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Analgesic effect of electroacupuncture on chronic neuropathic pain mediated by $P2X_3$ receptors in rat dorsal root ganglion neurons

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ABSTRACT

Adenosine 5′-triphosphate disodium (ATP) gated P2X receptors, especially the subtype $P2X_3$, play a key role in transmission of pain signals in neuropathic pain, ATP has been documented to play a significant role in the progression of pain signals, suggesting that control of these pathways through electroacupuncture (EA) is potentially an effective treatment for chronic neuropathic pain. EA has been accepted to effectively manage chronic pain by applying the stimulating current to acupoints through acupuncture needles. To determine the significance of EA on neuropathic pain mediated by $P2X_3$ receptors in the dorsal root ganglion (DRG) neurons, mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were recorded, and the expression of $P2X_3$ receptors in the DRG neurons was assessed by immunohistochemistry (IHC) and in situ hybridization (ISH). In addition, the currents which were evoked in DRG neurons isolated from rats following chronic constriction injury (CCI) by the $P2X_3$ receptors agonists i.e. ATP and α,β-methylen-ATP (α,β-meATP) were examined through the experimental use of whole cell patch clamp recording. The present study demonstrates that EA treatment can increase the MWT and TWL values and decrease the expression of $P2X_3$ receptors in DRG neurons in CCI rats. Simultaneously, EA treatment attenuates the ATP and α,β-meATP evoked currents. EA may be expected to induce an apparent induce analgesic effect by decreasing expression and inhibiting $P2X_3$ receptors in DRG neurons of CCI rats. There is a similar effect on analgesic effect between rats with contralateral EA and those with ipsilateral EA.

1. Introduction

EA is a procedure in which fine needles are inserted into an individual at discrete points and then electrical stimulation was applied, aiming to relieve chronic pain (Zhao, 2008). Contralateral acupuncture involves inserting needles on the side opposite the disease location and is commonly used for management of neuropathic pain. Pain threshold after nerve injury is reduced by the appearance of sensitized nociceptive neurons in the peripheral nervous system. It has been proved that ATP is an important transmitter that communicates sensory information in DRG neurons associated with neuropathic pain. It activates purinergic homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors in DRG neurons and transmits sensory information from the periphery to the spinal cord (Gu, 2003; Pan et al., 2009). EA analgesia is a complex physiological process mediated by many distinct transmitters and modulators including opioid peptides, glutamate and its receptors, γ-amino-butyric acid (GABA) and its receptors, substance P and various other related peptides. EA may have effect on different types of afferent nerve fibers to produce analgesic effect *in vivo* (Zhao, 2008). However, the exact neurophysiological mechanism of EA and the distinction between ipsilateral and contralateral applications remain poorly documented. Previous reports have shown that the analgesic effect of EA was closely correlated with the purines and purinergic receptors (Burnstock, 2009; Xiao et al., 2010). Despite this documented correlation, the role of EA treatment on neuropathic pain mediated by $P2X_3$ receptors in DRG remains largely unexplored. The relationship between the

Abbreviations: EA, electroacupuncture; CCI, chronic constriction injury; DRG, dorsal root ganglia; MWT, mechanical withdrawal threshold; TWL, thermal withdrawal latency; ATP, adenosine 5′-triphosphate disodium; α,β-meATP, α,β-methylen-ATP; IHC, immunohistochemistry; ISH, in situ hybridization; PBS, phosphate buffered saline; AOD, average optical density; ASCF, artificial cerebrospinal fluid.

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analgesic effect of EA and the $P2X_3$ receptor is uncertain. Alteration in pain threshold values and changes in expression of $P2X_3$ receptors in sensory neurons in DRG has been demonstrated in CCI rats. Electrophysiological approach was selected for investigating the change of the amplitudes of the inward currents of $P2X_3$ receptors to ATP and α,β-meATP. The primary aim of the present study was investigation of potentially clinically significant benefits of EA treatment in relieving neuropathic pain mediated by $P2X_3$ when applied at "zusanli" (ST-36) and "yanglingquan" (GB-34) points.

2. Materials and methods

2.1. Animals and drugs

All experiments were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the guidelines of the International Association for the Study of Pain. Male Sprague Dawley rats (150–180 g) were used for the studies. Rats were housed at a temperature of 21–25 °C with a 12 h light/dark cycle and were fed food and water *ad libitum*. The behavioral testing was conducted between 8:00 and 12:00 a.m. Rats were divided into 4 groups at random, including sham CCI group, CCI group, CCI plus contralateral EA group and CCI plus ipsilateral EA group. There are 8 rats in each group. ATP and α,β-meATP were obtained from Sigma. The ATP and α,β-meATP were dissolved and diluted in 0.9% saline and subsequently stored at −20 °C.

2.2. Chronic constriction injury model

CCI model as one of neuropathic pain models was prepared based on previous description (Fox et al., 2001; Patel et al., 2001). Rats were anesthetized with 4% chloral hydrate (10 ml/ kg, i.p.) during surgical procedures. The right sciatic nerve was exposed at mid-thigh level. Proximal to the sciatic trifurcation, four ligature knots (4–0 chromic gut) were performed loosely with microsurgical techniques. Intervals among ligature knots were about 1 mm. In the sham CCI group, the sciatic nerve was exposed but was not ligatured by chromic gut. Pain threshold was measured as an assessment of nociception at days 0, 3, 5, 7, 10, 12, and 14 after CCI operation.

