Nicotine alleviates chronic stress-induced anxiety and depressive-like behavior and hippocampal neuropathology via regulating autophagy signaling

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ABSTRACT
Recently, we reported that chronic nicotine significantly improved chronic stress-induced impairments of cognition and the hippocampal synaptic plasticity in mice, however, the underlying mechanism still needs to be explored. In the present study, 32 male C57BL/6 mice were divided into four groups: control (CON), stress (CUS), stress with chronic nicotine administration (CUS + Nic) and chronic nicotine administration (Nic). The anxiety-like behavior and neuropathological alteration of DG neurons were examined. Moreover, PC12 cells were examined with corticosterone in the presence or absence of nicotine. Both cell viability and apoptosis were determined. When treated simultaneously with an unpredictable chronic mild stress (CUS), nicotine (0.2 mg/kg/d) attenuated behavioral deficits and neuropathological alterations of DG neurons. Moreover, Western blotting showed that chronic nicotine also elevated the level of autophagy makers including Beclin-1 and LC3 II triggered by CUS. In addition, concomitant treatment with nicotine (10 μM) significantly attenuated the loss of PC12 cell viability (p < .01) and apoptosis compared to that of corticosterone treatment alone. Besides, chronic nicotine also enhanced the protein and RNA expression levels of autophagy markers triggered by corticosterone, such as Beclin-1, LC3 II and p62/SQSTM1. However, the above improvements were significantly blocked by autophagy inhibitor 3-MA. Importantly, the activation of the PI3K/Akt/mTOR signaling was carefully tested to illuminate the effects of chronic nicotine. Consequently, chronic nicotine played a role of neuroprotection in either CUS mice or corticosterone cells associating with the enhancement of the autophagy signaling, which was involved in activating the PI3K/Akt/mTOR signaling.

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1. Introduction
Depression is a common, life-threatening psychiatric disorder, which is a tragedy for patients and families (Leonard, 2010). It is widely accepted that environmental stress plays an important role in mental disorders. For instance, vulnerable individuals experiencing stressful life events may lead to clinical depression. The hippocampus is one of the brain structures that is involved in emotional and cognitive function. Recent studies illustrated that major depression and anxiety were closely associated with reduced hippocampal volumes (Biala and Kruk, 2009a; Li et al., 2003b; Saylam et al., 2006). Postmortem analyses showed a decrease in the neuronal cells in the hippocampus of patients with major depression (Stockmeier et al., 2004). Therefore, it is generally believed that neuronal atrophy and destruction in hippocampus play a causal role in the development and progress of depression and anxiety (Fuchs et al., 2004; Manji and Duman, 2001). These interrelated results establish a link between hippocampal damages and the subsequent formation of depressive symptoms (Sapolsky, 2001). While, an unpredictable chronic mild stress (CUS) model of mice is a widely established rodent model of depression, which employs various stressors to mimic the anxiety and depressive behaviors of depression in humans (Kumar et al., 2011; Laugeray et al., 2011; Shang et al., 2017).

To date, the mechanism of neuronal degeneration in depression still remains to be uncovered. Several lines of evidence support the involvement of the hyperactivation of hypothalamic–pituitary–adrenal (HPA) axis in response to stress, with the latter being characterized by elevated levels of circulating corticosterone (Sapolsky, 2001). The HPA axis response plays a crucial role in the development of depression and anxiety (Holsboer and De Kloet, 2007). The HPA axis is a neuroendocrine system that integrates neural, endocrine, and immune factors to control the neuroendocrine response to stress, in order to maintain homeostasis (Holsboer, 2000).

The HPA axis comprises three major components: the hypothalamus, the pituitary gland, and the adrenal gland. The hypothalamus, through the secretion of corticotropin-releasing hormone (CRH) and neuropeptide Y (NPY), stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH then stimulates the adrenal gland to release cortisol (Kopin, 2000). Cortisol plays a central role in regulating the neuroendocrine response to stress. However, cortisol has been shown to have negative feedback on the HPA axis, which may lead to the hyperactivity of the HPA axis in depression (Sapolsky, 2001). The hyperactivity of the HPA axis may be associated with the pathogenesis of depression and anxiety (Holsboer, 2000). The HPA axis response plays a crucial role in the development of depression and anxiety (Holsboer and De Kloet, 2007). The HPA axis is a neuroendocrine system that integrates neural, endocrine, and immune factors to control the neuroendocrine response to stress, in order to maintain homeostasis (Holsboer, 2000).

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To date, the mechanism of neuronal degeneration in depression still remains to be uncovered. Several lines of evidence support the involvement of the hyperactivation of hypothalamic–pituitary–adrenal (HPA) axis in response to stress, with the latter being characterized by elevated levels of circulating
glucocorticoids in blood (Aihara et al., 2007; Murray et al., 2008). The HPA axis and its end-effectors glucocorticoid hormones are a vital neuroendocrine signaling system in the regulation of physiological response to stress (Du and Pang, 2015). Glucocorticoids are steroid hormones produced by the adrenal cortex and important for the regulation of development, metabolism and immune function (Zhou et al., 2009). Under normal conditions, the HPA axis secretes large amounts of glucocorticoids when exposed to stressful situation or depression occurs, and then resets its activity to normal tone by HPA feedback regulation (Chrousos, 2009). Dysregulation of the HPA-axis caused by traumatic or chronic stress usually results in the development of behavioral deficits and/or mental disorders (Kino, 2015). Exposure to environmental and work-related stress has been viewed as the main cause of this change, since physiological adaptation fails to maintain the response to the negative effects of the stress or and sustained homeostasis during the repeated bouts of severe aggression (Hammen et al., 2009; Holsen et al., 2011; Yoon et al., 2012). The persistently high concentration of blood glucocorticoids causes the dysfunction of HPA axis, exacerbates the lesion in the nervous system (Hortnagl et al., 1993), and damages the normal hippocampal neurons both in vitro and in vivo (Li et al., 2007; Zhu et al., 2006), and even aggravates the depression (Murphy et al., 2006; Sapolsky, 2000), which can be reversed by antidepressants (Chen et al., 2006; Hellsten et al., 2002; Lee and Gustafsson, 2009).

