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RESEARCH ARTICLE

Preemptive Analgesia with Acupuncture Monitored by c-Fos Expression in Rats

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Abstract
Pain behavior and awareness are characterized by heightened alertness and anxiety, which begin to disappear as soon as the curative process starts. The present study aimed to quantify c-fos expression in rat spinal cords and brains after a surgical stimulus and with preoperative or postoperative acupuncture. Animals were randomly divided into preoperative and postoperative groups and were then further divided into control, manual acupuncture (MA), or electroacupuncture (EA) groups. Expression of c-fos was quantified using immunohistochemistry. The collected data were analyzed using the *t* test at a 5% probability level. Presurgery and postsurgery spinal cord c-fos expressions were similar in all of the treatment groups. In the control rats, c-fos expression was higher before surgery than after surgery, contradicting the expected outcome of acupuncture and preemptive analgesia. After treatment, the expression of c-fos in the brains of the

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rats in the MA and the EA groups was reduced compared with that of the rats in the control group. These findings suggest that acupuncture used as preemptive analgesia in rats is a useful model for studying its application in human treatment.

1. Introduction

Pain is an uncomfortable sensorial and emotional experience associated with an actual or psychogenic lesion, and an important mediator of response to harmful agents. Pain control can involve a multimodal approach that includes pharmacological and nonpharmacological techniques, promoting relaxation and reduction of the painful stimulus [1]. Among the nonpharmacological techniques with recognized analgesic effects are manual acupuncture (MA) and electroacupuncture (EA), which activate the sensitive-discriminative system to stimulate a suppressive pain response [2,3]. Pain control can be started prophylactically, and the preemptive administration of opioids or local anesthetics can reduce postoperative pain, hyperalgesia, and morphine intake [4,5]. This suggests that an analgesic intervention before surgery will produce a better outcome than the same intervention after surgery [6].

Pain response may influence the cellular genome, causing important changes in gene transcription and protein synthesis. Genes that show a rapid change in expression after neuronal activity are known as early or immediate response genes. The structural events that occur in the nervous system after a pain stimulus are related to the activation of these genes, which include proto-oncogenes such as *c-fos* and *c-jun*. The identification of c-fos expression in the spinal cord and brain is a reliable method for assessing the efficacy of analgesic treatments [7].

The repetition of a stimulus is capable of reducing c-fos expression [8,9]. Mapping of the brain areas associated with the analgesic effects of acupuncture by means of c-fos expression in either anaesthetized or restrained animals has helped to elucidate its mechanisms of action [7].

This study aimed to quantify c-fos expression in the spinal cord and brain of rats after a painful stimulus in order to evaluate the preoperative and postoperative effects of acupuncture.

2. Materials and methods

2.1. Animal treatments

Thirty-six adult male Wistar rats, from the Animal Department of Universidade Federal de São Paulo (UNIFESP), Brazil, weighing 210—235 g were maintained five to each cage in order to avoid stress caused by overcrowding, under normal temperature and controlled light, and with food and water provided *ad libitum*. All experimental protocols were approved by the Animal Care and Use Committee of UNIFESP and were in accordance with National Institutes of Health guidelines on animal care.

Animals were distributed randomly to control, preoperative, and postoperative groups, with 12 animals in each group, and then subdivided into MA and EA groups, with six animals in each group. All animals were anesthetized with 45 mg/kg sodium thiopental until no motor sensibility could be observed. The preoperative groups were submitted to MA or EA at 100 Hz frequency in the following points: Stomach 36, Kidney 1, and Bladder 67, bilaterally (Fig. 1). The needles were removed after 20 minutes and surgery was started. An approximately 1-cm long incision was made in the plantar region of the left paw using a scalpel blade. After this, superficial and deep tissues were dissected. After 60 minutes, animals were sacrificed and perfused. In the postoperative groups, animals were stimulated for 20 minutes, at the same points after surgery, and then sacrificed and perfused.

