

DREADD in Parvalbumin Interneurons of the Dentate Gyrus Modulates Anxiety, Social Interaction and Memory Extinction

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Abstract: Parvalbumin (PV)-positive interneurons in the hippocampus play a critical role in animal memory, such as spatial working memory. However, how PV-positive interneurons in the subregions of the hippocampus affect animal behaviors remains poorly defined. Here, we achieved specific and reversible activation of PV-positive interneurons using designer receptors exclusively activated by designer drugs (DREADD) technology. Inducible DREADD expression was demonstrated *in vitro* in cultured neurons, in which co-transfection of the hM3D-Gq-mCherry vector with a Cre plasmid resulted in a cellular response to hM3Dq ligand clozapine-N-oxide (CNO) stimulation. In addition, the dentate gyrus (DG) of PV-Cre mice received bilateral injection of control lentivirus or lentivirus expressing double floxed hM3D-Gq-mCherry. Selective activation of PV-positive interneurons in the DG did not affect locomotor activity or depression-related behavior in mice. Interestingly, stimulation of PV-positive interneurons induced an anxiolytic effect. Activation of PV-positive interneurons appears to impair social interaction to novelty, but has no effect on social motivation. However, this defect is likely due to the anxiolytic effect as the exploratory behavior of mice expressing hM3D-Gq is significantly increased. Mice expressing hM3D-Gq did not affect novel object recognition. Activation of PV-positive interneurons in the DG maintains intact cued and contextual fear memory but facilitates fear extinction. Collectively, our results demonstrated that proper control of PV interneurons activity in the DG is critical for regulation of the anxiety, social interaction and fear extinction. These results improve our fundamental understanding of the physiological role of PV-positive interneurons in the hippocampus.

Keywords: Parvalbumin-positive interneurons, hippocampus, Cre transgenic mice, DREADD, social memory, recognition memory.

INTRODUCTION

To date, multiple sub-types of interneurons have been identified in the hippocampal region [1]. Approximately 24% of interneurons in the CA1 region express parvalbumin (PV) and are known as PV-positive interneurons [2]. Accumulating evidence shows that PV-positive interneurons are involved in the regulation of diverse brain functions and animal behaviors, including gain of sensory responses,

plasticity, and learning [3]. PV-positive interneurons in the dentate gyrus (DG) area are fast-spiking cells known to play essential roles in the regulation of network oscillation and synchrony [1]. In addition, abnormal activities of PV-expressing interneurons contribute to a variety of human neurological and psychiatric diseases, including epilepsy, schizophrenia, autism, depression, and Alzheimer's disease [4]. Importantly, the disruption of social behavior and social recognition is noted in many different types of neurological and/or neuropsychiatric complications [5]. Some of these neurological disorders are also associated with symptoms of cognitive impairments [6]. Inhibition of PV and GAD65 interneurons in the ventral hippocampus leads to schizophrenia like behaviors in mice [7]. Although impairment of PV-positive interneurons has been linked to abnormalities in social behavior [8], deficits in spatial learning [9], and increased sensitivity to epileptic seizures [10], it is still unknown how the activation of PV-positive neurons in

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the subregions of the brain, such as the dentate gyrus, will influence the behaviors of social interaction and cognitive performance.

Optogenetic and pharmacogenetic tools have been widely used in modulating neuronal activities of different brain regions and allow to examine specific neural circuits. Optogenetics introduces genes encoding light-responsive transmembrane ion conductance regulators (such as opsins) to activate or inhibit targeted cells [11]. Pharmacogenetics applies designer receptors that response to specific chemicals to provide a stimulation/inhibition control of selective neuronal populations. Designer receptors exclusively activated by designer drugs (DREADD) technology was recently developed by Bryan Roth group [12] and has been quickly applied in animal studies to control the neuronal activities [13, 14]. This technique allows for manipulation of changes in diverse discrete populations of neurons [15-18]. However, the DREADD method to modulate PV-positive interneurons has not yet been widely applied.

In this study, we investigated the functional role of PV-positive interneurons in the mouse hippocampus by selectively simulating by DREADD technology. The gene encoding hM3Dq (human M3 muscarinic DREADD receptor coupled to Gq) was targeted selectively in PV-positive hippocampal neurons in Cre-mice with a Cre-inducible lentiviral vector that carried a double-floxed inverted open reading frame of hM3Dq and RFP (mCherry). PV-positive neurons were activated by injecting the hM3Dq ligand clozapine-N-oxide (CNO) [19, 20]. Our findings provide insight to better understand how suppression of the DG neuronal activity through activation of PV-positive interneurons regulates anxiety and memory.

MATERIALS AND METHODS

Animals

Male PV-Cre transgenic mice (B6; 129P2-Pvalb^{tm1(cre)Arbr}/J, stock number : 008069), 8–16 weeks old, were obtained from Jackson Laboratory (ME, USA). All mice were housed at controlled room temperature (22–24°C) with a 12-hour light (light on 7: 00 am to 6: 00 pm) and 12-hour dark cycle. Mice had *ad libitum* access to food and water. The animal experiments were approved by the Animal Ethical Committee of Pennsylvania State University.

Cell Culture

Human embryonic kidney (HEK) 293T and mouse CAD cells were culture in Dulbecco's Modified Eagle Mmedium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies, USA). Cells were maintained in a 37°C incubator with humidified air and 5% CO₂.

DNA Construction and Preparation of Lentivirus

AAV-DIO-hM3Dq-2A-mCherry vector [21], kindly provided by Professor Minmin Luo, was used for the

construction of recombinant DNA. Briefly, the DIO-hM3q-mCherry or DIO-mCherry sequence was cut from the AAV-DIO-hM3q-mCherry template vector and inserted into the pSIN-EF1 α -IRES-Puro vector to obtain the pSIN-EF1 α -DIO-hM3q-mCherry and pSIN-EF1 α -DIO-mCherry (control) vectors. The pSIN-EF1 α -DIO-hM3q-mCherry or control plasmid was co-transfected with packaging plasmids (psPAX2 and pCMV-VSVG) into HEK 293T cells by the phosphate precipitation method. Forty-eight hours after transfection, the VSVG-pseudotyped lentiviruses were collected and concentrated by ultracentrifugation at 25,000 rpm for 90 min as described previously [22-24]. The viral titer was greater than 1×10^8 unit/ml.

Transfection and Immunocytochemistry

To confirm the function of the constructed DNA plasmid, mouse CAD cells were seeded on glass coverslips in a 24-well plate. Cells were transfected with the PSIN-EF1 α -DIO-hM3q-mCherry or control plasmid using polyethylenimine according to a procedure reported previously [25]. Forty-eight hours after transfection, cells were fixed in 4% paraformaldehyde (PFA) and stained with anti-red fluorescent protein (RFP) antibody. Nuclei were counterstained with DAPI (4', 6-diamidino-2-phenylindole).

