The effects of parachlorophenylalanine and naloxone on acupuncture and electroacupuncture modulation of capsaicin-induced neurogenic edema in the rat hind paw.
A controlled blind study

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Abstract

Objective
The aim of this work was to study the effect of pre-treatment with parachlorophenylalanine (PCPA) and post-treatment with naloxone on the modulating action on neurogenic inflammation of manual acupuncture and low intensity (5 mAmp), low frequency (5 Hz) electroacupuncture (EA).

Methods
Edema was induced by the subcutaneous administration of 50 µg capsaicin in rat paws. Pre-treatment with intraperitoneal PCPA was given for 3 days: 200 mg/Kg on the first day and 100 mg/Kg on the second and third days. Naloxone (1 mg/Kg) was administered at the end of the stimulation.

Results
The results show that naloxone and PCPA reduce the anti-edema effect of both manual acupuncture and EA. Combined administration of the two drugs completely eliminated the effect of manual acupuncture, and decreased but did not abolish the effect of electroacupuncture.

Conclusion
These results indicate that both the opioid and the serotonergic inhibitory control systems are involved in the modulating action of acupuntural stimulation on neurogenic inflammation.

Key words
Inflammation, parachlorophenylalanine, naloxone, acupuncture, electrostimulation, capsaicin, pain, rat, animal.
Naloxone and PCPA on acupuncture analgesia / F. Ceccherelli et al.

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Introduction
Since the beginning of this century it has been suggested that the nervous system is involved in the genesis of inflammatory responses. In 1901 Bayliss (1) showed that antidromic stimulation of the sensory nerve trunks produced cutaneous vasodilation. More recently Jancso et al. (2) observed that antidromic stimulation provokes, in addition to vasodilation, increased vascular permeability and even tissue plasma extravasation. Vasodilation and edema, similar to those produced by the antidromic stimulation of nerve trunks, can be induced by the subcutaneous (3) or intra-arterial (4) administration of substance P, thus suggesting that substance P is involved in the inflammatory response (5). “Neurogenic inflammation” is defined as comprising vasodilation, increased vascular permeability and tissue edema (6). The analgesic effect of acupuncture is attributed to an increased synthesis and release of opioid peptides at the spinal and supraspinal levels and to the activation of serotonergic inhibitory descending pathways (7). The involvement of serotonergic descending pathways is also demonstrated by the antagonizing effect of parachlorophenylalanine (PCPA) on acupunctural analgesia (8). PCPA provokes the depletion of 5HT in ascending and descending 5HT pathways (9).

In a previous study on an experimental model of neurogenic inflammation induced by the subcutaneous injection of capsaicin in rat paws, we observed a reduced edema response with the administration of either morphine (5 mg/Kg i.p.) or acupuncture at points 30 VB and 36 St (10). This could indicate the existence of both opioid and acupunctural modulation on neurogenic inflammation. There are to date no studies in the literature on the serotonergic and opioid-dependent inhibition of neurogenic inflammation using acupuncture with manual or electrical stimulation of the needles. The aims of this study were to compare the effects of manual and electrical stimulation of acupuncture needles on the modulation of neurogenic inflammation and to determine whether the modulating effect of both of these modes of stimulation is mediated via the opioid or the serotonergic systems.

Materials and methods
One hundred and forty-four male Sprague-Dawley rats weighing between 180 - 220 g each were divided into 9 groups according to the treatment given. Sixteen animals, given no treatment, constituted the control group (Group 1). Group 2 (16 rats) received manual acupuncture only. Group 3 (16 rats) received electrical acupuncture only. Group 4 (14 rats) received manual acupuncture and naloxone and group 5 (14 rats) received electrical acupuncture and naloxone. Group 6 (18 rats) received pre-treatment with PCPA and then was stimulated with manual acupuncture. Group 7 (18 rats) was pre-treated with PCPA and then stimulated with electrical acupuncture. Group 8 (16 rats) was pre-treated with PCPA, given manual acupuncture and then naloxone, while Group 9 (16 rats) was pre-treated with PCPA, given electrical acupuncture and then naloxone.

Drugs
PCPA, an inhibitor of tryptophan hydroxilase, was administered intraperitoneally (i.p.) for three days: 200 mg/Kg on the first day, and 100 mg/Kg on the second and third day. This three-day treatment was necessary to obtain 5-HT depletion and to abolish the serotonergic component of stimulation-induced analgesia (9).

Naloxone, 1 mg/Kg i.p., was given 30 minutes after the injection of capsaicin. It was administered in order to verify directly the inversion of the edema progression; a second rationale for its administration was that the anti-edema effect of acupuncture is more pronounced 60 minutes after capsaicin administration. In groups 8 and 9, which were pre-treated with PCPA, naloxone was injected at the end of the stimulation session, at the same time as the administration of capsaicin. The edema was induced by the subcutaneous injection of 50 µg capsaicin in 50 µl saline solution into the right hind paw of the rats.

