

RESEARCH ARTICLE

Asymmetric activation of microglia in the hippocampus drives anxiodepressive consequences of trigeminal neuralgia in rodents

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Background and Purpose: Patients suffering from trigeminal neuralgia are often accompanied by anxiety and depression. Microglia-mediated neuroinflammation is involved in the development of neuropathic pain and anxiodepression pathogenesis. Whether and how microglia are involved in trigeminal neuralgia-induced anxiodepression remains unclear.

Experimental Approach: Unilateral constriction of the infraorbital nerve (CION) was performed to establish trigeminal neuralgia in rat and mouse models. Mechanical allodynia and anxiodepressive-like behaviours were measured. Optogenetic and pharmacological manipulations were employed to investigate the role of hippocampal microglia in anxiety and depression caused by trigeminal neuralgia.

Key Results: Trigeminal neuralgia activated ipsilateral but not contralateral hippocampal microglia, up-regulated ipsilateral hippocampal ATP and interleukin-1 β (IL-1 β) levels, impaired ipsilateral hippocampal long-term potentiation (LTP) and induced anxiodepressive-like behaviours in a time-dependent manner in rodents. Pharmacological or optogenetic inhibition of ipsilateral hippocampal microglia completely blocked trigeminal neuralgia-induced anxiodepressive-like behaviours. Activation of unilateral hippocampal microglia directly elicited an anxiodepressive state and impaired hippocampal LTP. Knockdown of ipsilateral hippocampal P2X7 receptors prevented trigeminal neuralgia-induced microglial activation and anxiodepressive-like behaviours. Furthermore, we demonstrated that microglia-derived IL-1 β mediated microglial activation-induced anxiodepressive-like behaviours and LTP impairment.

Conclusion and Implications: These findings suggest that priming of microglia with ATP/P2X7 receptors in the ipsilateral hippocampus drives pain-related anxiodepressive-like behaviours via IL-1 β . An asymmetric role of the bilateral hippocampus in trigeminal neuralgia-induced anxiety and depression was uncovered. The approaches targeting microglia and P2X7 signalling might offer novel therapies for trigeminal neuralgia-related anxiety and depressive disorder.

Abbreviations: 1-MT, 1-methyl-D-tryptophan; ChR2, channelrhodopsin-2; CION, constriction of the infraorbital nerve; fEPSPs, field excitatory postsynaptic potentials; IBA-1, ionized calcium-binding adapter molecule 1 or allograft inflammatory factor 1 (AIF-1); Mac-1-SAP, macrophage antigen complex-1-saporin.

KEYWORDS

anxiodepression, hippocampus, long-term potentiation (LTP), microglial activation, trigeminal neuralgia

1 | INTRODUCTION

Clinically, depressions are frequently observed in patients suffering from chronic pain, with a prevalence rate of 30% in neuropathic pain patients (Stubbs et al., 2017). This consequence of neuropathic pain can be preclinically modelled (Alba-Delgado et al., 2013; Humo et al., 2019; Yalcin et al., 2011, 2014). Patients with chronic pain and depression are poorly responsive to current antidepressant and analgesic treatment, which greatly increases the patients' burden on healthcare services (Rayner et al., 2016). Although the co-existence of chronic pain and depression has long been recognized clinically, mechanism-based preclinical studies are still rare.

In the past decade, accumulating evidence has shown that microglia-mediated neuroinflammation is involved in the development of affective disorders including pathogenesis of anxiety and depression (Cao et al., 2021; Kimura et al., 2021; Klawonn et al., 2021). As the primary immune cells of the central nervous system, microglia can be activated by various extracellular stimuli and promote inflammatory responses by expressing and releasing pro-inflammatory cytokines, which as powerful neuromodulators regulate synaptic transmission and plasticity (Chen et al., 2018; Ji et al., 2016). Patients with depression exhibited up-regulated inflammatory cytokines, such as **interleukin-1 β (IL-1 β)** in several brain areas, including the hippocampus (Kovacs et al., 2016; Wager-Smith & Markou, 2011). Imaging and post-mortem analysis of depressive patients, especially suicide patients with depression, showed a significant activation of microglia in several emotion-related brain regions, such as the hippocampus, anterior cingulate cortex and prefrontal cortex (Rajkowska & Miguel-Hidalgo, 2007; Stockmeier et al., 2004). Similar increases in microglia activity in chronic pain patients (Loggia et al., 2015) and animal models (Hisaoka-Nakashima et al., 2019; Zhang et al., 2020) were observed in emotion-related brain regions including the hippocampus, a brain region that is strongly linked with depression and chronic pain (Abdallah & Geha, 2017; Ezzati et al., 2019). The anti-depression effects of microglia activity inhibition were demonstrated in both human and animals (Hinwood et al., 2012; Miyaoka et al., 2012; Peng et al., 2021), suggesting an important role of microglial activation in the depression pathogenesis. However, there are few reports regarding the mechanism of microglia in anxiodepressive consequences of chronic pain.

Here, we will reveal new insights into the role of microglia-to-neurons communication in neuropathic pain-induced depression. We used optogenetic approach in **CX₃CR1::channelrhodopsin-2 (ChR2)** and **CX₃CR1::Arch** mice to investigate the role of microglial activity for anxiodepressive state and long-term potentiation (LTP) of neuronal activity in the hippocampal CA1 area. We found that hippocampal microglial activation is necessary for the anxiety and depression induced by neuropathic pain and that activating microglial

What is already known

- Chronic neuropathic pain often causes anxiety and depression in both clinical and animal models.
- Microglial activation and pro-inflammatory cytokine release are involved in pathogenesis of chronic pain and anxiodepression.

What does this study add

- Bilateral hippocampal microglia play an asymmetric role in trigeminal neuralgia-induced anxiodepression.
- Ipsilateral hippocampal microglia P2X7 receptors triggers IL-1 β release to impair hippocampal LTP driving anxiodepressive-like behaviours.

What is the clinical significance

- Microglia P2X7 receptor signalling is a potential therapeutic target of trigeminal neuralgia-related anxiety and depression.

is sufficient to induce an anxiodepressive state. Moreover, we also show that hippocampal microglia **P2X7 receptor** triggers up-regulation of IL-1 β to impair hippocampal LTP and drive depressive-like behaviours. In addition, a laterality effect has been reported in association of hippocampal volume and function with depression (Videbech & Ravnkilde, 2004) and pain (Ezzati et al., 2014), indicating a specialized role for hippocampal subfields. In this study, we provide very good evidence that ipsilateral but not contralateral hippocampal microglia are involved in depressive consequences of neuropathic pain.

2 | METHODS

2.1 | Animals

Adult male and female Wistar rats (4 rat190–220 g) and male C57BL/6 mice (10–12 weeks) were provided by the Shanghai Experimental

Animal Center of the Chinese Academy of Sciences. Both male and female P2X7 receptor knockout (KO) mice (JAX#005576) and CX₃CR1-CreER-GFP (JAX#021160), Flox (JAX#012735) and Arch-Flox (JAX#012567) mice were used. CX₃CR1-GFP::ChR2 and CX₃CR1-GFP::Arch mice were bred by CX₃CR1-CreER-GFP and ChR2-Flox and Arch-Flox mice, respectively. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020). All animals were housed (2 rats per cage, length × width × height, 45 × 30 × 20, clear plastic cage; 4 mice per cage, length × width × height, 33 × 21 × 15, clear plastic cage) on a 12/12 h light-dark cycle within a colony room at 22°C (humidity ≈ 60%; illumination intensity ≈ 100 lx). Food (purchased from SLACOM Inc. Shanghai, China) and water were provided *ad libitum*. The sexes of transgenic animals were randomly assigned to experimental and control groups. In all the behavioural testing, quantification of immunohistochemistry and western blot, and electrophysiological recording, the investigators were blinded to the treatments. All the animal experiments were approved by the Committee on the Use of Animal Experiments of Fudan University (Permit No. SYXK 2009-0082).

2.2 | Experimental design

The goal of the study was to investigate the roles of hippocampal microglia in the development of anxiety and depression caused by trigeminal neuralgia. We first confirmed the time course of the development of anxiodepressive-like behaviours and activation of hippocampal microglia in trigeminal neuralgia animals. Then, we assessed the hippocampal microglia responsible for anxiety and depression by conducting pharmacological and optogenetic inhibition of ipsilateral, contralateral or bilateral hippocampal microglia. Moreover, we examined the effect of trigeminal neuralgia and microglial activation on hippocampal LTP. Finally, using gene KO/knockdown and pharmacological methods, we examined the role of the P2X7 receptor/IL-1 β signalling in the development of anxiety and depression caused by trigeminal neuralgia. Animals were randomly assigned into different cages and groups and tested by an investigator blinded to rat treatments, mouse genotypes/treatments and group assignments. The sample sizes were at least five. All experiments have been replicated successfully and all data were included in analysis. All the experimental protocol and group sizes are shown in the figures or figure legends.

2.3 | Trigeminal neuralgia surgery

Trigeminal neuralgia model was constructed by constriction of the infraorbital nerve (CION) as reported in previous study (Sheng et al., 2020). In brief, rats or mice were anaesthetized with intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg·kg⁻¹) and the head was fixed, keeping the body supine and mouth wide open. During surgery, anaesthesia depth was maintained at a level, that is, the corneal

reflex disappears and noxious stimuli did not produce flexion and heart rate changes. The rectal temperature was monitored by a thermostatically adjusted heating pad (Homeothermic System, Harvard, USA) and maintained between 37°C and 38°C. A surgical incision was made at 0.1 cm proximal to the first molar along the left gingivobuccal margin, and the left or right infraorbital nerve was exposed. Two (for rat) and one (for mouse) ligatures with 4-0 chromic gut ligatures were tied loosely around the nerve at approximately 1.5 mm apart. Sham-operated animals received only nerve exposure but not ligation. Subsequently, the overlying mucosa was closed. All surgical procedures were performed aseptically.

2.4 | Intra-CA1 drug infusions

Rats were anaesthetized with sodium pentobarbital and then securely placed into a rat stereotaxic device with bregma and lambda horizontally level. A stainless steel cannula (OD 0.41 × ID 0.25, RWD Life Science Co., Ltd, Shenzhen, China) with a stainless steel stylet plug (OD 0.21 × ID 0.11, RWD Life Science Co., Ltd) was unilaterally or bilaterally implanted 0.5 mm above the CA1 of dorsal hippocampal injection site (from bregma: anteroposterior [AP] −3.2 mm, mediolateral [ML] ±1.8 mm; and dorsoventral [DV] −2.5 mm) according to the rat brain in stereotaxic coordinates (Paxinos & Watson, 2007). The cannula was fixed to the cranium with denture acrylic cement. Animals were allowed to recover for 7 days. Microinjection was carried out under isoflurane brief anaesthesia through a 33-gauge stainless steel injection cannula that extended 0.5 mm beyond the tip of the guide cannula. The injection cannula was connected to a 1 μ l Hamilton syringe through PE-10 tubing. A volume of 0.6 μ l of either vehicle or drug was injected with a steady speed (0.1 μ l·min⁻¹). Small interfering RNA (siRNA) targeting the rat P2X7 receptor mRNA (L-091415-00-0020) or control siRNA (D-001220-01-20) was prepared (1 μ g RNA/1.8 μ l polyethyleneimine [PEI]; 10 mM) as reported before (Yang et al., 2015). Macrophage antigen complex-1-saporin (Mac-1-SAP) toxin (15 μ g in 8.8 μ l), a specific microglia cytotoxin targeting CD11b-expressing cells (Sorge et al., 2015; Zhao et al., 2007), and saporin control (8.8 μ g in 8.8 μ l) were purchased in solution from Advanced Targeting Systems. Doses were determined from pilot experiments. According to our pre-experiment results and previous study from other laboratory (Huang et al., 2018), Mac-1-SAP (2.5 μ g/1 μ l, per side) was bilaterally injected into the hippocampal CA1 area on Day 0 and Day 7 after CION, respectively. The injection cannula was left in the place for additional 5 min to minimize drug overflow along the injection track.

The other drugs were continuously delivered to the hippocampal CA1 using micro-osmotic pumps. Implantation of the unilateral or bilateral hippocampal CA1 cannula was performed as described above. A micro-osmotic pump (Model 1002, Alzet, Cupertino, CA, USA) with a delivery rate of 0.25 μ l·h⁻¹ during 14 days, connected via polyethylene tubing to a brain infusion kit (Alzet), was embedded under the skin of the neck (Yang et al., 2016). **A740003, IL-1 receptor antagonist,**

IL-1 β and **indoximod (1-methyl-D-tryptophan; 1-MT)** were delivered in a dosage of 9 $\mu\text{g}\cdot\text{day}^{-1}$, 5 $\mu\text{g}\cdot\text{day}^{-1}$, 3 $\text{ng}\cdot\text{day}^{-1}$ and 5 $\mu\text{g}\cdot\text{day}^{-1}$ during 14 days, respectively. 1-MT, IL-1 receptor antagonist and IL-1 β were dissolved in 0.9% saline. A740003 was dissolved in 10% dimethyl sulfoxide (DMSO) and then diluted with normal saline to a final DMSO concentration of 1%.

