Acta Pharmaceutica Sinica B 2021;11(7):1903−1913

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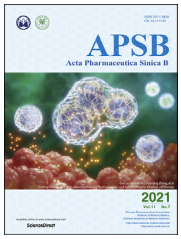

ORIGINAL ARTICLE

Protective effects of VMY-2-95 on corticosterone-induced injuries in mice and cellular models

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Received 9 August 2020; received in revised form 29 September 2020; accepted 21 October 2020

KEY WORDS

α4β2 nAChR antagonist;
VMY-2-95;
Cholinergic−adrenergic theory;
Depression;
Corticosterone;
SH-SY5Y cells;
PKA-CREB-BDNF signaling pathway

Abstract Nicotinic α4β2 receptor antagonists have drawn increasing attention in the development of new antidepressants. In this study, we aimed to investigate the protective effect of VMY-2-95, the new selective antagonist of α4β2 nicotinic acetylcholine receptor (nAChR) on corticosterone (CORT) injured mice and cellular models. Fluoxetine was applied as a positive control, and the effects of VMY-2-95 were investigated with three different doses or concentrations (1, 3, 10 mg/kg in mice, and 0.003, 0.03, 0.1 μmol/L in cells). As a result, VMY-2-95 showed significant antidepressant-like effects in the CORT injured mice by improving neuromorphic function, promoting hippocampal nerve proliferation, and regulating the contents of monoamine transmitters. Meanwhile, VMY-2-95 exhibited protective effects on cell viability, cell oxidant, cell apoptosis, and mitochondrial energy metabolism on corticosterone-impaired SH-SY5Y cells. Also, the PKA−CREB−BDNF signaling pathway was up-regulated by VMY-2-95 both *in vitro* and *in vivo*, and pathway blockers were also combined with VMY-2-95 to verify the effects furtherly. Therefore, we preliminarily proved that VMY-2-95 had protective effects in depressed mice and SH-SY5Y cells against injuries induced by corticosterone. This work indicated that the application of VMY-2-95 is a potential pharmacological solution for depression. This study also supported the development of α4β2 nAChR antagonists towards neuropsychiatric dysfunctions.

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2021.03.002

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1. Introduction

Depression is a public health threat because it is one primary reason for a person to suicide, and is one of the leading causes of disability around the world[1]. Approximately 800,000 people die of suicide each year due to depressive disorders, and such disorders cause a considerable long-term burden to the general economy and public health system[2]. Although cumulative studies have elucidated the pathophysiology of depression, the antidepressants are still limited to targeting the monoaminergic systems. In clinical studies, current antidepressants are found to be effective in only two-third of depression cases[3], and they typically need to take a long period to attenuate the depressive symptoms in patients[4].

The cholinergic−adrenergic theory of depression was first proposed in 1972. It has drawn more and more attention[5]. The hyperactivation of the cholinergic system breaks the homeostasis between cholinergic and noradrenergic systems, leading to the inactivation of choline receptors and triggering depressive symptoms. Receptors are the material basis for the action of the acetylcholine system. $\alpha4\beta2$ nicotinic acetylcholine receptor (nAChR)[6] and $\alpha7$ nAChR[7] are the two antidepressant drug targets mostly discussed in recent years. Most $\alpha4\beta2$ nAChR antagonists and a part of $\alpha4\beta2$ nAChR agonists were found to exhibit antidepressant effects in both clinical and preclinical studies[8−11]. Varenicline, a smoking cessation drug targeting $\alpha4\beta2$ nAChR, was observed to ease the emotion in depressed patients[12]. In animal studies, sazetidine-A and A-85380, the $\alpha4\beta2$ nAChR ligands, showed antidepressant-like effects in tail suspension and forced swimming tests[13−21]. Thus, as it is necessary to develop the new rapid-acting antidepressants with fewer side-effects and better efficacy, and $\alpha4\beta2$ nAChR might be a potential therapeutic target on antidepressants. However, the effects of $\alpha4\beta2$ nAChR in the central nervous system are still not apparent, and the research on efficacy and mechanism of $\alpha4\beta2$ nAChR ligands is not detailed.

Stress is considered as the most common and essential factor that leads to depression, and stress paradigms have long been used to mimic critical symptoms of depression[22]. Repeated corticosterone (CORT) treatment induces depression-like behaviors that marked by significant changes in behavioral traits, neurochemistry, and brain morphology[23,24]. The hypothalamic−pituitary−adrenal (HPA) axis activated in response to stress in depressed patients. A high concentration of blood glucocorticoids (GCs) is reported due to the dysfunction in the feedback mechanism, and then the elevated GCs cause further dysfunction of the HPA axis[25]. Another result is the reduced expression of the brain-derived neurotrophic factor (BDNF), and BDNF is a critical regulator of synaptic plasticity[26,27]. Therefore, CORT-induced stress-based mouse model and cell injury model are suitable for evaluating the efficacy and mechanisms of potential antidepressants.

VMY-2-95 (Fig. 1), a nAChRs ligand with picomolar affinity and high selectivity for $\alpha4\beta2$ receptor, has drawn our attention[28]. We prepared the compound of VMY-2-95·2HCl to get better solubility. Research showed that VMY-2-95·2HCl was soluble in water and chemically stable, and VMY-2-95·2HCl had right drug-like properties and can penetrate the blood−brain barrier with oral administration. The discovery and development of VMY-2-95 may provide new ligand for evaluation in models of depression[29]. In this study, the effects of selective nicotinic $\alpha4\beta2$ receptor antagonist VMY-2-95 and the mechanism after CORT treatment are also discussed.

2. Materials and methods

2.1. Drugs and reagents

Compound VMY-2-95·2HCl (purity>99%) was synthesized by the Medical Center for Drug Discovery of Georgetown University (Washington DC, USA), and the structure is shown in Fig. 1. Fluoxetine was purchased from Eli Lilly and Co., USA. CORT was purchased from Aladdin, China. H89 and J147 were purchased from Targetmolecule Corp., USA.

