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Induction of the P2X7 receptor in spinal microglia in a neuropathic pain model

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ABSTRACT

Peripheral nerve injury causes a progressive series of morphological changes in spinal microglia, and extracellular ATP stimulates proliferation of microglia and may be involved in neuropathic pain. We defined the precise expression of P2X7 in the spinal cord following peripheral nerve injury. We found that both P2X7 mRNA and protein increased in the spinal cord, with a peak at 7 d after injury. Double labeling studies revealed that cells expressing increased P2X7 mRNA and protein after nerve injury were predominantly microglia in dorsal horn. Pharmacological blockades by intrathecal administration of a P2X7 antagonist (A 438079 hydrochloride) suppressed the development of mechanical hypersensitivity. We present distinct evidence that increases in the number of P2X7 receptors in spinal microglia may play an important role in neuropathic pain.

Peripheral nerve injury can trigger neurological abnormalities, including neuropathic pain. Recent findings demonstrate the important role of spinal microglial activation following peripheral nerve injury in the pathomechanism of neuropathic pain. Extracellular ATP causes many pathological reactions of microglia, one of which is microglial activation [19]. ATP receptors (P2 receptors) are divided into 7 subtypes of ionotropic P2X receptors (P2X1–P2X7) and 8 subtypes of metabotropic P2Y receptors [5]. P2X receptors are assembled from different subunits into homo- or heterotrimers and the ATP binding opens the pore permeable to Na^+, K^+, and Ca^{2+}. It has been shown that the P2X4 receptor in microglia plays crucial roles in neuropathic pain development [20]. Recently, the possible involvement of spinal P2X7 in neuropathic pain has been suggested using pharmacological examinations [2,11,17]. In addition, the study with mice with genetic knockout of the P2X7 gene showed a suppression of pain related behaviors following peripheral nerve injury [7]. Although these studies suggest that P2X7 plays an important role in the pathology of neuropathic pain, the morphological of P2X7 expression in spinal cord has not been determined. In this study, we examined the precise expression of P2X7 in the spinal cord following peripheral nerve injury, both in terms of mRNA and in terms of protein levels, and examined the involvement of P2X7 in neuropathic pain behavior using a specific inhibitor of P2X7.

All animal experimental procedures were approved by the Hyogo College of Medicine Committee on Animal Research and were carried out in accordance with the National Institutes of Health guidelines on animal care. Male Sprague Dawley rats weighing 200–250 g were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the tibial and common peroneal nerves were transected, while the sural nerve was left intact (spared nerve injury; SNI model). The wounds were then closed and the rats were allowed to recover. At several time points (1, 3, 7, 14 and 30 days) following the surgery, groups of rats were processed for analysis. Every effort was made to minimize animal suffering and reduce the number of animals used.

PCR primers for P2X7 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA were designed as follows: P2X7 primers (accession number X95882, 949–1342), sense 5′-CCGGCTGGACGACAAGTACA-3′ and antisense 5′-GGGCTCGTCCACAAAGGACA3′; GAPDH primers (accession number M17701, 80–350), sense 5′-TGCTGGTGCTGAGTATGTCG-3′ and antisense 5′-GCATGTCAGATCCACAACGG-3′. Samples for RT-PCR and ISHH were prepared as described before [13]. For ISHH, the bilateral L4-5 spinal cord was dissected out, rapidly frozen in powdered dry ice, and cut on a cryostat at a 12 μm thickness. These sections were processed for ISHH.

In order to examine the distribution of mRNAs for P2X7 receptors, we used a combined IHC with ISHH. The bilateral L4-5 spinal cord was dissected out, rapidly frozen in powdered dry ice, and

Abbreviations: ATP, adenosine 5′-triphosphate; SNI, spared nerve injury; RT-PCR, reverse transcription-polymerase chain reaction; ISHH, in situ hybridization; TBS, Tris buffered saline; CDP-Star, disodium 2-chloro-5-(methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate; SEM, standard error; IHC, immunohistochemistry; Iba1, ionized calcium binding adaptor molecule 1; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; mRNA, messenger ribonucleic acid; PB, phosphate buffer; TNFα, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; BDNF, brain-derived neurotrophic factor.

