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4-F-PCP, a Novel PCP Analog Ameliorates the Depressive-Like Behavior of Chronic Social Defeat Stress Mice via NMDA Receptor Antagonism

Darlene Mae D. Ortiz[1], Mikyung Kim[1,2], Hyun Jun Lee[1], Chrislean Jun Botanas[3], Raly James Perez Custodio[4], Leandro Val Sayson[1], Nicole Bon Campomayor[2], Chaeyeon Lee[5], Yong Sup Lee[5], Jae Hoon Cheong[6,*] and Hee Jin Kim[1,*]

[1]*Uimyung Research Institute for Neuroscience, Department of Pharmacy, Sahmyook University, Seoul 01795,*
[2]*Department of Chemistry & Life Science, Sahmyook University, Seoul 01795, Republic of Korea*
[3]*Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA*
[4]*Department of Ergonomics, Leibniz Research Centre for Working Environment and Human Factors, 44139 Dortmund, Germany*
[5]*Department of Fundamental Pharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Seoul 02447,*
[6]*Institute for New Drug Development, School of Pharmacy, Jeonbuk National University, Jeonju 54896, Republic of Korea*

Abstract

Major depressive disorder is a leading cause of disability in more than 280 million people worldwide. Monoamine-based antidepressants are currently used to treat depression, but delays in treatment effects and lack of responses are major reasons for the need to develop faster and more efficient antidepressants. Studies show that ketamine (KET), a PCP analog, produces antidepressant effects within a few hours of administration that lasts up to a week. However, the use of KET has raised concerns about side effects, as well as the risk of abuse. 4 -F-PCP analog is a novel PCP analog that is also an NMDA receptor antagonist, structurally similar to KET, and might potentially elicit similar antidepressant effects, however, there has been no study on this subject yet. Herein, we investigate whether 4-F-PCP displays antidepressant effects and explored their potential therapeutic mechanisms. 4-F-PCP at 3 and 10 mg/kg doses showed antidepressant-like effects and repeated treatments maintained its effects. Furthermore, treatment with 4-F-PCP rescued the decreased expression of proteins most likely involved in depression and synaptic plasticity. Changes in the excitatory amino acid transporters (EAAT2, EAAT3, EAAT4) were also seen following drug treatment. Lastly, we assessed the possible side effects of 4-F-PCP after long-term treatment (up to 21 days). Results show that 4-F-PCP at 3 mg/kg dose did not alter the cognitive function of mice. Overall, current findings provide significant implications for future research not only with PCP analogs but also on the next generation of different types of antidepressants.

Key Words: 4-F-PCP, Ketamine, Depression, NMDA receptor antagonist, PCP analogs

INTRODUCTION

Major depressive disorder (MDD) is the leading cause of disability, affecting more than 280 million people worldwide. In the worst cases, depression can lead to suicide. According to the World Health Organization, approximately 700,000 people die by suicide because of depression (World Health Organization, 2021). Depression is associated with chronicity, relapse, and recurrent symptoms and is usually treated with behavioral or pharmacological interventions or a combination of both. Currently, monoamine-based antidepressants (e.g., serotonin and norepinephrine reuptake inhibitors) are the most commonly used pharmacological treatments to alleviate depressive symptoms. However, the main problem with these conventional medications is the delay between treatment and its therapeutic effects, which is often several weeks or more (Gaynes *et al*., 2009). In addition, high rates of lack of response or partial response to these drugs have been reported, and a large proportion of patients remain treatment-resistant (Rush *et al*., 2006). These limitations have resulted in many

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***Corresponding Authors**
E-mail: cheongjh@jbnu.ac.kr (Cheong JH), hjkim@syu.ac.kr (Kim HJ)
Tel: +82-2-2339-1605 (Cheong JH), +82-2-3399-1609 (Kim HJ)
Fax: +82-2-2339-1619 (Cheong JH), +82-2-3399-1617 (Kim HJ)

patients being treated inadequately, highlighting the urgent need for rapid-acting and potent antidepressants. Currently, researchers are attempting to formulate antidepressants that target different signaling pathways. This includes the theory of glutamatergic-based antidepressants (Pałucha-Poniewiera and Pilc, 2016). Phencyclidine (PCP) and its analogs are non-competitive inhibitors of N-Methyl-D-aspartate (NMDA) receptors. Preclinical studies have shown that chronic administration of NMDA antagonists (e.g., MK-801, CGP 37849, and ACPC) can reduce behavioral deficits (decreased sucrose consumption and increased immobility time) in an animal model of depression (Papp and Moryl, 1994; Puran *et al*., 2014; Zanos *et al*., 2016). However, each NMDA antagonist has disadvantages, such as the abuse potential reported for MK-801, hallucinations, drowsiness, and even coma for some NMDA antagonists with excessive dwell times or off rates (Lipton, 2004; Zarate *et al*., 2006; Hillhouse *et al*., 2014). These antagonists act directly on the glutamatergic system by inhibiting GABAergic neurons, resulting in an increase in glutamate levels in presynaptic neurons. This effect is thought to contribute to the pharmacological action of antagonists, particularly their antidepressant-like effects (Autry *et al*., 2011). In addition, ketamine (KET), a PCP analog, has been found to have an antidepressant effect within a few hours of administration that can last up to a week. Most importantly, KET rapidly antagonizes suicidal ideation and is effective in treatment-resistant depression and treatment-resistant bipolar depression (Jelen *et al*., 2021). The recent discovery of the antidepressant effects of KET has generated a great deal of excitement in the depression research community. The US FDA recently approved the S-enantiomer of KET, esketamine, as a drug for MDD. However, the use of KET for the treatment of MDD has raised concerns about its psychomimetic and neuropsychological side effects, as well as the risk of abuse (Polis *et al*., 2019; Ballard and Zarate, 2020). Due to these concerns, further efforts are being made to develop new agents that target the glutamatergic system, have tolerable side effects, and have a lower potential for abuse. Other PCP analogs with a similar structure to KET and that are NMDA receptor antagonists have been developed, including 4-F-PCP, a novel PCP analog. The present study investigated the antidepressant-like effects of 4-F-PCP after acute and repeated treatments in a mouse model of chronic social defeat stress (CSDS). The behavioral and molecular effects of the drug were explored to further understand the mechanisms underlying its antidepressant effects.

MATERIALS AND METHODS

Animals

Male mice were purchased from Hanlim Animal Laboratory Co. (Hwasung, Korea) and Raon Bio Co. (Yongin, Korea). They were housed in 5 per small and 10 per large cages and kept in a temperature-(22 ± 2°C) and humidity-controlled (55 ± 5%) vivarium on a 12-h light/dark (7:00–19:00 light) schedule. Food and water were provided ad libitum. Before the start of the experiments, the animals were acclimated to the laboratory for five days. Different groups of mice were used for each behavioral experiment. Retired ICR mice (12 weeks old) and C57BL6/N mice were used for the chronic social defeat stress experiments with ICR being the aggressive mice and the C57BL6/N the subject mice. The remaining experiments utilized C57BL6/N mice (7-8 weeks old). Animal studies reported abided with the ARRIVE guidelines (Kilkenny *et al*., 2010). All experimental procedures, maintenance, and animal care were carried out under the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985) and approved by the Animal Care and Use Guidelines of Sahmyook University, Korea (SYUIACUC2020-021).

Drugs

4′-F-PCP hydrochloride, 4-MeO-PCP, 4-Keto-PCP, and 3-MeO-PCP were generously provided and synthesized by the Medicinal Chemistry Laboratory at Kyunghee University, Seoul, Korea as described in previous papers (Abiero *et al*., 2020; Ortiz *et al*., 2021). KET was purchased from BVSPharm Co., Hanam, Korea. All drugs were diluted with saline (0.9% w/v NaCl) and administered intraperitoneally (i.p.).

Chronic social defeat stress (CSDS)

Before social defeat stress, retired ICR male mice were tested for aggressive behavior (aggressor) using older C57BL6/N mice (>12 weeks old) as described by Golden *et al*. (2011). Using different aggressors daily, the subject (8 weeks old C57BL6/N) was then introduced once into the cage of the aggressive mouse (ICR) for 10 minutes. Subsequently, the subject was left overnight on the other side of the resident cage with a transparent divider to separate them.