Experimental design
All experiments were carried out between 9 a.m. and 1 p.m. All animals were lightly anaesthetised with ether and then given an initial dose of 400 mg/kg chloral hydrate intraperitoneally followed by
a supplementary dose of 30 mg i.p. if necessary. This dosage was sufficient to keep the animals asleep for the entire duration of the experiment. The right hind paw of each animal was then shaved. A reference thread was placed above the tibio-tarsal joint in order to measure the paw volume. For each observation time the value of the volume was represented by the mean of 3 consecutive readings.

The baseline volume (TBas) was measured and then a 20-minute session of either manual acupuncture or electroacupuncture was performed. After this 20 minute session (T1), the paw volume was recorded, the capsaicin was injected, and measurements were taken at 15 minute intervals thereafter (T2, T3, T4, T5, T6, T7, T8, T9). The control group underwent exactly the same procedure as the groups receiving acupuncture or combined drugs and acupuncture, but without receiving any treatment. The acupuncture and drug administration were carried out by two different technicians; the measurements were made by a third technician, and all three were blinded to the treatments that the rats were receiving (if any).

**Manual acupuncture**
Each animal was stimulated at two anatomical points; one called 36 Stomach (Zu San Li) located on the anterior tibia-lis muscle just below the knee joint; the other called Gallbladder 30 (Huan Tiao) situated in the middle third of a line connecting the trochanter and the sacral hiatus. The points were identified using anatomical landmarks as well as a rheostat which gave an acoustic signal at the point of greatest conductance. These points can be found, in analogous anatomical locations, in man.

The needles (1.5 cm long and 0.25 mm thick) were fixed in the body of the muscle at a depth of 5 mm. They were inserted and rotated to the right and then to the left for 30 seconds at the beginning of the session and then every 5 minutes for the following 20 minutes, after which they were removed.

We excluded any comparison between acupuncture points and sham points (that is, points outside the acupuncture meridians) in determining the analgesic effect, and on no occasion did we stimulate points other than the two mentioned above.

**Electroacupuncture**
The needle site localization and fixing method were the same as for the manual acupuncture group. Once the needles had been fixed they were connected to an electrical stimulator which emitted a 5 Hz pulsed current. The impulse was a triangular biphasic wave, positive and negative, lasting 500 msec. There was a 1.8 msec pause between the positive and negative impulses. The total impulse lasted 2.8 msec. The mean current was zero. The width of the impulse was monitored by an oscilloscope (Hameg mod. HM 408).

**Measurement of the paw volume**
A calibrated plethysmometer (U. Basile, Mod 7150) was used to measure the edema in the animals (11). The plethysmometer consisted of a Perspex cell filled with solution into which the rat’s paw was dipped. The solution consisted of twice-distilled water, 3 g/l sodium chloride and 5 ml of a surface-active agent to reduce the surface tension. A digital display was connected to a transducer which recorded the minute differences in water level caused by the volume displacement. The digital read-out showed the exact volume of the paw.

**Statistical analysis**
The changes from baseline were calculated in percentages for the measurements taken at the various time intervals for each of the groups using the following formula:

$$\%V = (V_{TBAS+n} - V_{TBAS})/V_{TBAS} \times 100$$

where $V_{TBAS+n}$ was the volume measured at each time interval after the baseline measurement and $V_{TBAS}$ was the volume measured at baseline before the start of the acupuncture stimulation.

The mean and standard error of the data obtained from each treatment group were compared. The statistical significance of the differences was evaluated using the Bonferroni t-test.

**Results**
The results are summarized in Tables I and II. In the control group, where animals underwent all manipulations without drug and/or acupuncture treatment, there was an increase in paw volume reaching a maximum of 10.35% (S.E.M. 1.54) at T8 compared to baseline.

**Manual acupuncture**
The results are shown in Table I. In the group treated with acupuncture only the increase in paw volume reached a maximum of $1.95 \pm 0.70$ at T6. Comparison with the control group showed a significant statistical difference at all observation times (Fig. 1). Manual acupuncture, according to these results, is demonstrably effective in preventing neurogenic edema.

A double pattern can be observed in the group treated with acupuncture and, 30 minutes after the irritant injection, naloxone (Fig. 1). While in the first 30 minutes the anti-edema effect of acupuncture was apparent, after naloxone was administered the edema increased once again and the difference between the treated and control rats was no longer statistically significant. We can therefore conclude that intraperitoneal naloxone reduces but does not abolish the effectiveness of acupuncture.

