SUPPLEMENTARY MATERIAL

The antidepressant effect of 4-hydroxybenzyl alcohol 2-naphthoate through monoaminergic, GABAergic system and BDNF signaling pathway

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Gastrodigenin, also known as 4-hydroxybenzyl alcohol (HBA), is one of the main components of Gastrodia elata, which is a perfect lead compound of natural products. In order to get new active compounds, we modified the structure of HBA through esterification with carboxylic acid, and got a series of derivatives in which 4-hydroxybenzyl alcohol 2-naphthoate (NHBA) showed stronger antidepressant activity than HBA. In this paper, we firstly evaluated the antidepressant activity of NHBA by tail suspension test (TST) and forced swimming test (FST). Then, we carried out the biochemical assay and western blot to determine its mechanism. The results displayed that NHBA could increase the content of serotonin, dopamine, norepinephrine, γ-aminobutyric acid, brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) in mice brain. It suggested that NHBA exhibited an antidepressant-like effect through monoaminergic system, GABAergic system and BDNF/TrkB signaling pathways.
Keywords: gastrodigenin; antidepressant effect; monoaminergic system; BDNF/TrkB signaling pathways
Experimental Methods

Animals

Adult male ICR mice (weighting 18~22 g) were supplied by Changchun Yisi experimental animal technology Co. Ltd., who were group-housed (10 per cage) with under stable temperature (25 ± 2°C) and relative humidity (60 ± 2 %), kept in 12 h light/dark cycles (with light on at 7:00 a.m.) with food and water available ad libitum for the duration of study. Animals were adjusted for at least 7 days before tests performing. All tests were followed up in accordance with the Guide for Animal Experimentation of Jilin Agricultural University. The protocol was approved by the Jilin Agricultural University Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals used and any pain and discomfort experienced by the subjects.

Drugs

Fluoxetine, tianeptine, reboxetine, imipramine, p-chlorophenylalanine (pCPA), prazosin, haloperidol and bicucullin were purchased from Melone Pharma Co. Ltd. (Dalian, China). NHBA was synthesized by ourselves. All the reagents used in this study were of analytical grade.

Treatment

NHBA was suspended in 0.05% carboxymethylcellulose sodium (CMC-Na) physiological saline and was intragastrically (i.g.) administered. Other drugs were dissolved in physiological saline and administered by intraperitoneal injection (i.p.). All the drug solutions were freshly prepared before use, and all the tests were carried out between 9:00 and 11:00 hours once daily for three days. Fluoxetine (5 mg/kg), tianeptine (15 mg/kg), reboxetine (2.5 mg/kg) and imipramine (10 mg/kg) used as the sub-effective antidepressant drugs were administered after sub-effective NHBA (10 mg/kg) treatment 30 min before the behavioral test performed. pCPA (100 mg/kg) was
injected for 4 days, the last administration was given 30 min prior to NHBA (25 mg/kg). Prazosin (1 mg/kg), haloperidol (0.2 mg/kg) and bicucullin (4 mg/kg) were all given 30 min prior to NHBA (25 mg/kg). Mice in control group were treated for the same volume of 0.05% CMC-Na physiological saline. All administrations were given in a constant volume of 10 ml per kg body weight to mice.

**Tail suspension test (TST)**

Mice were suspended, 10 cm above the floor, for a 6 min test session using mastic tape affixed 1 cm from the tip of the tail. When mice gave up their struggle, we considered as the complete halting of movement while suspended. The immobility time was recorded during the last 4 min of a total 6 min trial. The changes in immobility duration were studied after administrating drugs in separate groups of animals. Each animal was used only once.

**Forced swimming test (FST)**

Mice were forced to swim alone in a glass jar (height 25 cm, diameter 14 cm), containing 18 cm of fresh water at 25 ± 2°C. After an initial 2 min period of powerful activity, each animal assumed a typical immobile posture. It was considered to be immobile when the mouse remained floating in the water without struggling, making only minimum movements of its limbs necessary to keep its head above the water. The total duration of immobility was recorded during the next 4 min of a total 6 min test. The changes in immobility duration were studied after administrating drugs in separate groups of animals. Each animal was used only once. After the FST, the mouse brain was taken out and stored in -80 °C refrigerator for biochemical measurements.

**Spontaneous locomotion activity test (SLT)**

In order to exclude the possibility that the alteration of the immobility time in TST and FST was owing to interference of motor effect, spontaneous locomotor
activity of each mouse was observed in a ZZ-6 mouse autonomic activity test instrument. The behavioral evaluation was performed between 8:00 AM and 11:00 AM before the start of the behavioral tests the animals were allowed to acclimate to the testing rooms for at least 1 h. During the experiment, keep the lab quiet. The times of autonomous activities were evaluated over a 5 min period.

**Biochemical measurements**

The brain of mouse was washed with ice-cold physiological saline and homogenized, shaken for 10 s and centrifuged at 10,000×g for 10 min at 4 °C. The supernatant was retained. The monoamine neurotransmitters including 5-HT, DA, NE, and GABA were all measured according to the instructions of the commercially available, competitive enzyme-linked immunosorbent assay kits which were bought from US R&D systems, Ltd. (Minneapolis, USA). The optical density (OD) of each well was quantified at 450 nm with a microplate reader.

