



Research Article

Isoflurane and the Analgesic Effect of Acupuncture and Electroacupuncture in an Animal Model of Neuropathic Pain

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Abstract

The present study aimed to determine whether isoflurane interferes with the analgesic effects of acupuncture (Ac) and electroacupuncture (EA), using a neuropathic pain (NP) rat model. In total, 140 male Wistar rats were used; isoflurane-induced nociceptive response was evaluated using the von Frey test, serum calcium-binding protein β (S100 β) levels and nerve growth factor (NGF) levels in the left sciatic nerve. The NP model was induced by chronic constriction injury of the sciatic nerve at 14 days after surgery.

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neuropathic pain

Treatment was initiated after NP induction with or without isoflurane anesthesia (20 min/day/8 days). The von Frey test was performed at baseline, 14 days postoperatively, and immediately, 24 h, and 48 h after the last treatment. Results of the nociceptive test and three-way analysis of variance were analyzed by generalized estimating equations, the Bonferroni test, followed by Student–Newman–Keuls or Fisher’s least significant difference tests for comparing biochemical parameters (significance defined as $p \leq 0.05$). At baseline, no difference was noted in the nociceptive response threshold among all groups. Fourteen days after surgery, compared with other groups, NP groups showed a decreased pain threshold, confirming establishment of NP. Ac and EA enhanced the mechanical pain threshold immediately after the last session in the NP groups, without anesthesia. Isoflurane administration caused increased nociceptive threshold in all groups, and this effect persisted for 48 h after the last treatment. There was an interaction between the independent variables: pain, treatments, and anesthesia in serum S100 β levels and NGF levels in the left sciatic nerve. Isoflurane enhanced the analgesic effects of Ac and EA and altered serum S100 β and left sciatic nerve NGF levels in rats with NP.

1. Introduction

Acupuncture (Ac) and electroacupuncture (EA) treatments have yielded good results for alleviating neuropathic pain (NP) pain in humans and experimental animals [1,2], though the exact mechanisms of action are unknown. Owing to the practical difficulties in Ac and EA application to awake and freely moving animals, most studies have used restraint [3] or anesthesia [4]. However, anesthetization or immobilization during treatment may lead to physiological changes which could potentially affect treatment efficacy.

The evaluation of Ac and EA may be biased by restraint stress or habituation in conscious animals [5] or by the anesthetics used in sedated animals. The application of Ac or EA treatment to awake animals (insertion of a needle or manual or electric stimulation of animals) may be viewed as stressors. Restraint, shock, and fear are known to trigger stress-induced analgesia when animals are awake, as shown in models of Ac and EA analgesia [6]. Therefore, Ac and EA analgesia can be significantly reduced if concomitant stressors are not adequately controlled.

In animal studies of Ac or EA, isoflurane is the most commonly used anesthetic [4]. It is easily administered and produces the behavioral and physiological characteristics of general anesthesia without an adjunct [7]. Nevertheless, whether it can alter the analgesic response to Ac or EA treatment is unknown. Interestingly, anesthesia may influence biomarker expression. Mice exposed to isoflurane during postnatal brain development showed increased serum levels of calcium-binding protein β (S100 β), a protein used as a neurodegenerative biomarker [8], though this has not been studied in adult rats.

Neurotrophins help in neuronal survival, growth, and differentiation and may also be affected by isoflurane. Nerve growth factor (NGF) is a pain-related neurotrophin that can exert pronociceptive or antinociceptive effects, depending on concentration and site of administration [9]. Chen et al. demonstrated the neuroprotective effect of EA-induced neurotrophins in an animal model of spinal cord injury [10], indicating that EA may reduce pain by neurotrophic modulation.

Based on these findings, we believe that isoflurane can potentiate the analgesic effect of Ac and EA treatments and modify neuromodulation parameters. To test this hypothesis, we evaluated the nociceptive response induced by isoflurane, the serum levels of S100 β and NGF in the left sciatic nerve of Ac- or EA-treated NP rats, using the von Frey test. Concurrently, we assessed locomotor behavior to demonstrate the extent to which the animals were affected by anesthesia.

2. Materials and Methods

2.1. Animals

A total of 140 male Wistar rats (weight ≥ 250 g) aged 55–65 days were used in the experiment. Based on our previous studies, 140 animals were deemed to produce reliable scientific data [11, 12, 13]. Animals housed individually in polypropylene cages (49 × 34 × 16 cm) in a controlled environment (22 ± 2°C) under a standard light–dark cycle (lights-on/lights-off: 0700 h/1900 h), with free access to water and chow (Nuvital, Porto Alegre, Brazil). All experimental procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol no. 13-0298) and conformed to the Guide for the Care and Use of Laboratory Animals (8th ed., 2011). Animal maintenance followed the Brazilian Law 11794 (specifying procedures for the use of animals in scientific research). The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines [14].

2.2. Experimental design

The animals were acclimated to the study environment for 2 weeks before experimental initiation. It is important to emphasize that the application of Ac and EA in awake animals is complex and generates discomfort which may alter treatment-induced analgesia. Furthermore, anesthesia with isoflurane was used during treatments, and its use may have generated a bias in the study, considering the possible interference of the drug in behavioral and

neurochemical results. To avoid possible bias, the animals were randomized and divided into 14 groups: control (no manipulation), sham surgery (Ss; surgery without nerve constriction), sham surgery + Ac (surgery without nerve constriction + 8 Ac sessions), sham surgery + EA (surgery without nerve constriction + 8 EA sessions), NP (Np; surgery with nerve constriction), NP + Ac (NpAc; surgery with nerve constriction + 8 sessions of Ac freely movements), NP + EA (NpEA; surgery with nerve constriction + 8 sessions of EA freely movements), control + anesthesia (no manipulation + 8 days of isoflurane anesthesia), sham surgery + anesthesia (surgery without nerve constriction + 8 days of isoflurane anesthesia), sham surgery + Ac + anesthesia (surgery without nerve constriction + 8 Ac sessions with isoflurane anesthesia), sham surgery + EA + anesthesia (surgery without nerve constriction + 8 EA sessions with isoflurane anesthesia), NP + anesthesia (NpAn; surgery with nerve constriction + 8 days of isoflurane anesthesia), NP + Ac + anesthesia (surgery with nerve constriction + 8 Ac sessions with isoflurane anesthesia), and NP + EA + anesthesia (surgery with nerve constriction + 8 EA sessions with isoflurane anesthesia) (Fig. 1). Subsequently, the Np and Ss groups received