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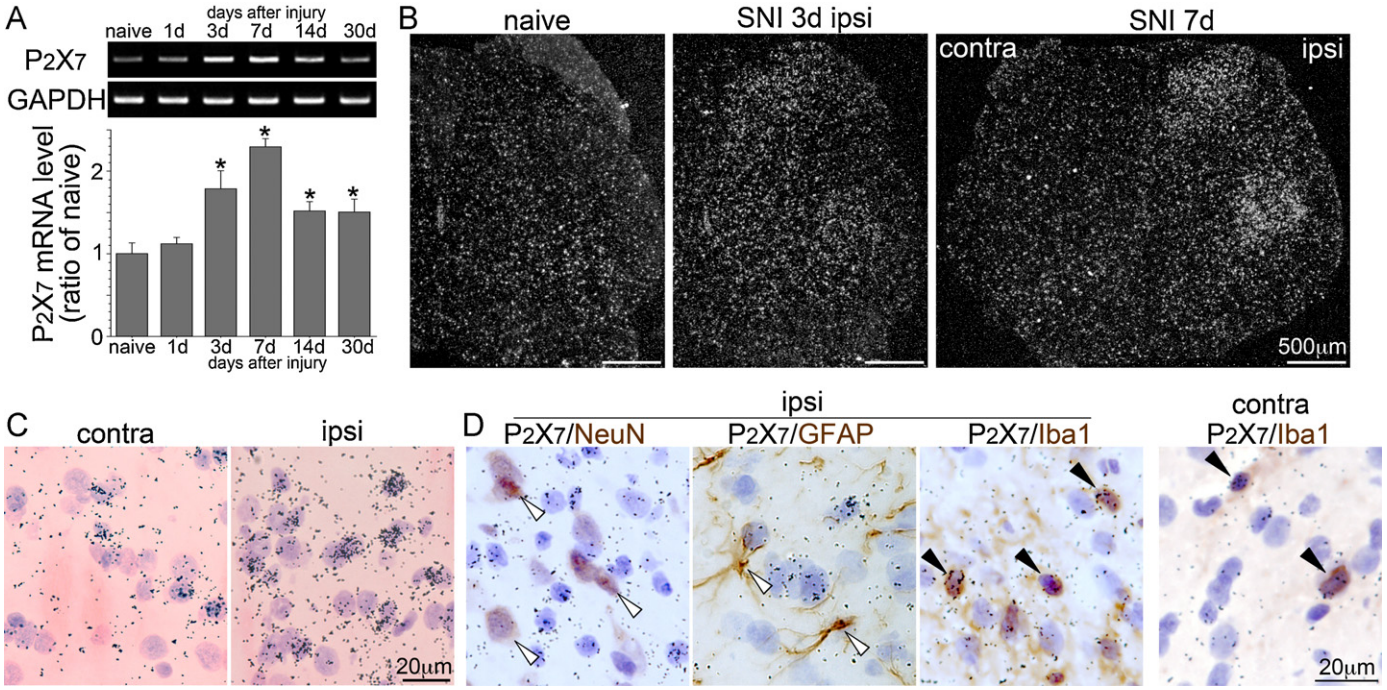

Fig. 1. Peripheral nerve injury in the spared nerve injury model (SNI) induces mRNAs of the P2X7 receptor in ipsilateral spinal microglia. (A) Gel panels show RT-PCR products from the L4–L5 spinal cord that were naive, 1, 3, 7, 14 and 30 days after surgery, and the lower graph shows the quantification of the relative mRNA levels of P2X7 in the spinal cord. P2X7 mRNA levels were normalized against the corresponding control, and ANOVA followed by Fisher's PLSD post hoc comparison were used for statistical analysis (mean ± SEM, each time points $n = 5$) (*$p < 0.05$ compared with naive). (B) Dark-field images of ISHH show P2X7 mRNA expression in the spinal cord of a naive rat, and 3 and 7 days after nerve injury. Scale bars, 500 μm. (C) Bright field photographs of laminae I–II in the contralateral and ipsilateral spinal cord 7 days after nerve injury. The sections were stained with hematoxylin–eosin. Scale bar, 20 μm. (D) Bright-field photomicrographs of laminae I–II in the contralateral and ipsilateral spinal cord 7 days after nerve injury shown by combined ISHH for P2X7 receptor mRNA with IHC for NeuN, GFAP, and Iba1. The sections were stained with hematoxylin. Black arrowheads indicate double-labeled cells and open arrowheads indicate single-immunostained cells (brown staining). Scale bar, 20 μm.

