FKN/CX3CR1 Axis Facilitated Migraine-like Behaviors via Activating Thalamic-cortical Network Microglia in SE Rat Models

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Abstract

Background: The incidence of migraines is higher among people with epilepsy than healthy people, and these two common diseases are proposed to have some shared pathophysiological mechanisms. Excitation/inhibition imbalance plays an essential role in the comorbidity of epilepsy and migraine. Microglia activation is crucial for abnormal neuronal signal transmission. However, whether and how microglia are activated, and their role in comorbidities after activation remains unclear. This study aimed to explore the characteristics and mechanism of microglia activation after seizures and its effect on migraine.

Methods: Status epilepticus (SE) rat models induced by lithium chloride (LiCl)-pilocarpine intraperitoneal injection and migraine rat models induced by repeated inflammatory soup (IS) dural injections were generated and assessed for molecular and histopathologic evidence of microglial activation target of fractalkine (FKN) signaling. HT22-BV2 transwell coculture was used to explore the interaction between neurons and microglia. LPS (a microglia agonist) and FKN stimulation of BV2 microglia cells were used to evaluate changes in BDNF content after microglia activation.

Results: Microglia were specifically hyperplasia and activation in the cortical-thalamus-sp5c neural circuit, which were pain-related brain regions, accompanied by the upregulation of FKN and CX3CR1 four days after seizures. Meanwhile, SE-induced increased nociceptive behavior and the FKN/CX3CR1 axis in migraine rat models. AZD8797 (a CX3CR1 inhibitor) prevented the worsening of hyperalgesia and microglia activation in migraine rat models after seizures, while FKN infusion in migraine rat models exacerbated hyperalgesia and microglia activation associated with BDNF-Trkb signaling. Furthermore, in neuron-BV2 coculture, microglial activation and FKN/CX3CR1/BDNF/iba1 expression were increased. Activating microglia with LPS and FKN stimulation increased BDNF synthesis in BV2 microglia.

Conclusions: Our results indicated that epilepsy facilitated migraine through the cortical-thalamus-sp5c microglia activated and interactions with neurons by the FKN/CX3CR1 axis, resulting in BDNF release. Blocking the FKN/CX3CR1 axis and microglia activation are potential therapeutic targets for preventing and treating migraine in patients with epilepsy.

Introduction

Migraine and epilepsy are among the most frequently encountered diseases of the nervous system. A large number of studies have highlighted the complex relationship between migraine and epilepsy. Patients with epilepsy have a 52% greater lifetime prevalence of migraines than those without epilepsy [3]. The underlying mechanisms are linked to many factors, such as the excitatory/inh6ibitory imbalance in the brain[37]. Neuronal excitability abnormalities exist in the cortex and thalamus of both epileptic and migraine patients[17, 34]. This suggests that the thalamic-cortical network may play some role in their comorbidity.

Human resting-state functional magnetic resonance imaging (fMRI) has exposed diverse connections between the thalamus and cortex [5]. Epilepsy originates from abnormal dynamics of neuronal networks. The sudden discharge of action potentials during epileptic seizures increases neural synchronization and enhances the rhythmic network activity of the cortex and thalamus[22]. In migraine, cortical spreading depression (CSD) and trigeminal neurovascular theory have been implicated in the pathogenesis. Numerous studies have suggested that the trigeminal neurovascular system changes may lead to neuronal abnormalities in genetically susceptible individuals directly related to thalamic cortical rhythmicity[9]. Rhythmic cortical feedback to the thalamus is significant in amplifying thalamocortical activity, making it a strong candidate for influencing neuronal excitability[17].

Microglia and neuron–microglia interactions play a crucial role in the central nervous system characterized by altered neural network excitability[15]. Numerous studies have indicated that microglia activation is associated with epileptogenesis and migraine attacks[11, 18]. Fractalkine is a chemokine expressed by both neurons and glia, while its receptor, CX3CR1, is primarily expressed on microglia[6]. Given the distinct expression of fractalkine and its receptor in neurons and microglia, reducing the FKN/CX3CR1 axis is sufficient to affect some physiological processes[27]. Recent studies have shown that FKN signaling is associated with epilepsy and neuropathic pain[12, 31]. Brain-derived neurotrophic factor (BDNF), including a precursor molecule (proBDNF) and a mature form (BDNF), is mainly expressed in the central nervous system, is released by neurons and microglia, and plays a crucial role in learning and memory formation[32]. BDNF is involved in neuroinflammation and participates in many nervous system diseases[24]. Some studies indicate that activated microglia can release increased amounts of BDNF[25]. This suggests that BDNF may be involved in the comorbidities of epilepsy and migraine.

Our research aimed to investigate whether microglia and their interactions with neurons by the FKN/CX3CR1 axis in the thalamic cortical network are involved in the comorbidities of epilepsy and migraine by regulating the expression BDNF. Here, we used a comorbidity model of epilepsy and migraine established by our previous research[13]. The migraine and epilepsy relevant behavioral and molecular biological alterations were investigated in the cortex-thalamus-trigeminal caudate nuclear circuit.

Materials And Methods

Animals

Adult male Sprague–Dawley rats weighing between 200 and 250 g were procured from the Laboratory Animal Center of Renmin Hospital, Wuhan University. All rats were housed under specific pathogen-free (SPF) laboratory conditions with a 12-h light/dark cycle (lights on at 8:00 A.M., humidity 60 ± 5%, temperature 22 ± two °C), ad libitum food, and water. After one week acclimatized, animals were randomly assigned to different groups. The investigators were blinded to the group allocation during the animal experiments. The protocol was reviewed and approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. Animal studies were performed in compliance with the ARRIVE guidelines and the Basel Declaration, including the “3R” concept[21].

Status epilepticus induction and seizure quantification

The induction of status epilepticus using pilocarpine was performed as previously described[23]. Experimental rats were injected with lithium chloride (125 mg/kg, i.p. Sigma-Aldrich, St. Louis, MO). 18 to 24 hours later, atropine sulfate (1mg/kg, i.p. Beijing reagent) was administered to antagonize the peripheral cholinergic effect. Thirty minutes later, SE was induced by injecting pilocarpine hydrochloride (350mg/kg, i.p.; Sigma). Pilocarpine hydrochloride was given repeatedly (10mg/kg, i.p.) every 30 minutes until the rats developed seizures. Sham rats received the same treatment with lithium chloride and atropine sulfate, but an equivalent amount of normal saline replaced pilocarpine.

Seizure behavior was scored by the modified Racine scale with the following stages: (0) no abnormality, (1) mouth and facial movements, (2) head nodding, (3) forelimb clonus, (4) rearing, (5) rearing and falling[29]. Only rats progressing to stages four or more were selected.

Inflammatory Soup dural Injections and nociceptive behavioral tests

The experimental procedures were carried out as previously reported in our laboratory[13]. After the skull was exposed, a 1 mm hole in the middle of the superior sagittal sinus (between bregma and lambda) was made with a precooled dental drill (DH-0 Pin Vise, Plastics One) to expose the dura carefully. A 0.5 mm guide cannula (22 GA, #C313G, Plastics One) was inserted into the hole and sealed into place with a mixture of dental cement powder and superglue. A dummy cannula (#C313DC, Plastics One Inc.) was inserted to ensure the patency of the guide cannula. The skin incision was closed with a silk suture. After one day of recovery from the surgery, 30 μl IS (1 mM bradykinin, serotonin, and histamine and 0.1 mM prostaglandin E2 or two mM bradykinin, serotonin, and histamine and 0.2 mM prostaglandin E2; pH 5.5) was delivered from the annular tubes to stimulate the dural once a day for four days.

