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·Original Article·

Pharmacogenetic activation of midbrain dopaminergic neurons induces hyperactivity

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ABSTRACT

Dopaminergic neurons regulate and organize numerous important behavioral processes including motor activity. Consistently, manipulation of brain dopamine concentrations changes animal activity levels. Dopamine is synthesized by several neuronal populations in the brain. This study was carried out to directly test whether selective activation of dopamine neurons in the midbrain induces hyperactivity. A pharmacogenetic approach was used to activate midbrain dopamine neurons, and behavioral assays were conducted to determine the effects on mouse activity levels. Transgenic expression of the evolved hM3Dq receptor was achieved by infusing Cre-inducible AAV viral vectors into the midbrain of DAT-Cre mice. Neurons were excited by injecting the hM3Dq ligand clozapine-N-oxide (CNO). Mouse locomotor activity was measured in an open field. The results showed that CNO selectively activated midbrain dopaminergic neurons and induced hyperactivity in a dose-dependent manner, supporting the idea that these neurons play an important role in regulating motor activity.

Keywords: pharmacogenetics; electrophysiology; motor activity; clozapine-N-oxide

INTRODUCTION

As an important neurotransmitter, dopamine is believed to modulate neuronal excitability and neurotransmission in numerous brain areas[1]. It is synthesized by a subset of neurons in the ventral tegmental area, the substantia nigra pars compacta (VTA/SNc) of the midbrain, as well as in the hypothalamus and olfactory bulb[2]. Midbrain dopaminergic neurons project axons extensively to the cortex and striatum and their activity is believed to be closely related to fundamental behavioral processes such as cognition, motor activity, and reward[2,3]. The dopaminergic projection from the VTA/SNc to the neostriatum in the basal ganglia is believed to be particularly critical for motor control[2].

Several lines of evidence suggest that brain dopamine signals are closely associated with the regulation of motor activity. Acute administration of drugs such as amphetamine and cocaine increases brain dopamine levels, leading to striking hyperactivity in animals and humans[4,5]. Genetic manipulations that change brain dopamine levels also alter motor activity levels[5,6]. In humans, malfunctions of the brain dopamine system are associated with several neurological and neuropsychiatric disorders with the disruption of motor activity as one of the core symptoms[7,8]. However, the conclusions of these studies are often confounded by drug effects on targets outside the dopamine system, or potential disruption of neural circuits during development after gene knockout. Whether increasing the activity of midbrain dopaminergic neurons induces hyperactivity remains to be directly tested.

Selective activation of midbrain dopaminergic neurons has been technically challenging because dopaminergic and non-dopaminergic neurons are intermixed in a

relatively large area of the midbrain[2]. In this study, we tested whether the activation of midbrain dopaminergic neurons affects motor activity with a pharmacogenetic tool[9,10]. The gene encoding the evolved hM3Dq receptor was targeted into midbrain dopaminergic neurons by stereotaxic injection of a Cre-inducible AAV viral vector[11,12]. Dopaminergic neurons were then remotely excited by intraperitoneal injection of the hM3Dq ligand clozapine-N-oxide (CNO). We found that selective pharmacogenetic activation induced a dramatic increase in mouse motor activity, directly supporting a role for midbrain dopaminergic neurons in hyperactivity.

MATERIALS AND METHODS

Mice

Animal protocols were approved by the institutional Animal Care and Use Committee and conformed to the institutional guidelines of the National Institute of Biological Sciences, Beijing as well as governmental regulations. All experiments were performed on adult (8–16 weeks old) DAT-Cre mice [Jackson Laboratory, strain B6.SJL-Slc6a3tm1.1(cre)Bkmn/J]. Animals were housed at 22–24°C with freely-available food and water and a 12-h light/12-h dark cycle (lights on 21:30–09:30).

