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Original Article

Activation of glial α7 nAChR promotes neurogenesis by suppressing Aβ-mediated neuroinflammation in Alzheimer's disease

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Abstract: Here, we report the effects of glial cells α7 nicotinic acetylcholine receptor (α7-nAChR) activation on neuroinflammation and proliferation as well as on the differentiation of implanted neural stem cells (NSCs). It was observed that nicotine reduced the levels of inflammatory mediators in the glial cell and NSCs co-culture system, and the rate of NSCs apoptosis demonstrated a comparable decline. In addition, both the *in vitro* and the *in vivo* study showed that nicotine amplified the proliferation of NSCs, and the neuronal and cholinergic neuronal differentiation of NSCs increased significantly, supplementing the nerve cells of the central nervous system. The anti-inflammatory and nerve regeneration-promoting effect of nicotine was reversed upon treatment with α-bungarotoxin, an inhibitor of α7-nAChR. These results suggest that, activation of α7-nAChR on glial cells attenuates the effects of neuroinflammation on the proliferation and differentiation of NSCs by reducing the secretion of inflammatory factors. In conclusion, the promotion of neurogenesis and simultaneous reduction of neuroinflammation is a promising strategy for therapy in Alzheimer's disease.

Keywords: Glial cells, β amyloid, α7-nAChR, inflammation, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is characterized by a progressive impairment of cognitive function and atrophy of the cerebral cortex resulting from the loss of numerous cholinergic neurons [1]. Adult neural stem cells (NSCs) are ubiquitous in almost all regions of the brain, and undifferentiated and multipotent NSCs differentiate into functionally mature neurons and glial cells to maintain homeostasis of the central nervous system (CNS) when nerve cells are lost [2, 3]. However, numerous pro-inflammatory cytokines and chemokines secreted by overactivated microglial or astroglial cells induce the apoptosis of neurons and suppress the proliferation and differentiation of inherent NSCs, resulting in a loss of neurons and glial cells that cannot be replenished over time in patients with AD [4, 5].

Neuronal loss accelerates the pathological changes associated with AD and predicts a degree of cognitive impairment [6]. In previous decades, neural stem cell transplantation (NSCT) piqued the interest of many researchers due to the supply of nerve cells [7, 8]. Unfortunately, NSCT never achieved success because the differentiation and proliferation of NSCs was suppressed in an inflammatory microenvironment [9, 10]. It has been shown that TNF-α inhibits neuronal differentiation and induces neuronal cell death in neural progenitor cell culture, and IL-6 secreted by astrocytes decreases hippocampal dentate gyrus neurogenesis in transgenic mice [11, 12]. Therefore, strategies to unravel the chronic inflammatory response are becoming a primary prerequisite to promote the survival of implanted NSCs.

Wang et al. found that macrophage α7-nAChR activation reduced the synthesis and secretion

of inflammatory mediators [13], as macrophage-like cells of the CNS, microglia and astrocytes express α7-nAChR [6, 14]. In a previous study, we showed that activation of astrocyte α7-nAChR reduced β amyloid (Aβ)-mediated inflammation *in vitro* [5]. However, the concrete effect of glial cell α7-nAChR activation on neuro-inflammation is not yet completely understood *in vivo*, and the role of activated α7-nAChR on the proliferation and differentiation of NSCs also remains unresolved.

Here, microglia and astrocytes, the main immune cells in the CNS, were treated with Aβ and co-cultured with NSCs to imitate the inflammatory microenvironment of the AD brain. Nicotine was used to activate glial cell α7-nAChR to explore its role in neuro-inflammation. BrdU-labeled NSCs were implanted into the hippocampi in a model of inflamed AD brain, by which the proliferation and differentiation of implanted NSCs could be measured clearly. This report provides evidence to support the notion that, glial cell α7-nAChR activation reduces inflammatory mediator secretion, and the proliferation and differentiation of implanted NSCs are improved in the presence of reduced neuro-inflammation.

Materials and methods

Materials and antibodies

Nicotine and α-bungarotoxin were obtained from Sigma-Aldrich (St. Louis, MO, USA), and $A\beta_{1-42}$ was obtained from Usbiological (Swampscott, MA, USA). Mouse monoclonal MAP-2, CHAT, and nestin antibodies, rat monoclonal anti-BrdU antibody and FITC and TRITC-labeled secondary antibodies were purchased from Abcam (Cambridge, MA, UK). The bFGF, EGF and B27 were from R&D systems (Minneapolis, Minnesota, USA). The rat IL-6, MCP-1α, MIP-1α, and TNF-α ELISA detection kits were obtained from Invitrogen (Carlsbad, CA, USA). The stereotaxic apparatus was provided by Stoelting (Wood Dale, IL, USA) and the Y-maze was provided by Styling (Changsha, China).

Primary cell culture

The isolation of primary NSCs from the hippocampi of SD rats (new-born 24 hours after birth) was performed according to a standard protocol as mentioned below. SD rats were immersed in 75% ethanol and disinfected for 30 s. Then the rats were decapitated under sterile conditions, removed the skull and the whole brain was dissected out and placed in ice-cold PBS. Meninges were separated, hippocampus was exposed and hippocampal tissues were pipetted out gently. The dissected tissue was digested with 0.25% trypsin for 20 minutes and then neutralized in culture medium containing 10% fetal bovine serum (FBS). The cells were cultured in culture medium containing EGF, bFGF and B27 supplements at a density of 1×10^6 cells/mL, and the third generation of NSCs was used for co-culturing or assay [15, 16].