Forced swimming test (FST)

This test followed the method described by Zanos *et al*. (2016) with few modifications. Mice were subjected to a 6-min swim session in clear Plexiglas cylinders (30 cm height×20 cm diameter) filled with 15 cm of tap water (23 ± 1°C) under normal light conditions. Each trial was recorded using a digital video camera. Two trained observers (blinded to the grouping) determined immobility time during the last 4 min of the 6-min test. The mice were considered immobile when they floated passively with no additional activity other than that necessary to keep their head above water (Botanas *et al*., 2021). To assess antidepressant-like effects, mice were treated with 4-MeO-PCP, 4-Keto-PCP, 3-MeO-PCP, 4-F-PCP, and KET (1, 3, and 10 mg/kg) or saline and tested after 1 h, or followed by repeated treatments for 3 and 7 days.

Tail-suspension test (TST)

This procedure was performed as described previously (Koike *et al*., 2011; Sayson *et al*., 2019) with modifications. Mice were suspended by the tail from a metal rod (45 cm above the table) using adhesive tape and positioned at least 20 cm away from the nearest object. The test sessions were recorded for 6 min, and the immobility time was measured by two trained observers (blinded to the grouping). Mice were considered immobile when they hung passively and motionless. As in the FST, we administered mice with 4-MeO-PCP, 4-Keto-PCP, 3-MeO-PCP, 4-F-PCP, and KET (1, 3, and 10 mg/kg) or saline and tested after 1 h, or after repeated treatments for 3 and 7 days later to assess the antidepressant-like effects.

Locomotor activity

Mouse locomotor activity was assessed in a square black Plexiglas container with an open field measuring 42 cm×42 cm×42 cm. Then, 30 min after 4-F-PCP (1, 3, and 10 mg/kg), KET (1, 3, and 10 mg/kg), or saline treatment, the mice were

placed in the open field arena. The distance moved and movement duration was recorded using a computer system (Ethovision system; Noldus I.T., Gelderland, The Netherlands) during the last 30 min of a 32-min test.

Three-chamber social test (3-CST)

This procedure was performed as described by Kim *et al*., (2019) with few modifications. Instruments include three connected rectangular chambers (each compartment of 230 mm ×400 mm ×220 mm). Two openings measuring 9 cm provided access to each of the compartments. The central compartment was the starting point of each test. Wire cages with a 10 cm diameter were placed in the middle of the compartment. The subject mouse was allowed to roam around the three chambers for 5 min during the habituation period. The sociability test begins by placing the stranger (stimulus) mouse into one of the two wire cages while the other wire cage was left empty. The subjects were then allowed to explore the three chambers for 10 min. The experiment operationalized sociability by measuring the time spent by the mouse between the empty and stranger wire cage compartments. After every trial, chambers and wire cages were cleaned with 70% ethanol. The Ethovision software (Ethovision system; Noldus I.T.) was linked via an overhead camera, and the movement of each subject in each compartment was tracked. The duration of stay in each compartment and the duration of stay in the approach area (5 cm around the wire cage) were measured automatically using Ethovision.

Y-maze

After acclimatization, the mice were tested in the Y-maze task following the methods described in previous studies (Custodio *et al*., 2021). Each arm of the Y-maze was 45 cm long, 10 cm wide, and 20 cm high, with both arms positioned at equal angles. Mice were placed individually at the end of an arm and allowed to enter the maze freely for a 10-min test session. An arm entry was defined as the entry of all four paws and the tail into one arm. Arm entries were recorded using an automated system (Ethovision system; Noldus I.T.). The alternation behavior (actual alternations) was defined as consecutive entry into three arms: the combination of three different arms with stepwise combinations in the sequence. Thus, the maximum number of alternations was the total number of arms entered –2, and the percentage of alternation behavior was calculated as (actual alternations/maximum alternations)×100. Actual alternations are the number of consecutive entries into three different arms (A, B, and C), such as ABC, ACB, BAC, BCA, CAB, or CBA, during the test session. The results are presented as the mean ± SEM. Data were analyzed using the D'Agostino and Pearson omnibus normality test, followed by a one-way analysis of variance (ANOVA) with Dunnett's test for post hoc comparisons. The normality test showed no significant deviation from a normal distribution. Pairwise comparisons were performed using unpaired t-tests. Statistical significance was set at $p<0.05$. Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA).

Quantitative real-time polymerase chain reaction

The prefrontal cortex (PFC) and hippocampus (Hipp) of the mouse brain (n=6) were isolated after 7 days of treatment with 4-F-PCP, KET, and SAL. Total RNA was obtained using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). A Hybrid-RTM Kit (GeneAll Biotechnology Co., LTD, Seoul, Korea) was used for further RNA purification. The total RNA concentration was determined with a Colibri Microvolume Spectrometer (Titertek-Berthold, Pforzheim, Germany). The qRT-PCR was utilized to measure the mRNA expression levels of Excitatory amino acid transporters (e.g. EAAT2, EAAT3, EAAT4) and the mTOR. One microgram (ug) of total RNA was reversely transcribed into cDNA using AccuPower CycleScript RT Premix (Bioneer, Seoul, Korea). The cDNA amplification was performed with custom-made sequence-specific primers (Cosmogenetech, Seoul, Korea). The sequences of the primers are provided in the supplementary information (Supplementary Data 2). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Tissue collection and western blotting

Mice (n=6/group) injected with 4-F-PCP, KET, or SAL were sacrificed by cervical dislocation and decapitation 7 days after administration for western blotting. The brains were rapidly and cautiously removed and placed in ice-cold saline to prevent brain injury. The hippocampus (Hipp) and pre-frontal cortex (PFC) were dissected from the appropriate slices and immediately frozen at –70°C until further use. Tissue samples were lysed with 300-μL homogenization buffer (RIPA buffer [Biosesang Inc., Seongnam, Korea], complete™ ULTRA protease inhibitor cocktail tablets [5,892,791,001; Sigma-Aldrich, Burlington, MA, USA], and PhosSTOP™ phosphatase inhibitor cocktail tablets [04906845001, Sigma-Aldrich]). The tissue extracts were centrifuged at 16,000 rpm at 4°C for 20 min. The samples were then heated to 95°C for 5 min. Twenty micrograms of protein lysate of each sample were loaded onto 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) gels, separated, and transferred to nitrocellulose membranes. The blots were blocked with 5% BSA in Tris-buffered saline in 0.1% Tween-20 (TBST) solution for 1 h and then incubated overnight at 4°C with specific primary antibodies (brain-derived neurotrophic factor (BDNF) rabbit polyclonal antibody [Abcam, Cambridge, UK; ab108319], phospho- mammalian target of rapamycin (mTOR) [Cell signaling, Beverly, MA, USA; #5536], mTOR antibody [Cell signaling; #2972], phosphorylated CREB [p-CREB] [Ser133] monoclonal antibody [Cell signaling; #9198], CREB monoclonal antibody [Cell signaling; #9197], Phospho-ERK1/ERK2 (Thr185, Tyr187) polyclonal antibody [Thermofisher, Waltham, MA, USA; #44-680G], ERK1/2 Monoclonal Antibody [Thermofisher; #14-9108-82], Phospho-TrkB (Tyr 705) Polyclonal antibody [Thermofisher; #PA5-38077; 1:1000], Anti-TrkB antibody [Abcam, ab18987; 1:1000], and beta-actin mouse monoclonal antibody [A5441; Bio-Rad laboratories, CA, USA]). The following day, the blots were washed three times in TBST and incubated with horseradish peroxidase-conjugated anti-rabbit (1:3000) or anti-mouse secondary antibodies (1:5000) for 1 h. After three final washes with TBST, the blots were visualized using enhanced chemiluminescence (Clarity Western ECL; Bio-Rad laboratories) and ChemicDoc Imaging System (Image Lab software, version 6.0; Bio-Rad). The values for phosphorylation-independent levels of proteins were normalized to those of β-actin. Levels of the phosphorylated forms of proteins were normalized to phosphorylation-dependent levels of the same proteins. The fold change of proteins was calculated by normalizing the values to those of the phosphorylated or saline group.