appropriate interventions (chronic constriction injury [CCI] or sham surgery). Fourteen days after surgery, hyperalgesia was evaluated by the von Frey test to confirm NP establishment. Treatments were initiated soon after the establishment of NP; animals were treated for 8 days according to group-specific protocols (Ac, EA, or no treatment). The von Frey test was performed at baseline, 14 days post-operatively, and immediately, 24 h and 48 h after the last treatment session.

2.3. NP model: CCI of the sciatic nerve

The CCI of the sciatic nerve was performed (as per Bennett and Xie [15]) to induce NP. The animals were anesthetized with isoflurane (5% for induction, 2.5% for maintenance), the surgical site shaved, and the skin cleaned with 2% alcoholic iodine [15]. The left sciatic nerve was approached in the mid-thigh by removing part of the biceps femoris muscle. Three ligatures (4-0 Vicryl) were tied 1 mm apart, close to the sciatic trifurcation, and tightened until muscle contraction of the leg could be observed, ensuring epineural blood flow. The same investigator performed the ligatures in all animals. In the Ss

Groups	
Control (C)	No manipulation
Sham Surgery (Ss)	surgery without nerve constriction
Sham Surgery + Ac (SsAc)	surgery without nerve constriction+ 8 sessions of acupuncture
Sham Surgery + EA (SsEA)	surgery without nerve constriction+ 8 sessions of electroacupuncture
Neuropathic Pain (Np)	surgery with nerve constriction
Neuropathic Pain + Ac (NpAc)	surgery with nerve constriction+ 8 sessions of acupuncture freely movements
Neuropathic Pain + EA (NpEA)	surgery with nerve constriction+ 8 sessions of electroacupuncture freely movements
Control+Anesthesia (CAn)	No manipulation + 8 days of isoflurane
Sham Surgery+ Anesthesia (SsAn)	surgery without nerve constriction+ 8 days of isoflurane anesthesia
Sham Surgery + Ac + Anesthesia (SsAcAn),	surgery without nerve constriction+8 sessions of acupuncture with isoflurane anesthesia
Sham Surgery + EA+ Anesthesia (SsEAAn)	surgery without nerve constriction+8 sessions of electroacupuncture with isoflurane anesthesia
Neuropathic Pain+ Anesthesia (NpAn)	surgery with nerve constriction+ 8 days of isoflurane anesthesia
Neuropathic Pain + Ac + Anesthesia (NpAcAn)	surgery with nerve constriction+8 sessions of acupuncture with isoflurane anesthesia
Neuropathic Pain + EA+ Anesthesia (NpEAAn)	surgery with nerve constriction+8 sessions of electroacupuncture with isoflurane anesthesia

Figure 1 Groups. Description of the procedures performed in the animals in each study group.

groups, animals were anesthetized and the left sciatic nerve was exposed but not constricted. The control groups did not undergo any surgical procedure.

2.4. Acupuncture

Two stainless steel Ac needles with guide tubes (Suzhou Huanqiu Acupuncture Medical Appliance Co. Ltd., 218, China; length: 0.18 mm; diameter: 0.8 mm) were inserted bilaterally approximately 2–3 mm into the acupoint BL-24, which is located on the side depression of the lower edge of the L3 spinous process of the third lumbar vertebra (Fig. 2).

2.5. Electroacupuncture

In rats that were either freely moving or anesthetized with isoflurane (under oxygen flow; 2% for induction, 0.5% for maintenance), Ac needles were connected bilaterally to an electro-stimulation device (Model EL 608 NKL, Brusque,

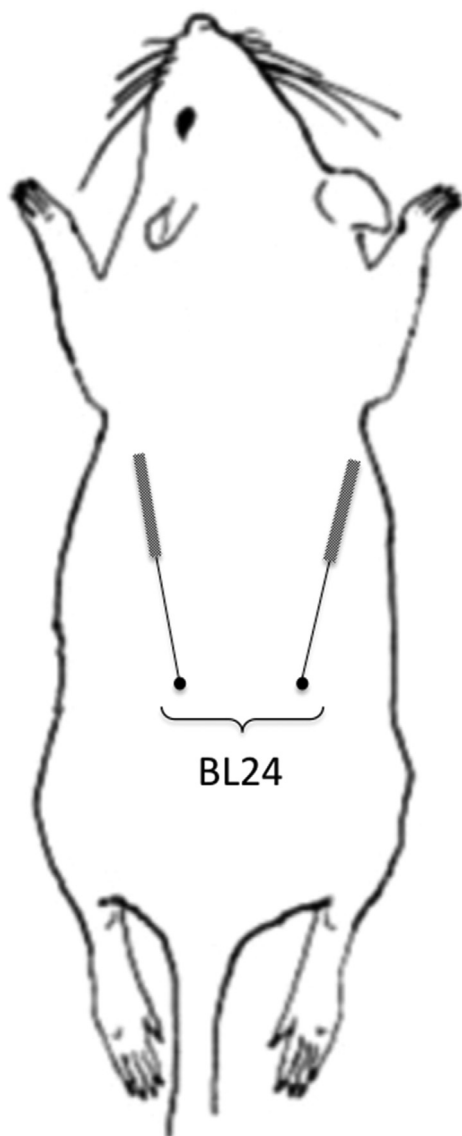


Figure 2 Acupoint. Description of the acupoint BL-24 in the rat.

SC, Brazil), with an alternating frequency of 2 Hz and 100 Hz (2/10 Hz, 0.3 ms width), and treatment performed for 20 min.

