Essential oil of *Perilla frutescens*-induced change in hippocampal expression of brain-derived neurotrophic factor in chronic unpredictable mild stress in mice

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**Abstract**

*Ethnopharmacological relevance:* *Perilla frutescens* (*Perilla leaf*), a traditional Chinese medicinal herb, has been used for centuries to treat various conditions including depression. A previous study of the authors demonstrated that essential oil of *Perilla frutescens* (EOPF) attenuated the depressive-like behavior in mice.

**Aim of the study:** This study was undertaken to explore the dynamic change of behaviors and brain-derived neurotrophic factor (BDNF) expression induced by chronic unpredictable mild stress (CUMS), and improved by EOPF.

**Materials and methods:** Four separate CUMS experimental groups (1-week, 2-week, 3-week and 4-week treatment) were treated with EOPF (3 mg/kg and 6 mg/kg, p.o.) or fluoxetine (20 mg/kg, p.o.), followed by sucrose preference, locomotor activity, immobility and hippocampal BDNF measurement.

**Results:** EOPF, as well as fluoxetine, restored the CUMS-induced decreased sucrose preference and increased immobility time, without affecting body weight gain and locomotor activity. Furthermore, CUMS (3 or 4-week) produced a reduction in both BDNF mRNA and protein expression in the hippocampus, which were ameliorated by EOPF (4-week) and fluoxetine (3 or 4-week) treatment.

**Conclusion:** These results presented here show that BDNF is expressed depending on length of CUMS procedure and EOPF administration. And this study might contribute to the underlying reason for the slow onset of antidepressant activity in clinic.

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1. Introduction

Depression, one of the major causes of disability worldwide, is a mood or affective disorder caused by many factors, from the psychological, social, and environmental to the genetic, and metabolic (World Health Organisation (WHO), 2007). The mechanisms underlying depression as well as antidepressant activity are still not fully understood. Stress is well known to be one of the most important factors responsible for depressive disorders (Zhu et al., 2012). Recently, a novel hypothesis has emerged suggesting a role for neurotrophin in the pathogenesis of depression and in its treatment (Sen et al., 2008). For example, preclinical and clinical studies have demonstrated that stress and depression resulted in neurogenesis impairment and down-expression of neurotrophin in the brain (Tanis et al., 2007).

Brain-derived neurotrophic factor (BDNF), one of the most extensively neurotrophins studied in relation to depression, has been shown to promote neuronal survival, differentiation, function, and plasticity (Huang and Reichardt, 2001), suggesting that BDNF plays a key role in the pathophysiology of depression (Castrén and Rantamäki, 2010). In addition, a growing number of clinical and experimental evidence reports that alterations in BDNF levels are associated with the mechanisms of action underlying the favorable therapeutic activity of antidepressant drugs (Schmidt and Duman, 2010; Thompson Ray et al., 2011). As a result, BDNF has been considered as a possible target for antidepressants.

*Perilla frutescens* (*Perilla leaf*), a traditional Chinese medicinal herb, has been used for centuries to treat various conditions including depression. A previous study of the authors demonstrated that essential oil of *Perilla frutescens* (EOPF) treatment decreased the immobility time in the forced swimming test (FST) via monoaminergic systems (Yi et al., 2010), suggesting that EOPF might be useful for the prevention of depression. However, the antidepressant-like mechanism involved in the aspect of BDNF remains unknown. Given the importance of BDNF in the
pathophysiology of depression and antidepressant treatment, the aim of the present study was to determine how duration of treatment influences hippocampal BDNF levels in mice exposed to chronic unpredictable mild stress (CUMS) procedure.