Sodium nitroprusside and 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma−Aldrich Chemical Co., USA. Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from PAN Seratech Co., Germany. All other reagents were of commercially available analytical grade. Antibodies, including PKA/P-PKA and CREB/P-CREB, were purchased from Cell Signaling Technology, USA. GAPDH was from Proteintech, USA; Ki67, dopamine 2 receptor (D2R), c-FOS and BDNF were from Abcam, UK; $\beta2$ nAChR was from Alomone Labs, Israel; 5-hydroxytryptamine 1 receptor (5-HT1AR) was from Novus, USA; and anti-rabbit IgG (H+L) was purchased from Proteintech, USA.

Lactate dehydrogenase (LDH) Cytotoxicity Assay Kit, Total Glutathione Peroxidase (GPs) Assay Kit with NADPH, Lipid Peroxidation Malondialdehyde (MDA) Assay Kit, Cu/Zn-superoxide dismutase (SOD) and Mn-SOD Assay Kit, Total Antioxidant Capacity (T-AOC) Assay Kit with a rapid ABTS method, Total Nitric Oxide (NO) Assay Kit and Annexin V-FITC Apoptosis Detection Kit were purchased from Beyotime Biotechnology, China. Mitochondrial Complex I Detection Kit was purchased from Jiang Lai Biotechnology, China. ELISA kits were purchased from Beijing Dongge Biotechnology Co., China.

2.2. Animals and grouping

C57BL/6 male mice (6−8 weeks old, weighing 18−22 g) were purchased from Laboratory Animal Centre of Beijing Hua-Fu-Kang Bioscience Co., Ltd. [Beijing, China; Animal certification number was SCXK (Jing) 2014-0004] at the beginning of the experiments. All animals were housed in a room with a controlled temperature of 23 ± 2 °C and humidity of 55 ± 5% with a regular 12 h light−dark cycle and allowed to have water and food *ad libitum*. Each experimental group consisted of 5−6 animals and all animals were adapted to the laboratory conditions for three days. All animal care and experimental procedures regarding the animals were approved by the Ethic Committees of the Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China.

A total of 72 C57BL/6 mice were randomly divided into 6 groups: control, model, fluoxetine (3 mg/kg), VMY-2-95 low dose (1 mg/kg), VMY-2-95 middle dose (3 mg/kg) and VMY-2-95 high

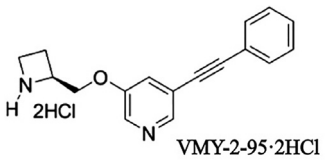

Figure 1 The structure of VMY-2-95·2HCl.

dose (10 mg/kg) groups. Each group except the control group was intraperitoneally injected with 20 mg/kg CORT daily, and intragastrically administrated of fluoxetine and VMY-2-95 at 1 μL/10 g daily after CORT injection from Day 21 to Day 28. The control group mice were injected or administrated with volume-equal saline.

2.3. Behavioral tests

All the behavioral tests were carried out between 08:30 and 17:30 and were matched between the groups. The observers were blind to the treatment. On Day 28, tail suspension test (TST) and forced swimming test (FST) were detected, and on Day 29, sucrose preference test (SPT) and open-field test (OFT) were conducted. See specific steps in Supporting Information.

2.4. Sacrifice and sample preparation

On Day 30, mice were anesthetized with ether, and blood samples were collected by retro orbital puncture. Then the eyeball blood was coagulated for 30 min at room temperature, centrifuged to separate plasma, and stored at −20 °C. Among them, three mice of every group transcardially perfused with PBS, followed by 4% formaldehyde fixation and the whole brain samples were post-fixed in 4% paraformaldehyde to perform immunohistochemistry analyses, and the hippocampus of the rest mice brains were preserved after blood collection and stored at −80 °C for Western blotting.

2.5. Neuronal morphology testing

Histologic staining was performed using cresyl violet Nissl stain to study the number, structural integrity of neurons, and the cell fullness in the hippocampus of mice.

2.6. Assay of neurotransmitter contents

The content of dopamine (DA), 5-hydroxytryptamine (5-HT), acetylcholine (ACh), norepinephrine (NE), CORT, adreno-corticotropic-hormone (ACTH), and corticotropin releasing hormone (CRH) in the plasma supernatant was estimated on a microplate reader of which the absorbance was read at 450 nm according to the guidelines of the ELISA kit as per the methodology provided by the manufacturer.

2.7. Immunohistochemistry analyses

After 4% formaldehyde fixation of the whole brain, immunohistochemistry analyses were used to detect the expression of the 5-HT receptor, DA receptor, c-FOS, Ki67, and BDNF in the dentate gyrus (DG) region of the hippocampus.

2.8. Cell culture and treatment

SH-SY5Y cell line was obtained from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere incubator. The medium was refreshed every three days.

2.9. Cell viability and LDH cytotoxicity assay

The cells were collected when they had grown to 80%−90% confluence, then the cells were treated with the different concentrations of CORT for 24 h or preincubated with VMY-2-95 at gradient concentration (0.003, 0.03 and 0.1 μmol/L) for 2 h before the CORT exposure. The control group cells were added with volume-equal medium without CORT, and the model group had no preincubation with VMY-2-95. Then the supernatant was aspirated for LDH assay, and MTT assay (0.5 g/L) was conducted to 96-well plate for 4 h at 37 °C. The LDH was measured at 490 nm and cell viability at 570 nm using a microplate reader. Cell viability and cytotoxicity/mortality were calculated according Eqs. (1) and (2):

$$\text{Cell viability } (\%) = (A_{\text{experiment}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100 \quad (1)$$

$$\text{Cytotoxicity or mortality } (\%) = (A_{\text{experiment}} - A_{\text{blank}})/(A_{\text{absorbance of cell maximum enzyme activity}} - A_{\text{blank}}) \times 100 \quad (2)$$

2.10. Assay of extracellular NO

The content of NO was measured following the instructions of the kit using the Griess reaction. The absorbance was measured at 540 nm.

2.11. Assay of GPs, MDA, SOD, T-AOC and adenosine triphosphate (ATP) in SH-SY5Y cells

After incubating the cells with drug for a corresponding period of time, the contents of GPs, MDA, SOD as antioxidant indexes and the T-AOC were tested, and the contents of ATP and the activity of mitochondrial complex I were also tested on a microplate reader according to the guidelines of assay kits.

2.12. Assay of cytochrome c (Cyt-c) contents

The contents of Cyt-*c* in the cell culture fluid were estimated on a microplate reader of which the absorbance was read at 450 nm according to the guidelines of the ELISA kit as per the methodology provided by the manufacturer.

