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Neuroinflammation drives sex-dependent effects on pain and negative affect in a murine model of repeated mild traumatic brain injury

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Abstract

The Center for Disease Control and Prevention estimates that 75% of reported cases of traumatic brain injury (TBI) are mild, where chronic pain and depression are 2 of the most common symptoms. In this study, we used a murine model of repeated mild TBI to characterize the associated pain hypersensitivity and affective-like behavior and to what extent microglial reactivity contributes to these behavioral phenotypes. Male and female C57BL/6J mice underwent sham or repeated mild traumatic brain injury (rmTBI) and were tested for up to 9 weeks postinjury, where an anti-inflammatory/neuroprotective drug (minocycline) was introduced at 5 weeks postinjury in the drinking water. Repeated mild traumatic brain injury mice developed cold nociceptive hypersensitivity and negative affective states, as well as increased locomotor activity and risk-taking behavior. Minocycline reversed negative affect and pain hypersensitivities in male but not female mice. Repeated mild traumatic brain injury also produced an increase in microglial and brain-derived neurotropic factor mRNA transcripts in limbic structures known to be involved in nociception and affect, but many of these changes were sex dependent. Finally, we show that the antiepileptic drug, gabapentin, produced negative reinforcement in male rmTBI mice that was prevented by minocycline treatment, whereas rmTBI female mice showed a place aversion to gabapentin. Collectively, pain hypersensitivity, increased tonic-aversive pain components, and negative affective states were evident in both male and female rmTBI mice, but suppression of microglial reactivity was only sufficient to reverse behavioral changes in male mice. Neuroinflammation in limbic structures seems to be a contributing factor in behavioral changes resulting from rmTBI.

Keywords: Concussion, Brain injury, Closed head injury, Anterior cingulate cortex, Nucleus accumbens, Emotional pain, Conditioned place preference, Negative reinforcement, Affective-motivational pain, Neuroinflammation, Microglia

1. Introduction

As a result of aggressive interventions and rehabilitation, traumatic brain injury (TBI) patients live longer but commonly live with intractable pain; indeed, chronic pain is the most frequently reported complication.[33] The prevalence of chronic pain in TBI patients ranges from 22% to 95% depending on the pain location and type.[47] The most commonly reported pain includes headache (often migraine) and neuropathic pain. Mild TBI accounts for more than 75% of injuries every year and has a prolonged neurological impact.[72] Importantly, mild, rather than more severe, TBI appears more likely to cause chronic pain,[19,47] indicating that the global severity of brain injury is not the only formative factor to trigger pain. To date, few studies have assessed the underlying mechanisms leading to the genesis or persistence of pain associated with a TBI.

Comorbidity between chronic pain and Axis I disorders of The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) (including depression and anxiety disorders) is well documented in patients with mild TBI.[19,33] Posttraumatic stress disorder (PTSD), depression, and anxiety following TBI are the most commonly reported comorbidities with posttraumatic pain. Suicide ideation and completed suicide is associated with

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mild TBI, even after controlling for other risk factors, including depression, PTSD, and TBI symptom severity.[24] Significant associations between suicide and migraine pain (commonly reported in TBI patients) are also evident, even when controlling for concomitant psychiatric conditions.[26] Indeed, patients with severe headache had the highest risk for suicide (6.5-fold) over other chronic pain cohorts.[14] Thus, understanding the processes that influence TBI-induced chronic pain is essential to developing treatment strategies.

Traumatic brain injury in rodents and nonhuman primates causes microglial activation in various brain regions.[13,46] Microglia/macrophages remain reactive at the sites of injury and in areas of axonal degeneration/survival for at least 12 months after TBI in nonhuman primates[46] and years in humans.[30] It is well appreciated that immunocompetent cells are involved in the development of chronic pain states.[60] Although these non-neuronal cells contribute to chronic pain in both sexes, there is strong evidence that inhibiting microglia in male, but not female, rodents is sufficient to alleviate sensory hypersensitivities in models of chronic pain.[25,27,67] However, much of what we know about the glial contribution to chronic pain emanates from studies in the spinal cord, and how such cells modulate the sensory component of pain; little is known about glial involvement in the affective dimension of pain. In addition, how glial activation within the brain contributes to chronic pain is relatively unknown. In this study, we determined the extent repeated mild TBI induces pain, disruptions in affective-like behaviors, and the extent of microglial activation within limbic brain structures in male and female mice. Pharmacological approaches were used to modify microglial reactivity through minocycline, which in addition to antimicrobial activity, is well known for its anti-inflammatory and neuroprotective effects. In addition, we used minocycline to assess potential sex differences and for its translational value because minocycline is an FDA-approved medication.

2. Methods

2.1. Animals

All experimental protocols were approved by the UC Irvine Institutional Animal Care and Use Committee (IACUC) and/or the UCLA Animal Research Committee. C57BL/6J male and female mice were obtained from JAX Laboratories and housed 4 per cage in a temperature-controlled room with a reverse 12-hour light/dark cycle (lights off at 8:00 AM). Mice were allowed free access to food and water and were acclimated to the housing conditions for 1 week before any experimental manipulation. Estrous cycle phases were not tracked in female mice because it produces an added stressor to only the female mice. Moreover, it was recently reported that different estrous cycle phases: proestrus, estrous, metestrus, and diestrus had no effect on various affective-like behavior or sensory (thermal or mechanical) thresholds.[78] In all experiments, mice in the same cage received the same surgery and drug treatment. ARRIVE guidelines were strictly followed for animal welfare and designed to optimize rigor and reproducibility. Mice were randomly assigned to groups before any experimentation, and experimenters were blind to surgery and treatments during all behavioral and ex vivo molecular studies. Behavioral testing of male and female mice was conducted separately. All male mice underwent testing and were returned to housing, and all equipment was thoroughly cleaned before bringing female mice to the testing room. After a week of vivarium acclimatization, all mice were habituated to touch by handling the mice 10 minutes a day every day for a week before any experimentation. Mice were habituated to the behavior room daily by leaving them in their cages for 30 minutes after they were handled.

2.2. Repeated mild traumatic brain injury

Mice were anesthetized with isoflurane anesthesia before being placed on a Marmarou foam bed (Type E Bed, Foam to Size, Inc, Ashland, VA). Two different devices were used to induce the injury. Animals used in sensory nociceptive and affective components of pain experiments were subjected to the controlled cortical impact device (TBI-0310 Head Impactor; Precision Systems and Instrumentation, LLC, Fairfax Station, VA) with a mouse resting on a thick piece of Marmarou foam.[18] Mice that underwent conditioned place preference studies were injured through a NeuroPactor (Neuroscience Tools, O'Fallon, MO), again on the same Marmarou foam bed. Mice were anesthetized with isoflurane and secured to the foam bed with laboratory tape to prevent movement during breathing. Their heads were shaved to allow for a clean surface to zero the impactor tip. Isoflurane percentages (2.0% maintenance) were optimized to minimize head movement from respiration while zeroing the piston and keeping the head position constant during impact. The piston was placed at approximately midline just caudal to the eye socket. Each mouse was in the injury station for a total of 3 minutes before delivery of an impact with pneumatic piston to the center of cranium with speed 5.0 m/s, 1.0-mm depth, and 50-millisecond dwell time for both impactors. Immediately following impact, animals were removed from the device and returned to their cage. In total, all animals were under anesthesia for less than 10 minutes from start to finish, including shams. This process was repeated once per day for 5 consecutive days.

2.3. Sensory nociceptive testing

2.3.1. Cold plate test

Mice were habituated to the apparatus kept at ambient temperature at least twice before experimentation. During experimentation, the cold plate was set to 4°C and sprayed with a thin film of water. A pain response was characterized as repeated flinching, rapidly shaking paws, or jumping to escape the cold plate. Mice were removed from the cold stimulus 5 seconds after their initial response to minimize learning that they would be removed from the apparatus as soon as they perform one of the pain responses. A cutoff of 3 times the baseline response time was imposed to minimize tissue damage. Mice underwent baseline testing before injury, followed by weekly testing for 8 weeks following injury or sham surgeries.

2.3.2. Von Frey test

Mice were habituated to a mesh grid floor in a clear Plexiglas enclosure without the stimulation for 30 minutes per day for at least 3 days before experimentation. Withdrawal thresholds to a mechanical stimulus applied with calibrated von Frey filaments were measured in all groups, as described previously.[9,68] Von Frey filaments were applied to the plantar surface of the left hind paw in an up–down manner. After the first positive reaction, a lower force filament was applied. If no reaction was evident, the next higher force filament was applied, and so on until 4 additional responses were assessed following the initial difference in response. The 50% withdrawal

threshold was calculated.[9] Baseline measurements were taken for all animals before surgery and weekly following the injury or sham surgery.

2.4. Affective-like behavioral tests

2.4.1. Open field

Mice were placed in a box (50 cm L × 50 cm W × 40 cm H) with beta chip on the floor for 5 minutes, without any prior habituation. The center area was brightly lit (~1000 LUX). The primary outcome was thigmotaxis (mice remain at the periphery of the apparatus or against the walls and have minimal time in the center area (30% of the compartment)). An infrared video camera was positioned above the box to capture all movements; the video was analyzed with EthoVision software (Noldus, Version X.0). At the beginning of the test, the mouse was placed in the corner of the open field and allowed to explore the arena for 5 minutes. Parameters that were measured included distance traveled, time spent moving, number of rearing events, time spent grooming, and time in the center area. The apparatus was cleaned with disinfectant Vimboa 128 and water following each mouse test, and clean bedding was added.

2.4.2. Elevated plus maze

This test involves a maze with 2 closed arms (50 × 5 cm) that are enclosed on 3 sides by 15-cm high walls and 2 open arms (50 × 5 cm) with no walls, that intersect to form a "+" shape. The maze was on a platform that was 80 cm off the floor in the middle of a children's wading pool (as some repeated mild traumatic brain injury [rmTBI] animals jumped off and this allowed them to be contained to catch them). The test was conducted in a dimly lit room (5 LUX). Mice were placed in the center of the maze facing one of the closed arms and allowed to explore the entire apparatus for 5 minutes while being videorecorded (Noldus EthoVision). Immediately after testing, mice were returned to their home cage. The number of open and closed arm entries was measured, which was considered to have occurred once 3 of the animal's paws were across the border of an arm, as well as the total time spent in the open and closed arms. In this well-validated paradigm, anxious mice spend less time in the open arms of the maze, and thus, the objective measure of anxiety-like behavior is the decreased time spent in the open arms of the maze or, alternatively, decreased entries into the open arms of the maze.[53] The maze was cleaned with disinfectant Vimboa 128 and water following each mouse tested.

2.4.3. Splash test

Mice were habituated to a new shoe box cage similar to their home cage for 30 minutes before conducting this test. Because of its viscosity, the sucrose solution dirties the mouse fur, which initiates grooming behavior. The time spent grooming was recorded for a period of 5 minutes as an index of self-care and motivational behavior. The frequency and latency of the grooming behavior were scored over a 5-minute period after spraying a 10% sucrose (in water) solution on the dorsal coat of the mice. This test is considered a form of motivational behavior that parallels some symptoms of depression such as apathetic behavior.[73] Grooming perturbation is associated with hedonic reactivity in the sucrose preference test and increased immobility in the force swim test,[20,57] and it is present in chronic pain states.[78]

2.4.4. Forced swim test

The apparatus for this test was a water tank filled with water to 25 cm for male mice and 20 cm for female mice. Water temperature was maintained at 25°C and replaced between each mouse. This test evaluated depressive-like behavior as the time mice spend immobile when forced to swim in an inescapable water tank.[56] A mouse was considered to be immobile once swimming and escape behaviors ceased, and it was floating belly-down with its head above the water. The test session lasted 6 minutes, but only the last 4 minutes of the test were scored. Following the test session, the mouse was removed from the tank, dried with a towel, and placed in their home cage under a heat lamp for 15 minutes. An experimenter monitored the test session for signs of drowning, in which case the test would be immediately terminated.