2.6. Von Frey test

Twenty-four hours before the test, the rats were acclimated to the apparatus for 5 min. On the test day, rats were placed on the analgesiometer, and the sensor containment box was positioned next to the paw, guided by the reflection in the mirror. The test was based on the maximum pressure (grams) required for the animal to show sensitivity to a paw touch [16]. Three measurements were performed, and the mean value was considered the pain threshold. Measurements were made at baseline, 14 days postoperatively, and immediately, 24 h, and 48 h after the last treatment session. The same trained investigator, who was blinded to the treatment groups, performed all tests. All tests were performed in awake and freely moving animals, without any sedation.

2.7. Open field test

Behavioral assessment excluded acute phase effects and was performed 24 h after the sixth session and before the seventh session to determine the chronic effect of six sessions of Ac and EA with and without anesthesia. A 60 × 40 × 50 cm cage with the floor divided by lines into 12 squares of 13 × 13 cm each was used. The test started immediately after the animals were placed in the back left corner and allowed to explore the surroundings for 5 min [17,18]. The number of line crossings (all paws crossing the boundary into an adjacent marked-out area) was taken as a measure of locomotor activity [19].

2.8. S100 β measurement in blood serum

Animals were killed by decapitation 24 hours after the last treatment. Blood serum was collected and frozen at -80°C until the time of testing. Serum S100 β levels were measured by a competitive enzyme-linked immunosorbent assay kit (MyBiosource, California, USA), according to manufacturer's instructions.

2.9. NGF measurement in the left sciatic nerve

The nerve was removed and frozen at -80°C until the time of testing. NGF levels were determined by a sandwich enzyme-linked immunosorbent assay, using monoclonal antibodies specific for each measurement (R&D Systems, Minneapolis, United States). Total protein was measured by Bradford's method using bovine serum albumin as a standard.

2.10. Statistical analysis

Data were expressed as mean \pm standard error of the mean. Nociception data were analyzed by generalized estimating equations followed by a Bonferroni test. The biochemical and open-field data of all groups were compared using a three-way analysis of variance (ANOVA)

followed by Student–Newman–Keuls (SNK) test or Fisher's least significant difference (LSD) test. A $p \leq 0.05$ indicated significance. SPSS, version 20.0, for Windows was used for all statistical analyses.

3. Results

3.1. Von Frey test showed that isoflurane potentiates analgesia induced by Ac and EA

Generalized estimating equations revealed a significant interaction between time and treatments ($\chi^2 = 1419.33$; 52; $p < 0.001$) (Fig. 3). At baseline, all groups had a pain threshold similar to that of the control group ($p \geq 0.05$). Fourteen days postoperatively, the pain threshold in pain groups was different from that in the control and sham groups, confirming the establishment of NP ($p < 0.001$). Immediately after the last treatment session (21 days after surgery), both treatments (Ac and EA) enhanced the mechanical pain threshold of animals exposed to the NP model (NpAc and NpEA groups), but these results did not differ from those of animals exposed to the NP model without treatment (Np and NpAn groups) ($p > 0.05$). Conversely, when all groups received isoflurane anesthesia, the increase in pain threshold was significantly different between the Np and NpAn groups ($p < 0.001$). This outcome was maintained 24 h and 48 h after the last treatment session (22 and 23 days after surgery).

3.2. Isoflurane decreased locomotor activity

No interactions among pain, treatments, and anesthesia (open field test data) were observed (three-way ANOVA/SNK, $p > 0.05$). However, there was an interaction between

anesthesia and treatments (three-way ANOVA/SNK, $F_{(2,125)} = 3.03$; $p = 0.052$). Significant effects of anesthesia were observed (ANOVA/SNK, $F_{(1,125)} = 13.92$; $p < 0.01$). Ac and EA induced an increase in locomotor activity, but anesthesia was able to reverse this effect (Fig. 4).

3.3. Isoflurane altered serum S100 β

The analyses showed interactions among pain, treatments, and anesthesia (three-way ANOVA/SNK, $F_{(2,68)} = 4.21$; $p < 0.05$), between pain and treatments (three-way ANOVA/SNK, $F_{(2,68)} = 6.57$; $p < 0.05$) and pain and anesthesia (three-way ANOVA/SNK, $F_{(2,68)} = 8.05$; $p < 0.05$). Results indicate that animals exposed to pain and treatment showed increased S100 β , while animals exposed to pain and anesthesia showed decreased S100 β . Significant effects of anesthesia (three-way ANOVA/SNK, $F_{(1,68)} = 34.96$; $p < 0.01$) and treatments (three-way ANOVA/SNK, $F_{(2,68)} = 6.158$; $p < 0.01$) were also observed, with decreased serum S100 β seen in anesthetized animals compared to those in nonanesthetized animals. EA treatment induced an increase in serum S100 β , while Ac treatment did not affect the S100 β (Fig. 5).

3.4. Isoflurane altered NGF levels in the affected nerve

The analyses showed interactions among pain, treatments, and anesthesia (three-way ANOVA/LSD, $F_{(2,116)} = 9.47$; $p < 0.05$). Significant independent effects of anesthesia (three-way ANOVA/LSD, $F_{(1,116)} = 30.33$; $p < 0.05$), pain (three-way ANOVA/LSD, $F_{(1,126)} = 101.15$; $p < 0.05$), and treatments (three-way ANOVA/LSD,

