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Ascending Nociceptive Control Contributes to the Antinociceptive Effect of Acupuncture in a Rat Model of Acute Pain

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Abstract: Acupuncture-induced analgesia depends on the activation of endogenous pain modulation pathways. In this study, we asked whether ascending nociceptive control (ANC), a form of pain-induced analgesia, contributes to the antinociceptive effect of acupuncture. To answer this question, we tested the ability of procedures that block ANC-induced analgesia, at peripheral, spinal, nucleus accumbens and rostral ventral medulla levels, to block acupuncture-induced analgesia. Acupuncture at ST36 (*Zusanli*), a widely used acupoint located in the hind limb, induced potent heterosegmental antinociception in the orofacial formalin test. The magnitude of this antinociceptive effect was similar to that induced by an intraplantar injection of capsaicin, a procedure classically used to activate ANC. The antinociceptive effect of acupuncture was blocked by sciatic C-fibers depletion (1% perineural capsaicin), spinal administration of a μ-opioid (Cys2,Tyr3,Orn5,Pen7amide, .2 μg) or of a $GABA_A$ (bicuculline, .3 μg) receptor antagonist, intra–nucleus accumbens administration of a μ-opioid receptor antagonist (Cys2,Tyr3,Orn5,Pen7amide, 1 μg), or intrarostral ventral medulla administration of a nicotinic acetylcholine receptor antagonist (mecamylamine, .6 μg). In addition, acupuncture at ST36 and/or upper lip formalin induced c-Fos expression in the nucleus accumbens and in rostral ventral medulla. On the basis of these results, we propose that ANC contributes to the antinociceptive effect of acupuncture.

***Perspective:** This article presents a novel mechanism of acupuncture analgesia, contributing to the understanding of its scientific basis. Because ANC is a pain modulation pathway activated by peripheral noxious stimulation that ascends to supraspinal regions, it could be the link between acupoint stimulation and the central mechanisms underlying acupuncture analgesia.*

***Key words:** Acupuncture, ascending nociceptive control, analgesia, nociception, pain.*

Acupuncture is an ancient Chinese technique in which specific body points (acupoints) are stimulated to treat a variety of diseases, including pain.[63] Both human[11,35,47] and animal[7-9,24] studies have provided evidence that acupuncture induces analgesia mediated by the release of endogenous opioids. However, recently several large rigorous clinical studies indicated that pain reduction after acupuncture is largely due to nonspecific effects rather than to specific effects resulting from needle insertion.[10,56,57] Because the study of the specific mechanisms underlying acupuncture-induced analgesia is limited in humans for ethical and technical reasons, animal studies are of relevance to our understanding of the mechanisms underlying acupuncture-induced analgesia. Such studies have indicated that it depends on the activation of primary sensory fibers, especially C-fibers,[27] and subsequently of endogenous pain modulation pathways, such as endogenous descending inhibitory system[49] and diffuse noxious inhibitory controls (DNIC).[2] However, the neuronal link between

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acupoint stimulation and central pain modulation is not known.

Among endogenous pain modulation pathways, the ascending nociceptive control (ANC) is one of the most recently described.[16] It mediates a form of pain-induced analgesia, in which noxious stimulation induces potent antinociception that can be experimentally demonstrated in a body region remote from stimulation (heterosegmental antinociception). Under basal physiological conditions, ANC-mediated analgesia is inhibited by an ongoing neural activity ascending from the spinal cord. Peripheral C-fiber stimulation activates inhibitory spinal mechanisms—dependent on μ-opioid and gamma-aminobutyric acid (GABA) receptors—that suppress the ongoing ascending activity and involve an opioidergic link in nucleus accumbens to produce antinociception.[52] Recent evidence included a cholinergic mechanism in rostral ventral medulla (RVM) in this pathway.[17]

In the present study, we asked whether ANC contributes to the antinociceptive effect of acupuncture. Acupuncture at ST36 (*Zusanli*), a widely used acupoint located in the hind limb,[22] was used to induce antinociception in the orofacial formalin test.[42] The injection of formalin into the upper lip was employed because it induces nociception segmentally remote from the hind limb, where acupuncture was applied, thus allowing separation of heterosegmental effects from any intrasegmental effects that might influence assays. The involvement of ANC in acupuncture-induced analgesia was tested by evaluating the ability of procedures that block ANC, at peripheral, spinal, and supraspinal (nucleus accumbens, RVM) levels, to block acupuncture-induced analgesia. The effect of acupuncture and/or noxious stimulation in neuronal activity in nucleus accumbens and rostral ventral medulla was evaluated by c-Fos expression, to further characterize the involvement of ANC in acupuncture.

Methods

Animals

Experiments were performed on 200- to 280-g male Wistar rats (Federal University of Paraná, Curitiba, Paraná, Brazil). The animals were maintained in a temperature-controlled room (±23°C) on a 12:12 light-dark cycle with food and water available ad libitum. All animal experimental procedures and protocols were approved by the Committee on Animal Research of the Federal University of Parana (protocol # 480) and are in accordance with the International Association for the Study of Pain guidelines for the study of pain in animals.[65]

Drugs

Formalin (an aqueous solution of 37% of formaldehyde dissolved in .9% NaCl to a concentration of 2.5%); capsaicin, a transient receptor potential vanilloid 1 agonist (dissolved in 6% ethanol, 8% Tween 80, and 86% .9% NaCl); Cys2,Tyr3,Orn5,Pen7amide (CTOP), a μ-opioid receptor antagonist (dissolved in .9% NaCl); bicuculline, a $GABA_A$ receptor antagonist (dissolved in dimethyl sulfoxide .6%); and mecamylamine, a nicotinic acetylcholine receptor antagonist (dissolved in .9% NaCl), were obtained from Sigma-Aldrich (St. Louis, MO).

Acupuncture

Rats were gently handled for at least 1 week prior to the experiments. A small area of skin overlying the hind limb region was shaved with an electric razor. Manual acupuncture was performed by inserting a stainless steel needle (.18 × 8 mm) to a depth of about 4 to 5 mm at right ST36 (*Zusanli*) acupoint, located, in the rat, below the capitulum fibulae and lateral to the tibia.[2] Adhesive tape was placed over the needle to protect the area during treatment. The animals were allowed to rest, with the needle inserted, for 20 minutes in their home cages and into the test chamber for a 10-minute habituation period, totaling 30 minutes of acupuncture treatment. During this period, the animals were not restrained and no signs of distress were observed. Control animals (sham groups) underwent the same procedure but no needle was inserted.

Immediately after acupuncture or sham treatment, animals received an upper lip injection of formalin or its vehicle and underwent the orofacial formalin test. Each drug treatment (perineural capsaicin, intrathecal, intra–nucleus accumbens, or intra-RVM drug administration) was performed previous to the acupuncture or sham treatment, as described below.

