Harmine produces antidepressant-like effects via restoration of astrocytic functions

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ABSTRACT

Depression is a world-wide disease with no effective therapeutic methods. Increasing evidence indicates that astrocytic pathology contributes to the formation of depression. In this study, we investigated the effects of harmine, a natural β-carboline alkaloid and potent hallucinogen, known to modulate astrocytic glutamate transporters, on chronic unpredictable stress (CUS)-induced depressive-like behaviors and astrocytic dysfunctions. Results showed that harmine treatment (10, 20 mg/kg) protected the mice against the CUS-induced increases in the immobile time in the tail suspension test (TST) and forced swimming test (FST), and also reversed the reduction in sucrose intake in the sucrose preference experiment. Harmine treatment (20 mg/kg) prevented the reductions in brain-derived neurotrophic factor (BDNF) protein levels and hippocampal neurogenesis induced by CUS. In addition, harmine treatment (20 mg/kg) increased the protein expression levels of glutamate transporter 1 (GLT-1) and prevented the CUS-induced decreases in glial fibrillary acidic protein (GFAP) protein expressions in the prefrontal cortex and hippocampus, suggesting that restoration of astrocytic functions may be a potential mechanism underlying the antidepressant-like effects of harmine. This opinion was proved by the results that administration of mice with L-Alpha-Aminoadipic Acid (L-AAA), a gliotoxin specific for astrocytes, attenuated the antidepressant-like effects of harmine, and prevented the improvement effects of harmine on BDNF protein levels and hippocampal neurogenesis. These results provide further evidence to confirm that astrocytic dysfunction contributes critically to the development of depression and that harmine exerts antidepressant-like effects likely through restoration of astrocytic functions.

1. Introduction

Major depression is a common disease affecting numerous persons in the world-wide. At present, nearly all of the clinical antidepressants are developed out of the monoaminergic deficit hypothesis of depression, and these agents have been used as classical antidepressants for a very long time (Lanni et al., 2009; Kern et al., 2012). However, more and more studies and clinical reports show that these antidepressants exhibit numerous limitations (Fava, 2010; Fabbri et al., 2013; Sanchez et al., 2015). For example, the first application of traditional antidepressants is effective only in about one-third of patients, and approximately two-third of patients fail to achieve clinical improvements after trying several times (Schwartz et al., 2016). Thus, it is necessary to develop novel mechanism-based antidepressants in order to improve the present status of drug therapy of depression.

Increasing evidence indicates that astrocytic dysfunction is actively involved in the pathogenesis of depression. Decreased numbers of hippocampal astrocytes have been observed in rodents treated with chronic stresses or maternal deprivation (Ye et al., 2011; Leventopoulos et al., 2007). Post-mortem studies of tissues from depressed patients

Abbreviations: ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CUS, chronic unpredictable stress; DCX, doublecortin; DMSO, Dimethyl Sulphoxide; EAATs, excitatory amino-acid transporters; FITC, fluorescein isothiocyanate; FST, forced swimming test; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GLAST, glutamate/aspartate transporter; GLT-1, glutamate transporter-1; GS, glutamine synthetase; L-AAA, L-Alpha-Aminoadipic Acid; TST, tail suspension test

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describe reduced numbers of astrocytes in the brain (Oh et al., 2012; Ménard et al., 2016), and these reductions are supported by the finding that the glial fibrillary acidic protein (GFAP)-expressing cells are reduced by chronic stresses in both prefrontal cortex and hippocampus (Baner and Duman, 2008; Ardalan et al., 2017). In other studies, researchers show that expressions of glial-specific excitatory amino-acid transporters (EAATs: EAAT1/glutamate/aspartate transporter (GLAST), EAAT2/glutamate transporter-1 (GLT-1)) and glutamine synthetase (GS) can be altered in brain tissues from depressed patients (Choudary et al., 2005; Bernard et al., 2011). Functionally, the astrocytic abnormality has been shown to induce a decrease in glutamate uptake and cycling, an accumulation of glutamates, as well as an impairment in brain-derived neurotrophic factor (BDNF) signals and hippocampal neurogenesis (Lutgen et al., 2016; Martin et al., 2012). Thus, inhibition of astrocytic dysfunction may be a potential strategy for depression therapy.

Harmine is a hallucinogenic alkaloid found in the seed of Peganum harmala and Banisteriopsis caapi, both of which are traditionally used for ritual and medicinal preparations in the Middle East, Central Asia, and South America (Sourkes, 1999). In past years, a wide range of pharmacological effects of harmine, such as antioxidation (Moura et al., 2007; Kim et al., 2001), antigenotoxicity (Moura et al., 2007), and anti-diabetes (Waki et al., 2007), have been revealed. Preclinical findings show that harmine has potential antidepressant-like activities in acute and chronic depression models (Fortunato et al., 2009; Fortunato et al., 2010; Aricioglu and Altunbas, 2003; Farzin and Mansouri, 2006). More strong correlations between harmine and depression have been evidenced by recent studies in depressed patients, in which a single dose of ayaluusca, a harmine-containing hallucinogenic plant, has been reported to produce rapid and sustained antidepressant-like activities (Osório Fde et al., 2015; Sanches et al., 2016). Mechanistically, the antidepressant-like effects of harmine may be mediated by restoration of BDNF signals (Fortunato et al., 2009; Fortunato et al., 2010). An inverse-agonistic mechanism located in the benzodiazepine receptors may also mediate the antidepressant-like effects of harmine, as flumazenil, an inhibitor of the benzodiazepine receptor, has been shown to abrogate the antidepressant-like effects of harmine in the forced swimming test (FST) (Aricioglu and Altunbas, 2003). Recently, several different studies show that harmine increases GLT-1 gene and protein expression as well as glutamate uptake activity in animal models of amyotrophic lateral sclerosis (ALS) (Li et al., 2011) and cerebral ischemia (Sun et al., 2014), suggesting that harmine may exert neuroprotective effects via enhancement of GLT-1 functions.

