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Spinal Serotonin 1A Receptor Contributes to the Analgesia of Acupoint Catgut Embedding by Inhibiting Phosphorylation of the *N*-Methyl-D-Aspartate Receptor GluN1 Subunit in Complete Freund's Adjuvant-Induced Inflammatory Pain in Rats

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Abstract: **Acupoint catgut embedding (ACE) is a widely used traditional Chinese medicine method to manage various diseases, including chronic inflammatory pain. We sought to assess the possible analgesic effects of ACE in comparison with electroacupuncture (EA) and to study the analgesic mechanisms of ACE in a rat model of inflammatory pain induced by injection of complete Freund's adjuvant (CFA) into the hind paw of rats. The von Frey, radiant heat, and gait analysis tests were performed to evaluate the analgesic effects of ACE and EA, and Western blot and immunohistochemistry assays were carried out to determine the molecular mechanisms of ACE. ACE treatments were administered every 4 days or every week with different acupoints (ipsilateral, contralateral, or bilateral ST36 and GB30 acupoints). The most effective ACE strategy for attenuating the nocifensive response induced by CFA injection was performing ACE once a week at ipsilateral ST36 in combination with GB30. EA treatment every other day at ipsilateral ST36 and GB30 showed comparable analgesic effects. ACE inhibited the increased activation of the GluN1 subunit of the *N*-methyl-D-aspartate receptor and the subsequent Ca^{2+}-dependent signals (CaMKII, ERK, and CREB) that take place in response to CFA. The effects of ACE were similar to intrathecal injection of vilazodone (a serotonin 1A receptor [5-HT_{1A}R] agonist) and were blocked by WAY-100635 (a 5-HT_{1A}R antagonist). In summary, we show that ACE attenuates CFA-induced inflammatory pain in rats by activating spinal 5-HT_{1A}R and by inhibiting the phosphorylation of GluN1, thus, inhibiting the activation of Ca^{2+}-dependent signaling cascades.**

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Perspective: ***This article presents the novel evidence concerning the spinal 5-HT$_{1A}$R activation-related molecular signaling of ACE analgesia in a rat model of CFA-induced inflammatory pain. This work may help clinicians to verify the effectiveness of ACE analgesia and to better understand the underlying mechanism.***

Key words: *Acupoint stimulation, electroacupuncture, serotonin, Ca^{2+}-dependent signal cascade, spinal cord.*

Acupuncture, which can be performed in several different ways, has been widely used for managing many diseases, including chronic inflammatory pain, especially in drug-refractory patients.[3,4,55] Several large studies have also provided evidence that acupuncture is a relatively safe treatment.[34,47,50]

Acupoint catgut embedding (ACE) refers to injecting sutures made of absorbable materials at acupoints that are associated with different physiological processes or diseases. This treatment is a combination of ancient traditional acupuncture and modern tissue therapy[11,42,43] and, as a variant of acupuncture, it has been practiced along with traditional acupuncture in China for thousands of years. ACE stimulates the acupoint persistently for a week or longer, until the suture softens, liquifies, and absorps.[11] Therefore, ACE is more convenient than traditional acupuncture, which needs to be performed daily or every other day. Moreover, ACE is easier to perform than traditional acupuncture and is, thus, widely used to treat various disorders in China such as obesity[18] and allergic rhinitis.[28] In particular, it has been widely used to manage clinical pain.[13,31,51] However, the mechanisms behind ACE's analgesic effects remain unclear. Thus, establishing an animal model to study its analgesic mechanisms and to popularize its application is meaningful.

ACE generates mild and long-lasting stimulation at specific acupoints through the injection of catgut, which is similar in essence to electroacupuncture (EA). Therefore, it has been speculated that ACE might share some common mechanisms with EA analgesia.[18,28] Previous research suggests that the activation of serotonergic inhibition on the activities of spinal neurons is at least partly behind the analgesic effects of EA.[26,53,57] Moreover, the serotonin 1A receptor (5-HT$_{1A}$R), which is a subtype of the 5-HT receptors, plays an important role in serotonergic-mediated effects on the nervous system and inhibition of 5-HT$_{1A}$R prevents the analgesic effects of EA in a collagen-induced arthritis pain model.[2] Previous studies also showed that spinal 5-HT$_{1A}$R and the GluN1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor are involved in acupuncture's analgesic effects in a complete Freund's adjuvant (CFA)-induced inflammatory pain model[54] and that 5-HT$_{1A}$R activation prevents the phosphorylation of GluN1,[37] which plays an important role in the regulation of the Ca^{2+}-dependent signal cascade through the phosphorylation of CaMKII, ERK, and CREB.[29,49]

