

# Persistent Headache and Pain Sensitization After Traumatic Brain Injury Is Driven by Mu-Opioid Receptor-Positive Neurons in The Rostral Ventromedial Medulla for both Males and Females

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# Abstract

## Background

Persistent posttraumatic headache and widespread pain hypersensitivity are common following mild traumatic brain injury (mTBI). However, the central mechanisms that support posttraumatic pain sensitization and ongoing vulnerability to chronic pain (e.g., latent sensitization) are not fully understood. The rostral ventromedial medulla (RVM) is a key output node of the endogenous pain modulation system, which contains mu-opioid receptor-positive (Oprm+) pain-facilitating neurons that are important in chronic pain. We investigated whether these neurons are essential for posttraumatic pain behaviors, ongoing vulnerability to postsurgical and headache-like pain, and latent pain sensitization after mild traumatic brain injury in both male and female animals.

## Methods

Male and female C57BL/6J mice received microinjection of dermorphin-saporin (Derm-sap) to lesion RVM Oprm+ neurons, or Blank-saporin (Blank-sap) as controls. After recovery, mice underwent closed-head mTBI or sham-TBI. Periorbital mechanical sensitivity was used as a headache-related measure, and hindpaw mechanical withdrawal thresholds were used to assess distal pain sensitization. Reinstatement of periorbital allodynia and persistent postsurgical hindpaw allodynia was induced using bright-light stress (BLS) and hindpaw incision, respectively. Latent sensitization was assessed by systemic naloxone challenge 100 days after mTBI.

## Results

mTBI produced acute periorbital allodynia in both sexes, which was not prevented by lesioning RVM Oprm+ neurons. In contrast, lesioning these neurons abolished BLS-induced reinstatement of periorbital allodynia at 5 and 9 weeks after injury in both males and females. Lesioning RVM Oprm+ neurons also prevented acute bilateral hindpaw allodynia after mTBI and eliminated the prolonged bilateral postsurgical allodynia that followed hindpaw incision in animals with mTBI. Naloxone reinstated periorbital and hindpaw allodynia in mice with intact RVM after mTBI, whereas this latent sensitization was absent after targeted RVM lesioning. These effects were broadly conserved across sexes.

## Conclusion

Pain-facilitating neurons in RVM, marked by Oprm, are required for supporting post-mTBI persistent pain sensitization. Sensitization of an RVM pain facilitation circuit may contribute to the increased headache vulnerability, delayed postsurgical pain recovery, and latent sensitization in those with mTBI history. The

dysregulation of its descending pain facilitation function is a conserved mechanism in both sexes, linking mTBI to chronic headache and widespread pain vulnerability.

## INTRODUCTION

Both male and female victims of mild traumatic brain injury (mTBI), or concussion, commonly experience chronic pain, including headaches, spine and limb pain, and are at high risk of long-term disability and opioid misuse [1–11]. The neural mechanisms underlying *how* mTBI increases pain remain unknown, and we have no mechanism-based strategies for the prevention or treatment of chronic pain in this population.

Current preclinical research indicates that dysfunction in endogenous pain modulation systems is a common feature in diseases characterized by chronic pain as well as persistent pain after injury, including mTBI [12]. The rostral ventromedial medulla (RVM) serves as a principal output center for this system, which is capable of both facilitating and inhibiting nociception [13, 14]. Specifically, activation of the RVM's pain-facilitating neurons (e.g., "ON-cells," marked by *mu-opioid receptors* (Oprm<sup>+</sup>) [15, 16]), triggered by acute injury, contributes to the development of chronic pain through a pronociceptive positive feedback loop [17–21]. While ON-cell tone eventually returns to baseline to facilitate recovery, likely via increased endogenous opioids, this compensatory suppression may form the basis for "latent sensitization," [22, 23]. In this state, hyperalgesia is hypothesized to be masked but can be unmasked with subsequent exposure to stress or opioid antagonism [24].

Prior reports demonstrated that male mice with mTBI exhibit latent sensitization. In these cases, behavioral allodynia can be reinstated by stressors, such as surgical incision or bright light, as well as by opioid antagonism. This process is dependent on descending pain-facilitatory output to the spinal cord [25, 26]. These observations suggest that mTBI produces complex dysfunction in central pain modulation; however, the role of the RVM in pain sensitization after mTBI remains elusive. This study hypothesizes that RVM Oprm<sup>+</sup> neurons (e.g., ON-cells) drive acute pain hypersensitivity triggered by mTBI and underlie the subsequent latent sensitization responsible for post-mTBI headache and persistent postsurgical limb pain. We further propose that this mechanism is active in both sexes. By addressing this critical knowledge gap regarding how mTBI leads to chronic pain, this research may inform the design of novel therapies.

## METHODS

### Animal Models

All experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Palo Alto VA Institutional Animal Care and Use Committee [27]. This manuscript adheres to the applicable EQUATOR/ARRIVE guidelines. Male and female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, MA) and

acclimated for at least 10 days under standard vivarium conditions with a 12-hour light/dark cycle (6:30 am to 6:30 pm). All animals were at least 13 weeks old (average weight: males 30 g, females 22 g) at the initiation of experiments and surgical preparation. All mice were kept in their original cages and randomly assigned to groups of 7–9 to receive one or more of the treatments outlined below. Observers were blinded to treatments and pharmacological manipulations.

## **Stereotaxic intracranial microinjection**

Deeply anesthetized mice under isoflurane anesthesia (5% for induction and 2% for maintenance) were placed in a stereotaxic apparatus and maintained on a circulating warm-water pad. A craniotomy was performed approximately 1–2 mm caudal to the lambdoid suture. Dermophin-saporin or Blank-saporin (Derm-Sap or Blank-sap, Advanced Targeting Systems, CA, USA; 1.5 picomoles in 300 nL of phosphate-buffered saline) was delivered to the RVM (relative to bregma, midline, 5.60–5.80 mm AP, and ~ 5.50 mm DV) with a 30-gauge stainless-steel microinjection needle connected to a 1  $\mu$ L Hamilton syringe and pressure microinjector (Micro 4 system, World Precision Instruments) (Fig. 1a). The selected dose was previously reported as specific for Oprm receptor binding and neuronal lesioning in the RVM [18, 28]. The needle was left at the injection site for 5 minutes before withdrawal to minimize backflow. The craniotomy was sealed with Gelfoam, and the incision was closed using 6 – 0 nylon sutures and cyanoacrylate glue (Vetbond, 3M). Post-procedure, mice received lidocaine ointment, Neosporin, and 0.5% bupivacaine subcutaneously at the incision site before returning to their home cages. The mice were monitored daily and given approximately 6 weeks to recover before behavioral experiments commenced.

## **Closed-head mild traumatic brain injury model**

To induce closed-head mTBI [25, 26, 29, 30], mice were positioned prone on a foam mold and placed in the stereotaxic frame under deep isoflurane anesthesia. A stereotaxic impactor (MyNeurolab, St. Louis, MO), mounted on the frame (David Kopf Instruments, Tujunga, CA) at a 40-degree angle, was aimed at the S1 somatosensory cortex using a 5-mm impactor tip. The impact parameters were set to deliver a force at 5.8–6.0 m/s, with a 0.2-second dwell time and a 5-mm impact depth. These parameters were validated, and no skull fractures were reported in earlier studies [31–33]. Mice in the sham-mTBI group underwent anesthesia without impact. Post-procedure, animals were placed on a warming pad to recover before returning to their cages. Post-mTBI periorbital and hindpaw sensitivities were assessed in their respective study cohort as outlined above.

## **Reinstatement of Sensitization**

## **Bright Light Stress Headache Model**

A bright light stress (BLS) challenge was performed to reinstate periorbital allodynia in headache-prone mice as previously described [26, 34, 35]. Animals underwent BLS protocol, where LED lights (1000 lux output) were placed directly above the test chamber for 15 minutes. Animals' periorbital mechanical thresholds were obtained prior to BLS as baseline, then again on hours 1, 3, and 5 after BLS challenge.

## **Hindpaw Incision Postsurgical Pain Model**

Hindpaw incision was performed to induce postsurgical hindpaw allodynia [36], and the hindpaw mechanical sensitivity over the postsurgical recovery period was tracked as described in our previous study [25]. Briefly, under isoflurane anesthesia, a 5 mm longitudinal incision was made with a #11 scalpel on the plantar surface of the left hindpaw, contralateral to the mTBI. The plantaris muscle and its adjacent tissues were dissected to mimic surgical exploration. Following hemostasis, the incision was closed with 6 – 0 nylon sutures, and antibiotic ointment was applied.