Similarly, pre-treatment with PCPA decreased but did not abolish the effect of acupuncture (Fig. 2). Treatment with both drugs (Group 8) completely abolished the modulating effect of acupuncture stimulation, there being no difference in the edema volume between the treated group and the control group, while a significant difference was found with respect to group 2 (manual acupuncture only) (Fig. 3).

**Electroacupuncture**
These results are summarised in Table II. Electroacupuncture at 5 Hz and 5 mA was effective in containing neurogenic edema to a statistically significant degree compared to the control group (Fig. 4). The maximum value, $3.22 \pm 0.88$, was reached at T5. Treatment with naloxone reduced the anti-edema effect of electro-acupuncture. After naloxone injection no statistically significant difference was seen in comparison with the control group (Fig. 4). A comparable result was obtained in the group treated with PCPA.
**Table I.** The effects of acupuncture and drugs over time on capsaicin-induced edema in rat hind paws. The mean (± S.E.) percent increase in the paw volume vs the basal value are given for each time interval. Statistical analysis was carried out using the Bonferroni t-test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
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<tr>
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<td>±0.98</td>
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<td>±1.08</td>
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<td>1.95</td>
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<tr>
<td>Acup + Naloxone</td>
<td>Mean</td>
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<td>1.33</td>
<td>2.29</td>
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<tr>
<td>Acupuncture + PCPA</td>
<td>Mean</td>
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Acup. = Acupuncture; Nal. = Naloxone; PCPA = Parachlorophenylalanine.

**Table II.** The effects of electroacupuncture and drugs over time on capsaicin-induced edema in rat hind paws. The mean (± S.E.) percent increase in the paw volume vs. the basal value are given for each time interval. The statistical analysis was carried out using the Bonferroni t-test.

<table>
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Eap = Electroacupuncture; Nal. = Naloxone; PCPA = Parachlorophenylalanine.
Fig. 1. Edema response in the control, manual acupuncture and manual acupuncture + naloxone groups. 50 µg of capsaicin diluted in 50 µl of physiological solution were injected into each rat’s right paw in correspondence with the arrow (T 0). * indicates the significance of delta for p < 0.05 between the acupuncture and the control groups; + indicates the significance of delta for p < 0.05 between the manual acupuncture + naloxone vs. the control groups.

Fig. 2. Edema response in control, manual acupuncture and manual acupuncture + PCPA groups. 50 µg of capsaicin diluted in 50 µl of physiological solution were injected into each rat's right paw in correspondence with the arrow (T 0). * indicates the significance of delta for p < 0.05 between the acupuncture and the control groups; + indicates the significance of delta for p < 0.05 between the acupuncture and the acupuncture + PCPA + naloxone groups.

Fig. 3. Edema response in the control, manual acupuncture and manual acupuncture + PCPA + naloxone groups. 50 µg of capsaicin diluted in 50 µl of physiological solution were injected into each rat’s right paw in correspondence with the arrow (T 0). * indicates the significance of delta for p < 0.05 between the acupuncture and the control groups; + indicates the significance of delta for p < 0.05 between the acupuncture and the acupuncture + PCPA + naloxone groups.
Fig. 4. Edema response in the control, electroacupuncture and electroacupuncture + naloxone groups. 50 µg of capsaicin diluted in 50 µl of physiological solution were injected into each rat's right paw in correspondence with the arrow (T 0). * indicates the significance of delta for p < 0.05 between the electroacupuncture and the control groups; + indicates the significance of delta between the electroacupuncture + naloxone and the control groups.

Fig. 5. Edema response in the control, electroacupuncture and electroacupuncture + PCPA. 50 µg of capsaicin diluted in 50 µl of physiological solution were injected into each rat's right paw in correspondence with the arrow (T 0). * indicates the significance of delta for p < 0.05 between the electroacupuncture and the control groups; + indicates the significance of delta between the electroacupuncture and the electroacupuncture + PCPA + naloxone groups.

Fig. 6. Edema response in the control, electroacupuncture and electroacupuncture + PCPA + naloxone groups. 50 µg of capsaicin diluted in 50 µl of physiological solution were injected into each rat's right paw in correspondence with the arrow (T 0). * indicates the significance of delta for p < 0.05 between the electroacupuncture and the control groups.
(Fig. 5). Treatment with either drug reduced but did not abolish the effect of acupuncture stimulation (Fig. 6).

**Discussion**

The results of this study confirm the hypothesis of the efficacy of acupuncture stimulation in modulating the neurogenic inflammation experimentally induced by capsaicin.