Actually, there has been a close relationship between the elevated circulating glucocorticoids, induced by long-term excessive stress, and the impairment of neurons in the dentate gyrus (DG) of the hippocampus (Sapolsky, 1985, 2001). Therefore, treatments that can inhibit corticosterone-induced neuronal damage may provide potential protection against depression.

The rat pheochromocytoma (PC12) cells, which possesses typical neuronal features and expresses a high level of glucocorticoid receptors, has been widely used in a variety of studies (Morsink et al., 2006; Westerink and Ewing, 2008). In the recent years, the use of exogenously co-incubated corticosterone with PC12 cells was extensively used as an in vitro model to induce cellular damage (Li et al., 2003a; Mao et al., 2012; Mao et al., 2011) and for studying the depression-like syndrome during the chronic stress (Li et al., 2013; Zhang et al., 2010). Interestingly, many types of antidepressants have been demonstrated to have protective function against cytotoxicity induced by corticosterone in PC12 cells (Li et al., 2003a, 2003b), suggesting that antidepressants may act by mitigating the corticosterone-induced neurotoxicity. Nicotine is the main psychoactive component of tobacco (Berrendero et al., 2012), which has wide-ranging effects on the performance of behavioral tasks by animals and humans, and many impacts on the psychiatric disorders (Markou et al., 1998). It has been suggested that nicotine participates in modulating mood states and anxiety (Biala and Kruk, 2009a; Picciotto et al., 2015). Indeed, some reports showed the neuroprotective actions of nicotine against various stressors leading to neuronal death (Picciotto and Zoli, 2002, 2008). Nicotine in low dosage (10 μM) rescued the decreased rates of PC12 cell viability and inhibited the production of lipid peroxidation resulted from H2O2 and Aβ in the cultured cells (Guan et al., 2003).

The autophagy signaling plays irreplaceable role in promoting the survival of stressed or starving cells by eliminating damaged macromolecules, organelles and proteins (Glück et al., 2010). It is well known that LC3 I is conjugated with phosphatidylethanolamine to form LC3 II during the process of autophagy, and autophagosomes are formed in the process. LC3 II, an active membranous protein, is localized to both the inside and the outside of autophagosomes (Mizushima et al., 2001). Beclin-1 is also an important protein during the autophagy process. The autophagy level can be up-regulated by the over-expression of Beclin-1 (Kang et al., 2011). Recently, many antidepressants were found to be involved in regulating the marker LC3II and p62/beclin-1 ratio of the neuronal autophagy signaling (Chen et al., 2012; Kara et al., 2013; Zschokke et al., 2011). In order to determine the role of autophagy, both protein level and mRNA level were measured in the present study. Furthermore, neuronal autophagy signaling was reported to regulate nicotine-mediated neuroprotection in a mouse model of Parkinson’s disease (Takeuchi et al., 2011). More importantly, a previous study showed that nicotine generated neuroprotection against Aβ-induced neurotoxicity in SH-SY5Y cell and hippocampal neurons through influencing the conversion of LC3 I to LC3 II (Hung et al., 2009). Nevertheless, there are few reports whether nicotinic amelioration of the anxiety-like behavior is concerned with the neuronal autophagy.

As is well known, the PI3K/AKT/mTOR signaling pathway plays an important role in autophagy modulation, synaptic development and function in brain (Heras-Sandoval et al., 2014). Furthermore, the PI3K/AKT/mTOR-dependent signaling pathway modulates the protein translation efficiency necessary for establishing long-term synaptic plasticity (Kelleher et al., 2004). It was reported that creatine, similar to ketamine, counteracted depressive-like behavior induced by corticosterone via PI3K/AKT/mTOR pathway (Pazini et al., 2016). Meaningfully, nicotine participation in activating the PI3K/AKT/mTOR pathway (Clark et al., 2010; Yuge et al., 2015). It was necessary to point out that the autophagy regulated Notch degradation and modulated stem cell development and neurogenesis (Wu et al., 2016).

Accordingly, the present study was aimed to investigate the underlying mechanism of chronic nicotine mood-enhancing effect on the emotional behavior change, induced by CUS in mice. It was upon one of our previous studies that nicotine cognition improvement was possibly associated with activating Notch signaling. We hypothesized that chronic nicotine ameliorated anxiety-like symptom via increasing the expression of Beclin-1 and LC3 proteins in the CUS group. This was done by establishing a CUS mouse model, conducting the open field test (OFT) and the elevated plus-maze (EPM) test. Moreover, PC12 cells exposed to high concentrations of corticosterone to verify if the autophagy signaling participated in the neuroprotective action of nicotine. The protein and mRNA expressions of Beclin-1, LC3 II and p62/SQSTM1 in both the hippocampus and PC12 cells were assayed by Western blotting and qPCR to explore the possible mechanisms. Finally, the phosphorylation of components in PI3K/AKT/mTOR pathway was checked to explore the possible mechanisms of chronic nicotine effect on corticosterone-induced impairment.

2. Materials and methods

2.1. Animals and drug treatments

32 male C57BL/6 mice (15–20 g body weight) were purchased from the Laboratory Animal Center of Academy of Military Medical Science of People’s Liberation Army, and reared in standard rodent cages in the animal house of Medical School, Nankai University, under the condition of a constant temperature of 25 °C (±2 °C) and a 12 h light/dark cycle (lights on at 7 a.m.). The mice took food and water freely during all phases of the experiment, with the exception of model establishing. After 4 days of habituation to the environment, the mice were randomly divided into four groups, which were control group (CON, n = 8), chronic mild unpredictable stressed group (CUS, n = 8), stressed + chronic nicotine group (CUS + Nic, n = 8) and chronic nicotine administration group (Nic, n = 8). The mice in the CUS and CUS + Nic groups were housed separately with each individual in one cage, while the animals in...
the CON and Nic groups were housed with three individuals in one cage. All efforts have been made to minimize the number of animals used and their suffering. Experiments in the present study were carried out according to protocols approved by the Ethical Commission at Nankai University and abiding by the practices outlined in the NIH Guide for the Care and Use of Laboratory Animals.