AAV Viral Vectors for hM3Dq and mCherry

We used Cre-dependent AAV vectors with two pairs of loxP recombination sites covering the specific genes in a 3'-to-5' orientation (AAV DIO)[12]. The inverted coding sequence of hM3Dq-2A-mCherry or mCherry was placed into an AAV DIO vector with a CMV early enhancer/chicken β-actin (CAG) promoter (Fig. 1A, B). The vectors were then packaged in AAV serotype 2/9 vectors consisting of the AAV2 ITR genomes and the AAV9 serotype capsid gene. The AAV2/9 viral vectors were produced in HEK293 cells by the 3-plasmid transfection method and purified by cesium chloride density-gradient centrifugation and then desalination by dialysis[13], producing AAV viral vector titers of $>10^{12}$ particles/mL.

Stereotaxic Virus Injection

Mice were anesthetized with pentobarbital (80 mg/kg; i.p.). After opening two small holes above the VTA/SNc area of a DAT-Cre mouse, a glass micropipette (~20 μm tip diameter) was filled with AAV virus and lowered into the VTA/SNc (coordinates relative to bregma: AP = –3.0 mm; DV = –4.0 mm; ML = 0.50 mm). AAV viruses (500 nL) were pressure-injected into each side with a WPI Nanoliter 2000 Injector at a very slow speed (23 nL/min). Mice were allowed to recover for 2 weeks before behavioral assays.

Immunohistochemistry

Mice were anesthetized with an overdose of pentobarbital (100 mg/kg; Merck) and then perfused with ice-cold 0.9% saline followed by 4% paraformaldehyde in 0.1 mol/L PBS containing 0.2% picric acid at pH 7.3. The brain was rapidly removed and postfixed in the same paraformaldehyde overnight at 4°C, then transferred to 30% sucrose in 0.1 mol/L PBS until settled. Coronal sections at 20 μm were cut on a cryostat (Leica CR 1900) and collected in 0.1 mol/L PBS containing 0.3% Triton X-100. Sections were pre-blocked for 1 h with 3% bovine serum albumin (BSA, AMRESCO Inc.) in 0.1 mol/L PBS. They were washed with wash buffer (0.1 mol/L PBS and 0.3% Triton X-100) and then incubated with a rabbit polyclonal anti-c-Fos antibody (1:500, EMD Chemicals, San Diego, CA) or rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (1:500, Chemicon) overnight at 4°C and incubated with Cy2-conjugated secondary antibody (1:500, Jackson ImmunoResearch) for 1 h at room temperature. After staining, sections were mounted on gelatin-coated slides with 50% buffered glycerol. Microscopic images were captured with a confocal microscope (DIGITAL ECLIPSE A1, Nikon) and processed with Adobe Photoshop.

Circadian Locomotor Activity

Circadian global activity was assessed by measuring the daily locomotor activity (24 h) in a test cage (30 cm × 30 cm) with an overhead infrared camera. Each animal was first habituated to the test cage for 24 h. Saline or CNO (1.0 mg/kg) was then injected at the onset of the dark phase of the second and third days before monitoring locomotor activity.

Open-Field Test

We used the open-field test to investigate the effect of CNO on locomotor activity. In this test, an individual mouse was placed in the center of a clean open-field arena (40 cm × 40 cm, wall height 24 cm) and allowed to move freely for

15 min. Animal positions were tracked with an overhead infrared video camera (25 frames per second) and moving distance was calculated at 5 Hz with a customized Matlab program. Saline was intraperitoneally injected 30 min prior to the test on the first day and saline or CNO was administered with increasing concentrations (0.5, 1.0, and 2.0 mg/kg) during the subsequent days.

Statistical Analysis

All data are presented as mean ± SEM. Data analysis was performed with Matlab (MathWorks). *T*-test and ANOVA were performed to determine the significance of variable contributions and $P < 0.05$ was considered as statistically significant.