Primary Culture of Hippocampal Neurons

Primary culture of mouse hippocampal neurons was carried out as previously described [26]. In brief, hippocampal tissues were dissected from the brain of postnatal C57BL/6J mouse (postnatal day 1, within 24 hours after birth). Tissues were minced into small pieces and dissociated with 0.05% trypsin-EDTA for 30 min, followed by mechanical trituration. Single-cell suspensions were prepared in Minimal Essential Medium (MEM, Invitrogen) supplemented with 5% FBS (HyClone), 2% B27 supplement (Invitrogen), 0.2 mg/mL NaHCO₃, 20 mM D-glucose, 2 mM GlutaMAX (Invitrogen), and 25 U/mL penicillin/streptomycin. Cells were seeded on culture dishes over a monolayer of primary cortical astrocytes at a density of 8,000–12,000 cells/cm². Hippocampal neurons were maintained at 37°C in a humidified incubator containing 5% CO₂.

Electrophysiology

Hippocampal neurons were transfected with lentiviral DIO-mCherry-hM3D-Gq vector alone or in combination with Cre plasmid as described above. The firing of action currents in mouse hippocampal neurons was recorded using a patch-clamp assay as previously described [27, 28]. Briefly, the electrophysiology recordings were performed using a Multiclamp700A amplifier (Molecular Devices). The resistances of patch-pipettes were 3–5 M Ω , and the typical access resistance was <20 M Ω . Neurons were perfused continuously with a bathing solution containing 123 mM NaCl, 30 mM glucose, 25 mM HEPES, 5 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂ (pH 7.3). Chloride-free pipette solution was supplemented with 135 mM KGluconate, 10 mM Tris-phosphocreatine, 5 mM

EGTA, 10 mM HEPES, 4 mM MgATP, and 0.5 mM Na₂GTP (pH 7.3). Spontaneous cell action potential firing was recorded under current clamp mode holding at -65 mV. After 5 minutes of recording, 10 μM CNO was added, and CNO was washed away 10 min after addition. The total recording time for each cell was 20 minutes.

Stereotaxic Virus Injection

Stereotaxic injection of lentivirus was carried out according to procedures reported previously [26, 29]. Mice were anesthetized by intraperitoneal injection of Avertin (100 mg/kg) and then fixed in a stereotaxic apparatus (Stoelting Co. cat# 51725). Artificial eye ointment was used to prevent damage to the animals' eyes. A midline scalp incision was made, and holes were drilled on the skulls at the following coordinates: -2.2 mm ventral to the skull from the bregma, AP-1.9, ML±1.5, DV-2.0. The lentivirus was then injected bilaterally into the hippocampus slowly over more than 10 min. For each injection, animals were given 1.5 μL of lentivirus. Control mice were given DIO-mCherry control. After that, the needle was removed and tissue glue was used to seal the wound. Then mice were returned to their cages and allowed to recover for at least 14 days before behavioral testing.

Immunohistochemistry

Immunohistochemistry was performed as described previously [30]. Two weeks after viral injection, animals were perfused with 4% PFA, and the brain samples were harvested and embedded in paraffin. The paraffin-embedded samples were sectioned, blocked, and stained with primary antibody against PV (Abcam, ab11427, 1: 500) and mCherry (Thermo Scientific, MA5-15257, 1: 500). After washing, samples were labeled with secondary antibodies (Life Technologies, USA). Slices were stained with TO-PRO-3 dye to label nuclei (Life Technologies, USA). The immunolabeled samples were examined under a confocal laser scanning microscope (Zeiss, Pascal LSM). To quantify the positive neurons in the DG, 6 slices per animal were used according to the stereology.

Behavior Tests

For all behavior tests, mice were allowed to acclimatize in the testing room for 1 hour before testing, and all of the tests were conducted during the day. The action of CNO peaks about 30-45 min after administration. Hence, thirty minutes before testing, animals were given a dose of CNO (0.5 mg/kg) by intra-peritoneal injection. After testing, mice were returned to cages and allowed to recover for at least 7 days. The same mice were used for all behavioral tasks, and followed the order of the task (first Open field, then, social interaction, novel object recognition, elevated plus maze, tail suspension, fear conditioning and extinction test, with one week delay between tasks).

Open Field Test

The open field test was performed as reported previously [22]. In brief, each mouse was placed in the center of an opaque square open field area (40 × 40 × 40 cm). Mice were allowed to move freely in the open field for 5 min, and their movements were tracked with an overhead video camera. The center area was defined as a circle 24 cm in diameter. The total distance traveled, cumulative duration in the center, center entry frequency, duration spent immobile, mean velocity and maximum velocity were recorded over a 5-min test period.

Elevated Plus Maze

To assess exploratory and anxiety-like behaviors, the elevated plus maze test was performed [31, 32]. The elevated plus maze used was (+)-shaped and 50 cm elevated from the floor with two open arms and two enclosed arms (30 cm long and 5 cm wide). Mice were placed in the center of the maze and allowed to freely move into the four arms of the maze for 5 min. The mice were videotaped, and the amounts of time spent in the open and closed arms were recorded. The number of entry into open arms, the number of head dips, vertical rears and wall leans were analyzed as previously reported.

Tail Suspension Test

The tail suspension test was conducted as previously described [33]. Animals were suspended on the tail suspension device (Penn State Behavioral Core) for 6 min. The immobility time during the middle 5 min (excluding the initial 30 sec and the last 30 sec) was calculated.

Social Interaction Test

The social interaction test was conducted according to the procedure used in previous studies [26, 34]. The apparatus was composed of a rectangular, three-chamber box. The dividing wall was made from clear Plexiglass, and the size of each chamber was 19 × 45 cm. There was an open middle section in the dividing walls, which allowed the mouse to have free access to each chamber. Two wire cup-like containers were placed in the left and right chambers, one in each side. During the habituation (adaptation) session, two empty wire containment cups were placed in the middle of the left and right chambers, and the mouse was placed at the center of the middle chamber to let the mouse freely move for 5 min. Then, a stranger male mouse (Stranger 1) was placed in a wire container located in either the left or right chamber. The interaction time between the two mice and the containment cup housing or not housing the Stranger 1 mouse was recorded using an overhead video. Ten minutes later, a second male mouse (Stranger 2) was placed in an identical wire container in the opposite chamber. The duration of contact was recorded over 10 min in this session. The three chambers were cleaned by 70% ethanol after each trial. The total duration of contact between the experimental mouse and empty cup vs the male Stranger 1 mouse, or between the experimental

mouse and the male Stranger 1 mouse vs the Stranger 2 mouse was calculated.

Novel Object Recognition Test

The novel object recognition test was performed according to a method reported previously [35]. Briefly, mice were first familiarized with the testing environment without objects for more than 10 min. CNO was administered 30 min before the familiarization, then, they were received a 10-min habituation trial with two identical objects. One hour after the habituation trial, the mice were placed into a testing chamber with one familiar object, and a novel object where they were kept for 5 min. The amount of time the animals spent exploring the object was recorded and used as a reflection of the short-term memory of the mice. Twenty-four hours after the initial training session, the familiar object used before was replaced with another novel object. The novel and familiar objects were randomly assigned to each mouse in order to control for object preference. The amount of time the mice spent exploring the familiar object and novel object within 5 min was recorded and used as a reflection of the long-term memory.