Formalin Test

The orofacial formalin test was conducted as described by Raboisson and Dallel,[42] with minor modifications. Briefly, animals were placed individually in a test chamber (30 × 30 × 30 cm mirrored-wood chamber with glass at the front side) for a 10-minute habituation period to minimize stress. Then the animals were gently held and received a subcutaneous injection (30 μL) of formalin (2.5%[42]) or its vehicle into the upper lip using a 26-gauge needle connected to a polyethylene tube (PE-50; BD INTRAMEDIC Polyethylene Tubing, Clay Adams; Becton-Dickinson, Franklin Lakes, NJ) and also to a 50-μL syringe (Hamilton, Reno, NV). The animals were immediately returned to the test chamber for counting the nociceptive behavior characterized by rubbing the injected area with its fore or hind paws. The period of time during which each animal spent rubbing the injected area was recorded cumulatively (using a stopwatch), in consecutive 3-minute blocks, over a period of 45 or 60 minutes. The duration of face rubbing behavior in seconds was used as a quantitative measure of nociception and was expressed in figures as mean ± standard deviation.

Intraplantar Injections

The injections into the plantar surface of the right hind paw (30 μL) were performed using a 26-gauge needle connected to a PE-50 and also to a 50-μL Hamilton syringe. Animals were gently held and capsaicin or its vehicle was injected at 2 different doses, dependent on the experimental purpose.

To activate ANC, we used capsaicin at 100 μg, which is the lowest dose able to induced heterosegmental antinociception, as previously standardized.[18] The formalin test was performed 30 minutes later.

To confirm whether perineural capsaicin treatment effectively depleted sciatic C-fibers, we used capsaicin at 1 μg,[19] which is a low dose able to induce significant nociceptive flinch behavior. Before the injection, the animals were placed individually in a test chamber (30 × 30 × 30 cm mirrored-wood chamber with glass at the front side) for a 10-minute habituation period to minimize stress. After injection, animals were immediately returned to the test chamber for counting the nociceptive behavior characterized by flinching the injected paw over a period of 5 minutes, after which the behavior ceased. Data are expressed in results as the cumulative number of flinches during the 5 minutes' observation period. The absence of flinch behavior in animals pretreated with perineural capsaicin (compared to those pretreated with perineural vehicle) was used as indicative of C-fiber depletion.

Perineural Capsaicin

The protocol for perineural administration of capsaicin, a procedure known to selectively deplete C-fibers, was performed as previously described.[48] Briefly, the rats were anesthetized with an intramuscular injection of xylazine (3 mg/kg) and ketamine (100 mg/kg). A small area of the skin overlying the right hind limb was shaved with an electric razor. The right sciatic nerve, which innervates the ST36 area[27] and the hind paw, was exposed high in the thigh and a small piece of cotton was moistened with a solution of capsaicin (1%, .1 mL) or its vehicle and wrapped around the nerve.[48] After 20 minutes, the cotton piece was removed and the wound was closed. After surgery, the animals received dipyrone (50 mg/kg) and enrofloxacin (.5 mg/kg) and the experiments were carried out 5 days later.

Intrathecal Drug Administration

The method for intrathecal injection was based on the technique of Papir-Kricheli and colleagues.[40] Briefly, for each injection, rats were anesthetized with halothane and positioned in dorsal recumbency. A small area of skin overlying the lumbar region was shaved with an electric razor. A 26-gauge needle was inserted in the subarachnoid space on the midline between L4 and L5 vertebrae, and the injections were performed at a rate of 1 μL/s. CTOP (.2 μg),[52] bicuculline (.3 μg),[52] or their vehicles were injected at a volume of 10 μL (1 μL/s). The animals regained consciousness approximately 1 minute after discontinuing the anesthetic. Immediately after intrathecal injection, animals received acupuncture or the sham procedure.

Nucleus Accumbens and Rostral Ventral Medulla Drug Administration

The rats were anesthetized by an intramuscular injection of xylazine (3 mg/kg) and ketamine (100 mg/kg). A 23-gauge stainless steel guide cannula was stereotaxically positioned and fixed into place with orthodontic resin (L.D. Caulk Co, Milford, DE). After surgery, the animals received dipyrone (50 mg/kg) and enrofloxacin (.5 mg/kg) and experiments were carried out 7 days later. Bilateral intra–nucleus accumbens CTOP (1 μg)[45] or vehicle administration was performed via insertion of a 30-gauge stainless steel injection cannula (1.3 mm rostral, 7.2 mm ventral, and ±1.8 mm lateral from bregma), which extended 2 mm beyond the guide cannulas, connected to a PE-10 polyethylene tube and also to a 2-μL Hamilton syringe.[45] Intra-RVM mecamylamine (.6 μg)[17] or vehicle administration was performed via insertion of a 30-gauge stainless steel injection cannula (2.3 mm caudal and .2 mm ventral to the intraaural line), which extended 5 mm beyond the guide cannula, connected to a 2-μL Hamilton syringe.[17] Injection volume in all experiments was .5 μL carried out over a period of 2 minutes. Injection cannula was left in place for an additional period of 30 seconds after injection. Immediately after intra–nucleus accumbens or intra-RVM injection, the animals received acupuncture or sham treatment. Injection sites were verified by injecting Evans blue dye (1%, .5 μL) and performing 50-μm postmortem coronal sections[41] to determine the location of the dye.[16]

c-Fos Immunohistochemistry

The rats were anesthetized by an intramuscular injection of xylazine (3 mg/kg) and ketamine (100 mg/kg) immediately after the behavioral test and were intracardially perfused with saline followed by 4% formaldehyde in .1 M phosphate buffer, pH 7.4. Brains were removed from the skulls and were immersed for 1 week in formaldehyde at 4°C. Subsequently, the brains were placed in 30% sucrose solution for 48 hours before sectioning. Six 40-μm sections per animal were taken between bregma 1.44-mm and 1.20-mm coordinates to nucleus accumbens and between intra-aural −2.16-mm and −2.40-mm coordinates to RVM.[41]

Tissue sections were incubated overnight at 4°C with rabbit anti-c-Fos primary antibody (1:500 in phosphate-buffered saline plus .3% Triton X-100; antibody catalogue #AB038; Chemicon, Temecula, CA). Sections were then incubated with a biotin-conjugated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 2 hours at room temperature. After several washes with phosphate-buffered saline, the antibody complex was localized using the ABC system (Vectastain ABC Elite kit, catalogue #PK6101; Vector Laboratories) followed by reaction with 3,3′-diaminobenzidine with nickel enhancement. The sections were then mounted onto gelatin-coated slides and cover-slipped after dehydration in ascending concentrations of ethanol-xylene solutions.

Quantification of c-Fos Immunoreactive Cells

The slides were digitized with a microscope scanner (Axio Imager Z2; Carl Zeiss, Jena, Germany) coupled with an imaging system (MetaSystems, Altlussheim, Germany). Quantification of c-Fos immunoreactive cells was performed using ImageJ 1.37c (National Institutes of

Health, Bethesda, MD) image analysis software. c-Fos immunoreactive (c-Fos-ir) cells in the nucleus accumbens were quantified automatically by optical density. Data are expressed in graphics as percentage of the mean optical density of the control group (animals receiving saline in the upper lip and sham procedure). In the RVM, c-Fos-ir cells were quantified manually using a reticular field. Data are expressed in graphics as percentage of the mean number of c-Fos-ir cells of the control group.

