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Original Article

L-theanine improves depressive behavioral deficits by suppressing microglial activation in a rat model of chronic unpredictable mild stress

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Abstract: L-Theanine (N-ethyl-L-glutamine) is an amino acid originally and uniquely found in green tea. Historically L-theanine has been recognized as a relaxing agent. Some studies have shown that L-theanine has anti-anxiety-like and anti-depressant-like effects. However, the underlying mechanisms of its action on microglia and neurogenesis have never been studied. In the present study, Sprague-Dawley male rats were randomly divided into four groups, including a normal control group, a model control group, a fluoxetine group and a theanine group. We established a chronic depression model using the chronic unpredictable mild stress (CUMS) rat model under solitary conditions for 21 days. To examine the effects of L-theanine and fluoxetine on the behavioral index, weight changes, total movement distance in the open-field test, sucrose preference values in the sucrose consumption test, and immobility time of the forced swimming test were determined. We also studied neurogenesis in the hippocampus and measured the microglial activation index in the limbic-cortical-striatal-pallidal-thalamic (LCSPT) circuit that was related to the encephalic region (prefrontal cortex, nucleus accumbens, striatum, amygdala, mid-brain and hippocampus). Compared with the model group, L-theanine significantly elevated the circulative locomotion path and sucrose-solution consumption, but it reduced the immobilization time in the FST, partially increased the BrdU-marked neurogenesis and decreased microglial activation in the six LCSPT circuit-related encephalic regions, which was almost in accordance with the fluoxetine-treated group. These results suggest that the mechanism of action of L-theanine, may partly depend on suppressing microglial activation in the LCSPT circuit related encephalic region and recovery of neurogenesis in the hippocampus.

Keywords: L-theanine, CUMS depression rats, behavioral test, microglia, neurogenesis

Introduction

Increasing evidence indicates that microglial activation and decreased levels of hippocampal neurogenesis may play important roles in the pathogenesis of depression [1-3]. Microglia are specialized macrophages of the central nervous system (CNS) that are the primary component of the brain immune system [4, 5]. Microglia rapidly responds to infect or stress stimuli and adopts an 'amoeboid' activated phenotype. Activated microglia have a deleterious effect on hippocampal neurons and are implicated in impaired neurogenesis and depressive-like behavior [6]. For example, activated microglia produces many pro-inflammatory mediators, including cytokines, chemokines, reactive oxygen species (ROS) and nitric oxide, which decrease cell proliferation and neurosphere growth. Thus, prolonged or excessive microglial activation may result in the pathophysiology of depression [1, 7]. Moreover, the abnormal neurogenesis caused by pro-inflammatory cytokines has been found to interact with many of the pathophysiological domains that characterize depression, including neurotransmitter metabolism, neuroendocrine function, synaptic plasticity and behavior [8, 9]. Thus, controlling microglial activation and facilitating effective neurogenesis in the hippocampus may prove to be therapeutically beneficial in the treatment of depression.

L-Theanine (N-ethyl-L-glutamine) is an amino acid originally and uniquely found in green tea and has historically been recognized as a relaxation agent [10, 11]. Others have reported [12] that L-theanine passes through the blood-brain barrier, and that it accumulates in the serum, the liver, and the brain one to five hours after administration.

Neurochemical studies suggest that L-theanine enhances the synthesis of nerve growth factor and neurotransmitters in infant rats, and exerts neuroprotective effects in a rat model of stroke by preventing glutamate receptor agonist-mediated brain injury [13, 14]. Others have indicated that L-theanine augments anti-psychotic therapy in schizophrenia and schizoaffective disorder patients [15]. More recent studies of psychiatric disorders indicate that L-theanine reduces immobility time in both the forced swim test and tail suspension test in mice, [16] and alters catecholamines in the hippocampus and the cerebral cortex in mice of chronic restraint stress-induced cognitive impairment [17].