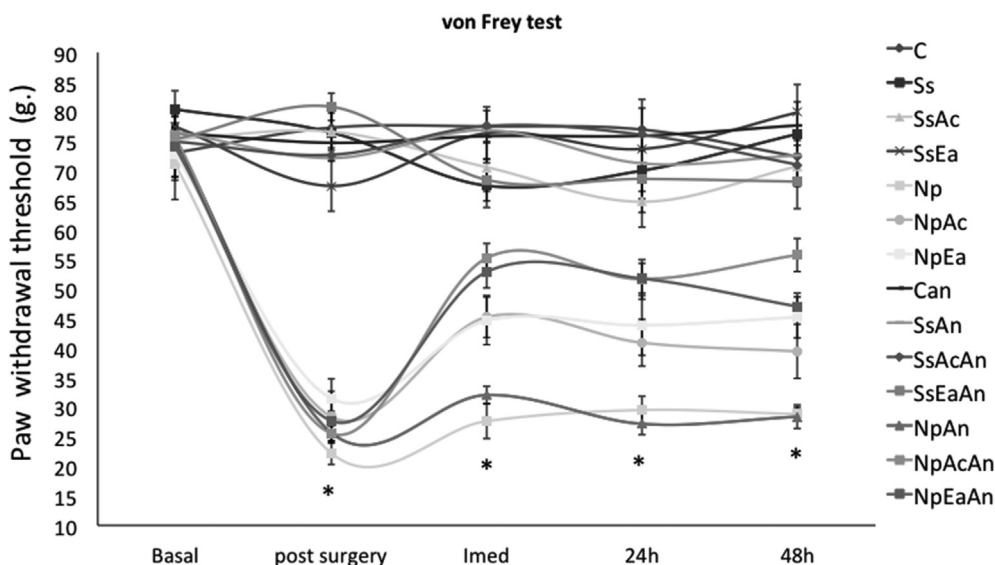


Figure 3 Von Frey test. Data are expressed as the mean \pm standard error of the mean (SEM) of pain threshold in grams. Generalized estimating equation (GEE) statistics showing interaction between time and treatments ($\chi^2 = 1419.33$; 52; $N = 8$ -10 animals per group). *Significantly different from control and sham groups. # Significantly different from Np and NpAn groups. Ac = acupuncture; EA = electroacupuncture; C = Control, Ss = sham surgery; SsAc = sham surgery + Ac; SsEA = sham surgery + EA; Np = neuropathic pain, NpAc = NP + Ac; NpEA = NP + EA, CAN = control + anesthesia; SsAn = sham surgery + anesthesia; SsAcAn = sham surgery + Ac + anesthesia; SsEaAn = sham surgery + EA + anesthesia, NpAn = NP + anesthesia; NpAcAn = NP + Ac + anesthesia; NpEaAn = NP + EA + anesthesia.

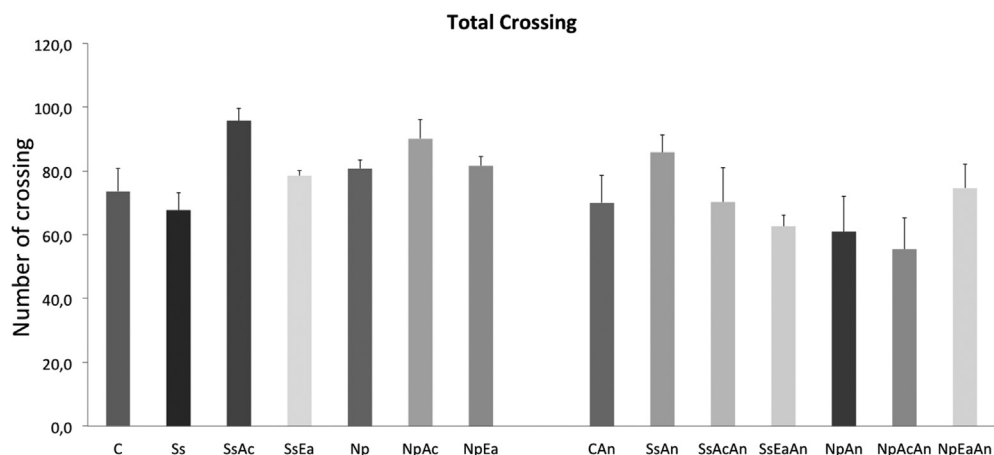


Figure 4 Open field test. Data are expressed as the mean \pm standard error of the mean (SEM) of total number of crossings. There was an interaction between anesthesia and treatments (three-way ANOVA/SNK, $F_{(2,165)} = 3.03$; $p = 0.05$), and a significant effect of anesthesia on treatments (ANOVA/SNK, $F_{(1,125)} = 13.92$; $p < 0.01$) ($N = 8-10$ animals per group).

Ac = acupuncture; EA = electroacupuncture; C = Control, Ss = sham surgery; SsAc = sham surgery + Ac; SsEA = sham surgery + EA; Np = neuropathic pain, NpAc = NP + Ac; NpEA = NP + EA, CAn = control + anesthesia; SsAn = sham surgery + anesthesia; SsAcAn = sham surgery + Ac + anesthesia; SsEaAn = sham surgery + EA + anesthesia, NpAn = NP + anesthesia; NpAcAn = NP + Ac + anesthesia; NpEaAn = NP + EA + anesthesia; ANOVA/SNK = analysis of variance/Student–Newman–Keuls test.

$F_{(2,116)} = 6.54$; $p < 0.05$) were observed. Animals receiving isoflurane had decreased NGF levels, similar to those found in animals in the pain model. Conversely, animals that received EA treatment had increased NGF levels in the affected nerve (three-way ANOVA/LSD, $p < 0.05$) (Fig. 6).

4. Discussion

We demonstrated that repeated exposure to isoflurane enhances the analgesic efficacy of Ac and EA, though

isoflurane alone did not have an analgesic effect. Use of needles in conscious rats may, however, complicate the interpretation of the results, since the needle may occasionally be erroneously inserted outside the acupoint in animals agitated during Ac. Additionally, needle insertion and electric stimulation are painful or uncomfortable stimuli [20], which could interfere with the analgesic response to Ac or EA. It is well established that Ac has segmental, extrasegmental, and central effects [21]. Therefore, a single Ac application may be an acute stressor leading to an analgesic response, though when applied