Data Analysis

Two-way repeated measures analysis of variance (ANOVA) with 1 between-subjects factor (treatment) and 1 within-subjects factor (time) followed by Tukey's post hoc test was used to determine if there were significant differences in nociceptive response among the groups in Fig 1. A t-test and 1-way ANOVA followed by Tukey's post hoc test were used to determine if there was significant difference in nociceptive response between the groups in Figs 2A and 2C, respectively. Two-way ANOVA with 2 between-subject factors (acupuncture and drug treatment) was used to determine if there were significant differences among the groups in the subsequent experiments. Tukey's post hoc test was employed to determine the basis of significant differences. Data are plotted in the figures as mean ± standard deviation. The level for statistical significance was $P < .05$. SigmaPlot software (SPSS, Chicago, IL) was used to perform data analysis and graphical representation.

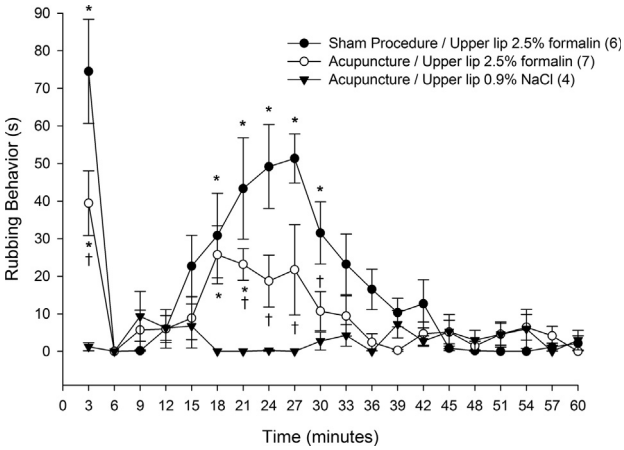

Figure 1. The antinociceptive effect of acupuncture at ST36 on the orofacial formalin test. Injection of formalin (2.5%) into the upper lip induced a biphasic nociceptive behavior (rubbing the injected area) that was significantly decreased by acupuncture (applied for 30 minutes before testing) at an acupoint located in the hind limb (ST36). The asterisk (*) indicates a behavioral response significantly higher than that induced by saline (.9% NaCl). The dagger symbol (†) indicates that formalin-induced nociceptive behavior was significantly lower in the acupuncture group than in the sham procedure one. The effect of different levels of treatment depends on what level of time is present, there is a statistically significant interaction between treatment and time (2-way repeated measures ANOVA and Tukey's test, F = 3.05, $P < .001$). In this and in subsequent figures, data are represented as mean ± standard deviation. Numbers in parenthesis indicate the number of animals in each group. See Methods for additional details regarding data presentation and analysis.

Results

Acupuncture at ST36, an Acupoint Located on the Hind Limb, Induces Antinociception in the Orofacial Region

The injection of formalin (2.5%) into the upper lip induced a significant nociceptive behavior characterized by rubbing the injected area. As shown in Fig 1, the nociceptive response was typically biphasic, with an early and short-lasting first phase followed, after a quiescent period, by a second prolonged tonic phase that lasted around 40 minutes after injection. Acupuncture pretreatment at ST36 significantly decreased formalin-induced orofacial nociception, an effect evident in the first and second phases. Two-way repeated-measures ANOVA showed a significant main effect of treatment (eg, acupuncture or sham procedure, F = 87.62, $P < .001$) and a significant treatment × time interaction (F = 3.05, $P < .001$; Fig 1). This finding shows that the stimulation of an acupoint located on the hind limb induces a heterosegmental antinociceptive effect in the orofacial region.

Because formalin-induced nociceptive behavior was absent 45 minutes after injection and the effects of acupuncture and of drug treatments were similar in the first and second phases, subsequent behavioral data were presented by the sum of nociceptive behavior (first plus second phases) observed up to 45 minutes after formalin injection.

Hind Limb Noxious Stimulation or Acupuncture Activates Local C-Fibers to Induce Antinociception in the Orofacial Region

To evaluate whether noxious stimulation of the hind paw is able to decrease nociception in the orofacial formalin test, we quantified formalin-induced orofacial nociception after an intraplantar injection of capsaicin (100 μg).[18] As shown in Fig 2A, the intraplantar injection of capsaicin significantly decreased formalin-induced orofacial nociception (t-test, $P = .01$). Because the intraplantar injection of capsaicin is a procedure classically used to activate ANC,[18] this finding suggests that ANC activation is able to decrease nociception in the orofacial formalin test.

To evaluate the contribution of peripheral C-fibers to acupuncture-induced antinociception, we tested the ability of perineural administration of capsaicin (1%), a procedure known to selectively deplete C-fibers,[5] to decrease acupuncture-induced antinociception. As shown in Fig 2B, the antinociceptive effect of acupuncture at ST36 on orofacial formalin test was prevented by perineural administration of capsaicin at sciatic nerve, which provides the sensory innervation to the ST36 area. There is a statistically significant interaction between acupuncture and capsaicin treatment (2-way ANOVA and Tukey's test, F = 6.35, $P = .017$; Fig 2B). This finding demonstrates that the antinociceptive effect induced by acupuncture at ST36 in the orofacial region depends on the activation of C-fibers (small fibers) located in the acupoint area.

