Helicid Ameliorates Learning and Cognitive Ability and Activities of Chronic Unpredictable Mild Stress Rats

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INTRODUCTION

Depression is the most common mental illness causing disability worldwide. Symptoms of depression include low mood, anhedonia, low self-worth, sleep and appetite disturbances, fatigue, changes in cognitive function and metabolic alterations. Additionally, persistent depression causes cognitive dysfunction which can negatively affect daily life and work performance.

The 5-hydroxytryptamine (5-HT) hypothesis of depression links depression with deficiency of 5-HT neurotransmission in the brain. Serotonin transporters (SERTs) located on presynaptic 5-HT neurons play a key role in maintaining 5-HT homeostasis; selective serotonin reuptake inhibitors bind to SERTs, blocking 5-HT uptake and enhancing synaptic 5-HT levels. In contrast, negative feedback control of 5-HT1A autoreceptors can decrease the concentration of 5-HT. Therefore, multi-targeted drugs which act on both 5-HT1A receptors and SERTs may offer a new strategy to improve antidepressant efficacy.

Depression and chronic stress disrupt the cAMP/protein kinase A (PKA)/cAMP response element-binding (CREB) signaling pathway. PKA, the upstream activator of CREB, is one of the most extensively studied transcription factors implicated in depression and antidepressant processes. Numerous studies have provided compelling evidence that the cAMP/PKA/CREB signaling pathway is involved in the regulation of synaptic plasticity and learning memory. The systemic perturbations of the cAMP/PKA/CREB signaling pathway could induce a cascade of neuropathological reactions in depression, including abnormalities in regional brain activity, changes in synaptic function and impaired neurogenesis. Antidepressants used in Western medicine are somewhat limited and can produce unacceptable side effects. The pharmacological potential of traditional Chinese medicines is very promising. Helicid (4-formylphenyl-β-D-allopyranoside, Fig. 1), an active ingredient extracted from the seeds of Helicia nilagirica, an indigenous herb found in western China, has been reported to have sedative, analgesic, hypnotic and antidepressant effects. Its low toxicity and high efficiency make this agent unique.

Previous studies have demonstrated the antidepressant-like effects of helicid in a chronic unpredictable mild stress (CUMS) model of depression in rats and to explore cAMP/protein kinase A (PKA)/cAMP response element-binding (CREB) signaling pathway. Sprague-Dawley rats were randomly assigned to six groups (n = 10): control; CUMS; CUMS + fluoxetine (5 mg/kg) and CUMS + helicid at 8, 16 and 32 mg/kg. All rats were subjected to 12 weeks of CUMS protocols and drug administration during the last 6 weeks of CUMS. Our results showed that helicid, at a dose of 32 mg/kg, significantly reversed decreases in body weight and sucrose consumption, increased the distance and number of crossings in the open-field test (OFT), reduced immobility times in the forced swimming test (FST) and improved spatial memory in the Morris water maze (MWM); all of these effects had been induced by CUMS paradigm. Immunohistochemistry showed that administration of helicid could promote the proliferation of neurons in the hippocampal CA1 and dentate gyrus (DG) regions. CUMS rats treated with helicid had dramatically decreased protein levels of serotonin transporters (SERTs). In addition, CUMS resulted in a significant reduction in the expression of cAMP, PKA C-α and p-CREB, each of which were partially attenuated by helicid administration. These results indicated that helicid could improve depressive behaviors, learning and cognitive deficits and increase hippocampal neurogenesis, which may be mediated by the regulation of SERTs, activation of the cAMP/PKA/CREB signaling pathway and upregulation of p-CREB levels in hippocampal.

Key words: serotonin transporter (SERT); learning ability; cognitive ability; chronic unpredictable mild stress (CUMS); depression

Fig. 1. Chemical Structure of Helicid
Effects of helicid in the chronic unpredictable mild stress (CUMS) model might be mediated through the regulation of the level of corticosterone (CORT), inflammatory cytokines and 5-HT, which is possibly associated with the expression of 5-HT1A receptor, and increased levels of signaling in extracellular signal-regulated kinase (ERK)/CREB/brain derived neurotrophic factor (BDNF) pathways. However, less research has focused on how helicid exerts its antidepressant-like effect to improve learning and cognition function. The present study investigated the effects of helicid on learning and cognitive function and the action associated with SERT in a CUMS model of depression in rats, and we also explored how the cAMP/PKA/CREB signaling pathway is related to a neurogenesis.

