Long-term antinociception by electroacupuncture is mediated via peripheral opioid receptors in free-moving rats with inflammatory hyperalgesia

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Abstract

Background: Electroacupuncture (EA) has been widely accepted and applied as an important acupuncture-related technique for acupuncture analgesia (AA) research. The involvement of opioid peptides and receptors in acute AA has been shown via pre-EA application of opioid receptor/peptide antagonists. In this study, we intended to reproducibly institute acupoint position and needling excluding influences from anaesthesia or restrainers on rats with complete Freund’s adjuvant (CFA) hind paw inflammatory pain, as well as to explore opioid-dependency and anti-inflammatory effects in sustained acupuncture analgesia.

Methods: Accurate position and needling approach on acupoint GB30 was modelled by computer-based three-dimensional (3D) images and followed by an optimal EA treatment protocol (100 Hz, 2–3 mA, 20 min) at 0 and 24 h post-CFA in conscious free-moving rats. Opioid receptor antagonists, naloxone (NLX) and naltrindole (NTI) were applied intraplantarly post-EA at late phase (96 h) of CFA. Nociceptive thresholds were assessed by paw pressure threshold (Randall-Sellito) or paw withdrawal latency (Hargreaves), and anti-inflammatory effects were evaluated by measurement of plantar temperature and paw volume.

Results: EA elicited significant sustained mechanical and thermal antinociception up to 144 h. Mechanical antinociception of EA was suppressed by peripheral intraplantar application of NLX and NTI. EA also reduced paw temperature and volume during the same time frame indicating anti-inflammatory effects.

Conclusions: By employing a reproducible EA treatment model on GB30 in free-moving rats, we demonstrated the involvement of peripheral opioid receptors mediated EA-induced long-term antinociception. Future studies should examine the specific neuroimmunological connection of EA-induced sustained antinociception in inflammation.

1. Introduction

Electroacupuncture (EA) as one of the main acupuncture-related techniques in traditional Chinese medicine (TCM) for treating varieties of acute and chronic pain-related disorders. Great interest was drawn from mechanism underlying acupuncture-elicited analgesia (AA) has been broadly explored via EA. According to TCM theory, the fundamental of acupuncture treatment is regarded as regulating and balancing Yin and Yang (Cheng, 1999), whereas research within the last couple of years revealed diverse molecular signalling pathways involved in AA (Zhao, 2008; Goldman et al., 2010; Zhang et al., 2010; Su et al., 2011). In addition, anti-inflammatory effects of EA were also seen in some previous studies (Li...
et al., 2005; Zhang et al., 2005b; Shiue et al., 2008; Kuai et al., 2009; Gondim et al., 2012; Su et al., 2012). Endogenous opioid-mediated central analgesia was found to be one of the important mechanisms of AA (Ha et al., 1981; Mayer et al., 1977; Pomeranz and Cheng, 1979; Shen et al., 1973). Subsequent studies identified the pivotal role of endogenous opioids in mediating the AA also at peripheral level (Ceccherelli et al., 2002; Han, 2004; Lao et al., 2004; Zhang et al., 2005a; Taguchi et al., 2010). However, previous publications only studied opioid-related acute effects of AA using pre-EA application of antagonists. The question remains how sustained AA and opioid-mediated antinociception are connected.

Accurate acupoint position is one of the necessary premises for optimal effects of acupuncture. GB30 (Huantiao) is one of the classic acupoints commonly used for relieving pain-related disorders of lumbar region, thigh and lower limbs in both clinic and scientific research (Lao et al., 2004; Fu et al., 2006; Zou et al., 2009; Su et al., 2011). The anatomical location of GB30 is clearly known in humans whereas the exact procedures to accurately position the comparable point in rat is less well described by previous researchers (Lao et al., 2004; Fu et al., 2006; Zhang et al., 2010; Su et al., 2011).

In the local inflammatory pain model induced by complete Freund’s adjuvant (CFA), opioid peptide-containing leukocytes migrate into the inflamed tissue and release opioid peptides. Opioid peptides bind to opioid receptors on peripheral nociceptive neurons and mediate a peripheral antinociceptive effect. This antinociceptive effect elicited by exogenous triggers, e.g., cold-water swim stress or local intraplantar (i.pl.) injection of certain cytokines (e.g., TNF-α, IL-1β), chemokines (CXCL2/3), corticotrophin releasing hormone or formyl peptides (Czlonkowski et al., 1993; Schafer et al., 1994; Rittner et al., 2001, 2006) could only last for a short period (10–20 min), which partly hampers clinical application. However, our previous studies showed secretion of opioid peptides could also be stimulated continuously under the basal conditions (Rittner et al., 2006, 2009).