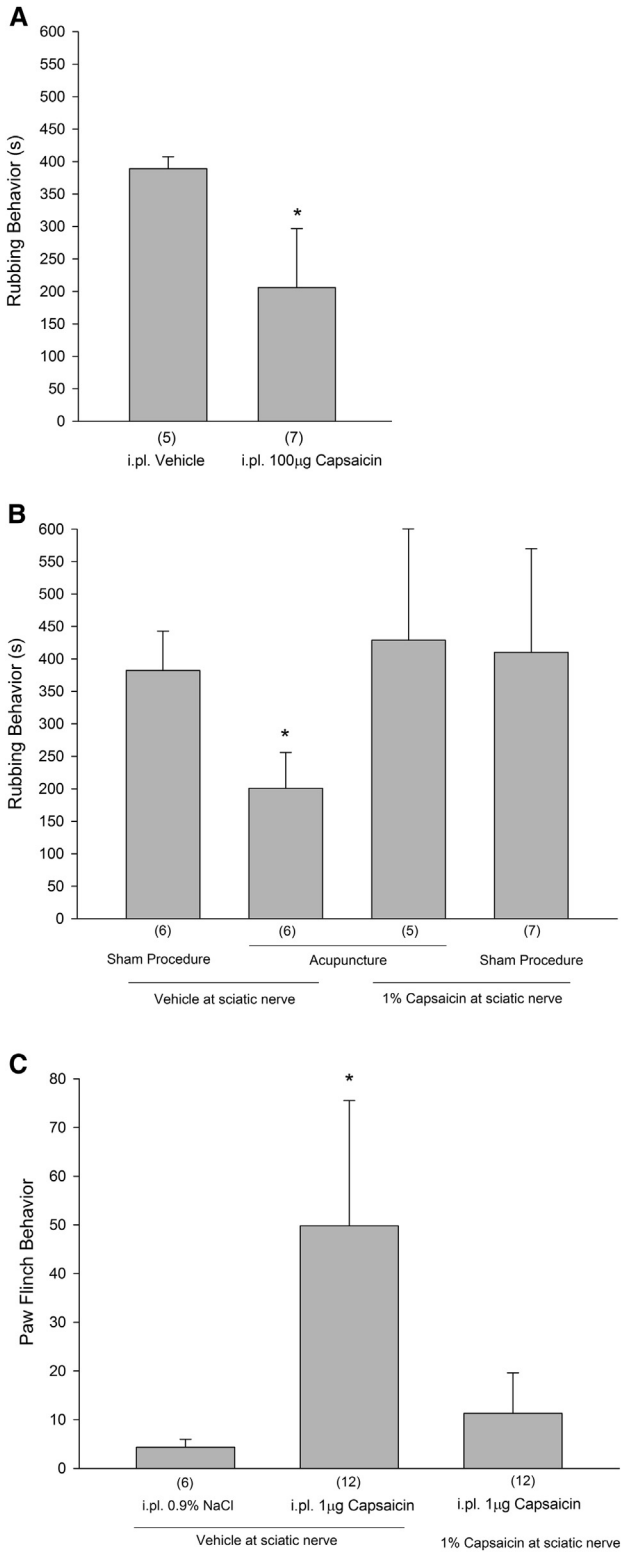

Figure 2. Stimulation of peripheral C-fibers at the hind limb induces antinociception in the orofacial region and is essential to acupuncture-induced antinociception. **(A)** The intraplantar (i.pl.) injection of capsaicin into the hind paw (30 minutes before formalin) significantly decreased the nociceptive behavior (rubbing the injected area) induced by the injection of formalin (2.5%) into the upper lip, as indicated by the asterisk (*) (t-test, $P = .01$). **(B)** The perineural administration of capsaicin (1%) at the sciatic nerve (5 days before testing) prevented acupuncture-induced antinociception in the orofacial region. The asterisk (*) indicates a nociceptive response significantly lower than that of the other groups. There is a statistically significant interaction between acupuncture and capsaicin treatment (2-way ANOVA and Tukey's test, F = 6.35, $P = .017$). **(C)** The perineural administration of capsaicin (1%) at sciatic nerve prevented the flinch behavior induced by an intraplantar injection of capsaicin (1 μg). The asterisk (*) indicates a nociceptive response significantly greater than that of the other groups. (1-way ANOVA and Tukey's test, $P < .001$). Vehicle = 6% ethanol, 8% Tween 80, and 86% .9% NaCl.

After this experiment, local depletion of C-fibers was confirmed by comparing the nociceptive behavior induced by an intraplantar hind paw injection of capsaicin (1 μg). As shown in Fig 2C, the cumulative number of flinches induced by the intraplantar injection of capsaicin was significantly greater in animals pretreated with perineural vehicle than in those pretreated with perineural capsaicin, which was similar to that of animals pretreated with perineural and intraplantar vehicle (1-way ANOVA and Tukey's test, $P < .001$). The absence of flinch behavior in animals pretreated with perineural capsaicin is indicative of C-fiber depletion.

Figs 1 and 2A respectively demonstrate that stimulation of an acupoint located on the hind limb and noxious stimulation of the hind limb significantly decreased nociception in the orofacial region. Figs 2A and 2B suggest that the first step for analgesia in both cases is local C-fiber stimulation, because it is the basis of the noxious effect of intraplantar capsaicin and essential to acupuncture-induced antinociception. Taken together, these findings support the idea that the antinociceptive effect of acupuncture and of ANC could be mediated by the same mechanisms.

Acupuncture-Induced Antinociception Is Prevented by Procedures Known to Block Ascending Nociceptive Control at the Spinal Cord

To evaluate whether the blockade of ANC at the spinal cord affects acupuncture-induced antinociception, we tested the ability of intrathecal administration of CTOP (.2 μg) or of bicuculline (.3 μg), at doses known to block ANC-induced antinociception,[52] to decrease acupuncture-induced antinociception. As shown in Fig 3A, the antinociceptive effect of acupuncture at ST36 on orofacial formalin test was prevented by previous intrathecal administration of the μ-opioid receptor antagonist CTOP (.2 μg). There is a statistically significant interaction between acupuncture and CTOP treatment (2-way ANOVA and Tukey's test, F = 5.38, $P = .032$; Fig 3A). Fig 3B shows that the antinociceptive effect of acupuncture at ST36 on orofacial formalin test was also prevented by intrathecal administration of the $GABA_A$ receptor antagonist bicuculline (.3 μg). There is a statistically significant interaction between acupuncture and bicuculline treatment (2-way ANOVA and Tukey's test, F = 6.96, $P = .014$; Fig 3B). Taken together, these findings demonstrate that, like ANC-induced analgesia, acupuncture-induced analgesia depends on spinal μ-opioid and $GABA_A$ receptor mechanisms, supporting the idea that ANC contributes to acupuncture-induced analgesia.

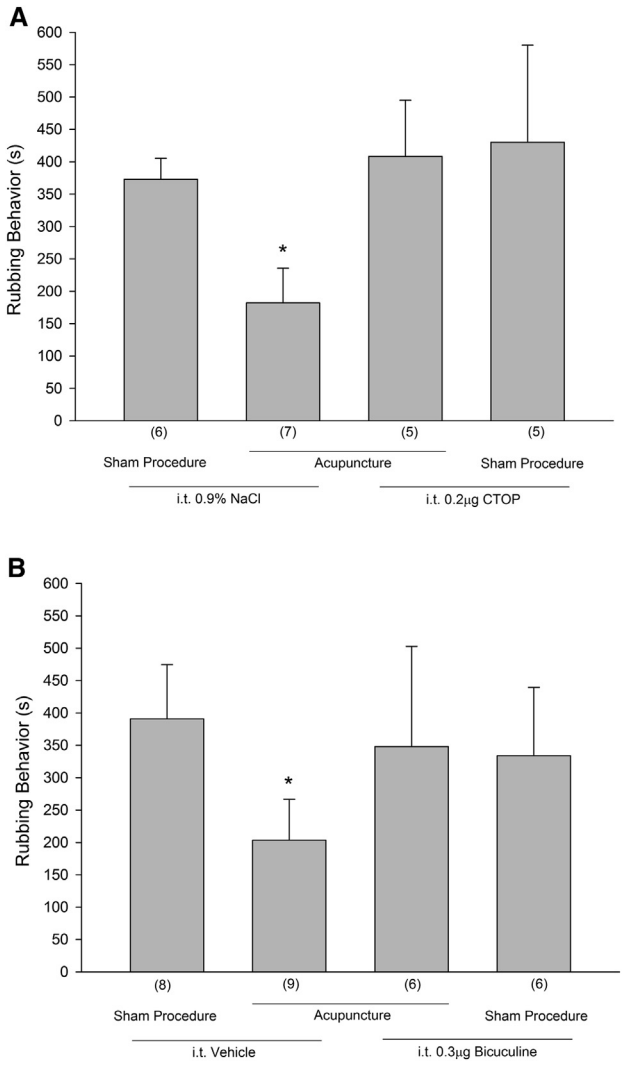

Figure 3. Pharmacologic blockade of spinal μ-opioid or $GABA_A$ receptors blocked acupuncture-induced antinociception. The intrathecal (i.t.) administration of CTOP **(A)** or of bicuculline **(B)** (immediately before acupuncture or sham treatment), at a dose known to block ANC-induced antinociception, prevented the antinociceptive effect of acupuncture on the orofacial formalin test. The asterisk (*) indicates a nociceptive behavior significantly lower than that of the other groups. There is a statistically significant interaction between acupuncture and CTOP or bicuculline treatment (2-way ANOVA, Tukey's test; **A**: F = 5.38, *P* = .032; **B**: F = 6.96, *P* = .014). Vehicle = dimethyl sulfoxide .8%.

