Intact mast cell content during mild head injury is required for the development of latent pain sensitization - implications for mechanisms underlying posttraumatic headache

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Disclosures: The study was funded by NIH grants NS086830, NS078263, NS101405 to DL and in part by Teva Pharmaceuticals, through a grant to DL. DB has no conflict of interest to declare.

Authorship: Authorship has been granted only to those individuals who have contributed substantially to the research or manuscript.

Highlights

- Ongoing activation of meningeal mast cells (MCs) following a mild closed head injury (mCHI) is independent of peripheral CGRP signaling.

- Prophylactic depletion of meningeal MC content prior to mCHI does not affect the development of acute posttraumatic headache-like behavior.

- Prophylactic depletion of meningeal MC content prior to mCHI induction blocks the establishment of chronic latent sensitization to glyceryl trinitrate (GTN), a major headache trigger.

- Latent sensitization to GTN post-mCHI does not involve acute activation of meningeal MCs.

Abstract

Posttraumatic headache (PTH) is one of the most common, debilitating and difficult symptoms to manage after a mild traumatic brain injury, or concussion. While the mechanisms underlying PTH remain elusive, recent studies suggest the potential involvement of calcitonin gene-related peptide (CGRP), a mediator of neurogenic inflammation, and the ensuing activation of meningeal mast cells (MCs), key pro-algesic resident immune cells that can lead to the activation of the headache pain pathway. The following study investigated the relative contribution of MCs to the development of PTH-like headache and pain behaviors using a recently developed rat model of mild closed head injury (mCHI). We initially employed a monoclonal antibody against CGRP and used histological methods and to test the relative contribution of peripheral CGRP signaling to the activation of meningeal MCs following mCHI. We then used a prophylactic, pharmacological MC granule depletion protocol, combined with behavioral nociceptive testing, to address the hypotheses that intact meningeal MC granule content is necessary for the development of PTH-related acute and persistent headache and pain-like behaviors following mCHI. The data suggest that following mCHI, ongoing meningeal MC degranulation does not involve peripheral CGRP signaling, and that the cephalic mechanical pain hypersensitivity that develops following mCHI does not depend upon acute meningeal MC degranulation. Our data, however, also reveals that the development of latent sensitization, a key chronic pain-like phenomenon, manifested as persistent hypersensitivity upon the recovery from mCHI-evoked acute cranial hyperalgesia to the headache trigger glyceryl trinitrate (GTN) requires intact MC content during and immediately after mCHI. Collectively, our data implicate the acute activation of meningeal MCs as mediator of chronic pain hypersensitivity following a concussion or mCHI. Targeting MCs may be explored for early prophylactic treatment of PTH.

Key words: posttraumatic headache, concussion, CGRP, mast cells, latent sensitization, cranial meninges

1. Introduction

One of the most common and disabling symptoms of traumatic brain injury (TBI) is posttraumatic headache (PTH). The estimated prevalence of PTH ranges between 30% to 90% (Lucas et al., 2014; Vargas and Dodick, 2012). Chronic PTH (lasting more than 3 months) has been reported in about 40% of individuals diagnosed with TBI (Hoffman et al., 2011). While the symptom profiles of individuals suffering from PTH vary, it most often resembles the most common types of primary headaches, namely migraine or tension-type headache (Lew et al., 2006; Lucas and Ahn, 2018). The resemblances between PTH and primary headaches points to the possibility that the two conditions are mediated by similar, or shared mechanisms.

A large body of preclinical evidence now supports the view that primary headaches, and particularly migraine, involve the activation and sensitization of trigeminal primary afferent neurons that innervate the intracranial meninges (Levy, 2010; Messlinger, 2009; Olesen et al., 2009). Persistent activation of meningeal afferents leading to the sensitization of second-order trigeminal dorsal horn neurons, which receive convergent sensory input from the meninges and cephalic skin has been reported in preclinical models and is thought to underlie the cephalic pain hypersensitivity in primary headaches (Noseda and Burstein, 2013). The finding of cephalic mechanical hypersensitivity in chronic PTH (Defrin et al., 2010; Defrin et al., 2015) as well as in preclinical models of mild TBI (mTBI) (Bree and Levy, 2016; Macolino et al., 2014; Moye et al., 2018) further supports the notion that PTH and primary headache involve a shared mechanism.

The endogenous processes responsible for driving meningeal primary afferent neurons in primary headache and potentially also in PTH remain poorly understood. One key hypothesis implicates sterile meningeal inflammation, involving the local activation of proinflammatory immune cells such as mast cells (MCs), precipitated by local release of sensory neuropeptides from meningeal afferent nerve endings via an axon-reflex (Levy, 2009; Levy et al., 2018; Russo, 2015). Acute activation and degranulation of meningeal MCs (the extrusion and release of preformed granule-associated mediators) and local action of numerous pro-inflammatory MC mediators have been shown to promote the activation and sensitization of meningeal afferents and the headache pain pathway (Levy et al., 2007; Zhang et al., 2007). Degranulation of meningeal MCs can also lead to the development of cephalic mechanical pain hypersensitivity (Levy et al., 2012). Our previous finding of persistent degranulation of meningeal MCs in two distinct mouse models of mild TBI (Levy et al., 2016) has led us to hypothesize that such head trauma-related proinflammatory is also involved in mediating PTH. The earlier findings that CGRP can promote meningeal MC degranulation (Ottosson and Edvinsson, 1997; Reynier-Rebuffel et al., 1994) and that peripheral CGRP signaling mediates PTH-like behaviors (Bree and Levy, 2016) further points to a potential link between meningeal neurogenic inflammation, MCs, and the development of PTH.