In this study, we wanted to establish a reproducible model for EA in CFA-induced hind paw inflammation exploring (1) how to reliably model the needling approach on acupoint GB30 and (2) which EA settings could produce sustained antinociception and anti-inflammatory effects as well as (3) possible mechanisms involved including peripheral opioid receptor-mediated long-term antinociception.

2. Materials and methods

2.1 Animals

Animal protocols (REG 69/10) were approved by the governmental animal care committee (Regierung von Unterfranken, Wuerzburg) and are in accordance with the International Association for the Study of Pain (Zimmermann, 1983). Male Wistar rats (280–350 g) were used. Experimental procedures except EA treatment were performed under isoflurane anaesthesia. For induction of local inflammation rats received 150 μl CFA i.pl. in the right paw according as described before (Stein et al., 1988). Due to regulations for animal welfare and requirements from the local animal care committee, experiments were terminated after 6 days (144 h) to minimize potential animal suffering. Per treatment group, we used six to eight animals. In total, 200 rats were used for all the experiment.

2.2 Reagents

CFA was purchased from Calbiochem, San Diego, CA, USA. Naloxone hydrochloride dehydrate (NLX) and naltrindole hydrochloride (NTI) were purchased from Sigma-Aldrich Chemie, Hamburg, Germany. Dose ranges of NLX (μ-opioid receptor; MOR) and NTI (δ-opioid receptor; DOR) were selected according to the previous studies (Rittner et al., 2009).

2.3 Creation of computer based three-dimensional (3D) rat model

In order to accurately and reproducibly find the acupoint position and EA performance on GB30, 3D rat model was created by Maya 2012 (Autodesk, San Raphael, CA, USA), with node-based theory plus NURBs (non-uniform rational B-spline), polygon and subdivision based 3D image. Firstly,
an original photography of the experimental rat was smoothed and polished by Adobe Photoshop 7.0 and then imported into Maya 2012, a preliminary background image based on the original rat photo was automatically generated. Each part of the model was created referring to the background image, the trunk of the model was transformed from a polygon sphere, the limbs or tail was transformed from a column, and each vertebra (part of the thoracic vertebrae, six lumbar vertebrae, five sacral vertebrae and entire coccyx) was transformed from a cube. During the procedures, the related parameters for each node (unit composing the entire model) including width (X axis), height (Y axis) and depth (Z axis) for determining the shape and angles of each part of the model (head and body, limbs, tails, eyes) were separately adjusted in the same 3D space and then were further properly combined to shape an entire model. Sciatic nerve was inserted based on its anatomical location. Lastly, bilateral acupuncture needles were precisely inserted on the junction of lateral two-thirds and medial one-third on the line between the great trochanter and last sacral vertebra; an entire rat model was ultimately displayed in a 3D format.

2.4 EA treatment

Rats were habituated within the sterilized disposable paper cap 3 days before experiment. Rats were randomly divided into CFA + EA, CFA + sham-EA (sham) and CFA control (CFA) group. Before needling, the fur above GB30 was shaved on the lower back and disinfected. Acupoint GB30 was first accurately located according to our 3D model. The hiatus sacral on the rat was comparable to the last sacral vertebra. Important landmarks to position GB30, the great trochanter and last sacral vertebrae, were palpated and marked (Supporting Information Fig. S1a, b). Disposable acupuncture needles (Ø = 0.20 mm, length = 25 mm, Schwa-Medico, Ehringshausen, Germany) were connected with an electrical stimulator (AS Super_4_digital, Schwa-Medico). The direction of the needle was slightly adjusted if sign of direct irritation on nerve or blood vessel were noted. A slight twitching of the needle was observed when the current intensity was adjusted to 1 mA in the first minute (Supporting Information Fig. S1c). Minor changes in the position were necessary if signs of distress occurred at this time point. In order to ensure the fully free-moving mode, the intensity of EA was delivered in a gradual and intermittent manner from 2 to 2.5 to 3 mA (Supporting Information Fig. S1d). The exact intensity for different individuals was flexibly kept between 2 and 3 mA: Muscle twitching of the entire hind limb including the paw could be observed as a sign of accurate needling on sciatic nerve underneath of GB30. Food was catered around in order to distract the attention from needling and to comfort the rats. Sham control rats were needled in a same way like EA rats without electric current.