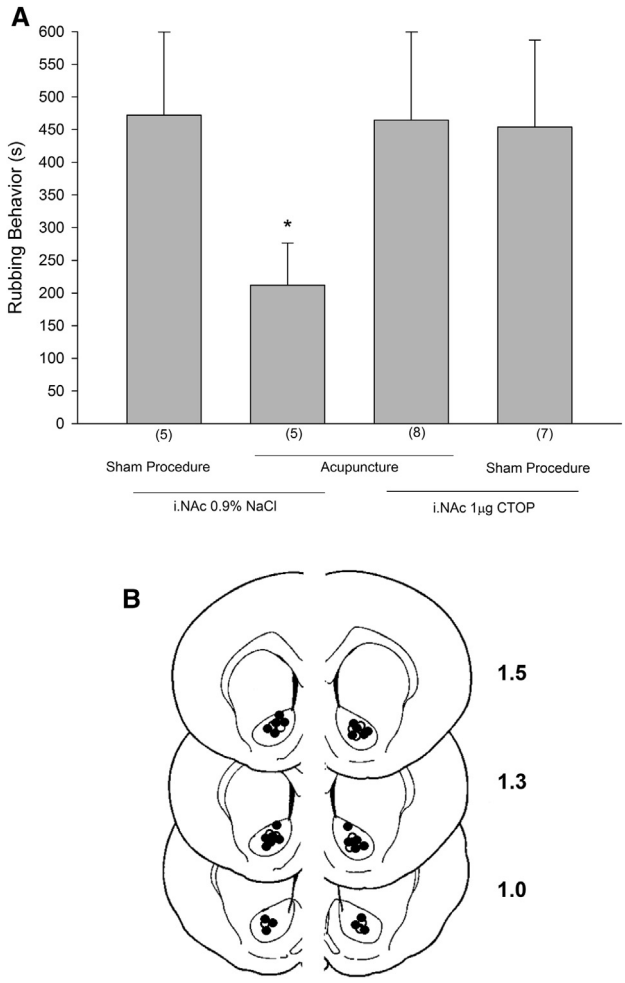

Figure 4. Pharmacologic blockade of μ-opioid receptors located in nucleus accumbens blocked acupuncture-induced antinociception. **(A)** The intra–nucleus accumbens (i.NAc) microinjection of CTOP (immediately before acupuncture or sham treatment), at a dose known to block ANC-induced antinociception, prevented the antinociceptive effect of acupuncture on orofacial formalin test. The asterisk (*) indicates a nociceptive behavior significantly lower than that of the other groups. There is a statistically significant interaction between acupuncture and CTOP treatment (2-way ANOVA, Tukey's test, F = 7.33, *P* = .013). **(B)** Nucleus accumbens injection sites plotted on drawings adapted from the atlas of Paxinos and Watson (2007[41]). Numbers represent distance caudal from bregma. Some symbols overlap others. Closed circles, injection of CTOP; open circles, injection of .9% NaCl.

Acupuncture-Induced Antinociception Is Prevented by a Procedure Known to Block Ascending Nociceptive Control at the Nucleus Accumbens

To evaluate whether the blockade of ANC at the nucleus accumbens affects acupuncture-induced antinociception, we evaluated the ability of intra–nucleus accumbens administration of CTOP, at a dose known to block ANC-induced antinociception,[51] to decrease acupuncture-induced antinociception. As shown in Fig 4A, the antinociceptive effect of acupuncture at ST36 on orofacial formalin test was prevented by previous intra–nucleus accumbens administration (prior to acupuncture) of the μ-opioid receptor antagonist CTOP (1 μg). There is a statistically significant interaction between acupuncture and CTOP treatment (2-way ANOVA and Tukey's test, F = 7.33, *P* = .013; Fig 4A). Fig 4B shows the correct nucleus accumbens injection sites. This finding demonstrates that like ANC-induced analgesia, acupuncture-induced analgesia depends on μ-opioid receptor mechanisms on nucleus accumbens, supporting the idea that ANC contributes to acupuncture-induced analgesia.

Acupuncture-Induced Antinociception Is Prevented by a Procedure Known to Block Ascending Nociceptive Control at Rostral Ventral Medulla

To evaluate whether the blockade of ANC at RVM affects acupuncture-induced antinociception, we

evaluated the ability of intra-RVM administration of mecamylamine, at a dose known to block ANC-induced antinociception,[17] to decrease acupuncture-induced antinociception. As shown in Fig 5A, the antinociceptive effect of acupuncture at ST36 on orofacial formalin test was prevented by previous intra-RVM administration of the nicotinic acetylcholine receptor antagonist mecamylamine (.6 μg). There is a statistically significant interaction between acupuncture and mecamylamine treatment (2-way ANOVA and Tukey's test, F = 6.07, *P* = .03; Fig 5A). Fig 5B shows the correct RVM injection sites. This finding demonstrated that, like ANC-induced analgesia, acupuncture-induced analgesia depends on nicotinic cholinergic receptor mechanisms on RVM, supporting the idea that ANC contributes to acupuncture-induced analgesia.

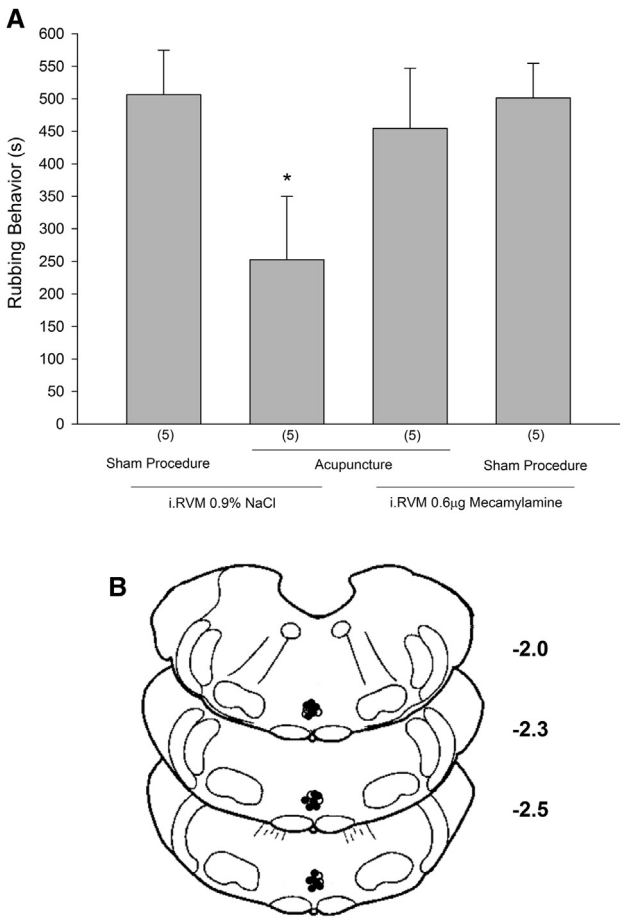

Figure 5. Pharmacologic blockade of nicotinic acetylcholine receptors located in rostral ventral medulla blocked acupuncture-induced antinociception. **(A)** The intra-RVM (i.RVM) microinjection of mecamylamine (immediately before acupuncture or sham treatment), at a dose known to block ANC-induced antinociception, prevented the antinociceptive effect of acupuncture on orofacial formalin test. The asterisk (*) indicates a nociceptive behavior significantly lower than that of the other groups. There is a statistically significant interaction between acupuncture and mecamylamine treatment (2-way ANOVA, Tukey's test, F = 6.07, *P* = .03). **(B)** RVM injection sites plotted on drawings adapted from the atlas of Paxinos and Watson.[41] Numbers represent distance caudal to the intra-aural line. Some symbols overlap others. Closed circles, injection of mecamylamine; open circles, injection of .9% NaCl.

