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Cytisine, a Partial Agonist of α4β2 Nicotinic Acetylcholine Receptors, Reduced Unpredictable Chronic Mild Stress-Induced Depression-Like Behaviors

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Abstract

Cytisine (CYT), a partial agonist of α4β2-nicotinic receptors, has been used for antidepressant efficacy in several tests. Nicotinic receptors have been shown to be closely associated with depression. However, little is known about the effects of CYT on the depression. In the present study, a mouse model of depression, the unpredictable chronic mild stress (UCMS), was used to evaluate the activities of CYT. UCMS caused significant depression-like behaviors, as shown by the decrease of total distances in open field test, and the prolonged duration of immobility in tail suspension test and forced swimming test. Treatment with CYT for two weeks notably relieved the depression-like behaviors in the UCMS mice. Next, proteins related to depressive disorder in the brain region of hippocampus and amygdala were analyzed to elucidate the underlying mechanisms of CYT. CYT significantly reversed the decreases of 5-HT1A, BDNF, and mTOR levels in the hippocampus and amygdala. These results imply that CYT may act as a potential anti-depressant in the animals under chronic stress.

Key Words: Cytisine, Depression, Nicotinic receptor, Chronic stress

INTRODUCTION

Neuron nicotinic acetylcholine receptors (nAChRs), belonging to the ligand-gated ion channel superfamily of neurotransmitter receptors. Varying combinations of nAChR subunits (α1-α10, β1-β4, γ, δ, and ε; α2-α7 and β2-β4 are expressed in the brain) assemble into pentameric ion channels, allowing for diverse pharmacological properties (Lukas *et al*., 1999), have been investigated for developing drugs that can potentially treat various central nervous system disorders. The cholinergic hypothesis of depression proposes that hyperactivity of the cholinergic system over that of the adrenergic system leads to depression. Several lines of evidence from rodent and human studies support this hypothesis (Janowsky *et al*., 1972). Additionally, a number of key antidepressants such as selective serotonin reuptake inhibitor antidepressants (SSRIs, e.g. fluoxetine, sertraline, paroxetine, and citalopram), the norepinephrine reuptake inhibitor reboxetine, the norepinephrine dopamine reuptake inhibitor bupropion, and tricyclics (amitriptyline, imipramine, and nortriptyline) have been shown to possess partial antagonistic activities at nAChRs (Hennings *et al*., 1997; Fryer and Lukas, 1999; López-Valdés and Garcia-Colunga, 2001; Arias *et al*., 2010). The α4β2 heteropentameric and α7 homopentameric subtypes are the two major nAChR subtypes expressed in the brain (Picciotto *et al*., 1998). The α4β2-nAChRs are widely distributed in the brain regions implicated in depression, including the thalamus, hippocampus, striatum, hypothalamus, amygdala, ventral tegmental area (VTA), locus coeruleus, and dorsal raphe nucleus (Philip *et al*., 2010), and thought to regulate the release of monoamine neurotransmitters through action in these areas (Hogg *et al*., 2003; Gotti *et al*., 2006).

Cytisine (CYT), a natural plant alkaloid, has been used as an inexpensive smoking-cessation aid for 50 years in Eastern Europe (Picciotto *et al*., 1995; Tutka and Zatoński, 2006). Cytisine, like varenicline, is a partial agonist selectively binding to the α4β2-nAChRs that appear to mediate nicotine dependence (Cahill *et al*., 2013). CYT behaves like a weak

