Antidepressant Activity of Astilbin: Involvement of Monoaminergic Neurotransmitters and BDNF Signal Pathway

Qiong-Qiong Lv,*,# Wen-Jie Wu,*,# Xiao-Liang Guo,† Rui-Liu, Yu-Ping Yang,∗
Du-Shuang Zhou,† Ji-Xia Zhang,‡ and Ju-Yuan Liu,*,*

School of Pharmacy, Xinxiang Medical University; The Third Affiliated Hospital, Xinxiang Medical University; Xinxiang 453000, China; and *The First Affiliated Hospital, Xinxiang Medical University; Weihui 453100, China.

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Depression and related mood disorders are among the world’s greatest public health problems. Previous studies have demonstrated that astilbin (AST) has broad pharmacological functions which may modulate numerous pathways, such as antioxidant, scavenging free radicals, anti-inflammatory and so on, similarly to some of other flavonoids. In this study, the antidepressant-like effect of AST was investigated using chronic unpredictable mild stress (CUMS) model of depression in mice. The results showed that chronic administration of AST at doses of 10, 20 and 40 mg/kg (intraperitoneally (i.p.), 21 d) reduced depressive-like behaviors of mice in the forced swim test (FST), tail suspension test (TST) and sucrose preference test (SPT) without affecting locomotor activity. AST increased the contents of serotonin (5-HT) and dopamine (DA) in the frontal cortex of CUMS mice. Additionally, it was shown that AST treatment restored the CUMS-induced inhibition of extracellular signal-regulated kinase (ERK) 1/2 and AKT phosphorylation in the frontal cortex, conformed to the brain-derived neurotrophic factor (BDNF) expression. Our findings suggest that AST has antidepressant activities and the mechanisms, at least in part, relate to up-regulation of monoaminergic neurotransmitters (5-HT and DA) and activation of the BDNF signaling pathway.

MATERIALS AND METHODS

Animals Adult male C57BL/6J mice (18–22 g), obtained from the Experimental Animals Center of Zhengzhou University, were housed six per cage (320×180×160 cm) under standard conditions (12 h light–dark cycle; lights on 7 a.m.–7 p.m.;

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: liujuyuan56@hotmail.com

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temperature of 22.5°C with free access to food and water, and allowed to acclimatize to a week. All experimental procedures were performed in accordance with the published guidelines of the China Council on Animal Care.

**Drugs Administration and Experimental Groups** Imipramine was provided by Hunan Dongting Pharmaceutical Co., Ltd. (China) and diluted in normal saline. AST (>98%, molecular weight of 450.40) was purchased from Bellancom Chemistry (U.S.A.) and was dissolved in dimethylsulfoxide (DMSO) as stock solutions. The stock solutions were diluted to the final concentrations with normal saline before application and the final concentration of DMSO did not exceed 0.1%. The mice were randomly separated into the following six groups with N=12/group: (a) Control (saline), (b) CUMS+vehicle group (Model), (c) CUMS+imipramine (10 mg/kg) group (IMI), (d, e, f) CUMS+AST (10 mg/kg, 20 mg/kg, 40 mg/kg, respectively) groups. Dose and route of imipramine and AST administration used in the experiment were chosen as based on prevenient results. All these agents were administered intraperitoneally (i.p.) to the mice in a volume of 0.1 mL/10g/day.

**Open Field Test (OFT)** The OFT was used to study the spontaneous locomotor activity, and the experimental condition was similar to those described previously. The mice were placed individually in the center of a wooden box (100×100×40 cm) with the floor divided into 25 (5×5 cm) squares and allowed to act at liberty. The apparatus was covered with a black surface and illuminated with a red bulb (50 W). Horizontal locomotion (number of total squares crossed) and rearing frequencies (defined as standing on their hind paws) were scored over a 5 min period to evaluate the locomotor activity. These tests were conducted between 8:30–11:30 a.m. in a quiet room. Between each test, the apparatus was cleaned. The observers were unaware of the treatment of the mice.

**Chronic Unpredictable Mild Stress (CUMS)** The CUMS was performed as previously described with slightly modifications. During this experiment, the mice in the control group were left undisturbed in the home cages in a separate room with the exception of general handling (e.g. regular cage cleaning) that matched the CUMS groups. Briefly, the CUMS mice were subjected to one of the following eight kinds of mild stressors daily (in random order to make the stress procedure unpredictable) for consecutive 3 weeks: (1) food deprivation for 24 h, (2) water deprivation for 24 h, (3) cage tilting (30°) for 24 h, (4) tail pinch for 1 min (a clothespin placed at 1 cm from the base of tail), (5) physically restraint for 2 h (in a 9.5 cm×3 cm cylindrical vitric rodent restrainer) (6) swimming in 17°C cold water for 5 min (after which the mice were towed dry) (7) swimming in 40°C cold water for 5 min (after which the mice were towed dry) (8) alterations of the light-dark cycle. Control animals were not exposed to any stressors.