The major goal of the following study thus was to investigate the relative contribution of MCs to the development of PTH-like headache and pain behaviors using our recently developed rat model of mild closed head injury (mCHI) (Bree and Levy, 2016). We initially investigated the relative contribution of peripheral CGRP signaling to the activation of meningeal MCs following mCHI, using a blocking antibody that selectively targets peripheral CGRP signaling. We then

tested the hypothesis that intact meningeal MC granule content during the induction of mCHI is necessary for the development of PTH-related acute and persistent headache and pain-like behaviors following mCHI.

2. Material and Methods

2.1 Animals

All experiments were approved by the institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Centre, and were in compliance with the ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines (Kilkenny et al., 2012). Since the incidence of TBI is higher in males (Spani et al., 2018) and PTH is considered to be more prevalent in males (Carlson et al., 2013), we focused on studying male rats (Sprague-Dawley rats, Taconic, USA, weighing 220–250 g at time of arrival). Animals were housed in pairs with food and water available *ad libitum* under a constant 12 hour light/dark cycle (lights on at 7:00 am) at room temperature. All procedures and testing were conducted during the light phase of the cycle (9:00 am to 4:00 pm). Experimental animals (n = 8-12 per group) were randomly assigned to either sham or mCHI as well as to the different treatment groups.

2.2 Experimental Mild Closed Head Injury (mCHI)

mCHI was induced using the weight-drop concussive device as described previously (Bree and Levy, 2016). Briefly, rats were anesthetized with 3% isoflurane and placed chest down directly under a weight-drop concussive head trauma device. The device consisted of a hollow cylindrical tube (inner diameter 2.54 cm) placed vertically over the rat's head. To induce a head trauma, a 250 g projectile was dropped through the tube from a height of 80 cm, striking the center of the

head. To ensure consistency of the hit location, animals were placed under the weight drop apparatus so that the weight struck slightly anterior to the center point between the ears. A foam sponge (thickness 3.81 cm, density 1.1 g/cm^3) was placed under the animals to support the head while allowing some anterior-posterior motion without any rotational movement at the moment of impact. Immediately after the impact, animals were returned to their home cages for recovery. All animals regained consciousness within 2 min and were neurologically assessed in the early hours and days post-injury for any behavioral abnormalities suggestive of neurological impairment. Sham animals were anesthetized but not subjected to the weight drop. All animals subjected to behavioral testing did not display any major neurological deficits.

2.3 MC granule depletion protocol

Repeated systemic administration of escalating doses of the MC secretagogue compound 48/80 leads to depletion of connective tissue MC granules with ensuing reduction of MC mediators that undergoes a slow recovery over several weeks (Feldberg and Talesnik, 1953; Jaffery et al., 1994). A modified 48/80 injection protocol, used in our previous work {Zhang, 2008 #2269), was employed to deplete meningeal MC granules prior to mCHI. Briefly, rats were pretreated with compound 48/80 (0.1% w/v in sterile saline; Sigma-Aldrich) twice a day (morning and afternoon) for a total of eight doses, starting with 0.5 mg/kg injections for the first day, 1 mg/kg for the second, and 2, and 4 mg/kg for the third, and fourth days respectively. To minimize the inflammatory and potentially afferent sensitizing effects elicited by the 48/80-induced MC degranulation, mCHI was induced 4 days following the last 48/80 dosing. In control experiments, animals were subject to a similar injection protocol using only saline.

2.4 Anti-CGRP treatment.

Blockade of peripheral CGRP signaling was achieved by using a murine anti-CGRP mAb (TEVA Pharmaceutical Industries). Control treatment was a corresponding isotype IgG. Agents were formulated in phosphate buffered saline (PBS) and injected i.p at a dose of 30 mg/kg, immediately after the head injury and then again 6 days later.

2.5 Tissue preparation, immunohistochemistry and quantitative assessment of meningeal MC density and degranulation.

Animals were deeply anaesthetized with urethane (1.5 g/kg, i.p.) and perfused transcardially with 200 ml of heparinized PBS followed by 150 ml of 4% paraformaldehyde in PBS. The heads were post-fixed overnight in the same fixative solution and then transferred to PBS. Following removal of the calvaria, the intracranial dura was removed bilaterally and mounted on a glass slide. For histological assessment of meningeal MCs, fixed dural whole-mount tissues were stained with toluidine blue (TB, 0.1% in 2.5 pH, Sigma-Aldrich), which binds to glycosaminoglycans in connective tissue MC granules. TB-stained MCs were observed using bright-field illumination, under a 400X magnification (Eclipse Ci, Nikon, Tokyo, Japan). Because MC degranulation levels were uniform across the dura on each side, MC counts and degranulation levels on each side were averaged based on 20 different randomly chosen visual fields. MCs were considered degranulated if there was an extensive dispersion of more than 15 extruded granules localized near the cell, or when there was an extensive loss of granule staining, giving the cell a ‘ghostly’ appearance (Levy et al., 2016). Following 48/80 treatment, we also designated irregular-shaped MCs as cells at various stages of recovery, such as cells with

reduced number of granules, or granules with faint TB staining. Assessment of MCs were conducted in a blinded fashion.

