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Role of spinal adenosine A1 receptors in the analgesic effect of electroacupuncture in a rat model of neuropathic pain

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Abstract

Objective: The aim of this study was to determine the role of spinal adenosine A1 receptors (A1Rs) in the analgesic effects of electroacupuncture (EA) for neuropathic pain.

Methods: We performed EA for 30 minutes at the zusanli acupoint in the legs of rats with previously induced chronic constriction injuries and observed the mechanical and thermal pain thresholds 1 hour later. We also examined adenosine levels by high-performance liquid chromatography and A1R expression in the L4–6 spinal cord by western blot analysis. We then injected A1R short interfering RNA (AV-shA1RNA) into the L4–6 spinal cord to downregulate A1R expression and re-examined the mechanical and thermal pain thresholds.

Results: Adenosine levels and A1R expression in the L4–6 spinal cord were increased at 1 hour after EA. In addition, EA exhibited an analgesic effect that was reversed by AV-shA1RNA.

Conclusions: Our results suggest that EA at the zusanli acupoint elicits an analgesic effect against neuropathic pain, mediated by A1Rs in the spinal cord.

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Introduction

Neuropathic pain is associated with high morbidity and is relatively refractory to treatment, presenting challenges to clinicians and severely affecting patient quality of life.[1] Several studies have attempted to find an effective and nontoxic treatment for alleviating this type of pain. In the late 90s, the National Institutes of Health proposed acupuncture as an effective alternative and complementary treatment for lower back pain and associated leg pain;[2] however, the mechanism of acupuncture analgesia was unclear. Since then, numerous studies have investigated the mechanism responsible for acupuncture analgesia, with the main focus on the effect of acupuncture on endogenous opioids.[3] However, the mechanism of acupuncture analgesia remains unclear. Adenosine is a widespread neurotransmitter in the central nervous system and can elicit an analgesic effect by activating adenosine A1 receptors (A1Rs) in the dorsal horn of the spinal cord.[4] Goldman et al.[5] found a significant increase in local adenosine concentrations at the zusanli acupoint after acupuncture in mice, and showed that local injection of an A1R agonist at this location could simulate the analgesic effect of acupuncture. The authors confirmed that acupuncture-induced analgesia was closely related to an increase in adenosine concentrations and A1R levels in local tissues associated with the acupoints.[5] However, traditional Chinese medicine considers the effects of acupuncture to be systemic rather than local.[6] We accordingly hypothesized that spinal A1Rs may be involved in the analgesic effect of electroacupuncture (EA) for neuropathic pain, and tested this hypothesis in a rat model of neuropathic pain.

Materials and methods

Animals

The study protocol was approved by the Institutional Animal Experimental Ethics Committee at the First Affiliated Hospital of Wenzhou Medical University, China (approval No. 12045). Forty-two healthy, adult, male Sprague-Dawley rats (specific-pathogen-free grade) weighing 220 to 250 g were provided by the experimental animal center of Wenzhou Medical University (Animal No: 2007000517448). The rats were purchased 3 days before surgery and were fasted without water for 1 day before surgery.

Experimental protocols

The 42 rats were divided randomly into the following groups (n = 6 each): 1) sham surgery (S) group, 2) chronic constriction injury (CCI) model group, 3) EA (EA+CCI) group, 4) adenovirus small interfering RNA (AV-shA1RNA) group, 5) adenovirus empty vector control (AV-shCTRL) group, 6) EA combined with AV-shA1RNA (EA+AV-shA1RNA+CCI) group, and 7) EA combined with AV-shCTRL (EA+AV-shCTRL+CCI) group.