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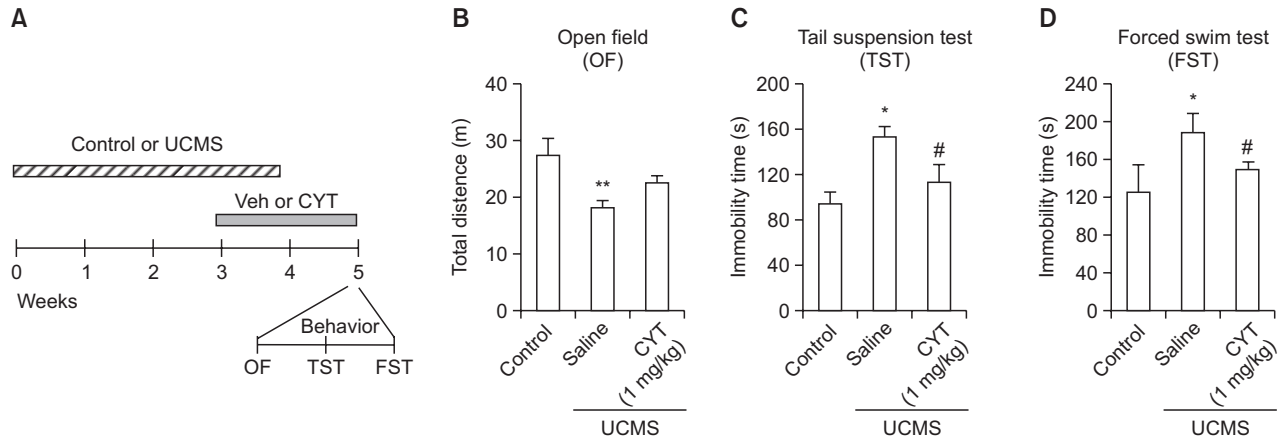

Fig. 1. Effects of CYT on depressive-like behaviors. CYT treatment blocked UCMS-induced depressive-like behaviors. (A) Timeline of the UCMS exposure, drug treatment, and behavioral tests. (B) Treatment of CYT (1 mg/kg) affected the total distance traveled in the OF. The total distance means the distance in whole area of the open field. (C) UCMS significantly increased the immobility time in the TST; CYT treatment decreased the immobility time in UCMS-exposed mice. (D) UCMS significantly increased the immobility time in the FST; CYT treatment decreased the immobility time in UCMS-exposed mice. n=6/group. *p<0.05, **p<0.01 compared with the control mice. $^{\#}p$<0.05 compared to the UCMS mice treated with saline.

partial agonist, mimicking effects of nicotine to a limited degree (Radchenko *et al*., 2015). Preclinical data support the antidepressant efficacy of CYT when used in conjunction with other primary antidepressants (e.g. SSRIs) (Philip *et al*., 2012). Cytisine is reported to reduce alcohol consumption and nicotine-induced alcohol drinking (Bell *et al*., 2009; Sajja and Rahman, 2013). Emerging evidence suggests that Cytisine significantly reduces depression-like behaviors in preclinical models that mimic major depressive disorder and co-morbid alcohol or nicotine use disorder (Rahman, 2015). However, little is known the effects of CYT on the chronic stress-induced depression-like behaviors.

Nicotine increases 5-hydroxytryptamine (5-HT) release in the cortex, striatum, hippocampus, dorsal raphe´ nucleus (DRN), hypothalamus, and spinal cord (Ma *et al*., 2005). In addition, nicotine has antidepressant, anxiolytic, anorexic, antinociceptive (Cheeta *et al*., 2001; Schmidt *et al*., 2001; Seth *et al*., 2002), and antihypnotic properties (Salín-Pascual *et al*., 1996). While the diverse and complex effects of nicotine are not yet fully understood, considerable evidence suggests that 5-hydroxytryptamine (5-HT) may play a role in affective disorders and drug addiction. The effects involve stimulation of 5-HT1A receptors. The 5-HT1A receptors play a role in mediating the anti-depression effects of nicotine (Seth *et al*., 2002), however, little has been reported on the alteration of antidepressant action under chronic treatment in hippocampus and amygdala. Brain-derived neurotrophic factor (BDNF) functions in both the peripheral and central nervous systems (CNS) and has a beneficial impact on supporting the survival of existing neurons and promoting the growth of newly differentiated neurons and synapses (Bergami and Berninger, 2012). Several lines of evidence indicate that chronic stress and low level of BNDF are the key components of depression pathology (Yan *et al*., 2015).