2.4.5. Novelty suppressed feeding

The novelty-suppressed feeding (hyponeophagia) test is a conflict-based test in which mice that are food deprived (24 hours) face a choice of approaching and consuming a piece of food in the center of a brightly lit, novel open arena. This is a measure of anxiety-related behavior that was previously validated with anxiolytics.[61] After fasting for 24 hours, the mice were transferred to the testing room in their home cage. The testing apparatus consisted of a large brightly lit (500 LUX) arena (50 cm W × 50 cm L × 40 cm H), where the floor was covered in approximately 3 cm of corn cob bedding and a piece of food chow on a filter paper was placed in the center of the arena. After 1 hour of habituation to the testing room environment, mice were individually placed in the corner of the arena and allowed to explore for 10 minutes with video capture (EthoVision software, Noldus). The trial ended when the mouse chewed/ate a part of the chow pellet. The time the mouse first started to consume the food pellet was recorded as the feeding latency. An increase in the latency to feed is considered a measure of anxiety.[51,78]

2.5. Conditioned place preference

The conditioned place preference (CPP) assay is a Pavlovian conditioning test to measure the motivational reward (or aversive) effects of a drug[12] and motivational negative reinforcement when a negative stimulus such as pain is alleviated.[23] This test allows for a within-subject analysis of the time spent in the vehicle compared with drug-paired conditioning chamber. The conditioning was conducted using an unbiased, counterbalanced, 3-chamber apparatus, where 2 conditioning chambers (28 × 28 × 19 cm) were distinguished with visual and tactile cues so that the animals could discriminate between the chambers. To counterbalance the groups before the conditioning sessions, animals were placed in the apparatus and allowed free access to all chambers. The time spent in each chamber was recorded for 30 minutes using an infrared video camera attached to a computer running behavioral tracking software (EthoVision; Noldus). The drug-paired chamber was assigned such that any innate bias for one chamber over the other was balanced between the treatment groups. During the conditioning session, animals were confined to only one of the compartments so that they would associate the contextual cues of the compartment with the motivational effects of the stimulus (drug or vehicle, counterbalanced every other day). Conditioning sessions consisted of 6 sessions with 3 trials of gabapentin (100 mg/kg, i.p.) and 3 trials of saline on alternating days; mice were confined to the chambers for 30 minutes

immediately after injections. On the postconditioning day (24 hours after the last conditioning trial), no treatment was given to the animals so that they could be tested in a drug-free (context-dependent) state. Animals were allowed free access to all chambers of the apparatus, and the time spent in the drug-paired chamber measured by motion capture Noldus software was determined over 30 minutes. Forty-eight hours after the postcondition test, all mice were administered gabapentin and immediately placed into the CPP apparatus and again allowed free access to all chambers, and the time spent in the drug-paired chamber was determined. This test is a state-dependent test that confirms the extent to which the animal could learn to associate the drug experience with the contextual cues. Data are presented as time in the chamber (raw data) and a CPP score which was calculated using the formula: (time in drug chamber − time in vehicle chamber) − (time in drug chamber during preconditioning − time in vehicle chamber during preconditioning).

2.6. Real-time quantitative polymerase chain reaction

Fresh brains were collected from a cohort of sham and rmTBI mice 2 weeks postinjury and were coronal sectioned through cryostat (150-m thick) at 20°C and mounted on Superfrost charged slides (Fisher Scientific). Tissue punches (1-mm diameter) were taken using a disposable biopsy plunger (Miltex) for the anterior cingulate cortex and nucleus accumbens. Total RNA was collected from the brain tissue punches through the Trizol extraction method (Ambion Life Technologies). RNA was converted to cDNA using 100 U of M-MulV Reverse Transcriptase, 1M Oligo d(T) 23VN, and 2 mM dNTP mix (New England Biolabs), annealed at 70°C and inactivated at 95°C. Real-time qPCR was conducted using primer sets for **IBA-1** (IBA-1-R CAG CAT TCG CTT CAA GGA CAT A, IBA-1-F ATC AAC AAG CAA TTC CTC GAT GA), **CD11b** (CD11b-R CAT CAT GTC CTT GTA CTG CCG CTT G, CD11b-F CAG ATC AAC AAT GTG ACC GTA TGG G), brain-derived neurotropic factor (**BDNF**) (BDNF-R CCG CCA GAC ATG TCC AC, BDNF-F CAC TCC GAC CCT GCC CGC), and **ß-actin** (actin-R CCA GTT GGT AAC AAT GCC ATG T, actin-F GGC TGT ATT CCC CTC CAT CG) genes. Using 96-well optical plates (Applied Biosystems), cDNA, and PerfeCTa SYBR Green FastMix containing the primer sets (Quanta Biosciences), samples were loaded and run with an ABI ViiA7 fast block qPCR machine using cycling conditions in the PerfeCTa SYBR Green FastMix manual. Cycle threshold outputs were calculated and normalized to the ß-actin housekeeping gene to compute delta cycle threshold. Relative expression levels were determined by normalizing sham and rmTBI groups to 3 age-matched sham mice brain samples through DDCT method.

2.7. RNAScope multiplex fluorescent in situ hybridization

Brains were collected from sham and rmTBI mice 8 weeks postinjury. Brains were perfused with saline and snap frozen with isopentane at −30°C and stored at −80°C until further processing. Brains were coronal sectioned through cryostat (18 μm thick) at −20°C and thaw-mounted on Superfrost charged slides. Custom fluorescent probe labels were designed to fit complementary sequences on mRNA strands for BDNF gene (*Bdnf*) carrying fluorescent AlexaFluor488, tyrosine hydroxylase (*TH*) carrying Atto555, and IBA-1 (*Itgam*) carrying Atto647. Brain slices were incubated with 4% paraformaldehyde and then dehydrated with 50%, 70%, and 100% ethanol washes for 5 minutes each. Slides were then incubated overnight at −20°C in 100% ethanol. Slides were then taken out and dried for 5 minutes. A hydrophobic barrier was drawn around the brain slices before the administration of the probes. Probes were activated in an oven at 40°C for 10 minutes. Slides were then incubated with probes following the RNAScope Multiplex processing kit instructions (ACD Biosciences). Slides were coverslipped and sealed with nail polish and stored in the dark at −20°C until visualization. Slides were visualized using a Nikon Ti-E wide-field inverted fluorescence microscope at 200x and NIS Elements software setup. Image fluorescence intensities were quantified using FIJI (ImageJ) software. Separate cohorts of animals were used to assess the effects of minocycline treatment (beginning 5 weeks postinjury) on markers of neuroinflammation. Brains were collected from sham vs rmTBI, male vs female, vehicle vs minocycline-treated mice at 8 weeks postinjury. This corresponded to a total of 8 distinct treatment groups with 6 animals in each group. At least 6 brain sections from each animal were processed and averaged for each N value.

2.8. Immunohistochemistry

Brains were isolated after cardiac perfusion with 4% paraformaldehyde, postfixed in the same fixative at 4°C overnight, and cryoprotected in 30% sucrose until the brains sunk. Brains were frozen in isopentane before being stored at −80°C before sectioning with a cryostat (Leica). Free-floating sections (40 μm) were incubated in blocking solution (5% normal goat serum and 1% bovine serum albumin), followed by overnight incubation at 4°C with antisera recognizing Iba-1 (1:1000; Abcam #019-19741) to label microglia. Sections were incubated either with a secondary antibody conjugated to an Alexa 488 fluorophore or an HRP-conjugated antibody. For light microscopy visualization, the HRP was visualized by incubating sections for 10 minutes in a solution of 3,3′-diaminobenzidine (0.05% DAB; Sigma-Aldrich), D-glucose (0.2%), and sodium azide (0.0005%) in 0.1 M Tris-buffer (TB), pH 7.4. Sections were incubated subsequently for 10 minutes with DAB solution containing 0.1% glucose oxidase to produce a brown precipitate. Imaging of fluorescent immunoreactive cells was performed as previously described.[41,42,69] A minimum of 12 sections were taken from each animal and averaged for N = 1. Brains from a minimum of 5 animals per condition (injury and sex) were processed for analysis. Immunofluorescence was captured using a confocal microscope (Leica DM5500 B). Images were captured at ×20 magnification. Image fluorescence intensities were quantified using FIJI (ImageJ) software.

2.9. Experimental protocol

All mice underwent sensory nociceptive threshold testing before sham and rmTBI surgeries. Following the surgery, mice were subjected to von Frey and cold plate testing weekly, where these tests were conducted at least 2 days apart to minimize sensitization of one test influencing outcomes of the other. At 6 weeks (day 42) postinjury or sham surgery, mice underwent 3 affective-like tests where the test chosen was randomized between successive cohorts. These tests included the open-field testing, followed by a splash test or novelty suppressed feeding at day 49, elevated plus maze testing on day 56, and a forced swim test on day 63. The timing of the affective-like behavior and the order of tests was taken from studies that assessed these behaviors in mice with neuropathic pain.[64,77] The order of these tests was set to minimize the influence of one test on another. Each of the affective-like behavioral tests was only conducted once. Separate cohorts of mice were used for

conditioned place preference studies and conducted 8 weeks postinjury. Mice were assigned to conditions in a randomized block design so that the running of subjects counterbalanced factors such as time of day over experimental conditions. We also completed experiments in successive replications where replications were balanced with respect to experimental groups. All behavior was performed in the dark (active) phase using a reverse light–dark cycle and recorded by a video infrared camera using an infrared illuminator under low lighting conditions (<10 LUX) except where bright light was required for a specific test. Data were acquired by automated unbiased software and analyzed by live tracking using EthoVision XT V.11.0 (Noldus). Raw data files amenable to reanalysis have been maintained.

To determine the extent to which minocycline, an anti-inflammatory/neuroprotective inhibitor of microglial reactivity, altered sensory or affective-like behaviors, mice received minocycline (0.544 mg/mL) in their drinking water beginning at week 5 postsurgery. Minocycline infused water was stored in light resistant water dispensers and was changed every 3 days.

2.10. Statistics and data analysis

The effect of rmTBI on pain hypersensitivities (mechanical and cold) were assessed by a 2-way analysis of variance (ANOVA) analysis (GraphPad Prizm v9.4.1). Data are reported for time, surgical/injury condition, and interaction. When significance was identified, a Tukey multiple comparison *post hoc* analysis was conducted to identify differences. For tests with an assessment at a single time point, the effect of the injury on affective-like behaviors and conditioned place preference was analyzed by a 2-way ANOVA (GraphPad Prizm v9.4.1) followed by Šídák multiple comparisons *post hoc* test. Immunohistochemistry was analyzed by a 2-way ANOVA (surgical/injury condition and drug intervention), whereas RNAScope and Quantitative real-time reverse-transcription PCR data were analyzed by an unpaired student t-test. One outlier, assessed by the Robust regression and Outlier removal method (Q = 1%), was removed from the Splash test data set (GraphPad Prizm v9).