Fear Conditioning

Fear conditioning was conducted as reported previously [36]. In the fear conditioning test, the mouse was placed in the fear conditioning apparatus and allowed to explore for 5 min to become familiar with the environment. After familiarization session, the mouse in the testing chamber will receive a three-tone stimuli at 58-second intervals (tone lasted 20 sec, 85 dB and 2.8 kHz). The tone co-terminated with a 2-sec, 0.55-mA foot shock. The mouse was removed immediately after the last shock. During the contextual testing, 24 hours after training, the mouse was placed in the testing chamber for 4 min to test memory recall. The freezing behavior was recorded. During cued fear testing, 3 hours after the contextual test, the mouse was placed in a novel decorated chamber. The tone (85 dB, 20 sec, 2.8 kHz) was presented, and the freezing behavior was recorded. During extinction testing, the mouse was placed in the testing chamber (not modified), and freezing behavior was measured. There was no shock in the extinction test, and the test continued for 7 days. Mice only received CNO on the training day.

Statistical Analysis

Data were analyzed using Excel software, SPSS19.0 software and are expressed as means \pm standard error of the mean (SEM). Lilliefors test was used to determine the normal distribution of our data sets. In some behavioral tests, animals that cannot complete task or do not fulfill the requirements according to the protocol will be excluded for all data analysis. Comparisons between the experimental group and control group were analyzed by Student's *t*-test. Social interaction and novel object recognition test were analyzed by ANOVA followed by planned comparisons among the relevant groups and other

factors with a Bonferroni correction. The *P* values were evaluated via ANOVA.

RESULTS

Selective Activation of PV-Positive Interneurons in the Mouse Hippocampus

To specifically activate PV-positive interneurons in the dentate gyrus (DG), hM3Dq, which has high binding capacity for CNO but not endogenous ligand, was fused with mCherry red fluorescence protein (RFP) and subcloned into a Cre-inducible lentiviral construct. The hM3Dq-mCherry DNA fragment was flanked with double loxp sites such that only when the Cre recombinase is present will hM3Dq be expressed (Fig. 1A). The lentiviral DIO-mCherry vector was used as a negative control. To confirm the inducibility, the DIO-hM3D-Gq-mCherry construct plasmid was transfected into neuroblastoma Cath.a-differentiated (CAD) cells without the Cre-expressing construct. We did not observe any mCherry expression (Fig. 1B), suggesting that there is no leakage of expression. When this transfection was combined with the Cre plasmid, notable expression of mCherry was observed in cells (Fig. 1B), confirming that the expression of hM3Dq in cells was Cre-dependent.

After verification of the lentiviral constructs in CAD cells, the DIO-hM3D-Gq-mCherry lentivirus was bilaterally injected into the DG of PV-Cre transgenic mice. Two weeks after injection, expression of hM3D-Gq-mCherry was evaluated by mCherry fluorescence and staining for PV. As shown in Fig. 1C, mCherry-expressing cells overlapped with PV-positive neurons, confirming the specific expression of hM3D-Gq in the PV-positive interneurons (Fig. 1C). We quantified the percentage of mCherry and PV double positive cells versus total mCherry positive cells in Table 1. Over 96% of mCherry positive cells are PV positive, demonstrating the high specificity. Moreover, to determine the efficiency of our viral infection in the DG, we found that over 70% of PV interneurons expressed mCherry, suggesting the high efficiency of viral transduction (Table 1). To confirm that the lentivirus correctly targeted to the DG, we found that mCherry positive cells are detected only in the DG, not in other regions as shown in a full view of an injected hemisphere (Fig. 1D), suggesting the high specificity of the injection. A wide viral spread has been detected from Bregma -1.5 mm to -2.92 mm, over 1.4 mm range.

To confirm whether the ligand CNO can activate primary cultured hippocampal neurons, the hippocampal neurons were transfected with lentiviral DIO-mCherry-hM3D-Gq vector alone or in combination with Cre plasmid. Electrophysiological analysis revealed that transfection with DIO-mCherry-hM3D-Gq vector alone had no significant effects on the firing frequency of action potential in mouse hippocampal neurons (Fig. 2A) even after addition of CNO. In contrast, in the presence of Cre-expressing plasmid, addition of CNO (10 μ M) remarkably increased the firing frequency of action potentials in the transfected