Acupuncture at ST36 and Upper Lip Formalin Induce c-Fos Expression in Nucleus Accumbens and in Rostral Ventral Medulla

The c-Fos protein is rapidly and transiently expressed in neurons in response to stimulation and, therefore, has been used as a marker of neuronal activity. Therefore, we quantified c-Fos expression in order to evaluate the effect of acupuncture and/or of the noxious stimulation used in this study (upper lip formalin) in the neuronal activity in nucleus accumbens and RVM. As shown in Fig 6, c-Fos expression in nucleus accumbens was significantly increased by acupuncture at ST36 (F = 12.34, *P* = .002) *or* by formalin injection into the upper lip (F = 6.21, *P* = .02). c-Fos expression was significantly higher in animals receiving both acupuncture in the hind paw *and* formalin into the upper lip (2-way ANOVA and Tukey's test). As shown in Fig 7, c-Fos expression in rostral ventral medulla was not significantly increased by acupuncture at ST36 (F = 1.62, *P* = .21) *or* by formalin injection into the upper lip (F = .74, *P* = .40; 2-way ANOVA). Although not statistically significant, the alterations in c-Fos expression in RVM in response to acupuncture and/or upper lip formalin exhibit the same rising tendency observed in nucleus accumbens.

Discussion

This study shows a novel mechanism involved in acupuncture-induced analgesia, the activation of the spino-striato-RVM pain modulation pathway ANC. This is supported by the findings that the pharmacologic blockade of ANC at the spinal cord, nucleus accumbens, or RVM prevents acupuncture-induced analgesia.

Although ancient, acupuncture came into scientific focus in the last 3 decades, when numerous experimental and clinical studies have demonstrated its effectiveness in reducing pain compared to no-acupuncture controls in different circumstances.[36,53,54,59,62] Recently, however, numerous large, rigorous, randomized controlled trials have shown that compared to real acupuncture, placebo controls such as sham (superficial needling of nonacupuncture points) or simulated (stimulation of the skin over the acupoint without needling) acupuncture is similarly effective in alleviating clinical pain.[4,10,55,56] These findings have led to the suggestion that the acupuncture-induced analgesia might be largely due to nonspecific effects rather than the specific effects of needling. However, it is important to point out that procedures involving any kind of skin stimulation have been considered not valid as inert controls to acupuncture because they evoke activity in cutaneous afferent nerves[34] resulting in emotional and hormonal reactions, which in turn alleviate the affective component of pain.[33] This could help to explain why clinical trials evaluating acupuncture for pain relief have failed to find that real acupuncture is more effective than sham or simulated acupuncture.[4,10,55,57] Further studies are needed to address this possibility as well as to investigate the differences in the neural basis of acupuncture- and

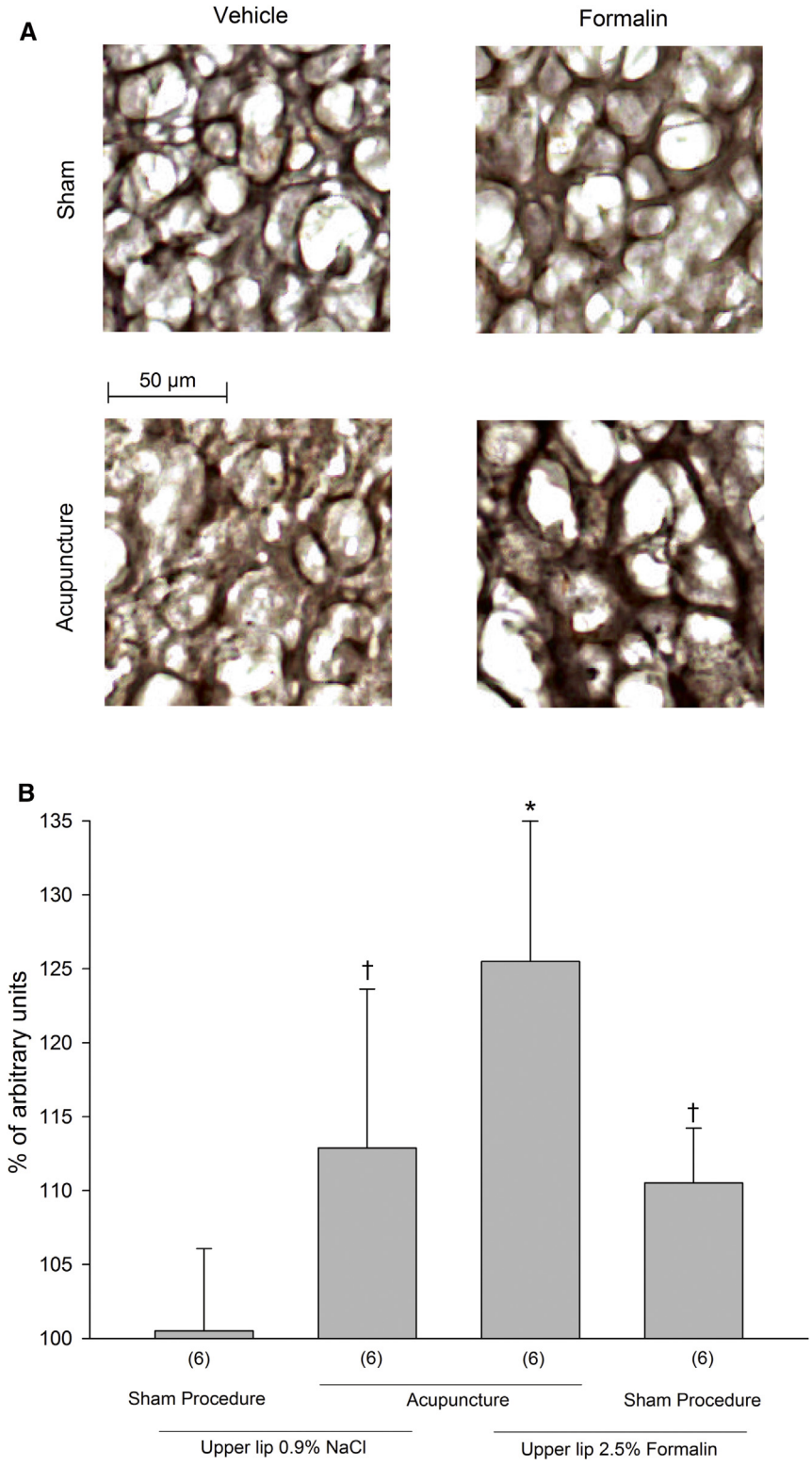

Figure 6. c-Fos expression in nucleus accumbens. **(A)** Photomicrograph of representative sections for each group of c-Fos immunoreactive (c-Fos-ir) cells in nucleus accumbens. **(B)** Optical density of c-Fos-ir cells in nucleus accumbens. The asterisk (*) indicates an optical density significantly higher than that of the other groups. The dagger symbol (†) indicates an optical density significant higher than that of the control group. There is no statistically significant interaction between acupuncture and formalin treatment (2-way ANOVA, Tukey's test, F = .291, *P* = .596). The optical density of c-Fos-ir cells in each group was expressed as a percentage of the mean optical density of the control group (animals receiving .9% NaCl in the upper lip and sham procedure).

