

REGULAR RESEARCH ARTICLE

DHF-7 Ameliorates Behavioral Disorders and White Matter Lesions by Regulating BDNF and Fyn in a Mouse Model of Schizophrenia Induced by Cuprizone and MK-801

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Abstract

Background: Schizophrenia is a psychiatric disorder including multiple clinical symptoms such as severe psychosis and cognitive dysfunction. DHF-7 is a novel dihydroflavanone derivative that was designed and synthesized to treat schizophrenia. This study aimed to investigate the effects and mechanisms of DHF-7 in a mouse model of schizophrenia induced by a combination of cuprizone and MK-801.

Methods: After intragastric administration of DHF-7 for 7 weeks, open field, Y-maze, and novel object recognition tests were performed to detect behavioral changes in the mouse model. White matter lesions and myelin loss were determined using transmission electron microscopy and oil red O staining. Western blotting and immunohistochemistry were used to detect the expression of the related proteins.

Results: The results showed that DHF-7 treatment significantly improved cognitive impairment and positive symptoms in the model mice. Moreover, DHF-7 alleviated white matter lesions and demyelination and promoted the differentiation and maturation of oligodendrocytes for remyelination in the corpus callosum of model mice. The mechanistic study showed that DHF-7 increased the expression of brain-derived neurotrophic factor and phosphorylated Fyn, thus activating the tyrosine kinase receptor B (Trk B)/Fyn/N-methyl-D-aspartate receptor subunit 2 B (NMDAR2B) and Raf/mitogen-activated protein kinase (MEK)/ extracellular signal-related kinase (ERK) signaling pathways.

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Significance Statement

Schizophrenia is a mental illness characterized by psychosis, apathy, social withdrawal, and cognitive impairment. White matter lesions and glutamatergic hypofunction are reported to be the key pathogeneses underlying multiple clinical symptoms of schizophrenia. New therapies that ameliorate these symptoms may help to improve the outcomes of patients with schizophrenia. In this study, we used a mouse model of schizophrenia induced by cuprizone and MK-801 to investigate the effects and mechanisms of DHF-7, a novel synthesized dihydroflavanone derivative. The results showed that DHF-7 improved multiple clinical symptoms, including cognitive impairment, in the model mice. DHF-7 alleviated white matter lesions and demyelination and promoted the differentiation and maturation of oligodendrocytes for remyelination. These mechanisms included the activation of TrkB/Fyn/NMDAR2B and Raf/MER/ERK signaling pathways by DHF-7. These results suggest that DHF-7 may improve the clinical symptoms of schizophrenia and may be a potential drug for the treatment of schizophrenia.

Conclusions: Our results provide an experimental basis for the development of DHF-7 as a novel therapeutic agent for schizophrenia.

Keywords: Dihydroflavanone, schizophrenia, cognitive impairment, white matter lesion, brain-derived neurotrophic factor, Fyn

Introduction

Schizophrenia is a severe psychiatric disorder affecting nearly 1% of the population worldwide (Fleischhacker et al., 2014). It is a heterogeneous mental illness with positive psychotic symptoms (delusions, hallucinations, and disorganized behavior), negative symptoms (anhedonia, avolition, social withdrawal, and poverty of expression), and cognitive dysfunction (impaired executive functions, memory, and speed of mental processing). Studies have shown that genetic factors (genes associated with the immune system, cytoskeletal development, and synaptic plasticity function) interact with environmental factors (including obstetrical complications, early-life adversity, and childhood residence in urban areas) to influence the liability to schizophrenia (Bennett, 2011; Radhakrishnan et al., 2017). Brain structural changes, increased dopamine activity, and glutamate and γ -aminobutyric acid (GABA) signaling are involved in the pathophysiology of schizophrenia (Marder and Cannon, 2019). Antipsychotic drugs have transformed the lives of people with schizophrenia mainly by ameliorating positive symptoms (Huhn et al., 2019). However, many patients with schizophrenia commonly present with additional symptoms such as cognitive impairment and negative symptoms that influence their social and occupational function, which remain untreated (Maric et al., 2016). New therapies that ameliorate cognitive impairment may improve the outcomes of patients with schizophrenia (Goff, 2021).

Currently, several animal models guide drug development for schizophrenia, including drugs targeting dysregulated dopamine transmission, hypofunction of glutamatergic N-methyl-D-aspartate receptors (NMDAR), and immune dysfunction (Nakazawa and Sapkota, 2020). Drugs targeting the N-methyl-D-aspartate (NMDA) cascade have been suggested to mitigate persistent psychotic and negative symptoms in patients with schizophrenia (Lopes Sakamoto et al., 2019; Nakazawa and Sapkota, 2020). It has been reported that animals exposed to MK-801, an NMDAR antagonist, exhibit schizophrenia-like behaviors and neurochemical alterations and are used as a schizophrenia model (Pinar et al., 2015). A recent study showed that MK-801 induced locomotor hyperactivity but failed to impair long-term recognition memory in mice (Chan et al., 2019).

Recent studies have found white matter abnormalities in patients with schizophrenia, which may be related to cognitive deficits (Hummer et al., 2018; Jiang et al., 2019). Cuprizone (CPZ) is a copper chelator that selectively damages oligodendrocytes

(OLs). Because OLs are myelin-forming cells, CPZ can induce demyelination and white matter lesions (Chandran, et al., 2012). CPZ-induced animal models have been used to mimic white matter lesions and behaviors, including cognitive impairments associated with schizophrenia (Wang et al., 2015).

Because schizophrenia shows multiple clinical symptoms and a complicated pathogenesis, an animal model induced by a combination of pathogenic factors is more suitable for therapeutic research (Winship et al., 2019). To better mimic the psychosis, multiple clinical symptoms, and complicated pathogenesis of schizophrenia, we established a novel mouse model with white matter lesions and glutamatergic hypofunction through exposure to CPZ in combination with MK-801 (Sun et al., 2021). The mouse model was established by feeding mice with 0.2 % CPZ forage for 5 weeks, followed by a 2-week i.p. injection of MK-801 (0.6 mg/kg/d). The behavioral data showed that this schizophrenia model exhibited impaired spatial and recognition memory in the Y-maze and novel object recognition tests, which mimicked cognitive impairment in schizophrenia. In the Y-maze and open field tests, the mouse model exhibited increased locomotor activity and hyperactivity, which mimicked the positive symptoms of schizophrenia. In the open field test, the mouse model showed less time in the central area and anxiety-like behavior, which mimicked the negative symptoms of schizophrenia (Sun et al., 2021).

We have previously described a novel dihydroflavanone derivative, DHF-7 (Fig. 1A), designed and synthesized by us (Gu et al., 2017). Our preliminary studies showed that DHF-7 decreased the activity of dopamine D2 receptors in D2 receptor/G protein 16a co-transfected HEK293 cells, as detected by the fluorescent method after dopamine stimulation. DHF-7 (0.1, 1, and 10 μ M) inhibited the Lipopolysaccharide (LPS)/Interferon (γ)-induced overproduction of nitric oxide in a dose-dependent manner in BV-2 microglial cells, indicating a potential anti-neuroinflammatory effect (Gu et al., 2017). In animal models of schizophrenia, DHF-7 reversed the increase in locomotor activity in the open field test induced by MK-801 and decreased the hyperactivity of climbing behavior induced by apomorphine, suggesting a potential antipsychotic effect of DHF-7 (Gu et al., 2017).

