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L-theanine inhibits nicotine-induced dependence via regulation of the nicotine acetylcholine receptor-dopamine reward pathway

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In this study, the inhibitory effect of L-theanine, an amino acid derivative of tea, on the rewarding effects of nicotine and its underlying mechanisms of action were studied. We found that L-theanine inhibited the rewarding effects of nicotine in a conditioned place preference (CPP) model of the mouse and reduced the excitatory status induced by nicotine in SH-SY5Y cells to the same extent as the nicotine receptor inhibitor dihydro-beta-erythroidine (DHβE). Further studies using high performance liquid chromatography, western blotting and immunofluorescence staining analyses showed that L-theanine significantly inhibited nicotine-induced tyrosine hydroxylase (TH) expression and dopamine production in the midbrain of mice. L-theanine treatment also reduced the upregulation of the α_4, β_2 and α_7 nicotine acetylcholine receptor (nAChR) subunits induced by nicotine in mouse brain regions that related to the dopamine reward pathway, thus decreasing the number of cells that could react to nicotine. In addition, L-theanine treatment inhibited nicotine-induced c-Fos expression in the reward circuit related areas of the mouse brain. Knockdown of c-Fos by siRNA inhibited the excitatory status of cells but not the upregulation of TH induced by nicotine in SH-SY5Y cells. Overall, the present study showed that L-theanine reduced the nicotine-induced reward effects via inhibition of the nAChR-dopamine reward pathway. These results may offer new therapeutic strategies for treatment of tobacco addiction.

nicotine addiction, L-theanine, nicotine acetylcholine receptor (nAChR), dopamine, conditioned place preference (CPP)

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Cigarette smoking has been linked to many life threatening diseases including heart disease, cancer and chronic obstructive pulmonary disease [1–3]. It is estimated that there are approximately 1.25 billion smokers in the world, with 5 million dying every year because of smoking-related diseases. Thus, cigarette smoking has become one of the largest international public health problems. Although many methods/campaigns have been developed for smoking cessation [4–6], quitting smoking is extremely difficult because of the addictive nature of nicotine.

Nicotine addiction is a chronic recurrent disease that is caused by repetitive uptake of nicotine [7]. Similar to other addictive drugs, nicotine addiction has an intimate connection with the mesostriatal dopamine reward circuit, in which the activity of dopamine neurons can be reinforced by addictive drugs including nicotine. The mesostriatal dopamine reward circuit, mainly composed of the ventral tegmental area (VTA), prefrontal cortex (PFC) and nucleus accumbens (NAc), expresses high levels of nicotine acetylcholine receptors (nAchRs). These receptors are thought to mediate several main behavioral effects of nicotine, including the

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regulation of conditioned place preference (CPP) formation [8,9]. Mounting data has indicated that the number of nAchRs increases after chronic nicotine uptake in both animal models and human subjects. *In vitro* experiments have shown that the expression of $\alpha_4\beta_2$ nAchRs increases significantly after prolonged nicotine treatment [10,11]. Moreover, the upregulation of $\alpha_4\beta_2$ nAchRs expression has also been found in the brains of smokers [12]. It is proposed that the $\alpha_4\beta_2$ nAchRs, α_7 nAchR and the transcription factor c-Fos are necessary for the formation of the stable neural adaptation during the exposure of nicotine [13,14]. These findings suggest that inhibition of the expression or the function of these subtypes of nAchRs in the mesostriatal reward circuit system may promote the cessation of nicotine addiction.

L-theanine is an amino acid derivative primarily found in tea. It has been reported to promote relaxation and have neuroprotective effects. It is also shown to have an antagonistic effect on the excitement induced by caffeine [15]. Recently, we have studied the effect of a newly developed tea filter and its components on smoking addiction and found an unprecedented smoke cessation effect of cigarette filters. Moreover, we identified that L-theanine had an inhibitory effect on nicotine addiction [16]. In this study, we further investigated whether L-theanine regulates the behavioral and biochemical processes underlying the nicotine-induced reward effects. With this aim, we used the mouse CPP model and human neuroblastoma SH-SY5Y cells to assess the inhibitory effect of L-theanine on the rewarding effects induced by nicotine and its underlying mechanisms of action.

1 Methods

1.1 Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and HEPES were purchased from GIBCO BRL (Grand Island, NY); ethylenediaminotetraacetic acid (EDTA), trypsin, penicillin, DHβE and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA); antibodies against AChRα_4 (sc-74519), AChR β_2 (sc-11372), AChRα_7 (sc-11372), TH (sc-25269), c-FOS (sc-52) and β-Actin (sc-1616-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 2-NBDG was purchased from Invitrogen Corporation (Invitrogen, Carlsbad, CA, USA); Vectastain ABC kit and DAB kit were purchased from Vector Corporation (Burlingame, USA). L-theanine, with a purity of 98.11% (analyzed by high performance liquid chromatography (HPLC)), was a generous gift from Hangzhou Gosun Biotechnologies Co., Ltd. (Hangzhou, China); nicotine, with a purity of 99%, was a generous gift from the tobacco Institute of Zhengzhou (Zhengzhou, China). All other chemicals were made in China and were of analytical grade.

1.2 Animal treatment

Animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee. Six- to eight-week-old female C57BL/6J mice were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Science and housed in an SPF (specific parasite free) environment at 22°C with a 12-h light-dark cycle. Food (Mouse Diet, Beijing Experiment Animal Center) and water were available *ad libitum*. Chemicals were dissolved in 0.9% (w/v) physiological saline and injected subcutaneously. Animals were randomly divided into the following groups (n=10) for treatment with different chemicals: control mice were injected with 0.9% (w/v) physiological saline subcutaneously (s.c.); nicotine mice were injected with nicotine (0.5 mg kg^{-1} d^{-1}, s.c.); Th-L mice were injected with L-theanine (250 mg kg^{-1} d^{-1}, s.c.); Th-H mice were injected with L-theanine (500 mg kg^{-1} d^{-1}, s.c.); Th-L(N) mice were injected with L-theanine (250 mg kg^{-1} d^{-1}, s.c.) and nicotine (0.5 mg kg^{-1} d^{-1}, s.c.); Th-H(N) mice were injected with L-theanine (500 mg kg^{-1} d^{-1}, s.c.) and nicotine (0.5 mg kg^{-1} d^{-1}, s.c.); DHβE(N) mice were injected with DHβE (2.0 mg kg^{-1} d^{-1}, s.c.) and nicotine (0.5 mg kg^{-1} d^{-1}, s.c.) [17]. Mice were injected daily with nicotine or physiological saline. L-theanine and DHβE were administered 15 min before nicotine injection.

