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Original Reports

Role of P2X7 Receptor-Mediated IL-18/IL-18R Signaling in Morphine Tolerance: Multiple Glial-Neuronal Dialogues in the Rat Spinal Cord

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Abstract: The glial function in morphine tolerance has been explored, but its mechanisms remain unclear. Our previous study has showed that microglia-expressed P2X7 receptors (P2X7R) contribute to the induction of tolerance to morphine analgesia in rats. This study further explored the potential downstream mechanisms of P2X7R underlying morphine tolerance. The results revealed that the blockade of P2X7 receptor by P2X7R antagonist or targeting small interfering RNA (siRNA) reduced tolerance to morphine analgesia in the pain behavioral test and spinal extracellular recordings in vivo and whole-cell recording of the spinal cord slice in vitro. Chronic morphine treatment induced an increase in the expression of interleukin (IL)-18 by microglia, IL-18 receptor (IL-18R) by astrocytes, and protein kinase Cγ (PKCγ) by neurons in the spinal dorsal horn, respectively, which was blocked by a P2X7R antagonist or targeting siRNA. Chronic morphine treatment also induced an increased release of D-serine from the spinal astrocytes. Further, both D-amino acid oxygenase (DAAO), a degrading enzyme of D-serine, and bisindolylmaleimide α (BIM), a PKC inhibitor, attenuated morphine tolerance. The present study demonstrated a spinal mechanism underlying morphine tolerance, in which chronic morphine triggered multiple dialogues between glial and neuronal cells in the spinal cord via a cascade involving a P2X7R–IL-18–D-serine–*N*-methyl-D-aspartate receptor (NMDAR)–PKCγ-mediated signaling pathway.

***Perspective:* The present study shows that glia-neuron interaction via a cascade (P2X7R–IL-18–D-serine–NMDAR–PKCγ) in the spinal cord plays an important role in morphine tolerance. This article may represent potential new therapeutic targets for preventing morphine analgesic tolerance in clinical management of chronic pain.**

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Key words: *P2X7 receptor, IL-18, glia-neuronal interaction, morphine tolerance.*

Morphine is a highly potent analgesic to treat diverse pains. However, its effectiveness gradually subsides following repetitive administration, leading to the diminished analgesic effect in the clinical setting.[2] The mechanism underlying morphine tolerance has been considered to be multifactorial, including desensitization of opioid receptors and functional changes in glutamate *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) and glutamate transporters.[25-29,51] Our early study first showed that disruption of activity of spinal glia blocked the induction of tolerance to morphine analgesia, suggesting a novel glial mechanism.[47] The glial function in morphine tolerance has been confirmed and extended by others.[6,40,55] Several studies have revealed that purinergic P2X7 receptor (P2X7R) is predominately expressed in microglia.[5,20,48,62] Our recent study reported a crucial role of P2X7R expressed by spinal microglia in the induction of morphine tolerance, showing that chronic morphine treatment upregulated expression of P2X7R in microglia in association with morphine

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tolerance and that a P2X7R antagonist and its targeting small interfering RNA (siRNA) blocked both P2X7R upregulation and morphine tolerance.[64] These findings explore a relevant signal molecule expressed by microglia.

Activation of P2X7R by extracellular adenosine triphosphate (ATP) is a crucial factor for the production and release of proinflammatory cytokine interleukin-1β (IL-1β).[4,9,52] Chronic morphine treatment induces upregulation of P2X7R[64] and the release of IL-1β along with morphine tolerance.[18,45] IL-1β and another cytokine, IL-18, are siblings with structural homology and share the IL-1β-converting enzyme. IL-18 is specifically expressed by microglia, and its receptor (IL-18R) is predominately expressed in astrocytes.[15,16,19,32] Given microglial P2X7R-mediated morphine tolerance, it would be of considerable interest to examine whether a dialogue between microglia expressing IL-18 and astrocyte expressing IL-18R is involved in tolerance to morphine analgesia.