2.6 Assessment of cephalic tactile pain hypersensitivity following mCHI.

Behavioral testing were performed during the light phase (09:00-15:00) using a method previously used by us and others to study PTH- and migraine-related pain behaviors (Bree and Levy, 2016; Edelmayer et al., 2012; Oshinsky and Gomonchareonsiri, 2007; Yan et al., 2012). Briefly, animals were placed in a transparent flat-bottomed acrylic holding apparatus (20.4 cm x 8.5 cm). The apparatus was large enough to enable the animals to escape the stimulus. Animals were habituated to the arena for 15 minutes prior to the initial testing. To determine if animals developed pericranial tactile hypersensitivity (i.e. mechanical allodynia) following mCHI, the skin region, including the midline area above the eyes and 2 cm posterior, was stimulated with different von Frey (VF) filaments (0.6 g-10 g) (18011 Semmes-Weinstein Anesthesiometer Kit). During the acute phase (3-14 days post-mCHI), we evaluated changes in withdrawal thresholds, as well as non-reflexive pain responses to stimulation using a method previously described (Levy et al., 2012; Zhao and Levy, 2014) by recording 4 behavioral responses adapted from Vos et al. (Vos et al., 1994). These behavioral responses included: 0) *No response*: rat did not display any response to stimulation 1) *Detection*: rat turned its head towards the stimulating object and explored it, usually by sniffing; 2) *Withdrawal*: rat turned its head away or pulled it briskly away from the stimulating object (which usually followed by scratching or grooming of stimulated region); 3) *Escape/Attack*: rat turned its body briskly in the holding apparatus in order to escape the stimulation or attacked (biting and grabbing movements) the stimulating object. Starting with the lowest force, each filament was applied 3 times with an intra-application interval of 5

seconds and the behavior that was observed at least twice was recorded. For statistical analysis, the score recorded was based on the most aversive behavior noted. The force that elicited three consecutive withdrawal responses was considered as threshold. To evaluate pain behavior in addition to changes in threshold, for each rat, at each time point, a cumulative response score was determined by combining the individual scores (0–3) for each one of the VF filaments tested. All tests were conducted and evaluated in a blinded manner. Responses to VF test stimuli were tested at baseline prior to mCHI, and then at 3, 7, and 14 days post-mCHI.

2.7 Latent mechanical sensitization following mCHI

The development of cephalic and hindpaw latent mechanical hyperalgesia in response to systemic administration of a previously subthreshold dose of the headache trigger GTN (100μg/kg i.p., American Reagents, USA) was assessed as described (Bree and Levy, 2016). Briefly, rats depleted of their MC granules pre-mCHI, using the 48/80 protocol, and rats receiving saline as vehicle (control group) were subjected to baseline testing of cephalic mechanical pain thresholds at day 29 post mCHI, as described above. Baseline hindpaw mechanical sensitivity was also tested by assessing withdrawal responses following mechanical VF stimulation of the mid-dorsal part of the hindpaw. On the next day, the animals received GTN and were assessed for changes in cephalic and hindpaw mechanical pain thresholds 4 hours later.

2.8 Data analyses

Data are presented as mean + standard error of the mean. Statistical analyses were conducted using GraphPad Prism (version 7.0). Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene tests, respectively. Repeated measures analysis of variance (ANOVA) was performed to determine effects of time and treatment followed by post hoc tests. Correction for multiple comparisons was controlled by the false discovery rate (FDR). Between groups analyses were conducted using unpaired two-tailed t-test. P values, and FDR-corrected p values (q-values) < 0.05 were considered statistically significant.

3. Results

3.1 mCHI-induced meningeal MC response does not involve peripheral CGRP signaling

We have shown recently that mCHI leads to prolonged degranulation of meningeal MCs and proposed that such immune response involves meningeal neurogenic inflammation, potentially facilitated by peripheral CGRP signaling (Levy et al., 2016). Meningeal MCs express CGRP receptors (Eftekhari et al., 2013; Lennerz et al., 2008) and CGRP has been shown to promote meningeal MC degranulation (Ottosson and Edvinsson, 1997; Reynier-Rebuffel et al., 1994). Blockade of peripheral CGRP signaling, using a monoclonal antibody (mAb), can inhibit PTH-like headache and pain behaviors following mCHI (Bree and Levy, 2016), suggesting the potential involvement of meningeal MCs in mediating the nociceptive, PTH-like effect of CGRP. We therefore asked whether blocking peripheral CGRP signaling, using the same regimen of treatment with the anti-CGRP mAb, might inhibit the degranulation of meningeal MCs in response to mCHI. To test the relative contribution of CGRP to the response of meningeal MCs following mCHI we focused on day 7 post-mCHI because it was the earliest time point that anti-

CGRP mAb treatment had an anti-hyperalgesic effect in this mTBI model (Bree and Levy, 2016). As Figure 1 demonstrates, administration of the anti-CGRP mAb, immediately post mCHI, and again 6 days later, did not decrease the level of meningeal MC degranulation, which was significantly higher than that observed in animals following a sham mCHI ($p<0.0001$ vs sham), and not different than that observed at the same time point in untreated, or control, IgG-treated mCHI animals ($p = 0.45$ and $p = 0.44$ respectively). This data suggests therefore that peripheral CGRP signaling does not mediate the ongoing meningeal MC degranulation in this mCHI model.

