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Name	*Affiliations*
Jingjing Zhou	1. Laboratory of Neuropharmacology and Neurotoxicology, School of Life science, Shanghai University, Shanghai, China
Danting Feng	1. Laboratory of Neuropharmacology and Neurotoxicology, School of Life science, Shanghai University, Shanghai, China
Xiaoxue Zhang	1. Laboratory of Neuropharmacology and Neurotoxicology, School of Life science, Shanghai University, Shanghai, China
Chenchen Xia	1. Laboratory of Neuropharmacology and Neurotoxicology, School of Life science, Shanghai University, Shanghai, China
Zhiping Zhang	1. Laboratory of Neuropharmacology and Neurotoxicology, School of Life science, Shanghai University, Shanghai, China
Jiahao Kang	1. Laboratory of Neuropharmacology and Neurotoxicology, School of Life science, Shanghai University, Shanghai, China
Research Assistant Professor Zhiyong Tan	1. Department of Pharmacology and Toxicology and Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN, United States
Bin Wu	1. Laboratory of Neuropharmacology and Neurotoxicology, School of Life science, Shanghai University, Shanghai, China 2. Department of Pharmacology and Toxicology and Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN, United States

Corresponding author: Research Assistant Professor Zhiyong Tan <zt2@iupui.edu>

Corresponding author: Bin Wu <bwu1@iu.edu>

Abstract

The P2X7 receptor (P2X7R) plays an important role in inflammatory and neuropathic pain. Our recent study indicated that activation of P2X7R in microglial cells of spinal cord contributes to the inflammatory pain induced by BmK I□the major active compound from Buthusmartensi Karsch (BmK). In the present study, we further investigated whether P2X7R in satellite glial cells (SGCs) of dorsal root ganglion (DRG) is involved in the BmK I-induced pain in rats. The results found that the expression of P2X7R in SGCs was increased in the ipsilateral side of L4–L5 DRGs after intraplantar injection of BmK I. Moreover, the expression of an inflammatory cytokine IL-1β was increased in DRG after BmK I injection. Systemic administration of an inhibitor of P2X7R (A-438079) significantly inhibited both spontaneous and evoked nociceptive behaviors induced by BmK I. These results suggest that the P2X7R in SGCs of DRG might contribute to pain induced by toxins that sensitize peripheral sensory nerves.

Involvement of P2X7 receptors in satellite glial cells of dorsal root ganglia in the BmK I -induced pain model of rats

Jingjing Zhou[1], Danting Feng[1], Xiaoxue Zhang[1], Chenchen Xia[1], Zhiping Zhang[1], Jiahao Kang[1], Zhiyong Tan[2]*, Bin Wu[1,2],*

[1], Laboratory of Neuropharmacology and Neurotoxicology, Shanghai University, Shanghai 200444, P.R. China; [2], Department of Pharmacology and Toxicology and Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN, 46202, USA.

*Corresponding author

Address for Corresponding author:

Zhiyong Tan, PhD

Research Assistant Professor of Pharmacology and Toxicology

Stark Neurosciences Research Institute

Indiana University School of Medicine

Indiana University – Purdue University Indianapolis

320 W. 15th Street, NB-514E

Indianapolis, Indiana 46202

Phone: (317)278-6310

Fax: (317)274-0067

E-mail: zt2@iupui.edu

Bin Wu, PhD

Stark Neurosciences Research Institute

Indiana University School of Medicine

Indiana University – Purdue University Indianapolis

320 W. 15th Street, NB-514E

Indianapolis, Indiana 46202

Phone: (317)7788665

E-mail: bwu1@iu.edu

Abstract

The P2X7 receptor (P2X7R) plays an important role in inflammatory and neuropathic pain. Our recent study indicated that activation of P2X7R in microglial cells of spinal cord contributes to the inflammatory pain induced by BmK I , the major active compound from *Buthusmartensi Karsch* (BmK). In the present study, we further investigated whether P2X7R in satellite glial cells (SGCs) of dorsal root ganglion (DRG) is involved in the BmK I-induced pain in rats. The results found that the expression of P2X7R in SGCs was increased in the ipsilateral side of L4–L5 DRGs after intraplantar injection of BmK I. Moreover, the expression of an inflammatory cytokine IL-1β was increased in DRG after BmK I injection. Systemic administration of an inhibitor of P2X7R (A-438079) significantly inhibited both spontaneous and evoked nociceptive behaviors induced by BmK I. These results suggest that the P2X7R in SGCs of DRG might contribute to pain induced by toxins that sensitize peripheral sensory nerves.