Rats were euthanized by injecting 75 mg/kg sodium thiopental intraperitoneally, and perfusion was started 60

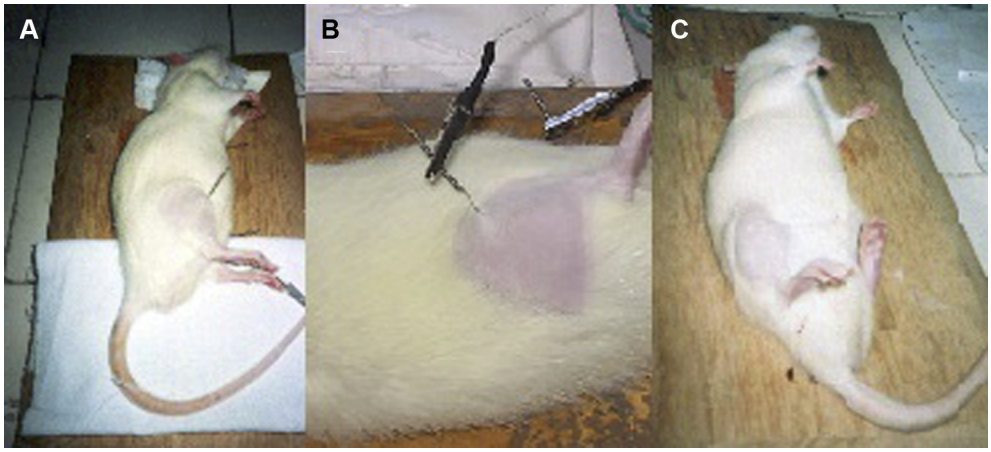

Figure 1 Manual acupuncture (MA) and electroacupuncture (EA) in rats. (A) MA in E36. (B) EA in E36. (C) EA in B67 (Ting).

minutes later if the animals did not manifest any signs of consciousness. To remove the spinal cord and brain, animals were placed in a horizontal ventral decubitus position above a wooden platform.

2.2. Immunohistochemistry

The c-fos immunoreactive (Fos-ir) cells were detected using a conventional avidin—biotin—immunoperoxidase technique to localize an antiserum raised against a synthetic N-terminal fragment of human Fos protein (Ab-5; Oncogene Science, Cambrige, MA, USA) [10]. Briefly, free-floating sections were pretreated with hydrogen peroxidase, followed by sodium borohydride. Sections were then treated with normal goat serum (1:100) and 0.3% Triton X-100 for 2 hours and incubated with the primary antiserum at a dilution of 1:3,000 in potassium phosphate buffer at room temperature for 24 hours. Subsequently, the sections were incubated with a secondary antibody (goat anti-rabbit IgG, 1:200; Vector Laboratories, Inc., Burlingame, CA, USA) for 90 minutes at room temperature and treated with the avidin—biotin complex (1:100; Vector) for a further 90 minutes. Staining was completed using the nickel-intensified diaminobenzidine reaction. Between steps, the sections were rinsed in 0.002 M potassium phosphate buffer (pH 7.4). The tissue was agitated on a rotator between each incubation and rinse step. Sections were mounted on gelatin-coated slides, dried, dehydrated, and covered with a coverslip [7].

2.3. Counting c-fos-positive nuclei

As described in previous studies [11], the nomenclature and nuclear boundaries defined in Swanson's Stereotaxic Rat Brain Atlas were used in this study [12]. C-fos-immunoreactive nuclear profiles in different areas of the brain were counted using a BX50 Olympus microscope (Olympus America Inc., Melville, NY, USA) coupled to a Macintosh-based image analysis system (Apple Computer Inc., Cupertino, CA, USA) and Neurozoom software (The Scripps Research Institute/Mount Sinai School of Medicine, La Jolla, CA, USA). The boundaries of the brain areas were identified using adjoining Nissl-stained sections. A template or outline was constructed for each brain nucleus or subnucleus based on the shape and size of the region. The location and relative size of each template is shown in Fig. 2. The c-fos-positive nuclei within each area were counted bilaterally (where possible) in two consecutive sections/animal and the mean value is reported as the number of Fos-ir cells/10,000 μm^2. This counting procedure allowed a reliable, time-effective analysis of c-fos expression in 41 brain areas [11].

For spinal cord c-fos-positive nuclei counting, the dorsal horn was analyzed. It was previously shown [13] that physiological stimulation of rat primary sensory neurons causes increased Fos-like immunoreactivity in the nuclei of postsynaptic neurons of the spinal dorsal horn.

2.4. Statistical analyses

The average, median, and standard deviation of each group (control, preoperative and postoperative) were calculated. To verify the effect of surgery and of each treatment (control, MA, and EA), a variance analysis was conducted, calculating the F statistics and p values for comparisons among the three treatments in pre- and postoperative conditions, and t and p values for pre- and postoperative comparisons. The results were considered statistically significant when $p < 0.05$.