## **Endogenous opioid blockade**

To evaluate the effect of Derm-sap and Blank-sap treatment on post-mTBI latent sensitization, naloxone, a mu-opioid receptor antagonist[25]. Naloxone hydrochloride dihydrate ((N7758, Millipore-Sigma) 1 mg/kg was given intraperitoneally (i.p.)[25, 37, 38]. Periorbital allodynia or hindpaw withdrawal thresholds were measured in their respective groups at pre-naloxone baseline, and then at 15 and 70 minutes post-naloxone administration.

## **Nociceptive behavioral assessment**

All animals were 20–24 weeks old ( $21.8 \pm 1.5$  weeks) at the time of behavioral experiments. Mice were habituated to the testing facility, experimental platforms, enclosures, and handlers for a minimum of three days, two hours per day, before the initiation of experiments.

## **Periorbital Mechanical Sensitivity**

On the day of testing, mice were acclimated in their individual plexiglass enclosure (10 cm x 10 cm) with mash flooring for 2 hours. Periorbital mechanical sensitivity was assessed by applying a 0.4 g von Frey filament (Stoelting Co, IL) to the periorbital region at the center of the face between the orbits. The filament was applied 10 times and left in place for 1 second. A 30-second interval was maintained between each application. A positive response was defined as the animal swiping its face, shaking its head, and/or turning its head away after the filament application. However, any of these behaviors performed before filament applications, or running away or rearing up following filament applications, are not considered a positive response. Response frequency was calculated with the formula “# positive responses/10 x 100%”. Animals with a hypersensitive baseline response frequency of 50% or more were excluded from the rest of the experiments.

## **Hindpaw Mechanical Sensitivity**

Hindpaw mechanical nociceptive thresholds were measured using von Frey filaments (Stoelting Co, IL) with the “up-down” algorithm previously described by Chaplan et al [39]. On the day of testing, mice were acclimated to their individual plastic enclosure (10 cm radius) on a wire-mesh platform for 1–2 hours. Increasing weight filaments were applied to the plantar surface and kept for 5 seconds. If a withdrawal in response to a filament, the next filament one sequent lower-weight filament; if no withdrawal occurred, the next higher-weight filament. After an initial withdrawal, four more filaments were tested following this protocol. The withdrawal threshold was then determined with a data-fitting algorithm for statistical analysis [25, 29, 39, 40].

## Post-hoc immunohistochemistry

At the completion of all behavioral experiments, animals were perfused transcardially with 20 ml of PBS followed by 20 ml of precooled 4% (w/v) paraformaldehyde (PFA) solution. The brains were removed, fixed in 4% PFA overnight, and then transferred to 30% sucrose at 4°C for at least 24 hours until they sank. The brains were then embedded in optimal cutting temperature (OCT) solution and cut into 20-micron transverse sections using a cryostat (CM3050 S, Leica, IL, US). After washing with PBS with 0.2% Triton X-100 (PBST), the sections were blocked for 1 hour in 10% goat serum (w/v, ThermoFisher) with 0.2% Triton X-100, incubated for 24 hours at 4°C with primary antibodies against Oprm receptors (Invitrogen, rabbit polyclonal IgG, 1:5,000 dilution). Followed by washing with PBST, sections were incubated for 1 hour at 4°C with secondary antibodies (Invitrogen, goat anti-rabbit IgG, Alexa Fluor 594, 1:10,000 dilution). After three additional washes with PBST, the sections were mounted with Fluoroshield™ containing DAPI (Millipore-Sigma, F6057) and imaged using a Keyence fluorescence microscope. The RVM was defined as the nucleus raphe magnus and within 0.2 mm of the adjacent reticular formation at the level of the facial nucleus. The tips of injection sites and Oprm+ neurons were confirmed histologically (data not shown), and only data from "on-target" injection sites were analyzed. Sites in the RVM were plotted on hand-drawn sections in Adobe Illustrator using landmarks defined by Paxinos and Watson [41].

## Statistical Analysis

All statistical analyses were performed in Prism 11.0 (GraphPad, San Diego, CA) and Excel (Microsoft, Redmond, WA). Data are expressed in mean  $\pm$  standard error. A priori power analysis calculation using G\* Power software demonstrated that ~ 6–8 mice per group would be needed for the targeted 80% power to detect an effect size of 20% between groups for periorbital allodynia (primary outcome) after RVM Derm-sap Lesion or Blank-sap control followed by mTBI or Sham-TBI (primary exposure) using a corrected alpha of 0.05 (4 groups for each sex, minimal of 16 measurements for the periorbital allodynia cohort and 20 measurements for the hindpaw allodynia cohort, correlation among repeated measures of 0.5, and nonsphericity correction of 1) for ANOVA testing with repeated measures and within-between subject interactions. The effect size was chosen based on observations from our previous studies [25, 26, 29, 30]. The normality of behavioral data was confirmed using D’Agostino & Pearson tests, and the data were subsequently analyzed using two-tailed paired t-test or 2-way repeated-measures ANOVA followed by Tukey’s multiple comparisons between treatment groups, time, and interactions between

treatment X time. Sex differences, as secondary measures, were compared using two-tailed unpaired t-tests for males and females from their respective parallel treatment groups at times of peak treatment effects based on previous reports [25, 26, 29, 30]. For all tests,  $p < 0.05$  was considered significant.

## RESULTS

Animals' injection sites are outlined in Fig. 1b. Approximately 6 weeks after microinjection of Derm-sap or Blank-sap into the RVM, baseline periorbital mechanical sensitivity and hindpaw mechanical sensitivity were obtained. Animals were randomly assigned to either the periorbital sensitivity testing cohort or the hindpaw sensitivity testing cohort. A summary of the testing cohorts (e.g., periorbital sensitivity testing and hindpaw sensitivity testing) and their respective subgroup assignments and treatment series is outlined in Fig. 1c-d.

### Acute periorbital allodynia after mTBI is unaffected by RVM Oprm+ neurons lesioning

Blank-sap pretreated male mice, in which RVM Oprm+ neurons are intact, exhibited acute periorbital allodynia for ~ 7 days after mTBI exposure (Blank-sap/mTBI), compared to their counterparts that underwent sham-TBI (Blank-sap/sham-TBI), which remained at baseline level (Fig. 2a). Additionally, lesioning RVM Oprm+ neurons with Derm-sap had no effect on mTBI-induced acute periorbital allodynia (Fig. 2b). Similarly, female mice with intact RVM are affected by mTBI and exhibited acute periorbital allodynia for 7 days after mTBI exposure (Blank-sap/mTBI, Fig. 2c). Lesioning of RVM Oprm+ neurons by Derm-sap had no effect on acute periorbital allodynia after mTBI (Derm-sap/mTBI, Fig. 2d). At the time of peak periorbital allodynia after mTBI (day 3 post-mTBI)[26], there was no sex difference between the Derm-sap/mTBI groups ( $t = 0.39$ ,  $p = 0.70$ ) or the Blank-sap/mTBI groups ( $t = 0.20$ ,  $p = 0.84$ ). Together, these findings indicate that RVM Oprm+ neurons are not necessary for the development of this mTBI-induced acute heightened periorbital sensitivity, regardless of sex.

### Lesioning of RVM Oprm+ neurons abolishes BLS-induced periorbital allodynia in mice with mTBI

To assess the role of RVM Oprm+ neurons in post-mTBI persistent vulnerability to headache, all animals were subjected to BLS as an acute stressor at 5 and 9 weeks post-mTBI, which aims to reestablish periorbital allodynia in latently sensitized animals as described in previous reports [26, 35]. In week 5 after the initial mTBI or sham-TBI exposure, 15 minutes of BLS challenge caused a 5-hour transient increase of periorbital allodynia in the male mice with intact RVM and mTBI exposure (Fig. 3a). In contrast, lesioned RVM Oprm+ neurons completely abolished BLS-induced periorbital allodynia in male mice 5 weeks after mTBI (Fig. 2b vs Fig. 3b). Similar results were observed at 9 weeks (Fig. 3c, 3d).

Previous reports on BLS-induced headache focused solely on male mice with mTBI despite high rates of headaches in females [26, 35]. In female mice with intact RVM, BLS challenge at week 5 (Fig. 3e-f) and

week 9 (Fig. 3g-h) after mTBI also caused transient periorbital allodynia that was sensitive to ablation of *Oprm*-expressing neurons in the RVM. There was no sex difference at the time of peak BLS-induced allodynia (1 hour post BLS) for the Derm-sap/mTBI groups (post-mTBI week 5:  $t = 0.32$ ,  $p = 0.75$ ; week 9:  $t = 1.08$ ,  $p = 0.30$ ) and the Blank-sap/mTBI groups (week 5:  $t = 0.59$ ,  $p = 0.56$ ; week 9:  $t = 0.60$ ,  $p = 0.56$ ).