Within these contexts, we hypothesized that harmine exerts antidepressant-like effects likely through restoration of astrocytic functions. As anticipated, we showed that harmine not only prevented mice depressive-like behaviors, but also reversed the reductions in BDNF protein levels and hippocampal neurogenesis induced by CUS. Harmine also prevented CUS-induced decreases in GFAP protein expressions and up-regulated GLT-1 protein expressions in mice hippocampus and prefrontal cortex. Specific inhibition of astrocytic functions abrogated the protective effects of harmine in depressed mice. Since astrocytes contribute critically to the integrity of neuronal functions (Ben Haim and Rowitch, 2017; Yang et al., 2015), our studies indicate that the astrocyte may be a potential target for the antidepressant-like effects of harmine.

2. Materials and methods

2.1. Animals

8–10 weeks old male C57BL/6J mice were housed five per cage under standard conditions (12-h light/dark cycle; lights on from 07:00 to 19:00; 23 ± 1 °C ambient temperature; 55 ± 10% relative humidity) for 1 week with free access to food and water. Each experimental group consisted of 10 mice. Behavioral experiments were carried out during the light phase. Animal experiments were conducted in accordance with internationally accepted guidelines for the use of animals in toxicology as adopted by the Society of Toxicology in 1999 and approved by the University Animal Ethics Committee of Nantong University (Permit Number: 2110836).

2.2. Materials

Harmine and fluoxetine were purchased from MedChem Express (Princeton, NJ, USA). L-AlkAminoacidic Acid (L-AAA) was the product of Sigma (Saint Louis, MO, USA). The doses of fluoxetine, harmine and L-AAA were chosen as previous studies (Takada and Hattori, 1986; Khurgel et al., 1996; Jiang et al., 2015a, 2015b). Harmine and fluoxetine were prepared in 10% dimethyl sulphoxide (DMSO) and were administered intraperitoneally (i.p.) in a volume of 10 mL/kg. L-AAA was dissolved in a phosphate buffer and was administered intracerebroventricularily (i.c.v.). Antibodies against doublecortin (DCX), GFAP, GS, GLAST, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against GLT-1 was the product of Abcam (Cambridge, MA, USA). Hoechst 33,258 was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

2.3. Tail suspension test (TST) and FST

The TST and FST were performed according to the methods of Steru (Steru et al., 1985) and Porsolt (Porsolt et al., 1977), respectively. In the FST, the experimental mice were individually placed in a clear glass cylinder (height 25 cm, diameter 10 cm) filled to 10 cm with water at 25 ± 1 °C for 6 min. In the TST, the experimental mice were suspended 50 cm above the floor for 6 min by adhesive tape placed approximately 1 cm from the tip of the tail 2 h after the last injection. The immobile time was recorded during the last 4 min by an investigator blind to the study. For FST, the immobile time was defined as the time spent by the mouse floating in the water without struggling and making only those movements necessary to keep its head above the water. For TST, the mice were considered immobile only when they hung passively and were completely motionless, and any mice that try to climb their tails were removed from statistical analysis. The concrete schematic diagram for the experimental timeline in the TST and FST in CUS models was presented in Fig. 1A.

2.4. Chronic unpredictable stress (CUS)

The CUS, consisting of daily exposure to two of the following stressors in a random order over a 5-week period: cage shaking (1 h), lights on during the entire night (12 h), placement in cold room (4 °C, 1 h), mild restraint in small cages (2 h), 45° cage tilt (14 h), lights-off during the daylight phase (3 h), wet cage (14 h), flushing light (6 h), noise in the room (3 h), and water deprivation during the dark period (12 h), was used to induce depressive-like behaviors in C57BL/6J mice.

