The opioid placebo analgesia is mediated exclusively through \( \mu \)-opioid receptor in rat

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Abstract

Placebo analgesia is one of the most robust and best-studied placebo effects. Recent researches suggest that placebo analgesia activated the \( \mu \)-opioid receptor signalling in the human brain. However, whether other opioid receptors are involved in the placebo analgesia remains unclear. We have previously evoked placebo responses in mice (Guo et al. 2010, 2011) and these mice may serve as a model for investigating placebo analgesia. In the present study, we tried to explore the site of action and types of opioid receptors involved in placebo response. Male Sprague–Dawley rats were trained with 10 mg/kg morphine for 4 d to establish the placebo analgesia model. This placebo analgesia can be blocked by injection of 5 mg/kg dose naloxone or by microinjection with naloxone (1, 3 or 10 \( \mu \)g/rat) into rostral anterior cingulate cortex (rACC). Then, animals were tested after intra-rACC microinjection of D-Phe-Cys-Tyr-Trp-Orn-Thr-NH\(_2\) (CTOP, a selective \( \mu \)-opioid receptor antagonist) or naltrindole (NTI, a highly selective \( \delta \)-opioid receptor antagonist) or nor-binaltorphimine (nor-BNI, a highly selective \( \kappa \)-opioid receptor antagonist). Our results showed that CTOP, but not NTI or nor-BNI, could reduce the pain threshold in placebo analgesia rats. It may be concluded that rACC is the key brain region involved in placebo analgesia and the opioid placebo analgesia is mediated exclusively through \( \mu \)-opioid receptor in rat.

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Introduction

Behavioural context can modulate neuronal activity in nociceptive and non-nociceptive somatosensory pathways (Schiff et al. 1999). Placebo analgesia is one of the most striking examples of the cognitive modulation of pain perception (Colloca & Benedetti, 2009). It represents an instance in which neural processes, through positive cognitive expectations, influence physical and neuropsychiatric states, a veritable example of ‘mind \( \times \) body’ interactions (Colloca et al. 2010, 2011). It is therefore not surprising that interest is emerging in understanding their underlying mechanisms. Although ample evidence exists that placebo analgesia reduce reported pain, the neurobiology of how placebo analgesia interact with nociceptive brain processes is relatively unexplored.

Pharmacological studies indicate that placebo analgesia can be antagonized by the opioid-antagonist naloxone, implicating that at least some aspects of placebo analgesia depend on the endogenous opioid system (Amanzio & Benedetti, 1999; Benedetti & Amanzio, 1997; Gracely et al. 1983; Levine et al. 1978). Recent neuroimaging data point towards the rostral anterior cingulate cortex (rACC) as a crucial cortical region involved in placebo analgesia. In a positron emission tomography study, Petrovic et al. (2002) demonstrated similarity in regional brain activation of exogenous opioid administration and systemic placebo analgesia, thus providing evidence of a link between placebo analgesia and the opioid system. According to their study, the rACC yielded increased activity during both placebo and opioid analgesia (Petrovic et al. 2002). Wager et al. (2004) investigated functional magnetic resonance imaging (fMRI) signal changes during the anticipation and experience of pain and found changed activity in pain-sensitive regions such as rACC when comparing the response to noxious stimuli applied to control and placebo cream-treated areas of the skin. In a recent fMRI study...
investigating the activation of the opioidergic descending pain control system in placebo analgesia, Eippert et al. (2008) have also found that the rACC was the important pain-modulatory cortical structures in behavioural and neural placebo effects as well as placebo-induced responses.

With a µ-opioid receptor-selective radiotrace, Zubieta et al. (2005) showed significant placebo-induced activation of µ-opioid receptor-mediated neurotransmission observed in both higher-order and subcortical brain regions. Regional activations were paralleled by lower ratings of pain intensity, reductions in its sensory and affective qualities. Wager et al. (2007) have demonstrated that the administration of a placebo with implied analgesic properties regionally activates a pain and stress inhibitory neurotransmitter system, the endogenous opioid system, through direct effects on the µ-opioid receptors. Also, the activation of the µ-opioid receptor system is associated with reductions in the sensory and affective ratings of the pain experience. In addition, Scott et al. (2008) reported that regional opioid activity was associated with the anticipated and subjectively perceived effectiveness of the placebo and reductions in continuous pain ratings. High placebo responses were associated with greater µ-opioid receptor-induced activity and nocebo responses were associated with a deactivation of µ-opioid receptor activity. However, whether δ- or κ-opioid receptors are involved in the placebo analgesia remains unclear.

