Effect of electroacupuncture on the cervicospinal P2X7 receptor/fractalkine/CX3CR1 signaling pathway in a rat neck-incision pain model

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Abstract Increasing evidence supports that acupuncture intervention is an effective approach for intraoperative and postoperative pain. Neuron–microglia crosstalk, mediated by the purinergic P2X7 receptor (R)/fractalkine/CX3CR1 cascade in the spinal cord dorsal horn, plays a pivotal role in pain processing. However, its involvement in the analgesic effect of electroacupuncture (EA) remains unclear. In this study, a rat neck-incision pain model was established by making a longitudinal incision along the midline of the neck and subsequent repeated mechanical stimulation. EA stimulation was applied to bilateral LI18, LI4-PC6, or ST36-GB34. The thermal pain threshold, cervicospinal ATP concentration, expression levels of purinergic P2XR and P2YR subunits mRNAs, and fractalkine, CX3CR1 and p38 MAPK proteins, were detected separately. The neck incision induced strong thermal hyperalgesia and upregulation of spinal ATP within 48 h. No significant change was found in thermal hyperalgesia after a single session of EA intervention. However, a single session of EA dramatically enhanced the neck incision-induced

upregulation of ATP and upregulated the expression of P2X7R, which was reversed by two sessions of EA. Two sessions of EA at bilateral LI18 or LI4-PC6 attenuated hyperalgesia significantly, accompanied with downregulation of P2X7R/fractalkine/CX3CR1 signaling after three sessions of EA. EA stimulation of LI18 or LI4-PC6 alleviates thermal hyperalgesia in neck-incision pain rats, which may be associated with its effects in regulating the neck incision-induced increase of ATP and P2X7R and subsequently suppressing fractalkine/CX3CR1 signaling in the cervical spinal cord.

Keywords Neck-incision pain · Electroacupuncture · ATP · P2X₇ receptor · Neuron-microglia interaction · Fractalkine/CX3CR1 signaling

Introduction

Postoperative pain management remains a significant challenge for healthcare providers because of the common side effects of anesthetics. Many patients experience pain after surgery, with about 86% reporting moderate, severe, or extreme pain [1]. Accumulating evidence suggests the efficacy of acupuncture and related techniques as an adjuvant method for postoperative analgesia [2, 3].

Thyroidectomy represents one of the most frequent procedures in endocrine surgery. Postoperative pain in patients undergoing thyroidectomy is common, and administration of opioid or non-opioid drugs is often required during the first day after the procedure. However, these treatments often worsen anesthetics-induced nausea and vomiting and other side effects [4]. Acupuncture anesthesia was used in 95.4% of the removal of thyroid adenomas in the 1970s in China [5]. It has been well documented from clinical practice of acupuncture analgesia and anesthesia that Futu (LI18) and Hegu (LI4)

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-Neiguan (PC6) are the most effective acupoints in reducing intraoperative and postoperative pain for thyroid surgery [6, 7]. But its mechanism is unclear.

One of the underlying causes of postoperative pain is the surgery-induced production and release of various pronociceptive mediators, which directly activate primary afferent neurons [8]. Therefore, intraoperative pain can be regarded as an acute homeostatic and adaptive process by which the organism becomes aware of harmful stimuli, guarding against actual or potential tissue injury [9]. As such, the physiological transduction and transmission of noxious stimuli are a vital protective mechanism, allowing withdrawal from potential damage by environmental factors. However, if this pain is not managed effectively, it may become chronic pain, harming the related organs and leading to dysfunction [10, 11].

In the dorsal horn of the spinal cord, disruption of homeostasis and exaggerated primary afferent inputs cause microglia to transform from the “quiescent” state to a pain-related activated state. This transition modifies the nature of neuron–microglia communication and promotes a maladaptive augmentation of nociceptive transmission that underlies the chronicity of acute pain [12, 13]. Following injury, microglia begin presenting antigens, upregulate various receptors including purinergic receptors for ATP, and release mediators such as proinflammatory cytokines and proteases, which activate neurons, thereby establishing a positive feedback loop which contributes to the development of pain [8, 14]. Recent studies suggest that neuron–microglia interaction mediated by purinergic receptors, and subsequently by chemokine fractalkine (FKN) and its sole receptor CX3C chemokine receptor 1 (CX3CR1), plays a pivotal role in nociceptive signal transmission [9]. In addition, it was reported that ATP-gated purinergic receptor P2X7 of the dorsal root ganglia (DRG) was involved in the analgesia effect of moxibustion in rats with chronic visceral inflammatory pain [15]. ATP and P2X3 receptor in DRGs and the spinal cord were implicated in the pain relief of electroacupuncture (EA) in rats with neuropathic pain [16, 17].

