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Acupuncture Resolves Persistent Pain and Neuroinflammation in a Mouse Model of Chronic Overlapping Pain Conditions

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Abstract

Patients with chronic overlapping pain conditions have decreased levels of catechol-O-methyltransferase (COMT), an enzyme that metabolizes catecholamines. Consistent with clinical syndromes, we previously demonstrated that COMT inhibition in rodents produces persistent pain and heightened immune responses. Here, we sought to determine the efficacy of manual acupuncture in resolving persistent pain and neuroinflammation in the classic inbred C57BL/6 strain and the 'rapid healing' MRL/MpJ strain. Mice received subcutaneous osmotic minipumps to deliver the COMT inhibitor OR486 or vehicle for 13 days. On day 7 following pump implantation, acupuncture was performed at the Zusanli (ST36) point or a non-acupoint for 6 consecutive days. Behavioral responses to mechanical stimuli were measured throughout the experiment. Immunohistochemical analysis of spinal phosphorylated p38 mitogen-activated kinase (p-p38 MAPK), a marker of inflammation, and glial fibrillary acidic protein (GFAP), a marker of astrogliosis, was performed on day 13. Results demonstrated that ST36, but not sham, acupuncture resolved mechanical hypersensitivity and reduced OR486-dependent increases in p-p38 and GFAP in both strains. The magnitude of the analgesic response was greater in MRL/MpJ mice. These findings indicate acupuncture as an effective treatment for persistent pain linked to abnormalities

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in catecholamine signaling and, further, that analgesic efficacy may be influenced by genetic differences.

Keywords

chronic pain; catecholamine; catechol-O-methyltransferase; glia; mitogen-activated kinase

Introduction

Chronic overlapping pain conditions (COPCs), such as fibromyalgia (FM), temporomandibular disorder (TMD), episodic migraine (EM), tension-type headache (TTH), and low back pain (LBP) affect over 100 million Americans and costs the US economy billions of dollars each year²². A hallmark of the COPCs is their idiopathic pathogenesis²⁰. Each individual condition produces pain unexplained by clinical findings such as injury or inflammation⁹⁷. Furthermore, each is characterized by pain that persists “beyond the normal time of healing”, thereby meeting the IASP definition of chronicity⁶⁷. While many studies report statistically significant overlap of chronic pain conditions¹, the clinical significance of the association is due to their common etiologic pathways⁹⁸. A pathway cited prominently in the literature is that regulating catecholamine bioavailability. Many patients with COPCs have increased levels of the catecholamines epinephrine and norepinephrine^{6, 23, 76, 96} alongside decreased levels of the catecholamine inactivating enzyme catechol-O-methyltransferase (COMT)^{62, 88}. Approximately 2/3 of patients with COPCs carry variants in the *COMT* gene that code for decreased levels of the COMT enzyme^{60, 70, 89} and predict increased risk of FM^{4, 17, 32, 63, 99} and TMD²¹. In pre-clinical studies, our lab showed that sustained COMT inhibition results in persistent pain evoked at multiple body sites and altered pain-related volitional behaviors^{37, 43, 71}. We also demonstrated that COMT-dependent pain is initiated by peripheral β_2 - and β_3 -adrenergic receptors (β_2 - and β_3 ARs) through release of the pro-inflammatory cytokines tumor necrosis factor- α (TNF α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6)^{15, 37}. Additionally, sustained stimulation of β_2 - and β_3 ARs leads to increased activation of astrocytes and microglia in the spinal cord¹¹⁴. Together, these findings suggest that heightened adrenergic tone, resulting from reduced COMT, promotes pain and inflammation in patients with COPCs.

Conventional pharmacologic treatments for COPCs include cyclo-oxygenase inhibitors, opioids, antidepressants, and anticonvulsants. These medications have poor efficacy for managing chronic pain and produce adverse side-effects, such as altered mental state, addiction, and life-threatening respiratory depression⁶⁴. Thus, there is urgent demand for safe and effective alternative treatments that can be used in the clinical setting to promote the resolution pain.

Acupuncture has traditionally been described as a therapeutic technique whereby needles are inserted at specific target points along meridians in the body. Despite outstanding questions regarding the specificity of acupuncture points and variability in treatment response, accumulating evidence from clinical trials conducted in the past decade demonstrate the efficacy of acupuncture for COPCs¹¹⁰. Acupuncture has been shown to alleviate pain and

improve function in individuals with FM¹⁰⁰, TMD¹⁰⁶, EM^{59, 116}, TTH⁵⁸, and LBP⁷⁹. Results from preclinical studies of neuropathic and inflammatory pain disorders suggest that the analgesic effects of acupuncture are due, in large part, to reducing the activity of glial cells involved in neuroinflammation^{54, 57}. However, to date, no preclinical studies have investigated the mechanisms underlying acupuncture for COPCs, and whether it minimizes neuroinflammation.