At the end of the experiment, brains were sectioned to verify cannula and injection site.

2.5 | Optogenetic experiments

CX₃CR1-GFP::ChR2 and CX₃CR1-GFP::Arch mice were used in the optogenetic experiments. We crossed CX₃CR1-CreER mice, harbouring a tamoxifen-inducible Cre recombinase, with Ai35 mice carrying the floxed stop-Arch-EGFP gene in the ROSA26 locus, to generate the CX₃CR1::Arch mice and also crossed CX₃CR1-CreER line with floxed ChR2-tdTomato mice to generate the CX₃CR1::ChR2 mice. Optical fibres (Newdoon Technology Co., Ltd, Hangzhou, China) were unilaterally implanted upon the mouse hippocampal CA1 (from bregma: AP -2.05 mm; ML ± 1.25 mm; and DV -1.05 mm). Experiments were performed at 1 week after optical fibre implantation. **Tamoxifen** (50 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, i.p.) was injected into CX₃CR1-GFP::Arch mice before CION or CX₃CR1-GFP::ChR2 mice for 3 days (Parkhurst et al., 2013). For the experiments of microglia inhibition, yellow light (580 nm, 6 mW with a pulse frequency of 50 s light on and 10 s light off for 1 h every day) was delivered into the ipsilateral hippocampus from Day 8 to Day 15 after CION. For the experiments of microglia activation, blue light (473 nm, 6 mW, pulse width 5 ms, 20 Hz, 30 $\text{min}\cdot\text{day}^{-1}$) was delivered into the unilateral hippocampus for three continuous days.

2.6 | Behavioural assessment

2.6.1 | von Frey test

Animals were handled and habituated to the testing environment for 30 min at least 3 days before testing. Rats were placed into a customized cage individually, and a series of calibrated von Frey filaments (2, 4, 6, 8, 10, 15 and 26 g, Stoelting Company, Wood Dale, Illinois, USA) were lightly applied to the skin within the infraorbital territory, near the centre of the vibrissal pad on hairy skin surrounding the mystacial vibrissae, in ascending order of force (Liang et al., 2007). The mouse was gently held by an experimenter wearing a regular leather work glove, and mechanical sensitivity was determined with a series of von Frey hairs (0.07, 0.16, 0.4, 0.6, 1, 1.4, 2 and 4 g). The filaments were performed in an increasing order from the lowest force, and a brisk or active withdrawal of the head from the filament was considered a response (Wang et al., 2020). Each filament was tested five times at 5 s intervals. The withdrawal threshold was defined as the lowest force in grams that produced at least three withdrawal responses in five consecutive applications.

2.6.2 | Assessment of cold allodynia

After acclimatized to the experimental environment for 30 min, the rat was gently grasped and restrained by tester with leather work glove for at least 10 min to minimize stress. Acetone (90%, 30 μl) was dropped onto the unilateral vibrissal pad skin, using a 25-gauge blunt needle attached to a 50 μl micro-syringe. Immediately after acetone administration, the rat was placed back to their home cage and the nociceptive behaviour was observed within 5 min. Asymmetric orofacial grooming was defined as nociceptive behaviours, and the time animals spent in this behaviour was recorded.

2.6.3 | Open field test

Open field test was performed in the open box (length \times width \times height: 60 \times 60 \times 35 cm for rat and 40 \times 40 \times 30 cm for mouse). The observation arena was divided into the angle zone (15 \times 15 cm at the four corners for rat; 10 \times 10 cm at the four corners for mouse) and the centre of the arena (30 \times 30 cm for rat; 20 \times 20 cm for mouse). The surrounding was maintained in a dim illumination (25 lx) with no noise. Rats or mice were gently placed into the centre of the arena and allowed to explore for 5 min. Video tracking software (EthoVision XT v11.5, Noldus BV) was used to record and analyse animal activities.

2.6.4 | Elevated plus maze (EPM)

The elevated plus maze consisted of four arms (10 \times 50 cm for rats; 6 \times 35 cm for mice) and a central platform (10 \times 10 cm for rats; 6 \times 6 cm for mice) elevated 50 cm above the floor. Two closed arms were enclosed by a 45-cm-high (rats) or 10-cm-high (mice) walls and crossed with two open arms (without walls). The room was maintained in a dim illumination (25 lx). Animals were placed in the central of the maze facing an open arm and were allowed to explore for 5 min. The time spent in open/closed arm and latency of the first entry into the closed arm were recorded by the video tracking system (EthoVision XT v11.5, Noldus BV).

2.6.5 | Forced swimming (FS) test

Animals were placed into a clear cylindrical glass container (height \times diameter, rats: 60 \times 30 cm; mice: 45 \times 20 cm) filled with water ($24 \pm 2^\circ\text{C}$) to a depth adjusted for the weight and body length of the individual animal, so that its hind paws could just touch the bottom of the container. Animals were trained for 10 min on the first day, and then animals were replaced in the cylinders on the second day. The total duration of immobility and activities of the animals were recorded during a 5 min test by the video. Immobility was defined as the animal not making any active movements other than that necessary to keep the head and nose above the water. The

animals were dried immediately and returned to their home cages after the test.

2.6.6 | Rotarod test

A rotarod test was used to determine whether CION surgery influences the motor function of animals. Animals were placed on an accelerating rotating rod (IITC Life Science), and the rotating rod underwent a linear acceleration from 0 to 40 rpm within 5 min. Animals were trained for three trials with a break of 10 min between the trials per day for two consecutive days before test. On Day 3, animal behaviours on the accelerating rotating rod were scored for their latency (in seconds) to fall for each trial.

2.7 | Immunofluorescence staining and microglia morphological analysis

Animals were killed with overdoses of urethane and were transcardially perfused with normal saline followed by pre-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was removed carefully and postfixed in 4% paraformaldehyde for additional 8–12 h at 4°C. After dehydrated with gradient (10–30%) sucrose in PB at 4°C, coronal sections (30 µm) were cut on a cryostat microtome (Leica CM1950, Germany). The sections containing the hippocampus were blocked with 10% donkey serum with 0.3% Triton X-100 for 2 h at room temperature (RT) and incubation overnight at 4°C with corresponding primary antibodies: goat anti-ionized calcium-binding adapter molecule 1 (IBA-1); 1:1000, #ab5076, [RRID:AB_2224402](#), Abcam, Cambridge, UK), chicken anti-YFP/GFP (1:500, #1020, [RRID:AB_10000240](#), Aves, Tigard, OR, USA), rabbit anti-CD68 (1:500, #ab125212, [RRID:AB_10975465](#), Abcam) or rabbit anti-P2X7 receptor (1:500, #APR-004, [RRID:AB_2040068](#), Alomone Labs, Israel). The sections were then incubated for 2 h at 4°C with Alexa Fluor 488/546-conjugated secondary antibody (1:200, #A21206, [RRID:AB_2535792](#); #A-11055, [RRID:AB_2534102](#); or #A-11056, [RRID:AB_2534103](#); Invitrogen, Carlsbad, CA, USA; #703-545-155, [RRID:AB_2340375](#), or #703-585-155, [RRID:AB_2340377](#), Jackson ImmunoResearch, West Grove, PA, USA) or 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:30,000, Sigma, #32670). For double immunofluorescence, the sections were incubated with a mixture of rabbit anti-P2X7 receptor (1:500) and goat anti-IBA-1 (1:1000)/mouse anti-GFAP (1:2000, #G6171, [RRID:AB_1840893](#), Sigma-Aldrich, St. Louis, MO, USA)/rabbit anti-NeuN (1:2000, #MABN140, [RRID:AB_2571567](#), Millipore, Billerica, MA, USA) primary antibodies. The sections were then incubated with a mixture of Alexa Fluor 488- and 546-conjugated secondary antibodies (1:200, #A-21202, [RRID:AB_141607](#); #A-11056, [RRID:AB_2534103](#); #A-10040, [RRID:AB_2534016](#); or #A-10040, [RRID:AB_2534016](#), Invitrogen) for 2 h at 4°C. The sources of the antibodies are detailed in Table S1. The specificity of immunostaining and primary antibodies was verified by omitting the primary antibodies and gene KO mice

(e.g. P2X7 receptor KO mice). The images were captured with a confocal microscope (Model FV1000, Olympus, Japan). The Immunorelated procedures used comply with the recommendations made by the British Journal of Pharmacology (Alexander et al., 2018).

Hippocampal IBA-1 immunopositive microglia were imaged with a confocal laser scanning microscope at 60× magnification. The Neurolucida software (MBF Bioscience, Williston, VT, USA) was applied for three-dimensional (3D) reconstruction of microglia within CA1 area of the hippocampus. Sholl analysis was performed to analyse the morphology of microglia by placing 3D concentric circles in 5 mm increments starting at 5 mm from the soma using the NeuroExplorer software.

2.8 | Western blotting

Animals were killed with overdoses of urethane and the hippocampal CA1 area was rapidly isolated. The hippocampal tissues were homogenized in lysis buffer (12.5 µl·mg⁻¹) containing a mixture of protease inhibitors and phenylmethylsulfonyl fluoride (Roche Diagnostics, Indianapolis, USA). The protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, Illinois, USA) according to its instruction provided by manufacturers. Protein samples (~15 µg) were loaded and separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA, USA). After blocked with 10% non-fat milk at RT for 2 h, the membranes were incubated overnight at 4°C with primary antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000; Pierce, Rockford, Illinois, USA) for 2 h at 4°C. GAPDH antibody was probed as a loading control. Signals were detected by enhanced chemiluminescence (Pierce) and captured by ChemiDoc XRS System (Bio-Rad). We used the following primary antibodies: rabbit anti-P2X7 receptor (1:2000), goat anti-IBA-1 (1:1000, #ab5076, Abcam) and rabbit anti-IL-1β (1:500, #A-10040, [RRID:AB_2534016](#), PeproTech, Rocky Hill, NJ, USA). All the Western blot analysis was performed three to four times and consistent results were obtained. A Bio-Rad image analysis system was then used to measure the integrated optic density of the specific bands. Western blotting procedures involved comply with the editorial on immunoblotting (Alexander et al., 2018).

2.9 | Microdialysis and ATP assay in the hippocampus

The microdialysis probe (MD-2211, BASi, West Lafayette, IN, USA) was inserted into the bilateral hippocampal CA1 area via the guide cannula (MD-2255, BASi) to 1 mm beyond the tip of the guide cannula under anaesthesia (sodium pentobarbital). The rat was spontaneously breathing and was kept warm. The dialysis probe was connected to a microinfusing pump (BASi, Microdialysis Syringe 1.0 ml). The probe was perfused with artificial cerebrospinal fluid

(ACSF) at a flow rate of $2 \mu\text{l}\cdot\text{min}^{-1}$. After dialysate levels stabilized (about 1 h), sample was collected. To prevent degradation of **ATP**, ecto-ATPase ($1 \text{ mmol}/20 \mu\text{l}$; Sigma-Aldrich) was applied as the perfusate. At the end of the experiments, the rats were killed with overdoses of urethane, and brains were sectioned to verify the position of the cannula. The ATP concentration was assessed by an ENLITEN ATP Assay System with a bioluminescence detection kit (Promega, Madison, WI, USA). According to the manufacturer's protocols, samples ($90 \mu\text{l}$) were neutralized to pH 7.4 with $10 \mu\text{l}$ of 4 M Tris and the luciferase reagent was added 1 s before a 5 s measurement in a luminometer. Light photons were measured by the luminometer and compared with the standard curve to calculate ATP concentration.

2.10 | Hippocampal slice preparation and electrophysiological recording

Coronal brain slices containing the hippocampus were obtained from mouse. After anaesthetizing with isoflurane, mice were killed by cervical dislocation in accordance with the guidelines of the animal ethics committee of Fudan University. Following decapitation, the brain was quickly removed and immediately submerged in pre-oxygenated (95% O_2 and 5% CO_2) cold cutting solution (93 mM *N*-methyl-D-glucamine, 93 mM HCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4 , 30 mM NaHCO_3 , 20 mM HEPES, 25 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 10 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.5 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ and 12 mM *N*-acetyl-L-cysteine). The osmolarity was adjusted to $290\text{--}320 \text{ mOsmol}\cdot\text{L}^{-1}$ and the pH to 7.3–7.4. A tissue block containing the hippocampus was fixed on the stage using cyanoacrylate glue and then horizontally cut into $350 \mu\text{m}$ sections by a vibratome (Leica VT1200 S, Leica, Germany). Subsequently, hippocampal slices were transferred to an oxygenated chamber filled with holding artificial cerebrospinal fluid (94 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4 , 30 mM NaHCO_3 , 20 mM HEPES, 25 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 2 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 2 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ and 12 mM *N*-acetyl-L-cysteine) at RT for 30 min and then transferred to regular artificial cerebrospinal fluid (126 mM NaCl, 4.0 mM KCl, 1.25 mM MgCl_2 , 26 mM NaHCO_3 , 1.25 mM NaH_2PO_4 , 2.5 mM CaCl_2 and 10 mM glucose) at RT for 1 h before recording. The slices could be maintained in a healthy state for up to 8 h.