In the present study, we established a rat model of neck incision pain to mimic the thyroid surgery pain, and investigated the role of the purinergic P2X7 receptor/KN/CX3CR1 pathway in the analgesic effect of EA. This research may help reveal a new target of EA in relieving postoperative pain.

Material and methods

Animals

Experiments were performed on adult male Sprague–Dawley rats weighing 200–250 g (purchased from the Experimental Animal Center of Peking Union Medical College, Beijing,

China). These rats were housed in a temperature- $(22 \pm 2^\circ\text{C})$ and light-controlled (12:12-h light–dark cycle) room with free access to food and water. Before experimental manipulation, the rats were allowed to acclimate to the housing facilities and the animals were handled for 10 min, once daily for at least 3 days to reduce their stress responses. Animals were divided into five groups: control, pain model, EA LI18, EA LI4-PC6 and EA ST36-GB34, which were further divided into 4-, 24-, and 48-h subgroups, with ten rats in each group at the three time points. In addition, six rats were used for immunofluorescence double labeling. A total of 156 rats were used. All experimental procedures were approved by the ethics committee of the Institute of Acupuncture and Moxibustion of China Academy of Chinese Medical Sciences and performed according to the “Guidelines for Laboratory Animal Care and Use” of the Chinese Ministry of Science and Technology (2006).

Establishment of neck-incision pain model

The animal neck around the bilateral thyroid gland area was treated topically with depilatory cream 1 day before operation. After detecting the baseline thermal pain threshold, a 1.5-cm longitudinal incision was made along the midline of the neck under isoflurane (0.5–1% in oxygen) inhalational anesthesia using a table-top isoflurane anesthesia unit, Matrx VIP 3000 (Midmark Corporation, Orchard Park, NY). This was followed by repeated blunt dissection stimulation along the bilateral sternohyoideus around the thyroid gland regions for 30 min with a pair of forceps. The incision was then sutured in layers. Rats in the sham operation (control) group were similarly anesthetized but without incision and dissection stimulation.

Measurement of thermal pain threshold

Thermal pain thresholds were tested before and 4, 24 and 48 h after the neck-incision surgery (4 h after each EA intervention). The thermal pain threshold of the neck incision area was measured using a tail-flick apparatus (UGO Basile, Comerio, Italy). The heat intensity was set to 25 units and the cutoff time was set to 30 s to avoid tissue damage in the absence of a response. The thermal neck-withdrawal latency was recorded automatically when the rat swiftly moved its neck from the heat source. Each rat was tested three times, with a 3–5-min interval between tests. The average of the three trials was then determined. If the basic thermal withdrawal latency was >24 or <12 s, the rat was excluded from the groups.

Electroacupuncture stimulation

Rats of the EA treatment groups were administered EA stimulation during the process of neck-incision surgery (single session of EA), at 20 h (two sessions of EA) and 44 h (three

sessions of EA) following the neck incision with the following procedure. Filiform needles (Gauge-32, Suzhou Acumoxibustion Products Factory, Suzhou, China) were inserted into bilateral LI18 or LI4-PC6 or bilateral Zusanli (ST36)-Yanglingquan (GB34). After insertion, the needle handles were connected to a HANS EA Apparatus (Hans-100A, Jisheng Medical Technology, Co., Ltd., Nanjing, China). The rats were then administered EA stimulation (1 mA, alternative 2 Hz/100 Hz) under light anesthesia with isoflurane (0.5–1.0% in oxygen) via a nose cone of a table-top isoflurane anesthesia unit (Midmark Corporation) for 30 min.

In rats, LI4 is located between the first and second metacarpal bones; PC6, about 3 mm to the wrist transverse stripe on the axopetal end; ST36, about 5 mm inferior of the capitulum fibulae and posterior-lateral to the hind-limb knee joint; and GB34, about 5 mm superior-lateral to ST36 [18]. No descriptions about LI18 of rats were found in acupuncture books. With reference to the position of the human body, the LI18 in the rat is located at the postborder of sternocleidomastoid, about 1 cm lateral to the laryngeal prominence (Fig. 1). Animals in the control group and pain model group were treated with the same anesthesia but without EA stimulation.