Although DHF-7 showed potential antipsychotic effects in previous drug screening, the effects of DHF-7 on the symptoms

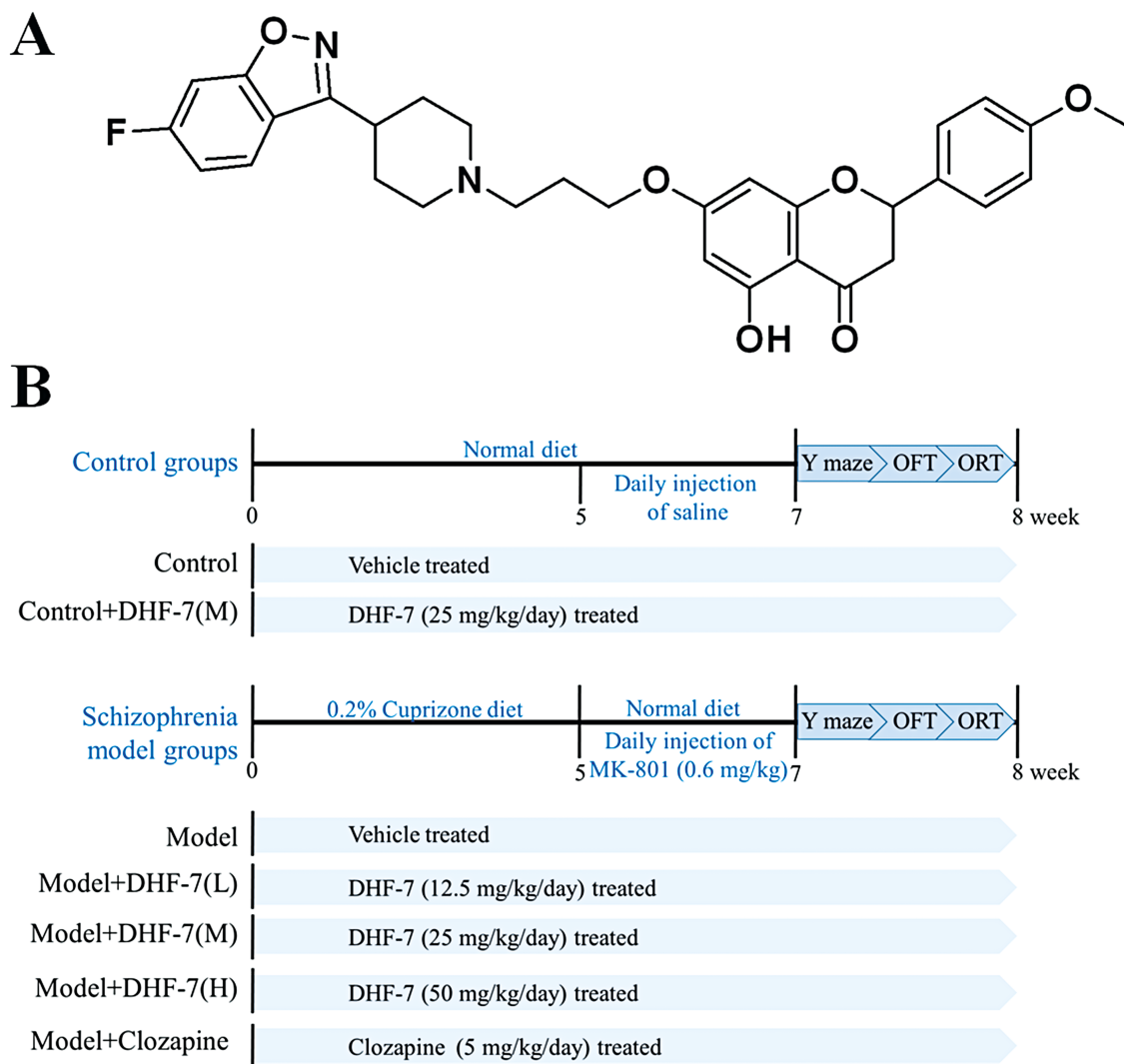


Figure 1. Structure of compound DHF-7 and the experimental design. (A) Structure of compound DHF-7. (B) Schematic illustration of the experimental design and groups allocation. The mouse model was established by feeding mice with diet containing 0.2 % cuprizone (CPZ) for 5 weeks, followed by a 2-week i.p. injection of MK-801 (0.6 mg/kg/d). Among the model mice, 1 group was vehicle-treated, 3 groups were intragastrically treated with 3 doses of DHF-7 (L: low dose, 12.5 mg/kg/d; M: medium dose, 25 mg/kg/d; H: high dose, 50 mg/kg/d), and 1 group received clozapine (CLZ) (5 mg/kg/d). Behavioral tests were conducted, including Y maze test, open field test (OFT), and object recognition test (ORT).

and pathologies of schizophrenia require further investigation, especially on cognitive impairments. Because white matter lesions and hypofunction of NMDARs are reported to be associated with the cognitive impairments, we used a novel mouse model of schizophrenia induced by a combination of CPZ and MK-801 and investigated the effects of DHF-7 on the related pathologies and signaling pathways in this model.

MATERIALS AND METHODS

Animals

Six-week-old male C57BL/6 mice weighing 20 ± 2 g were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice were housed under a 12-hour-light/dark cycle at a constant temperature of $22^\circ\text{C} \pm 1^\circ\text{C}$ and relative humidity of 55%–60%, with free access to food and water. This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and

was approved by the Bioethics Committee of Xuanwu Hospital of Capital Medical University.

Drugs

DHF-7 was synthesized as described previously (Gu et al., 2017), and the purity was over 98%, as determined by high-performance liquid chromatography assay. Clozapine was purchased from Harveybio (Beijing) Gene Technology Co., Ltd. (Beijing, China). DHF-7 (1.25/2.5/5 mg/mL) and clozapine (0.5 mg/mL) were micro-suspended in 2% Tween-80 in normal saline. The volume of drugs administered was calculated based on the body weight of the mice (10 mL/kg).

CPZ and MK-801 were purchased from Sigma-Aldrich (St. Louis, MO, USA). CPZ was mixed with the milled Lab Diet rodent chow at a final concentration of 0.2% (w/w) by Beijing Keao Xieli Feed Co., Ltd. (Beijing, China). MK-801 was diluted in normal saline to a concentration of 0.06 mg/mL prior use. The volume of MK-801 was calculated by the body weight of

the mice (10 mL/kg) and i.p. injected (0.6 mg/kg/d), as previously described (Sun et al., 2021).

Animal Treatment and Grouping

As described in our previous study, we established a novel mouse model of schizophrenia induced by a combination of CPZ and MK-801 (Sun et al., 2021). Briefly, the mice in the model group were fed a diet containing 0.2% CPZ for 5 weeks, followed by a normal diet, and simultaneously received an i.p. injection of MK-801 0.6 mg/kg/d for 2 weeks. Mice in the control group were fed a normal diet for 7 weeks and received an i.p. injection of an equal volume of normal saline in the last 2 weeks (Fig. 1B).

Twenty mice were randomly assigned to the vehicle-treated control group (Control, $n = 10$) and 25 mg/kg DHF-7-treated control group (medium dose, Control+DHF-7(M), $n = 10$). Fifty mice in the model group were randomly assigned and equally divided into 5 groups: vehicle-treated model group ($n = 10$, Model), 12.5 mg/kg DHF-7-treated model group [low dose, Model+DHF-7(L)], 25 mg/kg DHF-7 treated model group [medium dose, Model+DHF-7(M)], 50 mg/kg DHF-7 treated model group [high dose, Model+DHF-7(H)], and 5 mg/kg clozapine-treated model group (Model+Clozapine). DHF-7 and clozapine were diluted in the vehicle (2% Tween-80 in saline) and intragastrically administered once a day for 7 weeks. Behavioral tests were conducted as shown in Figure 1B.

Y-Maze Test

The Y-maze test was used to evaluate spatial memory and locomotor activity of the mice. The apparatus consisted of 3 arms ($30 \times 10 \times 25$ cm, length \times width \times height). The apparatus was placed 40 cm above the floor and was surrounded by various extra-maze cues. The mice were placed at the end of one arm and allowed to move freely through the maze for 8 minutes. The sequence of arm entries and total number of arms for each mouse were recorded. The percentage of alternations was calculated: (actual alternations/maximum alternations) \times 100%. A series of entries into all 3 arms on consecutive occasions was defined as actual alternation. The maximum alternation was the total number of arm entries minus 2.

Open Field Test

The open field test was used to evaluate locomotor activity and anxiety behavior in mice. The apparatus consisted of a Plexiglas box (size: $41.5 \text{ cm} \times 41.5 \text{ cm} \times 41.5 \text{ cm}$). The mice were placed at the center of the box and allowed to explore the apparatus for 10 minutes. The apparatus was cleaned with 75% ethanol and permitted to dry between tests. The total distance traveled and the distance traveled in the central square were recorded and calculated using a video-based Ethovision system (Noldus, Wageningen, the Netherlands).

Novel Object Recognition Test

The novel object recognition test was applied to detect the recognition memory of the mice, as previously described (Ma et al., 2019). Briefly, the test lasted for 3 days: on day 1, mice were allowed to explore the arena freely for 10 minutes; on day 2, they were re-introduced into the chamber for 10 minutes with 2 identical objects placed on opposite sides of the arena; on day 3 (the testing day), one of the objects was replaced with a novel object, and mice were placed in the chamber for a 10-minute object recognition test. Object exploration was defined as the orientation where the mouse snouts toward the object and stands out

within 2 cm or less from the object. The objects were cleaned with 75% ethanol after each trial to eliminate olfactory cues. The cumulative time spent exploring the objects on the testing day was recorded and used to calculate the discrimination index (DI): $DI = (N - F) / (N + F)$, where N is the elapsed time spent exploring the novel object, and F is the time spent exploring the familiar object. A higher DI value indicates better memory ability.