placebo-induced analgesia. However, the study of the mechanisms underlying the specific effects of acupuncture analgesia is limited in humans for ethical and technical reasons, such as the difficulty in defining a valid placebo control, because any kind of sensory stimulus may have a specific effect. In this regard, animal studies

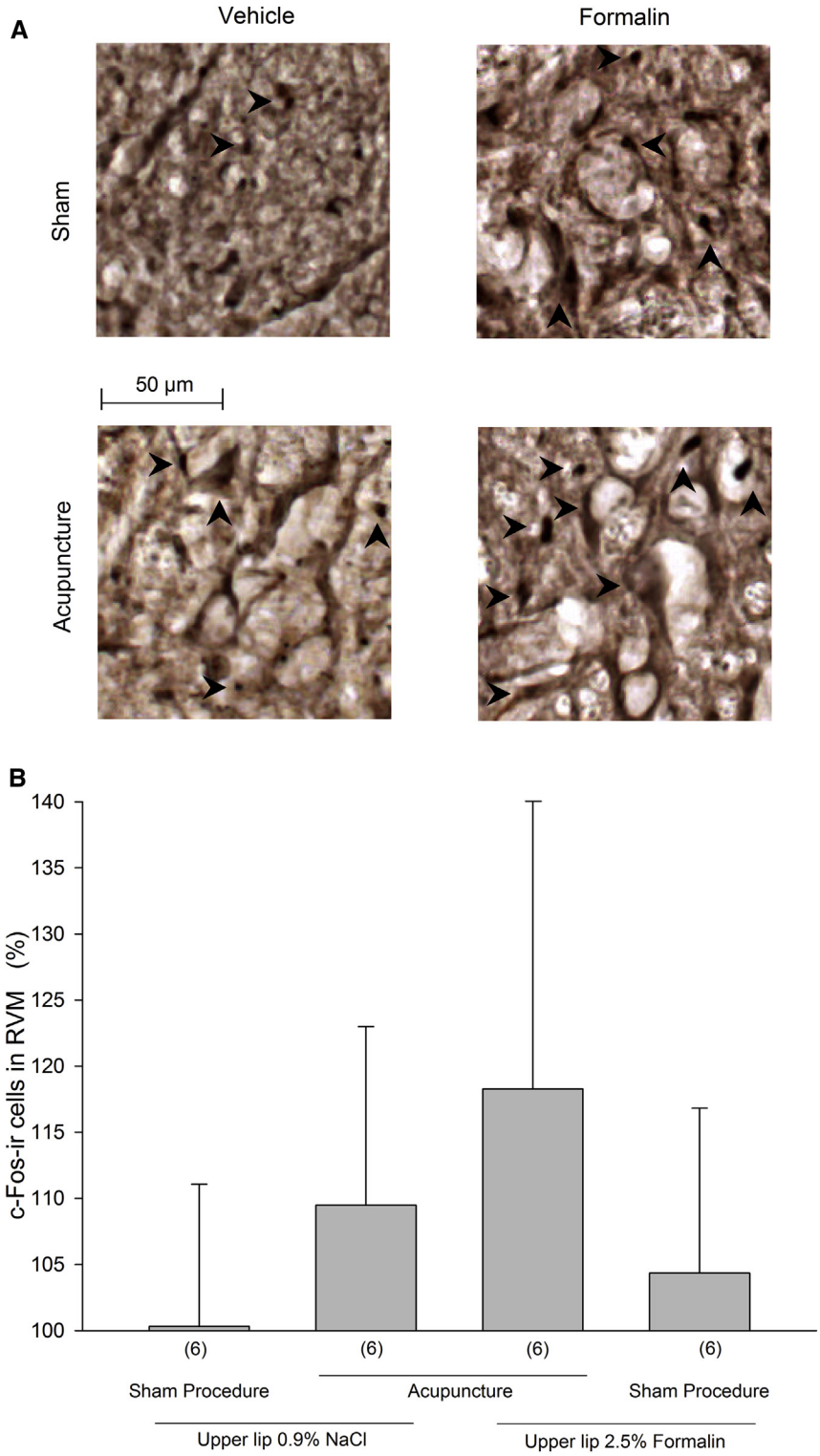

Figure 7. c-Fos expression in rostral ventral medulla. **(A)** Photomicrograph of representative sections for each group of c-Fos immunoreactive (c-Fos-ir) cells in RVM. Arrows indicate the c-Fos-ir neurons within RVM. **(B)** Number of c-Fos-ir cells in RVM. There is no statistically significant interaction between acupuncture and formalin treatment (2-way ANOVA, Tukey's test, $F = .0333$, $P = .857$). The number of c-Fos-ir cells in each group was expressed as a percentage of the mean number of the control group (animals receiving .9% NaCl in the upper lip and sham procedure).

may be of great value. Some of the mechanisms mediating acupuncture analgesia in animal studies, such as endogenous opioids release,[7-9,24] have also been demonstrated in human studies.[11,35,47] In the present study, we demonstrated that in a rat model of experimentally induced acute pain, acupuncture reduces nociception by activating the ANC. Although difficult to test experimentally, it is possible that ANC also contributes to acupuncture analgesia in humans. However, it is important to point out that even if it

occurs, ANC certainly is one of the neural mechanisms underlying acupuncture analgesia. How it interacts with the other underlying mechanisms, such as endogenous opioid release,[21] descending inhibitory system,[49] DNIC,[2] or the nonspecific effects attributed to acupuncture[4,10,55,57] is unclear.

In this study, we demonstrated that unilateral manual acupuncture at ST36, an acupoint located in the hind limb of rats, induces a potent heterosegmental antinociceptive effect in the orofacial formalin test (Fig 1). The magnitude of this antinociceptive effect was similar to that induced by noxious stimulation of the hind paw via an intraplantar injection of capsaicin (Fig 2A), which is classically used to trigger ANC[18] and to selectively activate C-fibers.[5] Therefore, peripheral C-fiber stimulation is the first step in inducing heterosegmental antinociception mediated by ANC. Stimulation of C-fibers in the acupoint region is also the first step in inducing analgesia at a site remote from the acupoint.[27,32,39,64] In fact, we showed that sciatic C-fiber depletion prevented the antinociceptive effect of acupuncture at ST36 on the orofacial region (Fig 2B). Although primary C-fiber inputs are necessary for the initiation of ANC- and acupuncture-mediated analgesia, they are not required for maintenance. Thus, the analgesia mediated by acupuncture lasts for hours after needle removal,[60] and the analgesia mediated by ANC also lasts for hours and is prevented but not reversed by peripheral nerve block.[18,50] The simplest explanation is that once initiated, the mechanisms underlying analgesia undergo centralization and no longer require ongoing peripheral input. The similarities in magnitude, time course, and mechanisms for initiation and maintenance support the idea that the mechanisms mediating ANC- and acupuncture-induced analgesia utilize the same pathway. To determine that ANC mediates the antinociceptive effect of acupuncture, we used pharmacologic tools to test the ability of procedures known to block ANC at different levels to block acupuncture-induced analgesia.

