Journal of Acupuncture and Meridian Studies 2021;14(4):167-172
pISSN 2005-2901 • eISSN 2093-8152
https://doi.org/10.51507/j.jams.2021.14.4.167
RESEARCH ARTICLE

# Age Differences in Naloxone Reversibility of Electroacupuncture on the Jaw Opening Reflex in Rats

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**Received** April 5, 2021
**Revised** May 11, 2021
**Accepted** June 29, 2021

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**Background:** Electroacupuncture is one of the most popular physical treatments for clinical pain, but the potential influence of a patient's age on the effectiveness of electroacupuncture treatment has not been clearly established.
**Objectives**: The present study aimed to detect a potential difference in electroacupuncture-induced analgesia between juvenile and adult rats.
**Methods:** In this study, we investigated the effects of electroacupuncture treatment on the nociceptive jaw-opening reflex evoked by tooth-pulp stimulation in juvenile and adult rats.
**Results:** Our results showed there were age differences in electroacupuncture-induced analgesic effects in rats, especially with naloxone antagonization. The ratio of naloxone-reversibility against electroacupuncture analgesia was greater in adult rats than in juvenile rats.
**Conclusion:** These results suggest that electroacupuncture analgesia is produced mainly by the non-opioid system in juvenile rats and by the opioid system in adult rats.

**Keywords:** Jaw-opening reflex, Tooth pulp, Electroacupuncture, Age, Naloxone, Rat

## INTRODUCTION

Acupuncture is often used for the treatment of various types of pain, such as headaches, temporomandibular joint dysfunction, dental pain, postoperative pain, fibromyalgia, low-back pain, menstrual cramps, myofascial pain, and osteoarthritis [1,2]. In animal studies, it is generally accepted that acupuncture stimulation can suppress nociceptive reflexes, such as the tail-flick reflex, paw-withdrawal reflex, and writhing reflex in cats, dogs, and rats [3,4]. However, pain is an extremely subjective experience, which can vary in relation to age, sex, and environment-related effects [5,6]. In addition, these effects interact with each other through unknown, but likely complex, mechanisms.

Clinical studies show that children experience pain differently from adults [7]. However, animal studies in rats and mice using reflex-based assessment strategies showed inconsistent results in relation to pain sensitivity and age. For example, Hess et al. reported that thermal and electrical sensitivities were decreased with increasing age using paw-lick and tail-flick latencies [8,9]. Contrary to these results, Chan and Lai reported a decrease in response latencies using the hot-plate test for rats aged 1.6 to 11.2 months old [10]. Similarly, conclusions from various studies addressing age-related changes in pain sensitivity showed decreased sensitivity, increased sensitivity, or no change in relation to age [11]. Possible reasons for these inconsistent results include different species, testing methods, sexes, and environmental conditions. Although there have been several studies on pain sensitivity in relation to age, few of these have investigated the endogenous analgesic systems. In stress-induced analgesia, Washington et al. [12] reported an age-related decline in the endogenous pain inhibitory system. The endogenous analgesic system can be activated by a variety of conditioning stimuli; tested and proved examples include foot-shock, cold-water swimming, and food deprivation [13,14]. No studies have examined whether acupuncture, a traditional method used against various painful conditions, is subject to age-related effects. A critical mechanism involved in acupuncture treatment is the activation of a descending endogenous analgesic action through the periaqueductal gray matter to produce an important analgesic effect [15-18].

In the present study, the jaw-opening reflex (JOR) evoked by tooth-pulp stimulation was used as a nociceptive reflex

[19-21]. The magnitude of the JOR in juvenile and adult rats after acupuncture-like conditioning was studied in order to reveal the potential role of age in acupuncture mechanisms.

## MATERIALS AND METHODS

### 1. General

The methods described here follow the ethical guidelines of Nagasaki University and received approval by the Animal Welfare Committee of Nagasaki University.

