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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.

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,

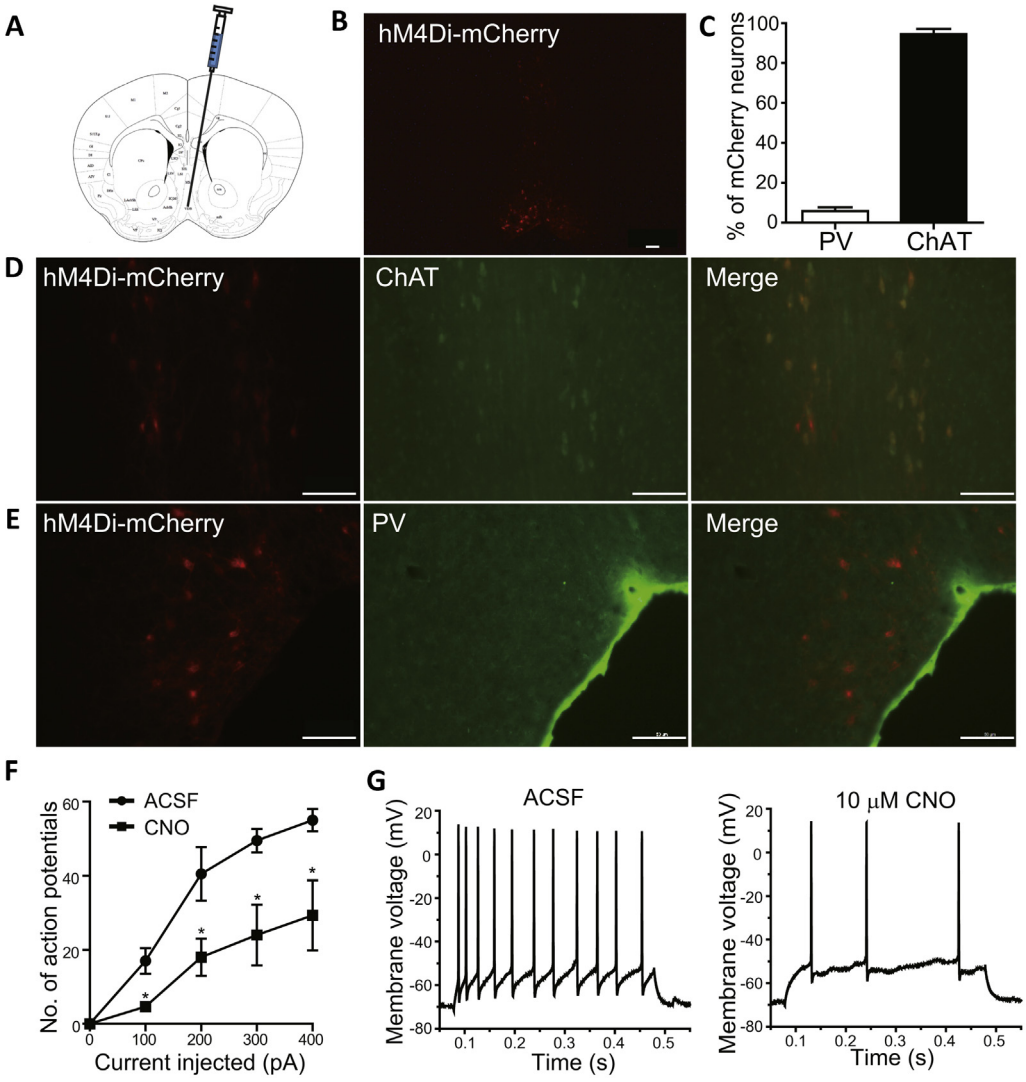

Fig. 1. Validation of the DREADD system. (A) Illustration of virus injection into the MS. (B) Restricted virus expression (red) in the MS. (C) A large proportion of infected MS neurons (red) were co-labelled with ChAT (green), a marker for cholinergic neurons (D), but few with PV (green), a marker for GABAergic neurons (E) (8 slices). (F) With whole-cell patch clamping, the number of action potentials induced by current injection significantly decreased with CNO treatment in MS cholinergic neurons (n = 8). (G) Sample recording traces of one MS cholinergic neuron with patch-clamping before (left) and after (right) 10 μm CNO bath application at 400 ms current of 100 pA. Scale bar = 100 μm. * $p < 0.05$, paired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pH 7.4). Whole-cell patch-clamp recordings started 30 min after slicing and the temperature was maintained at 34–36 °C both in the holding chamber and during the recording. Glass capillaries were pulled on a P-97 puller (Sutter Instrument, Novato, CA). Recording electrodes (5–8 MΩ) contained internal pipette solution (in mM): 118 $KMeSO_4$, 15 KCl, 10 HEPES, 2 $MgCl_2$, 0.2 EGTA and 4 Na_2ATP, 0.3 Tris-GTP, and 14 Tris-phosphocreatinin (pH 7.3 with CsOH).