2.13. Flow cytometry detection of apoptosis

The cells were trypsinized and resuspended by centrifugation to make cell suspension. Annexin V-FITC binding fluid, Annexin V-FITC, and propidium iodide staining solution were added in order and then tested in BD FACSAria II flow cytometry within 1 h.

2.14. Western blotting

The lysate was added into mice hippocampus tissue and SH-SY5Y cells, and then supernatant after centrifugation was collected, and the protein concentration was determined using bicinchoninic acid protein assay kits. After mixed with 5× loading buffer and heated at 100 °C for 15 min, protein extracts were separated by a sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The blots were blocked using 5% bovine serum albumin in Tris buffered saline with TBST buffer for 1.5 h and then were incubated at 4 °C overnight with respective primary antibodies diluted in 1% bovine serum albumin for anti-P-PKA

antibody (1:1000), anti-P-CREB (1:1000), anti-BDNF (1:1000) or GAPDH (internal control, 1:1000). Subsequently, membranes were washed using TBST for 4 × 5 min and were incubated with secondary antibody conjugated to horseradish peroxidase (1:5000) for 1 h at room temperature. Then protein membranes were washed in the same way, and finally, bands were detected by enhanced chemiluminescence Western blotting detection reagents. Membranes marking P-PKA and P-CREB were re-incubated with primary antibodies for PKA (1:1000) and CREB (1:1000) after stripping for 15 min, and the next steps were as mentioned above.

2.15. *Statistical analysis*

All data were analyzed using the SPSS 22.0 software package and expressed as mean ± standard error means (SEM) at the animal

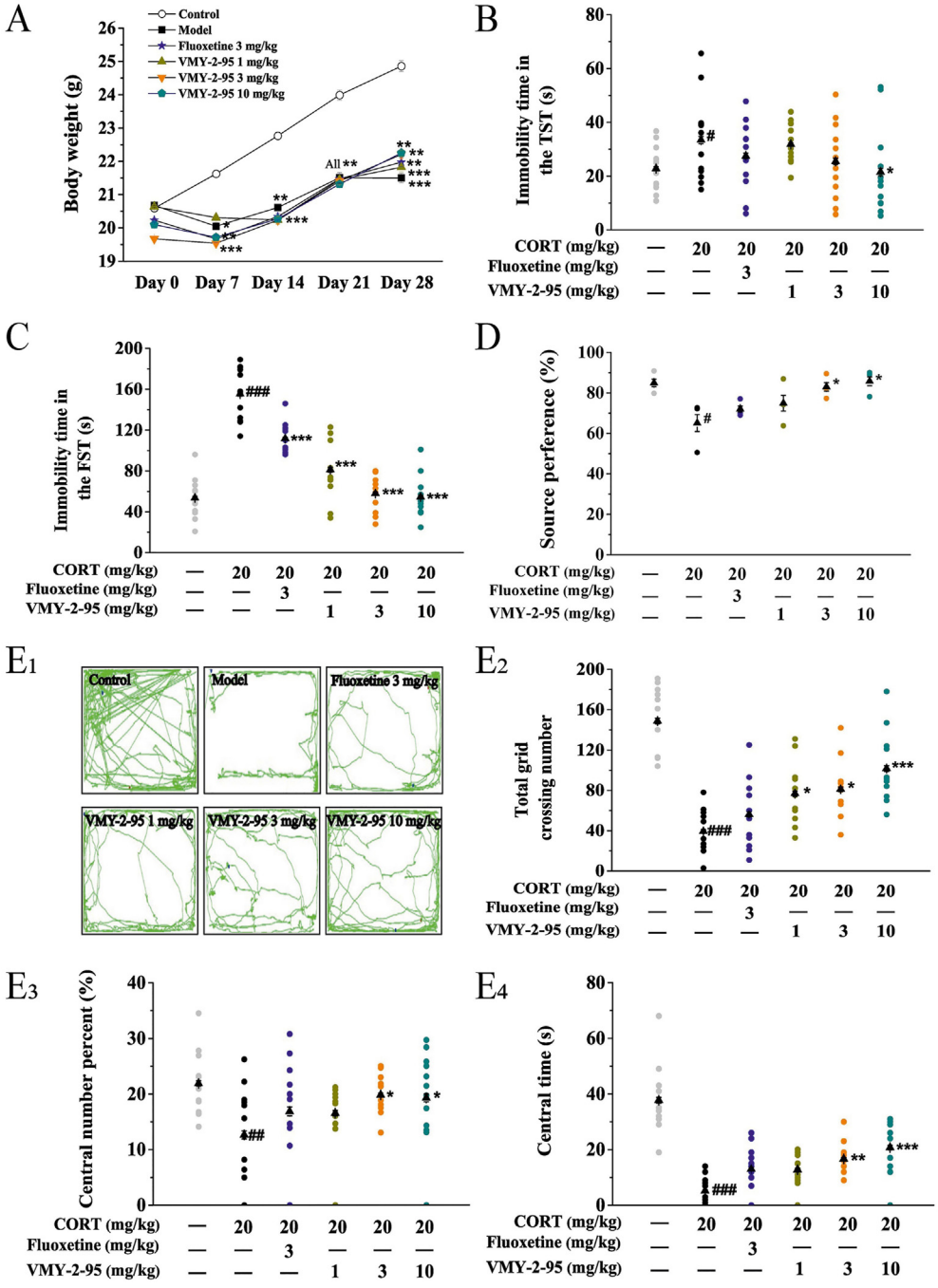

Figure 2 Behavior improving effects of VMY-2-95 in mice induced by corticosterone (CORT). (A) Changes in body weight, (B) immobility time in the tail suspension test (TST), (C) immobility time in the forced swimming test (FST), (D) changes in percent of sucrose preference, (E_1) motion track in the open-field test (OFT), (E_2) total crossing number in the OFT, (E_3) percent of central grid crossing number in the OFT, and (E_4) central grid crossing time in the OFT. Data are presented as mean ± SEM, $n = 12$. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* control group in A; and $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *versus* control group, and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* model group in B−E.

