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Psilocybin facilitates fear extinction in mice by promoting hippocampal neuroplasticity

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Abstract

Background: Posttraumatic stress disorder (PTSD) and depression are highly comorbid. Psilocybin exerts substantial therapeutic effects on depression by promoting neuroplasticity. Fear extinction is a key process in the mechanism of first-line exposure-based therapies for PTSD. We hypothesized that psilocybin would facilitate fear extinction by promoting hippocampal neuroplasticity.

Methods: First, we assessed the effects of psilocybin on percentage of freezing time in an auditory cued fear conditioning (FC) and fear extinction paradigm in mice. Psilocybin was administered 30 min before extinction training. Fear extinction testing was performed on the first day; fear extinction retrieval and fear renewal were tested on the sixth and seventh days, respectively. Furthermore, we verified the effect of psilocybin on hippocampal neuroplasticity using Golgi staining for the dendritic complexity and spine density, Western blotting for the protein levels of brain derived neurotrophic factor (BDNF) and mechanistic target of rapamycin (mTOR), and immunofluorescence staining for the numbers of doublecortin (DCX)- and bromodeoxyuridine (BrdU)-positive cells.

Results: A single dose of psilocybin (2.5 mg/kg, i.p.) reduced the increase in the percentage of freezing time induced by FC at 24 h, 6th day and 7th day after administration. In terms of structural neuroplasticity, psilocybin rescued the decrease in hippocampal dendritic complexity and spine density induced by FC; in terms of neuroplasticity related proteins, psilocybin rescued the decrease in the protein levels of hippocampal BDNF and mTOR induced by FC; in terms of neurogenesis, psilocybin rescued the decrease in the numbers of DCX- and BrdU-positive cells in the hippocampal dentate gyrus induced by FC.

Conclusions: A single dose of psilocybin facilitated rapid and sustained fear extinction; this effect might be partially mediated by the promotion of hippocampal neuroplasticity. This study indicates that psilocybin may be a useful adjunct to exposure-based therapies for PTSD and other mental disorders characterized by failure of fear extinction.

Keywords: Brain-derived neurotrophic factor; Bromodeoxyuridine; Depression; Doublecortin domain proteins; Extinction, psychological; Freezing; Hippocampus; Neuronal plasticity; Psilocybin; Stress disorders, post-traumatic

Introduction

Posttraumatic stress disorder (PTSD) is a chronic mental disorder triggered by a traumatic event.[1] Impaired hippocampal neuroplasticity is a notable feature of PTSD,[2,3] and patients with PTSD have a smaller hippocampal volume than healthy individuals.[4] Fear extinction is a process that involves learning and memory, which depend on neuroplasticity.[5,6] PTSD is characterized by failure of fear extinction, the process by which patients learn that a conditioned stimulus (CS) associated with fear memory no longer poses an acute threat.[7] This disease feature has motivated research on pharmacological agents to facilitate fear extinction.[8] Only two kinds of selective serotonin reuptake inhibitors (SSRIs), paroxetine and sertraline, have been approved for the treatment of PTSD by the US Food and Drug Administration (FDA).[9] Preclinical studies have indicated that paroxetine and sertraline facilitate fear extinction.[10,11] Unfortunately, these two SSRIs have limited efficacy and severe side effects.[12] Hence, new drugs that facilitate

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fear extinction with high efficiency and mild side effects are urgently needed.

Psilocybin is a natural serotonergic psychedelic drug that is the primary psychoactive compound in "magic mushrooms". Clinical studies have shown that psilocybin elicits rapid, substantial, and sustained alleviation of depression.[13,14] In 2019, the US FDA designated psilocybin-assisted psychotherapy a "breakthrough therapy" for the treatment of resistant major depressive disorder.[15] Multiple preclinical studies have shown that psilocybin can promote neuroplasticity both *in vivo* and *in vitro* by increasing neurogenesis, dendritogenesis, and synaptogenesis, which might contribute to its rapid antidepressant effects.[16-19] Although PTSD and major depressive disorder are different categories of mental disease, they are highly comorbid and have some genetic risk factors in common.[20] In 2013, a team from the University of South Florida showed that psilocybin facilitated fear extinction.[21] However, the neuroprotective mechanism of psilocybin in treating PTSD remains unclear.[22]

Given the abovementioned factors, it is not surprising that multiple studies have provided converging evidence of the therapeutic potential of psilocybin for PTSD.[22-25] Fear conditioning (FC) and fear extinction paradigms can be used to study the mechanisms of exposure therapy and the biological underpinnings of fear-related disorders, such as PTSD.[26] Given the existing evidence, we hypothesized that psilocybin would facilitate fear extinction in fear-conditioned mice, and the promotion of hippocampal neuroplasticity might be involved in the psilocybin-induced facilitation of fear extinction.

Methods

Animals and housing

Eight-week-old male C57BL/6J mice (Beijing SPF Biotechnology, Beijing, China) were individually housed in a room with a constant temperature (22°C ± 1°C), a relative humidity of 50–60%, and a 12–12 h light–dark cycle (lights on from 8:00 A.M. to 8:00 P.M.), with water and food freely available. Prior to FC, all mice were acclimated to the environment and handled for 7 days. All behavioral tests were performed between 9:00 A.M. and 5:00 P.M. Throughout the experiment, we made every effort to reduce animal suffering and the number of mice used. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Beijing Key Laboratory of Neuropsychopharmacology (No. IACUC-DWZX-2022-661). This study followed version 2.0 of the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. A hundred of mice were randomly divided into two groups, non-fear-conditioned group [FC(–) Veh, n = 20] and fear-conditioned group[FC(+), n = 80]. Then, after FC, 80 fear-conditioned mice were randomly divided into four groups, n = 20 for each group, including vehicle-treated fear-conditioned group [FC(+) Veh], and three psilocybin [0.1 mg/kg, 0.5 mg/kg, or 2.5 mg/kg]-treated fear-conditioned groups.