Therefore, in the present study, we examine whether rACC is the key brain structure in the placebo analgesia and the effect of µ-, δ- and κ-opioid receptors in the placebo analgesia. To do this, we used an animal model of placebo response after morphine preconditioning, which is known to be mediated by endogenous opioids both in rodents (Guo et al. 2010) and in humans (Amanzio & Benedetti, 1999; Benedetti et al. 2007, 2011). Our results showed that µ-, but not δ- or κ-opioid receptors, could reduce the pain threshold in placebo analgesia rats. It may be concluded that rACC is the key brain region involved in placebo analgesia and the opioid placebo analgesia is mediated exclusively through µ-opioid receptor in rat.

**Material and method**

**Animal**

Male Sprague–Dawley rats weighing 200–220 g obtained from Beijing Vital River Laboratory Animal Technology Co. (China) were used in all experiments following an acclimation period of 1 wk to laboratory conditions. The animals were caged individually and maintained under a 12-h light–dark schedule (lights on 8:00 hours). Food and water were provided ad libitum. The rats were handled daily (4–5 min) by an experimenter over the last 5 d of this habituation time. All protocol presented in the study were approved by the Institutional Review Board of the Institute of Psychology, Chinese Academy of Sciences and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

**Drugs**

Morphine hydrochloride (First Pharmaceutical Factory of Shenyang, China) was dissolved in 0.9% saline to a final concentration of 2 mg/ml. Naloxone, the non-selective opioid receptor agonist, d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), a selective µ-opioid receptor antagonist (Quang & Schmidt, 2010), naltrindole (NTI), a highly selective δ-opioid receptor antagonist (Steinmiller & Young, 2008), norbinaltorphimine (nor-BNI), a highly selective κ-opioid receptor antagonist (Broadbear et al. 1994) were purchased from Sigma Company (USA).

**Apparatus**

The place compartments (30.5 × 24.1 × 21 cm) were purchased from Med Associates Inc. (USA). This unit includes a grid floor and blue light to produce the conditioned cue. The hot-plate test employed a commercially available apparatus consisting of an acrylic resin cage (diameter: 20 cm; height: 31 cm) and a thermo-controlled aluminium plate (Model RB-200; Chengdu TME Technology Co., China).

**Surgical and microinjection procedures**

The animals were anaesthetized with sodium pentobarbital (30 mg/kg i.p.) and positioned in a Kopf stereotaxic instrument. The 24-gauge stainless steel guide cannulae were placed unilaterally or bilaterally to the intended site of injection according to the atlas of Paxinos & Watson (1986). The rACC was set as follows: 2.7 mm anterior to bregma; 0.6 mm lateral to midline; 2.8 mm ventral to the surface of the cortex. Cannulae were secured to the skull by jewellers’ screws with dental acrylic. To prevent clogging, the stainless steel stylets (27-gauge) were placed in the guide cannulae until the animals were given the rACC microinjection. All animals were allowed 5 d to recover from surgery. For drug microinjection, the animals were gently restrained by hand; the stylets were removed from the
guide cannulae and replaced by 27-gauge injection needles, extending to 0.5 mm below the tip of the guide cannula in rACCs. Each microinjection unit was connected by polyethylene tubing to 1-μl Hamilton syringe. The left and right rACC were microinjected with a 0.5 μl solution (1 μl/rat) over a 2-min period. The injection cannula remained untouched for an additional 60 s to allow for absorption into the brain region and to minimize injectate along the track of the cannula.

**Experimental procedure**

**Placebo analgesia procedure**

The animal training procedure was performed as previously described (Guo et al. 2010, 2011). Briefly, rats were treated with saline (i.p.) a.m. on day 1 and p.m. on day 2 and with morphine (10 mg/kg i.p.) p.m. on day 1 and a.m. on day 2. They were then put into distinct cue chambers for 30 min before receiving the hot-plate test. The same procedure was repeated on days 3 and 4. On day 5, they were treated with saline or drug at 08:00 hours but placed into the cue compartments previously paired with morphine for 30 min before the scheduled hot-plate test.