Transmission Electron Microscopy

After behavioral testing, 3 mice from each group were anesthetized and perfused with 2.5% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The brains were harvested and small brain blocks of 1 mm^3 were cut from the corpus callosum and post-fixed with 2.5 % glutaraldehyde and 1% osmium tetroxide. Ultrathin sections were prepared using ethanol dehydration and epoxy resin embedding. Serial ultrathin sections (50 nm) were prepared, stained with lead citrate and uranyl acetate, and placed under a Hitachi H-7650 transmission electron microscope (Tokyo, Japan) to observe the ultrastructure of myelin sheaths and demyelinated axons. From each section, 10 fields of vision were randomly photographed at a magnification of 15 000 \times . Ten fields of vision were captured in a "zigzag" fashion by moving the field using equidistance movement (Xiu et al., 2015). The ratio of demyelinated axons to the total axons (%) was calculated.

Oil Red O Staining

For histochemical analysis of the brain, 4 mice from each group were anesthetized after behavioral testing and perfused intracardially with 0.01 mol/L PBS followed by 4% paraformaldehyde (pH 7.4). The brains were removed and post-fixed in 4% paraformaldehyde solution, followed by cryoprotection in 30% sucrose at 4°C for 24 to 48 hours. Then, the specimens were cut into serial coronary slices (30 μm) using a cryostat microtome (Thermo Fisher Scientific Corp., Fairlawn, NJ, USA).

The brain sections were mounted and air-dried, followed by incubation in Oil Red O solution (0.5% in 60% ethanol) for 1 hour at room temperature, rinsed in 60% ethanol, and washed in water for 1 to 2 minutes. The sections were differentiated in an acid alcohol solution and mounted in gelatin mounting medium (ZSGB-Bio, Beijing, China). Images were digitally recorded using an Olympus microscope (Richmond Hill, ON, Canada) and analyzed using Image-Pro Plus software. The same conditions were maintained when performing the measurements on all selected brain sections.

Immunohistochemical Staining

Brain slices (Bregma -1.94 mm) were exposed to 3% H_2O_2 for 10 minutes to reduce endogenous peroxidase activity and blocked with blocking solution (5% normal goat serum in PBS) for 30 minutes at 37°C. The slices were subsequently incubated with rat anti-Myelin basic protein (MBP) antibody (Merck Millipore, Germany) or anti-NG2 antibody (Merck Millipore) at 4°C overnight. On the second day, the slices were incubated with a non-biotin detection system (Zsbio, Beijing, China) at 37°C for 1 hour and visualized using a 3,3'-Diaminobenzidine tetrahydrochloride (DAB) substrate kit (ZSGB-bio, Beijing, China). All slices were washed with PBS, mounted, dried, dehydrated, and cover slipped. Images were digitally recorded using an Olympus microscope (Richmond Hill, ON, Canada) and analyzed using the Image-Pro Plus software.

For immunofluorescence double staining of Adenomatous polyposis coli protein (APC) and phosphorylated Fyn (p-Fyn), brain slices were blocked in blocking solution (5% normal goat serum in PBS) for 60 minutes and incubated with the anti-APC antibody (CC-1, Merck Millipore) and p-Fyn (phospho Tyr-416, Abcam, Cambridge, UK) at 4°C overnight and followed by incubation with Alexa Fluor secondary antibodies (Thermo Fisher Scientific Corp., Fairlawn, NJ, USA) for 1 hour at room temperature. The slides were then covered with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (ZSGB-bio, Beijing, China). Fluorescent images were captured using a Nikon 80i microscope (Nikon Instruments, Japan) with the same fluorescence settings. Image-Pro Plus software was used to count the positive cells.

Western Blotting

After behavioral testing, 3 mice from each group were anesthetized and killed. The corpus callosum from the brains was rapidly removed and homogenized in lysis buffer, which contained 20 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM ethylenediaminetetraacetic acid, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China).

Equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The following primary antibodies were used in this study: rat anti-MBP (Merck Millipore), rabbit anti-2',3'-Cyclic nucleotide-3'-phosphodiesterase (CNPase) (Merck Millipore), rabbit anti-brain-derived neurotrophic factor (BDNF) (Abcam), rabbit anti-tyrosine kinase receptor B (TrkB; Abcam), rabbit anti-p-TrkB (phospho Tyr-705, Abcam), rabbit anti-Fyn (Abcam), rabbit anti-Fyn (phospho-Tyr-416; Abcam), rabbit anti-NMDA receptor subunit 2 B (NMDAR2B; Abcam), rabbit anti-NMDAR2B (phospho-Tyr-1472) (Abcam), rabbit anti-Braf, rabbit anti-p-Braf (Ser-445), rabbit anti-MEK1/2, rabbit anti-p-MEK1/2 (Ser217/221), rabbit anti-ERK1/2, and rabbit anti-p-ERK1/2 (Thr202/Try204) from Cell Signaling Technology, USA, and mouse anti- β -actin (Sigma-Aldrich). Membranes were then incubated with the corresponding secondary antibodies and visualized using enhanced chemiluminescence (ECL system; Merck Millipore). Images were captured and analyzed using AlphaView for FluorChem Systems (Protein Simple, CT, USA). β -actin was used as the internal loading control.

Statistical Analyses

All statistical analyses were performed using SPSS software version 19.0. Data were assessed using 1-way ANOVA to verify the significance between means, followed by Student-Newman-Keuls post hoc comparisons of the difference between 2 groups. Numerical data are presented as mean \pm SEM. Differences were considered statistically significant at $P < .05$.

RESULTS

Effects of DHF-7 on Locomotor Activity and Cognitive Function in the Mouse Model of Schizophrenia Induced by Combination of CPZ and MK-801

The open field test was used to detect locomotor activity in a schizophrenia mouse model induced by CPZ and MK-801 after

intragastric administration of DHF-7 for 7 weeks. In the open field test, the total distance was recorded to determine locomotor activity (ANOVA: $F_{(6, 63)} = 16.29$, $P < .01$, Fig. 2A). Compared with the control group, the total distance during the 10-minute test was significantly increased in the model group ($P < .01$). Furthermore, DHF-7 (25 and 50 mg/kg) resulted in decreased total distance traveled by the mice in the open field test ($P < .01$; Fig. 2A).

Moreover, a significant difference was found in the number of total arm entries in the Y-maze test, representing locomotor hyperactivity of the mice ($F_{(6, 63)} = 9.613$, $P < .01$, Fig. 2B). Further analysis revealed that the model group showed an increased number of total arm entries in the Y-maze ($P < .01$) and DHF-7 (25 and 50 mg/kg) significantly decreased the number of total arm entries ($P < .01$; Fig. 2B). These results indicate that DHF-7 treatment suppressed locomotor hyperactivity in the mouse model, representing a potential effect on ameliorating the positive symptoms of schizophrenia.

Alternative behavior in the Y-maze test was recorded to detect the spatial memory of the schizophrenia mouse model ($F_{(6, 63)} = 9.436$, $P < .01$, Fig. 2C). The results showed that the alternative index decreased in the model group ($P < .01$), and DHF-7 (25 and 50 mg/kg) significantly increased the alternative index of the model mice ($P < .01$; Fig. 2C). In addition, the DI in the novel object recognition test was used to measure the recognition memory of the mice ($F_{(6, 63)} = 6.792$, $P < .001$, Fig. 2D). The model group showed lower DI compared with the control group ($P < .01$), and DHF-7 (25 and 50 mg/kg) significantly increased the DI of the model mice ($P < .01$; Fig. 2D). These results indicate that DHF-7 might improve cognitive function in a mouse model of schizophrenia induced by CPZ and MK-801.

Effects of DHF-7 on Myelin Sheath Ultrastructure and Demyelination in the Corpus Callosum of the Mouse Model

The ultrastructure of myelin in the corpus callosum of mice was observed using transmission electron microscopy. The ratio of demyelinated axons in the corpus callosum was calculated to represent myelin loss (ANOVA: $F_{(6, 14)} = 73.90$, $P < .001$, Fig. 3A,B). Compared with the control group, the model group displayed severe myelin loss, axon damage, and a significant increase in demyelinated axons in the corpus callosum ($P < .01$). DHF-7 (25 and 50 mg/kg) treatment protected the structure of the myelin sheath and decreased the ratio of demyelinated axons in the mouse model ($P < .01$; Fig. 3A and C).