**Western blotting analysis**

The hippocampus tissue was washed and lysed with buffer. The protein concentration was determined using the BCA Protein Kit (Beyotime, Nanjing, China). The protein extract was decomposed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to a transferred polyvinylidene fluoride (PVDF) membrane. The membranes were sequentially incubated with primary and secondary antibodies and enhanced chemiluminescence (ECL) solutions, followed by autoradiography. The intensity of the blot was analyzed using Image Pro Plus 6.0.

**Statistical analysis**

All figures were performed using GraphPad Prism, version 6.00. Data were expressed as the mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) was used for statistical analysis of data, followed by Tukey’s post-hoc
multiple comparison test. Statistical significance was defined as $p < 0.05$.

**Discussion**

TST and FST were both known as the well-validated behavioral despair tests which commonly used to be reliable animal models of acute administration (Porsolt et al. 1977; Steru et al. 1985). A series of researches had demonstrated that the two tests were both highly sensitive to most of clinically effective anti-depressants, including tricyclic anti-depressants, selective serotonin reuptake inhibitors and monoamine oxidase inhibitors (Cryan et al. 2002; Detke et al. 1995). Therefore, we used TST and FST to measure the antidepressant effects of HBA derivatives we had synthesized from HBA and carboxylic acids (Zhu et al. 2018). Among the 10 new HBA derivatives, we found that NHBA showed the strongest antidepressant activity than HBA and other derivatives. In order to avoid the false positive effects induced by motor effect, then we detected the locomotor activities of NHBA (Rodrigues et al. 2002). The results showed that NHBA neither in different doses nor synergy with other antidepressants exhibited obvious effect on the locomotor activities of mice. So it suggested that the antidepressant effect of NHBA was not due to a psychostimulant or motor enhancing effect.

Depression was an extremely complex disease with unknown pathomechanism. Presently, the monoaminergic hypothesis was recognized as the main cause of depression. It was reported that the monoamine neurotransmitters imbalance in brain, such as 5-HT, NE and DA, could lead to mood disorders (Clausius et al. 2009). Antidepressants were developed to maintain these monoamines concentration in brain (Ma et al. 2016). Many studies also revealed that concurrent treatment with well-known antidepressant drugs might produce better effect rates or work faster than monotherapy (Tardito et al. 2012; Molina et al. 2004). Therefore, in order to verify the antidepressant activity of NHBA and study its possible action pathway, we chose some classical antidepressants like fluoxetine, the first selective 5-HT reuptake inhibitor (SSRI); tianeptine, the tricyclic antidepressants (TCA); reboxetine, NE
reuptake inhibitor and imipramine, NE and 5-HT reuptake inhibitor, to treat with NHBA simultaneously. The sub-effective dose of NHBA (10 mg/kg) was used to cooperate with the sub-effective dose of fluoxetine (5 mg/kg), tianeptine (15 mg/kg), reboxetine (2.5 mg/kg) and imipramine (10 mg/kg) in TST and FST. The results displayed that the immobility time in each co-administration group was significantly reduced. It suggested that the antidepressant effect of NHBA might be related to serotonergic and noradrenergic receptors.

GABAergic system which was known as the inhibitory neurotransmitter also played a pivotal role in the neurobiology of depression (Salari et al. 2015). GABA in major depressive disorder (MDD) patients exhibited a decreased level in the central nervous system (CNS), so effective treatment with antidepressants would lead to an increase in GABA concentration (Pehrson et al. 2015). Therefore, we studied the antidepressant effect of NHBA by related antagonist receptors of GABAergic system to reveal its possible mechanism. Furthermore, there were several studies suggested that fluoxetine could induce significant increasing in GABA neurotransmission independently of its serotonergic signaling, and pharmacological antagonism of GABA_A receptors could partially block fluoxetine’s antidepressant-like effects (Khisti et al. 2000). Ultimately, in our results we found that GABA levels in NHBA group were increased, while the bicuculline group had no increase compared with the control group, meanwhile NHBA played a dramatic synergy effect with fluoxetine, so it suggested that the antidepressant effect of NHBA was similar to fluoxetine. Further investigations would be focused on the specific associated subtype receptors.

In the biochemical test in vitro, in order to verify whether the antidepressant-like effect of NHBA was connected with the monoaminergic and GABAergic system, we detected the levels of 5-HT, NE, DA and GABA in mice brain of the relevant administration groups respectively. While, the biochemical measurements of 5-HT, NE, DA and GABA coincided with the behavioral tests.