2.3. Assessment of mechanical withdrawal threshold (MWT)

The 2392 Electronic von Frey Anesthesiometer (IITC Life Science, USA) was used to test the MWT to evaluate mechanical allodynia. Rats were placed individually into wire mesh-bottom cages (20 × 14 × 16 cm) allowed to acclimatize for 30 min. The probe was positioned below the plantar surface of the paw with Von Frey filaments at range of 0.1–70 g, increasing force until the rat twitches its paw. The maximum force was recorded at the time of paw withdrawal. Each paw was tested alternately in 5 min intervals and each rat was tested six times over the course of the experiment. The average value attained by this method was expressed as the MWT.

2.4. Assessment of thermal withdrawal latency (TWL)

The TWL was applied to estimate the thermal hyperalgesia by the 37370 Plantar Test Apparatus (Ugo-Basile, Milan, Italy). Rats were placed in a transparent, square, bottomless acrylic box (17 × 11.5 × 14 cm). After an initial 15 min acclimation period, the infrared source placed under a glass plate is positioned by the operator directly beneath the plantar surface of the hind paw. Radiant heat was set at 50 °C. Withdrawal of the paw, indicating sensation of pain in the rat, caused the infrared source to switch to the off position and the reaction time counter to stop. The hind paw was tested alternately at 15 min intervals. The cut off time for the heat stimulation was 40 s. Each rat was tested five times and the average value expressed as the TWL.

2.5. Electroacupuncture (EA) stimulation

In EA groups, EA began at day 7 after CCI operation. Rats were kept in an immobilization apparatus designed by our laboratory (Patent application number: 201110021482.5, State Intellectual Property Office) without anesthesia, a system both convenient for acupuncture research and comfortable to experimental rats to reduce stress (Fig. 1A). Two stainless steel needles were inserted to a depth of approximately 2–3 mm at ST-36 and GB-34 (Fig. 1B). Two needles were connected with the output terminals of an EA apparatus (HANS-200E, Jisheng Medical Instruments). The frequency of stimulation applied was a square wave alternately at

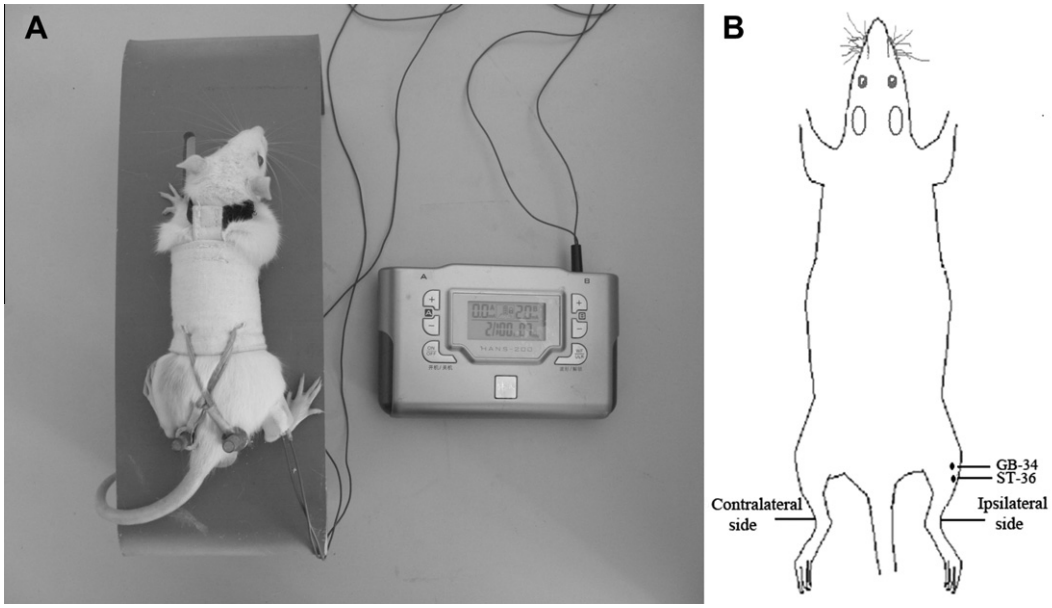

Fig. 1. (A) The new immobilization apparatus designed by our laboratory does not require anesthesia. The apparatus is comfortable to experimental rats which can reduce stress and is very convenient for acupuncture research. (B) Schematic diagrams of the "zusanli" (ST-36) and "yanglingquan" (GB-34) ipsilateral and contralateral EA acupoints.

2/100 Hz, and the intensity of the stimulation was set at 2 mA, lasting for 30 min for 7 days.

2.6. Tissue and section preparation

At day 14, the rats were deeply anesthetized by injection of 4% chloralhydrate (20 ml/kg, i.p.) into the abdominal cavity and then perfused with 100 mL normal saline into the aorta through the left ventricle, followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The L_4–L_6 DRGs of the right side were dissected, postfixed for 4–5 h, and transferred into 15% sucrose in 0.1 M PBS for overnight fixation at a temperature of 4 °C. Tissues were subsequently cut to a thickness of 15 μm using a cryostat for IHC and ISH analysis (Wotherspoon and Winter, 2000).