Keywords: Pain, Dorsal root ganglia, Interleukin 1 beta, Satellite glial cell, P2X7 receptor, BmK I

Introduction

Pathological pain is a common symptom of many conditions, and severely reduces quality of life and health status of millions of patients. It has been shown that adenosine triphosphate (ATP) receptors play important role in neuropathic and inflammatory pain conditions (Chizh and Illes 2001, Burnstock 2009, Burnstock 2013). Among the ATP receptors, the P2X7 receptor (P2X7R) can form a large, macromolecular pore upon repetitive or prolonged exposure to high concentrations of ATP (North 2002). Moreover, the P2X7R plays an important role in the initiation and maintenance of inflammatory and neuropathic pain (Chizh and Illes 2001, Sperlagh, Vizi et al. 2006, Skaper, Debetto et al. 2010). Particularly, recent study indicated that activation of P2X7R in microglial cells of spinal cord contributes to the inflammatory pain induced by BmK I, an activator of sodium channel and a major toxin component of the venom of Asian scorpion *Buthusmartensi Karsch* (BmK) (Zhou, Zhang et al. 2019). In dorsal root ganglion (DRG), P2X7R is selectively expressed in satellite glial cells (SGCs), and is involved in the modulation of nociceptive signals in DRGs (North 2002, Liu and Salter 2005, Nakatsuka and Gu 2006, Chen, Zhang et al. 2008). For instance, the P2X7R in SGCs promotes the release of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-a), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) (Arulkumaran, Unwin et al. 2011).

The inflammatory pain behaviors induced by BmK venom include spontaneous

pain, ipsilateral thermal hypersensitivity, and bilateral mechanical hypersensitivity in rats (Bai, Liu et al. 2010). The active compound BmK I purified from the venom of the BmK plays a major role in the inflammatory pain caused by the BmK venom (Bai, Zhang et al. 2003, Bai, Liu et al. 2010). The bilateral mechanical hypersensitivity is a characteristic feature of pain induced by BmK I venom or BmK I that highlights the importance of utilizing natural toxins in pain models. The DRG neuron is the primary neuron that transmits noxious stimuli from the periphery to the central nervous system (Basbaum, Bautista et al. 2009). The neuronal soma of DRG neurons communicate bilaterally with their surrounding SGCs in DRGs (Zhang, Chen et al. 2007, Chen, Zhang et al. 2008). However, it is not clear whether or how SGCs might interact with neurons in DRG in the pain model induced by toxins. In the current study, we investigated the role of P2X7R in SGCs of DRG in the BmK I-induced pain model of rat.

Materials and Methods

Experimental animals

Adult male Sprague-Dawley rats (210±10 g) used in this study were provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences. All experiments had been done according to the guidelines of International Association for the Study of Pain (IASP) for pain research in conscious animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Preparation and administration of BmK I

Crude BmK venom was purchased from an individual scorpion culture farm in Henan Province, China. BmK I used in this study was purified from the venom of scorpion BmK following the process described by Ji *et al.* (Ji, Mansuelle et al. 1996), and then assessed by both mass spectrum and high-performance liquid chromatography. Fifty microliters of BmK I (0.2 μg/μLin saline) was intraplantarly (i.pl.) injected into the left hind paw (Jiang, Pang et al. 2013). Saline solution of the same volume was used in control animals.

Preparation and administration of A-438079

A-438079 (MedChemExpress, Princeton, NJ, USA), an inhibitor of P2X7R was dissolved in saline (30.6mg/ml, 100mM). 100 microliters of A-438079 (15mg/kg) was intraperitoneal injected into rats 30 minutes before BmK I injection.

Behavioral testing

In the study, behavioral tests were used to evaluate the suppressive effect of A-438079 on BmK I-induced pain responses. The measurement of spontaneous nociceptive responses, paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTL) were performed according to the methods described by Bai *et al.* (Bai, Zhang et al. 2003).

Measurement of spontaneous nociceptive behaviors

The test box with a glass floor was placed on a steel frame above the

experimental table covered with a mirror. Before administration, rats were placed in the test box separately for habituation. 30 min after the intraperitoneal injection of A-438079, BmK I was injected into the rats' left hind paws. Spontaneous nociceptive behaviors are determined by the number of the injected hind paw flinches during 5 min interval for 2 h (Chen, Luo et al. 1999). Evaluation of spontaneous nociceptive behaviors was performed by an experimenter unaware of the experimental condition.

Measurement of PWMT

Mechanical sensitivity was detected by using a series of 10 calibrated von Frey filaments with forces ranging from 0.6 to 26 g (58011, Stoelting Co., Wood Dale, Illinois, U.S.A.). Each filaments were applied bilaterally to hind paws. Each filament was probed for same duration of 2-3 s with an inter-stimulus interval of 10 s. The positive response was indicated by brisk withdrawal and/or flinching. Each subject's PWMT was defined as the lowest force that caused at least five withdrawals out of ten consecutive applications (Chen, Luo et al. 1999). Baseline PWMT measures for each subject were taken 24 h prior to testing. Evaluation of PWMT was performed by an experimenter unaware of the experimental condition.