All virus injection sites were checked and visually identified with the microscope (BX51; Olympus, Center Valley, PA) equipped with both transmitted light illumination and epifluorescence illumination. MS cholinergic neurons were identified by AAV-expressed mCherry-fluorescence. All recordings were made using a Multiclamp 700 B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 10 kHz and sampled at 50 kHz. Data were acquired and analyzed using pClamp 10.0 (Molecular Devices). Series resistance was in the order of 10–30 MΩ and was approximately 60–80% compensated. Recordings were discarded if the series resistance increased by more than 20% during the course of the recordings. ACSF and drug were applied to the slice via a peristaltic pump (Minipuls3; Gilson, Middleton, WI) at 2 ml/min.

A series of 400 ms current pulses varying in intensities between −200 to 400 pA in 100 pA increments were injected into mCherry-positive neurons and the frequencies of resultant action potentials were recorded. After the recording of baseline action potentials in ACSF, hM4Di channels were activated by 10 μM CNO-containing ACSF. 30 min after CNO perfusion, action potentials were recorded again in the same neurons. Off-line analysis was performed using Clampfit software (Molecular Devices, Sunnyvale, CA).

2.9. Statistics

Data were analyzed by Student *t*-tests. All results were presented as means ± S.E.M. In all statistical comparisons, p values < 0.05 were considered to be significant.

3. Results

3.1. Validation of the DREADD system

The virus expression was restricted to the MS (Fig. 1B). The fluorescence mCherry (red) from the virus showed strong co-

