ORIGINAL ARTICLE

Failure of Placebo Analgesia Model in Rats with Inflammatory Pain

Xiang-Sha Yin¹ · Jin-Yu Yang¹ · Shuai Cao¹ · Yun Wang^{1,2}

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Abstract With the shifting role of placebos, there is a need to develop animal models of placebo analgesia and elucidate the mechanisms underlying the effect. In the present study, male Sprague-Dawley rats with chronic inflammatory pain caused by complete Freund's adjuvant (CFA) underwent a series of conditioning procedures, in which morphine was associated with different cues, but they failed to induce placebo analgesia. Then, conditioning with the conditioned place preference apparatus successfully induced analgesic expectancy and placebo analgesia in naïve rats but only induced analgesic expectancy and no analgesic effect in CFA rats. Subsequently, we found enhanced c-fos expression in the nucleus accumbens and reduced expression in the anterior cingulate cortex in naïve rats while c-fos expression in the anterior cingulate cortex in CFA rats was not altered. In summary, the behavioral conditioning model demonstrated the difficulty of

establishing a placebo analgesia model in rats with a pathological condition.

Keywords Placebo analgesia · Morphine · Conditioning · Inflammatory pain · Rat

Introduction

Chronic pain affects at least 20% of people worldwide and is a difficult problem to solve [1]. It affects many aspects of patients' quality of life and has caused the crisis of prescription opioid-related addiction and overdose [2, 3]. Recently, clinical and experimental studies have shown the efficacy of placebo analgesia, which could contribute to addressing this growing problem [4, 5]. In randomized controlled trials, placebo-associated improvements occur in 10%–60% of individuals with chronic pain [6–9]. The proper application of placebos may result in patients needing a lower cumulative amount of medication and thus having less severe side-effects [10, 11].

With the increasing interest in placebo treatments [12], a number of potential mechanisms have been proposed, including neurobiological mechanisms and psychological theories such as expectancy and classical conditioning [13]. Modern neuroimaging techniques suggest that placebos reduce pain-related brain responses and engage endogenous pain modulation circuitry *via* a μ -opioid or non-opioid mechanism [14–16]. Although human studies have successfully offered new insights into the mechanisms of placebo effects, there is still a strong need for animal models of placebo analgesia [17]. In human research, it is difficult to investigate mechanisms at the molecular and cellular levels while in animal models it is possible to conduct studies using techniques such as brain lesioning,

Xiang-Sha Yin and Jin-Yu Yang have contributed equally to this work.

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✉ Yun Wang
wangy66@bjmu.edu.cn

¹ Department of Neurobiology, School of Basic Medical Sciences and Neuroscience Research Institute, Key Laboratory for Neuroscience, Ministry of Education of China, and National Health Commission, State key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100083, China

² PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing 100871, China

genetic modification, and direct measurement of pain-related neurochemicals in the brain. Furthermore, it is easier to control environmental factors, drug administration, and previous experience in animal studies [18, 19].

Although a number of studies have reported non-analgesic placebo effects in animals in the past few decades [20, 21], there have been few reports on analgesic responses in mice or rats, and almost all existing animal models employ Pavlovian conditioning paradigms and measure behavioral pain responses in naïve animals [22–27]. However, studies using physiological pain do not resemble the real clinical situation. So far, only one study has used rats with severe neuropathic pain, but the results were not in agreement with other studies and they concluded that placebo analgesia in rats is not particularly robust [28]. Thus, there is still a need for a robust and easily-reproducible model of placebo analgesia in animals with pathological pain.

To overcome the limitations of previous models and elucidate the specific mechanisms underlying placebo analgesia in patients, we conducted a series of experiments in an attempt to develop a model of placebo analgesia in rats with inflammatory pain.

Materials and Methods

Animals

Male Sprague-Dawley rats (150–200 g) were used. The animals were housed under a 12 h light/dark cycle and the temperature was maintained at $(23 \pm 1)^\circ\text{C}$. Food and water were supplied to every cage *ad libitum*. The animals were given 5 days to acclimate after arrival. All experimental procedures were approved by the Animal Care and Use Committee of Peking University. Complete Freund's adjuvant (CFA) (100 μL per rat, Sigma-Aldrich, St. Louis, MO) was injected into the plantar surface of the hind paw while animals were under brief isoflurane anesthesia. The 50% CFA was a 1:1 mixture of complete and incomplete Freund's adjuvants. Morphine hydrochloride was dissolved in saline solution and administered by intraperitoneal injection at 5 mg/kg. Animals were randomly assigned into groups using a Microsoft Excel random number generator.