## **Acute hindpaw allodynia after mTBI is attenuated by RVM *Oprm*<sup>+</sup> neurons lesioning**

To examine the role of RVM *Oprm*<sup>+</sup> neurons in mTBI-induced generalized allodynia at a distal location from the head injury, bilateral hindpaw mechanical sensitivity was measured. Compared to the Blank-sap/sham-TBI group, male mice exhibited acute allodynia for ~ 5 days after mTBI (e.g., Blank-sap/mTBI) in both hindpaws contralateral (left) and ipsilateral (right) to the side of head injury (Fig. 4a,c). In contrast, mTBI failed to induce bilateral hindpaw allodynia up to 14 days post-mTBI in mice pre-treated with Derm-sap (e.g., Derm-sap/mTBI group) (Fig. 4b,d). Results were similar for female mice (Fig. 4e-h). On comparison between sexes at the time of peak mTBI-induced allodynia, there was no sex difference in Derm-sap/mTBI groups for both hindpaws (contralateral hindpaw:  $t = 0.93$ ,  $p = 0.37$ ; ipsilateral hindpaw:  $t = 0.71$ ,  $p = 0.49$ ). The Blank-sap/mTBI-treated females exhibited slightly higher mTBI-induced hindpaw allodynia than males (contralateral hindpaw:  $t = 3.32$ ,  $p = 0.005$ ; ipsilateral hindpaw:  $t = 0.58$ ,  $p = 0.57$ ). Together, these observations suggest that, unlike acute post-mTBI periorbital allodynia, RVM *Oprm*<sup>+</sup> neurons are necessary, in both sexes, for the development of acute allodynia in the hindpaws, a body part that is distal from the initial head injury.

## **Lesioning of RVM *Oprm*<sup>+</sup> neurons prevents persistent postsurgical allodynia in mice with mTBI**

To establish the role of RVM *Oprm*<sup>+</sup> neurons in postsurgical allodynia, an incision injury was performed on the hindpaw contralateral to the mTBI at 14 days post-injury, at which time hindpaw mechanical sensitivity had returned to preinjury levels. Incision surgery caused acute allodynia in the injured hindpaw for ~ 5 days in male mice in the Blank-sap/sham-TBI group, whereas the conspecifics with a history of mTBI exhibited persistent postsurgical allodynia in the injured hindpaw for up to 38 days and recovered by 52 days post-incision (Fig. 5a). Although uninjured, the ipsilateral hindpaw of Blank-sap/mTBI males also exhibited persistent allodynia after incision surgery (Fig. 5c). On the contrary, in male mice with lesioned RVM *Oprm*<sup>+</sup> neurons, the persistent allodynia of the injured contralateral hindpaw (Fig. 5b) and uninjured ipsilateral hindpaw (Fig. 5d) was no longer observed in the Derm-sap-treated groups. Results were similar for female mice (Fig. 5e-h).

There were no sex differences at the acute postsurgical pain phase (1 day post-incision) for both the Derm-sap/mTBI groups (contralateral hindpaw:  $t = 0.16$ ,  $p = 0.88$ ; ipsilateral hindpaw:  $t = 0.93$ ,  $p = 0.37$ ) and the Blank-sap/mTBI groups (contralateral hindpaw:  $t = 0.91$ ,  $p = 0.38$ ; ipsilateral hindpaw:  $t = 0.25$ ,  $p = 0.80$ ). During the persistent postsurgical pain phase, no sex differences were detected (Derm-sap/mTBI groups contralateral hindpaw:  $t = 0.38$ ,  $p = 0.91$ ; ipsilateral hindpaw:  $t = 0.36$ ,  $p = 0.94$ . Blank-sap/mTBI

groups contralateral hindpaw:  $t = 0.05$ ,  $p = 0.97$ ; ipsilateral hindpaw:  $t = 0.96$ ,  $p = 0.35$ ). Collectively, both sexes of mice exhibited mTBI-related persistent allodynia after hindpaw incision, and this delayed pain recovery is driven by RVM Oprm<sup>+</sup> neurons.

## Periorbital and hindpaw latent sensitization after mTBI is driven by RVM Oprm<sup>+</sup> Neurons

To examine the role of RVM Oprm<sup>+</sup> neurons during mTBI-induced latent sensitization, both male and female mice were treated with naloxone at 100 days post-mTBI.

Periorbital sensitivity of the male and female mice in the Blank-sap/sham-TBI group were unaffected by endogenous opioid blockade, while the male and Blank-sap/mTBI group exhibited acute periorbital allodynia 15–20 minutes after naloxone (Fig. 6). In contrast, when RVM Oprm<sup>+</sup> neurons were lesioned by Derm-sap pretreatment, blockade of endogenous opioids with naloxone failed to reinstate periorbital allodynia in both sexes of mice with previous mTBI (Fig. 6). In both male and female Blank-sap/mTBI mice, naloxone administration induced hypersensitivity in both the contralateral (left) and ipsilateral (right) hindpaws within 5 minutes of injection. Normal hindpaw sensitivity was subsequently reestablished by 90 minutes post-treatment (Fig. 7). On the contrary, hindpaw withdrawal thresholds of the Derm-sap/mTBI-treated mice or the sham-TBI mice were unaffected by naloxone (Fig. 7).

Together, these results indicate that the mTBI-induced latent sensitization of the periorbital area and the hindlimbs following naloxone treatment is dependent on the presence of intact RVM Oprm<sup>+</sup> neurons.

## DISCUSSION

The prevalence of chronic pain after post-mTBI pain is high, and it affects both sexes across all age groups. Recent experimental evidence suggests that mTBI leads to persistent sensitivity to pain by disrupting the interplay between descending pain-facilitating and pain-inhibiting controls, specifically dependent on the descending serotonergic pain-facilitating output to the spinal cord [25, 30, 42–44]. The current research further refines this understanding by pinpointing RVM as a key upstream element contributing to persistent pain after mTBI.

The RVM has long been recognized as a major output site of the endogenous pain modulation system [13, 14]. It facilitates and inhibits nociception through its two principal neurons, ON- and OFF-cells, which project down to nociceptive laminae in the dorsal horns [13, 14]. Functionally, activation of pain-facilitating ON-cells triggered by injury or inflammation contributes to chronic pain as part of a pronociceptive positive feedback loop, contributing to pathological pain states [17–20, 45]. Combining with the known critical role of the descending pain-facilitating circuit in mTBI-induced pain sensitization, the current data extend the framework by showing that an intact pain-facilitating ON-cell-enriched population, marked by Oprm in the RVM, is necessary for the expression of persistent allodynia after mTBI [25].

Building on this framework, one key aspect of the current results is the temporal distinction in post-mTBI pain-modulating mechanisms between acute pain and persistent pain vulnerability, which became unmasked by later stressors. Specifically, despite lesioning of RVM Oprm<sup>+</sup> neurons, the acute phase of periorbital allodynia after head injury and hindpaw allodynia after incision injury was preserved, whereas the persistent phase of pain sensitivity, triggered by subsequent BLS and hindpaw incision respectively, was fully dependent on this pain-facilitating neuronal population in RVM being intact. This observation points to the shift from local mechanisms to central pain facilitation in driving the transition from acute to chronic pain in subjects with mTBI. When head and limb injuries first occurred, local trigeminal/peripheral neuroplasticity, afferent activation, and inflammatory cytokine and peptide induction mechanisms likely dominate, resulting in acute allodynia at the injured site [46–49]. However, these immediate consequences of injury are not identical to those maintaining susceptibility to later persistent allodynia in animals with mTBI, at which point central pain-facilitating circuits become the key contributor to pain behaviors [25, 30, 42–44]. The current results demonstrated that RVM Oprm<sup>+</sup> neurons are a necessary component of the system responsible for post-mTBI persistent pain sensitization, supporting their identity as RVM ON-cells and a likely source of augmented descending serotonergic pain-facilitatory signals after mTBI [15, 16, 25, 50]. This notion of RVM-mediated persistent central pain facilitation after mTBI is further evidenced by the transient bilateral hindpaw allodynia immediately after mTBI, and the parallel persistent postsurgical allodynia in the uninjured hindpaw in mTBI animals [25]. RVM pain-modulating neurons project broadly to the dorsal horns, which can support centrally amplified and spatially diffuse sensitization in various pain models [14, 15, 50–52]. Indeed, permanent lesioning of RVM Oprm<sup>+</sup> neurons prevented the diffuse allodynia after mTBI and subsequent persistent bilateral postsurgical allodynia after hindpaw surgery, suggesting that the mTBI-induced persistent pain state is primarily driven by RVM pain-facilitating neurons.