2.7. $P2X_3$ immunohistochemistry(IHC)

The sections were fixed in 4% paraformaldehyde for 30 min at room temperature. After washing in 0.01 M PBS for three times (5 min each), the sections were incubated in 3% H_2O_2 for 10 min to block the endogenous peroxidase activity and then incubated in goat serum albumin for 15 min at 37 °C. The DRG sections were incubated in rabbit anti-rat $P2X_3$ polyclonal antiserum (1:750 diluted in primary antibody diluent; Chemicon, USA) for 2–3 h at 37 °C. After rinsed and washed in 0.01 M PBS for 3 times, the 5% biotinylated goat anti-rabbit serum (1:200; Beijing Zhongshan Biotech Co, China) was applied as a secondary antibody for 20 min at 37 °C. The sections were rinsed in 0.01 M PBS for 3 times (3 min each) and then added with streptavidin horseradish peroxidase (1:200; Beijing Zhongshan Biotech Co., China) for 20 min of incubation at 37 °C. After three times of rinses in 0.01 M PBS, the sections were incubated with diaminobenzidine tetrahydrochloride up to one minute in order to a visibly opaque brown reaction product. After dehydrationn and transparentization by ascending series of ethanol solutions and xylene, the sections were cover slipped. The positive neurons were identified and immunoreactivity was determined by average optical density (AOD) of $P2X_3$ receptor expressed in DRG using Image Pro Plus 5.1 software.

2.8. In situ hybridization (ISH)

The tissue slides were fixed in 4% paraformaldehyde for 30 min at room temperature (approximately 20–30 °C) and washed using PBS for three times (5 min each) and incubated in 3% H_2O_2 in methanol for 30 min. After washing with PBS, the slides were treated with proteinase K for 30 min at 37 °C. Following hybridization, the slides were incubated in prehybridization buffer for 2–4 h at 37–42 °C and then in hybridization buffer with biotinylated antisense probe overnight at 37–42 °C. After hybridization, excess probe was removed by washing 3 times with gradient saline sodium citrate (SSC) (2 × SSC for 10 min, 0.5 × SSC for 30 min and 0.2 × SSC for 30 min). Slides were then incubated in alkaline phosphatase labelled streptavidin for 60 min at 37 °C. The slides were rinsed for three times (3 min each) with PBS. Color development was performed in the 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)/Nitrotretrazolium nblue chloride (NBT) for 5–8 h and terminated with distilled water. After color development, slides were dehydrated and transparentized by ascendingseries of ethanol solutions and xylene, and then cover slipped (Eisenstat et al., 1999). In situ hybridization images were quantified using the method described above.

2.9. Acute isolation of DRG neurons

At day 14, the rats were deeply anesthetized with 4% chloral hydrate. L_4–L_6 DRGs were dissected out and put in an ice-cold, oxygenated artificial cerebrospinal fluid (ACSF). After removal of the connective tissue, the ganglia were minced with dissecting spring scissors and incubated with a 0.5 ml 0.125% collagenase type I (Sigma) in Dulbecco' modified eagle medium (DMEM) (GIBCO) for 1 h at 36.5 °C, and then 0.5 ml 0.25% trypsin in DMEM was added to the above solution for about 10 min, and then the ganglia were taken from the enzyme solution, and incubated in 2 ml ACSF at least 1 h at 36.5 °C to stop the enzymatic digestion (Zhang et al., 2008). Neurons were subsequently dissociated and dispersed with glass pipettes, and then placed on acid-cleaned glass coverslips and kept still for 20–30 min. Experiments were performed at room temperature (approximately 20–30 °C).

2.10. Electrophysiological recordings

Neurons were superfused (2–3 ml/min) at room temperature (approximately 20–30 °C) with ACSF, containing (in mM): NaCl 154, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10, glucose 5.6; pH was adjusted to 7.2–7.4 with NaOH (Ou et al., 2011). Recordings were made using the conventional whole-cell patch-clamp method. Membrane potential was held at −60 mV, and liquid–liquid junction potentials were corrected. The pipette tip was initially filled with pipette solution which contain (in mM) K-gluconate 145, $MgCl_2$ 2, K_2ATP 5, ethyleneglycol tetraacetic acid (EGTA) 0.5, HEPES 5; pH was adjusted to 7.2–7.4 using KOH. The resistance of recording electrodes was in the range of 2.5–6.5 MΩ. Membrane currents were filtered at 10 kHz and sampled at 1 kHz using pClamp software (Axon Instruments, USA). The experiment was carried out using an Axopatch 700B amplifier (Axon Instruments, USA). Clampfit 9.2 (Axon Instruments, USA) was used for analysis and plotting of data.

2.11. Statistical analysis

All results were expressed as mean ± standard deviation (S.D). Statistical analyses were evaluated using SPSS16.0. Statistical differences were analysed by one-way ANOVA of variance with Tukey or Dunnett's post hoc test for multiple comparisons. Comparison of the two groups was made using the Student's *t* test. A *P*-value <0.05 was considered to be statistically significant.

3. Results

3.1. Effect of EA on mechanical and thermal hyperalgesia of CCI rats

The MWT and TWL were measured before CCI operation and at day 3, 5, 7, 10, 12 and 14 after the CCI operation respectively. The MWT and TWL were measured initially at day 3 after operation, instead of an earlier point, in order to avoid potential error due to the effects of postoperative pain. Mechanical and thermal allodynia were developed at day 5–7. In CCI group, the MWT was markedly reduced from 32.5 ± 1.45 g to 10.8 ± 1.80 g compared with preoperative levels ($P < 0.01$). The TWL was reduced from 12.3 ± 1.22 to 4.4 ± 0.57 s ($P < 0.01$) at day 7. The MWT and TWL of the CCI group (10.1 ± 1.14 g, 4.1 ± 0.42 s) were less than those in sham CCI (32.5 ± 0.99 g, 12.4 ± 0.67 s) group at day 14 ($P < 0.01$, $P < 0.01$). EA had increased the mechanical and thermal threshold in the rats suffering from neuropathic pain after CCI operation in the ipsilateral paw. In the two EA groups, all of the rats were evaluated 30 min after EA treatment. The MWT of the CCI plus contralateral EA group elevated from 11.4 ± 1.29 g to 17.3 ± 1.15 g ($P < 0.01$), whereas the TWL increased from 3.8 ± 0.77 to 7.9 ± 0.78 s ($P < 0.01$). In the CCI plus ipsilateral EA group, EA can relieve mechanical allodynia in rats with the MWT values from 11.6 ± 1.17 g to 20.4 ± 1.33 g ($P < 0.01$). It also was