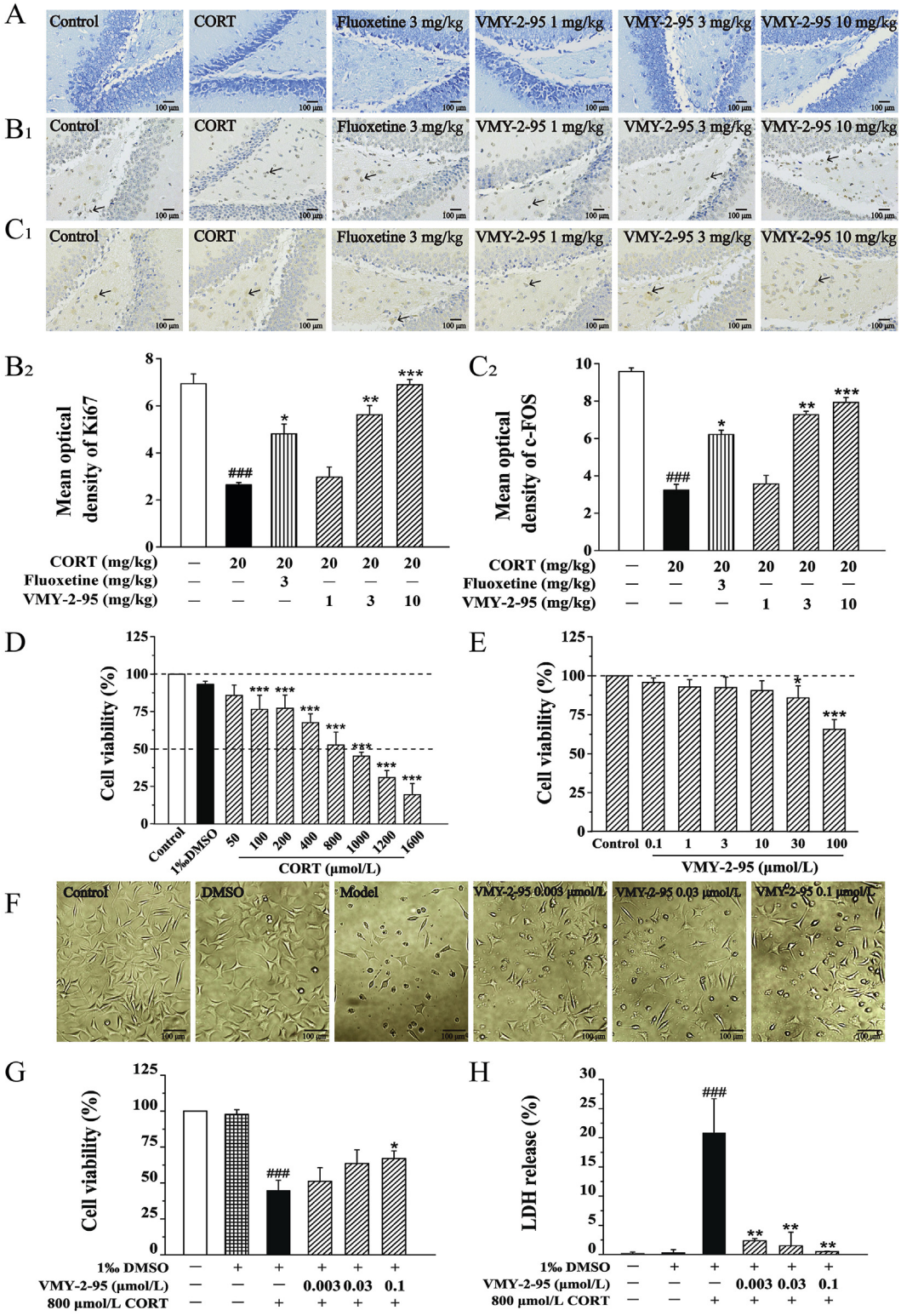

Figure 3 The effects of VMY-2-95 on neuron morphology and proliferation. (A) Nissl denaturation staining in the hippocampus (400×, scale bar indicates 100 μm), (B_1) immunohistochemistry pictures of Ki67 in different groups of mice (400×, scale bar indicates 100 μm), (B_2) the mean optical density of Ki67 in hippocampus, (C_1) immunohistochemistry pictures of c-FOS in different groups of mice (400×, scale bar indicates 100 μm), (C_2) the mean optical density of c-FOS in hippocampus, (D) the effect of different concentrations of corticosterone (CORT) on the viability of cells, (E) the effect of different concentrations of VMY-2-95 on the viability of cells, (F) the effect of VMY-2-95 on the cell morphology (200×, scale bar indicates 100 μm), (G) the protect effect of VMY-2-95 on the cell viability, and (H) the protect effect of VMY-2-95 on the cytotoxicity. Data are presented as mean ± SEM, $n = 3$ in mice; and data are represented as the mean ± SD, $n = 3$ in SH-SY5Y cells. $^{*}P < 0.05$, $^{***}P < 0.001$ *versus* control cells in D and E; and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* control group, and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* model group in the others.

behavior tests and mean ± standard deviation (SD) at the tissue and cell level. The figure was drawn by Origin 2018, Adobe Photoshop CC 2018, and Adobe Illustrator 2018 software. Results *in vitro* were analyzed by T-test and one-way ANOVA followed by Tukey's *post hoc* test. Probability (P) values less than 0.05 were considered statistically significant.

3. Results

3.1. VMY-2-95 modified the depression-like and anxiety-like behaviors of mice with CORT treatment

After CORT injection, the mice showed significant weight drop. While VMY-2-95 and fluoxetine were observed to increase the mice body weight after one week of treatment (Fig. 2A). Mice received CORT injection also showed distinct depression-like behaviors in the TST, FST, and SPT. VMY-2-95 significantly decreased the immobility time in TST (Fig. 2B) and FST (Fig. 2C), and increased the percent of sucrose preference (Fig. 2D) in SPT. Moreover, the antidepressant-like effects of VMY-2-95 at 3 and 10 mg/kg dose were similar in FST, SPT, and were better than those of fluoxetine. In the open field test, the effect of VMY-2-95 and fluoxetine treatment can be visually reflected by the mice's motion track (Fig. 2E_1). After 4 weeks of CORT injection, mice of the model group showed significantly reduced total crossing number, central grid crossing number percent and time compared with the control mice. VMY-2-95 at three different doses all significantly reversed these changes reflecting avoidance behaviors of mice, and the effect of fluoxetine was similar as that of low-dose VMY-2-95 (Fig. 2E_2−E_4).