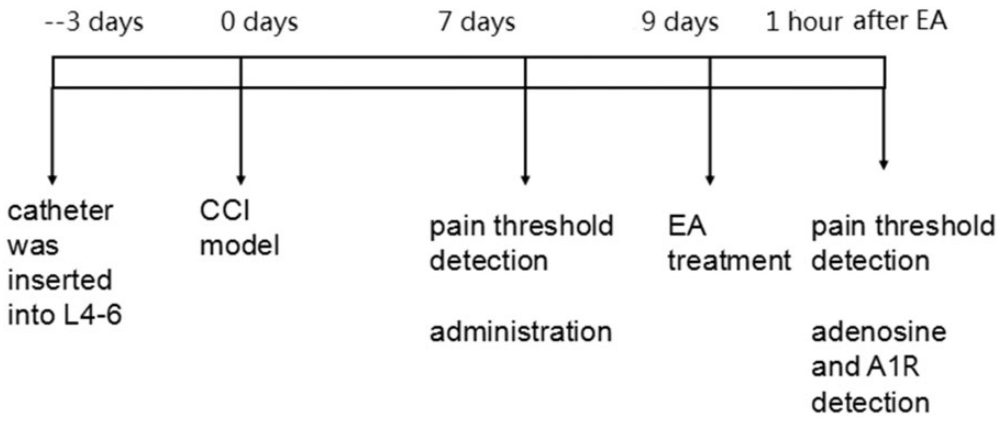

Figure 1. Schematic diagram of experimental scheme. CCI, chronic constriction injury; EA, electroacupuncture.

CCI model

Rats were anesthetized with intraperitoneal chloral hydrate (10%, 350 mg/kg) and routinely disinfected. The femoral biceps muscles in the right lower extremities were separated, and the sciatic nerve trunks were exposed and tied at four sites at 1-mm intervals using 4-0 chromium-containing catgut. The tying force was sufficient to induce a slight leg muscle or toe jerk. The incisions were then sutured and the rats were returned to their cages. In the sham group, the sciatic nerve was exposed but no knots were tied, while the other procedures remained the same. After surgery, the rats showed foot eversion, walking with claudication, and occasional licking and hovering, and the injured foot was held in a defensive position when the animals were quiet, which confirmed the successful induction of chronic sciatic nerve compression.[7] Rats with autophagy or wound infection were excluded and replaced.

EA treatment

The zusanli acupoint is located approximately 5 mm below the fibula head in the knee joint, according to the acupoint map for experimental acupuncture in rats.[8] A #30, 0.5-inch acupuncture needle was used to puncture the zusanli acupoints in the injured and uninjured legs, followed by electric stimulation at a current intensity, frequency, and duration of 1 mA, 2/100 Hz, and 30 minutes, respectively.

Administration method

According to the method of Yaksh and Rudy,[9] the large cisterna occipitalis was exposed under anesthesia 3 days before establishment of the CCI model (Figure 1). A sterile PE-10 catheter was inserted slowly into the subarachnoid space to prevent lumbar enlargement (L4–6). The rats were observed closely to detect any neurobehavioral defects before the model was established and rats with any such defects were excluded and replaced. Rats in the AV-shA1RNA and EA+AV-shA1RNA+CCI groups received AV-shA1RNA (1×10^{11} plaque-forming units/mL, 10 μL) via the catheter 48 hours before EA treatment, while rats in the AV-shCTRL and EA+AV-shCTRL+CCI groups received AV-shCTRL (10 μL). The administration times in the AV-shA1RNA and AV-shCTRL groups were the same as in the EA+AV-shA1RNA+CCI and

EA+AV-shCTRL+CCI groups, with no EA treatment in these groups.

Determination of mechanical and thermal pain thresholds

Mechanical pain threshold measurement. The pain field induced by touching the affected hindlimbs was determined using an electronic von Frey pain gauge (IITC Life Science, CA, USA). The rats were placed randomly at the bottom of a glass partition containing barbed wire and allowed to adapt to the environment for 20 minutes, and then stimulated with a plantar skin probe. Because of the pain stimulus, the rats rapidly removed their hindlimbs from the wire or exhibited licking behavior. The maximum value at this time was determined as the mechanical pain threshold. Mean values were calculated from three measurements recorded at 5-minute intervals for each foot.[10]

