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Research article

Cholinergic neurons in medial septum maintain anxiety-like behaviors induced by chronic inflammatory pain

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ABSTRACT

Cholinergic neurons in the medial septum (MS) participate in various cognitive and emotional behaviors, including innate anxiety. Chronic pain involves perceptual, cognitive and emotional components. Whether MS cholinergic system modulates pain-induced anxiety and the underlying neural circuits are involved remain unclear. In the present study, we showed that chemogenetic (DREADD) inhibition of MS cholinergic neurons relieved pain-induced anxiety-like behaviors in open field and elevated plus maze tests. Inhibiting the MS–rostral anterior cingulate cortex (rACC), but not the MS–ventral hippocampal CA1 pathway, achieved anxiolysis. These findings indicate the involvement of MS cholinergic system in modulating pain-induced anxiety-like behaviors.

1. Introduction

Acetylcholine has a broad range of neuromodulatory influences on neuronal properties and plays an active role in information processing and behaviors such as working memory and motivation [1–3]. Medial septum and diagonal band complex (MS), an important part of basal forebrain cholinergic system, is the major source of cholinergic (as well as non-cholinergic) projections to cingulate cortex, entorhinal cortex and hippocampus [4].

Anxiety is a common co-morbidity in various neurological disorders including chronic pain [5–7]. Though MS cholinergic system maintains innate anxiety in several rodent behavioral paradigms [8], whether MS cholinergic system modulates pathological anxiety and the underlying neural circuits are involved remain unclear.

In the present study, we examined the potential involvement of MS cholinergic neurons in pain-induced anxiety-like behaviors with chemogenetics [9].

2. Materials and methods

2.1. Animals

Adult male ChAT (choline acetyltransferase)-Cre transgenic mice (8–10 weeks of age, Jax Lab #006410, subsequently referred to as ChAT mice) were housed in 4–6 cohorts with stable room temperature (22–23 °C), humidity (40–60%), and circadian cycle (12 h light/dark cycles, starting at 07:00). All experimental procedures followed the Guidelines of the Committee for Research and Ethical Issues of International Association for the Study of Pain [10]. Mice were handled for at least three days before experiments. All behavioral testing was performed in a blind manner.

2.2. Virus injection

For intracranial virus injection, mice were anaesthetized with 1% pentobarbital sodium, positioned in a stereotaxic instrument (RWD, Shenzhen, China), and injected with 0.35 μl AAV5-hSyn-DIO-hM4D (Gi)-mCherry virus [11] solution (1×10^{12} virus particles/ml, the University of North Carolina Vector Core Facilities) into MS (1.2 mm anterior-posterior (AP), 0.73 mm medial-lateral (ML), −4.13 mm dorsal-ventral (DV) from bregma, 10° angle towards the midline) at a rate of 0.1 μl/min for 5 min through a 1-μL Hamilton microsyringe. After injection, needles were left in place for an additional 5 min before it was slowly withdrawn to minimize spread of virus solution. For chemogenetic manipulation (designer receptors exclusively activated by designer drugs, DREADD) of the MS, 1 mg/kg clozapine *N*-oxide (CNO, 0.2 mg/ml dissolved in normal saline, Tocris) [8,12] was injected intraperitoneally 30 min before behavioral testing. For MS–rostral anterior cingulate cortex (rACC) or MS–ventral hippocampal CA1 (vCA1) pathway manipulation, ACC (+1.6 mm AP, +0.4 mm ML, −0.7 mm DV) or vCA1 (-3.0 mm AP, +/−3.2 mm ML, −2.7 mm DV) were implanted with metal cannula (RWD, Shenzhen, China) for CNO

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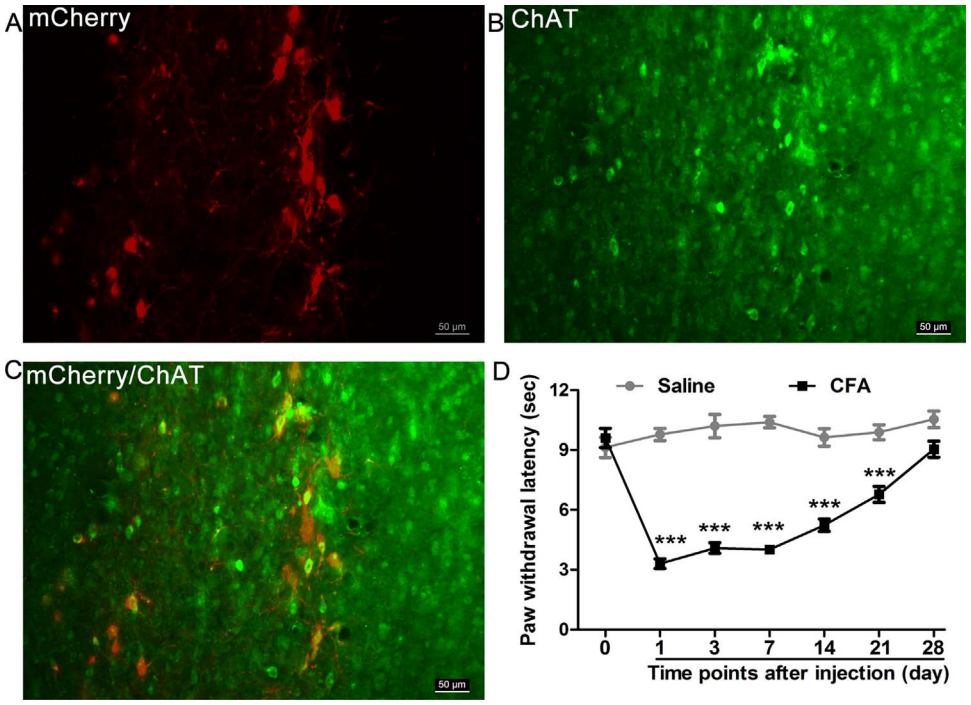