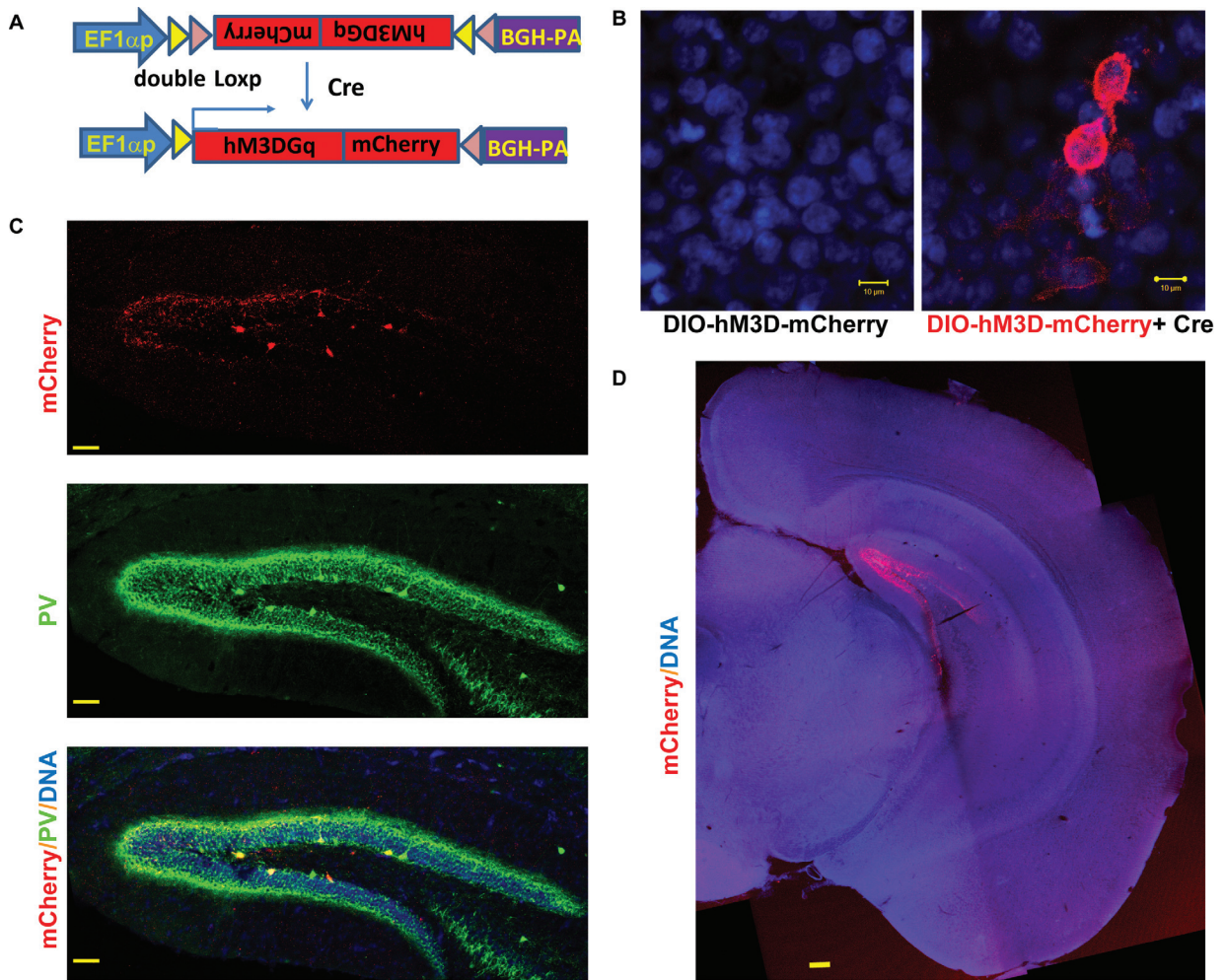


Fig. (1). Selective expression of the DREADD receptor hM3Dq in PV-positive interneurons in the mouse hippocampus. (A) The lentiviral vector carrying the hM3D-Gq coding sequence under the control of the human elongation factor 1 α promoter (EF1 α p) was designed for DIO-hM3D-Gq-mCherry. BGH-PA, bovine growth hormone-polyadenylation site. (B) CAD cells transfected with the DIO-hM3D-Gq-mCherry lentiviral vector alone did not exhibit mCherry fluorescence under a fluorescent microscope. However, co-transfection of the viral vector with Cre plasmid resulted in the expression of hM3D-Gq and mCherry (red). Nuclei were counterstained with DAPI. Scale bars = 10 μ m. (C) Cre mice received bilateral injections of lentiviral DIO-hM3D-Gq-mCherry vectors into the hippocampus area. Two weeks after viral injection, brain slices were stained with TO-PRO dye to label nuclei (blue). Under a fluorescent microscope, the expression of hM3D-Gq (red) and PV (green) was detected in the hippocampus. Scale bars = 50 μ m. (D) A full view of a hemisphere injected with lentivirus expressing mCherry shows the specific targeting. Scale bars = 200 μ m. (Color figure available online).

neurons (Fig. 2B). Once CNO was washed away, the firing frequency returned to the level observed before the addition of CNO, which demonstrates that this increase in firing frequency was dependent on CNO.

Table 1. Quantification of the percentage of mCherry positive cells that are PV+ interneurons and the percentage of infected PV interneurons in the DG.

mCherry+PV+/ Total mCherry +%	Infection Efficiency %
96.4 \pm 1.5%	70.9 \pm 5.1%

Percentage of mCherry and PV double positive cells was calculated by number of mCherry and PV double positive cells divided by total mCherry positive cells in the DG. The infection efficiency was calculated by number of mCherry and PV double positive cells.

Selective Activation of PV-Positive Interneurons in the DG Does Not Affect the Locomotion of Mice

To determine the potential influence of PV-positive interneurons in the DG on locomotor activity, PV-Cre mice were injected with either DIO-mCherry control vector or DIO-hM3D-Gq-mCherry lentivirus. The overall motor function of injected mice was examined via the open field test. After the activation of PV-positive interneurons by intraperitoneal injection of CNO, the open field test showed no significant differences in the total distance traveled, cumulative duration in the center, center entry frequency, duration spent immobile, mean velocity or maximum velocity between two groups of animals ($p > 0.05$ for all indexes; Fig. 3A-F), indicating that selective activation of PV-positive

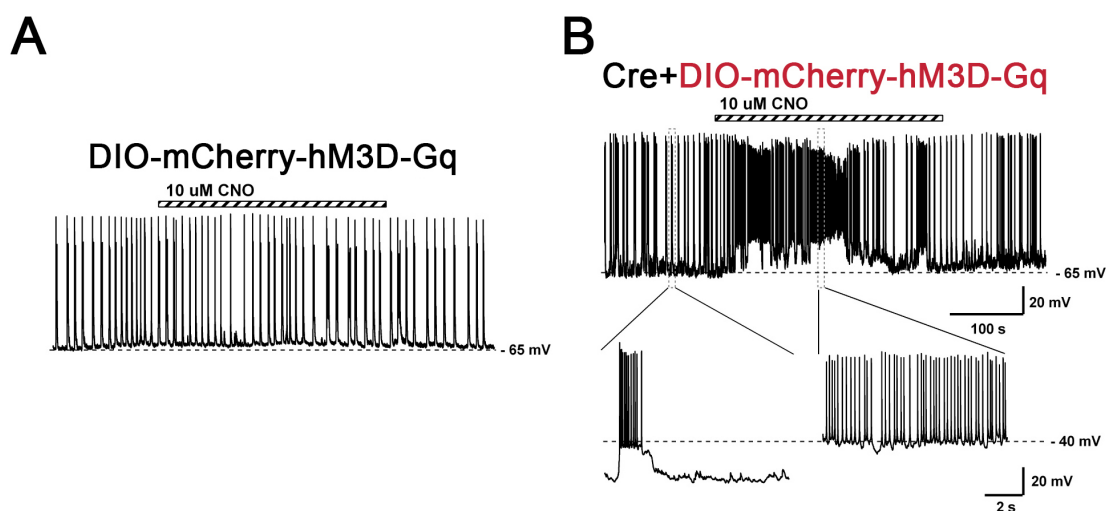


Fig. (2). Patch-clamp recording of cells after CNO exposure. Hippocampal neurons were transfected with the lentiviral DIO-mCherry-hM3D-Gq vector alone (A) or in combination with Cre plasmid (B). Then 10 μ M CNO (horizontal bar) was added to cell cultures, and the firing of action currents in mouse hippocampal neurons was recorded.