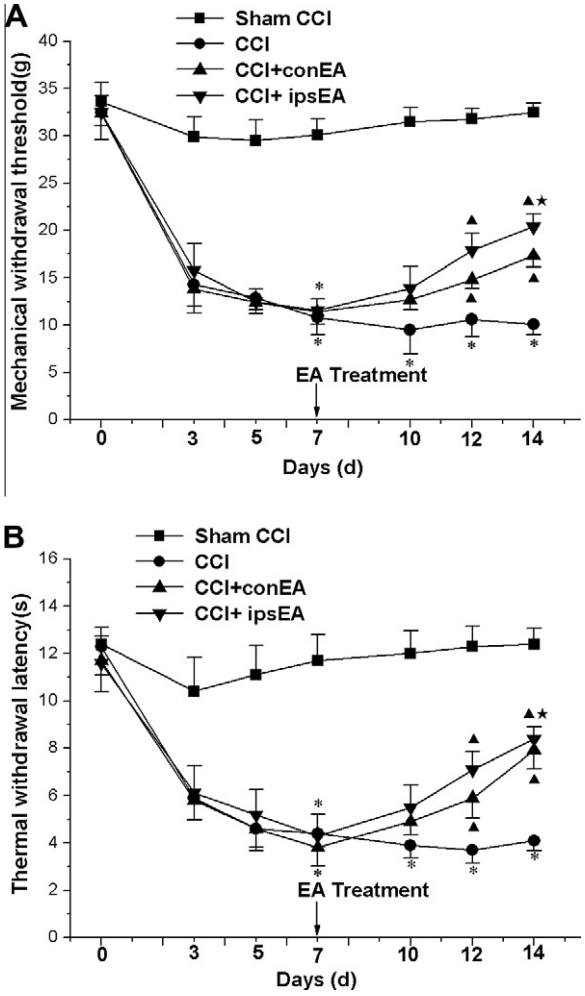

Fig. 2. Analgesic effects of EA treatment in the rats with chronic constrictive injury (CCI) ($n = 8$, each group). There is a significant difference in post-operative CCI group in the changes on mechanical allodynia (touch test, A) and thermal hyperalgesia (thermal test, B), compared with the same group pre-operatively and sham controls ($^{*}P < 0.01$). In the two EA groups, all of the rats were evaluated 30 min after EA treatment. There is a significant difference between EA groups on mechanical and thermal hypersensitivity and CCI group ($^{\blacktriangle}P < 0.01$), but no difference between ipsilateral and contralateral EA groups ($^{\bigstar}P > 0.05$). Error bars indicate s.e.m.

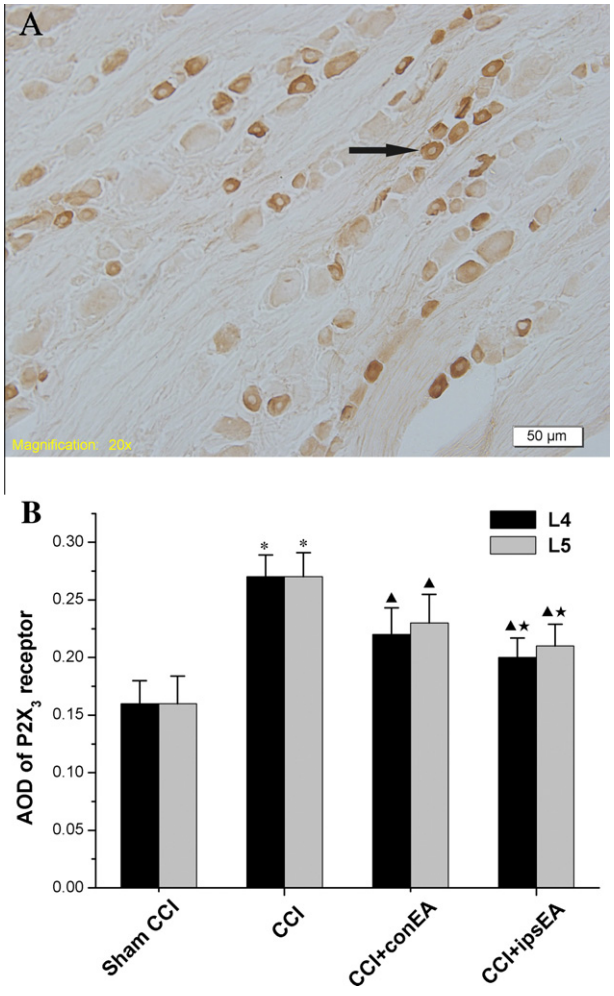

Fig. 3. Effect of EA on the expression of the $P2X_3$ receptor in the rat DRG neurons. (A) Immunostaining showing the expression of the $P2X_3$ receptors in DRG (arrow; positive neurons; scale bars, 50 μm). (B) Histogram comparing the average optical density (AOD) value of $P2X_3$ receptor expression in $L_{4\text{-}5}$ DRGs among the four groups ($n = 8$, each group). $^{*}P < 0.01$ for CCI group compared with sham CCI group. EA can decrease the expression of the $P2X_3$ receptor. $^{\blacktriangle}P < 0.01$ for CCI group compared with two EA groups; $^{\blacktriangle\bigstar}P > 0.05$ for CCI plus ipsilateral EA group compared with CCI plus contralateral EA group. Error bars indicate s.e.m.

demonstrated to reduce sensitivity to thermal pain, with the TWL increasing from 4.3 ± 0.92 to 8.4 ± 0.51 s ($P < 0.01$). Similarly, the MWT and TWL of the CCI plus contralateral EA group (17.3 ± 1.15 g, 7.9 ± 0.78 s) were higher than those of the CCI group (10.1 ± 1.14 g, 4.1 ± 0.42 s) at day 14 ($P < 0.01$, $P < 0.01$). In the CCI plus ipsilateral EA group, the MWT and TWL (20.4 ± 1.33 g, 8.4 ± 0.51 s) were greater than those in CCI group at day 14 ($P < 0.01$, $P < 0.01$) (Fig. 2 A and B).