3.2. VMY-2-95 protected the morphology and vitality of neurons against the injury induces by CORT

As shown in Fig. 3A, the neurons in the hippocampus were sparse in the CORT model group, the number of cell layers significantly reduced, and cell shrinkage and fragmentation occurred. VMY-2-95 administration conferred excellent protection in tissue morphology. The expression of Ki67, an indicator for detecting cell proliferation activity in mice, was significantly decreased, and the expression of c-FOS was also significantly decreased induced by the stress response. VMY-2-95 significantly alleviated these changes (Fig. 3B and C). As shown in Fig. 3D, we chose different concentrations at 50, 100, 200, 400, 800, 1000, 1200, and 1600 μmol/L of CORT to detect the cell viability. The CORT was found to inhibit the proliferation of SH-SY5Y cells with a significant concentration-dependent manner. Furthermore, we chose 800 μmol/L CORT, of which the inhabitation was around 50% to carry out the follow-up injury study. Then the effects of different concentrations at 0.1, 1, 3, 10, 30, and 100 μmol/L of VMY-2-95 on the cell viability were detected, which were toxic to cells only when concentrations were above 30 μmol/L (Fig. 3E). As shown in Fig. 3F and G, 0.1 μmol/L VMY-2-95 showed significant protection on the cell viability and morphology against injury induced by CORT, and consistent with this, VMY-2-95 significantly decreased the LDH content of CORT-injury SH-SY5Y cells (Fig. 3H).

3.3. VMY-2-95 regulated transmitter contents and receptor expression

As shown in Fig. 4A and B, the ACh content in the mice injected with CORT significantly increased compared with the control group, and the NE content significantly decreased. Fluoxetine and VMY-2-95 increased the content of NE, but fluoxetine had no effect on the content of ACh, and VMY-2-95 made the content of ACh higher than that of the model group. As shown in Fig. 4D, the 5-HT content of the mice injected with CORT significantly decreased compared with that of the control group, and VMY-2-95 alleviated the change. The mean optical density of DA receptors and 5-HT receptors in the DG region of the hippocampus was also detected, and we found that the expression of D2R and 5-HT1AR were both significantly decreased. VMY-2-95 increased the expression of 5-HT1AR, but fluoxetine did not affect the changes of both D2R and 5-HT1AR (Fig. 4E and F). Around the HPA axis, we tested the contents of related transmitters in mice. Results show that CRH, ACTH and CORT of the model mice significantly increased, and VMY-2-95 effectively reduced the excessive release of these three transmitters (Fig. 4G).

3.4. VMY-2-95 exerted neuroprotective effects on SH-SY5Y cells through different mechanisms

As shown in Fig. 5A−E, VMY-2-95 significantly inhibited the increased NO and MDA content induced by CORT, and increased GPs and SOD content with a concentration-dependent manner, thus significantly increased the total antioxidant capacity of SH-SY5Y cells. As shown in Fig. 5F, flow cytometry showed that CORT caused significant cell apoptosis, and VMY-2-95 significantly increased cell viability and inhibited the apoptosis with a good dose−effect relationship. Meanwhile, CORT caused a significant increase in the content of Cyt-*c*, and VMY-2-95 significantly decreased this change in SH-SY5Y cells (Fig. 5G). Furthermore, as shown in Fig. 5H and I, VMY-2-95 significantly increased the relative value of intracellular ATP level and the activity of mitochondrial complex I on SH-SY5Y cells, which provided reliable protection for mitochondrial energy metabolism.

3.5. VMY-2-95 had effects on the PKA−CREB−BDNF signaling pathway

As shown in Fig. 6A and B, both the PKA and CREB phosphorylation levels in the hippocampus of mice injected with CORT were significantly decreased compared with those of the control group, and the expression levels of BDNF was also significantly decreased (Fig. 6C and D). Fluoxetine and VMY-2-95 ameliorated these changes, and VMY-2-95 was found to significantly increase the expression of P-PKA, P-CREB, and BDNF. Consistent with the mice experiment results, pretreatment with VMY-2-95 significantly ameliorated this down-regulation of P-PKA, P-CREB and BDNF expression in SH-SY5Y cells (Fig. 6E−G). We retested the protective effect of VMY-2-95 when combined with PKA inhibitor H89 and BDNF inhibitor J147. Results show that compound VMY-2-95 almost lost its protection on SH-SY5Y cells when the PKA-BDNF pathway blocked. Both the cell viability and NO release content had no more significant difference compared with those of the model group (Fig. 6H and I).

4. Discussion

Depression, the most prevalent psychiatric disorder, is characterized by increased negative affect (*i.e.*, depressed mood) and reduced positive affect (*i.e.*, anhedonia)[30]. The development of antidepressants with new targets needs to be studied as soon as