2. Materials and methods

2.1. Animals

Male ICR mice (22–26 g; 4 weeks old) were purchased from Laboratory Animal Centre, Fujian Medical University, Fujian Province, PR China. Animals were housed 8 per cage (320 cm × 180 cm × 160 cm) under a normal 12-h/12-h light/dark schedule with the lights on at 07:00 a.m. and had free access to tap water and food pellets. Ambient temperature and relative humidity were maintained at 22 ± 2 °C and at 55 ± 5%, and given a standard chow and water ad libitum for the duration of the study. The animals were allowed 1 week to acclimatize themselves to the housing conditions before the beginning of the experiments. All procedures were performed in accordance with the published guidelines of the China Council on Animal Care (Regulations for the Administration of Affairs Concerning Experimental Animals, approved by the State Council on October 31, 1988 and promulgated by Decree No. 2 of the State Science and Technology Commission on November 14, 1988).

2.2. Reagents

Fluoxetine hydrochloride was purchased from Changzhou Siyao Pharmaceuticals Co., Ltd (Changzhou, PR China). All primers used in this study were designed and synthesized by Sangon Biotech Co. Ltd. (Shanghai, PR China). Trizol reagent was purchased from Invitrogen (Carlsbad, USA). Reverse transcriptase Moloney Murine Leukemia Virus (M-MLV) used for cDNA synthesis was from Promega Corporation (Madison, USA). All other reagents used in RT-PCR were purchased from Sangon Biotech Co. Ltd. (Shanghai, PR China).

2.3. Plant material and preparation of EOPF

The best quality commercial Perilla leaf [Perilla frutescens (L.) Britt (Labiatae)] was purchased from Fujian Pharmaceutical Co., LTD, PR China, which was collected in Zhejiang province of China and authenticated by Cheng-Fu Li, Department of Pharmacy, Xiamen Hospital of Traditional Chinese Medicine, PR China (voucher specimen number HU/CE-10251).

EOPF was prepared from 250 g of Perilla frutescens using supercritical equipment (Zeng et al., 2003; Yi et al., 2010). The extract was collected for 100 min under the condition of 45 °C and 30 MPa (3.1% w/w yield).

As described by Ling, the main constituents of EOPF extracted by supercritical fluid are L-perillaldehyde, limonene, trans-caryophyllene, selinene, santalene and bergamotene (Ling, 2005).

Table 1

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<thead>
<tr>
<th>Food and water deprivation</th>
<th>Exposure to an empty bottle</th>
<th>Soiled cage</th>
<th>Light/dark succession every 2 h</th>
<th>Space reduction</th>
<th>45° cage tilt</th>
<th>Overnight illumination</th>
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2.4. CUMS procedure

The procedure of CUMS was performed as described previously (Mao et al., 2009; Schweizer et al., 2009), with some modifications. Briefly, the weekly stress regime consisted of food and water deprivation, exposure to an empty bottle, soiled cage, light/dark succession every 2 h, space reduction, 45° cage tilt, overnight illumination and predator sounds (Table 1). All stressors were applied individually and continuously, day and night. Control animals were housed in separate room and had no contact with the stressed groups. They were deprived of food and water for the 24 h preceding sucrose preference test (SPT), but otherwise food and water were freely available in the home cage.

2.5. Drug treatments and experimental design

Four separate experiments (1-week, 2-week, 3-week and 4-week treatment) were carried out and in each case a total of 40 mice were used. In each experiment, animals were divided into five treatment groups as follows: one vehicle-control (0.9% physiological saline), one vehicle-CUMS (0.9% physiological saline), one CUMS-fluoxetine (20 mg/kg) and two CUMS-EOPF treatments (3, 6 mg/kg). Thus, different groups of mice, 8 animals per group, were used for drug treatment and for each experiment. All treatments were administered by oral (p.o.) gavage in a volume of 10 ml/kg body weight. Drugs were administered successively for 9, 16, 23 or 30 days, respectively (Fig. 1). The treatment protocols of dose and administration route used for EOPF and fluoxetine was adopted according to the literature and our previous study (Mao et al., 2009; Yi et al., 2010). Body weights of all animals were weighed at the beginning and end of the separate experiment.