Detection of ATP concentrations

The rats were euthanized immediately after the last thermal pain test. The upper segments of the cervical spinal cord (C2–C5) were excised rapidly on an ice-plate, followed by semitranssection and placing the dorsal part of C2–C5 in liquid nitrogen, and then stored at -80°C for ATP bioluminescent assay, quantitative RT-PCR, and western blot, separately. The content of ATP was measured using the ATP Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO). Briefly, every 20 mg of the tissue was homogenized by mechanical disruption in 100–200 μL of lysing buffer (provided by the manufacturer). After cleavage at $12,000\times g$ for 10 min at 4°C , supernatants were collected and stored at -80°C until use. A standard solution of ATP was prepared by making serial dilutions ranging from 10^{-3} to 10^{-11} M to quantify the test samples. The assay was performed in a white, flat-bottom, 96-well plate where 25 μL of the ATP assay mixture (provided by the manufacturer) was added to each well and incubated at room

temperature for 3 min. This was followed by rapid addition of 25 μL of each of the standards and samples in triplicate to the wells containing ATP assay mixture and measurement of the luminescence using a Synergy HT (BioTek, Winooski, VT) plate reader. ATP concentrations were calculated from a standard curve. Levels of ATP were measured in three independent experiments then averaged.

RNA preparation and quantitative real-time PCR detection

After the rats were euthanized, the upper segments of the cervical spinal cord (C2–C5) were isolated rapidly. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). RNA was reverse transcribed with oligo dT and M-MLV reverse transcriptase (Promega, Madison, WI). Primers were designed using Primer 3.0 and synthesized by Sangon (Shanghai, China). Sequences of the chosen primers are listed in Supplemental Table 1. Reactions were performed in triplicate using 10 ng of cDNA in a 25- μL reaction that contained 1 μL of each amplification primer (5 μM) and 12.5 μL of real-time PCR Master Mix (Applied Biosystems, Carlsbad, CA). Samples were amplified on a standard cycling condition, and data were collected and analyzed with SDS 2.3 software (Applied Biosystems). All samples for each reference gene were run on the same plate to avoid between-run variations. Relative mRNA expression levels were measured. Each assay was evaluated by a comparative method validated by Applied Biosystems with the formula $2^{-\Delta\Delta C_T}$.

Western blotting

The collected cervical spinal cord samples were placed into phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Roche, Mannheim, Germany), then homogenized in lysis buffer. The homogenate was centrifuged at $20,200\times g$ for 10 min at 4°C . The supernatant was collected for assaying the protein concentration using the BCA method with a bovine serum albumin standard. Each sample contained protein from one animal. The proteins (40 μg) were separated by 7.5% SDS-PAGE on a Bio-Rad system (Bio-Rad, Hercules, CA) and blotted on a PVDF membrane (GE Healthcare, Piscataway, NJ). The blots were blocked with

Fig. 1 Schematic of the locations of the acupoints in rats

5% milk in tris-buffered saline (TBS) and then incubated overnight at 4 °C with the respective primary antibodies: rabbit anti-P2X7R (1:1000; Abcam, New Territories, HK), rabbit anti-CX3CR1 (1:1000; Abcam), rabbit anti-FKN (1:500; Torrey Pines Biolabs, Houston, TX), goat anti-GAPDH (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p38 MAPK antibody (1:1000; Cell Signaling, Beverly, MA), and anti-phospho-p38 MAPK antibody (1:500; Cell Signaling). The membrane was washed with TBS and incubated with horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG (1:3000; Santa Cruz) for 2 h at room temperature. The immunoreactivity was detected using enhanced chemiluminescence (Pierce, Rochford, IL). The quantification of band intensity was carried out using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Band densities were normalized to individual GAPDH internal controls.