Measurement of PWTL

Each subject's PWTL to radiant heat stimuli was determined as previously described (Hargreaves, Dubner et al. 1988). Heat stimuli were provided with radiant heat stimulator (RTY-3, Xi'an Fenglan Instrument Factory, Xi an, Shaanxi province,

China). The heat source was a high intensity projector halogen lamp bulb (150 W, 24 V). For one rat, five stimuli were performed with a stimuli interval of 10 min, and the rat's PWTL was determined by averaging the last three values of the five consecutive stimuli. Baseline PWTL measures were taken 24h before testing. Evaluation of PWTL was performed by an experimenter unaware of the experimental condition.

Western blot

At different time points (1h, 2h, 4h, 8h, 24h) after i.p. Bmk I injection, the rats were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/kg), while naive rats were considered as control group. The L4–L5 DRGs protein lapping liquids were obtained by homogenization in ice-cold RIPA Lysis Buffer (Beyotime, Shanghai, China). After 30 min ice-water bath and centrifugation at 14000 rpm for 15 min, the supernate containing total cellular protein was collected. Then each protein concentrations were measured by Bradford Protein Assay Kit (Beyotime, Shanghai, China). Finally, SDS-PAGE Sample Loading Buffer was mixed into the supernate by proportion until heated for 5 min at boiling water. Protein samples (45μg) were separated on 5% SDS-PAGE and blotted on a PVDF membrane (0.45 μm; Millipore, Billerica, Massachusetts, U.S.A.). The membranes were then incubated in 5% non-fat milk at room temperature for 2 h. The primary antibodies listed in supplement were then individually diluted in PBS with Tween-20 (0.05%PBST) containing 1% BSA and incubated overnight at 4°C.

The blots were detected in ECL detection reagent (WBKLS0050; Millipore, Billerica, Massachusetts, U.S.A.) with a fully automatic chemiluminescence image analysis system (Tanon-5200; Tanon Science&Technology Co.,Ltd., Shanghai, China). The bands were captured with the image analysis system and quantified using Image J (National Institutes of Health, Bethesda, Maryland, U.S.A.).

Immunohistochemistry

Rats were anesthetized and perfused intracardially with 200 mL sterile saline after intraplantar injection of BmK I at different time points(2h,4h,8h, and 24h), followed by 400 mL fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PBS; pH7.4). Bilateral DRGs from L4-L5 were post-fixed in 0.1 M PBS containing 20% sucrose for dehydration until precipitates, then DRG tissues were cryoprotected in 0.1mol/L PBS containing 30% sucrose until they subsided again. Frozen serial coronal sections (14μm in thickness) were cut with a Cryostat Microtome(HM525;Thermo Fisher Microm, Walldorf, Germany) and mounted on gelatin-coated glass slides.

Frozen sections were air dried and incubated with 5% bovine serum albumin (in PBS) for 1 h at room temperature, followed by incubation with primary antibody diluents overnight at 4 °C. After rinsed in 0.01M PBS, the sections were incubated with secondary antibodies for 1.5h. Then DRG sections were rinsed again and coverslipped. The digital images were captured from fluorescent microscopy

(LSM710; Carl Zeiss, Jena, Germany) and merged by Image J software. The primary and secondary antibodies are listed in supplement.

Real-time quantitative polymerase chain reaction

Each 1 h, 2 h, 4 h, 8 h, and 24 h after intraplantar injection of 50 µl diluted BmK I solution (0.2 µg/µl) into adult male rats (n = 4 for each group), total RNA was isolated from bilateral L4-L5 DRGs with Total RNA Extractor(Trizol) (Sangon Biotech, Shanghai, China). Then the RNA was reverse-transcribed with Prime-Script® RT Master Mix (TaKaRa, Dalian, China). Primer sequences targeted to P2X7R were designed by Primer Premier 6.0 software (Premier Biosoft, California, U.S.A.) while the primers for β-actin was designed referring to a previous publication (Qin, Jiang et al. 2017). The primer sequences are listed in supplement.

Quantitative PCR was performed by CFX Connect™ Real-Time PCR System (Bio-Rad, California, U.S.A.) in SYBR® remix Ex Taq™ (TaKaRa, Dalian, China). The P2X7 subtypes mRNA was normalized to the β-actin mRNA level and the data were analyzed using the $2^{-\Delta\Delta Ct}$method (Adnan, Morton et al. 2011).