The nociceptive behavior was assessed by head-scratching numbers, and the facial mechanical sensitivity threshold of a von-Frey filament was measured by the Dixon "up-and-down" method[2]. Testing began with the 1gram (g) filament on the face and increased weight until a rat quickly retracted its head. Mechanical thresholds were detected after the last IS dural infusion. The head-scratching numbers were recorded for one hour after the fourth IS administration. All investigators were blinded to experimental conditions.

Drug administration

AZD8797 administration was carried out as described previously[7]. AZD8797 (HY-13848, MCE, USA) was dissolved in a DMSO solution to yield a final concentration of 2 mg/ml according to the instructions. Rats received either an intraperitoneal injection of AZD8797 (1mg/kg) or an equal amount of PBS once a day for four days before IS dural administration.

Rat Fractalkine (PeproTech, USA) was dissolved in 0.9% NaCl to yield a final concentration of 1ug/ul; bilateral intracerebral injection of fractalkine was performed stereotactically to TNC at coordinates of 14.08 mm, lateral 2.75 mm, and ventral 8.65 mm relative to the bregma in the rat. A total of 2.5 ul was

injected into each site using a 5 μL glass syringe with a fixed needle[1]. At the same time, the sham groups received 2.5 ul of 0.9% saline at each site.

Cell Cultures and drug treatments

BV2 mouse microglial cell line ICLCATL03001 (kindly provided by the emergency department Laboratory, Renmin Hospital of Wuhan University) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 incubator at 37°C. BV2 cell cultures were activated by LPS (1μg/ml, MCE, USA) for 90 minutes[19]. To examine the influence of FKN on microglia and the role of microglial cell release and synthesis of BDNF, we also give 0.1mg/ml FKN (PeproTech, USA) to the cell cultures for 90 minutes according to the morphological changes of BV2 cells.

HT22 mouse hippocampal neuronal cells were purchased from Wuhan Procell Century Technology. They were cultured in DMEM medium, supplemented with 10% FBS, 100 U/ml penicillin, and 100U/ml streptomycin at 37°C in a humidified environment containing 5% CO2.

Neuron-BV2 microglia transwell coculture

Neuron-BV2 microglia transwell cultures were used to investigate the role of neuron-mediated FKN on microglia. BV2 microglial cells were plated into 6-well dishes (106 cells/well). HT22 mouse hippocampal neurons were plated (5x105 cells/well) in the transwell chambers (0.4um, Cell Biolabs, Inc.), and these inserts were placed on top of the wells containing the microglia culture. After 24 h of coculture, the morphologic change of microglia was observed. Microglia on the lower membrane were collected to test related protein content.

Western blot

The rat brain tissue or cell samples were lysed in RIPA buffer containing phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail as previously described. Lysates were centrifuged and collected. Total protein concentration was determined using a BCA Protein Assay Kit (Beyotime). An equal amount of protein ranging from 15 to 30 μg total protein was separated by SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore). The following antibodies were used: rabbit anti-CX3CR1 antibody (1:3000, Abcam), goat anti-CX3CL1 antibody (1:200, Abcam), rabbit anti-BDNF antibody (1:3000, Abcam), rabbit anti-GAPDH antibody (1:3000, Service), goat anti-iba1 (1:500, Woko). Protein bands were visualized using a chemiluminescence system (ChemiDocTM XRS+, BioRad). The protein expressions were semi-quantitatively analyzed with ImageJ software.

Immunohistochemistry and Immunofluorescence

Rats were anesthetized and transcardially perfused with 4% paraformaldehyde. 20-μm-thick coronal sections were cut through the midbrain at the thalamus or caudal medulla level and the upper cervical spinal cord at the sp5c level. Microwave heating performed deparaffinization, gradient alcohol dehydration, and antigen repair on paraffin sections were deparaffinized and rehydrated. Then, 3% H2O2

was used to reduce nonspecific background staining caused by endogenous peroxidase. After 20 minutes of incubation with 2% Triton X-100 and 1 hour of blocking with 3% bovine serum albumin (BSA), the sections were incubated overnight at 4°C with rabbit-anti-cx3cr1 (1:500, Abcam), goat-anti-cx3cl1 (1:100, R & D), goat-anti-iba1 (1:500, Woko), and mouse-anti-Neun (1:200, Abcam, England). For the Immunohistochemistry (IHC) experiment, the signal was developed following the manufacturer's instructions for the High-Efficiency IHC Detection System Kit (sharp, Wuhan China). For the Immunofluorescence experiment, the sections were incubated with the corresponding florescent-labeled secondary antibodies (FITC or CY3-labeled donkey anti-goat IgG, FITC-labeled donkey anti-rabbit IgG, CY3-labeled donkey anti-mouse) and examined under a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

Immunocytochemistry staining

Microglia cells were fixed in 4% paraformaldehyde and 0.1% Triton X-100 for 15 min. The cells were washed with PBS, blocked with 3% BSA for 30 min, and incubated with goat-anti-iba1 (1:500, Woko) and rabbit-anti-BDNF (1:400, Abcam) antibodies overnight at 4℃. After washing with PBS and incubation with Alexa Fluor 594-conjugated anti-goat and Alexa Fluor 488-conjugated anti-rabbit for two hours at room temperature in the dark, the sections were washed with PBS followed by nuclear staining with DAPI. After a brief rinse in PBS, the sections were then examined under a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

Sholl analysis

The complexity of microglia was analyzed by setting a group of sequential concentric circles covering the cells in the ImageJ software. The innermost circle (35μm radius of activated microglia and 21μm radius for resting microglia) is located right in the nucleus. The other circles were arranged following a step length of 10μm. The diameter of the outermost circles is adaptive according to each cell. Then, the number of intersections was counted at individual concentric circles.

Statistical analysis

Statistical analyses were performed with SPSS version 22.0 and GraphPad Prism 8.0. Unpaired Student's t-tests were used to compare the means of two groups. One-way ANOVA followed by Bonferroni post-hoc tests was used for comparisons among three or more groups. All data were presented as the mean ± SEM. The results were considered significant at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, ****$p<0.0001$.

Results

1. SE increased the nociceptive behavior in migraine rat models

To investigate the impact of seizures on migraine, we established a comorbidity model for migraine and epilepsy as we previously described18. In brief, the repeated inflammatory soup was injected into the dura mater of the lithium chloride (LiCl)-pilocarpine rat models once a day for four consecutive days (Fig. 1A). Consistent with our previous results, the comorbidity group showed significantly higher head-scratching and markedly decreased von-Frey filament forces than the migraine group without pilocarpine (Fig. 1B-C). The immunostaining and protein level of cfos, an immediate early gene product which is usually used as a marker of neuronal activation, were also increased in the sp5c of the comorbidity group (Fig. 1D-G).