3. Results

3.1. Effects of repeated mild traumatic brain injury on sensory and affective behavior

To investigate the effects of rmTBI in mice on sensory nociceptive thresholds, mice were tested weekly (**Fig. 1A**). In contrast to our hypothesis, rmTBI had no effect on hind paw mechanical withdrawal thresholds in male or female mice when compared with sham control animals (**Fig. 1B**). Statistical analysis for this data set is presented in **Table 1**. However, both male and female mice developed pain hypersensitivity to cold thermal stimuli as evidenced by a decrease in withdrawal threshold (**Fig. 1C**, 2-way ANOVA: $^{****}P < 0.0001$ for injury, $^{****}P < 0.0001$ for time).

Considering the common occurrence of negative affective states associated with rmTBI, we asked whether our model produced behaviors indicative of an anxiogenic or depressive state. Mice were subjected to affective-like behavioral tests beginning 6 weeks postinjury or sham surgery (**Fig. 1D**). One of the most common sensorimotor tests of anxiety and emotionality is the open-field test.[63] Both male and female rmTBI mice spent less time in the inner/center compartment compared with the sham cohorts (**Fig. 1E**, 2-way ANOVA: $^{***}P = 0.0005$ for injury, but there was no effect of sex, $P = 0.6352$). This test also allowed assessment of locomotor activity to a novel environment. Repeated mild traumatic brain injury increased locomotor activity, as determined by total distance travelled ($^{****}P < 0.0001$), compared with sham animals; again, there was no effect of sex ($P = 0.8152$). The splash test was conducted 7 days after the open-field test. The splash test provides insight into self-care, an interoceptive behavior that is often disrupted in chronic pain conditions.[77] Latency to groom following sucrose application was significantly increased in male rmTBI mice compared with sham, but there was no difference between injury and sham in female mice (**Fig. 1F**, $P = 0.0043$ compares rmTBI with sham mice, $P = 0.0006$ compares males with females). In the nestlet test, there was an effect of rmTBI in male but not in female mice, although female mice showed reduced nestlet shredding and nest building compared with male mice (Supplementary Figure 1, available at http://links.lww.com/PAIN/B935). At 8 weeks postsurgery, mice were tested on the elevated plus maze. In contrast to our hypothesis, rmTBI mice exhibited increased open-arm time ($^{****}P < 0.0001$) and increased frequency ($^{***}P = 0.0003$) of entering the open arms (**Fig. 1G**). Some rmTBI male and female mice also jumped from the open-arm platform. Statistical analysis showed an effect of sex on open arm time ($^{*}P = 0.0366$), where female mice, independent of injury, spent more time in the open arms than male mice. Finally, mice underwent a forced swim test at 9 weeks postsurgery. Consistent with increased locomotion in the open-field test, rmTBI showed reduced immobility in this test for both sexes ($^{****}P < 0.0001$).

3.2. Effects of repeated mild traumatic brain injury on markers of neuroinflammation

Two brain regions important for affective dimensions of the pain experience were chosen for analysis: the anterior cingulate cortex (ACC) and the nucleus accumbens (NAc). RNAScope multiplex fluorescent in situ hybridization of the ACC revealed that *itgam* (mRNA for Iba-1 protein found on microglia) and *bdnf* (mRNA for brain-derived neurotropic factor) were both significantly upregulated in rmTBI compared with sham male mice (**Figs. 2A–D**). Similar results were found with quantitative RT-PCR of brain tissue from the ACC. There was an increase in the relative expression of Iba-1 (all Iba-1 protein in microglia) and CD11b (cell surface Iba-1 protein on microglia) and an increase in *bdnf* (**Fig. 2E**). A summary of statistical analysis is presented in **Table 2** for data presented in **Figure 2**.

RNAScope multiplex fluorescent in situ hybridization of the NAc revealed that both *itgam* and *bdnf* were significantly upregulated in rmTBI compared with sham mice (**Figs. 3A–D**). This result was confirmed by quantitative RT-PCR of brain tissue from the NAc. There was an increase in the relative expression of Iba-1, CD11b, and BDNF (**Fig. 3E**). A summary of statistical analysis is presented in **Table 2** for data presented in **Figure 3**. Together, these data demonstrate changes in mRNA transcripts of microglial proteins and BDNF that are known to be upregulated in neuroinflammation.

3.3. Inhibiting microglial activation recovered sensory and affective-like behaviors in a sex-dependent manner

To determine the extent to which neuroinflammation contributed to the sensory and affective behavioral changes induced by rmTBI, we treated mice with vehicle (autoclaved water) or minocycline (0.544 mg/mL) administered in their drinking water. We were specifically interested in the extent that inhibiting neuroinflammation would recover this behavior rather than prevent it, therefore treatment commenced 5 weeks after injury or sham surgery (**Fig. 4A**). At 7 weeks postsurgery and after 2 weeks of vehicle treatment, both male and female mice

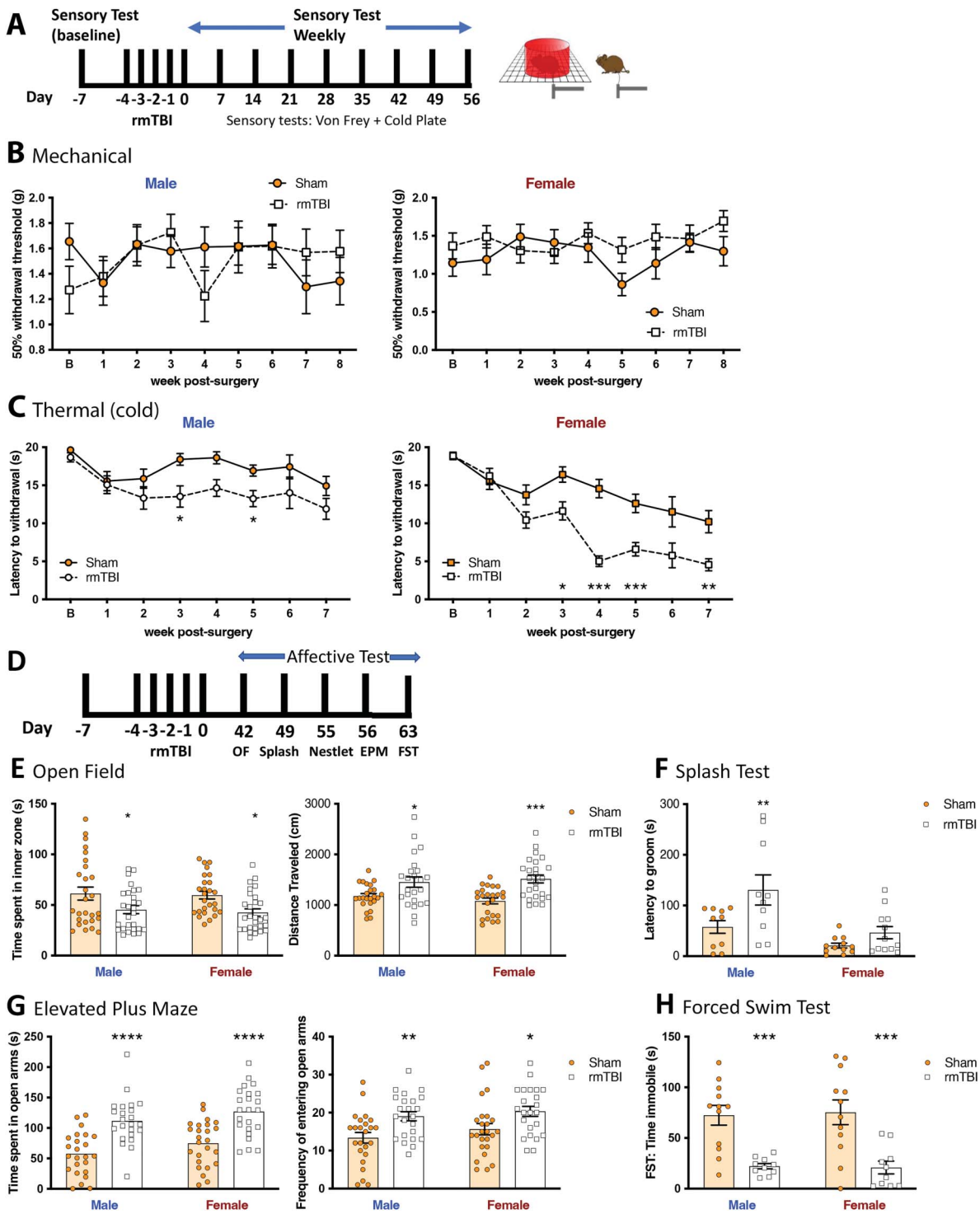

Figure 1. Repeated mild traumatic brain injury (rmTBI)–induced cold pain hyperalgesia and negative affective-like behavior. (A) Time course of sensory threshold testing following sham or rmTBI for 8 weeks. (B) Neither male or female mice developed mechanical sensitivity to hind paw stimulation with Von Frey monofilaments in rmTBI and sham mice. Statistics are presented in Table 1. (C) Cold thermal thresholds developed in both male and female rmTBI when compared with thresholds in sham animals. There was an effect of time and injury in both sexes. $^{****}P < 0.0001$, and an interaction was identified in female ($^{****}P < 0.0001$) but not in male ($P = 0.4411$) mice. N = 22 per group for male mice, N = 25 female sham, and N = 26 rmTBI female. (D) Time course of affective-like behavior testing following sham or rmTBI that began 6 weeks postinjury with open-field testing (OF), splash test on week 7, nestlet and elevated plus maze (EPM) test on week 8, conducted 1 day apart, and forced swim test (FST) on week 9 post injury. (E) rmTBI male ($^{***}P = 0.0005$) and female ($^{****}P < 0.0001$) mice showed reduced time in the inner 30% of the open field when subjected to the novel environment yet demonstrated an increase in locomotion as evidenced by an increase in distance traveled. N = 27 (sham) and 28 (rmTBI) females, and N = 26 (sham) and N= 28 (rmTBI) males per group. (F) rmTBI male but not female mice exhibited an increase in latency to groom in the splash test ($^{***}P = 0.0006$ for sex, $^{**}P = 0.0043$) for injury but no interaction ($P = 0.1521$). N = 10 females per group and N = 12 males per group. (G) rmTBI male and female mice exhibited both an increase in time spent in the open arms ($^{*}P = 0.0366$ for sex and $^{****}P < 0.0001$ for injury) and an increase in frequency of entering the open arms ($^{***}P = 0.0003$ for injury, but no sex effect $P = 0.1939$). N = 24 rmTBI male and N = 24 rmTBI female per group, and N = 25 female sham and N = 25 male sham per group. (H) Time sent immobile was lower in both male and female rmTBI mice compared with shams ($^{****}P < 0.0001$ for injury, $P = 0.9310$ for sex). N = 11 rmTBI male and N = 11 rmTBI female per group, and N = 12 female sham and N = 12 male sham per group. Data are expressed as mean ± SEM for all data sets. All statistics for this figure are presented in Table 1.

Table 1

Statistical analysis of Figure 1.