Immunofluorescence double labeling

Four hours after neck incision, three animals in the control group and three animals in the pain model group were terminally anesthetized with the same anesthetics mentioned above and received a transcardiac perfusion with normal saline (250 mL), and then with 4% paraformaldehyde solution (200 mL) containing 1.5% picric acid in 0.1 M PB (pH 7.4). After the perfusion, the C2–C5 spinal cord segments were removed and postfixed in the same fixative solution overnight, then replaced with 15% sucrose solution overnight. The cervical spinal cord was sectioned on a cryostat microtome (Thermo, Microm International GmbH, Germany) at 40 µm for immunofluorescence double-labeling. Free-floating tissue sections were placed in 0.01 M PBS, washed with PBS Tween-20 (PBST) three times, incubated in 1 N HCL for 20 min, followed by 3% H₂O₂ in distilled water for 15 min, then subsequently blocked with 5% goat serum for 30 min at room temperature to block the unspecific staining. The sections were then incubated with the relevant primary antibodies: mouse anti-NeuN (1:50; GeneTex, CA, USA) and rabbit anti-P2X7R (1:500; Millipore, Billerica, MA), goat anti-Iba1 (1:1000; Abcam) and rabbit anti-P2X7 (1:500; Millipore), mouse anti-GFAP (1:1000; Millipore) and rabbit anti-P2X7 (1:500; Millipore) for 24 h at 4 °C. The sections were washed three times with PBS, and then incubated in the secondary antibodies, goat anti-rabbit IgG-conjugated Alexa Fluor 561, goat anti-rabbit IgG-conjugated Alexa Fluor 488, or goat anti-mouse IgG-conjugated Alexa Fluor 488 (1:2000; all from Invitrogen) on a rocking bed (away from light) for 2 h at room temperature. Images of the spinal cord slices were acquired using a fluorescence microscope (Olympus AX70, Olympus Corporation, Tokyo, Japan) with an objective magnification of ×40 and software analysis Pro 3.1. The fluorescence intensity was measured by Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

Intraperitoneal injection of P2X7R antagonist

To observe the effect of P2X7R on EA analgesia in the neck incision pain model, we used A-740003 [N-(1-[(Cyanoimino)(5-quinolinylamino) methyl] amino)-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel competitive antagonist of P2X7R. Forty Wistar rats were equally divided into 4 groups: pain model + DMSO, pain model + A-740003, EA + DMSO, and EA + A-740003. The procedures of the neck incision and EA were the same as those previously described. Rats of the EA treatment groups were administered EA stimulation of bilateral LI4-PC6 during the process of the neck-incision surgery (single session of EA) and at 20 h (two sessions of EA) after the neck incision. A-740003 (MedChem Expression, Monmouth junction, NJ) was dissolved in 2% DMSO for intraperitoneal injection at a dose of 142 mg/kg at 3.5 h after the neck-incision surgery (0.5 h before the postincision 4 h thermal pain test) [19]. An equal volume of DMSO was used as a control.

Statistical analysis

The data of the present study were expressed as mean ± SD, and those of quantitative real-time PCR, ATP concentrations, and western blot were analyzed by one-way measure analysis of variance (ANOVA), and those of thermal pain thresholds analyzed by two-way repeated ANOVA, followed by post hoc analysis. All *P* values were two-sided, and *P* < 0.05 was considered significant.

Results

EA at LI18 and LI4-PC6, but not at ST36-GB34, attenuated neck incision-induced thermal hyperalgesia (Fig. 2)

Following neck incision, a thermal hyperalgesia was observed within 4 h and persisted over 48 h, having no significant differences among the 4, 24, and 48 h time-points (*p* > 0.05). Two-way repeated ANOVA showed a significant analgesic effect of EA intervention; and there was no significant interaction effect between EA treatment and time. Following one session of EA stimulation (4 h postincision), no significant difference was found in thermal withdrawal latency between the EA and pain model groups (Fig. 2). The thermal withdrawal latencies were significantly increased from the second session of EA intervention in both the LI18 and LI4-PC6 groups (*p* < 0.05, compared with the pain model group, one-way ANOVA) and returned to the normal control level after three sessions of EA (48 h, *p* > 0.05, compared with the control group). After three sessions of EA intervention, the thermal withdrawal latency was significantly increased in the ST36-GB34 group but still lower than the control level (*p* < 0.05).

Fig. 2 Effect of EA stimulation of LI18, LI4-PC6, and ST36-GB34 on thermal pain thresholds in rats with neck incision pain. Pain thresholds were measured at the time points of preincision, 4, 24, and 48 h postincision (Mean $\pm$ SD; $n = 10$ per group). * $p < 0.05$, versus the control group; # $p < 0.05$, versus the pain model group

EA regulated ATP levels in the cervical spinal cord (Fig. 3)