Primary microglial cells were obtained from the hippocampi of newborn SD rats. The cells were suspended in 10% FBS and plated on poly-L-lysine-coated flasks. The culture medium was renewed 2 days after plating. Nine days later, the flasks were gently shaken, and the supernatant was collected for centrifugation. Finally, microglial cells were plated at a density of 5×10^5 cells/mL [17].

The dissociated cells from the hippocampi of newborn SD rats were plated in flasks for primary astrocyte culturing. After 8-10 days, the flasks were shaken at 37°C for 4 h in a rotator, and then the adherent astrocytes were collected for further co-culturing [5].

Transwell co-culture system

To study the effect of glial cell-mediated inflammation on NSCs, primary NSCs and glial cells (microglia and astrocyte) were co-cultured in the upper and lower layer of a transwell plate, respectively, with a 1:1:1 ratio of NSCs, microglia and astrocytes. The upper and lower layer of the transwell was partitioned with a semipermeable membrane through which cytokines and chemokines secreted by glial cells could permeate to the NSC layer without any direct effects of glial cells on NSCs. Four groups were assessed in the *in vitro* study: 1) Control group: NSCs and glial cells were co-cultured in a transwell plate. 2) Aβ group: cells were cultured as described for the control group, and 10 μmol/L $A\beta_{1-42}$ was added to the glial cells; 3) α7-nAChR pre-activated group: cells were cultured as described for the control group, and nicotine (final concentration of 1 μM) was added to the glial cells 1 h before the addition of $A\beta_{1-42}$. 4)

α7-nAChR blocked group: cells were cultured as described for the control group, α-bungarotoxin was used to block glial α7-nAChR, and nicotine was added to the glial cell layer 1 h before the addition of $A\beta_{1-42}$.

Flow cytometry assay to assess the apoptosis and differentiation of NSCs

The apoptosis rate of NSCs was measured using a Fluorescein FragEL™ DNA Fragmentation Detection Kit according to the manufacturer's instructions (Calbiochem, USA). After treatment with $A\beta_{1-42}$ for 72 h, the apoptosis of NSCs was analyzed using a FACScan flow cytometer (Becton-Dickinson, USA). The data were analyzed using CellQuest 3.0 software (Becton-Dickinson, USA). The differentiation of NSCs was also detected by Flow cytometry. Briefly, the cells were collected 72 h after co-culture and incubated with MAP-2 (1:200) and CHAT (1:200) primary antibodies overnight at 4°C, and then the relevant secondary antibodies were added to the cells prior to the evaluation.

Hippocampal injection of Aβ and NSC implantation

Adult SD rats were randomly assigned to four groups: NSCs, Aβ, Aβ and NSCs and activated α7-nAChR (n=6 per group). $A\beta_{1-42}$ was dissolved in freshly prepared 0.1 M phosphate-buffered saline (PBS, pH 7.4), diluted to a concentration of 1 μg/μL, and then incubated in the dark for 7 days at 37°C prior to injection. Animals were anesthetized with 5% chloral hydrate and placed in a stereotaxic instrument (Wood Dale, IL, USA). Subsequently, 5 μl of $A\beta_{1-42}$ solution or vehicle was injected over 10 min into the hippocampi via Hamilton syringe based on the following coordinates: -3.8 mm anteroposterior to the bregma, +2.0 mm medial to lateral and -2.8 mm ventral to dura (AP -3.8, L +2.0, V -2.8), according to the atlas of Paxinos and Watson, and the syringe was left in place for another 10 min after injection to allow diffusion away from the tip [18-20]. The nuclei of the NSCs were labeled with BrdU (final concentration of 10 μmol/L) for 2 days, and approximately 1×10^6 individual NSCs were implanted into the hippocampi (AP -3.6, L +2.0, V -3.0) 7 days after $A\beta_{1-42}$ injection. In the α7-nAChR-activated group, nicotine (daily free base nicotine at a dose of 0.8 mg/kg) was administered by subcutaneous injection at 9 am and 5 pm from 5 days before $A\beta_{1-42}$ injection to the time of rat sacrifice. The rats in the other groups received normal saline. The rats were sacrificed 14 days after NSC implantation for ELISA and immunofluorescence detection [21].

Immunofluorescence

Immunofluorescence was performed using 30-μm-thick coronal brain tissue sections, and one was selected from every four to five sections (~150 μm apart) in the hippocampal region, which were stained according to the following procedure. The tissue sections were blocked with 5% (vol/vol) normal goat sera and incubated with the respective primary antibodies, such as nestin (ab6142, 1:50), MAP-2 (ab-11267, 1:100), or CHAT (ab6168, 1:100), with 0.3% Triton X-100 overnight at 4°C. Anti-BrdU primary antibody was added to the sections after incubation with FITC-labeled secondary antibodies for 30 min. Finally, the brain sections were treated with TRITC-labeled secondary antibody and then stained with DAPI. Blocking sera without primary/secondary antibodies and secondary antibody without primary antibody were used as negative controls to determine the specificity of the immunofluorescence.

ELISA

The level of IL-6, MCP-1α, MIP-1α and TNF-α in the co-culture system and rat hippocampi was quantified by ELISA according to the manufacturer's instructions. The supernatant of the co-culture system were collected at 72 h after $A\beta_{1-42}$ treatment for the detection of inflammatory mediators. A standard curve was performed in duplicate, and the levels of cytokines and chemokines were evaluated by use of the standard curve as reference.

