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Research paper

Effects of riluzole on P2X7R expression in the spinal cord in rat model of neuropathic pain

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HIGHLIGHTS

- P2X7 receptor expression in the spinal cord was upregulated during neuropathic pain process.
- P2X7 receptor mainly expressed in microglia.
- Riluzole attenuated neuropathic pain, mainly via the downregulation of P2X7R expression and inhibition of microglial activation.
- Anti-P2X7R effects of riluzole were not mediated via voltage-dependent sodium channels.

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ABSTRACT

Neuropathic pain is becoming an intractable health threat, with its profound effect on quality of life, thus posing a major challenge to clinical therapy. Despite the reported efficacy of riluzole in some pain models, the underlying mechanism remains largely unknown. The present study aimed to assess the effects of riluzole in a rat model of neuropathic pain induced by chronic constriction injury (CCI). Subsequent to model establishment, paw withdrawal latencies (PWLs) and the paw withdrawal mecha threshold (PWT) rapidly decreased, coupled with inhibited microglial activation and upregulated P2X7R expression in the spinal cord dorsal horn (SCDH). Following intraperitoneal administration of riluzole (4 mg/kg) once daily for 5 consecutive days as from day 3 after surgery, the mechanical allodynia and thermal hyperalgesia in the hind limbs were significantly attenuated. In addition, riluzole downregulated P2X7R expression and inhibited microglial activation in SCDH. Our results indicated that riluzole is effective in alleviating neuropathic pain and inhibiting microglial activation, presumably via the downregulated P2X7R expression in SCDH.

1. Introduction

Neuropathic pain is defined as pain resulting from injury or dysfunction of the peripheral nerves [16,19]. However, effective treatment is hampered by an incomplete understanding of pathogenesis of neuropathic pain. With a view to exploring novel therapies available for neuropathic pain, studies at the bench and bedside are contributing to the exploration of novel anticonvulsants and neuroprotective agents.

Riluzole is an anticonvulsant with neuroprotective properties. In addition to its clinical applications in traumatic spinal cord injury [18], riluzole can also alleviate symptoms in animal models of neurodegenerative disease, which is attributable to its inhibition of presynaptic glutamate release by blockage of voltage-gated sodium channels [3,15,23]. Furthermore, increasing evidence has confirmed the anti-nociceptive and anti-allodynic efficacies of riluzole in rat models of SCI and in other pain models [24,27], whereas the underlying mechanisms remain unfolded whatsoever.

Neuroimmune alterations reportedly contribute to pain after injury to the nervous system [28,31,33]. Glial cells are shown to mediate inflammatory processes, and microglia notably plays a cru-

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cial role in the initiation of peripheral injury-induced pain [22,34]. These cells express receptors for the neurotransmitters released from the central terminals of primary afferents, such as purinergic receptors including P2X7 and P2X4 [36]. The ATP-sensitive homomeric P2X7 receptor (P2X7R) is a nonselective cation channel involved in the development of inflammatory or neuropathic pain and neuronal sensitization, via the regulation of the expression and release of inflammatory cytokines (i.e. IL-1β and TNF) from microglia [10,17]. Riluzole reportedly attenuates microglial activation in animal models of cervical spondylotic myelopathy (CSM) [26]. However, there is a paucity of literature regarding the effects of riluzole on microglia. P2X7 receptors are overexpressed in microglia and are involved in regulating microglial activation via release of cytokines [11,25]. Hence, it would be of interest to reveal the means by which riluzole alleviates neuropathic pain and microglial activation as well as the role of P2X7 receptors in SCDH. Accordingly, we affirmed that riluzole mitigated neuropathic pain and microglial activation via the inhibition of P2X7R expression.

2. Material and methods

2.1. Animals, drugs and drug administration

Male Sprague-Dawley rats (200–250 g) from the Experimental Animal Center, Xuzhou Medical College were housed at a constant ambient temperature of 24 ± 1 °C under a 12 h light/dark cycle with ad libitum access to food and water. All experiments were in conformance with the guidelines of the International Association for the Study of Pain, with the approval of the institutional Committee for the Ethical Use of Laboratory Animals. Briefly, riluzole dissolved in 5% DMSO (2.5 mg/ml) was intraperitoneally (i.p.) administered (4 mg/kg) once daily for 5 consecutive days as from day 3 after surgery.

2.2. Neuropathic pain model: chronic constrictive injury

With CCI model established [4], rats were anesthetized under sodium pentobarbital (300 mg/kg, i.p.). The left sciatic nerve was exposed, followed by loose ligations with 4–0 silk suture (at a 1-mm interval), with identical procedures in the sham group except ligations for nerve injury.