Immunofluorescence

The chronic social defeat-stressed mice administered with SAL, 4-F-PCP, and KET were sacrificed immediately after the last experiment was performed. Standard protocols were used for brain-tissue fixation. Mice were anesthetized by injecting 0.02 mL of Zoletil® (50 mg·mL-1) and Rompun® (xylazine 23.32 mg·mL-1). Perfusion reagents, protocols, and brain sectioning were specifically used and performed as described previously (Custodio *et al*., 2021). The brain slices (40 μm) were then placed in a storage solution and maintained at 4°C. The PFC and Hipp were the brain areas considered in this experiment, as these brain regions are consistently affected across studies of depression. The primary antibodies used were BDNF (1:250) rabbit polyclonal antibody [Abcam; ab108319] and mTOR antibody with a dilution of 1:200 [Cell signaling; #2972] and goat anti-rabbit Alexa fluor-488 (Thermofisher; #A-11008) for the secondary antibody. Cells immunoreactive to the target antibodies were observed under a Leica TCS-SP8 confocal microscope with a zoom factor of 2.75x oil immersion objective (Leica Microsystems, Wetzlar, Germany).

Receptor binding assay

HEK-293 cells were transfected with the expression constructs containing the NMDA receptor's NR1 and NR2B subunits, which were purchased from Addgene (Cambridge, MA, USA). After 24 h, the cells were split into a 24-well plate. On the next day, the cells were incubated with 10 nM [3 H]-TCP (40 Ci/mmol), purchased from PerkinElmer (Waltham, MA, USA), for 1 h at room temperature. Cells were washed three times with ice-cold serum-free media, dissolved with 1% SDS, mixed with a liquid scintillation cocktail, and counted with a Wallac 1450 MicroBeta® TriLux liquid scintillation counter (PerkinElmer). Binding of the remaining [3 H]-TCP incubated with 10 μM MK-801 was defined as non-specific. The IC50 values were determined with GraphPad Prism version 7 (GraphPad Software), using non-linear regression with log concentration plotted against the percent-specific binding. Ki values were calculated. The Kd value for [3 H]-TCP, 54.3 nM, was based on previous studies (Mitrovic *et al*., 1991).

Data analysis

All values are presented as mean ± standard error of the mean (SEM). Sample sizes were based on our prior experience using the same paradigms. Mice were randomly assigned to each treatment group. The experiments and data analysis were performed in a manner completely blind to experimental groups. To determine the anti-depressant effects of 4-F-PCP and KET in the FST, TST, western blotting, immunofluorescence, and qRT-PCR, one-way analysis of variance (ANOVA) were employed, followed by Dunnet's post-hoc comparison. Data from the open field test, novel object recognition test, y-maze test, 3-chamber social test, and elevated plus maze test were analyzed also using One-way ANOVA. Statistical significance was set at $p<0.05$. All statistical analyses were performed using GraphPad Prism v. 8.01 (GraphPad Software, Inc., La Jolla, CA, USA) (RRID: SCR_002798). The statistical analyses (sample sizes, main or treatment effects, interactions, and *p* values) for each experiment are provided in the manuscript results. All immunofluorescence images were processed using Adobe Photoshop CS6 version 13.0×64 (Adobe Inc., San Jose, CA, USA).

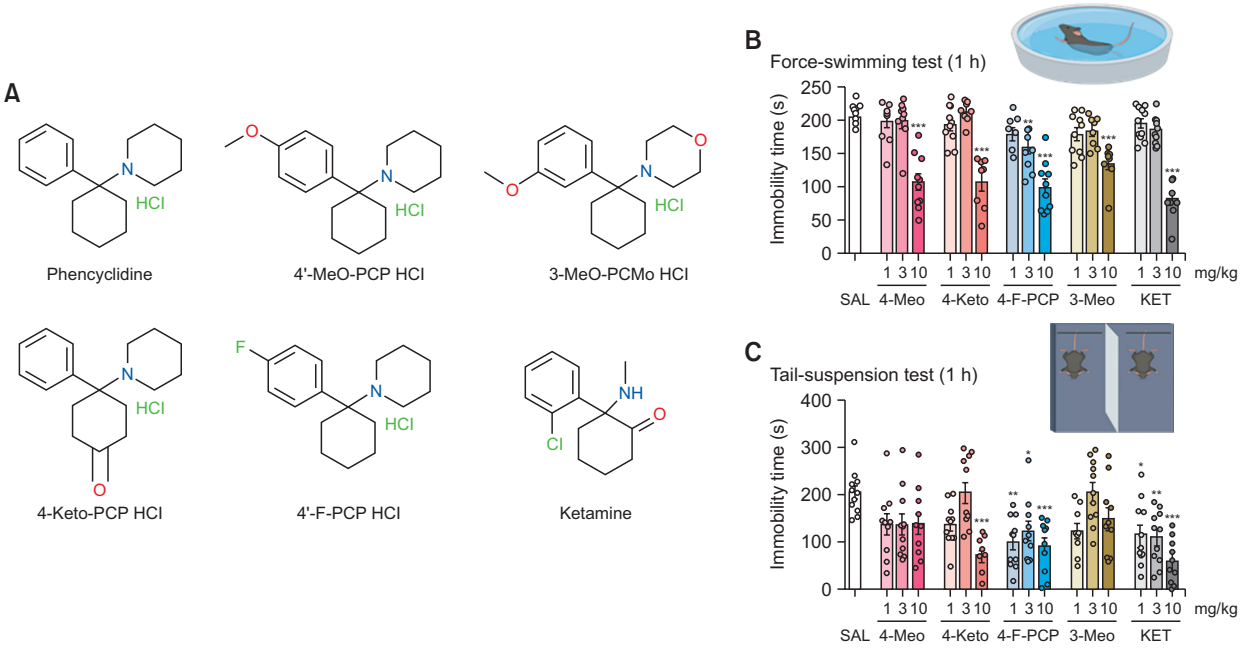

Fig. 1. Evaluation of PCP analogs. (A) Chemical structures of PCP and its analogs. Phencyclidine consists of an aromatic ring, cyclohexane ring, and basic amine function. The chemical structures of the PCP analogs showed modifications from the structure of PCP by substitution and addition of other functional groups. (B-C) Evaluation of the PCP analogs in the forced-swimming test and tail suspension test. Values are mean ± SEM. n=10 animals per group. *$p<0.05$, **$p<0.01$, and ***$p<0.001$, significantly different from the SAL group (Dunnett's posttest).

RESULTS

Antidepressant-like effects of PCP analogs

To determine whether PCP analogs such as 3-Meo, 4-Meo, 4-Keto, and 4-F-PCP produce antidepressant-like properties (Fig. 1A), we performed the FST and TST tests 1 h after injection in mice. Parallel experiments were conducted using KET. The 10 mg/kg dose of both the analogs and KET significantly reduced the immobility time of mice in the FST ($F(15, 143)=22.1$, $p<0.001$) and TST ($F(15, 141)=5.75$, $p<0.001$) Only 4-F-PCP decreased the immobility time in FST at 3 mg/kg (Fig. 1B) and both 4-F-PCP and KET showed a significant decline in the immobility of mice in TST at 3 and 10 mg/kg (Fig. 1C). Post hoc analysis showed significant differences between the PCP analogs and SAL.