2.6. SPT

SPT was carried out at the end of 1-week, 2-week, 3-week or 4-week CUMS exposure. The test was performed as described previously (Mao et al., 2009). Briefly, before the test, mice were trained to adapt to sucrose solution (1%, w/v): two bottles of sucrose solution were placed in each cage for 24 h, and then one bottle of sucrose solution was replaced with water for 24 h. After the adaptation, mice were deprived of water and food for 24 h. SPT was conducted at 9:30 a.m. in which mice were housed in individual cages and were free to access to two bottles containing 100 ml of sucrose solution (1% w/v) and 100 ml of water, respectively. After 24 h, the volumes of consumed sucrose solution and water were recorded and sucrose preference was calculated as sucrose preference (%) = sucrose consumption (ml)/[sucrose consumption (ml) + water consumption (ml)] × 100%.

2.7. Open-field test (OFT)

The mice were treated with EOPF or fluoxetine 60 min before the exposure to the OFT on 8th, 15th, 22nd or 29th day, in order to...
assess possible effects of drug treatment on spontaneous locomotor activity. The apparatus consisted of a wooden box measuring 40 cm × 40 cm × 30 cm, with the floor divided into 25 equal squares (8 cm × 8 cm). The numbers of squares crossed with all paws (crossings) and of standing on the hind legs (rearings) were counted in a 3-min session (Mao et al., 2008). The apparatus was cleaned with a detergent and dried after occupancy by each mouse. The test sessions were recorded by a video camera and scored by an observer blind to treatment.

2.8. FST

The mice were treated with EOPF or fluoxetine 60 min before the exposure to the FST on 9th, 16th, 23rd or 30th day according to the traditional method described by Porsolt et al. (1977), with some modifications. Briefly, mice were individually placed in a glass cylinder (20 cm in height, 14 cm in diameter) filled with 10-cm high water (25 ± 2 °C). All animals were forced to swim for 6 min, and the duration of immobility was recorded during the final 4 min interval of the test. The immobility period was regarded as the time spent by the mouse floating in the water without struggling and making only those movements necessary to keep its head above the water. The test sessions were recorded by a video camera and scored by an observer blind to treatment.

2.9. Tissue sample collection

Twenty-four hours after completion of the FST, mice were sacrificed by decapitation. Whole brains were rapidly removed from mice and chilled in an ice-cold saline solution. Brain region of hippocampus was dissected on a cold plate and frozen in liquid nitrogen immediately. The tissue samples were stored at −80 °C until assay.

2.10. Real-time RT-PCR

Total RNA was isolated from hippocampus using Trizol reagent following the manufacturer’s instructions. Reverse transcription was performed using M-MLV reverse transcriptase for cDNA synthesis. Real-time PCR reactions were performed using a SYBR Premix Ex Taq Kit in ABI-7500 system. The BDNF (forward 5′-TTATTTTCATACTTGGTTGC-3′; reverse 5′-GTGCAGCCAGTGATGTCG-3′) and the internal control GAPDH (forward 5′-GGGTGTGAACAGGTCTTCT-3′; reverse 5′-CAGCGAGAAATCGGC-3′) primers were used. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products. The results were analyzed by the 2−ΔΔCT method (Livak and Schmittgen, 2001). The results were normalized to the mRNA expression level of GAPDH in each sample.

2.11. BDNF ELISA

Hippocampus samples were homogenized in lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate. The homogenate was centrifuged at 16,000 × g for 30 min at 4 °C, and the supernatant was collected and stored at −80 °C until assay.

Protein levels of samples were measured using the Lowry method (Lowry et al., 1951). BDNF protein was measured using BDNF ELISA kit (Boster, Wuhan, PR China) according to the protocol of the manufacturer.

2.12. Statistical analyses

All data were expressed as mean ± S.E.M. Data was analyzed using a one-way ANOVA, followed by post-hoc Dunnett’s test. A value of P < 0.05 was considered statistically significant for analysis.