2.3. Behavioral test

Paw withdrawal latencies (PWLs) in response to thermal hyperalgesia were measured by the IITC Plantar Analgesia Meter (IITC Life Science) [20]. Animals were habituated for 2 days prior to blinded behavioral test. Rats were placed in transparent acrylic enclosures (7 cm by 9 cm by 11 cm) with a glass plate and acclimatized for 1 h in a temperature-controlled (23–26 °C) and noise-free room. The thermal stimulus was focused on a portion of the hind paws with a radiant source, which could be switched off responsive to hind paw movement or 25 s afterwards to obviate tissue damage, with the test performed in triplicate at an interval of 5 min.

Paw withdrawal threshold (PWT) in response to mechanical allodynia was assessed with von Frey filaments (North Coast Medical). Rats were placed in individual plastic boxes (20 cm by 25 cm by 15 cm) on a metal mesh floor and acclimated for 1 h. The filaments were presented, in ascending order of strength, perpendicular to the plantar surface with sufficient force to cause slight bending against the paw for 6–8 s. Presence of paw withdrawal or flinching was considered as a positive response. The PWT was determined by the "up-and-down" method, with the data analyzed using the nonparametric method of Dixon [9].

2.4. Western blotting

Rats were sacrificed immediately after behavioral test, followed by isolation of the lumbar spinal cords, which were frozen in liquid nitrogen and stored at −80 °C. Tissue samples were homogenized in RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail. Lysates were centrifuged at 12000 rpm for 15 min at 4 °C, and protein concentration was determined by the bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). Samples with equal amount of protein were separated by 8% PAGE (40 μg total protein per lane) and transferred onto polyvinylidene difluoride membranes (Millipore, USA), followed by blockage with 5% skim milk for 2 h and incubation overnight with primary polyclonal antibody rabbit anti-P2X7R (1:250; Alomone, Israel). The blots were extensively rinsed with washing buffer and incubated for 2 h with the secondary antibody mouse anti-β-actin conjugated with alkaline phosphatase (1:1000; ZSGB-Bio, China) at room temperature (r/t). The immune complexes were detected using a NBT/BCIP assay kit (Sigma-Aldrich). Western blot densitometry analysis of signal intensity was performed using ImageJ software.

2.5. Immunohistofluorescence

Rats were anesthetized under 10% chloral hydrate (300 mg/kg, i.p.) and underwent sternotomy followed by intracardial perfusion with 200 ml normal saline and 200 ml 4% ice-cold paraformaldehyde (PFA; 4% in 0.1 M phosphate buffer). The lumbar spinal cord tissues were isolated, post-fixed in 4% PFA overnight and subsequently allowed to equilibrate in 30% sucrose in phosphate buffer overnight at 4 °C. Transverse sections (40 μm) were sliced on a cryostat and stored in phosphate buffer. After three washes in PBS, the sections were incubated in PBS containing 10% normal donkey serum and 0.3% Triton X-100 (PBST) at r/t for 2 h, and incubated with primary antibodies, rabbit anti-PP2X7R (1:100; Alomone, Israel) and goat anti-Iba1(1:400; Abcam) in PBST overnight at 4 °C. Thereafter, slices were rinsed thrice with PBS at a 5-min interval and incubated with Alexa Fluor 488- or 594-conjugated secondary antibody (1:200; Invitrogen) for 2 h at r/t. Images were captured using a high-resolution digital confocal microscope (Olympus, Japan).

2.6. Statistical analysis

Data were represented as mean ± SEM of 3 individual experiments. Statistical analysis was performed using Prism 5.0 (GraphPad) software. One-way ANOVA for data from different groups and two-way ANOVA with one repeated factor (time) for data of TWLs and PWT were employed ($p < 0.05$ was considered significant).

3. Results

3.1. Upregulation of P2X7R in the spinal cord in CCI rats

Compared with the control and sham groups, PWLs in CCI group were significantly decreased from day 3 after the CCI surgery to day 14 ($p < 0.01$; Fig. 1A). Similar results were observed for the PWT values ($p < 0.01$; Fig. 1B), with no significant difference between control group (Con) and sham group (Sham), indicating successful establishment of CCI models. Since P2X7R is involved in the development of inflammatory or neuropathic pain [21], we performed western blotting analysis to determine P2X7R expression, which revealed that P2X7R expression in the spinal cord in the CCI rat model was significantly upregulated on day 7, and maintained till day 14 ($P < 0.001$; Fig. 1C and D).