Validation of the depressive-like mice using chronic social defeat stress

Chronic social defeat stress (CSDS) was introduced to C57BL6/N mice before the start of the experiments to induce depressive-like behavior for 10 consecutive days (Fig. 2A). On the 11th day, the subjects were evaluated for depressive-like behaviors through the FST, TST, sociability test, and locomotor activity (Fig. 2B, 2C). The FST showed a significant increase in the immobility time of CSDS mice ($t=4.37$; $p<0.001$). Similarly, CSDS mice exhibited a significantly higher immobility time in the TST compared to normal mice ($t=4.74$; $p<0.001$). There was also a significant decrease in locomotor activity and movement duration of the mice ($t=2.93$; $p<0.05$) ($t=3.94$; $p<0.05$). In the sociability test, stressed mice spent significantly more time near the empty cage than in a cage with a stranger mouse [$F(1, 21)=1.55$] (Fig. 2D). These results suggest that CSDS mice exhibited depressive-like behavior, as evidenced by the

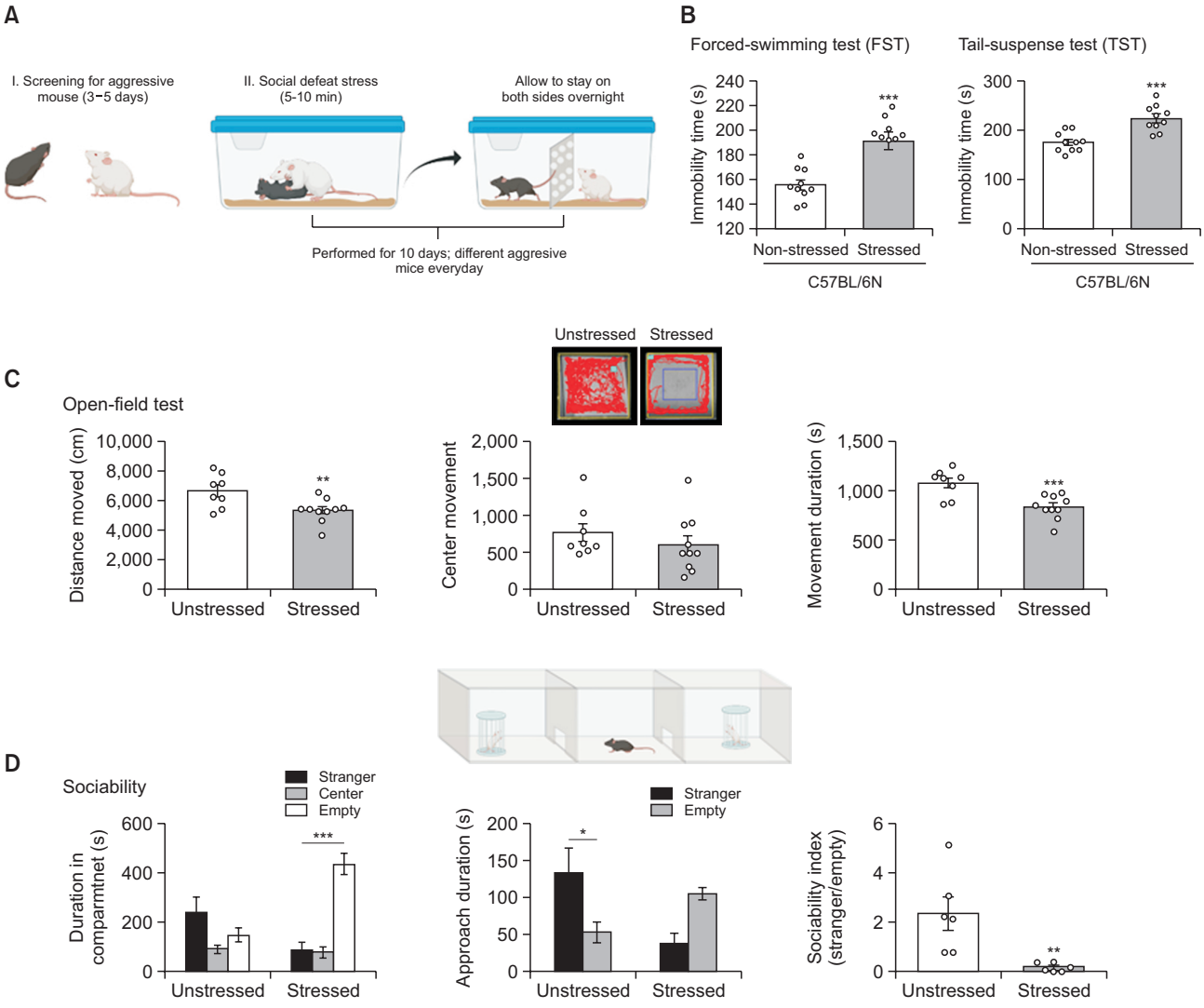

Fig. 2. Validation of the depressive-like behavior of chronic social defeat stressed (CSDS) mice. (A) Before the start of all behavioral experiments, subject mice undergo CSDS for 10 days. Series of experiments such as (B) tail suspension test and forced swimming test, (C) open-field test to evaluate the locomotor activity, and three-chamber test (3CST) to assess the sociability of the subjects (D). Values are mean ± SEM. n=8-10 animals per group *$p<0.05$, **$p<0.01$, and ***$p<0.001$ significantly different from the control (unstressed) (Dunnett's posttest).

reduced immobility time in the FST and TST and a decrease in their locomotor activity as well as their social behavior.

Acute antidepressant-like effects of 4-F-PCP

Out of all the PCP analogs, 4-F-PCP showed a more promising antidepressant-like effect as it showed decreased immobility time in TST and FST at both 3 and 10 mg/kg doses. Thus, using CSDS mice, we evaluated further the antidepressant-like effects of 4-F-PCP in a dose-dependent manner not only with FST and TST but also with the open-field test. Acute treatment with 4-F-PCP showed a significant decrease in the immobility time in FST [F (7, 77)=31.5], p<0.001) at 3 and 10mg/kg doses with KET at only 10 mg/kg (Fig. 3A). Similarly, 4-F-PCP- and KET- (3,10 mg/kg) treated mice exhibited significantly lower immobility time in TST [F (7, 73)=5.2], p<0.001) than SAL-treated mice (Fig. 3B). In the open-field test, only 4-F-PCP produced a significant increase in the locomotor activity of mice at 10mg/kg [F (5, 29)=7.36], p<0.01) (Fig. 3C).

Repeated treatment of 4-F-PCP maintains its antidepressant-like effects

To determine whether 4-F-PCP does not produce tolerance after repeated treatment, we assess its antidepressant-like effects following repeated treatment for 3 and 7 days at a 3 mg/kg dose. As presented in Fig. 4A and 4B, treatments with 4-F-PCP and KET showed a decreased immobility time in FST ([F (6, 75)=1.25], p<0.001) and TST ([F (3, 31)=5.16], p<0.001) after 1 and 3 days of treatment, and only 4-F-PCP on the 7th day of treatment. Furthermore, there is a significant increase in the locomotor activity of CSDS mice after 3 ([F (3, 30)=10.3], p<0.001 and 7 days [F (3, 31)=5.16], p<0.001) of repeated treatment with 4-F-PCP but not with KET (Fig. 4C).

4-F-PCP is an NMDA antagonist that is possibly involved in the inhibition of the glutamatergic system

To determine whether 4-F-PCP is indeed an NMDA receptor antagonist, we conducted a radioligand binding assay to check the inhibitory activity of 4-F-PCP on the NMDA receptor and calculated the IC50 of the compound. Fig. 5A reveals the percent (%) of NMDA receptor binding of 4-F-PCP and KET with 4-F-PCP reaching an IC50 of 0.023 μM. This evidence is proof of the inhibitory activity of the 4-F-PCP in the NMDA receptors. Furthermore, inhibition of the NMDA receptor can lead to changes in glutamate levels. Therefore, to check for the possible involvement of the glutamatergic system, we investigate the expression of the glutamate transporter genes such as EAAT2, EAAT3, and EAAT4 in both the PFC and Hipp, brain regions that are most likely involved in MDD (Fig. 5B). Decreased expression of the EAAT3 is significantly seen in the Hipp ([F (3, 16)=18.3], p<0.001). Similarly, decreased expression on the EAAT4 was observed ([F (3, 15)=4.84], p<0.015) after treatment with 4-F-PCP and KET. Although not significant, 4-F-PCP and KET treatment reduced EAAT3 expression in the PFC of mice compared with mice treated with saline; however, a decreasing trend in EAAT4 was only observed with 4-F-PCP treatment.

4-F-PCP rescues the reduced expression of proteins implicated in synaptic plasticity and major depressive disorder.