1.3 Conditioned place preference (CPP) test

CPP is a behavioral test that is usually used to study the rewarding properties of nicotine and other drugs. This procedure consists of three phases: preconditioning, conditioning, and post conditioning [18,19]. In our procedure, days 1 and 2 were the preconditioning days and mice were allowed to roam freely for 900 s in a rectangular box, which was separated by a narrow compartment. The time that an individual mouse spent in each compartment was recorded. Mice were then divided into groups with an unconditioned preference (n=10 mice per group) according to these data. On days 3–9, the mice were injected s.c. with saline or chemicals as described earlier and immediately placed in one of the pairing compartments for 30 min. Five hours later, the mice were injected with a combination of chemicals or saline and immediately placed in the opposite chamber for 30 min. Control groups received saline on both sides of the chamber. On day 10, the animals were once again allowed to roam freely among the three compartments for 900 s and the time spent in each compartment was recorded. The animals were drug-free on preconditioning and post conditioning days.

1.4 Cell culture and glucose-uptake using the 2-NBDG assay

Human neuroblastoma SH-SY5Y cells were maintained in a

medium consisting of DMEM supplemented with fetal bovine serum (10%, v/v), penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}) in a humidified 5% CO_2/95% air incubator at 37°C. Cells pretreated with DHβE (50 μmol L^{-1}) or L-theanine (100 μmol L^{-1}) for 30 min were treated with or without nicotine (10 μmol L^{-1}) for 24 h. The cells were then washed with KRB buffer (Krebs-Ringer Bicarbonate Buffer) three times, incubated with 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]; Invitrogen, CA, USA) for 20 min, and washed with KRB buffer three times. The fluorescence was examined under a fluorescence microscope (Olympus, kx14e).

1.5 Dopamine metabolism measured by HPLC with electrochemical detection

Thirty minutes after subcutaneous injection of saline or different chemicals, mice were killed by cervical vertebra dislocation and both sides of the striatum were carefully isolated. Samples were weighed and homogenized in 1 mL of 0.2 mol L^{-1} perchloric acid. Tissue homogenates were then centrifuged at 20000×g for 15 min at 4°C and the supernatants were collected for further analyses. The levels of striatal dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined by HPLC ESA-5600A Coularray system (ESA, USA) using a previously described method [20,21].

1.6 Tyrosine hydroxylase (TH) immunohistochemistry

Coronal mesencephalic sections (14 μm) were processed for TH immunohistochemistry [22,23]. TH-positive neurons were immunolabeled by incubating the tissue sections successively with a mouse monoclonal anti-TH antibody (1∶200) and biotinylated rabbit anti-mouse IgG (1:100; Vector Laboratories) following the staining procedure outlined by the manufactures of the Vectastain ABC kit (Vector Laboratories) in combination with DAB reagents. The total positive TH deposits in the substantia nigra pars compacta were examined under bright field using a Leica DM2500 microscope and images were obtained with a DFC 300 FX camera.

1.7 Western blotting assay

The tissues of the ventral tegmental nucleus (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC) were homogenized on ice in buffer (50 mmol L^{-1} Tris-Cl, 150 mmol L^{-1} NaCl, 0.02 mmol L^{-1} NaN_2, 100 μg mL^{-1} PMSF, 1 μg mL^{-1} Aprotinin and 1% (v/v) Triton X-100) for 30 min. Tissue homogenates and cell lysates were then centrifuged at 12000×*g* for 25 min at 4°C. Supernatants were collected and analyzed by Western blotting using the standard protocol. Band intensities were quantified using image analyzing software (NIH Image).

1.8 Immunofluorescence staining

SH-SY5Y cells were fixed in methanol/acetone (1∶1). The fixed cells were then permeabilized in 1% (v/v) Triton X-100, and blocked with 1% (v/v) normal goat serum for 30 min. Immunostaining was done by incubating the samples successively with antibodies specifically recognizing TH (sc-25269), c-FOS (sc-52) and fluorescein-conjugated goat anti-mouse IgG. The fluorescence was examined under a confocal laser scanning microscope (LSCMFV500).

1.9 siRNA transfection

siRNAs were designed and synthesized by GenePharma. The sequences against c-Fos were 5′-GGUGGAACAGUUAUCUCCATT-3′ (sense) and 5′-UGGAGAUAACUGUUCCACCTT-3′ (antisense). An antisense siRNA provided by the manufacturer that does not target rat, mouse, or human genes was used as a negative control (Nc); the sequences were 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (antisense). In brief, 1×10^5 SH-SY5Y cells/35 mm dish were plated and cultured under normal conditions as described earlier until the cells reached about 80% confluency. The siRNAs were then transfected using Lipofectamine 2000 (Invitrogen Inc., USA) following the manufacturer's instructions.

1.10 Statistical analysis

One-way analysis of variance (ANOVA) was used to estimate the overall significance followed by post hoc Tukey's tests corrected for multiple comparisons [24]. Data are presented as the mean±SEM. A probability level of 5% (P<0.05) was considered statistically significant.

2 Results

2.1 L-theanine reduces the rewarding effects of nicotine

The effect of L-theanine on nicotine-induced rewarding was studied using the CPP test in a mouse model. Mice were treated with nicotine (0.5 mg kg^{-1} d^{-1}, s.c.) for 14 d to induce the rewarding effects. As shown in Figure 1A, the time that the mice spent in the drug-paired compartment increased significantly after the treatment when compared with the control group. The preference for the drug-paired compartment induced by nicotine treatment was significantly inhibited when mice were pre-treated with L- theanine (250 or 500 mg kg^{-1} d^{-1}; s.c.) or the inhibitor of nicotine receptor (DHβE) before nicotine treatment (Figure 1B),

