Molecular Pain
MPX Express
Accepted on 18, February 2019
This article can be cited as 10.1177/1744806919838947

Memantine selectively prevented the induction of dynamic allodynia by blocking Kir2.1 channel and inhibiting the activation of microglia in spinal dorsal horn of mice in SNI model

Yangyang Chen*, Yiqian Shi*, Guoxiang Wang, Yimei Li, Longzhen Cheng, Yun Wang

Neurology Department, Institutes of Brain science, the State Key Laboratory of Medical Neurobiology, Zhongshan Hospital, Fudan University, Shanghai, China.

*They are co-authors

Corresponding author: Yun Wang, Institutes of Brain Science, Fudan University, Shanghai 200032, China.

E-mail: yunwang@fudan.edu.cn .

MPX Express
Accepted on 18, February 2019
This article can be cited as 10.1177/1744806919838947

ABSTRACT

Memantine (MEM) is one of the important clinical medications in treating moderate to severe Alzheimer disease. The effect of MEM on preventing or treating punctate allodynia has been thoroughly studied but not on the induction of dynamic allodynia. The aim of this study is to investigate whether MEM could prevent the induction of dynamic allodynia and its underlying spinal mechanisms.

1)in vivo SNI pain model, pretreatment with MEM at a lower dose (10nmol, i.t.; MEM-10) selectively prevented the induction of dynamic allodynia, but not the punctate allodynia. 2) Pretreatment with either MK801-10 (MK801-10nmol, i.t.) or higher dose of MEM (30nmol, i.t.; MEM-30) prevented the induction of both dynamic and punctate allodynia. 3) MEM-10 showed significant effect on the inhibition of the SNI induced overactivation of microglia in spinal dorsal horn. 4) In contrast, in CFA model, MEM-10 neither affected the CFA injection induced activation of microglia in spinal dorsal horn nor the induction of dynamic allodynia. 5) Immunohistology studies showed Kir2.1 channel distributed widely and co-localized with microglia in the spinal dorsal horn of mice. 6) Pretreatment with either minocycline, a microglia inhibitor, or

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ML133, a Kir2.1 inhibitor, both selectively prevented the overactivation of microglia in spinal dorsal horn, and the induction of dynamic allodynia following SNI.

The selectively inhibitory effect on the induction of dynamic allodynia in SNI model by low dose of the memantine (MEM-10) was tightly correlated with the blockade of microglia Kir2.1 channel to suppress the microglia activation.

KEYWORDS: memantine, microglia, dynamic allodynia, spared nerve injury, Kir2.1 channel

INTRODUCTION

Herpes zoster is characterized by clustered vesicles accompanied by severe pain in human subjects [1]. After healing from the skin lesions, postherpetic neuralgia established for long days which is often resistant to conventional analgesics [2, 3] even morphine. Among various types of pain (burning pain, aching pain, periodic piercing pain, allodynia, etc.), previous study revealed that appropriately 90% of the patients with postherpetic neuralgia suffered from dynamic allodynia, a painful sensation elicited by gentle stroking stimulation [4]. Different from punctate, different neural circuits were

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Accepted on 18, February 2019
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found to mediate the development of dynamic allodynia including primary afferent fibers, interneurons in spinal cord, etc [5, 6]. Though many researches have focused more attention on the establishing procedure of dynamic allodynia, no precise mechanisms and effective therapies documented clearly yet. In our previous studies, we proved that dynamic mechanical hypersensitivity induced by nerve injury or inflammation was compromised in mice with ablation of spinal $VT3^{Lbx1}$ neurons and preserved somatostatin lineage neurons were still largely sufficient to mediate punctate mechanical hypersensitivity [5]. In the present study, we investigated the clinical drug – memantine (MEM) 's effect on preventing the induction of dynamic allodynia after nerve injury.

MEM is approved to treat moderate to severe Alzheimer's disease because of its ability of blocking excessive NMDAR activity in central nervous system [7]. NMDAR also plays pivotal role in central sensitization and pain-related hyperexcitability during the development of neuropathic pain. In some animal neuropathic pain models, MEM alleviated [8-10] or did not change [11, 12] punctate allodynia. Clinical efficacy of MEM on painful patients also did not prove clearly targeting various types of pain [13]. Previous studies above mainly studied the MEM's effect on punctate mechanical allodynia and the results remained somewhat contentious. However, the efficacy of MEM on the

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induction of dynamic mechanical allodynia was less studied at present.

Microglia was strongly activated by neuropathic pain and treatment with MEM could suppress the activation of microglia [10]. Kir channel family has been found in a wide variety of cells and show inward rectification which not only orchestrate the passive and active electrical properties of cells but also link cellular metabolic state and membrane excitability [14]. Kir2.1 channel was especially abundant in the lamina I and II of the spinal dorsal horn [15]. Another research proved that MEM could block Kir2.1 channels which further affected the functional activities of microglia [16]. In the present study, we explored whether MEM prevented the induction of dynamic allodynia after spared nerve injury (SNI) and whether the underlying mechanism was tightly related with microglia and Kir2.1 channel in the dorsal spinal cord. Our results demonstrated that the selective prevention of dynamic allodynia by low dose MEM (10nmol, i.t.; MEM-10) was conducted by blocking of the Kir2.1 channel and inhibiting the activation of the microglia in spinal dorsal horn of mice.

METHODS

Animals

Male C57BL/6J mice (6w-8w) were purchased from Shanghai Slac laboratory animal Co. LTD and housed in the animal facilities of Institutes of Brain Science, Fudan

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University in the current study. The animal cages were under controlled conditions of room temperature (25℃) and humidity (65%-70%), on a 12h light-dark cycle (light: 7:00am-19:00pm; dark:19:00pm-7:00am) and allowed free access to food and water. After one week in the animal cages, the mice were injected drugs and operation.

Drug application

Drug administration was performed 30min before the surgery. Normal saline (10μl), MEM (10nmol, 30nmol), MK801 (3nmol, 10nmol), minocycline (30nmol) were all dissolved in 10μl normal saline and directly injected into spinal cavity by intrathecal injection method [17]. ML133 (Selleck) were dissolved in DMSO (at concentration of 200 mM) and diluted with normal saline (final concentration 3 mM) before intrathecal administrated (10 μL) to mice. In the CFA injection experiment, MEM-10 was dissolved in artificial cerebrospinal fluid (ACSF). Either normal saline or ACSF were used as vehicle control respectively. MEM, MK801 and minocycline were all purchased from Sigma-Aldrich company (USA).