Increased BDNF protein is suggested to contribute to the antidepressant-like effects of KET while mTOR is also a marker of synaptic plasticity that can also lead to antidepressant-like effects. Moreover, studies show that both BDNF and mTOR play a major role in the pathogenesis and pharmacotherapy of depression (Fig. 6A). With this, we measured the protein levels of

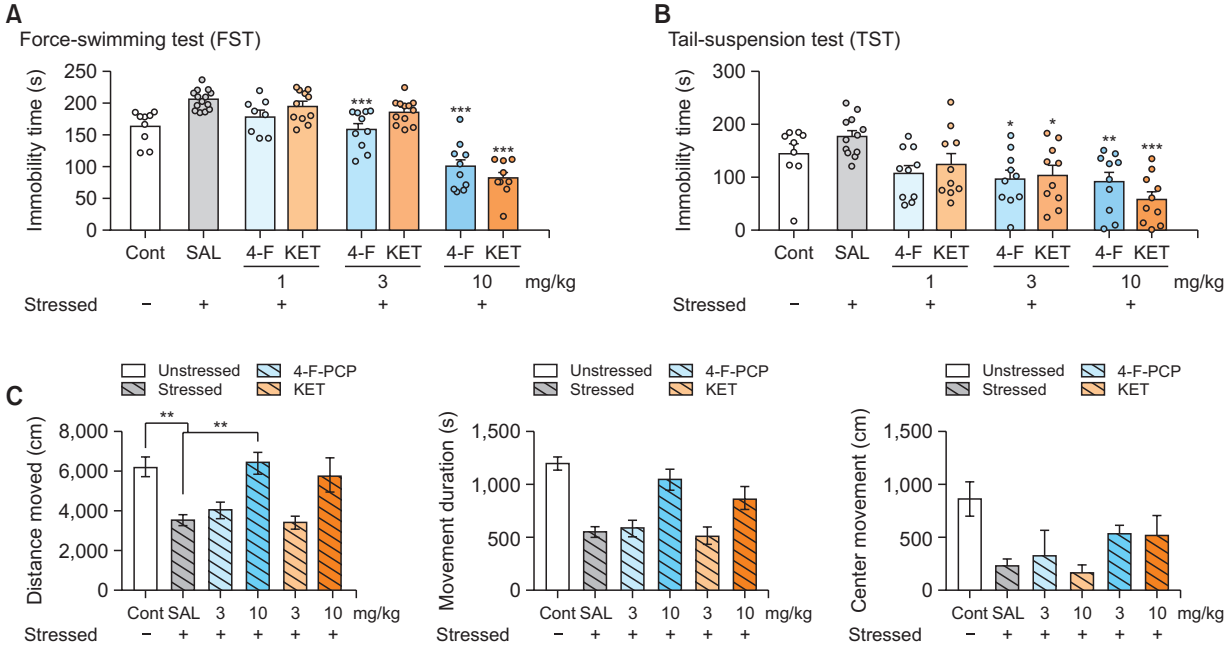

Fig. 3. Rapid effects of 4-F-PCP and ketamine (KET) after acute treatment. Forced-swimming test (A) the tail-suspension test (B) and (C) the open-field test showed a significant increase in the mobility and locomotor activity of CSDS mice an hour after treatment. Values are mean ± SEM. n=8-10 animals per group *p<0.05, **p<0.01, and ***p<0.001 significantly different from the saline (SAL) (Bonferroni's posttest).

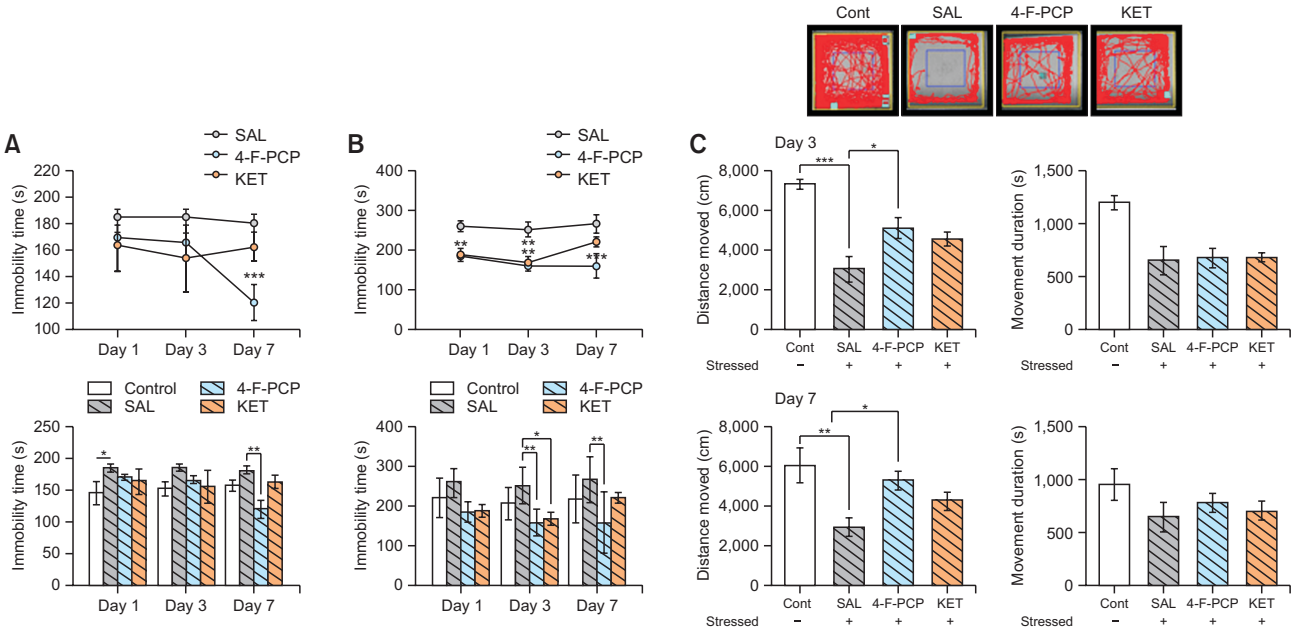

Fig. 4. Effects of 4-F-PCP and ketamine after repeated treatment in the forced swimming test (FST) (A), tail suspension test (TST) (B), and open-field test (OFT) (C) after 1, 3, and 7 days of repeated treatment. Values are mean ± SEM. n=10-11 animals per group. *p<0.05, **p<0.01, and ***p<0.001 significantly different from the saline (SAL) (Bonferroni's posttest).