3. Results

3.1. Effects of EOPF on the sucrose preference and body weight in the CUMS

Fig. 2 illustrates the sucrose preference of mice during 1-week (A), 2-week (B), 3-week (C) or 4-week (D) of the CUMS regimen. The onset of anhedonia, i.e. a significant reduction in sucrose preference (P < 0.05) was seen after one week of CUMS and remained in subsequent weeks (2-week: P < 0.05; 3-week: P < 0.01; 4-week: P < 0.01). However, only significant effect of treatment was observed after 3-week (Fluoxetine: P < 0.01; 3 mg/kg EOPF: P < 0.05; 6 mg/kg EOPF: P < 0.01) or 4-week (Fluoxetine: P < 0.01; 3 mg/kg EOPF: P < 0.05; 6 mg/kg EOPF: P < 0.01) administration.

In addition, we did not observe a statistically significant difference between the body weight gains of all groups at the end of CUMS regimen after 1-week, 2-week, 3-week or 4-week treatment (Fig. 3).
3.2. Effects of EOPF on the immobility time in the OFT

As shown in Fig. 4, neither EOPF (3, 6 mg/kg) nor fluoxetine (20 mg/kg) altered the locomotor activity of CUMS mice in the OFT after 1-week, 2-week, 3-week or 4-week treatment.

3.3. Effects of EOPF on the immobility time in the FST

The effects of EOPF and fluoxetine on the immobility time in the mouse FST are shown in Fig. 5. Our results showed that CUMS did not produce any effect on the immobility time after 1-week administration (Fig. 5A), while after 2-week (Fig. 5B), 3-week (Fig. 5C) or 4-week (Fig. 5D) administration, a one-way ANOVA revealed a significant effect in the FST ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively). Post hoc analysis indicated a significant decrease in the immobility time was elicited by the administration of fluoxetine ($P < 0.05$) after 2-week treatment, EOPF (6 mg/kg: $P < 0.05$) or fluoxetine ($P < 0.01$) after 3-week treatment, as well as EOPF (3 mg/kg: $P < 0.01$; 6 mg/kg: $P < 0.01$) or fluoxetine ($P < 0.01$) after 4-week treatment.

3.4. Effects of EOPF on the hippocampal BDNF mRNA expression

No significant effect of stress or treatment on BDNF mRNA expression in hippocampus was observed after 1- or 2-week. CUMS induced a decrease of hippocampal BDNF mRNA expression after 3- or 4-week ($P < 0.05$, $P < 0.01$, respectively). In addition, repeated daily administration of fluoxetine ($P < 0.01$) but not EOPF for 3-week increased BDNF mRNA expression. Moreover, after administration for 4-week, both EOPF (3 mg/kg: $P < 0.01$; 6 mg/kg: $P < 0.01$) and fluoxetine ($P < 0.01$) resulted in a marked increase in the hippocampal BDNF mRNA expression according to the Dunnett’s test Figs. 6 and 7.

3.5. Effects of EOPF on the hippocampal BDNF protein levels

Parallel to the effects on BDNF mRNA expression, 1- or 2-week CUMS or treatment failed to alter BDNF protein levels in the hippocampus. After 3-week procedure, CUMS induced a tendency to suppress the BDNF levels in hippocampus ($P = 0.055$). And 4-week CUMS procedure completely resulted in decrease of BDNF levels ($P < 0.05$). On the other hand, fluoxetine ($P < 0.05$) significantly elevated the BDNF protein levels after a 3-week period. And post hoc test revealed that 6 mg/kg EOPF ($P < 0.05$) and 20 mg/kg fluoxetine ($P < 0.01$) increased the hippocampal BDNF protein levels compared to vehicle-CUMS group after 4-week treatment.