3. Results

3.1. Spinal cord

Results from the statistical analysis of Fos-ir cell counts in the spinal cord and brain are shown in Table 1, and

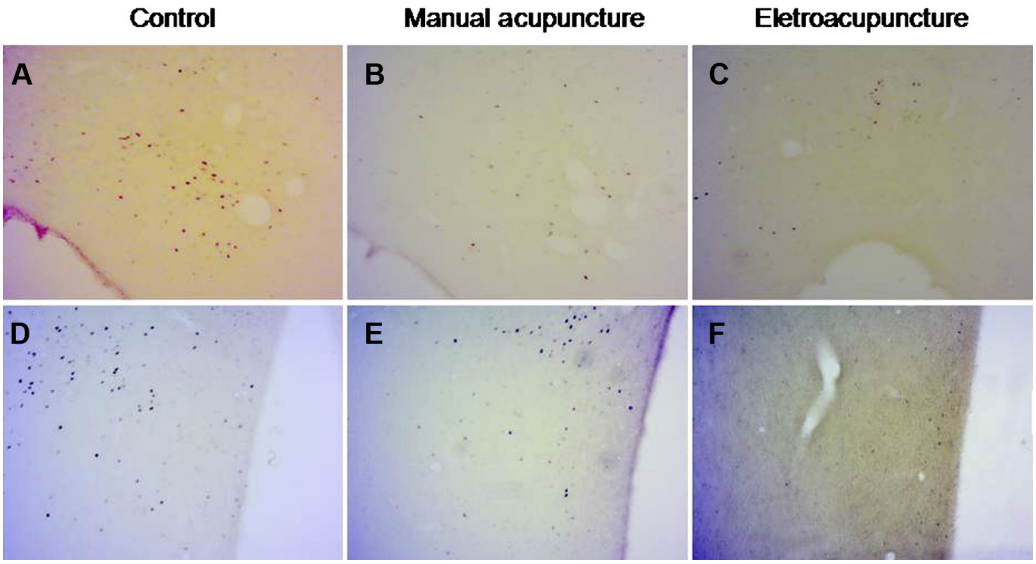

Figure 2 Photomicrograph illustrating stress-induced c-fos-immunoreactive cells. (A—C) In the spinal cord. (D—F) In the brain. Original magnifications 40×.

Table 1 The average number of c-fos-immunoreactive cells/10^4 μm^2 ($\pm$ standard deviation) in the spinal cord and brain.

	Control	Pre MA	Pre EA	Post MA	Post EA
Spinal cord	9.36 $\pm$ 5.51	8.12 $\pm$ 4.62	14.33 $\pm$ 11.75	12.23 $\pm$ 8.11	13.38 $\pm$ 15.84
Brain	94.31 $\pm$ 13.9[b,*]	47.94 $\pm$ 12.32[a]	42.98 $\pm$ 5.85[a]	46.26 $\pm$ 4.45[a]	39.49 $\pm$ 8.49[a]

* Different lowercase letters (a and b) indicate statistically significant differences between experimental groups.
EA = electroacupuncture; MA = manual acupuncture.

immunohistochemistry photomicrographs illustrating the effect of different treatments on c-fos expression are shown in Fig. 3. There were no significant differences in c-fos expression after treatment, and likewise in the pre- and posttreatment groups, neither MA nor EA caused a significant change in c-fos positivity. There was also no difference in c-fos expression when MA or EA were compared during the pre- and postsurgical period.

3.2. Brain

Table 2 summarizes the results of a comparative analysis of c-fos expression between control animals and those treated with MA or EA. Significant differences between all treatment groups compared with the control group were observed. Comparisons between the same treatments in the pre- or postsurgical periods revealed no significant

