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Short Communication

Insular muscarinic signaling regulates anxiety-like behaviors in rats on the elevated plus-maze

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HIGHLIGHTS

- Insular microinjection and rat elevated plus-maze are the main methods.
- Insular mAChR activation by nonselective and M_1-, M_4-selective agonists down-regulate anxiety-like behaviors.
- Insular mAChR inhibition by nonselective and M_1-, M_4-selective antagonists up-regulate anxiety-like behaviors.

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ABSTRACT

Anxiety is one of the most prevalent neuropsychiatric disorders, and little is known about its pathogenesis. In order to investigate the neural mechanisms of this mental disorder, we used rat behavior in the elevated plus-maze as an animal model of anxiety and the insular cortex (insula) as a brain target. The microinjection of non-selective and selective M_1 and M_4 muscarinic acetylcholine receptor (mAChR) agonists or antagonists was used to explore whether the insular muscarinic receptor and its subtypes regulate levels of anxiety. The results showed that both non-selective and selective M_1 and M_4 mAChR agonists increased the time spent on exploring in the open arms, whereas antagonists decreased exploration. Our results indicate that activation of insular mAChRs could produce anxiolytic effects, whereas inhibition of insular mAChRs could increase anxiety. We concluded that the insular muscarinic system plays a role in the modulation of anxiety, and dysfunction of mAChR signaling may be involved in the mechanism of anxiogenesis.

Anxiety is a prevalent and growing mental health problem in the general public. Despite its prevalence, the pathophysiological mechanisms and etiology remain poorly understood. The insular cortex (insula) links emotions to cognitive processes and behavioral responses. Dysfunction of the insula is linked to the likely emergence of anxiety [1]. Functional neuroimaging technique has revealed that people with increased anxiety have increased regional cerebral blood flow in the insula [2]. In addition, patients with generalized anxiety disorder show a reduced activation in the insula after their symptoms are relieved by pharmacological treatment [3]. Moreover, few animal studies also support a role of the insula in anxiety [4,5].

Researches on the muscarinic acetylcholine receptor (mAChR) antagonist scopolamine indicated that systemic usage increases ratings of anxiety in rats [6] and mice [7]. On the other side, systemic injection of the mAChR agonist pilocarpine induces long-lasting anxiogenic responses in rats [8]. Studies with scopolamine and pilocarpine indicate that the functional integrity of mAChR is essential for anxiety regulation. It is known that insular mAChR takes part in conditioned taste aversion, attenuation of neophobia, inhibitory avoidance, and object recognition memory [9]. However, the role of insular mAChR and its subtypes (M_1–M_5) in anxiety is hardly explored. In one of our previous studies [10], we showed that activation of M_1 and deactivation of M_4 mAChRs up-regulated morphine conditioned place preference (CPP), and that the deactivation of M_1 and activation of M_4 mAChRs down-regulated CPP expression. Since both the insula and the muscarinic system are respectively play key roles in the anxiety disorder, we wonder whether insular mAChR participate in the modulation of anxiety with a subtype-specific manner. In this study, we adapted a typical anxiety animal model, the elevated plus-maze (EPM) of rats, and the

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technology of microinjections of mAChR antagonists and agonists into the insula. On the arrangement, we firstly used the nonselective mAChR agonist and antagonist to validate the involvement of insular mAChR in anxiety. After it was verified, the roles of M_1 and M_4 subtypes were investigated.

Adult male Sprague-Dawley rats (Vital River Laboratory Animal Technology Co. Ltd., Beijing, China), weighing 260–280 g before surgery, were individually housed after surgery in a temperature- and humidity-controlled room (22 °C and ~60%, respectively) under a 12-h light/dark cycle (lights on at 8:00 A.M.) with *ad libitum* access to food and water. All rats were allowed to become accustomed to the housing environment for at least 3 days before the stereotaxic surgery. The study procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Ethical Committee of Animal Use and Protection of Peking University Health Science Center (No. LA2012-34).

The non-selective mAChR agonist (pilocarpine) and antagonist (scopolamine) were respectively provided by Guangzhou Pharmaceutical Co., Ltd and Shanghai Pharmaceutical Supply Station (China). The selective agonists MCN-A-343 (M_1 preferring) and LY-2033298 (M_4 preferring) and the selective mAChR antagonists pirenzepine (M_1 preferring) and tropicamide (M_4 preferring) were purchased from Sigma–Aldrich, USA. The LY-2033298 was dissolved in 100% DMSO, tropicamide in 10% DMSO, and all other drugs were prepared in 0.9% saline.

Three experiments were carried out in this study. In experiment 1, pilocarpine (0.4 μg/site and 0.5 μl/site) and scopolamine (30 μg/site and 0.5 μl/site) were tested. In experiment 2, MCN-A-343 (30 ng/site and 0.5 μl/site) pirenzepine (21.2 mg/site and 0.5 μl/site) were tested. In experiment 3, LY-2033298 (0.4 μg/site and 0.5 μl/site) and tropicamide (1 mM and 0.5 μl/site) were tested. The doses of agonists and antagonists were adapted from one of our previous studies [10] and references [11]. Because the anxiety level in EPMs is positively correlated with light illumination, alternation of light illumination to optimize reference anxiety level can make behavioral observations more effective [12]. In all above three experiments, EPMs with microinjections of agonists and their vehicles were performed under a high illumination condition (150 lx) for assessing anxiolytic effects and EPMs with microinjections of antagonists and their vehicles were performed under a low illumination condition (1 lx) for assessing anxiogenic effects.