The experiments were performed using 40 male Wistar albino rats, which were lightly anesthetized with intraperitoneal injections of thiamylal sodium (Isozol™, Nippon Iko, Toyama, Japan) at an initial dose of 80 mg/kg. Juvenile rats were aged 3 weeks old with a body weight of 46 ± 8.3 g (mean ± standard error [SE], n = 20 total) and adult rats were 12 weeks of age with a body weight of 227 ± 5.4 g (mean ± SE, n = 20). Juvenile and adult rats were then divided into Control (saline) and Naloxone groups (n = 10, each). Respiratory conditions (unassisted), blood pressure, and rectal temperature were continuously monitored and maintained within normal physiological levels throughout the recording session. The rectal temperature of the animals was maintained at 37 ± 0.2℃ with a heating pad.

### 2. Test stimulation

A pair of Teflon-coated stainless steel wire electrodes (interpolar distance of 2.0 mm) of 0.1 mm diameter, insulated except for the tips, was inserted into the lower incisor at a depth of 20 mm from the tip as reported previously [22]. Test stimulation of the pulp nerve for estimating the threshold value of the JOR was applied electrically with constant current rectangular pulses of 0.1 ms in duration. The JOR was monitored by a digastric electromyogram, which was recorded from the anterior belly of the digastric muscle amplified by a differential amplifier (DAM-5A, Sarasota, USA, Bandpass filter: 10 Hz, 10 KHz) with a gain of 1000 times and displayed on an oscillograph (Nihon Kohden, Tokyo, Japan). Our previous study indicated that a minimum electromyographic response very near the threshold was evoked at a latency of about 5-6 ms. In the present study, the intensity that evoked the minimum apparent response with a latency of about 6 ms was determined as the threshold current (Fig. 1).

### 3. Acupuncture stimulation

Cathodal electroacupuncture stimulation was delivered to bilateral Ho-Ku (Large Intestine 4) points by inserting stainless steel needles (0.2 mm in diameter) to a depth of 1.0 mm in juvenile rats and of 2.0 mm in adult rats. An anodal silver-plated electrode (10 mm × 10 mm for juveniles, 30 mm × 30 mm for adults) was placed on the center of the abdomen. Rectangular constant current pulses of 0.1 ms in duration were delivered at 45 Hz for 15 min. The intensity of electroacupuncture stimulation was determined as that which evoked moderate muscle contractions very near to the Ho-ku point. These intensities (ranging from 40 to 70 μA) were about 5 times the threshold required to evoked a minimum twitch observed in the forepad. These parameters were chosen based on previous studies where electroacupuncture analgesia was achieved successfully in rats [4,16,17,21].

### 4. Naloxone effects

In order to confirm whether endogenous pain inhibitory mechanisms were involved in the electroacupuncture effects, naloxone-HCl (Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally at a dose of 0.1 mg/kg. Naloxone was prepared at 0.1 mg/ml and injected at a dose of 1 ml/kg. For the controls, another group of rats received the same volume of saline at 1 ml/kg. Before and 5 min after naloxone or saline injection, threshold values were measured (Fig. 2, 0 min), and immediately afterward electroacupuncture stimulation was started (Fig. 2, under bars).

