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Acupuncture effects on the hippocampal cholinergic system in a rat model of neuropathic pain[☆]

Junying Wang, Junling Liu, Shuping Chen, Yonghui Gao, Fanying Meng, Lina Qiao

Institute of Acu-moxibustion, China Academy of Chinese Medical Sciences, Beijing 100700, China

Abstract

The present study observed the effects of repeated electroacupuncture of *Zusanli* (ST36) and *Yanglingquan* (GB34) on expression of hippocampal acetylcholinesterase, vesicular acetylcholine transporter, and muscarinic M₁ receptor mRNA in chronic constrictive injury (neuropathic pain) and/or ovariectomy rats. Results demonstrated increased expression of hippocampal acetylcholinesterase, vesicular acetylcholine transporter, and muscarinic M₁ receptor mRNA, as well as decreased pain threshold, in a rat model of chronic neuropathic pain after electroacupuncture. The effects of electroacupuncture increased with prolonged time, but the above-mentioned effects decreased in memory-deficient animals. Results indicated that repeated electroacupuncture has a cumulative analgesic effect, which is closely associated with upregulation of acetylcholinesterase and vesicular acetylcholine transporter activity, as well as M₁ receptor mRNA expression and memory.

Key Words: acetylcholinesterase; chronic pain; cumulative effect; electroacupuncture analgesia; hippocampus; M₁R mRNA expression; vesicular acetylcholine transporter

Junying Wang[☆], M.D.,
Institute of Acu-moxibustion,
China Academy of Chinese
Medical Sciences, Beijing
100700, China

Corresponding author: Junling Liu, Professor, Institute of Acu-moxibustion, China Academy of Chinese Medical Sciences, Beijing 100700, China
Jlliu2765@yahoo.com.cn

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INTRODUCTION

In rats with a transected sciatic nerve, local hippocampal damage considerably accelerates the development of pain syndromes, while hippocampal electrostimulation delays it^[1]. In normal and morphine-addicted rats, nociceptive electrical stimulation of the sciatic nerve potentiates electrical activity in pain-excited neurons and suppresses pain-inhibitory neuronal discharges in the hippocampal CA1 area; microinjection of acetylcholine (ACh) suppresses pain-excited neuronal discharges and activates pain-inhibitory neurons, suggesting a cholinergic mechanism in neuronal pain processing in the hippocampal CA1 area^[2-3].

Because of the delayed effect when utilizing acupuncture therapy for the treatment of chronic pain, multiple treatment sessions are necessary to achieve stable, cumulative efficacy^[4-5]. Several experimental studies on acupuncture analgesia have shown that the hippocampal and cholinergic system are involved in acupuncture analgesia^[6-7]. When ACh receptors are activated, the analgesic effect of acupuncture is significantly increased^[8-10]. In addition, the cholinergic system plays an important role in learning and memory pathways^[11]. Our previous studies have demonstrated that protein kinase A and synaptophysin expression in

hippocampal cells induces a repeated electroacupuncture (EA)-induced analgesic effect; when learning and memory ability are impaired by ovariectomy (OVX), the cumulative analgesic effect is decreased^[12-13]. We hypothesized that a repeated, EA-induced, cumulative, analgesic effect could be closely associated with memory and information storage in central nervous system neurons. The present study was designed to analyze the relationship between the cumulative analgesic effect of repeated EA and hippocampal cholinergic nerve activity in a rat model of chronic constriction injury (CCI) to the sciatic nerve and OVX.

RESULTS

Quantitative analysis of experimental animals

A total of 105 Wistar female rats were randomly assigned to normal control, CCI, CCI + EA 2 days (2D), CCI + EA 2 weeks (2W), CCI + OVX, CCI + OVX + EA 2D, and CCI + OVX + EA 2W groups ($n = 15$). Models of chronic neuropathic pain were established in CCI, CCI + EA 2D, CCI + EA 2W, CCI + OVX, CCI + OVX + EA 2D, and CCI + OVX + EA 2W groups. Models of OVX-induced learning-memory attenuation were generated in the CCI + OVX, CCI + OVX + EA 2D, and CCI + OVX + EA 2W groups before model establishment of

chronic neuropathic pain. Rats in the EA 2D and EA 2W groups were subjected to electroacupuncture at *Zusanli* (ST36) and *Yanglingquan* (GB34) at 4 and 16 days after surgery for 2 days and 2 weeks, respectively. Two rats in the CCI + OVX group died during OVX, and the remaining rats were included in the final analysis.