SNI model

SNI model was performed as described by Decosterd and Woolf [18]. Briefly, anesthesia was applied with isoflurane (0.2%), followed by 1cm skin incision exposing the femur left thigh. Next, left tibial and common peroneal branches of sciatic nerve were ligated

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and transected distally while the sural nerve was left intact. In the sham controls, the sciatic nerve and its branches were exposed without any ligation or transection. Following all procedures, the mice were returned to recovery cage and observed till they were fully ambulatory and able to take food and water. All experiment mice were handled gently to ensure that animal distress is minimized or eliminated during examination.

CFA model

Chronic inflammatory pain was induced by subplantar injection of complete form of Freund's adjuvant (CFA, 10μl; Sigma-Aldrich, St. Louis, MO) into the left hind paw under anesthetic conditions with isoflurane according to previous studies [5]. The subplantar of Mice in control group was injected with normal saline (10μl). Mice were checked in behavior test at 1, 3 days after CFA injection.

Behavior test

Mice were placed on the apparatus for consecutive 3 days before SNI surgery or CFA injection in order to acclimatize the new environment. After habituation and baseline sensitivity measurements, mice were examined punctate and dynamic allodynia in day 1, 3 post SNI surgery and CFA injection. The interval was at least 15min for two different tests. **Punctate allodynia**: Von Frey filaments were used to measure paw withdrawal

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threshold of experimental mice using Dixon's up-down method [19]. The mice were placed on an elevated wire grid and the lateral plantar surface of the hindpaw was stimulated with calibrated von Frey monofilaments (0.02～1g). Positive responses were recognized 3 lifts of 5 trials with 3 min intervals for each stimulus intensity. **Dynamic allodynia**: The protocol of evaluating dynamic allodynia was referred to the previous published studies [20]. Briefly, the operated left hindpaw was lightly stroked with paint brush (appropriately 10 hairs) in the direction from heel to toe. Scoring system was employed to determine the light touch sensitivity of mice. For each test, score 0: walking away or occasionally brief paw lifting (<1s or less); score 1: sustained lifting (>2s) of the stimulated paw towards the body; score 2: strong lateral lifting above the level of the body; score 3: flinching or licking of the operated paw. The test was repeated three times, with intervals of 10s.

Tissue preparation

The experimental mice were perfused transcardially with 0.9% normal saline followed by 4% paraformaldehyde under anesthesia with chloral hydrate (SNI at day 5). The whole spinal cord was removed and post-fixed in 4% paraformaldehyde for 2h and subjected to 15%, 30% sucrose solution in 0.01M phosphate-buffered saline (PBS) for dehydration, until it sunk to the bottom. Coronal lumbar spinal sections were cut into

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20μm by freezing microtome (Leica, Germany).

Immunohistochemistry

Spinal cord tissue sections (20μm) were processed for visualization of the Iba-1 protein and Kir2.1 channel expression by immunofluorescent labeling in the dorsal horn. Sections were incubated overnight at 4℃ in PBS containing 10% donkey serum, a mixture of two antibodies, rabbit monoclonal anti-Iba-1 antibody (dilution 1:500, GeneTex) and mouse polyclonal anti-Kir2.1 (dilution 1:200, Abcam). After thoroughly rinsed, the sections were incubated for 2h at room temperature in a mixture of two corresponding secondary fluorescently tagged antibodies (dilution at 1:500, Alexa Fluor 488 for Iba-1 and Alexa Fluor 594 for Kir2.1, Invitrogen). Sections were covered with glass coverslips and pictured under the fluorescence microscope (Olympus, Japan). The fluorescence intensities of Iba-1 and Kir2.1 channel in spinal dorsal horn were analyzed by ImageJ software.

Statistical analysis

Data are expressed as mean ± SEM; the “n” values refer to the number of experimental mice in each group. Two-way ANOVA with Bonferroni post hoc tests were used to detect the difference among different groups of data. Statistical analysis was employed using GraphPad Prism 7 software.

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This article can be cited as 10.1177/1744806919838947

RESULTS

MEM-10 selectively prevented the induction of dynamic, but not punctate, allodynia following SNI in mice

SNI could induce long-lasting ipsilateral mechanical allodynia. Consistent with our previous study [5], SNI operation induced robust punctate allodynia (sensitive to von Frey filaments) and dynamic allodynia (sensitive to paintbrush)) on the ipsilateral hind paw 3 days after surgery (n=12). In order to study the effect of MEM on punctate and dynamic allodynia, we intrathecal administrated MEM before SNI surgery. Interestingly, intrathecal pretreatment with low dose of MEM (10nmol, i.t.; MEM-10) before SNI surgery selectively prevented the induction of the dynamic allodynia ($p=0.0038$, n=11), but had no effect on the punctate allodynia ($p=0.1702$, Fig 1).

Either MK801-10 or MEM-30 prevented the induction of both punctate and dynamic allodynia

MEM was widely believed to be uncompetitive antagonist of NMDA receptors with a favorable pharmacokinetic profile and approved for the treatment of Alzheimer's Disease [21]. In order to confirm whether the MEM-10's analgesic effect toward dynamic allodynia was due to the blockade of NMDA receptor, MK801, another potent uncompetitive antagonist of NMDA receptor, and higher dose of MEM (30nmol, i.t.;

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MEM-30) were employed in the present study.

As showed in Fig.2A, pre-administration with MK801 (3 nmol, i.t., n=14) increased the withdrawal threshold (p=0.0088) but showed no effects on the dynamic score (p=0.1583) following SNI. Pretreatment with MK801 (10 nmol, i.t., n=5) prevented the induction of both punctate (p=0.0096) and dynamic (p=0.0057) allodynia compared with the vehicle group (n=12) in both postoperative day 1 and day 3. Similarly, MEM-30 (n=6) prevented the induction of both punctate (p=0.0039) and dynamic (p=0.0001) allodynia in both postoperative day 1 and day 3 following SNI (Fig 2B). These results indicated that the selective inhibitory effect of MEM-10 on the induction of dynamic, but not punctate, allodynia might not be correlated with the blockade of NMDA receptors in the spinal cord of mice.

MEM-10 prevented microglia overactivation during SNI injury in the dorsal spinal cord

Studies have implicated that the over activation of microglia cells in response to nerve injury is important in the development of neuropathic pain [22]. Thus, immunohistochemistry staining was performed to investigate whether activation of microglia was inhibited by MEM-10 in spinal dorsal horn following SNI and to verify the role of microglia in the induction of dynamic allodynia.