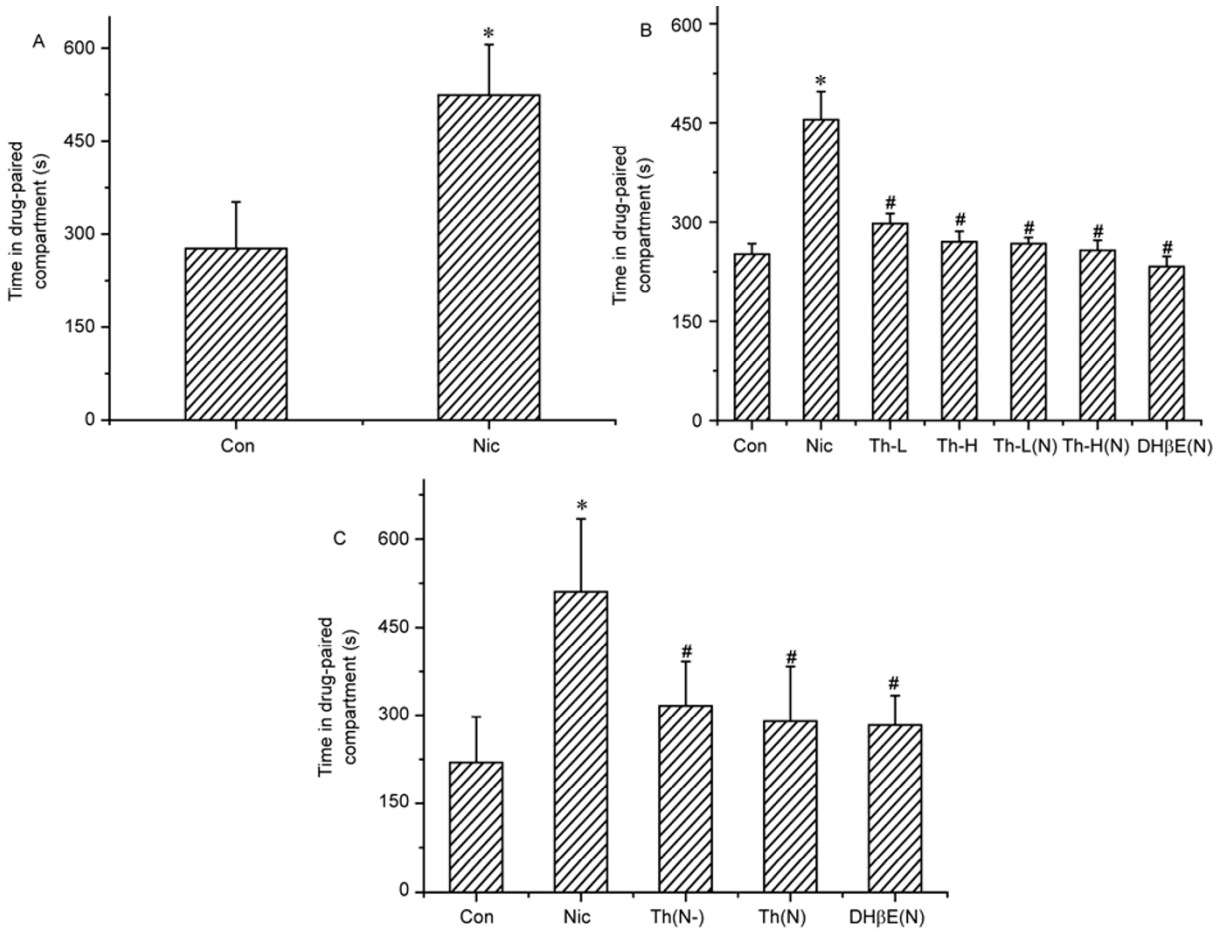

Figure 1 Effects of L-theanine on the rewarding effects induced by nicotine evaluated by the conditioned place preference (CPP) test. A, The rewarding effects induced by nicotine. Mice were injected with nicotine (0.5 mg kg^{-1} d^{-1}) or physiological saline every day for 14 d. The nicotine-induced rewarding effects were examined on day 17 using the CPP test. B, Effects of pretreatment of L-theanine on the rewarding effects induced by nicotine. Mice treated with nicotine were injected with different concentrations of L-theanine or DHβE in physiological saline every day for 14 d. L-theanine or DHβE was administered 15 min before nicotine injection. The nicotine-induced rewarding effects on the mice were examined on day 17 using the CPP test. C, Effects of L-theanine on the rewarding effects already induced by nicotine. Mice were injected with nicotine or physiological saline every day for 14 d. The nicotine-induced rewarding effects on mice were examined on day 17 using the CPP test. Starting from day 18, mice were administered with L-theanine (500 mg kg^{-1} d^{-1}) alone, or with L-theanine or DHβE 15 min before nicotine injection. The nicotine rewarding effects on mice were examined on day 31 using the CPP test. Con, mice treated with physiological saline; Nic, mice treated with nicotine (0.5 mg kg^{-1} d^{-1}); Th-L(N), mice treated with nicotine and L-theanine (250 mg kg^{-1} d^{-1}); Th-H(N), mice treated with nicotine and L-theanine (500 mg kg^{-1} d^{-1}); DHβE (N), mice treated with nicotine and DHβE; Th (N-), mice that had already developed rewarding effects in response to nicotine were treated with L-theanine (500 mg kg^{-1} d^{-1}) alone (nicotine abstinence). The results are presented as mean±SEM, n=8. *, P<0.05 compared with control group; #, P<0.05 compared with nicotine group.

suggesting that both dosages of L-theanine and DHβE pre-treatment inhibited the CPP formation induced by nicotine. To further test whether L-theanine treatment reduces the CPP that has been already formed, mice were first injected with nicotine (0.5 mg kg^{-1} d^{-1}, s.c.) for 14 d to induce the CPP preference, then treated with L-theanine alone (without nicotine), or injected with nicotine unceasingly with simultaneous pre-treatment of L-theanine or DHβE. The results showed that DHβE as well as L-theanine treatment attenuated the preference of mice for the drug-paired compartment that had already been induced by nicotine (Figure 1C). These data suggest that L-theanine can reduce the nicotine-induced rewarding effects both before and after the rewarding effects have been developed.