Drugs

Psilocybin was synthesized by Beijing Institute of Pharmacology and Toxicology; the drug was dissolved in sterile 0.9% saline solution and administered intraperitoneally (i.p.) in a volume of 0.1 mL/10 g of mouse body mass. Based on our pilot study and a study using psilocybin to treat depression,[27] we selected three different drug doses: 0.1 mg/kg, 0.5 mg/kg, and 2.5 mg/kg. One day before FC, four doses of bromodeoxyuridine (BrdU, 100 mg/kg per injection, 2.5-h interval, Sigma–Aldrich, St. Louis, USA, B9285) were injected i.p.[28]

Auditory cued FC and fear extinction paradigm

We subjected mice to an auditory cued FC regimen (d–2) consisting of five pairings of a tone (80 dB, 5000 Hz, 30 s) as the CS with a foot shock (0.8 mA, 2 s) as the unconditioned stimulus (US) in the FC context, context A, which consisted of a square chamber made of transparent plexiglas with a metal grid floor (Beijing Zhongshidichuang Science and Technology Development Co., Ltd., Beijing, China). The intertrial interval (ITI) from one CS–US pairing to the next was 120 s, and the US was terminated at the same time as the CS.

Vehicle (sterile 0.9% saline) or psilocybin (0.1 mg/kg, 0.5 mg/kg, or 2.5 mg/kg) was administered 30 min prior to fear extinction training, and fear extinction testing was performed on the first day. Both extinction training and extinction testing were performed in the fear extinction context, context B, a triangular chamber made of non-transparent black and white plexiglas with a flat, solid floor, which was distinct from context A. During both fear extinction training and fear extinction testing, mice received 12 CS presentations each day (ITI: 30 s) without foot shock. On the sixth day, fear extinction retrieval was tested with four CS presentations (ITI: 30 s) in context B. On the seventh day, fear renewal was tested with four CS presentations (ITI: 30 s) in context A. Freezing behavior was defined as the cessation of all movement except respiration and heartbeat; this behavior was used as a measure of the fear response. The variable of interest was the percentage of time spent freezing relative to the total duration of CS presentation. Freezing time was recorded and analyzed by a FC video analysis system (Beijing Zhongshidichuang Science and Technology Development Co., Ltd., Beijing, China).

Open field test (OFT)

To evaluate whether the therapeutic effect of psilocybin on percentage of freezing time was due to a change in locomotor activity, we assessed the total distance traveled by mice in the OFT 24 h after psilocybin administration. On the first day, we performed the OFT 2 h before fear extinction testing. In a slightly modified version of the procedure described in our previous research,[29] mice were placed in the center of a plastic box (40 cm × 40 cm × 40 cm) with a base divided into 16 equal sectors and allowed to move freely. First, the mice were allowed to acclimate for 2 min, and then the total distance traveled over the next 5 min was recorded

and analyzed by the Smart Video Tracing System (Smart version 3.0, Panlab, Barcelona, Spain).

Golgi–cox staining and dendrite analysis

Four mice per group were euthanized on the seventh day after administration of a single dose of psilocybin. Golgi staining was performed according to the protocol of the Rapid GolgiStain™ Kit (FD Neurotechnologies, Ellicott City, MD, USA). All steps were carried out in the dark. Fresh whole mouse brains were immersed in Golgi staining solution for 2 weeks at room temperature. Coronal sections (from 1.46 to 1.94 mm posterior to bregma) of the hippocampus (100 μm thick) were cut on a freezing microtome (CM1860 UV, Leica Microsystem Ltd., Wetzlar, Germany) and collected on gelatin-coated microscope slides. After being dried, these coronal sections were rehydrated, stained, and dehydrated. Finally, the coronal sections were cleared in xylene and covered with a resin-based mounting medium.

Hippocampal neurons were photographed with a white light microscope (Eclipse Ci-L, Nikon, Tokyo, Japan) oriented in the anteroposterior direction. The field of view was filled with tissue such that the background light was consistent in each image. We selected well-infiltrated cornu ammonis 1 (CA1), cornu ammonis 2 (CA2), and cornu ammonis 3 (CA3) pyramidal cells and dentate gyrus (DG) granule cells with clearly distinguishable and intact dendrites. Neuronal tracing and digital reconstruction were performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The total number of dendritic branches was analyzed at 200× power, a magnification at which the complexity of the dendrites is readily observable. Using the Sholl analysis method, 10 concentric circles 10 μm apart and centered around the cell body were drawn. Then, the number of intersections between dendrites and the concentric circles was calculated, and the total number of crossings was calculated. Dendritic spines were photographed at high power (1000× original magnification). The number of dendritic spines on the second or third dendritic branch of an intact neuron between 30 μm and 90 μm from the cell body was measured; additionally, the length of each dendritic branch was measured, and the number of dendritic spines along that length was counted. The number of spines was calculated along the length of dendritic branches using ImageJ software, and the spine density is expressed as the number of spines/10 μm. Four mice per experimental group were analyzed, and four representative images (CA1, CA2, CA3, and DG) were obtained from a total of four tissue sections per mouse; all representative images and mice were randomly selected.