ATP is one of the important transmitters released from the injured tissue after periphery tissue injury and may activate its receptors to initiate pain signaling by regulating neuronal and glial functions in the nervous system [20]. Acupuncture stimulation is also supposed to trigger release of ATP, e.g., from keratinocytes and mast cells [21]. In the present study, the neck incision induced an increase in ATP contents in the dorsal part of the cervicospinal cord 4, 24, and 48 h after modeling ($p < 0.05$, relative to the control level). One session of EA intervention further upregulated the ATP levels in all the three EA groups compared with the pain model group ($p < 0.05$). However, after two sessions of EA intervention, the ATP levels were considerably downregulated in both LI18 and LI4-PC6 groups ($p < 0.05$, relative to one session of EA intervention) and returned to the normal level after three sessions of EA. No significant difference was found between the ST36-GB34 and pain model groups 24 and 48 h after modeling ($p > 0.05$, Fig. 3).

Fig. 3 Comparison of ATP levels in the dorsal cervicospinal cord (C2–C5) after neck-incision and EA intervention. Data are presented as the mean $\pm$ SD ($n = 10$ per group). * $p < 0.05$, versus the control group; # $p < 0.05$, versus the pain model group

Expression of ATP receptors after modeling and EA (Fig. 4)

In peripheral noxious signal transmission, the released ATP acts on two major types P2X receptors (P2X1–P2X7) and P2Y receptors (P2Y1, 2, 4, 6, 11, 12, 13, and 14) [22]. To identify which subunit of the purinergic receptor family is implicated in the neck-incision pain, we profiled them using quantitative real-time PCR analysis (P2Y11 was not detected as no *Rattus norvegicus* P2Y11 mRNA sequence found in the NCBI database). Upregulations of P2X3R and P2Y12R mRNAs 4-h postmodeling, and P2X3, P2X4, P2X7, and P2Y12 mRNAs 24-h postmodeling, were found ($p < 0.05$, relative to the control level; Fig. 4a). The other receptor subunit mRNAs had no obvious changes ($p > 0.05$, relative to the control level). After one session of EA intervention of LI4-PC6, the incision-induced increase of P2X3R and P2Y12R mRNAs remained unchanged relative to the model group ($p > 0.05$). However, P2X7R mRNA, which had no statistical upregulation after modeling, showed a significant upregulation following one session of EA intervention relative to the control level ($p < 0.05$). After two sessions of EA, the upregulated P2X3R, P2X4R, P2X7R, and P2Y12R mRNAs were considerably downregulated compared with the model group ($p < 0.05$; Fig. 4a) and close to the normal levels. As P2X7R was the only receptor that had no statistical upregulation after modeling but showed a significant upregulation following one session of EA intervention relative to the control level, we used western blot and double immunofluorescence staining to further confirm the result. Western blot results (Fig. 4b) showed that the expression of P2X7R protein in the dorsal part of the cervical spinal cord had no obvious changes 4 h after neck incision ($p > 0.05$) and was significantly upregulated 24 and 48 h after incision in comparison with the control group ($p < 0.05$). Following one session of EA intervention, the expression level of P2X7R protein was notably upregulated in the LI18 and LI4-PC6

Fig. 4 Effect of EA stimulation on expression of cervical spinal purinergic receptors. **a** mRNA expression levels of purinergic receptor subunits after EA stimulation of LI4-PC6 (mean $\pm$ SD, $n = 10$ per group).

b Expression levels of purinergic receptor P2X7 protein in the five groups (mean $\pm$ SD, $n = 10$ per group). * $p < 0.05$, versus the control group; # $p < 0.05$, versus the pain model group

groups versus the pain model level ($p < 0.05$). However, after two sessions of EA intervention, P2X7R in the LI18 and LI4-PC6 groups was decreased to the control level. P2X7R expressions in the ST36-GB34 group and in the model group were still higher than the control level 48 h after modeling ($p < 0.05$). Double immunofluorescence staining indicated that the upregulated P2X7R was localized to microglia but not to astrocytes and neurons (Fig. 5), which is consistent with a previous study [23].

The effect of A-740003 on pain threshold and P2X7R expression following neck incision is shown in Fig. 6

Four hours after inducing postincision pain, the P2X7R antagonist A-740003 exerted an analgesic effect. At the same time,

EA failed to cause analgesia (comparison of the first and third columns in Fig. 6a, left panel) in spite of the persistence of the A-740003 effect on pain. However, 24 h after inducing postincision pain, A-740003 was ineffective on the thermal withdrawal latency. At this time point, EA was clearly analgesic (comparison of the first and third columns in Fig. 6a, right panel), and its effect was antagonized by A-740003. Thus, EA caused a clear cut increase in thermal latency via the stimulation of P2X7 receptors only 24 h after inducing neck incision pain.