In addition, persistent allodynia after mTBI in the current model is unique in its engagement of latent sensitization, in which hyperalgesia is masked by activation of the endogenous opioid system after mTBI (but not minor incision surgery) and can be unmasked with subsequent exposure to surgical stress or opioid antagonism [25]. The current data further refined this concept by identifying RVM Oprm<sup>+</sup> neurons as a key site of endogenous opioid action after mTBI. With endogenous opioid blockade reinstating behavioral allodynia in mTBI animals, but only when RVM Oprm<sup>+</sup> neurons were intact, the current data strongly support the mechanism that post-mTBI pain state is maintained by the interaction between ongoing endogenous opioid suppression and persistent sensitized descending pain-facilitating circuit in RVM [24, 25, 37, 52, 53].

The current study also expanded the mechanistic implications of RVM's involvement in mTBI-induced latent sensitization to chronic postsurgical allodynia and posttraumatic headache in both males and females. While previous studies reported that mTBI supports persistent head and limb pain by causing dysfunction of the descending pain-modulating system in male mice [25, 26, 43], the current data demonstrated that the current closed-head mTBI model *equally* impacted female mice's head and limb pain sensitivity. Furthermore, lesioning of RVM Oprm<sup>+</sup> pain-facilitating neurons in female mice also prevented mTBI-induced persistent pain behaviors in both head and hindpaw, as it did in males. While

the core function of RVM pain-modulating neurons is preserved in both sexes[21] and the current finding suggests that the brainstem mechanism of latent pain sensitization after mTBI is conserved across sexes in mice, it is worth emphasizing the nuances that underlie sex differences in chronic pain across different rodent mTBI models. For example, previous preclinical studies have reported sex differences in post-mTBI neuroinflammatory, structural, and hormonal responses, as well as across different injury types (such as single vs. repeat injuries, closed head vs. lateral fluid percussion), which could directly and indirectly influence the central pain-modulating system [54–56]. Therefore, the current finding highlights an important concept that although sex-related factors influence top-down modulatory input and responses, the essential descending pain-facilitating function of RVM Oprm+ neurons in post-mTBI pain sensitization may remain conserved in males and females.

Several study limitations should be considered. First, Derm-sap-mediated lesioning is a powerful loss-of-function approach targeting Oprm + RVM neurons, but it does not distinguish whether RVM pain-modulating neurons are important for the initiation or maintenance of latent sensitization after mTBI, nor does it reveal how neuronal activity in these neurons evolves over time post-mTBI, during BLS, or after subsequent surgery. Secondly, although RVM Oprm+ neurons likely assumed the identity of ON-cells, particularly given the loss of persistent hyperalgesia after lesioning, this molecular identity is still not synonymous with the classic electrophysiologically defined ON-cells. Furthermore, the role of pain-inhibiting OFF-cells in mTBI-related pain has remained elusive. Therefore, future studies using cell-type-specific chemogenetic manipulation, calcium imaging, and electrophysiological recordings in the RVM will be necessary to elucidate the neurophysiological mechanisms underlying chronic pain following mTBI. Lastly, although the mechanism underlying post-mTBI pain phenotypes is qualitatively concordant between male and female mice at the level of the RVM, defining sex-dependent upstream inputs to this brainstem pain-modulating circuit will be important for delineating the factors that modulate the sex differences in post-mTBI pain.

## CONCLUSIONS

In summary, the present study identified a critical central mechanism underlying the persistent latent state of pain sensitization after mTBI in both males and females. Driven by RVM Oprm+ pain-facilitating neurons, although animals' pain sensitivity may appear “recovered” after the initial head injury, they remain more susceptible to headache and persistent postsurgical pain with subsequent stressors. Furthermore, the similar dependence on this RVM circuit across sexes suggests that dysregulated descending pain facilitation is a shared and potentially targetable mediator of chronic pain after head injury for both males and females.

## Declarations

### Ethics approval and consent to participate

All experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Palo Alto VA Institutional Animal Care and Use Committee. Consent to participate not applicable: no human participants were involved in the current study.

### **Consent for publication**

Not applicable

### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

Name: QiLiang Chen, MD, PhD

Contribution: This author helped with designing the study, data collection, data analysis, interpretation of data, drafting of the manuscript, and critical revision of the manuscript.

Peyman Sahbaie, MD

Contribution: This author helped with data collection, interpretation of data, and critical revision of the manuscript.

Karen-Amanda Irvine, PhD

Contribution: This author helped with interpretation of data, and critical revision of the manuscript.

J. David Clark, MD, PhD

Contribution: This author helped with designing the study, interpretation of data, drafting of the manuscript, and critical revision of the manuscript.

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# Figures

Figure 1

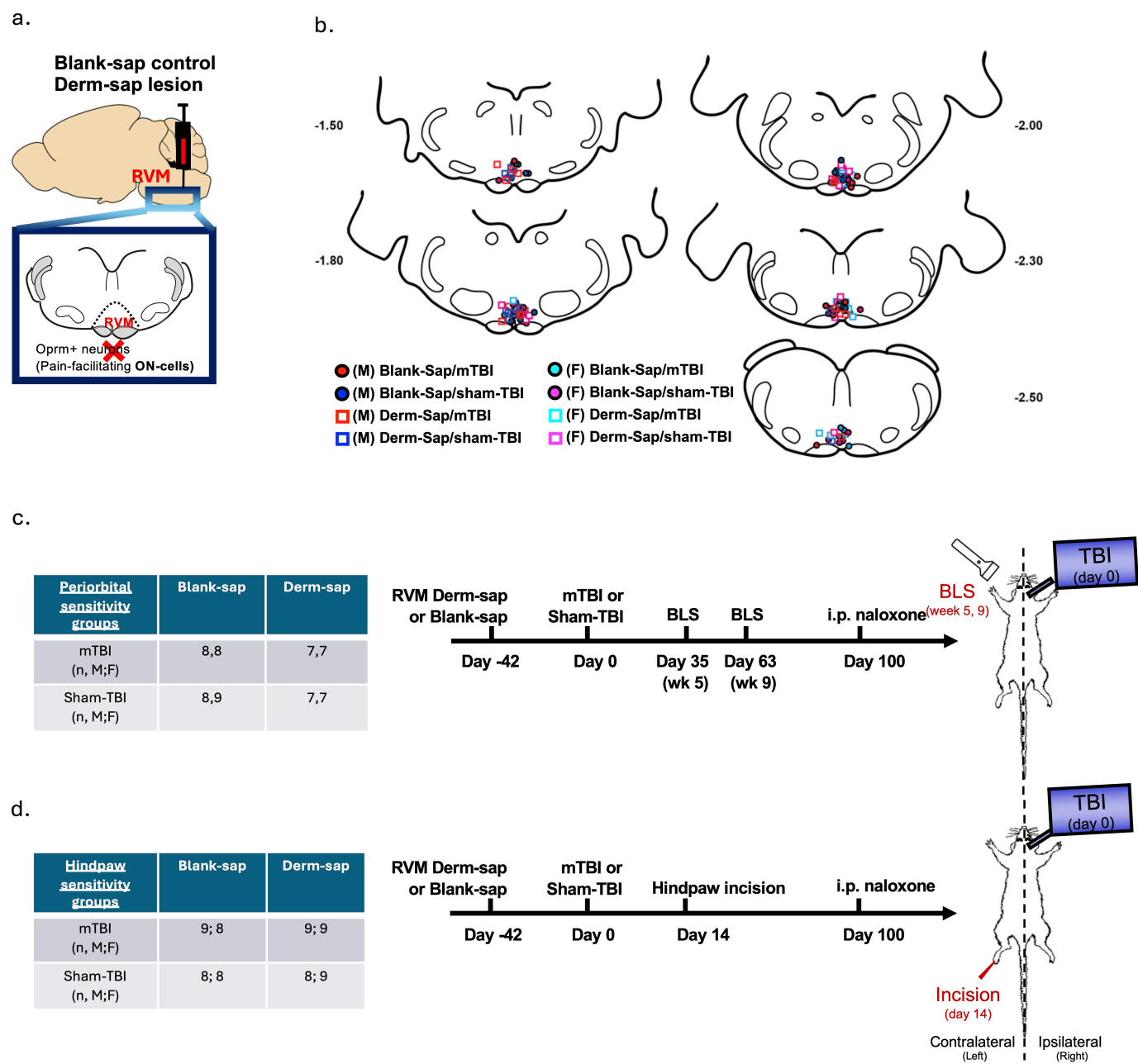


Figure 1

a) Schematic of intracranial injection of Derm-sap toxin/Blank-sap control. b) Map of RVM injection sites. Summary of c) the periorbital sensitivity testing groups and d) the hindpaw sensitivity testing groups, including their respective subgroup assignments, sample sizes, and treatment series.