Hargreaves Test and Hot Plate Test

Thermal hyperalgesia was evaluated by measuring the paw-withdrawal latency in response to a radiant heat stimulus applied to the core of the plantar surface of the hind paw. Rats were placed on a clean glass platform and each rat had a transparent restraint shell. The radiant heat

source was adjusted to a range of 12 s–15 s as the baseline latency with a cutoff time of 30 s to prevent tissue damage. All rats were tested 3 times at 5-min intervals. The mean latency was used for statistical analysis.

The hot plate test was performed using a previously-described protocol [29]. The apparatus contained a metal plate and a removable transparent wall. The plate temperature was set at 50°C or 52°C and the latency to the first lick of the hind paw was recorded, with 30 s as the cutoff time. The tester of pain behavior assays was blinded to the animal groups (another experimenter injected drugs or saline).

Spontaneous Paw-Licking Measurements

The rats were placed on the apparatus used for the Hargreaves test and a video camera was set up under the transparent glass platform. Rats were left in the test room for 40 min of video recording. The first 10 min of video were not used, as during this time the rats were acclimating to the glass apparatus and test room. The video analyst was blinded to animal groups (another experimenter used a random number list to re-number the videos).

Conditioning Paradigms

Context Conditioning

Conditioning paradigms were simplified from a previous study [26]. Briefly, after CFA injection into the left hind paw, rats had two days to recover and reach a state of steady hyperalgesia after inflammation (Fig. 2A). The context conditioning included 4 consecutive days of training, with two injections of morphine (5 mg/kg) or saline at 08:00 or 20:00 each day. For instance, rats received a saline injection at 08:00 on day 1, 20:00 on day 2, 08:00 on day 3, and 20:00 on day 4 (Fig. 2A), with morphine injection at the vacant times. The interval between injections was 12 h to ensure complete metabolism and excretion of morphine. To associate the context cues with morphine analgesia, rats were placed in their home cages after saline injection, while after morphine injection, they were placed on the Hargreaves apparatus in the testing room for 30 min, after which they underwent the Hargreaves test.

After training, the rats received saline injections in the same routine as the 4 days of training at 08:00 on day 5. The Hargreaves test was used to measure the conditioned response to saline injection. The 30 min on the Hargreaves apparatus in a particular room along with the injection was assumed to be the conditioned stimulus (mainly context stimulus), and the morphine injection was the

unconditioned stimulus; the analgesia induced by morphine was considered to be the unconditioned response, whereas pain relief from the saline injection was the conditioned response (placebo effect).

At 20:00 on day 5, rats were given an injection of saline and the Hargreaves test to ensure that the CFA inflammation prevailed at the end of the context paradigms.

Refined Context Conditioning

To validate the context conditioning paradigms and enhance the link between morphine injection (unconditioned stimulus) and its analgesic effect (unconditioned response), we further refined the context conditioning (Fig. 2C) by eliminating the saline injections and adding a positive control (morphine injection every day) and a negative control (saline injection every day).

Before CFA injection, the baseline (day 1) thresholds for the Hargreaves test were measured. On day 3 before training, the thresholds were recorded to ensure equal inflammatory pain and the rats were randomly assigned into three groups. The morphine and placebo groups received morphine (5 mg/kg) each day and were placed on the Hargreaves apparatus for 30 min, whereas the saline group received saline each day. On day 8, the morphine group received a morphine injection, while the placebo and saline groups received a saline injection. The conditioned response was measured as the pain threshold of the Hargreaves test and compared with the placebo and saline groups.

Refined Context Conditioning Without the Hargreaves Test in Training

To avoid the unpleasant stimulus of the Hargreaves test every day, we further deleted Hargreaves test from training (Fig. 2E). Training and testing on day 7 was exactly same as previously. At 20:00 on day 7, all rats were given an injection of saline and the Hargreaves test to ensure that CFA inflammation prevailed at the end of the context paradigms. On day 8, the morphine and placebo groups were given morphine and the Hargreaves test to ensure that there was no tolerance to the repetitive injection of morphine.