The mammalian target of rapamycin (mTOR) signaling pathway is disturbed with major depressive disorder, and the activation of mTOR signaling is required for rapid antidepressant action in the hippocampus and other brain regions (Zhong *et al*., 2014). Unpredictable chronic mild stress (UCMS) decreases the phosphorylation levels of mTOR and its downstream signaling components, i.e., Akt-1 (Wang *et al*., 2015). This is accompanied by suppressed synaptic plasticity.

In the present study, we sought to understand whether CYT, a partial agonist of $\alpha4\beta2$-nAChRs, attenuated depression-like behaviors in the UCMS mice and the underlying mechanisms. Here, we showed that CYT acted as an effective anti-depressive drug by regulating the serotonin receptor, BDNF, and mTOR signaling.

MATERIALS AND METHODS

Drugs

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. CYT was purchased from the ShanghaiPureone Biotechnology Co., Ltd (Shanghai, China). Anti-5-HT1A and anti-BDNF antibodies were purchased from Chemicon (Temecula, CA, USA). Anti-mTOR, p-mTOR, anti-AKT, p-AKT, anti-S6K, p-S6K, anti-CREB, and p-CREB antibodies were purchased from Abcam (Cambridge, UK). Anti-β-actin antibody was purchased from Sigma. All of the chemicals and reagents used were commercially available and of standard biochemical quality.

Animals

Male C57BL/6J mice (6-8 weeks, Laboratory Animal Center of the Fourth Military Medical University) were habituated for 1 week. Mice were housed under standard conditions (temperature 24 ± 2ºC, humidity 50-60%, 12:12-h light/dark cycle, lights on at 08:00 h). Food and water were available *ad libitum*. Mice were marked on their tail with a permanent marker for identification and were randomly assigned to one of the different treatment groups (control, UCMS-saline, and UCMS-CYT; n=6/group). Each animal received the same treatment throughout the experiments. The Fourth Military Medical University Animal Care and Use Committee approved the animal protocols.

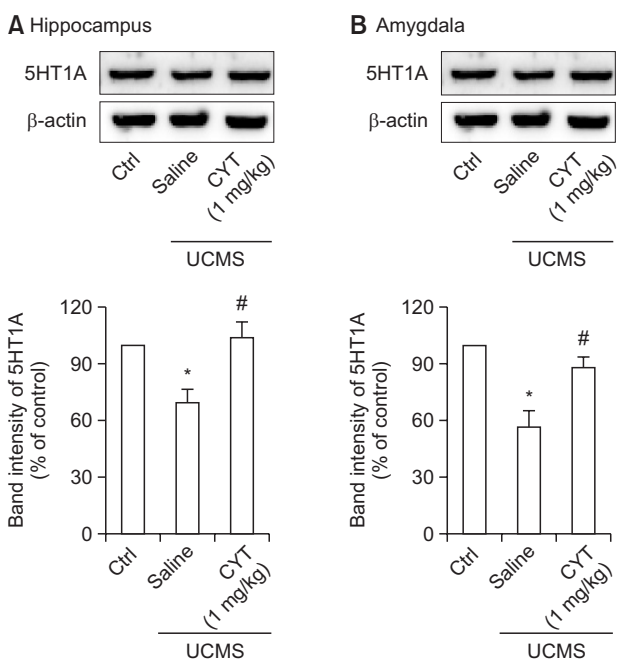

Fig. 2. CYT reversed levels of serotonin receptors in the UCMS mice. (A) Upper: representative Western blot of 5-HT1A in hippocampus. Lower: treatment of CYT (1 mg/kg) reversed the levels of 5-HT1A in UCMS mice. (B) Upper: representative Western blot of 5-HT1A in amygdala. Lower: treatment of CYT (1 mg/kg) reversed the levels of 5-HT1A in UCMS mice. n=6/group. **p*<0.05 compared with the control mice. #*p*<0.05 compared to the UCMS mice treated with saline.