Statistical analysis

All results were expressed as mean±S.E.M. (standard error of the mean) and analyzed by GraphPad Prism 6 software(GraphPad Software, Inc., La Jolla, California, U.S.A.). Data of immunostaining also used the Image-Pro Plus 6.0 software(Media Cybernetics, Inc. Rockville, Maryland, U.S.A.). The differences between groups were compared by Two-way ANOVA followed by Dunnett's post hoc test. The data of

behavior tests were analyzed using One-way ANOVA followed by a Dunnett's post hoc test and Two-way ANOVA followed by a Bonferroni's post hoc test. The relative densities of Western blots were analyzed by one-way ANOVA followed by Dunnett's post hoc test and One-way ANOVA followed by a Tukey's post hoc test, $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Effects of BmK I on P2X7R in SGCs of DRG

Immunohistochemistry experiments were conducted to study the effects of BmK I on the expression of P2X7R in DRG. Immunoreactivity (IR) for P2X7R was stronger at the ipsilateral DRG of BmK I group (Fig.1B-E, G-J) compared to the control group (Fig.1A,F) following BmK I injection. The increase in the P2X7R reactivity started from 2h after BmK I injection (Fig.1B), peaked at 4h (Fig.1 C), decreased at 8h (Fig.1 D), and further decreased at 24h (Fig.1E). On the other hand, the staining of contralateral P2X7R did not have significant change during the same period (Fig. 1G-J, M).

The protein expression levels of the P2X7R in the DRG were further analyzed by Western blot analysis. The expression of P2X7R in the BmK I group was significantly increased compared to the control group. The P2X7R in the ipsilateral dorsal root ganglia was significantly increased at 2, 4, and 8h after BmK I administration (Fig.1 K). Compared to the ipsilateral side (Fig.1 K), a significant change of P2X7R

expression was only observed at 4 h after BmK I injection at the contralateral side of the dorsal root ganglia (Fig.1 L). Moreover, the increase at 4h after BmK I injection was more than 2x larger at the ipsilateral side compared to contralateral side of DRG.

To study if transcriptional mechanism might be involved in the increase of P2X7R receptors, we also performed qPCR experiments to study the mRNA expression of P2X7R. It was observed that mRNA expression of P2X7R was selectively increased at the ipsilateral side, but not the contralateral side of DRG at 4 and 8 h after BmK I administration (Fig.1 N&O).

To study if the increased P2X7Rs are expressed in the SGCs of DRG, the co-localization of the P2X7R and GFAP (a marker of SGCs) was measured by double immunofluorescence staining. Positive staining of P2X7R was shown in Fig.2 A,D and GFAP was shown in Fig.2 B,E. Confocal microphotography indicated that the P2X7R and GFAP were co-localized in the DRG SGCs (Fig.2 C,F). The percentage of SCGs co-labeled P2X7R and GFAP was 28.6% and 88.8% for control and BmK I groups, respectively.

3.2 Effects of BmK I on IL-1β in DRG.

The immunoreactivity of IL-1β was studied in DRG 4h after BmK I administration. In the sections of the L4-5 dorsal root ganglia from the control rats (Fig.3 A and B), only few immunoreactivity for IL-1β could be detected. The immunoreactivity of IL-1β increased significantly after BmK I administration (Fig.3

C-F).

The protein expression of IL-1β in DRG was further detected by Western blot. It was found that IL-1β was increased at both sides of DRG after BmK I injection (Fig.3 G-H). Compared to the control group, a significant increase of IL-1β was detected at 4h after BmK I administration.

3.3 Effects of a P2X7R antagonist A-438079 on BmK I-induced pain behaviors

To study the functional relevance of P2X7R in the development of BmK I-induced pain, we examined whether A-438079 reduces the BmK I-induced pain behaviors. We administrated the A-438079 (100μM, intraperitoneal) 30 minutes before BmK I or saline administration. Compared to the control group, 100μM A-438079 significantly suppressed the spontaneous pain responses (Fig.4 A,B). The suppression of flinches by A-438079 lasted for 2h(Fig.4 A). Furthermore, the BmK I-induced hypersensitivity was also reduced by A-438079. Bilateral mechanical hypersensitivity (Fig.4 C,D) and ipsilateral thermal hypersensitivity (Fig.4 E) were reduced at 4 h and 8h after BmK I administration.However, A-438079 had no effects on the contralateral thermal sensitivity (Fig.4 F).

4. Discussion

DRG neurons produce primary sensory action potentials upon peripheral stimuli and transmit the action potential signal to the spinal cord .The P2X7 receptor in DRG modulates afferent nerve activation and is involved in both neuropathic (Wu, Ma et al.

2017, Xie, Liu et al. 2017) and inflammatory pain conditions (Liu, Tao et al. 2017). Our recent study indicates that activation of P2X7R in microglial cells of spinal cord contributes to the inflammatory pain induced by BmK I (Zhou, Zhang et al. 2019). In the present study, we examined the expression of P2X7 receptors in the SGCs of DRG in the BmK I-induced pain model.