Figure 2

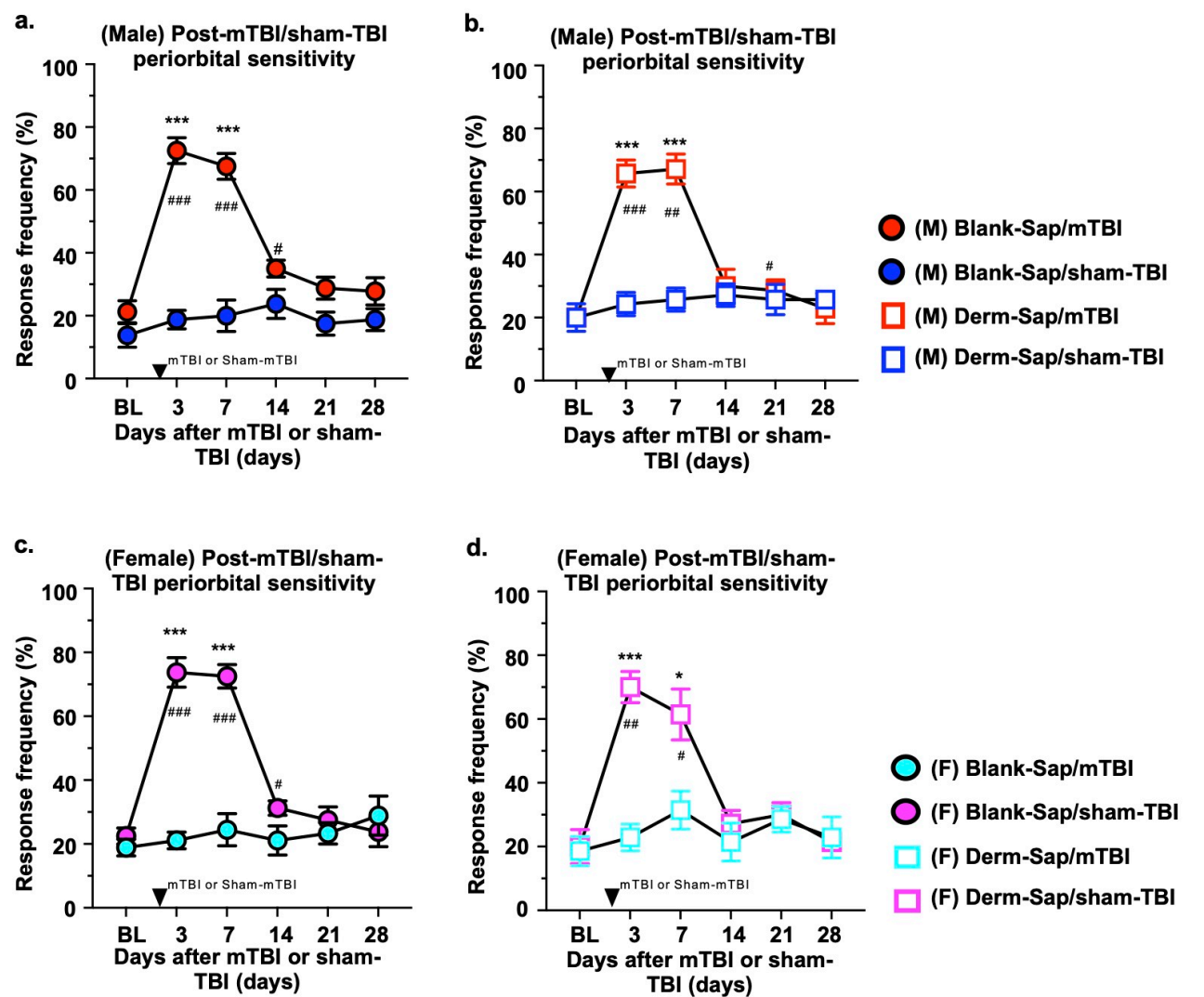


Figure 2

Effect of mTBI on acute periorbital allodynia in mice with microinjection of Blank-sap or Derm-sap pretreatment.

Periorbital response frequency at baseline and after undergoing mTBI or sham-TBI in male mice with A) intact RVM Oprm+ neurons (Blank-sap treated), and B) lesioned RVM Oprm+ neurons (Derm-sap treated). There were significant effects of Blank-sap vs Derm-sap treatment ( $F_{3,26} = 21.68, p < 0.0001$ ), Time ( $F_{5,130} = 42.99, p < 0.0001$ ) and time X treatment interaction ( $F_{15,130} = 12.64, p < 0.0001$ ).

Similarly, periorbital response frequency at baseline and after undergoing mTBI or sham-TBI in female mice with C) intact RVM Oprm<sup>+</sup> neurons (Blank-sap treated), and D) lesioned RVM Oprm<sup>+</sup> neurons (Derm-sap treated). There were significant effects of treatment ( $F_{3,27} = 11.24$ ,  $p < 0.0001$ ), Time ( $F_{5,135} = 39.87$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{15,135} = 12.00$ ,  $p < 0.0001$ ).

Two-way ANOVA with repeated measures on Blank/Derm-sap treatment, mTBI/sham-TBI, and time, followed by Turkey's test for multiple comparisons if significant. Values are the mean+SEM. For post-hoc Turkey's multiple comparisons between treatment groups: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; and within-subject comparisons to baseline within treatment group: #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ .

Figure 3

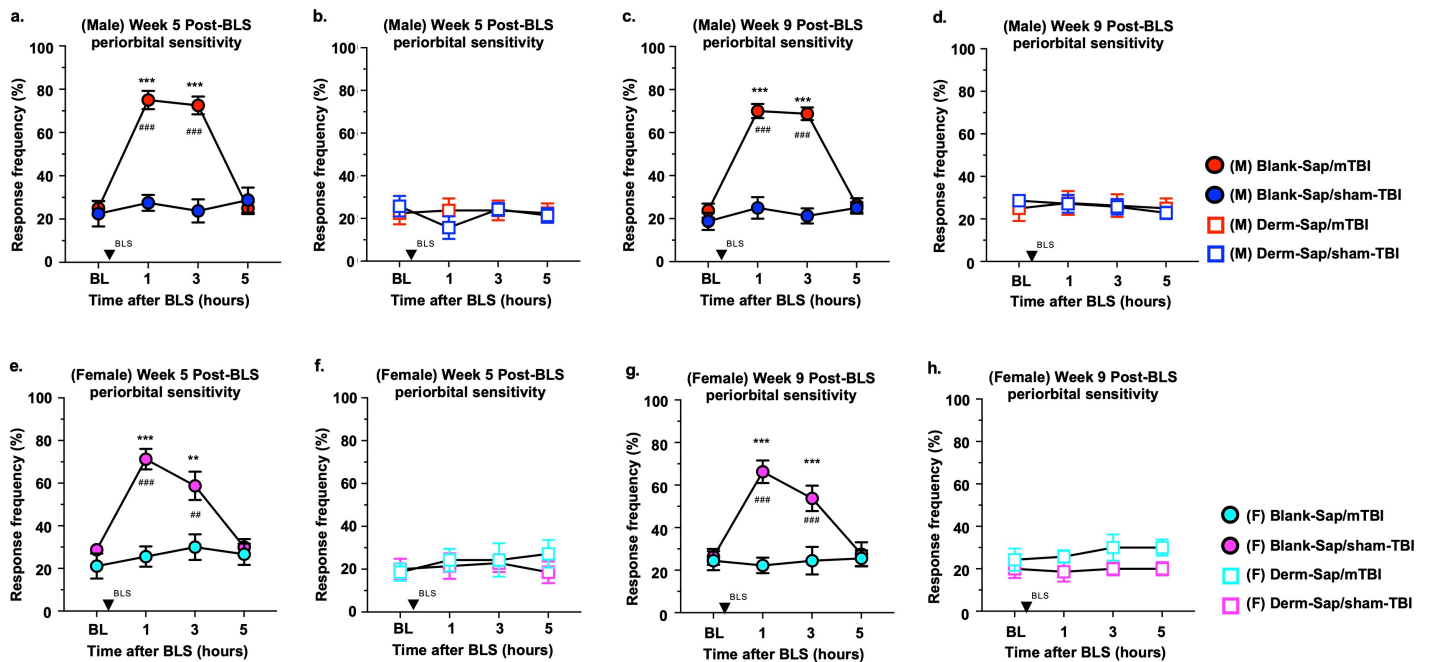


Figure 3

Effect of microinjection of Blank-sap or Derm-sap pretreatment in RVM on acute bright-light stress (BLS) induced periorbital allodynia.