3.2. Effect of EA on the expression of $P2X_3$ immunoreactivity in DRG of CCI rats

$P2X_3$ receptors expression within the L_4–L_5 DRGs neurons was observed by immunohistochemistry using anti-$P2X_3$ antibody treatment. The $P2X_3$ protein was primarily located in the cytoplasm of small and medium DRG neurons, with no significant amount located in the nucleus and even less in the large DRG neurons and peripheral nerve fibers (Fig. 3A). The immunoreactivity was confirmed in $P2X_3$ positive neurons with significantly increased numbers in CCI group when compared with sham CCI group. A significant decrease of $P2X_3$ positive neuron numbers was seen in the two EA groups when compared with levels in CCI group ($P < 0.01$), but there was no significant difference for $P2X_3$ positive neuron numbers between the two EA groups ($P > 0.05$). The average optical density (AOD) value of $P2X_3$ receptors expression in the CCI group was greater than that observed in sham CCI group. The AOD values of $P2X_3$ receptors expression in the two EA groups were less than those observed in CCI group ($P < 0.01$), but no difference was found between the two EA groups ($P > 0.05$) (Fig. 3B).

3.3. Effect of EA on the expression of $P2X_3$ mRNA in DRG of CCI rats

Assessment of alteration observed in $P2X_3$ mRNA was conducted by ISH analysis performed on L_4–L_5 DRGs neurons. The $P2X_3$ mRNA was also mainly expressed in the cytoplasm of small and medium diameter DRG neurons (Fig. 4A). There was a significant increased in $P2X_3$ mRNA positive neuron number in CCI group when compared with that in sham CCI group ($P < 0.01$), but there was no significant difference of $P2X_3$ positive neuron number between the two EA groups ($P > 0.05$). The average

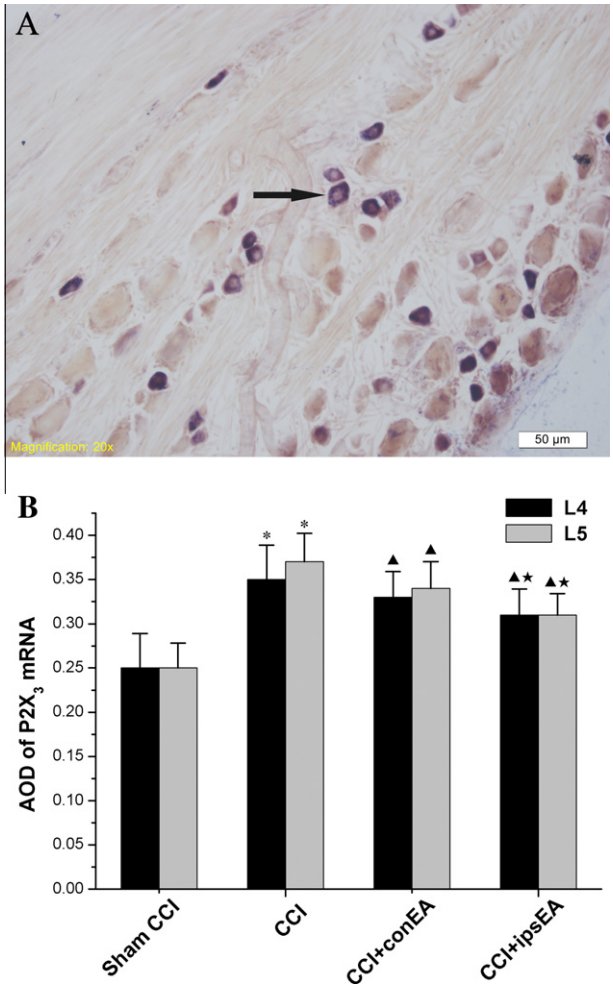

Fig. 4. Analysis of $P2X_3$ receptor mRNA expression levels in rat DRG neurons by in situ hybridization (ISH). (A) Expression of the expression of $P2X_3$ receptors mRNA in $L_{4\text{-}5}$ DRGs neurons (arrow; positive neurons; scale bars, 50 μm). (B) $^{*}P < 0.01$ for CCI group compared with sham CCI group. EA reduced the expression of the $P2X_3$ receptor mRNA. $^{▲}P < 0.01$ for CCI group compared with two EA groups; $^{▲★}P > 0.05$ for CCI plus ipsilateral EA group compared with CCI plus contralateral EA group. Error bars indicate s.e.m.

density value of $P2X_3$ receptors expression in the CCI group was greatly higher than that in the sham CCI group. The average density values of $P2X_3$ receptors expression in the two EA groups were lower than that in CCI group ($P < 0.01$). No significant difference was observed in the intensity of the $P2X_3$ receptors expression in DRG neurons between the two EA groups (Fig. 4B).