2.2 L-theanine inhibits the excitatory status of SH-SY5Y cells induced by nicotine

Nicotine has been shown to activate cells and lead to their excitatory status [11]. The excitability of dopaminergic neurons induced by nicotine is considered part of the mechanism for the rewarding effects of nicotine [25]. Glucose is the key molecule of energy metabolism in cells and its uptake is an indicator of the excitatory status of the cells. Therefore, we next studied the effect of L-theanine on the nicotine-induced excitatory status of SH-SY5Y cells by assessing the glucose uptake of cells using the fluorescent probe 2-NBDG. As shown in Figure 2, the fluorescent signal intensity in SH-SY5Y cells increased significantly after nicotine treatment, indicating that nicotine-induced excita-

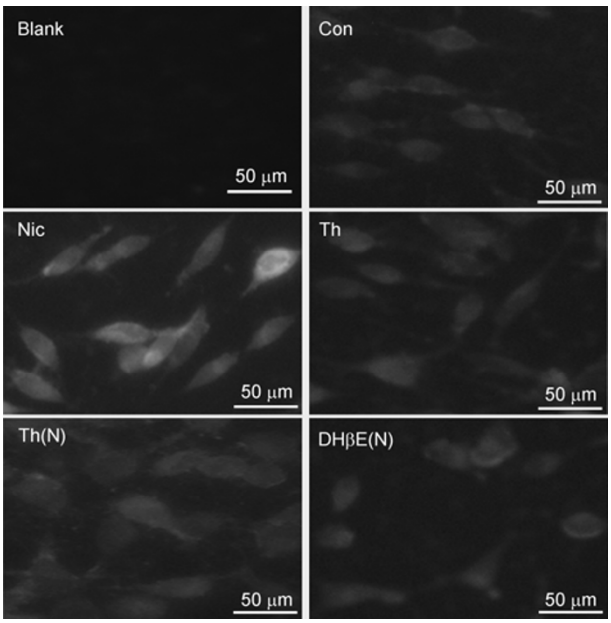

Figure 2 Effects of L-theanine and nicotine on glucose uptake in SH-SY5Y cells. A, Glucose uptake was measured using the 2-NBDG probe and fluorescence microscopy. Blank, cells without 2-NBDG; Con, cells treated with phosphate buffered saline (PBS) and measured using the 2-NBDG probe; Nic, cells treated with nicotine, PBS and measured using the 2-NBDG probe; Th, cells treated with L-theanine, PBS and measured using the 2-NBDG probe; Th(N), cells treated with nicotine, L-theanine, PBS measured using the 2-NBDG probe; DHβE(N), cells treated with nicotine, DHβE, PBS and measured using the 2-NBDG probe. B, Statistical analysis results from the fluorescence in (A). The data were expressed as the mean of ratio±SEM, n=3. *, P<0.05 compared with control group; #, P<0.05 compared with nicotine group.

tory status and glucose uptake were increased in cells. When cells were pre-treated with L-theanine or the inhibitor of the nicotine receptor (DHβE), the fluorescence intensity of cells reduced significantly when compared with cells treated with nicotine alone (P<0.05) (Figure 2). These data indicated that the nicotine-induced excitatory status of SH-SY5Y cells was inhibited by L-theanine.

2.3 L-theanine inhibits the upregulation of tyrosine hydroxylase (TH) expression and dopamine levels in the mouse midbrain

The increase of dopamine release is an important reward process caused by nicotine [20,21]. We next examined whether L-theanine had any effect on the DA release induced by nicotine in mice. The results showed that levels of dopamine in the midbrain increased significantly after two weeks of nicotine treatment, while pretreatment with the inhibitor of the nicotine receptor (DHβE) or L-theanine inhibited this increase (P<0.05) (Figure 3).

TH is a key enzyme responsible for the synthesis of dopamine. Immunohistochemical staining of TH showed that the number of TH-positive neurons was significantly increased following nicotine treatment, whereas this increase

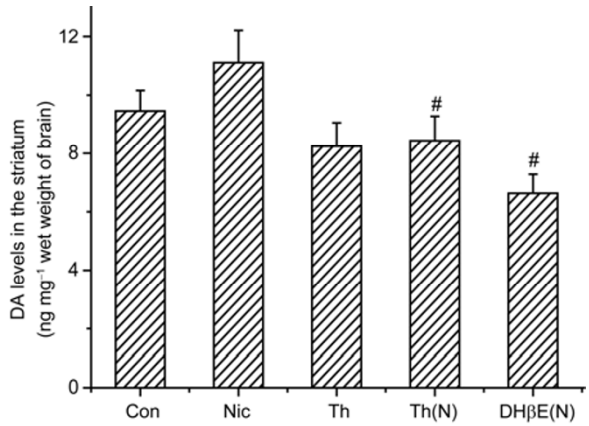

Figure 3 Effects of L-theanine on the nicotine-induced increase of dopamine (DA) in the midbrain. The DA levels in the mouse midbrain were measured by high performance liquid chromatography with electrochemical detection. Con, mice were treated with physiological saline; Nic, mice were treated with nicotine; Th, mice were treated with L-theanine (500 mg kg^{-1} d^{-1}, s.c.); Th(N), mice were treated with nicotine and L-theanine (500 mg kg^{-1}); DHβE(N), mice were treated with nicotine and DHβE. The data were expressed as the mean of ratio±SEM, n=3. #, P<0.05 compared with nicotine group.

was blocked in the brains of L-theanine pretreated animals (Figure 4A). Meanwhile, western blotting showed that the expression of TH in the ventral tegmental area (VTA) increased significantly after nicotine treatment when compared with the control group, while pre-treatment with L-theanine reduced the expression of TH in a dose-dependent manner. As shown in Figure 4, L-theanine treatment alone did not affect the production of dopamine in the midbrain and the expression of TH in the VTA, suggesting that the inhibitory effects of L-theanine on the production of DA and the expression of TH in mice only occurred when mice were stimulated by nicotine.