Refined Context Conditioning with the Hot Plate Test

The hot plate was introduced to replace the Hargreaves test in a later training paradigm (Fig. 2G). Rats were randomly assigned into two groups. The 4 days of training and pain threshold testing were same as previously (refined context conditioning without the Hargreaves test in training).

Refined Context Conditioning with Conditioned Place Preference (CPP) Chambers

The conditioning paradigms were simplified from a previous study [29]. Briefly, rats were conditioned using a two-chamber CPP apparatus, with differences in litter (crushed corncob or sawdust) and in the shape of the lights. Before training, a preference test was performed. For the placebo group, the preferred chamber of an individual rat was paired with a saline injection; and the other chamber was paired with a morphine injection. The control group had the same pairing as the placebo group for two days and the opposite pairing for the other two days to avoid CPP for morphine. In 4 days of training, rats received one morphine and one saline injection each day at 08:00 and at 20:00. The morphine and saline injections were randomly arranged to avoid unwanted pairing to the injection time. After injection, each rat was placed in the paired chamber for 40 min and removed for the hot plate test. On day 5, the control and placebo groups received a saline injection and were placed in the saline-paired chamber and morphine-paired chamber, respectively, before the hot plate test. The test for place preference was carried out before the placebo test. The time in the morphine-paired chamber (t_1) and time in the saline-paired chamber (t_2) were used to calculate the CPP coefficient = $(t_1 - t_2)/(t_1 + t_2) \times 100$.

Rats with a CPP coefficient larger than 10 or smaller than -10 were given morphine in the least-preferred chamber in a 15-min pre-test, while rats with a CPP coefficient between -10 and 10 were paired in a counterbalanced pattern.

This experimental design assumed that morphine injection was the unconditioned stimulus, the analgesic effect induced by morphine was the unconditioned response, the morphine-paired chamber was the conditioned stimulus, and the pain relief after saline injection and being in the morphine-paired chamber were the conditioned response.

The tester in the pain behavior assays was blinded to animal groups (another experimenter injected drugs and placed animals in different chambers in CPP).

c-fos Immunofluorescence Assay

At 120 min after the placebo test, rats were anesthetized with 10% chloral hydrate (0.4 g/kg, i.p.) and perfused with 0.1 mol/L phosphate-buffered saline (PBS) followed by perfusion with 4% paraformaldehyde. The brains were then removed, post-fixed for 12 h, and cryo-protected in 20% and 30% sucrose in 0.1 mol/L PBS. Coronal sections were cut at 30 μ m on a cryostat (Leica 1900, Leica Microsystems Ltd., Nussloch, Germany). Nucleus accumbens (NAc) and anterior cingulate cortex (ACC) sections were determined using the Paxinos and Watson rat brain atlas.

Sections were rinsed 3 times in 0.1 mol/L PBS (5 min each) before blocking with a blocking solution (1% bovine serum albumin (BSA), 0.3% Triton X-100, 0.1 mol/L PBS) for 1 h at room temperature. The sections were then incubated with c-fos antibody (cat26192-1-AP-50, 1:200 dilution in 1% BSA, 0.3% Triton X-100, 0.1 mol/L PBS) for 24 h at 4 °C. The sections were rinsed 3 times in PBS (10 min each) and incubated with antibody tagged with Alexa Fluor 488 nm (catO-11038, 1:1000, 1% BSA, 0.3% Triton X-100, 0.1 mol/L PBS) for 12 h at 4 °C, then rinsed 3 times in PBS (10 min each) and covered with 1% glycerol.

Statistical Analysis

Data are presented as the mean $\pm$ SEM. Statistics were calculated using GraphPad Prism 6.0. Comparisons between two groups were made using Student's unpaired two-tailed *t*-test, or one-way ANOVA, or two-way repeated-measures ANOVA. Post-tests are named individually and were used in comparing two groups in particular columns. Statistical significance was defined as $P < 0.05$.