RESULTS

Selective Activation of Midbrain Dopaminergic Neurons

We used Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology to selectively excite midbrain dopaminergic neurons. AAV viral vectors were bilaterally injected into the VTA/SNC areas of DAT-Cre mice to express the evolved human muscarinic receptor hM3Dq, which is activated by its ligand CNO but not endogenous neurotransmitters[10]. The coding sequence of hM3Dq was linked with those of the 2A peptide and a red fluorescent protein mCherry and then packaged into a Cre-recombinase-dependent AAV vector to allow bicistronic expression of hM3Dq and mCherry (AAV DIO hM3Dq-2A-mCherry; Fig. 1A). AAV DIO mCherry vectors were used as control (Fig. 1B). Following stereotaxic infusion of either viral vector into the midbrain of DAT-Cre mice, we observed strong mCherry expression selectively in the midbrain VTA/SNc, where most dopaminergic neurons are located (Fig. 1C–E). The distribution of $mCherry^+$ neurons resembled that of TH-immunopositive neurons. By using the bicistronically-expressed mCherry as a marker of hM3Dq expression and TH-immunoreactivity as a marker of dopaminergic neurons, we found that ~78% of dopaminergic neurons were infected with AAV virus and produced clear mCherry signals (425 ± 75 $mCherry^+$ neurons out of 540 ± 75 TH^+ neurons from one coronal section of each of seven mice examined), suggesting high efficiency of transgenic expression. More importantly, the vast majority of $mCherry^+$ neurons (>95%) were also TH-immunopositive (Fig. 1D). These results thus demonstrated that the application of Cre-dependent AAV virus produces efficient and selective expression of hM3Dq/mCherry in midbrain dopaminergic neurons.

To determine whether CNO excites midbrain dopaminergic neurons, we first carried out target recordings from hM3Dq/$mCherry^+$ dopaminergic neurons in brain slices (n = 6 cells). Cell-attached recordings showed that pressure-injection of CNO (10 μmol/L) elicited vigorous firing of action potentials (Fig. 2A). Whole-cell current-clamp recordings demonstrated that CNO depolarized these neurons and evoked action-potential firing (Fig. 2B). As a control, CNO did not evoke responses from DAT-Cre mice injected with AAV DIO mCherry virus (data not shown). We further tested whether CNO activated neurons in the VTA/SNc *in vivo* by using c-Fos expression as the indicator of neuronal activity. Mice were injected with CNO (1 mg/kg body weight, i.p.) and were then killed 60 min later for immunohistological analysis of c-Fos expression. CNO induced c-Fos signals in many neurons in the VTA/SNc area of DAT-Cre mice injected with AAV DIO hM3Dq-2A-mCherry vectors but not in those injected with AAV DIO mCherry vectors (Fig. 2C–E), suggesting that hM3Dq expression elicits selective neuronal activation. In the mice expressing hM3Dq/mCherry, c-Fos signals were detected in many $mCherry^+$ neurons. A substantial number of $c\text{-}Fos^+$ neurons lacked clear mCherry expression, suggesting that CNO activates hM3Dq-expressing neurons as well as nearby neurons through synaptic connections.

Activation of Midbrain Dopaminergic Neurons Results in Hyperactivity

After confirming the effectiveness of the pharmacogenetic approach, we tested whether the selective activation of midbrain dopaminergic neurons affects motor activity levels. AAV DIO hM3Dq-2A-mCherry vectors or the AAV DIO mCherry control vectors were bilaterally infused into the VTA/SNc of DAT-Cre mice. After two weeks' recovery, these mice were injected with either saline or CNO (1 mg/kg, i.p.) at the onset of the dark phase and their locomotor activity in a test arena was video-recorded and analyzed online for 24 h. Horizontal travel distance was used as the index of activity levels. In hM3Dq-expressing mice, the activity was immediately elevated after CNO injection but not saline. A more than eight-fold increase was induced during the first hour after drug injection (Fig. 3). In contrast,