MATERIALS AND METHODS

Animals Adult male Sprague–Dawley (SD) rats (210–250 g) were obtained from Qinglong Mountain Animal Breeding Farm (Nanjing, China). Animals were housed in plastic cage under standard conditions; room temperature was maintained at 24 ± 2°C with a 12 h/12 h light–dark cycle (lights on at 07:00). All animals were fed adaptively for one week prior to experiments. All procedures of the study were performed in line with institutional guidelines and the P. R. China legislation for the care and use of laboratory animals.

Drugs and Reagents Helicid (Kunming Baker Norton Pharmaceutical, Kunming, China) and Fluoxetine hydrochloride (Sigma-Aldrich, St. Louis, MO, U.S.A.) were both dissolved in saline (0.9%). The drug or saline was injected intraperitoneally once daily at a volume of 10 mL/kg.

Chronic Unpredictable Mild Stress Procedure and Drug Administration A total of 60 SD rats were randomly assigned to six groups (n = 10) using a randomization method based on body weights. The groups were as follows: control; CUMS; Fluoxetine (5 mg/kg); Helicid (32, 16 and 8 mg/kg). Control rats were housed in a separate room and had no contact with the CUMS rats. The CUMS procedure was conducted as previously described by Willner et al.11) with slight modifications. Briefly, the CUMS rats were subjected to the following stressors once daily for 12 weeks: (1) water/food deprivation (24 h), (2) cold water swimming (4°C for 5 min), (3) inversion of day/night light cycle (light on in the night and light off in day time), (4) 45° cage tilt (24 h), (5) wet caging (200 mL of water into the sawdust bedding for 24 h), (6) 1 min tail pinch (1 cm from the beginning of the tail) and (7) foot shock. Rats were individually exposed to one stressor, selected at random, per day. All drugs were administered intragastrically once daily from week 6 to 12 of the CUMS procedure (Fig. 2).

Behavior Tests

Body Weight and Sucrose Preference Test

Body weight and sucrose preference were recorded every week during the experiment paradigm. The sucrose preference test (SPT) has been employed to evaluate the state of anhedonia-like behavior in rats.12) For the sucrose intake test, rats were trained to adapt to the sugary drink. On the first day, each rat was given two bottles of 1% sugar water. The following day, each rat was given one bottle of water and one bottle of 1% sugar water. The sequence of bottles were changed every 2 h. After training, fasting water treatment was performed for 24 h. Each rat was then given one bottle of water and one bottle of 1% sugar water, which were weighed in advance, and the consumption of each bottle was measured 1 h later. The sucrose preference rate of the rats was calculated according to the formula: sucrose consumption (g)/[sugar water consumption (g) + water consumption (g)] × 100%.

Open-Field Test (OFT)

The OFT was used to evaluate locomotor activity and exploratory behavior among the rats.13) Prior to forced swimming test, the rats were individually placed in the center of the open field (100 cm long × 100 cm wide × 50 cm deep box) for 5 min. The speed and the distance of movements were monitored by an automated video-tracking system using the XINRUMAN program (Shanghai, China). The floor surface of each chamber was thoroughly cleaned with 75% ethanol between tests.

Forced Swimming Test (FST)

The FST was used to evaluate depressive-like behavior by recording the time spent immobile.14) The forced swim test was conducted in a cylindrical Plexiglas swimming device (height: 60 cm, diameter: 20 cm), filled to a depth of 30 cm with water at 24 ± 2°C. Behavior was recorded with a camera mounted above the swimming device. Each rat was individually placed into the forced swim device for 6 min. The rat was then removed from the swimming device and dried with a towel before being returned to the cages. The water in the swimming device was changed between tests. Immobility times were measured for the last 4 min of the test.