Acupuncture clinicians assert that needling restores the flow of vitality (or healing power), which is stagnant in patients with persistent pain and that those with higher vitality respond to acupuncture better. On this ground, we chose to study the effects of acupuncture in a mouse model of COPCs using two strains of mice: the classic inbred C57BL/6 strain and the MRL/MpJ strain known for their unique capacity to heal and regenerate following injury to nerves, muscles, or cartilage^{16, 26, 47, 95}. We hypothesized that acupuncture would alleviate pain and neuroinflammation in an animal model of COPCs and that ‘high vitality’ MRL/MpJ mice would respond to acupuncture better than C57BL/6 mice.

Methods

Animals

Adult 12–16 week-old male and female C57BL/6 (17–30 g, N=39) and MRL/MpJ (32–43 g, N=39) mice were bred in-house. They had *ad libitum* access to standard laboratory chow and water. All animal procedures were approved by the Institutional Animal Care and Use Committee at Duke University and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

General experimental conditions

Previous work has characterized a rodent model for the initiation of persistent pain associated with enhanced catecholaminergic tone, demonstrating that sustained administration of the COMT inhibitor OR486 leads to enhanced mechanical pain *via* β_2 - and β_3 AR-dependent increases in pro-inflammatory cytokines^{3, 37, 71}. Here, we aimed to determine the efficacy of acupuncture treatment in reversing COMT-dependent pain and inflammation in two strains of mice: the classic C57BL/6 inbred mouse strain and the rapid wound-healing MRL/MpJ strain. Mice from each strain were randomly assigned to one of four experimental groups (N=6–7 per group), to receive 1) vehicle alone (Veh), 2) OR486 alone (OR486), 3) OR486 + ST36 acupuncture (OR486+ST36), or 4) OR486 + sham acupuncture (OR486+sham). Additional mice were randomly assigned to one of two control groups (N=6–7 per group) to receive vehicle + Zusanli ST36 acupuncture (Veh+ST36) or vehicle + sham acupuncture (Veh+sham). Experimenters providing acupuncture were blinded to the drug condition. Experimenters performing pain assessments and quantifying p-p38 and GFAP fluorescence intensity were blinded to both the drug and acupuncture conditions.

Drug preparation

The COMT inhibitor OR486 (Tocris Bioscience, Bristol, UK) was dissolved in a vehicle consisting of a 5:2:3 ratio of dimethylsulfoxide (DMSO), ethanol and 0.9% saline. OR486 or vehicle solutions were injected into Alzet osmotic minipumps (model 1002, Durect Corporation, Cupertino, CA, USA) with a 100 µl reservoir volume expelled over 14 days, and a 0.25 µl/h infusion rate. Mini-pumps were placed in 15-ml conical tubes containing sterile 0.9% saline and primed overnight in a dry heat bath (Lab Armor, Cornelius, OR, USA) at 37°C. Subcutaneous delivery of OR486 was at a constant rate of 15 mg/kg/day over 13 days.

Surgical procedure

Mice were anesthetized by isoflurane (Baxter, San Juan, Puerto Rico) inhalation (5% induction, 2.5–3% maintenance). Incision sites were shaved and disinfected with ethanol and betadine. For systemic delivery of OR486 or vehicle, an approximately 1 cm incision was made over the back of the mouse. Hemostats were used to create a small subcutaneous pocket in which the pump was placed. After implanting the pump subcutaneously, stainless steel wound clips (Kent Scientific Corporation, Torrington, CT, USA) were used to close the wounds.

Behavioral testing

Mice were handled and habituated to the experimenter and environment for 4 days prior to testing. Behavioral testing was performed 1 day prior to osmotic pump implantation and 7, 9, 11 and 13 days after the implantation (Fig. 1A). Paw withdrawal threshold was assessed using the von Frey up–down method. Nine calibrated and logarithmically spaced von Frey monofilaments (bending forces: 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1 and 1.5 g; Stoelting, Wood Dale, IL, USA) were applied to the plantar hind paw. First, the middle filament (0.16 g) was applied to the hind paw for 3 s. If the mouse responded with a withdrawal, an incrementally lower filament was applied. In the absence of a withdrawal, an incrementally higher filament was applied. A series of six total responses were recorded for each paw. Results were entered into the Paw Flick module within the LabVIEW 2.0 software (National Instruments, Austin, TX, USA), which uses a logarithmic algorithm to determine the gram-force value that would elicit paw withdrawal in 50% of trials $(10(X_f + k\delta)/10,000)$, where X_f = value [in log units] of the final von Frey hair used; k = tabular value of positive and negative responses, and δ = mean difference [in log units] between stimuli). Mechanical allodynia was defined as a heightened response to a normally innocuous stimulus, as determined by a decrease in paw withdrawal threshold.

Mechanical hyperalgesia was assessed using a 1.5 g von Frey filament. This filament was chosen as a normally noxious stimulus, as it has a gram-force value well over the 50% withdraw threshold for animals tested in this study. The filament was applied to the hind paw 10 times for a duration of 1 s, with an interstimulus interval of 1 s. The number of paw withdrawals (which could range from 0 to 10) was recorded for each hind paw at each time point. Mechanical hyperalgesia was defined as an increase in the number of paw withdrawals in response to a normally noxious mechanical stimulus.