2. The number of and activation of microglia was boosted in the cortex-thalamus-sp5c neural circuit after status epilepticus

Gliosis is considered a molecular hallmark of status epilepticus[14]. Previous studies have demonstrated that microglial activation occurs both in epilepsy and migraine. To explore microglia changes after epileptic seizures and whether these changes are associated with the occurrence of migraine, the immunohistochemistry of iba1, a marker of microglia, was performed to assess microgliosis. Notably, the number of microglia showed a specific selectivity increased along the trigeminovascular pathway, which is related to the pathogenesis of migraine, comparison between sham group and SE group. These sites include the temporal lobe cortex, paraventricular thalamic nucleus (PV), laterodorsal thalamic nucleus, ventrolateral part (LDVL), ventromedial thalamic nucleus (VM), and spinal trigeminal nucleus, caudal part (sp5C, Fig. 2A-C). Moreover, Sholl analysis of microglia showed that the SE group has a larger cell body, more synapses, and shorter branches (Fig. 2D-E). These indicated that the complexity and activation of microglia were increased after seizures. To further verify the expression of microglia in the cortex-thalamus-sp5c neural circuit, we also measured levels of iba1 protein (Fig. 2F-G). The results were consistent with immunohistochemistry. The expressions of Iba1 in the SE group were higher than in the sham group.

3. Epileptic seizures increased FKN/CX3CR1 expression in cortex-thalamus-sp5c of migraine rat models

To determine whether microglial activation was associated with the FKN/CX3CR1 axis, we measured the protein level of FKN and CX3CR1 in the temporal cortex, thalamus, and sp5c of SE rat models four days after the seizure (Fig. 3A). Both the expression of CX3CR1 and FKN was increased in cortical-thalamus-sp5c (Fig. 3B-C). To further explore the relationship between epilepsy and migraine, western blot evaluation of sp5c tissues in the comorbidity rat models was also conducted. Compared with the control group, the expression of CX3CR1/FKN was increased in the migraine group and further increased in the comorbidity group (Fig. 3D-E). The sp5c area from the sham group, SE group, and comorbidity were examined by immunofluorescence staining. With Iba1 immunoreactivity serving as a marker for microglia, a remarkable visual overlapping between iba1 and CX3CR1 immunoreactivities was commonly

observed in the sp5c sections. CX3CR1 was mainly expressed in the membrane of microglia (Fig. 3F). Immunoreactivities of Iba1 and CX3CR1 were significantly increased in the migraine group and further increased in the comorbidity group compared to the sham group (Fig. 3H-I). Correspondingly, FKN largely co-localized with Neun, a marker for neurons, significantly increased in the migraine group and further increased in the comorbidity group compared to the sham group (Fig. 3G, J). FKN was mainly expressed in the membrane and cytoplasm of neuron cells (Fig. 3G).

4. Neuron activated microglia by releasing FKN and increased BDNF synthesis in HT22-BV2 coculture cell

To determine the relationship between activation of microglia and the FKN/CX3CR1 axis, we indirectly cocultured BV2 microglia cells in transwell plates with HT22 neuron cultures. In the transwell coculture system, the neurons and microglial cells shared the same medium, but no direct cell-cell interactions were possible (Fig. 4A). After 24 hours of coculture, the protein levels of underlying BV2 cells were measured by western blot. The content of CX3CR1/FKN/proBDNF/BDNF/iba1 was remarkably increased compared with simple BV2 cell lines (Fig. 4B-C). To investigate whether the change of proBDNF/BDNF content is related to microglial activation, we studied microglia in BV2 cells. Lipopolysaccharide (LPS) is a recognized activator of microglia. The cells were treated with 0.1mg/ml LPS for 90 minutes according to the morphological changes observed to activate BV2 cells, while the control BV2 cells were given a matching vehicle (VEH). Immunofluorescence staining showed a high cell co-localization between iba1(red) and BDNF (green) (Fig. 4D). Meanwhile, we measured proBDNF/BDNF secreted from BV2 cells into the supernatant by western blot. Compared with resting microglia, the content of proBDNF/BDNF secreted by activated microglia was significantly increased (Fig. 4E-F). To further determine whether microglial activation and BDNF level upregulation are caused by FKN released by neurons in the upper layer, we also stimulated BV2 microglia with 0.1mg/ml FKN for 24 hours according to BV2 morphologic change. The results were in line with LPS stimulation: FKN treatment increased the expression of proBDNF/BDNF (Fig. 4G-H).

5. AZD8797 treatment reduced nociceptive behavior and proBDNF/BDNF/Iba1 expression in migraine rat models after SE

AZD8797 is one of the most commonly used antagonists of CX3CR1[7]. To further explore the effects of the FKN/CX3CR1 axis on nociceptive behavior after an epileptic seizure, AZD8797 was injected into the SE rat models once a day for four days before IS dural administration to inhibit the FKN/CX3CR1 axis (Fig. 5A). AZD8797 reversed the decrease in pain threshold in comorbidity rats (Fig. 5B). Meanwhile, AZD8797 reduced the increase in head-scratching in migraine rat models after SE (Fig. 5C). Iba1 immunofluorescence staining showed that AZD8797 reversed epileptic microglia activation and

increased in number (Fig. 5D). We also detected the protein expression of proBDNF/BDNF/Iba1 by western blot. AZD8797 reduced the protein levels compared to the comorbidity-PBS group (Fig. 5E-F).

6. FKN infusion increased nociceptive behavior as well as proBDNF/BDNF/Iba1 expression in migraine rat models

To further investigate the role of the FKN/CX3CR1 axis in migraine rat models after a seizure, we also injected FKN into sp5c of migraine rat models using stereotactic brain injection while the sham groups received the same amount of PBS. The experimental procedure is shown below (Fig. 6A). Consistent with the post-epileptic migraine model, the mechanical pain threshold of the PBS-migraine group was decreased and decreased further in the FKN-migraine group compared with the PBS-sham group (Fig. 6B). Meanwhile, FKN infusion increased the head-scratching numbers of migraine rat models (Fig. 6C). Iba1 immunofluorescence showed that FKN infusion activated microglia of SP5C in migraine rats (Fig. 6D). The protein levels of proBDNF/BDNF/iba1 further increased in the FKN-migraine group (Fig. 6E-F). These results indicated that FKN infusion might have the same effect as epileptic seizures in the migraine rat models.

Discussion

In line with the hypothesis, the current study supports the idea by showing that neurons activate microglia in the cortical-thalamic-sp5c neural circuit via the FKN/cx3cr1 axis, which leads to enhanced BDNF production in migraine rat models following an epileptic episode. Furthermore, the FKN/CX3CR1 axis intervention regulated microglia activation, proBDNF/BDNF expression, and nociceptive behavior in the migraine rat models exposed to seizures. These findings add to our understanding of a pathophysiological process and a possible preventative target for migraine after seizures.

Due to high neuronal excitability and neuronal damage in epilepsy, we focus on the interaction between the neurons and microglia. In the CNS, neurons send signals to nearby astrocytes and microglia cells, which become activated and can sustain neuroinflammation under pathological conditions[37]. FKN/CX3CR1 plays an indispensable role in neuronal microglia's communication[27]. There is a monogamous association between FKN and CX3CR1[8]. Numerous studies have revealed that improved fractalkine signaling plays a role in the epil eptic brain. In patients with TLE, immunoreactivity and protein levels of CX3CL1 were increased in the temporal neocortex and hippocampus compared to non-epileptic autopsy controls[16]. In animal models of epilepsy, FKN levels increase 1-3 days after seizures, but FKN expression three days later is controversial. Yeo et al show that FKN decreases after three days[36]. whereas Wu et al believe that FKN continues to rise after three days but peaks at 72 hours and remains relatively high for the next 60 days[35]. In our study, FKN levels at four days after seizure were still higher than those in the control group (Fig. 3A, C). The difference may be due to the location of the measurement. With either analysis, it should be emphasized that FKN plays a role in the pathophysiological mechanism of epilepsy. In agreement with the previous study, the immunoreactivity

and protein levels of CX3CR1 remained higher than control at four days after SE (Fig. 3A-B). Furthermore, our study first revealed that SE increased the FKN/CX3CR1 axis expression in migraine rat models (Fig. 3D-E). This may indicate that activated microglia and elevated FKN/CX3CR1 after a seizure make the epileptic brain hypersensitive and are more likely to cause migraine headaches in an externally stimulated or hyperimmune environment compared to a healthy brain. To our knowledge, we are the first group to link FKN signaling to post-seizure migraine. Our study provides a new target for the treatment and prevention of migraine after epileptic seizures.