			F value	*P*
Figure 1B	Male VF	Time	F (4.650, 55.80) = 1.952	0.1050
		Injury	F (1, 12) = 0.4294	0.5246
		Interaction	F (8, 96) = 0.6759	0.7116
	Female VF	Time	F (4.852, 58.22) = 1.056	0.3931
		Injury	F (1, 12) = 1.494	0.2450
		Interaction	F (8, 96) = 1.655	0.1194
Figure 1C	Male cold plate	Time	F (7, 372) = 6.010	<0.0001****
		Injury	F (1, 372) = 25.43	<0.0001****
		Interaction	F (7, 372) = 0.9859	0.4411
	Female cold plate	Time	F (7, 423) = 31.53	<0.0001****
		Injury	F (1, 423) = 59.93	<0.0001****
		Interaction	F (7, 423) = 5.315	<0.0001****
Figure 1E	Open field	Sex	F (1, 105) = 0.2264	0.6352
	% time in inner	Injury	F (1, 105) = 12.99	0.0005***
	Zone	Interaction	F (1, 105) = 0.01918	0.8901
	Distance	Sex	F (1, 93) = 0.05493	0.8152
	Traveled	Injury	F (1, 93) = 22.53	<0.0001****
		Interaction	F (1, 93) = 1.079	0.3017
Figure 1F	Splash test	Sex	F (1, 40) = 13.80	0.0006***
		Injury	F (1, 40) = 9.188	0.0043**
		Interaction	F (1, 40) = 2.131	0.1521
Figure 1G	EPM	Sex	F (1, 91) = 4.500	0.0366*
	Open arm time	Injury	F (1, 91) = 47.04	<0.0001****
		Interaction	F (1, 91) = 0.01527	0.9019
	Frequency of entering open arm	Sex	F (1, 91) = 1.713	0.1939
		Injury	F (1, 91) = 14.48	0.0003***
		Interaction	F (1, 91) = 0.1241	0.7255
Figure 1H	FST	Sex	F (1, 40) = 0.007589	0.9310
		Injury	F (1, 40) = 33.02	<0.0001****
		Interaction	F (1, 40) = 0.05310	0.8189

All data were analyzed by a 2-way analysis of variance. *P* values are provided for each of the factors (time, surgical condition, and if there was an interaction between time and surgical condition) for sensory thresholds and affective-like behaviors.
FST, forced swim test; EPM, elevated plus maze.

developed pain hypersensitivity to cold thermal stimuli, as evidenced by a decrease in withdrawal threshold (**Fig. 4B**), similar to data presented in **Figure 1**. The rmTBI-induced change in cold thermal thresholds were recovered by minocycline treatment in male, but not female, mice, although there was no difference in total liquid consumed (**Fig. 4B**). Statistical analysis of this data set is presented in **Table 3**.

Mice were subjected to affective-like behavioral tests beginning 6 weeks postinjury or sham surgery (**Fig. 4A**). In the open-field test, both male and female rmTBI mice treated with vehicle spent less time in the inner/center compartment compared with the sham cohorts (**Fig. 4C**). Minocycline treatment recovered this anxiogenic-like effect in male but not female mice (**Fig. 4C**). The novelty suppressed feeding test was conducted 7 days after the open-field test. The latency to approach the food and consume it was suppressed in male rmTBI but not in female rmTBI mice when compared with their sham counterparts (**Fig. 4D**). Minocycline treatment recovered this hyponeophasia effect in the male mice (**Fig. 4D**). At 8 weeks postsurgery, mice were subjected to the elevated plus maze. Similar to the previous cohorts (**Fig. 1**), rmTBI increased the time spent in the open arms in both male and female mice, and this effect was not blocked by minocycline treatment (**Fig. 4F**). Repeated mild traumatic brain injury reduced forced swim test immobility time in both male and female mice; minocycline had no effect on this outcome (**Fig. 4E**). Finally, locomotor activity obtained from the open-field test is presented in **Figure 4G**. Repeated mild traumatic brain injury increased locomotor activity (as in the previous cohorts in **Fig. 1**), but again this effect was not blocked by minocycline treatment.

To confirm that the minocycline treatment regimen reduced markers of neuroinflammation, we conducted immunohistochemistry and RNAScope fluorescent in situ hybridization for Iba-1 on brains from rmTBI and sham mice treated with vehicle or minocycline. **Figure 5A** illustrates the extent of upregulation of Iba-1 (brown precipitate) in rmTBI compared with sham at both low

Table 2

Statistical analysis of Figures 2and 3.

Figure 2D	Iba-1	t = 7.408, df = 6	*P* = 0.0003***
	BDNF	t = 18.75, df = 6	*P* < 0.0001****
Figure 2E	Iba-1	t = 3.756, df = 24	*P* = 0.0010***
	CD11b	t = 2.844, df = 25	*P* = 0.008**
	BDNF	t = 2.556, df = 30	*P* = 0.0159*
Figure 3D	Iba-1	t = 7.803, df = 6	*P* = 0.0002***
	BDNF	t = 9.097, df = 6	*P* < 0.0001****
Figure 3E	Iba-1	t = 2.276, df = 25	*P* = 0.0317*
	CD11b	t = 2.395, df = 23	*P* = 0.0251*
	BDNF	t = 3.010, df = 30	*P* = 0.0053**

Data in both figures were from male mice. All data were analyzed by an unpaired student *t* test.
BDNF, brain-derived neurotropic factor.

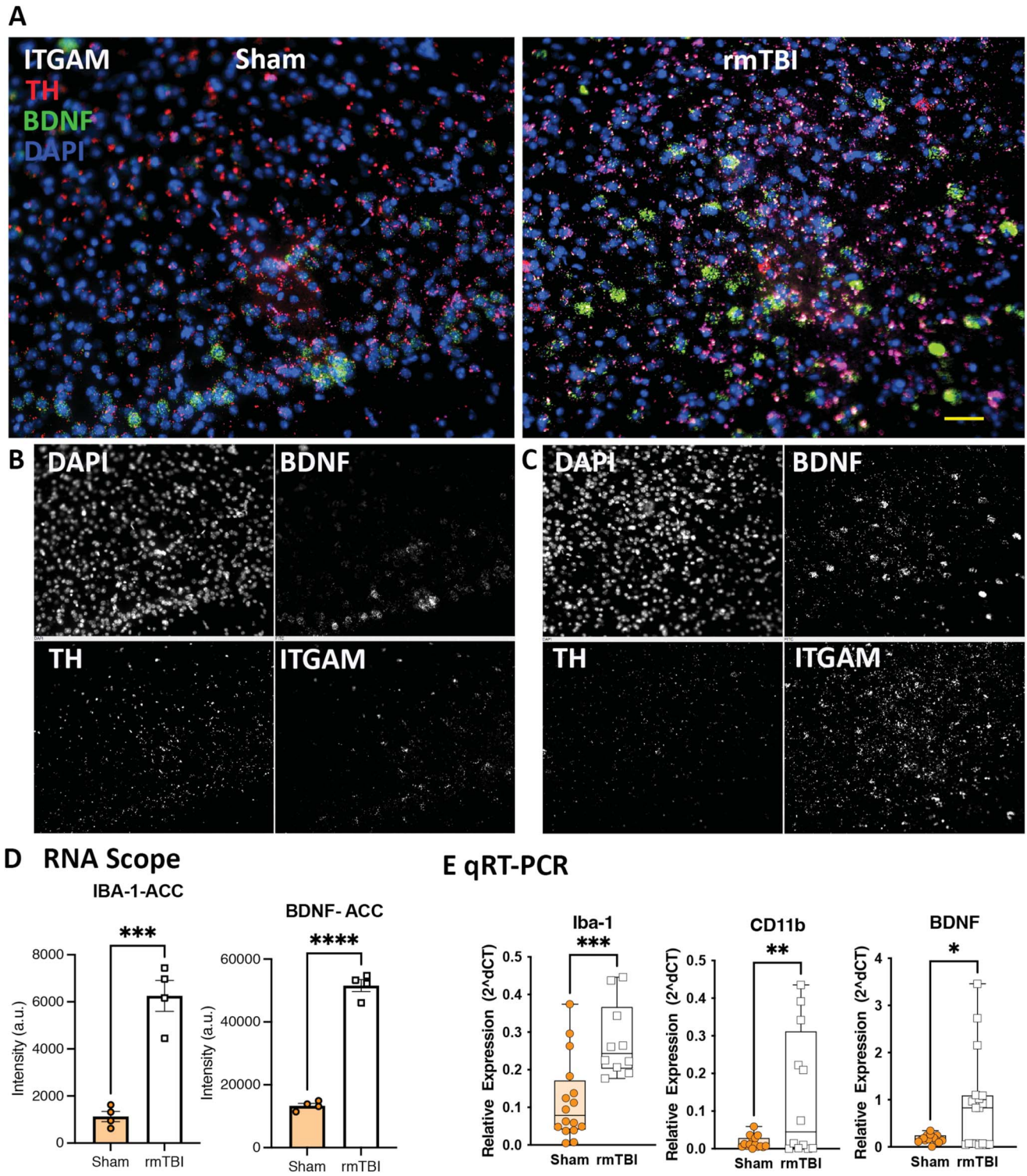

Figure 2. mRNA transcript for microglial protein Iba-1 and brain-derived neurotropic factor (BDNF) is increased in the anterior cingulate cortex (ACC) of rmTBI mice. (A–C) *Itgam* (white) and *bdnf* (green) gene expression detected through RNAScope fluorescent multiplex in situ hybridization was increased in the ACC of male rmTBI mice where tissue was collected 8 weeks postinjury. Dapi is show in blue. (D) Quantification of RNAScope fluorescent intensity shows an increase in Iba-1 (***$P = 0.0003$) and BDNF (****$P < 0.0001$) in rmTBI mice. Data represent N = 4 to 5 per group, with a minimum of 6 sections imaged and analyzed per N. (E) Quantification of mRNA transcript detected by quantitative RT-PCR shows an increase in Iba-1 (***$P = 0.0010$), CD11b (**$P = 0.008$), and BDNF (*$P = 0.0159$) in rmTBI compared with sham mice. Tissue for qRT-PCR was collected 2 weeks postinjury. N = 10 to 16 per group. RNAScope data are presented as mean ± SEM. qRT-PCR data are expressed as box plots with all points reported, and statistics for this figure are presented in Table 2. TH (red) is not highlighted in text here or in results section. Scale bar 50 μm. ACC, anterior cingulate cortex; rmTBI, repeated mild traumatic brain injury; TH, tyrosine hydroxylase.

and higher magnification. Immunofluorescence labeling of Iba-1 (green) was conducted on brain sections to assess neuroinflammation in the NAc. This brain region was chosen because it is important in both sensory and affective dimensions of pain.[22] Four representative images from all the treatment groups are presented. Quantification of the immunofluorescence is presented