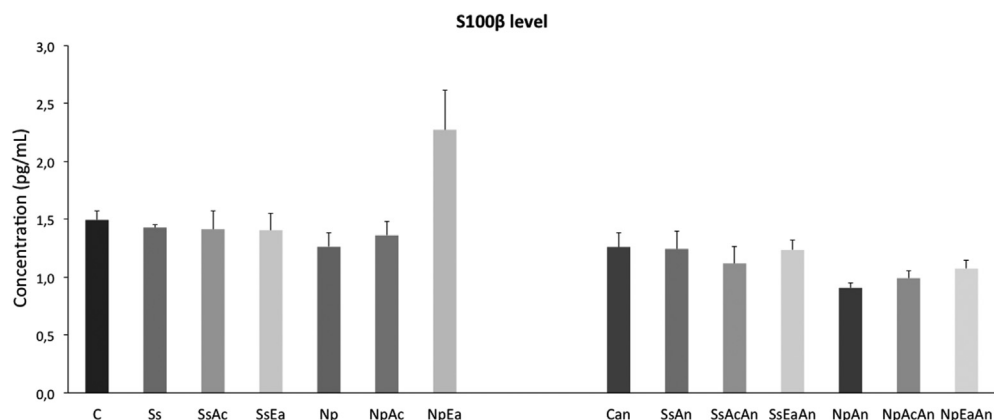


Figure 5 S100 β levels in blood serum. Data are expressed as the mean \pm standard error of the mean (SEM) of serum S100 β levels in pg/mL. There was an interaction among pain, treatments, and anesthesia (three-way ANOVA/SNK, $F_{(2,68)} = 4.21$; $p < 0.05$), between pain and treatments (three-way ANOVA/SNK, $F_{(2,68)} = 6.57$; $p < 0.05$), and pain and anesthesia (three-way ANOVA/SNK, $F_{(2,68)} = 8.05$; $p < 0.05$) ($N = 5-7$ animals per group).

Ac = acupuncture; EA = electroacupuncture; C = Control, Ss = sham surgery; SsAc = sham surgery + Ac; SsEA = sham surgery + EA; Np = neuropathic pain, NpAc = NP + Ac; NpEA = NP + EA, CAn = control + anesthesia; SsAn = sham surgery + anesthesia; SsAcAn = sham surgery + Ac + anesthesia; SsEaAn = sham surgery + EA + anesthesia, NpAn = NP + anesthesia; NpAcAn = NP + Ac + anesthesia; NpEaAn = NP + EA + anesthesia; ANOVA/SNK = analysis of variance/Student–Newman–Keuls test.

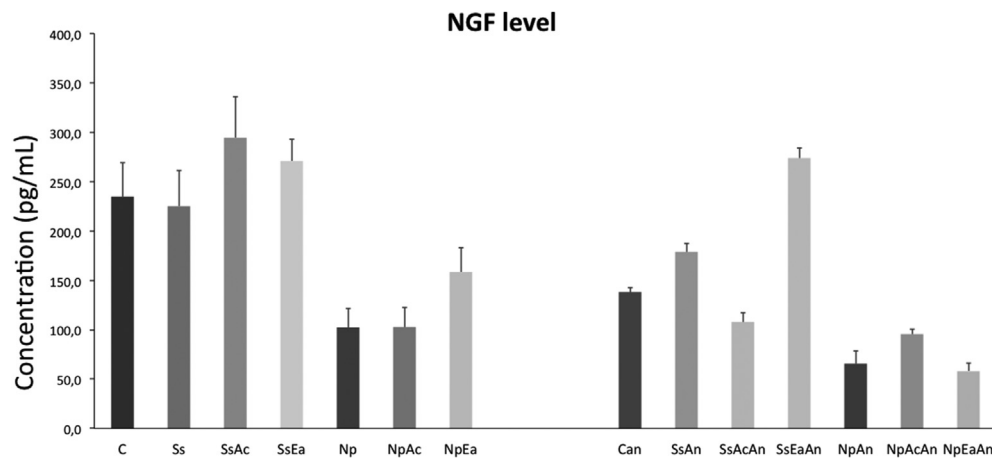


Figure 6 Nerve growth factor (NGF) levels in the left sciatic nerve. Data are expressed as the mean \pm standard error of the mean (SEM) of left sciatic nerve growth factor (NGF) levels in pg/mL. Interactions among pain, treatments, and anesthesia (three-way ANOVA/LSD, $F_{(2,116)} = 9.47$; $p < 0.05$) were apparent. There were significant independent effects of anesthesia (three-way ANOVA/LSD, $F_{(1,116)} = 30.33$; $p < 0.05$), pain (three-way ANOVA/LSD, $F_{(1,126)} = 101.15$; $p < 0.05$), and treatments (three-way ANOVA/LSD, $F_{(2,116)} = 6.54$; $p < 0.05$) ($N = 5$ -7 animals per group).

Ac = acupuncture; EA = electroacupuncture; C = Control, Ss = sham surgery; SsAc = sham surgery + Ac; SsEA = sham surgery + EA; Np = neuropathic pain, NpAc = NP + Ac; NpEA = NP + EA, CAN = control + anesthesia; SsAn = sham surgery + anesthesia; SsAcAn = sham surgery + Ac + anesthesia; SsEaAn = sham surgery + EA + anesthesia, NpAn = NP + anesthesia; NpAcAn = NP + Ac + anesthesia; NpEaAn = NP + EA + anesthesia; ANOVA/LSD = analysis of variance/Fisher's least significant difference test.

several times, Ac stimuli may act as chronic stressors [22], potentially altering the analgesic effect of Ac or EA.

Acute stress reduces pain sensitivity (stress-induced analgesia), which is probably mediated by brainstem modulation. In contrast, chronic stress increases pain sensitivity, inducing hyperalgesia [12,23] and allodynia [12,23]. This relationship between stress and pain sensitivity probably occurs because of activation of the hypothalamic–pituitary–adrenal axis, leading to glucocorticoid release, which can alter the serotonergic and dopaminergic systems involved in the nociceptive response [24]. Previous data from our group suggest the involvement of the opioid system in the hyperalgesic response induced by repeated restraint stress [12]. It is known that the Ac and EA analgesia pathways are also related to the opioid system [25]. Therefore, chronic stress caused by needle insertion and electric current may lead to decreased analgesic efficacy of Ac and EA in awake rats, since they are most likely to have reverse actions in the opioid system.