However, the general anti-depressive effect and mechanism of action of L-theanine on cells in the CNS, and specifically microglia and neurogenesis in the hippocampus have remained elusive. Indeed, the effects of L-theanine on microglia have not been reported. Chronic unpredictable mild stress (CUMS) with solitary condition exposed rats exhibits several neurobehavioral alterations that resemble symptoms of chronic depression in humans. This model has been widely employed for preclinical screening of anti-depressants [18]. Therefore, the aim of this study was to evaluate the general anti-depressive effects of L-theanine in a rat model of CUMS with solitary condition-induced depression, and to determine the effects of L-theanine in controlling microglial activation and facilitating neurogenesis in the hippocampus of CUMS rats.

Materials and methods

Animals

In this study, we used 72 adult male Sprague-Dawley rats (SPF, nearly 8 weeks old, weighing 180-220 g, license number: SCXK (Hunan) 2011-0003, Hunan Lake Scenery Company Limited), and maintained them under standard laboratory conditions (12 hrs light-dark cycle, light on at 19:00; temperature of 22±1°C, and free access to food and water except when the CUMS procedure was performed). Prior to the experiment, all rats were handled singly and handled daily to adapt to the experimental procedure for 1 week. All behavioral experiments were performed in the behavioral experimentation room during the dark phase of the light cycle. All experimental procedures were completed in accord with the guidelines of the United State's National Institutes of Health and the institutional Animal Care and Use Committee approved the procedures.

Drugs and treatment

L-theanine was supplied as a white powder by the National Research Center of Engineering Technology for Utilization of Functional Ingredients from Botanicals (Hunan, China) and prepared with sodium carboxymethylcellulose. We selected a dose of L-theanine according to similar reports [17, 19]. The selective serotonin reuptake inhibitor fluoxetine hydrochloride was supplied by Patheon France (Bourgoin-Jallieu).

L-Theanine (2.0 mg/kg/d) and Fluoxetine (1.54 mg/kg/d) was freshly prepared every day in 0.5% sodium carboxymethylcellulose and was administered by gavage in a volume of 1 ml/100 g body weight.

Animal model

Rats in the normal group were fed normally, and rats in the other groups were used to establish a rat model of chronic stress depression. The animal model was established as chronic unpredictable mild stressors (CUMS).

The CUMS procedure consisted of a variety of unpredictable mild stressors (CMS) for 21 days, additionally one of the stimuli was chosen randomly and the rats treated in such a way that they could not expect the stimulus. Every stimulus was used 2-3 times in total. The stressors included 24 hrs water deprivation, 1 min tail pinch, 5 min thermal stimulus by placing rats in a 45°C oven, 5 min cold swim at 4°C, 24 hrs reversed light/dark, 24 hrs food deprivation, electric shock in the foot (10 mA electricity was given, for 10 s for each occasion for a total of 30 times), shaking the rats (180 RPM, lasting for 5 min), and constraining rats in a narrow cage (2 hrs) [20, 21]. The rats were weighed on the 1st and 22nd day of the experiment.

Open-field test

The Open-field test was performed according to the improved method recorded on documents at 7:00 AM-9:00 AM on the 1st and 22nd day of the experiment in a quiet room [22]. Application of the ENV-520 animal behavior video tracking system was used to record the total movement distance of rats for a period of 5 min.

Sucrose-solution consumption test

The test was done according to the method recorded in the document in a quiet room 72 hrs before each open-field test. Before the test, the rats were trained to adapt to drinking water that was supplemented with sugar. Two bottles were placed in every cage. In the first 24 hrs both bottles were filled with 1% sucrose solution, and over the next 24 hrs, one of the bottles was filled with 1% sucrose solution, and the other was filled with pure water. Then the basic energy expenditure test and water consumption test were measured after 24 hrs of food and water deprivation.

Meanwhile, the rats were given 2 bottles of liquid that had first been weighed. One of the bottles was filled with 1% sucrose solution, and the other with pure water. At 60 min later, both bottles were weighed. Then the total liquid consumed and the percentage sucrose consumed were calculated [23].