A single hippocampal slice was transferred to a recording chamber and continuously perfused with regular artificial cerebrospinal fluid at a rate of $5 \text{ ml}\cdot\text{min}^{-1}$ at RT. For recording field excitatory post-synaptic potentials (fEPSPs), a single glass pipette filled with 4 M NaCl was used and a bipolar tungsten stimulating electrode placed in CA1 stratum radiatum of the hippocampus was used to stimulate excitatory response at Schaffer collateral–CA1 synapses. Electrical stimuli ($100 \mu\text{s}$ pulses) were delivered to Schaffer collateral fibres by a square pulse stimulator (Master-8; AMPI, Jerusalem, Israel) and a stimulus isolator (ISO-Flex; AMPI) at an interval of 30 s. Stimulation intensities were adjusted to evoke approximately half of the maximal field potentials. The specific fEPSP signals were acquired using a patch clamp

amplifier (Axopatch 700B; Axon Instruments, Foster City, CA, USA) and digitized with an axon Digidata 1440A. After obtaining a stable baseline of 10 min, LTP was induced by a theta burst stimulation (four bursts of four pulses at 100 Hz delivered at 200 ms intervals, repeated four times with intervals of 10 s). Following theta burst stimulation, the intensity was then adjusted to the level previously utilized to produce baseline fEPSPs and recorded for additional 1 h.

2.11 | ELISA assay

Hippocampal slices were prepared as described above under sterile condition and maintained in sterile culture medium at cell incubator with 95% O_2 and 5% CO_2 for 2 weeks. Hippocampal slices were treated with different agents: **lipopolysaccharide** (LPS; $100 \text{ ng}\cdot\text{ml}^{-1}$), **BzATP** (μM) + LPS ($100 \text{ ng}\cdot\text{ml}^{-1}$) and A740003 ($100 \mu\text{M}$) + BzATP (μM) + LPS ($100 \text{ ng}\cdot\text{ml}^{-1}$). Twenty-four hours later, hippocampal slices were collected and homogenized in the lysis buffer ($12.5 \mu\text{l}\cdot\text{mg}^{-1}$) containing protease and phosphatase inhibitors (Sigma Chemical Company) at 4°C . Then, the tissue lysate was centrifuged with $12,000 \times g$ for 15 min to get the supernatant. ELISA was conducted according to manufacturer's instructions (R&D Systems, #RLB00, Minneapolis, MN, USA) to assay IL-1 β content in the supernatant.

2.12 | Materials

BzATP (a P2X7 agonist), A740003 (a P2X7 antagonist) and indoximod (1-MT; a selective inhibitor of **indoleamine 2,3-dioxygenase 1** [IDO1]) were purchased from Tocris Bioscience. DAPI, DMSO, pentobarbital sodium, **LPS**, **minocycline**, **brilliant blue G (BBG)**, **tamoxifen** and PEI were obtained from Sigma, USA. IL-1 receptor antagonist was purchased from R&D Systems. IL-1 β was obtained from PeproTech, USA. Mac-1–SAP was provided by Advanced Targeting Systems. Details of other materials and suppliers were provided in the specific sections and the sources of other the materials are detailed in Table S1.

2.13 | Data analysis

All data were summarized as the mean \pm SEM. The group sizes were at least five ($n \geq 5$), where group size is the number of independent values and that statistical analysis was done using these independent values. All data from different groups were verified for normality (Shapiro–Wilk) test and homogeneity of variance (Levene's test) before analysis, where no data were transformed. No data were excluded from statistical analysis due to outlier status. Behavioural data, immunohistochemistry, western blot data and electrophysiological recording were analysed using Student's *t* test when comparing two groups, or one-way ANOVA followed by post hoc Dunnett's test or two-way repeated-measures (RM) ANOVA followed by post hoc Student–Newman–Keuls test when comparing more than two groups

using GraphPad 7.0 software (GraphPad Software, San Diego, CA, USA). The post hoc tests were conducted only if the *F* value in ANOVA achieved statistical significance and there was no significant variance in homogeneity. All the hypothesis testing was two-tailed with *P* value less than 0.05 considered statistically significant. The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.14 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY <http://www.guidetopharmacology.org> and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

3.1 | Activation of microglia in the ipsilateral hippocampal CA1 area contributes to trigeminal neuralgia-induced anxiodepressive-like behaviours in Wistar rats

Trigeminal neuralgia rat model was established by CION (Figure 1a) to mimic clinical trigeminal neuropathic pain. Following CION, mechanical allodynia developed with 4 days and persisted for at least 20 days in the ipsilateral, but not contralateral, vibrissal pad (Figures 1b,c and S1A). We also observed significant cold allodynia in the ipsilateral vibrissal pad of CION rats (Figure S1B,C).

The anxiodepressive-like behavioural tests were performed in different groups from Day 4 to Day 7 and Day 14 to Day 17, respectively (Figure 1b). In the anxiety-like behavioural tests, the rats with CION for more than 14 days but not for 4/5 days spent less time in the centre arena of open field (OF, Figures 1d,e and S1D) and in the open arm of elevated plus maze, as well as shorter latency into the closed arm in elevated plus maze (Figures 1f,g and S1D). CION rats also exhibited depressive-like behaviour at Day 17 but not Day 7 after surgery in the forced swimming (Figure 1h) test. We also observed similar anxiodepressive-like behaviours in female rats after CION (Figure S2A–F). These results indicated that anxiodepressive-like behaviours caused by neuropathic pain were time dependent. There was no difference among the groups for total travel distance in open field (Figure 1d) and latency to fall in rotarod test (Figure S1E), indicating that the above behavioural phenotypic differences were not due to motor impairment.

It has been reported that microglia in the brain, such as anterior cingulate cortex (Steiner et al., 2011) and dorsal striatum (Klawonn et al., 2021), contribute to the pathogenesis of anxiety and depression. In the present study, we further examined whether hippocampal microglia were involved in CION-induced anxiety and depression.

Western blot analysis showed a robust elevation of IBA-1 (a microglial marker) in the ipsilateral hippocampal CA1 area on Day 14, but not Day 4 after CION, which was associated with the development of anxiodepressive-like behaviours (Figure 1i,j). CION-induced activation of microglia was further confirmed by immunohistochemistry (Figures 1k,l and S2G,H). Activated microglia exhibited large cell bodies and short or thick processes in the rat hippocampal CA1 area (Figure 2a–d). Consistently, CD68 (a microglial activation marker) expression in the hippocampal CA1 area was also significantly increased at Day 14 after CION (Figure 2e,f).

Next, we examined the effects of temporary partial deletion of hippocampal microglia on development of CION-induced anxiodepressive-like behaviours in rats. Mac-1–SAP was used to induce microglial depletion. Following injection of Mac-1–SAP into the bilateral hippocampal CA1 area, CION-induced IBA-1 up-regulation was significantly suppressed (Figure 3a–d) and the CION rats failed to develop anxiodepressive-like behaviours (Figure 3e–i), suggesting that hippocampal microglia play an important role in chronic pain-induced anxiety and depression. The anti-anxiodepressive effect of inhibiting microglia was further confirmed by systematic administration of minocycline (20 mg·kg^{−1}, i.p.), a semi-synthetic second-generation tetracycline that has emerged as a potent inhibitor of microglial activation (Figure S3).

Intriguingly, administration of Mac-1–SAP to the left (ipsilateral to CION) but not right (contralateral to CION) hippocampus can mimic the anxiolytic and antidepressant effect of bilateral Mac-1–SAP. To further confirm that ipsilateral rather than left hippocampal microglia are involved in the development of CION-induced anxiodepressive-like behaviours, we performed right CION surgery and compared the effects of Mac-1–SAP given to the ipsilateral and contralateral hippocampal CA1 area. As shown in Figure 3e–i, partial deletion of ipsilateral hippocampal microglia prevented the development of anxiodepressive-like behaviours by neuropathic pain, regardless of left or right CION. Differently, neither ipsilateral nor contralateral administration of Mac-1–SAP alleviated CION-induced mechanical allodynia (Figure 3j). These results indicate that ipsilateral hippocampal microglia are required for the development of neuropathic pain-induced anxiodepressive-like behaviours in trigeminal injury rats.

3.2 | Optogenetic manipulation of ipsilateral hippocampal microglia in the CA1 area alters the anxiodepressive state in mice

Just like Wistar rats, C57BL/6 mice with CION exhibited similar anxiodepressive-like behaviours after 2 weeks but not within 1 week (Figure S4). To obtain selective control of microglial activity, the optic fibre was implanted in the ipsilateral hippocampal CA1 area of CX₃CR1::Arch mice, and yellow light (580 nm) illumination was applied from Days 7 to 15 after CION (Figure 4a,b). As shown in Figure 4c, Arch-EGFP signals were co-localized with IBA-1-immunoreactivity almost without exception after tamoxifen injection. Following yellow light treatment, CION-induced microglial

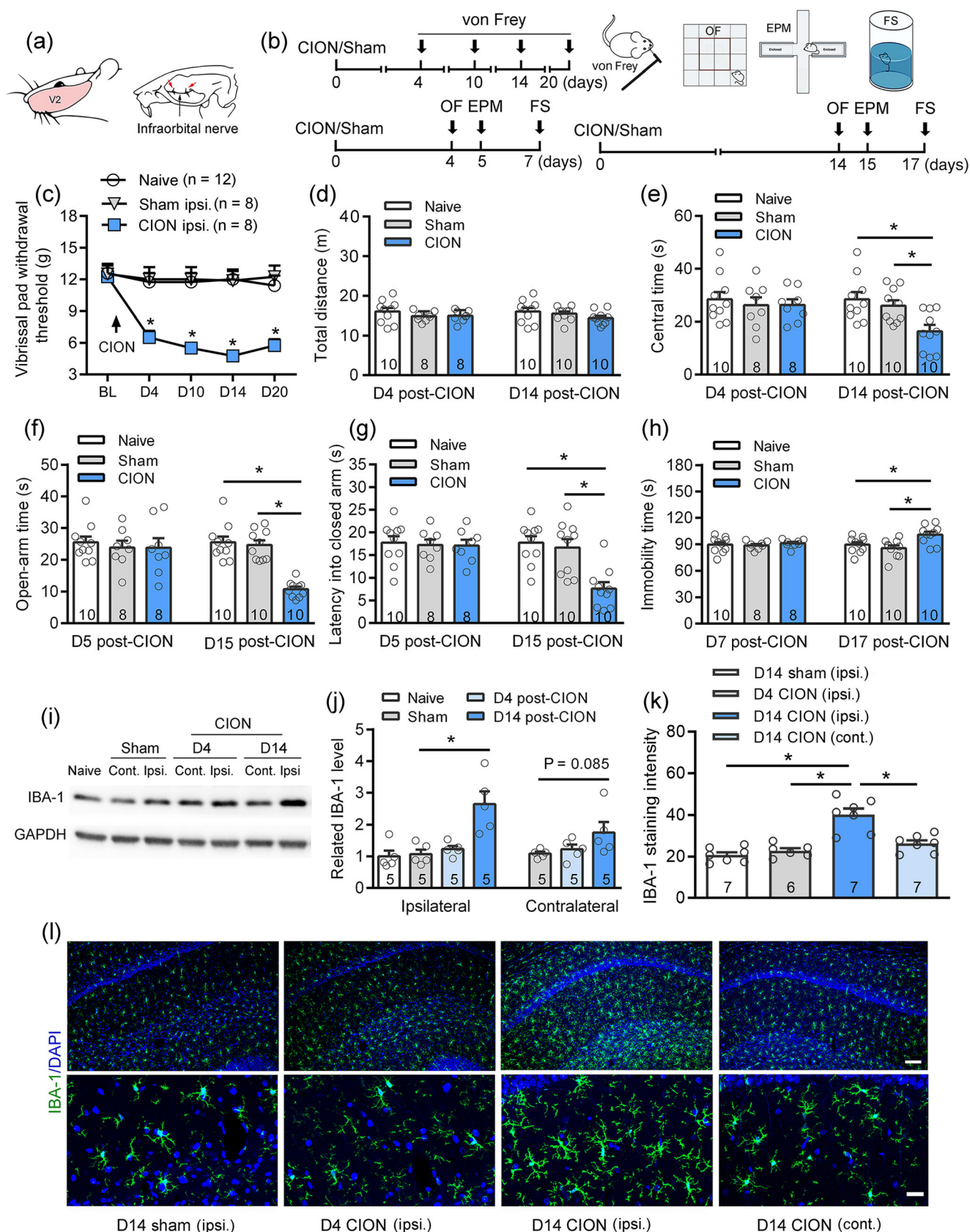


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activation of the hippocampal CA1 area was suppressed in CX₃CR1::Arch mice (Figure 4d,e). Consistent with the inhibition of microglia by Mac-1-SAP into ipsilateral CA1, optogenetic inhibition of ipsilateral hippocampal microglia significantly blocked CION-induced anxiodepressive-like behaviours in open field, elevated plus maze and forced swimming tests (Figure 4f–j).