2.5. Sucrose preference experiment

The sucrose preference experiment was performed at day 36. The experimental mice were given the choice to drink from two bottles in individual cages, one with 1% sucrose solution and the other with water (Yang et al., 2017). All were acclimatized for 2 days to two-bottle choice conditions, and the position of two bottles was changed every 6 h to prevent possible effects of side preference in drinking behavior. The experimental mice were then deprived of food and water for 24 h, and on day 39, the mice were exposed to pre-weighed bottles for 1 h with their position interchanged. Sucrose preference was calculated as a percentage of the consumed sucrose solution relative to the total amount of liquid intake. The schematic diagram for sucrose preference experiment was presented in Fig. 1A.
2.6. Chronic intracerebroventricular infusions of L-AAA

In this experiment, L-AAA was used to block astrocytic functions. Briefly, C57BL/6J mice were anesthetized with pentobarbital sodium and placed in a stereotaxic frame (Jiang et al., 2015a, Quintessential Stereotaxic Injector, STELING Corporation, DALE, IL, USA). The cannulas were implanted into the left lateral brain ventricle (−0.2 mm anterior and 1.0 mm lateral relative to bregma and 2.3 mm below the surface of the skull, Kleinridders et al., 2009) and connected to an osmotic minipump (Alzet model 2002 for chronic injections and Alzet model 1003D for acute injections, Alza Corporation, Cupertino, CA, USA) according to manufacturer’s protocols. Minipumps were filled with 1 μg/μL L-AAA or 1 μg/μL vehicle in artificial cerebrospinal fluid (ACSF) and were implanted subcutaneously in the interscapular region. The speed of chronic (10 days) injections was 1 μL/h. The total dosage of L-AAA in this experiment was within the range used in the literature (Takada and Hattori, 1986; Khurgel et al., 1996).

2.7. BDNF analysis

Immediately after the behavioral experiments mice were killed and the hippocampal and prefrontal cortical tissues were dissected and stored at −70 °C. The BDNF protein levels were measured by anti-BDNF sandwich-ELISA according to manufacturer’s protocols (Chemicon, USA). Briefly, brain tissues were homogenized in phosphate buffers containing phenylmethylsulfonyl fluoride (1 mM) and EGTA (1 mM). The 96-well plates were pre-coated for 24 h with the samples diluted (1:2) in sample diluent and standard curve ranged from 10 to 500 pg/mL of BDNF. The plates were then washed three times by sample diluent, and a monoclonal anti-BNDF antibody (1:500) prepared in sample diluents was added into each well and incubated for 3 h at room temperature. After washing, a horseradish peroxidase conjugated anti-rabbit secondary antibody (1:1000) was added into each well and incubated at room temperature for 1 h, and then the streptavidin-enzyme, substrate, and stop solution were added into the 96-well plates. The amounts of BDNF protein were determined by absorbance in 450 nm, and measured by Lowry’s method using bovine serum albumin as a standard, as previously described by Frey et al. (2006). The standard curve demonstrates a direct correlation between Optical Density and BDNF protein concentrations.

2.8. Western blot

This experiment was conducted according to previous studies with some modifications (Yao et al., 2016; Huang et al., 2017). Briefly, brain tissues were dissected and homogenized in lyses buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 20 mM NaF, 3 mM Na3VO4, 1 mM PMSF, 1% NP-40 and protease inhibitor cocktail on ice for 30 min. The lysates were centrifuged at 12,000 × g for 15 min, and the supernatants were harvested. After being separated in 10% SDS/PAGE, 30–50 μg of denatured protein were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The milk-blocked nitrocellulose membranes were incubated overnight at 4 °C with 1:500 primary antibodies against DCX, GFAP, GS, GLAST, GLT-1 or GAPDH, and then further incubated for 2 h at room temperature with IRDye 680-labeled secondary antibodies (1:5000). Finally, immunoblots were visualized by scanning using the Odyssey CLx Western blot detection system. The band density was quantified by Image J software.

2.9. Immunofluorescence

Animals were anesthetized with sodium pentobarbital (50 mg/kg) and perfused transcardially with 37 °C saline and 4% paraformaldehyde in 0.01 M phosphate buffer. The brains were removed, frozen, and sectioned at 25 μm, and consecutive sections were collected in 24-well plates containing PBS. The sections were first permeabilized with 0.3% Triton X-100 for 30 min, then incubated with 3% bovine serum albumin in PBS for another 30 min at room temperature, and followed by incubation in diluted goat anti-DCX (1:100) primary antibody in PBS containing 0.3% Triton X-100 and 1% BSA overnight at 4 °C. After that, the primary antibody was removed by washing the sections three times in PBS. The sections were further incubated in fluorescein isothiocyanate (FITC)-labeled horse anti-rabbit IgG (1:50) for 2 h at room temperature. The cellular nuclei were marked with Hoechst 33,258 (10 min). After being washed with PBS, sections were mounted on slides and coverslipped and finally examined under a confocal fluorescence microscope (FV500; Olympus, Tokyo, Japan).

2.10. Statistical analysis

All analyses were performed using SPSS 13.0 software (SPSS Inc.,
USA), and data are presented as mean ± SEM. Differences between mean values were evaluated using two-way analysis of variance (ANOVA), and the Bonferroni’s post hoc test was used to assess isolated comparisons. *p < 0.05* was considered statistically significant.