(−)-Nicotine ditartrate was bought from Merck Millipore, Germany. In the CUS + Nic and Nic groups, the mice were administrated nicotine daily by intraperitoneal injection at a dose of 0.2 mg/kg (free-weight) from the 1st to 28th day (Matta et al., 2007; Suemaru et al., 2006). At the same time, animals in the both CON and CUS groups received vehicles by intraperitoneal injection at the same dose.

2.2. Chronic mild unpredictable stress procedure

The stress regimen was conducted for 4 weeks, according to the minor modification method of other researchers (Peng et al., 2012; Williner, 2005). The types of stressors include reversed ice water swimming (4°C, 1 min), confinement in tube (2 h), light/dark cycle (24 h), cage tilt (45°, 12 h), oven (45°C, 5 min), dam bed (150 ml water + 200 g bedding, 24 h), tail pinch (1 min), white noise (85 dB, 5 min), water and food deprivation (24 h). They were applied on purpose, at random times of both night and day, in order to be completely unpredictable (Shang et al., 2017). All mice in both the CUS and CUS + Nic groups were exposed to the same single stressor simultaneously in one day. No single stressor was applied for two days consecutively.

2.3. Body weight measurement and sucrose preference test

The body weight of each mouse in all groups was measured every day at 10 a.m. and recorded on the 4th, 7th, 10th, 13th, 16th, 19th, 22nd, 25th, 28th and 31st day. The sucrose preference test (SPT) was performed as previously described (Dhingra and Bhananker, 2014). Animals were habituated to drink 1% sucrose solution for 48 h before SPT. Afterwards, food and water were deprived for 24 h. On the test day, each animal was provided with 1% sucrose solution and water for 1 h. The sucrose preference value was calculated as sucrose solution consumption (g)/[ sucrose solution consumption (g) + water intake (g)] × 100%.

2.4. Behavioral tests

2.4.1. Forced swim test

Mice were separately forced to swim in an open cylinder container (30 cm in diameter, 24 cm in deep, contained 12-cm water at 25 ± 1°C). Each mouse was placed into water gently and the total duration of immobility was recorded during the last 4 min of a 6-min period. During the test, an animal was considered immobile when it stopped moving and floated motionless in the water. A mouse makes only the brief movements to keep its head above water (Porsolt et al., 1977b).

2.4.2. Tail suspension test

Using adhesive tape, mice were suspended on the edge of a rod above the table top, placed approximately 1 cm from the tip of the tail. Immobility time was manually recorded during the test. Mice were judged to be immobile when they were completely motionless and hung down passively (Haj-Mirzaian et al., 2015).

2.4.3. Open field test (OFT)

The method of OFT was modified on the base of our previous study (Shang et al., 2017). The test was conducted in a bare square box (48 cm long × 48 cm wide × 36 cm high) made of compressed wood and carried out 12 h after the last nicotine treatment avoiding the immediate effect of stressor and pharmacological treatment (Fig. 1). The floor was partitioned into 16 equal squares by white-colored grids, including around area (12 around squares) and central area (4 middle squares). Mice were placed in a particular middle square of the center area and tracked by a CCD camera connected to a personal computer, through which data were collected and analyzed (Ethovision 2.0, Noldus, Wageningen, Netherlands). Mice were allowed to explore the maze for 5 min, after which they were returned to their home age. The maze was cleaned with 75% ethanol wipes before starting testing with the next mouse. Around area distance was analyzed as measurement of anxiety emotion.

2.4.4. Elevated plus-maze (EPM) test

EPM was performed in the dark room of the light cycle. The elevated plus-maze consists of two open and two closed arms (16 × 5 cm). Closed arms has 12 cm high walls at the sides and end. The maze made of plywood and elevated to a height of 25 cm above the floor. Each mouse was placed in the central square (5 × 5 cm) facing an open arm and allowed to explore the maze freely for 5 min (Sharma and Kulkarni, 1992). During the test, animals were tracked by a CCD camera connected to a personal computer, through which data were collected and analyzed (Ethovision 2.0, Noldus, Wageningen, Netherlands). Entry into one arm was measured as all four paws of a mouse crossed from the central region into an arm, and the number and time of total open arm entries was recorded. The percentage of open arm entries and time spent in the open arms were expressed as a ratio to the total number entries in any arms and all time spent on any arms.

2.5. Hematoxylin/eosin staining

The brains isolated from the sacrificed mice were perfused with 0.1 mol/l phosphate buffer (pH = 7.4) immediately. Then the brains were removed and immersed in 4% paraformaldehyde and fixed at 4°C for at least 24 h. After that, they were dehydrated through a graded sucrose solution and embedded in OCT compound (Tissue-Tek, Miles) for tissue sectioning. The coronal slices (5 μm) were stained with hematoxylin/eosin (HE) and photographed on a Leica microscope (Wetzlar, Germany). The density of DG cells was measured. The number of cells per mm² area, which was counted in three randomly chosen fields from one slide, was employed to represent the density of DG cells. There are five mice for each group and with five slides of one mouse.

2.6. PC12 cell culture

The cells were routinely thawed and grown in RPMI-1640 supplemented with 10% FBS, 1% penicillin, and 1% streptomycin under a humidified atmosphere containing 5% CO2 and 95% air at 37°C. The medium was changed three times a week. PC12 cells were seeded in 6-well plates coated with poly-D-lysine/laminin. It was replaced with RPMI-1640 medium containing 0.5% FBS, 1% penicillin, and 1% streptomycin with NGF (nerve growth factor, 2.5 ng/ml) 24 h after plating (Nishimura et al., 2008). The second generation of differentiated cells was employed in next step.

2.7. Cell viability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) reduction assay was used to assess the cell viability. Briefly, 1 × 10⁵ cells were seeded into each well in 96-well plates then cultured 24 h for the stabilization. And then, the medium containing different drugs was added in each well. Cells were
incubated for a certain time. Subsequently, MTT (20 μL, 5 mg/ml) was added to each culture well. Following cultured at 37 °C for 4 h, the medium was removed carefully and 150 μL DMSO was added in each well to dissolve the formazan product. Finally, formazan absorbance was assessed by using a microplate reader (Multiskan MK3; Thermo Labsystems, Helsinki, Finland) with a wave length of 492 nm. Each experiment was repeated thrice.