EA increased hippocampal vesicular acetylcholine transporter (VACHT) expression

A larger gray value represents lower VACHT expression. Compared with the normal control group, VACHT expression significantly decreased in the hippocampal CA1 region ($P < 0.05$; Figure 1). At 2 weeks after EA, VACHT expression significantly increased in the CCI + EA 2W group ($P < 0.05$). Following CCI, VACHT expression significantly decreased in the OVX + CCI group compared with the CCI group ($P < 0.05$; Figure 1). In comparison with the OVX + CCI group, VACHT expression significantly increased in the OVX + CCI + EA 2D and OVX + CCI + EA 2W groups ($P < 0.05$). In addition, VACHT expression was significantly less in the CCI + EA 2W group than in the OVX + CCI + EA 2W group ($P < 0.05$), suggesting a weakened effect of repeated EA in upregulating hippocampal VACHT expression in OVX rats. There were no significant differences between CCI and CCI + EA 2D groups, or between OVX + CCI + EA 2D and OVX + CCI + EA 2W groups ($P > 0.05$; Figure 1).

EA increased hippocampal acetylcholinesterase (AChE) activity

Compared with the normal control group, acetylcholinesterase (AChE) expression did not change in the hippocampal CA1 region of the CCI and OVX + CCI groups ($P > 0.05$). However, compared with the CCI group, AChE expression significantly increased in the CCI + EA 2W group ($P < 0.05$). In addition, AChE activity significantly increased in the OVX + CCI + EA 2W group compared with the OVX + CCI group ($P < 0.05$). In the

CCI + EA 2W group, AChE expression was significantly greater than in the OVX + CCI + EA 2W group ($P < 0.05$; Figure 2).

EA increased hippocampal muscarinic M1 receptor (r) mRNA expression

Compared with the normal control group, expression of hippocampal muscarinic M₁ receptor (M₁R) mRNA in the CCI and OVX + CCI groups was significantly downregulated ($P < 0.05$). Compared with the CCI group, M₁R mRNA expression levels were significantly greater in the CCI + EA 2D and CCI + EA 2W groups ($P < 0.05$), and the effect of 2W EA was significantly greater than 2D EA ($P < 0.05$). Compared with the OVX + CCI group, M₁R mRNA expression was significantly increased in the OVX + CCI + EA 2W group ($P < 0.05$). In addition, M₁R mRNA expression was significantly greater in the CCI + EA 2W group compared with the OVX + CCI + EA 2W group ($P < 0.05$), suggesting a weakened cumulative effect of EA on M₁R mRNA expression in OVX rats. There was no significant difference in M₁R mRNA expression between the OVX + CCI and OVX + CCI + EA 2D groups ($P > 0.05$; Figure 3).

EA decreased pain responses after CCI

In the CCI and OVX + CCI rat groups, pain scores were significantly greater than the normal control group ($P < 0.05$), suggesting decreased pain threshold. The pain scores were significantly less in the CCI + EA 2W group and OVX + CCI + EA 2W group compared with in the CCI group and OVX + CCI group, respectively ($P < 0.05$). There was no significant difference in pain scores between the CCI + EA 2D and CCI groups or between the OVX + CCI and OVX + CCI + EA 2D groups ($P > 0.05$). However, pain scores were significantly less in the CCI + EA 2W group than in the OVX + CCI + EA 2W group ($P < 0.05$), suggesting a weakened analgesic effect of repeated EA in OVX rats compared with simple CCI rats (Figure 4).