Thermal pain threshold measurement. The thermosensitive pain threshold was determined by a 336 tail flap plantar tester (IITC Life Science series 8, model 336). The rats were placed in a transparent glass cage and allowed to adapt to the environment for 20 minutes, and the time required for the development of a thermal pain reaction in the plantar aspect of the injured hindlimbs was then recorded and determined as the thermal pain threshold. The same measurement was repeated three times at 5-minute intervals and the mean value was calculated. Each exposure was not allowed to exceed 25 seconds, to avoid thermal radiation-induced damage.[10]

Detection of adenosine levels by high-performance liquid chromatography (HPLC)

Rats in the S, CCI, and EA groups were sacrificed immediately after determination of the pain thresholds. The L4–6 spinal segments were removed and perchloric acid (0.4 mol/L, 10 mL/g) was added according to the weight of the sample. The sample was homogenized at high speed and centrifuged at $1778.8 \times g$ for 15 minutes. The supernatant was removed and its pH was adjusted to 6.0 to 7.0 by adding potassium hydroxide (4 mol/L). The supernatant was then centrifuged again for 15 minutes and collected and stored at -80°C until HPLC analysis.

All specimens were analyzed using HPLC (Agilent 1100 system; Agilent Technologies Inc., Santa Clara, CA, USA), and chromatographic separation was performed on an Atlantis T3 column (Yilite Analytical Instrument Co. Ltd., Dalian, China) ods2-c18, 4.6 mm 150 mm, 5 m). The mobile phase for adenosine detection was 25 mM KH_2PO_4:acetonitrile, 94:6 (vol:vol; pH 6.0), the flow rate was 1.0 mL/minute, the detection wavelength was 260 nm, and the infusion volume was 20 L. An adenosine standard was used to determine the peak time of adenosine at 7.4 seconds. At the same time, an adenosine standard was diluted to 0.425, 0.85, 1.7, 3.4, 6.8, and 13.6 μg/mL in double-distilled water and a standard curve was established according to the peak area for the six standard concentrations as follows: area $= 71.68815 \times$ concentration $(\text{ng}/\mu\text{L}) + 20.57132$, $r^2 = 0.9976$. The peak area for each sample was recorded and substituted into this formula to calculate the corresponding concentration.

Detection of A1R expression by western blot analysis

Rats in each group were sacrificed 1 hour after acupuncture, the L4–6 spinal segments were removed, and the expression levels of A1R protein were determined by western blot. Total protein was extracted from the spinal cord tissue using a cell protein extraction kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), according to

the manufacturer's instructions. Sample amounts of protein were transferred from the polyvinylidene fluoride membrane to a nitrocellulose membrane and incubated with bovine serum albumin for 1 hour. Bovine serum was added to block the antibodies. Rabbit monoclonal anti-A1R antibodies (Abcam, Cambridge, UK) were added and the sample was incubated overnight at 4°C, followed by rinsing with shaking at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:500, Biogot Technology Co. Ltd., Nanjing, China) were then added and incubated, followed by chemiluminescence, development, and imaging. β-actin was used as an internal reference. The target protein was analyzed using an AlphaImager 2200 gel image processing system (ProteinSimple, CA, USA). The ratio of the optical densities of the A1R and β-actin bands indicated the level of protein expression.

Statistical analysis

All statistical analyses were carried out using IBM SPSS Statistics for Windows, version 17.0 (IBM, Armonk, NY, USA). Data were presented as mean ± standard deviation (mean ± SD). Data that met the assumption for homogeneity of variance were analyzed by one-way analysis of variance (ANOVA) followed by *post-hoc* least significant difference tests. Behavioral data were analyzed using repeated measurement ANOVA, and between-group differences were measured by one-way ANOVA. A *P*-value of <0.05 was considered statistically significant.

Results

EA upregulated adenosine levels in L4–6 spinal cord

Adenosine levels were significantly higher in the EA+CCI group compared with the S ($P < 0.05$) and CCI ($P < 0.05$) groups without EA (Figure 2). There was no significant difference in adenosine levels between the S and CCI groups.