**Forced Swimming Test (FST)** The FST was carried out in mice according to previous reports with slight modification. Briefly, 30 min after injection, mice were individually placed into a glass cylinders (20 cm in height, 15 cm in diameter) filled with 15 cm high water (24±1°C). The water was exchanged after each trial. All mice were forced to swim for 6 min, and the immobility time during the final 4 min of the test was recorded. Immobility time was defined as the time spent by the mouse floating in the water without struggling, and making only small movements necessary to keep its head above the water. After the test, the mice were towed dry and placed into their cages. As in previous tests, the FST was performed by the observers blind to the treatment groups in a quiet room with soft light, in order to ensure the objectivity of the experimental data.

**Tail Suspension Test (TST)** The total duration of immobility induced by tail suspension was measured according to the methods described previously. A reduction in the duration of immobility is indicative of an antidepressant-like effect in this test. The TST was carried out on the basis of previous procedures with a little modification. Mice, visually isolated, were suspended by adhesive tape (approximately 1 cm from the tip of the tail) with the head 35 cm above the floor for 6 min. The total duration of immobility was registered during the final 5 min of the test by the observers blind to the treatment groups. Immobility was defined as lack of all movement except for whisker movement and respiration.

**Sucrose Preference Test (SPT)** The SPT is considered to detect the levels of anhedonia and the procedure was performed as previously described with minor modifications. Before the test, mice were divided individually to cages for acclimatization and given free access during two days to two bottles, one bottle containing water and the other 1% (w/v) sucrose solution. Bottles were exchanged every 12 h to prevent possible position preference in drinking behavior. Following the adaptation procedure, the mice were deprived of water and food for 24 h. The SPT was conducted at 9:00 a.m. The mice were given free access to two bottles containing 100 mL of sucrose solution (1%, w/v) and 100 mL of water, respectively. After 1 h, the volume of both the consumed water and sucrose solution were measured, and sucrose preference was evaluated as sucrose uptake rate, namely, a percentage of sucrose consumption relative to the total liquid ingested.

**High Performance Liquid Chromatography (HPLC)** To confirm monoamine depletion in mice, the frontal cortex contents of DA and 5-HT were measured using HPLC (Varian Prostar 210, U.S.A.) following previously reported methodology. Mice were sacrificed by decapitation and brains were removed and dissected over ice; brain area sample was placed into an Eppendorf tube, weighed and homogenized on ice in 3 times volume of 0.1 M perchloric acid (containing 0.004 M sodium metabisulfite) and centrifuged at 10000×g for 30 min at 0°C. The supernatants were filtered by 0.45 μm hydrophilic membranes and transferred to centrifuge tubes and stored at −80°C until analysis. The standard of DA and 5-HT were purchased from Sigma-Aldrich (U.S.A.). All other chemicals used in this study were of HPLC grade. The HPLC procedures were established according to previous experiments. The measurements were made by injecting 20 μL of the supernatant into the Varian prostar210 series HPLC system (U.S.A.) with fluorescence detector, which employed a Varian C18 column (4.6 mm×250 mm, 5 μm). The mobile phase was composed of 30% methanol, 4% ultrapure water and 66% phosphate buffer, where the buffer consisted of 0.02 M NaH 2PO 4 and 0.005 M sodium 1-octanesulfonate (pH 3.3). The flow rate was maintained at 1.0 mL/min.

**Western Blot Analysis** The mice were randomly selected and sacrificed by decapitation at the end of the last stressor and drug exposure. The frozen cortex from the 3 mice in each
group were homogenized in 6 times volume of lysis buffer (50 mm Tris-base, (pH 7.4); 100 mm NaCl; 1 mm ethylenediaminetetraacetic acid (EDTA); 1% NP-40, 1 mm phenylmethylsulfonfyl fluoride (PMSF), 3 mm Na3VO4, and protease inhibitor) and then lysed on ice for 30 min. The lysate was centrifuged at 12000×g at 4°C for 15 min and supernatant was recovered and protein concentration was measured by using the BCA protein assay kit (Beyotime, China). Equal amount of proteins (20 µg) were separated by 10% or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels electrophoresis and then transferred to nitrocellulose membranes (Pall, U.S.A.). After being blocked with 5% nonfat milk or 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 h at room temperature, membranes were incubated overnight at 4°C with anti-β-actin (1:2000, Boster) anti-BDNF (1:1000, Abcam, U.K.), anti-ERK1/2 (1:1000, Abcam, U.K.), anti-phospho-ERK1/2 (1:5000, Abcam, U.K.), anti-Akt (1:1000, Abcam, U.K.), anti-phosphor-Akt (1:750, Abcam, U.K.), all with 1% BSA in TBST. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:4000, Boster) at room temperature for 1 h. Immunoblots were visualized with enhanced chemiluminescent reagents (ECL Plus kit, Beyotime, China). The optical density of the bands was determined using Image-J Software (NIH).