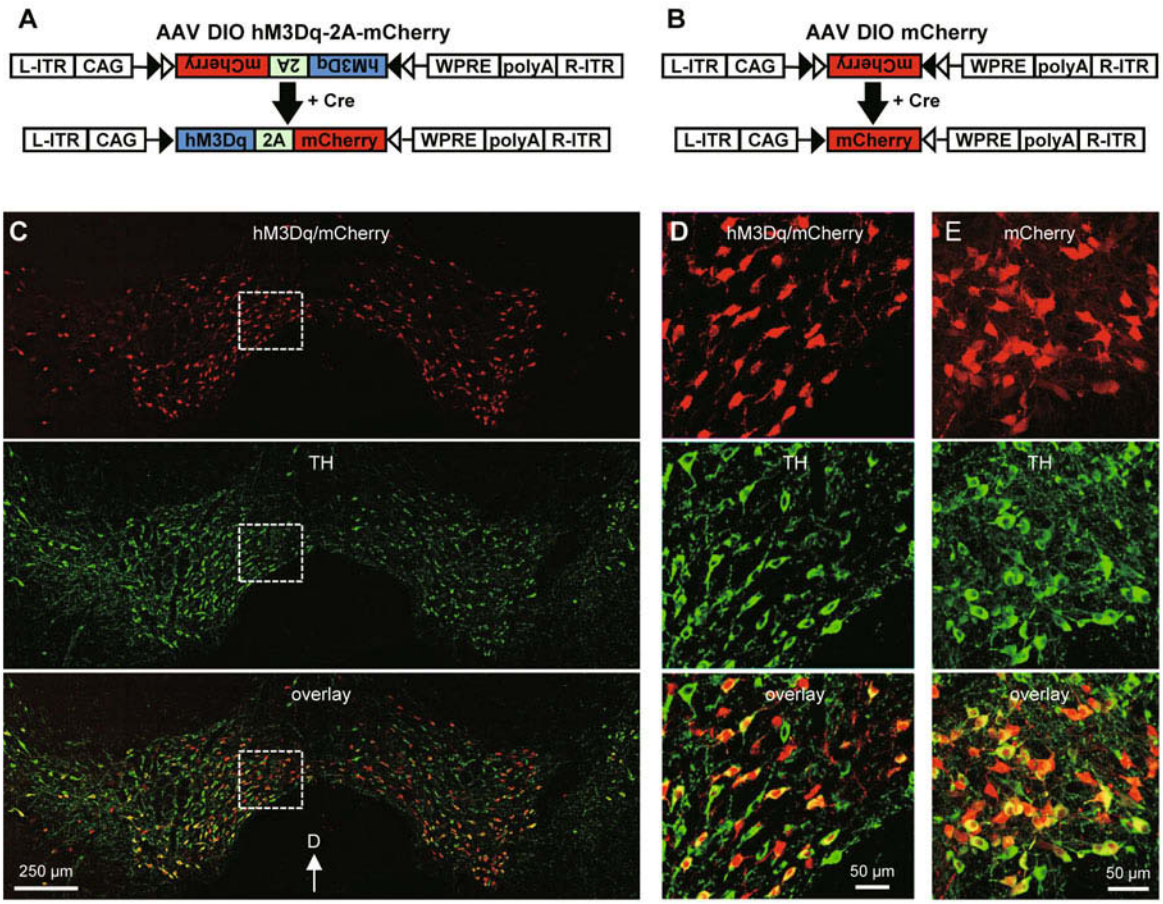

Fig. 1. Selective expression of the DREADD receptor hM3Dq in midbrain dopaminergic neurons. A and B: AAV viral vector designs for AAV DIO hM3Dq-2A-mCherry (A) and AAV DIO mCherry (B). CAG: CMV enhancer/chicken β-actin promoter; L-ITR and R-ITR: left and right inverted terminal repeat; WPRE: woodchuck hepatitis virus post-transcriptional regulatory element. C: Bilateral infusion of AAV DIO hM3Dq-2A-mCherry vectors into the VTA/SNC of DAT-Cre mice resulted in bicistronic expression of hM3Dq and mCherry (red) in tyrosine hydroxylase (TH)-immunopositive (green) midbrain dopaminergic neurons (*n* = 7 mice). The label D and arrow indicate dorsal up. D: High-power view of the mCherry fluorescence signal and TH immunoreactivity in the dashed boxes shown in (C). E: Selective expression of mCherry (red) in TH-immunopositive neurons (green) in the VTA following the injection of AAV DIO mCherry control vectors into a DTA-Cre mouse (*n* = 12 mice).