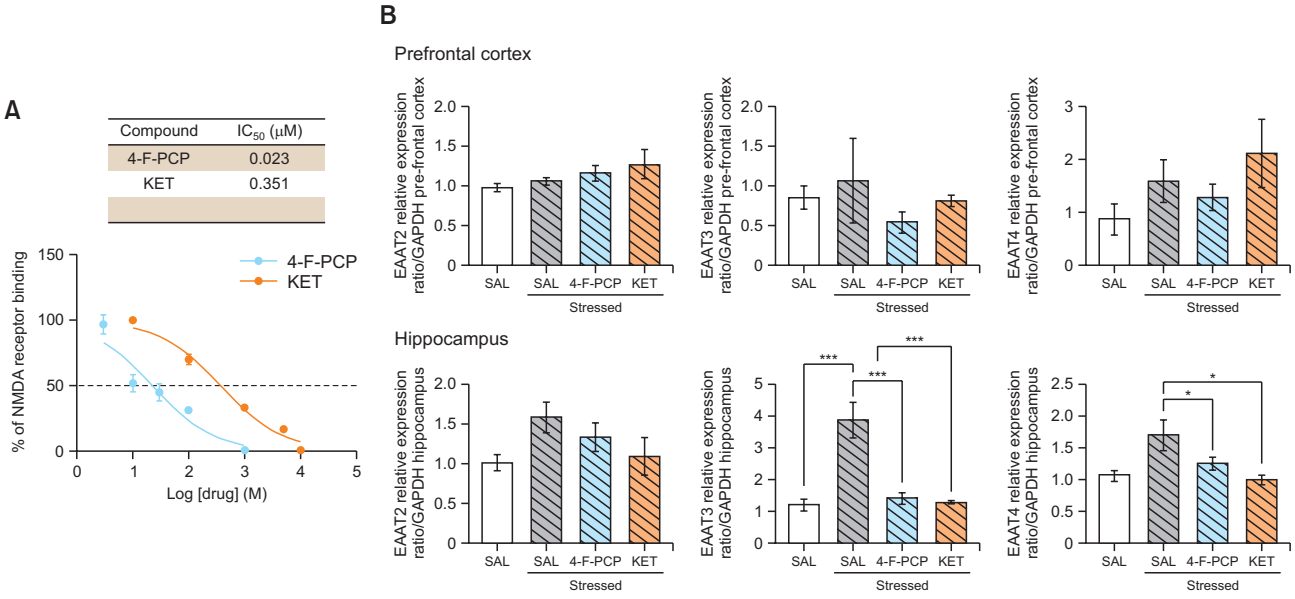

Fig. 5. (A) Competition binding affinity of [3H]-TCP with 4-F-PCP and KET (10 nM, 100 nM, and 1 μM final concentration) for NMDA receptor. 4-F-PCP showed a strong affinity to NMDA receptors at IC50 with 0.023 μM. (B) Effects of 4-F-PCP and KET on the excitatory amino acid transporters (EAAT2, EAAT3, EAAT4) gene expression in the hippocampus and prefrontal cortex. Values are mean ± SEM. n=6 animals per group. *p<0.05 and ***p<0.001 significantly different from saline (SAL-stressed) (Bonferroni's posttest).

BDNF and mTOR in the PFC and HIPP. A significant decrease in the expression of BDNF was observed in the PFC (t=2.7 df=6, p<0.094) and Hipp (t=2.27 df=4, p<0.043) of CSDS mice (Fig. 6B). Downstream signaling proteins of BDNF including p-TrkB, p-ERK, p-CREB, and p-mTOR showed decreased expressions as well. Interestingly, repeated treatments with 4-F-PCP and KET (3 mg/kg) for 7 days significantly enhanced the initial reduced expression of BDNF ([F (3, 23)=4.57], p<0.05) in the PFC (Fig. 6C). A significant increase in p-ERK levels is also observed following 4-F-PCP and KET treatment ([F (3, 16)=4.34], p<0.020). No significant difference was observed in p-TrkB levels, but an increasing trend was observed following treatment with 4-F-PCP and KET. There was also a decline in p-mTOR expression in CSDS mice, followed by

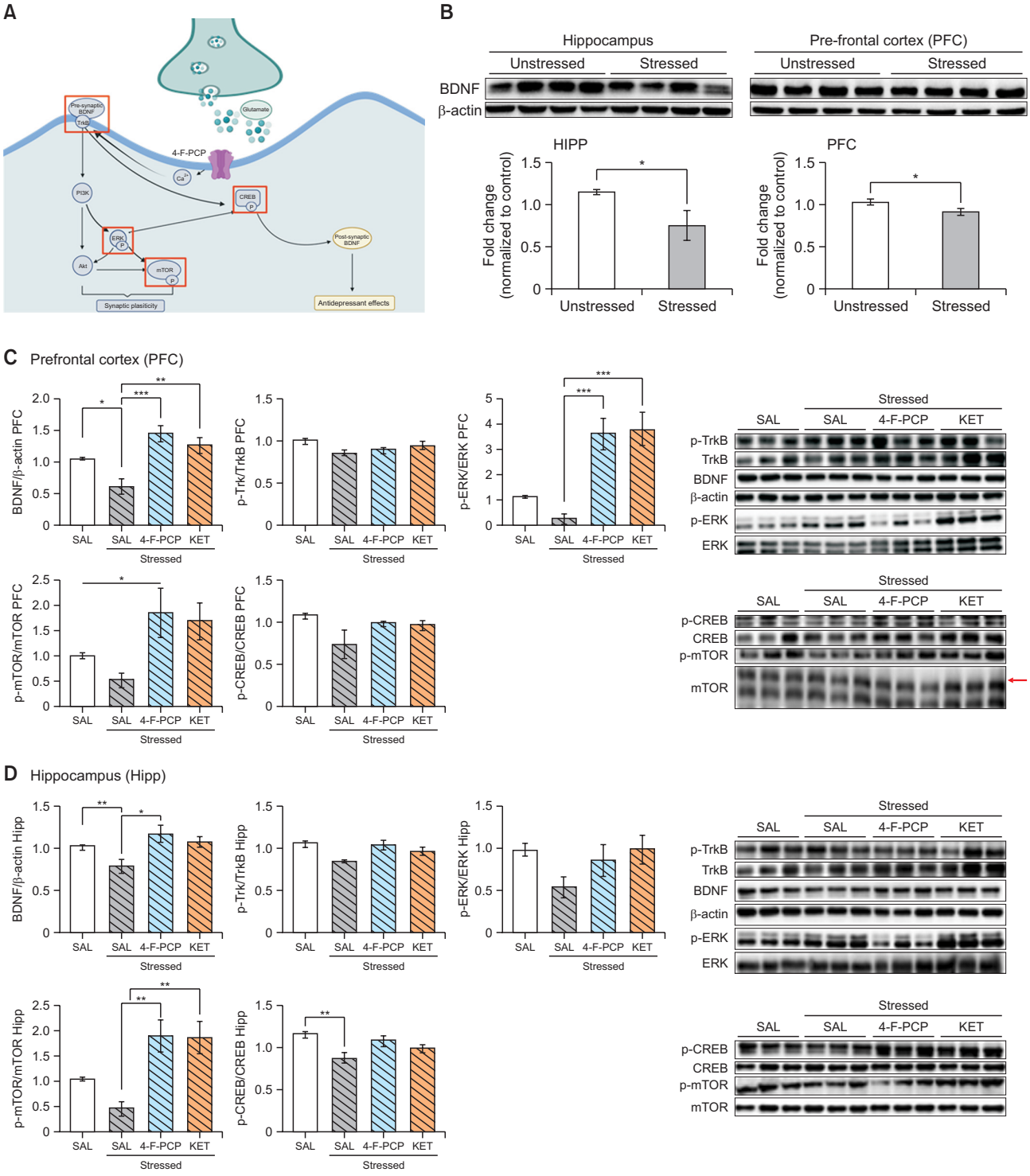

Fig. 6. (A) Possible mechanism of Effects of 4-F-PCP and ketamine on proteins implicated in depression and synaptic plasticity. (B) Decreased expression of BDNF protein is seen in depressed mice in both PFC and HIPP. (C, D) Effects of 4-F-PCP (KET) (3 mg/kg) treatment on BDNF, p-TrkB/TrkB, p-ERK/ERK, p-mTOR/mTOR, p-CREB/CREB protein levels in hippocampus and prefrontal cortex, respectively. Specific brain regions were isolated right after the last experiment was performed (TST, FST, OFT). Brain lysates were resolved and analyzed using the western blot analysis. Each bar represents the mean ± SEM. n=6 animals per group. *p<0.05, **p<0.01, and ***p<0.001 were significantly different from the stressed group (one-way ANOVA with Dunnett's test).

a significant increase in p-mTOR expression between treatments [F (3, 14)=5.59, $p<0.05$]. The p-CREB protein showed no significant difference between treatments, but a reduced expression was seen between normal and stressed mice [F (3, 8)=3.57], $p<0.05$]. Similarly, one-way ANOVA showed a significant difference in BDNF between treatments ([F (3, 20)=3.91, $p<0.05$]) in the Hipp (Fig. 6D). Post-test revealed augmented BDNF expression in mice treated with 4-F-PCP and KET.