We also examined the effects of activating hippocampal microglia on anxiety and depression levels in normal mice. Implantation of optic fibre per se did not cause obviously microglial activation in the hippocampal CA1 area CX₃CR1::ChR2 mice (Figure 4k). ChR2-tdTomato signals were specifically co-localized with microglial markers, but not astrocytic and neuronal markers, after tamoxifen injection (Figures 4l and S5A). Blue light (473 nm) illumination evoked a robust activation of microglia in the hippocampal CA1 area without neuronal damage (Figures 4l,m and S5B). Optogenetic activation of unilateral hippocampal microglia elicited an obvious anxiodepressive-like behavioural phenotype, marked by less centre time and distance in open field test and higher immobile duration in forced swimming test than that of controls (Figure 4n–q). Taken together, the above results suggest that unilateral hippocampal CA1 microglial activation is sufficient to drive mouse anxiodepressive-like behaviours.

3.3 | Trigeminal neuralgia impairs ipsilateral hippocampal LTP by activated microglia in mice

Impaired hippocampal LTP has been found in a variety of animal models of depression (Innes et al., 2019; Li et al., 2012; Liu et al., 2019; Riga et al., 2017). To investigate whether hippocampal LTP is affected by trigeminal neuralgia and related anxiodepression, we recorded hippocampal CA1 area LTP in post-CION 4 and 14 day mice. Hippocampal LTP of fEPSP was successfully induced by theta burst stimulation in naive, sham and post-CION 4 day mice (Figure 5a,b). However, by 14 days after CION, ipsilateral hippocampal LTP was impaired, with only short-term potentiation, regardless of the left or right CION (Figure 5b,c). Most evidence showed that microglia regulate synaptic plasticity and respond to multiple behavioural consequences, such as depression and chronic pain (Cao et al., 2021; Yin

et al., 2021). To investigate whether the suppression of hippocampal LTP in ipsilateral to CION can be rescued by inhibition of microglia, we recorded LTP in hippocampal slices from the ipsilateral side of CION in the presence of minocycline (5 μ M) or vehicle (Figure 5d). The suppression of LTP was completely rescued by minocycline treatment (Figure 5e). We also recorded hippocampal LTP in CX₃CR1::Arch mice. Yellow light (580 nm) illumination was applied from Days 7 to 14 after CION with tamoxifen or without tamoxifen (control) treatment mice (Figure 5f). Optogenetic inhibition of microglia in the ipsilateral hippocampal CA1 area significantly blocked CION-induced LTP suppression (Figure 5g). Furthermore, we examine the effects of directly activating microglia on hippocampal LTP by optogenetically stimulating the hippocampal slices from CX₃CR1::ChR2 mice (Figure 5h). Blue light (473 nm) illumination suppressed hippocampal LTP, with a decreased fEPSP amplitude as compared to no light control and no tamoxifen control groups (Figure 5i). These results suggest that microglial activation in the hippocampal CA1 area may be involved in mediating the impairment of LTP.

3.4 | P2X7 receptor mediates constriction of the infraorbital nerve (CION)-induced hippocampal microglial activation and anxiodepressive-like behaviours

Our previous studies indicated that P2X7 receptor was expressed in spinal and hippocampal microglia and mediated bone cancer pain and chronic stress-induced depression (Yang et al., 2015; Yue et al., 2017). Herein, we further identified whether P2X7 receptor mediates hippocampal microglial activation and neuropathic pain-induced anxiodepressive-like behaviours. Western blot analysis revealed a significant up-regulation of P2X7 receptors on Day 14 but not Day 4 after CION in the ipsilateral hippocampus (Figure 6a,b). The increased P2X7 receptors expression in the ipsilateral CA1 was further confirmed by immunohistochemistry (Figures 6c and S6). Double immunostaining showed that P2X7 receptor-immunoreactivity was almost co-expressed with IBA-1 (microglial marker) but not with GFAP (astrocytic marker) and NeuN (neuronal marker) in the hippocampus

FIGURE 1 Trigeminal neuralgia (TN) time dependently induces anxiodepressive-like behaviours, mechanical allodynia of the ipsilateral vibrissal pad and microglial activation in ipsilateral hippocampal CA1. (a) Schematic showing chronic constriction injury of infraorbital nerve (CION). Arrows point to the ligation sites. (b) Schematic of the protocol for experiments (c)–(h). (c) Following the TN, mechanical response threshold of the ipsilateral vibrissal pad decreases markedly on Day (D) 4 and lasted for more than 20 days. $^*P < 0.05$ versus sham and naive controls, two-way RM ANOVA followed by post hoc Student–Newman–Keuls test ($n = 12/8/8$, naive/sham/CION, rats/group). The TN rats exhibit anxiety-like behaviours in open field test (OF, d, e) and elevated plus maze (EPM) test (f, g) and depressive-like behaviour in forced swimming test (FS, h) on Days 14–17 but not Days 4–7 after CION. $^*P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 10/8/8$, naive/sham-/CION-4/5/7d; $n = 10/10/10$, naive/sham-/CION-14/15/17d, rats/group). In order to avoid the impact of repeated testing on animal behavioural phenotypes in the same experimental scenes, we performed these experiments in different groups. (i, j) Western blot analysis reveals significant up-regulation of ionized calcium-binding adapter molecule 1 (IBA-1; microglial marker) level on Day 14 after CION in the ipsilateral hippocampal CA1 area. $^*P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 5$ rats for all the groups). (k, l) Immunohistochemistry data show microglial activation, as indicated by intense IBA-1 immunofluorescence and large cell bodies of microglia in the ipsilateral hippocampal CA1 area. Microglial activation occurs on Day 14 but not Day 4 after CION. $^*P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 7/6/7/7$, D14 sham/D4 CION-ipsi./D14 CION-ipsi./D14 CION-cont., rats/group). Scale bars indicate 100 and 25 μ m for upper and bottom rows of (l), respectively. Error bars indicate standard error of the mean.

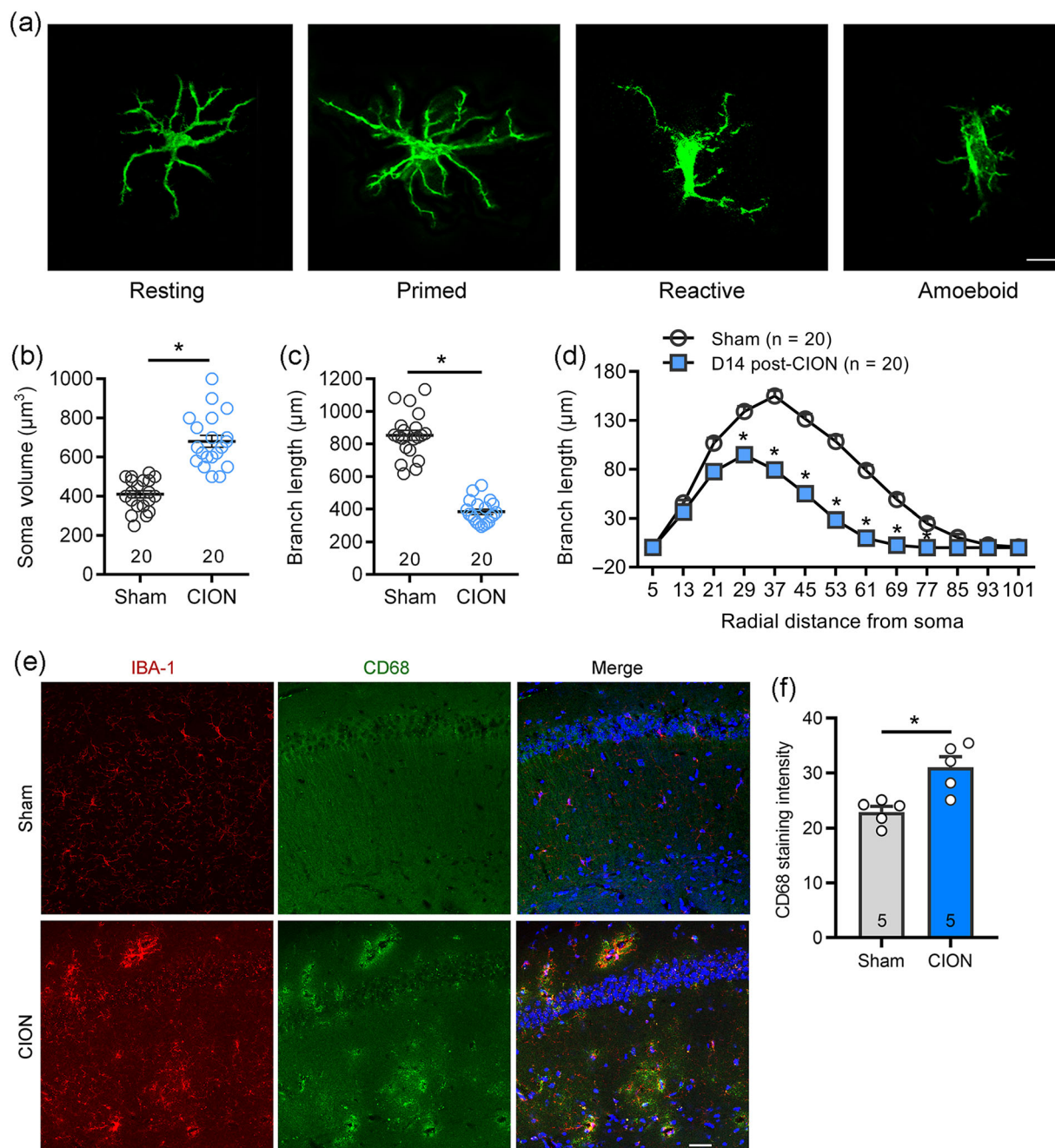


FIGURE 2 Morphological changes and activation of hippocampal microglia at 14 days after constriction of the infraorbital nerve (CION). (a) Representative immunofluorescence images showing the morphology of microglia in resting (radial branching), primed (thickened processes and increased polarity), reactive (thickened stout processes with highly reduced branching) and amoeboid (few-to-no processes and enlarged cell body). Scale bar indicates 20 μm . Sholl analysis showing enlarged soma size (b) and shortened branches (c, d) of hippocampal microglia on Day (D) 14 after CION. Two-tailed Student's *t* test (b, c) or two-way RM ANOVA followed by post hoc Student–Newman–Keuls test (d), $^*P < 0.05$ versus sham (n = 20 cells for sham and CION-14d). (e, f) Double immunofluorescence staining of CD68 and ionized calcium-binding adapter molecule 1 (IBA-1) showing CD68-immunoreactivity (IR) co-expressed with IBA-1 and increased CD68-positive signal in the hippocampal CA1 area on Day 14 after CION in mice. Scale bars indicate 30 μm . $^*P < 0.05$, two-tailed Student's *t* test (n = 5/5, sham/CION, rats/group). Error bars indicate standard error of the mean.