Both mRNA and immunohistochemistry experiments found that the P2X7R was significantly increased at the ipsilateral side, but not contralateral side of DRG. On the other hand, Western blot experiments found that the P2X7R was significantly increased at both the ipsilateral and contralateral side of DRG. However, the increase at the contralateral side was moderate compared to the ipsilateral side. These results suggest that BmK I induces profound and preferential increases in the P2X7R at the injection side of DRG. Moreover, the increase in the P2X7R was from 2-8 hours after BmK I injection. Therefore, it is suggested that BmK I induces a transient activation of P2X7 receptors. Double staining experiments found that the P2X7R was co-localized with GFAP suggesting that BmK I activates the P2X7R in the SGCs. Taken together, it is suggested that BmK I induces a transient, ipsilateral side preferentially increase in the expression of P2X7R in the SGCs of DRG in rats.

Notably, our results indicate that the BmK I-induced increase in the mRNA expression is earlier than that in the protein expression of P2X7R (Fig. 1N&K) This phenomenon suggests that a post-transcriptional mechanism that enhances translation of P2X7R mRNA might be involved in the early up-regulation of P2X7R protein induced by BmK I. On the other hand, the BmK I-induced increase in mRNA peaked

289 at 8 h while the increase in protein peaked at 4 h (Fig. 1N&K). This result suggests a
290 negative post-transcriptional mechanism might be also involved in the modulation of
291 BmK I on the expression of P2X7R. Interestingly, a brain enriched microRNA,
292 miR-22 was recently identified to control the expression of P2X7R in hippocampus. It
293 can selectively silence the mRNA of P2X7R resulting in the decreased expression of
294 protein, but not mRNA level of P2X7R (Jimenez-Mateos, Arribas-Blazquez et al.
295 2015, Engel, Brennan et al. 2017). It might be suspected that there may be a similar
296 post-transcriptional feedback mechanism in DRG which inhibits the continual
297 increasing of P2X7R protein in the BmK I model.

298 It has been generally assumed that pro-inflammatory cytokines, including IL-1β
299 and TNF-α, play an important role in the initiation and maintenance of inflammatory
300 (Albuquerque, Fonteles et al. 2017) and neuropathic pain (Wu, Peng et al. 2017, Xie,
301 Liu et al. 2017). It has been demonstrated that P2X7 receptors can mediate the release
302 of IL-1β (Burnstock and Knight 2018). Our results found that the expression of IL-1β
303 was increased in the BmK I-induced rats. Therefore, it was suggested that the
304 activation of the P2X7R might lead to the release of IL-1β in the DRG following
305 BmK I injection. However, our results found that the increase in IL-1β was similar
306 between ipsilateral and contralateral sides while the increase in P2X7R was
307 preferentially on the ipsilateral side. The results suggest that BmK I-induced
308 up-regulation of P2X7R might preferentially contribute to the release of IL-1β at the
309 ipsilateral side of DRG, and that there might have other mechanism contributing to
310 the up-regulation of IL-1β at the contralateral side of DRG.

In addition to the increased expression of P2X7R and IL-1β in DRG, we also found that systemic administration of A-438079 reduced both evoked and spontaneous pain behaviors induced by BmK I. These results suggest that P2X7R in SGCs of DRG might contribute to the pain hypersensitivity in the BmK I –induced pain model. Moreover, our recent study suggested that microglial P2X7R in spinal cord might contribute to the BmK I –induced pain. Therefore, both P2X7R in SGCs of DRG and in microglial cells of spinal cord might contribute to the pain hypersensitivity induced by BmK I.

The effects of peripheral SGCs on pain have been studied in the DRG (Hanani, Huang et al. 2002, Hanani 2005). Spontaneous pain activity originating at the injured side or DRG neurons may be a cause of glial activation (Chung and Chung 2002, Xie, Strong et al. 2009). It is well known that the soma of neurons in primary sensory ganglia are tightly enwrapped by SGCs. The SGCs in DRG express P2X7 receptors (Gu, Chen et al. 2010, Chen, Li et al. 2012, Puchalowicz, Baranowska-Bosiacka et al. 2015) and can communicate with neurons by signaling molecules. P2X7Rs in SGCs are endogenously active in the DRG (Chen, Zhang et al. 2008, Huang, Gu et al. 2013). Therefore, increased P2X7Rs in SGCs of DRG might contribute to the activation of SGCs following BmK I injection. Activated SGCs might release excitatory neuropeptides such as IL-1β that can increase the excitability of DRG neurons, and the pain sensitivity in the BmK I pain model.

5. Conclusion

In conclusion, the present study provides first evidence to support an involvement of peripheral P2X7R (expressed in the SGCs in DRG) in the pain induced by a toxin (BmK I).

Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure Legends:

Fig.1: BmK I induces P2X7R activation in DRG

The spatiotemporal distribution of P2X7R in DRG following the injection of BmK I (A-K). Compared with the saline group (A,F), BmK I-treated groups (B-E) showed largely increased P2X7R immunoreactivity in the ipsilateral DRGs. Increased ipsilateral P2X7R immunoreactivity began at 2 h and peaked at 4 h following the administration of BmK I. Scale bar: (A-J) 100μm (K). The histogram represents the statistic results of P2X7R expression in bilateral DRG.***p< 0.001, **p< 0.01, and *p< 0.05 (n=3), when compared with control group by Two-way ANOVA, Dunnett's post hoc test, Error bars indicate SEM (K).Western blot analysis of P2X7R in DRG after intraplantar injection of BmK I(G,H). Representative Western blots show levels of P2X7R and β-actin in both ipsilateral (G) and contralateral (H) sides of DRG, histograms represent the mean levels with respect to each control group at different time points after intraplantar BmK I injection. QPCR results of P2X7R mRNA expression on the ipsilateral (N) and contralateral (O) sides of spinal cord. The data are presented as mean ± S.E.M. of three rats per group. *p<0.05, **p<0.01, ***p<0.001, when compared with control group and assessed using a One-way ANOVA, Dunnett's post hoc test.

Fig 2: Cellular localization of P2X7R immunoreactivity in DRG.

Double immunofluorescence of P2X7R in DRG after intraplantar administration of BmK I. (A-F) (A, D) showed the positive staining of P2X7R while (B, E) showed the positive staining of GFAP. (C, F) showed the colocalization of P2X7R with GFAP. Scale bars: (A-F) 50 μm;

Fig 3: Effects of BmK I on the release of IL-1β in DRG.

Immunoreactivity of IL-1β in the rat DRG following the injection of BmK I. (A-F) Compared with the saline group (A-B), bilateral IL-1β immunoreactivity of DRG increased significantly in

BmK I-treated rats (C-F). White open squares (in C, D) indicate the corresponding scope of the amplified images (E, F) in the confocal images. Scale bars: (A-D) 100 μm; (E, F) 50 μm. Western blot analysis of IL-1β in DRG in the presence of BmK I (G, H). (G) and (H), representative Western blots showing levels of IL-1β and β-actin in both ipsilateral (G) and contralateral (H) sides of DRG, histograms represent the mean levels with respect to each control group at different time points after intraplantar BmK I injection. The data are presented as mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 (n=3), when compared with control group and assessed using a one-way ANOVA, followed by Dunnett's post hoc test.

Fig 4: P2X7R antagonist A-438079 inhibits the BmK I-induced pain.

(A) Attenuated spontaneous pain behavior was observed after pretreatment of 100 μM A-438079 (i.p.) 30 min before local administration of BmK I. Total number of paw flinches (B) was suppressed by the pretreatment of A-438079 within the 2 h following the injection of BmK I. A-438079 reduced both ipsilateral (C) and contralateral (D) mechanical hypersensitivity as well as ipsilateral (E) thermal hypersensitivity. A-438079 had no effect on contralateral basal thermal latency values (F). *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA, Dunnett's post hoc test and Two-way ANOVA, Bonfereoni's post hoc test, when compared with saline vehicle group. n=6 for each group.