2.4 L-theanine inhibits the expression of the α_4, β_2 and α_7 nAChRs subunits induced by nicotine in the reward circuit related areas of the mouse brain

To dissect the mechanisms by which L-theanine inhibits the rewarding effects induced by nicotine, we next examined the expression of the α_4, β_2 and α_7 nAChR subunits in the reward circuit related areas of the mouse brain (VTA, PFC and NAc) following different treatments using Western blotting. Our results revealed that the expression of these three forms of nAChR subunits in these three areas increased significantly after treatment with nicotine (P<0.05). When mice were pre-treated with L-theanine, the upregulation of the α_4 and β_2 nAChR subunits were inhibited in the VTA, PFC and NAc, and the upregulation of the α_7 nAChR subunit induced by nicotine were inhibited in the VTA and PFC but not in the NAc. The effects of L-theanine treatment alone on the expression of α_4, β_2 and α_7 nAChR in the VTA, PFC and NAc areas were examined in mice that were not

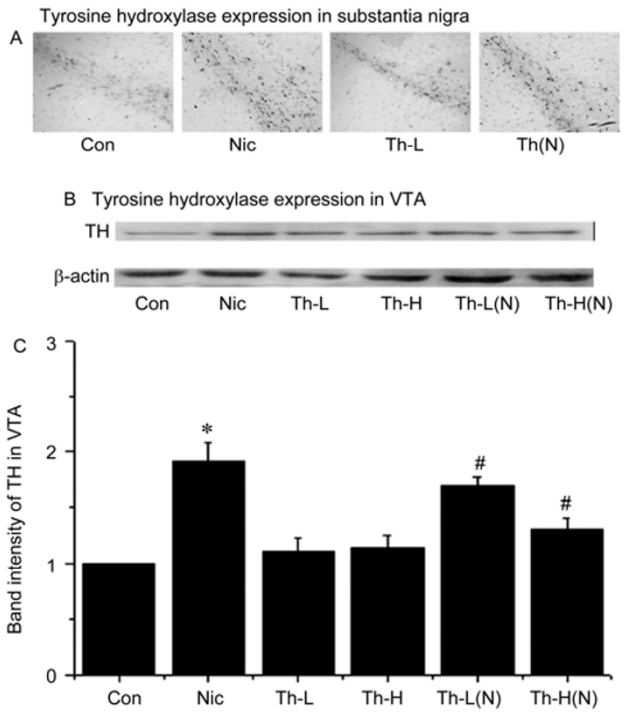

Figure 4 Effects of L-theanine on the nicotine-induced expression of tyrosine hydroxylase (TH) in the mouse brain. A, Immunohistochemical staining of tyrosine hydroxylase in the mouse substantia nigra. A1, The quantification results of immunohistochemical staining from 3 independent experiments. B, Effects of L-theanine on the expression of tyrosine hydroxylase (TH) analyzed by western blotting in the ventral tegmental area (VTA). B1, The quantification results of TH expression in the VTA from 3 independent experiments. Con, mice were treated with physiological saline; Nic, mice were treated with nicotine; Th-L, mice were treated with L-theanine (250 mg kg^{-1}); Th-H, mice were treated with L-theanine (500 mg kg^{-1}); Th-L(N), mice were pre-treated with L-theanine (250 mg kg^{-1}) then were treated with nicotine; Th(N), Th-H(N), mice were pre-treated with L-theanine (500 mg kg^{-1}) then were treated with nicotine. The data were expressed as the mean of ratio±SEM, n=3. *, P<0.05 compared with control group; #, P<0.05 compared with nicotine group.

treated with nicotine. L-theanine treatment alone only affected the expression of the α_4 nAChR subunit, which decreased in the NAc (Figure 5A–C, A1–C1). These data suggested that the L-theanine-induced downregulation of α_4, β_2 and α_7 nAChR subunit expression in the three reward circuit-related areas primarily happened when mice were stimulated by nicotine.

2.5 L-theanine inhibits nicotine-induced c-Fos expression in the reward circuit related areas of the mouse brain

Addictive substance abuse may induce neuron-specific adaptation and lead to physical and psychological dependence [25,26]. Recent investigations indicate that changes in c-Fos may play a crucial role in nicotine addiction [26]. Our study showed that the expression of c-Fos was upregulated in the three reward circuit related areas of mice after treatment with nicotine, while this phenomenon was inhibited after pretreatment with L-theanine (Figure 6). Meanwhile, double-immunofluorescence staining showed that both TH and c-Fos expression were co-localized and upregulated in SH-SY5Y cells after treatment with nicotine, while these nicotine-stimulated effects were inhibited by L-theanine pretreatment (Figure 7).

2.6 Knockdown of c-Fos inhibits the excitatory status of the cell but not the upregulation of TH induced by nicotine in SH-SY5Y cells

To study the importance of c-Fos in the nicotine-induced excitatory status of cells, we performed c-Fos siRNA experiments in SH-SY5Y cells and measured glucose intake using the 2-NBDG assay. As shown in Figure 8A and B, the expression of c-Fos decreased by 47% following c-Fos siRNA treatment. Meanwhile, glucose uptake induced by nicotine decreased significantly in cells treated with c-Fos siRNA (P<0.05; Figure 8C). This indicated that knockdown of c-Fos inhibited the excitatory status of cells. However, the expression of TH induced by nicotine was not changed by c-Fos siRNA treatment (Figure 8A and B).

3 Discussion

In this study, we found that L-theanine, an amino acid derivative of tea, reduced the nicotine-induced rewarding effects in a mouse CPP model and inhibited the nicotine-induced excitatory status of SH-SY5Y cells. The inhibitory effect of L-theanine was as effective as the inhibitor of the nicotine receptor (DHβE). This is in accordance with previous findings that reveal L-theanine had an antagonistic effect on the excitement induced by caffeine [16].

The increase in TH expression and dopamine release are important factors in the development of the rewarding effects of nicotine [27,28]. In our experiments, it was found that TH expression increased significantly after treatment with nicotine in the mouse midbrain, while it was inhibited after pretreatment with L-theanine. Interestingly, TH expression was not different between rats treated with L-theanine alone and the control group (Figure 4). These data suggest that the inhibitory effects of L-theanine on the production of dopamine and the expression of TH in mice only occurred when mice were stimulated with nicotine.

Mounting data indicates that the increase in the number of α_4, β_2 and α_7 nAchRs and their activation play a crucial role in the rewarding effects of nicotine[10–12]. Nicotine-seeking behavior was observed to decrease when the function of the nicotine receptors and their related pathways were inhibited [29,30]. Thus, nicotine receptors are responsible for nicotine addiction. Our present data showed that expression of α_4, β_2 and α_7 nAChR subunits increased significantly after treatment with nicotine in the reward circuit-related areas of mice. It has been shown previously that