Oil Red O staining was used to detect intact myelin phospholipids in the corpus callosum. The optical density (OD) of Oil Red O staining was calculated and analyzed (ANOVA: $F_{(6, 21)} = 24.69$, $P < .001$, Fig. 3B and D). The OD of the model group was lower compared with the control group ($P < .01$). Further, DHF-7 (25 and 50 mg/kg) treatment elevated the OD of the mouse model ($P < .01$, Fig. 3C, D), indicating that it can alleviate demyelination and white matter lesions in the corpus callosum of the mouse model.

Effects of DHF-7 on the Expression of Myelin-Associated Proteins in the Corpus Callosum of the Mouse Model

MBP is a key component of myelin produced in OLs and maintains the structural stability and function of the myelin sheath. CNPase is another OL-specific protein used to represent the integrity of myelin and the level of mature OLs. The OD of MBP

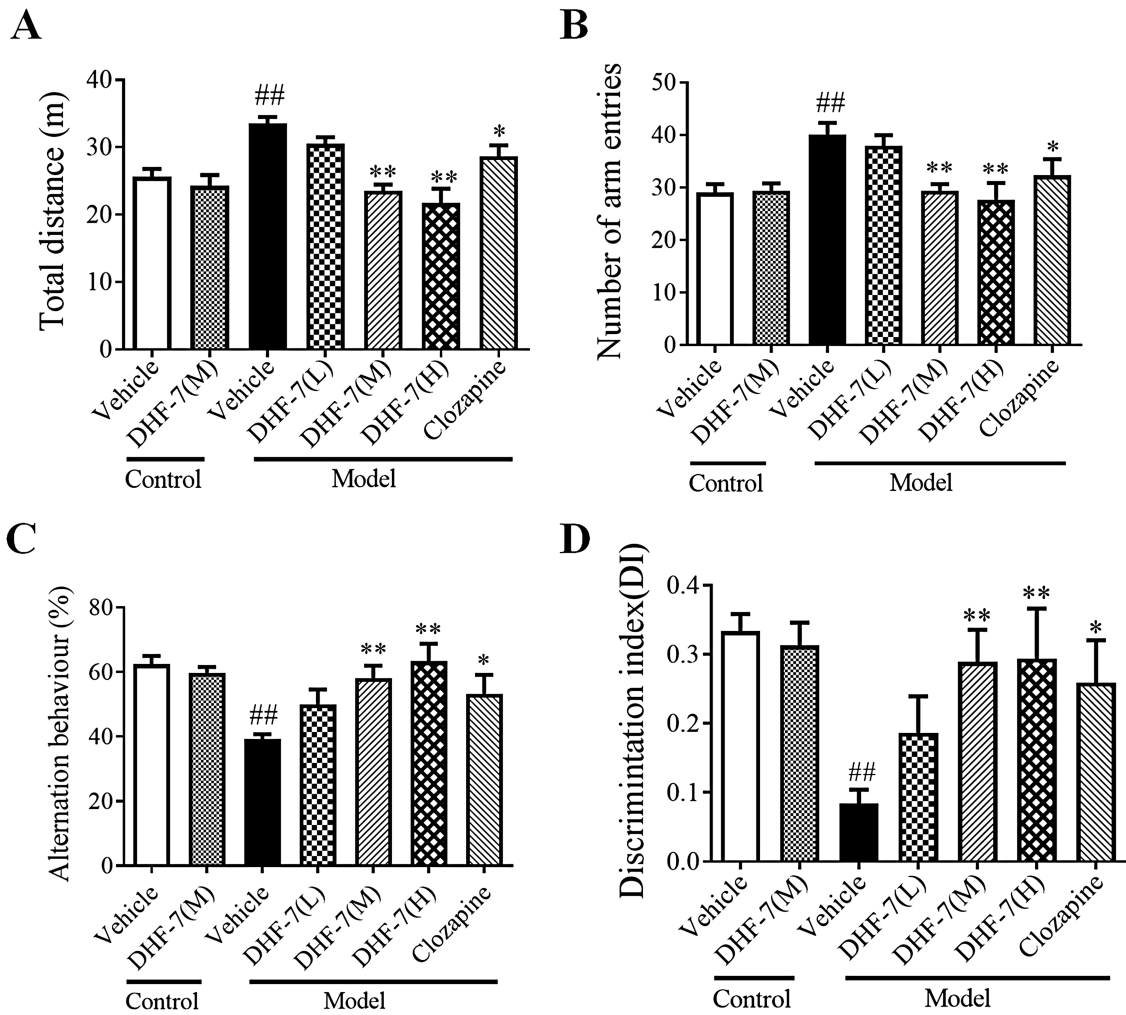


Figure 2. Effects of DHF-7 on locomotor activity and cognitive function in the mouse model of schizophrenia induced by cuprizone and MK-801. (A) The total movement distance during a 10-minute test in the open field. (B) The number of total arm entries in the Y-maze test. (C) The percentage of alternation behaviour (alternation index, %) in the Y-maze test. (D) Discrimination index (DI) in object recognition test. Data are expressed as mean \pm SEM; $n = 10$ each group. ^{##} $P < .01$, model group vs. control group; ^{*} $P < .05$, ^{**} $P < .01$, drug-treated groups vs. model group.

immunohistochemistry was analyzed to represent MBP expression and intact myelin in the corpus callosum ($F_{(6, 21)} = 15.73$, $P < .001$, Fig. 4A,B). Results from immunohistochemical staining showed a decreased level of MBP staining in the corpus callosum of the model groups ($P < .01$), and DHF-7 at the 3 doses increased the expression of MBP ($P < .05$, $P < .01$; Fig. 4A,B).

Western-blotting analysis was conducted to detect the expression levels of MBP ($F_{(6, 21)} = 16.82$, $P < .001$; Fig. 4C,D) and CNPase ($F_{(6, 14)} = 12.58$, $P < .001$; Fig. 4C and E) in the corpus callosum of mice. Further analysis revealed that the expression levels of MBP and CNPase in the corpus callosum were lower in the model group than in the control group ($P < .01$; Fig. 4C-E). DHF-7 treatment elevated the expression levels of MBP and CNPase in the mouse model ($P < .05$, $P < .01$; Fig. 4C-E).

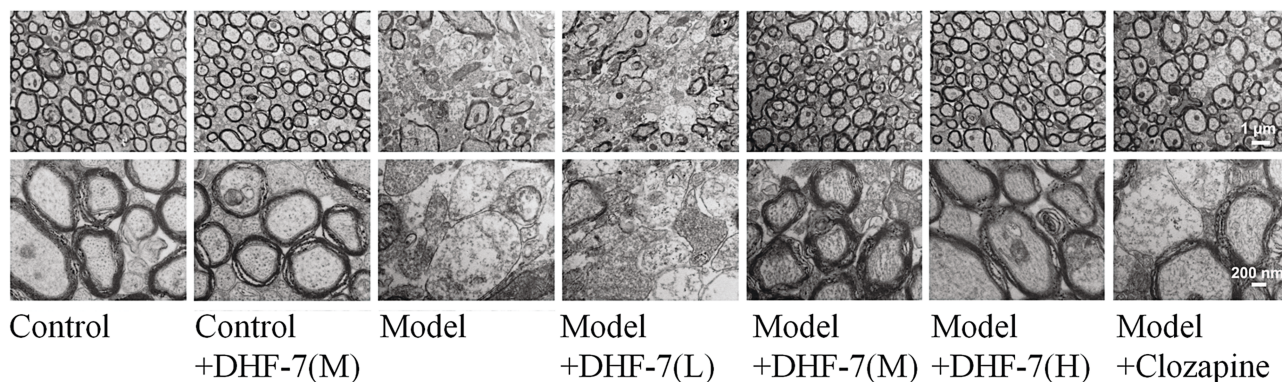
Effects of DHF-7 on OLs and Fyn in the Corpus Callosum of the Mouse Model

OL precursor cells (OPCs, labeled by NG2) have the potential to differentiate into mature OLs in the mouse brain. The number of NG2⁺ cells representing OPCs was analyzed ($F_{(6, 14)} = 29.10$, $P < .001$; Fig. 5A,B). An increase in the number of NG2⁺ cells was found in the corpus callosum of model mice compared with the