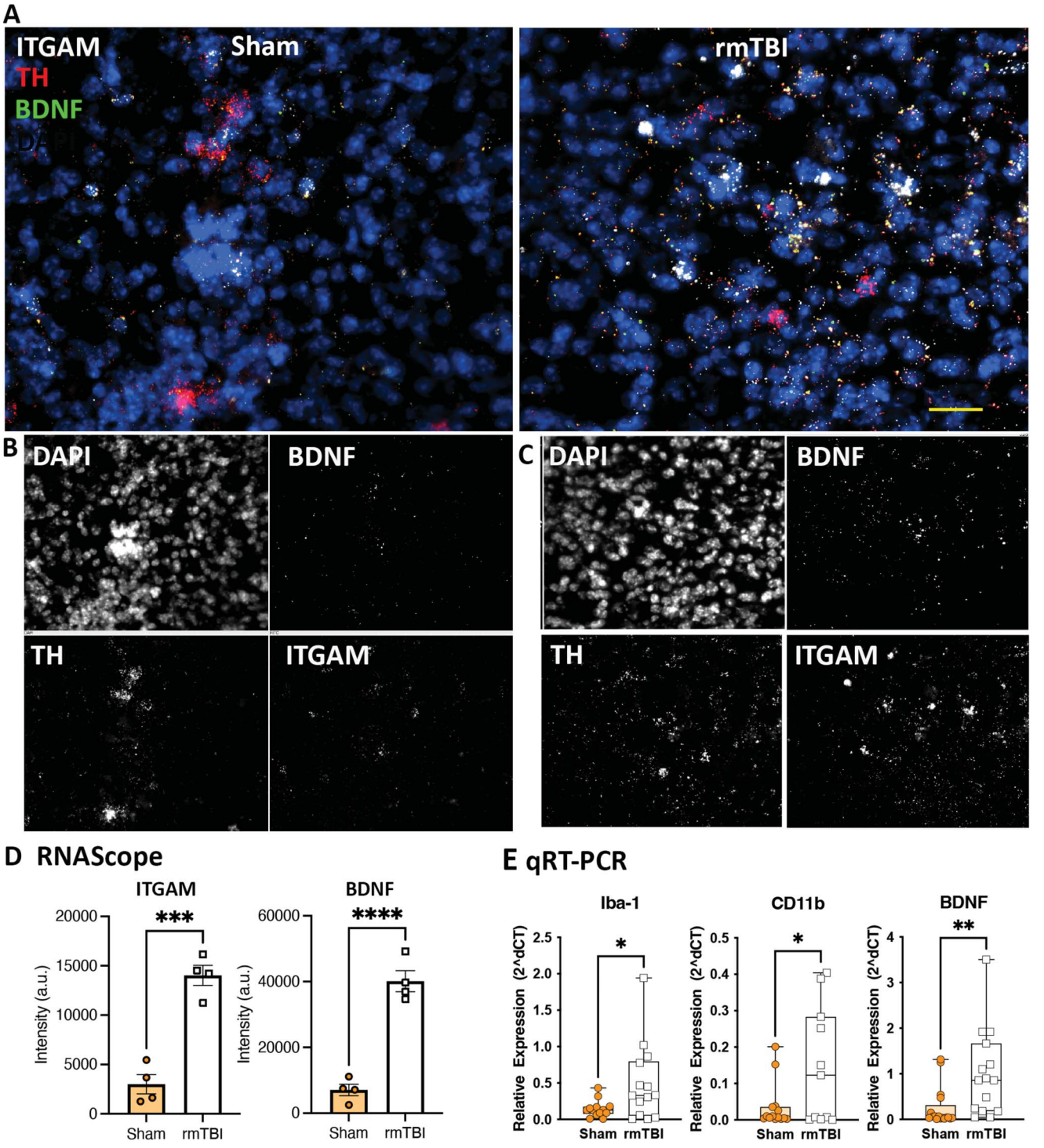

Figure 3. mRNA transcript for microglial protein Iba-1 and brain-derived neurotropic factor (BDNF) is increased in the nucleus accumbens (NAc) of rmTBI mice. (A–C) *Itgam* (white) and *bdnf* (green) gene expression detected through RNAScope fluorescent multiplex in situ hybridization was increased in the NAc of male rmTBI mice, where tissue was collected 8 weeks postinjury. Dapi is show in blue. (D) Quantification of RNAScope fluorescent intensity shows an increase in Iba-1 (***$P = 0.0002$) and BDNF (****$P < 0.0001$) in rmTBI mice. Data represent N = 4 to 5 per group, with a minimum of 6 sections imaged and analyzed per N. (E) Quantification of mRNA transcript detected by quantitative RT-PCR shows an increase in Iba-1 (*$P = 0.0317$), CD11b (*$P = 0.0251$), and BDNF (**$P = 0.0053$) in rmTBI compared with sham mice. Tissue for qRT-PCR was collected 2 weeks postinjury. N = 10 to 16 per group. RNAScope data are presented as mean ± SEM. qRT-PCR data are expressed as box plots with all points reported. Statistics for this figure are presented in Table 2. TH (red) is not highlighted in text here or in results section. Scale bar 50 μm. rmTBI, repeated mild traumatic brain injury; TH, tyrosine hydroxylase.

in **Figure 5B**. rmTBI significantly increased immunofluorescent labeling of Iba-1 compared with sham animals ($F(1, 16) = 12.60$, **$P = 0.0027$), and this increase was reversed by minocycline treatment ($F(1, 16) = 12.60$, $P = 0.4214$). Statistical analysis by 2-way ANOVA also identified an interaction (treatment × surgery, $F(1, 16) = 11.28$, **$P = 0.0040$).

To examine potential sex differences, separate cohorts of male and female sham and rmTBI mice were processed. Male and

Table 3

Statistical analysis of Figure 4.

			F value	*P*
Figure 4B	Male cold plate	Treatment	F (2, 110) = 25.47	<0.0001****
		Injury	F (1, 110) = 13.62	0.0003***
		Interaction	F (2, 110) = 7.544	0.0009***
	Female cold plate	Treatment	F (2, 128) = 45.53	<0.0001****
		Injury	F (1, 128) = 19.73	<0.0001****
		Interaction	F (2, 128) = 2.891	0.0592
Figure 4C	Male open field	Treatment	F (1, 60) = 2.224	0.1411
	% time in inner	Injury	F (1, 60) = 6.291	0.0149*
	Zone	Interaction	F (1, 60) = 2.816	0.0985
	Female open field	Treatment	F (1, 56) = 0.06919	0.7935
	% time in inner	Injury	F (1, 56) = 14.12	0.0004***
	Zone	Interaction	F (1, 56) = 0.08500	0.7717
Figure 4D	Male hyponeophagia	Treatment	F (1, 20) = 3.519	0.0407*
		Injury	F (1, 20) = 6.677	0.0177*
		Interaction	F (1, 20) = 4.735	0.0417*
	Female	Treatment	F (1, 20) = 0.1166	0.7363
	Hyponeophagia	Injury	F (1, 20) = 2.867	0.1059
		Interaction	F (1, 20) = 0.07459	0.7876
Figure 4E	Male FST	Treatment	F (1, 42) = 0.3570	0.5534
		Injury	F (1, 42) = 16.69	0.0002***
		Interaction	F (1, 42) = 0.3310	0.5682
	Female FST	Treatment	F (1, 44) = 0.3852	0.5381
		Injury	F (1, 44) = 14.62	0.0004***
		Interaction	F (1, 44) = 0.0003445	0.9853
Figure 4F	Male EPM	Treatment	F (1, 41) = 2.245	0.1417
		Injury	F (1, 41) = 74.26	<0.0001****
		Interaction	F (1, 41) = 5.650	0.0222*
	Female EPM	Treatment	F (1, 42) = 0.1348	0.7154
		Injury	F (1, 42) = 18.26	0.0001****
		Interaction	F (1, 42) = 0.07144	0.7906
Figure 4G	Male distance traveled	Treatment	F (1, 42) = 0.01098	0.9170
		Injury	F (1, 42) = 20.24	<0.0001****
		Interaction	F (1, 42) = 0.3753	0.5434
	Female distance travelled	Treatment	F (1, 43) = 3.884e-005	0.9951
		Injury	F (1, 43) = 26.09	<0.0001****
		Interaction	F (1, 43) = 0.1213	0.7293

All data were analyzed by a 2-way analysis of variance with treatment (minocycline or vehicle), injury, or interaction of treatment × injury. Sexes were analyzed separately. *P* values are provided for each of the factors (time, surgical condition, and if there was an interaction between time and surgical condition) for sensory thresholds and affective-like behaviors. *Post hoc* analyses were conducted with a Šídák's multiple comparisons test. FST, forced swim test; EPM, elevated plus maze.

female mice exhibited an increase in Iba-1 mRNA in the ACC and NAc (**Fig. 6A**). Statistical analysis of this data set is presented in **Table 4**. Male and female mice also showed an increase in BDNF mRNA in the ACC, but only males showed an increase in the NAc in response to rmTBI (**Fig. 6B**). Minocycline treatment recovered rmTBI-induced increases in Iba-1 and bdnf mRNA transcript in all cohorts without modifying the expression in sham animals (**Figs. 6A and B**).

3.4. Neuroinflammation contributes to the tonic-aversive component of pain induced by repeated mild traumatic brain injury

To provide evidence that neuroinflammation contributed to the tonic-aversive component of chronic pain, we took advantage of a conditioned place preference (CPP) protocol (**Fig. 7A**). Previous research reported that gabapentin analgesic treatment produced a CPP (negative reinforcement) in chronic pain animals but not sham cohorts.[49] Therefore, we asked to what extent gabapentin may produce a CPP in rmTBI mice. Data from these cohorts are expressed as time spent in the drug or vehicle chamber following conditioning to test context-specific learning and memory to assess the effects in a drug-free state (**Figs. 7B and F**) or in the presence of the drug (state dependent) that allows one to test the ability of the animal to learn the contextual cues with drug experience (**Figs. 7C and G**). Data are also presented as transformed CPP scores that provides information on the magnitude of the rewarding effect in both the context (**Figs. 7D and H**) and state dependent (**Figs. 7E and I**) testing—where a negative score means X and a positive score means Y. Gabapentin produced a significant CPP in male rmTBI mice compared with sham controls (**Figs. 7B–E**), demonstrating that this antiepileptic drug used for managing clinical neuropathic pain produced a negative reinforcement effect in rmTBI male mice. Because gabapentin produced a CPP in rmTBI but not in sham animals, we next determined to what extent chronic minocycline treatment may prevent this motivation to seek gabapentin, rationalizing that if minocycline alleviated the affective component of pain, then the animal would no longer have motivation to seek the gabapentin associated chamber. Minocycline significantly attenuated the gabapentin-induced reinforcement effect in