Results

Intraperitoneal Administration of Morphine Relieves the Thermal Hyperalgesia Induced by CFA Injection into the Hind Paw of Rats

A previous report indicated that Pavlovian conditioning with a cue induces placebo analgesia in naïve animals [26]. We used a similar paradigm to measure placebo analgesia in rats with inflammatory pain. We used CFA injection as an inflammatory pain model and the Hargreaves and hot plate tests (Fig. 1A). The latency to withdraw or lick the paw decreased after CFA injection, and this effect persisted for 12 days (Fig. 1B, $n = 5$ animals, two-way ANOVA, group effect: $F(1, 8) = 234.9$, $P < 0.001$; Sidak multiple comparisons test: $P > 0.05$ at baseline and $P < 0.001$ for 1–12 days). The degree of swelling had no correlation with the hyperalgesia (Fig. 1D). We measured changes in the thermal pain response after different doses of morphine in CFA rats (Fig. 1C, $n = 4$ animals, two-way ANOVA, group effect: $F(3,12) = 19.44$, $P < 0.001$; Turkey's multiple comparisons test: $P > 0.05$ for each group compared to saline pre-treatment, $P = 0.13$ for 2.5 mg/kg compared to saline treatment, $P < 0.001$ for 5.0 mg/kg and 10.0 mg/kg compared to saline treatment); 2.5 mg/kg morphine did not reduce the paw withdrawal latency and 10 mg/kg morphine had an apparent sedative effect. Moreover, 5 mg/kg morphine had a modest analgesic effect and little sedative

effect, so we used 5 mg/kg for the conditioning training paradigms.

Conditioning with Distinct Environmental Cues does not Elicit Placebo Analgesia in Rats

We found that i.p. morphine had a significant analgesic effect on CFA rats, with no accumulation or tolerance side-effects during training. However, the placebo injection group had no significant increase in paw withdrawal latency in the morning on day 5 compared to the saline injection in the afternoon on day 4 (Fig. 2B, $n = 12$ animals, $F(9,110) = 60.62$, one-way ANOVA with Dunnett's multiple comparison test, compared to baseline in the morning on day 1, $P < 0.001$, adjusted $P_{(5AM)} = 0.71$), indicating that conditioning with context does not induce placebo analgesia.

In the inflammatory hyperalgesia model, rats with cognitive deficits may ignore context in training so it is more difficult to induce placebo analgesia in them than in naïve rats [30, 31]. Thus we simplified the training paradigm by removing the saline injection (Figs. 2C and S1A). Rats received a morphine injection once a day and the pain response was measured 30 min after injection. We added a positive control group to verify morphine potency and a negative control group to confirm persistent hyperalgesia induced by CFA. We found that the negative control group had a shorter latency to withdraw the CFA-injected hind paw than the morphine-injected groups, and the positive control group had a longer latency to withdraw the hind paw, indicating a steady analgesic effect without any tolerance. The placebo group received saline at the same time as the morphine-injection training; the rats had no significant increase of paw withdrawal latency compared to the negative control group in CFA rats (Fig. 2D, $n = 8$ animals/group, two-way ANOVA, Turkey's multiple comparison test: $P < 0.01$ on days 4–7 for morphine *versus* saline and placebo *vs* saline, $P < 0.01$, morphine *vs* saline and morphine *vs* placebo at day 8, adjusted $P_{(8)} = 0.998$, placebo *vs* saline on day 8). However, we trained naïve rats with the training paradigm and found the placebo group had longer paw withdrawal latency than the control group on the test day (Fig. S1B, $n = 6$ animals/group, two-way ANOVA, Turkey's multiple comparison test: $P < 0.01$ on days 2–5 morphine *vs* saline and placebo *vs* saline; $P < 0.01$ on day 6, morphine *vs* saline and placebo *vs* saline; $P < 0.01$ on day 6, placebo *vs* morphine). In addition, there was no significant increase of paw withdrawal latency in 50% of CFA rats (Fig. S1C–D, $n = 8$ animals, two-way ANOVA, group effect: $F(1, 14) = 185.3$, $P < 0.01$; Sidak multiple comparison test: $P > 0.05$ on days 1, 3, and 8, and $P < 0.001$ on days 4–7).