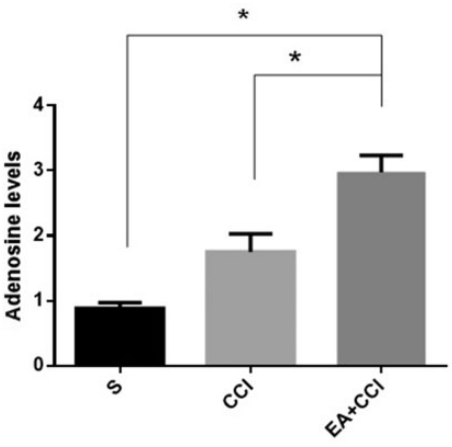

Figure 2. EA upregulated adenosine levels in L4–6 spinal cord. Detection of adenosine levels in different groups by HPLC. Data are presented as mean ± SD (n = 6) and analyzed by one-way ANOVA followed by a *post-hoc* least significant difference test. *$P < 0.05$ between the indicated groups. CCI, chronic constriction injury; EA, electroacupuncture.

EA upregulated A1R expression in L4–6 spinal cord

A1R protein expression levels at 1 hour after EA treatment were significantly higher in the EA+CCI group compared with the S ($P < 0.05$) and CCI ($P < 0.05$) groups (Figure 3). There was no significant difference in A1R expression levels between the S and CCI groups.

EA induced analgesia against neuropathic pain

We verified the analgesic effect of EA by observing the mechanical and thermal pain thresholds in rats with CCI at 7 days after surgery and 1 hour after EA treatment (Figure 4). The mechanical and

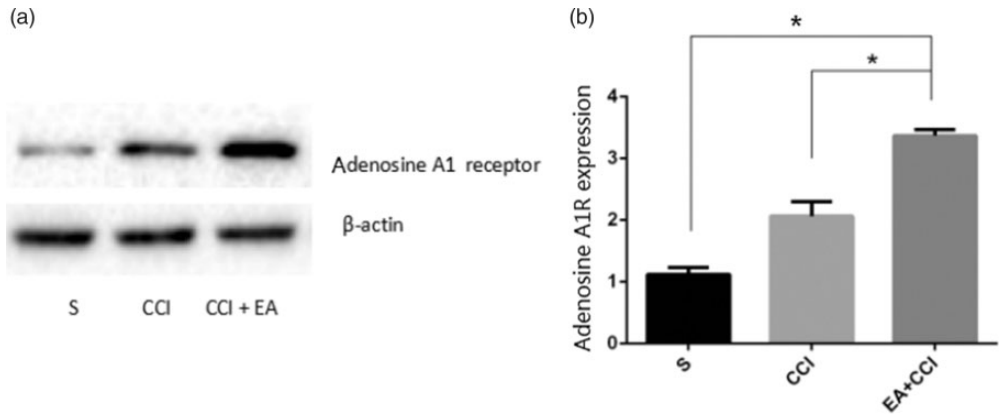

Figure 3. EA upregulated adenosine A1 receptor expression in L4–6 spinal cord. (a) Detection of adenosine A1 receptor expression in different groups by western blotting. (b) Quantitative analysis of adenosine A1 receptor expression in the different groups. Data are presented as mean ± SD (n = 6) and analyzed by one-way ANOVA variance followed by a *post-hoc* least significant difference test. *$P < 0.05$ between the indicated groups. S, sham surgery; CCI, chronic constriction injury; EA, electroacupuncture.