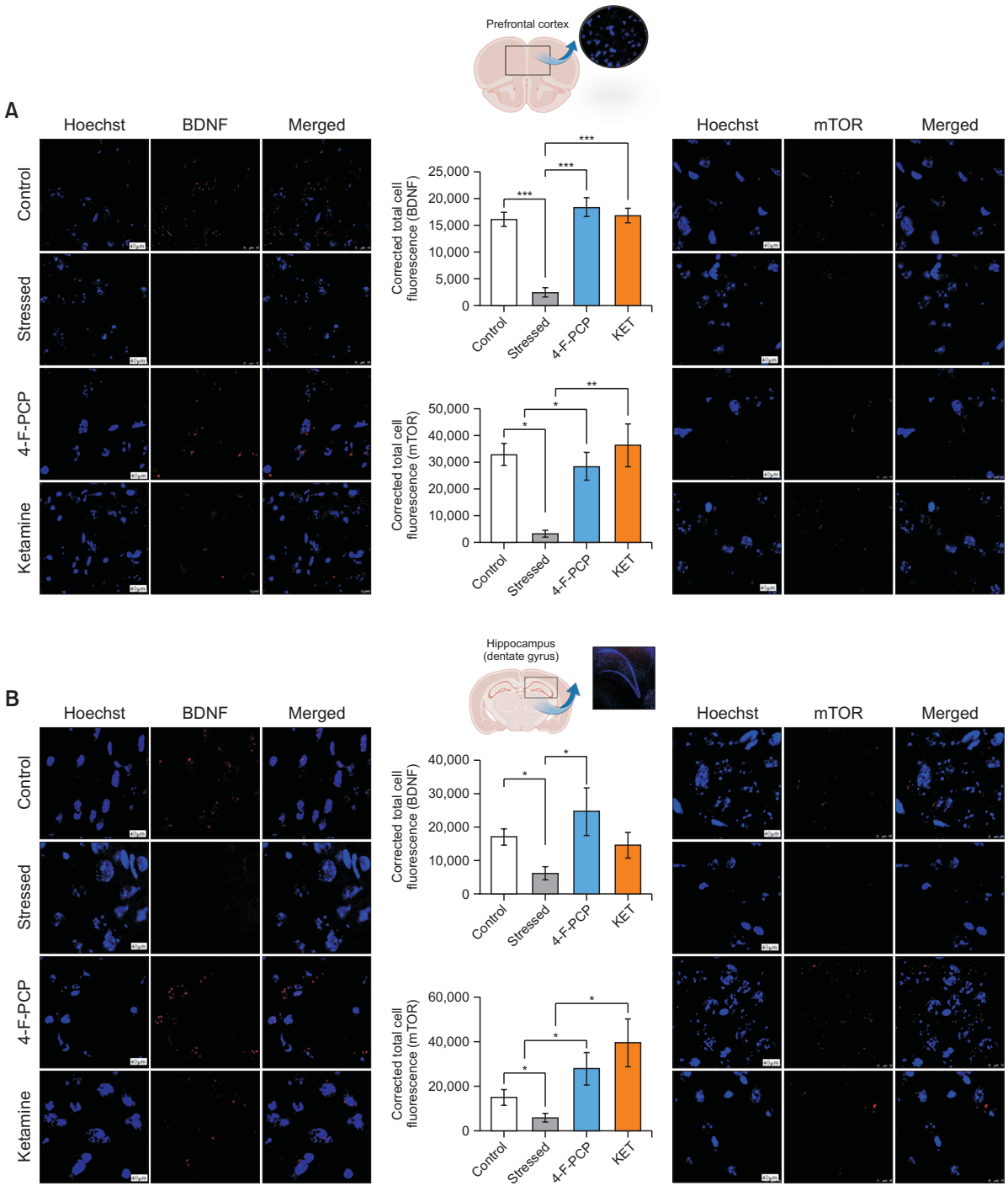

Fig. 7. Immunofluorescence assay for the detection of BDNF and mTOR proteins. (A) Visual representation of the presence of the proteins in the sample groups in both the prefrontal cortex (B) and hippocampus brain tissues (40 μm, zoom factor: 2.75x oil immersion objective). Quantitative analysis shows a decreased expression of BDNF and mTOR in stressed samples and ameliorating effects of 4-F-PCP and KET after treatment in both regions. Values are mean ± SEM. n=10-11 animals per group. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ significantly different from stressed group.

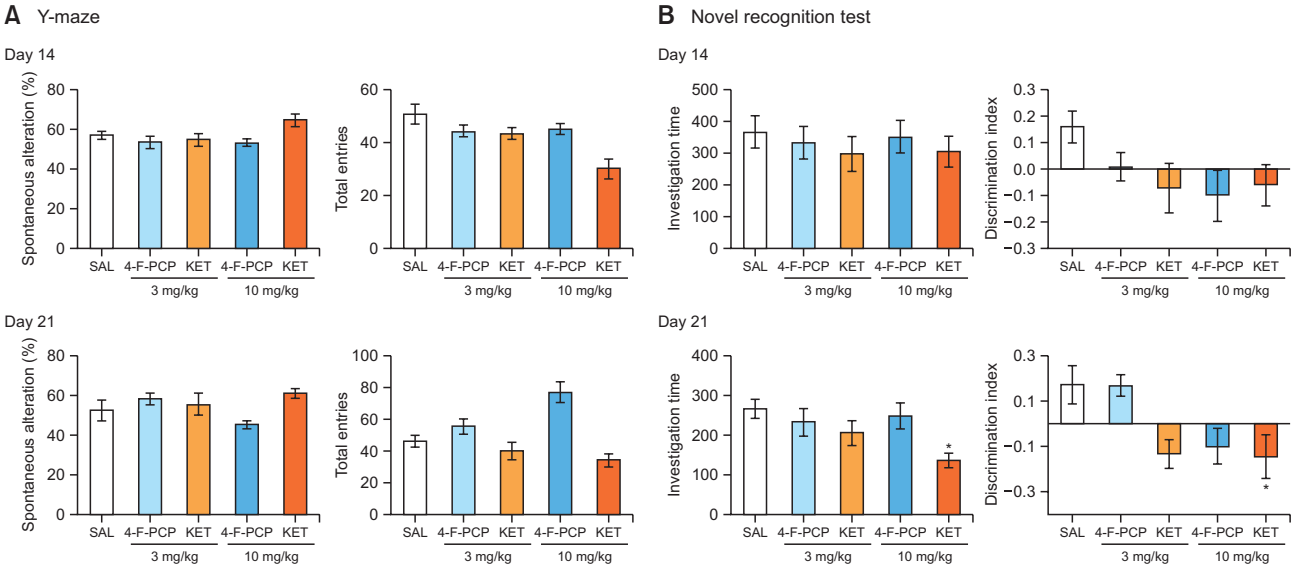

Fig. 8. Effects of 4-F-PCP and ketamine (KET) on the cognitive function of mice following long-term treatment (3mg/kg & 10 mg/kg) through y-maze (A) and novel object recognition test (B). Values are mean ± SEM. n= 6-10 animals per group. *p<0.05 significantly different from saline (SAL) (Bonferroni's posttest).

There was no significant difference between treatments in the expression of the p-ERK protein in Hipp, but an increasing trend was observed following treatments. A similar trend was observed for p-TrkB protein expression. Additionally, there is also a significant difference in the expression of p-CREB between normal and CSDS mice ([F (3, 16)=5.39], p<0.05). Further analysis showed decreased levels of p-CREB protein in stressed mice compared to those in normal mice. Furthermore, there was a significant difference between the treatment groups in the protein level of p-mTOR in Hipp ([F (3, 20)=3.91], p<0.024). Post hoc analysis revealed that 4-F-PCP and KET improved the decreased expression of p-mTOR in the Hipp of CSDS mice. To further evaluate the effects of 4-F-PCP and KET, we performed the immunofluorescence labeling of BDNF after 7 days of repeated treatments at 3 mg/kg (Fig. 7). Results showed a decreased expression of BDNF in CSDS mice in comparison to the control group. Treatments with 4-F-PCP and KET showed a rescuing effect as shown through its increased expression in both PFC (Fig. 7A) ([F (3, 8)=29.9], p<0.001) and Hipp (Fig. 7B) ([F (3, 12)=6.46], p<0.008) with 4-F-PCP showing a stronger BDNF signal than KET. Fluorescence-labeled expression of mTOR, a protein involved in synaptic plasticity, also showed a significant increase in groups treated with 4-F-PCP and KET in both PFC ([F (3, 8)=8.08], p<0.008) and Hipp ([F (3, 12)=8.53], p<0.003) These results further prove the possible mechanism of the antidepressant-like effects 4-F-PCP which is quite similar if not the same to that of KET.