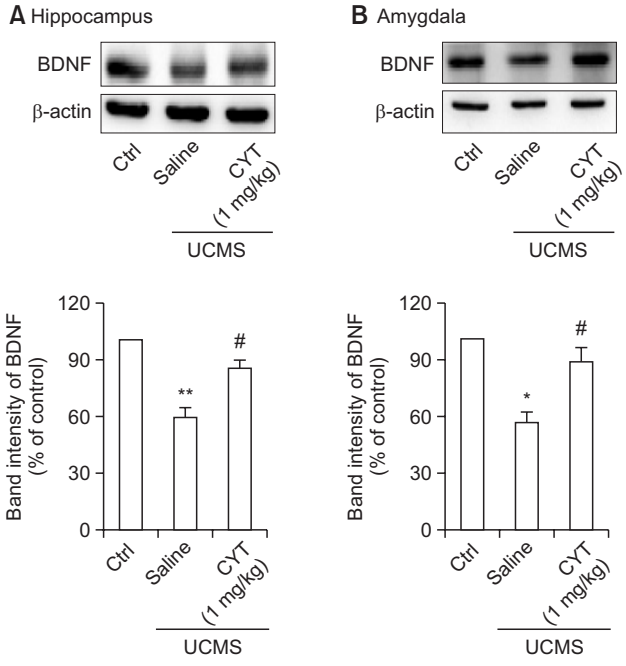

Fig. 3. CYT reversed the levels of BDNF in the UCMS mice. (A) Upper: representative Western blot of BDNF in hippocampus. Lower: treatment of CYT (1 mg/kg) reversed the levels of BDNF in UCMS mice. (B) Upper: representative Western blot of BDNF in amygdala. Lower: treatment of CYT (1 mg/kg) revered the levels of BDNF in UCMS mice. n=6/group. **p*<0.05, ***p*<0.01compared with the control mice. #*p*<0.05 compared to the UCMS mice treated with saline.

UCMS paradigm and drug treatments

UCMS mice were exposed to various stressors for 5 weeks, and time-matched control mice did not receive any stressors. The stressors include restraint (2 h), inversion of day/night light cycle (light off and light on), cold (in a cold room at 4°C for 1 h), 45° tilted cage (overnight), cage rotation (20 min), wet bedding (250 ml of water added into cage, overnight), no bedding (overnight), food and water deprivation (overnight), forced swimming (cold water 4ºC for 6 min), and overcrowding (overnight) (Willner *et al*., 1987; Koo and Duman, 2008). On average, two stressors were administered per day. Non-stressed controls were handled only for cage changes and behavioral tests. The stressors and time course of UCMS have been detailed in Fig. 1. Mice were exposed to UCMS for a total of 5 weeks. At the beginning of the third week, UCMS-exposed mice were given CYT (1 mg/kg, i.p.) for two weeks. Cytisine (1-3 mg/kg) partially substituted for nicotine and at the highest dose tended to antagonize nicotine's discriminative stimulus effects (Radchenko *et al*., 2015). In present study, 1 mg/kg of CYT (i.p.) was selected for the behavioral experiments.

Open field test (OF)

Mice were placed individually in one corner of the open field (50 cm length×45 cm wide×30 cm deep box)(Shanghai Jiliang Software Technology, JLBehv-LAR-1, Shanghai, China) and allowed to freely explore the arena during a 15-min test session.

Tail suspension test (TST)

Mice were suspended by the tail with a paper clip attached with adhesive tape about 5 mm from the end of the tail (Shanghai Bio-will Co., Ltd., BW-DTS203, Shanghai, China). Time spent immobile was recorded over the 6 min test. The time spent immobile during the last 4 min was scored by an observer blind to treatments. After completion of the test, mice were returned to the home cage a holding cage until all cagemates were tested.

Forced swim test (FST)

Mice were placed individually into glass cylinders (13 cm diameter, 25 cm tall) filled to a depth of 18 cm with water (25 ± 1.0ºC) (Shanghai Jiliang Software Technology, JLBehv-FSR-1, Shanghai, China). The mice were placed in the cylinders for 6 min. The time spent immobile during the last 4 min was scored by an observer blind to treatments. Immobility was defined as the cessation of all movements (*e.g*., climbing, swimming) except those necessary for the mouse to keep its head above water (*i.e*., floating).