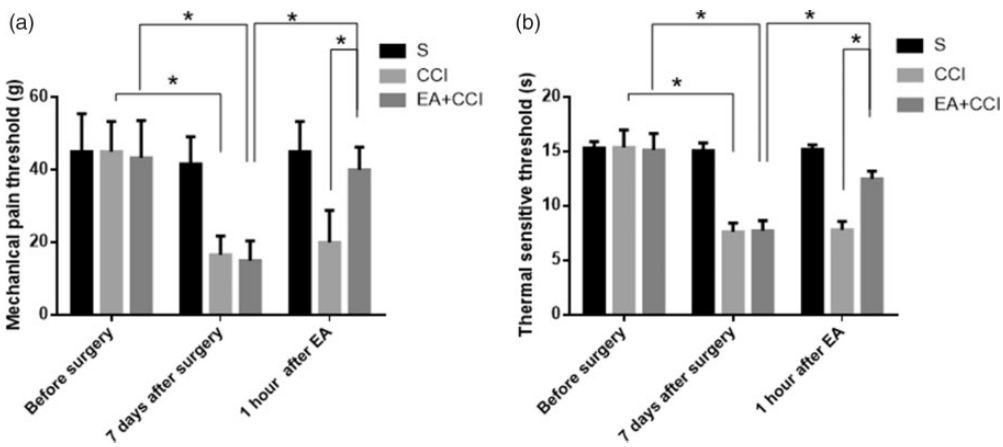

Figure 4. EA induced analgesia against neuropathic injury. (a) Mechanical and (b) thermal pain thresholds in CCI rats. Data are presented as mean ± SD (n = 6 rats) and analyzed by repeated measurement ANOVA, and one-way ANOVA between groups. *$P < 0.05$ between the indicated groups. S, sham surgery; CCI, chronic constriction injury; EA, electroacupuncture.

thermosensitive pain thresholds at 7 days after surgery were the same as the preoperative levels in rats in the S group, but these thresholds were significantly reduced compared with preoperative levels in rats in the EA + CCI and CCI groups (all $P < 0.05$). These results indicated that the CCI model had been established successfully. The mechanical and thermal pain thresholds were significantly higher at 1 hour after EA than at 7 days after surgery in the EA+CCI group ($P < 0.01$), and were also higher than in rats in the CCI group at 1 hour after EA ($P < 0.05$). There were no

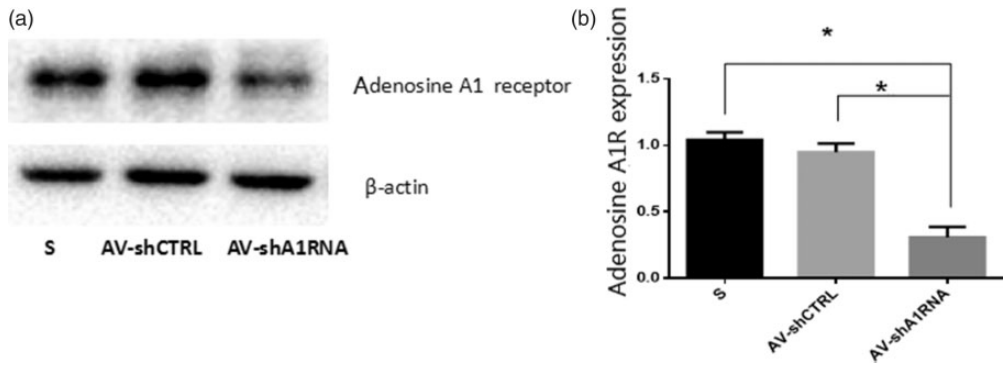

Figure 5. Adenosine A1 receptor expression in L4–6 spinal cord was downregulated by AV-shA1RNA. (a) Detection of adenosine A1 receptor expression in different groups by western blotting. (b) Quantitative analysis of adenosine A1 receptor expression in the different groups. Data are presented as mean ± SD (n = 8) and analyzed by one-way ANOVA followed by a *post-hoc* least significant difference test. *$P < 0.05$ between the indicated groups. S, sham surgery; AV-shCTRL, adenovirus empty vector control; AV-shA1RNA, adenovirus short interfering RNA.

significant differences in the mechanical and thermal pain thresholds between 7 days after surgery and 1 hour after EA in the CCI group.