2.5 Measurement of nociceptive threshold

Thermal nociceptive thresholds (paw withdrawal latency) were obtained by the Hargreaves test (IITC Inc/Life Science, USA) as described before (Rittner et al., 2009). Rats were habituated in the plastic box with a glass plate underneath for 2 to 3 days before experiments. The required time (s) for paw withdrawal was taken as thermal nociceptive threshold. The heat of a radiant bulb was adjusted to 20 s on for measurement of in paw withdrawal latencies the normal paw. The cut-off was set at 30 s to avoid tissue damage. The average of two measurements (with 20 s intervals) was calculated for analysis.

Mechanical nociceptive thresholds (paw pressure threshold) were evaluated with the paw pressure algosimeter (modified Randall-Selitto test; Ugo Basile, Comerio, Italy) as described before (Rittner et al., 2009). Rats were gently habituated into a sterilized disposable man-made cap for several days before experiments (Supporting Information Fig. S1a) and were gently held in the cap during the pain measurements. Increasing pressure (g) was applied to the dorsal surface of paw until the rat withdrew its paw. The cut-off point was set at 250 g to avoid tissue damage. Measurements were performed three times (10 s intervals) and averages were calculated. A decreased value of nociceptive threshold represents hyperalgesia, whereas an increased value represents antinociception. The experimenter was blinded for all the behavioural measurements.

2.6 Measurement of temperature and paw volume

The surface temperature of plantar skin was measured with a contact thermometer sensor before (0 h) and 6 d (144 h) post CFA injection (TM99A, Cooper-Aktins, Middlefield, CT, USA) (Schmitt et al., 2003). The volume of hind paw was measured by submerging the hind paw till the tibiotarsal joint inside the water-filled Perspex cell of a plethysmometer at the same time points (37140, Ugo Basile, Comerio, Italy) (Schmitt et al., 2003; Brack et al., 2004).

2.7 Statistical analysis

All the data were presented as mean ± standard error of the mean. Data of pain threshold changes was shown with raw values while the data of plantar temperature and paw volume were presented as % change (values/baseline * 100). Multiple measurements at different time points between two or more than two groups were analysed by two-way repeated measures (RM) analysis of variance (ANOVA). $p < 0.05$ was regarded as statistically significant.

3. Results

3.1 Computer-based 3D rat model for acupoint positioning and needling

In order to accurately and reproducibly find the acupoint position, and angle of the needle for EA performance on GB30, a 3D rat model was created by...
Maya 2012 (Fig. 1A). By exhibiting specific parameters (depth, angle, distance) of the needling on GB30 from three different perspectives (rear, vertical or side position), accurate needling on GB30 with appropriate needle is anatomically in close proximity to the sciatic nerve (Fig. 1B–D).

### 3.2 Optimal program selection by comparison of different frequencies

First, we established EA in free-moving rats in CFA-induced hind paw inflammation using measurement of thermal nociceptive thresholds. Four frequencies (10, 2/100, 120 and 100 Hz) with a fixed time duration (20 min) and pulse width (0.1 ms) were selected according to the literature (Lao et al., 2004) and our pilot experiments (data not shown). Single treatment with 10 Hz frequencies immediately after CFA administration did not elicit antinociception at the measured time points (Fig. 2A). Single treatment with either mixed frequencies (2/100 Hz) or higher frequency (100 Hz) could inhibit hyperalgesia at 72 and 96 h post CFA (Fig. 2B, D). In contrast, the highest frequency (120 Hz) was only able to reduce hyperalgesia at early phase (2 h) of CFA (Fig. 2C). A second EA treatment with 100 Hz at 24 h was further performed in order to enhance the effect. This delayed the maximal antinociceptive effect from 72 to 96 h post CFA. Double 100 Hz treatments (pulse wide: 0.1 ms, duration: 20 min), at 0 h (immediately after CFA injection) and 24 h (post-CFA), exhibited the best antinociceptive effect at 96 h post CFA and was chosen for following studies (Fig. 2E).