The first site for the transmission of primary afferent input is the spinal cord, where sensory information is integrated and processed. The demonstration of spinal mechanisms in acupuncture-induced analgesia dates from the time of the first studies on acupuncture. Although spinal inhibitory mechanisms are involved in the antinociceptive effect of acupuncture,[63] whether neuronal inputs that ascend from the spinal cord are important to acupuncture-induced analgesia is not clear.[6,20,30,43 44,46] Like acupuncture, ANC-mediated analgesia also depends on inhibitory mechanisms in the spinal cord because it is prevented by intrathecal administration of selective μ-opioid or $GABA_A$ receptor antagonists.[52] Here we showed that the antinociceptive effect induced by acupuncture at ST36 on the orofacial region is also prevented by previous intrathecal administration of selective μ-opioid (Fig 3A) or $GABA_A$ (Fig 3B) receptor antagonists, which is in accordance with previous studies using other experimental models.[8,29] These findings represent the first evidence to suggest that the mechanisms underlying ANC- and acupuncture-induced analgesia involve the same pathway.

Several supraspinal regions have been implicated in acupuncture-induced analgesia and among them is the nucleus accumbens,[63] a major component of the ventral striatum, involved in pain modulation and in substance abuse. The nucleus accumbens is also implicated in ANC-mediated antinociception, as it is prevented by intra–nucleus accumbens administration of a μ-opioid receptor antagonist. Here we showed that the antinociceptive effect induced by acupuncture at ST36 on the orofacial region is also prevented by intra–nucleus accumbens administration of a μ-opioid receptor antagonist (Fig 4A), which is in accordance with previous studies using other experimental models.[26,31] This finding represents the second piece of evidence to suggest that the mechanisms underlying ANC- and acupuncture-induced analgesia involve the same pathway.

A key supraspinal region involved in pain modulation[15,23] and in the mechanisms underlying acupuncture-induced analgesia[49] is the RVM, a brainstem region that includes the nucleus raphe magnus. ANC-mediated analgesia is also dependent on mechanisms in RVM, as it is prevented by the microinjection of a selective nicotinic acetylcholine receptor antagonist into the RVM.[17] Here we showed that the antinociceptive effect induced by acupuncture at ST36 on the orofacial region is also prevented by the administration of a selective nicotinic acetylcholine receptor antagonist into the RVM region (Fig 5A). To our knowledge, this is the first demonstration of a cholinergic mechanism in RVM mediating acupuncture-induced antinociception. This finding represents the third piece of evidence to suggest that the mechanisms underlying ANC- and acupuncture-induced analgesia involve the same pathway.

Acupuncture increased c-Fos expression at nucleus accumbens (Fig 6) and RVM (Fig 7, not statistically significant), which supports the idea that acupuncture by itself activates neuronal mechanisms at these regions. Numerous studies have demonstrated that acupuncture increases c-Fos expression in different brain areas involved in pain modulation,[12,13] including the RVM.[25] However, to our knowledge this is the first study showing that acupuncture by itself increases c-Fos expression at nucleus accumbens. The injection of formalin into the upper lip also increased c-Fos expression in the nucleus accumbens (Fig 6) and RVM (Fig 7, not statistically significant), which supports the idea that noxious stimulation of the upper lip by itself activates neuronal mechanisms at these regions. Noxious stimulation in several models is associated with an increase in c-Fos expression in different areas involved in pain modulation,[37] including RVM and nucleus accumbens.[38] When the animals received acupuncture *plus* formalin into the upper lip, c-Fos expression was higher than that induced by each one of these treatments alone. A cumulative effect of acupuncture and noxious stimulation on c-Fos expression had been previously demonstrated in other brain areas[58] and supports the idea that the stimuli interact to increase neuronal activity in nucleus accumbens and RVM.

The most investigated endogenous pain modulation mechanism is the descending system, in which periaqueductal gray matter (PAG) receives inputs from different brain areas and indirectly controls nociceptive transmission in the dorsal horn through the RVM. This system has been implicated in acupuncture analgesia.[49] Although it is possible that ANC represents an unrecognized element of the PAG-RVM pain modulation system, this system is mediated by endogenous opioids in both PAG and RVM whereas ANC is not. In addition, available evidence argues against PAG participation in RVM cholinergic mechanisms.[1] Another pain modulation system implicated in acupuncture-induced analgesia is the DNIC.[2] The analgesia mediated by DNIC resembles that of ANC because it also is activated by noxious stimulation. However, ANC-mediated analgesia lasts for hours and persists long after a local anesthetic blockade at the site of the initiating noxious stimulus,[18,50] whereas DNIC lasts less than 5 minutes in most studies and is dependent on the duration of the noxious stimulus.[28] DNIC- and ANC-mediated analgesia also differ in that DNIC is mediated in the caudal medulla and does not require brain circuitry rostral to that point.[3] Therefore, the antinociception produced by ANC and that produced by DNIC are likely to result from different mechanisms. This body of data, taken together with results of the present study, suggests that ANC is a newly identified mechanism underlying acupuncture-induced analgesia.

Recently, it was demonstrated by using animal models of diverse chronic pain syndromes, including cancer chemotherapy, alcoholic neuropathy, and stress-induced chronic widespread pain, that chronic pain adversely attenuates the activity of ANC by shortening the duration of capsaicin-induced analgesia.[14] These findings taken together with the current findings that ANC contributes to acupuncture analgesia suggest that some chronic pain syndromes can degrade the efficacy of acupuncture. Therefore, one important implication of our finding that acupuncture analgesia is mediated by ANC is that it helps to explain the variable efficacy and the short-lasting benefits,[61] as well as the limited therapeutic effect[4,10,57] of acupuncture in some chronic pain conditions. In addition, the attenuation in ANC activity by some chronic pain syndromes is sympathoadrenal dependent.[14] Thus, another important implication of this study is that treatments designed to mitigate the adverse effects of sympathoadrenal activation may be useful adjuncts to acupuncture and increase the duration of acupuncture analgesia across diverse pain syndromes.

In summary, this study demonstrated that acupuncture at an acupoint located on the hind limb induces heterosegmental antinociception similar to that induced by an intraplantar injection of capsaicin, a procedure classically used to activate ANC. The analgesia induced by acupuncture is blocked by procedures known to block ANC-mediated analgesia: 1) sciatic C-fiber depletion, 2) spinal administration of μ-opioid or $GABA_A$ receptor antagonists, 3) intra–nucleus accumbens administration of a μ-opioid receptor antagonist, or 4) intra-RVM administration of a nicotinic acetylcholine receptor antagonist. In addition, acupuncture and/or upper lip formalin induced c-Fos expression in nucleus accumbens and in rostral ventral medulla. Based on these results, we propose that ANC mediates the analgesic effect of acupuncture. Because ANC is a pain modulation pathway peripherally activated that ascends to supraspinal regions, it could be the link between the acupoint stimulation and the central mechanisms underlying acupuncture analgesia. Such findings contribute to understand the scientific basis underlying acupuncture analgesia.

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