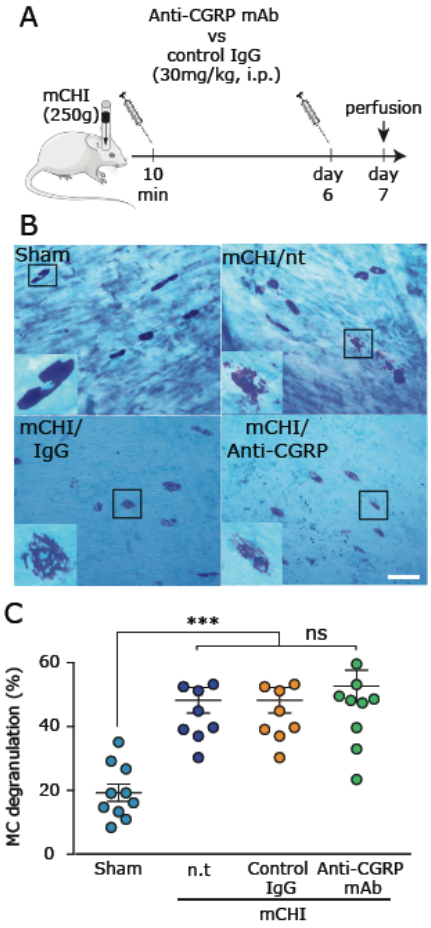

Figure. 1 Blockade of peripheral CGRP signaling using systemic administration of a blocking mAb does not inhibit mCHI-evoked changes in dural MC. (A) Rats were injected i.p. with anti-CGRP mAb, or control IgG, immediately following mCHI and then 6 days later followed by perfusion a day later. (B) Representative images of TB-stained meningeal whole-mounts showing healthy, non-degranulated MCs in sham animals and degranulated MCs in treated, and non-treated (n.t) mCHI animals. Scale bar = 50 μm. (C) Quantification of meningeal MC degranulation level at baseline, and at 7 days following a sham head injury or mCHI, in non-treated animals, and animals treated with the anti-CGRP mAb, or a control IgG. ns = not significant; *** $p<0.0001$.

3.2 Functional meningeal MCs are not required for development of acute cephalic mechanical hypersensitivity following mCHI

While our data suggests that CGRP contributes to PTH-like pain via a mechanism unrelated to the degranulation of meningeal MCs, it does not rule out the possibility CGRP acts downstream, or in parallel to a MC related process: that acute post-mCHI MC response provides sufficient meningeal nociceptive stimulus that contributes to the development of PTH-like cephalic pain hypersensitivity behavior, but independent of CGRP signaling. We therefore asked whether limiting the acute mCHI-evoked meningeal MC response, using prophylactic depletion of their content, might affect the development of cephalic mechanical pain hypersensitivity. Meningeal MC granule content was depleted using a 4 day treatment protocol with ascending doses of compound 48/80 as described in the Methods section. Histological examination revealed that the 48/80 treatment protocol was effective in depleting meningeal MC granules at the time of mCHI induction (Figure 2B, C).

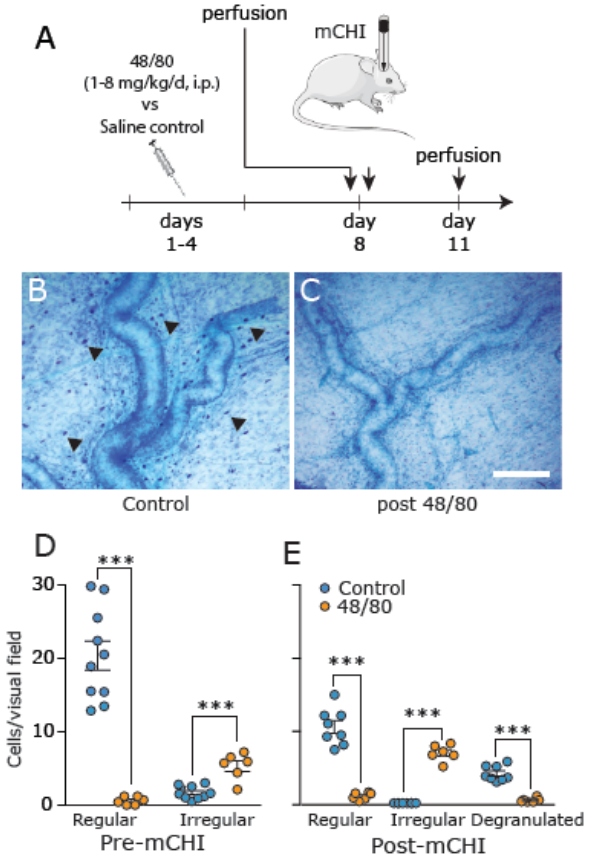

Figure. 2 Depletion of meningeal MCs using 48/80 treatment. (A) After 4 days of repeated 48/80 injections, rats were perfused prior to mCHI, or 3 days post-mCHI. Representative examples of TB-stained meningeal whole-mounts depicting MCs (arrow heads) near the middle meningeal artery in control (B), and 48/80 pre-treated (C) animals prior to mCHI. Scale bar 500 μm. (D-E) Quantification of meningeal MCs density in control and 48/80-treated animals. *** $p<0.0001$, post-hoc test.

In 48/80-treated rats, the total number of non-degranulated meningeal MCs was reduced by 84.6% when compared to that observed in saline-treated animals ($p < 0.0001$; Figure 2D) and was comprised primarily of irregular-shaped cells. At 3 days post mCHI (7 days following the last 48/80 injection, Figure 2E), the MC population in the 48/80-treated group was primarily comprised of irregular MCs ($p < 0.001$ vs the control group, which was primarily comprised of degranulated and non-degranulated cells), suggesting cells at various stages of recovery. In this group, we also observed reduced number of degranulated cells ($p < 0.001$ vs the control group).

Because MC degranulation releases proinflammatory nociceptive factors that can activate and sensitize meningeal nociceptive afferents (Levy et al., 2007; Zhang et al., 2007), as well as give rise to cephalic tactile hypersensitivity (Levy et al., 2012), we sought to confirm that the 48/80 injection protocol used for MC granule depletion does not result in tactile hypersensitivity, which could serve as a confounding variable in the study of behavioral changes post mCHI. As Figures 3 B&C depict, at the time of baseline testing (prior to mCHI) mechanical pain sensitivity in animals pretreated with 48/80 was not different that that observed in the control animals, indicating that the MC granule depletion protocol *per se* was not pro-nociceptive.