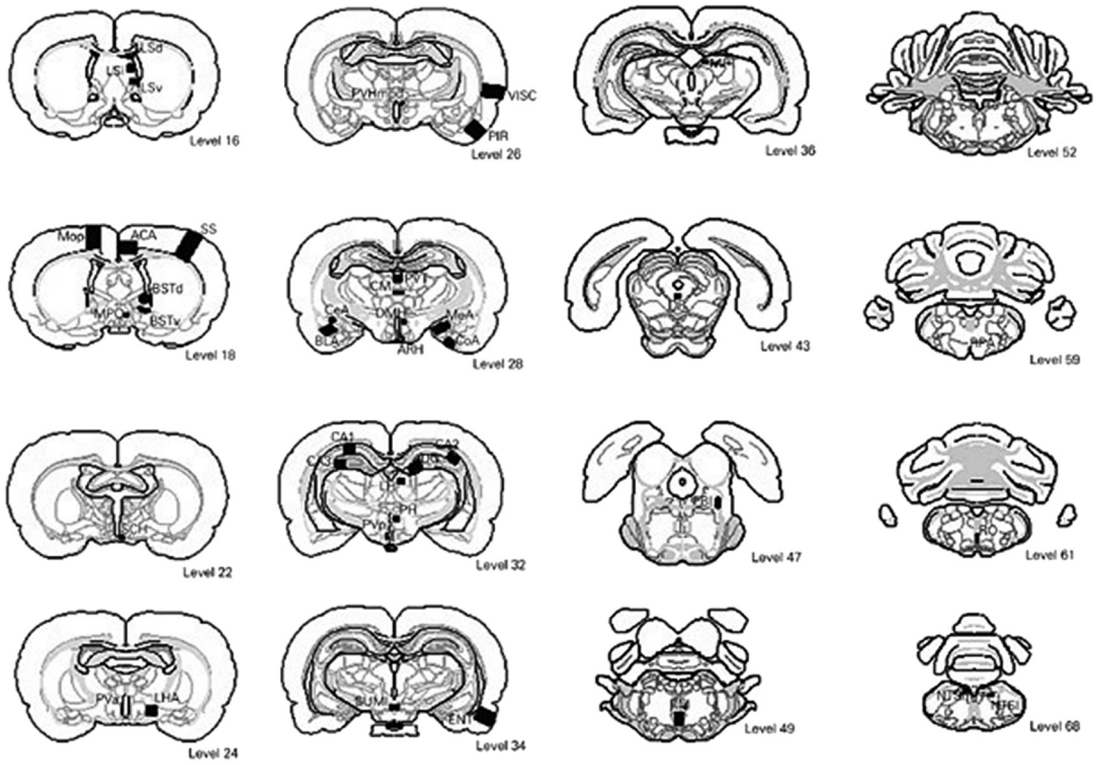

Figure 3 Diagrams illustrating the templates and relative sizes of the different brain areas in which c-fos-immunoreactive cells were counted [11]. The levels were based on Swanson's Stereotaxic Atlas of the Brain [12]. Abbreviations list: ACA = anterior cingulate area; ARH = arcuate nucleus of the hypothalamus; BLA = basolateral nucleus of the amygdala; BST = bed nucleus of the stria terminalis; CA1 = field CA1; CA2 = field CA2; CA3 = field CA3; CeA = central nucleus of the amygdala; CM = central medial nucleus of the thalamus; CoA = cortical nucleus of the amygdala; DG = dentate gyrus; DMH = dorsomedial nucleus of the hypothalamus; DR = dorsal raphe nucleus; ENT = entorhinal area; LC = locus coeruleus; LH = lateral habenula; LHA = lateral hypothalamic area; LSd = lateral septal nucleus, dorsal part; LSi = lateral septal nucleus, intermediate part; LSv = lateral septal nucleus, ventral part; MeA = medial nucleus of the amygdala; Mop = primary motor area; MPT = medial pretectal nucleus; MPO = medial preoptic area; NTS = nucleus tractus solitarius; PBl = parabrachial nucleus, lateral part; PH = posterior hypothalamus; PIR = piriform area; PVT = paraventricular nucleus of the thalamus; PVa = anterior periventricular nucleus of the hypothalamus; PVp = posterior periventricular nucleus of the hypothalamus; PVHmpd = paraventricular nucleus of the hypothalamus, medial parvicellular part, dorsal zone; RM = nucleus raphe magna; RO = nucleus raphe obscurus; RPA = nucleus raphe pallidus. SCH = suprachiasmatic nucleus; SS = somatosensory area; SUMl = supramammillary nucleus; VISC = visceral area.

Table 2 Comparative analysis between control and treatment groups with respect to the number of c-fos-immunoreactive cells in the brain.