Y maze

Y maze experiment for testing learning and memory functions are carried out according to the protocol mentioned in a previous study [22]. Immediate spatial working memory performance was assessed by recording spontaneous alternation behavior in a single-session Y-maze test. Each rat was placed at the end of one arm and allowed to move freely for 10 min. An arm entry was recorded when a rat moved all 4 feet into the arm. The measured parame-

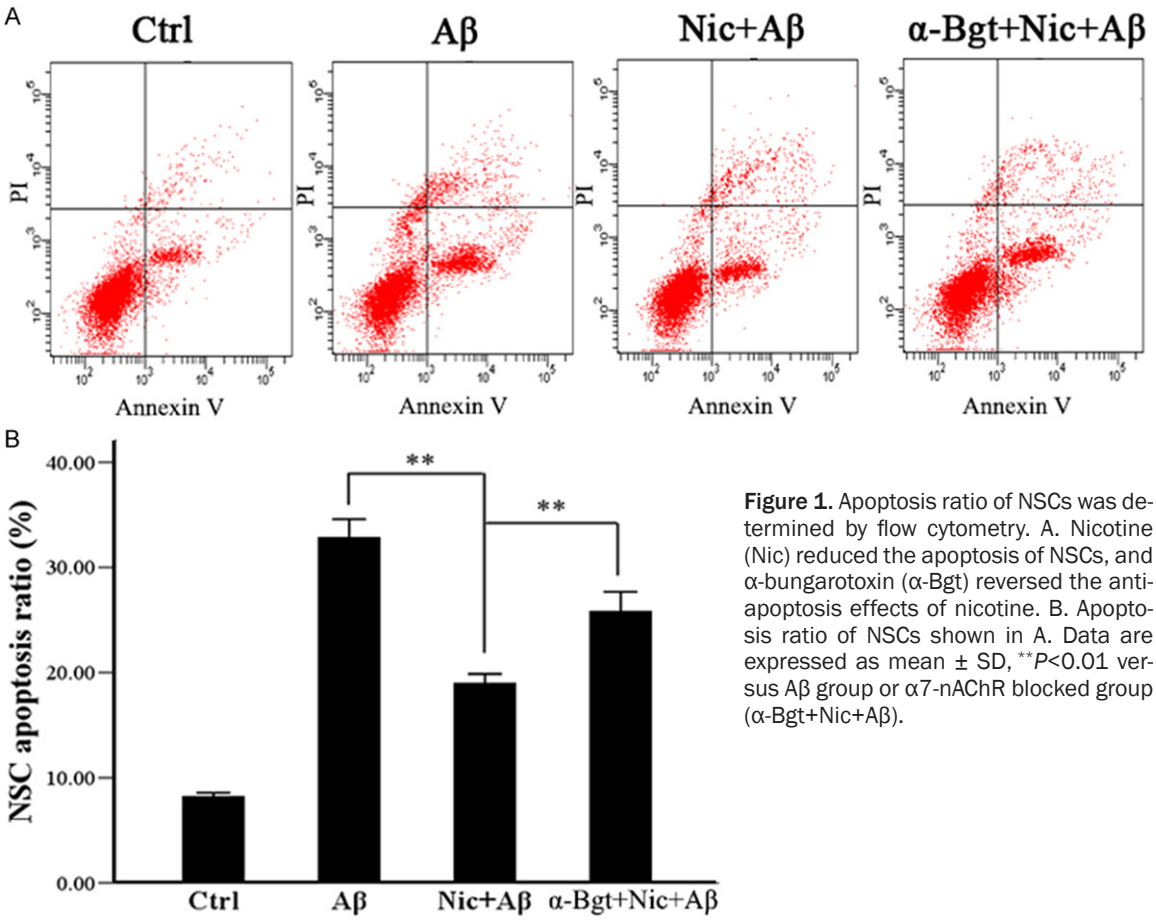

Figure 1. Apoptosis ratio of NSCs was determined by flow cytometry. A. Nicotine (Nic) reduced the apoptosis of NSCs, and α-bungarotoxin (α-Bgt) reversed the anti-apoptosis effects of nicotine. B. Apoptosis ratio of NSCs shown in A. Data are expressed as mean ± SD, $^{**}P<0.01$ versus Aβ group or α7-nAChR blocked group (α-Bgt+Nic+Aβ).

ters were the total number of arm entries and the sequence of entries into the three arms.

Statistical analysis

Data were analyzed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA) and expressed as the mean ± S.E.M. Statistical analyses were performed using one-way ANOVA with Fisher's LSD post-hoc test for multiple comparisons of the means. Differences were considered to be statistically significant at $P < 0.05$.

Results

Nicotine protects NSCs against Aβ-induced toxicity

To determine whether activation of glial α7-nAChR would affect the apoptosis and differentiation of Aβ-treated NSCs, a co-culture system of NSCs and glial cells was introduced in this study. The flow cytometry results showed that, apoptotic NSCs in the Aβ group increased 4-fold compared to the control group. Nicotine significantly decreased the NSC apoptosis ratio and showed a potent anti-apoptotic effect; however, this effect was reversed by treatment with α-bungarotoxin, which is a widely used blocker of α7-nAChR (**Figure 1**). Therefore, activation of glial cells carrying α7-nAChR decreased NSC apoptosis induced by Aβ.