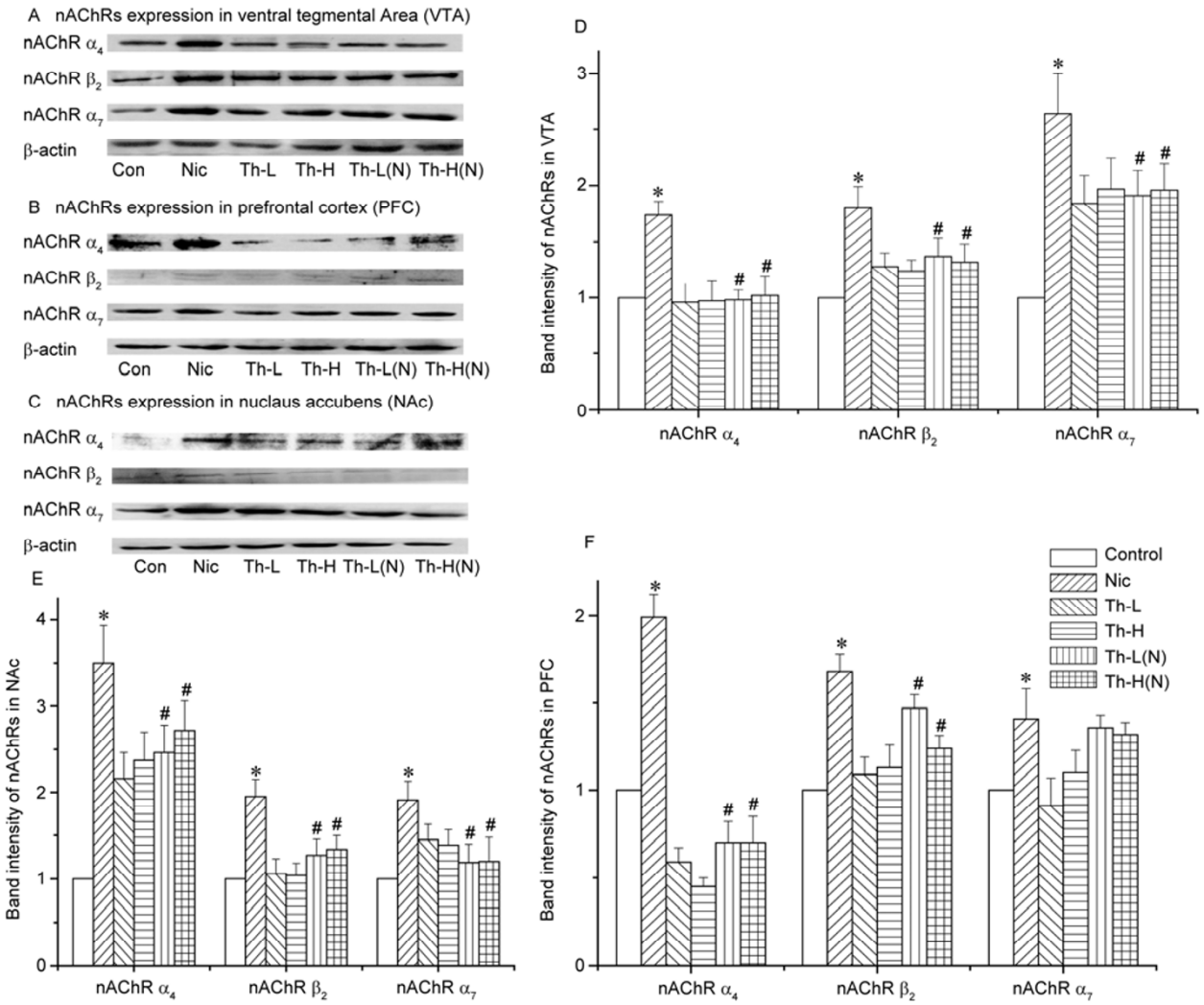

Figure 5 Effects of L-theanine on the expression of the nicotine receptor (nAChR) in the mouse brain. Protein extracts prepared from different parts of mouse brain were analyzed using Western blotting. The expression of nAChR was examined in the ventral tegmental area (VTA) (A), prefrontal cortex (PFC) (B) and nuclear accumbens core (NAc). C, The quantification results of nAChR expression in the VTA (A1), PFC (B1) and NAc (C1) from 3 independent experiments. Con, mice were treated with physiological saline; Nic, mice were treated with nicotine; Th-L, mice were treated with L-theanine (250 mg kg^{-1}); Th-H, mice were treated with L-theanine (500 mg kg^{-1}); Th-L(N), mice were pre-treated with L-theanine (250 mg kg^{-1}) then were treated with nicotine; Th-H(N), mice were pre-treated with L-theanine (500 mg kg^{-1}) then were treated with nicotine. The data were expressed as the mean of the ratio±SEM, n=3. *, P<0.05 compared with control group; #, P<0.05 compared with nicotine group.

prolonged nicotine exposure causes the functional desensitization of the nicotine-sensitive nAChRs [31–34], as a result, nAChR expression is upregulated while tolerance of nAChRs to nicotine increases. This is consistent with our results that the expression of the nAChRs was upregulated in the reward circuit-related areas of mice after nicotine treatment. Our data also showed that L-theanine pretreatment inhibited the nicotine-induced upregulation of α_4, β_2 nAChR subunits in the VTA, PFC and NAc, and the α_7 nAChR subunit in the VTA and NAc. This is a specific process of the nAChRs, which causes the upregulation of the nAChR. The expression of the above nAChR subunits were not downregulated in these three reward circuit-related areas of mice by L-theanine treatment alone, except for the expression α_4 nAChR, which was found to decrease in the NAc. These data suggest that the inhibitory effects of L-theanine on the expression of α_4, β_2 and α_7 nAChR subunits in the reward circuits primarily occurred when mice were stimulated with nicotine. Overall, the data indicates that L-theanine may inhibit midbrain dopamine production through inhibiting the nicotine-induced upregulation of the α_4, β_2 and α_7 nAChR subunits in the reward circuits, thus inhibiting the rewarding effects. However, it is unclear how L-theanine inhibits the nicotine-induced upregulation of nAChR subunit expression, for example, whether L-theanine inhibits nicotine through competitive or uncompetitive binding to the receptors. This area requires further investigation.