(Figure 6d). To verify whether neuropathic pain results in increased release of ATP, we measured ATP concentration in extracellular dialysate of the hippocampal CA1 on Day 14 after CION. The extracellular ATP level was significantly increased in the hippocampus ipsilateral to

CION (Figure 6e). To address whether P2X7 receptor participates in CION-induced anxiodepressive-like behaviours, we examined the effects of blocking hippocampal P2X7 receptors by pharmacological approach. After A740003 (0.375 $\mu\text{g}\cdot\text{h}^{-1}$), a P2X7 receptor-selective

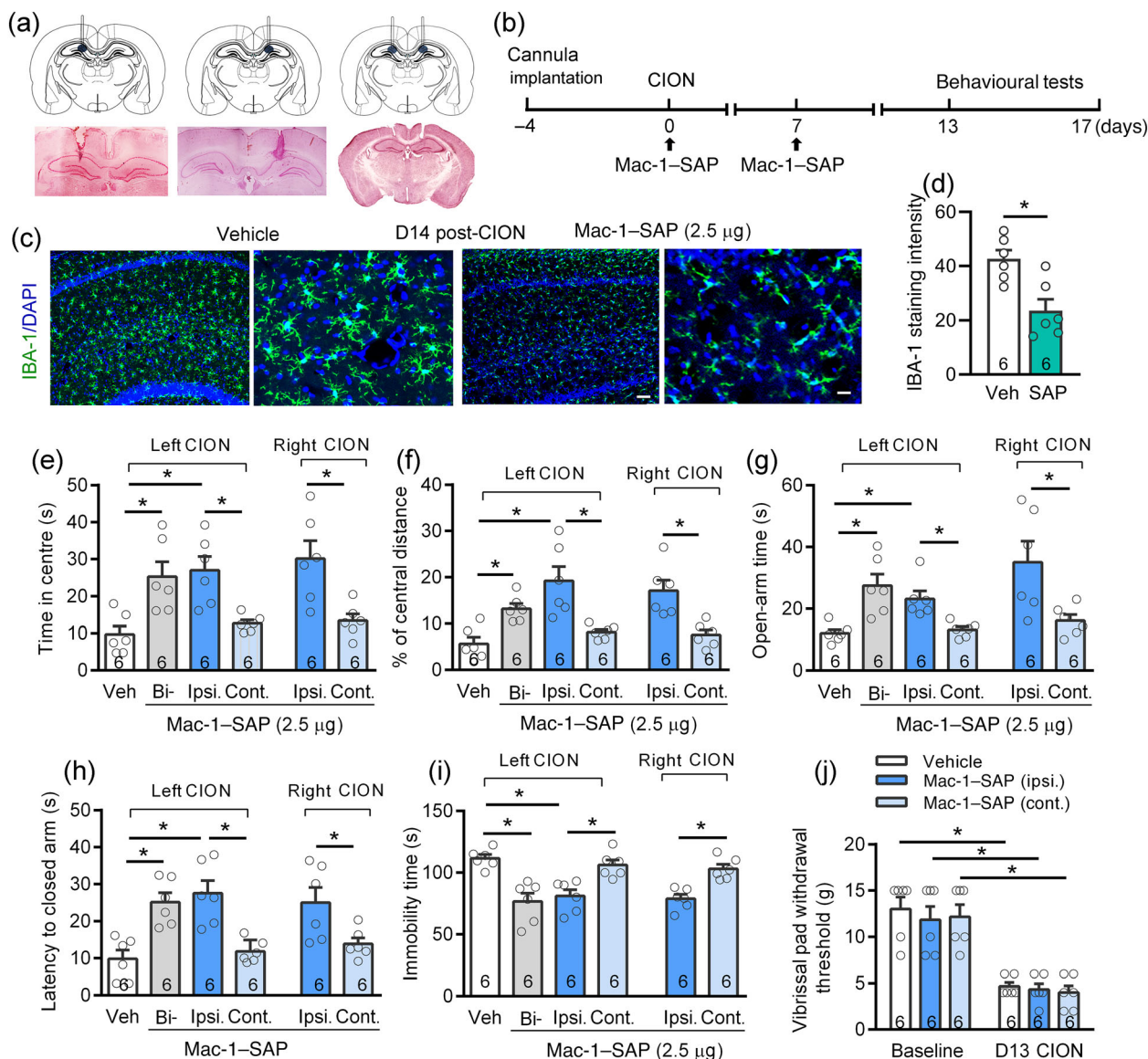


FIGURE 3 Temporary depletion of hippocampal microglia blocks the constriction of the infraorbital nerve (CION)-induced anxiodepressive-like behaviours. (a) Schematic and photomicrograph of coronal section showing cannula placement in the unilateral and bilateral hippocampus. (b) Schematic of the protocol for experiments (e)–(j). Mac-1-SAP (2.5 µg/1 µl, per side) was injected into the hippocampal CA1 area at Day (D) 0 and Day 7 after CION, respectively. (c, d) Selective targeting of hippocampal microglia with Mac-1-SAP (2.5 µg, once a week for twice) results in a robust decrease in the immunofluorescence staining signal of ionized calcium-binding adapter molecule 1 (IBA-1) on Day 14. Scale bars indicate 100 µm (low magnification) and 25 µm (high magnification). **P* < 0.05, two-tailed Student's *t* test (*n* = 6/6, vehicle/Mac-1-SAP, rats/group). Ipsilateral or bilateral injection of Mac-1-SAP (2.5 µg, twice) into the CA1 area of the hippocampus leads to a significant anti-anxiodepressive effect in OF (e, f), elevated plus maze (EPM; g, h) and forced swimming (FS; i) tests in CION rats. Administration of Mac-1-SAP into the contralateral hippocampal CA1 fails to block the development of anxiodepressive-like behaviours, no matter the CION on the left or right. **P* < 0.05, one-way ANOVA followed by post hoc Student–Newman–Keuls test (*n* = 6 rats for all the groups). (j) Neither ipsilateral nor contralateral intra-CA1 of Mac-1-SAP affects CION-induced mechanical allodynia. **P* < 0.05, one-way ANOVA followed by post hoc Student–Newman–Keuls test (*n* = 6 rats for all the groups). Error bars indicate standard error of the mean.

antagonist, bilaterally or ipsilaterally into the hippocampal CA1 area by osmotic pump system for 14 days, CION rats failed to develop anxiodepressive-like behaviours; injection of A740003 contralaterally into the hippocampus had no effect (Figure 6f–i). Neither ipsilaterally nor contralaterally blockade of P2X7 receptors in the hippocampal CA1 alleviated CION-induced mechanical allodynia (Figure 6m). The effects of the ipsilateral hippocampal P2X7 receptors on microglial

activation and behavioural responses were further confirmed by siRNA targeted against rat P2X7 receptors. CION-induced IBA-1 elevation on Day 14 was significantly suppressed by P2X7 receptor-siRNA (0.4 µg, Figure 7a–c). Infusion of P2X7 receptor-siRNA ipsilaterally but not contralaterally into the CA1 completely blocked CION-induced anxiodepressive-like behaviours (Figure 7d–h). Either ipsilaterally or contralaterally knocking down P2X7 receptor in the

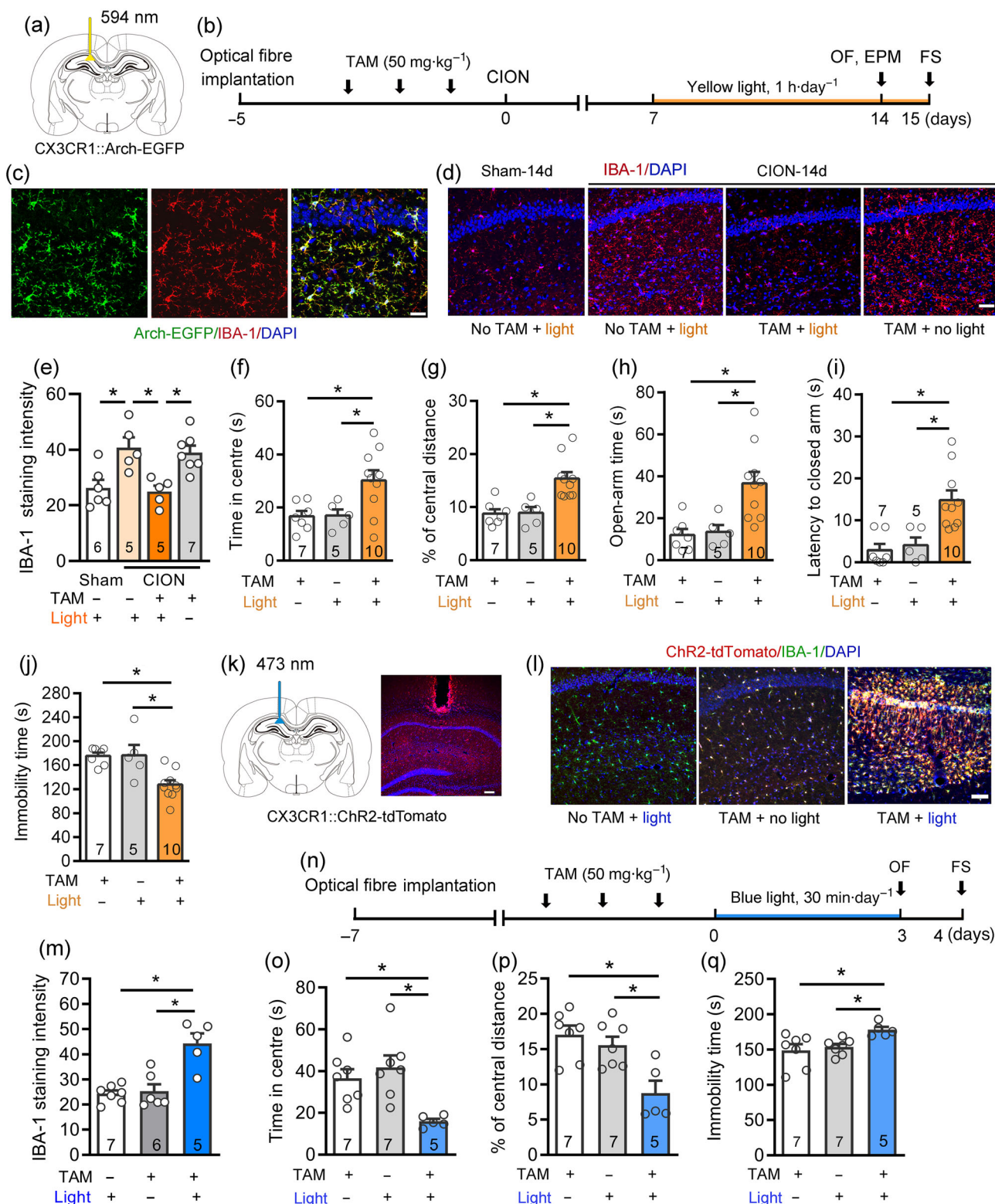


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hippocampal CA1 did not affect CION-induced mechanical allodynia (Figure 7i). These results suggest that P2X7 receptor in the ipsilateral hippocampus to trigeminal neuralgia was specifically involved in trigeminal neuralgia-induced microglial activation and anxiodepression.

Similarly, repeated intraperitoneal injection of a selective P2X7 antagonist BBG (40 mg·kg⁻¹·day⁻¹) also prevented the development of anxiodepressive-like behaviours and attenuated mechanical allodynia in CION rats (Figure S7A–I). Additionally, in *P2x7r* KO mice,

all the CION-induced mechanical allodynia, LTP impairment and anxiodepressive-like behaviours in wild-type mice were not developed (Figure S8A–H).

3.5 | P2X7 receptors contributed to constriction of the infraorbital nerve (CION)-induced anxiodepressive-like behaviours via IL-1 β

Activated microglia synthesize and release various cytokines, such as IL-1 β and facilitate the development of neuropathic pain (Chen et al., 2018). Therefore, we reasoned that P2X7 receptor activation could trigger IL-1 β release in the hippocampal CA1. ELISA analysis revealed that P2X7 agonist BzATP (100 ng·ml⁻¹) significantly increased LPS (100 ng·ml⁻¹)-stimulated IL-1 β level in hippocampal slices, which was blocked by P2X7 antagonist A740003 (Figure 7j). Knockdown of P2X7 receptors in the hippocampus obviously decreased the CION-induced IL-1 β on Day 14 (Figure 7k). Consistent with the temporal profiles of increased IBA-1 and P2X7 receptors, significant up-regulation of IL-1 β level occurred in the ipsilateral hippocampus on Day 14 after CION (Figure 7l). Furthermore, we tested whether the LTP impairment caused by microglial activation is mediated by IL-1 β . The presence of (IL-1 β receptor antagonist, 5.7×10^{-9} M) significantly blocked optogenetic activating microglia-induced hippocampal LTP impairment in CX₃CR1::ChR2 mice (Figure 7m). Behaviourally, intra-CA1 of IL-1 receptor antagonist (5 μ g) effectively prevented the anxiodepressive-like behaviours caused by optogenetic activation of microglia (Figure 7n–q). Importantly, intra-CA1 of IL-1 receptor antagonist (0.2 μ g·h⁻¹) by osmotic pump system for 14 days effectively prevented CION-induced anxiodepressive-like behaviours (Figure 8a–f). CION-induced hippocampal LTP impairment was also rescued by IL-1 receptor antagonist (5.7×10^{-9} M) treatment (Figure 8g).

Next, we tested whether IL-1 β per se directly impairs LTP and elicits anxiodepressive-like behaviours. Acute perfusion of IL-1 β (5.8×10^{-10} M) robustly impaired the maintenance of hippocampal

LTP (Figure 8h). Consistently, intra-CA1 of IL-1 β (0.125 ng·h⁻¹) by osmotic pump system produced anxiodepressive-like behaviours (Figure 8i–n). These results suggested that hippocampal microglial-derived IL-1 β contributes to CION-induced anxiety and depression. IL-1 β has been demonstrated to increase the activity of IDO1, a rate-limiting enzyme in tryptophan metabolism (Miura et al., 2008). Increased IDO1 resulted in the increased kynurenine (KYN)/tryptophan ratio and decreased 5-hydroxytryptamine (5-HT; serotonin)/tryptophan ratio in the bilateral hippocampus (Kim et al., 2012). Therefore, we also examined the effect of IDO1 inhibitor on CION-induced anxiodepressive-like behaviours. When 1-MT (0.2 μ g·h⁻¹), a selective inhibitor of IDO1, was delivered into ipsilateral hippocampal CA1 area for consecutive 14 days after CION by osmotic pump system, CION rats failed to develop anxiodepressive-like behaviours in 14 days (Figure S9).