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As depicted in Fig 3, sham group had no change in microglia activation (p=0.9999，n=5) compared with naïve group (n=3), whereas vehicle group (n=11) showed significant activation of microglia in the spinal dorsal horn after SNI surgery compared with sham group (p=0.0005, n=11). Pretreatment with MEM-10 significantly inhibited the activation of microglia induced by SNI surgery in day 5 (p=0.0166, n=8). The activation of microglia in the spinal cord was similar between sham and MEM-10 groups (p=0.9704). Therefore, the inhibition effect of MEM-10 on activation of microglia may be corelated with the inhibitory effect displayed only in the induction of dynamic allodynia.

MEM-10 did not affect the induction of dynamic allodynia in CFA inflammation pain model

Previous study has proved that CFA treatment did not increase the proliferation of microglia in the ipsilateral side [23]. Therefore, we employed CFA model as a negative control of microglia activation model to evaluate the relationship between microglia over activation and MEM-10 inhibition of dynamic allodynia. As shown in Fig 4A-B, CFA injection did not increase the proliferation of microglia in spinal dorsal horn of mice, which is in consistent with previous study [23]. The behavior test results proved that pretreatment with MEM-10 inhibited the induction of punctate allodynia (p=0.0096,

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n=9) while showed no significant effects on CFA induced dynamic allodynia (p=0.2512, n=9, Fig 4C). Combined with above results, we speculated that the inhibitory effect of microglia cells in dorsal spinal cord induced by MEM-10 may be correlated with the prevention effect toward the induction of dynamic allodynia.

Kir 2.1 channel blocker (ML133) selectively inhibited the induction of dynamic allodynia, as well as the overactivation of microglia after SNI injury

Despite of the NMDA receptor, Kir2.1 channel was also important target of MEM in microglia cells. MEM could suppress the $I_{kir2.1}$ amplitude of microglia cells by decreasing the slow component of mean open time and increasing the closed time of Kir2.1 channels and then depolarize the membrane potential in BV2 microglia cells.[16]. In the spinal cord, Kir2.1 channels are present in gray matter of dorsal and ventral horn [24]. Our immunochemistry staining result also proved that, similar with former published study, Kir2.1 channel distributed widely on the gray matter and located in the membrane of microglia on the spinal dorsal horn of control mice (Fig 5A).

Previous study has reported that blocking Kir2.1 channel by Ba^{2+} could inhibit microglia proliferation [25]. In order to identify the precise role of Kir2.1 channel on induction of dynamic allodynia after SNI surgery, Kir channel blocker-ML133 used in the present study was initially investigated for preventing the induction of mechanical

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allodynia. Pre-treatment with ML133 (30nmol, i.t.; n=6) selectively inhibited the induction of dynamic allodynia (p=0.0002) while showed no significant changes on punctate allodynia compared with vehicle group (p=0.7237, n=7, Fig 5B). The activation state of microglia in spinal cord following pretreatment with ML133 was also tested following SNI surgery. As depicted in Fig 6A and 6B, compared with vehicle group (n=11), pretreatment with ML133 inhibited the activation of microglia cells in spinal dorsal horn (p=0.0370, n=6). This result mimicked the effect of MEM-10 pre-treatment on the inhibitory induction effect of dynamic allodynia and activation of microglia cells in spinal dorsal horn after SNI surgery.

Minocycline inhibited the overactivation of microglia and prevented the induction of dynamic allodynia following SNI surgery

Previous study has been shown that minocycline acts as an inhibitor of microglial activation[26]. SNI surgery could induce significant overactivation of microglia in spinal dorsal horn (Fig 3). Whether pretreatment with minocycline (Mino) could inhibit the activation of microglia in the spinal dorsal horn and the behavior test of dynamic allodynia following SNI were evaluated in the present study. As shown in Fig 6A and 6B, the results revealed that minocycline (p=0.0002, n=6) significantly inhibited the activation of microglia after SNI injury. This phenomenon also matched the effects of

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pretreatment with MEM-10 on the inhibition effect toward activation of microglia cells in dorsal spinal cord. As shown in the Fig 6C, pretreatment with minocycline significantly inhibited the induction of dynamic allodynia in day 1 ($p=0.0001$, $n=6$) but had no significant changes in day 3 after SNI surgery compared with vehicle group ($n=11$). The punctate allodynia showed no significant changes in minocycline pretreatment group after SNI surgery ($p=0.2280$, $n=6$). This part of research revealed that microglia played crucial role in the development of dynamic allodynia, and blockage of Kir2.1 could inhibit microglia activation, which further inhibited dynamic allodynia.

DISCUSSION

The animal pain behavior results from the present study revealed that pretreatment with low dose of memantine (MEM-10) has selectively analgesic property towards the induction of dynamic allodynia, but not punctate allodynia. Further immunohistochemistry staining results indicate that this selective analgesic property toward dynamic allodynia by MEM-10 is probably mediated by inhibiting of microglia overactivity induced by peripheral nerve injury of SNI through blockade of Kir2.1 channels localized in microglia in the spinal dorsal horn. This conclusion is based on the observation that: (1) In *in vivo* SNI pain model, pretreatment with MEM-10 selectively

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prevented the induction of dynamic allodynia while showed no significant changes on punctate allodynia; (2) Pretreatment with either MK801 or MEM-30 prevented the induction of both dynamic and punctate allodynia; (3) MEM-10 significantly inhibited the overactivation of microglia in spinal dorsal horn in SNI model; (4) Microglia was not overactivated by CFA injection. MEM-10 pretreatment before CFA injection did not affect the induction of dynamic allodynia; (5) Both Minocycline and ML133 selectively prevented the overactivation of microglia in spinal dorsal horn as well as the induction of dynamic, but not punctate, allodynia following SNI.