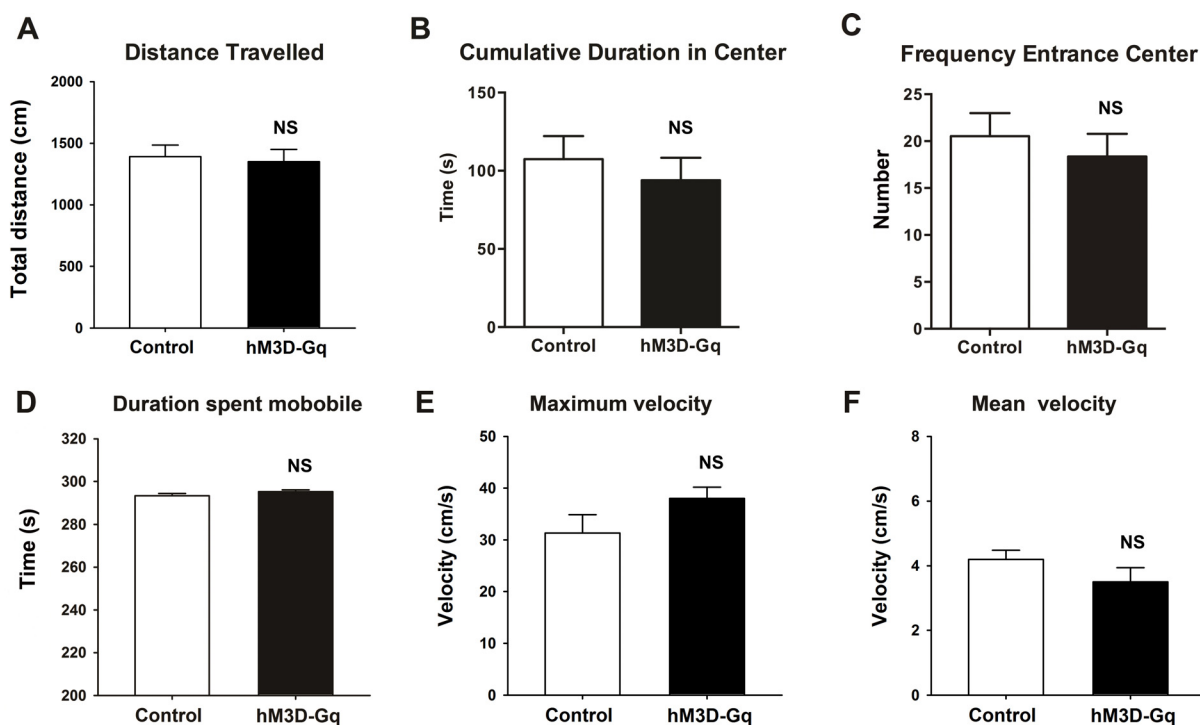


Fig. (3). Animal behavior analysis in the open field test. PV-Cre mice were injected with the lentiviral control vector or lentiviral DIO-hM3D-Gq-mCherry vector. Thirty minutes before behavioral testing, mice were given CNO (0.5 mg/kg body weight) by intraperitoneal injection. During the open field test, the total distance traveled (cm) (A), cumulative duration in the center (s) (B), center entry frequency (C), duration spent mobile (s) (D), maximum velocity (cm/s) (E) or mean velocity (cm/s) (F) were recorded over 5 min. $n = 26$ for the control group; $n = 27$ for the hM3D-Gq group. NS, not significant.

interneurons in the mouse DG does not affect the locomotor activity of mice.

Selective Activation of PV-Positive Interneurons in the DG Affects Anxiety-Like Behavior But Not Depression-Related Behavior

As PV-positive interneurons have been implicated in anxiety- and stress-related disorders [37-39], we analyzed how PV-positive interneurons in the DG

modulate anxiety-related behaviors using the elevated plus maze test. As shown in Fig. 4A, the time in the open arm was significantly increased about 2 folds for mice with activated PV-positive interneurons compared to that for control mice. Our data suggest that activation of PV-positive interneurons in the DG produces an anxiolytic effect, even though the numbers of entries into the open arms were similar between the two groups (Fig. 4B). In addition, we observed a significantly changed number of head dips in mice with

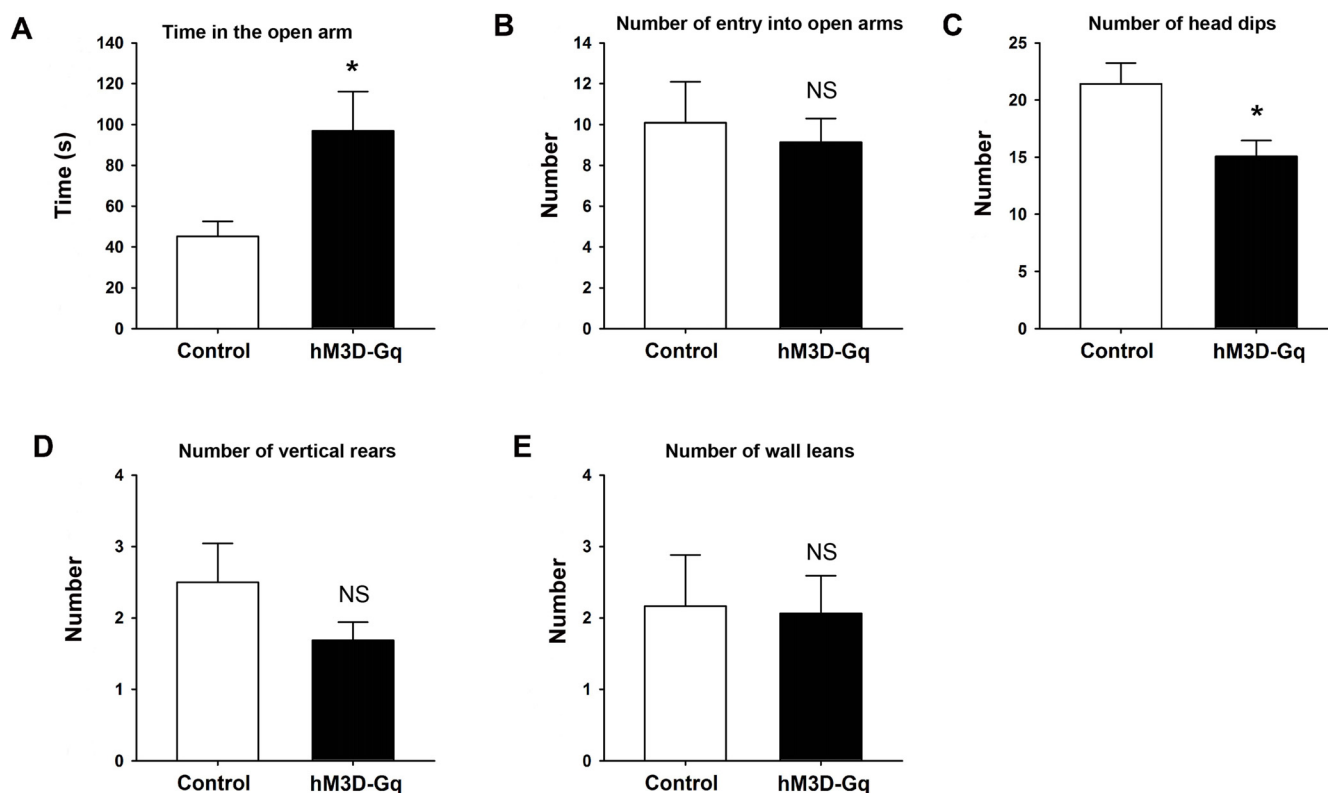


Fig. (4). Elevated maze test. PV-Cre mice were injected with the lentiviral control vector or lentiviral DIO-hM3D-Gq-mCherry vector. Thirty minutes before behavioral testing, mice were given CNO (0.5 mg/kg body weight) by intraperitoneal injection. During the elevated maze test, the time the mice spent in the open arms (**A**), the number of entries into the open arms (**B**), the number of head dips (**C**), the number of vertical rears (**D**) and the number of wall leans (**E**) was recorded. $n = 21$ for the control group; $n = 24$ for the hM3D-Gq group. * $p < 0.05$. NS, not significant.

activated PV-positive interneurons when compared with control (Fig. 4C). No significant difference was found in the number of vertical rears or wall leans between two groups (Fig. 4D, E).