As Figure 3B depicts, induction of mCHI in control, saline-treated animals gave rise to a pronounced, time-dependent decrease in cephalic mechanical pain thresholds ($F_{(3,28)} = 13.08$; $p = 0.0001$). When compared to baseline values, withdrawal thresholds were significantly reduced at 3 days ($p = 0.003$) and 7 days ($p = 0.0001$) post-mCHI, and returned to baseline levels at 14 days ($p = 0.15$). In MC depleted animals, mCHI also gave rise to decreased cephalic thresholds ($F_{(3,24)} = 9.73$; $p = 0.0004$), with significant decreases observed at 3 days ($p=0.002$), 7 days ($p<0.0001$), and also at 14 days ($p = 0.01$). Two-way ANOVA reveled no significant difference of treatment ($F_{(1,15)} = 0.02$; $p = 0.89$) indicating that the prophylactic MC depletion did not have an anti-

hyperalgesic effect. As Figure 3C depicts, in saline-treated control animals, mCHI also led to increase in cephalic pain scores ($F_{(3,28)} = 13.08$; $p = 0.0001$), at 3 days ($p = 0.0003$), and 7 days ($p = 0.0003$), which similar to the change in threshold responses also returned to baseline values at 14 days ($p = 0.55$). MC granule depletion was also ineffective in blocking the increase in cephalic pain scores following mCHI ($F_{(3,24)} = 19.17$; $p < 0.0001$). Significant increases in nociceptive responses were observed at 3 days ($p < 0.0001$), 7 days ($p < 0.0001$), and 14 days ($p < 0.001$). Two-way ANOVA revealed no significant difference of treatment ($F_{(1,14)} = 0.3$; $p=0.59$) indicating that functional MCs are not critical for the development of the acute cephalic pain hypersensitivity following mCHI.

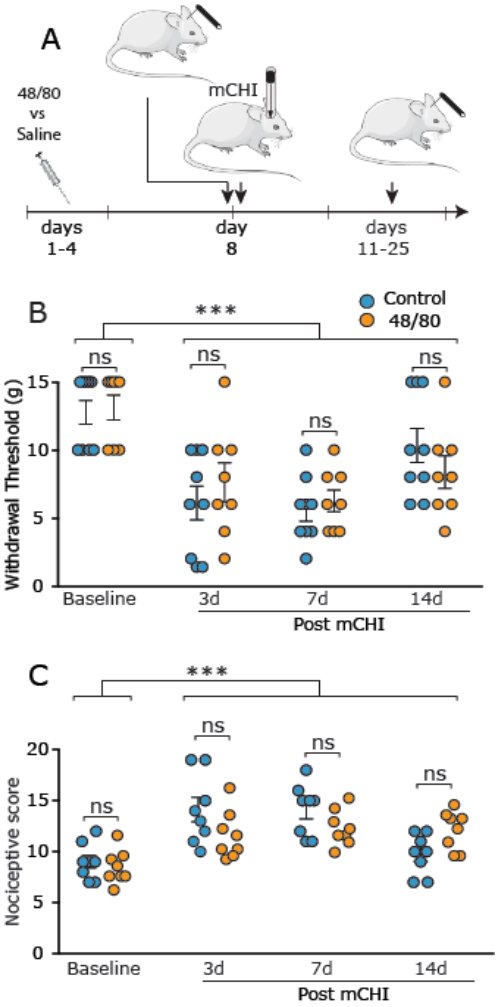

Figure. 3 Prophylactic MC depletion does not prevent the development of mechanical pain hypersensitivity following mCHI. (A) At the end of the MC depletion protocol, or control saline treatment, rats were subjected to baseline behavioral nociceptive testing, followed by mCHI and post-mCHI testing. Cephalic mechanical pain withdrawal thresholds (B) and corresponding cumulative pain response scores (C) at baseline, 3, 7, and 14 days post mCHI. *** $p < 0.0001$ vs baseline values.

3.3 Granule-containing MCs during mCHI induction are required for the development of latent mechanical sensitization

We have shown previously that mCHI also gives rise to a prolonged latent mechanical sensitization, manifested as decreased cephalic and extracephalic (hind paw) mechanical pain thresholds in response to systemic administration of a subthreshold dose of the migraine trigger GTN, following the recovery from the acute cephalic hypersensitivity phase (Bree and Levy, 2016). Latent sensitization has been suggested to play a role in pain chronification; it likely involves peripheral changes, particularly at the level of the nociceptor's peripheral nerve endings, and signaling cascades distinct from those underlying acute inflammatory hyperalgesia (Reichling and Levine, 2009). We therefore investigated the possibility that the prolonged latent sensitization that develops in the wake of mCHI might involve a MC-related process. Prior to GTN administration, at day 29 following mCHI, baseline cephalic and hind paw mechanical thresholds were not different between animals pretreated with saline or 48/80 prior to mCHI ($p = 0.23$ and $p = 0.88$ respectively). In control, saline pre-treated animals, administration of GTN led to a significant reduction in mechanical pain thresholds in both the cephalic ($p < 0.0001$, Figure 4B) and hind paw ($p < 0.0001$, Figure 4D) regions. In animals subjected to the MC granule depletion protocol, the latent sensitization was blocked. Overall, mechanical withdrawal thresholds tested at 4 hours post GTN treatment were not different than at baseline, in both the cephalic ($p = 0.99$; Figure 5C) and hind paw ($p = 0.42$; Figure 4E) regions.