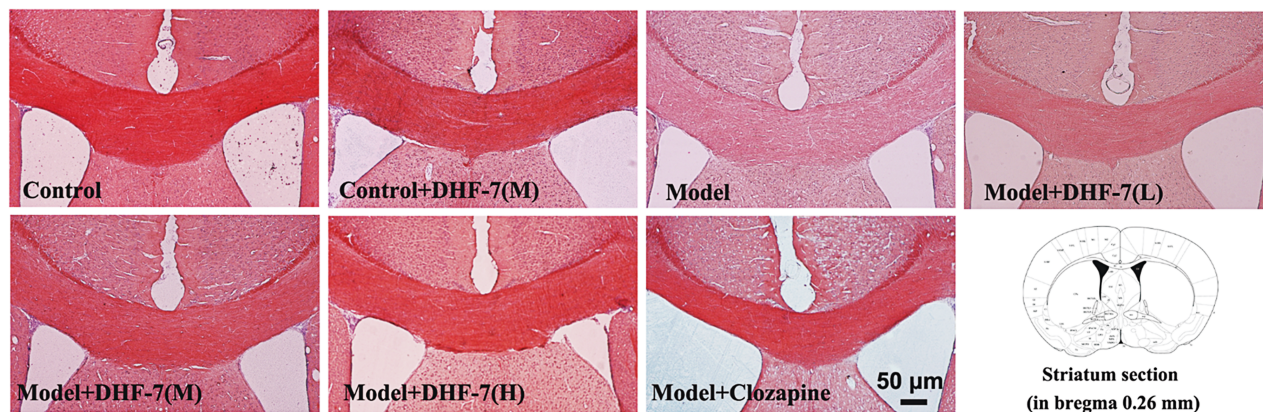
control mice ($P < .01$), suggesting a possible failure in the process of OPC differentiation in the mouse model. DHF-7 (25 and 50 mg/kg) treatment reduced the number of OPCs in the corpus callosum of the model mice ($P < .01$; Fig. 5A,B). These results indicate that DHF-7 may promote the differentiation of OPCs into mature OLs.

Moreover, we used immunofluorescence double staining for mature OLs labeled with APC (red color, $F_{(6, 14)} = 62.26$, $P < .001$; Fig. 5C,D) and p-Fyn (green color) as well as their colocalization (merge) in the corpus callosum of mice. The results showed that the number of APC⁺ mature OLs in the model group was significantly lower than that in the control group ($P < .01$), and DHF-7 (25 and 50 mg/kg) treatment increased the number of APC⁺ mature OLs ($P < .01$; Fig. 5C,D). p-Fyn was co-immunolabeled with APC, indicating the possible role of tyrosine kinase Fyn in promoting the maturation of OLs ($F_{(6, 14)} = 29.10$, $P < .001$; Fig. 5C and E). The model group showed a decline in the number of APC⁺/p-Fyn⁺ OLs in the corpus callosum ($P < .01$), and DHF-7 (25 and 50 mg/kg) significantly increased the number of APC⁺/p-Fyn⁺ OLs in the corpus callosum of the model mice ($P < .01$; Fig. 5C and E). These results indicate that DHF-7 enhances the maturation of OLs, and the mechanism might be associated with the activation of Fyn.

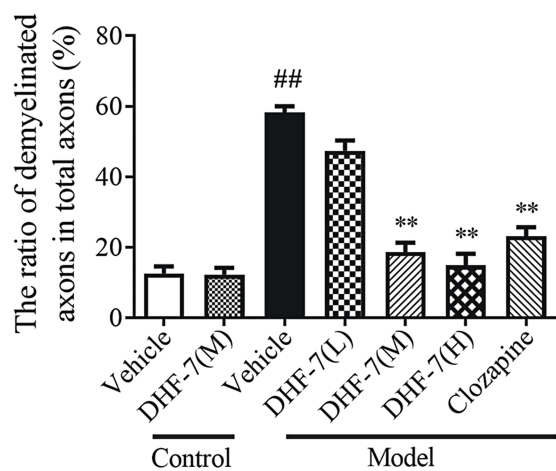
A



B



C



D

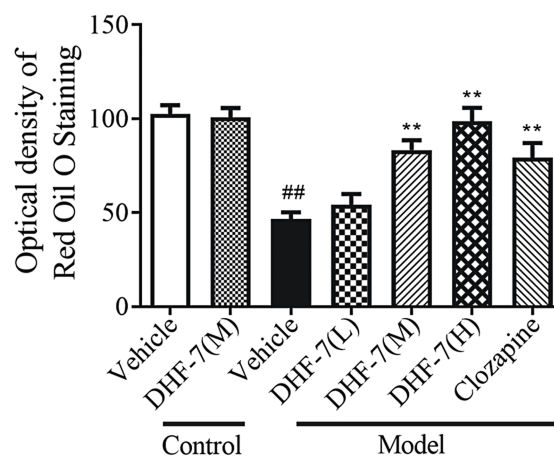


Figure 3. Effects of DHF-7 on myelin sheath ultrastructure and demyelination in the corpus callosum of the mouse model induced by cuprizone and MK-801. Representative images of myelin sheaths and axons (A) and quantitative analysis for the ratio of demyelinated axons in total axons (%) (C) detected by transmission electron microscope. Scale bar = 1 μ m, scale bar (magnified images) = 200 nm; $n = 3$. Representative images of Oil Red O staining for myelin sheath (B) and quantitative analysis for the optical density of Oil Red O staining (D). Scale bar = 50 μ m. The optical density in the control group is taken as 100%, $n = 4$. Data are expressed as the mean \pm SEM. ** $P < .01$, model group vs. control group; ** $P < .01$, drug-treated groups vs. model group.

Effects of DHF-7 on the Expression of BDNF and Activation of TrkB/Fyn/NMDAR2B Signaling Pathway in the Corpus Callosum

Reduced levels of BDNF in the plasma and brain have been reported as potential biomarkers for schizophrenia, and BDNF reduction is associated with poor cognitive function in patients

with chronic schizophrenia (Man et al., 2018). We detected the expression of BDNF and the activation of its downstream kinase TrkB/Fyn and NMDAR2B in the corpus callosum of mice by western blotting (BDNF: $F_{(6,14)} = 26.33$, $P < .001$; p-TrkB/T-TrkB: $F_{(6,14)} = 17.66$, $P < .001$; p-Fyn/T-Fyn: $F_{(6,14)} = 17.41$, $P < .001$; p-NMDAR2B/T-NMDAR2B: $F_{(6,14)} = 24.72$, $P < .001$). The model group showed a significant decrease in the expression level of BDNF in