Thus, we hypothesized that spinal 5-HT$_{1A}$R, GluN1, and the Ca^{2+}-dependent signaling cascade are all involved in ACE analgesia. In the present study, we found that ACE showed long-lasting analgesic effects in the CFA models similar to the effects of EA and that blocking 5-HT$_{1A}$R activity markedly decreased the analgesic effects of ACE. Moreover, the decreased phosphorylation of the GluN1 subunit of the NMDA receptor and subsequent decrease in phosphorylation of CaMKII, ERK, and CREB were involved in the 5-HT$_{1A}$R-mediated analgesic effect of ACE.

Methods

Animals

The experiments were performed on adult male Sprague-Dawley rats weighing 200 to 300 g. The rats were supplied by the Experimental Animal Center (Chinese Academy of Sciences, Shanghai, China). The rats were maintained under constant conditions (22°C−24°C and a 12-hour light-dark cycle) with food and water available ad libitum. Four animals were housed per cage and allowed to acclimate to these conditions for $\geq$1 week before inclusion in the experiments. All animal procedures in this study were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ethical standards of the International Association for the Study of Pain[59] Best efforts were exerted to minimize both the number of animals used and their suffering. For each experiment, the animals were randomly divided into groups. The sample size was calculated based on our previous work.

Induction of Inflammatory Pain

To induce inflammatory pain, CFA (Sigma, St. Louis, MO; suspended in a 1:1 oil/saline emulsion, .1 mL, 50 μg *Mycobacterium tuberculosis*) was subcutaneously injected into the right hind paw of the rats using a 500-μL BD syringe with a 30 1/2-G needle. Saline (.9%, .1 mL) was used for the vehicle control group. Except for measuring the time course of CFA-induced inflammatory pain, all other experiments were performed 1 to 15 days after saline or CFA injection when the tissue inflammation in the hind paws was obvious, including erythema, edema, and hyperpathia.

Behavioral Studies

Mechanical allodynia was measured with a series of von Frey hairs (.4, .6, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g; Stoelting, Wisconsin, USA) according to the up-and-down method described in a previous study.[33] Briefly, the rats were placed individually into a Plexiglas cage with a wire net floor and allowed to acclimate for 15 to 20 minutes. The animals were acclimated to this

environment for 2 to 3 days by recording a series of baseline measurements. The von Frey hairs were held against the skin for about 6 to 7 seconds with a 10-minute interval between applications. A trial began with the application of the 2.0-g hair. A positive response was defined as the brisk withdrawal of the hind paw upon stimulation. When there was a positive response to a hair, the smaller hair was used next; when there was a negative response, the larger hair was used next. Five more stimulations were administrated after the first positive response was observed. The final score was converted to a 50% paw-withdrawal threshold (PWT) using an adaptation of the Dixon up-down paradigm as previously described.[33]

Thermal hyperalgesia was reflected by the paw-withdrawal latency (PWL) to radiant heat. The PWL was measured by using an IITC Model 390 Paw Stimulator Analgesia Meter (Life Science Instruments, Woodland Hills, CA, USA) as previously described.[21] The rats were allowed to acclimate to the environment for 15 to 20 minutes in a Plexiglas cage set on the elevated special diabatic glass. Radiant heat was applied to irradiate the plantar surface of each paw until the rat lifted its paw from the glass. The intensity of radiant heat was adjusted to elicit the response at around 12 to 14 s in control rats, and the heat was maintained at a constant intensity. A 20 seconds cut-off time and a 10-minute interval between trials were imposed to avoid tissue damage. Five trials were administrated for each rat. The longest and shortest times were removed, and the average of the 3 remaining data points was calculated. The mean time from the beginning of the heating to the lifting of the rat's hind paw was defined as the PWL.