Western blot analysis

Tissue samples from the bilateral amygdala and hippocampus were dissected from the brain slices (300 μm) under the anatomical microscope. Total homogenates of amygdala and hippocampus sample were got from 6 mice respectively, and each sample of the mouse was examined by Western blot. Equal amounts of protein (30 μg) from the hippocampus and amygdala were separated and electro-transferred onto PDVF membranes (Invitrogen), which were probed with antibodies for 5-HT1A (dilution ratio 1:1,000), BDNF (dilution ratio1:1,000), AKT (dilution ratio 1:1,000), p-AKT (dilution ra-

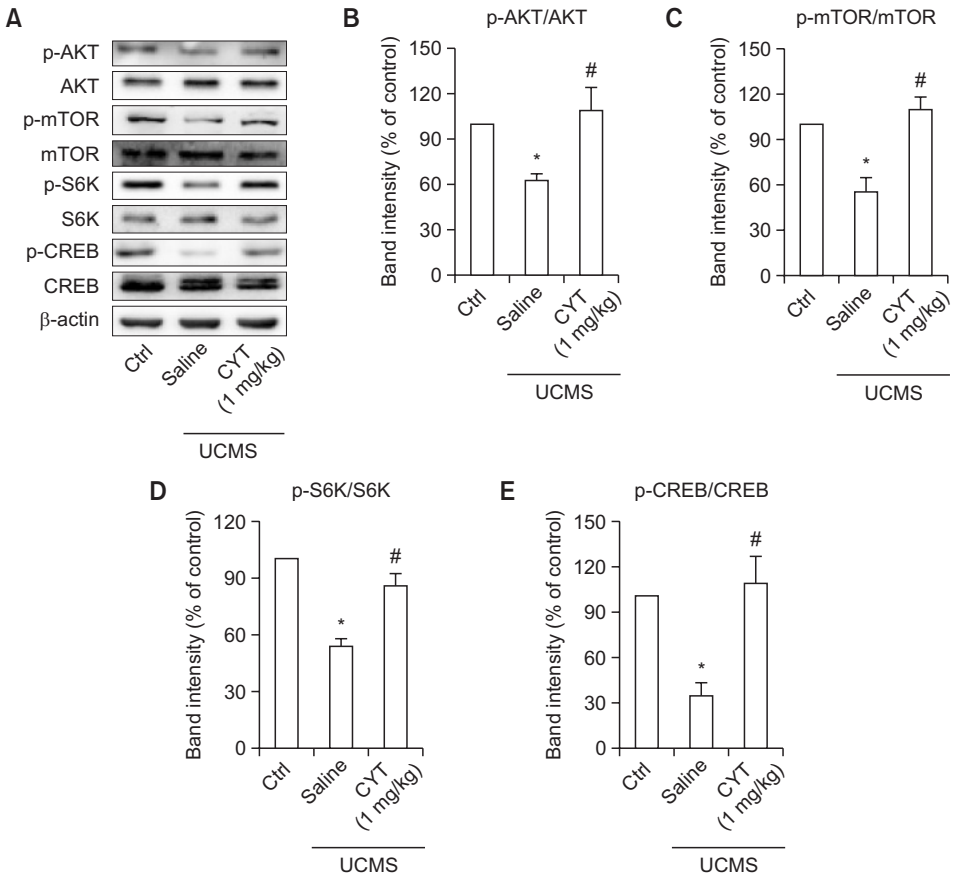

Fig. 4. CYT increased mTOR signaling activities in the hippocampus. (A) Representative Western blot of proteins in hippocampus. (B) Treatment of CYT (1 mg/kg) up-regulated the ratio of p-AKT/AKT in UCMS mice. (C) Treatment of CYT (1 mg/kg) up-regulated the ratio of p-mTOR/mTOR in UCMS mice. (D) Treatment of CYT (1 mg/kg) up-regulated the ratio of p-S6K/S6K in UCMS mice. (E) Treatment of CYT (1 mg/kg) up-regulated the ratio of p-CREB/CREB in UCMS mice. n=6/group. *p<0.05 compared with the control mice. $^{\#}p$<0.05 compared to the UCMS mice treated with saline.