3. Results

3.1. Chronic harmine treatment reverses the CUS-induced depressive-like behaviors in C57BL/6J mice

To characterize the antidepressant-like effects of harmine, we employed a widely-used animal model of depression (Jiang et al., 2015b), CUS, in this study. TST and FST have been applied to detect the antidepressant-like activities of potential antidepressants (Steru et al., 1985; Porsolt et al., 1977). In the FST, two-way ANOVA revealed significant effects for stress [F(1, 72) = 39.70, *p < 0.001] and drug treatment [F(3, 72) = 7.85, *p < 0.001], but not for stress × drug treatment interaction [F(3, 72) = 0.01, *p = 0.44] (Fig. 1B). Post hoc analysis showed that CUS markedly increased the immobile time of mice in the FST (Fig. 1B, *n = 10, *p < 0.05 vs. vehicle-alone-treated group), and this increase was reversed by 10 days of harmine treatment (10, 20 mg/kg, Fig. 1B, *n = 10, *p < 0.05 or *p < 0.01 vs. vehicle + CUS group), similar to fluoxetine treatment (Fig. 1B, *n = 10, *p < 0.05 vs. vehicle + CUS group). Chronic harmine treatment also reduced the immobile duration of naive mice in the FST (Fig. 1B, *n = 10, *p < 0.05 vs. vehicle-alone-treated group).

In the TST, two-way ANOVA revealed significant effects for stress [F(1, 72) = 35.47, *p < 0.001] and drug treatment [F(3, 72) = 7.55, *p < 0.001], but not for stress × drug treatment interaction [F(3, 72) = 0.15, *p = 0.93] (Fig. 1C). Post hoc analysis showed that CUS robustly increased the immobile time of mice in the TST (Fig. 1C, *n = 10, *p < 0.01 vs. vehicle-alone-treated group), and this increase was reversed by 10 days of harmine treatment (10, 20 mg/kg) (Fig. 1C, *n = 10, *p < 0.05 vs. vehicle + CUS group). Harmine treatment also reduced the immobile duration of naive mice in the TST (Fig. 1C, *n = 10, *p < 0.05 vs. vehicle-alone-treated group).

We then evaluated the antidepressant-like effects of harmine using the sucrose preference experiment. Two-way ANOVA revealed significant effects for stress [F(1, 72) = 44.64, *p < 0.001], drug treatment [F(3, 72) = 4.32, *p < 0.01], but not for stress × drug treatment interaction [F(3, 72) = 2.73, *p = 0.05] (Fig. 1D). Post hoc analysis showed that CUS induced a significant decrease in sucrose consumption, compared with control group (Fig. 1D, *n = 10, *p < 0.01 vs. vehicle-alone-treated group). While harmine produced no significant effects in naive mice, similar with fluoxetine treatment, 10 days of harmine (10, 20 mg/kg) treatment caused a significant increase in sucrose intake in CUS-treated mice (Fig. 1D, *n = 10, *p < 0.05 vs. vehicle + CUS group). Taken together, these results suggest that harmine is able to reverse the CUS-induced depressive-like behaviors in mice.

3.2. Harmine attenuates the CUS-induced reductions in BDNF protein levels and hippocampal neurogenesis

Adult normal mice were received daily injections of harmine (10, 20 mg/kg) for 10 days, and the protein levels of BDNF in mice hippocampus and prefrontal cortex were detected. In the hippocampus, two-way ANOVA revealed significant effects for stress [F(1, 56) = 20.64, *p < 0.001] and drug treatment [F(3, 56) = 4.83, *p < 0.01], but not for stress × drug treatment interaction [F(3, 56) = 0.04, *p = 0.99] (Fig. 2A). Post hoc analysis showed that the protein level of hippocampal BDNF was down-regulated by CUS (Fig. 2A, *n = 8, *p < 0.05 vs. vehicle-alone-treated group), and this downregulation was reversed by harmine treatment at the doses of 10 and 20 mg/kg (Fig. 2A, *n = 5, *p < 0.05 vs. vehicle + CUS group). In accordance with previous reports, chronic harmine treatment also increased the protein level of hippocampal BDNF in naive mice (Fig. 2A, *n = 8, *p < 0.05 vs. vehicle-alone-treated group).

In the prefrontal cortex, two-way ANOVA revealed significant effects for stress [F(1, 56) = 18.45, *p < 0.001] and drug treatment [F(3, 56) = 3.59, *p < 0.05], but not for stress × drug treatment interaction [F(3, 56) = 0.25, *p = 0.86] (Fig. 2B). Post hoc analysis showed that CUS reduced the protein level of BDNF in the prefrontal cortex (Fig. 2B, *n = 8, *p < 0.05 vs. vehicle-alone-treated group), and this reduction was attenuated by harmine treatment (10, 20 mg/kg, Fig. 2B, *n = 5, *p < 0.05 vs. vehicle + CUS group). Chronic harmine treatment also increased the prefrontal cortical BDNF protein level in naive mice (Fig. 2B, *n = 8, *p < 0.05 vs. vehicle-alone-treated group).