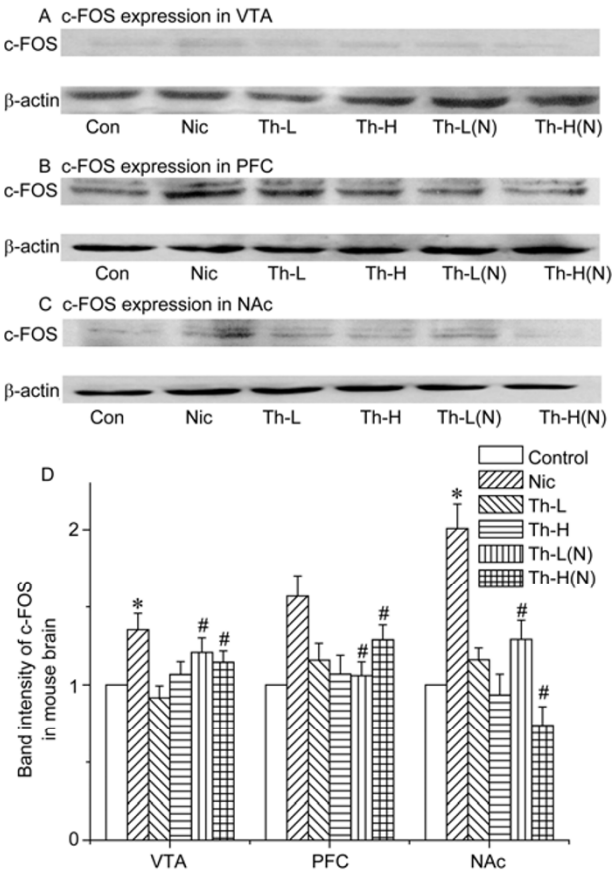

Figure 6 Effects of L-theanine on the expression of c-Fos in the mouse brain. Protein extracts prepared from different parts of mouse brain were analyzed using Western blotting. The expression of c-Fos was examined in the ventral tegmental area (VTA) (A), prefrontal cortex (PFC) (B), and nuclear accumbens core (NAc) (C). D, The quantification results of c-Fos expression in the VTA, PFC and NAc from 3 independent experiments. Con, mice were treated with physiological saline; Nic, mice were treated with nicotine; Th-L, mice were treated with L-theanine (250 mg kg^{-1}); Th-H, mice were treated with L-theanine (500 mg kg^{-1}); Th-L(N), mice were pre-treated with L-theanine (250 mg kg^{-1}) and then with nicotine; Th-H(N), mice were pretreated with L-theanine (500 mg kg^{-1}) and then with nicotine. The data were expressed as the mean of the ratio±SEM, n=3. *, P<0.05 compared with control group; #, P<0.05 compared with nicotine group.

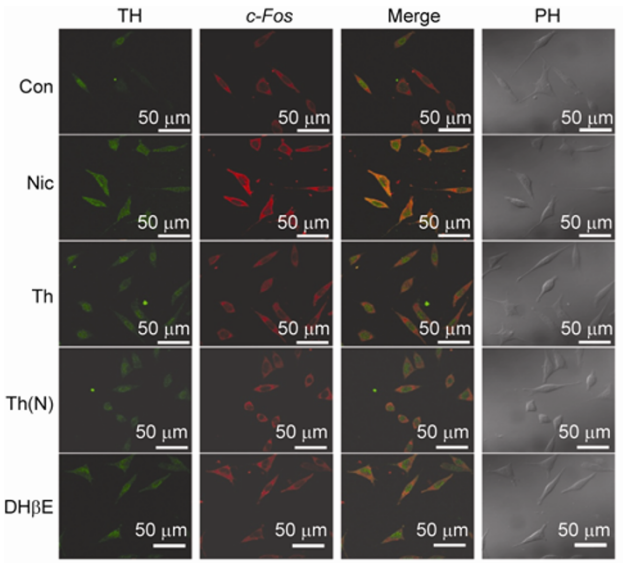

Figure 7 Effects of L-theanine on tyrosine hydroxylase (TH) and c-Fos expression examined by immunofluorescence staining. SH-SY5Y cells were treated and double-immunofluorescence staining was performed as described in Methods. Fluorescence was examined under a confocal laser scanning microscope (LSCMFV500). TH, TH immunostaining (green); c-Fos, c-Fos immunostaining (red); Merge, double-immunostaining of TH and c-Fos (yellow); PH, cell optical micrograph. Con, cells were treated with physiological saline; Nic, cells were treated with nicotine; Th, cells were treated with L-theanine (100 μmol L^{-1}); Th(N), cells were treated with nicotine and L-theanine (100 μmol L^{-1}); DHβE(N), cells were treated with nicotine and DHßE.

As a member of the fos transcription factor family, c-Fos has been shown to be associated with the neuron-specific adaptation induced by nicotine [14,26]. The expression of c-Fos increased significantly in the reward circuit-related areas after acute treatment with nicotine [35,36]. Moreover, the expression of c-Fos increased significantly in the NAc after nicotine was injected into the VTA directly [26]. In our study, the expression of c-Fos was upregulated in the three reward circuit-related areas by nicotine treatment, while this phenomenon was inhibited by L-theanine pre-treatment (Figure 6). In SH-SY5Y cells, double-immunofluorescence staining revealed that the expression of c-Fos and TH increased significantly in the same cells after nicotine treatment. This effect of nicotine was inhibited by L-theanine pre-treatment (Figure 7). The data suggest that the inhibitory effects of L-theanine on nicotine-induced c-Fos expression may contribute to the regulation of L-theanine on nicotine-induced neuron-specific adaptation. We found that knock-down of c-Fos inhibited the nicotine-induced excitatory status of cells but not the expression of TH (Figure 8A and A1), indicating that the effect of c-Fos on the excitatory status of cells and neuron-specific adaptation induced by nicotine was not mediated by the regulation of TH expression.

The binding sites of the ^{3}H nicotine in the central nervous system as well as the number of cells that can react to nicotine were increased after prolonged nicotine exposure [37,38]. Our present study indicates that L-theanine can inhibit the rewarding effects in the mouse CPP model and reduce the excitatory status of cells induced by nicotine. The inhibitory effects of L-theanine may be achieved by a complex mechanism. First, L-theanine can inhibit the nicotine-induced up-regulation of the α_4, β_2 and α_7 nAChR subunits in regions that are related to the dopamine reward pathway and decrease the number of cells that can react to nicotine. Second, L-theanine can inhibit the nicotine-induced upregulation of dopamine production in the midbrain. Third, L-theanine can inhibit nicotine-induced expression of c-Fos in the regions that are related to the dopamine reward pathway, ultimately inhibiting the re-