Otherwise, even more unexpected is that the activation and proliferation of microglia after epileptic seizures are region-specific, mainly concentrated in the cortex, thalamic nucleus and sp5c (Fig. 2A), which are closely related to pain attacks. Consistent with our results, a recent study reported that mean iba1 labeling in all brain regions was significantly higher in patients with human epilepsy. Espiceally when epilepsy causes sudden and unexpected death, the medial thalamic nucleus and superior temporal gyrus alternative increased[30]. Furthermore, another study that used translocator protein-targeted molecular imaging found microglial activation in the hippocampus, thalamus, and piriform cortex in LiCl-pico induced SE rat models[4]. Compared to these studies, our study is more specific in describing the site of activation and proliferation of microglia in the thalamus after epileptic seizures. At the same time, we were the first to pay attention to the changes of microglia in sp5c after epileptic seizures. The thalamus acts as the main gate, filtering and processing sensory inputs from sensory and associative neocortical projections[8]. This means the thalamus plays a liaison role in seizures and migraines. During a migraine attack, the major suppression of depolarization occurs in the cortex. The thalamus monitors cortical signal changes and sends them to peripheral sensory areas such as the trigeminal nerve[26]. Microglia monitor changes in the thalamic cortical network and make adaptive adjustments, such as releasing inflammatory factors, which in turn worsen seizures and migraines. Notably, in our study, the microglia in cortical and thalamic nuclei showed apparent aggregation and proliferation, while the sp5c only had an increase in number without microglia recruitment. Maybe epilepsy patients can trigger a migraine only activated microglia in sp5c reached a specific number. This might explain why not all epilepsy patients suffer from migraines.

Our previous studies have shown that BDNF is involved in comorbidities of epilepsy and migraine[13]. This study further proves that BDNF is mainly synthesized and released by microglia (Fig. 4D). Activation of microglia boosted the expression of proBDNF/BDNF in BV2 cells (Fig. 4E-F). When combined with our findings of post-epileptic microglial activation in a rat model, it is reasonable to speculate that microglia-released BDNF plays a vital role in developing post-epileptic migraine. Support our speculation, blocking CX3CR1 can lower migraine nociception and down-regulate BDNF production and release (Fig. 5B-F). In contrast, stereotaxic injection of FKN into sp5c is consistent with the behavioral and molecular changes in the rat model of migraine after seizures. Meanwhile, we discovered that neurons could interact with microglia by releasing FKN to promote microglia activation and the synthesis and release of proBDNF/BDNF in the HT22-BV2 coculture cell (Fig. 4A-C). These results suggest that FKN can mimic the effect of epileptic seizures on migraine to some extent suggesting that hyperexcited neurons after epileptic seizures may interact with microglia through the FKN/CX3CR1 axis to promote migraine.

While the present study has provided evidence that modulating microglia activation can affect epileptic seizure-induced migraine-relevant phenotypes, it has limitations that must be acknowledged. First, microglia may play a bipolar role in the pathogenesis of epilepsy. Wu et al. discovered that microglial depletion aggravates the severity of acute and chronic seizures in Cx3cr1-KO mice[33]. Christopher Käufer also proved that the lack of CX3CR1 receptors did not prevent seizure development after viral CNS infection in Cx3cr1-KO mice[20]. This suggests that complete ablation of microglia can also impair the development and function of the central nervous system. It also suggests that using large doses of FKN/CX3CR1 inhibitors in the comorbidity of epilepsy and migraines may not alleviate migraines but worsen seizures. Therefore, it is still necessary to further explore the concentration and use method of FKN/CX3CR1 inhibitors in clinical and animal experiments. Second, only male rats were included in the analysis of neural mechanisms. Sexual dimorphism in FKN and BDNF in the central nervous system has been suggested in some studies[10, 28]. It is now generally accepted that migraines are more common in women. The role and neural function of FKN in female migraine still need to be further investigated.

In summary, our study deepens people's perception of microglia in epilepsy, fills the gap in the research of microglia in the comorbidity of epilepsy and migraine, and gives a new thought for managing migraine sufferers after epilepsy.

Abbreviations

SE: Status epilepticus

LiCl: lithium chloride

IS: inflammatory soup

FKN: fraction

FKN: Fractalkine

fMRI: functional magnetic resonance imaging

CSD: cortical spreading depression

BDNF: Brain-derived neurotrophic factor

proBDNF: precursor molecule Brain-derived neurotrophic factor

VEH: vehicle

Declarations

Ethics approval and consent to participate

Ethics approval and consent to participate was obtained.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analyzed in the present study are available from the corresponding author on reasonable request.

Competing interests

The authors have no conflicts of interest to declare.

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Authors' contributions

YJ. Zhou designed the experiments and wrote the paper. YJ. Zhou, YY, Hao and L.Y performed the experiments and analyzed data. YJ. Zhou and LL. Zhang wrote the manuscript. ZM. Xiao guided the completion of this article. All authors read and approved the final manuscript.