Bioavailability of MEM

MEM is a novel class of Alzheimer's disease medications acting as a moderate-affinity voltage-dependent noncompetitive antagonist at glutaminergic NMDA receptors [27]. Except for NMDA receptor, Kir2.1 channel was another important target of MEM in the microglia cells. Previous study proved that MEM could inhibit the amplitude of inwardly rectifying K^+ current through the Kir channels in BV2 microglia cells [16]. From previous studies, MEM's IC50 toward NMDA receptor was 0.54μM (in rat wild type NMDA receptor clones) [28] and Kir2.1 channel was 12μM (in clonal strain BV2 microglia cell line) [16]. The volume of cerebrospinal fluid (CSF) in mice was around 37μL[29]. At therapeutic concentrations, memantine effectively blocks excessive

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extrasynaptic NMDAR-mediated currents, while relatively sparing normal synaptic activity [7]. Therefore, the inhibitory effect toward synaptic NMDARs may much higher than 0.54μM reported before. It is possible that MEM (i.t.) only blocks the Kir2.1 channel while show enough effects toward synaptic NMDAR to influence mEPSC and AP number in the spinal cord slices (Fig S1).

Antinociceptive effect toward dynamic allodynia after SNI surgery

Neuropathic pain was initiated or caused by a primary lesion or dysfunction in the nervous system [30]. Spared nerve injury model was established to induce neuropathic pain behaviors including punctate, dynamic and thermal allodynia [20]. Previous studies mostly paid attention to studying the analgesic effect of MEM toward punctate or thermal allodynia after nerve injury on animal [31]. The precise mechanism underlying dynamic allodynia was not very clear and the effective therapy was still under exploration. In current study, we demonstrated that pretreatment with MEM-10 inhibited the induction of dynamic allodynia but not the punctate allodynia. This result is the first time to show the selective analgesic role of low dose of MEM toward dynamic allodynia in the mice SNI model. At the same time, MK801 was also pretreated to verify the role of NMDA receptor in the induction of dynamic allodynia. The result revealed that MK801-3 only inhibited the induction of punctate allodynia and

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MK801-10 prevented the induction of both punctate and dynamic allodynia, which indicating that NMDA receptor is more likely to be involved in the formation of punctate allodynia, but not dynamic allodynia. Therefore, the selective analgesic effect of low dose of MEM in dynamic allodynia may be mediated through a NMDA-independent pathway. In our study, we also found that MEM-10 could inhibit the activation of microglia. Kir2.1 channel is another target for MEM [16]. Therefore, ML133 was also pretreated before SNI surgery to examine the role of Kir2.1 channel in the induction of dynamic allodynia. Both dynamic allodynia behavior and immunohistostaining results were consistent with MEM-10 pre-administration in mice. What's more, minocycline, one of important inhibitor of microglia, prevented the induction of dynamic allodynia after SNI surgery. This part of results revealed that the selectively inhibition effect of MEM-10 toward dynamic allodynia was likely mediated by blockade of Kir2.1 channel in spinal cord. Moreover, previous studies have reported that microglia were activated following nerve injury and punctate mechanical allodynia could also be abolished via microglia silencing [32]. Combined with our results together, the activation of microglia participated in the establishment of both punctate and dynamic allodynia.

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Spinal mechanism of inhibition effect of MEM toward dynamic allodynia

Previous study proved that Kir2 channels composed of Kir2.1-2.3 subunits were expressed widely in neuronal and glia cells in spinal cord, contributing to sensory transduction and motor control [15]. Kir2.1 channel in which cell types of spinal dorsal horn was further studied to verify the precise analgesic effect of MEM-10 toward prevention of dynamic allodynia. Consistent with former studies, nerve injury would induce significant activation of microglia in spinal dorsal horn [23]. Pretreatment with MEM-10 inhibited this overactivation of microglia induced by SNI injury (Fig 3). Previous studies have revealed that Kir channel blocker Ba^{2+} potently inhibited the proliferation of microglia [25]. MEM-10 was also acted as one of Kir2.1 channel blockers and the inhibition toward proliferation of microglia could mediated by Kir2.1 channel. On the contrary, microglia cells were not activated by CFA injection into plantar of mice. Pretreatment with MEM-10 also did not affect the induction of dynamic allodynia after CFA injection. Kir 2.1 channel in microglia might be important target in preventing the induction of dynamic allodynia.

Except for NMDAR and Kir2.1 channel，several potassium channels are also targets of MEM in the spinal cord, K_{ATP}, $K_V1.3$, $K_{Ca}3.1$ etc. But the K_{ATP} channel opener treatment is an effective therapy for postoperative pain in animals while MEM was a

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Accepted on 18, February 2019
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KATP channel blocker. MEM was believed to aggravate the pain sensation. Kv1.3 channel was weakly expressed in the spinal cord in animals. KCa3.1 did not play role in the neuropathic pain processing but in the noxious chemical stimuli in mice. Therefore, we believe that Kir2.1 channel may be one of major targets of MEM conducting the inhibition effect towards the induction of dynamic allodynia.

It's well-recognized that peripheral nerve injury could lead to rapid and vigorous microglial activation. Once activated, the microglia could release excitatory amino acids, interleukin-1β, and prostaglandin E2, which all involved in the induction of central sensitization of pain sensation [10]. MEM could reduce microglia-associated inflammation by decreasing the production of inflammatory factors, such as extracelluar superoxide anion, intracellular reactive oxygen species, nitric oxide, and TNF-α [33]. In the present study, besides Kir2.1 channel in the spinal cord, inflammatory factors may also contribute to the analgesia effect toward dynamic allodynia which need to be further studied.

Previous studies have revealed that punctate and dynamic allodynia shared different afferent fibers and neuronal circuits in spinal cord [5, 34]. Our studies which pretreatment with MEM-10 selectively prevented the induction of dynamic allodynia provided direct evidence toward the former theory. Previous study also reported that glycine inhibitory

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Accepted on 18, February 2019
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dysfunction induced a selectively dynamic, morphine-resistant mechanical allodynia [35]. However, our whole-cell patch clamp recordings' final results showed that bath application of MEM (10μM and 100μM) did not change the spontaneous glycinergic inhibitory postsynaptic currents (gly-sIPSCs) in the lamina II neurons (data was not shown). Activities of glycinergic interneurons were also excluded in the mechanism underlying intrathecal administration with MEM on the inhibition effect of the induction of dynamic allodynia in our study.

MEM-10 did not alleviate punctate and dynamic allodynia 7 days after SNI

We also evaluated the MEM's treatment effect after totally forming of dynamic allodynia at day 7 post SNI surgery (Fig S3). However, the effective prevention dose of MEM on the induction of dynamic allodynia did not alleviate punctate and dynamic allodynia at all. 7 days after SNI injury, a large number of microglia cells have already been in activated stage and the dynamic allodynia have also established. The activities of Kir2.1 channel blocked by MEM-10 or ML133 would not affect the proliferation of microglia cells even further broke the maintenance of dynamic allodynia. Our studies will further focus on the mechanism underlying the treatment doses of MEM after the forming of dynamic allodynia following nerve injury.