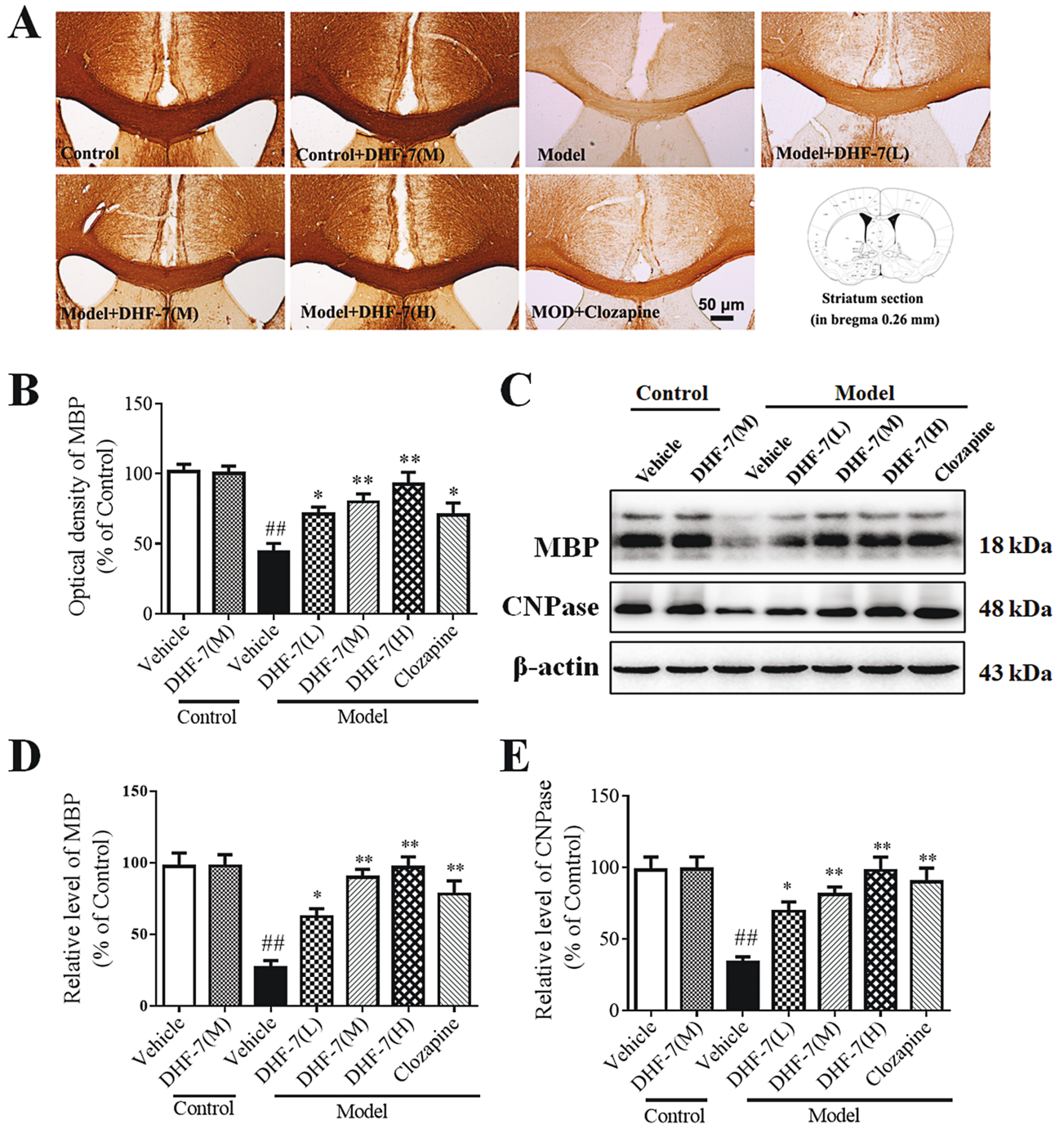


Figure 4. Effects of DHF-7 on the expression of myelin-associated proteins in the corpus callosum of the mouse model induced by cuprizone and MK-801. (A) Representative images of Myelin basic protein (MBP) immunohistochemistry staining. Scale bar = 50 μ m. (B) Optical density of MBP immunohistochemistry. The optical density in the control group is taken as 100%, $n = 4$. (C) Representative western-blot images of MBP and 2',3'-Cyclic nucleotide-3'-phosphodiesterase (CNP). (D, E) Quantitative analysis of MBP and CNPase expression from the western-blot images, respectively. The ratio of MBP and CNPase to β -actin in the control group was taken as 100%, $n = 3$. Data are expressed as mean \pm SEM. ^{##} $P < .01$, model group vs. control group; ^{*} $P < .05$, ^{**} $P < .01$, drug groups vs. model group.

the corpus callosum ($P < .01$); DHF-7 (25 and 50 mg/kg) treatment increased the expression level of BDNF in model mice ($P < .01$; Fig. 6A & B). Moreover, the phosphorylation levels of TrkB, Fyn, and NMDAR2B were decreased in the corpus callosum of model mice ($P < .01$); DHF-7 at the three doses significantly elevated the phosphorylation levels of TrkB, Fyn, and NMDAR2B compared with the untreated model group ($P < .05$, $P < .01$). However, the total expression levels of TrkB, Fyn, and NMDAR2B did not show significant changes (Fig. 6C-F). These results indicate that DHF-7

promotes the expression of BDNF and activation of its downstream TrkB/Fyn/NMDAR2B signaling pathway.

Effects of DHF-7 on the Activation of Raf/MER/ERK Signaling Pathway in the Corpus Callosum of the Mouse Model

The Raf/mitogen-activated protein kinase (MEK)/extracellular signal-related kinase (ERK) signaling pathway has been

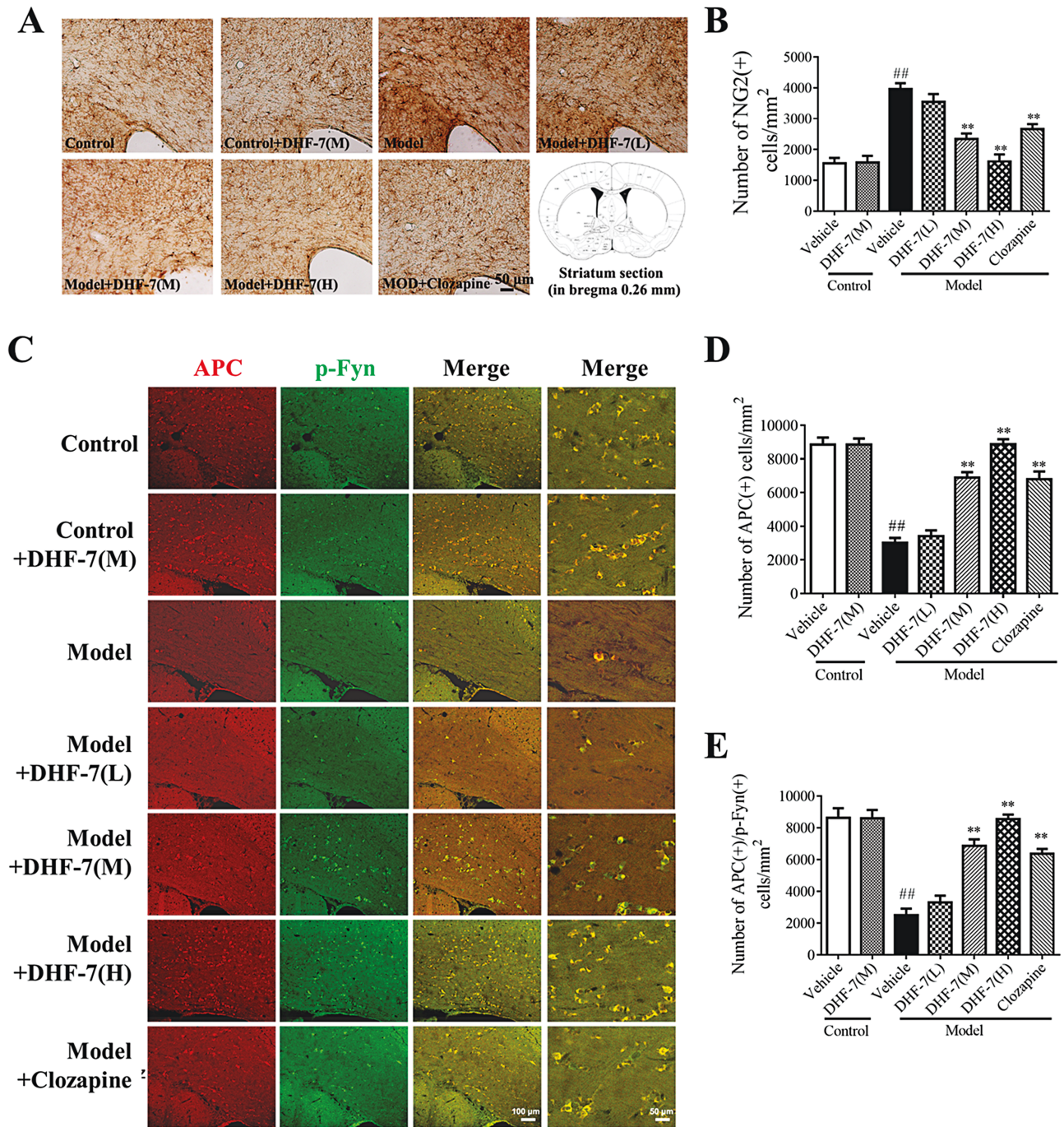


Figure 5. Effects of DHF-7 on the number of oligodendrocyte precursor cells and the co-localization of oligodendrocytes with phosphorylated Fyn in the corpus callosum of the mouse model. (A) Representative images of immunohistochemistry for oligodendrocyte precursor cells (OPCs) labeled by NG2. Scale bar = 50 μ m. (B) Quantitative analysis of the number of OPCs per mm^2 , $n = 4$. (C) Representative images of immunofluorescence double staining for mature oligodendrocytes (OLs) labeled by Adenomatous polyposis coli protein (APC) (red color) and phosphorylated Fyn (p-Fyn) at Tyr-416 (green color) as well as the merge images; scale bar = 100 μ m, scale bar (higher magnification) = 50 μ m. (D) Quantitative analysis of the number of APC⁺ mature OLs/ mm^2 . (E) Quantitative analysis of the number of APC⁺/p-Fyn⁺ cells per mm^2 , $n = 3$. Data are expressed as the mean \pm SEM. ^{##} $P < .01$, model group vs. control group; ^{**} $P < .01$, drug-treated groups vs. model group.

reported to regulate cellular proliferation and survival and promote OL myelination, which can be regulated by Fyn (Xiao et al., 2012). In the present study, the total and phosphorylation levels of Raf (B-Raf), MER1/2, and ERK1/2 were detected using western blotting (p-B-Raf/T-B-Raf: $F_{(6,14)} = 20.60$, $P < .001$; p-MER1/2: $F_{(6,14)} = 18.44$, $P < .001$; p-ERK1/2: $F_{(6,14)} = 32.84$, $P < .001$). The model group showed a decrease in the phosphorylation levels of B-Raf, MER1/2, and ERK1/2 in the corpus

callosum compared with the control group ($P < .01$). DHF-7 at the 3 doses significantly elevated the phosphorylation levels of B-Raf, MER1/2, and ERK1/2 compared with the model group not receiving DHF-7 ($P < .05$, $P < .01$), while the total expression levels of B-Raf, MER1/2, and ERK1/2 did not show significant changes (Fig. 7). These results indicate that DHF-7 treatment activated the Raf/MER/ERK signaling pathway in the corpus callosum of a mouse model.