**The effect of naloxone (i.p.) on the placebo analgesia**

Animals underwent placebo analgesia training for 4 d. On day 5, rats were treated with an injection of naloxone (0.5, 1.5 and 5.0 mg/kg i.p.) to test its effects on the morphine-induced placebo analgesia.

**The effects of microinjection of naloxone into the rACC on the placebo analgesia**

After recovery from surgery, four groups of animals underwent the placebo analgesia training for 4 d. On day 5, rats were given an intra-rACC injection with naloxone (1, 3 or 10 μg/rat) or saline 30-min before the hot-plate tests, respectively, and then paw withdraw latency data were collected.

**The effects of microinjection of CTOP, NTI and nor-BNI into the rACC shell on the placebo analgesia**

After recovery from surgery, animals underwent the placebo analgesia training for 4 d. On day 5, rats were given intra-rACC injection with CTOP (0.6, 2 or 6 μg/rat), NTI (3, 10 or 30 μg/rat), nor-BNI (6, 20 or 60 μg/rat) or saline 30-min before the hot-plate tests, respectively. Then, the paw withdraw latency data were collected.

**Fig. 1.** Morphine-induced placebo effect. After the procedure of morphine conditioning (10 mg/kg i.p.) on days 1–4, rats were injected with saline and put into the conditioned cue box for 30 min on day 5 at 08:00 hours. Paw withdrawal latency was significantly elevated, which mimics the morphine analgesic response. n = 14 rats per group. *p < 0.05, **p < 0.01, compared to the placebo analgesia group, ANOVA followed by post-hoc test.

**Histology**

At the end of the experiments, the rats were killed by decapitation, after which the location of the injection site was confirmed. Data from the individuals that were not verified by the presence of injection needle tips in the correct sites were discarded. The number of rats used for data analysis is shown in each figure.

**Statistical analysis**

All data are expressed as mean ± S.E.M. One-way analysis of variance followed by post-hoc test was used for multiple comparisons. A p value ≤ 0.05 was considered as indicative of a significant difference.

**Results**

**Induction of placebo analgesia**

As shown in Fig. 1, a significant difference was seen when the paw latency was measured for 5 consecutive days (F5,126 = 12.73, p < 0.001). The mean paw latency after saline injection on day 1 at 08:30 hours and day 2 at 20:30 hours was 12.20 ± 1.37 and 13.32 ± 1.19 s, respectively. When morphine was administered on day 1 at 20:00 hours and day 2 at 08:00 hours, a significant increase in pain tolerance was found (23.31 ± 2.66 and 23.86 ± 1.83 s, respectively; p < 0.01). Similar results were obtained on days 3 and 4 (p < 0.01). After saline treatment on day 5 at 08:00 hours and a 30-min exposure to the cue compartment, pain tolerance was significantly elevated.
Pain latency (all animals) and pain threshold were measured after three doses of naloxone (1, 3, 10 mg/kg i.p.) 30 min prior to the test. Paw withdrawal latency scores were expressed as mean ± S.E.M. *p < 0.05, **p < 0.01, compared to the placebo analgesia group, ANOVA followed by post-hoc test.

(18.99 ± 2.20 s) compared with a.m. on day 1 (the baseline, p < 0.01), indicating that previous morphine conditioning was sufficient to evoke a placebo effect.

**Effects of systemic naloxone (i.p.) on the placebo analgesia**

Figure 2 shows that systemic injection of naloxone produces a dose-related inhibition on the paw withdrawal latency (F$_{3,32}$=3.261, p < 0.01). Post-hoc analysis showed that naloxone at 5 mg/kg (p < 0.01) inhibited the placebo response. However, the 0.5 and 1.5 mg/kg doses of naloxone did not reduce the paw withdrawal latency.

**Effects of microinjection of naloxone into the rACC on the placebo analgesia**

To further locate the site of action, we performed microinjection of naloxone into the rACC and observed the effects on the morphine-induced placebo analgesia. Figure 3 shows that microinjection of naloxone also produces a dose-related inhibition on the paw withdrawal latency (F$_{3,34}$=9.933, p < 0.01). All three doses of naloxone (1, 3, 10 µg/rat) reduced the pain threshold (p < 0.05, p < 0.01 and p < 0.01, respectively). Moreover, drug injection outside rACC (in the prelimbic cortex or secondary motor cortex, 1 µg/rat: n = 6; 3 µg/rat: n = 7; 10 µg/rat: n = 7) did not alter the pain latency (all p > 0.05).