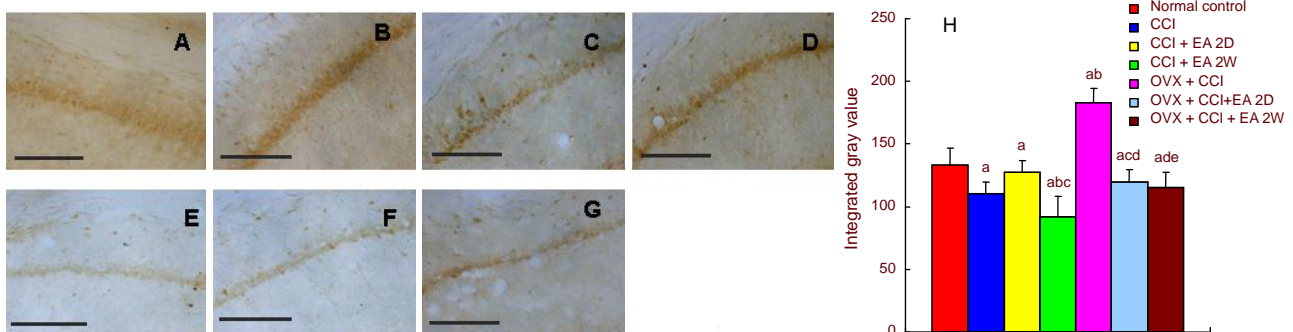


Figure 1 Comparison of vesicular acetylcholine transporter (VACHT) expression in the hippocampal CA1 region.

VACHT expression in the hippocampal CA1 region in the control (A), CCI (B), CCI + EA 2D (C), CCI + EA 2W (D), OVX + CCI (E), OVX + CCI + EA 2D (F), and OVX + CCI + EA 2W (G) groups. Scale bars: 200 μ m, brown staining reflects positive VACHT expression. (H) The integrated gray values of VACHT immunoreaction-positive products (immunohistochemical staining, mean \pm SEM; $n = 10$, except for OVX + CCI group which contains eight rats). Five regions were randomly selected from the CA1 region for each staining to calculate the total gray value; an average value was subsequently obtained. A larger gray value represents lower VACHT expression. ^a $P < 0.05$, vs. normal control group; ^b $P < 0.05$, vs. CCI group; ^c $P < 0.05$, vs. CCI + EA 2D group; ^d $P < 0.05$, vs. OVX + CCI group; ^e $P < 0.05$, vs. CCI + EA 2W group. Least significant difference-*t* was used to compare data between groups. CCI: Chronic constriction injury; EA: electroacupuncture; OVX: ovariectomy; 2D: 2 days; 2W: 2 weeks.

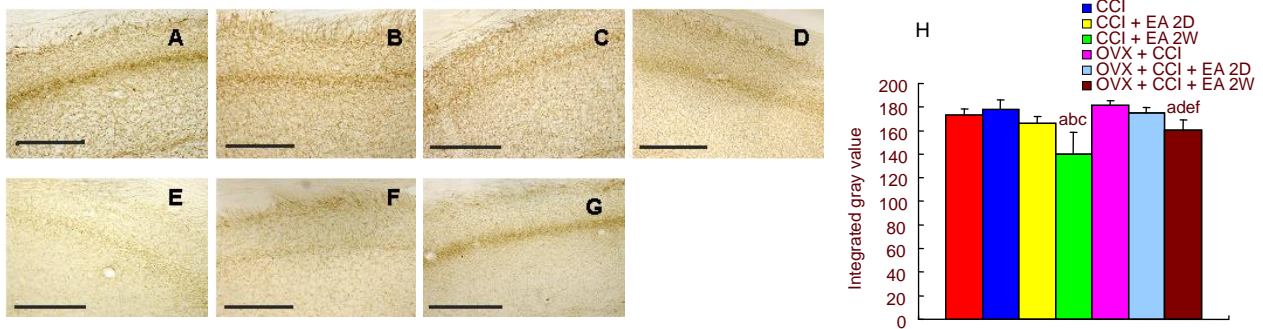


Figure 2 Comparison of acetylcholinesterase (AChE) expression in the hippocampal CA1 region.