Fig. 1. Validation of the chemogenetics system in the MS and establishment of CFA-induced inflammatory pain model in mice. (A) mCherry (red) stands for the AAV5-hSyn-DIO-hM4D(Gi)-mCherry virus expression. (B) ChAT (green). (C) mCherry/ChAT merge. It is noted that restricted expression of AAV5-hSyn-DIO-hM4D(Gi)-mCherry virus in the MS of ChAT-Cre mice. A large proportion (86.5 ± 3.1%) of infected MS neurons (red) (A) were co-labelled with ChAT (green), a marker for cholinergic neurons (B, C). (D) CFA injection produced thermal hyperalgesia that lasted approximately 3 weeks. ***p < 0.001, two-way ANOVA with Bonferroni's test, n = 9 animals/group.

(3.0 μM in aCSF, 0.5 μl) delivery. All virus injection sites were verified histologically.

2.3. Establishment of CFA-induced inflammatory pain

The complete Freund's adjuvant (CFA) model of chronic pain in mice was established as previously described [13]. Three to 4 weeks after virus injection, mice were anaesthetized with 1% sodium pentobarbitone, and the left hindpaw was intraplantarly injected with 40 μl CFA to induce inflammatory pain. Equal volumes of normal saline (NS) were used as the control.

2.4. Assessment of thermal hyperalgesia

Each mouse was handled for 10 min, and adapted in a Plexiglas cube for 30 min for 3 days before experiments. Thermal pain threshold was measured while the animal stayed calm and awake. Paw withdrawal latencies (PWLs) to thermal stimuli were measured with a focused radiant heat (15 W of power) applied onto the central region of the hind paw (Hargreaves Method, IITC 390) [13]. PWLs were measured 3 times at minimal 5-min intervals and averaged. To avoid tissue damage, a cut-off time of 20 s was imposed.

2.5. Open field test

Twenty-eight days after CFA or saline injection, each mouse was placed in a 60 cm × 60 cm × 60 cm open field chamber with 30 lx illumination and allowed to explore for 5 min freely. The area was divided into 16 quadrants (4 central and 12 peripheral). Time, entries and distance in the central zone and total distance travelled during the test were recorded using a digital video camera and measured using the SMART software (v2.5.21, Panlab, Harvard Apparatus. SMART Video-tracking, RRID:SCR_002852). The apparatus was cleaned using 75% ethanol after each exploration [8,13].

2.6. Elevated plus maze test

The elevated plus maze test was carried out two days after open field test. The maze was made of clear Plexiglas with two open arms and two identical closed arms (5 cm × 30 cm each arm, and 15 cm wall height for closed arms) and was placed 50 cm above the floor in a room (30 lx illumination). Each mouse was placed onto the center area with the head toward one open arm, and allowed to explore the maze for 5 min recorded with a digital video camera. Time in open arms, and entries into open and closed arms were analyzed with the SMART software. The apparatus was cleaned using 75% ethanol after each exploration [8,13,14].

2.7. Immunostaining

Mice were deeply anaesthetized with 1% pentobarbital (100 mg/kg, *i.p.*) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.01 M phosphate buffer (PB), pH 7.4. After removal from the skull, the brain was post-fixed for 4–6 h, and dehydrated in graded sucrose (20% to 30%). After embedding in optimal cutting temperature compound, the brain was sectioned coronally at 50 μm on a freezing microtome (Model 1950, Leica Instrument Co, Ltd).

For the ChAT immunofluorescent staining, sections were rinsed with 0.5% Triton-X in 0.1 M PBS and blocked with 10% BSA for 1 h. Sections were then incubated with the primary antibody (rabbit anti-ChAT, 1: 200, Millipore AB143) in 0.5% PBST for 30 h at 4 °C. Sections were rinsed, incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (1: 500, Abcam, 1 h at 24 °C), rinsed in PBS, mounted and coverslipped. Images were taken by a fluorescence microscope (Leica DMI4000B) [13].

2.8. Data analysis

Data were expressed as means ± SEMs (standard error of means). All data were analyzed and plotted using the GraphPad Prism 5 software. Statistical analysis was performed using two-way ANOVA with Bonfferoni *post hoc* test among groups, and unpaired Student's *t*-test between two groups. Numbers of animals used were indicated by n. Probability values of $P < 0.05$ were considered to represent significant differences.