Careful consideration should be given to the possibility that electrostimulation might be able to influence the release of neurotransmitters by means of a peripheral mechanism triggered by the current flowing through the limb. Pertovaara investigated the mechanism of transcutaneous electrical nerve stimulation at high intensity and high frequency in human volunteers and observed an elevation of the pain threshold for thermal stimuli only when the stimuli were applied at a site distal to the TENS electrodes (12). The author concluded that pain threshold elevation during high-frequency TENS is caused either by peripheral electrogenic blockade or by the fatigue of pain-mediating fibers.

Jensen et al. (13) later reported vasodilation in the distal portion of the dermatome where electroacupuncture was applied with an intensity of 20 V. The authors ascribed this vasodilation to the release of polypeptides from nerve endings. We also observed in an earlier study based on the same experimental model as the present one (14) that low frequency, high intensity stimulation (70 mA) was sufficient to induce edema in the rat paw in addition to the edema provoked later by capsaicin injection.

The intensity of stimulation employed in the present study, on the contrary, did not seem to influence the peripheral release of substance P, since it antagonized edema formation. In an earlier study using the same animal model, we observed a modulation of capsaicin-induced edema irrespective of whether the stimulation was applied ipsilaterally or contralaterally to the paw injected with capsaicin (15).

Both manual acupuncture and low frequency, low intensity electroacupuncture produced a statistically significant effect in comparison with the controls. There were, however, a few differences in the effects between the two techniques: the administration of naloxone or PCPA reduced the effect of acupuncture but did not abolish it wholly. The drugs administered either singly or in combination reduced but did not completely abolish the modulating effect of electroacupuncture. In fact, there was no significant difference between the group treated with electroacupuncture and the group treated with electroacupuncture and drugs.

The modulation of neurogenic inflammation by morphine and the synthetic analogues of enkephalins highlights the role of opioids in the control of the release of mediators from capsaicin-sensitive fibers. The opiate agonists inhibit the vasodilation evoked by antidiromic nerve stimulation, but do not change the effect of the intra-arterial infusion of substance P. This shows that opioids can inhibit the release of substance P, but cannot antagonize its effect (16). Whether the opioids act centrally or peripherally remains to be clarified (17). There are two hypotheses regarding this point, one suggesting that the opioid action is exerted at a pre-synaptic level (18), while the other favours a peripheral site of action (19) located directly at the nerve endings, and in fact opioid receptors have been identified in the peripheral nervous system (20).

The serotonergic systems would also seem to play an important role in inflammatory response modulation. 5-HT turnover is increased in the mouse paw after an electric shock (21), and an increase in the amount of spinal serotonin in rats with polyarthritis has also been observed (22). The administration of 5-HT1 receptor agonists, acting at the pre-junction, inhibits the release of the neuropeptides implicated in the inflammatory response (23). Electroacupuncture acts by increasing the release of 5-HT in the CNS due to the administration of substance P (24).

A reasonable hypothesis could therefore be formulated regarding the anti-edema effect of acupuncture stimulation: i.e., it may be caused by the activation of both the opioid and the serotonergic systems. Manual acupuncture acts at the spinal medullary level, segmental and/or extra-segmental to the zone of stimulation, through an opioid mechanism. Indeed, there are data indicating an increase in Met-enkephalin-like substances at the lumbar and cervical trigeminal levels after acupuncture stimulation of the 36 St (Zusani) (25).

The results of the PCPA-treated groups in the present study show the activation of a serotonergic control system, as well, with a supraspinal integration level. However, the partial reduction of the modulating effect of electroacupuncture indicates that another subsystem, such as for example one involving the ACTH (26), could also play a role. Electrostimulation causes an increase in the release of ACTH through the activation of the hypothalamus-hypophysis axis.

In conclusion, it seems that manual acupuncture and low intensity electroacupuncture are effective in modulating the edema response, at least in this animal model of neurogenic inflammation. The opioid and monoaminergic pathways play a primary role in the effect of electroacupuncture and manual acupuncture on the control of the release of inflammatory neuromediators by capsaicin-sensitive afferent fibers. These results offer a theoretical basis for hypotheses regarding the possible therapeutic effects of acupuncture in syndromes whose pathogenesis is related to neurogenic inflammation, as for example in migraine and headache (27), in rheumatological syndromes such as primary fibromyalgia (28), in chronic arthropathies (29), and finally in syndromes not directly related to pain, but to other medical conditions such as asthma (30) or chronic bronchitis (31).

We suggest that acupuncture should be considered not only as a technique capable of producing some degree of pain control in chronic pain patients, but also as having a probable therapeutic effect on inflammation in general. This effect could modulate the inflammatory phase through the peripheral release of substance P. Further experimental and clinical studies could help to clarify the mechanisms involved and optimize the rational use of this technique.

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