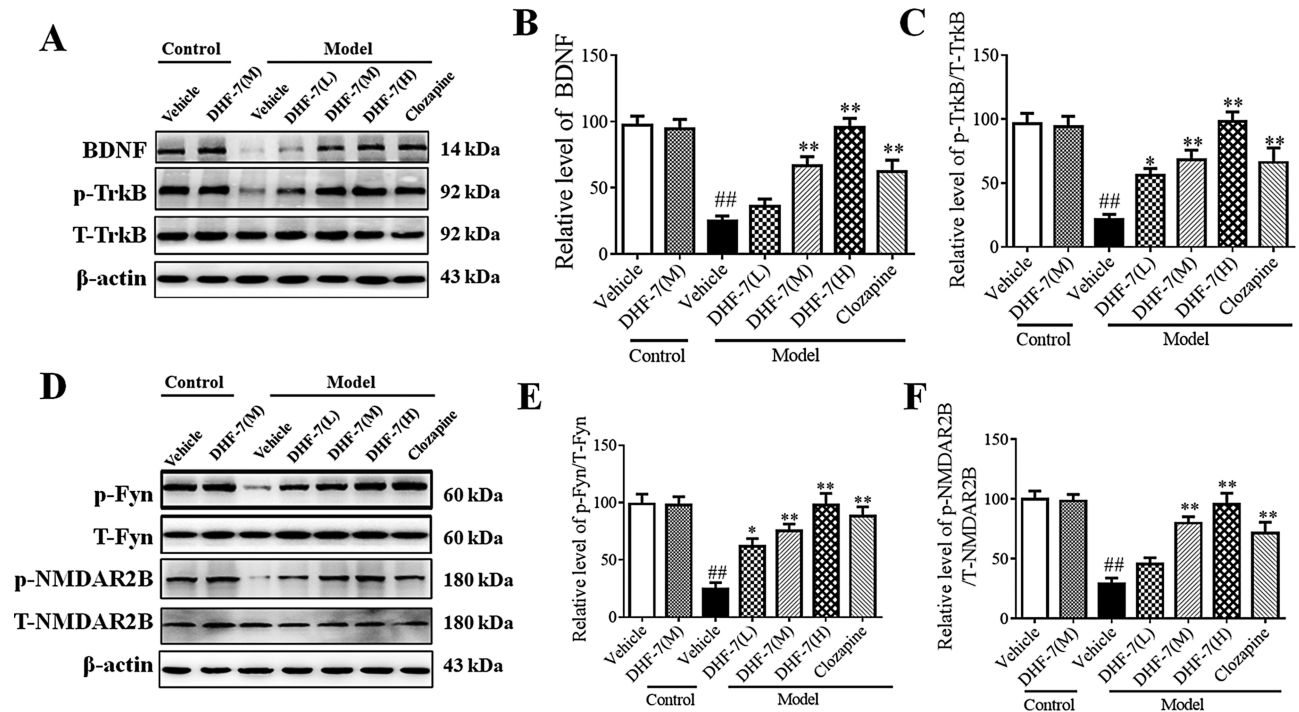


Figure 6. Effects of DHF-7 on the expressions of brain-derived neurotrophic factor (BDNF) and phosphorylation of tyrosine kinase receptor B (TrkB)/Fyn/N-methyl-D-aspartate receptor subunit 2 B (NMDAR2B) in the corpus callosum of the mouse model (western blotting). (A) Representative blot images of BDNF, phosphorylated and total TrkB. (B) The quantitative analysis of BDNF expression level. The ratio of BDNF to β -actin in the control group was taken as 100%. (C) The quantitative analysis of phosphorylated and total TrkB. (D) Representative blot images of phosphorylated and total Fyn, phosphorylated and total NMDAR2B. (E) The quantitative analysis of phosphorylated and total Fyn. (F) The quantitative analysis of phosphorylated and total NMDAR2B. The ratio of phosphorylated to total level in the control group was taken as 100%. Data are expressed as the mean \pm SEM, $n = 3$. $^{##}P < .01$, model group vs. control group; $^{*}P < .05$, $^{**}P < .01$, drug-treated groups vs. model group.

DISCUSSION

The present study showed that intragastric administration of DHF-7 for 7 weeks improved spatial and recognition memory dysfunction, ameliorated locomotor hyperactivity, alleviated white matter lesions and demyelination, increased the expression of myelin-associated proteins, and increased the number of mature OLs in the corpus callosum in a mouse model of schizophrenia induced by a combination of CPZ and MK-801. This mechanism might be related to the potential effects of DHF-7 on promoting OPC differentiation via the BDNF/TrkB/Fyn/NMDAR2B and Raf/MER/ERK signaling pathways.

The white matter of the brain is mainly composed of nerve fibers and myelin sheaths surrounding the nerve fibers, which play an important role in signal transmission and normal brain functions. The integrity of white matter depends on the complete structure of myelin sheaths, which influences the processing speed and strength of signal propagation (Takahashi et al., 2011). White matter abnormalities are observed in patients with schizophrenia, which is closely related to learning, memory, and overall cognitive abilities of the patients with schizophrenia (Hummer et al., 2018; Jiang et al., 2019). The corpus callosum is a bundle of fibers connecting the left and right hemispheres and is related to cognitive ability and the connection of the cerebral hemispheres (Ohoshi et al., 2019). In the present study, white matter lesions were found in the corpus callosum of the brain of a schizophrenia mouse model induced by MK-801 and CPZ. Transmission electron microscopy and Oil Red O staining showed that DHF-7 ameliorated myelin loss and white matter lesions in the corpus callosum of the mouse model, which may explain the improvement in cognitive impairment in the mouse model by DHF-7.

OLs are the myelin-forming cells in the CNS, and OPCs have the ability to differentiate into mature OLs for regeneration and repair of myelin during the adult life (Wheeler and Fuss, 2016). NG2 is a marker of immature OPCs, whereas APC is a marker for labeling mature OLs. MBP and CNPase are proteins expressed in mature OLs that maintain the structural stability and functions of the myelin sheath (Alizadeh et al., 2015). Myelin-related proteins, including MBP and CNPase, show reduced expression in the brains of patients with schizophrenia (Dracheva et al., 2006; Parlapani et al., 2009). In the present study, the mouse model exhibited an increased number of OPCs and a decrease in mature OLs, indicating a failure in the differentiation of OPCs into OLs. DHF-7 treatment reduced the number of OPCs, elevated the number of mature OLs, and increased the expression of MBP and CNPase in the corpus callosum of the mouse model. These results indicate that DHF-7 may play a role in promoting the differentiation of OPCs into mature OLs, which partly explains the protective effects of DHF-7 on myelin sheaths and white matter in an animal model of schizophrenia.

Specific growth and transcription factors have been reported to be involved in the differentiation and maturation of OPCs into OLs (Alizadeh et al., 2015). Among these, BDNF plays a key role in promoting OL genesis, differentiation, and myelination (Niu et al., 2020; Langhnoja et al., 2021). BDNF is the most abundant neurotrophic factor in the CNS and promotes cellular development and survival by activating its receptor, TrkB. In addition, decreased BDNF levels and dysfunction in the BDNF/TrkB signaling pathway are reported to be closely related to schizophrenia (Mohammadi et al., 2018). Moreover, lower levels of serum BDNF are positively associated with negative cognitive impairments in patients with schizophrenia (Binford et al., 2018;