**Effects of microinjection of CTOP, nor-NBI or NTI into the rACC on the placebo analgesia**

To further study the types of opioid receptors involved in the placebo analgesia, we performed microinjection of CTOP, nor-NBI or NTI into the rACC. As can be seen in Fig. 4, CTOP dose-dependently inhibited the placebo analgesia (F$_{3,34}$=3.773, p < 0.01). CTOP at doses of 2 and 6 µg/rat significantly reduced the paw latency compared to the placebo group (p < 0.05 and p < 0.01, respectively). However, nor-NBI or NTI did not affect the placebo analgesia in rats.

**Histology**

Cannula and injection sites were located in the rACC as shown in Fig. 5.

**Discussion**

Following previous studies that showed the action of rACC (Eippert et al. 2009; Kong et al. 2006; Sarinopoulos et al. 2006; Scott et al. 2008; Wager et al. 2004) and the involvement of µ-opioid in placebo analgesia (Bingel et al. 2006; Scott et al. 2008; Wager et al. 2007; Zubieta et al. 2005), in the present study we tested the site of action and types of opioid receptors involved in placebo response in rats. Consistent with
previous research in humans, this study also showed that rACC plays a key role in opioid placebo analgesia. Moreover, the opioid placebo analgesia was blocked by microinjection of CTOP into rACC, a selective μ-opioid receptor antagonist, but not the δ- or κ-opioid receptor antagonists, NTI and nor-BNI, respectively, indicating that the opioid placebo analgesia is mediated exclusively through μ-opioid receptors in rat.

In order to evoke opioid-mediated placebo responses, we used the animal model of morphine pre-conditioning, whereby pharmacological pre-conditioning with an opioid drug is known to elicit placebo responses that are naloxone-reversible in mice (Guo et al. 2010) and in humans (Amanzio & Benedetti, 1999; Benedetti et al. 2007, 2011). After rats were given morphine for 4 d with the conditioned cue stimulus, saline injection with the contextual cue could produce placebo analgesia at day 5 and this placebo response was completely antagonized by naloxone. Therefore, this represents a nice animal model of opioid-mediated placebo responses that is amenable to experimental manipulation.

The rACC is located on the medial surface of the brain, ventral (subgenual) and anterior (pregenual) to the genu of the corpus callosum (Pizzagalli, 2011). This cortical territory has a well-established role in mood and emotional processing as well as being a key site of convergence for several neural pathways, neurotransmitters and neuromodulator systems implicated in pain (Bennett, 2010; Boes et al. 2008). The neural basis of placebo analgesia was first established by Levine et al. (1978), who discovered that the placebo response could be blocked by the opioid receptor antagonist naloxone. This indicates the involvement of the endogenous opioid system. Following this finding, complex experiments have been designed to confirm
this exciting and provocative hypothesis (Amanzio & Benedetti, 1999; Benedetti, 1996; Gracely et al. 1983; Zubieta et al. 2005). In line with this, a similar functional anatomy of opioid and placebo analgesia was found and the opioid-receptor-rich rACC is activated in both opioid and placebo analgesia conditions (Bingel et al. 2006; Eippert et al. 2009; Petrovic et al. 2002). Although the human literature described above is strongly suggestive of a role for the rACC in placebo analgesia, the evidence therein is indirect and/or correlative. The literature is conspicuously devoid of a definitive study, demonstrating the necessity of neural activity in the rACC for processing the placebo-induced analgesia. This deficiency is due partly to the limitations of placebo analgesia animal models. In the current study, we induced placebo analgesia in rats at the beginning of the experiment using a 4-d schedule of morphine conditioning (Guo et al. 2010, 2011). This placebo analgesia could be blocked by systemic injection of naloxone (5 mg/kg but not 0.5 or 1.5 mg/kg) administered 30 min prior to the hot-plate testing session. However, all three doses of naloxone (1, 3, 10 μg/rat) could reduce the pain threshold and inhibit the placebo response after microinjection into rACC in placebo analgesia rats. Moreover, drug injection outside this region did not alter the pain latency. This is the first study that has directly observed rACC activity involved in placebo analgesia. Our results suggest that rACC did play an important role in placebo analgesia.