2.8. Hoechst33258 staining assay for cell apoptosis

Hoechst33258 staining assay was used to detect PC12 cells apoptosis. The cells with the condensation of nuclear chromatin and fragmentation were scored as apoptotic. They were plated in 6-well plates. After 24 h drug treatment, the cells were stained with nuclear dye Hoechst 33258 as described previously (Tang et al., 2005). Briefly, cells were fixed with 4% paraformaldehyde in PBS for 10 min and incubated with 5 mg/L of Hoechst 33258 for 15 min at room temperature. Cells were visualized and photographed under ultraviolet illumination using a fluorescence microscope (BX50-FLA, Olympus, Tokyo, Japan).

2.9. Western blotting assay

The method of Western blotting assay was modified on the base of a previous study (Wang et al., 2016). Each hippocampus was dissected from the brain after behavioral tests and immediately stored at −80 °C until needed. Firstly, it was mashed with a grinder and 200 μL lysis buffer (Beyotime Biotechnology, Haimen, China) containing Phenylmethanesulfonyl fluoride (PMSF, 1:100 dilutions). PC12 cells were treated by various compounds, which were divided into several groups, such as CON group, CORT group, CORT + Nic group and CORT + Nic+3-MA group. After incubation, they were harvested and lysed in lysis buffer (50 mM Tris-HCl [pH = 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], and 0.5% deoxycholic acid sodium salt [DOC]) at 0 °C. The hippocampus/PC12 cells lysates were centrifuged at 12000 r/min for 10 min with Tris-buffered saline/Tween 20 (TBST) and incubated for a certain time. Subsequently, MTT (20 μL, 5 mg/ml) was added to each culture well. Following cultured at 37 °C for 4 h, the medium was removed carefully and 150 μL DMSO was added in each well to dissolve the formazan product. Finally, formazan absorbance was assessed by using a microplate reader (Multiskan MK3; Thermo Labsystems, Helsinki, Finland) with a wave length of 492 nm. Each experiment was repeated thrice.

2.10. mRNA extraction and real-time quantitative PCR (qPCR)

Total mRNA isolation and qPCR for detecting Beclin-1 and p62/SQSTM1 expressions were adopted and modified on the basis of previous studies (Li et al., 2016). Briefly, total RNA was extracted using TRIZOL reagent (RNAiso Plus Reagent, TaKaRa, Japan) according to manufacturer’s instructions. The cDNA was synthesized by PrimeScript RT reagent Kit (TaKaRa, Japan). Primers for target genes along with 150 ng of cDNA products were used to perform SYBR green qPCR. The oligonucleotide primers for Beclin-1, p62/SQSTM1 and GAPDH were as follows: Beclin-1 (accession: NM_053739.2), forward- TTGGCCAATAAGATGGTGCTGA; reverse-TTGCCCAAATAAGATGGTGCTGA; p62/SQSTM1 (accession: NM_175843.4), forward- CTGAAAATGATGGTTGCTGG, reverse- CAGGAAATTGCATGTGATCCA; GAPDH (accession: NM_017008.4), forward- GGCAACAGTCAAGGCTGAGAATG, reverse- ATGGTGTGTTGAA-GACGCCGTA. GAPDH was used as an internal standard. All reactions were done in triplicate using Mastercycler ep realplex (Eppendorf AG, Hamburg, Germany) and mRNA levels were calculated using the comparative CT method (2−ΔΔCT).

2.11. Immunofluorescence

The cells were treated with different drugs in 6-well plates for 24 h, which were named as CON, CORT, CORT + Nic and CORT + Nic+3-MA groups respectively. Following 30 min fixation in 4% paraformaldehyde, they were washed with PBS, and then permeabilized with 0.5% Triton X-100 and blocked with 10% NGS in 4% paraformaldehyde, they were washed with PBS, and then permeabilized with 0.5% Triton X-100 and blocked with 10% NGS for 2 h at room temperature. Subsequently, the cells were incubated with LC3 primary antibody (１:1 000). After washing with PBS, they were incubated with the Alexa 488-conjugated goat anti-mouse IgG secondary antibody (１:1 000). Thereafter, the cell nuclei were stained by DAPI. Samples were examined under a fluorescence microscope (Olympus FX1000, Japan).

2.12. Data presentation and statistics

All data were presented as mean ± S.E.M. Differences in cell viability and behavioral data including force swim test, tail suspension test, open field and elevated plus-maze between the groups were measured by the two-way ANOVA to detected the interaction effect. If the interaction effect was statistically significant, the significant differences between the groups would be
detected. This was performed by ANOVAs supporting by least significant difference (LSD) or Tamhane post hoc tests. Based on if Levene's test of homogeneity of variance was significant, different post hoc test was used. Western blotting and qPCR were assessed by a one-way ANOVA for multiple comparisons with stress or chronic nicotine. All analyses were performed using SPSS (20.0) software and differences were considered significant when \( p < .05 \).

3. Results

The hypothesis was going to be verified by following evidences, which were obtained from behavioral tests, histopathological observation, fluorescence staining and molecular measurements.

3.1. Effects of chronic nicotine administration on the body weight and sucrose test in stressed mice

As shown in Fig. 2a, the body weight in all groups was increased during the four weeks. There was a significant CUS × nicotine interaction \( (F(1, 28) = 8.224, p < .01) \) on the body weight. Post-hoc tests showed that the mice weight was statistically lower in the CUS group compared to that in the CON group \( (p < .05) \) on the 22nd day and the 31st day; \( p < .01 \) from the 13th-19th day; \( p < .001 \) from the 7th-10th day and the 25th-28th day). Furthermore, the body weight was significantly enhanced in the CUS + Nic group compared to that in the CUS group \( (p < .05) \) from the 10th-13th day and the 28th day; \( p < .001 \) on the 7th day).