4. Discussion

Aromatherapy is a therapeutic use of essential oils naturally extracted from plant material, such as flowers, trees, grasses, roots, seeds and bark. It is a "branch" of herbal medicine that centers on using fragrant substances, particularly oily plant extracts, to alter mood or to improve individuals’ health or appearance, especially depression. Despite several studies examining the role of aromatherapy in the treatment of depression (Seol et al., 2010; Sah et al., 2011), the molecular alterations that occur during its antidepressant-like action are very poorly understood. In the present study, the behavioral and neurotrophic improvements of oral EOPF administration were investigated to clarify the treatment duration and the time after CUMS beginning. As a widely used model for researching and developing antidepressants (Willner et al., 1987), the measurement index sucrose preference reduction of the CUMS procedure, which reflects a state of anhedonia to produce a condition similar to human depression, can be restored by therapeutically effective drugs for the treatment of depression (Zhang et al., 2010). In the present experiment, we used an efficacious mouse CUMS procedure to mimic stress in daily life. A reduction of sucrose preference induced by CUMS procedure was significantly restored by EOPF, which confirmed...
again the antidepressant-like effect of EOPF. In addition, the results demonstrated that 3 weeks of EOPF treatment was needed for a complete recovery of anhedonia.

While comparing body weight from the beginning to the end of the stress procedure, we observed that the body weight gain was not affected by either CUMS or treatment in our study, which is consistent with the previous studies (Li et al., 2007; Yalcin et al., 2008). However, contrary to the previous reports and our findings, several studies also reported that CUMS caused a reduction in body weight gain (Schweizer et al., 2009; Nollet et al., 2011). This discrepancy may be produced by many parameters in the experimental design, mainly including strains of mice, experimental conditions, stressed time and so on (Pothion et al., 2004; Mutlu et al., 2009).

Crossing and rearing numbers were also assessed in a 3-min session in an OFT apparatus 1 day after CUMS. However, no
significant difference emerged from our data. The effect of stress on locomotor activity is still controversial. Although various studies found a decrease in the parameters induced by CUMS (Mao et al., 2010; Liu et al., 2012; Liu and Zhou, 2012), some recent studies indicated an increase (Harris et al., 1997) or no change (Li et al., 2008; Yalcin et al., 2008; Mutlu et al., 2009), especially in different time points of one CUMS procedure (Liu et al., 2011).

FST is one of the most common animal tests specifically used for screening new antidepressant drugs and their mechanisms of action, because of its good reliability and predictive validity (Petit-Demouliere et al., 2005). Currently used antidepressant medications, after single doses, often rapidly reduce the immobility time in the FST. However, the clinical antidepressant response develops slowly over the first weeks of treatment (Krishnan and Nestler,
produce long-lasting behavioral pathology (Krishnan and Nestler, 2008). For these reasons, they prefer the word “test” to “model”, which corresponds to an examination of a critically key aspect of either the response to stress or to antidepressant drug action (Petit-Demouliere et al., 2005; Krishnan and Nestler, 2008). To summarize these ideas, FST as a very specific test, where behaviors are induced by stress with no direct relation to symptoms in humans, but which are extremely sensitive to antidepressants. Additionally, this test also provides a useful tool to study neuropharmacological mechanisms underlying stress and antidepressant response (Hascoët and Bourin, 2009). Moreover, new approaches of research for antidepressant treatments continue to use the FST as a preliminary test. For example, some authors used the FST and showed that BDNF infusion in the brain resulted in 57% shorter latency to immobility relative to control rats (Eisch et al., 2003). According to the previous studies, chronic repeated stress has been shown to dramatically increase the immobility time in the mouse FST (Zhou et al., 2007; Mao et al., 2009). Consistent with the literatures, our present study also found CUMS resulted in an increased immobility time in FST in mice. Long-term EOPF treatment (3- or 4-week treatment) counteracted the CUMS-induced elevation in immobility time. Taken together, results obtained from behavioral studies indicated a 3-week treatment may be essential for onset of antidepressant-like activity related to abnormal behaviors by EOPF.