4 | DISCUSSION

4.1 | Activation of microglia is involved in the pathogenesis of neuropathic pain and depression

As the primary immune cells in the central nervous system, microglia have been well documented to be involved in the pathogenesis of both depression and chronic pain. The implication of microglia in depression is supported by the magnetic resonance imaging showing the obvious activation of microglia in multiple brain regions of depressed patients (Ramirez et al., 2017). Microglia activation in the hippocampus was also revealed in multiple animal models of depression induced by chronic stress (Frank et al., 2012; Franklin et al., 2018). A recent study from Engblom Lab. suggests that microglia might be an interference target for treatment of depressive symptoms (Klawonn et al., 2021). The causal relationship between microglia activation and neuropathic pain has been well established in the spinal cord level (Tsuda, 2016), but the role of supraspinal microglia in neuropathic pain is still debated. Spared nerve injury

FIGURE 4 Optogenetic manipulation of unilateral hippocampal microglia in the CA1 area alters the anxiodepressive-like behaviours in mice. (a) Schematic showing the site of optical fibre implantation in the unilateral hippocampal CA1 area of CX₃CR1::Arch mouse. (b) Schematic of the protocol for experiments (f)–(j). (c) Double immunofluorescence staining reveals that Arch-EGFP co-expressed with ionized calcium-binding adapter molecule 1 (IBA-1). Scale bar: 50 μ m. (d, e) IBA-1 immunoreactivity in the hippocampal CA1 area is reduced by yellow light (580 nm, 6 mW with a pulse frequency of 50 s light on and 10 s light off) illumination on Day 14 after constriction of the infraorbital nerve (CION) in CX₃CR1::Arch mice. Scale bar: 50 μ m. * P < 0.05, one-way ANOVA followed by post hoc Student–Newman–Keuls test (n = 6/5/5/7, sham + light/CION + light without tamoxifen (TAM)/CION + TAM + light/CION + TAM without light, mice/group). Optogenetic inhibition of ipsilateral hippocampal microglia prevents the anxiodepressive-like behaviours in open field (OF; f, g), elevated plus maze (EPM; h, i) and forced swimming (FS; j) tests on Day 14/15 after CION in CX₃CR1::Arch mice. * P < 0.05, one-way ANOVA followed by post hoc Student–Newman–Keuls test (n = 7/5/10, no light/no TAM/TAM + light, mice/group). (k) Schematic and photomicrograph of coronal section showing the site of optical fibre implantation in the unilateral hippocampal CA1 area of CX₃CR1::channelrhodopsin-2 (ChR2) mouse. Scale bar: 100 μ m. (l, m) Double immunofluorescence staining reveals that ChR2-tdTomato co-expressed with IBA1 and IBA-1 immunoreactivity in the hippocampal CA1 area is increased by blue light (473 nm, 20 Hz, 25 ms) illumination on Day 14 after CION in CX₃CR1::ChR2 mice. Scale bar: 50 μ m. * P < 0.05, one-way ANOVA followed by post hoc Student–Newman–Keuls test (n = 7/6/5, no TAM/no light/TAM + light, mice/group). (n) Schematic of the protocol for experiments (o)–(q). Optogenetic activation of unilateral hippocampal microglia induces anxiodepressive-like behaviours in open field (OF; o, p) and FS (q) tests in naive CX₃CR1::ChR2 mice. * P < 0.05, one-way ANOVA followed by post hoc Student–Newman–Keuls test (n = 7/7/5, no light/no TAM/TAM + light, mice/group). Error bars indicate standard error of the mean.

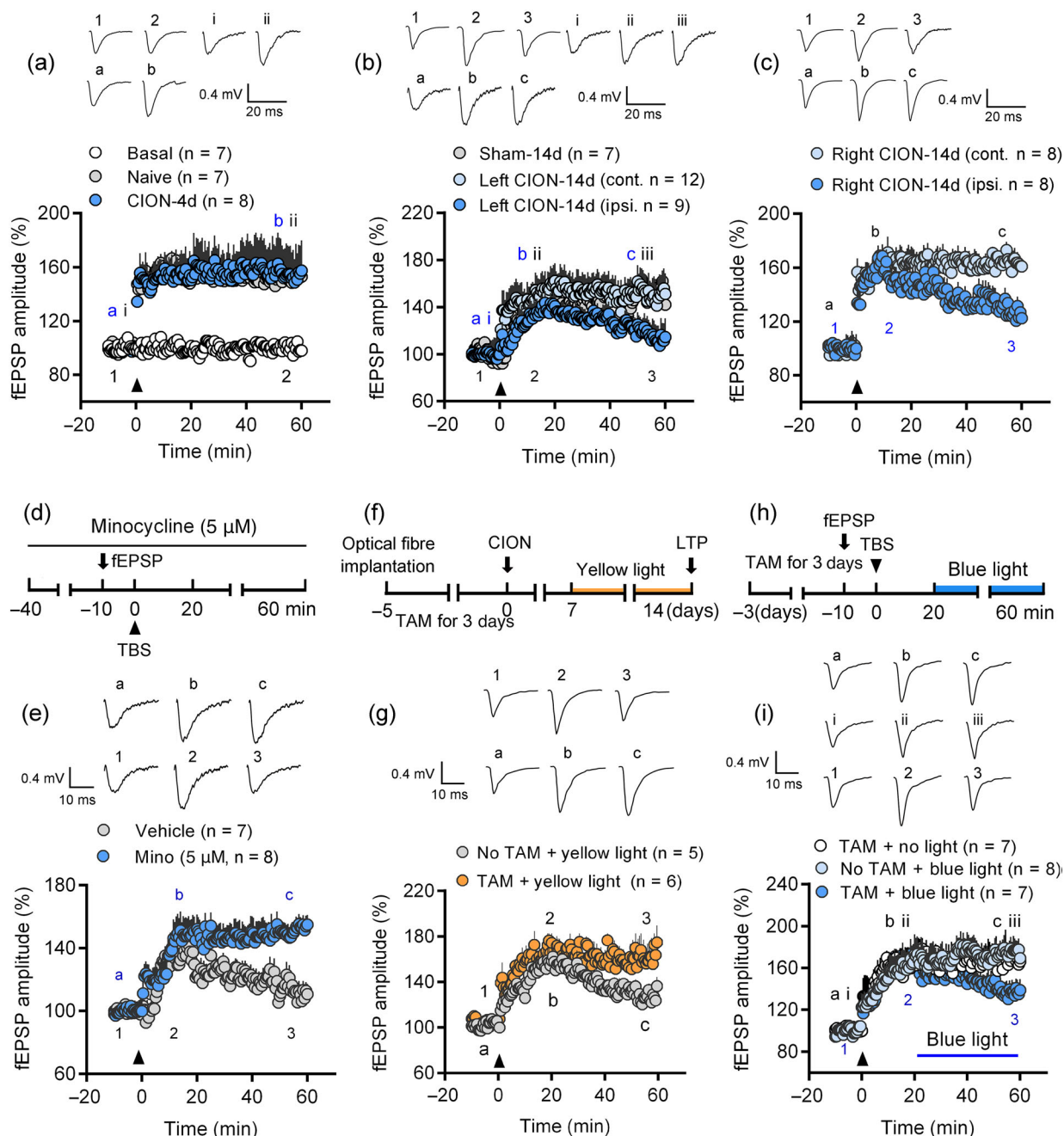


FIGURE 5 Trigeminal neuralgia time dependently impairs ipsilateral hippocampal long-term potentiation by activated microglia. (a) Long-term potentiation (LTP) of fEPSP in area CA1 of the hippocampus is successfully induced by the theta burst stimulation (TBS) protocol (indicated by an arrow head) in hippocampal slice from both naive and constriction of the infraorbital nerve (CION)-4d mice (n = 7/7/8, basal/naive/CION-4d, slices/group). Stable basal recording (without TBS) suggested a stable electric stimulation. The LTP in the ipsilateral hippocampal CA1 area is impaired on Day (D) 14, regardless of the left (b) or right (c) CION (n = 7/12/9, sham/contralateral/ipsilateral hippocampal in left CION on Day 14; n = 8/8, contralateral/ipsilateral hippocampal in right CION on Day 14, slices/group). (d, e) Pre-application of minocycline (Mino; 5 μM) reverses CION-induced LTP impairment (n = 7/8, vehicle/minocycline, slices/group). (f, g) Optogenetic inhibition of hippocampal microglia partially blocks CION-induced ipsilateral hippocampal LTP impairment in CX₃CR1::Arch mice (n = 5/6, no tamoxifen (TAM) + yellow light/TAM + yellow light, slices/group). (h, i) Optogenetic activation of hippocampal microglia suppressed LTP amplitude in CX₃CR1::ChR2 mice (n = 7/8/7, TAM + no blue light/no TAM + blue light/TAM + blue light, slices/group). The insets showing the traces of fEPSPs before and after TBS stimulation in different treatment groups (points 1–3, points i–iii or points a–c). Error bars indicate standard error of the mean.

significantly increased the expression levels of IBA-1 and IL-1β on Day 12 not only in spinal dorsal horn but also in the hippocampal CA1, prefrontal cortex, nucleus accumbens and amygdala (Gui

et al., 2016). Following spinal nerve ligation, hippocampal microglia were robustly activated and inhibition of hippocampal microglia could relieve neuropathic pain (Zhu et al., 2018). Differently, some studies

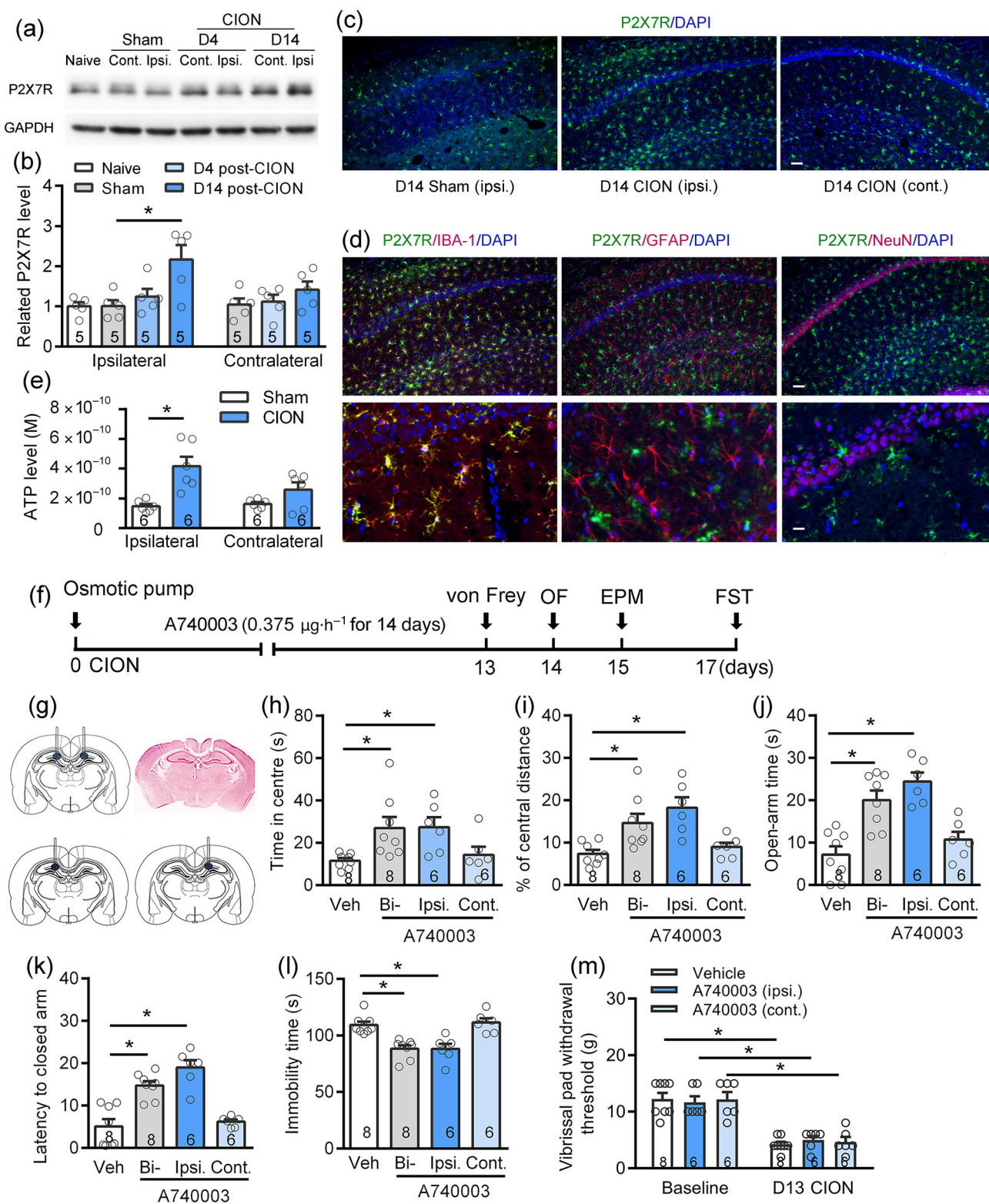


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showed that peripheral nerve injury selectively activated microglia in the spinal dorsal horn cord without affecting the supraspinal structures (Marcello et al., 2013; Zhang et al., 2008). These contradictions might result from the differences in animal strains or species, animal

models and time point of experimental observation and so forth. Indeed, our current study demonstrated that significant activation of hippocampal microglia occurred only 2 weeks after constriction of the infraorbital nerve (CION), not within 1 week. More importantly, the

time window of hippocampal microglial activation coincides with the development of anxiodepressive-like consequences of neuropathic pain, implying the involvement of microglia activation in anxiodepressive consequences of neuropathic pain. Consistent with previous studies (Yalcin et al., 2011; Zhou et al., 2019), the present results showed that the different symptoms of trigeminal neuralgia, including mechanical allodynia and anxiodepressive-like consequences, exhibited different time courses after nerve injury. Animals developed mechanical allodynia within 3 days after CION and anxiodepressive-like behaviours occurred after 2 weeks, which allows us to study the time-dependent evolution of hippocampal microglial activation with anxiodepressive-like symptom of trigeminal neuralgia.