The tail suspension test initially proposed by Steru *et al.* is a well-established behavioral test for determining the efficacy of anti-depressant drugs and is also used to determine the effects of genetic and neurobiological manipulations [33]. Using the tail suspension test, we examined depression-related behavior in mice and found that the depression-related behavior of mice was not affected by CNO-induced activation of PV-positive interneurons in the DG. The immobility time (lack of escape-related behavior) was not significantly altered for mice with activated PV-positive interneurons compared with that for control mice (Fig. 5). These data indicate that selective activation of PV-positive interneurons in the mouse DG does not affect the depression-related behaviors of mice.

Selective Activation of PV-Positive Interneurons in the Mouse DG Affects Social Interaction

Because PV-positive interneurons play an important role in pathophysiological changes in autism, which is characterized by social and communication deficits, we next examined the potential actions of PV-positive

interneurons in affecting social interaction in mice. In the first session of the social interaction test, the social affiliation and motivation of the animals were investigated in both control mice and mice injected with DIO-hM3D-Gq-mCherry lentivirus. Under normal conditions, the control mice spent more time in the chamber containing the Stranger 1 mouse than in the chamber with an empty cup (Fig. 6A), suggesting that control animals had normal sociability, social motivation, and affiliation. Notably, mice with activated PV-positive interneurons (hM3D-Gq group) exhibited a similar behavioral pattern, indicating that activation of PV-positive interneurons did not affect the social affiliation and motivation in mice. (Two-way analysis of variance (ANOVA): treatment \times chamber $F(1,84)=0.350$, $P=0.556$; treatment $F(1,84)=5.922$, $P=0.017$; chamber $F(1,84)=11.768$, $P=0.001$). In the second session of this test, the control animals tended to spend more time with a newly encountered mouse (Stranger 2; vs Stranger 1), with the capability to recall their previous contact with Stranger 1 (Fig. 6B) (Control group, $P=0.033$; hM3D-Gq group, $P=0.947$). Intriguingly, mice expressing hM3D-Gq, however, appeared to have difficulty to recognize the new mouse by spending the same amount of time in both chambers (Fig. 6B) (Two-way analysis of variance (ANOVA): treatment \times chamber $F(1,84)=2.016$, $P=0.159$; $F(1,84)=8.806$, $P=0.004$; chamber $F(1,84)=0.982$, $P=0.325$). Moreover,

interestingly, although the mice expressing hM3D-Gq keep relative normal exploratory behavior during social affiliation trial, they significantly increase the social interaction time when the stranger 2 is present (Fig. 6B). This phenotype may be possibly explained by anxiolytic effect directly affecting their social recognition. These results reveal that activation of PV-positive interneurons does not affect social affiliation and motivation but greatly impaired the social interaction to novel mice.

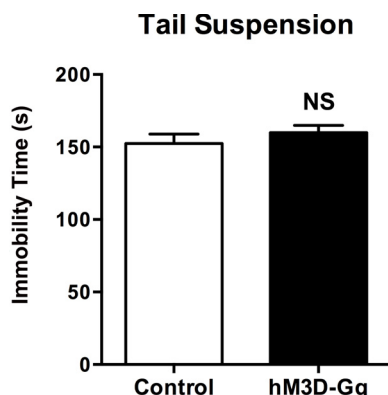


Fig. (5). Comparison of the immobile time between control mice and mice with hM3D-Gq-expressing PV-positive interneurons in the tail suspension test. PV-Cre mice were injected with the lentiviral control vector or lentiviral DIO-hM3D-Gq-mCherry vector. Thirty minutes before behavioral testing, mice were given CNO (0.5 mg/kg body weight) by intraperitoneal injection. During the tail suspension test, the immobility time (sec) was recorded. $n = 26$ for each group. NS, not significant.

Selective Activation of PV-Positive Interneurons in the Mouse DG Does Not Affect Recognition Performance

As we detected a deficit in social recognition, we decided to test how activation of PV interneurons in the DG affect the ability to recognize novel objects. Alteration of PV-interneuron activity has been shown to affect recognition [40, 41]. To further evaluate the effects of PV-positive interneurons on short- and long-term recognition memory in mice, we applied the novel object recognition test. The novel object recognition test showed no significant differences in both the test session for short-term memory (1 h) (Fig. 7A) (treatment $F(1,86)=2.762$, $P=0.100$; object $F(1,86)=2.050$, $P=0.156$) and the test session for long-term memory (24 h) (Fig. 7B) (treatment $F(1,86)=0.099$, $P=0.754$; object $F(1,86)=0.078$, $P=0.780$) between two groups of animals, indicating that selective activation of PV-positive interneurons in the mouse DG does not affect the object recognition activity of mice. Our data suggested that the deficits in the social recognition is not caused by the defect in recognition performance.

Selective Activation of PV-Positive Interneurons in the Mouse DG Results in Enhanced Memory Extinction

PV interneurons are required for synchronization of spiking activity in neuronal networks. Abnormal PV interneuron activity is known to affect fear memory [42, 43]. However, how local PV interneuron activity in the DG affect fear learning is not clear. Because we detected that activation of PV-positive interneurons in the DG leads to deficits in social interaction but not novelty recognition, we next investigated the fear