Fig. 1 Morphine injection relieved CFA-induced hyperalgesia in rats. **A** Illustration of the CFA injection and Hargreaves test. **B** Paw withdrawal latency of the left hind paw in naïve and CFA rats ($n = 5$ animals, $***P < 0.001$, two-way ANOVA with Bonferroni *post hoc* tests). All the training paradigms and tests were performed from 3 to 8 days after CFA injection, when the thermal hyperalgesia was stable. **C** Paw withdrawal latency of the left hind paw in CFA rats after morphine injection ($n = 4$ animals, $***P < 0.001$, two-way ANOVA with Bonferroni *post hoc* tests). **D** Correlations between volume of swelling in the hind paw and paw withdrawal latency ($n = 24$ animals, Pearson $r = -0.05$, $P = 0.82$). The data are presented as the mean $\pm$ SEM.

Considering that the measurement of pain response is a negative experience for rats and interferes with attention and context-pairing learning during training [32], we further omitted everyday Hargreaves testing. However, we still found similar results on the test day (Fig. 2E–F, $n = 14$ animals/group, two-way ANOVA, Turkey's multiple comparisons test: $P < 0.001$ on day 7 (AM) for morphine *vs* saline and morphine *vs* placebo, adjusted $P_{(7PM)} = 0.68$ for placebo *vs* saline, $P < 0.001$ on day 8 for morphine *vs* saline and placebo *vs* saline).

The Hargreaves test measures paw-withdrawal behavior, and indicates a mainly reflexive response at the spinal level. However, the hot plate test integrates the pain response of the spinal cord and brain, and placebo analgesia is thought to occur in the brain [33]. Hence, we used the hot plate test to replace the Hargreaves test, but still did not succeed in conditioning placebo analgesia (Fig. 2G–H, $n = 15$ animals, two-way ANOVA, group effect: $F(1,28) = 10.39$, $P < 0.01$; Sidak multiple comparisons test: $P > 0.05$ on days 1, 3, and 8, and $P < 0.001$ on day 9).

In summary, all four paradigms of conditioning with context failed to elicit placebo analgesia in CFA rats.

Conditioning with CPP Chambers does not Elicit Placebo Analgesia in CFA Rats, while Reward Expectation is Intact

In a study of healthy rats [29], researchers divided the conditioning placebo model into two continuous segments: reward learning and placebo analgesia. Reward expectation is necessary for placebo analgesia formation. In CFA rats, consistent inflammation causes deficit in some parts of the establishment of placebo analgesia compared to naïve rats.

To further investigate which part is weakened in conditioning training models, we performed conditioning similar to that described by Lee *et al.* [29]. First, we trained naïve rats with conditioning procedures (Fig. 3A) and found that the placebo group had a longer paw licking latency than the control group on the test day when saline was injected (Fig. 3B–E). In particular, the placebo group had a significantly longer latency to lick the hind paw on the test day ($n = 10$ animals/group. Control, 9.68 ± 0.44 ; placebo, 14.39 ± 0.84 . Unpaired two tailed t -test: $t_{18} = 4.962$, $P < 0.001$), indicating a placebo analgesia in naïve rats. The CPP coefficient indicates a preference for the morphine-paired chamber; before training, the rats had no preference, and afterwards they established preference as presented by an increase in the CPP coefficient. The placebo group had an increased preference for the morphine-paired chamber (which represented the formation of

Fig. 2 Context conditioning did not produce placebo analgesia in CFA rats. **A** Schematic of the first context conditioning paradigm. **B** Paw withdrawal latency in CFA rats after first conditioning with context ($n = 12$ animals, $***P < 0.001$, one-way ANOVA with Turkey's test compared 1PM, 2AM, 3PM, 4AM to 1AM). **C** Schematic of the second context conditioning paradigm. **D** Paw withdrawal latency in CFA rats after second conditioning with context ($n = 8$ animals/group, $**P < 0.01$, two-way ANOVA with Turkey's test compared to day 8). **E** Schematic of the third context conditioning paradigm. **F** Paw withdrawal latency in CFA rats after third conditioning with context ($n = 14$ animals/group, $***P < 0.001$, two-way ANOVA with Turkey's test compared to day 6). **G** Schematic of fourth context conditioning paradigm. **H** Paw withdrawal latency in CFA rats after fourth conditioning with context ($n = 15$ animals/group, $***P < 0.001$, two-way ANOVA with Turkey's test compared to day 8). The data are presented as the mean $\pm$ SEM.