Morris Water Maze

Spatial learning and memory were evaluated using the

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Morris water maze (MWM). The MWM device is composed of a black circular pool (150 cm diameter and 60 cm height) which is divided into four visual quadrants and filled with water (24 ± 2°C). The round platform (10 cm diameter) was placed in the center of the third quadrant, 2 cm below the surface of the water. Over four consecutive days, rats were placed into the pool facing the wall, each rat entered the water basin from four quadrants in turn and entered the water points in the same order. A different entry site was used for each session and trials were duplicated on each rat. Rats who failed to find the platform within 60 s were guided and allowed to stay on the platform for 10 s. On day five, the time that rats spent finding the platform was recorded as the latency. On day six, the platform was removed and the animals were given 60 s to determine the extent to which they had learned the location of the platform. Rat were placed in the water at the entry site where the last training session was performed and allowed to swim for 60 s. The number of crosses in the quadrant where the platform was located were recorded. The latency, travel orbit and number of crosses were recorded using the XINRUAN program (Shanghai, China).

5-Bromo-2-deoxyuridine (BrdU) Labeling and Immunohistochemical Staining To label newly proliferated and survived cells in rat hippocampal CA1 and dentate gyrus (DG) regions, rats (n = 4) were given an intraperitoneal injection of BrdU (Sigma-Aldrich, 100 mg/kg) once daily for 3 d after the last day of helicid or fluoxetine treatment. Rats were anesthetized and perfused with 100 mL of 0.1 M cold 3 d after the last day of helicid or fluoxetine treatment. Rats were euthanatized by decapitation at 4°C for 4 h. The round platform (10 cm diameter) ± 2°C. The round platform (10 cm diameter) with water (24 ± 2°C). The round platform (10 cm diameter) was placed in the center of the third quadrant, 2 cm below the surface of the water. Over four consecutive days, rats were placed into the pool facing the wall, each rat entered the water basin from four quadrants in turn and entered the water points in the same order. A different entry site was used for each session and trials were duplicated on each rat. Rats who failed to find the platform within 60 s were guided and allowed to stay on the platform for 10 s. On day five, the time that rats spent finding the platform was recorded as the latency. On day six, the platform was removed and the animals were given 60 s to determine the extent to which they had learned the location of the platform. Rat were placed in the water at the entry site where the last training session was performed and allowed to swim for 60 s. The number of crosses in the quadrant where the platform was located were recorded. The latency, travel orbit and number of crosses were recorded using the XINRUAN program (Shanghai, China).

RESULTS

Effects of Helicid on Body Weight and Sucrose Preference As shown in Fig. 3A, CUMS rats exhibited decreased body weight after six weeks of the CUMS procedure \([F(5, 54) = 5.14, p < 0.01]\). Administration of helicid (32, 16, 8 mg/kg) and fluoxetine (5 mg/kg) for six weeks significantly increased the body weights of CUMS rats \((p < 0.01)\). Sucrose preference is used as a measure to detect anhedonia. The effect of helicid on sucrose preference is presented in Fig. 3B, prior to testing no obvious group differences in sucrose intake were noted \([F(5, 54) = 0.14, p > 0.05]\). After six weeks of CUMS treatment, a significant decrease in sucrose intake was observed in the CUMS groups compared with the control group \([F(5, 54) = 183.77, p < 0.01]\). Helicid (32, 16, 8 mg/kg) and fluoxetine (5 mg/kg) treatment reversed the CUMS-induced changes in sucrose preference \((p < 0.01)\).

Effects of Helicid on OFT The OFT was conducted following the SPT. The total distance travelled during the 5-min test was indicative of rat locomotive activity. Figure 4A shows that CUMS exposure induced a significant decrease in distance traveled compared to the control group \([F(5, 54) = 69.56, p < 0.01]\), the treatment with helicid (32 mg/kg) and fluoxetine (5 mg/kg) dramatically increased locomotive activity compared with the CUMS rats \((p < 0.01)\). The number of crossings and rearings of CUMS rats was significantly decreased compared to controls \([F(5, 54) = 5.99, p < 0.01; F(5, 54) = 22.17, p < 0.01]\), whereas administration of helicid (32 mg/kg) or fluoxetine (5 mg/kg) for six weeks increased the number of crossings and rearings compared to the CUMS group \((p < 0.01)\). The effects of helicid at a dose of 32 mg/kg were more pronounced than doses of 16 and 8 mg/kg.