cut on a cryostat at a 12 μm thickness. The cryostat sections were fixed in 4% formaldehyde in 0.1 M phosphate buffer (PB) for 20 min. The spinal cord sections were processed for immunohistochemistry (IHC) using the ABC method. The following antibodies were used: rabbit anti-Iba1 polyclonal antiserum (Wako Chemicals, Tokyo, Japan), mouse anti-NeuN monoclonal antiserum (1:1000, Chemicon) and rabbit anti-GFAP polyclonal antiserum (1:1000, Chemicon). After IHC, these sections were immediately processed for ISHH. These sections were fixed again in 4% formaldehyde in PB for 5 min, treated with 10 μg/mL protease K in 50 mM Tris–5 mM EDTA (pH 7.5) for 5 min, postfixed in the same fixative, acetylated with acetic anhydride in 0.1 M triethanolamine, rinsed in PB, and dehydrated through an ascending ethanol series. The following processes are the same as those mentioned in ISHH. More details of the treatment of sections and methods of double labeling with IHC and ISHH were described in a previous paper [14].

The spinal samples preparation and Western blotting were performed as described before [15]. Membranes were incubated with Blocking One P (Nakarai, Kyoto, Japan) in Tris buffer containing Tween 20 (TBS-T) (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.2% Tween 20) for 20 min at room temperature and incubated with the polyclonal primary antibody for P2X7 (1:2000, corresponding to residues 576–595 of rat P2X7; Alomone, Israel) at 4 °C overnight. Membranes were then washed twice with TBST and probed with goat anti-rabbit IgG conjugated with alkaliphosphatase (1:2000, Chemicon, California, USA) at room temperature for 2 h and visualized by chemiluminescence using CDP-Star (Roche, Indianapolis, USA). The loading and blotting of the amount of protein was verified by reprobing the membrane with anti-β-actin antiserum (1:2000; Sigma, Missouri, USA). Films were scanned and quantified using NIH Image, version 1.61 and normalized against a loading control (beta-actin). Data are expressed as mean ± SEM. Differences in changes of values over time of each group were tested using one-way ANOVA, followed by individual post hoc comparisons (Fisher's exact test). A difference was accepted as significant if $p < 0.05$.

IHC was performed as described before [15]. The tissue was frozen in powdered dry ice, cut on a cryostat at a 25 μm thickness. The following antibodies were used for IHC: rabbit anti P2X7 polyclonal antiserum (1:250, Alomone), goat anti Iba1 polyclonal antiserum (1:500, abcam, Cambridge, MA), mouse anti NeuN monoclonal antiserum (1:2000, Chemicon) and mouse anti GFAP polyclonal antiserum (1:2000, Chemicon). In brief, spinal cord sections were incubated with a primary antibody over night at 4 °C and followed by secondary antibodies; anti rabbit Alexa Fluor 594 IgG (1:1000; Invitrogen, CA, USA), anti goat Alexa Fluor 488 IgG (1:1000) and anti mouse Alexa Fluor 488 IgG (1:1000) after incubation with respective primary antibodies.

A preabsorption control experiment with the P2X7 receptor peptide (corresponding to amino acid residues 576–595 of rat P2X7, Alomone) was performed to test the specificity of the anti P2X7 antibody. Anti P2X7 IgG was incubated over night at 4 °C with the P2X7 receptor peptide that was used to generate the antibody. Dilutions of 1:250 (P2X7 antibody, final concentration: 2.4 μg/mL) and 1:1 for the P2X7 receptor peptide (final concentration: 2.4 μg/mL) were used in this study.

After the SNI, the L5 vertebra was laminectomized under adequate anesthesia with sodium pentobarbital, and a soft tube (Silastic laboratory tubing, Dow Corning, Midland, MI, USA, outer diameter, 0.64 mm) filled with 5 μl of saline was inserted into the subarachnoid space for an ~0.5 cm length. After the muscle incision was closed, the mini-osmotic pumps (Alzet model 2001; 7d pump, 1 μl/h, CA, USA) filled with P2X7 antagonist A438079 hydrochloride (100 n mol/μl, TOCRIS, MO, USA) diluted by saline were connected