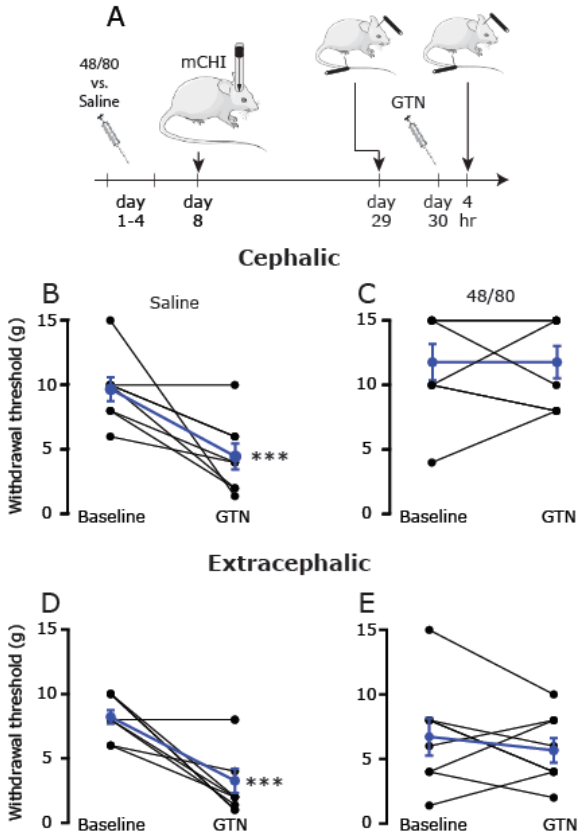

Figure. 4 Latent sensitization following mCHI requires intact MC population during and immediately after the induction of mCHI. (A) 48/80 and saline-pretreated mCHI rats were subjected to behavioral testing on day 29 following mCHI, and then, a day later (Day 30), 4 hrs after GTN administration. Changes in cephalic (B & C) and hindpaw (D & E) mechanical pain thresholds, 4 hours following administration of GTN . *** $p < 0.0001$ vs pre-GTN baseline values on Day 29 post mCHI.

3.4 Inhibition of the latent sensitization by prophylactic MC granule depletion does not involve altered acute meningeal MC response to GTN

Systemic administration of GTN has been shown to promote delayed meningeal MC degranulation (Pedersen et al., 2015; Reuter et al., 2001). However, the relative contribution of such proinflammatory response to the activation and sensitization of meningeal afferents and the ensuing behavioral nociceptive effects of GTN in naïve animals is questionable (Zhang et al., 2013). We therefore tested whether the blockade of the latent sensitization we observed in 48/80-pretreated mCHI animals might involve altered response of meningeal MCs, including reduction in the number of cells amenable to degranulation by GTN, as well as their overall level of degranulation in response to GTN treatment. As Figure 5B demonstrates, prior to GTN administration, the density of non-degranulated meningeal MCs in 48/80-pretereated mCHI animals (34 days following the last 48/80 injection) was not different than in control mCHI

animals that received only saline prior to mCHI ($p = 0.49$). Both the 48/80 and saline-treated groups, however had lower density of meningeal MCs when compared to pre-mCHI values ($p = 0.0003$ and $p = 0.0053$, respectively) suggesting that the persistent mCHI-evoked MC response, as seen in a mouse model (Levy et al., 2016) was not curtailed by the prophylactic MC depletion post-mCHI. As Figure 5C depicts, the level of meningeal MC degranulation at 4 hr following GTN treatment was similar in mCHI-treated prophylactically with 48/80 or saline ($p = 0.44$), suggesting that the inhibitory effect of prophylactic 48/80 treatment protocol on the latent sensitization did not involve diminished acute MC degranulating response to GTN.

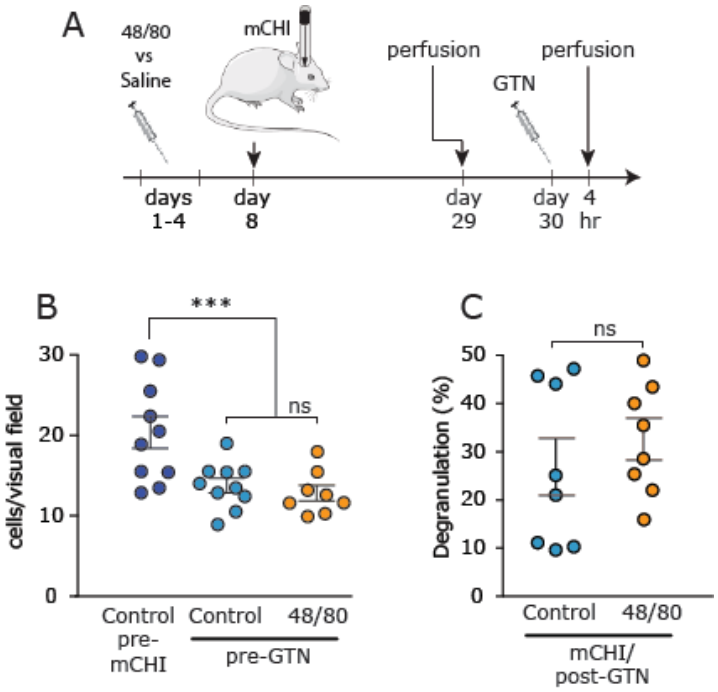

Figure. 5 Latent sensitization following mCHI does not involve GTN-evoked acute MC degranulation. (A) 48/80 and saline-pretreated mCHI rats were perfused at 29 days post-mCHI or 4 hrs after GTN treatment, on day 30 following mCHI. (B) Density of meningeal MCs in mCHI animals prior to GTN administration in animals treated prophylactically with 48/80 or saline. (C) Meningeal MC degranulation level at 4 hrs following GTN treatment in animals pretreated with 48/80 or saline. *** $p < 0.001$.