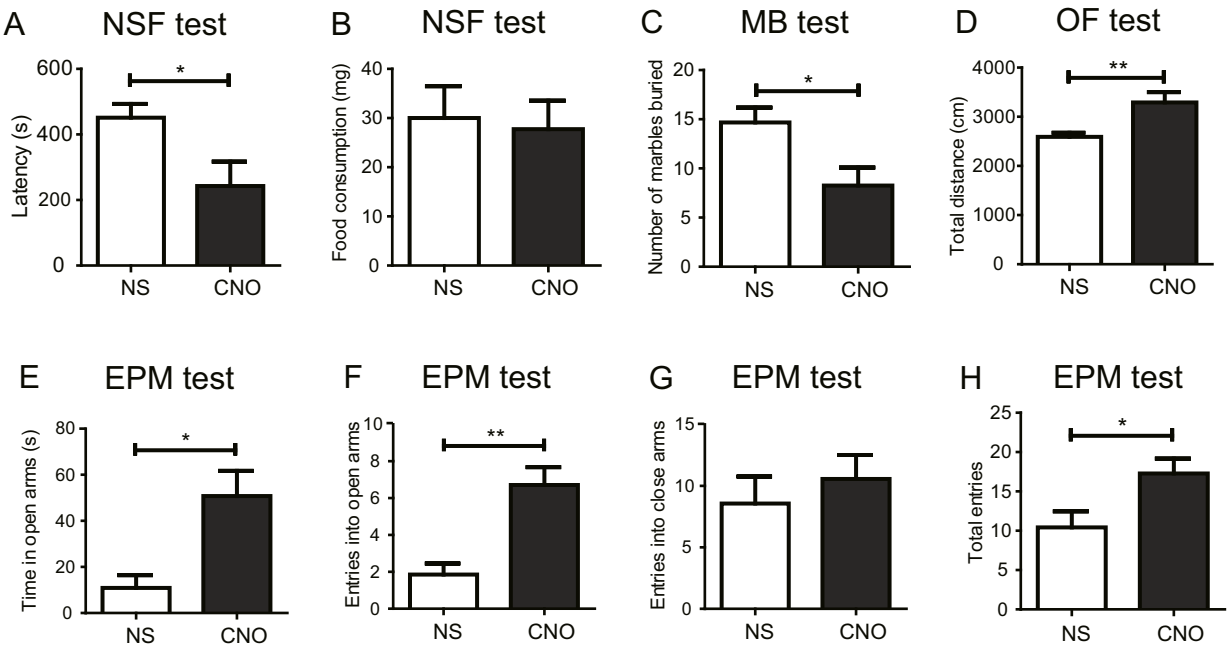

Fig. 2. DREADD inhibition of MS cholinergic neurons relieved innate anxiety-like behaviors. DREADD inhibition relieved novelty-suppressed feeding, indicated by a shorter latency to contact and eat the food pellet in the box (A), but similar amount of food consumed within 5 min after they returned to their home cages (B). (C) DREADD inhibition decreased the number of buried marbles in CNO-injected mice in the marble burying test. (D) DREADD inhibition of MS cholinergic neurons promoted exploration in the open field test. (E)–(H) DREADD inhibition relieved anxiety in the elevated plus-maze test. CNO-injected mice spent more time (E) and exhibited more entries (F) into the open arms, but similar entries into close arms (G). The total number of arm entries also increased in CNO-injected mice (H). * $p < 0.05$, ** $p < 0.01$, unpaired *t*-test.

labelling with ChAT, the marker for cholinergic neurons (green) ($94.5 \pm 2.6\%$), but not with PV, the marker for interneurons (green) ($5.8 \pm 1.9\%$), another major neuronal type in the MS (Fig. 1C–E).

To confirm the inhibitory effect of the DREADD system, 8 cholinergic neurons from 8 slices were selected to receive a series of 400 ms current injection from −200 pA to 400 pA with an increment of 100 pA. Bath application of CNO in brain slices significantly decreased the number of action potentials upon current injection (Fig. 1F). Recording traces from one sample neuron with 400 ms current of 100 pA were shown in Fig. 1G.

Together, these results validate the specificity and the inhibitory effect of the DREADD system applied in the present study.

3.2. Novelty-suppressed feeding (NSF) test

16 mice with DREADD virus expression in the MS were tested in four serial behavioral tests. 8 mice were administered *i.p.* with CNO for MS cholinergic inhibition 30 min before behavioral testing, and 8 with saline served as the control group. In the NSF test, CNO-injected mice showed a significant decrease in the latency to contact and eat the food pellet placed in the central area of the box ($t_{(14)} = 2.516$, $p < 0.05$, Fig. 2A), indicating reduced anxiety. By contrast, there were no differences between the amount of food consumed after the mouse returned to its home cage ($t_{(14)} = 0.167$, $p > 0.05$, Fig. 2B), suggesting that the above difference was not a result of altered food preference.

3.3. Marble burying (MB) test

Anxiolytic effects of cholinergic inhibition were also observed in the MB test. Significantly fewer marbles were buried in CNO-injected mice after the 30 min test session ($t_{(14)} = 2.231$, $p < 0.05$, Fig. 2C).

3.4. Open field (OF) test

The OF test is commonly used to assess exploratory behaviors in rodents. We detected enhanced locomotive exploration in CNO-injected mice in this test, indicated by significantly increased distance travelled in 5 min ($t_{(14)} = 3.068$, $p < 0.01$, Fig. 2D).

3.5. Elevated plus-maze (EPM) test

Finally in the EPM test, CNO-injected mice spent more time ($t_{(14)} = 3.089$, $p < 0.05$, Fig. 2E) and performed more entries ($t_{(14)} = 4.272$, $p < 0.01$, Fig. 2F) into the open arms, indicating decreased anxiety. The total number of arm entries also increased in CNO-injected mice ($t_{(14)} = 2.465$, $p < 0.05$, Fig. 2H), despite similar close arm entries ($t_{(14)} = 0.682$, $p > 0.05$, Fig. 2G). The generally increased maze exploration paralleled with findings from the OF test.