Meanwhile, Fig. 2b showed that there was a significant CUS × nicotine interaction \( (F(1, 28) = 8.027, p < .001) \) on the sucrose consumption percentage. Post-hoc tests showed that the sucrose consumption was considerably lower in the CUS group than that in the CON group \( (p < .01) \) on the 17th day; \( p < .001 \) on the 24th day and the 33rd day). Additionally, it was significantly increased by nicotine in the CUS + Nic group compared to that in the CUS group \( (p < .05) \) on the 17th day; \( p < .01 \) on the 24th day; \( p < .001 \) on the 33rd day). There was no statistical difference of the sucrose consumption percentage between the CON and the Nic groups \( (p > .05) \).

3.2. Effects of chronic nicotine administration on the force swim test (FST) and tail suspension test (TST) in stressed mice

In order to measure the depressive-like behavior in both the CUS group and the CUS + Nic group, the experiments of FST and TST were performed. Fig. 2c showed the immobility time in the FST in all groups. There was a significant CUS × nicotine interaction \( (F(1, 28) = 4.640, p < .05) \) on the immobility time in the FST. Post-hoc tests revealed that the immobility time was longer in the CUS group than that in the CON group \( (p < .05) \). Furthermore, it was significantly shortened by chronic nicotine administration in the CUS + Nic group compared to that in the CUS group \( (p < .05) \). However, there was no statistical difference of immobility time between the CON and the CUS + Nic groups \( (p > .05) \).

Meanwhile, Fig. 2d showed the immobility time in the TST. There was a significant CUS × nicotine interaction \( (F(1, 28) = 4.351, p < .05) \) on the immobility time in the TST. Post-hoc tests showed that the immobility time was longer in the CUS group than that in the CON group \( (p < .01) \). Furthermore, it was significantly shortened by nicotine in the CUS + Nic group compared to that in the CUS group \( (p < .01) \). There was also no statistical difference of immobility time between the CON and the CUS + Nic groups \( (p > .05) \).

3.3. Effects of chronic nicotine administration on the open field test (OFT) and the elevated plus-maze (EPM) in stressed mice

The status of anxiety in mice was evaluated by OFT and EPM tests. There was a significant CUS × nicotine interaction \( (F(1, 28) = 4.900, p < .05) \) on the percentage of time spent in central areas. Post hoc test showed that a significant decrease of the percentage of time spent in central areas in the CUS group compared to that in the CON group \( (p < .01, \text{Fig. 2e}) \). The time spent in central area was longer in the CUS + Nic group than that in the CUS group \( (p < .05) \). There was no statistical difference of the percentage of time spent in central area between the CON and the CUS + Nic groups \( (p > .05) \).

For the EPM test (Fig. 2f), there were much less open arms entries in the CUS group compared to that in other three ones. There was a significant CUS × nicotine interaction \( (F(1, 28) = 11.383, p < .01) \) on the open arms entries. One-way ANOVA revealed the effect of the chronic nicotine treatment \( (F(3, 28) = 4.481, p < .05) \) on the percentage of open arms entries. The LSD post hoc comparisons showed that there was a significant difference of the open arms entries in the CUS group compared to that in the CON group \( (p < .01, \text{Fig. 2f}) \). Furthermore, it was also significantly increased by chronic nicotine in the CUS + Nic group compared to that in the CUS group \( (p < .01, \text{Fig. 2f}) \). However, Post hoc test showed that there was no statistical difference of the open arms entries between the CON group and the Nic group \( (p > .05, \text{Fig. 2f}) \).

3.4. Effects of chronic nicotine administration on the autophagy level in stressed mice

Two types of proteins that were related to the autophagy signaling, bands at about 60 kDa and 16 kDa were detected by Beclin-1 and LC3 antibodies, respectively (Fig. 3a). One way ANOVA showed that the protein levels of Beclin-1 and LC3 II were significantly different among groups in the hippocampus (Beclin-1: \( F(3, 8) = 127.084, p < .001, \text{Fig. 3b}; \) LC3: \( F(3, 8) = 12.700, p < .01, \text{Fig. 3c} \)). The LSD post hoc comparisons showed that the expression of Beclin-1 was statistically increased in the CUS group compared to that in the CON group \( (p < .001, \text{Fig. 3b}) \). Similarly, the expression of LC3 II was also statistically increased in the CUS group compared to that in the CON group \( (p < .05, \text{Fig. 3b}) \). However, it was found that the Beclin-1 and LC3 II were significantly enhanced by chronic nicotine (Beclin-1: \( p < .01, \text{between the CUS + Nic group and the CUS group, Fig. 3b}; \) LC3 II: \( p < .05, \text{between the CUS + Nic group and the CUS group, Fig. 3c} \)). Nevertheless, the protein levels of Beclin-1 and LC3 II were not changed in the Nic group compared to that in the CON group \( (p > .05, \text{Fig. 3b and c}) \).

3.5. Effects of chronic nicotine administration on the morphology of DG neuron in stressed mice

Histological analysis was used to evaluate the neuropathological alterations of DG neurons in each group. As seen in Fig. 3d and e, there was a significant effect of the chronic nicotine treatment on the density of cells in the DG region \( (F(3, 16) = 4.295, p < .05, \text{Fig. 3e}) \). Post hoc test showed that the density of cells was lower in the CUS group than that in the CON group \( (p < .01) \). Meanwhile, the density of cells was higher in the CUS + Nic group than that in the CUS group \( (p < .01) \). Whereas, there was no distinct change in the morphology and structure of the pyramidal cells in the Nic group compared to that in the CON group.
3.6. Corticosterone significantly reduced the viability of PC12 cells while nicotine reversed the effect of corticosterone by enhancing autophagy signaling

As shown in Fig. 4a, corticosterone induced cell death in a corticosterone-dependent manner in PC12 cells. It was found that the cell death existed at all concentrations of corticosterone with approximately 60–70% of the cells left viable at 400 μM. Accordingly, the concentration of 400 μM was selected for the following study. Fig. 4b showed that nicotine significantly improved the cell viability in corticosterone treated cells at different concentrations (F(5, 24) = 50.492, p < .001).
To further validate the protective effect of nicotine and the role of the autophagy signaling pathway in nicotine-mediated protection, PC12 cells were administrated with autophagy inhibitor 3-MA (5 mM), with cells exposed to 400 μM of corticosterone in the presence or absence of 10 μM of nicotine for 24 h. And then, the cell viability was determined. Fig. 4c showed that there were a significant corticosterone/C2/nicotine interaction \( F(1, 16) = 27.233, p < .001 \) and a significant nicotine/C2/3-MA interaction \( F(1, 16) = 6.603, p < .05 \) on the cell viability. These results further suggest the role of autophagy in the protective effect of nicotine.