Effect of nicotine on the differentiation of neural stem cells in vitro

To investigate the effects of glial α7-nAChR activation on the differentiation of NSCs, flow cytometry was used to detect the expression of MAP-2 and CHAT. The results showed that, the ratio of differentiating NSCs to neurons and cholinergic neurons decreased due to the effects of Aβ. Interestingly, when the glial cells in the NSC co-culture were pre-treated with nicotine both neuronal and cholinergic neuronal differentiation increased significantly. To determine whether the protective effect of nicotine on NSC differentiation was dependent on α7-nAChR, α-bungarotoxin was used to block

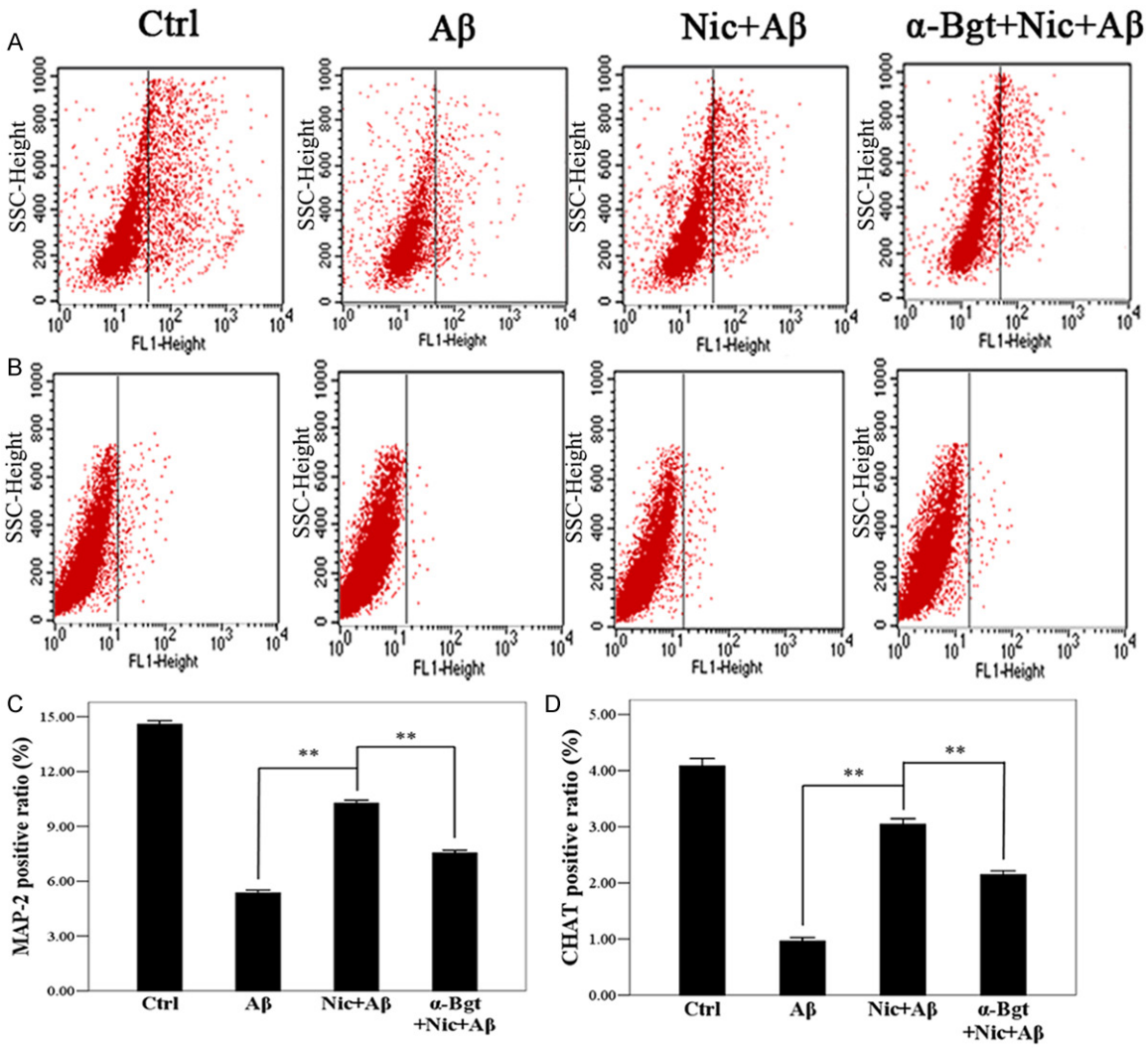

Figure 2. Expression of MAP-2 and CHAT was detected by flow cytometry. A. The ratio of NSCs that differentiated into neurons. B. The ratio of NSCs that differentiated into cholinergic neurons. The cells which are differentiated into neurons are represented here on the right side half of the figure. C. Differentiation ratio of NSCs shown in A. D. Differentiation ratio of NSCs shown in B. $^{**}P<0.01$ versus the Aβ group or the α7-nAChR blocked group (α-Bgt+Nic+Aβ).

α7-nAChR. As shown in **Figure 2**, α-bungarotoxin suppressed the differentiation-promoting effect of nicotine. Thus, Aβ inhibited the differentiation of NSCs, and the activation of glial cell α7-nAChR increased the neuronal and cholinergic neuronal differentiation of NSCs.