Acupuncture stimulation

ST36 acupuncture or sham acupuncture was performed immediately following behavioral testing on days 7–12 to determine the ability of these treatments to reverse COMT-dependent pain and inflammation (Fig. 1A). The Zusanli (ST36) acupoint was chosen because it is one of the most frequently used acupoints for the treatment of pain. Stimulation of ST36 has a systemic analgesic effect, suppressing pain in patients with conditions that affect many different body sites^{11, 12}. In pre-clinical studies, stimulation of ST36 suppresses neuropathic pain induced by paclitaxel¹⁴, oxaliplatin⁶⁹, inferior caudal trunk injury⁴², and spinal nerve ligation⁵⁴, as well as inflammatory pain induced by Complete Freund's Adjuvant^{24, 61}, collagenase⁸⁴, and carrageenan¹⁰⁸, and visceral pain induced by acetic acid¹⁰⁷. The sham point was chosen because of the scarcity of acupoints located in the region^{30, 35, 56}. The ST36 acupoint is near the knee joint of the hind limb and 1.5 mm lateral to the distal end of the anterior tubercle of the tibia, while the sham non-acupoint is located 1 cm distal to the tail base toward the semitendinosus (Fig. 1B). Compared to the sham point, ST36 is 1) highly vascularized⁴⁴, 2) innervated^{44, 86}, and 3) and rich with lymph nodes receiving sympathetic innervation⁷⁸.

Mice in the acupuncture-treated groups were gently held in an assistant's hand, while the experimenter inserted a stainless steel acupuncture needle (0.18 mm × 8mm; DongBang acupuncture, Boryeong, Korea) to a depth of 2 mm at the ST36 acupoint or sham non-acupoint, turned it at a rate of 3 revolutions per second for 20 s, and removed it immediately afterward. Mice first received acupuncture on the left side, followed by the right side (treatment lasting for a duration of 40 s). This treatment continued at 24-h intervals for 6 consecutive days (total of 6 times). Mice in the Veh and the OR486 alone groups were also lightly immobilized for 40 s without acupuncture and then returned to their cage.

Immunohistochemical staining

Following behavioral assessments on day 13, mice were deeply anesthetized with Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI, USA) (65 mg/kg i.p.) and perfused intracardially with 50 ml of ice-cold heparinized 0.1 M phosphate-buffered saline (Life Technologies, Carlsbad, CA, USA) followed by 25–50 ml of fixative (4% paraformaldehyde (ThermoFisher, Carlsbad, CA, USA) in 0.1 M phosphate-buffered saline) at a rate of 20 ml/min. The lumbar-sacral segment of the spinal cord was removed, post-fixed in 4% paraformaldehyde solution for 48 h, and immersed in 30% sucrose solution for storage at 4°C until sectioning. Transverse sections were cut to a thickness of 30 µm using a cryostat (HM 550, Microm International GmbH, Walldorf, Germany), incubated with 3% fetal bovine blocking serum in 0.01 M phosphate-buffered saline at room temperature for 1 h, then stained overnight at room temperature using a rabbit anti-phospho-p38 mitogen-activated protein kinase (p-p38 MAPK) primary antibody (1:800; Cell Signaling Technology, Beverly, MA, USA) or a mouse anti-glial fibrillary acidic protein (GFAP) primary antibody (1:800; Cell Signaling Technology, Beverly, MA, USA). On the following day, the sections were reacted with anti-rabbit Alexa-488 IgG (1:200; Jackson ImmunoResearch Inc., West Grove, PA, USA) or anti-mouse Alexa-594 IgG (1:200; Jackson ImmunoResearch Inc., West Grove, PA, USA) secondary antibodies for 1 h at room temperature. The tissues were then mounted on gelatin-coated slides, air-dried, dehydrated,

and coverslipped. Histological features of the dorsal horn of the spinal cord were captured using an Olympus IX51 microscope (Tokyo, Japan). The activation of astrocytes and the expression of p-p38 were determined by optical density using ImageJ software.

Statistical analyses

Group differences in mechanical allodynia and mechanical hyperalgesia over the 13-day experimental time course were analyzed by repeated measures 2-way ANOVA (group $\times$ time). Group differences in p-p38 or GFAP fluorescent intensity on day 13 following OR486 or vehicle delivery were analyzed by 1-way ANOVA. Post hoc comparisons of daily differences were performed using the Bonferroni test, which corrected for multiple comparisons. C57BL/6 and MRL/MpJ strain differences in baseline behavioral responses on day 0, in OR486-induced changes in behavioral responses on days 7-13, and in responses to acupuncture on days 9-13 were analyzed by t-test. Sex differences were analyzed by repeated measures MANOVA accounting for group and sex. Normality was assessed for each metric by group and day and when necessary log transformations were applied for hypothesis testing.