Effects of Helicid on FST Test The FST was conducted following the OFT. As shown in Fig. 4D, the immobility times in the CUMS group were significantly increased when compared to the control group \([F(5, 50) = 45.20, p < 0.01]\). The treatment groups with helicid (32, 16 and 8 mg/kg) or fluoxetine (10 mg/kg) for six weeks dramatically reduced the duration of immobility in the CUMS group \((p < 0.01)\).

Effects of Helicid on MWM Test To investigate the...
The effect of helicid on spatial learning and memory, the MWM was employed. In the place navigation test (Fig. 5A), the escape latency of each group was shortened following the four consecutive days of training. On the fifth day, the CUMS rats had a longer escape latency than the control rats $[F(5, 54) = 24.68, p < 0.01]$, compared with the CUMS rats, the latency of the helicid (32, 16, 8 mg/kg) or fluoxetine (10 mg/kg) groups was significantly reduced ($p < 0.01$). In the probe test (Fig. 5C), the CUMS rats crossed the platform position significantly less often compared to the control rats $[F(5, 54) = 16.682, p < 0.01]$; however, after helicid (32, 16 and 8 mg/kg) and fluoxetine (10 mg/kg) treatment the crossing number was significantly increased compared to the CUMS rats ($p < 0.01$). As demonstrated in Figs. 5C and D, rats treated with helicid (32, 16 and 8 mg/kg) and fluoxetine (5 mg/kg) had better performances than the CUMS rats. Overall, the results indicate that helicid can ameliorate learning and memory impairment in CUMS rats.

Helicid Increased Hippocampal Neuronal Neurogenesis in CUMS Rats In order to determine the influence of helicid on the survival of cells in the hippocampus CA1 and DG, BrdU was administered after CUMS. Levels of BrdU-positive cells were moderate in the adult hippocampus. In the hippocampal CA1 region, the CUMS rats were significant reduction in the number of viable (i.e., surviving) neurons $[F(5, 18) = 10.86, p < 0.01]$; Figs. 6A, B]. Administration of helicid (32, 16 or 8 mg/kg) or fluoxetine (5 mg/kg) resulted in a significant increase in BrdU-positive cells in the CA1 region compared with the CUMS-exposed rats ($p < 0.01$). As shown in Figs. 7A and B, in the hippocampal DG, we noticed a significant decrease in the number of BrdU-positive cells in CUMS rats compared with control rats $[F(5, 18) = 8.66, p < 0.01]$. Administration of helicid (32, 16, 8 mg/kg) or fluoxetine (5 mg/kg) resulted in a significant increase in BrdU-positive cells in the hippocampus compared with the CUMS-exposed rats ($p < 0.01$). Therefore, helicid may improve learning...
cognitive ability and exert antidepressant effects by inducing the proliferation of neurons.

**The Effects of Helicid on SERT Protein Levels in CUMS Rats**

As shown in Figs. 8B and C, SERT levels in the CUMS-exposed rats increased compared to the control group ($F(5, 18) = 52.60, p < 0.01$), whereas the treatment with helicid
(32, 16 or 8 mg/kg) or fluoxetine (5 mg/kg) resulted in a decrease in SERT in the hippocampus compared with the CUMS group ($p < 0.01$).

**The Effects of Helicid on the cAMP/PKA/CREB Signaling Pathway.** To investigate the effect of helicid on the cAMP/PKA/CREB pathway in the hippocampus of CUMS rats, we examined changes in cAMP, PKA C-α, CREB, p-CREB protein expressions via ELISA and Western blot assays. As shown in Fig. 8A, a significant decrease in cAMP levels was observed in the hippocampus of the CUMS group compared with control animals ($F(5, 24) = 8.42$, $p < 0.01$), but daily administration of helicid (32 or 16 mg/kg) or fluoxetine (5 mg/kg) significantly elevated cAMP levels in the hippocampus ($p < 0.01$) compared with the CUMS rats. As shown in Figs. 8B, D and E, PKA C-α and p-CREB expression levels in the CUMS group were decreased compared with...
the control group [respectively, $F(5, 18) = 13.05$, $p < 0.01$; $F(5, 18) = 4.50$, $p < 0.01$]. Following treatment with helicid (32, 16 or 8 mg/kg) or fluoxetine (5 mg/kg), expression of hippocampal PKA C-α and p-CREB in the helicid and fluoxetine groups were significantly upregulated compared with the CUMS group ($p < 0.05$). No significant differences were found between the helicid and fluoxetine groups ($p > 0.05$). Thus, these results suggest that helicid regulates the cAMP/PKA/CREB pathway to exert antidepressant effects.

