
Time (days)
(e)
Periorbital allodynia post-mTBI PTH
Female
CGRP 0.1 pg
Tactile frequency of response (%)
Time after supradural injection (min)
(f)
Hindpaw allodynia post-mTBI PTH
Female
CGRP 0.1 pg
Tactile frequency of response (%)
Time after supradural injection (min)
(g)
Hindpaw allodynia post-mTBI PTH
Male
CGRP 1 pg
Tactile frequency of response (%)
Time after supradural injection (min)
(h)
Periorbital allodynia post-mTBI PTH
Male
CGRP 1 pg
Tactile frequency of response (%)
Time after supradural injection (min)

Figure 9. mTBI-induced PTH sensitizes to CGRP to promote CM-like pain behavior. Periorbital and hind paw tactile frequency responses were collected at baseline (BL) and on the following day, mTBI or sham was induced. CA was evaluated on days 1, 3, 5, 7, 10, and 12 post-mTBI induction in female (a, b) and male (c, d) mice. On day 13 post-injury onset (e, f) female and (g, h) male mice received a supradural injection of the respective subthreshold dose of CGRP, followed by periorbital and hind paw tactile frequency response measurements. Data are presented as mean ± SEM and were analyzed using two-way repeated measures ANOVA followed by Tukey's multiple comparison test (n = 8 per group), with * representing $p < 0.05$ for comparisons between groups, and # representing $p < 0.05$ for comparisons to BL within the mTBI-treated group. Details of the statistical analysis can be found in Table 1.

Table 1. Summary of statistical analysis

Figures	Analysis	Test statistic	p-value	n
2a	Two-way repeated-measures ANOVA	F (6, 84) = 10.19	<0.0001	8
2b	Two-way repeated-measures ANOVA	F (6, 84) = 4.543	0.0005	8
2c	Two-way repeated-measures ANOVA	F (6, 84) = 1.105	0.3664	8
2d	Two-way repeated-measures ANOVA	F (6, 84) = 0.7697	0.5958	8
3e	Unpaired two-tailed Student t-test	t = 5.031, df = 6	0.0024	4
3f	Unpaired two-tailed Student t-test	t = 0.5714, df=6	0.5885	4
4e	Two-way repeated-measures ANOVA	F (9, 126) = 4.978	<0.0001	8
4f	Two-way repeated-measures ANOVA	F (9, 126) = 3.021	0.0027	8
4g	Two-way repeated-measures ANOVA	F (9, 126) = 5.645	<0.0001	8
4h	Two-way repeated-measures ANOVA	F (9, 126) = 4.204	<0.0001	8
4i	Two-way repeated-measures ANOVA	F (6, 84) = 2.550	0.0256	8
4j	Two-way repeated-measures ANOVA	F (6, 84) = 2.426	0.0327	8
4k	Two-way repeated-measures ANOVA	F (6, 84) = 6.252	<0.0001	8
4l	Two-way repeated-measures ANOVA	F (6, 84) = 5.640	0.0002	8
5b	Unpaired two-tailed Student t-test	t = 3.175, df = 6	0.0192	4
5d	Unpaired two-tailed Student t-test	t = 2.575, df = 6	0.0421	4
6b	Unpaired two-tailed Student t-test	t = 1.451, df=6	0.1969	4
6d	Unpaired two-tailed Student t-test	t = 1.045, df=6	0.3361	4
7c	Two-way repeated-measures ANOVA	F (10, 140) = 5.246	<0.0001	8
7d	Two-way repeated-measures ANOVA	F (10, 140) = 10.59	<0.0001	8
7e	Two-way repeated-measures ANOVA	F (6, 84) = 9.623	<0.0001	8
7f	Two-way repeated-measures ANOVA	F (6, 84) = 2.662	0.0206	8
8a	Two-way repeated-measures ANOVA	F (10, 140) = 3.676	<0.0001	8
8b	Two-way repeated-measures ANOVA	F (10, 140) = 2.705	0.0046	8
8c	Two-way repeated-measures ANOVA	F (6, 84) = 5.404	<0.0001	8
8d	Two-way repeated-measures ANOVA	F (1, 14) = 14.33[a]	0.0020[b]	8
9a	Two-way repeated-measures ANOVA	F (6, 84) = 7.770	<0.0001	8
9b	Two-way repeated-measures ANOVA	F (6, 84) = 10.58	<0.0001	8
9c	Two-way repeated-measures ANOVA	F (6, 84) = 5.546	<0.0001	8
9d	Two-way repeated-measures ANOVA	F (6, 84) = 1.022	<0.4166	8
9e	Two-way repeated-measures ANOVA	F (6, 84) = 5.492	<0.0001	8
9f	Two-way repeated-measures ANOVA	F (6, 84) = 3.106[a]	<0.0085[b]	8
9g	Two-way repeated-measures ANOVA	F (6, 84) = 4.375	<0.0007	8

9h	Two-way repeated-measures ANOVA	$F(6, 84) = 2.570$	<0.0247	8

F-values are shown for interaction factor (treatment and time), unless otherwise noted.
[a]Treatment F-value.
[b]Treatment p-value.

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