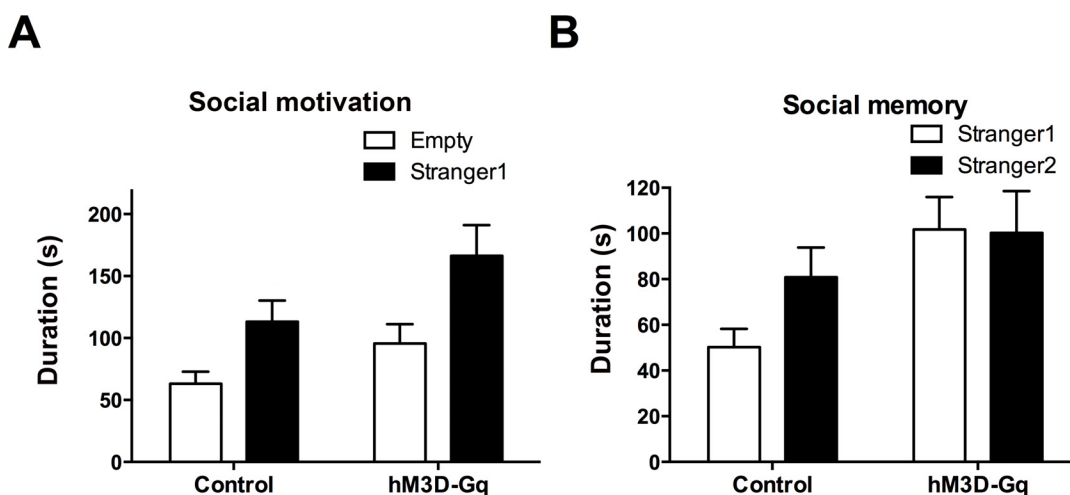


Fig. (6). Social interaction test. PV-Cre mice were injected with the lentiviral control vector or lentiviral DIO-hM3D-Gq-mCherry vector. Thirty minutes before behavioral testing, mice were given CNO (0.5 mg/kg body weight) by intraperitoneal injection. (A) In the first session of the social interaction test, the average duration of time (sec) spent in the chamber with the Stranger 1 mouse or with the empty cup (empty) was recorded. Both of the cherry and hM3D-Gq group mice preferred the stranger 1 mouse. Statistical analysis was conducted using two-way analysis of variance (ANOVA). Treatment vs chamber $F(1,84)=0.350$, $P=0.556$; treatment $F(1,84)=5.922$, $P=0.017$; chamber $F(1,84)=11.768$, $P=0.001$. (B) In the second session, the average duration of time (sec) spent in the chamber with the Stranger 1 mouse or with a new unfamiliar mouse (Stranger 2) was recorded. $n = 22$ for the control group; $n = 22$ for the hM3D-Gq group. Control group, $P=0.033$; hM3D-Gq group, $P=0.947$. Treatment \times chamber $F(1,84)=2.016$, $P=0.159$; $F(1,84)=8.806$, $P=0.004$; chamber $F(1,84)=0.982$, $P=0.325$.

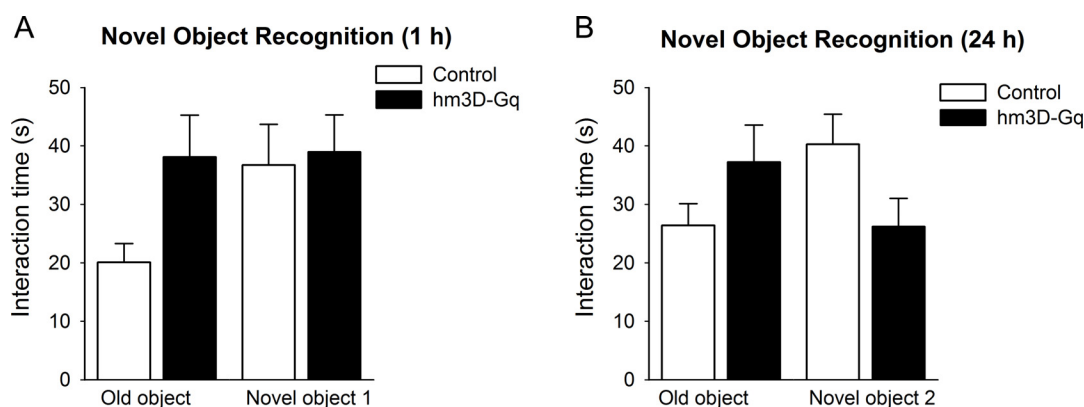


Fig. (7). Novel object recognition test. PV-Cre mice were injected with the lentiviral control vector or lentiviral DIO-hM3D-Gq-mCherry vector. Thirty minutes before behavioral testing, mice were given CNO (0.5 mg/kg body weight) by intraperitoneal injection. During the novel object recognition test, the duration of contact (sec) over 5 min was recorded. $n = 23$ for the control group; $n = 22$ for the hM3D-Gq group. **(A)** Short-term memory (1 h). Statistical analysis was conducted using two-way analysis of variance (ANOVA). Treatment $F(1,86)=2.762$, $P=0.100$; object $F(1,86)=2.050$, $P=0.156$. **(B)** Long-term memory (24 h). Treatment $F(1,86)=0.099$, $P=0.754$; object $F(1,86)=0.078$, $P=0.780$.

memory in mice in the different experimental groups using a fear conditioning and extinction paradigm (Fig. 8A). No significant difference was detected in the contextual fear conditioning test during the first 6 days, suggesting that the hM3D-Gq mice had an intact memory. Interestingly, the hM3D-Gq mice show enhanced extinction formation on day 7 (Fig. 8B). No statistical difference in the cued fear test, a hippocampal-independent learning test, between the two groups (Fig. 8C, D) supports the enhancement in memory extinction is the DG-dependent.

DISCUSSION

As a major subset of GABAergic interneurons, PV-positive interneurons are known to play key roles in the neural network responsible for many essential behaviors and deficits of PV interneurons will contribute to pathological changes of neurological disorders [44-46]. Because of the importance of PV-positive interneurons in circuitry and behavioral regulation, we seek to better understand the role of PV interneurons in the dentate gyrus, a critical subregion for learning and memory. As PV interneurons exhibit an inhibitory effect on local circuitry [47], selective activation of PV-positive interneurons in the hippocampus using lentivirus-mediated DREADD technology can suppress neuronal activity in the DG in this study. Our data indicated that the activation of PV-positive interneurons impaired social memory performance in mice, without significantly affecting locomotor activity, depression-related behavior or object recognition.

The efficacy of a Cre-inducible lentiviral vector carrying mCherry and hM3D-Gq was verified by transfecting neuroblastoma CAD cells [48] with viral vector and Cre plasmid. In the presence of Cre, mCherry expression was observed in cells, whereas no fluorescence was detected in cells transfected with viral vector alone, indicating the successful construction of the recombinant lentiviral vector. Additionally, the activity of neurons in response to CNO treatment was

confirmed using patch-clamp technology. The firing of action currents in mouse hippocampal neurons was greatly increased upon CNO exposure. As most of these PV-positive interneurons are GABAergic interneurons (GABA, γ -aminobutyric acid) [2], it is possible that activation of these inhibitory neurons may yield increased inhibition in activity of excitatory neurons in the DG [49-51]. Indeed, hM3D-Gq was expressed in PV-positive neurons in Cre-mice that received lentiviral injection and CNO administration.

Several neuropsychiatric diseases, including autism spectrum disorders, share similar neurobehavioral deficits that are characterized by dysfunctional social interaction and failure in social communication. Such social behavioral deficits are known to be associated with the impaired hippocampal function in rodent experiments [52]. The neural circuitry regulating social interaction just start to emerge [34, 53]. Nevertheless, whether social interaction behaviors are controlled by a special population of neurons in the hippocampus remains unclear. To further investigate the possible role of PV-positive interneurons in modulating social behavior, the social interaction test was conducted. We found that selective activation of PV-positive interneurons did not affect social affiliation and motivation but greatly impaired social memory performance in mice. We observed the increase exploratory behaviors, which could affect their ability to recognize new mice.