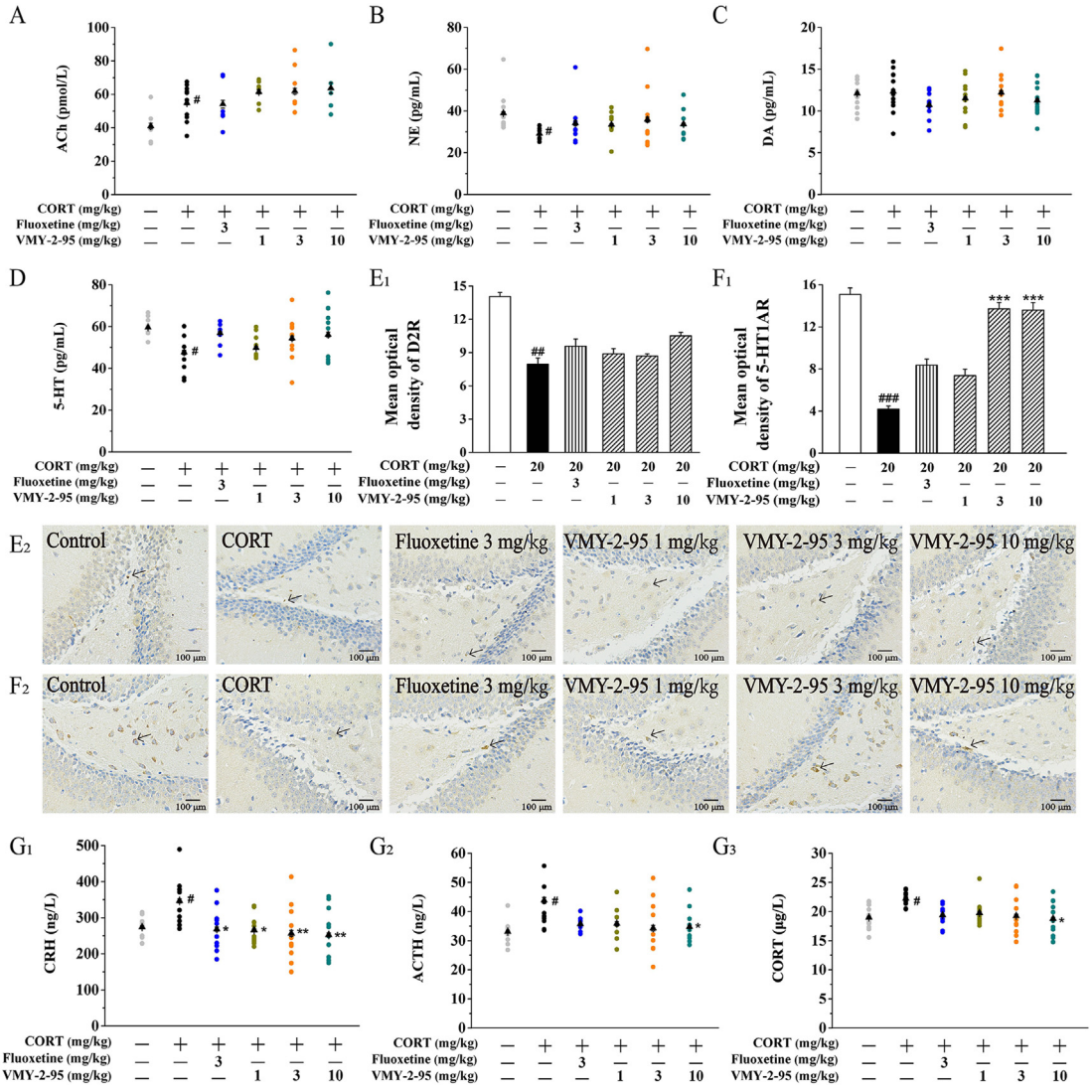

Figure 4 The effects of VMY-2-95 on contents of transmitter and expression of the receptors. The contents of acetylcholine (ACh, A) and norepinephrine (NE, B) in the plasma supernatant of mice, the contents of dopamine (DA, C) and 5-hydroxytryptamine (5-HT, D), and the mean optical density of dopamine 2 receptor (D2R, E_1) in dentate gyrus region of hippocampus, (E_2) immunohistochemistry pictures of D2R in different groups of mice (400×, scale bar indicates 100 μm), (F_1) the mean optical density of 5-hydroxytryptamine 1 receptor (5-HT1AR) in dentate gyrus region of hippocampus, (F_2) immunohistochemistry pictures of 5-HT1AR in different groups of mice (400×, scale bar indicates 100 μm), contents of corticotropin releasing hormone (CRH, G_1), adreno-cortico-tropic-hormone (ACTH, G_2), and corticosterone (CORT, G_3). Data are presented as mean ± SEM, $n = 3$ in E and F, and $n = 10-12$ in others. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* control group, and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* model group.

possible. In this area, choline receptor antagonists show great potential[31].

Neuronal $\alpha4\beta2$ nAChR is a receptor involved in the role of neurotransmitters regulation and release, and this ionic channel participates in the biological process of memory, learning, and attention. The $\alpha4\beta2$ nAChR subtype is a remarkable therapeutic target since this is one of the most abundant receptors in the central nervous system[32]. Many selective $\alpha4\beta2$ nAChR partial agonists such as sazetidine-A[19] and nAChRs antagonist such as mecamylamine[33] can regulate depression-like behaviors, and researches showed that VMY-2-95 as a selective $\alpha4\beta2$ nAChR antagonist could be developed for an antidepressant[23].

Stress in our daily life severely results in the dysregulation of the HPA axis and then implicates in the development of depression[34]. It has demonstrated that CORT-mediated hormonal disorders are the direct cause of depressed patients, and exogenous CORT administration could develop mouse depression model mimicking HPA-axis induced depression-like state[35,36], leading to depression-like indexes such as reduced sucrose intake and increased immobility time in TST and FST[37,38]. We established the depression model by a daily injection of 20 mg/kg CORT for 21 consecutive days as reported[39]. In our experiments, VMY-2-95 showed significant antidepressant-like effects in SPT, TST, FST, OFT with an excellent dose-effect relationship, and the effects were more pronounced compared with fluoxetine. As the transmitters play an essential effect on neural activity, and significant changes occurred in the signal pathway involving both pathology and pharmacology, hence, we investigated the mechanism of