3.4. Effect of EA on ATP-activated currents and α,β-meATP-activated currents in DRG neuron of CCI rats

The small and medium sized neurons (between 12 and 35 μm in diameter) were chosen as subjects of the present study based on their documented role in mediating transmission of the nociceptive signals. ATP can evoke a fast desensitizing inward current (I_{ATP}) in the DRG neurons of rats (Fig. 5A). ATP was observed to have the capacity to evoke inward current in the DRG neurons in concentration dependent manner (Fig. 5D). Similar to ATP, α, β-meATP was shown to evoke a fast desensitizing inward current ($I_{\alpha,\beta\text{-meATP}}$) as well. The present study conducted the electrophysiological experiment using concentrations of ATP at 1, 10, 100, 1000 μM and α,β-meATP at 10 μM. The percentage of the DRG neurons responsed to the external application of ATP and α,β-meATP was 61.0% (25/41) in sham CCI group, 65.5% (19/29) in CCI group, 66.7% (20/30) in CCI plus contralateral EA group, and 54.0% (27/50) in CCI plus ipsilateral EA group. The amplitudes of the I_{ATP} at 10 and 100 μM in CCI group were greater than those obtained in the sham CCI group ($P = 0.027$, $P = 0.04$). The amplitudes of the currents in the CCI group were greater than those in CCI plus ipsilateral EA ($P < 0.001$, $P < 0.001$) and CCI plus contralateral EA groups ($P < 0.001$, $P < 0.001$). The amplitudes of the I_{ATP} in CCI group were reduced with EA treatment. There were no significant difference ($P = 0.750$, $P = 0.672$) in amplitudes of I_{ATP} at 10 and 100 μM between CCI plus ipsilateral EA and CCI plus contralateral EA groups. However, the amplitudes of the I_{ATP} at 1 and 1000 μM were no significant difference ($P > 0.05$) among the groups (Fig. 5B). As a selective agonist of $P2X_3$ receptors, the α,β-meATP was applied to verify the effect of EA in $P2X_3$ receptors (Fig. 5A). The amplitudes of the $I_{\alpha,\beta\text{-meATP}}$ in CCI group were greater than those in sham CCI ($P = 0.022$), CCI plus ipsilateral EA ($P = 0.001$), and CCI plus contralateral EA ($P < 0.001$) groups while the amplitudes of the $I_{\alpha,\beta\text{-meATP}}$ between CCI plus contralateral group EA and CCI plus ipsilateral EA group were not significantly different ($P = 0.934$) (Fig. 5C). Thus, the amplitudes of the I_{ATP} at moderate concentration in the CCI group were inhibited by EA treatment, and the amplitudes of the $I_{\alpha,\beta\text{-meATP}}$ in the CCI group were also inhibited by EA treatment; however, no significant difference between CCI plus ipsilateral EA group and CCI plus contralateral EA group was observed.

4. Discussion

EA treatment is a modern form of acupuncture that is a viable complementary or alternative therapy for treatment of chronic pain conditions. The technique characterized by electrical stimulation of certain sites on the body commonly referred to as acupoints, has become more prevalent (Kelly, 2009; Jiang and Yang, 2008; White, 2006). The stimulating current at various parameters applied to acupoints through acupuncture needles can produce analgesic effect in human being and experimental animals (Zhao, 2008). CCI rats have been shown to exhibit behavioral signs of spontaneous pain and hyperalgesia due to noxious thermal and mechanical stimuli (Zhao, 2008). In the present study, the CCI model, a classic model of neuropathic pain, was used to explore these observations. EA treatment on ST-36 and GB-34 acupoints could increase the mechanical and thermal nociceptive thresholds as well as providing relief of the allodynia symptoms in CCI plus EA groups, indicating that EA treatment has a beneficial effect on the pain threshold of CCI rats. This experimental evidence suggests that EA analgesia is a viable treatment with true clinical potential, as shown in Fig. 2. However, it still remains unclear about the potential mechanism between the EA acting as a suppressive effect on hyperalgesia and the $P2X_3$ receptors in DRG neurons.

Purinergic signaling, purines and their receptors are now widely accepted as an important local communication system (Burnstock, 2006a). In neuropathic pain, ATP released after tissue injury can activate primary sensory afferent neurons via purinergic receptors, including $P2X_3$ and $P2X_{2/3}$ receptors in the nerve injury models (Abbracchio et al., 2009). DRG plays an important role in initiation and transmission via purinergic receptors, especially the $P2X_3$ receptors (Chen et al., 2005; Hamilton and McMahon, 2000). Neuropathic pain results in up-regulation of $P2X_3$ receptor expression on DRG neurons (Burnstock, 2006b; Novakovic et al., 1999; Gao et al., 2008; Ou et al., 2011). In this study, the expression of $P2X_3$ receptors in the DRG neurons was examined by IHC and ISH. The experimental evidence demonstrated that the expression of $P2X_3$ receptors in small and medium sized DRG neurons was increased after CCI. Similar to previous studies, the present study observed that pathological pain enhanced the expression of $P2X_3$