AV-shA1RNA downregulated A1R expression in L4–6 spinal cord

We determined the effects of AV-shA1R on A1R expression levels in the L4–6 spinal cord using western blot analysis (Figures 4 and 5). A1R expression levels were downregulated in rats in the AV-shA1RNA group compared with the S ($P < 0.05$) and AV-shCTRL ($P < 0.05$) groups. However, there was no significant difference in expression levels between the S and AV-shCTRL groups. These results suggested that the empty adenovirus vector did not affect A1R expression, while AV-shA1RNA downregulated the expression of A1R in the L4–6 spinal cord.

Effects of AV-shA1RNA and AV-shCTRL on analgesic effect of EA

We investigated the effects of AV-shA1R on the analgesic effect of EA in control rats by determining its effects on the mechanical and thermal pain thresholds (Figure 6). There were no significant differences among the S, AV-shA1RNA, and AV-shCTRL groups suggesting that AV-shA1R and AV-shCTRL did not alter the analgesic effect of EA.

Analgesic effect of EA in CCI was reversed by AV-shA1RNA

We measured the mechanical and thermal pain thresholds in rats with downregulation of A1R in the L4–6 spinal cord (Figure 7), to determine if the analgesic effect of EA still occurred. The mechanical and thermal pain thresholds of rats in the S group were similar to those before surgery, but were significantly lower than preoperative levels in the other groups ($P < 0.05$). This confirmed that the CCI model had been established successfully. Furthermore, the pain thresholds at 1 hour after EA were significantly lower in the EA+CCI+AV-shA1RNA compared with the EA+CCI+AV-shCTRL ($P < 0.05$) and EA+CCI ($P < 0.05$) groups, with no significant differences between these latter two groups or between the EA+CCI+AV-shA1RNA and

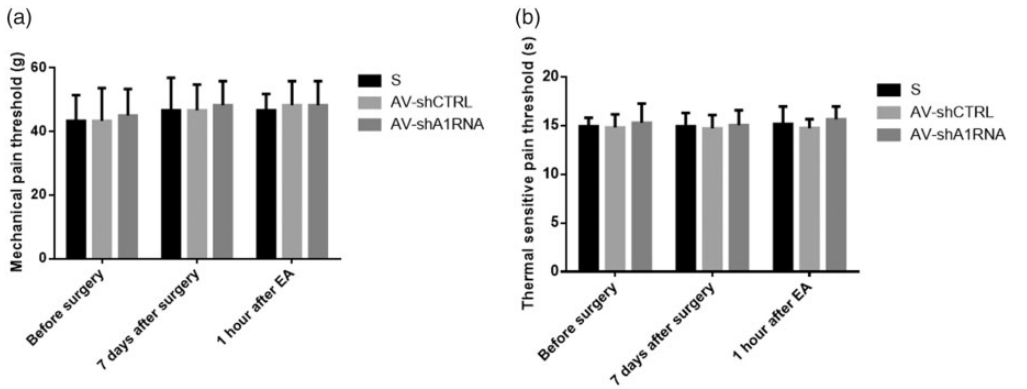

Figure 6. Effects of AV-shA1RNA and AV-shCTRL on analgesic effect of electroacupuncture. (a) Mechanical and (b) thermal pain thresholds in control rats. Data are presented as mean ± SD (n = 6 rats) and analyzed by repeated measurement ANOVA, and one-way ANOVA between groups. There were no significant differences among the groups, suggesting that AV-shA1R and AV-shCTRL did not alter the analgesic effect of electroacupuncture. S, sham surgery; AV-shCTRL, adenovirus empty vector control; AV-shA1RNA, adenovirus short interfering RNA.