The primary analysis of mechanical allodynia and mechanical hyperalgesia in 4 groups with 6–7 mice each in a repeated measures ANOVA with 5 time points will achieve >84% power to detect a minimum F effect size of 0.30 (critical F value of 1.88) for the group by time interaction analysis at significance level 0.05. Previous studies have shown F statistics of this size and larger to indicate margins of difference that are worthy of future study^{13, 15, 71}, hence treatment of 6–7 mice in each group provides sufficient power to study similar magnitude effects. Statistical significance was defined as $P < 0.05$. Power and effect size calculations were performed a priori in G*Power v 3.1.9.2 and all statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) or SAS v9.4 (SAS Inc. Cary, NC).

Results

Acupuncture alleviates persistent pain in both C57BL/6 and MRL/MpJ mice

Here, we evaluated the analgesic efficacy of acupuncture in resolving persistent pain in two genetically-diverse strains of mice. Responses to mechanical stimuli were measured in separate groups of C57BL/6 or MRL/MpJ mice receiving continuous systemic delivery of either OR486 or vehicle over a 13-day period together with either ST36 or sham acupuncture treatment on days 7–12.

Among C57BL/6 mice we identified significant group differences in both mechanical allodynia ($F_{12,92}=7.93$, $P < 0.0001$) and mechanical hyperalgesia ($F_{12,92}=8.85$, $P < 0.0001$). Compared to C57BL/6 mice receiving vehicle, those receiving continuous systemic delivery of OR486 exhibited mechanical allodynia (Fig. 2A) and mechanical hyperalgesia (Fig. 2B) on days 7, 9, 11, and 13. ST36 acupuncture, but not sham acupuncture, alleviated mechanical allodynia measured on day 13 and mechanical hyperalgesia measured on days 11 and 13. No significant differences in behavioral responses were observed at any time among C57BL/6 mice in the Veh, Veh+ST36, and Veh+sham control groups (Supplemental

Fig. 1A and B), and, while the sample size was small, no significant differences in treatment effects were observed between males and females in any of the experimental groups (Supplemental Fig. 2).

Similar to the C57BL/6 mice, the MRL/MpJ mice exhibited significant group differences in mechanical allodynia ($F_{12,92}=10$, $P < 0.0001$) and mechanical hyperalgesia ($F_{12,92}=6.30$, $P < 0.0001$). The MRL/MpJ mice receiving continuous systemic delivery of OR486 exhibited mechanical allodynia (Fig. 2C) and mechanical hyperalgesia (Fig. 2D) on days 7, 9, 11, and 13, compared to those receiving vehicle. ST36 acupuncture, but not sham acupuncture, reversed mechanical allodynia and mechanical hyperalgesia measured on days 9, 11, and 13. No significant differences in behavioral responses were observed at any time among MRL/MpJ mice in the Veh, Veh+ST36, and Veh+sham control groups (Supplemental Fig. 1C and D), and, while the sample size was small, no significant differences in treatment effects were observed between males and females in any of the experimental groups (Supplemental Fig. 3).

The analgesic effect of acupuncture on persistent pain is greater in MRL/MpJ mice compared to C57BL/6 mice

To determine the contribution of individual variability to persistent pain and ST36 analgesic efficacy, we compared behavioral responses in C57BL/6 to those in MRL/MpJ mice, a genetically diverse strain with increased healing capacity, throughout the course of the experimental timeline. At baseline, MRL/MpJ mice exhibited lower pain responses compared to C57BL/6 mice, evidenced by a higher 50% paw withdrawal threshold ($t_{11}=3.67$, $P < 0.004$; Fig. 3A) and fewer number of paw withdrawals to the noxious 1.5 g monofilament ($t_{11}=2.21$, $P < 0.05$; Fig. 3B). Following COMT inhibition, MRL/MpJ mice had more pronounced pain. Compared to C57BL/6 mice, MRL/MpJ mice receiving OR486 exhibited a greater degree of mechanical allodynia ($t_{12}=7.73$, $P < 0.0001$; Fig. 3C) and mechanical hyperalgesia ($t_{12}=2.73$, $P < 0.02$; Fig. 3D) on days 7–13. Despite having more pronounced pain, MRL/MpJ mice were more responsive to acupuncture treatment. Compared to C57BL/6 mice, MRL/MpJ mice receiving ST36 acupuncture exhibited greater reductions in mechanical allodynia ($t_{12}=2.27$, $P < 0.05$; Fig. 3C) and mechanical hyperalgesia ($t_{12}=3.05$, $P < 0.02$; Fig. 3D) on days 9–13.