Forced swim test

This test was performed as previously described [24]. Individual rats were placed in a clear plastic cylinder with a diameter of 23 cm. The height of the cylinder was 65 cm, which was filled with 40 cm of clear water at 25°C. The duration of the test was 5 min and a trained investigator scored the behavior. The duration of the immobility was determined during the test. Immobility was defined as the absence of all movement except minor movement that is normally required for the mouse to keep its head above the surface. Afterwards the rat was towel dried and returned to its home cage. The water used in the test was replaced between each animal testing cycle.

Western blotting analysis

After a 21-day intervention, rats were decapitated and their brains quickly removed for dissection. The prefrontal cortex (PFC), accumbens (NAC), striatum (ST), amygdala (AM), midbrain (MB) and hippocampus (HIP) were dissected, flash frozen and stored at -80°C until preparing them for immunoblotting. Each sample was made four to six pooled slices and homogenized in 160 μl of ice-cold buffer consisting of 50 mM 3-(N-morpholino) propane sulfonic acid (pH7.4), 2 mM sodium orthovanadate, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM each of dithiothreitol, EDTA, ouabain, leupeptin and pepstatin A. The homogenates were centrifuged at 12,000×g for 10 min at 4°C. Supernatants was incubated in sample buffer (2% sodium dodecyl sulfate, 20% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris-HCl, pH6.8 and 0.01% bromphenol blue at 100°C for 5 min. Prepared samples were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto PVDF membranes as previously described [25]. The membrane was probed with anti-Iba1 antibody (1:500, Abcam, U.K.) or anti-β-tubuline (1:2000, Abcam). Then, membranes were incubated with anti-rabbit or anti-mouse IgG horseradish peroxidase (HRP) (1:3000, Abcam), respectively. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL-Pierce). Densitometric analysis was conducted using Molecular Analysis software obtained from Bio-Rad. The same experiments (with multiple experimental conditions) were repeated at least four times.

BrdU labeling

Four groups of rats were administered BrdU (Sigma-Aldrich, 4×75 mg/kg IP, every 2 hrs) for 21 days and sacrificed 24 hrs after the last BrdU injection. Then, rats were deeply anesthetized and transcardially perfused with 0.01 M phosphate buffer solution (PBS, pH7.4) followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed, post-fixed in 4% PFA for 4 to 6 hrs, cryoprotected with 20% sucrose in PBS overnight, embedded with OCT, cut into 12 μm-thick sections using a freezing microtome (CM1950; Leica, Germany) and mounted onto superfrost plus glass slides, and then stored at -70°C for immunohistochemistry.

The BrdU immunohistochemistry was performed as previously described [26, 27]. After being washed in PBS, endogenous peroxidase was

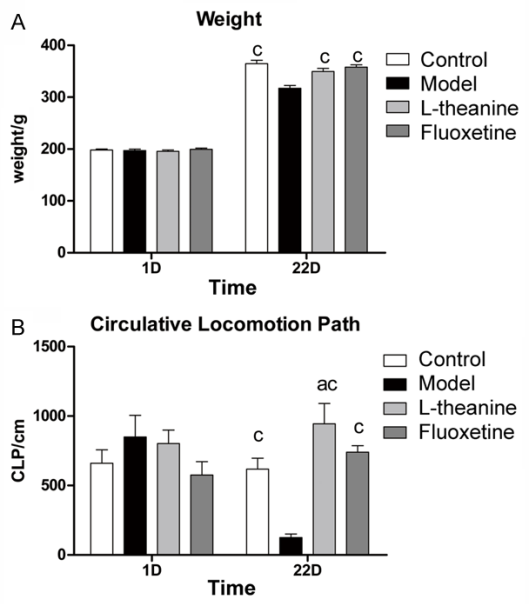

Figure 1. The effects of L-theanine and fluoxetine on weight (A) and circulative locomotion path (B) in CUMS-induced rats. Values are calculated as mean ± SEM in the first day before and after the CUMS administration. Compared with the model group, the weights of the rats were much higher (A), and the locomotion path was significantly improved in L-theanine and fluoxetine groups (B) after the CUMS and separation administration of 21 successive days. But when compared with the model group, L-theanine and fluoxetine, which served as a positive control in this experiment, the weights of the rats were much higher (A), and the locomotion path was significantly improved (B) after the CUMS and separation administration of 21 successive days.