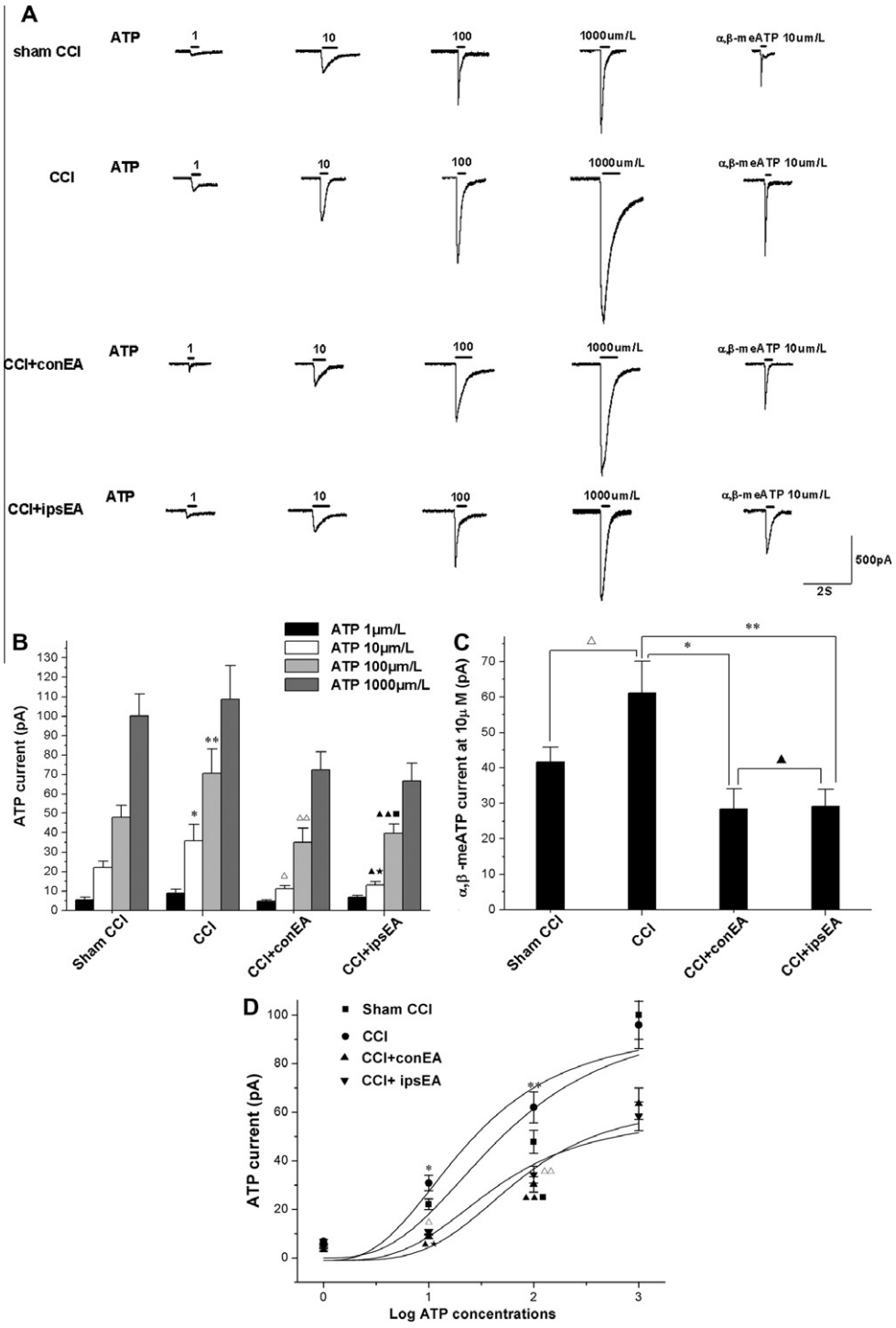

Fig. 5. Effects of EA treatment on $P2X_3$ receptor agonists ATP and α,β-meATP evoked inward currents in DRG neurons. (A) Representative ATP at 1–1000 μm/L and α,β-meATP at 10 μm/L evoked currents in each group. Histograms (B) and (C) comparing the amplitude of the currents evoked by ATP at 1–1000 μm/L and α,β-meATP at 10 μm/L among sham CCI, CCI, CCI plus ipsilateral EA and CCI plus ipsilateral EA groups. At 10 μm/L ATP, $^{*}P < 0.05$ for CCI group compared with sham CCI group; $^{\triangle}P < 0.01$, $^{\blacktriangle}P < 0.01$ for CCI group compared with two EA groups; $^{\bigstar}P > 0.05$ for CCI plus ipsilateral EA group compared with CCI plus contralateral EA group. At 100 μm/L ATP, $^{**}P < 0.05$ for CCI group compared with sham CCI group; $^{\triangle\triangle}P < 0.01$,$^{\blacktriangle\blacktriangle}P < 0.01$ for CCI group compared with two EA groups; $\bigtriangleup P > 0.05$ for CCI plus ipsilateral EA group compared with CCI plus contralateral EA group. (C) At 10 μm/L α,β-meATP, $^{\triangle}P < 0.05$ for CCI group compared with sham CCI group; $^{*}P < 0.01$, $^{**}P < 0.01$ for CCI group compared with two EA groups; $^{\blacktriangle}P > 0.05$ for CCI plus ipsilateral EA group compared with CCI plus contralateral EA group. Error bars indicate s.e.m. (D) The concentration dependent ATP evoked currents. The graph shows that the concentration–response curves for ATP evoked currents in DRG neurons. Comparing the amplitude of the currents evoked by ATP at 1–1000 μm/L among sham CCI, CCI, CCI with ipsilateral and contralateral EA groups. At 10 μm/L ATP, there is a significant difference between CCI and sham group ($^{*}P < 0.05$); There is a significant difference between CCI group and two EA groups ($^{\triangle}P < 0.01$, $^{\blacktriangle}P < 0.01$); There is no significant difference between ipsilateral and contralateral EA groups ($P > 0.05$). At 100 μm/L ATP, there is a significant difference between CCI and sham CCI groups ($^{**}P < 0.05$); There is a significant difference between CCI group and two EA groups ($^{\triangle\triangle}P < 0.01$, $^{\blacktriangle\blacktriangle}P < 0.01$); There is no significant difference between ipsilateral and contralateral EA groups ($\bigtriangleup P > 0.05$).

receptors in DRG neurons. In the model of neuropathic pain, inhibition of the development of mechanical hyperalgesia as well as significant reversal of established hyperalgesia was observed with down-regulation of $P2X_3$ receptor protein (Barclay et al., 2002).