3.7. Nicotine inhibited the apoptosis induced by corticosterone in PC12 cells

To further investigate the protective effects of nicotine, Hoechst 33458 staining was performed to examine morphological changes in PC12 cells. The fluorescence images revealed oval-shaped nuclei with homogeneous fluorescence intensity in the normal cells (Fig. 4d), while heterogeneous intensities and chromatin condensation were observed in the nuclei of the corticosterone-injured PC12 cells (Fig. 4d). The nuclei of the apoptotic cells, i.e., the Hoechst-positive cells, was indicated by arrowheads in the images. The results showed that nicotine evidently alleviated the morphological changes induced by corticosterone in PC12 cells. The number of apoptotic cells was significantly decreased after treatment with 10 μM nicotine (CORT + Nic group). However, the improvement of nicotine was blocked by 3-MA to some extent (CORT + Nic + 3-MA group).

3.8. Effects of nicotine on Beclin-1 and LC3 II expressions in PC12 cells

Two types of proteins that were related to the autophagy signaling, bands at about 60 kDa and 18 kDa were detected by Beclin-1 and LC3 antibodies, respectively (Fig. 5a). One way ANOVA showed that the protein levels of Beclin-1 and LC3 II were significantly different among groups in PC12 cell (Beclin-1: \( F(3, 8) = 308.730, p < .001 \), Fig. 5b; LC3 II: \( F(3, 8) = 24.285, p < .001 \), Fig. 5c). Tamhane post-hoc analysis showed that the expression of Beclin-1 was statistically increased in the CORT group compared to that in the CON group (\( p < .01 \), Fig. 5b). Similarly, the expression of LC3 II was also increased (\( p < .01 \), Fig. 5c). Interestingly, it was found that the expression of LC3 II was significantly elevated by nicotine in the CORT + Nic group compared to that in the CORT group (LC3 II: \( p < .01 \), Fig. 5c). Furthermore, 3-MA significantly suppressed the Beclin-1 and LC3 II levels, and elevated by nicotine treatment (Beclin-1: \( p < .05 \), Fig. 5b; LC3 II: \( p < .01 \), Fig. 5c).

3.9. Morphological evaluation of autophagy

Fig. 5d shows the morphological analysis of autophagy with different compounds treatments for 24 h. Staining with anti-LC3 is
Fig. 4. Corticosterone concentration-dependently induced cell death and apoptosis in PC12 cells.

a: PC12 cells were treated with different concentrations of corticosterone for 24 h, and the viability of cells was determined by MTT assay;
b: PC12 cells were pretreated with various concentrations of nicotine for 24 h, which were named as nicotine-treated group (0.1 μM), nicotine-treated group (1 μM), nicotine-treated group (10 μM), and nicotine-treated group (100 μM), and then 400 μM corticosterone was added into the medium; 24 h later, cells viabilities were measured by MTT assay;
c: PC12 cells were pretreated with various drugs, such as CORT (400 μM), CORT + Nic (10 μM) and CORT + Nic + 3-MA (5 mM) for 24 h, which were named as CON group, CORT group, CORT + Nic group and CORT + Nic + 3-MA group, and the cells viabilities were measured by MTT assay.
d: Protective effects of nicotine on apoptosis of PC12 cells induced by corticosterone. Cells pretreated with or without nicotine were incubated with corticosterone. Apoptosis of cells were measured by staining with Hoechst33258. The nuclear pyknosis and aggregates could be observed (arrow). Scale bar 20 μm.

Data are expressed as a percentage of the corresponding CON value. Results are shown as the mean ± SEM. ***p < .001 versus control, n = 3; **p < .01 and ### p < .001 versus CORT (400 μM); ***p < .001 comparison between the CON group and the CORT group, ### p < .001 comparison between the CORT group and the CORT + Nic group, %p < .05 and ### p < .001 comparison between the CORT + Nic group and the CORT + Nic + 3-MA group. n = 3 in each group.

Fig. 5. Nicotine significantly enhances the expressions of Beclin-1 and LC3 II in the PC12 cells.

Nicotine and 3-MA altered the expression of Beclin-1 and the ratio of LC3 II in corticosterone-treated PC12 cells. Results are immunoblots from single representative experiments (a). The expression values of Beclin-1 (b) and LC3 II (c) were normalized with β-actin value, and then compared to CON. The representative microphotographs of PC12 cells in the CON group, CORT group, CORT + Nic group and CORT + Nic + 3-MA group by immunofluorescence (d). Data are presented as mean ± SEM. **p < .01 comparison between the CON group and the CORT group, ***p < .01 comparison between the CORT group and the CORT + Nic group, "p < .05 and ""p < .01 comparison between the CORT + Nic group and the CORT + Nic + 3-MA group. n = 3 in each group.
a recognized marker for autophagosomes. It was found that staining intensity and larger numbers of bright fluorescent particles in cells were visibly enhanced by corticosterone and nicotine treatment, suggesting that there was the presence of autophagosomes. The fluorescence intensity and the number of bright fluorescent particles were related to the extent of lysosome acidity, and used to predict autophagy level. Images, obtained from Anti-LC3 staining, showed an increase in autophagy process in both the corticosterone-treated cells (CORT group) and corticosterone plus nicotine-treated cells (CORT + Nic group, Fig. 5d). In these cells, a more intensive fluorescence was detected, and the number of stained lysosomes was much higher than that in the CON group. However, 3-MA significantly inhibited the effect of nicotine on enhancing the autophagy activity (CORT + Nic + 3-MA group).