reward expectation); the control group did not exhibit this preference (Fig. 3F, $n = 8$ and 10 animals, two-way ANOVA, group effect: $F(1,16) = 1.753$, $P = 0.20$; time effect (CPP training): $F(1,16) = 8.366$, $P = 0.01$; Sidak multiple comparisons test: Test1 vs Test2, $**P < 0.01$ for placebo group, $P > 0.05$ for control group; control vs placebo, $P_{\text{test2}} = 0.077$). These results in naïve rats showed that our conditioning course is convincing and reliable.

Interestingly, the placebo group had no significant change in paw licking latency when compared to the control group in CFA rats (Fig. 4A–F). Most importantly,

the placebo group showed no difference in paw licking latency on the test day ($n = 16$ animals/group, Control, 5.86 ± 0.43 s; placebo, 5.96 ± 0.63 s; $P = 0.89$, two-tailed unpaired t test), indicating that no placebo analgesia was induced by CPP training. However, only the placebo group shifted their preference to the morphine-paired chamber ($n = 16$ animals/group, two-way ANOVA, group effect: $F(1,30) = 0.997$, $P = 0.326$; time effect (CPP training): $F(1,16) = 13.72$, $P < 0.001$; Sidak multiple comparisons test: Test 1 vs Test 2, $P < 0.01$ for placebo group, $P > 0.05$ for control group; control vs placebo,

Fig. 3 The conditioning procedure induced placebo analgesia and preference for the morphine-associated chamber in naïve rats. **A** Cartoons of the conditioning procedure with the CPP apparatus. **B–D** Hind paw licking latency in naïve rats during conditioning training and testing in the control and placebo groups ($n = 10$ animals/group, $**P < 0.01$, $***P < 0.001$, two-way ANOVA with Sidak *post hoc* tests). **E** Hind paw licking latency in naïve rats that received conditioning training (control and placebo groups) on the test day after receiving saline injection ($***P < 0.001$, unpaired *t* test with Welch correction). **F** CPP scores of CFA rats before and after conditioning training ($**P < 0.01$, two-way ANOVA with Sidak *post hoc* tests). The data are presented as the mean $\pm$ SEM.

$P > 0.05$ for Test 1 and Test 2). This finding indicates that CFA rats do not experience placebo analgesia, yet they still display reward expectation.

To confirm the behavioral changes in naïve and CFA rats, we used immunofluorescence to investigate c-fos activation in the brain regions associated with reward-learning (NAc) and pain-processing (ACC) (Fig. 5A–B). In naïve rats (Fig. 5C–F), the placebo group had fewer c-fos-positive cells in the ACC than the control group (Fig. 5D; 11.4% of cells were c-fos-positive in the control group compared to 24% in the placebo group, $P < 0.01$). Naïve rats also had more c-fos-positive cells in the NAc (Fig. 5F; 20.8% of cells were c-fos-positive in the control group compared to 3.0% in the placebo group, $P < 0.01$). In CFA rats (Fig. 5G–J), the placebo group had more c-fos-positive cells in the NAc (Fig. 5H; 2.1% of cells were c-fos-positive in the control group compared to 17.1% in the placebo

group, $P < 0.001$). However, the c-fos-positive cells in the ACC were not significantly different from the control group (Fig. 5J; 31% of cells were c-fos-positive in the control group compared to 30.2% in the placebo group, $P > 0.05$). These findings were consistent with the behavioral changes.