4. Discussion

The main findings of this study suggest that: 1) Ongoing meningeal MC degranulation following mCHI is not blocked by treatment with a mAb that blocks peripheral CGRP signaling; 2) The reversible cephalic mechanical hypersensitivity that develops following mCHI does not depend

upon acute meningeal MC degranulation; 3) Upon the recovery from mCHI-evoked cranial hyperalgesia, the establishment of latent sensitization to GTN requires a MC-related process that occurs during or early after the induction of mCHI, and 4) Latent sensitization to GTN post mCHI does not involve GTN-induced acute meningeal MC degranulation.

The current study confirms our previous mouse data demonstrating persistent MC degranulation in an analogous rat model of mCHI. While the acute degranulation of meningeal MCs following mCHI likely involves blunt trauma to the calvaria that propagates to the underlying meningeal tissue (Stokely and Orr, 2008), the mechanism that maintains this post-mCHI meningeal inflammatory response is unclear. While CGRP has been a candidate signaling molecule, due to its role in meningeal neurogenic inflammation (Russo, 2015), and ability to promote meningeal MC degranulation (Ottosson and Edvinsson, 1997; Reynier-Rebuffel et al., 1994), the present study does not support a role for peripheral CGRP signaling in mediating the persistent mCHI-evoked meningeal MC degranulation. Whether mCHI leads to persistent activation of meningeal nociceptive afferents, and the ensuing release of other mediators of neurogenic inflammation, such as substance P that contribute to the prolonged activation of meningeal MCs following mCHI remains to be studied. The possibility that a non-neurogenic process promotes the activation of meningeal MCs following mCHI should also be considered.

We previously hypothesized that the CGRP-dependent cephalic hypersensitivity that develops in the wake of mCHI involves meningeal neurogenic inflammation (Bree and Levy, 2016). The current finding that the activation of meningeal MC following mCHI is not required for the development of cephalic hypersensitivity suggests, however, that CGRP mediates this PTH-related behavior through a mechanism independent of meningeal MC degranulation, and thus perhaps not involving neurogenic inflammation. Our data also suggests that the meningeal

MC degranulation response that occurs in the wake of mCHI is not sufficient to promote peripheral meningeal nociception, which likely drives the cephalic pain hypersensitivity after mCHI. While MCs have been implicated in nociceptor sensitization and pain hypersensitivity, some inflammatory conditions have been shown to promote pain hypersensitivity independent of MC activation (Lopes et al., 2017). Whether sterile inflammation propagated by the activation of other meningeal immune cells drives the cephalic hypersensitivity in the wake of mCHI remains to be determined. A preliminary study in our lab suggests, however, that prophylactic depletion of meningeal macrophages is also not sufficient to abolish the cephalic hypersensitivity following mCHI (Figure 6).

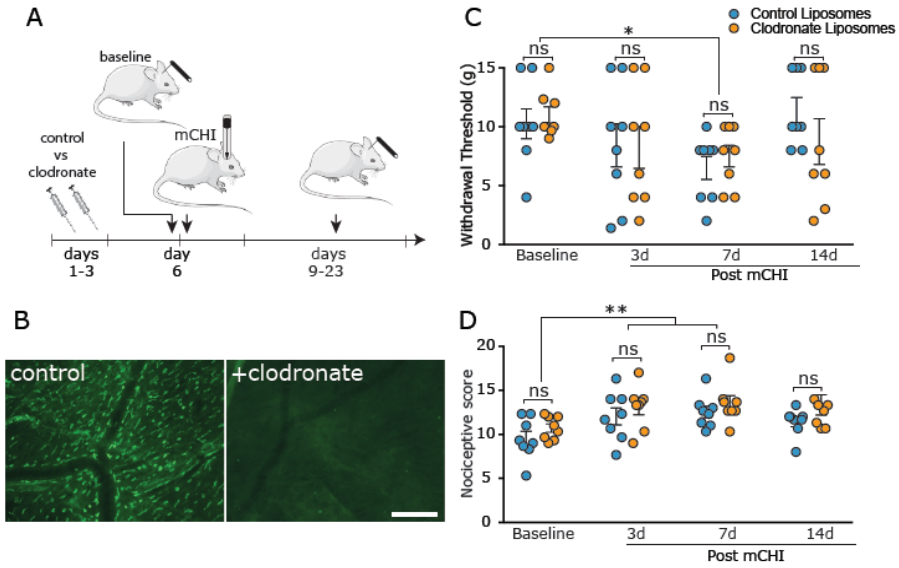

Figure 6. Prophylactic depletion of meningeal macrophages does not prevent the development of mechanical pain hypersensitivity following mCHI. (A) Rats were subjected to a macrophages depletion using i.p. injections of clodronate, or control liposomes at 1ml/kg. The concentration of clodronate is 5mg/kg. Two injections were made, 48 hrs apart and rats were subjected to baseline behavioral testing followed by mCHI 72 hrs later. Post-mCHI testing were conducted 3-14 days following mCHI. (B) Representative images of meningeal whole-mounts subjected to immunohistochemistry using a mouse anti-rat mAb against CD163 (clone ED2, 1:500, MCA342, BioRad) showing dural macrophages in animals treated with control liposomes and their depletion following treatment with clodronate. Scale bar = 500μm. Cephalic mechanical pain withdrawal thresholds (C) and corresponding cumulative pain response scores (D) at baseline, 3, 7, and 14 days post mCHI. * $p < 0.05$, ** $p < 0.001$.