The effects of inhaled anesthetics have been previously evaluated in Ac studies with rats [20,26]. Wen et al. [20] evaluated the role of reducing EA-induced stress and found that 0.5% halothane, at a sub-anesthetic minimum alveolar concentration (sub-MAC dose), reduces the influence of stress during EA, allowing strong needle stimulation and rapid recovery of the animal. Kung et al. [26] assessed the use of isoflurane and showed that 0.75% isoflurane (sub-MAC dose) is also an effective anesthetic dose for EA studies. However, an important issue regarding the use of anesthetics in animal models is motor impairment resulting from anesthetic effects. Antognini et al. [27] showed that the MAC of isoflurane (0.6–0.9%) is able to preserve the integrity of the motor system in rats receiving this anesthetic. Silva et al. 2010 [28], in a study evaluating the effects of EA on the role of the anterior pretectal nucleus,

used isoflurane at the same concentrations used in the present study for induction and maintenance of anesthesia (2% and 0.5% respectively) and, likewise, found no motor impairment in the animals.

Inhaled anesthetics hyperpolarize neuronal membranes and inhibit C-fiber latency, reducing neuronal excitability. However, isoflurane at sub-MAC doses does not affect wind-up phenomenon and thus is a candidate anesthetic for evaluating antinociceptive effects of Ac and EA [20,29]. Moreover, isoflurane alone at sub-MAC doses does not influence motor or nociceptive responses. It is important to highlight that the present study behavioral tests were performed in awake animals that had completely recovered from anesthesia.

In the open field test, all animals were able to move, indicating that they were not sedated. However, there was a significant effect of anesthesia on treatment, i.e., Ac induced an increase in locomotor activity, and anesthesia was able to reverse this effect. We observed that the anesthetic effect on locomotion depended on the state of the animal. Isoflurane led to decreased locomotion in animals with increased locomotion, while in sham animals, isoflurane had no significant effect on locomotion. It is possible that since isoflurane was administered for six consecutive days before the test; this effect was due to repeated exposure to the drug. Literature evidence suggests that isoflurane decreases locomotion and neuronal excitability by inhibiting glutamate release [30]. It is known that N-methyl-D-aspartate receptor, a glutamate receptor, is related to locomotor activity [31] and nociception response [32]. We may also speculate that isoflurane exerts a protective effect in animals submitted to the NP model. Ac-induced increase in locomotion in the Ss and Np groups is another interesting finding of this study. Isoflurane administration reversed this effect, indicating

an interaction between isoflurane and Ac. Manual Ac has been shown to increase dopamine release, improving locomotor function in mice [33]. Dopamine plays an important role in peripheral and central neurons, including those in the substantia nigra, hypothalamus, midbrain, and the ventral tegmental area [34]. In the present study, rats in the NpEaAn group showed greater locomotor activity than those in the NpAn and NP + Ac + anesthesia groups. This improvement may be due to stimulation that reached the motor threshold, maintaining skeletal muscle integrity in the NP model and improving locomotion. Another hypothesis is related to the increase of monoamines in the brain, which has been shown to block anesthetic action and increase locomotor activity in rabbits [35]. However, further studies are necessary to clarify Ac-induced locomotion.

Another important finding was the association between serum S100 β and pain, treatments, and anesthesia. It is known that chronic pain, such as fibromyalgia, correlates with higher serum S100 β and BDNF levels [36], but our results were not in agreement. Some studies suggest that rats exposed to isoflurane during postnatal brain development have increased serum S100 β [8], which could be related to brain damage. Nevertheless, according to the present results, isoflurane prevented an increase in S100 β in adult male rats with NP treated with EA. Although Ac and EA are known to reduce S100 β after neural injuries [37], our results showed the opposite effect. Since the animals underwent treatment while awake, the treatment may have mimicked a chronic stress condition, leading to increased S100 β , which would be consistent with literature data [38]. Considering that the NpEA group had the highest serum S100 β , our data suggest that animals submitted to NP and EA without isoflurane show increased stress levels due to NP induction and electric stimulation while awake. The NpAc and Np groups showed different results, indicating that electric stimulation has effects distinct from those of manual stimulation. The NP + EA + anesthesia group had S100 β levels equal to those of the control group, indicating a protective effect of isoflurane anesthesia possibly by maintaining serum S100 β similar to that of controls. Similarly, Garcia-Sanchez et al. (1993) [39] showed that isoflurane anesthesia increases plasma beta-endorphin after surgery, supporting the present study in that it suggests a protective effect of isoflurane against stress.

Isoflurane administration, NP, and Ac and EA treatments also altered NGF levels in the left sciatic nerve. When the control and control + anesthesia groups were compared, isoflurane clearly decreased NGF levels in the left sciatic nerve, as did NP, suggesting that isoflurane modifies the effect of Ac and EA in the left sciatic nerve (injured nerve). Although the groups receiving Ac or EA showed an increase in NGF levels, *post hoc* tests showed that this effect was linked to EA treatment, but not to Ac treatment. Despite extensive literature on the topic, there is still no consensus on the role of NGF in NP. It has been suggested that NGF, a neurotrophin involved in the growth, maintenance, and apoptosis of neurons [40], is decreased in sensory neurons of the dorsal root ganglion and of the spinal dorsal horn in diabetic NP models [41]. In contrast, Watson et al. (2008) [42] found increased NGF expression in an NP model [43].

This finding suggests that, after nerve injury, many substances, including NGF, are released mainly by the astrocytes of the central nervous system. In the dorsal horn of the spinal cord, increased NGF levels are associated with central sensitization [44], leading to NP. Similarly, increased NGF levels in the CNS [45] have been associated with an increase in pain. However, some studies suggest that the increase in NGF levels in the peripheral nervous system is associated with NP [46]. In contrast, another study suggests that neural mobilization increases the level of NGF in injured nerves, promoting nerve regeneration and reducing painful symptoms [47].