CNO did not have any clear effect on mice injected with mCherry control vectors. A three-way ANOVA analysis was conducted to compare the effects on travel distance of CNO, AAV vectors (AAV DIO hM3Dq-2A-mCherry or AAV DIO mCherry) and time. These variables had significant effects [CNO *vs* saline: $F(1, 23) = 74.1$; $P < 0.001$; vector: $F(1, 23) = 89.7$, $P < 0.001$; time: $F(1, 23) = 23.5$, $P < 0.001$; $n = 12$ mice for both hM3Dq/mCherry and mCherry groups]. The CNO-induced hyperactivity of hM3Dq-expressing mice lasted up to 11 h, suggesting that the selective activation of midbrain dopaminergic neurons induces dramatic and prolonged hyperactivity.

We then analyzed the dose-dependency of the CNO-induced hyperactivity. Mice expressing hM3Dq/mCherry or mCherry were given CNO at 0, 0.5, 1.0 and 2.0 mg/kg on consecutive days. Thirty minutes after injection, the activity levels in the open field were measured for 15 min. CNO at 0.5 mg/kg increased the activity levels of hM3Dq-expressing mice ($P < 0.001$; paired *t*-test between saline and CNO; $n = 12$ mice) but not mCherry-expressing mice ($P = 0.59$; paired *t*-test between saline and CNO; $n = 12$ mCherry-expressing mice) (Fig. 4A–C). The activity levels

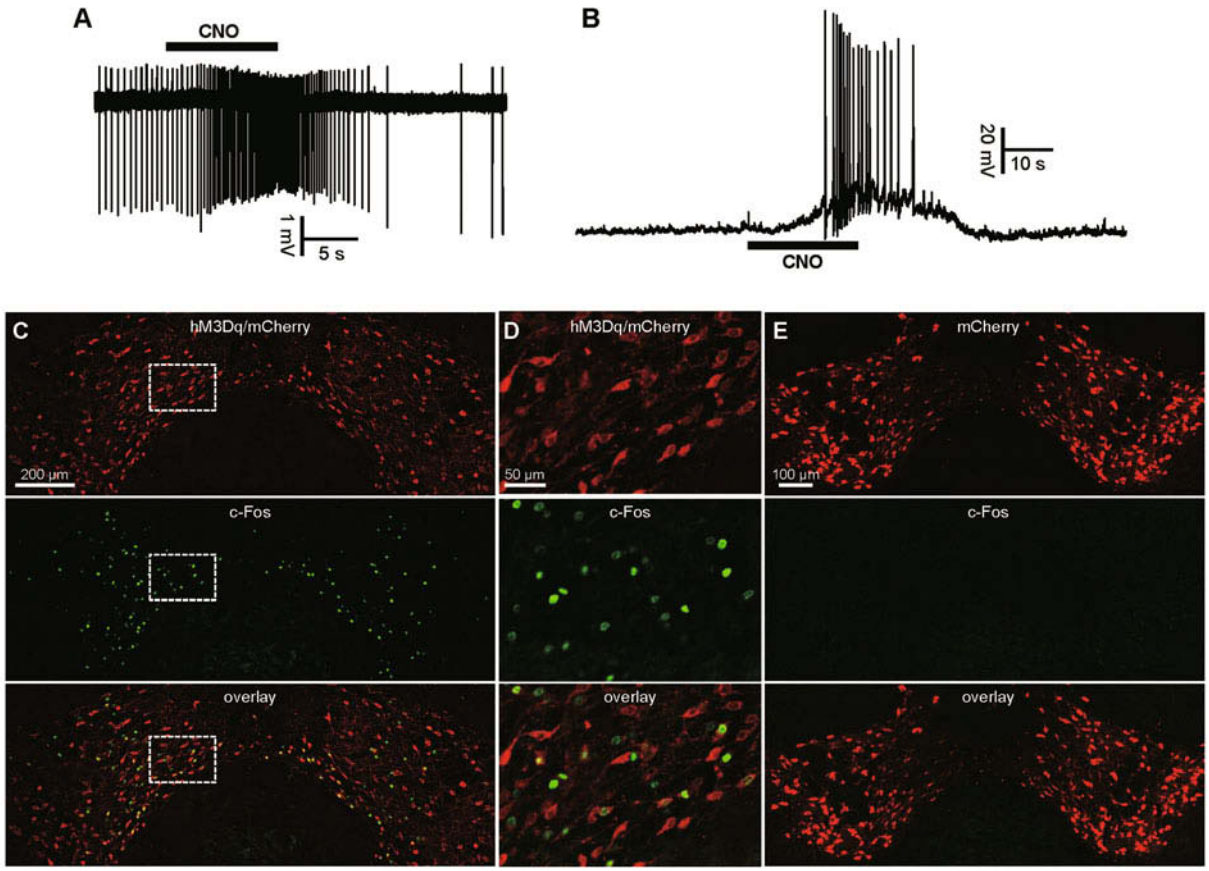

Fig. 2. Pharmacogenetic activation of midbrain dopaminergic neurons. A: Cell-attached patch recording showed that pressure-injection of the hM3Dq receptor ligand CNO (horizontal bar) evoked vigorous firing of action potentials from an hM3Dq/mCherry$^+$ dopaminergic neuron in the VTA. Cells were target recorded using mCherry fluorescent signals. B: Whole-cell current-clamp recording revealed that a hM3Dq/mCherry$^+$ dopaminergic neuron in the VTA was excited by CNO. C: Intraperitoneal injection of CNO (1 mg/kg, i.p.) induced c-Fos expression (green) following the expression of hM3Dq/mCherry (red) in the VTA/SNC (n = 7 mice). D: High-power view of mCherry signals and c-Fos immunoreactivity within the dashed rectangle in (C). E: In contrast, CNO injection did not produce any c-Fos signal in the VTA/SNc of mCherry-expressing mice (n = 7 mice).

of hM3Dq mice were further enhanced by injecting 1.0 mg/kg CNO and reached a plateau at 2.0 mg/kg (Fig. 4C). Two-way ANOVA analysis revealed significant effects of CNO doses, viral vector, and the interaction of these two variables [dose: $F(1, 3) = 27.3$; $P < 0.001$; vector: $F(1, 3) = 260.3$, $P < 0.001$; interaction: $F(1, 3) = 27.7$, $P < 0.001$; n = 12 mice/group]. The time spent exploring the central region of an open field is thought to be related to anxiety. Perhaps due to the hyperactivity, hM3Dq-expressing mice exhibited significantly more entries into the center of the open field following CNO injection [dose: $F(1,3) = 7.3$; $P < 0.001$; vector: $F(1, 3) = 16.0$, $P < 0.001$; interaction: $F(1, 3) = 5.9$, $P < 0.001$; 2-way ANOVA; n = 12 mice/group] (Fig. 4D). Although the number of entries into the center of an open field is associated with reduced anxiety, the proportion of time spent in central exploration was not affected by hM3Dq expression [vector: $F(1, 3) = 0.31$, $P = 0.58$; one-way ANOVA; n = 12 mice/group]. There was a significant contribution to variance by different CNO doses [$F(1, 3) = 4.0$, $P < 0.05$; one-way ANOVA; n = 12 mice/group], but the proportion of time spent in center exploration did not increase with higher CNO doses (Fig. 4E). These results thus suggest that pharmacogenetic activation of midbrain dopaminergic neurons induces hyperactivity in a dose-dependent manner but might not induce clear changes in anxiety levels.

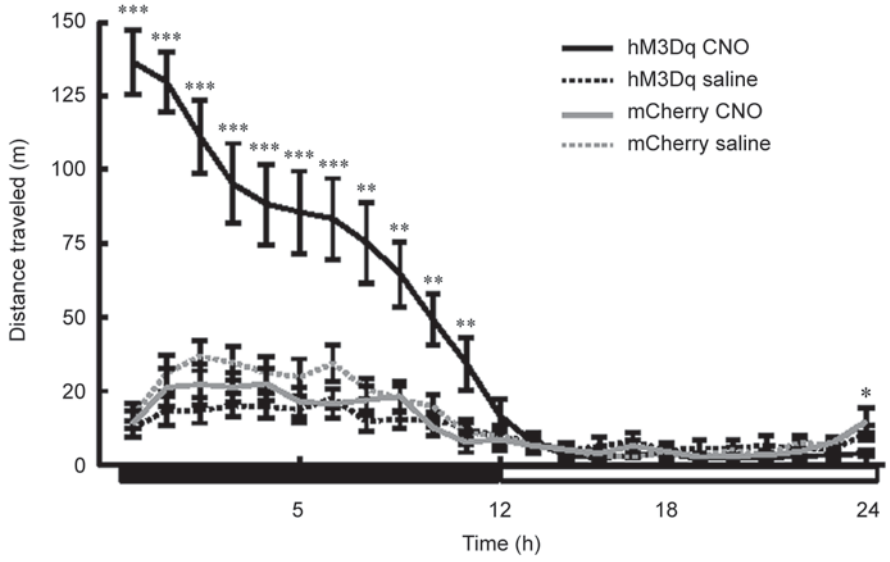

Fig. 3. Selective activation of midbrain dopaminergic neurons results in hyperactivity. Mouse locomotor activity was video-recorded and analyzed with a computer program. CNO (1 mg/kg) or saline control was injected to hM3Dq/mCherry-expressing or mCherry-expressing DAT-Cre mice at the onset of the dark phase. Horizontal travel distance was measured as the index of activity. CNO injection resulted in a drastic increase in activity for over 10 h. Error bars indicate SEM in this and the following figures. The filled rectangle below the horizontal axis represents the dark phase and the open rectangle indicates the light phase. ******P* **<0.001; *****P* **<0.01; ****P* **<0.05; *t*-test of CNO effects between hM3Dq/mCherry-expressing mice and mCherry-expressing mice (*n* = 12 mice/group).

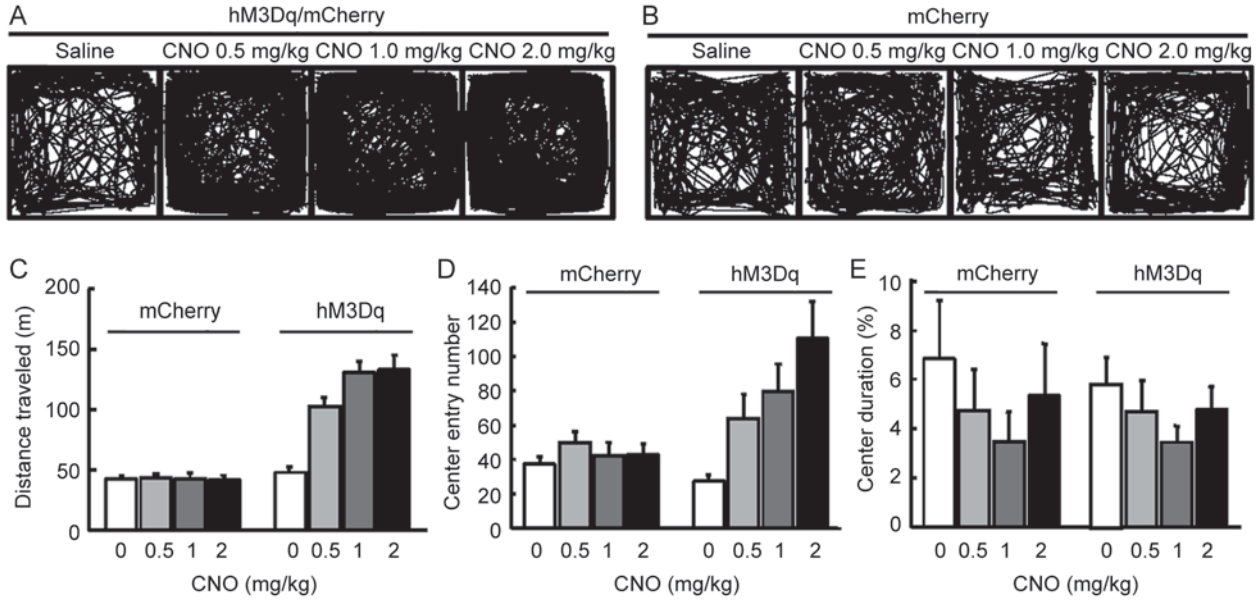

Fig. 4. CNO induces hyperactivity in a dose-dependent manner and does not affect anxiety levels. A: Representative 15-min locomotor tracks of an hM3Dq/mCherry-expressing mouse in response to injection of saline or CNO at 0.5, 1.0, and 2.0 mg/kg. B: Locomotor tracks of a mCherry-expressing control mouse following saline or CNO injection. C: Group data showing that CNO increased the locomotor activity of hM3Dq/mCherry-expressing mice but not mCherry-expressing control mice in a dose-dependent manner (*n* = 12 animals expressing hM3Dq and 12 expressing mCherry control). D: CNO increased the number of central entries in a dose-dependent manner. E: CNO did not affect the duration of central exploration.

DISCUSSION

Although higher brain dopamine levels are often associated with increased motor activity, it remained unclear whether the selective activation of dopaminergic neurons in the midbrain leads to hyperactivity. In this study, we used a pharmacogenetic method to selectively excite midbrain dopaminergic neurons in mice and showed that viral-delivery of the hM3Dq receptor allowed the selective activation of midbrain dopaminergic neurons with its inert ligand CNO and induced drastic hyperactivity, further supporting the role of the midbrain dopaminergic system in regulating motor activity levels.

Our results confirm and extend our understanding of the relationship between the dopaminergic system and the regulation of motor activity. High levels of dopamine are believed to induce hyperactivity[4,5]. However, previous studies did not establish a direct link between the activity of midbrain dopaminergic neurons and hyperactivity. The interpretation of drug-induced behavioral changes is commonly challenged by the presence of multiple populations of dopaminergic neurons within and outside the midbrain VTA/SNc area as well as by the effects of drugs on targets not directly related to dopamine signaling. Hyperactivity is manifested by hyperdopaminergic mutant mice, including dopamine transporter (DAT) knockout mice[5,6] and DAT knockdown mice[14]. Genetic knockout or knockdown of DATs increases dopamine levels inside and outside the nervous system and may change neural circuit formation during development. Our study benefits from the advantages of pharmacogenetic approaches, which allow acute, reversible, and selective activation of a given set of neurons. In addition, viral transgenic expression of the hM3Dq receptor bypasses the possibility of developmental change that might be associated with genetic manipulations.

Interestingly, hyperactivity can be induced by both increasing and decreasing brain dopamine levels. Human brain imaging and animal studies have strongly suggested that a hypofunctional dopamine system underlies the core symptoms of attention deficit/hyperactivity disorder (ADHD)[15-18]. The low-dopamine theory explains why low levels of stimulant drugs mildly increase brain dopamine levels but paradoxically reduce the hyperactivity in human ADHD. Nevertheless, the increase of activity in low-dopamine-related ADHD is substantially milder than that evoked by the selective activation of midbrain dopaminergic neurons. Rodent models of ADHD exhibit about a one-fold increase in locomotor activity[15,19]. In contrast, a nearly ten-fold increase can be induced by increasing dopamine levels with drugs[5,6]. This drastic hyperactivity evoked by pharmacogenetic activation might be more appropriately considered as a symptom of the mania-like behavior associated with high-dopamine[5,6] but not ADHD associated with low-dopamine[15,19].

Our study demonstrated the effectiveness of pharmacogenetic approaches in activating midbrain dopaminergic cells. CNO depolarized hM3Dq-expressing dopaminergic neurons and elicited vigorous action potential firing. Its application *in vivo* induced c-Fos expression in many VTA/SNc neurons, some of which lacked clear hM3Dq expression. This might reflect the extensive collateral connections in the midbrain. CNO failed to produce c-Fos signals in some hM3Dq-expressing neurons, possibly because c-Fos is a marker of strong neuronal activation and does not label weakly-excited neurons. Nevertheless, the robust behavioral effects of CNO validated the power of pharmacogenetic methods in activating a given set of neurons. Compared to optogenetic stimulation, pharmacogenetic methods offer the advantage of activating neurons in large bilateral areas. Moreover, they do not require the implantation of optic fibers and provide a more convenient way to monitor animal behavior. Since the midbrain dopaminergic system is involved in a diverse array of cognitive processes[2,3], pharmacogenetic methods can be used to study the effects of activating dopaminergic neurons on animal performance in other behavioral tests, such as conditioned place preference, drug self-administration, and operant conditioning.

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