4.2 | Microglia regulate hippocampal synapse plasticity

Microglia have been shown to participate in the process of synapse pruning and stripping by selectively contacting synapse with their vicinity and thus affecting synapse plasticity (Eroglu & Barres, 2010; Yi, Liu, Liu, et al., 2021). Evidence has shown that peripheral nerve injury can impair LTP at hippocampal CA3–CA1 synapse by increasing the level of $\text{TNF-}\alpha$ in a microglia-dependent mechanism (Liu et al., 2017; Ren et al., 2011). Microglia activation was also proved to directly suppress hippocampal LTP (Griffin et al., 2006). In the present study, we further revealed that trigeminal neuralgia impaired ipsilateral hippocampal LTP at 2 weeks after CION surgery, when the hippocampal microglia were activated and anxiodepression-like behaviours were developed. Optogenetic activation of microglia directly impaired hippocampal LTP. On the contrary, optogenetic inhibition of microglia prevented the trigeminal neuralgia-induced LTP impairment, suggesting a key role of microglia in regulating hippocampal synapse plasticity.

It should be mentioned that although optogenetics have been widely used to manipulate neuronal activity to study the brain circuit functions, the optogenetic manipulation of glia has only been reported in recent years. ChR2 has been specifically expressed by transgenic

mice (*Iba1-tTA:tetOChR2(CS128S)-EYFP*) and depolarized by blue light (Tanaka et al., 2012). Specific expression red-activatable-channelrhodopsin (ReaChR), a newer generation of non-selective cation channels, in microglia was successfully achieved using *Cx3cr1^{creER/+};R26^{LSL-ReaChR/+}* mice and light activation of ReaChR in the spinal cord could trigger chronic pain by releasing IL-1 β (Yi, Liu, Umpierre, et al., 2021). Similarly, ChETA, a modified form of ChR2, was also selectively expressed in microglia using *Cx3cr1^{creER/+};R26^{LSL-ChETA-tdTomato/+}* mice and light activation of ChETA was also found to depolarize microglia (Laprell et al., 2021). In the present study, we adopted the same strategy and successfully expressed ChR2 or Arch specifically in microglia using *Cx3cr1^{creER/+};ChR2^{Flox}* or *Cx3cr1^{creER/+};Arch^{Flox}* mice. Immunostaining results showed that ChR2-tdTomato or Arch-EGFP was almost exclusively co-labelled with IBA-1. After optogenetic activation of microglia, the expression of IBA-1 was significantly increased, but no activating transcription factor 3 (ATF3) positive signal (a marker of damaged cells) was observed. Moreover, impaired hippocampal LTP induced by optogenetic activation of microglia could be blocked by IL-1 β inhibitor, IL-1 receptor antagonist, suggesting that light-activated microglia trigger the release of IL-1 β .

4.3 | Microglial P2X7 receptor signalling contributes to neuropathic pain and depression

P2X7 receptor is an ATP-gated, non-selective cation channel that is widely expressed in immune-associated cells, including macrophages, mast cells and microglia (Visentin et al., 1999). Both our study and other studies suggested that P2X7 receptor was preferentially expressed in spinal microglia and plays an important role in the communication between neurons and microglia (Clark et al., 2010; Yang et al., 2015; Zhou et al., 2010). Herein, we further demonstrated P2X7 receptor up-regulation in the hippocampal microglia in chronic pain with anxiodepressive status. Knockdown of P2X7 receptors prevented CION-induced increase in IBA-1 and anxiodepressive-like behaviours. Analogously, P2X7 antagonist BBG also reversed the microglial activation in cortical and hippocampal regions and the basal

FIGURE 6 P2X7 receptors (R) in hippocampal microglia participates in constriction of the infraorbital nerve (CION)-induced anxiodepressive-like behaviours. (a, b) Western blot analysis reveals significant up-regulation of P2X7 receptor level on Day (D) 14 after CION in the ipsilateral hippocampal CA1 area of rats. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 5$ rats for all the groups). (c) Immunofluorescence staining of P2X7 receptors, showing increased P2X7 receptor-positive signal in the ipsilateral hippocampal CA1 area on Day 14 after CION in rats. Scale bars indicate 100 μm . (d) Double immunofluorescence staining reveals that P2X7-immunoreactivity (IR) co-expressed with IBA-1 (microglial marker) but not GFAP (astrocytic marker) and NeuN (neuronal marker) in the hippocampus. Scale bars indicate 100 μm (low magnification) and 25 μm (high magnification). (e) ATP concentration in extracellular dialysate of the ipsilateral hippocampal CA1 area is significantly increased on Day 14 after CION. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 6/6$, sham/CION, rats/group). (f) Schematic of the protocol for experiments (g)–(m). (g) Schematic and photomicrograph of coronal section showing cannula placement in the bilateral and unilateral hippocampus. Ipsilateral or bilateral injections of A740003 (a P2X7 selective antagonist, 9 $\mu\text{g}\cdot\text{day}^{-1}$) into the CA1 area of the hippocampus by osmotic pump system lead to a significant anti-anxiodepressive effect in open field (OF; h, i), elevated plus maze (EPM; j, k) and forced swimming (FS; l) tests in CION rats, whereas administration of A740003 into the contralateral hippocampal CA1 fails to block the development of anxiodepressive-like behaviours. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 8/8/6/6$, vehicle/bilateral A740003/ipsilateral/contralateral A740003, rats/group). (m) Either ipsilateral or contralateral intra-CA1 of A740003 does not affect CION-induced mechanical allodynia. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 8/6/6$, vehicle/ipsilateral/contralateral A740003, rats/group). Error bars indicate standard error of the mean.

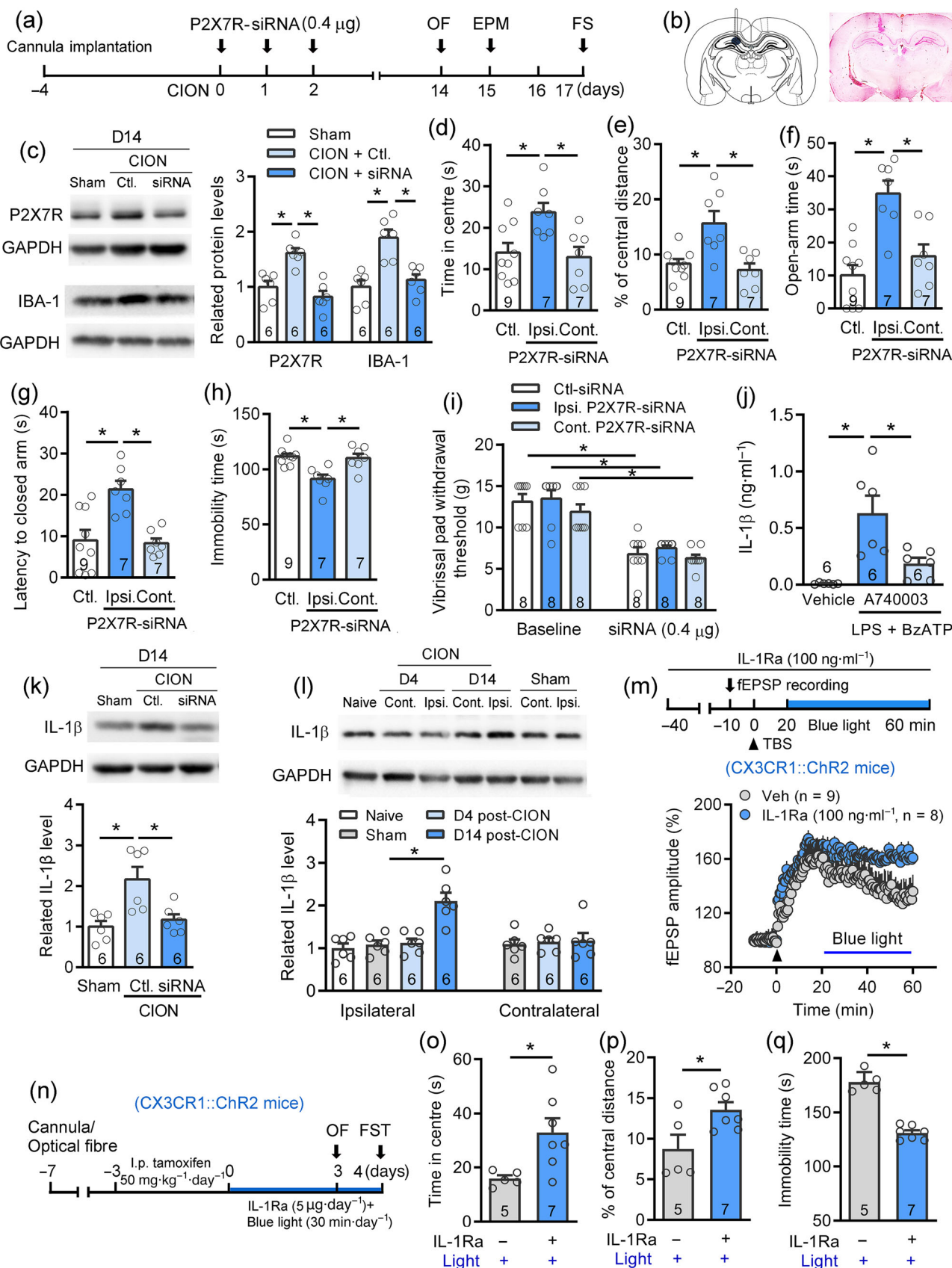


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nuclei of mouse brains in an unpredictable chronic mild stress (UCMS) model (Farooq et al., 2018; Yue et al., 2017). P2X7 selective antagonist, oxidized ATP (oxATP), was effective in markedly attenuating microgliosis (Monif et al., 2009). As the resident immune cells of central nervous system, microglia could initiate a pro-inflammatory response by detecting the up-regulation of damage-associated molecular patterns in the microenvironment (Falzoni et al., 2013; Janks et al., 2018). In recent years, activated microglia have been demonstrated to participate in the pathogenesis of multiple neuroinflammation-related diseases including depressive disorder by secreting IL-1 β and other inflammatory cytokines (Benatti et al., 2016; Hellwig et al., 2016; Tsuda, 2017). Activation of P2X7 receptors on murine and human neutrophils exacerbated inflammatory responses by NLRP3 inflammasome-dependent IL-1 β secretion (Karmakar et al., 2016). Our previous study has revealed that P2X7 receptor-mediated NLRP3 inflammasome assembly in hippocampal microglia contributes to unpredictable chronic mild stress-induced depression (Yue et al., 2017). The current study showed that CION-induced IL-1 β up-regulation in hippocampal CA1 area was blocked by P2x7r-siRNA. Microglial activation-induced anxiodepressive-like behaviours and hippocampal LTP impairment were prevented by IL-1 receptor antagonist. Taken together, it could indeed be authentic that P2X7 receptor mediated hippocampal microglial purinergic inflammatory responses leading to IL-1 β increase, which may be a pathogenesis of anxiodepression caused by trigeminal neuralgia (Figure 9).