tio 1:1,000), p-S6K (dilution ratio 1:1,000), S6K (dilution ratio 1:1,000), mTOR (dilution ratio 1:1,000), p-mTOR (dilution ratio 1:1,000), CREB (dilution ratio 1:1,000), p-CREB (dilution ratio 1:1,000), and with β-actin (dilution ratio 1:10,000) as a loading control. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit/anti-mouse IgG for the primary antibodies), and bands were visualized using an ECL system (Lightning Blot System, Perkin Elmer, Waltham , MA, USA). For data quantification, band intensity of each blot was calculated as ratio relative to the β-actin. The intensity ratio of control group was set as 100%, and the intensity ratios of other treatment groups were expressed as percentage to the control group.

Data analysis and statistics

Results are expressed as mean ± SD. Data were evaluated using one-way analysis of variance (ANOVA) for post hoc comparisons (SPSS 13.0). Data that passed the homogeneity test were analyzed by the one-way ANOVA least significant difference (LSD) test. Data that did not pass the homogeneity test were analyzed by the one-way ANOVA Dunnett's T3 test. p<0.05 was considered statistically significant.

RESULTS

Anti-depression like behaviors of CYT in UCMS model

Mice were exposed to UCMS for a total of 5 weeks. At the beginning of the third week, UCMS-exposed mice were given saline or CYT (i.p., 1 mg/kg) every day for 2 weeks (Fig. 1A). OF was used to determine the locomotor activity. Reduced activity in the total distance of an open field has been correlated with depression-like behaviors in rodents (El Yacoubi *et al*., 2003). Treatment of CYT increased the total distance traveled in UCMS mice during the 15-min test session in the OF ($F_{(2, 15)}$=5.861, p=0.013, LSD test; Fig. 1B). In the tail suspension test (TST) and forced swim test (FST), the mean immobility period of the UCMS mice was significantly longer than that of the control group (TST: $F_{(2, 15)}$=6.735, p=0.008, LSD test; Fig. 1C) (FST: $F_{(2, 15)}$=6.456, p=0.009, LSD test; Fig. 1D). Treatment of CYT (1 mg/kg) decreased immobility time of UCMS mice in the TST (Fig. 1C) and FST (Fig. 1D).

CYT increases the levels of 5-HT1A in UCMS mice

Serotonin receptors play pivotal roles in depression disorders (Seth *et al*., 2002). UCMS decreased the levels of 5-HT1A receptor in homogenates of hippocampus and amygdala, whereas treatment of CYT reversed the alteration of 5-HT1A

