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Research article

Inhibiting medial septal cholinergic neurons with DREADD alleviated anxiety-like behaviors in mice

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HIGHLIGHTS

- We investigated the role of medial septal cholinergic neurons in anxiety-like behaviors with DREADD.
- Temporal inhibition of medial septal cholinergic neurons produced consistent anxiolytic effects in three behavioral models.
- Temporal inhibition of medial septal cholinergic neurons increased voluntary exploration in the open field test.
- These results reconciled conflicting findings from previous studies using irreversible lesions or non-specific inhibition.

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ABSTRACT

Cholinergic neurons in the medial septum (MS) participate in a variety of cognitive and emotional behaviors. Some studies but not others show that lesions or inhibition of the MS reduce anxiety-like behaviors and locomotive exploration in rats. However, these conclusions come from manipulations that are either irreversible or non-specific to cholinergic neurons, casting doubt on their validity. With DREADD (designer receptors exclusively activated by designer drugs), we temporarily and reversibly inhibited cholinergic neurons in the MS. We observed consistent anxiolytic effects of MS cholinergic inhibition in the novelty-suppressed feeding test, the marble burying test and the elevated plus-maze test, as well as increased exploratory activities in the open field test. These findings confirm an excitatory role of the MS cholinergic neurons in the control of innate anxiety, and reconcile conflicting findings from previous studies using irreversible lesions or non-specific inhibition.

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1. Introduction

The forebrain cholinergic system actively modulates functional states of the brain [1–3]. Medial septum (MS), one important source of cholinergic neurons, innervates the prefrontal cortex, the hippocampus and the entorhinal cortex in rodents [4]. Cholinergic neurons in the MS set hippocampal network states by affecting theta oscillations, modulate local neuroplasticity [5], and participate in a variety of behaviors such as working memory, motivation and vegetative functions [1–3].

MS has also been suggested as a subcortical node in anxiety [6,7]. Non-selective ablation or pharmacological inhibition of the MS reduces rats' anxiety-like behaviors in several tasks including the elevated plus-maze and the defensive burying paradigms [8–17]. The anxiolytic effect of MS lesions is at least partially mediated by its cholinergic efferents to the prefrontal cortex and the hippocampus [4]. A number of more recent studies (e.g. [18–20]) apply selective ablation of MS cholinergic neurons with the specific cholinergic immunotoxin 192 IgG-saporin (SAP). However, the resultant effects on innate anxiety vary across studies: Pizzo et al. [21] reported anxiolytic effects of SAP treatment in the plus maze test, whereas others detected limited [22] or even slight anxiogenic effects [23]. One explanation for these conflicts lies in the fact that irreversible lesions induce either functional compensation or indi-

Abbreviations: ChAT, choline acetyltransferase; CNO, clozapine N-oxide; DREADD, designer receptors exclusively activated by designer drugs; EPM, elevated plus-maze; MB, marble burying; MS, medial septum; NSF, novelty-suppressed feeding; OF, open field; PFA, paraformaldehyde; PV, parvalbumin; SAP, 192 IgG-saporin.

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rect effects secondary to vegetative modulation on sleep, circadian variation, and thermoregulation [24–26].

DREADD (designer receptors exclusively activated by designer drugs) is a widely applied chemogenetic technique that reversibly activates or inhibits neurons. By expressing inhibitory designer receptors (hM4Di) in cholinergic neurons through the Cre-LoxP system, for example, local or systematic injection of the designer drug (CNO) could site- and neuronal type-specifically inhibit the activity of these neurons. In the present study, we examined innate anxiety and exploratory behaviors of transgenic mice under DREADD inhibition of MS cholinergic neurons in four ethological models: the novelty-suppressed feeding (NSF) test, the marble burying (MB) test, the open field (OF) test and the elevated plus-maze (EPM) test. We showed that temporary inhibition of MS cholinergic neurons with DREADD produced consistent anxiolytic and exploration-promoting effects in mice.

2. Materials and methods

2.1. Animals

Adult male ChAT-cre mice (Jax Lab #006410) were 8–10 weeks of age at the beginning of the experiment. Mice were housed 4–6 per cage in a temperature and light-controlled room under a 12:12 h light: dark cycle with water and food provided *ad lib*. The animals were handled and habituated 7–10 days before any experiments. All animal experimental procedures were approved by the Animal Care and Use Committee of the University. The behavioral experimenters were kept blind from the groupings of the mice.

2.2. DREADD

16 mice were anesthetized with 1% pentobarbital sodium (0.05 g/kg *i.p.*) and positioned in a stereotaxic instrument (RWD, Shenzhen, China). 0.3 μl of the AAV-DIO-hM4Di-mCherry virus solution (1×10^{12} virus particles/ml, the University of North Carolina Vector Core Facilities) was injected through a 2-μl Hamilton microsyringe in 5 min into the MS (AP +1.20, ML 0.73, DV −4.15 mm relative to Bregma with a 10° angle towards the midline, Fig. 1A). All injections were followed by an additional 5 min to allow for virus diffusion before removing the injection needle.