In the present study, treatment with EA induced an increase in NGF levels although a previous study showed that Ac and EA in NP decreased NGF levels, which was associated with decreased hyperalgesia [48]. Nevertheless, studies have shown a 52% increase in NGF levels after CCI and neural mobilization in rats, indicating that NGF contributes to regeneration of the sciatic nerve after CCI [47]. Considering this later study, the data from the present study suggest that the decrease in NGF levels in the injured nerve after NP may be due to reduced nerve regeneration involving pain. Although isoflurane also reduces pain, EA may have promoted an increase in the levels of this neurotrophin.

In conclusion, our data show that isoflurane administered during Ac or EA treatment in an animal model of NP decreased allodynia as determined by the von Frey test. Similarly, isoflurane prevented the increase in serum S100 β in rats with NP treated with EA while awake and, most likely, reduced the harmful effects of chronic stress exposure. Inhaled anesthesia decreased NGF levels in the left sciatic nerve, while EA increased the levels of this neurotrophin. These results suggest that these effects are related to nerve regeneration rather than to increased analgesia.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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References

- [1] Wang SM, Kain ZN, White PF. Acupuncture analgesia: II. Clinical considerations. *Anesth Analg* 2008;106:611–21.

- [2] Norrbrink C, Lundeberg T. Acupuncture and massage therapy for neuropathic pain following spinal cord injury: an exploratory study. *Acupunct Med* 2011;29:108–15.
- [3] Tu WZ, Cheng RD, Cheng B, Lu J, Cao F, Lin HY, et al. Analgesic effect of electroacupuncture on chronic neuropathic pain mediated by P2X3 receptors in rat dorsal root ganglion neurons. *Neurochem Int* 2012;60:379–86.
- [4] Cha MH, Nam TS, Kwak Y, Lee H, Lee BH. Changes in cytokine expression after electroacupuncture in neuropathic rats. *Evid Based Complement Alternat Med* 2012;2012:792765.
- [5] Park HJ, Kim HY, Hahm DH, Lee H, Kim KS, Shim I, et al. Electroacupuncture to ST36 ameliorates behavioral and biochemical responses to restraint stress in rats. *Neurol Res* 2010;32:111–5.
- [6] de Medeiros MA, Canteras NS, Suchecki D, Mello LE. Analgesia and c-Fos expression in the periaqueductal gray induced by electroacupuncture at the Zusanli point in rats. *Brain Res* 2003;973:196–204.
- [7] Purdon PL, Sampson A, Pavone KJ, Brown EN. Clinical electroencephalography for anesthesiologists: Part I: Background and basic signatures. *Anesthesiology* 2015;123:937–60.
- [8] Liang G, Ward C, Peng J, Zhao Y, Huang B, Wei H. Isoflurane causes greater neurodegeneration than an equivalent exposure of sevoflurane in the developing brain of neonatal mice. *Anesthesiology* 2010;112:1325–34.
- [9] Siuciak JA, Altar CA, Wiegand SJ, Lindsay RM. Antinociceptive effect of brain-derived neurotrophic factor and neurotrophin-3. *Brain Res* 1994;633:326–30.
- [10] Chen J, Qi JG, Zhang W, Zhou X, Meng QS, Zhang WM, et al. Electro-acupuncture induced NGF, BDNF and NT-3 expression in spared L6 dorsal root ganglion in cats subjected to removal of adjacent ganglia. *Neurosci Res* 2007;59:399–405.
- [11] Medeiros LF, Rozisky JR, de Souza A, Hidalgo MP, Netto CA, Caumo W, et al. Lifetime behavioural changes after exposure to anaesthetics in infant rats. *Behav Brain Res* 2011;218:51–6.
- [12] Spezia Adachi LN, Caumo W, Laste G, Fernandes Medeiros L, Ripoll Rozisky J, de Souza A, et al. Reversal of chronic stress-induced pain by transcranial direct current stimulation (tDCS) in an animal model. *Brain Res* 2012;1489:17–26.
- [13] Scarabelot VL, Medeiros LF, de Oliveira C, Adachi LN, de Macedo IC, Cioato SG, et al. Melatonin alters the mechanical and thermal hyperalgesia induced by orofacial pain model in rats. *Inflammation* 2016;39:1649–59.
- [14] Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *J Pharmacol Pharmacother* 2010;1:94–9.
- [15] Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988;33:87–107.
- [16] Fruhstorfer H, Gross W, Selbmann O. von Frey hairs: new materials for a new design. *Eur J Pain* 2001;5(3):341–2.
- [17] Filho PR, Vercelino R, Cioato SG, Medeiros LF, de Oliveira C, Scarabelot VL, et al. Transcranial direct current stimulation (tDCS) reverts behavioral alterations and brainstem BDNF level increase induced by neuropathic pain model: Long-lasting effect. *Prog Neuropsychopharmacol Biol Psychiatry* 2016;64:44–51.
- [18] Medeiros LF, de Souza AC, de Souza A, Cioato SG, Scarabelot VL, Caumo W, et al. Fentanyl administration in infant rats produces long-term behavioral responses. *Int J Dev Neurosci* 2012;30:25–30.
- [19] Roesler R, Walz R, Quevedo J, de-Paris F, Zanata SM, Graner E, Izquierdo I, et al. Normal inhibitory avoidance learning and anxiety, but increased locomotor activity in mice devoid of PrP(C). *Brain Res Mol Brain Res* 1999;71:349–53.
- [20] Wen YR, Yeh GC, Shyu BC, Ling QD, Wang KC, Chen TL, et al. A minimal stress model for the assessment of electroacupuncture analgesia in rats under halothane. *Eur J Pain* 2007;11:733–42.
- [21] Zhao ZQ. Neural mechanism underlying acupuncture analgesia. *Prog Neurobiol* 2008;85:355–75.
- [22] Torres IL, Gamaro GD, Silveira-Cucco SN, Michalowski MB, Corrêa JB, Perry ML, et al. Effect of acute and repeated restraint stress on glucose oxidation to CO₂ in hippocampal and cerebral cortex slices. *Braz J Med Biol Res* 2001;34:111–6.
- [23] Bardin L, Malfetes N, Newman-Tancredi A, Depoortère R. Chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rat: Relevance to human stress-associated painful pathologies. *Behav Brain Res* 2009;205:360–6.
- [24] Martenson ME, Cetas JS, Heinricher MM. A possible neural basis for stress-induced hyperalgesia. *Pain* 2009;142:236–44.
- [25] Wang Y, Hackel D, Peng F, Rittner HL. Long-term antinociception by electroacupuncture is mediated via peripheral opioid receptors in free-moving rats with inflammatory hyperalgesia. *Eur J Pain* 2013;17:1447–57.
- [26] Kung HH, Hsu SF, Hung YC, Chen KB, Chen JY, Wen YR, et al. Electroacupuncture analgesia, stress responses, and variations in sensitivity in rats anesthetized with different sub-MAC anesthetics. *Eur J Pain* 2011;15:600–7.
- [27] Antognini JF, Carstens E. Increasing isoflurane from 0.9 to 1.1 minimum alveolar concentration minimally affects dorsal horn cell responses to noxious stimulation. *Anesthesiology* 1999;90:208–14.
- [28] Silva ML, Silva JR, Prado WA. The integrity of the anterior pretectal nucleus and dorsolateral funiculus is necessary for electroacupuncture-induced analgesia in the rat tail-flick test. *Eur J Pain* 2010;14:249–54.
- [29] Cuellar JM, Dutton RC, Antognini JF, Carstens E. Differential effects of halothane and isoflurane on lumbar dorsal horn neuronal windup and excitability. *Br J Anaesth* 2005;94:617–25.
- [30] Sandstrom DJ. Isoflurane depresses glutamate release by reducing neuronal excitability at the Drosophila neuromuscular junction. *J Physiol* 2004;558:489–502.
- [31] Acton D, Miles GB. Differential regulation of NMDA receptors by D-serine and glycine in mammalian spinal locomotor networks. *J Neurophysiol* 2017;117:1877–93.
- [32] Saminen VK, Grajales-Reyes JG, Copits BA, O'Brien DE, Trigg SL, Gomez AM, et al. Divergent modulation of nociception by glutamatergic and GABAergic neuronal subpopulations in the periaqueductal gray. *eNeuro* 2017;4:1–13.
- [33] Kim SN, Doo AR, Park JY, Bae H, Chae Y, Shim I, et al. Acupuncture enhances the synaptic dopamine availability to improve motor function in a mouse model of Parkinson's disease. *PLoS One* 2011;6:e27566.
- [34] Dominguez-Mejide A, Rodriguez-Perez AI, Diaz-Ruiz C, Guerra MJ, Labandeira-Garcia JL. Dopamine modulates astroglial and microglial activity via glial renin-angiotensin system in cultures. *Brain Behav Immun* 2017;62:277–90.
- [35] Chang CL, Lee JC, Tseng CC, Chang YH, Cheng JT. Decrease of anesthetics activity by electroacupuncture on Jen-Chung point in rabbits. *Neurosci Lett* 1995;202:93–6.
- [36] Zanette SA, Dussan-Sarria JA, Souza A, Deitos A, Torres IL, Caumo W. Higher serum S100B and BDNF levels are correlated with a lower pressure-pain threshold in fibromyalgia. *Mol Pain* 2014;10:46.
- [37] Zhang H, Kang T, Li L, Zhang J. Electroacupuncture reduces hemiplegia following acute middle cerebral artery infarction with alteration of serum NSE, S-100B and endothelin. *Curr Neurovasc Res* 2013;10:216–21.
- [38] Veena J, Srikumar BN, Raju TR, Shankaranarayana Rao BS. Exposure to enriched environment restores the survival and differentiation of new born cells in the hippocampus and