### 3.3 High frequency EA produced persistent thermal and mechanical antinociception

In the next step, we analysed mechanical and thermal nociceptive thresholds in CFA inflammation and compared EA with sham EA. Double EA (100 Hz) treatments significantly suppressed thermal hyperalgesia at 96 h and mechanical hyperalgesia at 48, 72 and 96 h compared to CFA control. No changes in mechanical or thermal nociceptive thresholds in EA + CFA compared to CFA alone were seen at early time points (data not shown). Sham-EA elicited a small effect on mechanical hyperalgesia at 96 h but was not statistically different.
compared to EA (Fig. 3A, B). Antinociception from double EA (100 Hz) therapy lasted up to 144 h (Fig. 3C).

3.4 Peripheral opioid receptors contributed to sustained antinociception of EA

To address whether the peripheral opioid receptors were involved in antinociceptive effects of EA on mechanical hyperalgesia, MOR and DOR antagonists were applied (i.pl.) after EA treatment 96 h post CFA (Fig. 4A, B). Optimal doses of NLX (0.56 ng, i.pl.) or NTI (25 μg, i.pl.) significantly reversed mechanical antinociception of EA 5 min after administration of antagonists (Fig. 4C, D). The same dose of NLX failed to block sham-EA produced antinociception (Fig. 4C).

3.5 High frequency EA dampened increased temperature and swelling of the paw elicited by CFA

Based on the persistent antinociceptive effect of EA, we further examined whether the signs of inflammation induced by CFA could be attenuated by EA (Fig. 5A, B). Skin temperature of plantar surface and inflamed paw volume was daily assessed before CFA until 144 h post CFA. EA slightly but significantly reduced plantar temperature and paw volume at 72 h comparing with sham treatment and 144 h compared to CFA control. No difference of paw volume between EA treatment and CFA control or sham treatment was observed in plantar temperature and paw volume at 96 h post CFA.

4. Discussion

In this study, we established standard EA treatment modelling in 3D visualized image on fully free-moving rats with CFA-induced hind paw inflammatory pain and found: (1) Double application of high frequency (100 Hz) EA induced sustained thermal and mechanical antinociception. (2) Sustained mechanical antinociception produced by EA at late phase post CFA.
(96 h) was mediated by peripheral opioid receptors. (3) High frequency EA concurrently elicited anti-inflammatory effects simultaneously with antinociception at given time points.

4.1 3D modelling for EA treatment on conscious free-moving rats

Computer-based 3D modelling is widely used to answer questions regarding anatomy or mechanisms better than in two-dimensional presentations (Bolliger et al., 2012; Davies et al., 2012; Wang et al., 2012). We therefore used this approach to establish our model in order to reproducibly define position, angle and depth of the needle. According to our anatomical computer-based 3D modelling of GB30, perpendicularly needling could easily reach sciatic nerve located underneath of GB30 unavoidably stimulating the sciatic nerve leading to a twitch of the entire limb, which could be regarded as one of the important signs for accurate positioning. Therefore, our 3D model aimed to build a bridge between so far known simply descriptive needling protocol by other researchers (Lao et al., 2004; Su et al., 2011) and a practical guideline for exact positioning and needling.

Needling sensation ‘De Qi’ meaning soreness, numbness, heaviness and distension and/or aching...
Local blockade of opioid receptors by naloxone (MOR; NLX) and naltrindole (DOR; NTI) inhibited mechanical antinociception by electroacupuncture (EA). Rats were treated with complete Freund’s adjuvant (CFA) i.pl. for 96 h and double EA at 0 and 24 h as described before. (A, B) To find the best dose of the OR antagonists, mechanical nociceptive thresholds after different doses of NLX (0.28, 0.56 and 1.12 ng i.pl.) and NTI (25, 50 and 100 μg i.pl.) in EA treated rats were measured before (baseline: BL) and 5 min after injection (treated) (n = 3–5 per group). (C, D) Optimal doses of NLX (0.56 ng) and NTI (25 μg) were further applied (i.pl.) on EA or sham treated rats, mechanical nociceptive thresholds were evaluated (BL) before and 5 min (treated) after injection. Solvent (saline) was administrated (i.pl.) as control. Mechanical nociceptive thresholds of CFA rats without EA treatment at 96 h were shown for comparison. All the data were presented as mean ± standard error of the mean (n = 6 per group, *p < 0.05, CFA + EA vs. CFA; Two-way repeated measures analysis of variance, Student-Newman-Keuls).