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Figures

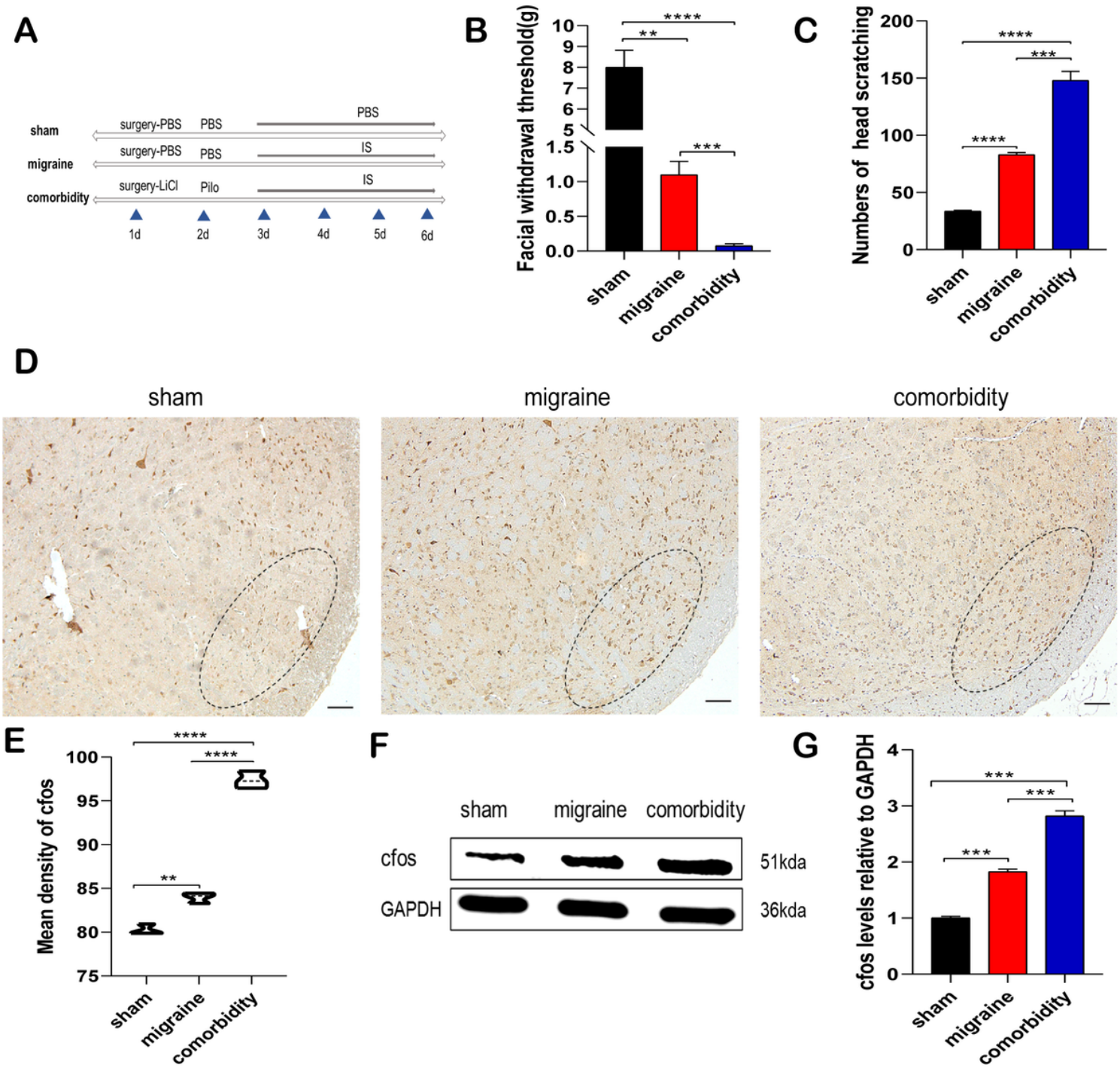

Figure 1

SE-induced increase in nociceptive behavior in migraine rat models. (A) Flow chart of the experimental groups for sham, migraine, and comorbidity. (B) The mechanical threshold in the migraine group was decreased compared to the sham group and further decreased in the comorbidity group. n = 6 rats per group; F (2, 15) = 127.727, P < 0.001, one-way ANOVA. (C) The number of head-scratches in the migraine group was increased compared to the sham group and further increased in the comorbidity group. F (2, 15) = 235.728, P < 0.001, one-way ANOVA; n = 6 rats per group. (D) Representative immunohistochemical staining of cfos in the sp5c. Scale bars=100μm. (E) The mean density of cfos was increased in the

migraine group and further increased in the comorbidity group. F (2, 15) = 325.358, P < 0.001, one-way ANOVA; n= 3 rats per group(F) Representative Western blot of cfos in the sp5c. (G) The protein levels of cfos were increased in the migraine group and further increased in the comorbidity group. F (2, 15) = 456.393, P 0.001, one-way ANOVA; n= 3 rats per group.

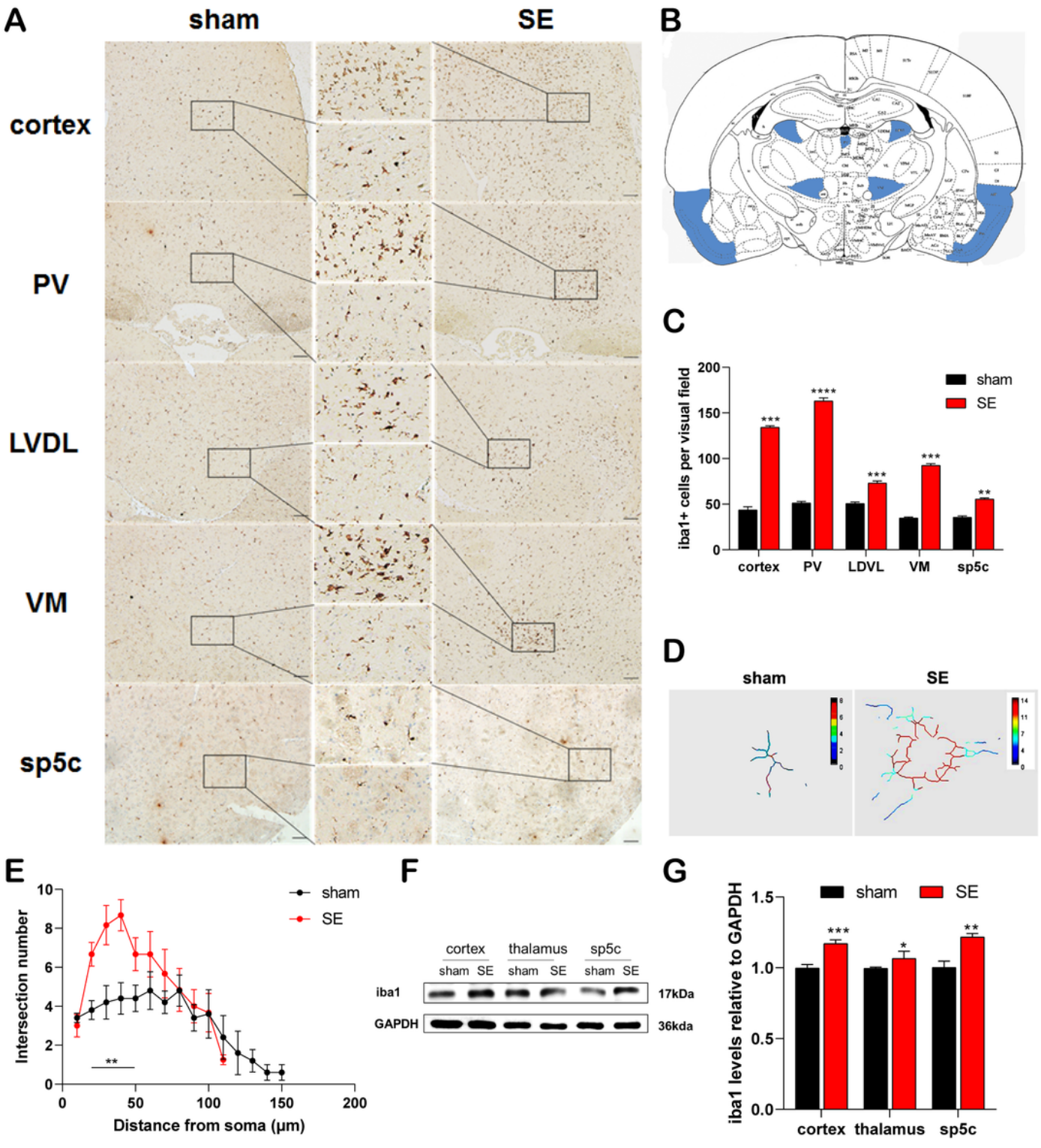

Figure 2

SE-induced increase in microglial activation and amount in the cortex-thalamus-sp5c neural circuit in adult rats. (A) Representative immunohistochemical staining of iba1 in the cortex, PV, LDVL, VM, and sp5c. Scale bars=100μm. (B) Iba1 positive (iba1+) area in different brain regions. (C) Quantification of the number of Iba1+ cells. n= 3 rats per group. **p< 0.01, ***p< 0.001, ****p<0.0001 compared to the sham group. (D-E) Sholl analysis of microglia showed that SE-induced branch shortening and synapses increased compared to the sham group. n = 6–11 per group; **p < 0.01, unpaired t-test versus sham group.(F) Western blot analysis of iba1 in the cortex, thalamus, and sp5c.(G) Western blot showing the protein levels of iba1 in the cortex-thalamus-sp5c neural circuit were increased compared to the sham group. n= 3 rats per group. *p <0.05, **p < 0.01, ***p<0.001 compared to the sham group.