Acupuncture reduces the expression of spinal p-p38 MAPK

The p38 MAPK is an important mediator of persistent pain and inflammation. To evaluate changes in the activation (phosphorylation; p) of p38, we performed immunostaining in spinal cord sections collected on day 13 using an anti-p-p38 antibody. C57BL/6 mice receiving continuous systemic delivery of OR486 exhibited a 40% increase in p-p38 expression compared to those receiving vehicle ($F_{3,37}=6.76$, $P < .0009$; Fig. 4B). Similarly, MRL/MpJ mice receiving continuous systemic delivery of OR486 exhibited a 70% increase in p-p38 expression compared to those receiving vehicle ($F_{3,43}=3.47$, $P < .03$; Fig. 4C). The percentage of OR486-induced p-p38 expression in both strains was reduced by ST36 acupuncture, but not sham acupuncture.

Direct comparison of p-p38 staining in C57BL/6 and MRL/MpJ strains, demonstrated that group differences in p-p38 expression were similar for the two strains (Supplemental Fig. 4C). No significant differences in p-p38 staining were observed among C57BL/6 or MRL/MpJ mice in the Veh, Veh+ST36, and Veh+sham control groups (Supplemental Fig. 5B and 5D).

Acupuncture reduces the activation of spinal astrocytes

Astrocytes are abundant immune cells in the central nervous system (CNS) that play a critical role in the development and maintenance of persistent pain following nerve injury or inflammation⁷³. To evaluate changes in the activation of astrocytes in our mouse model of COPCs, we performed immunostaining of spinal cord sections collected on day 13 using an anti-GFAP antibody as a marker of astrogliosis. C57BL/6 mice receiving continuous systemic delivery of OR486 exhibited a 130% increase in GFAP+ astrocytes compared to those receiving vehicle ($F_{3,41}=6.76$, $P<.0009$; Fig. 5B). Similarly, MRL/MpJ mice receiving continuous systemic delivery of OR486 exhibited a 70% increase in GFAP+ astrocytes compared to those receiving vehicle ($F_{3,41}=7.47$, $P<.0004$; Fig. 5C). The percentage of OR486-induced GFAP+ astrocytes in both strains was reduced by ST36 acupuncture, but not sham acupuncture.

Direct comparison of GFAP staining in C57BL/6 and MRL/MpJ strains, demonstrated comparable levels of astrocyte activation following systemic delivery of vehicle. However, systemic delivery of OR486 led to greater increases in GFAP+ astrocytes in C57BL/6 mice compared to MRL/MpJ mice (Supplemental Fig. 4D). No significant differences in GFAP staining were observed among C57BL/6 or MRL/MpJ mice in the Veh, Veh+ST36, and Veh+sham control groups (Supplemental Fig. 5A and 5B).

Discussion

Acupuncture represents an effective complementary and integrative approach for the treatment of a wide variety of pain conditions. Pre-clinical studies in animal models of nerve injury and inflammation have demonstrated that the analgesic effects of acupuncture are mediated, in large part, through the resolution of inflammation in the periphery and CNS. The mechanisms underlying the analgesic effects of acupuncture for COPCs, however, have remained unknown. Here, we report that acupuncture at ST36 alleviates persistent pain and neuroinflammation linked to abnormalities in catecholamine signaling, and that the analgesic efficacy of acupuncture is influenced by genetic background.

Acupuncture alleviates persistent pain

COPCs are linked to COMT-dependent increases in catecholamine signaling. An estimated 66% of patients with COPCs carry 'low COMT activity' genetic variants^{60, 70, 89} associated with increased pain in response to experimental stimuli^{21, 118} and enhanced pain following stressful events^{65, 87} that potentiate catecholamine release from sympathetic nerves. While these findings seem inconsistent with the ability of antidepressants to alleviate persistent pain by increasing synaptic levels of catecholamines, it is important to note that the analgesic effect of antidepressants is associated with descending inhibition of pain *via*

actions at α_2 ARs or D₂DARs in the spinal dorsal horn^{68, 81}, while COMT-dependent pain is mediated by peripheral β_2 - and β_3 ARs¹⁵. Thus, catecholamines can exert divergent influences on nociception as a function of localization and net influence on neuronal excitability.

Using an animal model of sustained COMT inhibition that captures genetic and phenotypic characteristics of patients with COPCs, we found that acupuncture alleviated mechanical pressure pain, a hallmark feature of these syndromes. ST36, but not sham, acupuncture beginning on day 7 reversed mechanical allodynia and mechanical hyperalgesia exhibited by C57BL/6 and MRL/MpJ mice on days 9–13. While numerous studies have demonstrated the analgesic efficacy of acupuncture in models of neuropathic, inflammatory, and cancer-related pain¹¹³, this is the first to establish the benefit of acupuncture in a model of COPCs.