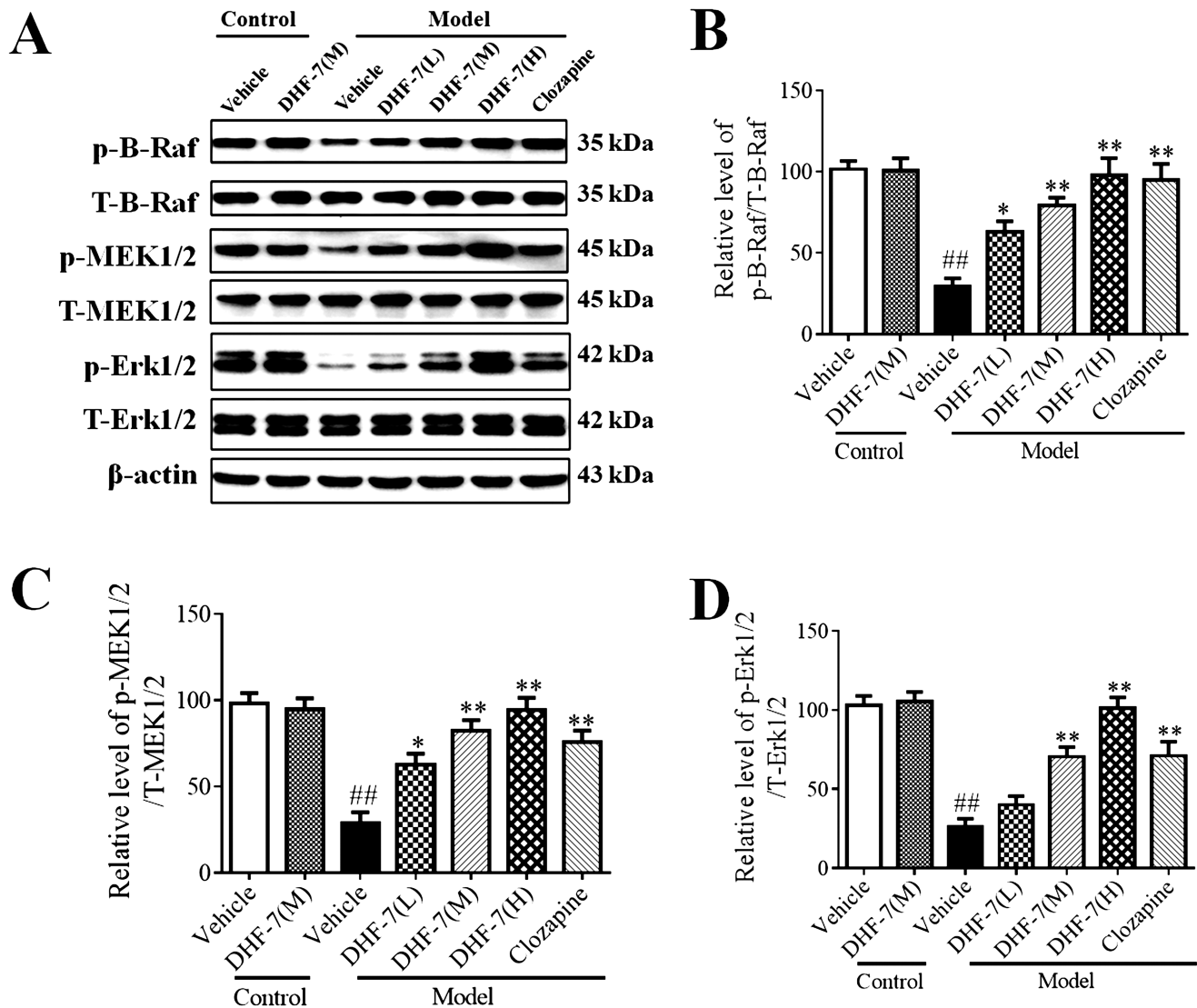


Figure 7. Effects of DHF-7 on the activation of Raf/mitogen-activated protein kinase (MEK)/extracellular signal-related kinase (ERK) signaling pathway in the corpus callosum of mouse model (western blotting). (A) Representative blot images of phosphorylated and total Raf (B-Raf), phosphorylated and total MEK1/2, phosphorylated and total Erk1/2. (B) The quantitative analysis of phosphorylated and total B-Raf expression level. (C) The quantitative analysis of phosphorylated and total MEK1/2. (D) The quantitative analysis of phosphorylated and total Erk1/2. The ratio of phosphorylated to total level in the control group was taken as 100%. Data are expressed as the mean \pm SEM, $n = 3$. ^{##} $P < .01$, model group vs. control group; ^{*} $P < .05$, ^{**} $P < .01$, drug-treated groups vs. model group.

Man et al., 2018). In the present study, the model group showed a decrease in the expression of BDNF and phosphorylated TrkB in the corpus callosum, whereas DHF-7 increased the expression of BDNF and phosphorylated TrkB (active form) in the mouse model. The effects of DHF-7 on BDNF expression provide a clue for its effects on white matter and cognitive impairment in a mouse model of schizophrenia.

Fyn is a member of the Src family of nonreceptor tyrosine kinases and has been implicated in axon-glia signal transduction and several cellular processes required for OL maturation and myelination (Krämer-Albers and White, 2011). A recent study showed that Fyn kinase exerted a key role in mediating the promyelinating influence of BDNF (Peckham et al., 2016). BDNF activation of oligodendroglial TrkB receptors stimulates the phosphorylation of Fyn at Tyr 416, a necessary step required to potentiate the phosphorylation of Erk1/2, which in turn regulates OL myelination (Peckham et al., 2016). To verify

the possible involvement of Fyn in the maturation of OPCs, double immunofluorescence staining for mature OLs and phosphorylated Fyn was performed in the brain of the mouse model. The results indicated that p-Fyn colocalized with mature OLs, and DHF-7 increased the number of APC⁺/p-Fyn⁺ OLs, suggesting that DHF-7 may promote the maturation of OPCs by activating Fyn.

Phosphorylated Fyn can activate the Raf/MEK/ERK signaling pathway, which plays a key role in cell survival, differentiation, and proliferation (Xie et al., 2016). Several studies have identified a critically important role of ERK1 and ERK2 in regulate oligodendroglial development and myelination by mediating the effects of several growth factors (Xiao et al., 2012). As well-characterized transcriptional activators, the MEK1/2-dependent phosphorylation of ERK1/2 induces their nuclear translocation, enabling them to phosphorylate and/or stabilize transcription factors and proteins

that then alter myelin-related gene expressions (Hazzalin and Mahadevan, 2002; Gonsalvez et al., 2016). In the present study, DHF-7 treatment increased the phosphorylation level of Fyn and activated its downstream Raf/MEK/ERK signaling pathway in the corpus callosum of model mice, indicating possible mechanisms by which DHF-7 promotes the maturation of OPCs.

Another hypothesis for the pathophysiology of schizophrenia includes glutamatergic hypofunction, which can be induced by MK-801 in a mouse model (Nakazawa and Sapkota, 2020). Interventions that enhance NMDAR function or downstream intracellular mediators of NMDAR are considered useful in the treatment of schizophrenia (Coyle et al., 2012; Liu et al., 2018). NMDAR2B is a regulatory subunit of NMDAR, which has been reported to be activated by Fyn through increased phosphorylation at Tyr-1472 (Li et al., 2017; Berrout and Isokawa, 2018). Activation of NMDARs promotes the maturation of OLs and favors myelination (Salter and Fern, 2005; Cavaliere et al., 2012). Moreover, Fyn can facilitate the accumulation of synaptic NMDAR2B-containing NMDARs and activation of neuroprotective cascades, including the elevated expression of BDNF (Gutierrez-Vargas et al., 2014). In the present study, the level of phosphorylated NMDAR2B at Tyr-1472 was decreased in the corpus callosum of model mice, and DHF-7 increased the phosphorylation level of NMDAR2B. The upregulating effects of DHF-7 on the activity of Fyn and NMDAR2B may partly explain its protective effects in ameliorating symptoms and white matter lesions in a mouse model of schizophrenia.

However, there may be some limitations to this study. The negative symptoms of schizophrenia include sociophobia, autism, depression, and anxiety (Miyamoto and Nitta, 2014). Although anxiety-like behavior could be detected by rearing, time, and latency in the center area in the open-field test, it might be influenced by increased locomotor activity. The present study did not evaluate the possible effects of DHF-7 on negative symptoms, which would be investigated in our future studies. Additionally, we used 10 mice per group to conduct the experiment and 3 to 4 mice per group for immunohistochemistry staining or western blotting assays, which might have influenced the interpretation of the results in the current study. Thus, we aim to investigate the efficacy of DHF-7 by using a larger sample size in future studies.

In conclusion, we used a mouse model of schizophrenia induced by a combination of CPZ and MK801 to investigate the pharmacological effects and mechanisms of DHF-7. We found that oral administration of DHF-7 for 7 weeks improved cognitive impairment and hyperactivity in the mouse model. DHF-7 alleviated white matter lesions and demyelination, possibly by promoting OL differentiation and maturation. The mechanisms included increased expression of BDNF and p-Fyn by DHF-7, thus activating the TrkB/Fyn/NMDAR2B and Raf/MER/ERK signaling pathways. These results suggest that DHF-7 may improve positive symptoms and cognitive impairment and be a potential drug candidate for the treatment of schizophrenia.

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Conflict of Interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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