AChE expression in hippocampal CA1 region in the control (A), CCI (B), CCI + EA 2D (C), CCI + EA 2W (D), OVX + CCI (E), OVX + CCI + EA 2D (F), and OVX + CCI + EA 2W (G) groups. Scale bars: 200 μ m; yellowish-green stain reflects positive AChE expression). (H) Integrated gray values (mean \pm SEM; $n = 10$, except for OVX + CCI group which contains eight rats) of AChE in the hippocampal CA1 region. A larger gray value represents lower AChE expression. ^a $P < 0.05$, vs. normal control group; ^b $P < 0.05$, vs. CCI group; ^c $P < 0.05$, vs. CCI + EA 2D group; ^d $P < 0.05$, vs. OVX + CCI group; ^e $P < 0.05$, vs. OVX + CCI + EA 2D group; ^f $P < 0.05$, vs. CCI + EA 2W group. Least significant difference- t was used to compare data between groups. CCI: Chronic constriction injury; EA: electroacupuncture; OVX: ovariectomy; 2D: 2 days; 2W: 2 weeks.

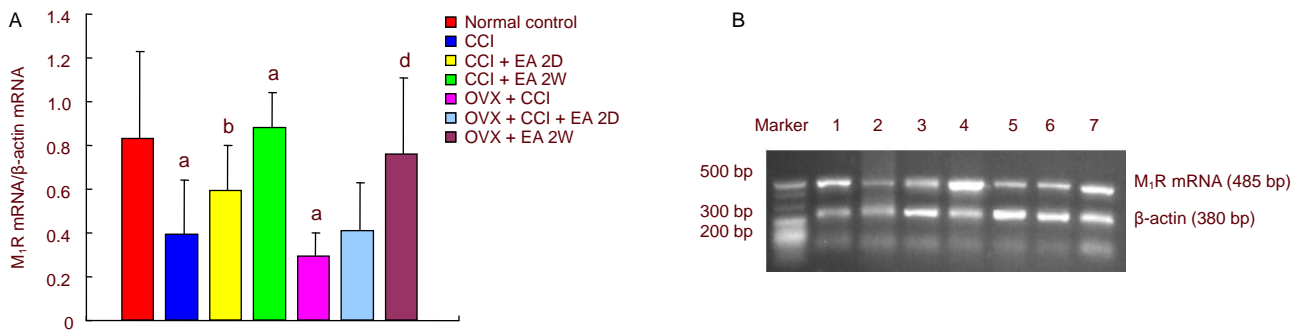


Figure 3 Comparison of the expression of muscarinic M₁ receptor (M₁R) mRNA in the rat hippocampus in each group.

(A) The expression levels (absorbance ratio, mean \pm SEM, $n = 10$, except for OVX + CCI group which contains eight rats) of hippocampal M₁R mRNA in various groups. The higher the absorbance, the higher the mRNA expression was. M₁R mRNA expression was significantly lower in the CCI and OVX + CCI groups compared with the normal control group ($P < 0.05$), and higher in the CCI + EA 2D and CCI + EA 2W groups compared to the CCI group ($P < 0.05$), and also higher significantly in the OVX + CCI + EA 2W group compared to the OVX + CCI group ($P < 0.05$). ^a $P < 0.05$, vs. the normal control group; ^b $P < 0.05$, vs. the CCI group; ^c $P < 0.05$, vs. the CCI + EA 2D group; ^d $P < 0.05$, vs. the OVX + CCI group; ^e $P < 0.05$, vs. the OVX + CCI + EA 2D group; ^f $P < 0.05$, vs. the CCI + EA 2W group. Differences in paw withdrawal latency were assessed by one-way analysis of variance with repeated measures when appropriate. Least significant difference- t was used to compare data between groups.

(B) Gel electrophoresis strips of hippocampal M₁R mRNA detected by western blot. 1: Normal control group; 2: CCI group; 3: CCI + EA 2D group; 4: CCI + EA 2W group; 5: OVX + CCI group; 6: OVX + CCI + EA 2D group; 7: OVX + CCI + EA 2W group. CCI: Chronic constriction injury; EA: electroacupuncture; OVX: ovariectomy; 2D: 2 days; 2W: 2 weeks.