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Functional implication

MEM-10 suppressed the function of Kir2.1 channel which further inhibited the over-activation of microglia in spinal dorsal horn, indicating a potential therapy in preventing the induction of dynamic allodynia. As one of clinical medications, the analgesic effect of MEM toward preventing induction of dynamic allodynia will provide directive evidence in exploring precise mechanism of dynamic allodynia. In clinical prevention of dynamic allodynia, MEM and spinal Kir2.1 channel blocker may have great potential in preventing the establishment of this disease before predictable injury, such as amputation in clinical treatment.

CONFLICT OF INTEREST STATEMENT

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by grants from the Nature Science Foundation of China (31771188, 31471027) to YW.

MPX Express
Accepted on 18, February 2019
This article can be cited as 10.1177/1744806919838947

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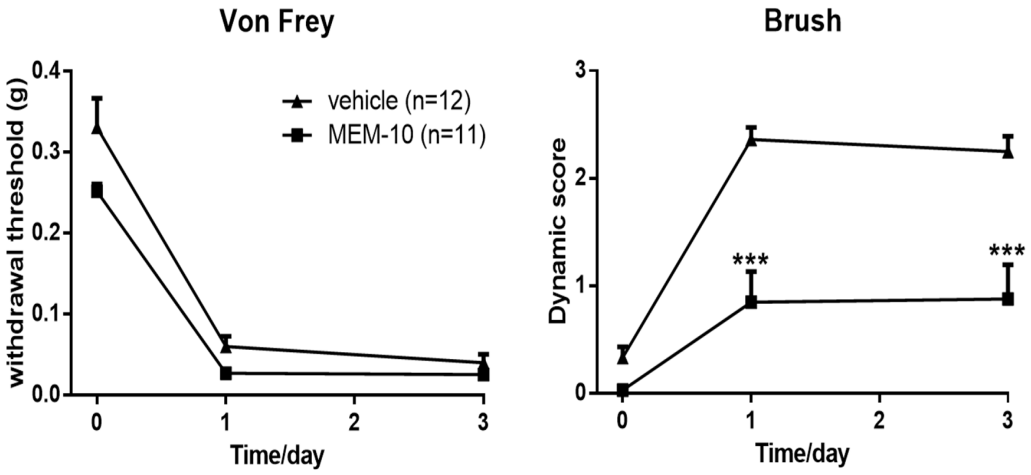

Figure 1.

Selective inhibition of dynamic, but not punctate allodynia, in MEM-10 pretreated mice following SNI. (vehicle, n=12; MEM-10, n=11; two-way ANOVA with Bonferroni posthoc. Von Frey test, vehicle vs MEM-10, p=0.1702; brush test, vehicle vs MEM-10, p=0.0038). Graphs represent the mean response ± SEM, ***, p<0.001.

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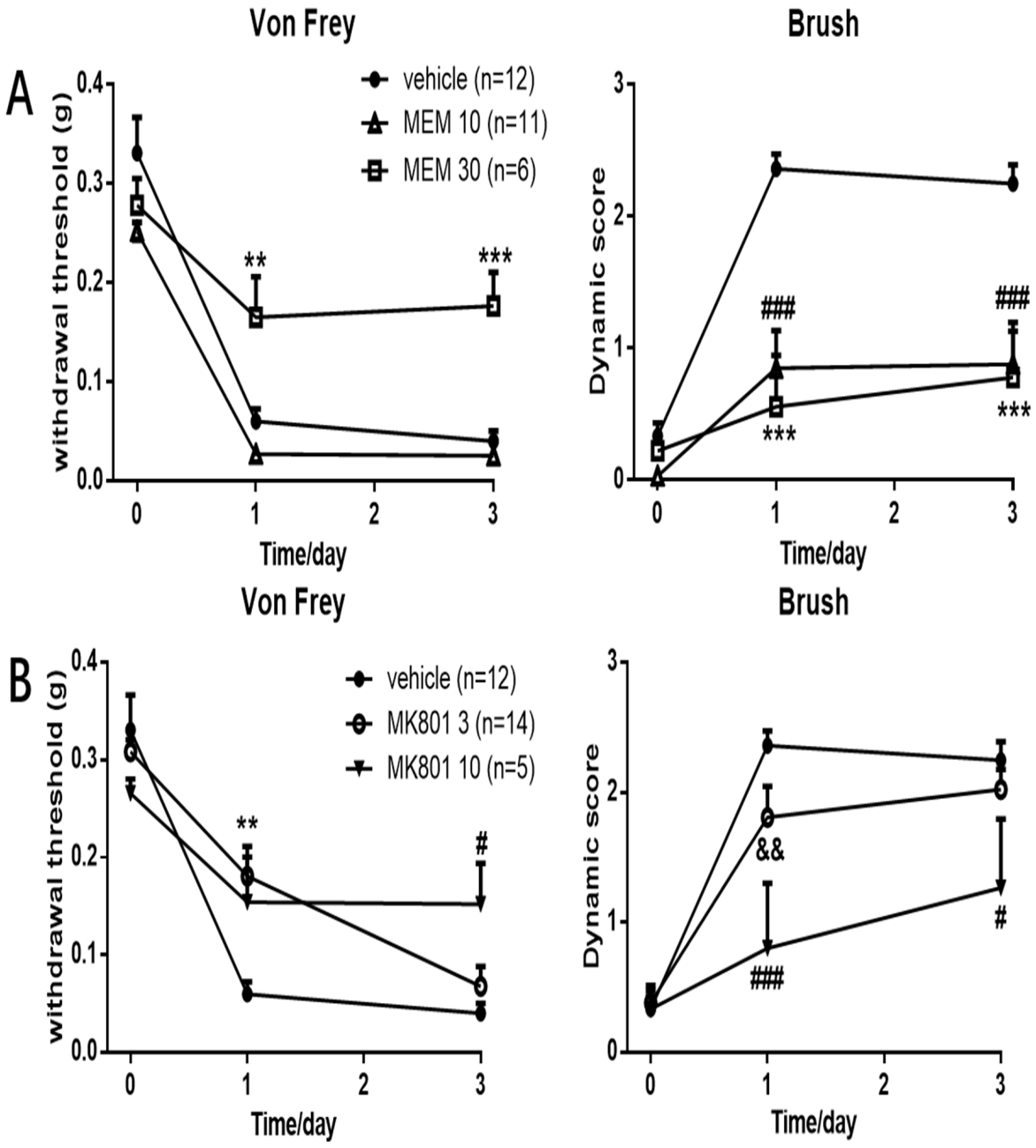

Figure 2.