Figure 6b demonstrates that 4 h after inducing postincision pain, EA increased the level of P2X7R protein expression, whereas after an additional 20 h, the P2X7R protein level was decreased. Both effects could be antagonized by A-740003. Moreover, P2X7R protein was strongly elevated

Fig. 5 Immunofluorescence images showing a colocalization of P2X7R at Iba1-labeled microglia (yellow/orange) in the dorsal horns of the cervicospinal cord (C2–C5) in rats with neck-incision pain (4 h after neck incision, $n = 3$ per group). *Iba1* ionized calcium-binding adapter molecule 1 (a marker of microglia), *NeuN* neuronal nuclei (a marker of neuron), *GFAP* glial fibrillary acidic protein (a marker of astrocyte)

during a period elapsing between 4 and 24 h postincision (comparison of Fig. 6b left and right panels).

Fig. 6 Effect of A-740003 on thermal pain thresholds (a) and P2X7R expression (b) following the neck incision and EA stimulation ($n = 10$ per group). # $p < 0.05$, versus the pain model + DMSO group; ▲ $p < 0.05$, vs the EA + DMSO group; * $p < 0.05$, vs the pain model + DMSO group 4 h postincision

EA suppressed neck incision-induced cleavage of FKN and upregulation of CX3CR1 (Fig. 7)

FKN, a newly discovered chemokine functioning as a cellular adhesion molecule in the central nervous system, has two forms: a large size (~100 kDa) and a small size (~80 kDa), which are the membrane-bound form (mFKN) and soluble secreted form (sFKN), respectively [24, 25]. Expression of mFKN was downregulated in the cervicospinal cord 48 h after neck incision (Fig. 7a), and sFKN was considerably upregulated ($p < 0.05$, versus the control level), with enhancement of its receptor CX3CR1 expression 48 h after modeling ($p < 0.05$; Fig. 7b). One and two sessions of EA intervention had no observable effect on the expression of both mFKN and sFKN proteins. After three sessions of EA intervention, the decreased mFKN protein level was upregulated to the normal level in the three EA groups ($p > 0.05$, versus the control group), and the increased sFKN protein levels were significantly decreased in both the LI18 and LI4-PC6 groups ($p < 0.05$, Fig. 7a), but not in the ST36-GB34 group ($p > 0.05$). Along with the reduction of sFKN after three sessions of EA, its microglia receptor CX3CR1 was also decreased but still remaining obviously higher than that of the control level ($p < 0.05$, Fig. 7b).

EA suppressed neck incision-induced p38 MAPK phosphorylation (Fig. 8)

p38 MAPK is an important intracellular signal transporter expressed in spinal microglia and is involved in pain processing [26, 27]. No changes were found in the expression levels of cervicospinal p38 MAPK after the neck incision compared

Fig. 7 Effect of EA on expression levels of cervicospinal mFKN and sFKN (**a**) and its receptor CX3CR1 (**b**) in neck-incision pain rats. Data are presented as mean $\pm$ SD ($n = 10$ per group). * $p < 0.05$, vs the control group; # $p < 0.05$, versus the pain model group

with the control group ($p > 0.05$, Fig. 8a). Further detection showed that the phosphorylated p38 MAPK (p-p38 MAPK) expression was considerably increased 24 and 48 h after neck incision in comparison with that of the control group ($p < 0.05$, Fig. 8b). The increased p-p38 MAPK expression levels were gradually downregulated to the control level after three sessions of EA stimulation of LI18 and LI4-PC6 ($p < 0.05$, compared with the pain model group). No significant difference was found between the pain model and ST36-GB34 groups in the expression of p-p38 MAPK protein ($p > 0.05$).

Discussion

In the present study, we demonstrated that two sessions of EA stimulation of both LI18 and LI4-PC6, but not ST36-GB34,

Fig. 8 Effect of EA on expression of p38 MAPK (**a**) and phosphorylated p38 MAPK (**b**) protein in neck-incision pain rats. Data are presented as mean $\pm$ SD ($n = 10$ per group). * $p < 0.05$, vs the control group; # $p < 0.05$, versus the pain model group

attenuated the thermal hyperalgesia of the local neck incision region in the neck-incision pain model. Moreover, we demonstrated that EA at both LI18 and LI4-PC6 significantly induced regulation of ATP and P2X7R levels in the spinal cord. In addition, EA at both LI18 and LI4-PC6 significantly attenuated neck-incision-induced upregulation of the P2X7R/FKN/CX3CR1 signaling pathway.