Acupuncture is a recommended treatment for FM¹⁰⁰, TMD¹⁰⁶, EM^{59, 116}, TTH⁵⁸, and LBP⁷⁹. Results from randomized controlled clinical trials have shown that manual or electrical stimulation of acupoints reduces pain and improves daily function, often long after the treatment is completed. Acupuncture has also been shown to outperform pregabalin and duloxetine for the treatment of FM¹⁰⁰. However, it is important to recognize that the effect sizes observed in clinical trials are typically smaller than those observed in this and other pre-clinical studies of acupuncture¹⁹. This may be attributed to multiple sources of variability inherent in human study participants, including variability in genetics, environment, disease etiology, symptom severity, and age, all of which may influence vitality, immune signaling, and analgesic response. This may also be attributed to methodological variability associated with clinical studies, including differences in acupuncture administration parameters, sham interventions, and blinding procedures. With the increasing availability and use of acupuncture in clinical practice^{80, 110, 115}, these represent urgent challenges faced by the acupuncture community. Thus, it is important that we investigate the genetic and biological basis of acupuncture in order to optimize, and possibly individually tailor, acupuncture interventions for the treatment of COPCs.

Acupuncture reduces neuroinflammation

COMT-dependent pain is initiated by peripheral β_2 - and β_3 ARs through release of the pro-inflammatory cytokines TNF α , IL-1 β , and IL-6 in plasma within hours^{15, 37}, and maintained by subsequent increases in pro-inflammatory cytokines and activation of glial cells in spinal tissues over the course of several weeks¹¹⁴. This transition from acute inflammation in peripheral tissues to persistent inflammation in neural tissues (neuroinflammation) is thought to play a critical role in the chronification of pain.

Thus, the present study evaluated the effect of acupuncture on neuroinflammation, characterized by increased p-p38 expression and astrocyte activation, in a mouse model of COPCs. Phospho-p38, expressed by primary afferent neurons⁷² and spinal cord microglia³⁹, drives the synthesis of pro-inflammatory cytokines and growth factors that sensitize nociceptive neurons and cause persistent pain. Of note, increased levels of p-p38 have also been found in patients with chronic TMD⁶⁶. Astrocytes are neuroectoderm-derived glial cells that regulate the release of glutamate, cytokines, and chemokines in spinal cord and brain¹⁸. Astrocytes remain active for prolonged periods and play a key role in the

maintenance of pain³⁸. We demonstrated that systemic delivery of OR486 for 13 days led to increased expression of p-p38, a marker of inflammation⁴⁰, and GFAP, a marker of activated astrocytes¹⁰¹, in the spinal dorsal horn of C57BL/6 and MRL/MpJ mice. Administration of ST36, but not sham, acupuncture on days 7-12 reduced OR486-dependent increases in p-p38 expression and astrocyte activation.

The ability of acupuncture to regulate p38 and glial activity in our animal model of COPCs is consistent with findings from studies of neuropathic and inflammatory pain. In models of nerve injury, ST36 stimulation reduces the activation of microglia and astrocytes and the production of TNF α and IL-1 β in spinal tissues^{28, 54}. Notably, the effect of ST36 acupuncture on injury-induced microglial activation was shown to be mediated by p-p38⁵³. In models of inflammation, stimulation of ST36 or other acupoints (e.g., Huantiao GB30 and Yanglingquan GB34) reduces the activation of glial cells in spinal tissues⁹³, the activation of macrophages and T cells in peripheral tissues^{103, 104} and the production of pro-inflammatory cytokines in circulating blood^{55, 103, 104}. Our results are also consistent with findings from clinical studies that link acupuncture's beneficial effects on pain to reduced inflammation. For example, intraoperative acupuncture has been shown to improve postoperative recovery and reduce analgesic use coincident with its ability to reduce plasma IL-1 β levels and increase production of the anti-inflammatory cytokine transforming growth factor- β 1 (TGF β 1)^{25, 31}. Studies of patients with painful inflammatory conditions such as rheumatoid arthritis and prostatitis have also demonstrated that the analgesic effects of acupuncture are associated with reduced plasma levels of TNF α and other pro-inflammatory cytokines following treatment^{74, 111}. By quieting the activity of spinal cord glia and peripheral immune cells, acupuncture may restore homeostasis in immune signaling so as to prevent or reverse pathological pain states.

Possible mechanisms underlying the effect of acupuncture on persistent pain and neuroinflammation

Acupuncture may produce its analgesic effects through dynamic alterations in the tissue and extracellular milieu which directly or indirectly influence the activity of innervating nociceptors. An acupuncture needle inserted to a depth of 2 mm, traverses the epidermis, dermis, adipose, fascia, and muscle. Manual stimulation at ST36 leads to activation of A α -, A β -, A δ -fibers innervating the skin and muscles of the hindlimb⁴¹. Manual twisting of the needle also generates micro injuries, characterized by cytoskeletal remodeling of subcutaneous connective tissue fibroblasts and deformation of extracellular matrix proteins^{45, 46}. Similar to other brief mechanical stimuli (e.g., shear stress and stretch), the twisting of acupuncture needles may induce the synthesis and release of cytokines, chemokines, and growth factors from skin, muscle, and endothelial cells^{2, 33, 82, 94}, which promote healing and resolve inflammation⁸⁵. Furthermore, acupuncture induces the local release of endogenous opioids (e.g., β -endorphin) and cannabinoids (e.g., anandamide) that act on peripheral nerve terminals to suppress the transduction of pain and on immune cells to decrease the production of pro-inflammatory cytokines in circulation^{9, 91, 92, 112}. Finally, acupuncture regulates the metabolism of catecholamines. Acupuncture decreases levels of norepinephrine and epinephrine in the plasma and renal tissues of spontaneously hypertensive rats¹⁰⁹ and levels of norepinephrine in the hypothalamus of alcoholic rats¹¹⁷.