Nicotine reduces glial cell IL-6, MCP-1α, MIP-1α and TNF-α levels in vitro

The supernatant of the co-culture system was collected to determine the effects of glial cell α7-nAChR activation on the levels of inflammatory mediators. Compared with the control group, the levels of inflammatory mediators in the Aβ group increased. Nicotine significantly decreased the levels of IL-6, MCP-1α, MIP-1α and TNF-α in the co-culture system. However, the anti-inflammatory effect of nicotine was blocked by α-bungarotoxin (**Figure 3**). Therefore, these results indicate that nicotine weakens neuroinflammation by activating glial cell α7-nAChR.

Nicotine reduces rat hippocampal IL-6, MCP-1α, MIP-1α and TNF-α level in vivo

Next, we examined whether nicotine play a role in regulating neuroinflammation *in vivo* by using ELISA to detect proinflammatory cytokines and chemokines. $A\beta_{1-42}$ was injected into the hippocampi of SD rat to recapitulate the chronic

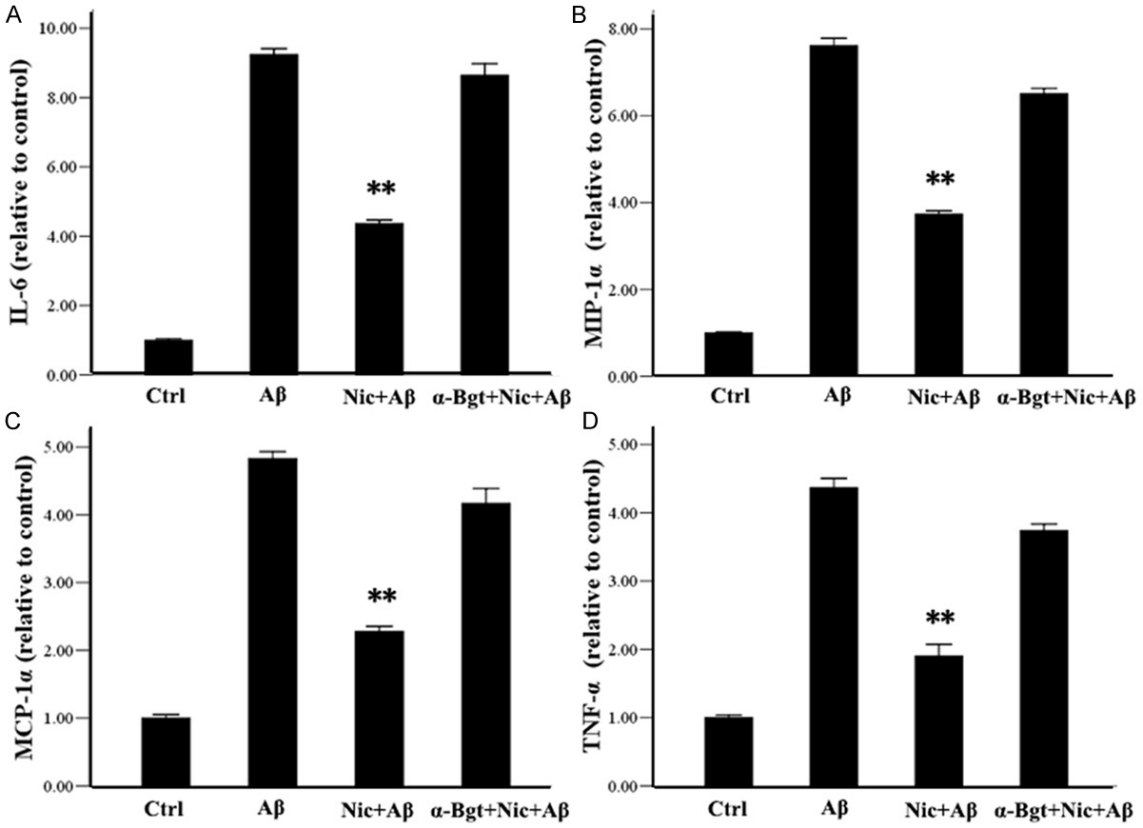

Figure 3. Nicotine (Nic) reduced the levels of glial cell IL-6 (A), MCP-1α (B), MIP-1α (C) and TNF-α (D) *in vitro*. The inflammatory factors in the glial cell and NSC co-culture system were detected 72 h after $A\beta_{1-42}$ treatment. Nicotine significantly decreased the levels of IL-6, MCP-1α, MIP-1α and TNF-α; and α-bungarotoxin (α-Bgt) reversed the anti-inflammatory effects of nicotine. $^{**}P<0.01$ versus the Aβ group or the α7-nAChR blocked group (α-Bgt+Nic+Aβ).