4. Discussion

Cholinergic neurons in the MS receive afferents from brainstem, midbrain and other areas, and further project to the frontal and hippocampal areas [4]. Most previous reports with non-specific pharmacological inhibition or electrolytic or excitotoxic MS lesions show anxiolytic effects [8–17]. However, we need to note that permanent MS lesions produce a number of non-specific effects. Srividya et al. [25,26] have shown that destruction of the MS significantly alters sleep-wakefulness, body temperature, locomotor activities and thermal preference. These changes could affect animal interoception in the long-term and hide the immediate MS modulation on innate anxiety. More importantly, these alterations change in a dynamic manner at various time points after the lesion surgery [25,26], providing direct evidence for functional compensation. These findings emphasize the significance of temporal and reversible modulation of specific neurons in investigating MS functions.

In the present study, we adopted DREADD, a mature chemogenetic technique to achieve temporal inhibition. The chemogenetic virus selectively infected MS cholinergic neurons in ChAT-cre mice and upon CNO application, inhibited their activities. Under DREADD inhibition, we examined the role of MS cholinergic neurons in NSF, MB and EPM tests, three commonly used models of anxiety-like behaviors in rodents. These tests adopt the rationale that innate fear in open arenas competes with the desire of feeding (NSF test), hiding (MB test) or exploring novel space (EPM test), to determine animal behaviors. We showed consistent anxiolytic effects of inhibiting MS cholinergic neurons in these tests, suggesting that this manipulation affects innate anxiety *per se*, but not specific behavioral aspects (*e.g.*, feeding or hiding) of it. DREADD technique also eliminates other confounding factors discussed above, and confirms MS's excitatory role in the control of anxiety.

One factor potentially affecting anxiety-like behaviors is exploration. A number of previous studies with non-specific lesions or inhibition of the MS report reductions or lack of changes in locomotor activities [22,24,28,29]. This is in sharp contrast to findings in the present study. The OF test is the most common test for locomotive exploration. We detected significant increases in the distance travelled in the field by CNO-injected mice. This finding is further supported by increased total arm entries in the EPM test. However, we do not consider these exploratory alternations as the cause of the anxiolytic effects. In the EPM test, CNO-injected mice performed significantly more open arm entries but similar entries into close arms. The higher proportion of open versus close arm entries is consistent with the longer proportion of time spent in the open arms. In addition, the MB test is relatively independent of locomotive exploration and still reveals reduced anxiety with cholinergic inhibition. Indeed, increased locomotive activities would predict more buried marbles by the end of test, which is clearly opposite to our findings. Thus, we conclude that cholinergic inhibition in the MS promotes exploration, and that the reduced locomotive activities from previous studies could be secondary to other non-specific changes [25,26].

The most possible mechanism of anxiolytic effects of MS cholinergic neurons is through their efferents to the prefrontal and hippocampal areas [4]. The ventral pole of the hippocampus is closely interconnected with amygdala and crucial for anxiety-like behaviors [30]. In particular, hippocampal acetylcholine modulates the amplitude of theta oscillations and affects synaptic plasticity and information encoding [1–3]. Correlation between theta oscillations in the prefrontal cortex and ventral hippocampus increases in anxiogenic environments, whereas increased theta power in the prefrontal cortex predicts avoidance of aversive environments [31]. Prefrontal neurons with anxiety-related activities are more strongly coupled to theta oscillations in the ventral hippocampus [32]. Optogenetic inhibition of the hippocampal-prefrontal pathway disrupts theta synchrony and reduces anxiety [33].

In conclusion, our findings confirm an excitatory role of the MS cholinergic neurons in innate anxiety, reconcile conflicting findings from previous studies using non-specific inhibition or irreversible lesions, and support the significance of applying temporal, reversible and neuronal-type-specific techniques including DREADD in investigating septal functions.

Conflict of interest

The authors claim no competing interests.

Authors' contributions

YZ, YYJ, SS and CZ performed the experiment; FYL, YW and MY designed the experiment; All authors wrote the manuscript.

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