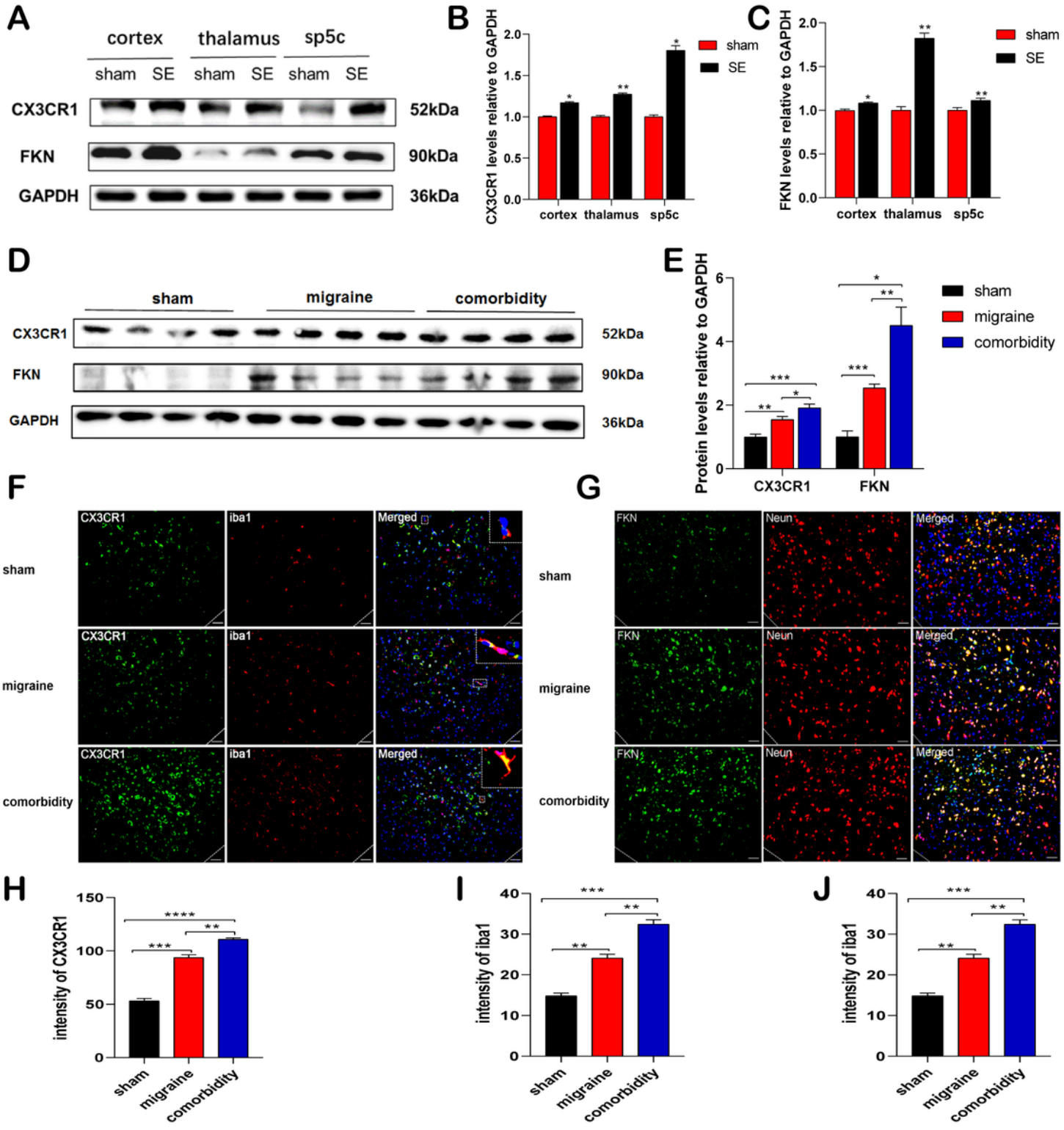

Figure 3

SE upregulates FKN/CX3CR1 in the sp5c of migraine rat models. (A) Representative western blot films for CX3CR1 and FKN protein expression in the cortex-thalamus-sp5c neural circuit of SE rat models. (B-C) Western blot analysis shows the CX3CR1 and FKN protein levels of the SE group are upregulated compared to the sham group in the cortex-thalamus-sp5c neural circuit. n= 3 rats per group. *p<0.05, **p < 0.01, un-paired t-test compared to sham group. (D) Representative Western blots of CX3CR1 and FKN in

different groups. (E) Western blot analysis showing the protein levels of CX3CR1/FKN are increased in the migraine group, and further increased in the comorbidity group. n= 4 rats per group; CX3CR1 protein levels: F (2,33) = 83.838, P < 0.001; FKN protein levels: F (2, 33) =47.823, P<0.001; one-way ANOVA. (F) Representative immunofluorescence of CX3CR1+ cells (green) co-localized with Iba1+ cells (red) in the sp5c of different groups. Scale bars=50μm. (G-H) Quantification of iba1 and CX3CR1 immunoreactivity (in arbitrary units) in the sp5c. n = 3 rats per group; iba1 intensity: F (2, 15) = 8.147, P< 0.01; CX3CR1 intensity: F (2, 15) = 12.521, P<0.01; one-way ANOVA. (I) Representative immunofluorescence of FKN+ cells (green) co-localized with Neun+ cells (red) in the sp5c of different groups. Scale bars=50μm. (J) Quantification of FKN immunoreactivity (in arbitrary units) in the sp5c. n = 3 rats per group; F (2, 15) = 23.153, P < 0.01, one-way ANOVA.