Statistical Analysis Data are presented as mean± standard deviation (S.D.). One-way ANOVA, followed by Dunnett’s post hoc tests is employed for the statistical analysis by using SPSS 12.0 software. A p<0.05 was considered to be statistically significant for analysis.

RESULTS

AST Treatment Produces No Effect on Spontaneous Locomotor Activity of Mice To explore the effect of AST on the excitement of central nervous system (CNS), the locomotion (number of crossings) and the rearing frequency of mice without stress were measured in the OFT. As shown in Fig. 1B, after 7 d, the number of crossings was 83±6 in the control group. After chronic treatment with AST (10, 20, 40 mg/kg, i.p.) and imipramine (10 mg/kg) once daily for 7 d, the number of crossings were 78±7, 82±7, 81±6 and 80±6, respectively (n=10). Consist with the result, no significant change of rearing frequency in non-stress mice treated with AST and imipramine was found as compared to the control group (n=10, Fig. 1C), indicating that AST produces no effect on the excitation of CNS.

Chronic AST Treatment Reverses the CUMS-Induced Depressive-Like Behaviors of Mice FST and TST in mice are the most widely used behavioural assays for detecting potential antidepressant-like activity and have a high predictive validity for antidepressant activity.2,36 Thus, the possible antidepressant effects of AST were first examined in the FST. AST (10, 20, 40 mg/kg) was given i.p. and imipramine (10 mg/kg, i.p.) was included as the positive control. The results showed that a single injection of AST produced a strong antidepressant effect in the FST. As shown in Fig. 1D, the duration of immobility of model mice exhibited a significant increase compared with control mice (n=10, p<0.01 vs. control). However, the immobility time of AST-treated mice was markedly shortened, as compared with the model mice (n=10, p<0.01 vs. model). Imipramine also produced an evident decrease of immobility time (n=10, p<0.01 vs. model), an effect that has been consistently described.37

The similar results were also performed to assess the antidepressant-like effects of AST in the TST (Fig. 1E). Data from this test revealed a significant main effect of drug treatment. The immobility time of model mice in TST was obviously increased to 141±19 s (n=10, p<0.01 vs. control). Nevertheless, AST at 10, 20, 40 mg/kg observably decreased the immobility time to 97±11 s, 85±12 s, 75±11 s (n=10, p<0.01 vs. model), respectively, which were similar to the effect of imipramine, and the immobility time in the latter group was reduced to 89±9 s (n=10, p<0.01 vs. model).

Chronic AST Treatment Reverses the CUMS-Induced Anhedonia To further characterize the antidepressant effects of AST, in the present study, we examined the effects of AST on the sucrose intake as indices of stress-induced responses. As shown in Fig. 1F, CUMS induced a 56±10% decrease in sucrose intake in the mice compared with control (n=10, p<0.01 vs. control). The effect was evidently reversed by chronic AST treatment. After receiving injection of 10, 20, 40 mg/kg AST, the sucrose consumption was increased by 72±24%, 83±28%, and 112±24% (n=10, p<0.01 vs. model), respectively, similarly to that observed with imipramine (n=10, p<0.01 vs. model), a classical antidepressant drug used as standard (positive control).

AST Reverses the CUMS-Induced Decrease of Cortical DA and 5-HT Level According to the proposed chromatography condition, HPLC chromatogram of a cocktail of DA (1 µg/mL) and 5-HT (0.5 µg/mL) standard was shown in Fig. 2A. HPLC chromatogram of a sample from one of the experimental groups was shown in Fig. 2B. A series of standard solutions were prepared with a concentration gradient and injected into HPLC. The values of peak areas and concentrations with reference to the DA and 5-HT standard curves, which were shown in Figs. 2C and D, respectively. As a result, the regression equation of DA is: Y = 1.23×10^3X−6.28×10^2, correlation coefficient (r)=0.998; the regression equation of 5-HT is: Y = 1.79×10^3X−3.34×10^2, r = 0.999. Results indicate that peak area have greatly significant linear relation with the level of DA or 5-HT. Then the contents of DA and 5-HT in the cortex of mice were calculated by the regression equations, respectively.