**DISCUSSION**

Depression is a common illness associated with long episodes, high rates of chronicity, relapse and recurrence, psychosocial and physical impairment, a high suicide rate, and has become a major public health issue. Although many types of antidepressant including selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOIs), and tricyclic antidepressants (TCAs) have beneficial effects on depression, many antidepressant drugs have a number of short-coming, including slow onset, low response rates, toxic effects on organs and drug resistance. Recent studies have found traditional herbal medicine to be effective complementary and alternative therapies for depression that are expected to induce less obsevere side effects such as insomnia, constipation, cardiovascular, and metabolic disorders compared with traditional antidepressant drugs. Traditional herbal medicine (e.g., St. John's wort) have demonstrated beneficial antidepressant-like effects in attenuating side-effects.

The CUMS model simulates the pathogenesis of clinical depression and is widely used to screen antidepressants and study the pathophysiology of depression. CUMS animals are exposed to different kinds of stress that imitate unpredictable stressful life events and caused depressive-like behaviors that mirror similar cognitive, physiological, emotional changes. In our behavioral tests, after 6 weeks of CUMS rats showed, reduced sucrose consumption, and SRT could reflect anhedonia (a decrease of interest to rewards), a core symptom of human major depression. With the administration of helicid, CUMS-induced anhedonia-like behavior in the SPT was clearly reversed. In the present study, CUMS resulted in a significant decrease in sucrose preference in rats, demonstrating that the rat depression model has been successfully established. Helicid had the same effects as fluoxetine and improve anhedonia and increase interest in rewords.

The OFT test is an experimental test used to assay general locomotor activity and exploratory behavior in experimental animals. The total distance traveled reflects the locomotor activity in a novel environment, suggesting changes in emotionality. The number of crossing and rearings are not purely a measure of activity but also an index of the animal’s behavior to explore its surroundings. The reduction in distance traveled and diminished number of crossing and rearings in the OFT mimic to some extent the symptoms of human major depression, suggesting a change in emotion or security. In addition, we found the CUMS procedure lengthened immobility times in the FST, a manifestation of ‘behavioral despair.’ However, these depression-like behaviors were reversed by chronic treatments with a traditional antidepressant agent (fluoxetine) as well as helicid, suggesting that helicid has the same antidepressant effect as fluoxetine and improves depressive symptoms in CUMS rats.

Rodents exposed to chronic stress also show reduced learning and cognitive flexibility in attentional set shifting and reversal learning tasks, and this effect can be alleviated after acute or subchronic antidepressant treatment. Fluoxetine, a common SSRI, was selected as a positive control drug in our study. Fluoxetine can improve cognition, learning and memory in patients or animals with depression. In the MWM test, chronic administration of helicid and fluoxetine reversed the increase in escape latency and reduced platform crossings induced by the CUMS procedure. Helicid treatment at a dose of 32 mg/kg enhanced learning and cognition to the same extent as fluoxetine at 5 mg/kg. Therefore, we speculate that helicid has equal abilities as fluoxetine to improve learning and cognition.