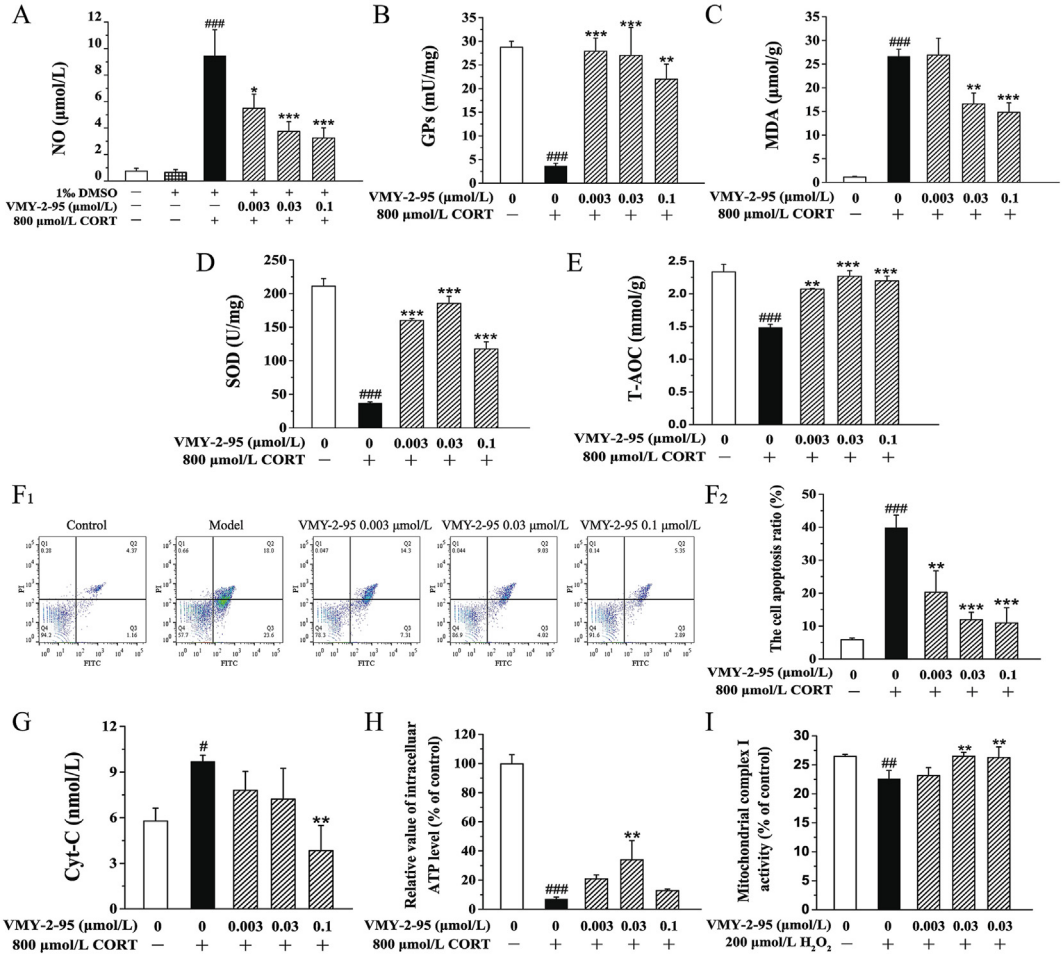

Figure 5 The neuroprotective mechanisms of VMY-2-95 on SH-SY5Y cells against injury induced by corticosterone (CORT). The effect of VMY-2-95 on the contents of nitric oxide (NO, A), glutathione peroxidase (GPs, B), malondialdehyde (MDA, C), superoxide dismutase (SOD, D), and total antioxidant capacity of cells (E); flowcytometry analysis of the cell apoptosis results (F_1); the effect of VMY-2-95 on cell apoptosis ratio (F_2), cytochrome *c* (Cyt-*c*) level (G), adenosine triphosphate (ATP) level (H), and the activity of mitochondrial complex I (I). Data are represented as mean ± SD, $n = 3$. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *versus* control cells, and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* model cells.

VMY-2-95 in terms of transmitters and pathway changes. Furtherly, we investigated the manifestations of anti-oxidant, anti-apoptosis and mitochondrial energy metabolism at the cellular level. Thereby the mechanisms of the antidepressant potential of VMY-2-95 were advanced.

CORT can cross the blood-brain barrier, and corticosteroid receptors enrich in the hippocampus. Therefore, certain hippocampal functions are susceptible to disruption by stress[40]. It is reported that GCs and antidepressants modulated neurogenesis in opposing directions. Chronic CORT exposure affected the proliferation of progenitor cells in the dentate gyrus of the hippocampus, and hippocampal neurogenesis is required for treatment in the depressed model induced by CORT[41]. Here, we showed that chronic CORT treatment damaged hippocampus morphology, reduced the expression of Ki67 and c-FOS, while VMY-2-95 effectively improved hippocampal neurogenesis in depressed mice. Consistent with this, VMY-2-95 protected the cell viability of SH-SY5Y cells well against injury induced by CORT. VMY-2-95 had significant protective effects at both tissue morphology and cell levels.

Many first-line antidepressants are 5-HT and norepinephrine reuptake inhibitors[42,43]. It is reported that neurotransmitter and metabolite disorder was involved in the process of depression, such as 5-HT, DA, monoamineoxidase, NE[44–46]. We showed that VMY-2-95 could significantly recover the level of 5-HT and the corresponding receptor, but the effect of VMY-2-95 on regulating the content of DA was not detected. Consistent with the cholinergic system theory of depression[47,48], we found the significantly increased ACh content and decreased NE content in model mice. However, the VMY-2-95 only alleviated the change of NE but further increased the content of ACh instead, and we thought it was due to the properties of the antagonist of nAChRs that VMY-2-95 increased the content of ACh directly. It is worth mentioning that the action of VMY-2-95 inhibited the entire over-activated cholinergic system.

Extensive clinical and animal research evidence has shown that chronic stress leads to over-activation of the HPA axis and concomitant elevation of GCs production[27,49]. Furthermore, the remarkable roles of the HPA axis and BDNF playing in the actions of antidepressants have also been demonstrated. BDNF is a neurogenesis marker involved in cell differentiation, neuronal survival, migration, and synaptic plasticity[50,51] and the PKA−CREB−BDNF signaling pathway is the classic signaling pathway studied in depression research[52,53]. In our experiments, VMY-2-95 was found to regulate the HPA axis function by decreasing the content of