Our finding that two sessions of EA stimulation of both LI18 and LI4-PC6 attenuates the thermal hyperalgesia of the local neck-incision region is similar to our past studies in the same pain model rats [28, 29] and in neuropathic pain rats [30]. The LI18 and the thyroid gland region are at the same spinal nerve segment, innervated by the major auricular nerve, superficial cervical nerve, minor occipital nerve, and the accessory nerve. Our past study using fluorescent double-labeling revealed that the afferent nerves from the thyroid gland region and LI18 region mainly concentrate in the DRGs of C1–C4, and those from LI4 and PC6 mainly distribute in the DRGs of C3–C7 in the rat [31]. The afferent nerve fibers from the ST36 region distribute mainly in DRGs of L3–L6–S1 segments in the rat [32]. The GB34 and ST36 have a similar innervation. Thus, it is understandable that the effects of EA stimulation of LI18 and LI4-PC6 were comparable in relieving neck-incision pain and superior to those of EA of ST36-GB34 at the lower limbs because of the nerve segmental difference under a lower (1 mA) electrical stimulation condition. Our results suggested that the effect of EA is at least in part related to peripheral nerve stimulation. A proper control

for acupuncture, including the location of non-acupoint, is a challenge, particularly in small animals. Our results demonstrate that EA exhibited therapeutic effects on specific acupoints, which may provide new insight for the proper control for acupuncture. In this study, ST36-GB34 design was just used for comparing the specificity of efficacies of different acupoint groups; therefore, no sham point stimulation is needed.

ATP is suggested to be one of the first mediators of tissue damage, which activates the primary sensory afferents [8]. In the present study, 4, 24, and 48 h after neck-incision, ATP levels in the dorsal part of the spinal cord were significantly increased and even further upregulated after the first session of EA stimulation of LI18 and LI4-PC6. This is identical to the expression of c-fos in other brain areas in response to acupuncture and formalin-induced acute pain [33]. In the present study, after the neck incision, ATP levels in the dorsal part of the spinal cord were significantly increased. However, this increase may not be high enough to trigger the low-affinity purinergic receptor P2X7 in microglia. In this situation, the first session of EA stimulation further upregulated the ATP level. This is consistent with previous research showing that acupuncture rapidly triggers extracellular adenosine and ATP metabolites [34]. However, under pathological conditions, when the effect of ATP had reached a plateau, EA stimulation had a decreasing effect on ATP concentration. This result showed that the EA effect may be related to the functional status of the body, which has been observed in other pathological models [35, 36]. This may be the reason that the ATP level declined from the peak and was lower than that in the incision model group after two sessions of EA treatment. These findings suggest that a transient upregulation of ATP may trigger an acute and intense homeostatic and adaptive process by which the organism becomes aware of harmful stimuli, thus initiating a signaling system to overcome the nociceptive signal.

To determine how the upregulated ATP was degraded or reuptaken into the intracellular pools, we profiled changes of the known ATP purinergic receptor subclasses after the neck-incision and EA stimulation of LI4-PC6. Our results showed that one session of EA induced a significant upregulation of P2X7R. We propose that EA intervention following the neck-incision may induce a transient accumulation of ATP in the dorsal horns of the spinal cord and trigger an upregulation of the low-affinity purinergic receptor P2X7 in microglia, which was demonstrated by our immunofluorescence double-staining in the present study. The most likely explanation for the lack of P2X7R changes after neck-incision or EA alone is that the ATP does not reach a high enough concentration to trigger P2X7R expression. This point is supported by the notion that only high concentrations of extracellular ATP can activate the ionotropic P2X7R in microglia [37]. A majority of the studies on P2 receptor functions were conducted under

chronic pathological conditions and suggest that blocking P2 receptor activation is a valid strategy to treat spinal cord injuries and control chronic pain. However, conflicting results have been published for some receptor subtypes. At least in DRG neurons, different types of P2Y receptors have the opposite effect in modulating painful sensations [38]. Chen and colleagues showed that activation of P2X7Rs in satellite glial cells reduced pain through downregulation of P2X3Rs in nociceptive neurons [26]. Only after a significant level of ATP was released from the satellite glial cells, the ATP acting upon P2X7R could reach a level to influence other receptors. By regulating P2X7R expression, P2X3R activity could be effectively controlled and was helpful in relieving pain. Another study by the same group demonstrated that activation of P2X7R–P2Y1R led to activation of p38 MAPK, which downregulated P2X3 and reduced nociception [39]. These findings are consistent with our observation that a higher level of ATP was accompanied by higher P2X7R protein expression and lowered P2X3R mRNA expression after EA stimulation of LI4-PC6 or LI18. However, the effect of EA-induced regulation of P2X7 protein expression on P2X3R and other receptors in the dorsal horns has yet to be determined.