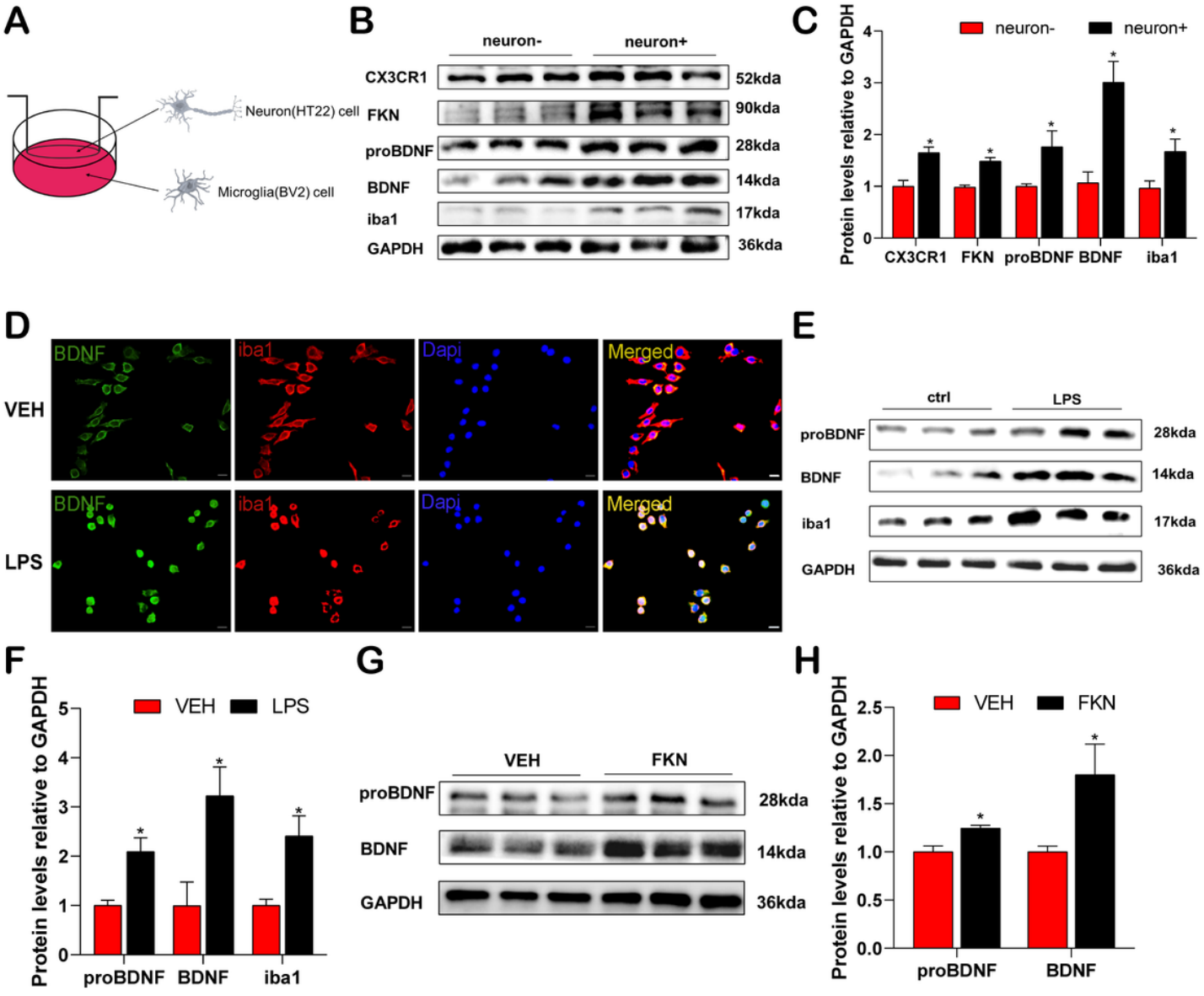

Figure 4

Neurons interact with microglia via the FKN/CX3CR1 axis in HT22-BV2 coculture cells and enhance the release of BDNF in BV2 microglia cells. (A) HT22-BV2 coculture pattern diagram. (B) Western blot images

of CX3CR1/FKN/proBDNF/BDNF/iba1 protein expression released from BV2 cells in the presence of HT22 cells. (C) Quantification of CX3CR1/FKN/proBDNF/BDNF/iba1 protein levels in neuron- and neuron+ cells. n=3 per group; *p < 0.05, unpaired t-test. (D) Representative cell immunofluorescence staining for BDNF (green) and iba1 (red) in the VEH and LPS groups. Scale bar = 20μm. (E) Representative western blot films for proBDNF, BDNF, and iba1 protein expression were released from BV2 cells in the VEH group and the LPS group. (F) Western blot analysis showed that the proBDNF, BDNF, and iba1 protein levels of the LPS group are upregulated compared to the VEH group. n=3 per group; *p < 0.05, unpaired t-test. (G) Western blot images of proBDNF/BDNF/iba1 protein expression released from BV2 cells in response to FKN stimulation. (H) Quantification of proBDNF and BDNF protein levels in the VEH group and FKN group. n= 3 per group; *p < 0.05, unpaired t-test.

