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Spinal p38 activity and analgesic effect after low- and high-intensity electroacupuncture stimulation in a plantar incision rat model

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ABSTRACT

Aims: Postoperative pain is a major problem. Electroacupuncture (EA) has been accepted as a useful and low-risk complementary therapy for post-operative pain. Animal studies indicate that surgical incision activates p38 MAPK in the spinal microglia, which critically contributes to post-incisional nociceptive development. How EA affects incision-induced p38 activation is important but yet to be fully elucidated.
Methods: Male adult rats received plantar incision (PI) at the right hind paw followed by 30-min EA of 4-Hz, one of two intensities (3 and 10 mA), and at right ST36 (Zusanli) acupoint immediately after PI and for 3 successive days. EA analgesia was evaluated by von Frey fibers and Hargreaves' tests. Spinal p38 activation was examined by immunostaining. In separate groups, SB203580, a p38 inhibitor, was intrathecally injected alone or with EA to test the combining effect on nociception and spinal phospho-p38.
Key findings: EA of 10-mA significantly ameliorated mechanical allodynia, but 3-mA did not. None of them altered thermal hyperalgesia. Repeated EA could not inhibit phospho-p38 in the PI rats, contrarily, EA per se significantly induced phospho-p38 in the normal rats. Intrathecal SB203580 injection dose-dependently prevented PI-induced allodynia. Combination of low-dose SB203580 and 3-mA EA, which were ineffective individually, profoundly reduce post-PI allodynia.
Significance: We demonstrated that 10-mA EA exerts a significant inhibition against post-PI mechanical hypersensitivity *via* a p38-independent pathway. Importantly, co-treatment with low-dose p38 inhibitor and 3-mA EA can counteract spinal phospho-p38 to exert strong analgesic effect. Our finding suggests a novel strategy to improve EA analgesic quality.

Introduction

Post-operative pain is a common acute pain problem. Poorly controlled pain increases the risks of heart attack, pneumonia, deep vein thrombosis, immune impairment, anxiety, and persistent neuropathic pain [30,42]. Standard opioid administration remains the mainstay [46], however, its use is complicated with opioid-related side effects which degrade analgesic quality. Our and other human studies demonstrated that perioperative acupuncture or electroacupuncture (EA) could simultaneously reduce postoperative opioid consumption, spare adverse symptoms, and meanwhile maintain adequate analgesia [40,61]. Although EA has been accepted to pose these advantages, its use is still unpopular due to unclear mechanisms and low therapeutic efficacies.

Opioid-dependent and opioid-independent mechanisms interactively underlie acupuncture-induced analgesia [18,56,60,65]. Following acupuncture stimulation, neuron-based releases of neuropeptides, including endogenous opiates-like endorphin, enkephalin, dynorphin, and endomorphin [3,11,17–19,62]; non-opioid substances like serotonin [1,3,4,39], noradrenaline [3,4,65], oxytocin [69], neuropeptide Y [35], neurotrophin-3 [44], somatostatin [8], spinal orexin A [10], and

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newly found peripheral release of adenosine [14] were all involved to play roles in regulating acupuncture effects. However, how these molecules participate and interact in various pain processes is complex and unclarified.

On the other hand, different lines of studies proved that neuroglia are powerful contributors or modulators in persistent pain states [16,26]. Using a plantar incision (PI) model, which imitates postoperative pain as a preclinical animal study [2], we showed that activation of p38 mitogen-activated protein kinase (MAPK) within microglia in the spinal dorsal horn contributed to nociceptive development at early post-PI stage [63]. Pretreatment with p38 inhibitor suppressed phosphorylated-p38 (p-p38), an activated form of p38, can dose-dependently prevent behavioral hypersensitivity and downstream proinflammatory cytokines and chemokines [43,63]. Emerging evidence indicated that EA effect may be partly mediated through microglial regulation by inhibiting intracellular signaling, including p-p38, and exocytosis of inflammatory mediators in many pain conditions [13,37,38,44,55,57,67]. Therefore, it would be of great value to investigate how EA controls p38 activation in surgical pain.

The present animal study aimed to answer the following questions: first, if EA could ameliorate PI-induced nociceptive behaviors; second, if EA suppresses incision-induced p-p38 in spinal microglia; and third, if combination of EA and p38 inhibitor could enhance analgesic effect.

Materials and methods

Experimental animals

Adult male Sprague–Dawley rats (200–250 g; BioLASCO, Taiwan) were tri-housed and allowed to freely access food and water. The animal facility maintained a 12-h light–dark cycle at 22–24 °C and humidity of 70%. Animal managements were approved by Institutional Animal Care and Use Committee of China Medical University and followed the institutional Animal Care Guidelines. Efforts were made to minimize animal number and suffering.

Anesthesia, PI model, and EA

Experiment design and protocols were referring to our previous PI and EA studies [2,63,66]. In brief, the rats were anesthetized with 2% isoflurane in oxygen *via* a nasal mask. A one-cm longitudinal incision was made at plantar surface of the right hind paw, 0.5 cm from the edge of the heel, through skin, plantar fascia, until flexor digitorum brevis muscle. The skin wound was sutured by skin layers with 5 — 0 nylon. Then the rats were transferred to transparent holders and were continuously anesthetized with 1% isoflurane in 100% oxygen *via* a cone nasal mask for EA stimulation.