Immunofluorescence staining

Four mice per group were euthanized on the seventh day after administration of a single dose of psilocybin. The mice were anesthetized with 1.0% pentobarbital after the fear renewal test and then transcardially perfused with 0.9% sterile saline followed by 4% paraformaldehyde. The brains were postfixed with cold paraformaldehyde overnight and dehydrated with 30% sucrose before preparing coronal sections (20 μm thick) using a freezing microtome (CM1860 UV, Leica Microsystem Ltd., Wetzlar, Germany). The coronal sections underwent permeabilization (phosphate-buffered saline with Tween 20 [PBST] for 30 min), blocking (PBST with 5% normal goat serum at room temperature for 1 h), and incubation with anti-doublecortin (anti-DCX, 1:800, Cell Signaling Technology, Boston, USA, 4604S) or anti-BrdU (1:200, Abcam, Cambridge, UK, ab1893) antibodies overnight at 4° C. Then, the sections were washed thrice in PBST and incubated with secondary antibodies (DCX: Alexa Fluor 594-conjugated goat anti-rabbit immunoglobulin G [IgG], 1:2000, Cell Signaling Technology 8889S; BrdU: Alexa Fluor 488-conjugated donkey anti-sheep IgG, 1:2000, Abcam, ab150177) at room temperature for 2 h. Finally, after three more washes with PBST, the sections were mounted with Fluoroshield ™ with 4′, 6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and imaged with upright fluorescence microscopy (BX63, Olympus, Tokyo, Japan). Four sections of the hippocampal DG were photographed at 400× original magnification. ImageJ software was used to count DCX- or BrdU-positive cells in the hippocampal DG in each section. Finally, the number of DCX- or BrdU-positive cells in four sections of the hippocampal DG (from 1.46 mm to 1.94 mm posterior to bregma) per mouse was statistically analyzed for four mice from each group.

Western bloting analysis

Six mice per group were euthanized on the seventh day, and the hippocampus was carefully dissected. The separated hippocampal tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (Millipore, Billerica, MA, USA) containing protease inhibitor (Boster, Wuhan, China, AR1182-1) and phosphatase inhibitor (Boster; AR1183). The lysed tissues were centrifuged, and the supernatants were collected for further analysis. A total of 30 μg of each protein sample per lane was loaded onto 10% acrylamide gels, and the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to 0.45 mm polyvinylidene difluoride membranes (Millipore) and blocked with 5% skim milk.[30] The membranes were incubated with primary anti-BDNF (1:1000, Abcam; 108319) and anti-mammalian target of rapamycin (anti-mTOR, 1:1000, Cell Signaling Technology; 2983) or anti-β-actin (1:1000, Cell Signaling Technology; 4970) antibodies at 4°C overnight. The membranes were incubated for 120 min at room temperature with fluorophore-conjugated goat anti-rabbit (IRDye 800CW, 1:5000, LI-COR Biosciences, Lincoln, USA, 926-32211) or goat anti-mouse (IRDye 680RD, 1:5000, LI-COR Biosciences; 926-68070) secondary antibody. The bands were detected using the Odyssey infrared imaging system (Odyssey Sa, Gene Company Limited, Hong Kong, China). The relative level of each protein is expressed as the density ratio of the target protein band to the β-actin band, and relative BDNF and mTOR expression levels were normalized to the levels in vehicle-treated mice not subjected to FC.

Statistical analysis

All data are presented as the means ± standard errors of the means (SEMs) and were analyzed with GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). Behavioral data were analyzed using an unpaired Student's *t* test to compare two groups (the vehicle-treated non-fear-conditioned group *vs*. the vehicle-treated fear-conditioned group) or one-way analysis of variance (ANOVA) followed by Dunnett's test to compare four groups (the vehicle-treated fear-conditioned group *vs*. the psilocybin [0.1 mg/kg, 0.5 mg/kg, or 2.5 mg/kg]-treated fear-conditioned group). Golgi–Cox staining, Western blot, and immunofluorescence staining data were analyzed using an unpaired Student's *t* test to compare two groups (the vehicle-treated non-fear-conditioned group *vs*. the vehicle-treated fear-conditioned group; or, the psilocybin [2.5 mg/kg]-treated fear-conditioned group *vs*. the vehicle-treated fear-conditioned group). The results were considered statistically significant if the two-tailed *P* value was <0.05.

Results

Psilocybin facilitated rapid and sustained fear extinction when administered before fear extinction training

The effect of psilocybin on fear extinction was investigated with an auditory cued FC and fear extinction paradigm [Figure 1A]. The baseline percentage of freezing time was not significantly different among the groups (t = 0.431, df = 98, P = 0.667, Supplementary Figure 1, http://links.lww.com/CM9/B494). On the day of FC (day–2, context A), the percentage of freezing time during the last CS presentation was significantly longer in fear-conditioned mice than in mice that did not undergo FC (t = 8.079, df = 98, P < 0.001, Figure 1B), indicating that FC was successful. Mice that underwent FC were randomly assigned to receive vehicle or different doses of psilocybin (0.1 mg/kg, 0.5 mg/kg, or 2.5 mg/kg).