As shown in Fig. 2E, the DA level in the cortex of model group was substantially reduced when compared with that of control mice (n=3, p<0.01 vs. control). However, AST at 10, 20, 40 mg/kg have an evident effect on increasing DA concentration by 67±17%, 121±15%, and 165±18% in the frontal cortex (n=3, p<0.05 vs. model), similar to that in the imipramine-treated group (n=3, p<0.01 vs. model).

Figure 2F exhibits the 5-HT level in the frontal cortex of mice. There was a marked change between control mice and model mice (816±130 ng/g vs. 353±21 ng/g, n=3, p<0.01). Nevertheless, the 5-HT level in the model mice was obviously restored by chronic AST administration, especially at the dose of 20 and 40 mg/kg (n=3, p<0.01 vs. model), which was no significant difference with that in positive control (n=3, p<0.01 vs. model).

Chronic AST Treatment Attenuates the CUMS-Induced BDNF-Regulation BDNF is an attractive candidate for neu-
ral growth in the brain and plays an important role in current theories of depression.\textsuperscript{38,39} There is growing evidence demonstrated that chronic stress result in the change in BDNF expression.\textsuperscript{40} Therefore, we first detect the expression of BDNF to explore the possible antidepressant mechanisms induced by AST. As shown in Fig. 3A, BDNF expression in the cortex was decreased in CUMS model. On the contrary, after treatment with 10, 20, and 40 mg/kg AST, the expression of BDNF was gradually reversed (Fig. 3B). This effect was also similar to that of classic antidepressant drug IMI.

**Chronic AST Treatment Restores the CUMS-Induced Decrease in the BDNF Signaling Pathway in the Cortex**

The ERK mitogen activated protein (MAP) kinase pathway plays an important role in the pathophysiology of the depressive-like behavior,\textsuperscript{41} and antidepressant efficacy in depressed humans and animal models of depression was associated with
the increase of BDNF expression in the brain, which was mediated by MAPK/ERK signaling pathway. We then examined the activation of ERK in the cortex of mice by western blot analysis. As shown in Figs. 4A and B, it was found that the mice exposed to CUMS showed a decrease in phospho-ERK and this change was reversed by the chronic AST treatment, especially at the dose of 40 mg/kg (n=3, p<0.01 vs. model). Similarly, imipramine also significantly elevated the level of p-ERK1/2 (n=3, p<0.05 vs. model). No differences were detected in total ERK in all groups.

Besides ERK, the phosphatidylinositol 3-kinase (PI3K)/AKT kinase pathway is also a key downstream signaling pathway of BDNF and has attracted attention for their important role in the actions of antidepressants. Therefore, we next examined whether AKT pathway participated in the action of antidepressant of AST. As shown in Figs. 4A and C, compared with the model group, imipramine restored the cortex p-AKT expression to the control level (n=3, p<0.01 vs. control). Similarly, chronic treatment with AST also signally elevated the pAKT level by 93±11%, 118±15% and 129±20%, respectively (n=3, p<0.01 vs. model), and the level of total AKT protein was not altered.

DISCUSSION

In the present study, we have demonstrated for the first
time that AST produced robust antidepressant effects in mice models of depression, which was similar to imipramine, and mediated by the monoaminergic neurotransmitter (5-HT and DA) and the BDNF signaling pathway.

The OFT is used to measure the locomotor activity and exploration of animals to a novel environment, standing for the excitability and curiosity of animals. It has been certified that some medicines, which themselves have the excitatory effect on the CNS, can not only elevate the number of crossings and rearings in OFT, but also decrease the immobility time in FST. However, these medicines actually have no antidepressant effect.44) To avoid false positives in the following test, normal mice were exposed to the open-field apparatus for 5 min. The results showed that AST did not increase or reduce locomotor activity of normal mice, indicating that AST does not produce neural excitability.

We know that both FST and TST have been widely used to assess new antidepressant drugs activity in the preclinical study.36,45) In this study, CUMS mice produced a substantial elevation of immobility time in the FST and TST, representing that the model of depression was established successfully. And our results showed that chronic AST treatment for 3 weeks reduced the immobility time of CUMS mice to control level in the FST and TST, similarly to that observed with the established antidepressant, imipramine, which provided powerful evidence for the antidepressant effects of AST.