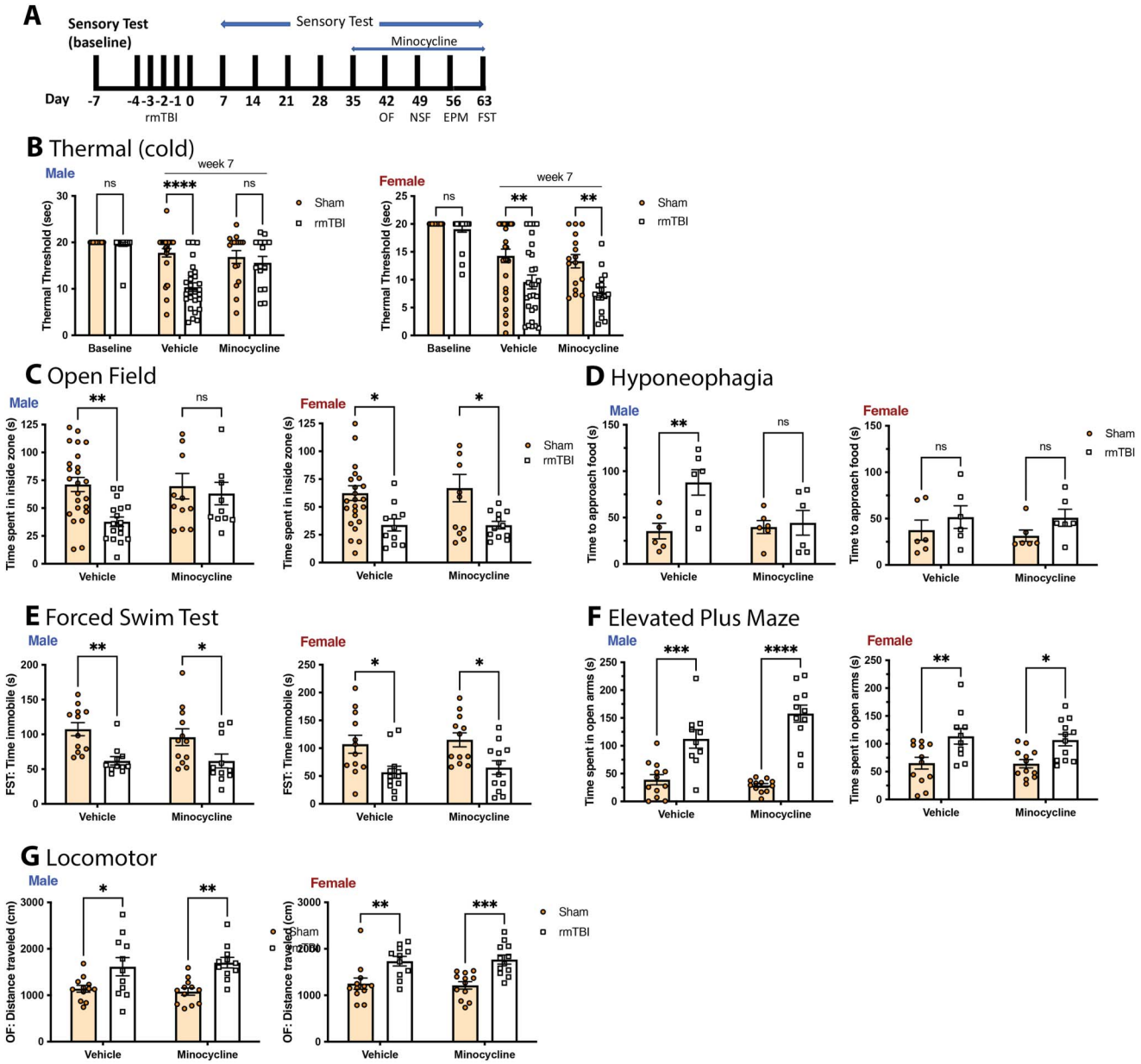

Figure 4. Minocycline treatment recovered cold pain hyperalgesia and negative affective-like behavior in male but not in female mice. (A) Time course of sensory threshold and affective-like behavior testing following sham or repeated mild traumatic brain injury (rmTBI) for 9 weeks, where drug intervention with minocycline (0.544 mg/mL [~6.3 mg/kg/day] in drinking water) began at week 5 post injury. (B) Both male and female mice exhibited cold pain hyperalgesia in response to rmTBI. Minocycline reversed cold hyperalgesia in male (****$P < 0.0001$) but not in female rmTBI mice. N = 24 sham male vehicle treatment per group, N = 16 sham male minocycline treatment per group, N = 26 sham female vehicle treatment per group, N = 20 sham female minocycline treatment per group, N = 27 rmTBI male vehicle treatment per group, N = 16 rmTBI male minocycline treatment per group, N = 26 rmTBI female vehicle treatment per group, and N = 16 rmTBI female minocycline treatment per group. (C) Both male and female mice displayed anxiogenic responses as a result of rmTBI. Minocycline reversed rmTBI effects of anxiogenic-like effects of the open-field test in male but not in female mice. N = 24 sham male vehicle treatment per group, N = 12 sham male minocycline treatment per group, N = 25 sham female vehicle treatment per group, N = 12 sham female minocycline treatment per group, N = 17 rmTBI male vehicle treatment per group, N = 12 rmTBI male minocycline treatment per group, N = 12 rmTBI female vehicle treatment per group, and N = 12 rmTBI female minocycline treatment per group. (D) Only male mice exhibited hyponeophagia as a result of rmTBI. Minocycline reversed rmTBI effects of anxiogenic-like effects of the novelty suppressed feeding test in male. N = 6 per group. (E–G) Both sexes exhibited a decrease in the time spent immobile during the forced swim test. Minocycline had no effect on rmTBI-induced decrease in immobility time in the forced swim test (N = 12 male sham per group, N = 11 male rmTBI per group, N= 12 female sham per group, and N = 12 female rmTBI per group), increased open arm time in the elevated plus maze (N = 12 male sham per group, N = 11 male rmTBI per group, N= 12 female sham per group, and N = 12 female rmTBI per group), or increased locomotor activity in the open-field test (OF) (N = 12 male sham per group, N = 11 male rmTBI per group, N= 12 female sham per group, and N = 12 female rmTBI per group). Data are expressed as mean ± SEM for all data sets. All statistics for this figure are presented in Table 3. FST, forced swim test; EPM, elevated plus maze; OF, open field; rmTBI, repeated mild traumatic brain injury.

rmTBI mice in both the context-dependent and state-dependent tests (**Figs. 7D and E**), suggesting that this effect was mediated by inflammation. By contrast, gabapentin had the opposite effect in female rmTBI mice, where it produced a place aversion rather than preference (**Figs. 7F–I**). Interestingly, this negative effect was also blocked by minocycline treatment in the state-dependent test (**Fig. 7I**). Statistical analysis for data in **Figure 7** is presented in **Table 5**.

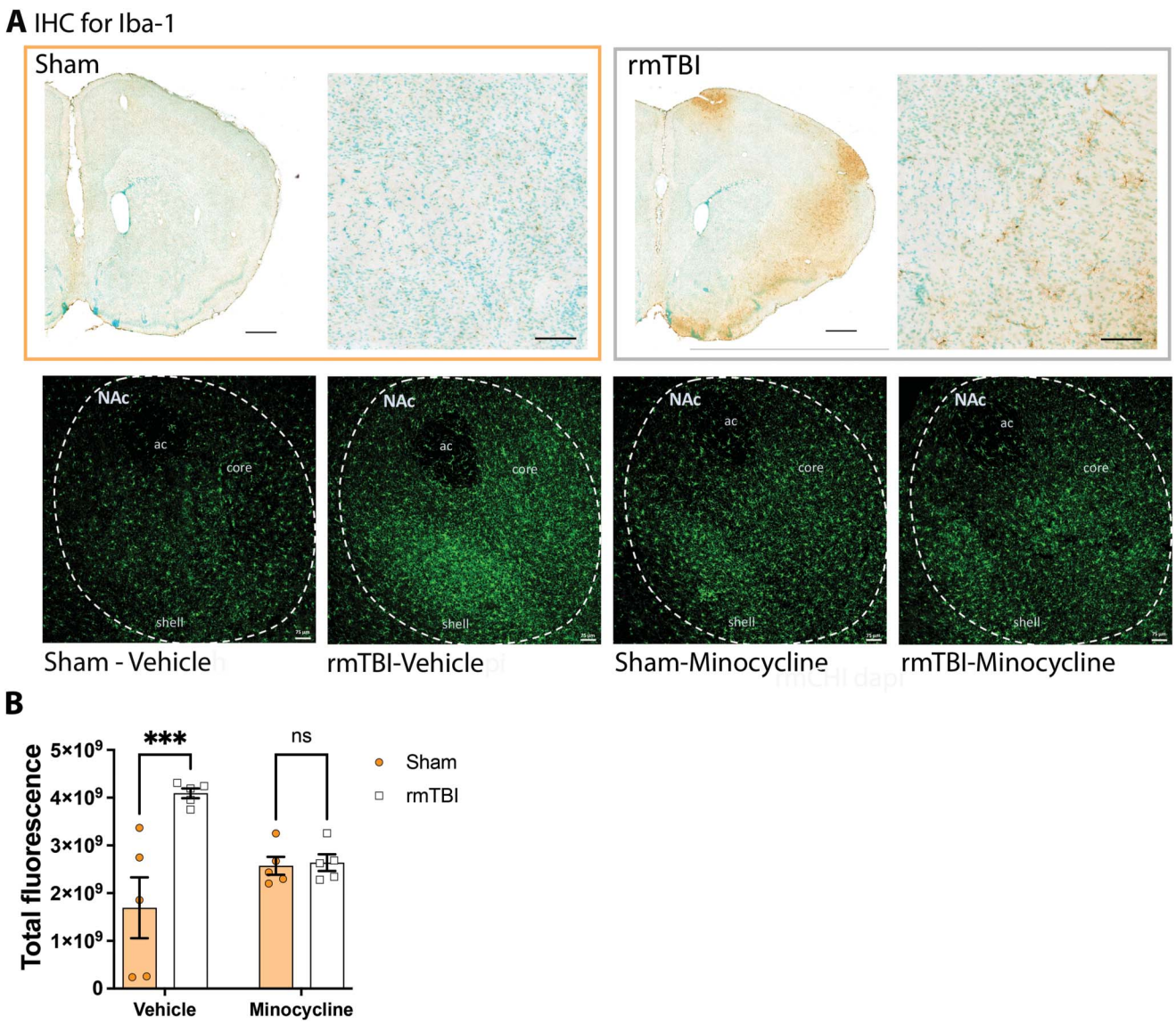

Figure 5. rmTBI-induced neuroinflammation was reversed by oral minocycline treatment. (A) Upper panel. Low magnification images of Iba-1 immunohistochemistry in sham and rmTBI mice, where tissue was collected at 8 weeks postinjury. Higher magnification images of the anterior cingulate cortex are presented as insets. An increase in Iba-1–positive cells is evident in the rmTBI tissue. Lower panel. Green fluorescent images of Iba-1 in the nucleus accumbens (NAc) of sham and rmTBI mice treated with vehicle or minocycline (0.544 mg/mL [~6.3 mg/kg/day] in drinking water) beginning 5 weeks postinjury. There is an increase in Iba-1–positive cells in response to rmTBI and an apparent decrease in Iba-1 in response to minocycline. (B) Quantification of immunohistochemistry fluorescent intensity of Iba-1 immunolabeling in sham and rmTBI male mice. rmTBI-induced increase in Iba-1 immunolabeling was recovered by minocycline treatment (***$P = 0.0$X, Student *t*-test or was this ANOVA). Data are expressed as mean ± SEM for N = 5 per group, where a minimum of 6 sections were imaged and analyzed per N. Scale bar for low magnification images is 1 mm. Scale bar for higher magnification in upper panels is 50 μm. Scale bar on lower panels is 75 μm. ANOVA, analysis of variance; rmTBI, repeated mild traumatic brain injury.

4. Discussion

We used a murine model of repeated mild TBI (rmTBI) to characterize the emergence of pain hypersensitivity and affective-like behavioral changes that occur in response to injury. In addition, we characterized to what extent microglial reactivity contributes to these behavioral phenotypes. There are extensive interactions between systems involved in pain processing and other affective and motivational systems. Our rmTBI model produced cold but not mechanical pain hypersensitivity, as early as 3 weeks postinjury in males and females, as well as an affective dimension of chronic pain that was revealed by the presence of gabapentin CPP in male rmTBI mice, a first-line therapy for chronic noncancer pain.[17] Repeated mild traumatic brain injury also resulted in increased anxiolytic and depressive behaviors, measured by the open-field, splash, and novelty suppressed feeding tests, but not forced swim test or nestlet test. The elevated plus maze revealed risk-taking behavior in both sexes.

Our findings demonstrate that chronic pain and negative affect are, in part, mediated by neuroinflammation; microglial activation in mesolimbic circuitry plays an important role in these sensory and affective phenotypes. Indeed, it is established that TBI results in upregulated neuroinflammatory markers and related behaviors that minocycline can reverse.[2,8,10,31,32,36,65,76] In our study, rmTBI-induced cold hyperalgesia developed over time and was reversed by treatment with minocycline—where drug intervention was initiated after the establishment of neuroinflammation and nociceptive changes. Similarly, rmTBI-induced affective-like behaviors indicative of anxiety and depression developed over

Table 4

Statistical analysis of Figure 6.