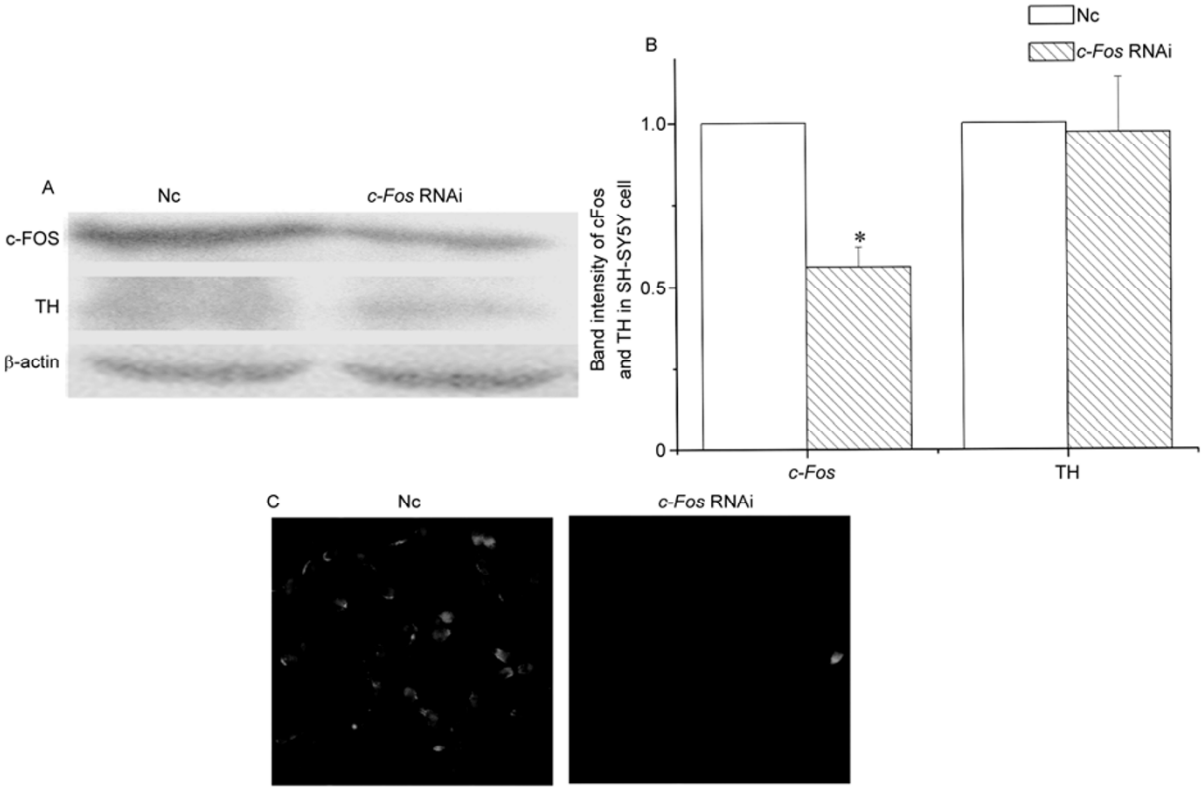

Figure 8 Effects of c-Fos knockdown on the expression of TH and glucose uptake in SH-SY5Y cells. A, c-Fos and TH expression levels after c-Fos knockdown. Protein extracts prepared from SH-SY5Y cells were analyzed using Western blotting. B, The quantification results of c-Fos and TH expression from 3 independent experiments. C, Glucose uptake status in SH-SY5Y cells induced by nicotine after c-Fos knockdown. D, Quantification results of fluorescence from 3 independent experiments. Nc, siRNA-Control; c-Fos RNAi, siRNA-c-Fos; The data were expressed as the mean of the ratio±SEM, n=3. *, P<0.05 compared with control group.

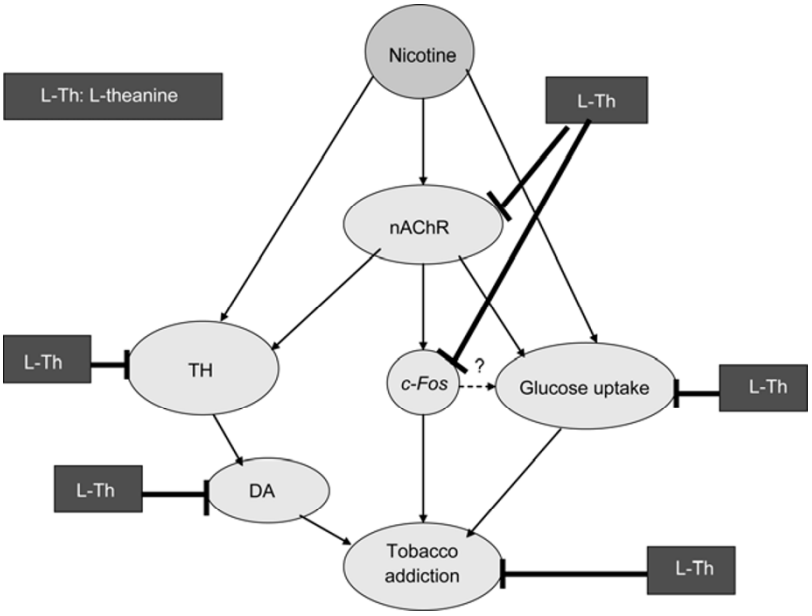

Figure 9 Schematic diagram: L-theanine inhibits nicotine-induced dependence through inhibiting the nAChR-dopamine reward pathway.

warding effects and the neuron-specific adaptation induced by nicotine (Figure 9).

Smoking cessation is the ultimate method for reducing smoking-related diseases. However, quitting smoking is extremely difficult because of the addictive nature of nicotine. Future application of L-theanine in smoking cessation

may provide an alternative therapeutic strategy for smokers to reduce and/or quit smoking by inhibition of nicotine dependence [39–43].

Some studies have suggested nicotine as a therapy for Alzheimer's disease (AD) and Parkinson's disease (PD) [44–49]; however, the dependence of nicotine limits the use of nicotine as a medication. Interestingly, L-theanine has been shown to have some benefits for the prevention and treatment of AD [50]. Thus, L-theanine may have a synergic effect with nicotine for AD therapy, and a combination of L-theanine and nicotine may prevent nicotine dependence, opening up new possibilities for novel therapeutics for neurodegenerative disorders such as AD and PD.

Overall, the present study showed that L-theanine reduced the nicotine-induced rewarding effects via inhibition of the nAChR-dopamine reward pathway. These results may offer new therapeutic strategies for the treatment of tobacco addiction.

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