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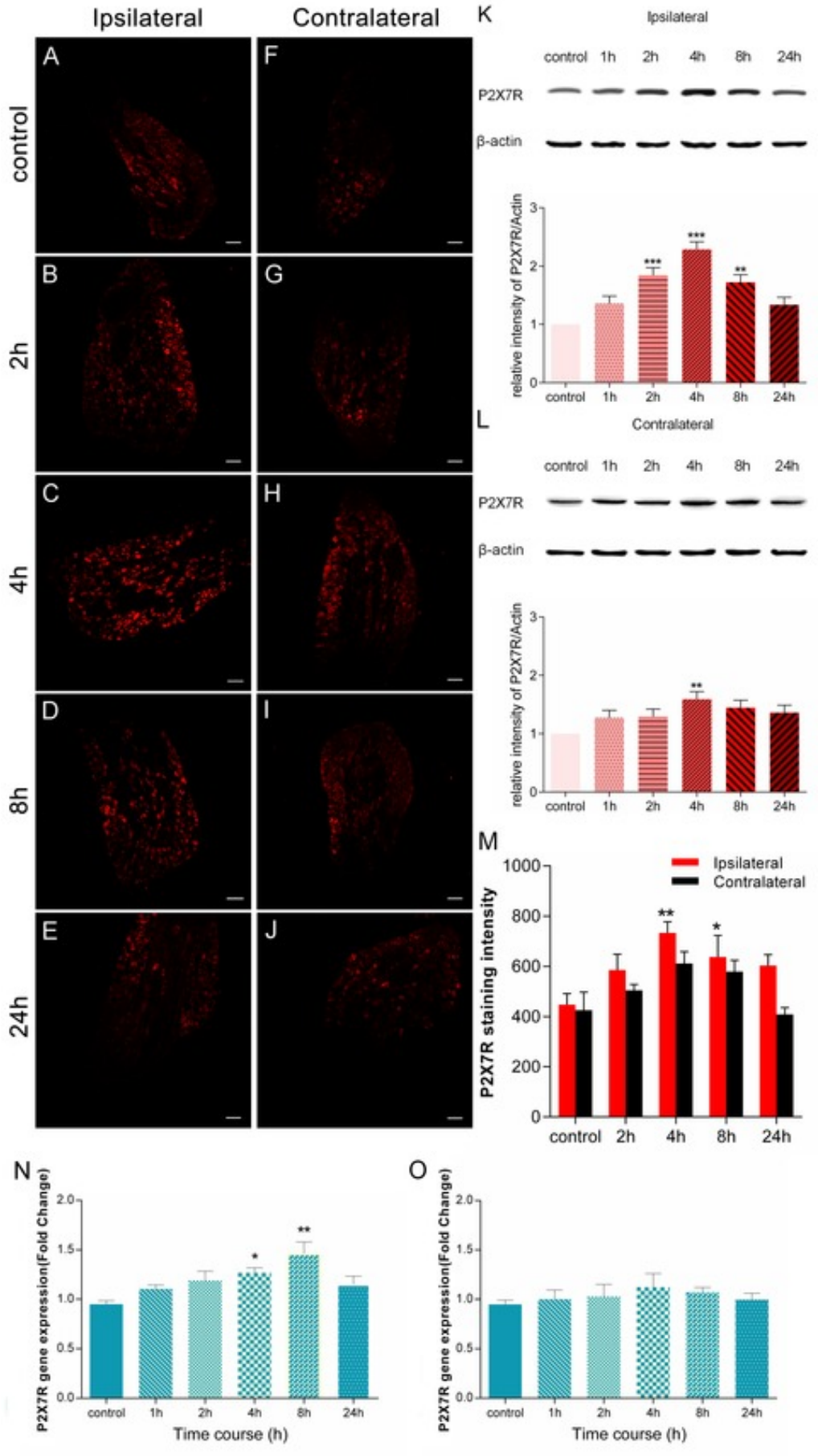

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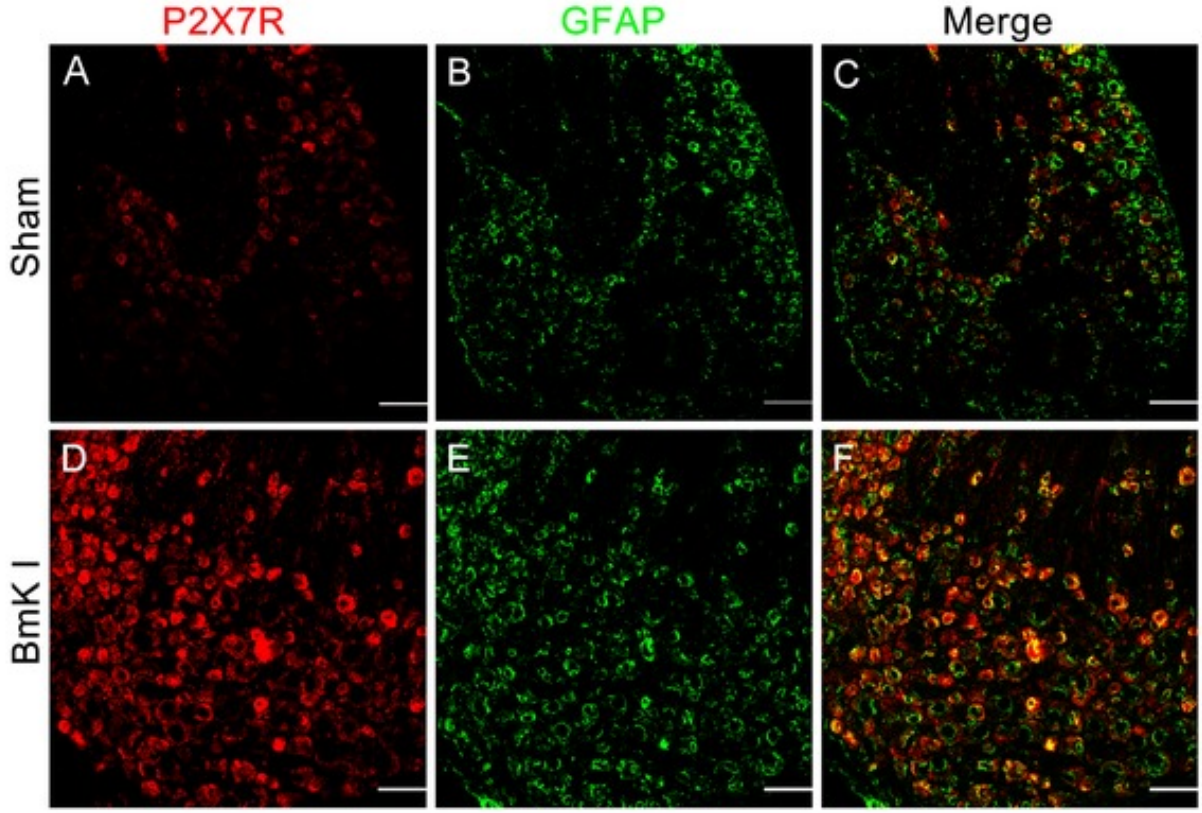