blocked by incubation in 0.3% hydrogen peroxide in 50% methanol in distilled water for 30 min. Sections were washed in PBS and pre-incubated in a blocking solution containing 6% bovine serum albumin (BSA) and 0.03% Triton X-100 in PBS for 60 min at room temperature. Then, sections were incubated in a humidified chamber with rabbit primary antibody against BrdU (1:500; Abnova, USA) in 3% BSA in PBS overnight at 4°C. After several washes in PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:300; CWBIO, China) for 60 min at room temperature, followed by incubation with an avidin-biotin complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA). Immunoreactive signals were detected using 3,3'-diaminobenzidinetetrahydro-chloride (Boster, China) as a chromogen. Images were captured using a digital camera (Olympus DP72) attached to an Olympus microscope (Olympus BX51) and Cellsens standard 1.6 computer program.

Statistical analysis

All statistical analyses were performed using the SPSS software, version 16.0. Values were expressed as mean ± SEM (Standard Error of the Mean). For the step-through, behavioral experimental data and microglia-Iba1 assays, these observations were analyzed with one-way analysis of variance (ANOVA). Levene test was used for homogeneity of variance test. If data were determined to be homogeneous, the intergroup variation was measured by Dunnett's post-hot test. If data were heterogeneous, the intergroup variation was measured by the Tamhane's T2 test. Statistical significance was set at an alpha value of $P<0.05$.

Results

Effects of sub-chronic administration of L-theanine on CUMS-induced behaviors in rats

Effect of L-theanine on weight changes and circulative locomotion path: The effects of L-theanine and fluoxetine on the CUMS induced changes in rat weights and circulative locomotion path are shown (**Figure 1**). The four groups did not show significant variance before the administration of the drug, but when compared with the model group, L-theanine and fluoxetine, which served as a positive control in this experiment, the weights of the rats were much higher (**Figure 1A**), and the locomotion path was significantly improved (**Figure 1B**) after the CUMS and separation administration of 21 successive days.

Effects of L-theanine on sucrose consumption percentage: There was no significant difference in total liquid consumption in four groups ($F(3, 32)=1.500$, $P=0.233$). As shown in **Figure 2A**, L-theanine and fluoxetine administered for 21 successive days significantly elevated the percent sucrose consumption of rats.

Effect of L-theanine on immobility duration in the forced swim test: As shown in **Figure 2B**, L-theanine and fluoxetine administered for 21 successive days significantly reduced the immobility time.

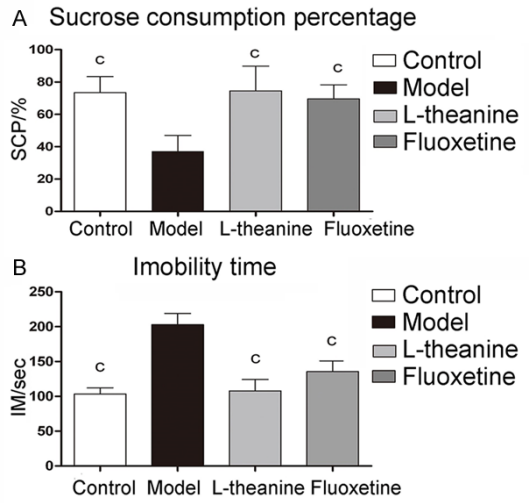

Figure 2. Effects of L-theanine and fluoxetine on sucrose consumption values in CUMS-induced rats, which are calculated as mean ± SEM in the first day after CUMS administration. A: Compared with model group, L-theanine and fluoxetine administered for 21 successive days significantly elevated the percent sucrose consumption of rats (P<0.001). B: Compared with other groups, the immobility time increased in model group (P<0.001). c: P<0.001 versus the model group.