In addition, there was an accumulating body of evidence linking impaired
neurogenesis and insufficient signaling of neurotrophic factors to the development of depression. BDNF was a most abundant and widely distributed neurotrophin in the brain which played critical roles in neuronal development, function, and survival (Park et al. 2013). BDNF/TrkB signaling provides beneficial microenvironment for neuronal survival, neurodevelopment, and neurogenesis. Previous studies revealed that classic antidepressant drugs benefited depression patients partially by improving neurogenesis and/or BDNF/TrkB pathway. Reduced levels of BDNF mRNA and protein were shown in the brains of Alzheimer’s disease patients (Phillips et al. 1991) and Parkinson’s disease patients as well as in depression (Scalzo et al. 2010; Tsankova et al. 2006). BDNF expression could be upregulated by antidepressant treatment (Koppel et al. 2013), suggesting that BDNF contributed to therapeutic action. In our study the results showed that NHBA could increase BDNF and TrkB contents in a dose-dependent manner. So we could speculate the underlying mechanism of antidepressant-like effects of NHBA to be involved with the neurogenesis through BDNF/TrkB.

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Figure S1 The chemical structure of NHBA.
Figure S2. The antidepressant effect of NHBA in different doses in TST (a) and FST (b). The values represent the mean ± SD (n=10 in each group), compared with the control group, * p<0.05, ** p<0.01, *** p<0.001. Two way ANOVA, post-hoc Tukey’s test.
Figure S3. The antidepressant effect of sub-effective dose of NHBA (10 mg/kg, i.g.) co-administered with fluoxetine (5 mg/kg, i.p.) (a), tianeptine (5 mg/kg, i.p.) (b), reboxetine (2.5 mg/kg, i.p.) (c) and imipramine (10 mg/kg, i.p.) (d) in TST and FST. The values represent the mean ± SD (n=10 in each group), compared with the control group, ** p<0.01, *** p<0.001, **** p<0.0001. Two way ANOVA, post-hoc Tukey’s test.
Figure S4. The antidepressant effect of effective dose of NHBA (25 mg/kg, i.g.) co-administered with pCPA (100 mg/kg, i.p.) (a), prazosin (1 mg/kg, i.p.) (b), haloperidol (0.2 mg/kg, i.p.) (c) and bicuculline (4 mg/kg, i.p.) (d) in TST and FST. The values represent the mean ± SD (n=10 in each group), compared with the control group, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; compared with the NHBA group, # $p<0.01$, ## $p<0.05$, ### $p<0.001$, #### $p<0.0001$. Two way ANOVA, post-hoc Tukey’s test.
Figure S5. The content of 5-HT in the brain of mice exposed to the FST (a), the content of DA in the brain of mice exposed to the FST (b), the content of NE in the brain of mice exposed to the FST (c), the content of GABA in the brain of mice exposed to the FST. The values represent the mean ± SD (n=10 in each group), compared with the control group, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; compared to NHBA (25 mg/kg, i.g.) group, # p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001. Two way ANOVA, post-hoc Tukey’s test.
Figure S6. Effects of NHBA on the protein expression of BDNF (a), TrkB (b). The protein expression was examined by western blotting analysis in hippocampus tissues: (I) control group, (II) fluoxetine group, (III) NHBA (100 mg/kg), (IV) NHBA (50 mg/kg), (V) NHBA (25 mg/kg) and (VI) NHBA (10 mg/kg). Values were the mean ± SD with 10 mice in each group. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), **** \( p < 0.0001 \), compared to the control group. Two way ANOVA, post-hoc Tukey’s test.
| Groups                             | Average value (x±SD) |
|-----------------------------------|----------------------|
| control                           | 156.67±32.08         |
| fluoxetine (25 mg/kg)             | 159.20±30.38         |
| HBA (50 mg/kg)                    | 150.00±31.74         |
| NHBA (10 mg/kg)                   | 135.67±20.08         |
| NHBA (25 mg/kg)                   | 128.17±7.96          |
| NHBA (50 mg/kg)                   | 149.40±38.93         |
| NHBA (100 mg/kg)                  | 153.50±32.18         |
| fluoxetine (5 mg/kg)              | 149.33±12.30         |
| fluoxetine (5 mg/kg) + NHBA (10 mg/kg) | 146.60±32.94     |
| reboxetine (2.5 mg/kg)            | 142.34±26.23         |
| reboxetine (2.5 mg/kg) + NHBA (10 mg/kg) | 127.83±13.80     |
| tianeptine (15 mg/kg)             | 131.62±9.61          |
| tianeptine (15 mg/kg) + NHBA (10 mg/kg) | 127.17±28.20     |
| imipramine (10 mg/kg)             | 128.33±18.41         |
| imipramine (10 mg/kg) + NHBA (10 mg/kg) | 122.37±22.80     |
| Treatment                                      | Value       |
|-----------------------------------------------|-------------|
| $p$CPA (100 mg/kg)                            | 143.17±19.03|
| $p$CPA (100 mg/kg) + NHBA (25 mg/kg)         | 138.83±32.37|
| prazosin (1 mg/kg)                            | 146.27±12.08|
| prazosin (1 mg/kg) + NHBA (25 mg/kg)         | 141.83±30.73|
| bicuculline (4 mg/kg)                         | 175.67±13.62|
| bicuculline (4 mg/kg) + NHBA (25 mg/kg)      | 160.40±20.47|
| haloperidol (0.2 mg/kg)                       | 167.33±32.71|
| haloperidol (0.2 mg/kg) + NHBA (25 mg/kg)    | 138.60±36.00|