Thus, acupuncture may alleviate pain and reduce neuroinflammation, in part, by restoring deficiencies in catecholamine metabolism.

Acupuncture analgesic efficacy is influenced by genetic variability

The analgesic effect of acupuncture varies among patients⁹⁰ and mouse strains¹⁰², such that some respond better than others. Identifying the intrinsic factors associated with acupuncture analgesic efficacy will help us understand the mechanisms by which acupuncture alleviates pain and guide treatment choices related to pain management.

According to traditional beliefs, chronic pain results from an insufficiency or blockage of vitality (similar to *Qi* or life force) and stimulation of acupuncture points along meridians (routes through which *Qi* travels) alleviates pain by restoring the flow of vitality⁴⁸.

Clinicians assert that patients with higher vitality respond to acupuncture better. Thus, the present study evaluated the effects of acupuncture on persistent pain in two strains of mice: the classic inbred C57BL/6 strain and the ‘rapid healing’ MRL/MpJ strain. MRL/MpJ mice have a unique regenerative capacity, observed following ear punch¹⁶ as well as injury to heart muscle⁴⁷, articular cartilage²⁶, digit tips⁸, and brain³⁴, suggestive of ‘high vitality.’ Compared to C57BL/6 mice, we found that MRL/MpJ mice exhibited a greater degree of COMT-dependent pain as well as a greater analgesic response to acupuncture, with behavioral responses returning to near-baseline levels after only two acupuncture treatments.

MRL/MpJ’s robust response to acupuncture is linked to their ability to effectively resolve inflammation. Compared to C57BL/6 mice, we found that MRL/MpJ mice exhibited reduced OR486-induced activation of astrocytes on day 13. These findings are in line with those from other studies demonstrating that MRL/MpJ mice exhibit reduced neural and peripheral inflammation relative to other strains. For example, MRL/MpJ mice have less astrocyte activation in spinal cord and brain following spinal cord injury^{34, 95} and reduced infiltration of macrophages and production of pro-inflammatory cytokines in local tissues following tibial fracture⁴⁹. Differences between MRL/MpJ and other inbred strains reflect variability in trait-relevant genes. Gene expression studies have found that MRL/MpJ mice have decreased expression of pro-inflammatory (e.g., calcitonin gene-related peptide β^{83}) and increased expression of anti-inflammatory (e.g., heme oxygenase-1⁷⁷) genes relative to C57BL/6 mice following injury^{51, 52, 95}. The mechanisms underlying MRL/MpJ’s increased response to OR486 in the absence of acupuncture are less clear, but may be related to their stronger initial innate immune response and secretion of cytokines and growth factors involved in both healing and nociception^{8, 52, 95}. The stronger initial immune response may then more effectively initiate the resolution of inflammation²⁷. Ultimately, these innate characteristics of MRL/MpJ mice, together with the anti-inflammatory effect of ST36 stimulation, likely work synergistically to more effectively resolve neuroinflammation and alleviate persistent pain.

Acupuncture analgesic efficacy is similar among males and females

As previous preclinical and clinical studies have reported sex differences in COMT-related phenotypes^{5, 29, 36, 75, 105} and COMT expression patterns^{7, 10}, we examined the effects of acupuncture in both males and females. We found that male and female mice in this study

exhibited comparable increases in mechanical pain following sustained delivery of OR486. While the sample size was limited, results were similar to our previous finding in a larger cohort of male and female rats³. Male and female mice also exhibited comparable reductions in pain following ST36 acupuncture. Nonetheless, future studies should continue to consider possible sex-specific effects of COMT-dependent pain and acupuncture treatment using larger sample sizes.

Conclusion

Based on findings obtained in our pre-clinical model of COPCs, we conclude that ST36 acupuncture reduces pain and neuroinflammation in two genetically-diverse strains of mice, with the rapid healing (higher vitality) MRL/MpJ strain exhibiting the greatest analgesic efficacy. Further studies are required to understand the genes, receptors, and signaling pathways that mediate the therapeutic effects of acupuncture as well as to identify biological markers and clinical characteristics predictive of treatment response in patients with COPCs or other pain syndromes (e.g. complex regional pain syndrome⁵⁰) linked to abnormalities in catecholamine signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sustained inhibition of COMT led to persistent pain and neuroinflammation in mice
- ST36, but not sham, acupuncture alleviated pain
- ST36 acupuncture reduced spinal p-p38 expression and astrocyte activation
- The magnitude of the analgesic response was greater in ‘rapid healing’ MRL/MpJ mice

Perspective

Chronic overlapping pain conditions (COPCs) remain ineffectively managed by conventional pharmacotherapies. Here, we demonstrate that acupuncture alleviates persistent pain and neuroinflammation linked to heightened catecholaminergic tone. Mice with superior healing capacity exhibit greater analgesic efficacy. Findings indicate acupuncture as an effective treatment for COPCs and provide insight into treatment response variability.