Effect of the L-theanine and fluoxetine on CUMS-stimulated microglial activation in hippocampus

There have been many studies showing that neuroimaging and neuropathological studies of major depressive disorder (MDD) and bipolar disorder (BD) have identified abnormalities of brain structure in areas of the prefrontal cortex (PFC), amygdala (AM), striatum (ST), hippocampus (HIP), parahippocampal gyrus, and the raphe nucleus, all in the limbic-cortical-striatal-pallidal-thalamic (LCSPT) circuit [28-31]. So in the study, we focused on assessing whether L-theanine and fluoxetine could inhibit microglial activation of CUMS induced in LCSPT circuit related encephalic region (prefrontal cortex (PFC), nucleus accumbens (NAC), striatum (ST), amygdala (AM) midbrain (MB) and hippocampus (HIP)).

It was previously reported that microglial activation is associated with marked increases in Iba1 immunolabeling expression [32, 33]. As shown in **Figure 3**, the model rats in the CUMS with solitary condition-induced depression showed a marked increase in the Iba1 levels after 21 days in the LCSPT circuit related to the encephalic region. Treatment with L-theanine and fluoxetine significantly inhibited Iba1 levels.

Effect of the L-theanine and fluoxetine on CUMS-stimulated neurogenesis in hippocampus

It was previously reported that neurogenesis could be demonstrated using an exogenous cell tracer-BrdU system [34, 35]. To evaluate whether the changes in hippocampal BrdU-positive cells induced by 21 days of CUMS with solitary conditions, immunohistochemical analysis was used and showed that the expression of BrdU had decreased in the model group. Treatment with L-theanine and fluoxetine partly recovered CUMS-mediated decreases in neurogenesis (**Figure 4**).

Discussion

In this study, we identify for the first time that L-theanine changes depressive-like behavior that were dependent on suppressing microglial activation and partly facilitating effective neurogenesis in the hippocampus *in vivo*. Specifically, the selective serotonin reuptake inhibitor and anti-depressant Fluoxetine, decreased microglial activation in the CUMS rat model, and increased neuronal differentiation of hippocampal neural stem cells.

We and others have previously shown that L-theanine alters the catecholamines norepinephrine and dopamine in the LCSPT circuit related to the encephalic region including the prefrontal cortex, nucleus accumbens, striatum, amygdala, midbrain and hippocampus in mice and rats of stress-induced chronic restraint [17].

To investigate whether L-theanine could change microglial function, we established a depression rat model by chronic unpredictable mild stress (CUMS) with rats separated alone. This model was widely used in research of the pathogenesis of depression and new drugs for the therapy of depression. This experimental model can satisfy core symptoms that are analogous to the human depressive symptom anhedonia, and compromised spontaneous activity and social communication [22, 36].

Interestingly we found that 21 days of sub-chronic treatment with L-theanine, elevated the