A key, clinically-relevant finding of the rat mCHI model is the development of latent sensitization. This chronic pain related phenomenon is manifested as a delayed and prolonged cephalic and hind paw mechanical allodynia following administration of a subthreshold dose of the headache trigger GTN, long after the resolution of the initial acute cephalic pain hypersensitivity state (Bree and Levy, 2016). A major finding of the current study was that prophylactic depletion of MC granule inhibited the mCHI-evoked latent sensitization. The finding that prior to GTN treatment, the number of meningeal MCs was similar in the control and 48/80-treated animals suggests that reduced number of meningeal MC was not responsible for the lack of hyperalgesic response to GTN in mCHI animals subjected to prophylactic MC granule depletion. Our data further suggest this inhibition of the latent sensitization was not due to a reduced acute meningeal MC degranulation response to GTN. This is in agreement with our previous finding suggesting that GTN can promote delayed sensitization of meningeal afferents via a mechanism independent of acute meningeal MC degranulation (Zhang et al., 2013). We propose therefore that the MC-related mechanism responsible for mediating the latent sensitization following mCHI involves events that occurred earlier, likely during the acute phase following the mCHI, and that are distinct from those mediating the acute cephalic hyperalgesia. Our previous finding that blocking peripheral CGRP signaling also inhibited the latent sensitization (Bree and Levy, 2016), together with our current data suggesting that CGRP does not mediate the mCHI-evoked meningeal MC degranulation, points to the possibility that parallel mechanisms account for the involvement of CGRP and MCs in mediating the latent sensitization. The possibility that this process involves a CGRP-related event that occurs downstream to meningeal MC activation may also be considered.

The latent hyper-responsiveness to GTN, beyond the resolution of the initial acute cephalic hypersensitivity, may be related to a state of hyperalgesic priming - a neuroplasticity response thought to involve persistent and latent hyper-responsiveness of primary afferent nociceptive neurons to inflammatory mediators subsequent to an inflammatory or nerve injury insult (Reichling and Levine, 2009). Numerous MC factors have been implicated in the hyperalgesic priming cascade, including the cytokines tumor necrosis factor alpha (Parada et al., 2003), interleukin-6 (Dina et al., 2008), and nerve growth factor (Joseph and Levine, 2010). Their exact contribution of these mediators and possibly of other MC-related factors following mCHI will require further studies.

In summary, we evaluated the relative contribution of MCs to the development of acute and persistent headache and pain behaviors reminiscent of PTH following a concussive head trauma in rats. The key findings point to a differential role of MCs in the genesis and maintenance of PTH: having no contribution to the development of the acute cephalic allodynia, but appearing to be critical for the sustained state of the latent sensitization that develops post-mCHI. While CGRP has been suggested to play a key role in mediating PTH-like behaviors, our data suggests that the mechanism underlying its involvement does not involve downstream signaling mediated by MCs, and thus likely unrelated to meningeal neurogenic inflammation. Early targeting of MCs and their related peripheral inflammatory and nociceptive signaling following mCHI may be useful to prevent the development of PTH and possibly complementary to treatment with anti-CGRP mAb.

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Figure Legends:

Figure. 1 Blockade of peripheral CGRP signaling using systemic administration of a blocking mAb does not inhibit mCHI-evoked changes in dural MC. (A) Rats were injected i.p. with anti-CGRP mAb, or control IgG, immediately following mCHI and then 6 days later followed by perfusion a day later. (B) Representative images of TB-stained meningeal whole-mounts showing healthy, non-degranulated MCs in sham animals and degranulated MCs in treated, and non-treated (n.t) mCHI animals. Scale bar = 50 μm. (C) Quantification of meningeal MC degranulation level at baseline, and at 7 days following a sham head injury or mCHI, in non-treated animals, and animals treated with the anti-CGRP mAb, or a control IgG. significant; *** $p<0.0001$. ns = not

Figure. 2 Depletion of meningeal MCs using 48/80 treatment. (A) After 4 days of repeated 48/80 injections, rats were perfused prior to mCHI, or 3 days post-mCHI. Representative examples of TB-stained meningeal whole-mounts depicting MCs (arrow heads) near the middle meningeal artery in control (B), and 48/80 pre-treated (C) animals prior to mCHI. Scale bar 500 μm. (D-E) Quantification of meningeal MCs density in control and 48/80-treated animals. *** $p<0.0001$.

Figure. 3 Prophylactic MC depletion does not prevent the development of mechanical pain hypersensitivity following mCHI. (A) At the end of the MC depletion protocol, or control saline treatment, rats were subjected to baseline behavioral nociceptive testing, followed by mCHI and post-mCHI testing. Cephalic mechanical pain withdrawal thresholds (B) and

corresponding cumulative pain response scores (C) at baseline, 3, 7, and 14 days post mCHI. *** $p < 0.0001$ vs baseline values.

Figure. 4 Latent sensitization following mCHI requires intact MC population during and immediately after the induction of mCHI. (A) 48/80 and saline-pretreated mCHI rats were subjected to behavioral testing on day 29 following mCHI, and then, a day later (Day 30), 4 hrs after GTN administration. Changes in cephalic (B & C) and hindpaw (D & E) mechanical pain thresholds, 4 hours following administration of GTN . *** $p < 0.0001$ vs pre-GTN baseline values on Day 29 post mCHI.

Figure. 5 Latent sensitization following mCHI does not involve GTN-evoked acute MC degranulation. (A) 48/80 and saline-pretreated mCHI rats were perfused at 29 days post-mCHI or 4 hrs after GTN treatment, on day 30 following mCHI. (B) Density of meningeal MCs in mCHI animals prior to GTN administration in animals treated prophylactically with 48/80 or saline. (C) Meningeal MC degranulation level at 4 hrs following GTN treatment in animals pretreated with 48/80 or saline. *** $p< 0.001$.