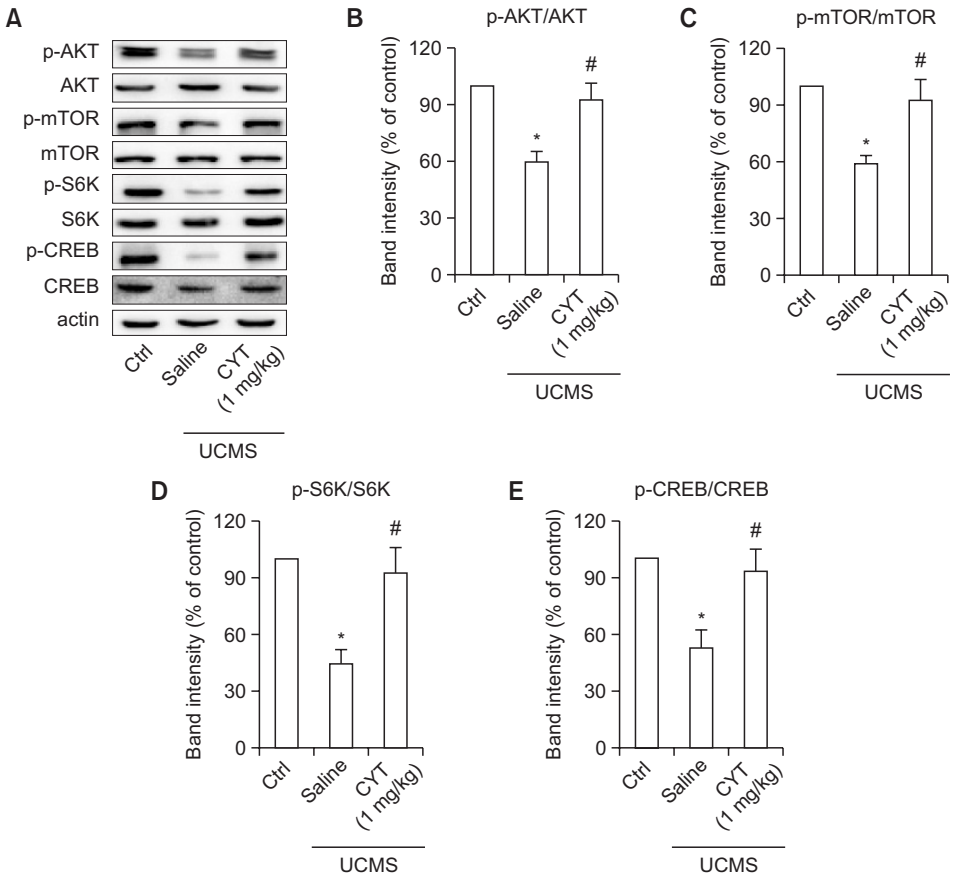

Fig. 5. CYT increased mTOR signaling activities in the amygdala. (A) Representative Western blot of proteins in amygdala. (B) Treatment of CYT (1 mg/kg) up-regulated the ratio of p-AKT/AKT in UCMS mice. (C) Treatment of CYT (1 mg/kg) up-regulated the ratio of p-mTOR/mTOR in UCMS mice. (D) Treatment of CYT (1 mg/kg) up-regulated the ratio of p-S6K/S6K in UCMS mice. (E) Treatment of CYT (1 mg/kg) up-regulated the ratio of p-CREB/CREB in UCMS mice. n=6/group. *p<0.05 compared with the control mice. $^{\#}p$<0.05 compared to the UCMS mice treated with saline.

receptor (Hippocampus: $F_{(2, 15)}$=10.29, p=0.002, LSD test; Fig. 2A) (Amygdala: $F_{(2, 15)}$=12.95, p=0.001, LSD test; Fig. 2B).

CYT up-regulates levels of BDNF in UCMS mice

Levels of BDNF is decreased in depression disorders (Yan *et al*., 2015). UCMS decreased the expression levels of BDNF in hippocampus and amygdala of UCMS mice, whereas treatment of CYT increased the BDNF levels in the hippocampus and amygdala (Hippocampus: $F_{(2, 15)}$=25.798, p<0.001, LSD test; Fig. 3A) (Amygdala: $F_{(2, 15)}$=17.735, p<0.001, LSD test; Fig. 3B).

Treatment of CYT increases the activities of mTOR signaling in UCMS mice

mTOR signaling pathway is down-regulated in the major depressive disorder (Zhong *et al*., 2014). Proteins related to the mTOR signaling in the hippocampus and amygdala were detected by the western blot. We found that the total levels of AKT, mTOR, S6K, and CREB were not changed in the hippocampus of UCMS mice, however, the ratio of p-AKT/AKT ($F_{(2, 15)}$=25.757, p<0.001, LSD test; Fig. 4A, 4B), p-mTOR/mTOR ($F_{(2, 15)}$=21.342, p<0.001, LSD test; Fig. 4C), p-S6K/S6K ($F_{(2, 15)}$=14.961, p<0.001, LSD test; Fig. 4D), or p-CREB/CREB ($F_{(2, 15)}$=6.682, p=0.008, LSD test; Fig. 4E) was significantly decreased. Treatment of CYT could reverse above alterations of p-AKT/AKT, p-mTOR/mTOR, p-S6K/S6K, or p-CREB/CREB. Similar results were found in the region of amygdala. Treatment of CYT could ameliorate the decrease of ratio of p-AKT/AKT ($F_{(2, 15)}$=12.01, p=0.001, LSD test; Fig. 5A, 5B), p-mTOR/mTOR ($F_{(2, 15)}$=9.452, p=0.002, LSD test; Fig. 5C), p-S6K/S6K ($F_{(2, 15)}$=9.293, p=0.002, LSD test; Fig. 5D), or p-CREB/CREB ($F_{(2, 15)}$=11.491, p=0.001, LSD test; Fig. 5E) in the amygdala.