			F value	P
Figure 6A Iba-1	Male ACC	Treatment	F (1, 20) = 8.250	0.0094**
		Injury	F (1, 20) = 22.27	0.0001****
		Interaction	F (1, 20) = 14.90	0.0010***
	Female ACC	Treatment	F (1, 20) = 2.925	0.1027
		Injury	F (1, 20) = 11.84	0.0026**
		Interaction	F (1, 20) = 2.654	0.1189
	Male NAc	Treatment	F (1, 20) = 6.310	0.0207*
		Injury	F (1, 20) = 38.40	<0.0001****
		Interaction	F (1, 20) = 24.55	<0.0001****
	Female NAc	Treatment	F (1, 20) = 34.04	<0.0001****
		Injury	F (1, 20) = 48.62	<0.0001****
		Interaction	F (1, 20) = 68.27	<0.0001****
Figure 6B BDNF	Male ACC	Treatment	F (1, 20) = 15.92	0.0007***
		Injury	F (1, 20) = 54.79	<0.0001****
		Interaction	F (1, 20) = 17.83	0.0004***
	Female ACC	Treatment	F (1, 20) = 0.5271	0.4762
		Injury	F (1, 20) = 17.24	0.0005***
		Interaction	F (1, 20) = 0.3206	0.5775
	Male NAc	Treatment	F (1, 20) = 23.77	<0.0001****
		Injury	F (1, 20) = 30.06	<0.0001****
		Interaction	F (1, 20) = 31.56	<0.0001****
	Female NAc	Treatment	F (1, 20) = 20.36	0.0002***
		Injury	F (1, 20) = 2.512	0.1287
		Interaction	F (1, 20) = 4.139	0.0554

All raw data were analyzed by a 2-way analysis of variance with treatment (minocycline or vehicle), injury, or interaction of treatment × injury. Sexes were analyzed separately. *P* values are provided for each of the factors (treatment, surgical condition, and if there was an interaction between treatment and surgical condition). *Post hoc* analyses were conducted with a Šídák's multiple comparisons test.
ACC, anterior cingulate cortex; BDNF, brain-derived neurotropic factor; NAc, nucleus accumbens.

the course of weeks that was also reversed by the administration of minocycline. Previous work has shown that minocycline treatment reduces TBI-induced neuroinflammation, blocks ethanol-induced microglial activation in the NAc, prevents the development of TBI-induced spatial memory impairments, and reduces anxiety.[8,31,36,65] We confirmed the effectiveness of minocycline treatment the by assessment of inflammatory molecular markers. It should be noted that minocycline has many effects in addition to inhibiting microglia, including antioxidant, antiapoptotic neuroprotective effects and other immunomodulatory actions.[66] Importantly, minocycline reduced the rmTBI-induced upregulation of Iba-1 protein in microglia and reduced mRNA transcripts for various neuroinflammatory markers. However, a limitation of this study is that although minocycline recovered behavioral phenotypes and the molecular changes induced by rmTBI in these groups, the molecular changes noted remain correlative with behavioral outcomes.

We focused our efforts on understanding to what extent rmTBI induced neuroinflammation in 2 limbic brain structures known to be involved in the affective emotional component of pain: NAc and ACC. The NAc integrates stress and reward systems to produce drug withdrawal–induced negative affective states,[34] promotes negative reinforcement,[35,71] and contributes to the affective emotional component of pain.[40,48,70] We show upregulation of Iba-1 immunolabeling and its transcript as well as CD11b transcript as measured by RNAScope and/or quantitative RT-PCR in NAc of rmTBI male and female mice. This upregulation of microglia was reversed by minocycline. A recent study reported that inflammatory pain modified microglia-derived cytokines in the NAc.[11] Moreover, an upregulation of the chemokine CCL2 and its major receptor CCR2 was shown in both dopamine D1 and D2 receptor (D1R and D2R)–containing neurons in the NAc of mice with neuropathic pain, where inhibiting CCR2 *through* lentivirus attenuated pain hypersensitivity and depressive behaviors.[75] The clinical evidence that neuroinflammation in this brain region can drive chronic pain states is supported by PET imaging, where patients with complex regional pain syndrome showed a higher distribution volume ratio of [C]-(R)-PK11195, a marker of neuroinflammation, compared with control subjects.[28] PET imaging of athletes with concussion also showed an increase in neuroinflammation in the striatum, where microglial activation persisted beyond clinical recovery.[50] Our study is consistent with these previous studies, but to our knowledge, this is the first report that rmTBI in adult mice induces neuroinflammation in the NAc that likely contributes to the negative affective state and pain hypersensitivity.

The ACC is another area with an important role in the affective component of pain. Lesions of the ACC eliminate the aversiveness of neuropathic pain,[29,58,59] and ACC lesions or deep brain stimulation performed in patients improve intractable chronic pain.[5,54] We identified increases in neuroinflammatory markers in the ACC of rmTBI mice, consistent with previous reports that mild TBI patients exhibit inflammation-induced structural changes in the default mode network, which includes the ACC.[52] It is unclear from this study to what extent neuroinflammation in the ACC and NAc contribute to these behavioral phenotypes, but inhibition of neuroinflammation recovered both sensory and affective dimensions of the pain experience as evidenced by the recovery of cold pain hypersensitivity in male rmTBI mice and the elimination of CPP to gabapentin in both sexes.[4] Although gabapentin had no effect in sham animals, it produced opposite effects in male and female rmTBI mice, where gabapentin produced a place preference in the male mice but an aversion in the female mice. Previous studies with chronic neuropathic pain also reported gabapentin-induced CPP in male rodents,[49] but to our knowledge, no study has examined its effects in female rodents. This was consistently seen in successive replicates, where replications were balanced with respect to experimental groups to obtain the appropriate N for statistical power. This is a curious finding given that gabapentin attenuates pain hypersensitivity in both male and female mice in models of chronic neuropathic pain.[49,74]

A behavioral phenotype identified in both male and female rmTBI mice was hyperlocomotion. Increased locomotor activity was identified in an open-field test, which likely underlies the increased swim time in the forced swim test. Neither of these effects were blocked by oral minocycline treatment and are most likely not maintained through neuroinflammatory mechanisms, although we cannot rule out the potential of neuroinflammation initiating changes in neural circuit activity that accounts for this behavior because drug intervention was not initiated until 5 weeks post-rmTBI. Others reported prolonged hyperlocomotion in a lateral fluid percussion and repeated mild closed head models of TBI[3,37] and is reminiscent of the hyperactivity in a mouse model of Alzheimer disease, which was attributed to behavioral disinhibition.[16] Disinhibition or impulsive behavior was previously reported in a closed-head TBI model where mice increased entries in the elevated plus maze.[45,55] In our study, we also identified increased open-arm time and open-arm entries where some mice jumped off the open arms, which may be because of increased risk taking, reduced fear avoidance, or changes in impulsivity. Risk-taking behavior and impairment in judgment are common

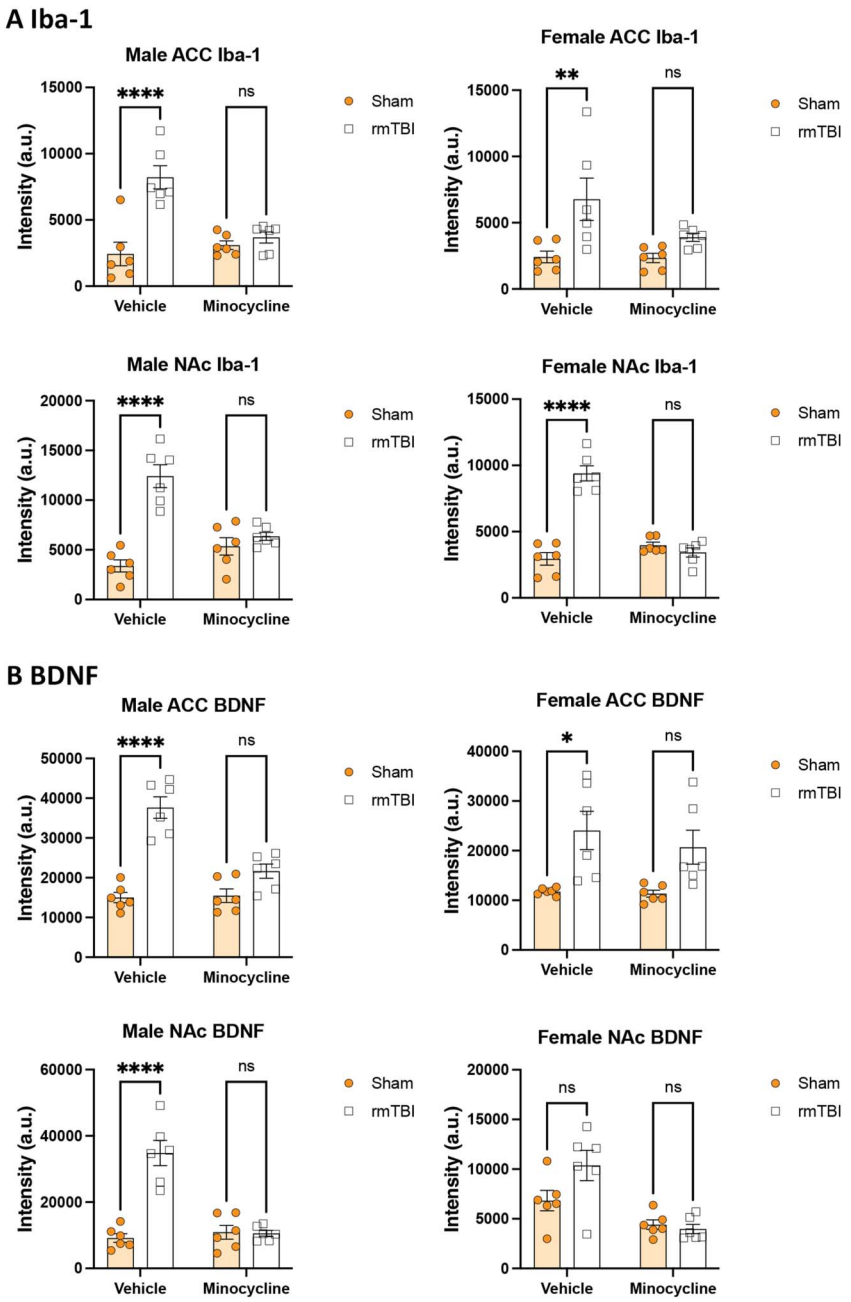

Figure 6. rmTBI-induced neuroinflammation was reversed by oral minocycline treatment. (A) Quantification of RNAScope multiplex fluorescent in situ hybridization of male and female rmTBI and sham mice treated with vehicle or minocycline (0.544 mg/mL [~6.3 mg/kg/day] in drinking water). Intensity of Iba-1 transcript was increased in the ACC of male and female rmTBI mice (****$P < 0.0001$, **$P < 0.01$). This increase in rmTBI mice was recovered by minocycline treatment. Intensity of Iba-1 transcript was increased in the NAc of male and female rmTBI mice (*$P < 0.05$; ****$P < 0.0001$), and minocycline treatment reversed this increase in both sexes. Tissue was collected at 8 weeks postinjury. (B) Quantification of RNAScope multiplex fluorescent in situ hybridization of male and female rmTBI and sham mice treated with vehicle or minocycline (0.544 mg/mL [~6.3 mg/kg/day] in drinking water). Tissue was collected at 8 weeks postinjury. Intensity of BDNF transcript was increased in the ACC and NAc of male and female rmTBI mice. This increase in rmTBI mice was reversed by minocycline treatment. Intensity of BDNF transcript was increased in the NAc of male but not female rmTBI mice (****$P < 0.0001$); minocycline treatment reversed this increase in the male mice. Data are expressed as mean ± SEM for N = 6 per group, where a minimum of 6 sections were imaged and analyzed per N. Statistics for data presented in this figure are found in Table 4. ACC, anterior cingulate cortex; BDNF, brain-derived neurotropic factor; NAc, nucleus accumbens; rmTBI, repeated mild traumatic brain injury.