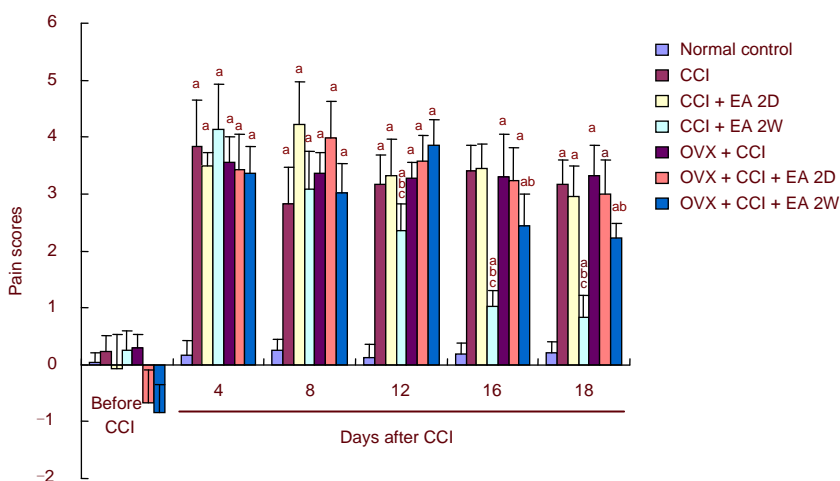


Figure 4 Comparison of pain scores (difference value of paw withdrawal latency between healthy and affected footplates) before and after CCI in the sciatic nerve in the following groups: normal control ($n = 10$), CCI ($n = 10$), CCI + EA 2D ($n = 10$), CCI + EA 2W ($n = 10$), OVX + CCI ($n = 8$), OVX + CCI + EA 2D ($n = 10$), and OVX + CCI + EA 2W ($n = 10$). Values represent mean \pm SEM. ^a $P < 0.05$, vs. normal control group; ^b $P < 0.05$, vs. CCI and OVX + CCI groups; ^c $P < 0.05$, vs. OVX + CCI + EA 2W group. Least significant difference- t was used to compare data between groups. CCI: Chronic constriction injury; EA: electroacupuncture; OVX: ovariectomy; 2D: 2 days; 2W: 2 weeks.

DISCUSSION

In the central nervous system, ACh, which is a major excitatory neurotransmitter, exhibits a variety of effects on sensory perception, plasticity, learning and memory, and in particular on perception and pain control^[14-15]. Previous results have shown that muscarinic acetylcholine receptors (mAChRs) are the pharmacological targets for the treatment of pain^[16]. In the basal forebrain, the cholinergic system originates from the basal optic nucleus of Meynert and medial septal nucleus, which acts mainly on M1R in the hippocampus and neocortex^[17]. Intrahippocampal microinjection of ACh or pilocarpine (mAChRs agonist) decreases the discharge frequency of pain-excited neurons, and increases the discharge frequency of pain-inhibitory neurons evoked by the noxious stimulation in the hippocampal CA1 region. In contrast, intrahippocampal administration of atropine (0.5 mg/mL) results in the opposite response, suggesting that ACh and mAChRs are involved in modulation of nociceptive information transmission in the hippocampal CA1 region^[3]. M1R is localized to brain regions involved in cognition, such as the hippocampus and cortex, but the relative contribution to function has been difficult to ascertain due to the lack of subtype specific ligands^[18]. The muscarinic M1R is abundantly expressed in higher brain regions, including the hippocampus and amygdala^[19-21]. Following inactivation of the M1 gene by intraventricular injection of a M1R antisense oligodeoxyribonucleotide in mice, results from thermal pain tests demonstrated that activation of the M1 muscarinic receptor subtype is fundamental for inducing central cholinergic analgesia^[22-23]. Results from the present study showed that CCI significantly reduced the pain threshold compared with normal control rats. The pain threshold evaluation on day 18 after CCI demonstrated that all the increased pain scores were significantly reduced in the rats undergoing 2-week EA treatment compared with those undergoing 2-day EA treatment, suggesting a cumulative analgesic effect after repeated EA. Findings of the present study showed that after simple CCI, AChE activity did not change in the hippocampal CA1 area, but VAcHT expression significantly increased and M1R mRNA expression significantly decreased. These results suggested that increased ACh synthesis was likely due to autonomic self-protection during chronic neuropathic pain stress stimulation. This study was not able to determine the reasons for the inconsistency between VAcHT expression and M1R mRNA expression, but dysfunction of the intracellular signaling pathway under chronic pain circumstances could play a role. Following two sessions of EA of ST36 and GB34 in simple CCI rats, there was no change in VAcHT or AChE expression, although M1R mRNA expression was significantly upregulated. After two weeks of EA, VAcHT and AChE