Since hippocampal neurogenesis is closely implicated in the pathogenesis of depression and can be modulated by BDNF (Kim and Leem, 2016; Sakharkar et al., 2016), we explored whether harmine affects hippocampal neurogenesis. For DCX positive cells, two-way ANOVA revealed significant effects for stress [F(1, 24) = 12.70, *p < 0.01] and drug treatment [F(2, 24) = 7.72, *p < 0.01], but not for stress × treatment interaction [F(2, 24) = 0.37, *p = 0.70] (Fig. 2C, D). For DCX protein expression levels, two-way ANOVA revealed significant effects for stress [F(1, 24) = 39.06, *p < 0.001] and drug treatment [F(2, 24) = 10.93, *p < 0.001], but not for stress × treatment interaction [F(2, 24) = 0.50, *p = 0.61] (Fig. 2E, F). Post hoc analysis showed that CUS induced a significant decrease in the number of DCX positive cells in the hippocampus (Fig. 2D, *n = 5, *p < 0.01 vs. vehicle-alone-treated group), and accordingly the expression level of DCX protein was also reduced (Fig. 2F, *n = 5, *p < 0.05 vs. control). Chronic treatment of mice with harmine (20 mg/kg) substantially reversed the CUS-induced decreases in the number of DCX positive cells (Fig. 2D, *p < 0.05 vs. vehicle + CUS group) as well as in the expression level of DCX protein in the hippocampus (Fig. 2E, *p < 0.05 vs. vehicle + CUS group).

3.3. Effects of CUS and harmine on expressions of brain GFAP, GLT-1, GS and GLAST

To determine whether astrocyte-associated markers are altered in this experiment, we investigated the effects of CUS and/or harmine on expressions of GFAP, GLAST, GLT-1, and GS protein in mice hippocampus and prefrontal cortex. For hippocampal GFAP, two-way ANOVA revealed significant effects for stress [F(1, 16) = 10.46 *p < 0.01], but not for drug treatment [F(1, 16) = 2.19, *p = 0.16] and stress × drug treatment interaction [F(1, 16) = 4.65, *p < 0.05] (Fig. 3A, B). For hippocampal GLT-1, two-way ANOVA revealed significant effects for drug treatment [F(1, 16) = 18.44, *p < 0.001], but not for stress [F(1, 16) = 1.23, *p = 0.28] and stress × drug treatment interaction [F(1, 16) = 0.02, *p = 0.97] (Fig. 3A, C). For hippocampal GS and GLAST, two-way ANOVA revealed no significant effects for stress, drug treatment and stress × drug treatment interaction (Fig. 3A, D). For prefrontal cortical GFAP, two-way ANOVA revealed significant effects for stress [F(1, 16) = 5.36, *p < 0.05] and stress × drug treatment interaction [F(1, 16) = 4.65, *p < 0.05], but not for drug treatment [F(1, 16) = 2.19, *p = 0.16] (Fig. 3E, F). For prefrontal cortical GLT-1, two-way ANOVA revealed significant effects for drug treatment [F(1, 16) = 21.55, *p < 0.001], but not for stress [F(1, 16) = 0.04, *p = 0.84] and stress × drug treatment interaction [F(1, 16) = 0.01, *p = 0.91] (Fig. 3E, G). For prefrontal cortical GS and GLAST, two-way ANOVA revealed no significant effects for stress, drug treatment and stress × drug treatment interaction (Fig. 3E, H). Post hoc analysis showed that CUS induced significant decreases in the protein expression levels of GFAP (Fig. 3A, *n = 5, *p < 0.05 vs. vehicle-alone-treated group) but not in other markers (Fig. 3A, C, D, *n = 5). No significant changes of GFAP protein expressions were observed after harmine treatment in non-stressed mice (Fig. 3A, *n = 5). However, chronic harmine treatment markedly prevented the CUS-induced decreases in GFAP protein expressions in mice hippocampus (Fig. 3A, *n = 5, *p < 0.05 vs. vehicle + CUS group) and prefrontal cortex.
Fig. 2. Effects of harmine on CUS-induced reductions in BDNF protein levels and hippocampal neurogenesis. (A, B) Statistical analysis showing the restoration effects of harmine (10, 20 mg/kg) on CUS-induced decreases in BDNF protein levels in mice hippocampus (A) and prefrontal cortex (B). (C, D) Representative images (C) and quantitative analysis (D) showing the restoration effect of harmine (20 mg/kg) on CUS-induced decrease in hippocampal DCX+ cells (n = 5, *p < 0.05, **p < 0.01 vs. vehicle alone-treated group). (E, F) Representative images (E) and quantitative analysis (F) showing the restoration effect of harmine on CUS-induced decrease in hippocampal DCX protein expressions (n = 5, *p < 0.05, **p < 0.01 vs. vehicle alone-treated group). The fluoxetine administration (20 mg/kg) was used as a positive control, and all data were shown as mean ± SEM.

(Fig. 3E, F, n = 5, p < 0.01 vs. vehicle + CUS group), demonstrating that the architecture and/or function of astrocytes may be altered by CUS and that chronic harmine treatment may block these changes. In addition, we found that harmine alone treatment induced significant increases in GLT-1 protein expressions in mice hippocampus (Fig. 3A, C, n = 5, p < 0.05 vs. vehicle alone-treated group) and prefrontal cortex (Fig. 3E, G, n = 5, p < 0.05 vs. vehicle alone-treated group). No changes were observed in hippocampal (Fig. 3A, D, n = 5) and prefrontal cortex (Fig. 3E, H, n = 5) GLAST and GS expressions following chronic harmine treatment in stressed and non-stressed mice. Increased GLT-1 protein expressions by harmine treatment were in accordance with previous studies and suggest that harmine treatment may attenuate glutamatergic hyperactivity.