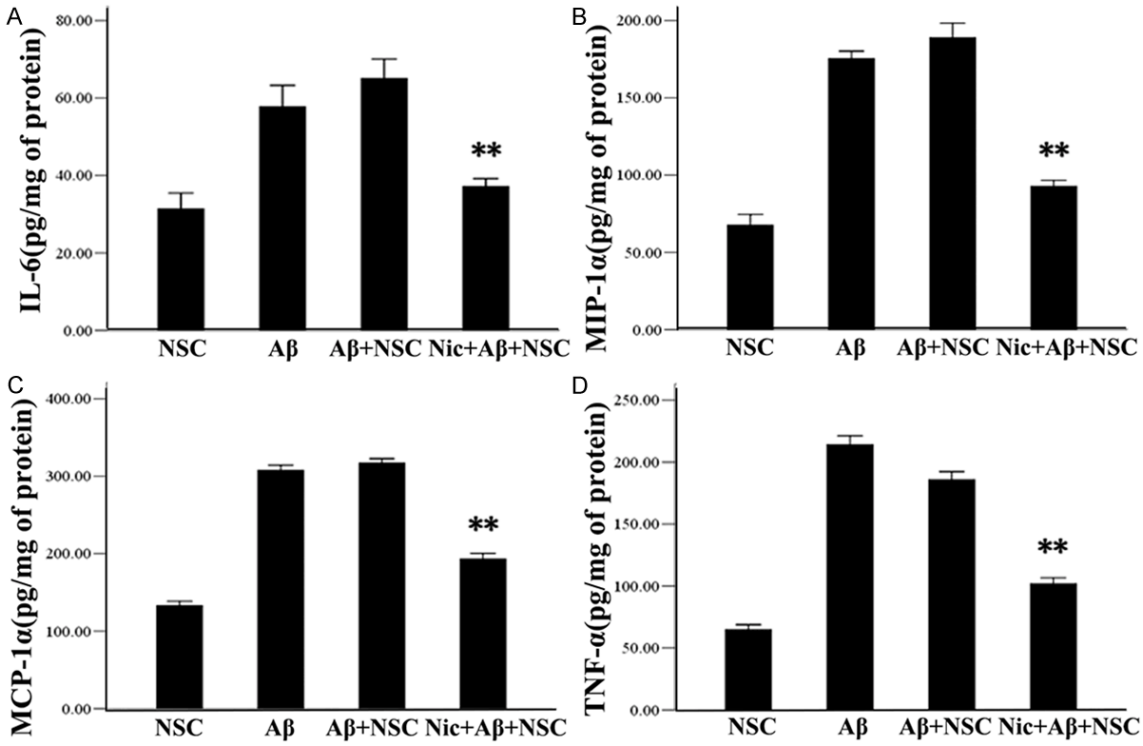

Figure 4. Nicotine (Nic) reduced the levels of rat hippocampal IL-6 (A), MCP-1α (B), MIP-1α (C) and TNF-α (D) *in vivo*. The proinflammatory cytokines and chemokines in AD rat hippocampi were detected by ELISA at 14 days after NSC implantation and nicotine significantly reduced the levels of hippocampal IL-6, MCP-1α, MIP-1α and TNF-α. $^{**}P<0.01$ versus the Aβ and NSC group (Aβ+NSC).

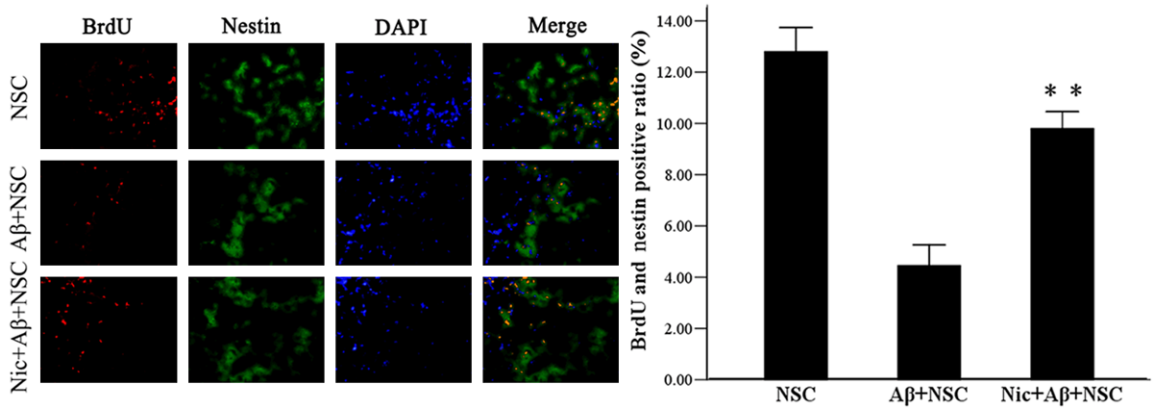

Figure 5. Nicotine improves the proliferation of transplanted NSCs in the inflamed AD brain. BrdU and nestin primary antibodies were used to stain hippocampal BrdU-labeled NSCs and NSCs, respectively, using double-labeling immunofluorescence. Nicotine significantly improved the proliferation ratio of implanted NSCs in the inflamed AD brain (n=6) (400×). $^{**}P<0.01$ versus the Aβ and NSC group.