### 5. Statistical analysis

Group differences in the JOR threshold were analyzed by

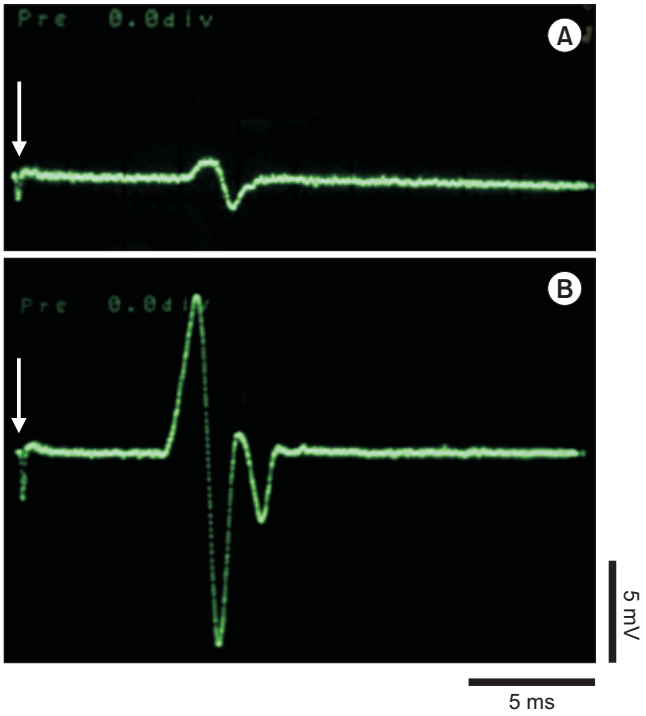


**Fig. 1.** Oscilloscope photos of jaw opening reflex (JOR) in adult rats. (A) Threshold intensity (101.0 μA). (B) 2.5 times threshold intensity (252.5 μA). Downward arrows indicate stimulus artifacts.

ANOVA followed by Fischer's PLSD for post-hoc pairwise comparisons. Repeated measures ANOVA was used to analyze differences in the time course of changes in the JOR threshold, followed by post-hoc Fischer's PLSD for pairwise comparisons between the saline and naloxone groups. JOR suppression at each time point was analyzed by a one-sample t-test with a hypothesized mean of 100 (i.e., 100% of the baseline) with Bonferroni correction for multiple comparisons. In all tests, the level of significance was set at the 5% level. Data are presented as the mean ± standard deviation.

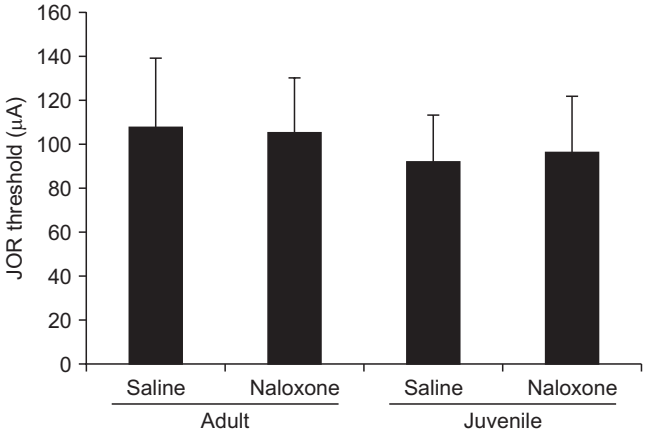


**Fig. 2.** Threshold intensity for JOR before electroacupuncture treatment. 1: Adult saline, 2: adult naloxone, 3: juvenile saline, and 4: juvenile naloxone. No significant differences were found between groups ($p > 0.05$ in ANOVA). Error bars indicate standard deviation.

## RESULTS

In adult rats, before the injection of saline or naloxone, the threshold values of current intensities for evoking JOR were 107 ± 10.0 μA (saline, n = 10) and 105.4 + 7.8 μA (naloxone, n = 10), respectively. In juvenile rats, these values were 91.7 ± 6.8 μA (saline, n = 10) and 95.8 ± 8.3 μA (naloxone, n = 10). There were no significant differences between the saline and naloxone groups (Fig. 2, ANOVA test).

Electroacupuncture treatment increased the threshold value for JOR in adult rats compared with baseline (Fig. 3A). Acupuncture effects gradually increased and reached a maximum just before the cessation of the 15-min electroacupuncture stimulation. After cessation of the electroacupuncture stimulation, the analgesic effect remained for 20-60 min. Naloxone clearly antagonized the analgesic effects in adult rats. In juvenile rats, the onset of the analgesic effect was slightly delayed (about five minutes) and the peak of the