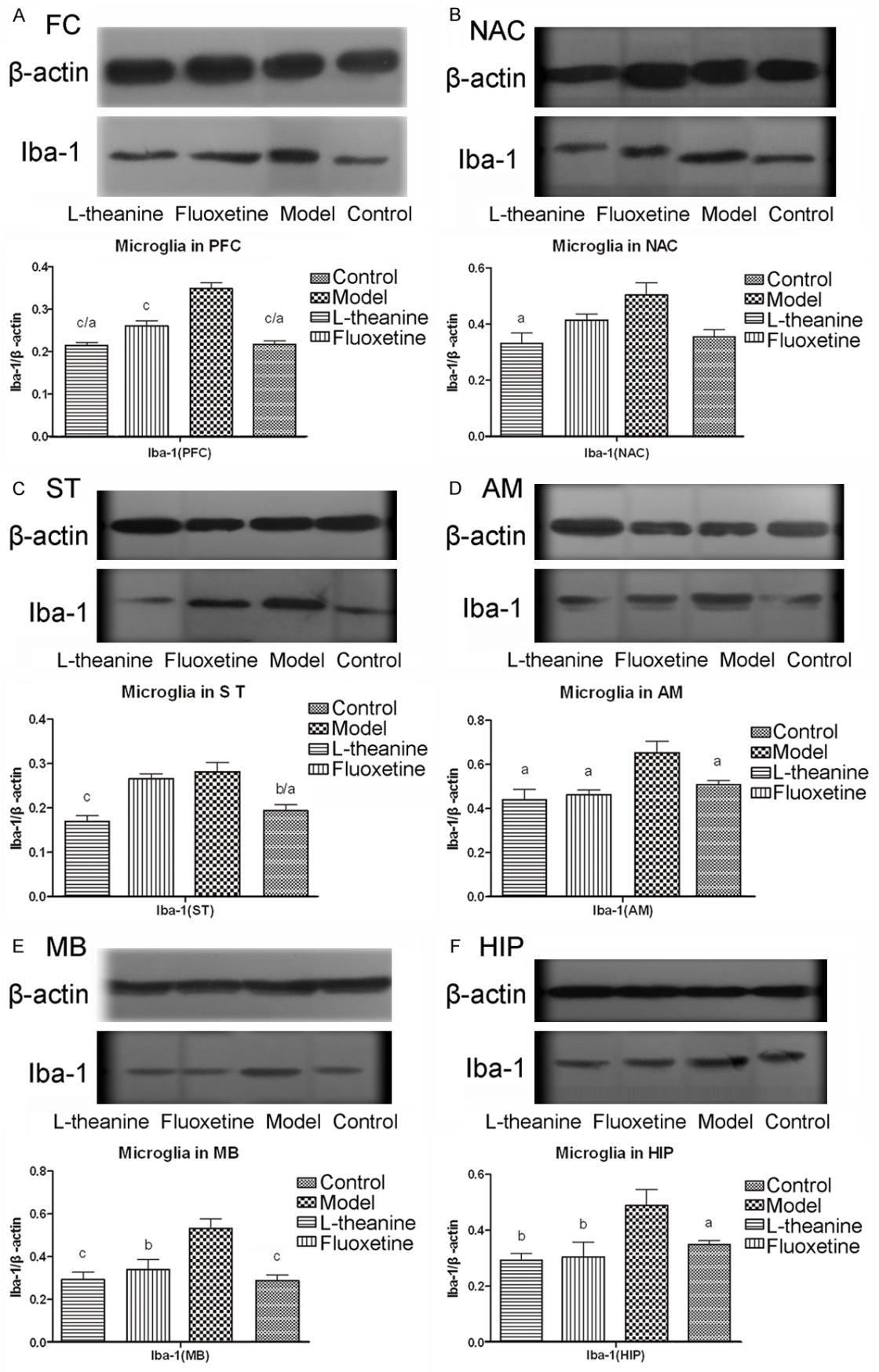
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Figure 3. Effects of L-theanine and fluoxetine on CUMS-induced microglial activation rats in the LCSPT circuit related encephalic region. Values are calculated as mean ± SEM, a: P<0.05, b: P<0.01, and c: P<0.001 versus the model group. The relative Iba-1 levels were quantified by scanning densitometry and normalized to β-actin protein. The model rats in the CUMS with solitary condition-induced depression showed a marked increase in the Iba1 levels after 21 days in the LCSPT circuit related to the encephalic region. Treatment with L-theanine and fluoxetine significantly inhibited Iba1 levels. The Iba-1 levels in PFC (A), the Iba-1 levels in NAC (B), the Iba-1 levels in ST (C), the Iba-1 levels in AM (D), the Iba-1 levels in MB (E), and the Iba-1 levels in HIP (F) are shown.

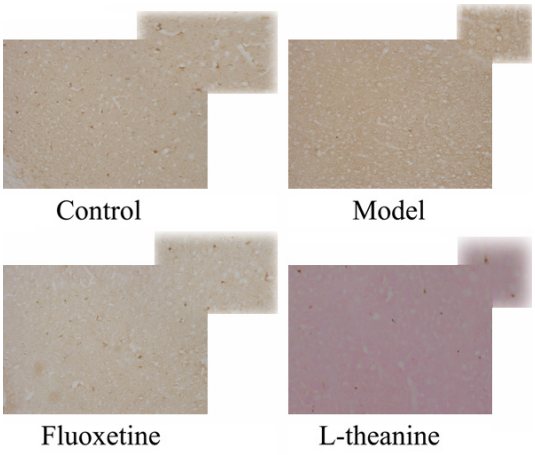

Figure 4. Effects of L-theanine and fluoxetine on CUMS induced neurogenesis in the rat hippocampus as detected by BrdU-marking. BrdU immunohistochemistry staining was decreased in the depressive model group. However, reduced BrdU immunohistochemistry was also evident in the fluoxetine group, and partially in the L-theanine group. Scale bar =50 μm. Images are representative of triplicate sets.