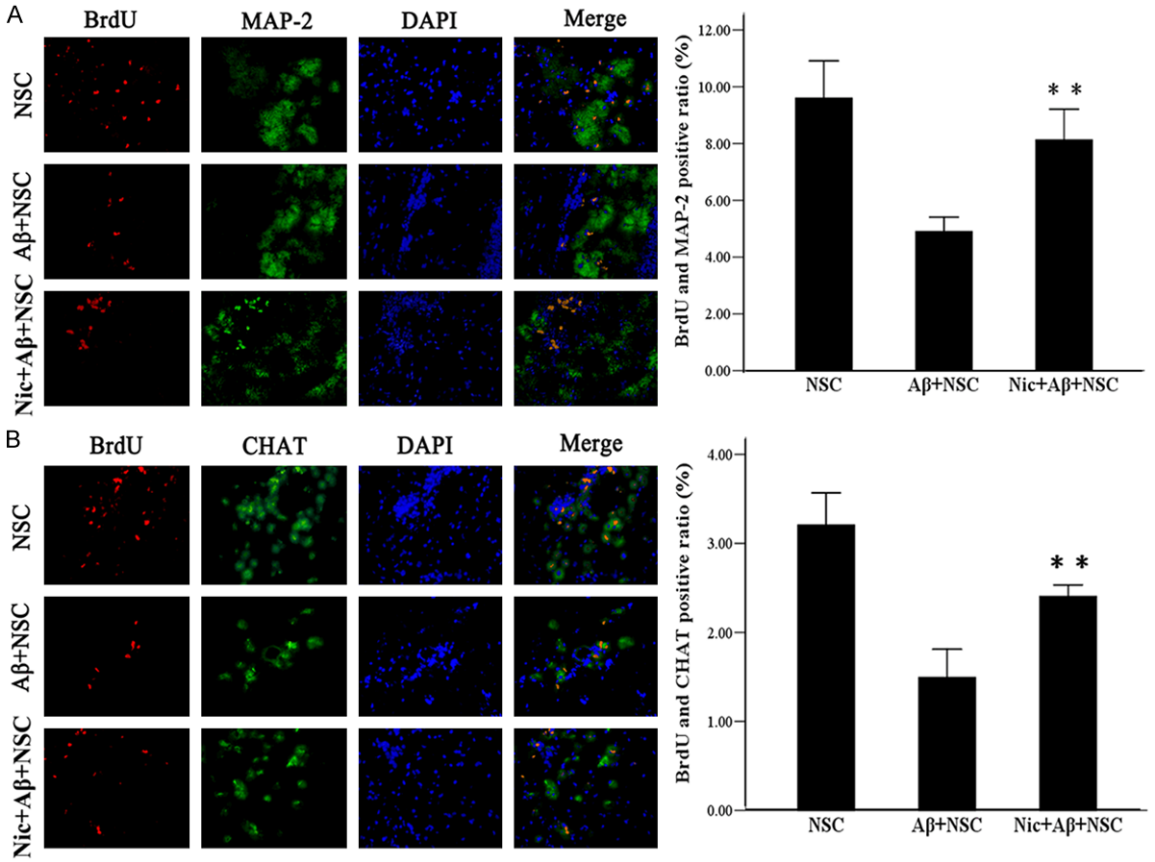

Figure 6. Nicotine improves the neuronal and cholinergic neuronal differentiation of transplanted NSCs. MAP-2 and CHAT primary antibodies were used to stain hippocampal neurons and cholinergic neurons, respectively, using double labeling immunofluorescence. Double-stained BrdU and MAP-2-positive neurons were differentiated from

BrdU-labeled NSCs, and double-stained BrdU and CHAT-positive cholinergic neurons were differentiated from BrdU-labeled NSCs. Nicotine significantly improved the differentiation ratio of neurons (A) and cholinergic neurons (B) in the inflamed AD brain (400×). $^{**}P<0.01$ versus the Aβ and NSC group.

Table 1. Learning and memory ability of each group

Group[a]	Learning (n1)	Memory (n2)
NSC	30.33±9.45	14.33±3.06
Aβ	66.67±10.01	35.33±4.16
NSC+Aβ	61.33±4.16	39.67±8.32
NSC+Aβ+nicotine	46.33±4.51[b,c,d]	22.32±3.57[b,c,d]

[a]All data are expressed as mean number of learning or memory ± S.E.M. n1, learning times; n2, memory times; [b]$P<0.05$ versus the NSC group, [c]$P<0.05$ versus the Aβ group, [d]$P<0.05$ versus the NSC and Aβ group.

inflammation, and then BrdU-labeled NSCs were implanted into the hippocampi 7 days after $A\beta_{1-42}$ injection. The statistical analysis revealed higher IL-6, MCP-1α, MIP-1α and TNF-α concentration in the Aβ group than in the control group, and the implanted NSCs did not reduce the levels of inflammatory factors (**Figure 4**). Reciprocally, the levels of IL-6, MCP-1α and MIP-1α in the NSC implantation group (NSC+Aβ) were higher than those in the Aβ group. When AD rats were treated with nicotine, the concentrations of inflammatory factors decreased significantly. Together, these results indicated that nicotine reduced the secretion of inflammatory factors *in vivo*.

Nicotine improves the proliferation of NSCs in vivo

To observe the effects of nicotine on the proliferation of NSCs, BrdU-labeled NSCs were implanted into the hippocampi of the inflamed AD brain. Immunofluorescence was used to determine the expression of nestin, which is an NSC-specific protein, and the proliferation ratio was expressed as double-stained BrdU and nestin-positive cells to nestin-positive cells. Following nicotine stimulation, the proliferation of NSCs was greatly increased compared with the Aβ group (**Figure 5**). These results suggested that, nicotine could influence the inhibition of Aβ-mediated neuroinflammation on the proliferation of NSCs in the inflamed AD brain.