On the day of fear extinction training (day 0), fear-conditioned mice exhibited a significantly longer percentage

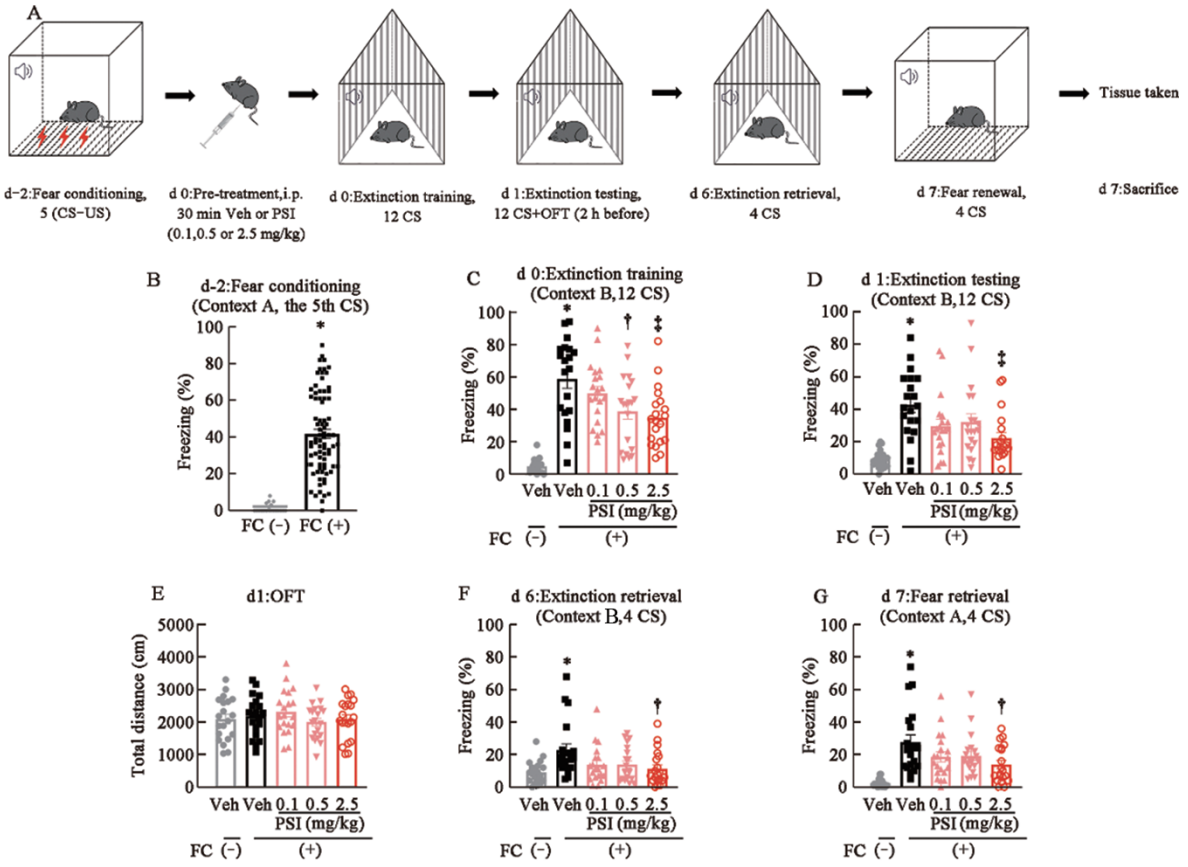

Figure 1: Effect of psilocybin on percentage of freezing time in an auditory cued FC and fear extinction paradigm. (A) The auditory cued FC and fear extinction paradigm and the experimental plan. (B) FC significantly prolonged the percentage of freezing time of mice in context A (fear conditioning context). (C) On the day of extinction training, administration of psilocybin (0.5 mg/kg and 2.5 mg/kg) reversed the increase in percentage of freezing time of fear-conditioned mice across 12 CS presentations during fear extinction training in context B (fear extinction training context). (D) On the day of extinction testing, 2.5 mg/kg psilocybin significantly rescued freezing time in response to 12 CS presentations 24 h after treatment. (E) In the OFT, the total distance traveled at 24 h after psilocybin treatment did not differ significantly among the groups. (F) In the extinction retrieval test context (context B), psilocybin (2.5 mg/kg) reversed the increase in percentage of freezing time on the sixth day. (G) The fear renewal test showed that in the FC context (context A), percentage of freezing time on the seventh day was significantly reduced by 2.5 mg/kg psilocybin. The data shown are the means ± SEMs; n = 20 or 80 per group for B; n = 20 per group for C–G; unpaired Student's *t*-test, $^{*}P < 0.001$, $^{§}P < 0.01$, *vs.* the vehicle-treated non-fear-conditioned group; one-way analysis of variance followed by Dunnett's test, $^{†}P < 0.05$, $^{‡}P < 0.01$ *vs.* the vehicle-treated fear-conditioned group. CS: Conditioned stimulus; FC: Fear conditioning; i.p.: Intraperitoneal; OFT: Open field test; PSI: Psilocybin; SEMs: Standard errors of the means; US: Unconditioned stimulus; Veh: Vehicle.

of freezing time during the 12 CS presentations than the mice not exposed to FC (t = 9.348, df = 38, P < 0.001, Figure 1C). We found that psilocybin treatment significantly decreased percentage of freezing time ($F_{3, 76}$ = 5.171, P = 0.003). Multiple comparisons using Dunnett's *post hoc* test showed that compared to vehicle, 0.5 mg/kg and 2.5 mg/kg psilocybin significantly reduced percentage of freezing time in fear-conditioned mice (P = 0.010 for 0.5 mg/kg; P = 0.002 for 2.5 mg/kg).