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Effects of intrathecal MK801-3， MK801-10 and MEM-30 following SNI.(A) Pretreatment with MK801-3 prevented the induction of punctate allodynia at day 1 after SNI surgery and MK801-10 prevented the induction of punctate allodynia at day 3 (two-way ANOVA with Bonferroni posthoc, vehicle vs MK801-3, $p=0.0088$; vehicle vs MK801-10, $p=0.0096$). MK801-3 exerted no effects on the induction of dynamic allodynia (two-way ANOVA with Bonferroni posthoc, $p=0.1583$). MK801-10 prevented the induction of dynamic allodynia (two-way ANOVA with Bonferroni posthoc, $p=0.0057$). Vehicle, n=12; MK801-3, n=14; MK801-10, n=5. (B) Pretreatment with MEM-30 prevented the induction of punctate allodynia (two-way ANOVA with Bonferroni posthoc, vehicle vs MEM-30, $p=0.0039$) and dynamic allodynia (two-way ANOVA with Bonferroni posthoc, vehicle vs MEM-30, $p=0.0001$). Graphs represent the mean response ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, MEM-30 or MK801-3 vs vehicle group. $^{\#}$, $p<0.05$; $^{\#\#\#}$, $p<0.001$, MK801-10 vs vehicle group; $^{\&\&}$, $p<0.01$, MK801-3 vs MK801-10 group.

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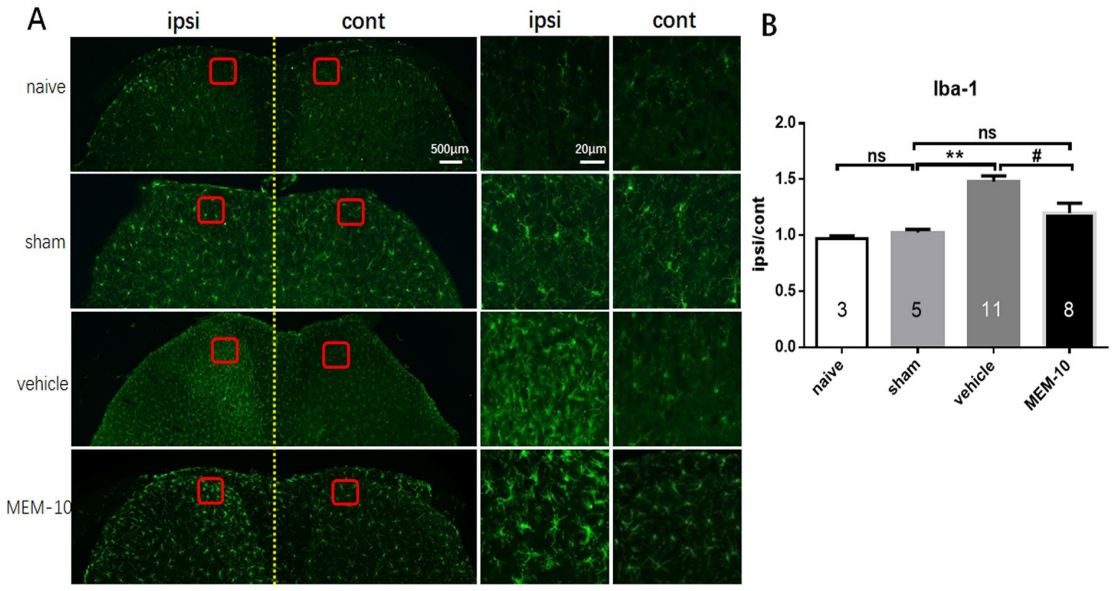

Figure 3.

Reduction of the microglia cells activation in the spinal cord horn of MEM-10 treatment mice after SNI. (A) Microglial immunostaining against Iba-1 in mice spinal cord dorsal horns after SNI surgery. (B) The histograms respectively show the quantification of ratio of immunostaining density on ipsilateral (ipsi) and control (cont) sides of dorsal spinal cord after SNI surgery (one-way ANOVA with Bonferroni posthoc, naive vs sham, $p>0.9999$; sham vs vehicle, $p=0.0005$; vehicle vs MEM-10, $p=0.0166$; sham vs MEM-10, $p=0.9704$). Naive, n=3; sham, n=5; vehicle, n=11; MEM-10, n=8. **, $p<0.01$, vehicle group vs sham group. $^{\#}$, $p<0.05$, MEM-10 group vs vehicle group.