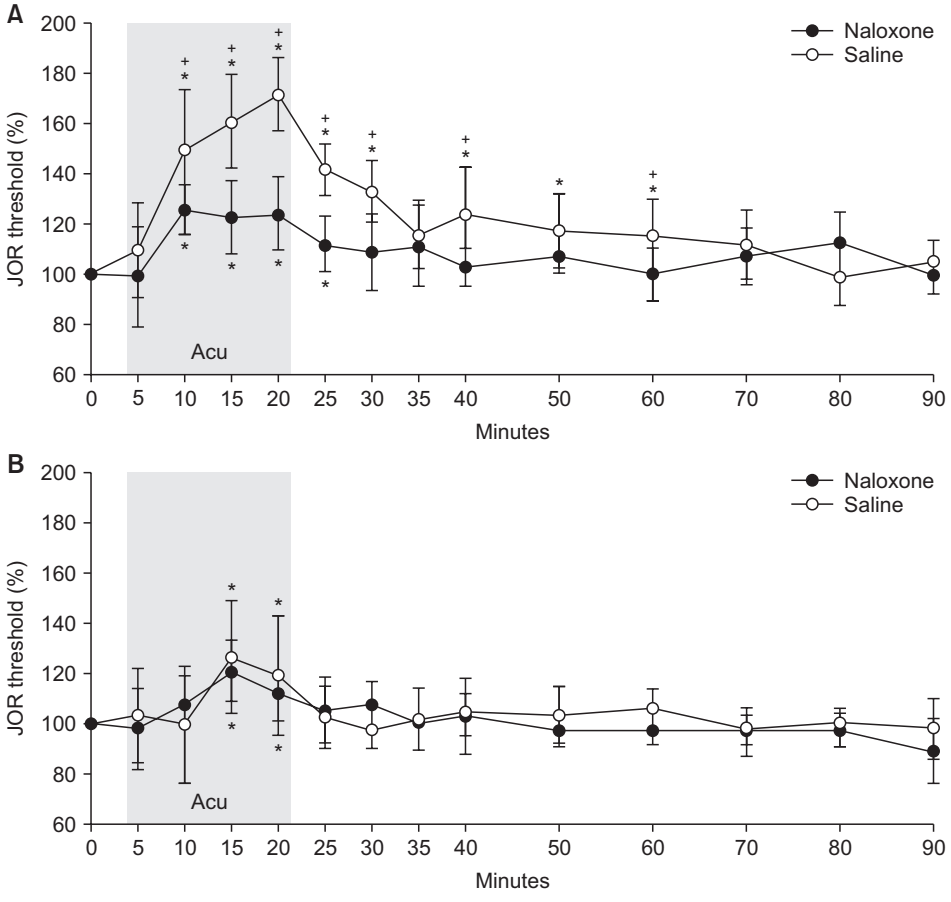


**Fig. 3.** Time course of electroacupuncture effects. (A) Adult rats. (B) Juvenile rats. Vertical axis indicates the threshold intensity for JOR normalized to a percentage of the initial value. Saline (white circles) or naloxone (black circles) was injected systemically after the initial JOR measurement (time zero min) and before electroacupuncture treatment (grayed area). Naloxone pretreatment blunted the response to electroacupuncture in adult rats, but not juvenile rats. Asterisks denote a significant difference from baseline at each time point ($p < 0.012$ in the one-sample t-test with a hypothesized mean of 100%). Plus signs denote a significant difference between the saline and naloxone groups ($p < 0.05$ by ANOVA, followed by Fischer's PLSD test). Error bars indicate standard deviation.

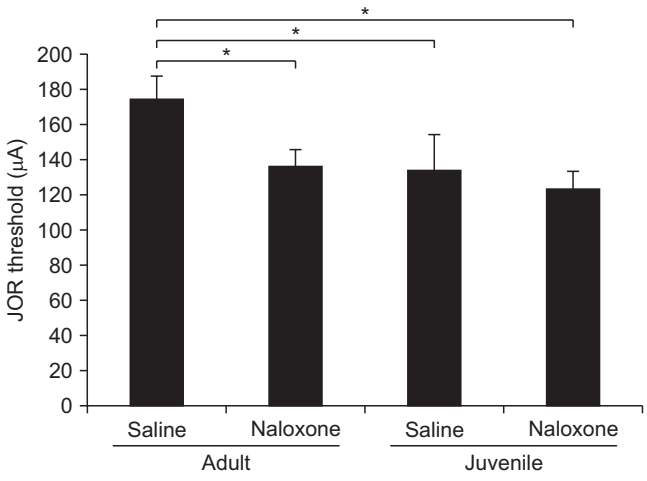


**Fig. 4.** Maximum effects on the JOR during and after electroacupuncture. The threshold value for evoking the JOR was significantly greater in adult rats without naloxone pre-treatment compared with all other groups. Asterisks denote $p < 0.05$ by ANOVA, followed by Fisher's PLSD post hoc test. Error bars indicate standard deviation.

effect was weaker when compared with adult rats (Fig. 3B). However, 1) the magnitude of the analgesic effect was smaller, 2) the peak of the analgesic effect was reached earlier, and 3) naloxone reversibility was not significant in juvenile rats when compared with adult rats.