3.4. Blockade of astrocytic functions abrogates the antidepressant-like effects of harmine in the FST, TST and sucrose preference experiment

To investigate the potential role of astrocytes in the antidepressant-like effects of harmine, a potent inhibitor of astrocytic functions, L-AAA (Takada and Hattori, 1986; Khurgel et al., 1996), was employed in the following experiment. The CUS-treated mice were co-injected with harmine (20 mg/kg) and L-AAA for 10 days, with behavioral tests performed 2 h after the last injection. Results showed that chronic harmine treatment markedly prevented the CUS-induced increases in the immobile time in the FST (Fig. 4A, p < 0.05 vs. vehicle + CUS group) and TST (Fig. 4B, p < 0.05 vs. vehicle + CUS group), as well as the CUS-induced decrease in sucrose intake (Fig. 4C, p < 0.01 vs. vehicle + CUS group). L-AAA co-administration abrogated these effects of harmine (Fig. 4A–C). For FST, two-way ANOVA revealed significant main effects for stress [F(1, 54) = 31.15, p < 0.001] and drug treatment [F(2, 54) = 9.09, p < 0.001], but not for stress × drug treatment interaction [F(2, 54) = 0.24, p = 0.78] (Fig. 4A). For TST, ANOVA revealed significant main effects for stress [F(1, 54) = 28.75, p < 0.001] and drug treatment [F(2, 54) = 4.87, p < 0.05], but not for stress × drug treatment interaction [F(2, 54) = 0.18, p < 0.83] (Fig. 4B). For sucrose preference, ANOVA revealed significant main effects for stress [F(1, 54) = 41.08, p < 0.001] and drug treatment [F(2, 54) = 6.45, p < 0.01], but not for stress × drug treatment interaction [F(2, 54) = 3.16, p = 0.05] (Fig. 4C).
3.5. Blockade of astrocytic functions prevents the improvement effects of harmine on brain BDNF protein levels and hippocampal neurogenesis

The harmine-induced increase in hippocampal BDNF protein levels (Fig. 5A, p < 0.05 vs. vehicle + CUS group) in stressed mice were blocked by L-AAA co-administration. In the prefrontal cortex of stressed mice, L-AAA co-administration also abolished the restoration effect of harmine on the protein level of BDNF (Fig. 5B, p < 0.05 vs. vehicle + CUS group). For hippocampal BDNF, two-way ANOVA revealed significant effects for stress [F(1, 42) = 20.04, p < 0.001] and drug treatment [F(2, 42) = 3.88, p < 0.05], but not for stress × drug treatment interaction [F(2, 42) = 0.12, p = 0.89] (Fig. 5B).

Further studies showed that the astrocyte was essential for the effect of harmine on hippocampal neurogenesis, as L-AAA co-administration blocked the increases in hippocampal neurogenesis (Fig. 5C, D, p < 0.05 vs. vehicle + CUS group) and DCX protein expressions (Fig. 5E, F, p < 0.05 vs. vehicle + CUS group) in harmine-treated mice. For DCX positive cells, two-way ANOVA revealed significant effects for stress [F(1, 24) = 104.10, p < 0.001] and drug treatment [F(2, 24) = 12.45, p < 0.001], but not for stress × drug treatment interaction [F(2, 24) = 0.15, p = 0.86] (Fig. 5D).

4. Discussion

One of the major contributions of this study is the identification of the antidepressant-like effects of harmine, which have been reported in previous studies (Fortunato et al., 2009; Fortunato et al., 2010; Aricioglu and Altunbas, 2003; Farzin and Mansouri, 2006). The major difference between our and other’s studies is that we evaluated the antidepressant-like effects of harmine in stressed conditions, while the others did it in normal animals. On the face of it, there seems to be nothing new for this difference. However, the disease-dependent effect existing in the evaluation of antidepressants suggests that evaluation of antidepressants in stressed conditions is more important and necessary than that in normal conditions. Fortunately, harmine, in normal conditions, has successfully been confirmed to have antidepressant-like effects, and thus our results provide further evidence to prove the antidepressant-like effects of harmine. But for others, the stress environment would help evaluate their antidepressant-like activities properly. For example, in untreated control mice, chronic treatment with fingo-lomod, the first oral drug approved for the treatment of relapsing-remitting multiple sclerosis, caused no changes in BDNF protein levels and even a significant reduction in BDNF mRNA levels in the hippocampal, whereas in mice treated with chronic stresses, fingo-lomod administration markedly prevented depressive-like behaviors (di Nuzzo et al., 2015).