Systemic psilocybin concentrations subside to negligible levels approximately 6 h after administration.[31] Hence, fear extinction testing was performed in the same context used for fear extinction training (context B) at 24 h after psilocybin administration (ie, in a drug-free state). Exposure to FC significantly increased percentage of freezing time (t = 6.995, df = 38, P < 0.001, Figure 1D), and this increase was significantly reversed by psilocybin ($F_{3, 76}$ = 3.839, P = 0.010); multiple comparisons using Dunnett's *post hoc* test showed that 2.5 mg/kg psilocybin had the strongest effect compared with the vehicle-treated fear-conditioned group (P = 0.004).

As locomotor activity may affect percentage of freezing time, we evaluated the locomotor activity of mice using the OFT 24 h after psilocybin treatment. Compared to mice not exposed to FC, the fear-conditioned mice showed no statistically significant difference in total distance traveled on the first day (t = 0.421, df = 38, P = 0.676, Figure 1E). Different doses of psilocybin had no effect on the total distance traveled ($F_{3, 76}$ = 0.957, P = 0.417). Details regarding percentage of freezing time during fear extinction training and fear extinction testing from the first to the last CS presentation are shown in Supplementary Figure 2, http://links.lww.com/CM9/B494.

To explore the duration of the effects induced by a single dose of psilocybin, we then tested whether these effects persisted in an extinction retrieval test (in context B, where the mice experienced fear extinction) on the sixth day and a fear renewal test (in context A, where the mice experienced FC) on the seventh day after psilocybin administration. The percentage of freezing time of fear-conditioned mice was significantly longer than that of mice not exposed to FC in the extinction retrieval test on the sixth day in context B (t = 3.374, df = 38, P = 0.002, Figure 1F) and in the fear renewal test on the seventh day in context A (t = 5.676, df = 38, P < 0.001, Figure 1G). A single dose of psilocybin significantly alleviated the increase in percentage of freezing time of fear-conditioned mice on the sixth day ($F_{3,76}$ = 3.240, P = 0.030, Figure 1F) and seventh day ($F_{3, 76}$ = 3.035, P = 0.030, Figure 1G). *Post hoc* analysis confirmed that 2.5 mg/kg was the optimal dose of psilocybin for facilitating fear extinction (sixth day, P = 0.010; seventh day, P = 0.010); therefore, we selected this dose for subsequent mechanistic research.

Psilocybin rescued the decrease in hippocampal dendritic complexity and spine density induced by FC

We performed Golgi–Cox staining to detect dendritic plasticity and Sholl analysis of the total number of dendritic branches to assess dendritic complexity. The total number of dendritic branches and the density of dendritic spines in the hippocampus were significantly decreased induced by FC (for total number of dendritic branches: t = 2.808, df = 30, P = 0.009, Figure 2A, C; for density of dendritic spines: t = 3.602, df = 30, P = 0.001, Figures 2B, D). Interestingly, these negative changes were rescued by 2.5 mg/kg psilocybin 7 days after treatment (for total number of dendritic branches: t = 2.266, df = 30, P = 0.031; for density of dendritic spines: t = 4.057, df = 30, P < 0.001), which suggested that psilocybin rescued hippocampal dendritic complexity and spine density, both of which were decreased by FC.

Psilocybin rescued the decrease in the protein levels of hippocampal BDNF and mTOR induced by FC

Psilocybin elicits profound changes in neuroplasticity through the BDNF and mTOR signaling pathways.[18] Western blot analysis indicated that FC significantly decreased the protein levels of BDNF (t = 4.340, df = 10, P = 0.002, Figure 3A) and mTOR (t = 2.431, df = 10, P = 0.035, Figure 3B) in the hippocampus. Compared to vehicle-treated mice, psilocybin-treated mice showed a reversal of these negative changes in BDNF (t = 3.196, df = 10, P = 0.009, Figure 3A) and mTOR (t = 2.243, df = 10, P = 0.049, Figure 3B) levels 7 days after treatment.

Psilocybin rescued the decrease in the numbers of DCX- and BrdU-positive cells in the hippocampal DG induced by FC

Doublecortin (DCX) is a neurogenesis marker associated with neuroplasticity.[32] We labeled DCX (red fluorescence)- and DAPI (blue fluorescence)-positive cells in the hippocampal DG by immunofluorescence staining; this staining showed that FC significantly reduced the number of DCX-positive cells in the hippocampal DG (t = 6.561, df = 30, P < 0.001, Figure 4). A single dose of psilocybin rescued the decrease in the number of DCX-positive cells (t = 4.300, df = 30, P < 0.001, Figure 4) induced by FC 7 days after administration.

As neurogenesis may be reduced in PTSD,[33] we labeled BrdU (green fluorescence)- and DAPI (blue fluorescence)-positive cells in the hippocampal DG using immunofluorescence staining [Figure 5], this quantification revealed that FC significantly reduced the number of BrdU-positive cells in the hippocampal DG (t = 2.783, df = 30, P = 0.009). Seven days after a single dose of psilocybin, the reduction in the number of BrdU-positive cells in the hippocampal DG induced by FC was alleviated (t = 3.287, df = 30, P = 0.003).