Our finding that the first session of EA induced a sharp upregulation of ATP and P2X7R, strongly favors the involvement of the P2X7 in mediating the effects of EA. This was further confirmed by the result that after depleting P2X7R by injection of A-740003, the increase in thermal withdrawal latency was not as obvious as that in the EA + DMSO group at 24 h after the neck-incision surgery. After peripheral tissue injury, microglia cells undergo proliferation and chemotaxis toward the spinal cord dorsal horns where the injured afferent fibers terminate. Chemokines such as FKN play a pivotal role in mediating neuron–microglia communication, which leads to increased nociception [40]. Interaction of FKN and its microglial receptor CX3CR1, initiated by the activation of P2X7R, is critically involved in the maintenance of pain [9]. Neurons are also the principal FKN expressing cells in the central nervous system. Under conditions of the persistent peripheral noxious stimulation, microglial P2X7R may be responsible for the liberation of sensory neuronal FKN, which stimulates p38 MAPK phosphorylation in microglia, thereby activating neurons via the release of pronociceptive mediators [37]. Therefore, we examined the expression of FKN, CX3CR1, and p38 MAPK using western blot. Our findings demonstrate that the expression levels of the sFKN protein and CX3CR1 in the dorsal part of the cervical spinal cord were considerably increased at 48 h after modeling. This is probably because the membrane-bound FKN is required to be cleaved before its release

[24, 25], and sFKN may represent a major signal for chemoattraction in the brain and may sensitize discharges of cervical wide dynamic range neurons in response to stimulation applied to the neck [41]. The abovementioned findings of the decreased mFKN and the increased sFKN may suggest a cleavage of FKN and a delayed activation of the secondary sensory neurons via microglia–neuronal interaction. After one session of EA stimulation of both LI18 and LI4-PC6, with the increased expression of P2X7R, there was no change in FKN protein level. However, along with the decreasing of P2X7, the neck incision-induced increase of sFKN and CX3CR1 levels were remarkably down-regulated. These results suggest that, to maintain homeostasis at the early stage of neck incision, EA may suppress sFKN/CX3CR1/p38 MAPK-induced neuronal–microglia crosstalk through regulation of ATP and its receptors in microglia. These results are similar to previous reports by Liang and colleagues that EA stimulation alleviates spinal nerve ligation-induced neuropathic pain, partially through inhibition of microglia and reduced p38 MAPK phosphorylation in rats [42, 43].

In summary, our results showed that EA stimulation of LI18 and LI4-PC6 can relieve thermal pain in a rat neck-incision pain model, regulate neck incision-induced increase of ATP and its microglial purinergic receptor P2X7, and inhibit its downstream FKN/CX3CR1/p38MAPK signaling in the upper cervical spinal cord. The upregulated ATP and P2X7R may be critical for the early initiation phase of EA analgesia in the neck incision pain model. The present study, for the first time, provides evidence for EA intervention-induced neck pain relief, which is at least in part related to the regulation of P2X7 receptor/FKN/ CX3CR1 signaling pathway.

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Compliance with ethical standards

Conflict of interests Yong-hui Gao declares that she has no conflict of interest.

Cheng-wen Li declares that he has no conflict of interest.

Jun-ying Wang declares that she has no conflict of interest.

Lian-hong Tan declares that she has no conflict of interest.

Cheng-lin Duanmu declares that she has no conflict of interest.

Xiang-hong Jing declares that she has no conflict of interest.

Xiao-rong declares that she has no conflict of interest.

Jun-ling Liu declares that he has no conflict of interest.

Ethical approval All experimental procedures were approved by the ethics committee of the Institute of Acupuncture and Moxibustion of China Academy of Chinese Medical Sciences and performed according to the “Guidelines for Laboratory Animal Care and Use” of the Chinese Ministry of Science and Technology (2006).

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