The changes in gait after CFA injection and ACE treatment were analyzed with the CatWalk Analysis System (Noldus Information Technology, Wageningen, the Netherlands) as previously described.[19] Briefly, this test was conducted in a dark and silent room. The rat was placed on a 1.5-m enclosed glass plate with a light beam from a fluorescent lamp projecting through the glass plate, and the rat was tempted by food to travel across the plate. A high-speed camera was placed under the plate with a focal length that allowed it to capture >3 complete gaits when the rat walked through the filming region. Each rat was tested ≥ 3 times, and the gait parameters were analyzed using the CatWalk Analysis software (CatWalk XT 10.0). Gait parameters were automatically labeled as right forepaw, right hind paw, left forepaw, and left hind paw, and 6 spatiotemporal parameters, including maximum contact area (the area contacted at the moment of maximum paw-floor contact during stance phase), mean contact area (the mean contact area of the complete paw), maximum contact maximum intensity (the maximum intensity of a paw at the moment of maximal paw-floor contact), mean intensity (the mean intensity of the complete paw), swing time (the duration of no contact between a paw with the glass CatWalk plate), and stand time (the duration in seconds of contact of a paw with the glass), were incorporated in our results to evaluate the motor function of the animals. The first 4 values were adjusted by the ratio of the affected side (right hind paw) to the unaffected side (left hind paw).

EA and ACE Treatment

EA treatment started on day 1 after CFA injection. Before EA, the rats were bound by a tailored apparatus so that their bodies were held still while their heads and 4 limbs could move freely. The rats were allowed to acclimate to this for 20 minutes and then a pair of stainless steel acupuncture needles (diameter .3 mm) were inserted vertically into the ipsilateral (right side) acupoint *Zusanli* (ST36, 5 mm below the anterior tubercle of tibia and 2 mm lateral to the knee joint; the lateral sural cutaneous nerve and the cutaneous branch of the saphenous nerve) and *huantiao* (GB30, the posterosuperior border of the hip joint of the hind limbs; underneath are the sciatic nerve, inferior gluteal nerve, and gluteal muscles) at a depth of 6 mm. To keep a consistent and reproducible depth, the needles were bent into an L shape. The handles of these needles were connected to the output terminals of a HANS Acupuncture Point Nerve Stimulator (LH-202H Huawei Co., Ltd., Beijing, China). Parameters of EA were as follow: square wave output current (pulse width of .2 ms), 3 to 4 mA (each current intensity for 15 minutes), and alternating dense −sparse frequencies (alternating between 100 Hz for 3 seconds and 2 Hz for 3 seconds). Sham EA rats received the same treatment but without electrical stimulation.

ACE treatment was administered from day 1 after CFA. A disposable catgut embedding needle (diameter .7 mm; Gaoguan Medical, Zhenjiang, China; Fig 1A) and absorbable catgut (.5 cm long as determined by preliminary experiments; collagen wire, 2-0, 2 cm*10, BD150101, [Boda Co., Ltd., Shandong, China]; Fig 1B) were used in our studies. The rats in the ACE treatment group were anesthetized with isoflurane (2% in a 1:1 mixture of oxygen and air; RWD Life Science Co., Shenzhen, China), and the absorbable catgut was implanted into ST36 and GB30 at a depth of .3 cm using the embedding needle (Fig 1C). Sham-treated ACE rats received the same treatment but without catgut injection.

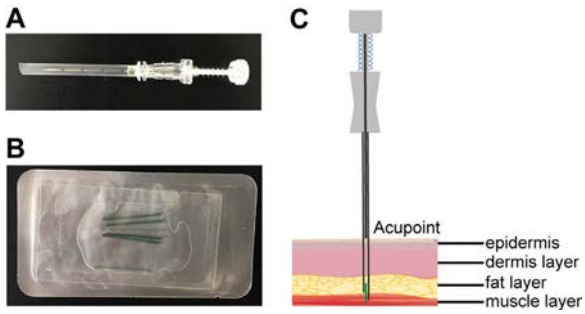

Figure 1. The needle (**A**), catgut (**B**), and schematic (**C**) of ACE.