3.10. Effects of nicotine on the expressions of Beclin-1 and p62/SQSTM1 mRNA in corticosterone-treated PC12 cells

The potential intracellular molecular mechanisms, which may subsequently result in cognitive impairment, have been further explored. As shown in Fig. 6, Beclin-1 and p62/SQSTM1 mRNA expressions in PC12 cells were detected by qRT-PCR assay. One-way ANOVA analysis showed that there was significant difference of Beclin-1 and p62/SQSTM1 mRNA expressions between these four groups (Beclin-1: F(3, 8) = 143.612, p < .001, Fig. 6a; p62/SQSTM1: F(3, 8) = 6.841, p < .05, Fig. 6b). The LSD post-hoc analysis further showed that Beclin-1 and p62 mRNA expressions were significantly increased in the CORT group compared with that in the CON group (Beclin-1: p < .001, Fig. 6a; p62/SQSTM1: p < .05, Fig. 6b). The effect of nicotine on Beclin-1 and p62/SQSTM1 mRNA expressions was evaluated ulteriorly. On the one hand, the LSD post-hoc analysis showed that Beclin-1 expression was significantly increased in the CORT + Nic group compared to that in the CORT group (p < .001, Fig. 6a). On the other hand, it was found that p62/SQSTM1 mRNA expression was not statistically changed in the CORT + Nic group compared to that in the CORT group (p > .05, Fig. 6b). It was further found that Beclin-1 and p62/SQSTM1 mRNA expressions were significantly inhibited in the CORT + Nic + 3-MA group compared to that in CORT + Nic group (Beclin-1: p < .001, Fig. 6a; p62/SQSTM1: p < .05, Fig. 6b).

4. Discussion

The present study verifies in vitro and in vivo evidences that there is a neuroprotective effect of chronic nicotine on a mouse model of CUS and a corticosterone-induced PC12 cells depression model. Behavioral deficits and neuropathological alterations of DG neurons in the CUS mouse model were effectively rescued by chronic nicotine treatment. Furthermore, the cytotoxicity induced by corticosterone in PC12 cells was also significantly improved by using nicotine.

Firstly, it is well known that the CUS animal model can simulate depressive-like symptoms with the decrease of both body weight and sucrose preference. Hence, the body weight and the SPT were employed to verify the validity of CUS as an animal model to investigate depression. It was found that there were significant differences of both body weight and SPT between the CUS group and the CON group. These results were in accordance with the previous findings (Andreasen and Redrobe, 2009; Yu et al., 2016). Thus, our data showed that CUS model simulated the depressive-like behavior in mice. Secondly, the FST and TST are commonly used as an approach for detecting antidepressant effect of drugs (Cryan et al., 2005; Porsolt et al., 1977a). The immobility time in the FST or TST has been considered as an index of despair, which is the core symptom of the depressive disorders (Lino-De-Oliveira et al., 2005).
CORT (Gassen et al., 2015; Ma et al., 2013). Several studies have suggested that chronic nicotine treatment could alleviate the animal’s depressive-like behavior effectively. A previous study showed that animals rapidly became tolerant to the stimulatory effects of nicotine on corticosterone secretion, therefore, the effect of nicotine was possibly related to its anti-depressant function in CUS model animals (Benwell and Balfour, 1979).

Our data showed that the immobility time in either the FST or the TST was statistically reduced by nicotine in the CUS + Nic group compared to that in the CUS group, suggesting that chronic nicotine treatment could alleviate the animal’s depressive-like behavior effectively. A previous study showed that animals rapidly became tolerant to the stimulatory effects of nicotine on corticosterone secretion, therefore, the effect of nicotine was possibly related to its anti-depressant function in CUS model animals (Benwell and Balfour, 1979). The data, obtained from the OFT experiment, showed that the time spent in central areas was shorter in the CUS + Nic group than that in the CUS group, suggesting that the anxiety symptoms were efficiently relieved by chronic nicotine. It was mostly similar to a standard antidepressant drug, fluoxetine. What’s more, mounts of studies have been further showed that CUS induced anxiety-like behavior in the EPM test. CUS-treated mice spent more time in the closed side of the EPM (Mineur et al., 2006). Chronic mild stress exposed mice demonstrated anxiety-like effects in the EPM (Gross and Pinhasov, 2016). 4-weeks CUS caused significant partial anxiety-like behavior in the EPM, while, etazolate produced partial anxiolytic-like behavior in the EPM (increased percentage of open arm entries) (Jindal et al., 2013). The data, obtained from our study, is in accordance with that of aforementioned reports, in which the percentage of open arms entries is decreased compared with CON group mice. However, it was significantly improved by chronic nicotine. This was supported by a previous study that acute nicotine hydrogen tartrate (0.25 mg/kg, i.p.) attenuated the 2-h restraint stress-induced anxiety-like behavior in the EPM (Hsu et al., 2007).

There are decreased thickness of granule cell layer, disturbed cell arrangement, increased intercellular space, obvious cell deformity and nucleus condensation in the hippocampal DG of CUS rats (Fan et al., 2011). Furthermore, previous investigations showed that hippocampus was sensitive to stress, as the main target of attack because of its high concentrations of glucocorticoids during stress (Haynes et al., 2001; Korte, 2001). The present study also demonstrated that the cells in the DG area of CUS mice became loose and disordered, and there were shortening and deformations in cell shape as well. Interestingly, nicotine blocked the adverse effect of CUS on the cell morphology in the DG region. This result was proofed by the report that acute intermittent nicotine treatment induced fibroblast growth factor-2 in the subventricular zone of the adult rat brain and enhanced neuronal precursor cell proliferation (Mudo et al., 2007).

Recent investigations evidenced that many antidepressants impacted autophagy pathways in the process of antidepressant (Gassen et al., 2015; Ma et al., 2013). Several studies have demonstrated that cellular autophagy markers, such as LC3 II and Beclin-1, are upregulated upon treatment with antidepressants, including amitriptyline and the selective serotonin re-uptake inhibitor citalopram (Gassen et al., 2014; Zschocke and Rein, 2011; Zschocke et al., 2011). Trehalose may have antidepressant-like properties through its enhancement of autophagy (Kara et al., 2013). Lithium, which has been used for several decades to treat manic-depressive illness, induces autophagy, thereby promoting the clearance of mutant huntingtin and alpha-synucleins from experimental systems (Heiseke et al., 2009).