Recently, harmine is becoming a star compound, as it has been
selected from > 100,000 different chemicals with β cell growth-promoting effects (Wang et al., 2015). However, harmine is also famous as one of the ingredients in the psychoactive mixture ayahuasca, and is traditionally used by some indigenous people for religious purposes. In this study, we used several different methods, including the FST, TST and sucrose preference experiment, to evaluate the antidepressant-like effects of harmine, and found that chronic daily injections of harmine (10, 20 mg/kg) markedly improved the behavioral impairments induced by CUS, and the antidepressant-like effects of harmine were similar to that of fluoxetine. Harmine's effects on major depression have been found in previous studies (Fortunato et al., 2009; Fortunato et al., 2010; Aricioglu and Altunbas, 2003; Farzin and Mansouri, 2006), and in those studies, only the FST was used to evaluate the antidepressant-like effects of harmine. Harmine's effects on major depression have been found in previous studies (Fortunato et al., 2009; Fortunato et al., 2010; Aricioglu and Altunbas, 2003; Farzin and Mansouri, 2006), and in those studies, only the FST was used to evaluate the antidepressant-like effects of harmine. In the current study, two other experiments with a high predictive validity for antidepressant activities, TST and sucrose preference experiments, were employed to assess the effect of harmine in depression. These experiments would help pay the way to understand the characteristic of harmine in depression therapy. Recently, ayahuasca, a harmine-containing plant, has been shown to produce a rapid and sustained antidepressant-like effect in depressed patients, establishing stronger correlations between harmine and depression (Osório Fde et al., 2015; Sanches et al., 2016). However, since ayahuasca also contains other substances, such as dimethyltryptamine, an agonist of serotonin (5-HT) 2A receptor actively involved in the pathogenesis of depression, evaluation of the correlation between harmine and depression using ayahuasca should be cautious, and thus although our results is beneficial for the development of harmine as a novel antidepressant, these results just provide preclinical evidence to strengthen the antidepressant-like activities of harmine.

The impairment of hippocampal neurogenesis is an important pathophysiological event in depression formation (Serafini et al., 2014; Rotheneichner et al., 2014). Most current clinical antidepressants have been shown to exert antidepressant-like effects via restoration of hippocampal neurogenesis (Pascual-Braza et al., 2014; Tang et al., 2012). We showed here that chronic stress impaired hippocampal neurogenesis, and harmine treatment markedly reversed this impairment. BDNF is a critical molecule upstream of hippocampal neurogenesis (Jiang et al., 2015a; Kim and Leem, 2016; Sakharkar et al., 2016).
Hippocampal BDNF has been found to be reduced in depressed patients (Schmidt and Duman, 2007). Some clinical and preclinical antidepressants regulate depressive-like behaviors via activation of BDNF signals (Jiang et al., 2015a; Alboni et al., 2017; Liu et al., 2016). Searching agents that increase BDNF expressions would help develop new antidepressants. Our results showed that harmine markedly reversed the CUS-induced decreases in hippocampal and prefrontal cortical BDNF protein levels, further underscoring the importance of BDNF in the antidepressant-like effects of harmine (Fortunato et al., 2009; Fortunato et al., 2010). However, how harmine increases BDNF protein levels remains to be determined in the future study.

Astrocytes are the most abundant form of cells in the brain. Numerous studies employing depressed patients and animals show that astrocyte pathology mediates the pathogenesis of depression. First, the decreases of astrocyte-expressing GFAP have been seen in genetically stress susceptible Wistar Kyoto rat (Gosselin et al., 2009), as well as in mice treated with CUS (Ye et al., 2011) and maternal deprivation (Leventopoulos et al., 2007). Second, the integrity of hippocampal astrocytic plasticity mediates the antidepressant-like effects of some clinical antidepressants (Zarei et al., 2014), and the rapid antidepressant-like effect of ketamine correlates astroglial plasticity in the hippocampus (Ardalan et al., 2017). Furthermore, local infusion of a gliotoxin in the prefrontal cortex resulting in the decrease in GLT-1 and astrocyte numbers is sufficient to induce depressive-like phenotypes similar to that observed in CUS conditions (Banasr and Duman, 2008). Third, strong evidences of stress-related impairments of astrocyte functions have been reported in recent years. For instance, the glial-specific EAATs and GS reflecting the glutamate clearance and metabolism ability of astrocytes are reduced in some regions of depressed brains (Choudary et al., 2005; Bernard et al., 2011). The impairment of astrocyte functions induces the brain incapable of clearing extracellular glutamate, ultimately resulting in an altered ratio of synaptic to extrasynaptic glutamate content as well as an altered neurotransmission that is considered to contribute to the pathogenesis of neuropsychiatric disorders including major depression. In this study, we showed that CUS reduced GFAP protein expressions in the prefrontal cortex and hippocampus, and these reductions were reversed by harmine treatment. GFAP is an intermediate filament protein that is traditionally considered a marker of astrocytes. Its expression can be modulated by neurodegenerative and neuropsychiatric stimuli (Olivera-Bravo et al., 2011; Schreiner et al., 2015). Although whether the reduction of astrocytic GFAP in depressed individuals is directly associated with depression remains unclear, the decrease of GFAP to some extent reflects the impairment of astrocytic functions and behavioral deficits. Thus, the regulating effect of harmine on GFAP expressions indicates that the astrocyte may be involved in the antidepressant-like effects of harmine. This hypothesis was further supported by the up-regulation effects of harmine on the protein expression levels of hippocampal and prefrontal cortical GLT-1, though CUS itself did not alter GLT-1 and EAATs expressions. It is well-known that the micro-environmental glutamate is increased abnormally in the brain in depressed animals and patients (Jia et al., 2015; Dávalos et al., 2000). Since the increased glutamate is postulated to mediate the pathogenesis of depression (Paul and Skolnick, 2003; Deutschenbaur et al., 2016), promotion of glutamate clearance would be beneficial for depression therapy. Harmine, because of its GLT-1 upregulation effect, is a potential candidate for that purpose. In fact, previous studies have already provided several similar candidates for that purpose. For example, ceftriaxone, a clinical available drug that increases glutamate transport, has been reported to exhibit antidepressant-like properties in the FST, TST, and novelty suppressed feeding experiment (Mineur et al., 2007). Riluzole, a Food and Drug Administration-approved drug for the treatment of ALS, also ameliorates depressive-like behaviors via up-regulation of GFAP and GLT-1 protein expression and promotion of glutamate clearance (Banasr et al., 2010).