DISCUSSION

Nicotinic agonists, through activation of neuronal nAChR, improve cognitive performance in both animals and humans. nAChRs, including $\alpha4\beta2$-nAChRs, influence synaptic plasticity through the facilitation of presynaptic and postsynaptic mechanisms (Glassman *et al*., 1990; Salín-Pascual *et al*., 1995; Semba *et al*., 1998). In present study, mice were exposed to UCMS for a total of 5 weeks exhibited significant depressive behaviors, as shown by the decreased locomotor activity and the longer mean immobility period in the tail suspension test and forced swim test. Cytisine (1 mg/kg) could alleviate the depressive-like behaviors in the chronic stress mice. Present study indicates that CYT, a partial agonist of $\alpha4\beta2$-nicotinic re-

ceptors, may act as a potential anti-depressant in the animals under chronic stress.

Nicotinic receptors are pentameric, ligand-gated ion channels comprised of heteromeric or homomeric subunits encoded by nine α (α2-10) and three β (β2-4) genes in the human brain. nAChR blockers potentiate the effects of selective serotonin reuptake inhibitors (SSRIs) in some treatment-resistant patients (Mineur *et al*., 2015). Interactions between the serotonergic and cholinergic systems are quite related to mood disorders. The effect of nicotine is attributed to an activation of several β2- and β4-containing receptors, as well as homomeric α7 nicotine receptors, which are broadly distributed in the CNS (Damaj *et al*., 2003; Millar, 2003; Wooltorton *et al*., 2003). Presynaptic nAChRs regulate neurotransmitter release, while postsynaptic nAChRs activate intracellular signaling and gene transcription. Nicotine exposure is known to have multiple effects on the 5-HT system, and thus the expression of nAChRs by 5-HT neurons may play an important role in the 5-HT abnormalities. CYT, as a partial agonist of α4β2-nAChRs, up-regulates the levels of 5-HT1A receptors in hippocampus and amygdala and produces antidepression-like effects in the present UCMS model.

Stress and depression are associated with neuronal atrophy and decrease of synaptic connections and leads to decreased expression and release of BDNF in the prefrontal cortex, limbic brain regions, and hippocampus (Nasca *et al*., 2013). Present study demonstrates that BDNF levels are decreased in the hippocampus and amygdala. Treatment of CYT up-regulates BDNF levels, and improves stress-induced cognitive and behavioral alterations.

The mTOR signaling pathway is implicated in the pathophysiology of depression and in the antidepressant-like effects of different compounds (Abelaira *et al*., 2014; Zhong *et al*., 2014). Our results found that UCMS induced a significant decrease of p-AKT, p-S6K, p-mTOR and p-CREB levels in the hippocampus and amygdala. CYT administration significantly ameliorated the decrease of ratio of p-AKT/AKT, p-mTOR/mTOR, p-S6K/S6K, or p-CREB/CREB. PI3K/AKT pathway and mTOR signaling play an important role in the production of BDNF, implicating the activities of CYT in the antidepressant response (Duman and Voleti, 2012).

In summary, the present study shows that CYT produces antidepressant-like effects through modulating the 5-HT1A, BDNF, and mTOR signaling in the hippocampus and amygdala of UCMS model. This study is helpful to elucidate the mechanisms underlying the antidepressant effects of CYT and for the clinical treatment of depression by traditional herbs.

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