Functional deficits on PV interneurons impair animal behavioral. A reduction of excitatory drive on whole brain PV interneurons by ablation of GluR leads to imprecise spike timing and damaged working/episodic like memory [54]. Loss of NMDA receptor on PV interneurons also alters the hippocampal network and affects working memory [55]. A previous report showed that the excitation of PV-positive interneurons contributes to learning and memory in mice, and deletion of the M1 muscarinic acetylcholine receptor (mAChR) from PV-positive interneurons leads to

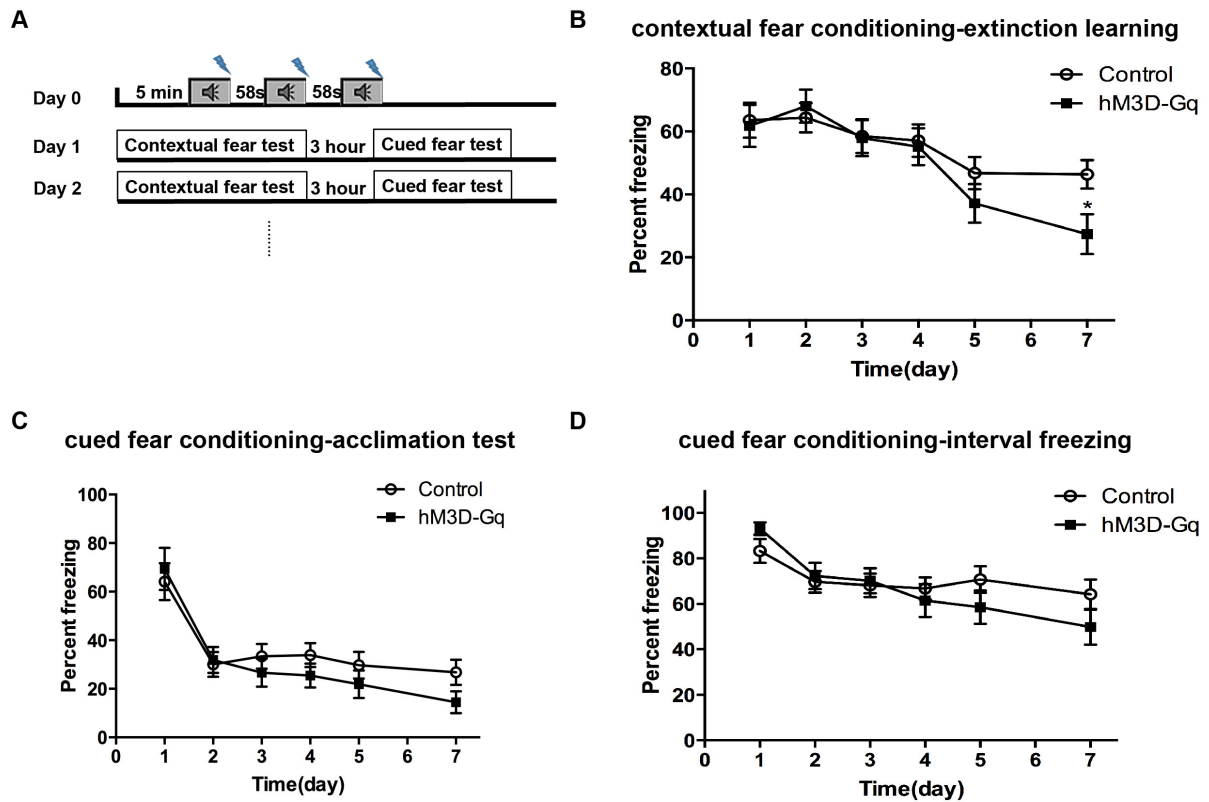


Fig. (8). Fear conditioning test. PV-Cre mice were injected with the lentiviral control vector or lentiviral DIO-hM3D-Gq-mCherry vector. Thirty minutes before behavioral testing, mice were given CNO (0.5 mg/kg body weight) by intraperitoneal injection. A sketch of experimental paradigm is shown (A). During the fear conditioning test, the percentage of freezing behavior in different trials, including (B) contextual fear conditioning-extinction learning, (C) cued fear conditioning-acclimation testing, and (D) cued fear conditioning-interval freezing, was calculated. $n = 23$ for the control group; $n = 19$ for the hM3D-Gq group. $*p < 0.05$.

impaired novel object recognition in animals [41]. Collectively, these results demonstrate that global PV interneurons play critical roles to maintain the excitation/inhibition balance and to modulate proper behaviors. However, it is still unclear how PV interneurons work at a defined sub-region of the brain. CA1 PV interneurons are required for spatial working but not for reference memory [9]. Inhibition of PV and GAD65 interneurons in the ventral hippocampus by hM4D leads to schizophrenia like behaviors in mice [7]. Here, we showed that selective activation of PV-positive interneurons in the DG does not affect object recognition in mice, suggesting that require PV interneurons in the other brain regions to complete the recognition task. Different types of memory such as social memory and object recognition memory may involve PV-positive interneurons in different ways using distinct underlying mechanisms. Nonetheless, further research is needed to determine the detailed molecular mechanism(s) by which PV-positive interneurons regulate each specific type of memory.

The patients with anxiety disorders, such as post-traumatic stress disorder (PTSD), in which fear extinction is compromised. The use of Pavlovian fear conditioning has significantly advanced our understanding of mechanism controlling the fear and anxiety in experimental animals. Conditioned fear reactions to the tone diminish in the absence of the

shock. The return of fear after extinction indicates that extinction does not wipe away fear memories, but generates a suppressive memory capable of temporarily blocking the expression of fear. Many defects in the DG and amygdala circuits impair the fear extinction [43, 56-58]. But to enhance extinction might be potentially beneficial for the treatment of PTSD. Our data suggest that simple activation of the PV interneuron in the DG can enhance fear extinction in mice, suggesting a critical role of PV interneuron in formation of inhibitory memory to conditioned fear. Activation of PV interneurons in the DG might be a potentially useful new strategy to alleviate PTSD symptoms.

CONCLUSION

Our current study showed that selective activation of PV-positive interneurons thereby suppressing the DG region of the mouse hippocampus affects social interaction, without significantly influencing locomotor activity and depression-related behavior, or social motivation in mice. These findings provide basic evidence to improve our understanding of the actions of PV-positive interneurons in controlling mouse behaviors. Our study also supports that abnormal activation of PV-positive interneurons may underlie the pathophysiology of some human neurological diseases. However, we could not rule out the possibility that

social memory is modulated collaboratively by other subgroups of neurons in addition to PV-positive neurons in the hippocampus. Future studies will be carried out to explore the precise molecular mechanism(s) involved in PV-positive interneuron-mediated actions in mice.

ABBREVIATIONS

CAD	=	Cath.a-differentiated
CNO	=	Clozapine-N-oxide
DG	=	Dentate gyrus
DMEM	=	Dulbecco's Modified Eagle Mmedium
DREADD	=	Designer receptors exclusively activated by designer drugs
FBS	=	Fetal bovine serum
GABA	=	γ -aminobutyric acid
HEK	=	Human embryonic kidney
hM3Dq	=	Human M3 muscarinic DREADD receptor coupled to Gq
mAChR	=	M1 muscarinic acetylcholine receptor
PV	=	Parvalbumin
RFP	=	Red fluorescent protein

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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