Accordingly, the autophagy related proteins Beclin-1 and LC3 were measured to evaluate the levels of autophagy. As is well known, there is a positive correlation between the ratio of LC3 II to LC3 I and the number of autophagosomes. Therefore, detecting LC3 conversion (LC3 I-LC3 II) to measure the level of autophagy is a crucial approach (Mizushima and Yoshimori, 2007). The results of the Western blotting assay showed that the LC3 II was increased in the CUS group than that in the CON group. Beclin-1 is also an important protein during the autophagy process. The autophagy level can be up-regulated by the over-expression of Beclin-1 (Kang et al., 2011). In this study, the level of Beclin-1 expression was also elevated in CUS mice. These results combined with the increased LC3 II expression suggest that autophagic activity is elevated under CUS in the hippocampus of mice compared to that in the CON group. After the exposure of chronic nicotine, the autophagic level was enhanced in the CUS + Nic group than the CUS group. The results showed that CUS resulted in autophagic level rise, while, the nicotine could further strengthened it. However, there was not an increase in autophagic level in the Nic group.

Previous studies showed that high concentrations (250–800 μM) of corticosterone could induce cellular damage in PC12 cells (Jiang et al., 2015; Zou et al., 2016). The increase in corticosterone concentration induced by sleep deprivation paralleled the reduced conversion of LC3 I to LC3 II and increased levels of p62/SQSTM1, suggesting that corticosterone triggered these changes (Monico-Neto et al., 2015). Calorie restriction increased serum corticosterone expression in rodents, accompanied higher levels of Beclin-1 and LC3 mRNA and/or protein (Yang et al., 2016). A previous report showed that corticosterone markedly facilitates Ca2+ influx into the hippocampal neuron leading to neurotoxicity (Zhou et al., 2000). Our results showed that corticosterone decreased the viability of PC12 cell in a concentration-dependent manner. More importantly, the corticosterone increased the level of autophagy in PC12 cells. It was found that nicotine significantly increased the viability of corticosterone-treated cells by MTT assay, but 3-MA considerably inhibited it. Moreover, nicotine up-

![Fig. 7. Nicotine significantly enhances the ratios of p-Akt/Akt and p-mTOR/mTOR in PC12 cells.](image-url)
regulated the increase of autophagic level in corticosterone-induced PC12 cell, which also inhibited by 3-MA. These results were corroborated mutually with the in vivo results.

The major finding of the present study is that corticosterone caused apoptotic cell death, whereas nicotine prevented cell death by stimulating the activation of the autophagy signaling pathway in PC12 cells. This is based on the following observations: (1) Corticosterone induced cell death in a corticosterone-dependent manner in PC12 cells; (2) Nicotine protected against corticosterone induced cell death in a nicotine-dependent manner; (3) 3-MA blocked the protective effect of nicotine against corticosterone induced cell death. Stress or depression increases the level of corticosterone, which subsequently activates glucocorticoid receptors (Smith et al., 1995). Corticosterone treatment is a well-known injury model under in vitro conditions to study the mechanism of antidepressants. Moreover, repeated exposure to mild stressors in rats leads to increased circulating levels of corticosterone and accelerated cell death in the hippocampal formation (Axelson et al., 1993). Corticosterone injection has been shown to cause depressant behavior which can be reversed by administering antidepressant drugs (Bijak et al., 2001).

It has been suggested that increased cell death is due to chronic stimulation of glucocorticoid receptors, which are particularly abundant in the hippocampus (Zheng et al., 2011). Its mechanisms may be due to increased Ca\(^{2+}\) levels, excessive glutamate release, and inhibition of autophagy signaling induced by corticosterone (Konradi and Heckers, 2003). The current study validates the ability of corticosterone to induce cell death in cultured PC12 cells and evaluates the use of this as a cell death model in these cells. mTOR is a vital negative regulator of autophagy (Dudek et al., 1997), and the PI3K/Akt pathway is an upstream major modulator of mTOR (Manning and Cantley, 2007). The PI3K/Akt pathway is involved in survival and inhibition of apoptosis in different cells (Dudek et al., 1997). A previous study illustrated that chronic stress reduced phosphorylation of the mTOR signaling pathway components in the amygdala of rats, accompanied by decreased phosphorylation of ERK-1/2, Akt-1, and GluR1 (Chandran et al., 2013). Results from our study further certified that corticosterone stress decreased the phosphorylation of Akt and mTOR (Fig. 6), which in accordance with an issued paper (Pazini et al., 2016). Interestingly, nicotine rescued the abnormal levels of PI3K/Akt/mTOR (Fig. 6), which was able to be one of the under mechanisms in enhancing the autophagy activity in corticosterone-treated PC12 cells. This was also proofed partially by another study that nicotine induced mTOR pathway activation in head and neck squamous cell carcinoma (Clark et al., 2010).

5. Conclusion

In summary, our results show that chronic nicotine at a dose of 0.2 mg/kg is effective in impeding the anxiety and depressive-like behavior, as well as neuropathological alteration of DG neurons in a mouse model of CUS, presenting potential antidepressant-like properties. It is along with enhancing the level of autophagy signaling proteins besides activating Nurtch signaling components. In addition, corticosterone increased autophagy signaling in PC12 cells. Nicotine significantly reduced corticosterone-induced cell apoptosis in PC12 cells via enhancing the autophagy signaling, which was inhibited by 3-MA. In conclusion, the results suggest that chronic nicotine ameliorates the anxiety and depressive-like symptoms, associating with the enhancement of autophagy signaling components, such as the levels of Beclin-1, LC3 II and p62/SQSTM1, which is closely associated with the activation of the PI3K/Akt/mTOR signaling.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuint.2018.01.004.

Conflicts of interest

No competing interests declared.

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