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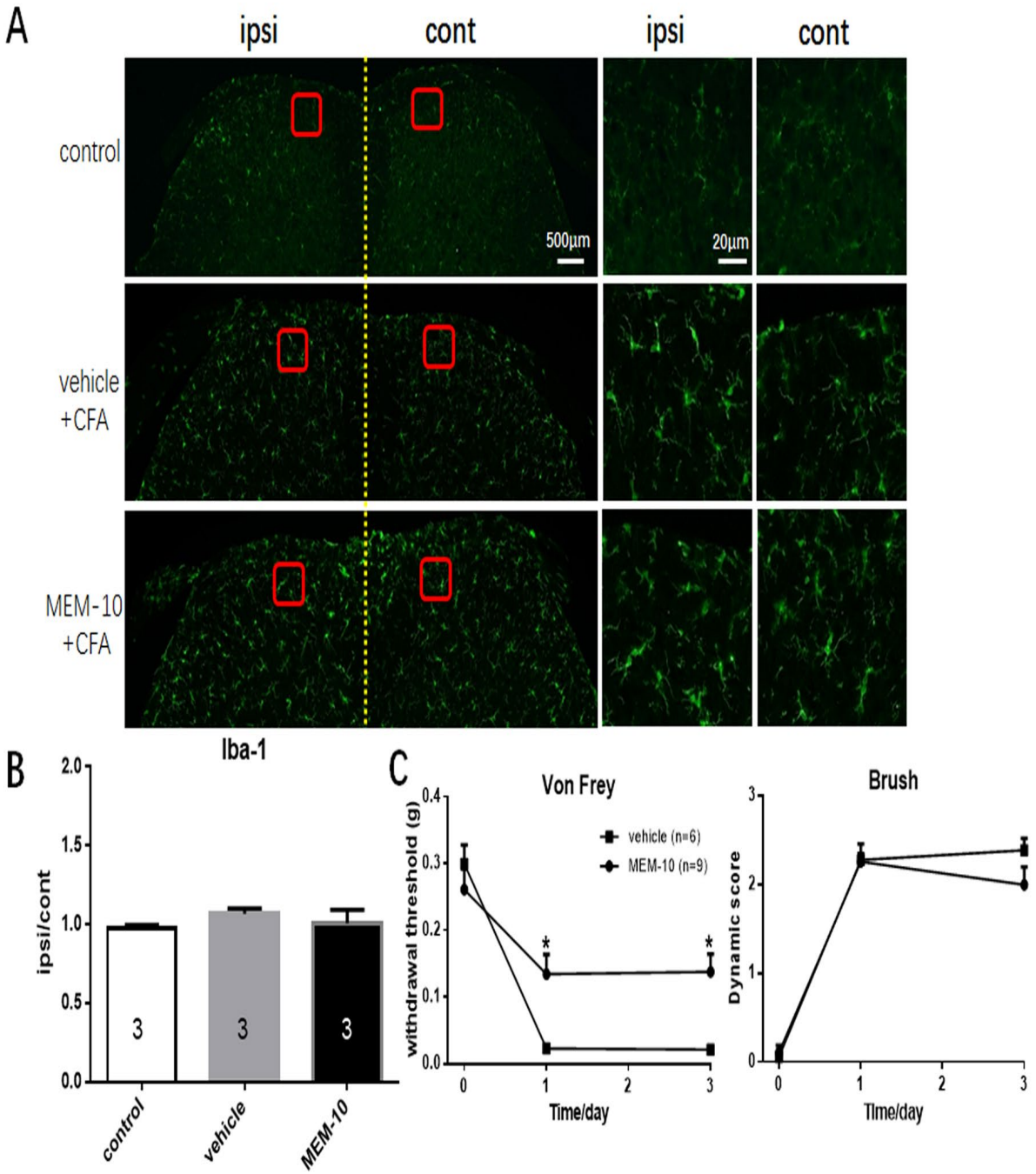

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Figure 4.

CFA injection did not increase the activation of microglia in dorsal spinal cord of mice and MEM-10 showed no changes on the induction of dynamic allodynia following CFA injection. (A) Microglial immunostaining against Iba-1 in mice spinal cord dorsal horns after CFA injection and MEM-10 administration. (B) The histograms respectively show the quantification of ratio of immunostaining density on ipsilateral (ipsi) and control (cont) sides of dorsal spinal cord after CFA injection. (C) Punctate allodynia was prevented by MEM-10 injection (one-way ANOVA with Bonferroni posthoc, $p=0.0096$) and dynamic allodynia was not affected by MEM-10 injection (one-way ANOVA with Bonferroni posthoc, $p=0.2512$). Vehicle, n=5; MEM-10, n=9. Graphs represent the mean response ± SEM. *, $p<0.05$.

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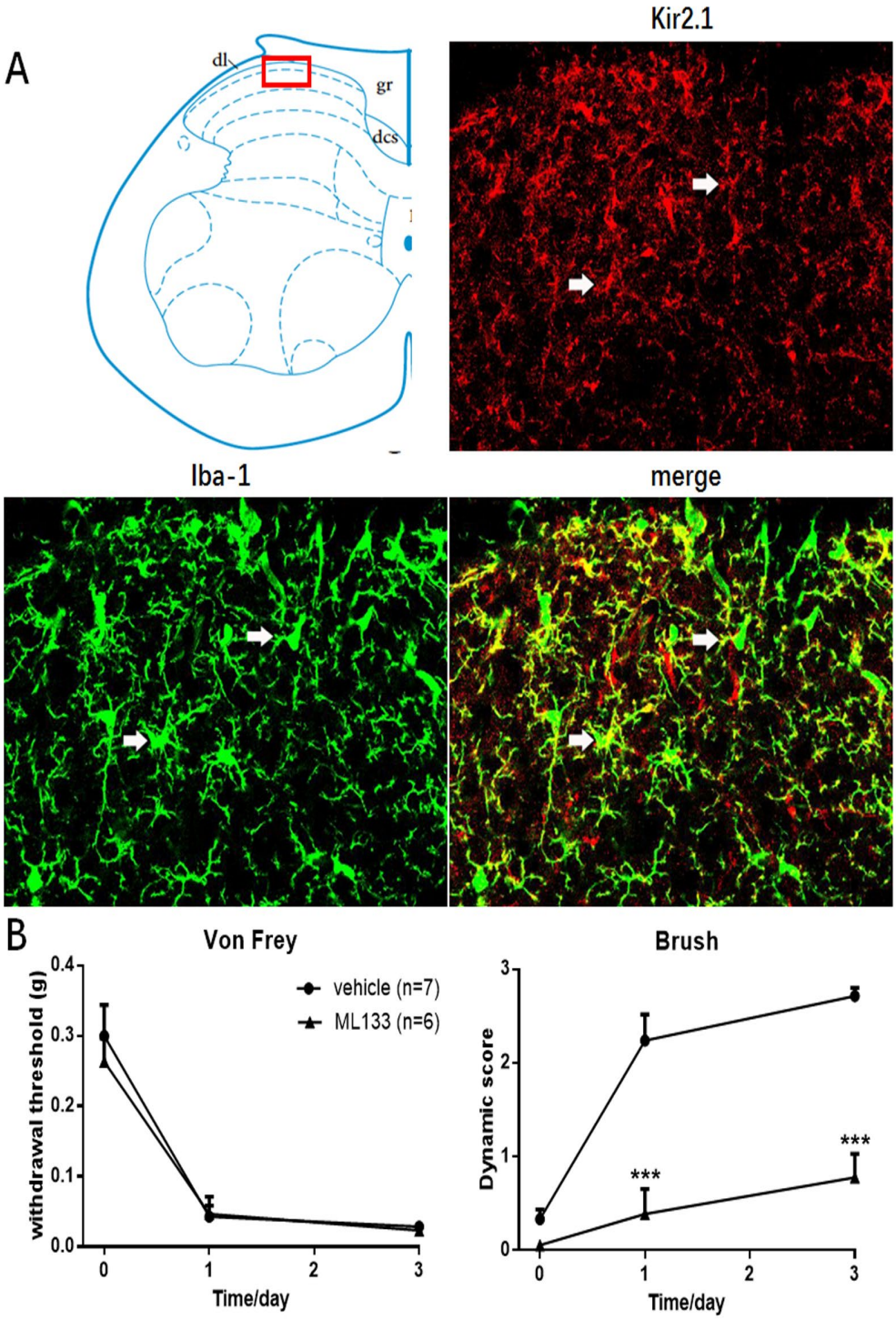

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Figure 5.