Anhedonia is the core symptom of human major depressive disorder and CUMS can induce anhedonia in rodents.46) So the mice was exposed to unpredictable stress for consecutive 21 d to model the depressive-like state of anhedonia, which was indicated by sucrose preference test, a validated index for anhedonia measure. Our results confirmed that the reduction in sucrose consumption induced by CUMS, consistent with the previous results,13 was reversed by the AST chronic treatment, although the dose-dependent was not obvious. The reduced sucrose preference of mice therefore reflected depression-like symptoms induced by present CUMS procedures.

A considerable amount of clinical and experimental evidences have accrued to demonstrate that monoaminergic systems (catecholamines and serotonin) play an important role in the pathophysiology of mental depression.47) It is known that the modulation of monoaminergic neurotransmission at a synaptic level is the basic mechanism of the different types of antidepressants currently available for clinical practice, including imipramine, fluoxetine etc.48) Numerous studies have reported that some antidepressant drugs act by inhibiting the uptake of 5-HT,49 participating in the regulation of mood,

![Fig. 3. Effects of AST Treatment on BDNF Expression in the Frontal Cortex](image)

![Fig. 4. AST Treatment Reverses the Change of ERK1/2 and AKT Expression in Frontal Cortex Induced by CUMS](image)
memory, sleep in patients with depression, which suggest that the increasing of 5-HT level seems to be connected with the behavioral deficits induced by CUMS. In addition, DA in catecholaminergic system has been considering as another important target in the action of antidepressants. In order to certify whether the catecholaminergic (DA) and serotonergic (5-HT) involved in or not the antidepressant-like activity of AST, we examined their levels by HPLC. Our results indicated that the CUMS-induced reductions of cortical 5-HT and DA levels were prevented by AST. This effect was measured after chronic treatment, and we didn’t measure extracellular (or release) levels of monoamine, so it is unclear whether the up-regulation of monoamine by AST is the initial stage of the antidepressant-like effects. The mechanisms of the up-regulation of monoamine contents are also not very clear. Maybe BDNF expression through promoter IV disturbs expression of monoamine genes in the frontal cortex, maybe through other mechanisms. A lot of flavonoids have been found to display antidepressant-like effect by inhibiting the reuptake of biogenic amines and monoamine oxidase (MAO) activity to increase the contents of 5-HT and DA in synaptic space. 52,53

BDNF signaling pathway, one of the important intraneuronal biochemical pathways, is closely related to neuronal survival and neurogenesis and plays a prominent role in the pathogenesis of depression. It is reported that BDNF level in some brain regions of limbic system is evidently lower in depression patients than normal people. Various stress procedures, including CUMS, result in obviously decreases of BDNF expression in the frontal cortex and hippocampus, while chronic administration of almost all kinds of antidepressants, including imipramine, regulates BDNF levels in those regions. 3,5 In the present study, AST could reverse the decreased BDNF protein level in the frontal cortex induced by CUMS, in line with the change of 5-HT and DA, which suggested that the up-regulation of BDNF induced by AST may be from the activation of monoaminergic system. In Fig. 3, BDNF level was not recovered by chronic treatment of 10 mg/kg AST despite the antidepressant-like activity in behavioral tests, suggesting that BDNF may participate in, may not be critical for the antidepressant-like effects of AST.

The MAPK/ERK and PI3K/AKT kinase pathways are the two important downstream signaling pathways of BDNF and closely related to antidepressants to modulate depressivelike behaviors in depressed humans and animal models of depression. It is known that the phosphorylation of ERK1/2 and AKT is involved in influencing neurotrophic actions and neuronal cell survival through activating various intracellular signaling cascades. Therefore, we observed the changes in ERK activity and in AKT activity as evaluated by the ratio of p-ERK/ERK and p-AKT/AKT, respectively. As expected, AST treatment restored the CUMS-induced inhibition of ERK and AKT phosphorylation in the frontal cortex, with the BDNF expression, suggesting that MAPK/ERK and PI3K/AKT signaling pathways are involved in antidepressant action of AST.

In summary, the present study shows that AST exhibited antidepressant-like properties in experimental animal models of depression, which appeared to be mediated, at least in part, through up-regulation BDNF signal pathway and monoaminergic neurotransmitters release. This newly discovered effect of AST provides a new insight to understand the pharmacological effects of AST, a further insight into the possible therapeutic use of AST for treating major depression, and more importantly, sheds light on the development of new antidepressants with higher efficacy and fewer side effects.

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