Our results suggested that the up-regulated expression of $P2X_3$ receptors in DRG neurons of CCI rats was reduced by EA treatment as demonstrated in Figs. 3 and 4. Thus, EA treatment might act by decreasing the expression of the $P2X_3$ receptors to attenuate the hyperalgesia and allodynia in neuropathic pain. Previous investigations showed that the P2X signal system was associated with some pain mediators including glutamate, GABA and substance P at spinal cord and DRG levels (Gu and MacDermott, 1997; Sokolova et al., 2003; Paukert et al., 2001), while it has been well documented that EA analgesia is a complex physiological process mediated by various transmitters and modulators including opioid peptides, glutamate and its receptors, GABA and its receptors, and substance P (Zhao, 2008). Therefore, we speculated that EA modulated these transmitters and modulators, which in turn decrease the expression of the $P2X_3$ receptors to alleviate the allodynia symptom. However, larger and more specific studies will be required to ascertain the mechanism with certainty. In addition, clinical observations and experimental studies suggest that the pathways of EA signals are interwoven with pain pathways and the convergence of impulses originating from pain sites and EA acupoints occur in the spinal dorsal horn and medial thalamus, where integration of two kinds of impulses takes place. EA impulses might inhibit the up-regulation of P2X receptors through a negative feedback loop in this manner.

Neuropathic injury causes primary sensory neurons to become hyperexcitable to ATP evoked inward currents (Mo et al., 2011). It then depolarizes DRG neurons by eliciting these inward currents (Evans et al., 1996; Khakh et al., 2001). The increased amplitudes of ATP and its analogues evoked currents have been shown to result in sensitization in sensory afferents (Gao et al., 2008; Ou et al., 2011; Zhang et al., 2008). It is the first time to apply patch clamp technique to explore the underlying electrophysiological mechanism of acupuncture analgesia. The present study investigated whether EA when applied to ST-36 and GB-34 acupoints could reduce the amplitudes of the currents in rats with CCI, and the study went onto further explore the effect of EA treatment on primary afferent transmission induced by $P2X_3$ receptors in neuropathic pain. Extracellular administration of ATP and its analogues elicited inward currents by cell depolarization in the sensory neurons (Grubb and Evans, 1999; YuV et al., 2001), which increased the excitability of the nociceptors and facilitated initiation and transmission of the nociceptive responses. Whether EA treatment is possible to alter the amplitudes of the inward currents of ATP and α,β-meATP. The present study used various concentrations of $P2X_3$ receptor agonists ATP and α,β-meATP to evaluate the change of the amplitudes of the inward currents. The currents of ATP in CCI group were higher than those in other groups after application of the concentration ATP at 10 μM and 100 μM and α,β-meATP at 10 μM. Our studies also showed that the amplitudes of the ATP at 10 μM and 100 μM and α,β-meATP at 10 μM in DRG neurons of CCI plus EA groups were much lower than those in CCI group. Thus, $P2X_3$ receptors agonist activated currents were reduced by EA treatment. Although the down-regulation of $P2X_3$ receptors which induced by EA treatment suppressed ATP responses are the primary causative factor in decreasing the amplitudes of inward currents, but it is unknown whether EA can decrease the sensitization of $P2X_3$ receptors to inhibit hyperalgesia of CCI rats. EA impulses may suppress the activities of primary afferent nerve fibers and block the signal transmission mediated by $P2X_3$ receptors in neuropathic pain, causing inhibition of the activation of $P2X_3$ receptors to decrease the sensitization of $P2X_3$ receptors.

It has been noted that EA, as one type of tissue injuries, could trigger an increase in the extracellular concentration of ATP (Goldman et al., 2010). The ATP cannot be transported into the DRG neurons, already rich in $P2X_3$ receptors, but is rapidly degraded by several ectonucleotidases before re-uptake (Nanna et al., 2010). Therefore, the ATP induced by EA cannot activate the $P2X_3$ receptors to act as anti-analgesic effect. In the model of nuropathic pain, it is notable that the pathological pain was relieved, the up-regulated expression of $P2X_3$ receptors was reduced and the amplitudes of the I_{ATP} and $I_{\alpha,\beta\text{-meATP}}$ of $P2X_3$ receptors were suppressed by EA treatment. This indicated that $P2X_3$ receptors were, in fact, critical in the development of neuropathic pain and that EA treatment could likely cause analgesia by decreasing the expression and the sensitization of $P2X_3$ receptors.

Acupuntrue treatment evoked acupuncture feeling "De-Qi" associated with the efficiency of acupuncture analgesia. Similar to other acupuncture techniques, EA can also evoke the same feeling. As mentioned before, ATP activates purinergic receptors, and transmits sensory information from the periphery to the spinal cord. Whether EA treatment could affect the $P2X_3$ receptors in the spinal cord, further investigation is required.

The experimental data from the present research study the EA treatment may decrease the expression of the $P2X_3$ receptors and inhibit the sensitization of the $P2X_3$ receptors in DRG neurons in neuropathic pain and reduce the thermal hyperalgesia, and tactile allodynia in CCI rats. However, further clinical research might be required to elucidate whether the same mechanism applied for human being in EA treatment in reduction of hyperalgesia and allodynia in neuropathic pain conditions.

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