Since the pain response induced by noxious stimuli like von Frey filaments can disturb the measurement of placebo analgesia [28], we recorded the number of occurrences as well as the total duration of spontaneous hind paw-licking behavior during a 30-min period (Fig. 6A–F). Intraperitoneal administration of morphine reduced the spontaneous licking time of CFA rats (53.8 s for the control group, 8.4 s for the placebo group at day 7, $P < 0.001$) and the counts of licking behaviors (2.1 for the control group, 0.7 for the placebo group at day 8, $P < 0.001$). Nevertheless, we

Fig. 4 Conditioning procedure induced preference for the morphine-associated chamber, with no placebo analgesia in CFA rats. **A** Cartoons of conditioning procedure with CPP apparatus. **B–D** Hind paw licking latency in CFA rats that received conditioning training (control and placebo groups) ($n = 16$ animals/group, $**P < 0.01$, two-way ANOVA with Sidak *post hoc* tests). **E** Paw licking latency of hind paws in CFA rats with conditioning training (control and placebo groups) on the test day after receiving a saline injection ($P = 0.89$, unpaired *t* test). **F** CPP score of CFA rats before and after conditioning training ($***P < 0.001$, two-way ANOVA with Sidak *post hoc* tests). Data are presented as the mean $\pm$ SEM.

found no success in repeated conditioning placebo analgesia in CFA rats.

Discussion

As previously noted, there is a strong need for animal models of placebo analgesia at present, especially in animals with pathological pain; these models will contribute to elucidating the mechanism underlying placebo effects in patients and may provide patients with additional placebo-based benefits [34, 35]. However, our experiments illustrate the difficulty in eliciting robust placebo analgesia in rats with pathological pain (Fig. 7). None of our

experiments found evidence of the placebo effect, which is in agreement with the research on rats with an L5 spinal nerve lesion [28] but contrasts with other experiments on healthy rats [23, 24, 26]. Therefore, our research confirms that a model of placebo analgesia in rats with pathological pain is quite elusive and that placebo analgesia is more difficult to induce in rats than in humans. The primary findings of our study can be separated into two parts.

In part one, we tried to establish a placebo analgesia model in rats with inflammatory pain using context conditioning paradigms, which have successfully induced placebo analgesia in naïve rats in previous studies as well as our experiments. Unfortunately, there were no statistically significant differences between the placebo and

Fig. 5 The conditioning procedure increased the percentage of c-fos-positive cells in the NAc in both naïve and CFA rats, but decreased it in ACC in naïve rats. **A, B** Illustration of the ACC and NAc according to the brain atlas. Scale bars, 500 μ m. **C–F** Left, representative images of c-fos immunofluorescence in the ACC (**C**) and NAc (**D**) in naïve rats and in the ACC (**E**) and NAc (**F**) of CFA rats; right, statistics for c-fos-positive cells in the corresponding groups. Scale bars, 100 μ m. ($n = 4$ animals/group, $**P < 0.01$, $***P < 0.001$, unpaired t test).

Fig. 6 Spontaneous pain behaviors did not change after the conditioning procedure in CFA rats. **A** Cartoon of the context conditioning paradigms. **B, D** Numbers (**B**) and durations (**D**) of spontaneous paw-licking behavior of CFA rats during conditioning and placebo testing ($*P < 0.05$, $**P < 0.01$ on day 7, two-way ANOVA with Sidak *post hoc* tests). **C, E** Statistics of **B** and **D**, respectively ($P > 0.05$, unpaired *t* test).

Fig. 7 Schematic of placebo analgesia in naive and CFA rats. Behavioral tests and immunostaining demonstrate that naive rats have both analgesia expectation and placebo analgesia, whereas CFA rats have intact brain function in reward learning yet diminished placebo analgesia under pathological conditions.

control groups on the test day. We explain these results as a lack of placebo analgesia in rats with pathological pain, which was further shown in the second part of our study. Here, we list some advantages of our models that contribute to the credibility of this study.

First, we used an appropriate model of pathological pain in rats. The previous model in rats with severe neuropathic pain that failed to induce the placebo effects recommended a milder pain condition [36, 37]. Most human studies of placebo analgesia were tested with mild experimental pain [38]. Furthermore, immune activation and inflammatory responses were involved in the processing of pathological pain states [39] and NSAIDs were used in healthy volunteers [40] and naïve animals [26] to induce robust placebo analgesia. Therefore, it can be inferred that placebo analgesia plays a role in the treatment of inflammatory pain. In addition, the CFA-induced chronic inflammatory pain model is relatively suitable. Our results suggest that this painful condition remained stable during the entire experiment. A challenge for most clinical experiments is controlling the spontaneous remission or fluctuation of disease symptoms [41–44].