expression significantly increased, and M1R mRNA expression was also significantly increased. These results suggested an increased synthesis and degradation of ACh in the hippocampal CA1 region as a result of the cumulative analgesic effect of repeated EA intervention. In OVX + CCI rats, hippocampal VAcHT and M1R mRNA expression was significantly decreased compared with normal control rats. However, this effect was reversed by 2 days and 2 weeks of EA, respectively. There were no significant changes in AChE activity in the hippocampal CA1 region after OVX + CCI. However, after 2 weeks of EA, AChE activity significantly increased. In the OVX + CCI rats, repeated EA increased synthesis and degradation of ACh in the hippocampal CA1 region. These results suggested that repeated EA-induced analgesia activated the cholinergic system in the hippocampal CA1 region. However, there is no direct evidence for the involvement of M1R in the hippocampal CA1 region as a result of acupuncture analgesia. Nevertheless, previous results have shown that hippocampal ChAT mRNA expression levels and plasma E2 content significantly decreased in OVX rats; these changes were reversed after EA of ST36^[24]. The metabolic turnover of ACh in the central nervous system increased as a result of acupuncture analgesia. When ACh biosynthesis was suppressed by intracerebroventricular injection of hemicholine, or the M receptor was blocked by atropine injection, acupuncture analgesia became significantly attenuated. In contrast, interrupted degradation of ACh by intracerebroventricular injection of calabarine or exogenous ACh, or by intrathecal injection of neostigmine, results in increased pain threshold and reinforced acupuncture analgesia^[25-27]. To date, there are no reports addressing the involvement of hippocampal M1R in acupuncture analgesia. However, at the spinal cord level, experimental study results have shown that EA of ST36 or transcutaneous electrical nerve stimulation significantly attenuates pain following intrathecal injection of M1 muscarinic antagonist (pirenzepine) in rat models of neuropathic and inflammatory pain^[28-29].

In the present study, comparisons between CCI + EA 2W and OVX + CCI + EA 2W groups revealed the effects of repeated EA, which increased hippocampal VAcHT and AChE expression, and significantly increased M1R mRNA expression, following OVX. These results suggested a reduced cumulative effect of EA in learning and memory attenuation. These results were consistent with our previous studies, showing that OVX attenuates EA-induced upregulation of protein kinase A expression and synaptophysin expression in the hippocampal CA1 region and paraventricular nucleus, arcuate nucleus, and supraoptic nucleus of hypothalamus^[12-13]. These results were also similar to previous results demonstrating that EA at *Guanyuan* (CV4) promotes recovery of prolactin-positive cells in the arcuate nucleus and paraventricular nucleus in OVX rats^[30]. OVX-induced learning and memory attenuation is a

result of significantly reduced estrogen (and progesterone) secretion from the ovary^[31]. Estrogen has a neuroprotective effect in the central nervous system^[32] and also plays an important role in modulation of hippocampal functions with regard to cell morphology, synapse formation, signaling, and excitability^[33]. Moreover, estradiol significantly potentiates hippocampal ACh release during place training in female rats^[34-35]. Therefore, in OCX rats, the cumulative effects of repeated EA on increased pain threshold, as well as hippocampal VAcHT and AChE expression, and decreased M₁R mRNA expression, are predictable. In summary, hippocampal CA1 acetylcholine and the M₁ muscarinic receptor are involved in the cumulative analgesic effect of repeated EA, which suggests a role for repeated EA in learning and memory.

MATERIALS AND METHODS

Design

Randomized, controlled, animal experiment.

Time and setting

The study was performed at the Institute of Acu-moxibustion, China Academy of Chinese Medical Sciences, from June 2007 to May 2008.

Materials

A total of 98 clean, female, Wistar rats, weighing 230–250 g, were purchased from the Experimental Animal Center of the Union Medical University (Animal license No. SCXK2007-004). All procedures were conducted in accordance with *Regulations for Laboratory Animal Care and Use*, issued by the Ministry of Science and Technology of China^[36].