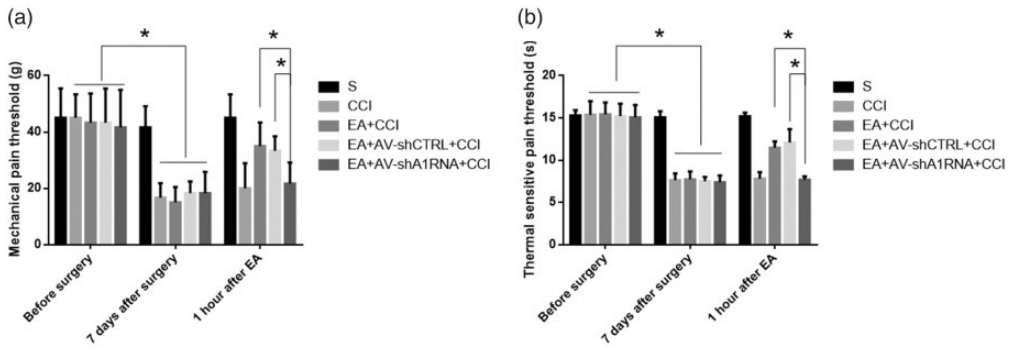

Figure 7. The analgesic effect of EA was abolished by AV-shA1RNA. (a) Mechanical and (b) thermal pain thresholds in CCI rats. Data are presented as mean ± SD (n = 6 rats) and analyzed by repeated measurement ANOVA, and one-way ANOVA between groups. *$P < 0.05$ between the indicated groups. S, sham surgery; CCI, chronic constriction injury; EA, electroacupuncture; AV-shCTRL, adenovirus empty vector control; AV-shA1RNA, adenovirus short interfering RNA.

CCI groups. These results suggested that AV-shA1RNA could reverse the analgesic effect of EA.

Discussion

The efficacy of EA for the treatment of neuropathic pain has been demonstrated in a variety of pain models and clinical trials.[11,12] Traditional Chinese medicine considers that repeated acupuncture at the zusanli acupoint has an analgesic effect, and studies have also shown that single acupuncture at the bilateral zusanli acupoints had an analgesic effect on neuropathic pain in rats.[13] Moreover, the analgesic effects of EA were similar to those of conventional acupuncture used in traditional Chinese medicine, with a frequency of 2/100 Hz resulting in the best analgesic effects

among all the frequencies used for electrostimulation (2, 100, 2/100, and 2/15 Hz).[14] We accordingly investigated the effects of single acupuncture at the zusanli acupoints using a frequency of 2/100 Hz in the present study.

We established a CCI model by sciatic nerve ligation in rats, to control the degree of damage. This technique also resulted in more stable changes in pain levels, with repeat surgeries based on this method showing similar results.[7] We observed the effect of EA performed at peak neuropathic pain (7 days after surgery) on adenosine and A1R levels in the L4–6 spinal cord of rats with CCI.[1] We measured the mechanical and thermal pain thresholds at 7 days after surgery and 1 hour after EA. We also examined the role of A1Rs in EA analgesia by downregulating their expression by intrathecal injection of shA1RNA. The present study thus introduced a new methodology for studying the biological mechanism of EA analgesia.

Neuropathic pain is a chronic pain syndrome caused by peripheral or central nervous system diseases and dysfunction, characterized by hypersensitivity to pain, touch-induced pain, and spontaneous pain. Its pathogenesis is extremely complex and current treatments are unsatisfactory, with serious effects on patient quality of life. Previous studies showed that the sensitivity of the spinal dorsal horn neurons increased after peripheral nerve injury,[15] and that the spinal nerves from the L4–L6 spinal segments constituted the sciatic nerve in rats.[16] In the present study, were therefore administered AV-shA1RNA locally into the L4–6 spinal segments and observed the effect of EA on adenosine levels in these spinal segments. Adenosine levels and A1R expression in these segments were significantly increased by EA in CCI rats ($P < 0.05$), indicating that EA could further increase levels of endogenous adenosine and upregulate the expression of A1R in the spinal cord in rats with sciatic nerve injury. Mechanical and thermal pain thresholds are commonly used as pain indicators in rats,[10,15] and have been shown to reach their lowest levels in the CCI rat model on the 7th postoperative day.[7] We therefore performed EA on the 7th day after the induction of CCI, and showed that both pain thresholds were significantly lower at 7 days after surgery compared with before surgery in rats in the CCI, EA + CCI, EA + AV-shA1RNA + CCI, and EA + AV-shCTRL + CCI groups, indicating that the CCI model had been established successfully in all rats in these groups. We also found that the mechanical and thermal pain thresholds were significantly higher in the EA + CCI compared with the CCI group, and were significantly different from those at 1 hour after EA, indicating that EA could alleviate neuropathic pain caused by CCI. The pain thresholds at 1 hour after EA were significantly lower in the EA + AV-shA1RNA + CCI than in the EA + CCI group, demonstrating that shA1RNA could partially reverse the analgesic effect of EA. We concluded that adenosine and A1Rs in the L4–6 spinal segments played a role in the analgesic effect of EA in rats with CCI.