Discussion

Our study is the first to investigate the long-term effect of psilocybin on the facilitation of fear extinction and to assess whether this effect is mediated by the promotion of hippocampal neuroplasticity. Encouragingly, our results suggested that a single dose of psilocybin facilitated rapid and sustained fear extinction starting at 24 h after administration and for up to 7 days. The psilocybin-induced facilitation of fear extinction coincided with the promotion of hippocampal neuroplasticity, including the

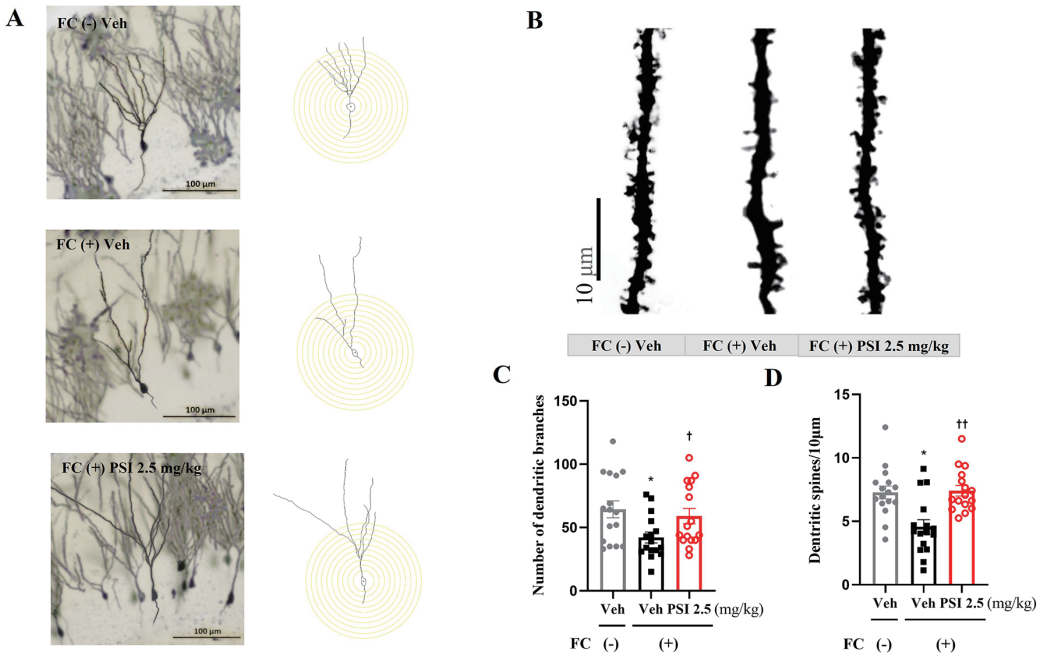

Figure 2: Effect of psilocybin on hippocampal dendritic complexity and spine density. (A) Hippocampal neurons were photographed with a light microscope, typical diagram of granule cells based on Golgi–Cox staining and NeuronJ tracing are shown. Scale bar = 100 μm. (B) Hippocampal neurons were photographed with a light microscope, representative photomicrograph of dendritic spines from each group are shown. Scale bar = 10 μm. (C) Psilocybin significantly reversed the reduction in the total number of dendritic branches induced by FC. (D) The decrease in the density of dendritic spines induced by FC was significantly reversed by psilocybin treatment. The data shown are the means ± SEMs; $n = 16$ neurons from four mice per group; unpaired Student's *t*-test, $^{*}P < 0.01$ *vs.* the vehicle-treated non-fear-conditioned group; unpaired Student's *t*-test, $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.001$ *vs.* the vehicle-treated fear-conditioned group. FC: Fear conditioning; PSI: Psilocybin; SEMs: Standard errors of the means; Veh: Vehicle.

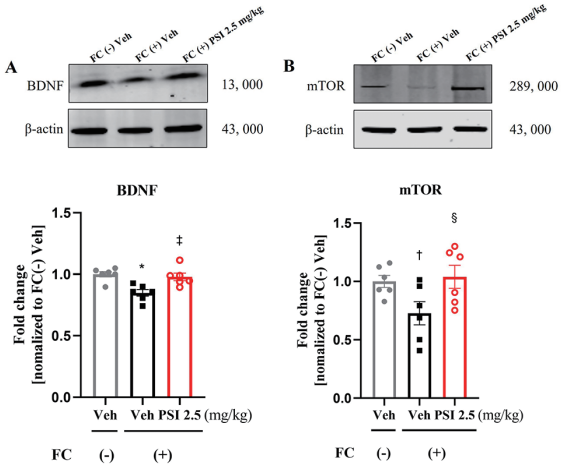

Figure 3: Effect of psilocybin on hippocampal BDNF and mTOR protein levels by Western bloting. (A) Psilocybin ameliorated the effect of FC on hippocampal BDNF levels. (B) Psilocybin rescued the decrease in hippocampal mTOR levels induced by FC. The data shown are the means ± SEMs; $n = 6$ per group; unpaired Student's *t*-test, $^{*}P < 0.01$, $^{\dagger}P < 0.05$ *vs.* the vehicle-treated non-fear-conditioned group; unpaired Student's *t* test, $^{\ddagger}P < 0.01$, $^{\S}P < 0.05$ *vs.* the vehicle-treated fear-conditioned group. BDNF: Brain-derived neurotrophic factor; FC: Fear conditioning; mTOR: Mammalian target of rapamycin; PSI: Psilocybin; SEMs: Standard errors of the means; Veh: Vehicle.

rescue of the decreases in dendritic complexity, spine density, the protein levels of BDNF and mTOR, and the number of DCX- and BrdU-positive cells induced by FC.