EA was conducted at Zusanli (ST36) of right hind limb by a pair of 36G (0.22 mm in diameter) acupuncture needles with electric stimulation generated from a Grass S88 electrostimulator (Astromed, Grass, West Warwick, RI, USA) *via* constant current units (Grass CCU1A, West Warwick, RI, USA). Stimulation parameters were set at 4-Hz square waves of 0.5 ms pulse width for a period of 30 min, following our lab protocol [66]. Two intensities, 3 mA (low-intensity) and 10 mA (high-intensity), were applied to rats according to the group allocation. ST36 is the most frequently used acupoint and has been shown to produce systemic analgesic effects in animals [50,66]. The rats in the sham EA groups received needle insertion in muscle layer (5 mm depth) of ST36 but without electrostimulation. All rats were removed from the anesthetic apparatus after stop of EA, and all of them rapidly recovered to a movable state within 1 — 2 min. The recovery was so fast that anesthesia would not interfere the first behavioral test conducted 1 h later. We had shown that this manipulation did not change behaviors or induce Fos expression [65].

Behavioral tests

Animals were acclimatized to experimental environment from at least 2 days prior to study. To test mechanical threshold, the rats were put in inverted plastic boxes (10 × 10 × 20 cm) on an elevated mesh floor and allowed 30 min for habituation. Tactile thresholds were measured by von Frey fibers (Stoelting, Wood Dale, IL, USA). The paw was pressed with one of a series of von Frey fibers with logarithmically incrementing stiffness (0.4, 1.0, 2.0, 4.0, 6.0, 10.0, 15.0, and 26.0 g) perpendicularly onto the plantar medial surface. Each fiber was applied for 5–6 s. The 50% withdrawal threshold was determined using up-down method [5]. The above protocol had been published [12,41]. To test thermal thresholds, animals were put in a plastic box placed on a glass plate pre-warmed to a constant 30 °C (Plantar Test Apparatus, IITC, *CA*, USA), and the plantar surface was exposed to a beam of radiant heat underneath the glass floor. The baseline latencies were adjusted to 8 — 10 s with a maximum of 20 s as cut off to prevent potential heat injury. The latencies were averaged over three trials, separated by a 5-min interval [21]. The experimenter who performed these behavioral tests was blind to the group allocation of the rats.

Intrathecal (i.t.) administration

To evaluate the role of p-p38 in response to plantar incision and EA, the rats received intrathecal administration of a p38 inhibitor, SB203580 (Cell signaling Technology, Danvers, MA, USA). One hour before plantar incision, the rats were transiently anesthetized with high concentration of sevoflurane and back hairs were shaved to expose the lower back skin. After careful identification of L4 — 5 or L5 — 6 interspace, dural puncture to the intrathecal space was performed with a 30 G needle and a Hamilton microsyringe (Hamilton Co., Nevada, USA). Correct placement of needle was confirmed by a sign of brisk tail flick. Three doses of SB203580 (0.2, 0.5 or 2.0 mM in 10 μL) were slowly injected to the study groups. Because SB203580 had to be dissolved in dimethyl sulfoxide (DMSO) which has anti-inflammatory [15] and analgesic properties [9], two concentrations of DMSO (2% and 20%) at the identical volume were injected as vehicle control to exclude bias of DMSO's potential effect.

Immunostaining

The animals were deeply anesthetized by isoflurane for transcardial perfusion of normal saline at room temperature (RT), followed by 4 °C, 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The L4 — 5 spinal cord segments were carefully removed, post-fixed overnight, and cryoprotected in 30% sucrose/normal saline for 3 days. The transverse spinal sections were cut in cryostat (LEICA CM3050S, Nussloch, Germany) at a thickness of 30 μm and collected in 0.1 M PB. After blocking with 3% normal goat serum for 1 h at RT, the sections were incubated with primary antibodies containing 1% normal goat serum and 0.3% Triton X-100 overnight. Sections were then incubated with biotinylated anti-rabbit IgG (1:400, Vector Laboratories, Burlingame, *CA*, USA) and subsequently in an avidin-biotin-peroxidase complex/diaminiobenzidine-H_2O_2 solution (Elite ABC kit, Peroxidase substrate kit, Vector Laboratory). Spinal free-floating sections were mounted onto gelatin-coated glass slides, air-dried, dehydrated, cleared with xylene and coverslipped with Entellan mounting medium (Merck, Darmstadt, Germany). For double immunofluorescence, sections were incubated with a mixture of two primary antibodies from different species at 4°C, followed by a mixture of Cy3-conjugated (1:400, Jackson ImmunoResearch, West Grove, PA, USA) and Alexa Fluor 488-conjugated (1:400, Jackson ImmunoResearch) secondary antibodies at RT. In this study, our primary antibodies include anti-phospho-p38 (rabbit, 1:400, Cell Signaling Technology), anti-neuronal nuclei (NeuN, mouse, 1:500, Millipore), anti-CD11B (OX-42, mouse, 1:50, Chemicon), and anti-glial fibrillary acidic protein (GFAP, mouse, 1:4000, Chemicon).