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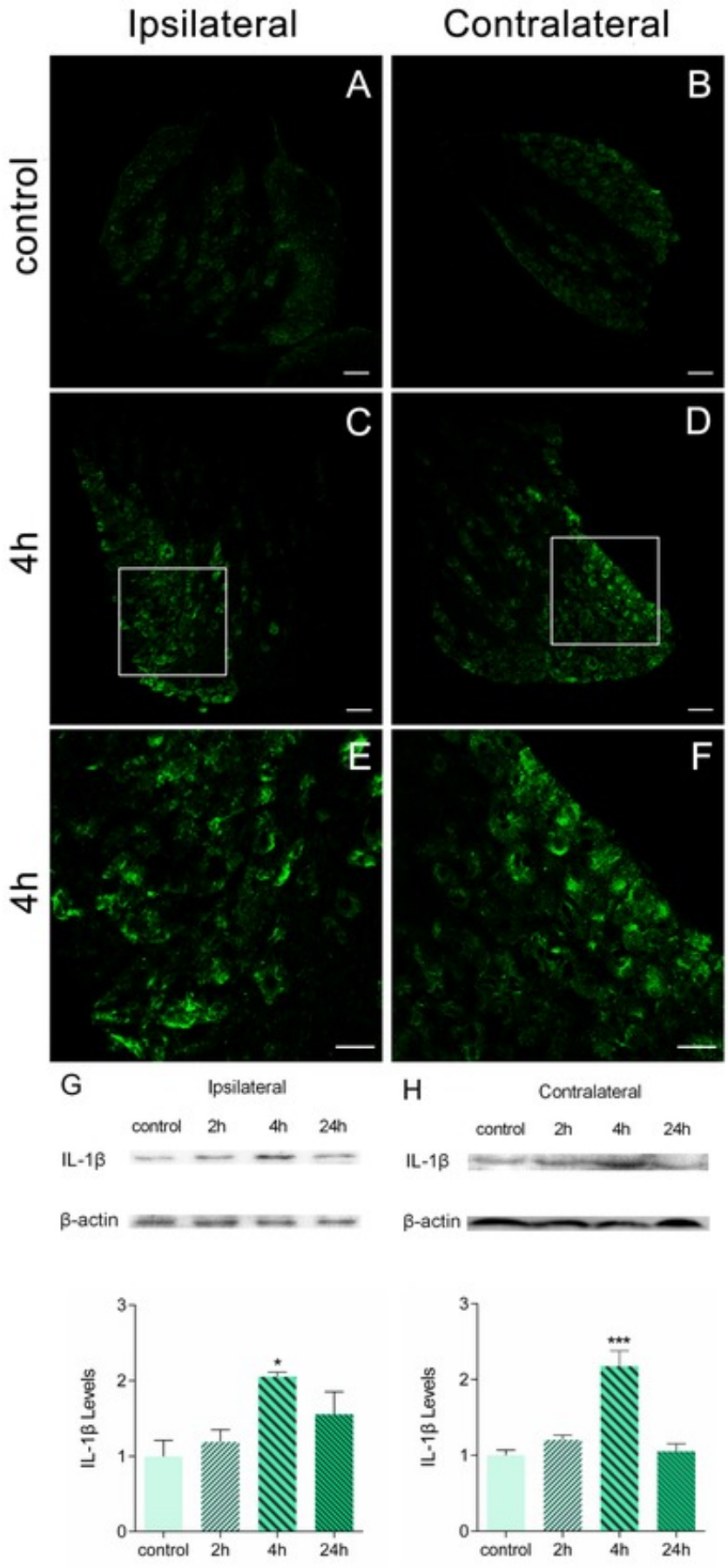

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