Fear extinction is a key process in exposure-based psychotherapy for PTSD;[34,35] therefore, we used the auditory cued FC and fear extinction paradigm, which is commonly used to study fear-related disorders, such as PTSD.[29,36,37] In the FC paradigm, pairing an initially neutral CS (a tone) with an aversive US (an inescapable foot shock) can lead to FC.[35] Therefore, subsequent presentations of the CS elicit a conditioned response, ie, freezing. Our results were consistent with a study conducted in 2013, which showed that psilocybin facilitated fear extinction after trace FC.[21] However, in the previous study, psilocybin was administered before FC; thus, the administration was prophylactic. In real life, it is impossible to predict the onset of traumatic events, and prophylactic administration is not feasible. Therefore, in our study, psilocybin was administered 2 days after FC; thus, the treatment was therapeutic rather than prophylactic. Our findings suggest that psilocybin facilitated rapid and sustained fear extinction when administered before fear extinction training. Another recent preclinical study published in 2021 reported that psilocybin reduced percentage of freezing time in a contextual FC paradigm.[27] That study evaluated the effects of psilocybin at only one time point: 0.5 h after administration; however, the hallucinogenic effects of psilocybin can last up to 6 h. Therefore, the previous study did not exclude the acute hallucinogenic effects of psilocybin. Additionally, that study did not explore the duration of effects of a single dose of psilocybin. In our

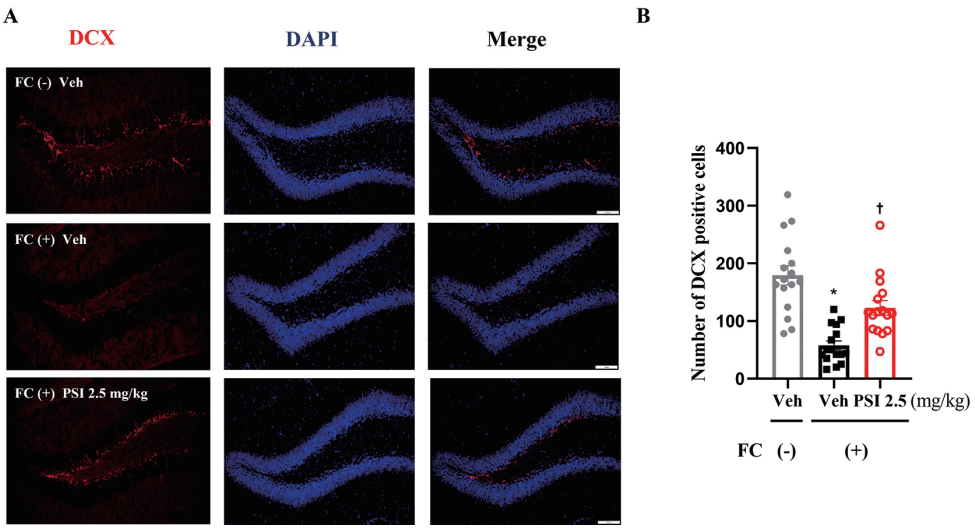

Figure 4: Effect of psilocybin on the number of DCX-positive cells in the hippocampal DG. (A) Immunofluorescence staining showed the typical diagram of DCX-positive cells (red) in the DG. Scale bar = 100 μm. (B) Psilocybin reversed the decrease in the number of DCX-positive cells in the DG induced by FC. The data shown are the means ± SEMs; $n = 16$ sections from four mice per group; unpaired Student's *t*-test, $^{*}P < 0.001$ *vs.* the vehicle-treated non-fear-conditioned group; unpaired Student's *t*-test, $^{\dagger}P < 0.001$ *vs.* the vehicle-treated fear-conditioned group. DAPI: 4′,6-Diamidino-2-phenylindole; DCX: Doublecortin; DG: Dentate gyrus; FC: Fear conditioning; PSI: Psilocybin; SEMs: Standard errors of the means; Veh: Vehicle.

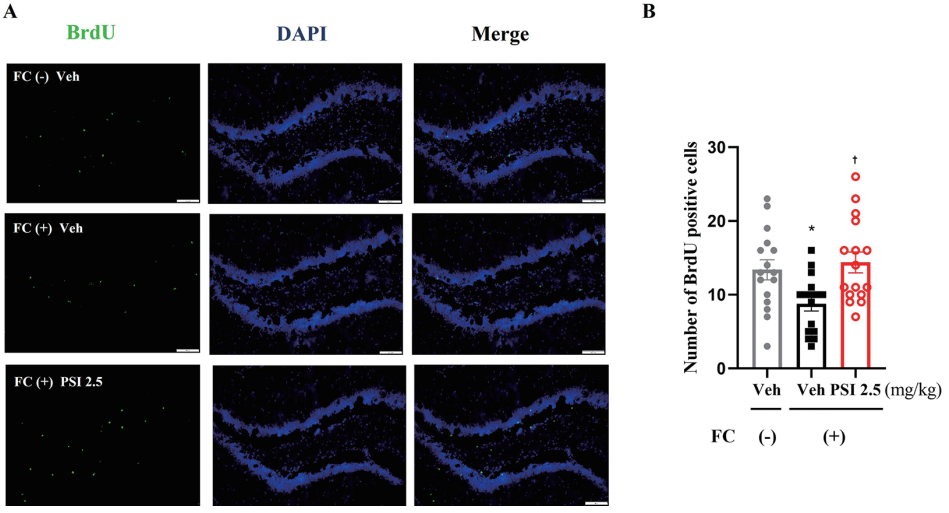

Figure 5: Effect of psilocybin on the number of BrdU-positive cells in the hippocampal DG. (A) Typical diagram of BrdU-positive cells in the DG (green). Scale bar = 100 μm. (B) Psilocybin reversed the reduction in the number of BrdU-positive cells in the DG induced by FC. The data shown are the means ± SEMs; $n = 16$ sections from four mice per group; unpaired Student's *t*-test, $^{*}P < 0.01$ *vs.* the vehicle-treated non-fear-conditioned group; $^{\dagger}P < 0.01$ *vs.* the vehicle-treated fear-conditioned group. BrdU: Bromodeoxyuridine; DAPI: 4′,6-Diamidino-2-phenylindole; DG: Dentate gyrus; FC: Fear conditioning; PSI: Psilocybin; SEMs: Standard errors of the means; Veh: Vehicle.