Reduction of complete Freund’s adjuvant (CFA)-induced increased temperature and paw oedema by electroacupuncture (EA). Wistar rats were injected (i.pl.) with CFA for 96 h and treated with double EA at 0 and 24 h as described before. (A) Plantar temperature of inflamed paws was daily measured before and up to 144 h post-CFA. (B) The volume of inflamed paw was assessed simultaneously. All the data were presented as mean ± standard error of the mean (n = 8 per group, *p < 0.05, CFA + EA vs. CFA; *p < 0.05, CFA + EA vs. CFA + sham; *p < 0.05, CFA + sham vs. CFA; Two-way repeated measures analysis of variance, Student-Newman-Keuls).
(dull pain in deep tissue) can only be well felt when conscious. Acupuncture in conscious rats, however, was regarded to be stressful. Therefore, application of anaesthetics or restrainers was often used during acupuncture in order to ease the treatment procedure (Fu et al., 2006; Lund et al., 2009; Kong et al., 2011; Niu et al., 2011). However, interferences from anaesthetics or restrainers might affect molecular signalling pathways and/or physiological and psychological conditions. A semi-free mode using conscious rats in a relative commodious restrainer was employed by Lao et al. (2004) and also used by others (Su et al., 2011). Free-moving rats without anesthesia for EA have been successfully used in a previous study by Iwa et al., 2006 to improve colonic motility.

Based on our pilot experiments, intensity above 2 mA was strong for normal rats independently of the level of consciousness, excessive intensity at given acupoint GB30 might convert EA stimulation into direct sciatic nerve stimulation or exaggerate electrical stimulating sensation and further elicit possibly opposing effects (Ceccherelli et al., 2008). According to our observation, different rats had a different level of tolerance to EA intensity. Besides, intensity above the rat’s endurance would hamper free-moving treatment and elicit stress-induced antinociception (Pomeranz, 1986; Takeshige et al., 1992; de Medeiros et al., 2003). Indeed, a flexible current intensity of 2–3 mA at GB30 was tolerable for the majority of rats weighing 280–350 g. Reasons for differences in the tolerance of current intensity could be the thickness of the tissue between skin and sciatic nerve underneath of GB30 dependent on the body weight or other inherited factors (individual differences). Flexible intensity based on the comfort and endurance of different individuals in electricity-based acupuncture related techniques was already recognized and verified in early clinical trials (Chapman et al., 1983).

We also observed that rats with a relaxed mentality or those who enjoyed the food offered during the treatment had better therapeutic effects, which might support psychological factors in EA-antinociception (Kong et al., 2005, 2009). In this line, we experienced that rats could be classified as non-responders, lower-responders and higher-responders, although the difference in AA responsiveness to experimental pain was only reported in healthy participants (Lee et al., 2002; Sekido et al., 2003; Kong et al., 2005; Chae et al., 2006). If individual variation is of genetic origin (Chae et al., 2006), different types of responders should be present in patients and pathological animal models as well.

4.2 Sustained peripheral antinociceptive and anti-inflammatory effect of EA

4.2.1 Features of antinociceptive properties of endogenous opioids in AA

In the central nervous system, low frequency (2 Hz) EA potentiated the release of enkephalin, β-endorphin (β-END) and endomorphin, while high frequency (100 Hz) selectively increased the release of dynorphin (Han, 2004). Further combination of the two frequencies 2/100 Hz produced a simultaneous release of all four opioid peptides, resulting in a maximal therapeutic effect (Han, 2004). In the spinal cord, 10 Hz EA-induced antinociception was reversed by antagonists of all three opioid receptors [MOR, DOR and κ-opioid receptor (KOR)] (Zhang et al., 2004). Peripheral opioid receptors in AA are less examined in short-term inflammatory pain. Taguchi et al. (2010) illustrated that i.pl. application of all three peripheral opioid receptors antagonists (MOR, DOR, KOR) dose- and time-dependently reversed EA-induced antinociception (3 Hz, 1–3 mA) in short-term carrageenan-elicited peripheral inflammatory pain.

Peripheral opioid peptides mediate antinociception at early phase (0–6 h) of CFA due to release of opioid peptides from neutrophils, while monocytes/macrophages are mainly responsible for the late phase (96 h) (Brack et al., 2004; Hackel et al., 2011). Opioid peptide release does not only occur after cold water swim stress or exogenous stimulations but also is present under basal conditions dependent on bacterial products via formyl peptide receptor stimulation (Rittner et al., 2009). Early research demonstrated pre-EA administration of MOR antagonists reversed EA-induced (30 Hz, 2 mA, and 0.1 ms for 30 min) antinociception at 5 days post-CFA inflammation (Zhang et al., 2005a). However, application of opioid receptor antagonists immediately before EA can only identify the critical role of opioid receptors in transient EA-induced antinociception. In contrast, our study supports the pivotal role of peripheral opioid receptors on persistent EA-induced antinociception by post-EA application of antagonists in inflammatory pain.