Hippocampus is expected to be particularly associated with cognitive abnormalities (learning and memory impairments) that are seen in depressive patients (Berton and Nestler, 2006). Regional distribution of the BDNF gene is highly variable throughout the brain, with parts of the hippocampus showing particularly high densities (De Foubert et al., 2004). As a result, it is generally accepted that hippocampal BDNF maintains and promotes neurons, enhances synaptic activity and regulates memory, and thus could possibly be an underlying cause for the development of depression (Angelucci et al., 2005). Antidepressants have been shown to reverse and protect against depression-induced BDNF down-regulation in the hippocampus (Molteni et al., 2006; Thompson Ray et al., 2011). On the other hand, the fact that the delayed increase in BDNF expression, like the clinical antidepressant response, has made BDNF a very popular molecule among antidepressant studies (Castrén and Rantamäki, 2010). These results, together with the antidepressant-like effects of BDNF in cellular and behavioral models of depression (Shirayama et al., 2002; Schmidt and Duman, 2010), suggest an important role for hippocampal BDNF in the molecular mechanisms of antidepressive therapy.

The main goal of the present study was to directly compare the effects on BDNF mRNA and protein levels in the hippocampus with the behavioral effects seen after different periods of CUMS and drug treatment. Different from the hypersecretion of corticosterone levels during the whole CUMS processing (Li et al., 2007), hippocampal BDNF levels were inhibited at the end of CUMS procedure in our study. Additionally, although CUMS induced a reduction in hippocampal BDNF mRNA expression after 3-week, the down-expression was restored by EOPF only after 4-week treatment. In parallel to the change of mRNA expression, BDNF protein levels remained unaltered until 3-week of CUMS procedure. The ELISA analysis demonstrated that only 6 mg/kg but not 3 mg/kg EOPF administration for 4-week period increased the hippocampal BDNF protein levels. The data above suggested that the effect of EOPF on hippocampal BDNF expression was dependent on treatment duration (at least 4-week treatment). It is basically consistent with the previous studies that BDNF levels following traditional antidepressant agents (not rapid antidepressant agents such as ketamine) take time to develop (Coppell et al., 2003; De Foubert et al., 2004; Lee et al., 2010). As the explanation for delayed response of clinical antidepressants may be delayed BDNF elevation (Castrén and Rantamäki, 2010), the results presented above implied that a period of 4-week EOPF treatment may be necessary for its full clinical response.
Interestingly, the increase in BDNF mRNA and protein with fluoxetine in the present study was both more marked and extensive compared with a previous study (De Foubert et al., 2004). The study reported by De Foubert et al. (2004) has shown that rats treated with fluoxetine for 4, 7, 14 and 21 days displayed unaltered hippocampal BDNF protein levels when assessed by ELISA assay. The reason for these disparate results on BDNF changes might be due to the variety in animal species, different dose and especially stress schedule. First, 20 mg/kg fluoxetine for mice was adopted in the present study, whilst 10 mg/kg for rats was administered in their study. Moreover, CUMS and FST procedures were performed before BDNF measurement in our study, while BDNF assay 24 h after last administration without stress-induced paradigm was performed in the previous study. For example, hippocampal BDNF levels were increased after repeated antidepressant treatment in CUMS-induced rat or mice (Li et al., 2007; Dang et al., 2009; Mao et al., 2010).

In addition, our results indicated that fluoxetine exerted a better profile concerning its ability to restore CUMS-induced BDNF alterations, as compared to EOPF. The therapeutic effects of stress are ambiguous. Animal and human studies are clearly undisputed, however, because of a prevalent belief that “natural is better”. Depressed patients turn to alternative therapies owing to the lower side effects of medication, time and effort (Pilkington et al., 2006; Schulz, 2006). For example, due to the concern about the effect of pharmacological treatment on breastfeeding, women with postpartum depression prefer to seek complementary and alternative medicine treatment (Weier and Beal, 2004). As a result, a significant amount of public interest in antidepressant development has focused on the herbal and natural products in recent years (Zhang, 2004; Dwyer et al., 2011). Therefore, a reduced profile of undesirable side effects will become very important if EOPF becomes a medicine.

In conclusion, to our knowledge, this study is the first to analyze dynamic hippocampal BDNF expression in a mice depression model of CUMS. The main finding from this study is that the BDNF gene and protein are expressed depending on the length of stress duration and EOPF administration (4-week treatment). This might contribute to the underlying reason for the slow onset of antidepressant activity in clinic.

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