Figure 1. Experimental timeline and treatments

(A) Mice were handled and habituated to the experimenter and environment for 4 days (days -5 to -2) and baseline behavioral testing performed 1 day prior (day -1) to implantation of osmotic minipumps (day 0) for continuous delivery of OR486 (15 mg/kg/day) or vehicle for a duration of 13 days. Behavioral responses were re-evaluated on days 7, 9, 11, and 13 following pump implantation. ST36 or sham acupuncture treatment was performed on days 7–12, immediately following behavioral testing on days 7, 9, and 11. Tissues were collected on day 13 for immunohistochemical analyses. (B) Stainless steel acupuncture needles (0.18 mm × 8mm) were inserted to a depth of 2 mm either at the ST36 acupoint (a highly vascularized, innervated, lymph node rich area that is located near the knee joint of the hind limb and 1.5 mm lateral to the distal end of the anterior tubercle of the tibia) or the sham non-acupoint (a poorly vascularized and innervated area that is located 1 cm distal to the tail base) and turned at a rate of 3 revolutions per second for 20 s, and removed immediately afterward. Mice underwent this procedure from left to right acupoints in sequence, with the treatment lasting 40 s. B, behavioral testing; A, acupuncture treatment.

Figure 2. Acupuncture alleviates persistent pain in C57BL/6 and MRL/MpJ mice receiving OR486

Compared to vehicle, continuous systemic delivery of OR486 produces (A) mechanical allodynia and (B) mechanical hyperalgesia in C57BL/6 mice. ST36 acupuncture, but not sham acupuncture, reduced mechanical allodynia on day 13 and mechanical hyperalgesia on days 11 and 13. Similarly, continuous systemic delivery of OR486 produces (C) mechanical allodynia and (D) mechanical hyperalgesia in MRL/MpJ mice. ST36 acupuncture, but not sham acupuncture, reversed mechanical allodynia and mechanical hyperalgesia on days 9-13. N=6-7 mice (3 males and 3-4 females) per group. Data are shown as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 different from the Veh group. # P < 0.05, ## P < 0.01, ### P < 0.001 different from the OR486 group.

Figure 3. C57BL/6 and MRL/MpJ strains exhibit differences in baseline pain, persistent pain, and acupuncture treatment efficacy

At baseline, MRL/MpJ mice have (A) higher paw withdrawal thresholds and (B) fewer responses to repeated presentation of a noxious 1.5 g monofilament compared to C57BL/6 mice. Following continuous systemic delivery of OR486, MRL/MpJ mice develop more pronounced (C) mechanical allodynia and (D) mechanical hyperalgesia on days 7–13 compared to C57BL/6 mice. MRL/MpJ mice are also more responsive to ST36 acupuncture, such that mechanical allodynia and mechanical hyperalgesia are completely reversed on days 9–13. This is illustrated by the shading on the line graphs in panels C and D, which corresponds to the difference in mechanical hypersensitivity following ST36 acupuncture treatment as compared to baseline. N=6–7 mice (3 males and 3–4 females) per group. Data are shown as mean $\pm$ SD. * P < 0.05, ** P < 0.01 different between groups.

Figure 4. Acupuncture reduces expression of p-p38 in the spinal cord

(A) Immunohistochemical staining showing the expression of p-p38 on day 13 in separate groups of C57BL/6 and MRL/MpJ mice receiving continuous systemic delivery of OR486 or vehicle with either ST36 or sham acupuncture. Quantitative analysis of immunofluorescence intensity demonstrates that both (B) C57BL/6 mice and (C) MRL/MpJ mice exhibit OR486-induced increases in p-p38 expression that are reduced by ST36, but not sham, acupuncture. N=6–7 mice (3 males and 3–4 females) per group. Scale bar = 50 μ m. Data are shown as mean $\pm$ SD. * P < 0.05, *** P < 0.001 different from the Veh group. # P < 0.05, ### P < 0.001 different from the OR486 group.

Figure 5. Acupuncture reduces activation of astrocytes in the spinal cord

(A) Immunohistochemical staining showing the expression of the astrogliosis marker GFAP on day 13 in separate groups of C57BL/6 and MRL/MpJ mice receiving continuous systemic delivery of OR486 or vehicle with either ST36 or sham acupuncture. Quantitative analysis of immunofluorescence intensity demonstrates that both (B) C57BL/6 mice and (C) MRL/MpJ mice exhibit OR486-induced increases in GFAP expression that are reduced by ST36, but not sham, acupuncture. N=6–7 mice (3 males and 3–4 females) per group. Scale bar = 50 μ m. Data are shown as mean $\pm$ SD. * P < 0.05, ** P < 0.01 different from the Veh group. # P < 0.05 different from the OR486 group.