Adenosine is a widely distributed and important neuromodulator in the central nervous system. It is a metabolite of ATP, which can be metabolized into ADP, AMP, and adenosine via enzymatic pathways. The final metabolite is creatinine, which can then be eliminated *in vitro*.[5] Adenosine receptors are G protein-coupled receptors that can be divided into A1, A2a, A2b, and A3 receptors.[15] A1Rs are closely related to analgesia[4] and are mainly distributed in the cerebral cortex, hippocampus, spinal cord, and other structures.[17] Studies in a rat model of neuropathic pain found that astrocytes in the spinal cord segment were in an active state, and that activation of A1Rs on these astrocytes inhibited their

activation, thereby reducing the release of inflammatory substances and resulting in an analgesic effect.[13] Numerous studies in different animal models of pain have shown that stimulation of A1Rs in the spinal cord or throughout the body can produce analgesic effects.[18] Delivery of a physiological stimulus that does not cause tissue damage, such as mechanical stimulation, hypoxia, or an electric current, is considered to promote the transport of ATP from many types of cells (including dermal tissue and neurons) to the extracellular environment,[19] where it is metabolized into adenosine. Previous studies also found that acupuncture induced a significant increase in adenosine levels around the zusanli acupoints in mice and excited A1Rs around this acupoint resulting in analgesia, suggesting that local A1Rs around the zusanli acupoints mediated the effect of EA analgesia.[5] However, this study did not examine the effects of acupuncture on the central nervous system. Moreover, electrodes implanted into the brain tissue of rats using a stereotactic positioning device increased the levels of ATP and adenosine in the brain tissue,[20] indicating that electrical stimulation could induce the release of adenosine in nerve tissue. These theoretical and experimental studies suggest that the mechanism underlying the EA-induced increase in adenosine levels in the L4–6 spinal segments may include the EA-induced release of ATP within the neurons or spinal glial cells into the extracellular space, with subsequent metabolism to adenosine by ATP lyase and other enzymes. The source of the adenosine was not determined in the present study, which represented a limitation. In addition, A1R expression in the L4–6 spinal segments was increased by EA, indicating that EA induced an increase in adenosine levels in the spinal cord, which in turn activated A1Rs to produce an analgesic effect.

Adenosine is easily and quickly metabolized into creatinine in the body, and it is therefore unclear why the analgesic effect of EA could still be observed at 1 hour after EA in rats with CCI in the current study. However, Goldman et al.[5] found that the analgesic effect of acupuncture was related to the acupuncture-induced release of adenosine, and they suggested that acupuncture could cause the release of ATP in tissues, which is then metabolized to ADP, AMP, and adenosine. AMP can be stored in tissues for a long time and is gradually metabolized to adenosine.[5] We therefore speculated that the analgesic effect of EA and increase in adenosine levels could be related to the *in vivo* accumulation of AMP at 1 hour after EA. However, this needs to be confirmed by the measurement of AMP levels.

In conclusion, the present study used a classic rat model of CCI and confirmed that adenosine levels and A1R expression were increased by EA treatment. Furthermore, intrathecal injection of AV-shA1RNA could partly reverse the analgesic effect of EA. These results suggest that A1Rs in the spinal cord play an important mechanistic role in the analgesic effect of EA in neuropathic pain.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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