All rats were first prepared with implantation of cannula. Rats were intraperitoneally anesthetized using 50 mg/kg pentobarbital sodium and positioned in a stereotaxic device (Narishige Co., Japan). Two stainless steel guide cannulas (length = 8 mm, outer diameter = 0.56 mm) were implanted bilaterally and aimed at the insula (AP: −2.3 mm, ML: ±6.5 mm, DV: −6.5 mm) [4]. The guide cannula was fixed to the skull with three anchoring screws and dental cement and a stainless stylet was introduced into each guide cannula to prevent occlusion. After completing the surgery, antibiotic penicillin (75000 U) was intraperitoneally administered for 3–5 days to prevent possible infections. One week after surgery, rats received bilateral infusions into the insula with needles introduced through the guide cannulas until their tips were 1 mm beyond the cannula end. A volume 0.5 μl/site of either the vehicle or mAChR agonists/antagonists was injected over 60 s, using two microsyringes connected to an infusion pump. The injection needles were left in place for an additional 60 s to allow for infusion into the brain tissue before removal.

The EPM was used to detect anxiety-like behavior in the rats. The apparatus was made of black Plexiglas, had four 50 cm × 10 cm arms, and was elevated 50 cm above the floor. The two closed arms were enclosed by 40 cm walls; the two open arms were surrounded by a 1-cm-high Plexiglas ledge, and all arms were illuminated equally. A 10 cm × 10 cm platform (the junction area of the four arms) at the center was considered the neutral area. Before each test, rats were placed in the testing room for at least 1 h to habituate to the test environment. When performing the test, the rats were put in the center of the maze facing one open arm and were allowed to explore the plus-maze for 5 min. An arm entry was defined as an animal entering the arm with its front two feet [13]. EPM tests were performed 5 min after the insular microinjection [14].

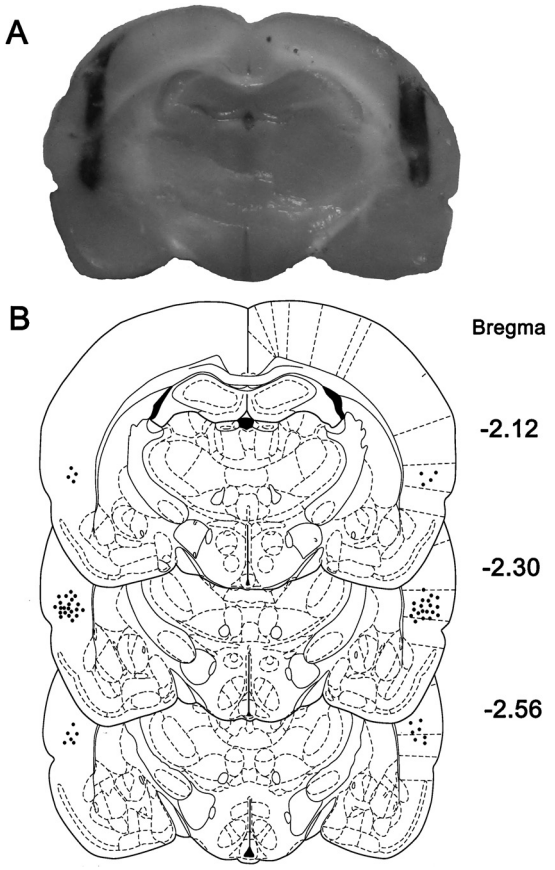

Fig. 1. (A) An illustration of the microinjection cannula track into the insula; (B) representative location of microinjection sites in the insula in experiment 1, indicated by solid black circles.

Locomotor activity was assessed in a clean wooden open field arena (80 cm × 80 cm × 80 cm). The floor was marked with lines forming 16 cm × 16 cm squares. When performing this test, the rats were initially placed in the center of the arena for 5 min, and the crossing of any of the lines with all four paws was recorded as locomotor activity. The locomotor activity tests were performed immediately after the EPM tests and under the same illumination conditions.

On completion of the behavioral testing, rats were immediately anesthetized with a lethal dose of pentobarbital sodium and 0.5 μl/site of 1% Evans blue was microinjected to mark the drug infusion sites. Then the rats were transcardially perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde. Each rat brain was removed and immersed in 4% paraformaldehyde overnight at 4 °C before sectioning. Fig. 1 shows photomicrographs of representative infusion placement sites in the insula in experiment 1. Rats receiving drug infusions outside this region were excluded from the analysis.

Data are expressed as the mean ± SEM. Means were compared with Student's *t* test. The statistical significance threshold adopted was $P < 0.05$.

Experiment 1 was first performed with microinjections of the non-selective mAChR agonist pilocarpine under high light