behavioral abnormalities in TBI patients diagnosed with chronic traumatic encephalopathy.[15,43]

Epidemiological evidence indicates that TBI occurs at a greater incidence in male mice compared with female mice.[21] We observed sex differences in affective and thermal behavioral data, particularly in the efficacy of minocycline for reversing rmTBI-induced changes. Male and female mice had a reduced threshold to cold stimuli, decreased time spent in the anxiogenic center zone of the open-field test, and increased latency to approach food in the anxiogenic zone of the novelty suppressed feeding test at 7 weeks post-rmTBI. In male mice, but not female mice, minocycline reversed reduced cold thermal thresholds and reversed the increased anxiety-like behavior produced by rmTBI. This is consistent with the reports that minocycline attenuated

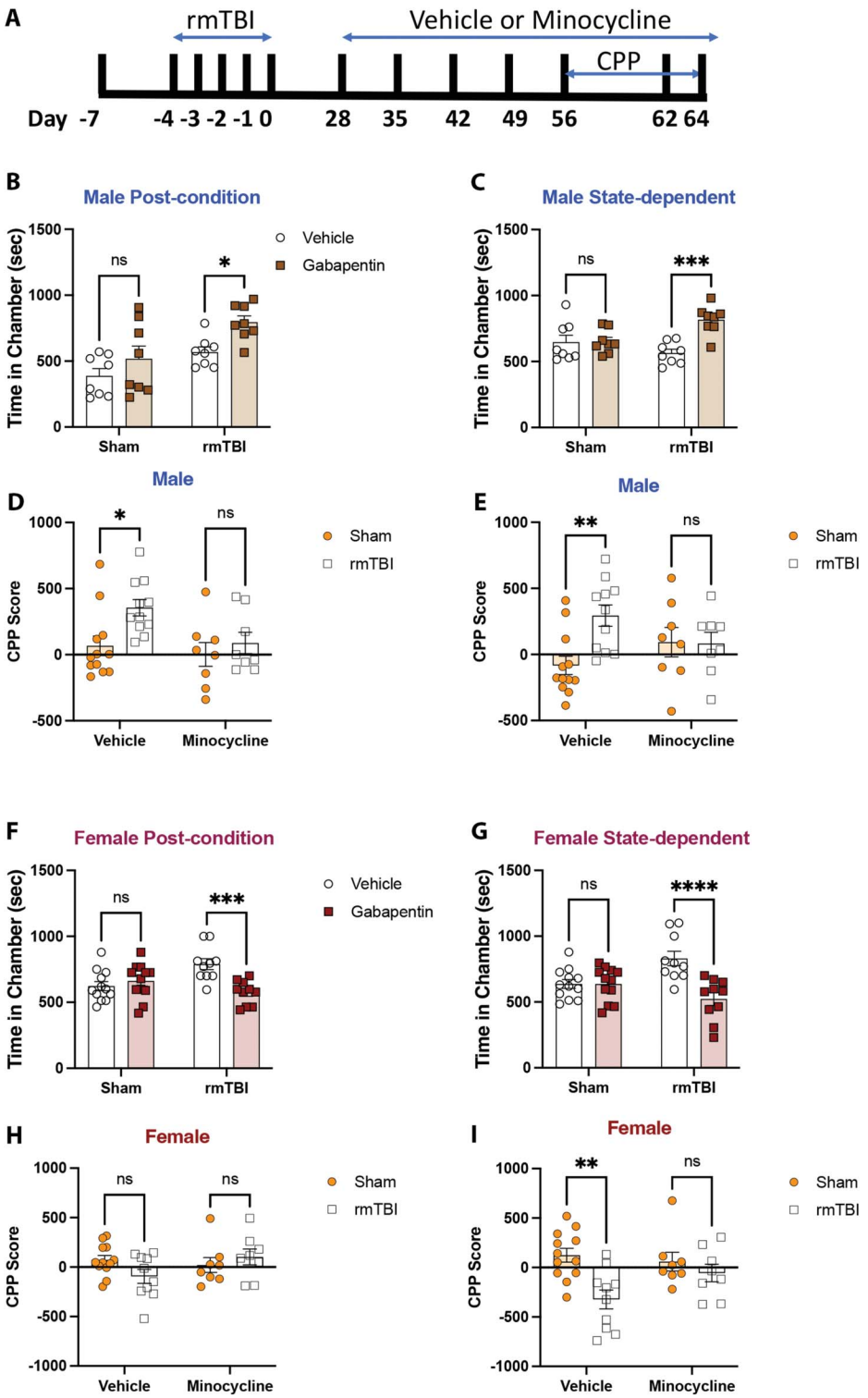

Figure 7. Gabapentin induces place preference in male and place aversion in female rmTBI mice. Minocycline treatment prevented both the preference and aversive effects. (A) Time course of injury, treatment, and conditioned place preference testing. (B and C) Gabapentin (100 mg/kg) induced a place preference in male rmTBI but not sham mice as evidenced by context-dependent and state-dependent testing. N = 8 per group. (D and E) Data are expressed as a conditioned place preference score for context-dependent and state-dependent testing in both vehicle and minocycline treated male mice. Gabapentin-induced place preference was absent in rmTBI mice treated with minocycline. Minocycline had no effect on the lack of preference in sham animals. N = 12 vehicle treatment per group and N = 8 minocycline treatment per group. (F and G) Gabapentin (100 mg/kg) induced a place aversion in female rmTBI but not sham mice as evidenced by context-dependent and state-dependent testing. N = 12 per group. (H and I) Data are expressed as a conditioned place preference score for context-dependent and state-dependent testing in both vehicle and minocycline treated female mice. Gabapentin-induced place aversion was absent in rmTBI mice treated with minocycline. Minocycline had no effect on the lack of preference in sham animals. N = 12 vehicle treatment per group and N = 8 minocycline treatment per group. All data are expressed as mean ± SEM. Statistics for data in this figure are presented in Table 5. CPP, conditioned place preference; rmTBI, repeated mild traumatic brain injury.

Table 5

Statistical analysis of Figure 7.

			F value	*P*
Figure 7B	Male post condition	Treatment	F (1, 28) = 7.908	0.0089**
		Injury	F (1, 28) = 13.00	0.0012**
		Interaction	F (1, 28) = 0.5824	0.4517
Figure 7C	Male state dependent	Treatment	F (1, 28) = 10.78	0.0028**
		Injury	F (1, 28) = 1.152	0.2924
		Interaction	F (1, 28) = 10.06	0.0037**
Figure 7D	Male CPP score context dependent	Treatment	F (1, 35) = 4.626	0.0385*
		Injury	F (1, 35) = 5.908	0.0203*
		Interaction	F (1, 35) = 1.717	0.1986
Figure 7E	Male CPP score state dependent	Treatment	F (1, 35) = 4.517	0.0407*
		Injury	F (1, 35) = 0.04295	0.8370
		Interaction	F (1, 35) = 5.012	0.0316*
Figure 7F	Female post condition	Treatment	F (1, 40) = 5.841	0.0203*
		Injury	F (1, 40) = 1.205	0.2788
		Interaction	F (1, 40) = 12.25	0.0012**
Figure 7G	Female state dependent	Treatment	F (1, 40) = 12.50	0.0010***
		Injury	F (1, 40) = 0.8303	0.3677
		Interaction	F (1, 40) = 12.49	0.0010***
Figure 7H	Female CPP score context dependent	Treatment	F (1, 34) = 1.190	0.2831
		Injury	F (1, 34) = 0.4155	0.5235
		Interaction	F (1, 34) = 3.512	0.0695
Figure 7I	Female CPP score state dependent	Treatment	F (1, 34) = 1.321	0.2585
		Injury	F (1, 34) = 10.23	0.0030**
		Interaction	F (1, 34) = 3.568	0.0675

All raw data were analyzed by a 2-way analysis of variance. Panels B, C, F, and G were analyzed as treatment (gabapentin), injury, or treatment × injury. Panels D, E, H, and I were analyzed by treatment (minocycline), injury or treatment × injury. *P* values are provided for each of the factors (treatment, surgical condition, and if there was an interaction between treatment and surgical condition). *Post hoc* analyses were conducted with a Šídák's multiple comparisons test.

CPP, conditioned place preference.

pain hypersensitivity associated with chronic neuropathic pain in male but not in female mice.[38,39,67] Despite the differences in behavioral effects of minocycline treatment, the upregulation of bdnf and Iba-1 in rmTBI mice, both sexes showed recovery to sham levels with minocycline treatment. Our findings also highlight the emergence of sex differences in microglial responses induced by TBI, which is important for the clinical translation of minocycline and other microglia-targeting therapeutics. Others have also shown that microglial responses differ in male and female mice. From preclinical evidence, male and female mice have differences in the quantity and phenotype specificity of microglial responses during early postnatal stages and into adulthood (for review, see Bordt et al.[6]). For example, Sorge et al.[67] showed that microglia are not sufficient for pain hypersensitivity in female mice, whereas they are in male mice. Moreover, there is evidence that sex hormones, eg, estrogen and progesterone, are protective post-TBI due to their role in reducing inflammation.[7] Our study, in addition to others, highlights the need to integrate female and male animals into TBI and chronic pain studies to better elucidate the mechanism(s) that drive these sex differences.

Clinical experiments support the translation of preclinical work, showing that minocycline can reduce TBI neuroinflammation and associated impairments. In a clinical setting measuring neuroinflammation 6 months after TBI, minocycline administered orally twice daily at 100 mg for 12 weeks decreased neuroinflammation measured by 11C-PBR28 binding but increased a marker of neurodegeneration (plasma neurofilament light chain)[62]; sample size and homogeneity (mostly male 81%-82%) prevented exploration of sex differences. Importantly, microglia plays a role in neuronal repair after injury, and decreasing neuroinflammation through microglial activation may impede repair. By contrast, a preclinical model of male rats with TBI and sepsis show that minocycline (45 mg/kg i.p.) for 3 days reduced not only microglial activation but also hippocampal CA3 cell death and lesion volume.[1] In a sample of mostly male (80%) patients with Glasgow coma scores between 3 and 12, the highest dose of minocycline (initially 800 mg and maintained at 400 mg for 7 days) showed trending improvement of Disability Rating Scale scores.[44] More research in the clinical setting is necessary to further understand neuronal and microglial effects of minocycline in these samples and at different time points post injury, as well as sex differences and accompanying biological contributors.

In conclusion, rmTBI results in phenotypes of pain hypersensitivity and depressive and anxiogenic behaviors that are sex-dependently reversed by microglial suppression. In our rmTBI model before and after minocycline treatment, sex differences were observed in behavioral and molecular markers of inflammation, supporting sex differences in injury-induced pain behaviors. Female rmTBI mice did not exhibit elevated BDNF levels in the NAc or ACC, or conditioned place preference for gabapentin, whereas male mice showed upregulation of Iba-1 in the ACC and gabapentin CPP, which were reversed by minocycline. Our study also reports that rmTBI female mice have an aversion to gabapentin. To our knowledge, no study has examined sex differences in gabapentin, which is problematic given that chronic pain has a higher prevalence in females than males. Future studies should investigate the mechanism of these sex differences. Our study supports the involvement of

mesolimbic structures in chronic pain states associated with TBI and highlights that the neural mechanisms should be further investigated.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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The data that support the findings of this study are available on request from the corresponding author, [C.M.C.].

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