4.4 | Asymmetric role of the bilateral hippocampus in trigeminal neuralgia-induced anxiodepression

The roles of the hippocampus in the pathogenesis of depression have been well established. However, most experimental observations and functional manipulation are directed at the bilateral hippocampus. Unconventionally, we found that long-term CION exposure resulted in microglial activation in the ipsilateral but not contralateral

hippocampus. CION-induced LTP impairment occurred only on the ipsilateral hippocampus. Pharmacological or optogenetic inhibition of ipsilateral hippocampal microglia significantly blocked trigeminal neuralgia-induced anxiodepressive-like behaviours. Optogenetic activation of unilateral hippocampal microglia is sufficient to evoke anxiodepressive-like states. These results suggest that the role of bilateral hippocampal CA1 area in trigeminal neuralgia-induced anxiodepression is asymmetric. As early as 1994, it has been reported that activation of the 5-HT_{1A} receptor in the right hippocampus rather than the left hippocampus produced anxiety (Belcheva et al., 1994). Delivering vasoactive intestinal peptide (VIP) into the left or bilateral hippocampus exhibited obvious antinociceptive effects in olfactory bulbectomy rats, a widely used animal model of depression, whereas delivering VIP into the right hippocampus has no effects (Belcheva et al., 2009). Recently, Hodaie's laboratory found that right-side trigeminal neuralgia patients have significant volumetric reductions in the ipsilateral CA1, CA4, dentate gyrus and hippocampus-amygdala transition area, compared with healthy controls (Vaculik et al., 2019). Our recent study in proteomic analysis found that differences in the biomolecules and signalling pathways between the ipsilateral and contralateral hippocampi 14 days after unilateral CION were evident. Neural Wiskott-Aldrich syndrome protein (WASL) that may play an important role in microglia activation (Sato et al., 2007) was up-regulated only in the ipsilateral hippocampal CA1 area (Guo et al., 2018). In addition, trigeminal ganglion (TG) sensory neurons have been revealed to provide a direct monosynaptic input to ipsilateral lateral parabrachial nucleus (PBL) and activating this ipsilateral TG \rightarrow PBL monosynaptic projection in awake animals can elicit robust aversive behaviours (Rodriguez et al., 2017). Thus, the ipsilateral projection of trigeminal ganglion neurons to the brain provides an anatomical basis for the connection between facial nociceptive inputs and emotion-related brain regions, which may partly explain why the ipsilateral hippocampus plays an important role in the trigeminal neuralgia-induced anxiodepression.

FIGURE 7 P2X7 receptor (R) mediates constriction of the infraorbital nerve (CION)-induced microglial activation and anxiodepressive-like behaviours via IL-1 β . (a) Schematic of the protocol for experiments (d)–(h). (b) Schematic and photomicrograph of coronal section showing cannula placement in the unilateral hippocampus. (c) Knockdown of P2X7 receptor by siRNA (0.4 μ g) blocks CION-induced P2X7 receptor and ionized calcium-binding adapter molecule 1 (IBA-1) up-regulation on Day (D) 14. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 6$ rats for all the groups). Knockdown of P2X7 receptors by siRNA in the ipsilateral but not contralateral hippocampus prevents CION-induced anxiodepressive-like behaviours in open field (OF; d, e), elevated plus maze (EPM; f, g) and forced swimming (FS; h) tests ($n = 9/7/7$, control siRNA/ipsilateral/contralateral P2X7R-siRNA, rats/group). (i) CION-induced mechanical allodynia of vibrissal pad is not blocked by both ipsilateral and contralateral intra-CA1 of P2X7R-siRNA. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 8$ rats for all the groups). (j) ELISA analysis reveals that P2X7 agonist BzATP (100 ng·ml^{−1}) significantly increases LPS (100 ng·ml^{−1})-stimulated IL-1 β level in hippocampal slices and A740003 blocks the IL-1 β elevation. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 6$ slices for all the groups). (k) Western blot analysis reveals that CION-induced IL-1 β up-regulation is blocked by knockdown of P2X7R on Day 14. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 6$ rats for all the groups). (l) Western blot analysis reveals significant up-regulation of IL-1 β level on Day 14 after CION in the ipsilateral hippocampal CA1 area of rats. * $P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls test ($n = 6$ rats for all the groups). (m) Microglial activation-induced hippocampal LTP impairment is reversed by IL-1 receptor antagonist (IL-1Ra) in CX₃CR1::ChR2 mice ($n = 9/8$, vehicle/IL-1Ra, slices/group). (n) Schematic of the protocol for experiments (o)–(q). (o–q) Injections of IL-1Ra (5 μ g·day^{−1}) ipsilaterally into the CA1 area of the hippocampus block optogenetic activation of microglia-induced anxiodepressive-like behaviours in CX₃CR1::ChR2 mice. * $P < 0.05$, two-tailed Student's t test ($n = 5/7$, vehicle/IL-1Ra, mice/group). Error bars indicate standard error of the mean.

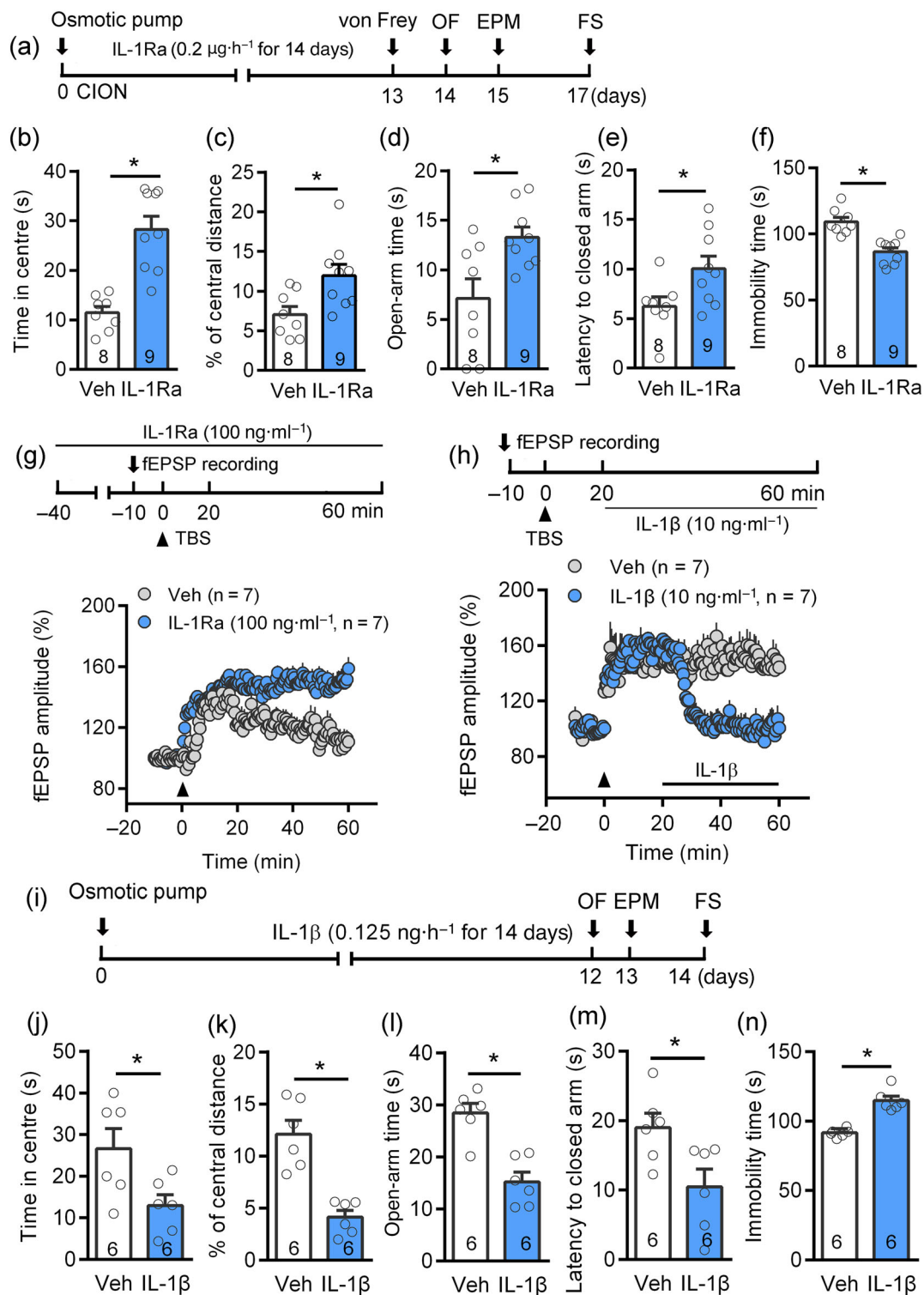


FIGURE 8 IL-1 β is involved in constriction of the infraorbital nerve (CION)-induced anxiodepressive-like behaviours. (a) Schematic of the protocol for experiments (b)–(f). Intra-CA1 of IL-1 receptor antagonist (IL-1Ra; an inhibitor of IL-1 β receptor, 0.2 $\mu\text{g}\cdot\text{h}^{-1}$) by osmotic pump system significantly blocked CION-induced anxiodepressive-like behaviours in open field (OF; b, c), elevated plus maze (EPM; d, e) and forced swimming (FS; f) tests in CION rats. * $P < 0.05$, two-tailed Student's t test ($n = 8/9$, vehicle/IL-1Ra, rats/group). (g) Perfusion of IL-1Ra (5.7×10^{-9} M) reverses CION-induced hippocampal LTP impairment in CION mice ($n = 7/7$, vehicle/IL-1Ra, slices/group). (h) Perfusion of IL-1 β (5.8×10^{-10} M) obviously impairs the hippocampal LTP of naive mice ($n = 7/7$, vehicle/IL-1 β , slices/group). (i) Schematic of the protocol for experiments (j)–(n). Intra-CA1 of IL-1 β (0.125 ng·h⁻¹) by osmotic pump system directly results in anxiodepressive-like behaviours in OF (j, k), EPM (l, m) and FS (n) tests in naive rats. * $P < 0.05$, two-tailed Student's t test ($n = 6/6$, vehicle/IL-1 β , rats/group). Error bars indicate standard error of the mean.

Depression and anxiety

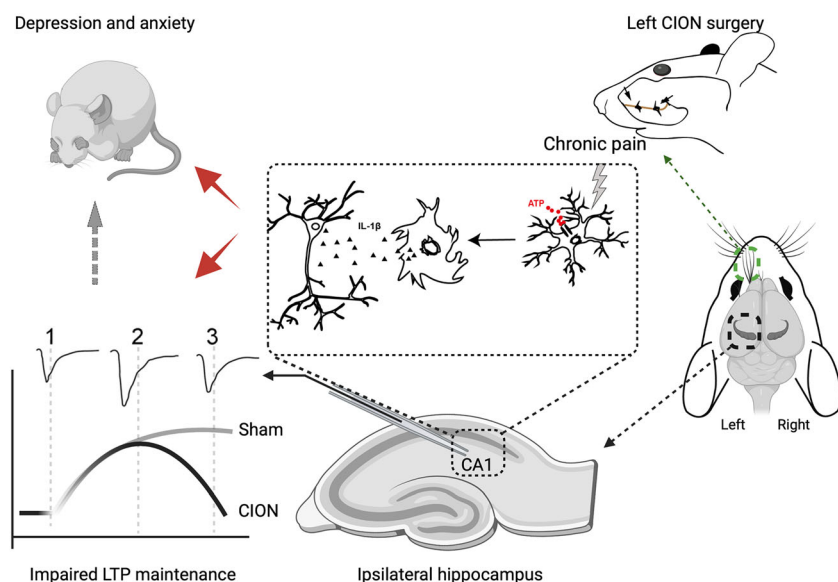


FIGURE 9 A schematic illustration of probable mechanisms for microglial activation in the ipsilateral hippocampal CA1 area impairing LTP and leading to anxiodepressive consequences of trigeminal neuralgia. Increased extracellular ATP acts on P2X7 receptors on microglia and activates microglia. Activation of microglia results in increased IL-1 β release, leading to LTP impairment and anxiodepressive-like behaviour.

5 | CONCLUSION

In summary, using genetic and pharmacological bidirectional manipulation of microglial activity in the hippocampal CA1 area, we found that ipsilateral activation of microglia is necessary for trigeminal neuralgia-induced anxiety and depression, and unilateral activation of hippocampal microglia is sufficient to induce an anxiodepressive state in rodents. Microglial purinergic inflammatory responses and the neuronal plasticity changes in the ipsilateral hippocampal CA1 area may be a key pathogenesis of anxiodepression caused by trigeminal neuralgia. The approaches targeting microglia and P2X7 receptor signalling might offer novel therapies for trigeminal neuralgia-related anxiety and depressive disorder.

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AUTHOR CONTRIBUTIONS

Y-QZ conceived the project and supervised all the experiments. L-QC, X-JL and Q-HG performed the behavioural experiments. L-QC and S-SL performed the electrophysiological recording. L-QC and X-JL performed the immunohistochemistry and optogenetic experiments. L-QC and Q-HG performed the ELISA experiment. Q-HG performed the microdialysis and western blotting experiments. L-QC, W-DX and

Y-QZ analysed the data. L-QC, JY and Y-QZ designed the experiments. L-QC and Y-QZ wrote the manuscript. All the authors read and discussed the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design & Analysis](#), [Immunoblotting and Immunochemistry](#) and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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