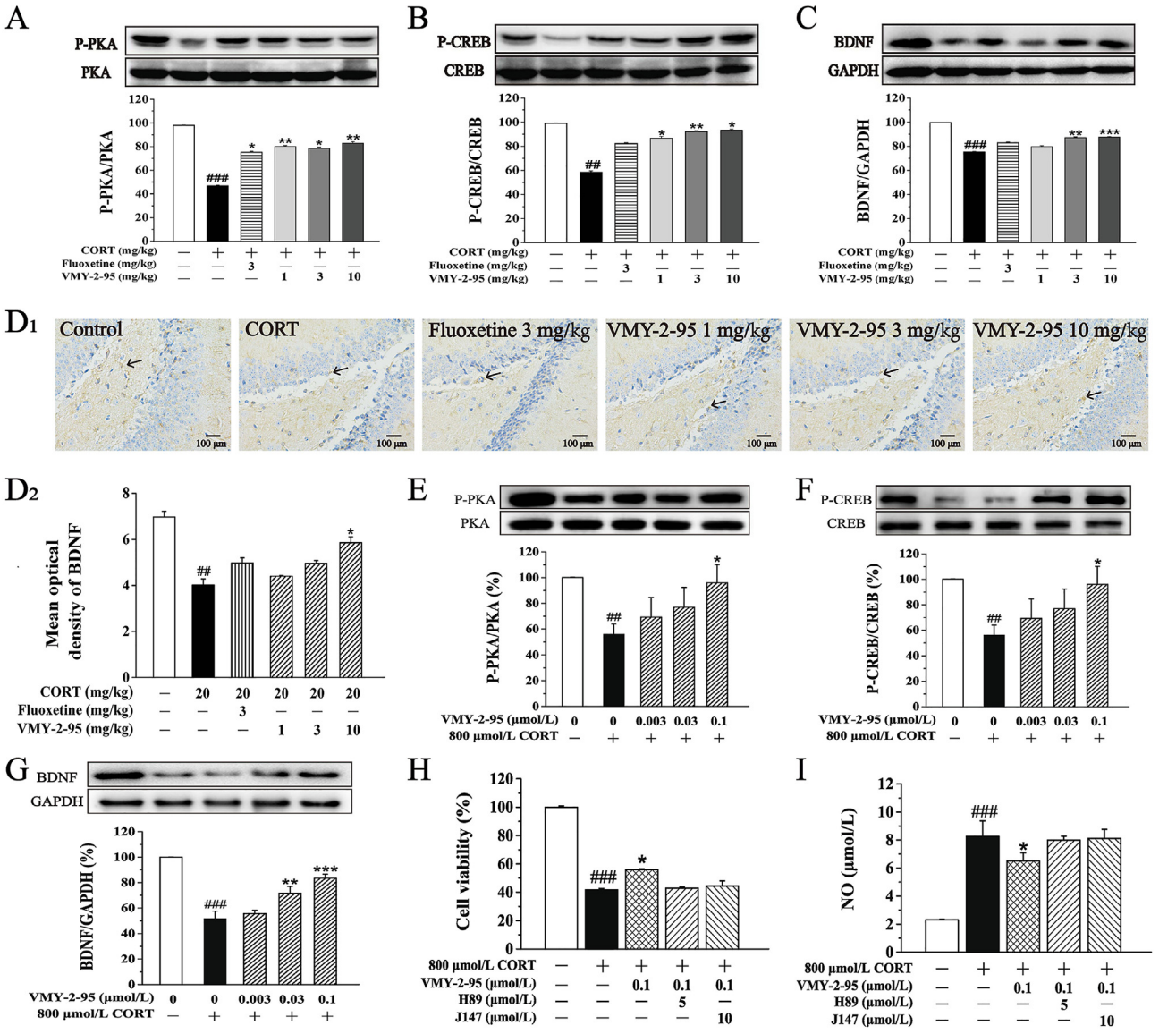

Figure 6 Effects of VMY-2-95 on the PKA−CREB−BDNF signaling pathways. The protein expressions of (A) P-PKA and PKA, (B) P-CREB and CREB, and (C) BDNF in hippocampus; (D_1) immunohistochemistry pictures of BDNF in different groups of mice (400×, scale bar indicates 100 μm); (D_2) the mean OD of BDNF in hippocampus; the protein expressions of (E) P-PKA and PKA, (F) P-CREB and CREB, and (G) BDNF in SH-SY5Y cells; (H) cell viability when VMY-2-95 combined with H89 or J147; and (I) NO content when VMY-2-95 combined with H89 or J147. Data are presented as mean ± SEM, $n = 12$ in A−C; and data are presented as mean ± SD, $n = 3$ in others. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* control group, and $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* model group.

CRH, ACTH and CORT. Meanwhile, VMY-2-95 was found to regulate the PKA−CREB−BDNF signaling pathway both in CORT injured mice and SH-SY5Y cells damaged by CORT.

Moreover, CORT causes nerve damage in many ways, such as the concomitant oxidative damage[54], energy metabolism damage[55], and cell apoptosis. We initially confirmed that VMY-2-95 significantly elevated the activity of antioxidants such as SOD and GPs, and reduced the levels of oxidative damage markers (NO and MDA) to increase the antioxidant capacity of SH-SY5Y cells. Also, VMY-2-95 effectively reduced apoptosis and decreased the content of Cyt-*c*. Besides, VMY-2-95 increased the level of ATP and the activity of mitochondrial complex I to rescue impaired mitochondrial function caused by CORT treatment. Thus, VMY-2-95 provided comprehensive protection of SH-SY5Y cells by various routes.

We have initially proved the antidepressant-like effect of the selective nicotinic $\alpha4\beta2$ receptor antagonist VMY-2-95 both *in vitro* and *in vivo*, suggesting that $\alpha4\beta2$ nAChR would be a new target for the development of antidepressants. Multiple targets and mechanisms involved in anti-depression and neuroprotection of VMY-2-95 should be further discussed.

5. Conclusions

In conclusion, our results reveal that VMY-2-95 reversed depression-like behaviors in chronic CORT-induced depression model in C57 mice by improving neuromorphic function, promoting hippocampal nerve proliferation, and regulating the contents of monoamine transmitters through the PKA−CREB−BDNF signaling pathway. Meanwhile, VMY-2-95 effectively protected

SH-SY5Y cells against injury induced by CORT through protecting the cell viability, improving antioxidant and anti-apoptosis capacity, improving mitochondrial energy metabolism. Our experiments suggested that VMY-2-95 is a promising lead compound as well as a drug candidate to be advanced through the antidepressant discovery pipeline. Further, the antagonists of $\alpha4\beta2$ nAChR would have potential in the development of novel antidepressants.

Acknowledgments

This project was supported by the National Natural Science Foundation of China (No. 81603100), the Drug Innovation Major Project (2018ZX09711001-003-005, 2018ZX09711001-012, China), CAMS Innovation Fund for Medical Sciences (2017-I2M-1-010, China), and Peking Union Medical College Graduate Innovation Fund Project (2017-1007-11, China).

Author contributions

Ziru Yu and Guanhua Du designed research; Ziru Yu, Yu Liang and Xiaoyue Zhao performed research; Ziru Yu, Dewen Kong analyzed data; Ziru Yu wrote the paper. All authors reviewed the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.03.002.

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