Second, we attempted to develop a model of placebo analgesia in rats. We followed the most common model and failed to induce it, which suggests that placebo analgesia is more difficult to elicit in rats with pathological pain than in healthy rats. Therefore, we simplified the training paradigm and maximized the association between sensory cues and the experience of analgesia [45]. However, this strategy was also ineffective. And we also chose a dose of 50% CFA to rule out the possibility that the degree of inflammatory pain affected the manifestation of the placebo effect. Then, we thought that the daily measurement might have been an interference factor, so we repeated the experiments without it. Finally, we used the hot plate test to replace the Hargreaves test, because the former integrates the spinal and brain levels of the pain response while the latter mainly tests the reflexive response at the spinal level [46, 47]. Although we did not obtain positive results, we provided sufficient evidence to demonstrate that it is difficult to create a placebo analgesia model in rats with pathological pain using conventional context conditioning procedures.

Finally, we carefully selected the proper experimental design. For example, we selected the optimum dose of morphine to suppress pain (5 mg/kg) and our results suggested that this dose did not induce tolerance effects and was appropriate for our repeated-dose conditioning paradigm [48, 49]. Furthermore, no opiate-induced hyperalgesia was found in any of the experiments, in agreement with other studies [28, 50]. We also designed multiple controls, including comparisons between groups and self-controls.

In part two, we tried to determine why we failed to develop a model of placebo analgesia in rats with inflammatory pain. It is known that expectation plays an important role in the human placebo response [51, 52], which mainly results from learning and previous experience [53, 54]. Similarly, one animal study divided the placebo effect model into two parts (reward learning and the expression of placebo analgesia) and suggested that reward expectation is necessary for the formation of placebo analgesia [29]. To further investigate which part was affected in our models in rats with inflammatory pain, we designed a more complicated model that successfully induced placebo analgesia and preference for the morphine-associated chamber in naïve rats. We found that the reward expectation was unaffected in the rats with inflammatory pain, but there was no analgesia. It is known that humans can be classified as high- or low-responders to placebo effects [55–57], and it is possible that such subpopulations may exist in rats. We determined the distribution of individual data on the test day which revealed no deference between the two groups. In addition, we quantified the expression of c-fos in the ACC and the NAc as these areas are important for the pain response and reward expectation [58–61]. We found increased c-fos expression in the NAc but no differences in c-fos expression in the ACC of the placebo groups with inflammatory pain, which directly corresponded with the observed behaviors. Yet we must acknowledge that our interpretation of the brain regions activated has limitations. One of the reasons is that ACC is involved not only in sensory pain, but also in attention, emotion, cognition, and motivation [62–64]. Moreover, as NAc function in reward expectation and noxious stimuli changes [65, 66], we could not rule out the possibility that ACC activation may reflect motivational and emotional changes, and NAc activation may have implication for pain responses.

In general, the most plausible explanation is that the conditioning paradigm successfully elicited an expectation of treatment in rats that was then broken by the altered treatment on the test day. Reviewing past studies, most placebo research in naïve rats used thermal assays to measure placebo effects while human studies used subjective self-reports to measure pain [67]. In our experiments, we used the established method of testing the thermal pain response, which is a better measurement of evoked or reflexive pain and which may therefore violate the expression of placebo effects. In light of this, we next tested spontaneous pain [68, 69], a better indicator of affective pain, without any other change on the test day but this did not produce placebo analgesia either. Given this result, we must consider that the expectation of reward in rats in pathological condition is not enough to elicit placebo effects even though expectation is strongly

associated with placebo analgesia in humans. Chronic inflammatory pain results in hyperalgesia, which decreases the heat pain threshold in patients and animals, therefore the placebo effect may be too weak to relieve the severe pathological pain. Moreover, since placebo analgesia is a complex process involving multiple brain functions and psychological factors, it is difficult to completely mimic placebo analgesia in rats due to the cognitive gap between human and rats, especially under pathological conditions.

In summary, this series of experiments demonstrates the failure of placebo analgesia in rats with inflammatory pain. Rodents are unable to adequately model a phenomenon as complex as humans. We conclude that, at least in the present paradigm, placebo analgesia in rats with pathological pain is not feasible, and therefore we recommend considering the possibility that the topic is best studied in humans.

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