behavioral experiment, fear extinction tests were performed 24 h after administering psilocybin to rule out any acute hallucinogenic effects. We also explored the long-term effect of a single dose of psilocybin at 7 days after administration. Surprisingly, we found that a single dose of psilocybin exerted fear extinction effects at 24 h after administration and that these effects lasted for 7 days.

To rule out potential false positives due to the general effect of psilocybin on activity levels, we assessed locomotor activity with the OFT. The total distance traveled by the mice was not influenced by psilocybin administration, suggesting that the effects of psilocybin on fear extinction were not solely attributable to a change in locomotor activity. Moreover, other studies also support the idea that psilocybin does not significantly affect the locomotor activity of mice.[21,38]

Failure of fear extinction is closely associated with aberrant regulation of hippocampal neuroplasticity.[2-4] Therefore, we examined neuroplasticity in the hippocampus with Golgi–Cox staining. Our results indicated that FC decreased the density of dendritic spines and the total number of dendritic branches. The dendritic arbor is responsible for receiving and consolidating neuronal inputs;[39] thus, decreases in dendritic complexity and

spine density may lead to negative behavioral changes, ie, PTSD-like symptoms. A single dose of psilocybin reversed these negative changes, which suggests that psilocybin-induced facilitation of fear extinction was at least partially mediated by the promotion of hippocampal neuroplasticity. Similarly, several other studies have shown that psilocybin promotes hippocampal neuroplasticity in depression, supporting our hypothesis.[17,40]

BDNF and mTOR signaling are crucial in fear extinction.[41] BDNF is an important protein associated with neuroplasticity, and its levels are often reduced in PTSD patients.[42] Furthermore, increasing BDNF levels can facilitate fear extinction by suppressing fear responses.[41,43] To explore the role of BDNF and mTOR in the effects of psilocybin on fear extinction, we measured the levels of these two proteins in the hippocampus. Psilocybin ameliorated the decreases in hippocampal BDNF and mTOR protein levels induced by FC, suggesting that BDNF and mTOR signaling is associated with the effects of psilocybin. This finding also suggests that hippocampal neuroplasticity is involved in the psilocybin-induced facilitation of fear extinction.

FC has been found to alter DCX protein levels, and higher DCX expression is associated with enhanced fear extinction.[32] Our results showed that a single dose of psilocybin reversed the decrease in the number of DCX-positive cells in the hippocampal DG induced by FC at 7 days after administration, which suggested that psilocybin promoted neurogenesis in the hippocampal DG. BrdU labeling is currently the other most commonly used technique for studying neurogenesis.[44] Interestingly, our results showed that psilocybin ameliorated the reduction in the number of BrdU-positive cells in the hippocampal DG induced by FC. In 2013, Catlow *et al*[21] found that a single dose of psilocybin (0.1 mg/kg) resulted in a trend toward an increased number of BrdU-positive cells in the hippocampal DG of mice. In another study by the same group, mice were administered once-weekly doses of psilocybin for 4 weeks; they showed that compared with administration of saline, chronic administration of psilocybin (1.5 mg/kg) led to a significantly increased number of BrdU-positive cells.[45] Our DCX and BrdU immunofluorescence staining results further supported the hypothesis that the promotion of hippocampal neuroplasticity may be the mechanism underlying psilocybin-induced facilitation of fear extinction. Malberg *et al*[46] showed that cell proliferation in the adult hippocampus is decreased by an inescapable stress, ie, foot shock. Our findings indicated that FC reduced hippocampal neurogenesis, which is consistent with the results of Malberg *et al*.[46] In contrast to our results, others have reported that various types of learning and memory can increase the number of newly born cells in the hippocampus.[47,48] The intensity of stress varies among the different models used, which may explain the contradictory findings.

Clinical trials using psilocybin to treat PTSD are ongoing, and the results have not yet been published. An interpretative phenomenological analysis of the use of psilocybin by veterans with symptoms of trauma showed that all the participants who had used psilocybin reported immediate and long-term amelioration of their symptoms.[49] In October 2020, it was reported that veterans suffering from PTSD experienced relief through psilocybin treatment.[50]A psilocybin nasal spray has been designed to make microdosing easier for people trying to treat their PTSD or depression.[51] Collectively, there is increasing evidence suggesting that psilocybin has the potential to treat PTSD.[22] Our findings suggest promising potentials of psilocybin for treatment of PTSD at the preclinical level and provide impetus for future clinical studies. But, it is not clear in which receptor psilocybin plays its role in facilitating fear extinction, which is worth further study.

In conclusion, a single dose of psilocybin facilitated rapid and sustained fear extinction; this effect may have been partially mediated by the promotion of hippocampal neuroplasticity. Our results suggest that psilocybin may be a useful adjunct to exposure-based therapies for PTSD and other mental disorders characterized by the failure of fear extinction.

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Conflicts of interest

None.

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