The hippocampus is the primary brain structure involved in emotion, learning and memory. CUMS causes a reduction in hippocampal volume, clinically manifested as increased depressive-like behaviors, learned helplessness, anhedonia and social withdrawal. Studies have shown that hippocampal lesions impair recognition memory and the ability to perform object recognition and spatial awareness tasks. Collectively, these results highlight the importance of hippocampal processing for both spatial and recognition memory and emphasize the critical role that CA1 neurons play in the consolidation of these processes. BrdU is an analogue of thymidine which is rapidly incorporated into dividing and proliferating cells. BrdU can accurately reflect the proliferation of cells and is widely used in neurogeneticrelated research. In our study, we have demonstrated that helicid or fluoxetine treatment significantly decreased hippocampal CA1 cell death in comparison to CUMS rats. We suggest that hippocampal CA1 neuroprotection may be responsible for the observed significant improvement in learning and cognitive function of the helicid-treated rats in Morris water maze tests.

Studies have reported a robust link between impaired neuroplasticity and depression, specifically because both decreases adult hippocampus neurogenesis, a process in which new granule cell neurons are born and incorporated into the DG of the hippocampus throughout life, suggesting a causal link between DG neurogenesis and antidepressant efficacy. Additionally, stress-induced hippocampal neuronal atrophy and loss of may lead to the pathogenesis of depression. In the present study, neuronal proliferation was attenuated in the hippocampal DG in CUMS rats. However, the decrease in number of BrdU-positive cells in CUMS rats was ameliorated after six weeks treatment with either helicid or fluoxetine. This suggests that both treatments increase hippocampal cell proliferation. Therefore, we may conclude that helicid has neuroprotective effects, inducing increase cell proliferation.

Impairment of 5-HT neurotransmission by depletion of 5-HT or inhibition of 5-HT synthesis can induce symptoms of depression. Activity of 5-HT neurons is, in turn, limited by homeostatic negative feedback control exerted by extracellular 5-HT via somatodendritic inhibitory 5-HT1A autoreceptors. The importance of 5-HT1A receptor function is further supported by the presumed mechanism of selective 5-HT reuptake inhibitor (SSRI) antidepressant action. Among various mediators of brain 5-HT signaling, SERT is present in 5-HT neuronal soma, dendrites and axon terminals, and plays a central role because it mediates the reuptake of 5-HT from the
extracellular space/synapse; moreover, SERTs are molecular targets of clinically effective antidepressants. In our previous study, we showed that helicid plays an antidepressant role by activating 5-HT1A receptors and modulating the concentration of 5-HT, similar to fluoxetine.\(^{30}\) In the present study, we demonstrated that CUMS-treated rats increased SERT protein levels, whereas treatment with helicid or fluoxetine significantly reduced SERT expression. These results suggest that the antidepressant-like effects of helicid are possibly mediated by regulation of 5-HT, 5-HT1A and SERT in the hippocampus.

The CAMP-signaling pathway is one of intracellular signaling pathways involved in neurogenesis of neural stem cell (NSC), learning and memory capacity. The levels of cAMP are affected by and modulate the activities of CAMP-dependent PKA, followed by reduced levels of p-CREB. CREB signaling is a crucial factor implicated in promoting synaptic and neural plasticity by regulating genes that increase synaptic and neural plasticity. It has also been shown that p-CREB may be a useful molecular markers for indicating the effectiveness of antidepressant treatment.\(^{33}\) The effects of antidepressant drugs may be mediated by the stimulation of CREB expression in depression patients, CREB activation following chronic SSRI administration is a sign of neuroplasticity.\(^{34,35}\)

In the present study, we found a decrease in the expression of hippocampal cAMP, PKA C-α and p-CREB, induced by CUMS, whilst chronic administration of helicid and fluoxetine significantly reversed the decrease in cAMP, PKA C-α and p-CREB expression. Therefore, we hypothesize that helicid regulates the CAMP/PKA/CREB signaling pathway to strengthen its neuroprotective effects in the CUMS model.

In summary, our experimental results indicated that helicid substantially improved learning and cognitive deficits and ameliorated depression-like behaviors. The effects of helicid are possibly mediated by the expression of SERT, stimulation of the CAMP/PKA/CREB pathway and enhancing the proliferation of hippocampal neurons. Our findings show that helicid may be a promising treatment for depression and lay the foundation for therapeutic development of depression drugs focusing on natural products. Future research will be required to confirm these mechanisms at the molecular and gene level and fully elucidate the antidepressant effects of helicid.

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Conflict of Interest The authors declare no conflict of interest.

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