- ameliorates depressive symptoms in chronically stressed rats. *Neurosci Lett* 2009;455:178–82.
- [39] Garcia-Sanchez MJ, Polo A, Peran F. Effects of halothane and isoflurane on beta-endorphin release in children. *Anaesthesia* 1993;48:38–40.
- [40] Ceni C, Unsain N, Zeinieh MP, Barker PA. Neurotrophins in the regulation of cellular survival and death. *Handb Exp Pharmacol* 2014;220:193–221.
- [41] Aloe L, Manni L, Properzi F, De Santis S, Fiore M. Evidence that nerve growth factor promotes the recovery of peripheral neuropathy induced in mice by cisplatin: behavioral, structural and biochemical analysis. *Auton Neurosci* 2000;86:84–93.
- [42] Watson JJ, Allen SJ, Dawbarn D. Targeting nerve growth factor in pain: what is the therapeutic potential? *BioDrugs* 2008;22:349–59.
- [43] Campbell JN, Raja SN, Meyer RA, Mackinnon SE. Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain* 1988;32:89–94.
- [44] Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science* 2000;288:1765–9.
- [45] Zhao B, Pan Y, Wang Z, Tan Y, Song X. Intrathecal administration of tempol reduces chronic constriction injury-induced neuropathic pain in rats by increasing SOD activity and inhibiting NGF. Expression. *Cell Mol Neurobiol* 2016;36:893–906.
- [46] Chiang CY, Sheu ML, Cheng FC, Chen CJ, Su HL, Sheehan J, et al. Comprehensive analysis of neurobehavior associated with histomorphological alterations in a chronic constrictive nerve injury model through use of the CatWalk XT system. *J Neurosurg* 2014;120:250–62.
- [47] da Silva JT, Santos FM, Giardini AC, Martins Dde O, de Oliveira ME, Ciena AP, et al. Neural mobilization promotes nerve regeneration by nerve growth factor and myelin protein zero increased after sciatic nerve injury. *Growth Factors* 2015;33:8–13.
- [48] Aloe L, Manni L. Low-frequency electro-acupuncture reduces the nociceptive response and the pain mediator enhancement induced by nerve growth factor. *Neurosci Lett* 2009;449:173–7.