Cognitive impairment after long-term treatment

Fig. 8 shows the effects of 4-F-PCP and KET after chronic treatment (14 and 21 days). One of the side effects of KET is a decline in memory function with long-term treatment. No significant decline in memory function was observed in the Y-maze test. Although no significant difference was seen after 14 days of repeated treatment, KET in 10 mg/kg showed a significant decrease in the investigation time ([F (4, 46)=3.08], p<0.025) after 21 days. A significant decrease in the discrimination index between KET and SAL groups is observed as well after 21 days of treatment ([F (4, 43)=3.54], p<0.014). The -1 discrimination index in KET represents a lower recognition index, translating to a failure to recognize between familiar and novel objects observed. In contrast, 4-F-PCP maintained its cognitive function even after 21 days of treatment. These results initially show that repeated treatment with 4-F-PCP might be possible even for a longer period.

DISCUSSION

To our knowledge, this is the first study to evaluate the antidepressant effects of 4-F-PCP. Of all the analogs, 4-F-PCP was chosen for further evaluation because of its more potent effects compared to 4-MeO, 3-MeO, and 4-Keto-PCP. CSDS mice showed decreased behavioral activity following exposure to stress, consistent with previous studies showing depressive-like behaviors in mice exposed to CSDS (Golden *et al*., 2011). Our current results show that 4-F-PCP exhibited rapid antidepressant effects similar to that of KET an hour post-treatment. Moreover, repeated treatment with 4-F-PCP (3 mg/kg) showed a more effective and consistent antidepressant-like effect over time. At the end of 7 days of treatment, 4-F-PCP was able to restore the depression-like behavior of CSDS mice while KET showed possible signs of tolerance after 7 days of repeated treatment. At 1 mg/kg dose, 4-F-PCP did not consistently produce a positive result, while a 10 mg/kg dose of 4-F-PCP showed alarmingly high locomotor activity (Supplementary Data 1). However, the 3 mg/kg dose showed a reliable antidepressant-like effect and a normal range of locomotor activity, similar to that of control mice (unstressed). Overall, our behavioral test results showed that 4-F-PCP does have antidepressant-like properties, with its efficacy increasing over time. NMDA receptors play a major role in the neurophysiology

of depression. Studies have shown that levels of glutamatergic metabolites are decreased in the medial frontal cortex of patients with depression and that dyshomeostasis of the glutamatergic system is associated with an imbalance between glutamatergic neurotransmission and synaptic plasticity in the clearance of mood disorders. However, the antagonism of NMDA could rapidly increase glutamate levels, leading to structural changes that might contribute to synaptogenesis and an increase in BDNF that eventually leads to the improvement of depressive symptoms. Further evidence points to the possibility that agents act on glutamate receptors, including NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and metabotropic glutamate (mGlu) receptors, to produce antidepressant-like effects that play a major role in depression management (Sanacora *et al*., 2008; Chowdhury *et al*., 2017). Our results showed that 4-F-PCP is an NMDA antagonist similar to KET. The rapid antidepressant-like effects of 4-F-PCP might be because of the inhibition of NMDA receptors. Moreover, the blockade caused by NMDA antagonists can also result in a glutamate surge and activation of downstream proteins involved in synaptic plasticity and depression. We assessed the involvement of glutamatergic circuitry by evaluating excitatory amino acid transporters (EAAT)s. Of the five subunits, we checked the three subunits linked to synaptic plasticity and depression. The role of EAATs is to control extracellular glutamate levels in response to the exocytosis of neurotransmitters due to neuronal activity. Research has shown that EAATs regulate NMDAR activation in the Hipp, and the activation of NMDAR and some AMPAR can lead to several forms of synaptic plasticity. EAAT3 specifically limits the activation of perisynaptic NMDARs that influence LTP and long-term depression (LTD). The inhibition of post-synaptic EAAT4 and EAAT3 significantly enhanced LTD. This resulted in decreased AMPAR expression and increased EAAT4 levels to compensate for the decreased number of receptors (Lin *et al*., 2012; Divito and Underhill, 2014). This is probably the reason for the increased level of EAAT4 in CSDS mice compared with that of normal mice. A decreasing trend of expression in EAAT3 and EAAT4 was observed post-treatment, indicating the possible alleviating effect of 4-F-PCP and KET in CSDS mice. Furthermore, this could also indicate a possible involvement of the glutamatergic system through the blockade of the NMDA receptors. As no study has ever been conducted regarding the mechanism of action of 4-F-PCP, it is most likely that 4-F-PCP does have a pathway similar to that of glutamatergic-based antidepressants, especially KET. We measured the proteins involved in the activation of AMPA receptors due to NMDA antagonism, which then modulates downstream signaling to mediate rapid antidepressant-like effects (Wang and Dwivedi, 2021). Studies have shown that an increase in the expression of AMPA results in the activation of AMPA receptors, which could further lead to synaptic plasticity through BDNF and mTOR production (Gong *et al*., 2006; Björkholm and Monteggia, 2016; Ignacio *et al*., 2016; Miranda *et al*., 2019). Patients with depression have been observed to show decreased BDNF levels (Duman and Aghajanian, 2012; Lang and Borgwardt, 2013). Similarly, CSDS mice showed decreased expression of BDNF protein as well. In addition, BDNF, ERK, mTOR, and CREB are proteins involved in synaptic plasticity as well as the growth and maturation of neuronal cells (Blendy, 2006; Gass and Riva, 2007; Pochwat *et al*., 2017). Our data showed that 4-F-PCP treatment enhanced the reduced expression of the target proteins, displaying the ability of 4-F-PCP to alleviate the molecular alterations associated with depression. Moreover, BDNF release in turn stimulates the expression of mTOR signaling and synaptic protein synthesis (Kishi *et al*., 2018; Botanas *et al*, 2021). One of the major drawbacks of therapeutic drugs is their potential to have abusive potential properties. KET has become a commonly abused drug since 2006 in different parts of the world, such as the US, Australia, China, and Hong Kong (Liu *et al*., 2016). Previous studies have demonstrated the addictive effects of KET in self-administration tests. Both drugs elicit reinforcing effects in the self-administration test; however, compared to KET, 4-F-PCP displays a much lower number of active presses and infusions. Moreover, this study also evaluated the involvement of the dopaminergic system in the rewarding effects of 4-F-PCP. Repeated treatment with a 10 mg/kg dose of 4-F-PCP and KET for 7 days resulted in the upregulation of dopamine receptor 1 followed by the downregulation of dopamine receptor 2 in the NAcc and ventral tegmental areas of the brain. Thus, repeated 4-F-PCP treatment with a high dose (10 mg/kg) may influence the dopaminergic system in the brain reward circuit, resulting in rewarding effects (Ortiz *et al*., 2021). Given this evidence, we cannot refute the fact that 4-F-PCP contains addictive properties however, based on our previous study, we could also conclude that 4-F-PCP is probably a better treatment option than KET in terms of having less abuse potential in a much lower dose. Further research is still needed to confirm the addictive properties of 4-F-PCP on a broader scale. Research has shown that long-term treatment with high doses of KET can lead to long-term impairments in cognition (Nikayin *et al*., 2022). However, other studies also support the notion that there are no changes in cognition; rather, KET has several other serious side effects if used for a long time (Murrough *et al*., 2014; Gass *et al*., 2019). Our data showed no impairment in the short-term memory with 4-F-PCP and KET treatment; however, KET showed an inverted result in the discrimination index of the NORT after 14 and 21 days of treatment, even at a low dose (3 mg/kg). In contrast, 4-F-PCP showed a potential of lesser ability to elicit memory impairment following chronic treatment. Overall, 4-F-PCP has the potential to elicit rapid antidepressant effects with consistent efficacy after repeated treatment. Moreover, 4-F-PCP showed less abuse than KET. In addition, the prolonged treatment showed a possibility of no adverse effects on cognitive impairment, making it a much better treatment option. However, 4-F-PCP should be further explored to assess other adverse effects. Nevertheless, current evidence provides valuable insights for future research on 4-F-PCP and other NMDA antagonists as well as for the development of a more effective and safe antidepressant.

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