Comparison	Statistical difference	p
Control *vs.* pre-MA	46.369	< 0.001
Control *vs.* pre-EA	51.327	< 0.001
Control *vs.* post-MA	48.047	< 0.001
Control *vs.* post-EA	54.813	< 0.001

EA = electroacupuncture; MA = manual acupuncture.

differences in c-fos expression. The difference in the average number of c-fos-immunoreactive cells/10^4 μm^2 ($\pm$ standard deviation) between pre- and postsurgical MA groups was 1.678 ($p > 0.05$), and between pre- and postsurgical EA groups it was 3.486 ($p > 0.05$). Likewise, there was no significant difference in c-fos positivity between different treatments in the same period (4.958 for preoperative MA and EA, and 6.766 for postoperative MA and EA; $p > 0.050$).

4. Discussion

4.1. Spinal cord

The absence of any significant difference ($p > 0.100$) in c-fos expression between pre- and postoperative treatments indicates that animals exposed to pre- and postoperative stimuli experienced a similar level of stress, and consequently, had similar c-fos expression levels in the dorsal horn of the spinal cord. Regardless of the treatment used, none of the animals were completely sedated, to the point where c-fos expression would be repressed. Although the differences in c-fos expression were not significant, the highest expression was in animals treated with preemptive EA, suggesting that this technique causes more stress than the other treatments. It is also noteworthy that the "Ting" points selected in this study (Bladder 67 and Kidney 1) are localized in the edges of the toes, which are more sensitive to pain stimuli due to intense innervations. It was previously reported [3] that 2 Hz electrical stimulation at traditional acupuncture sites induced the release of encephalin in the spinal cord of rats, providing a more effective analgesia than we observed in this study.

The absence of significant differences in c-fos expression between treatments in postoperative animals might be explained by individual variations, particularly in the animals treated with MA and EA, and may not necessarily be associated with the treatment effect *per se*. Other studies have shown conflicting results when using opioids to control conditional pain, probably as a result of methodological differences [14].

Against our expectations, MA in pre- and postoperative conditions did not cause a significant reduction in c-fos expression. The E36 point has been shown to have an analgesic and sedative effect (7), and is thus expected to reduce c-fos expression. This might indicate that the animals were subjected to too much stress in these experiments. Previous studies have shown that B67 stimulation, but not stimulation of less important visual function acupoints, resulted in a significant increase in the number of c-fos-positive cells in the primary visual cortex [15]. However, we observed no significant differences in c-fos positivity after B67 stimulation.

The pre- and postoperative acupuncture treatments failed to have any analgesic effect, probably because no endogenous opioids were released. There was, therefore, an increase in c-fos expression, mainly because the initial stimuli of analgesia by MA or EA can be painful. Analgesia by acupuncture relies on the manipulation of a physiological process, and thus may not lead to a complete loss of sensation or pain.

4.2. Brain

Changes in c-fos expression in the brain indicated that preemptive analgesia with either AM or EA reduced stress. Treated animals showed a significant reduction of c-fos expression, corroborating previous findings [3,16,17]. The comparison between control animals and those in the different treatment groups (Table 2) showed a very significant difference in c-fos expression ($p < 0.001$) for each treatment, proving that they are effective compared with the control. We observed that, regardless of the treatment, MA and EA were able to induce analgesia and increase the pain threshold of the animals, possibly with increased opioid and monoamine release.

With respect to the number of Fos-ir cells, our study revealed that there was no statistical difference between pre- and postoperative groups ($p > 0.050$) after either MA or EA treatment. This indicates that animals subjected to painful stimuli pre- and postoperatively suffered a similar level of stress and manifested a similar c-fos expression level in the brain.

When comparing groups treated at the same time (pre- or postoperatively) but with different treatments (MA or EA), there were indications that EA reduced pain (although the differences were not significant), in agreement with previous studies. Recent studies [18] have shown that EA is effective in the control of pain and symptoms for which MA has always been ineffective. Moreover, an increased number of Fos-ir neurons in the hippocampal region following EA was recently demonstrated [19]. Despite there being no significant differences in spinal cord c-fos expression, the brain results corroborate studies in which acupuncture needle penetration produced only a low level of stress [20—22], suggesting that it could be used as an alternative to, or in combination with, pharmacological intervention.

Our study provides strong evidence that acupuncture can induce analgesia by reducing c-fos expression. Using acupuncture to provide preemptive analgesia in rats represents an important model for studying the physiological effects and mechanisms of this technique, and the results of these studies could be applicable to future human treatment.

Disclosure statement

The authors declare that they have no conflicts of interest and no financial interests related to the material of this manuscript.

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