Nicotine improves the differentiation of NSCs in vivo

The primary cause of dementia is the massive apoptosis of neurons and cholinergic neurons. Therefore, we examined whether nicotine play a role in resisting the inhibitionory effect of neuroinflammation on NSC differentiation. The neuronal differentiation ratio of BrdU-labeled NSCs was expressed as double-stained BrdU and MAP-2 double-positive cells to MAP-2-positive cells. The results showed that, nicotine mitigated the deleterious effects of Aβ and improved the neuronal differentiation of implanted NSCs (**Figure 6A**). Furthermore, cholinergic neuronal differentiation was determined because these are the primary apoptotic neurons in AD. Interestingly, the cholinergic neuronal differentiation ratio of BrdU-labeled NSCs was also elevated in response to nicotine treatment (**Figure 6B**). Taken together, the aforementioned results indicated that nicotine improved the differentiation of NSCs to supplement neuronal and cholinergic neuronal loss due to apoptosis *in vivo*.

Nicotine improves learning and recall ability in the rats

The Y maze was used to detect the learning and recall ability of rats. Compared to the Aβ and NSC group, the learning and recall ability of the nicotine pretreatment group was significantly improved (**Table 1**). These results suggested that nicotine improved the behavior of the implanted NSCs in AD rats.

Discussion

In the present study, we found that glial α7-nAChR activation mitigated the inhibition of neuroinflammation on the proliferation and differentiation of NSCs by suppressing inflammatory mediator secretion, thus facilitating improvements in the learning and memory ability of the resembling AD rats.

It is known that accumulated Aβ activates glial cell constitutively and induces the secretion of proinflammatory cytokines and chemokines in AD. Moreover, the chronic inflammatory response in the CNS suppresses synaptic plasticity and the mitochondrion-related energy supply, subsequently inducing neuronal apoptosis. Neuroinflammation inhibits the proliferation and differentiation of NSCs [23, 24]. In this sce-

nario, the lost neurons are not replenished in time, which causes neurodegeneration. Therefore, techniques to weaken neuroinflammation are becoming key factors in the treatment of AD. Our previous study demonstrated that astrocytes express α7-nAChR, and activated astrocyte α7-nAChR has been shown to inhibit Aβ-induced inflammation *in vitro* [5]. Similarly, *De Simone et al.* found that, rat microglial cells expressed α7-nAChR [25]. In the present study, we extended the analysis of glial α7-nAChR to the long-term neuroinflammation and neurogenesis that occurs in AD. Both the *in vitro* and *in vivo* inflammatory model studies of AD showed that, the levels of proinflammatory cytokines and chemokines, such as IL-6, MCP-1α, MIP-1α and TNF-α were remarkably reduced in response to stimulation by nicotine. In addition, nicotinic suppression of inflammatory mediator production was prevented by pre-treatment with α-bungarotoxin, which is a widely applied antagonist of nicotine. The α1, α7 and α9 nAChR have been described as potential α-bungarotoxin-binding nicotinic receptor subunits; however, macrophages and monocytes do not express α1 and α9 nAChR [13, 26]. In addition, our research demonstrated that NSCs do not express α7-nAChR, as evidenced using RT-PCR and immunofluorescence (data not shown). Therefore, it is glial α7-nAChR that is bound by α-bungarotoxin in reversing of nicotinic inflammation inhibition effect. These finding are in agreement with those of *Wang*, who previously reported that, electrical stimulation of the vagus nerve inhibited cytokine synthesis in wild-type mice but not in α7 subunit-knockout mice [13].

Aberrant chronic neuroinflammation is believed to play a key role in the etiology of AD, which triggers nerve cell apoptosis [27]. Consistent with these finding, we found that NSC apoptosis increased in response to stimulation of Aβ. Interestingly, our results also indicated that nicotine markedly reduced the apoptosis of NSCs in the glial cell and NSC co-culture system, and this effect was reversed by the application of α-bungarotoxin. Therefore, one plausible explanation for the present finding is that nicotine activates α7-nAChR and weakens neuroinflammation, leading to a decrease in the NSC apoptotic ratio.

Neuroinflammation restricts the application of NSC implantation in the treatment of AD. Many experimental studies have demonstrated that the self-perpetuating inflammatory response markedly inhibited implanted NSC differentiation into neurons [28, 29]. In the present study, in order to measure the effects of the improved microenvironment on the differentiation and proliferation of NSCs, BrdU-labeled NSCs were injected into the hippocampi of the inflamed AD brain. Although the rat model based on the injection of Aβ into the hippocampus, does not recapitulate all of the common features of AD, an aberrant inflammatory response occurs that resembles the etiology of AD. The results showed that the neuronal and cholinergic neuronal differentiation of implanted NSCs increased following the activation of α7-nAChR. Neuron and cholinergic neuron disorders and loss accelerate the onset of dementia, and increased neuronal numbers provide a benefit in AD [30, 31]. Consistent with this research, we observed an enhanced learning and recall ability of the rats in the present research. *Hernandez* and his colleague claimed that nicotine improved memory function via its effects on high-affinity NGF receptors [21]; however, in the present study, nicotine may improved rat behavior by increasing neuronal differentiation of NSCs via the activation of α7-nAChR and attenuation of the inflammatory response.

In conclusion, the data presented herein suggest that, glial α7-nAChR activation weakens Aβ-mediated neuroinflammation and improves the proliferation and differentiation of implanted NSCs, which contribute to behavior in AD. However, due to the extensive role of nicotine, specific agonists must be developed to weaken neuroinflammation. Our results suggest that, the simultaneous promotion of neurogenesis and reduction of neuroinflammation is a promising strategy for therapy in AD.

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Disclosure of conflict of interest

None.

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