circulative locomotion path and the percentage of sucrose solution consumed, and reduced the immobilization time in the FST. Those behavior tests reflected the depressed state of the rat, such as anhedonia, desperate state, and helpless behavior. So they are used widely for their potential to screen anti-depressants [37, 38]. In our study, L-theanine had an antidepressant-like activity that was comparable to the fluoxetine in these behavioral tests. It is therefore possible that L-theanine exerts an anti-depressant-like effect, which is in accordance with some prior mouse models studies [16, 19].

Neuroimaging and neuropathological studies indicated that depression from stroke is etiologically related to the disruption of the left LCSPT circuit across the cortical and limbic circuits [29]. In our present study, it was demonstrated that L-theanine and fluoxetine inhibited CUMS activation of microglia in the LCSPT circuit related encephalic region. Activated microglia produce a wide range of proinflammatory mediators, including TNF-α, IL-1, IL-6, reactive oxygen species (ROS) and nitric oxide (NO) synthesis, which play a critical role in the pathology of depression [39]. Notably, recent studies have suggested that Fluoxetine significantly reduced inflammatory mediators such as TNF-α, IL-6 and NO production in LPS-stimulated microglia [40, 41]. Additionally, this is the first evidence describing L-theanine and fluoxetine reduced CUMS-stimulated microglia numbers in rats. However, the mechanism remains unclear, and it may be linked to a reduction in inflammatory mediators by antagonizing the glutamine receptor. Since prior mouse studies have shown that L-Theanine has anti-anxiety-like and anti-depressive-like functions, through induction of brain derived neurotrophic factor (BDNF) in the hippocampus, and the agonistic action of L-theanine on the NMDA receptor [13, 19].

The brain-derived neurotrophic factor (BDNF) might be a candidate molecule that regulates adult neurogenesis [42, 43]. In addition, maintenance of neurogenesis in the adult hippocampus is important for functions such as mood and memory. Exposure to unpredictable chronic stress (UCS) also results in decreased hippocampal neurogenesis [44, 45]. In further development of anti-depressants, neurogenesis may serve as an important parameter to examine the efficacy and mechanism of action of novel drugs. Further investigation of the effect of L-theanine and Fluoxetine on neurogenesis has shown that the CUMS procedure decreases hippocampal neurogenesis in SD rats, but chronic L-theanine and fluoxetine exposure partly increased SGZ cell proliferation in CUMS-treated as well as model rats. Thus, we hypothesized that decreased depression and anxiety behavior after CUMS are associated with increases in the production and growth of new neurons in the hippocampus. Notably, recent studies have suggested that chronic fluoxetine treatments can potentially recover psycho-behavioral function, deficits in hippocampal neurogenesis, and problems related to anxiety, depression, and cognition [46, 47].

In sum, this study demonstrated anti-depressant like effects of L-theanine and fluoxetine in a 21 day continuous consumption in the CUMS and separated rat model-induced depressive behavior in rats. In addition, the suppressive effects of L-theanine and fluoxetine treatment on the CUMS activation of microglia in the LCSPT circuit related encephalic region, and the facilitation of effective neurogenesis in the hippocampus has been determined. These observations suggested that the therapeutic effects of L-theanine and fluoxetine were partially mediated by the actions on microglial activation and neurogenesis in the hippocampus. However, as a potentially anti-anxiety and antidepressant herbal therapy, the specific molecular mechanisms of L-theanine on microglia and neurogenesis require further studies.

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Disclosure of conflict of interest

None.

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