In week 5 post mTBI/sham-TBI, periorbital response frequency at baseline and after acute BLS exposure in mTBI/sham-TBI male mice with A) Blank-sap treatment, and B) Derm-sap treatment. There were significant effects of treatment ( $F_{3,26} = 13.71$ ,  $P < 0.0001$ ), Time ( $F_{3,78} = 15.13$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{9,78} = 17.87$ ,  $p < 0.0001$ ). In week 9 post mTBI/sham-TBI, acute BLS-induced periorbital allodynia was assessed in mTBI/sham-TBI male mice with C) Blank-sap treatment, and D) Derm-sap treatment. There were significant effects of treatment ( $F_{3,26} = 15.05$ ,  $P < 0.0001$ ), Time ( $F_{3,78} = 15.05$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{9,78} = 14.30$ ,  $p < 0.0001$ ).

Similar results were observed in female mice. In week 5 post mTBI/sham-TBI, periorbital response frequency at baseline and after acute BLS exposure in mTBI/sham-TBI female mice with E) Blank-sap treatment, and F) Derm-sap treatment. There were significant effects of treatment ( $F_{3,27} = 8.73$ ,  $p=0.0003$ ), Time ( $F_{3,81} = 10.75$ ,  $p<0.0001$ ) and time X treatment interaction ( $F_{9,81} = 6.70$ ,  $p<0.0001$ ). In week 9 post mTBI/sham-TBI, acute BLS-induced periorbital allodynia was assessed again in mTBI/sham-TBI female mice with G) Blank-sap treatment, and H) Derm-sap treatment. There were significant effects of treatment ( $F_{3,27} = 10.98$ ,  $p<0.0001$ ), Time ( $F_{3,81} = 5.07$ ,  $p=0.004$ ) and time X treatment interaction ( $F_{9,81} = 6.45$ ,  $p<0.0001$ ).

Two-way ANOVA with repeated measures on Blank/Derm-sap treatment, mTBI/sham-TBI, and time, followed by Turkey's test for multiple comparisons if significant. Values are the mean+SEM. For post-hoc Turkey's multiple comparisons between treatment groups: \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ ; and within-subject comparisons to baseline within treatment group: #  $p<0.05$ , ##  $p<0.01$ , ###  $p<0.001$ .

Figure 4

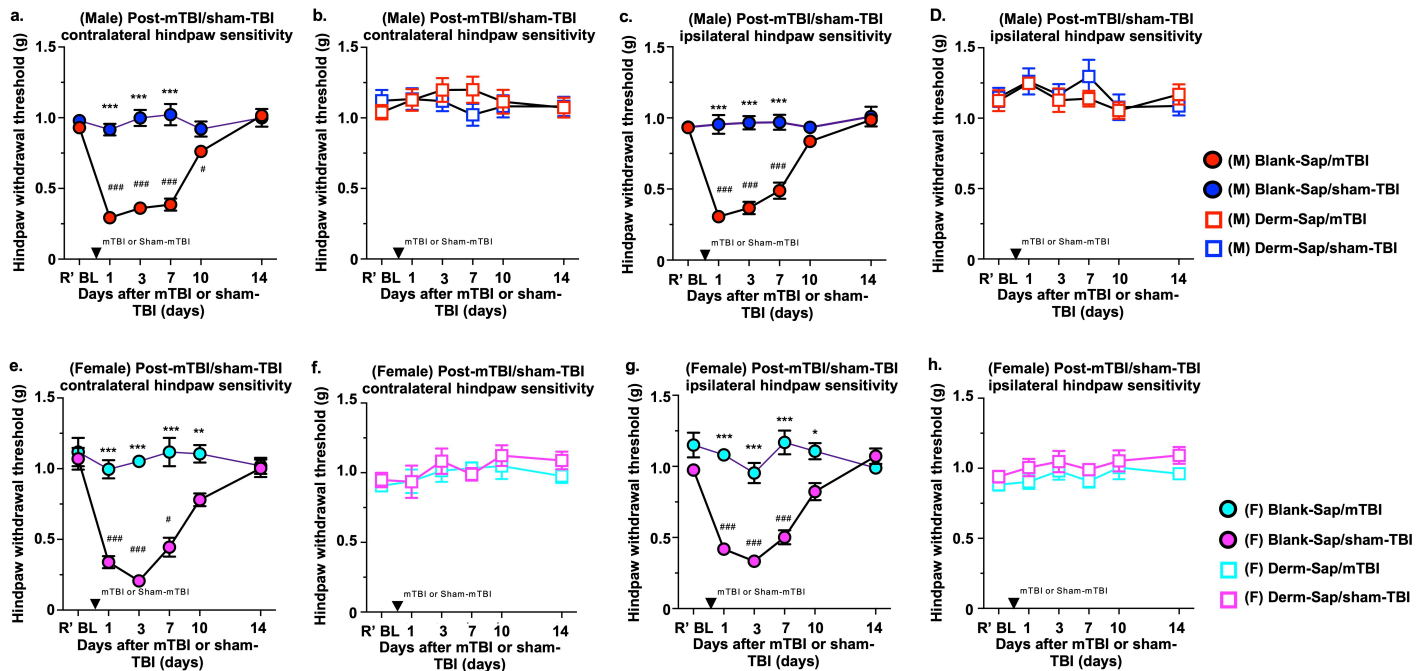


Figure 4

Effect of mTBI on acute hindpaw allodynia in mice with microinjection of Blank-sap or Derm-sap pretreatment.

Contralateral hindpaw (contralateral to the mTBI side) mechanical withdrawal thresholds after undergoing mTBI or sham-TBI in male mice with A) Blank-sap treatment, and B) Derm-sap treatment. There were significant effects of Blank-sap vs Derm-sap treatment ( $F_{3,30} = 34.73$ ,  $p<0.0001$ ), Time ( $F_{5,150} = 6.55$ ,  $p<0.0001$ ), and time X treatment interaction ( $F_{15,150} = 12.18$ ,  $p<0.0001$ ).

Ipsilateral hindpaw (ipsilateral to the mTBI side) mechanical withdrawal thresholds after undergoing mTBI or sham-TBI in male mice with C) Blank-sap treatment, and D) Derm-sap treatment.. There were significant effects of treatment ( $F_{3,30} = 46.28$ ,  $p < 0.0001$ ), Time ( $F_{5,150} = 4.20$ ,  $p < 0.0001$ ) and time X treatment interaction ( $F_{15,150} = 9.76$ ,  $p < 0.0001$ ).

Contralateral hindpaw (contralateral to the mTBI side) mechanical withdrawal thresholds after undergoing mTBI or sham-TBI in female mice with E) Blank-sap treatment, and F) Derm-sap treatment.. There were significant effects of treatment ( $F_{3,30} = 34.18$ ,  $p < 0.0001$ ), Time ( $F_{5,150} = 8.39$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{15,150} = 7.41$ ,  $p < 0.0001$ ).

Ipsilateral hindpaw (ipsilateral to the mTBI side) mechanical withdrawal thresholds after undergoing mTBI or sham-TBI in female mice with G) Blank-sap treatment, and H) Derm-sap treatment. There were significant effects of treatment ( $F_{3,30} = 34.18$ ,  $p < 0.0001$ ), Time ( $F_{5,150} = 8.39$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{15,150} = 7.41$ ,  $p < 0.0001$ ).

Two-way ANOVA with repeated measures on Blank/Derm-sap treatment, mTBI/sham-TBI, and time, followed by Turkey's test for multiple comparisons if significant. Values are the mean+SEM. For post-hoc Turkey's multiple comparisons between treatment groups: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; and within-subject comparisons to baseline within treatment group: #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ .

Figure 5

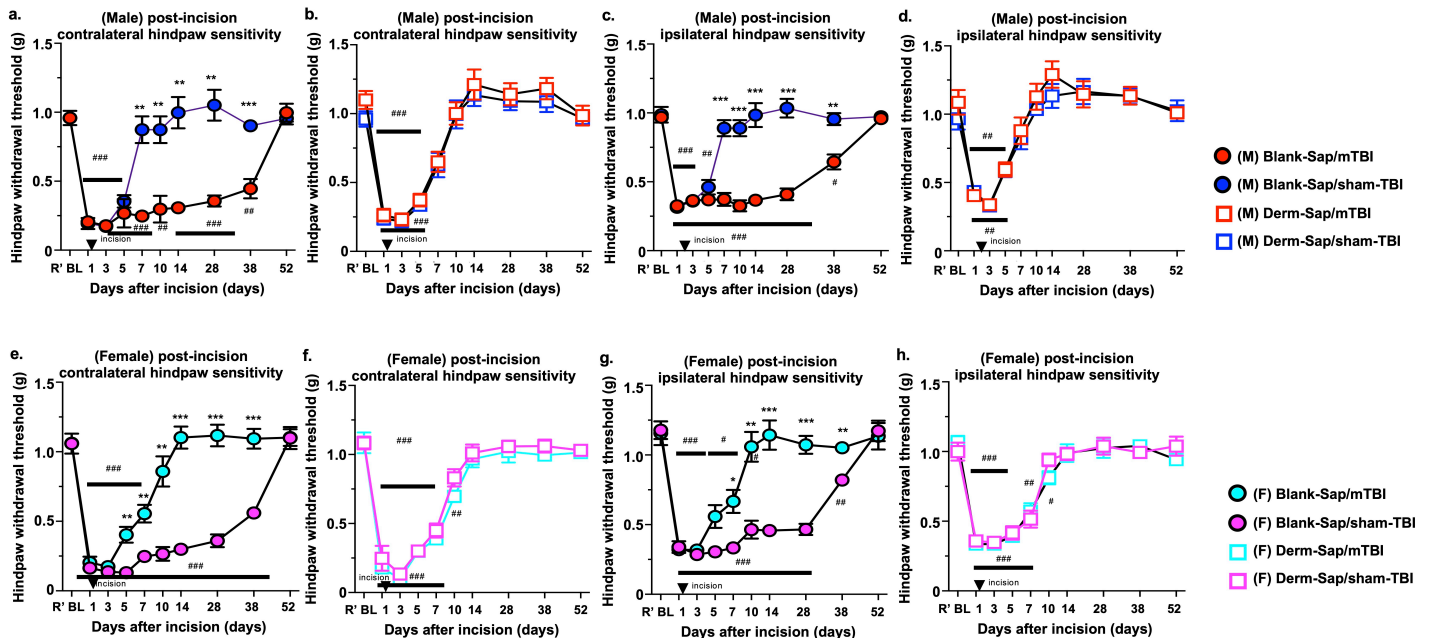


Figure 5

Two-way ANOVA with repeated measures on Blank/Derm-sap treatment, mTBI/sham-TBI, and time, followed by Turkey's test for multiple comparisons if significant. Values are the mean+SEM. For post-

hoc Turkey's multiple comparisons between treatment groups: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; and within-subject comparisons to baseline within treatment group: #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ .

Recovery course of post-incision surgery hindpaw allodynia in mTBI/sham-TBI mice after Blank-sap or Derm-sap pretreatment.

Post-incision surgery injured hindpaw (contralateral to the mTBI side) mechanical withdrawal thresholds in male mice with A) Blank-sap treatment, and B) Derm-sap treatment. There were significant effects of treatment ( $F_{3,30} = 31.15$ ,  $p < 0.0001$ ), Time ( $F_{9,270} = 101.84$ ,  $p < 0.0001$ ) and time X treatment interaction ( $F_{27,270} = 8.47$ ,  $p < 0.0001$ ).

Post-incision surgery uninjured hindpaw (ipsilateral to the mTBI side) mechanical withdrawal thresholds in male mice with C) Blank-sap treatment, and D) Derm-sap treatment. There were significant effects of treatment ( $F_{3,30} = 28.34$ ,  $p < 0.0001$ ), Time ( $F_{9,270} = 98.81$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{27,270} = 10.19$ ,  $p < 0.0001$ ).

Post-incision surgery injured hindpaw (contralateral to the mTBI side) mechanical withdrawal thresholds in female mice with E) Blank-sap treatment, and F) Derm-sap treatment. There were significant effects of treatment ( $F_{3,30} = 23.29$ ,  $p < 0.0001$ ), Time ( $F_{9,270} = 242.31$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{27,270} = 10.36$ ,  $p < 0.0001$ ).

Post-incision surgery injured hindpaw (contralateral to the mTBI side) mechanical withdrawal thresholds in female mice with G) Blank-sap treatment, and H) Derm-sap treatment. There were significant effects of treatment ( $F_{3,30} = 13.37$ ,  $p < 0.0001$ ), Time ( $F_{9,270} = 148.09$ ,  $p < 0.0001$ ) and time X treatment interaction ( $F_{27,270} = 7.52$ ,  $p < 0.0001$ ).

Figure 6

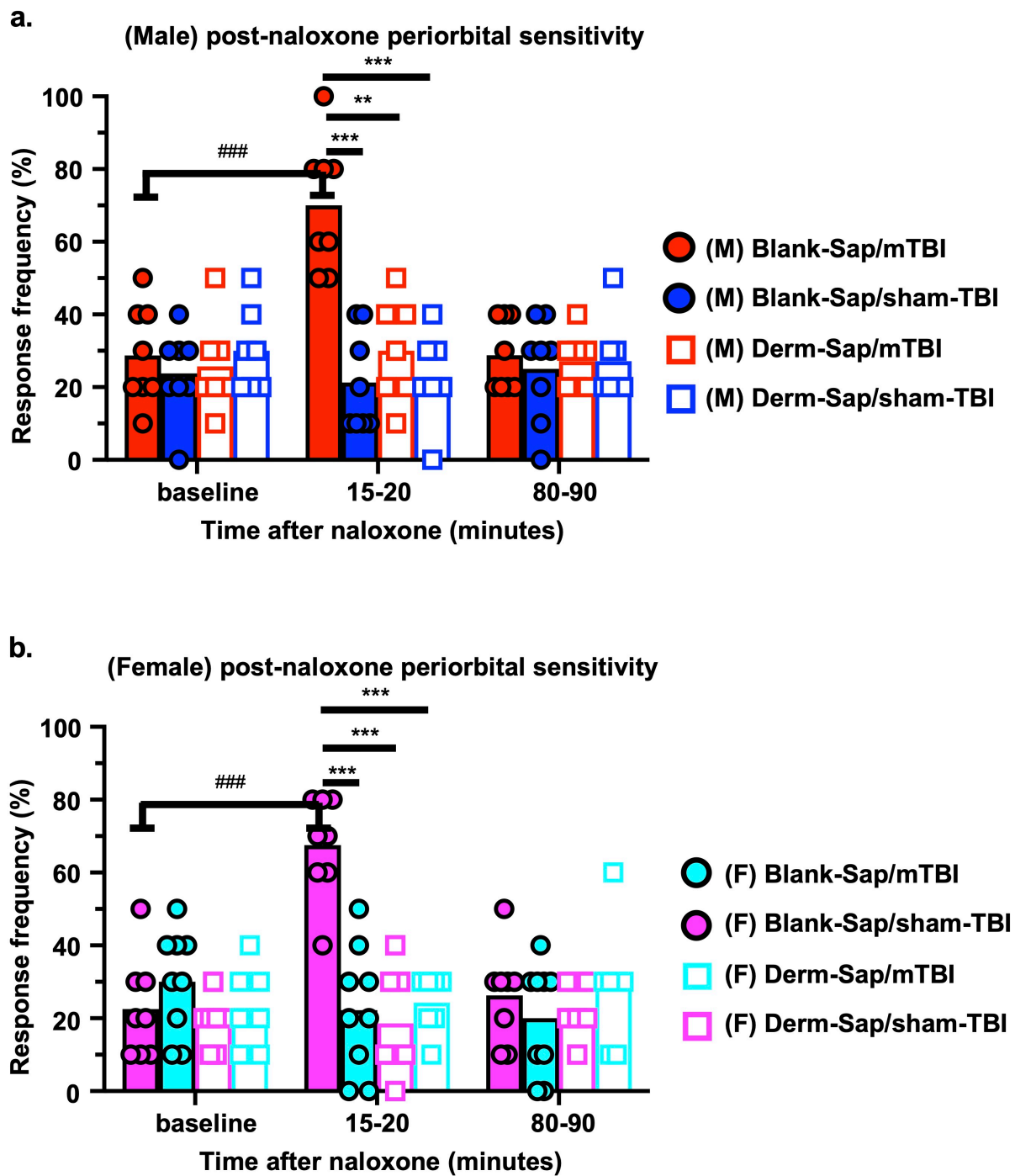


Figure 6

Two-way ANOVA with repeated measures on Blank/Derm-sap treatment, mTBI/sham-TBI, and time, followed by Turkey's test for multiple comparisons if significant. Values are the mean+SEM. For post-hoc Turkey's multiple comparisons between treatment groups: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; and within-subject comparisons to baseline within treatment group: #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ .

Effect of endogenous opioid blockade on periorbital mechanical sensitivity in mTBI/sham-TBI mice after Blank-sap or Derm-sap pretreatment.

Lesioning of RVM Oprm+ neurons abolished mTBI-induced latent sensitization in male mice compared to the mTBI mice with intact RVM. A) Mechanical withdrawal thresholds of contralateral hindpaw in mTBI/sham-TBI male mice with intact RVM Oprm+ neurons (Blank-sap treatment) vs lesioned RVM Oprm+ neurons (Derm-sap treatment). There were significant effects of treatment ( $F_{3,26} = 6.89, p = 0.0014$ ), Time ( $F_{2,52} = 6.59, p = 0.0036$ ), and time X treatment interaction ( $F_{6,52} = 10.25, p < 0.0001$ ).

Similar abolishment of mTBI-induced latent sensitization is also seen in female mice with lesioned RVM Oprm+ neurons. B) Mechanical withdrawal thresholds of contralateral hindpaw in mTBI/sham-TBI female mice with intact RVM Oprm+ neurons (Blank-sap treatment) vs lesioned RVM Oprm+ neurons (Derm-sap treatment). There were significant effects of treatment ( $F_{3,27} = 5.51, p = 0.0044$ ), Time ( $F_{2,54} = 39.87, p = 0.0023$ ) and time X treatment interaction ( $F_{6,54} = 11.68, p < 0.0001$ ).

Figure 7

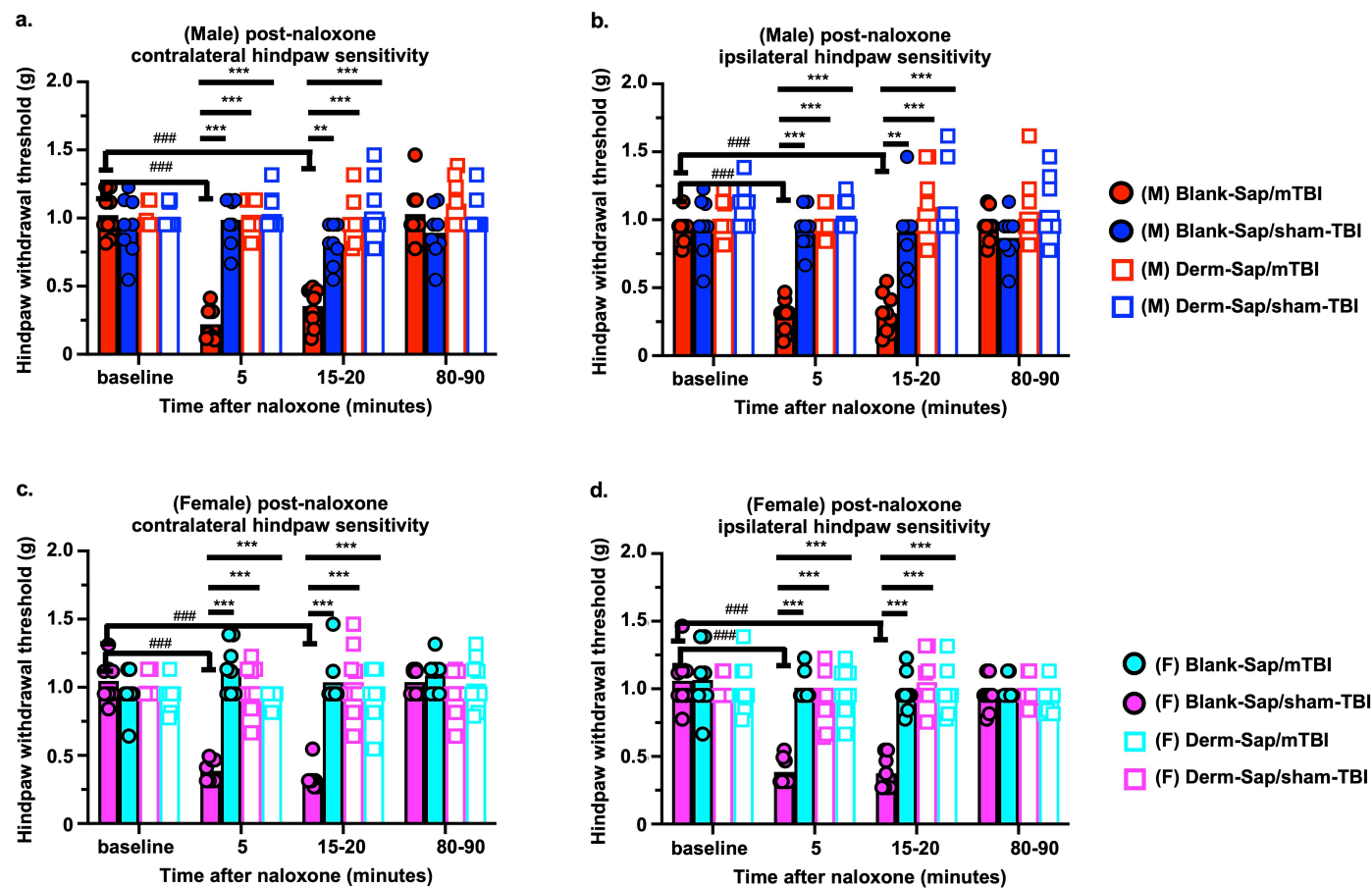


Figure 7

Effect of endogenous opioid blockade on hindpaw mechanical sensitivity in mTBI/sham-TBI mice after Blank-sap or Derm-sap pretreatment.

Lesioning of RVM Oprm+ neurons abolished mTBI-induced latent sensitization in male mice compared to the mTBI mice with intact RVM. A) Mechanical withdrawal thresholds of contralateral hindpaw in mTBI/sham-TBI male mice with intact RVM Oprm+ neurons (Blank-sap treatment) vs lesioned RVM Oprm+ neurons (Derm-sap treatment). There were significant effects of treatment ( $F_{3,30} = 19.04$ ,  $p < 0.0001$ ), Time ( $F_{3,90} = 29.36$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{3,90} = 28.38$ ,  $p < 0.0001$ ). B) Mechanical withdrawal thresholds of ipsilateral hindpaw in mTBI/sham-TBI male mice with intact RVM Oprm+ neurons (Blank-sap treatment) vs lesioned RVM Oprm+ neurons (Derm-sap treatment). There were significant effects of treatment ( $F_{3,30} = 20.94$ ,  $p < 0.0001$ ), Time ( $F_{3,90} = 15.44$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{3,90} = 15.28$ ,  $p < 0.0001$ ).

Similar abolishment of mTBI-induced latent sensitization is also seen in female mice with lesioned RVM Oprm+ neurons. C) Mechanical withdrawal thresholds of contralateral hindpaw in mTBI/sham-TBI female mice with intact RVM Oprm+ neurons (Blank-sap treatment) vs lesioned RVM Oprm+ neurons (Derm-sap treatment). There were significant effects of treatment ( $F_{3,30} = 21.45$ ,  $p < 0.0001$ ), Time ( $F_{3,90} = 16.22$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{3,90} = 20.08$ ,  $p < 0.0001$ ). D) Mechanical withdrawal thresholds of ipsilateral hindpaw in mTBI/sham-TBI male mice with intact RVM Oprm+ neurons (Blank-sap treatment) vs lesioned RVM Oprm+ neurons (Derm-sap treatment). There were significant effects of treatment ( $F_{3,30} = 20.60$ ,  $p < 0.0001$ ), Time ( $F_{3,90} = 16.70$ ,  $p < 0.0001$ ), and time X treatment interaction ( $F_{3,90} = 14.48$ ,  $p < 0.0001$ ).

Two-way ANOVA with repeated measures on Blank/Derm-sap treatment, mTBI/sham-TBI, and time, followed by Turkey's test for multiple comparisons if significant. Values are the mean+SEM. For post-hoc Turkey's multiple comparisons between treatment groups: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; and within-subject comparisons to baseline within treatment group: #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ .