SHORT COMMUNICATION

A novel dual serotonin transporter and M-channel inhibitor D01 for antidepression and cognitive improvement

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Abstract Cognitive dysfunction is a core symptom common in psychiatric disorders including depression that is primarily managed by antidepressants lacking efficacy in improving cognition. In this study, we report a novel dual serotonin transporter and voltage-gated potassium Kv7/KCNQ/M-channel inhibitor D01 (a 2-methyl-3-aryloxy-3-heteroarylpropylamines derivative) that exhibits both anti-depression effects and improvements in cognition. D01 inhibits serotonin transporters ($K_i = 30.1 \pm 6.9$ nmol/L) and M channels ($IC_{50} = 10.1 \pm 2.4$ μmol/L). D01 also reduces the immobility duration in the mouse FST and TST assays in a dose-dependent manner without a stimulatory effect on locomotion. Intragastric administrations of D01 (20 and 40 mg/kg) can significantly shorten the immobility time in a mouse model of chronic restraint stress (CRS)-induced depression-like behavior. Additionally, D01 dose-dependently improves the cognitive deficit induced by CRS in Morris water maze test and increases the exploration time with novel objects in normal or scopolamine-induced cognitive deficits in mice, but not fluoxetine. Furthermore, D01 reverses the long-term potentiation (LTP) inhibition induced by scopolamine. Taken together, our findings demonstrate that D01, a dual-target serotonin

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reuptake and M channel inhibitor, is highly effective in the treatment-resistant depression and cognitive deficits, thus holding potential for development as therapy of depression with cognitive deficits.

1. Introduction

Depression is a chronic, severe and recurrent mood disorder with multiple causes and complex clinical symptoms[1−4]. Cognitive dysfunction is a core symptom commonly observed in psychiatric disorders, including patients with major depressive disorder (MDD) exhibiting cognitive deficits in their first major depressive episode (MDE)[5,6]. The cognitive impairment in depression affects various domains, such as working memory, executive function, and social cognition[7,8]. The current mainstream approach for depression treatment includes selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs). However, most drugs in this class have little efficacy in improving the cognitive deficits experienced by MDD patients[9,10]. To date, only few antidepressants such as duloxetine, rivastigmine and vortioxetine have demonstrated procognitive effects in MDD[11,12]. Therefore, it is necessary to identify novel antidepressants capable of improving both mood and cognition.

Several biological pathways are implicated in cognitive deficits in MDD, including a hyperactive hypothalamic−pituitary−adrenal axis (HPA), an increase in oxidative and nitrosative stress, inflammation, mitochondrial dysfunction, increased apoptosis and diminished neurotrophic support [14]. Drugs targeting these pathways include vortioxetine[15], minocycline[16,17], statins[13], N-acetylcysteine[18,19], S-adenosylmethionine[20] and erythropoietin[21], which have shown some improvement in cognition in preclinical models. However, with the exception of vortioxetine, these drugs have not exhibited significant improvement in emotional or cognitive symptoms in MDD patients[9]. Vortioxetine is a multi-targeted antidepressant approved for the treatment of adult MDD, and it acts as an antagonist of 5-HT$_3$ (K_i = 3.7 nmol/L), 5-HT$_{1D}$ (K_i = 54 nmol/L) and 5-HT$_7$ (K_i = 19 nmol/L); a partial agonist of 5-HT$_{1B}$ (K_i = 33 nmol/L) and a 5-HT$_{1A}$ agonist (K_i = 15 nmol/L) as well as a serotonin transporter (SERT) inhibitor[22,23]. The improvement in emotional disorder and enhancement of cognition in MDD appears to result from the synergistic effects of vortioxetine on SERT and 5-HT receptors[24], suggesting that multi-targeted drugs may be beneficial for the treatment of MDD by improving both mood and cognition.

Hyperexcitability defines the fundamental mechanism of neuropsychiatric disorders, including depression[25] and memory impairment[26]. The neuronal voltage-gated potassium Kv7/KCNQ/M channel conducts a low-threshold and slowly activating K$^+$ current that plays a critical role in control of neural excitability. The native M-current consists of a heteromeric assembly of Kv7.2/Kv7.3 channel subunits that are inhibited by muscarine through the activation of acetylcholine (ACh) receptors[27] and also by small molecule XE991 or linopirdine[28]. Inhibition of Kv7/M-current improves cognitive deficits by increasing the excitability of cholinergic neurons[29−32]. In the central nervous system, cholinergic activity is associated with neural functions such as awareness, attention, learning and memory[33]. Several acetylcholinesterase inhibitors (AChEIs) are commonly used for the clinical treatment of cognitive deficits in Alzheimer's disease (AD), such as rivastigmine, donepezil and galantamine[34]. These observations further suggest that multi-targeted agents inhibiting both SERT and Kv7 channels may be an effective strategy for MDD patients with cognitive deficits.

To verify the hypothesis, we synthesized and identified a representative compound N,N,2-trimethyl-3-(1-naphthyloxy)-3-(thien-2-yl) propan-1-amine (D01), which exhibits a potent inhibition on both SERT and Kv7 channels. Compared with fluoxetine, a traditional antidepressant, D01 is more efficacious in antidepression and improvement of cognition in multiple models of depression and cognition. Therefore, D01 may hold developmental potential for therapy of emotional and cognitive disorders, such as depression.

2. Materials and methods

2.1. Animals

Healthy C57BL/6 mice (body weight, 18−22 g) and male ICR mice (body weight, 18−22 g) or male Sprague−Dawley (SD) rats (body weight, 180−350 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed in groups under standard conditions at room temperature of 22 ± 2 °C and humidity of 50 ± 10% with a 12-h light/dark cycle (lights on at 8:00 AM). Water and food were provided ad libitum except during the test. Animals were adapted to laboratory conditions for approximately 1 week prior to testing. Animal experimental protocols were approved by the Animal Ethics Committee of Nhwa Pharmaceutical Company and Qingdao University College of Medicine and the experiments were conducted under the guidelines of the bylaw for experiments on animals.

2.2. Chemicals

Compound D01, fluoxetine, rivastigmine were synthesized with a purity of above 95% by Jiangsu Nhwa Pharmaceutical Co., Ltd. (Jiangsu, China). Scopolamine, XE991 and retigabine (RTG) were purchased from Sigma−Aldrich (St. Louis, MO, USA). For electrophysiological experiments, compounds D01, Kv7 channel blocker XE991 and Kv7 opener RTG were dissolved in DMSO as a stock solution. Other compounds were dissolved in distilled water and administered either intragastrically or intraperitoneally, unless otherwise specified.

2.3. Cell culture

Human embryonic kidney 293 (HEK293) cells stably transfected with human KCNQ2/3 cDNAs were cultured in DMEM

supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, 600 μg/mL G418 and 600 μg/mL hygromycin B at 37 °C in a humidified atmosphere with 5% CO_2. Cells were removed from the culture flask through a 1-min digestion with 2.5 mg/mL trypsin (1:250) and plated at low density onto 8-mm-diameter glass coverslips for patch-clamp recordings within 48 h.

2.4. Electrophysiology

2.4.1. Whole-cell recordings in cultured cells
HEK293 cells transfected with Kv7 channel cDNAs were recorded using a perforated patch-clamp technique with amphotericin B (250 mg/mL; Millipore, Sigma) in the pipette solutions. Data were acquired at 10 kHz and filtered at 2.5 kHz using the HEKA EPC 10 amplifier (Harvard Bioscience, Holliston, MA, USA). Patch electrodes were pulled with a micropipette puller (Sutter Instrument, Novato, CA, USA) and fire-polished to a final resistance of 1−2 MΩ. Series resistances were compensated by 60%−80%. The internal pipette solution for recording was prepared as follows: 150 mmol/L KCl, 5 mmol/L MgCl, and 10 mmol/L HEPES at pH 7.4 adjusted with KOH. The external recording solution contained 160 mmol/L NaCl, 2.5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 10 mmol/L D-glucose, and 20 mmol/L HEPES and pH was adjusted to 7.4 using NaOH. Hippocampal neurons were isolated as previously described[35], cultured for 14−16 days, and then were recorded using a whole-cell patch-clamp configuration. The recording electrodes had a resistance of 2−6 MΩ when filled with the pipette solution containing 100 mmol/L K-gluconate, 50 mmol/L KCl, 10 mmol/L EGTA, 5 mmol/L $MgCl_2$, and 2 mmol/L HEPES at pH 7.3 adjusted with KOH. The extracellular solution was composed of 140 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L D-glucose at pH 7.4 adjusted with NaOH.

For the recordings of native M currents in hippocampal neurons, the extracellular solution was supplemented with ionotropic glutamate NMDA and non-NMDA receptor inhibitors, D,L-2-amino-5-phosphonovaleric acid and 6,7-dinitroquinoxaline-2,3-dione (each at 20 μmol/L); and GABA receptor inhibitors, bicuculline and baclofen (each at 10 μmol/L). All experiments were carried out in an air-conditioned room with a controlled temperature of 22 ± 2 °C.

2.4.2. Recording of LTP in hippocampal slices
Hippocampal slices were prepared from male C57/BL6 mice (22−30 g). Mice were divided into three groups: vehicle group (0.9 % saline, intraperitoneal injection, i.p.); scopolamine group (1 mg/kg, i.p.); D01 group (1 mg/kg scopolamine + 30 mg/kg D01, i.p.). Compounds were administrated 30 min before brain was removed. Transverse slices were cut with 400 μm thick on a vibration microtome (Leica VT1000S, Leica Microsystems, Wetzlar, Germany) in ice-cold dissection buffer (213.26 mmol/L sucrose, 2.5 mmol/L KCl, 2 mmol/L $MgSO_4$, 0.5 mmol/L $CaCl_2$, 1.25 mmol/L NaH_2PO_4, 26 mmol/L $NaHCO_3$, and 10 mmol/L D-glucose). Slices were equilibrated in artificial cerebrospinal fluid (aCSF, containing 124 mmol/L NaCl, 2.5 mmol/L KCl, 1 mmol/L $MgSO_4$, 2 mmol/L $CaCl_2$, 1.25 mmol/L NaH_2PO_4, 26 mmol/L $NaHCO_3$, and 10 mmol/L D-glucose) saturated with 95% O_2/5% CO_2 for at least 1 h before recording. Recordings were made in a submerge chamber at a flow rate of 1.5−2 mL/min with aCSF.

For the long-term potentiation (LTP) recordings, the bipolar platinum electrode (FHC, USA) was positioned at CA1 stratum radiatum to evoke field excitatory postsynaptic potentials (fEPSPs) every 20 s. The fEPSPs were monitored to be stable for 20 min as baseline before a high-frequency train of stimuli (100 Hz for 1 s) was applied, and fEPSPs were recorded for another 60 min. The average slope of fEPSPs at baseline was set at 100%, and changes in slope were expressed as percent change from baseline.

2.5. Binding affinity of D01 to monoamine transporters

Assays for competitive binding against serotonin transporter (SERT), norepinephrine transporter (NET) and dopamine transporter (DAT) were performed as previously described with minor modifications[1,36]. Briefly, rats were sacrificed by guillotine apparatus, and membrane proteins were prepared from frontal cortex (for SERT and NET) or striatum (for DAT). Competitive receptor binding assays were performed in reaction buffer containing 50 μg of membrane proteins, [³H]paroxetine (0.5 nmol/L), [³H]nisoxetine (0.5 nmol/L), or [³H]WIN-35428 (0.5 nmol/L) and various concentrations of D01 compound (0.1 nmol/L−10 μmol/L) at 23 °C for 60 min (for SERT), at 25 °C for 30 min (for NET), or at 4 °C for 120 min (for DAT). Nonspecific binding to SERT, NET, and DAT was determined using 10 μmol/L paroxetine, 10 μmol/L desipramine, and 10 μmol/L nomifensine, respectively. The inhibition rates were calculated by Eq. (1):

$$\text{Inhibition rate } (\%) = (\text{Total binding} - \text{Drug binding})/(\text{Total binding} - \text{Nonspecific binding}) \times 100 \quad (1)$$

All data was expressed as the mean ± SEM from three independent experiments.

2.6. 5-HT uptake transporter assay

The reuptake assays were performed using freshly prepared crude synaptosomes from rat brains, as previously described with minor modifications[1]. Briefly, the uptake of [³H]5-HT by SERT was carried out using membrane preparations of rat cerebral cortex. Crude synaptosomes were incubated in a solution of Krebs bicarbonate buffer that contains [³H]5-HT (20 nmol/L) and various concentrations of D01 compound (0.1 nmol/L−10 μmol/L) at 37 °C for 10 min. The nonspecific uptake to SERT was determined with 10 μmol/L paroxetine. All experiments were performed in triplicate from three independent tests.

2.7. Forced swimming test

The forced swimming test (FST) was conducted in mice according to the previously described procedures[1,37]. Briefly, a total of 50 mice were randomly divided into 5 groups consisting of vehicle control, fluoxetine (40 mg/kg), and three dosages of D01 (10, 30, and 100 mg/kg) and each group contained 10 mice. One hour after intragastric (i.g.) administration, each mouse was placed in an open cylindrical container (diameter of 10 cm, height of 25 cm, containing 15 cm of water with temperature maintained at 24 ± 1 °C). The duration of immobility during the last 4 min of a total 6-min time was recorded. Animals were considered immobile when they floated motionlessly, making only necessary movements to keep their heads above the water surface.

2.8.   Tail suspension test

The tail suspension test (TST) procedure in mice was adapted from previously described methods[1,38]. A total of 50 mice were randomly assigned to 5 groups: vehicle control, fluoxetine (40 mg/kg), and three dosages of D01 (10, 30, and 100 mg/kg), with each group consisting of 10 mice. After intragastric administration for 60 min, each mouse was suspended on the top of the apparatus using adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility during the last 4 min of a total 6-min period was recorded. Mice were considered immobile only when they hung passively motionless.

2.9.   Locomotor activity measurement

To assess the effect of D01 on locomotion, 20 naive mice were randomly divided into 2 groups with each group consisting of 10 mice. The animals were transferred to the testing room at least 2 h before drug administration. After intragastric administration of D01 (100 mg/kg) or vehicle for 55 min, mice were individually placed into a corner of each test chamber (length × width × height: 25 cm × 25 cm × 30 cm) for a 5-min acclimation period. Subsequently, the spontaneous locomotor activity of the mice was recorded for 30 min using a tracking and computerized analysis system (Clever Sys Inc., Leesburg, VA, USA).

2.10.   Novel object recognition assay

The novel object recognition (NOR) assay was performed in mice according to the procedures as previously described[39]. Briefly, a total of 70 mice were randomly assigned to 7 groups: vehicle control, rivastigmine (0.01 mg/kg), XE991 (1 mg/kg), fluoxetine (100 mg/kg), and three doses of D01 (10, 30 and 100 mg/kg) with each group consisting of 10 mice. The NOR assay was conducted over three days.

On the first day, mice were introduced to a test chamber (length × width × height: 50 cm × 20 cm × 35 cm) for 10 min of free exploration. On the second day, 60 min after intragastric administration of D01 (10, 30, and 100 mg/kg), fluoxetine or vehicle, 30 min after subcutaneous injections (s.c.) of rivastigmine or XE991, mice were placed to the test chamber in which two identical objects (wooden balls with a diameter of 3.5 cm) were positioned at a distance of more than 20 cm apart. The mice were allowed to explore the two objects for 10 min (acquisition phase trial, T1). 24 h following T1, one of the objects used in T1 was replaced with a novel object (square wooden block with a side length of 3.5 cm), and the mice were allowed to explore the objects for 10 min (retention phase trial, T2). The exploring behavior was defined as the mouse actively exploring the object with its nose facing the object at a distance of less than 1 cm. However, circling or sitting on the object was not considered exploration behavior. The test area was wiped and cleaned with 75% alcohol solution and dried before each test.

For the NOR assay in cognitive deficit induced by scopolamine, a total of 80 mice were randomly assigned to 8 groups, with 10 mice in each group. Group 1 received saline solution and served as normal control. Group 2 received scopolamine (1 mg/kg, i.p.) and served as the model. Group 3 received scopolamine (1 mg/kg, i.p.) and rivastigmine (0.01 mg/kg, s.c.), group 4 for scopolamine (1 mg/kg, i.p.) and XE991 (1 mg/kg, s.c.), and group 5 for scopolamine (1 mg/kg, i.p.) and fluoxetine (100 mg/kg, i.g.). Group 6 to group 8 received scopolamine (1 mg/kg, i.p.) with different doses of D01 (10, 30, and 100 mg/kg, i.g.), respectively.

The test protocol was similar to the NOR test in normal mice as described above with the following modifications for the scopolamine-induced cognitive impairment: scopolamine was injected 30 min before T1 to induce learning and memory impairment in mice, and the interval between T1 and T2 was changed to 1 h from 24 h. The discrimination index (DI) was used to access the memory activity, as calculated as Eq. (2):

$$DI = (\text{The time spent on the novel object} \\ - \text{The time spent on the familiar object})/\text{The total time}$$

(2)

2.11.   Chronic restraint stress model

The chronic restraint stress (CRS) model was established following the procedures as previously described[40]. A total of 84 mice were randomly assigned to 7 groups: sham control, CRS model with vehicle control, XE991 (1 mg/kg, s.c.), fluoxetine (20 mg/kg, i.g.) and three dosages of D01 (10, 20, and 40 mg/kg, i.g.) with each group consisting of 12 mice.

Compounds in a volume of 10 mL/kg were administered once a day for 21 days before the initiation of restraint stress. Mice under chronic stress were restrained for 6 h per day in the tunnel of a 50 mL conical transparent plastic centrifuge tube with multiple ostioles in tube walls for ventilation. During the restraint period, the mice were kept in individual cages with no access to food or water.

2.12.   Morris water maze test

The Morris water maze (MWM) test was used to evaluate spatial learning and memory in mice. The MWM device consisted of a circular tank measuring 120 cm in diameter and 60 cm in height, filled with water maintained at 24 °C. The pool was divided into four imaginary quadrants. In one of the quadrants (the target quadrant), a black platform with a diameter of 6 cm was placed 1 cm below the water surface. Water was dyed in black with non-toxic and odorless dye to make the platform invisible.

The experimental design comprised an acquisition trial conducted over four days and a probe trial on the fifth day. During the acquisition trial, mice were trained for four consecutive days with four trials per day to identify the invisible platform. Each mouse was given 60 s to search for the escape platform and was allowed to stay on the platform for 20 s. The swimming pathway and escape latency were recorded during the test. If a mouse failed to find the platform within 60 s, it was gently placed on the platform and allowed to stay for 20 s with the escape latency recorded as 60 s. On the fifth day, the probe trial was performed by removing the platform before the trial. Mice were placed into the pool from the opposite quadrant where the platform had been located. Mice were given 60 s to probe, and the time and distance spent in the target quadrant were recorded using a video camera and the data were digitized automatically into a computer with an image analyzer.

2.13.   Statistical analysis

All data are presented as the mean ± standard error of mean (SEM). Statistical analyses were conducted using GraphPad Prism version 5.0 (GraphPad Software). For binding/uptake assays, the transporter binding and monoamine uptake data

were analyzed using one-site nonlinear regression of the concentration−effect curve. The K_i values were calculated using the equation of Cheng and Prusoff (1973). For electrophysiology data, the dose-dependent effect curve was fitted by logistic function, meanwhile the statistical analysis for different groups was carried out using paired Student's t test. For data from behavioral tests, the analysis was conducted using one-way analysis of variance (ANOVA) followed by Dunnett's test. A difference with a $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Inhibitory effect of D01 on SERT

Compound D01 was synthesized at Jiangsu Nhwa Pharmaceutical Co., Ltd. (Xuzhou, China) and the chemical structure is depicted in Fig. 1A under the published Patent Cooperation Treaty (CN 106349211A). We started investigating the binding affinity of D01 to monoamine transporters, including SERT, NET, and DAT, using the radioligand receptor binding assay. As shown in Fig. 1B and C, D01 exhibited a potent binding affinity to SERT with a K_i value of 30.1 ± 6.9 nmol/L. This affinity is comparable to the SSRI fluoxetine that as a positive control also exhibited high bin ding affinity against SERT with a K_i value of 27.1 ± 4.9 nmol/L. D01 at 10 μmol/L displayed weak binding affinities to NET and DAT with less than 70 % of inhibition (Fig. 1B). Furthermore, a functional in vitro assay revealed that D01 was capable of blocking [³H]5-HT uptake by cerebral synaptosomes from rats with an IC_{50} value of 45.0 ± 9.0 nmol/L (Fig. 1D), consistent with its potent affinity of binding to SERT.

We next examined the selectivity of D01 over other receptors including 5-HT receptors, dopamine receptors, α receptors, histamine receptors, sigma receptors, NMDA and $GABA_A$ receptors (Table 1). D01 at the concentration of 10 μmol/L exhibited no obvious effects on these CNS-related receptors.

3.2. Inhibition of M current by D01

We further evaluated the effect of D01 on Kv7/KCNQ/M channel current using the perforated whole-cell patch-clamp recordings of Kv7.2/7.3 channels expressed in HEK293 cells. Perfusing D01 (10 μmol/L) decreased the steady-state current (-30 mV) about 55% (Table 1). D01 resulted in a dose-dependent inhibition of Kv7.2/7.3 currents elicited by depolarization potential at -40 mV with IC_{50} of 10.1 ± 2.4 μmol/L (Fig. 1E).
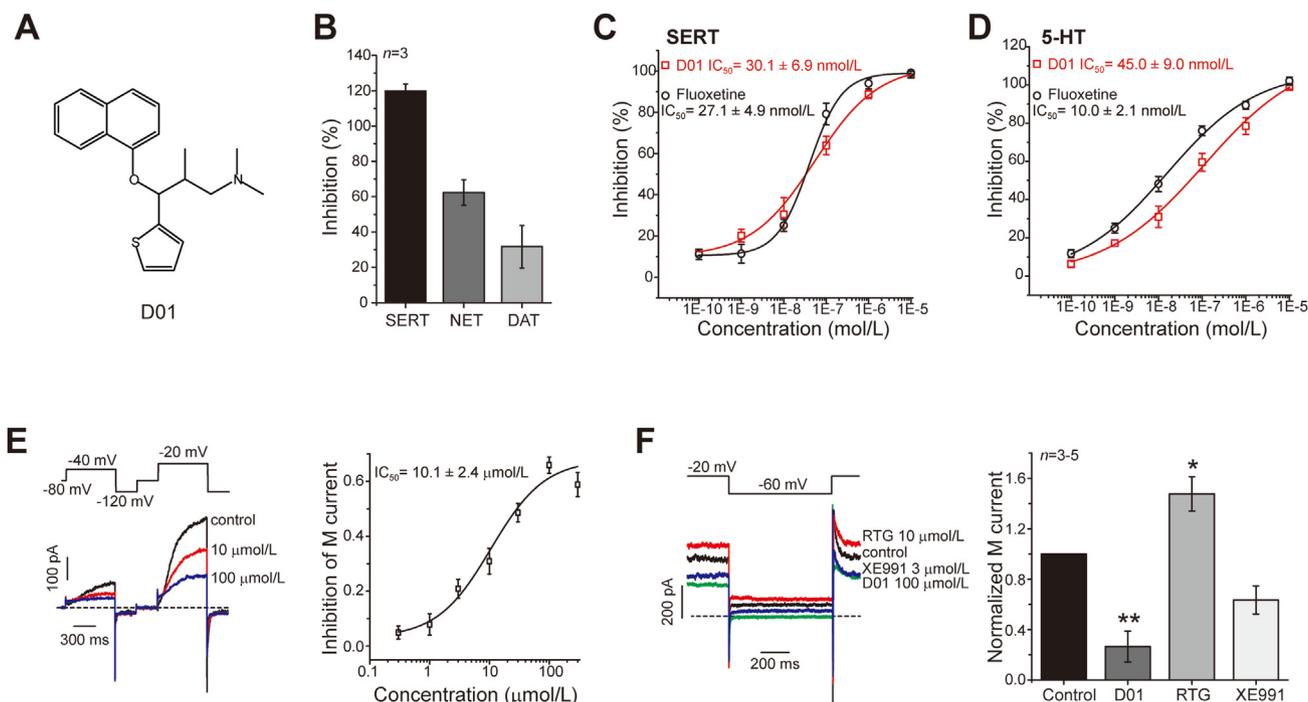

Figure 1 Concentration-dependent inhibition of SERT and M channel by compound D01. (A) Chemical structure of compound D01. (B) Binding affinity of D01 to monoamine transporters from crude membranes ($n = 3$). (C) Dose-dependent binding affinity of D01 and fluoxetine as a positive control to SERT from crude membranes ($n = 3$). The K_i value is 30.1 ± 6.9 nmol/L for D01 (square) and 27.1 ± 4.9 nmol/L for fluoxetine (circle). (D) Dose-dependent inhibition of [³H]5-HT uptakes into rat brain synaptosomes by D01 and fluoxetine ($n = 3$). The IC_{50} is 45.0 ± 9.0 nmol/L for D01 (square) and 10.0 ± 2.1 nmol/L for fluoxetine (circle). Data are presented as the mean $\pm$ SEM from three independent tests. (E) Left panel: Representative current traces for dose-dependent inhibition of KCNQ2/3 channels expressed in HEK293 cells by D01 using a step protocol at holding potential of -80 mV following a depolarized potential at -40 mV or -20 mV as indicated. Right panel: The dose−response curve was fitted by the logistic function for inhibition of KCNQ2/3 channels recorded at -40 mV by D01 with the IC_{50} value of 10.1 ± 2.4 μmol/L ($n = 3-7$). (F) Native M currents recorded on cultured hippocampal neurons using a step protocol at a holding potential at -20 mV and repolarized potential at -60 mV as indicated. Left panel: Representative current traces of native KCNQ/M channels inhibited by D01 (100 μmol/L), XE991 (3 μmol/L) or activated by RTG (10 μmol/L). Right panel: Summary effects of D01 (100 μmol/L), XE991 (3 μmol/L) or RTG (10 μmol/L) on M-current measured at -60 mV ($n = 3-5$). Data are expressed as the mean $\pm$ SEM. *$P < 0.05$ and **$P < 0.01$ indicate statistical significance in comparison with their controls.

Table 1   Binding affinities of D01 on receptors in the central nerve system.

| Target receptor	Radioligand	% Inhibition of binding by D01 (10 μmol/L)	IC$_{50}$ for D01 (nmol/L)
5-HT$_{1A}$	[^{3}H]-8-OH-DPAT	−24.12	—[b]
5-HT$_{1B}$	[^{3}H]-GR-125743	12.03	—[b]
5-HT$_{2A}$	[^{3}H]-Ketanserin	25.10	—[b]
5-HT$_{2C}$	[^{3}H]-Mesulergine	38.02	—[b]
D$_1$	[^{3}H]-SCH23390	16.30	—[b]
D$_2$	[^{3}H]-Spiperone	16.06	—[b]
D$_3$	[^{3}H]-Spiperone	32.8	—[b]
α$_1$	[^{3}H]-Prazosin	56.21	>10,000
α$_2$	[^{3}H]-Rauwolscine	38.43	—[b]
M$_1$	[^{3}H]-QNB	24.73	—[b]
H$_1$	[^{3}H]-Pyrilamine	61.82	>10,000
H$_3$	[^{3}H]-Methylhistamine	13.89	—[b]
Sigma-1	[^{3}H]-Pentazocine	34.05	—[b]
Sigma-2	[^{3}H]-DTG	−4.5	—[b]
NMDA	[^{3}H]-MK801	9.83	—[b]
GABA$_A$	[^{3}H]-Flunitrazepam	22.53	—[b]
KCNQ 2/3	—[a]	55.00	—[b]

[a]Not applicable.
[b]Not detected.
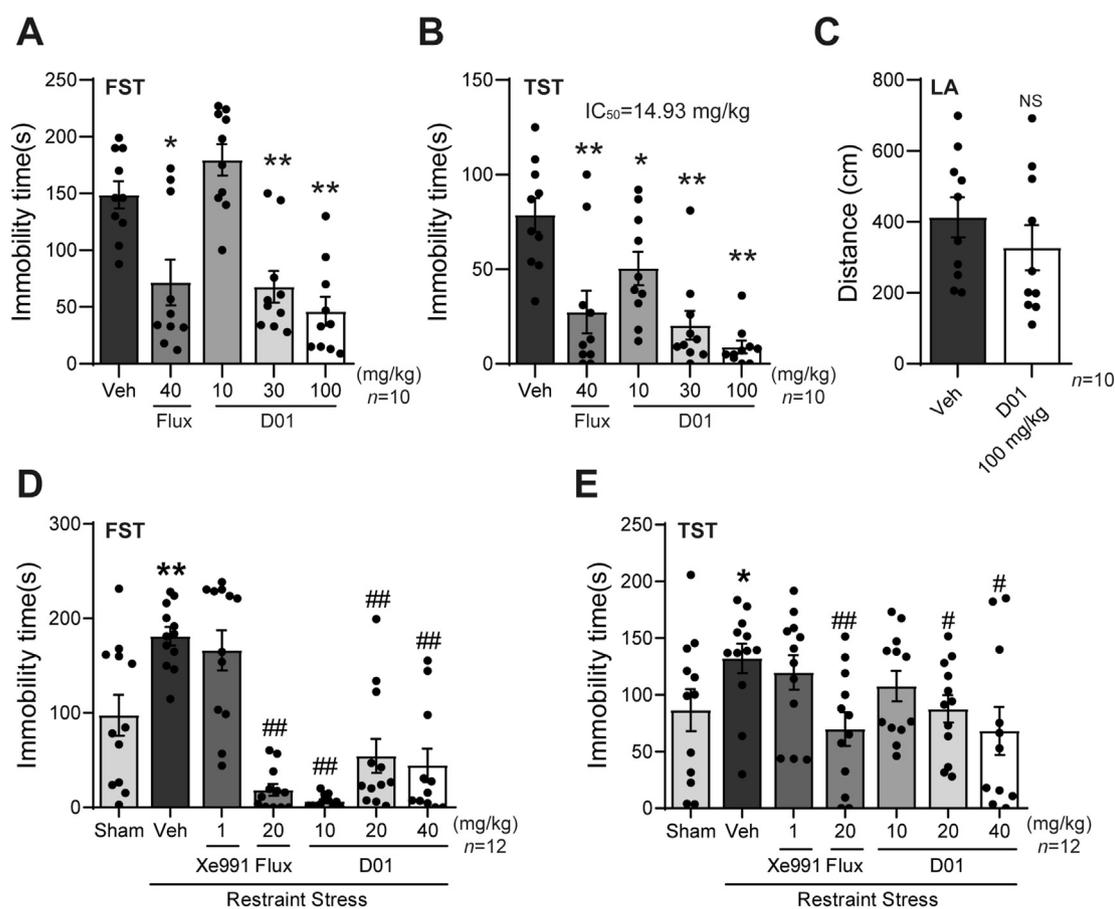
Figure 2   Anti-depressive activity of D01 in mouse tail suspension and forced swimming tests. (A, B) Depression behavior tests in wild-type mice. (A) Forced swimming test (FST), $n = 10$. (B) Tail suspension test (TST), $n = 10$. (C) Spontaneous locomotor test, $n = 10$. The total distance traveled in the open field for 5 min after injections of D01 (100 mg/kg, i.g.). *$P < 0.05$, **$P < 0.01$ indicate statistical significance compared to the control. (D, E) Depression behavior tests in restraint stress-induced depression models in mice, $n = 12$. (D) FST. (E) TST. *$P < 0.05$ and **$P < 0.01$ indicate statistical significance compared to the sham control. #$P < 0.05$ and ##$P < 0.01$ indicate the statistical significance compared to the vehicle control.

We also examined the effect of D01 on native M current in cultured neurons acutely isolated from rat hippocampus. The M-current of hippocampal neurons was activated by a depolarizing voltage of -20 mV, and the tail current was elicited at -60 mV (Fig. 1F). Applications of either D01 (100 µmol/L) or specific blocker XE991 (3 µmol/L) inhibited the M-current activated by a specific opener retigabine (RTG) at 10 µmol/L (Fig. 1F). Transgenic or pharmacological suppression of M-channel function can induce neuronal hyperexcitability and potentially lead to epilepsy[41,42]. Our data show that D01 at the concentration of 100 µmol/L dramatically inhibits native M current (Fig. 1F) and depolarizes the resting membrane potential of hippocampal neurons. We did not observe any significant increase of firing frequency in the presence of D01 at 100 µmol/L or epileptic behavior in animals treated with D01 even at a high dosage of 100 mg/kg.

3.3. Efficacious anti-depressant activity of D01 in mouse models of depression

The anti-depressive effects of D01 were evaluated in the mouse model of acute depression using forced swimming test (FST) and tail suspension test (TST) with fluoxetine used as a positive control. As shown in Fig. 2A and B, D01 (10, 30, and 100 mg/kg, i.g.) reduced the duration of immobility in a dose-dependent manner with minimal effective doses (MEDs) of 30 mg/kg in FST and 10 mg/kg in TST, as compared fluoxetine at 40 mg/kg. The spontaneous locomotion test indicated that D01 at dose of 100 mg/kg did not significantly alter the total distance traveled during the 30-min period. This suggests that the decrease in the immobility time induced by D01 in FST and TST was not a result of a psychostimulant effect, but rather attributed to its anti-depressive activity (Fig. 2C).

The anti-depressive activity of D01 was further evaluated in the mouse model of chronic restraint stress (CRS)-induced depression, where mice were subjected to 6 h of daily restraint stress for a period of 21 days using the FST and TST assays. The immobility time of restraint stress group mice was significantly longer in both FST and TST with 181.03 $\pm$ 9.82 s and 132.09 $\pm$ 13.03 s, respectively, as compared with the sham group mice with 97.57 $\pm$ 21.59 s and 86.52 $\pm$ 18.51 s, respectively. In contrast, D01 significantly reduced the immobility time with 6.67 $\pm$ 1.92 s (10 mg/kg), 54.53 $\pm$ 18.11 s (20 mg/kg), and 44.62 $\pm$ 17.03 s (40 mg/kg) in FST; and 87.61 $\pm$ 12.04 s
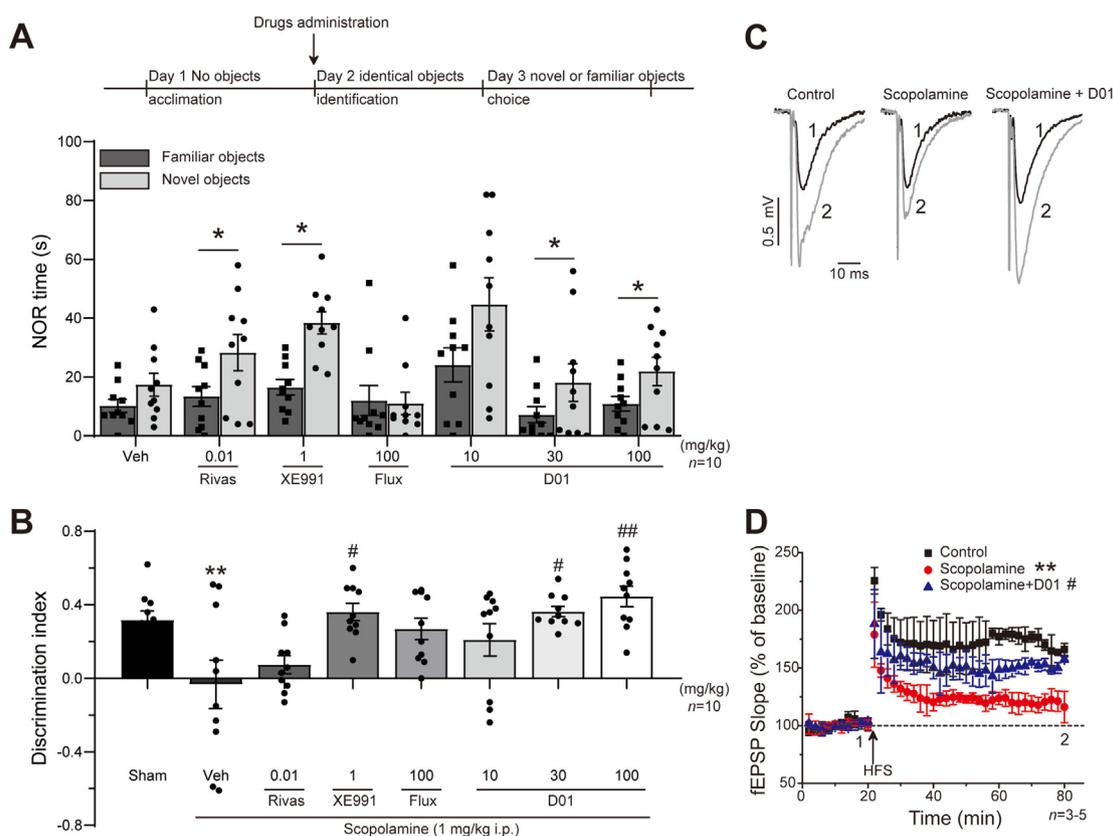

Figure 3 Alleviation of scopolamine-induced cognitive impairment by D01. (A) The exploration time after intragastric administrations of D01 and fluoxetine or subcutaneous injections of rivastigmine and XE991 in mice using novel object cognition (NOR) test assay. *$P < 0.05$ indicates statistical significance compared to the group of familiar objects, $n = 10$. (B) The discrimination index of intragastric administrations of D01 and fluoxetine, subcutaneous injections of rivastigmine and XE991 in scopolamine-induced depression mice. **$P < 0.01$ indicates statistical significance compared with the sham control. #$P < 0.05$, ##$P < 0.01$ indicate statistical significance compared with the vehicle control, $n = 10$. (C, D) Intraperitoneal administration of D01 (30 mg/kg) abolished scopolamine-induced LTP inhibition. (C) Representative fEPSPs for each group were presented taken at the end of the baseline (1) and 60 min after HFS (2). (D) Summary data of LTP. Arrow indicates the time of HFS delivery. **$P < 0.01$ indicates statistical significance compared with the control. #$P < 0.05$ indicates statistical significance compared with scopolamine group, $n = 3-5$.

(20 mg/kg) and 68.17 ± 20.30 s (40 mg/kg) in TST, respectively, as compared to fluoxetine (20 mg/kg) that showed immobility time of 18.7 ± 6.3 s in FST and 69.8 ± 14.9 s in TST (Fig. 2D and E). In addition, the M channel inhibitor XE991 at 1 mg/kg showed a trend towards reducing immobility, although the effect was not statistically significant.

3.4. Improvement on cognitive deficits by D01 in novel object recognition (NOR) assay

We further evaluated the effect of D01 on learning and memory in normal mice using NOR assay for learning and memory test[43]. As shown in Fig. 3A, different doses (30 and 100 mg/kg, i.g.) of D01 significantly increased the time spent on novel object with 18.10 ± 6.42 s and 21.90 ± 4.84 s, as compared with the time of 7.20 ± 2.73 s and 10.90 ± 2.50 s spent on familiar objects, respectively. As a positive control, rivastigmine (0.01 mg/kg, s.c.), a cholinesterase inhibitor, also increased the time spent exploring the novel arm with 28.30 ± 6.17 s, as compared with

13.40 ± 3.42 s (Fig. 3A). Similarly, mice administered with KCNQ/M channel inhibitor XE991 (1 mg/kg, s.c.) also spent more time on exploring the novel object with 38.40 ± 3.80 s, compared with time of 16.50 ± 2.63 s on the familiar one. In contrast, mice receiving fluoxetine (100 mg/kg, i.g.) showed no obvious difference in the time spent on the novel and familiar objects (Fig. 3A).

To further confirm the cognitive improvement effect of D01, we conducted an additional experiment using a mouse model of memory impairment caused by an anticholinergic, scopolamine. Scopolamine was administered at a dose of 1 mg/kg, intraperitoneally (i.p.), resulting in a significant decrease of time spent on exploring novel object with the discrimination index (DI) at −0.03 ± 0.12, as compared with the group of vehicle-treated mice with DI at 0.32 ± 0.05, suggesting the successful induction of scopolamine-induced cognitive impairment (Fig. 3B).

D01 was administered at different doses, and its effects on the time spent exploring the novel object were measured. The results show a dose-dependent increase in the time spent with DIs of 0.21 ± 0.08 for 10 mg/kg group, 0.36 ± 0.03 for 30 mg/kg group,
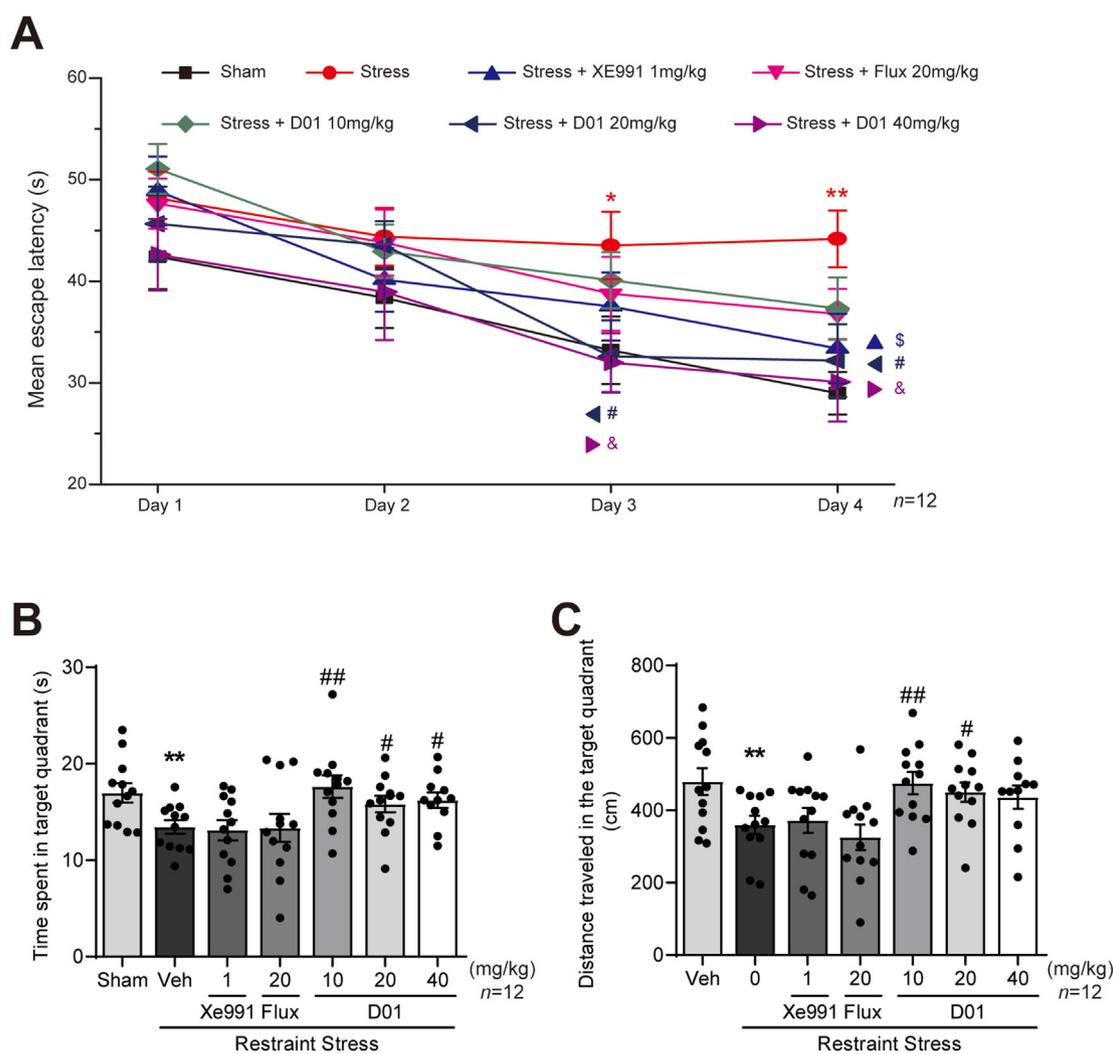

Figure 4 Improvement of spatial learning by D01 in the Morris water maze test. (A) An escape latency in the target quadrant of different groups as indicated, $n = 12$. *$P < 0.05$, **$P < 0.01$ vs. sham, [#]$P < 0.05$, [&]$P < 0.05$, [$]$P < 0.05$ vs. vehicle. (B, C) Spatial memory was assessed in a probe trial on training Day 5 with respect to: (B) time or (C) path length spent in the target quadrant relative to non-target quadrants, $n = 12$. Data are expressed as the mean ± SEM. *$P < 0.05$ and **$P < 0.01$ indicate statistical significance compared with the sham control. [#]$P < 0.05$ and [##]$P < 0.01$ indicate the statistical significance compared with their vehicle control.

and 0.45 ± 0.05 for 100 mg/kg group. As a positive control, XE991 also showed a significant increase of the time spent exploring the novel object with DI at 0.36 ± 0.04. In contrast, administration of rivastigmine (0.01 mg/kg) or fluoxetine (100 mg/kg) did not show any noticeable improvement in scopolamine-induced cognitive impairment.

Learning and memory formation involves the long-term potentiation (LTP) of synaptic strength in the hippocampus that plays a vital role in cognition[44]. We further investigated the effect of D01 on LTP induction using electrophysiological recordings of field excitatory post-synaptic potentials (fEPSPs) in hippocampal brain slices. As shown in Fig. 3C and D, scopolamine at 1 mg/kg caused a significant inhibition of fEPSPs evoked by high frequency stimulation (HFS). In contrast, pretreatment of D01 at 30 mg/kg significantly reversed scopolamine-induced LTP inhibition (Fig. 3C and D).

3.5. Improvement on cognitive impairment by D01 in Morris water maze test

Chronic stress has been linked to the development of various psychiatric conditions leading to cognitive and emotional impairments in MDD[40]. We next evaluated the effect of D01 on cognitive deficits induced by chronic restraint stress (CRS) using the Morris water maze (MWM) assay that assesses spatial learning in mice by measuring their ability to locate an invisible platform and escape from swimming in water.

In the sham control group, the escape latency decreased to 29.0 ± 2.1 s on the fourth day of testing from the first day of 42.4 ± 3.20 s (Fig. 4A), indicating successful learning and memory. Conversely, mice subjected to 21 days of restraint stress without any treatment exhibited an escape latency of approximately 45 s after four days of training, suggesting the induction of CRS-induced cognitive impairment (Fig. 4A). In contrast, treatment with D01 demonstrated a dose-dependent reduction in the escape latency. The 10 mg/kg group showed an escape latency of 37.3 ± 3.1 s, the 20 mg/kg group had an escape latency of 32.2 ± 3.6 s, and the 40 mg/kg group exhibited an escape latency of 30.1 ± 3.9 s, as compared to their respective initial escape latencies of 51.1 ± 2.5, 45.7 ± 3.7 and 42.6 ± 3.5 s, respectively (Fig. 4A). These results are similar to the observation with XE991 that reduced the escape latency to 33.4 ± 3.4 from 48.9 ± 3.4 s, whereas fluoxetine did not show any significant effect (Fig. 4A).

We also assessed the spatial memory. On Day 5 of training, a probe trial was conducted. As shown in Fig. 4B and C, mice subjected to chronic stress spent significantly less time (13.4 ± 0.7 s) in the target quadrant and covered a shorter travel distance (3594.0 ± 253.5 cm) while crossing the target zone, as compared with non-stress treated mice that spent 17.0 ± 1.0 s and covered distance 4790.0 ± 368.1 cm, respectively. These results suggest an impairment on spatial memory. In contrast, the administration of D01 significantly reversed the decreased time induced by chronic stress in the target quadrant with times of 17.6 ± 1.2 s (for 10 mg/kg), 15.8 ± 0.8 s (for 20 mg/kg), 16.2 ± 0.8 s (for 40 mg/kg). Similarly, the distance traveled while crossing the target zone were improved with 4748.7 ± 308.1 cm (10 mg/kg), 4501.3 ± 266.5 cm (20 mg/kg), and 4357.5 ± 310.9 cm (40 mg/kg) (Fig. 4B and C). On the other hand, XE991 and fluoxetine effectively did not exhibit any noticeable effects (Fig. 4B and C). These results indicate that D01 effectively attenuates the cognitive impairment caused by chronic stress.

4. Conclusions

We have successfully synthesized and evaluated a novel compound N,N,2-trimethyl-3- (1-naphthyloxy) -3-(thien-2-yl) propan-1-amine (D01), as a dual target inhibitor of SERT and KCNQ/M channels. Our findings demonstrate that D01 is efficacious in antidepression and cognition improvement. D01 may hole developmental potential for treatment of depression accompanied by cognitive impairment.

Author contributions

Yaqin Wei designed and performed binding assays and animal behavior experiments, analyzed data, and prepared the manuscript. Xiangqing Xu conceived and designed the research, analyzed data, and revised the manuscript. Qiang Guo, Song Zhao, Yinli Qiu and Dongli Wang participated in binding assays and animal behavior experiments. Wenwen Yu participated in electrophysiological experiments. Yani Liu performed electrophysiological experiments, analyzed data, prepared figures, and drafted the manuscript. KeWei Wang conceived and designed the research and revised the manuscript. All authors reviewed and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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