Methods

Establishment of memory impairment model

Following anesthesia with a mixed solution of 28 mg/100 g urethane (Beijing Chemistry Reagent, Beijing, China) and 3.3 mg/100 g chloralose (Sigma-Aldrich, St. Louis, MO, USA), rats from the CCI + OVX, CCI + OVX + EA 2D, and CCI + OVX + EA 2W groups underwent ovariectomy. Bilateral mid-abdominal dorsolateral incisions (about 2 cm long) were made, and both ovaries were removed. Four weeks after OVX, four animals from each group (CCI, CCI + EA 2D, CCI + EA 2W, CCI + OVX, CCI + OVX + EA 2D, and CCI + OVX + EA 2W) were subjected to a vaginal smear test to verify OVX success. Forty-five days after OVX, learning and memory was analyzed by escape latency in the Morris water maze for 7 days^[12]. The rats with learning and memory impairment were considered as successful models^[12-13].

Establishment of chronic neuropathic pain model (CCI)

After the Morris water maze test, a chronic pain model was established by ligation of the sciatic nerve according to previously described methods^[37]. Under anesthesia and routine sterilization, the left sciatic nerve was exposed at the mid-thigh level by blunt dissection

through the biceps femoris muscle. Four constrictive ligatures (4-0 surgical suture) were tied around the nerve at the distal end close to the nerve bifurcation at spaces about 1.0 mm apart. The ligature was considered successful if local and moderate muscular contraction of the leg was clearly observed. Following local application of antibiotics (sodium penicillin, 9 000–10 000 U/rat), the muscle and skin tissues were sutured in layers. To reduce experimental variability, all surgeries were performed by the same operator. All rats were exposed to the chronic pain model, except for the rats in the normal control group (supplementary Figure 1 online).

Electroacupuncture

Bilateral ST 36 (5 mm beneath the capitulum fibulae and lateral posterior to the knee-joint) and GB34 (about 5 mm superior-lateral to ST36) were punctured with filiform needles (Gauge 28), respectively, and electrically stimulated using a HANS EA Apparatus (LH202, Beijing Huawei Industrial Developing Company, Beijing, China). EA (2/15 Hz, 1 mA) was administered for 30 minutes, once/day for 2 weeks from day 4 after surgery, as well as for 2 days from day 16 after surgery, respectively.

Thermal hyperalgesia detection

Each rat was placed into a black, cloth bag with the hindlimbs and tail exposed to move freely. A mobile radiant heat source (high-intensity light beam with radiant heat dolorimeter) was focused on the plantar surface of the hind paw. Paw withdrawal latency (*i.e.*, pain threshold) of the bilateral footplates was measured three times with an interval of 3–5 minutes between detections. To avoid potential tissue damage, the cutoff time of the radiant heat radiation was set to 20 seconds. Thermal pain threshold detection was conducted prior to, as well as 4, 8, 12, 16, and 18 days after CCI. Pain thresholds were detected in rats from the CCI + EA and CCI + OVX + EA groups on the following day morning to observe post-effects of EA. To minimize individual differences between the rats, the difference value of paw withdrawal latency between the healthy and the affected footplates was used as a pain score (supplementary Figures 1, 2 online).

Sampling and sectioning

The animals were deeply anesthetized with the same anesthetics and were transcardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The brain (without cerebellum) was removed and post-fixed in 30% sucrose solution (containing 4% paraformaldehyde) at 4°C. Serial coronal sections (40 µm) were cut on a cryostat (whole body slicing microtome Leitz 400 with a chest mobile freezer Leitz OM, Leica, Germany) and stored in 0.01 M PBS solution at 4°C until further use.