Glutamate neurotransmission via NMDARs plays a critical role in chronic opioid-induced neuronal adaptation.[21,24,53] D-serine, an endogenous ligand for the glycine site of NMDARs, is synthesized and released by astrocytes and has been identified as a major gliotransmitter to trigger neuronal function in the central nervous system.[36,38] Therefore, it is critical to address whether D-serine released from IL-18-activated astrocytes would trigger pain-sensitive neurons in the spinal cord and mediate morphine tolerance.

This study was performed to investigate the potential downstream mechanisms of P2X7R underlying the induction of tolerance to morphine analgesia in rats by means of behavioral, electrophysiological, immunohistochemical, and pharmacological techniques. Our data provide first evidence for multiple dialogues among neurons–microglia–astrocytes–neurons through IL-18 signal transduction cascades mediating the development of morphine tolerance.

Methods

Animals

Experiments were performed on adult (weighing 180–200 g, or 250–350 g) and young (11- to 12-day-old) male Sprague Dawley rats for in vivo studies and spinal cord slice studies, respectively. Animals were obtained from the Experimental Animal Center, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, and were housed under a 12/12 hour light/dark cycle at a room temperature of 22 ± 1°C with food and water available ad libitum. All experiments were carried out with the approval of the Shanghai Animal Care and Use Committee and followed the policies issued by International Association for the Study of Pain on the use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experimenters were blind to drug treatments during the behavioral study.

Drugs

Morphine hydrochloride was purchased from Shenyang First Pharmaceutical Factory (Shenyang City, China). Brilliant Blue G (BBG), a noncompetitive selective antagonist of P2X7R, D-amino acid oxidase (DAAO), which depletes endogenous D-serine but not glycine, and bisindolylmaleimide α (BIM), a protein kinase C (PKC) inhibitor, were purchased from Sigma-Aldrich Co (St. Louis, MO). BBG (10 μM) and DAAO (50 μM) were diluted with saline. BIM (1.15 μM) was diluted in 10% demethylsulfoxide (DMSO). IL-18-binding protein (IL-18BP) and IL-18 antibodies were purchased from R&D Systems (Minneapolis, MN) and dissolved in sterilized phosphate buffered saline (PBS) (pH 7.4). Isofrane was purchased from Hebei Jiupai Pharmaceutical Company (Shijiazhuang City, China).

Induction of Morphine Tolerance

Morphine was given subcutaneously (s.c.) twice daily at 12-hour intervals, from day 1 to, at most, day 9 at 10 mg/kg body weight, to establish systemic analgesic tolerance. In the control group, saline injection (1 mL/kg, s.c.) was applied with the same schedule. To evaluate the development of morphine tolerance, morphine antinociception to thermal stimuli was assessed 30 minutes after an acute dose (5 mg/kg) of morphine given intraperitoneally (i.p.), and the analgesic effects before and after a defined period of tolerance induction were compared.

Tail Flick Tests

Rats were gently handled on the platform of tail flick testing apparatus (BME-410C; Institute of Biomedical Engineering, CAMS, Tianjin, China) with tails exposed to thermal stimuli. Radiant heat was applied to the caudal end (3.0 cm from the tip) of the rat's tail and the time till flicking the tail was recorded as the latency. The intensity of radiant heat was adjusted to elicit a baseline flick latency of approximately 2 to 3 seconds. Cutoff time was 10 seconds to avoid tissue damage. Baseline was tested before drug administration. Tail flick latency was tested 30 minutes after acute morphine administration (5 mg/kg, i.p.).

Western Blotting

Animals were euthanized by overdose (2 g/kg) of urethane (SCR Co, Shanghai, China), and the spinal cord tissue (L4–6) was rapidly removed. Tissues were collected at different time points according to different experiments. When we observed the time course of IL-18, IL-18 receptor, glial fibrillary acidic protein (GFAP), and PKCγ, tissues were collected on days 2, 4, 6, and 10, respectively. When we observed the effects of drugs on their downstream targets, day 6 after chronic morphine treatment was selected. Samples were homogenized in a lysis buffer (12.5 μL/mg tissue) containing a mixture of protease inhibitors (Roche, Mannheim, Baden-Württemberg, Germany) and phenylmethylsulfonyl fluoride (Sigma-Aldrich). Protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (5–12% gels) and transferred onto polyvinilidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked in 10% nonfat milk for 2 hours at room temperature and then incubated

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