The involvement of brain astrocytes in the antidepressant-like effects of harmine was further ascertained by the astrocyte inhibition experiment, which showed that injection of mice with L-AAA, a blocker of astrocytic functions (Banasr and Duman, 2008), prevented the amelioration effects of harmine on depressive-like behaviors in the experiments of FST, TST and sucrose preference. This finding provided a direct evidence to uncover the potential role of astrocytes in the antidepressant-like effects of harmine, and would help understand the indepth mechanism of action of harmine in major depression. The reason for that saying is that i) astrocyte dysfunction is now considered an important factor that determines depression formation (Ye et al., 2011; Leventopoulos et al., 2007); ii) some well-known targets for antidepressants, such as 5-HT receptors and monoamine oxidase (MAO), are tightly correlated with astrocyte functions. For example, the 5-HT1A (Eriksen et al., 2002) and 5-HT2A receptor (Maxishima et al., 2001) have been confirmed to be expressed in astrocytes. Their activation may protect neurons against injuries induced by transient global cerebral ischemia (Lee et al., 2015) or Parkinson’s disease (Miyazaki et al., 2013). Reactive astrocytes can produce the inhibitory glutamate transporter γ-aminobutyric acid (GABA) by MAO-B, and the released GABA reduces spike probability of granule cells by acting on presynaptic GABA receptors (Jo et al., 2014). Thus, it is reasonable speculate that the mutual interaction between astrocytes and antidepressant targets may co-ordinate neuronal functions during stress stimulation. Further investigation of the mutual interaction between astrocytes and antidepressant targets would promote the understanding about the role of harmine and other hallucinogens, such as lysergic acid diethylamide (LSD) (Dos Santos et al., 2016) and psilocybin (Carhart-Harris et al., 2016), in depression regulation.

Further analysis showed that the harmine-induced increases in hippocampal and prefrontal cortical BDNF protein levels in CUS-treated mice were blocked by L-AAA treatment, demonstrating that the BDNF signal may be necessary for the regulation of depressive-like behaviors by endogenous astrocytes. BDNF has been considered to regulate depressive-like behaviors through promotion of hippocampal neurogenesis (Jiang et al., 2015a; Kim and Leem, 2016; Sakharkar et al., 2016). Our results showed that L-AAA co-administration abrogated the promoting effect of harmine on hippocampal neurogenesis. Previous studies also showed that specific overexpression of BDNF in hippocampal astrocytes promotes local neurogenesis and elicits anxiolytic activities (Quesseveur et al., 2013), suggesting that BDNF improves depressive-like behaviors and hippocampal neurogenesis likely through enhancement of astrocytic functions. Taken together, all these findings emphasize the importance of brain astrocytes in harmine’s effects on depression.

5. Conclusions

Our results are in consistent with a growing number of studies showing that astrocyte dysfunction contributes critically to the development of depression (Ye et al., 2011; Leventopoulos et al., 2007; Oh et al., 2012; Ménard et al., 2016). Harmine, a natural-derived drug reported to increase GLT-1 expression (Li et al., 2011; Sun et al., 2014), reversed the behavioral deficits induced by CUS. The potential involvement of GLT-1 in harmine’s effects on depression is supported by previous studies, which showed that ceftriaxone (Mineur et al., 2007) and riluzole (Banasr et al., 2010), two compounds with GLT-1 increasing effects, ameliorates depressive-like behaviors in depressed animals or patients. The antidepressant-like effects of harmine were also evidenced by the astrocyte-mediated restoration of BDNF protein levels and hippocampal neurogenesis. These mechanistic findings not only provide a further validation for the hypothesis of astrocyte dysfunction in depression formation, but also help understand the potential role of harmine in depression therapy.
Ethical Statements

All animal experiments in this study were conducted in accordance with internationally accepted guidelines for the use of animals in toxicology as adopted by the Society of Toxicology in 1999 and approved by the University Animal Ethics Committee of Nantong University (Permit number: 2110836).

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