Immunohistochemical staining for VAcHT

The brain sections were mounted onto glass slides, immersed in PBS for 5 minutes, and incubated in 3% H₂O₂/deionized water for 12 minutes. Excess fluid was removed and the sections were incubated in 5% normal goat serum for 15 minutes at room temperature, followed

by rabbit anti-VACHT polyclonal antibody (1: 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37°C for 4 hours. The sections were then washed three times in PBS for 9 minutes each, incubated with goat anti-rabbit IgG (1: 300, Zhongshan Golden Bridge Biotechnology, Beijing, China) for 2 hours at room temperature, washed three times in PBS, incubated in avidin-biotin-horseradish peroxidase (1: 300, Zhongshan Golden Bridge Biotechnology) for 2 hours at room temperature, washed three times with PBS for 3 minutes each, and stained with 3, 3'-diaminobenzidine. The sections were then washed in running water, counterstained with 0.01% cresyl fast violet at 37°C for 15 minutes, incubated in a mixture of 100% alcohol:ether:chloroform (1: 1: 1) for 10 minutes, rinsed 2 minutes in water, dehydrated in alcohol, and then immersed twice in dimethyl benzene fluid for 10 minutes. The slides were sealed with neutral gel and dried at room temperature. The immunostained structure (gray scale values) of the CA1 area was analyzed using Image-Pro Plus 4.1 image analysis software (MediaCybernetics, Bethesda, MD, USA). Two sections were chosen from each rat, and three areas of the hippocampal CA1 of each slice were selected for detecting gray scale value.

AChE histochemistry

Brain sections containing the hippocampus were washed 3–4 times in distilled water and incubated overnight in a solution containing fluid A [1.3 mL acetic acid buffer solution (pH 6.0,) and 4 mM acetylthiocholine iodide 1 mg] and fluid B [0.1 mL citrate sodium, 0.2 mL 30 mM anhydrous cupric sulfate, 0.2 mL double-distilled water, and 0.2 mL 5 mM potassium ferricyanide]. The sections were placed in a saturated sodium sulfate aqueous solution at 37°C (the solution was replaced three times), washed 3–4 times in distilled water at 37°C, incubated in 2% ammonium sulfide for 15 minutes, and washed in distilled water. The sections were mounted to glass slides, dried at room temperature, and sealed with transparent neutral gum. Image-Pro Plus 4.1 image software was utilized to analyze gray value in each group.

RT-PCR of muscarinic acetylcholine M₁R mRNA

Total RNA for reverse transcription-PCR (RT-PCR) was extracted from hippocampal tissues from 5 rats in each group. RNA was prepared using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (20 µL) was utilized for reverse transcription using the M₁R 5'-sense primer, 5'-GCT TTG ACC GTT ACT TCT CG-3' and M₁R 3'-antisense primer, 5'-ATC CTC CTC TTC TTC CTT C-3'. PCR reactions were performed in a 2720 Thermal Cycler PCR Instrument (Applied Biosystems, Foster, CA, USA) using Taq polymerase (TaKaRa, Dalian, China). PCR amplification consisted of 30 cycles of denaturing (94°C, 5 minutes), 94°C for 30 seconds, 52°C for 30 seconds, annealing (55°C, 1 minute), and extension (72°C, 1 minute) and a final extension step (72°C, 10 minutes). The obtained gene fragments (PCR reaction products, 5 µL) were combined with 6 × electrophoresis

loading buffer (1 µL), and separated on 1.5% agarose gels (containing GV 0.5 µg/mL) at 100 V for 40 minutes. The gel images were analyzed under ultraviolet lamps [Amersham Pharmacia Biotech (SIM, Foster, CA, USA)], photographed, and absorbance values of the image bands were analyzed using the AlphaEaseFC Analyzer System. The ratio between absorbance value of the target gene (M₁R) band and the house-keeping gene (β-actin) was used to determine M₁R mRNA expression levels.

Statistical analysis

Data were expressed as mean ± SEM. Differences in paw withdrawal latency were assessed using one-way analysis of variance with repeated measures when appropriate. Least significant difference was used to compare data between groups. A value of $P < 0.05$ was considered statistically significant.

Author contributions: Junying Wang was responsible for data analysis and drafting of the manuscript in Chinese. Shuping Chen and Fanying Meng participated in the behavioral experiments and immunohistochemical staining. Yonghui Gao and Lina Qiao participated in RT-PCR experiments. Junling Liu designed the study and provided research funding, and finished the manuscript in both Chinese and English.

Conflicts of interest: None declared.

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Ethical approval: All procedures were approved by the Institute of Acu-moxibustion of China Academy of Chinese Medical Sciences.

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Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 3, 2012 item after selecting the "NRR Current Issue" button on the page.

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