Fig. 4 shows the maximum changes in the JOR magnitude during and up to 90 minutes after electroacupuncture stimulation. As is clearly seen, naloxone reversibility was observed in adult, but not juvenile, rats. Maximum effects in adult rats were about 2.3 times stronger than those in juvenile rats.

## DISCUSSION

The present study showed that there are age differences in electroacupuncture-induced analgesic effects in rats, especially for naloxone antagonization. The ratio of naloxone reversibility against electroacupuncture analgesia was larger in adult rats than in juvenile rats, suggesting that the non-opioid analgesic system may have a predominant role in juvenile rats.

The threshold values were not significantly different between adult and juvenile rats, indicating that nociceptive responses were similarly evoked in both age groups. However, despite no significant differences, the value of the threshold for JOR was slightly lower in juvenile rats than in adult rats. This may be due to peripheral and central factors. For example, Hess et al. [8] reported a decrease in thermal and electrical shock sensitivity with increasing age that correlated with a decrease in the number of opiate receptors in the central nervous system. In mice, it was reported that decreased sensitivity to thermal stimuli was due to a decrease in the expression of nerve growth factor receptor and the TRPV1 ion channel [23].

Tooth pulp stimulation is often used for the study of pain or analgesic mechanisms [24-26] because the tooth pulp is mainly innervated by small diameter nerve fibers. Furthermore, pain is almost the only sensation evoked when tooth pulp is stimulated by mechanical, heat, cold, or chemical stimuli [24,27]. We used electrical tooth-pulp stimulation in this study, which is a typical nociceptive reflex in the trigeminal area. However, the threshold for JOR was similar in both age groups, suggesting that dental innervation in the tooth pulp was already mature at 3 weeks of age, as shown in previous studies [28-30].

Multiple neuronal mechanisms involved in acupuncture analgesia can be largely grouped into opioid and non-opioid systems [31-33]. Although endogenous opioids appear to be key mediators in acupuncture analgesia or other stimulation-induced analgesia, more recent studies now support the existence of mediators that do not depend on the opioid system [34-36]. Our results in juvenile rats, which showed a lack of naloxone reversibility and faster peak time than in adult rats, indicate the predominant involvement of the non-opioid system. Indeed, a previous study found that the endogenous opioid system may be immature and therefore not fully functional during infancy [37]. It is apparent that a certain type of acupuncture analgesia observed in juvenile rats is not reversed by naloxone [38,39]. For example, evidence has accumulated that other neurohumoral networks including GABAergic, glutamatergic, and monoaminergic systems play an important role in mediating non-opioid acupuncture analgesia [35,40,41]. In addition, mechanisms of the acupuncture analgesia involve various neuroendocrine systems, most importantly the pituitary system [42,43]. Therefore, acupuncture analgesia during infancy is not produced by opioidergic mechanisms, but rather it is more likely produced by serotonergic and noradrenergic mechanisms descending from the brain stem. Furthermore, during infancy, there are rich serotonergic and noradrenergic terminals in the dorsal horn, which are lost with increasing age [44,45]. However, opioid-related analgesic mechanisms are mature and fully functional in adult rats, in line with our observed antagonization of electroacupuncture analgesia by naloxone in the adult group only. The residual analgesic effect of electroacupuncture after naloxone injection indicates that opioid and non-opioid systems are active in adult rats.

In conclusion, electroacupuncture analgesia is mainly produced by the opioid system in adult, but not juvenile, rats.

## FUNDING

This work was supported by a JSPS KAKENHI Grant. JLZ was supported by grant 04/2019 from DPI/University of Brasília.

## AUTHORS' CONTRIBUTIONS

HY: performed experiments, wrote manuscript; JLLZ: analyzed results, wrote manuscript; KT: planned study, analyzed results, wrote manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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