A likely candidate for the mediation of placebo-induced analgesia is an opioid neuronal network in the cerebral cortex and the brainstem (Benedetti et al. 2005; Craggs et al. 2008; Price et al. 2008; Zubieta et al. 2005). This opioid network belongs to a descending pain-modulating pathway that, either directly or indirectly, connects the cerebral cortex to the brainstem. In particular, the rACC and the orbitofrontal cortex project to the periaqueductal grey (PAG), which in turn modulates the activity of the rostral ventromedial medulla (RVM). The rACC and the PAG, together with some other nuclei in the brainstem (e.g. the parabrachial nuclei), are rich in opioid receptors and could play an important role in placebo-induced analgesia. In fact, context-related cognitive cues could activate this opioid network in the cerebral cortex and the brainstem. There was a significant covariation in activity between the rACC and the PAG, suggesting that the descending rACC/PAG/RVM pain-modulating circuit is involved in placebo analgesia (Eippert et al. 2009; Kong et al. 2006). Through descending projections, this circuit controls both spinal and trigeminal dorsal horn pain transmission neurons and mediates both opioid and stimulation-produced analgesia (Kong et al. 2006; Qiu et al. 2009; Scott et al. 2008). Therefore, the rACC might not directly modulate pain processing, but rather exert its effect through subcortical pain modulating circuitry during the placebo-induced analgesia process.

High placebo responders have shown more pronounced rACC blood flow responses to the systemic administration of a μ-opioid receptor agonist, remifentanil, suggesting the presence of variations in the responses of this receptor system as a function of placebo response (Petrovic et al. 2002). μ-Opioid receptor-mediated neurotransmission is one of the principal systems involved in the modulation of pain (Matthes et al. 1996). The μ-opioid system has also been implicated in the regulation of behavioural responses to novel environments and stimulus–reward associations in studies using animal models devoid of these receptors (Filliol et al. 2000; Moles et al. 2004), as well as in the regulation of affective responses in humans (Wager et al. 2007; Zubieta et al. 2003). However, so far there is no study on other types of opioid receptors that are involved in placebo analgesia. At present, three distinct classes of opioid receptors, namely μ-, δ- or κ, are pharmacologically characterized (Minami & Satoh, 1995). Naloxone is considered as a non-selective opioid receptor antagonist. To determine which subtype of the opioid receptors was blocked by naloxone to reveal the placebo-induced analgesia, we studied the pain latency in the presence of CTOP, NTI and nor-BNI, which are, respectively, μ-, δ- or κ-opioid receptor selective antagonists. Microinjection rACC with CTOP (2 and 6 μg/rat) significantly inhibited the paw withdrawal latency, indicating that blockade of the μ-opioid receptor is sufficient to suppress placebo analgesia. Since NTI and nor-BNI were without effect in this regard, our results suggest that the placebo analgesia is a μ-opioid receptor-mediated event, supporting recent researches that suggest that placebo analgesia activates μ-opioid receptor signalling in the human brain (Wager et al. 2007; Zubieta et al. 2005).

The comparatively high opioid-antagonists dosages and the small target area (rACC) were investigated in this study. It is possible that part of the drugs will diffuse outside of the local brain area. However, we found that the rats with the cannula outside rACC showed a similar pain latency as the placebo control. Moreover, even in that case the concentration of the drug should be low and the low dose did not have any effect on the placebo effect in the present study. It implicated that even diffusion of drugs to neighbouring regions might not have any significant effect on behaviour. In addition, the microinjection may cause some non-specific effect on the function of the
target brain area, but the control animals receiving the saline injection would balance the non-specific role. It is less likely that the findings observed in the present study attribute to the trauma associated with the microinjection procedure.

In summary, results obtained in the present study clearly show that \( \mu \)-opioid receptor antagonist, but not \( \delta \)- or \( \kappa \)-opioid receptor agonists, could reduce the pain threshold in placebo analgesia rats. It may be concluded that rACC is the key brain region involved in placebo analgesia and that the opioid placebo analgesia is mediated exclusively through \( \mu \)-opioid receptor in rat.

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Statement of Interest

None.

References