1 mg/kg clozapine N-oxide (CNO, 0.2 mg/ml dissolved in normal saline, Tocris) was administered *i.p.* 30 min before behavioral testing (n = 8). Normal saline was administered as the control group (n = 8). Previous reports (e.g., Ref. [27]) as well as our pilot experiments had shown that this amount of CNO was sufficient to induce behavioral and electrophysiological changes that lasted at least 3 h, covering the whole testing phase.

2.3. Novelty-suppressed feeding (NSF) test

Mice were first examined in the NSF test. The test apparatus consisted of a plastic arena (40 × 40 cm) filled with cob bedding materials at a depth of 5 cm. A single food pellet (5 g) was placed on a piece of white filter paper on a food platform (9 cm in diameter) positioned in the center of the arena. Mice were deprived of food in their home cages for 24 h before test. The mouse was gently placed in a random corner of the arena and the amount of time passed before the mouse approached and ate the pellet was recorded. After the first bite or a 10 min cut-off time, the mouse was immediately transferred to its home cage and the amount of food consumed there within the next 5 min was measured.

2.4. Marble burying (MB) test

The MB test was performed one week after the NSF test. Each mouse was placed in a polycarbonate testing chamber containing 5 cm of cob bedding for 30 min to habituate. The mouse was briefly removed and placed in a holding chamber while 20 clean, black glass marbles (1.5 cm in diameter) were placed in the testing chamber in a 5 × 4 configuration. The mouse was returned to the testing chamber for a 30 min testing session without food or water access. The number of marbles buried ≥67% by the end of the test was counted.

2.5. Open field (OF) test

One week after the MB test, each mouse was placed in a 60 × 60 × 60 cm open field under dim illumination and allowed to explore for 5 min. Their activities were videotaped and the total distance travelled in the field was measured using SMART software (v2.5.21, Panlab) to reflect exploratory activities. The field was cleaned by 75% ethanol between tests.

2.6. Elevated plus-maze (EPM) test

The EPM test was carried out one week after the OF test. The maze consisted of two open and two closed arms (5 × 30 cm, and 15 cm wall height for the closed arms) and was placed 50 cm height above the floor. Mice were tested in a dimly illuminated room. Each mouse was placed onto the central area heading towards the same open arm. Activities in the following 5 min were videotaped. Time spent in open arms, and numbers of entries into open and close arms were analyzed using the SMART software. The maze was cleaned by 75% ethanol between tests.

2.7. Immunofluorescence

After behavioral testing, mice in the CNO group (n = 8) were sacrificed for histological verification of the virus expression with immunofluorescence. Mice were anaesthetized with 1% pentobarbital sodium and intracardially perfused with 4% paraformaldehyde (PFA, in 0.1 M phosphate buffer, pH 7.4). Brains were post-fixed with PFA for 6 h, and cryoprotected in 20% and 30% sucrose solutions in turn. 30 μm sections were sliced coronally using a cryostat microtome (Leica 1950) through the entire MS. Free-floating sections were washed in PBST (5 min × 3 times), blocked for 30 min with blocking-buffer containing 3% bull serum albumin and 0.3% triton x-100 dissolved in PBS, and incubated with the primary choline acetyltransferase (ChAT) (Millipore) or parvalbumin (PV) (Sigma-Aldrich) antibodies in 4 °C for 24 h. The primary antibody was dissolved 1:200 in blocking-buffer. Sections were then washed in PBST (5 min × 3 times) and incubated with secondary antibodies (Alexa Fluor 488, Invitrogen) in room temperature for 60 min, followed by PBST-washing (5 min × 3 times). Images were taken by a fluorescence inverted research microscope (Leica DMI4000B). Subjects with virus expression in regions other than the MS were excluded from analysis.

2.8. Patch clamping

An *ex vivo* patch-clamp slice preparation was used to confirm the inhibitory effect of CNO to hM4Di-infected cholinergic neurons. Mice in the control group (n = 8) were sacrificed with their brains extracted. Coronal sections of 300 μm containing at the level of the MS were cut by vibrating blade microtome (VT1200S; Leica, Buffalo Grove, IL), with cutting chamber containing ice-cold cutting solution of (in mM): 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 6 $MgSO_4 \cdot 7H_2O$, 0.5 $CaCl_2$, 10 glucose, 1.7 L-ascorbate, and 252 sucrose. Coronal sections were collected in a holding chamber filled with 37 °C artificial cerebrospinal fluid (ACSF) saturated with 95% O_2 and 5% CO_2, containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $MgSO_4 \cdot 7H_2O$, 2 $CaCl_2$, 10 glucose, and 1.7 L-ascorbate (315 mOsm,

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