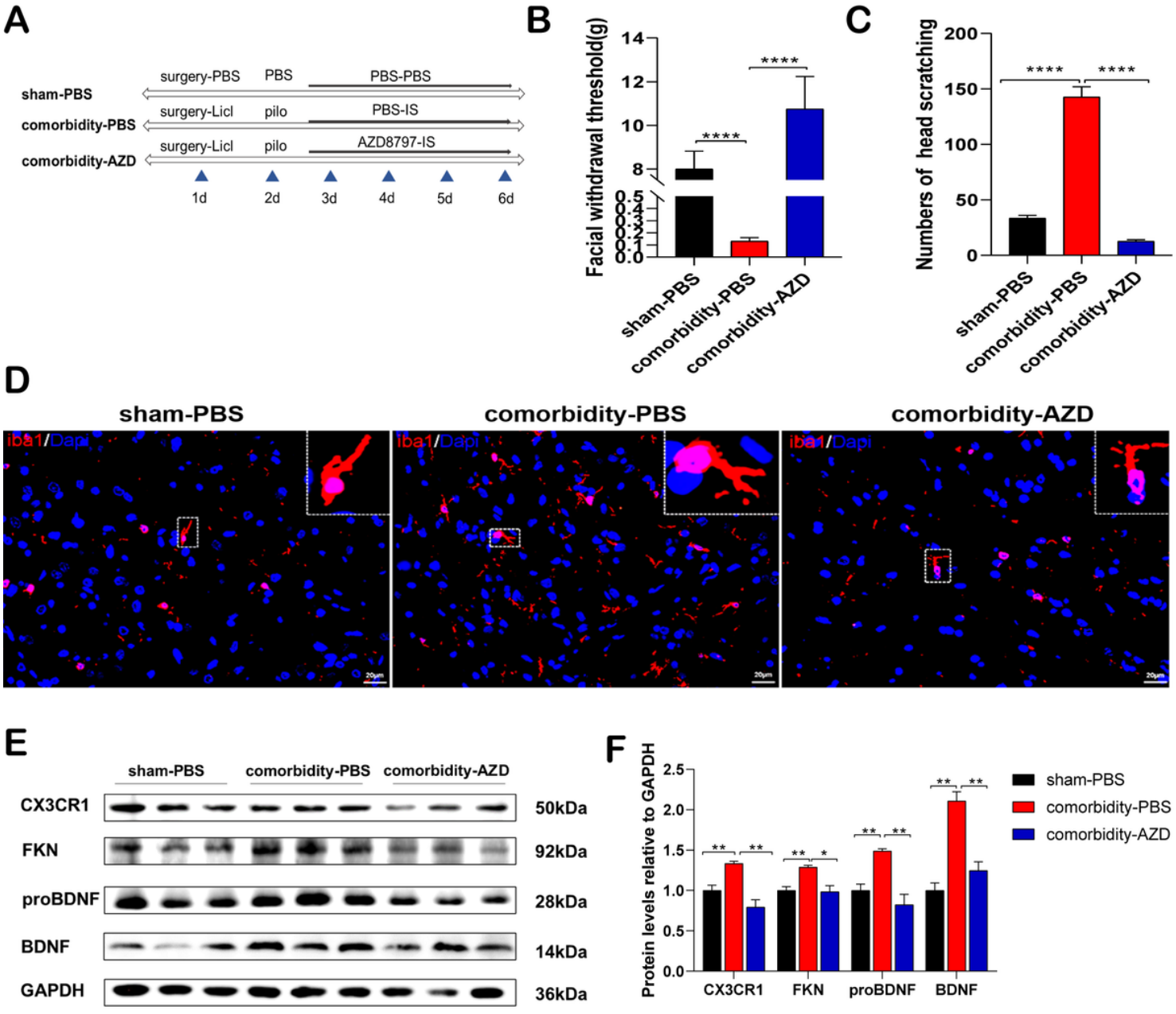

Figure 5

AZD8797 treatment attenuates SE-induced increase in nociceptive behavior, microglia activation, and protein change. (A) Flow chart of the experimental groups sham-PBS, comorbidity-PBS, and comorbidity-AZD. (B) Head-scratching numbers were increased in the comorbidity-PBS group, compared to the sham-PBS group, and recovered after AZD8797 treatment. F (2,15) = 320.040, P < 0.001, one-way ANOVA; n = 6 rats per group. (C) Mechanical threshold in the comorbidity-PBS group was decreased compared to the sham-PBS group and reversed in the comorbidity-AZD group. n = 6 rats per group; F (2, 15) = 41.495, P < 0.001, one-way ANOVA. (D) Immunofluorescence staining of iba1 shows AZD8797 reverses epileptic microglia activation and increases their number. Scares bar= 50μm. (E) Representative western blot films for CX3CR1/FKN/proBDNF/BDNF /iba1 protein expression in sp5c of different groups. (F) Quantification of proBDNF and BDNF protein levels in the sham-PBS group, comorbidity-PBS group, and comorbidity-AZD group. n= 3 rats per group; CX3CR1 protein levels: F (2, 15) = 42.018, P<0.001; FKN protein levels: F (2, 15) = 24.933, P<0.001; proBDNF protein levels: F (2, 15) = 37.266, P<0.001; BDNF protein levels: F (2, 15) = 75.130, P<0.001; one-way ANOVA.

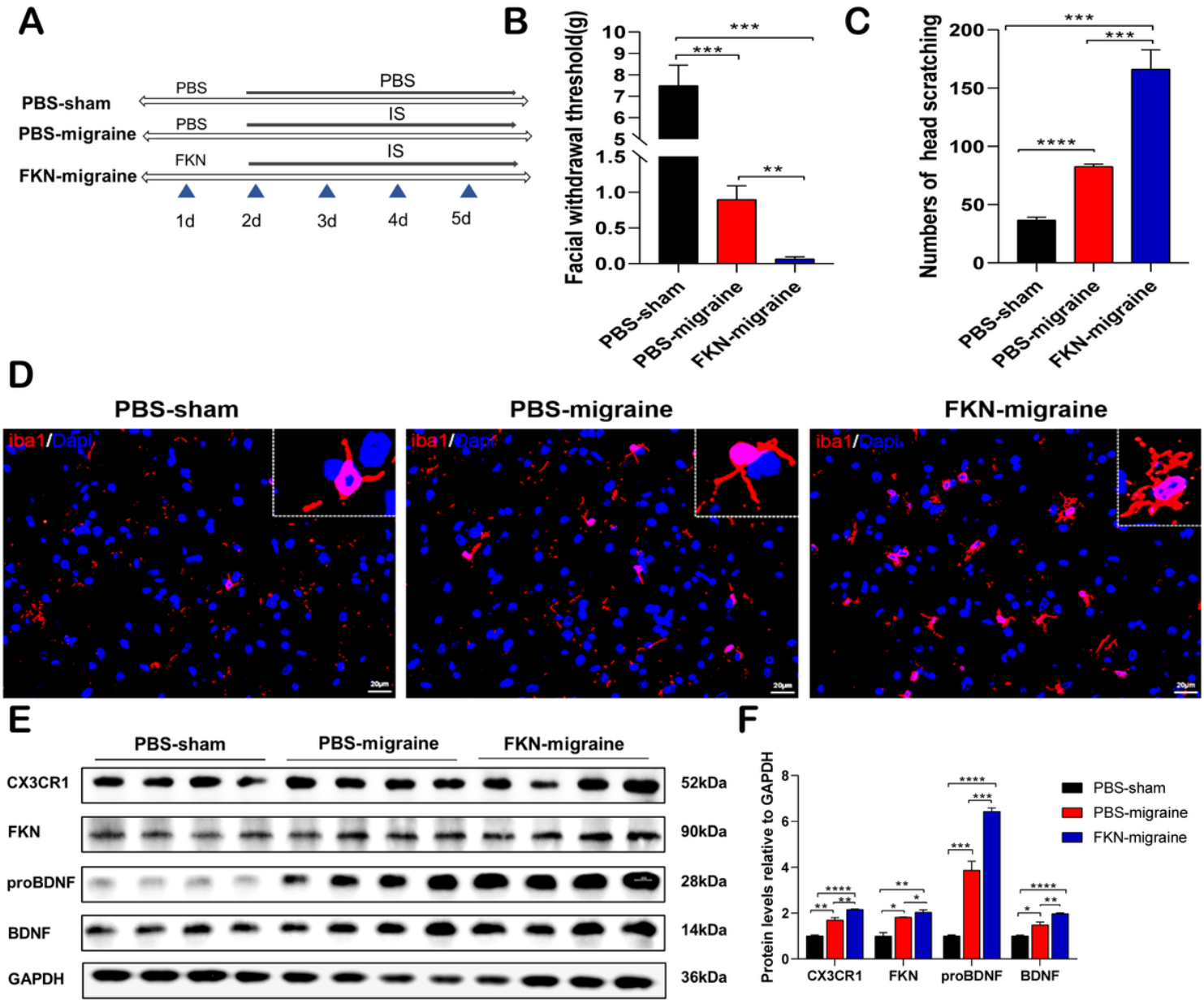

Figure 6

FKN infusion exacerbated the SE-induced increase in nociceptive behavior, microglia activation, and relative protein upregulation.(A) Flow chart of the experimental groups PBS-sham, PBS-migraine, and FKN-migraine.(B) The mechanical threshold in the PBS-migraine group was decreased compared to the PBS-sham group and decreased further in the FKN-migraine group. $F(2, 15) = 100.350$, $P < 0.001$, one-way ANOVA; n = 6 rats per group. (C) Head-scratching numbers were increased in the PBS-migraine group, compared to the PBS-sham group, and increased further after FKN infusion. n = 6 rats per group; $F(2, 15) = 86.596$, $P<0.001$, one-way ANOVA. (D) Immunofluorescence staining of iba1 shows FKN exacerbated epileptic microglia activation and an increase in number. Scares bar= 50μm. (E) Representative western blot films for CX3CR1/FKN/proBDNF/BDNF/iba1 protein expression in sp5c of different groups. (F) Quantification of proBDNF and BDNF protein levels in PBS-sham group, PBS-migraine group, and FKN-migraine group. n= 3 rats per group; CX3CR1 protein levels: $F(2, 15) = 45.278$, $P<0.001$; FKN protein levels: $F(2, 15) = 20.510$, $P<0.01$; proBDNF protein levels: $F(2, 15) = 172.627$, $P<0.001$; BDNF protein levels: $F(2, 15) = 76.943$, $P<0.001$; one-way ANOVA.