4.2.2 Comparison of antinociception produced by sham and verum acupuncture

In several pain-related diseases, clinical randomized controlled trials confirmed the specific analgesic effect of verum acupuncture compared to sham acupuncture in (Ezzo et al., 2001; Witt et al., 2005; Molsberger...
et al., 2010). However, some studies also showed that the sham needling could induce comparable analgesia as verum in patients (Dincer and Linde, 2003; Brinkhaus et al., 2006; Hutchinson et al., 2012) presumably due to placebo effects (Takeishi et al., 1992; Kong et al., 2005, 2009; Shi et al., 2012). The major open question remains unanswered in clinical research which sham control is appropriate (Bender et al., 2001; Yamashita and Tsukayama, 2001; Lund et al., 2009; Lundeberg et al., 2011). Animal studies on analgesic effects of sham acupuncture are relatively insufficient. Invasive sham-EA without electricity in rats is often regarded as an appropriate control for verum EA without comparable analgesic effects (Lao et al., 2004; Su et al., 2011, 2012). However, in present study, invasive sham-EA showed a small elevation of mechanical nociceptive thresholds at 96 h but not 144 h post-CFA. This could be explained by the notion that so-called ‘minimal needling’ of sham-EA is not sufficiently minimal. However, application of the same dose of NLX failed to block sham-EA-induced antinociception, indicating that sham-EA triggered sustained antinociception might not be peripheral opioid receptor-dependent.

4.2.3 Anti-inflammatory properties underlying sham and verum acupuncture

EA has been shown to elicit anti-inflammatory effects (Li et al., 2005; Zhang et al., 2005b; Hahm, 2007; Kuai et al., 2009; Su et al., 2012), which might be frequency-dependent: lower frequencies (2 or 10 Hz) but not higher frequencies EA (100 Hz) dampen inflammatory oedema, whereas both elicited comparable antinociceptive effects (Zhang et al., 2005b; Hahm, 2007). In our studies, increased plantar temperature and paw volume of the inflamed paws were significantly attenuated by EA at 72 and 144 h post-CFA within the same time frame of the antinociceptive effects.

Anti-inflammatory effects of EA were less pronounced than the antinociceptive effects because the difference between EA and CFA control was not presented in each time point and sham-EA elicited comparable effects on plantar temperature and paw volume at 144 h. In line, we have observed in CFA inflammation that inflammatory infiltrate, cytokines and hyperalgesia do not always correlate, e.g., pro-inflammatory chemokines can elicit antinociception (Rittner et al., 2006) and hyperalgesia and the number of infiltrating leukocytes are not in parallel (Brack et al., 2004). Therefore, the underlying peripheral pathophysiological mechanisms of acupuncture itself and sham versus verum acupuncture still needs be examined in further studies.

In summary, our 3D modelled acupoint position and needling approach could pave the way for rigorous and quantitative acupuncture research in the future. The local sustained antinociceptive effect of EA (100 Hz) was mediated by peripheral opioid receptors at late phase of CFA (96 h) together with anti-inflammatory effects. Future studies should be directed to specific neural pathways including both opioid and non-opioid mechanism regulating sustained AA.

Author contributions

Y.W., H.R. and D.H. designed the experiments; Y.W. performed the experiments and data analysis; F.P. designed and created 3D rat model; Y.W., H.R. wrote the manuscript. All authors discussed the results and commented on the manuscript.

References

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

![Figure S1](image-url) EA protocol on free-moving rats. (A) Wistar rats were gently handled using a paper cap 3 days before the experiments. (B) Spinous processes of last four vertebrae (four black dots in a row) and the great trochanter (bilateral black dots) were marked. The accurate anatomical position of GB30 (bilateral yellow dots) on rats was located at the junction of lateral one-third and medial two-thirds of the distance between the great trochanter and the last sacral vertebra. (C) The acupuncture needle connected a the cable delivering the electric current was swiftly punctured through the skin and vertically inserted till the entire body of the needle was within the subcutaneous tissue. The current intensity was adjusted to 1 mA within 1 min. (D) Rats were gently released from the paper cap allowing free movement in the cage. The current intensity was gradually elevated up to 2–3 mA in 0.1 mA step within the next 4 min.