Loss of brush induced dynamic allodynia in ML133 injection mice following SNI. (A) Kir 2.1 channel expressed in the membrane of microglia in dorsal spinal cord lamina Iand II. Immunohistochemistry confocal scanning in ipsilateral lumbar spinal cord between Kir2.1 (red) and Iba-1 (green) showed the distribution of Kir2.1 channel in spinal cord dorsal horn. Three confocal images are zoom-in images from area in top red square. White arrows showed the co-localization of Kir2.1 channel and microglia. (B) ML133 prevented the induction of dynamic allodynia following SNI (two-way ANOVA with Bonferroni posthoc, $p=0.0002$) and had no effect on the induction of punctate allodynia (two-way ANOVA with Bonferroni posthoc, $p=0.7237$). Vehicle, n=7; ML133, n=6. Graphs represent the mean response ± SEM. ***, $p<0.001$.

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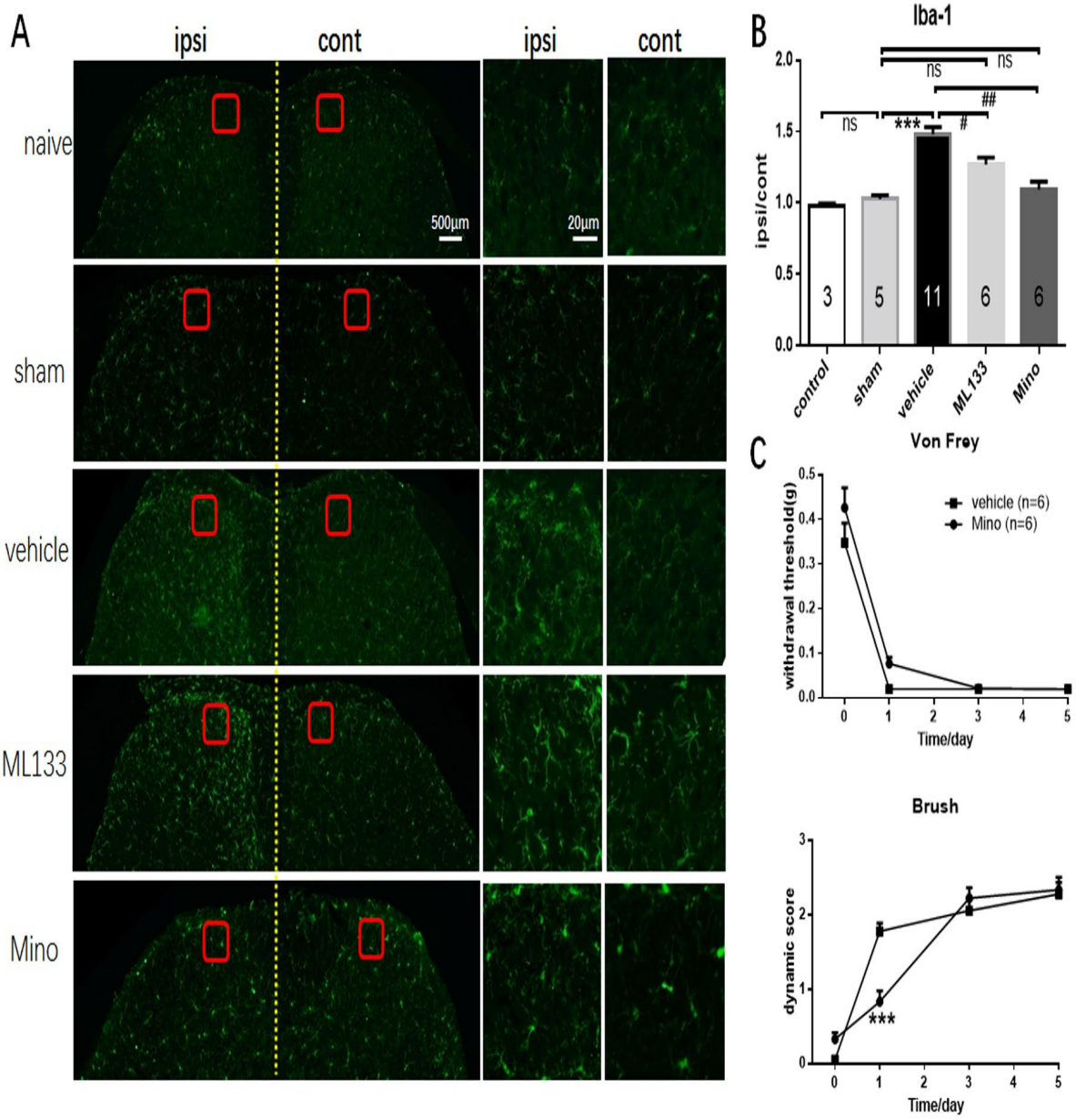

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Accepted on 18, February 2019
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Figure 6.

Loss of brush induced dynamic allodynia in minocycline (mino) injection mice following SNI. (A) Microglial immunostaining against Iba-1 in mice spinal cord dorsal horns after SNI surgery. (B) The histograms respectively show the quantification of ratio of immunostaining density on ipsilateral (ipsi) and control (cont) sides of dorsal spinal cord after SNI surgery (one-way ANOVA with Bonferroni posthoc, naive vs sham, $p>0.9999$; sham vs vehicle, $p<0.0001$; vehicle vs ML133, $p=0.0370$; vehicle vs minocycline, $p=0.0002$; sham vs mino, $p>0.9999$). (C) Mino delayed the forming of dynamic allodynia following SNI (two-way ANOVA with Bonferroni posthoc, $p=0.0001$) while showed no significant changes on punctate allodynia (two-way ANOVA with Bonferroni posthoc, $p=0.2280$). Graphs represent the mean response ± SEM. Ns, no significant changes; ***, $p<0.001$, vehicle group vs sham group in (B) and mino group vs vehicle group in (C); $^{\#}$, $p<0.05$, ML133 group vs vehicle group; $^{\#\#}$, $p<0.01$, Mino group vs vehicle group.