

Original Research

Acetylcholine suppresses microglial inflammatory response via $\alpha 7$ nAChR to protect hippocampal neurons

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Neuroinflammation is principally linked to glial function and has been demonstrated to participate in the pathogenesis of Alzheimer's disease, a neurodegenerative disorder characterized by beta-amyloid accumulation and neurotransmission disruption. Previous findings suggest acetylcholine exerts anti-inflammatory and neuroprotective properties in several neurodegenerative disorders. However, the underlying mechanisms remain elusive. Here evaluation of the influence of acetylcholine on neuroinflammation and neurodegeneration in Alzheimer's disease is reported and further neuroprotective mechanisms are investigated. Investigation of microglia in lipopolysaccharide-induced hippocampal neuronal toxicity employed $\alpha 7$ nAChR gene silencing and demonstrated that both the anti-inflammatory and neuroprotective effects of acetylcholine rely on $\alpha 7$ nAChR pathways. As expected, in neuron-microglia co-cultures lipopolysaccharide induced an increase in expression of pro-inflammatory factors, including inducible nitric oxide synthase, interleukin-1 β , and tumor necrosis factor- α , and decreased expression of neurotrophic factors such as insulin-like growth factor-1, and neuronal apoptosis. Acetylcholine protects against lipopolysaccharide-elicited neuronal injury by inhibiting the microglial inflammatory response and promoting microglial neurotrophic factor production via the action of $\alpha 7$ nAChR on microglia. These findings establish that ACh activates $\alpha 7$ nAChR in microglia, which in turn protects hippocampal neurons.

Keywords

Acetylcholine; $\alpha 7$ nAChR; hippocampal neuron; lipopolysaccharide; microglia

1. Introduction

Neuroinflammation has been demonstrated to play an important role in the neurodegeneration process of Alzheimer's disease (AD), a disease considered to be the most common neurodegenerative disorder of ageing (Depino et al., 2003; Agostinho et al., 2010; Sadigh-Eteghad et al., 2015b). Although microglia-driven neuroinflammation exerts a favourable effect on scavenging cell

debris and tissue repair; it is also widely accepted that chronic and excessive activation of these cells has a detrimental effect on the survival of neurons. Thus, microglia-driven neuroinflammation contributes to the progress of neurodegenerative diseases (Egea et al., 2015). Microglial cells show a typical resting phenotype in the healthy central nervous system (CNS); however, they can be activated in response to neurotoxicant or electrical stimulations (Hung et al., 2010). To further show the relevance of microglia and neuroinflammation in neurodegenerative diseases, this study employed a lipopolysaccharide (LPS)-elicited inflammatory AD cell model. LPS has a profound impact on peripheral immune cells (e.g. monocytes and macrophages) as well as brain microglial cells, which are stimulated to produce major immunoregulatory and pro-inflammatory mediators (such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and inducible nitric oxide synthase (iNOS) in response to LPS (González-Scarano et al., 1999; Medvedev et al., 2000; Sanlioglu et al., 2001; Choi et al., 2009; Cao et al., 2010). Excess accumulation of these pro-inflammatory mediators in the CNS leads to neuronal injury (McGuire et al., 2001; Liu et al., 2002; Ramesh et al., 2013). Besides being a potent non-specific stimulator of brain microglia, LPS has often been employed to induce the AD animal model used to investigate the association between neuroinflammation and neurodegeneration (Ogura et al., 2006; Nam et al., 2013).

It is known that the immune system is regulated via stimulation of the vagus nerve. The term "cholinergic anti-inflammatory pathway" has been associated with exploration of peripheral immunity (Gallowitsch-Puerta et al., 2007; Martelli et al., 2014). Other research has indicated that acetylcholine (ACh) significantly attenuates the elevation of pro-inflammatory mediators, including IL-6, IL-1 β , TNF- α , and IL-18, in human macrophage cultures exposed to LPS (Borovikova et al., 2000). In this study, ACh was applied to neuron-microglia co-cultures to further confirm its anti-inflammatory properties in response to microglia-derived neuroinflammation.

The activation of an $\alpha 7$ subtype of the nicotinic acetylcholine receptor ($\alpha 7$ nAChR) plays a vital role in the cholinergic anti-inflammatory pathway where macrophage/microglia function is regulated in the inflammatory response. Further, $\alpha 7$ nAChR subtype signalling participates in a diversity of biological activities

such as neuronal survival and synaptic plasticity (Egea et al., 2015). The involvement of $\alpha 7$ nAChR has also been reported when stimulation of the vagus nerve failed to suppress pro-inflammatory cytokine TNF- α synthesis in $\alpha 7$ nAChR knockout mice (Wang et al., 2003). Consequently, exploring the molecular pathways behind $\alpha 7$ nAChRs activation in microglia may provide new approaches for pharmacological regulation of microglial activation in AD. Further, in the current study, the regulation of the anti-inflammatory pathway of ACh by $\alpha 7$ nAChR in microglia has been demonstrated by means of genetic intervention.

2. Materials and methods

2.1 Ethics approval

All animals were obtained from the Center of Experimental Animals at Nantong University (Nantong, China). Animal procedures were strictly in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Nantong University (20160920_001).

2.2 Primary neuronal culture

Primary neuronal cultures were derived with minor modification from the hippocampal tissues of embryonic Day 18 (E18) Sprague-Dawley (SD) rats (Wayman et al., 2006). Briefly, hippocampal tissues were dissociated into single cells via appropriate mechanical shearing and 1.25 mg/ml trypsin (Gibco, Carlsbad, CA, USA) treatment. Cells were then seeded at a density of 5×10^5 cells/cm² on 6- or 24-well plates precoated with 0.01% poly-L-lysine (sigma-Aldrich, St. Louis, MO, USA) and incubated in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). 24 hours after seeding, 5 μ M cytarabine was added to the medium to inhibit glial cell proliferation.

2.3 Primary microglia-enriched culture

Neonatal one-day-old SD rat brains were dissected and cerebral cortical tissues were isolated in ice-cold PBS as described previously (Bachstetter et al., 2011). Tissues were trypsinized for 15 min at 37°C, cell mixtures were obtained by pipette blowing, then incubated in DMEM/F12 supplemented with 10% FBS in the flasks for 12-14 days. On reaching confluence, flasks were shaken for 4-6 hours at 240 rpm to isolate microglia. Floating microglia were obtained for further study and incubated on plates in DMEM/F12 supplemented with 10% FBS.

2.4 Primary neuron-microglia co-cultures

Hippocampal neuronal cultures were initially obtained as described above. The highly enriched microglial cells were then added to neuronal cultures four days after initial seeding at a density of 5×10^4 cells/cm². After a further three days LPS was applied to the reconstituted cell cultures.

2.5 Drug exposure

As described above, LPS (*Escherichia coli* 0111:B4, Sigma-Aldrich, St. Louis, MO, USA) was applied three days after microglia and hippocampal neurons were co-cultured for two hours at a concentration of 100 ng/mL. ACh (Sigma-Aldrich, St. Louis, MO, USA) was then added at a concentration of either 10^{-7} or 10^{-9} mol/L. The cultures were incubated for 24 hours, and then examined for inflammatory responses and neuronal apoptosis.

2.6 Transfection of the lentiviral vectors expressing $\alpha 7$ nAChR-shRNA into microglia

The microglia-enriched cultures obtained were transfected by lentiviral vectors expressing either Scr-shRNA or $\alpha 7$ nAChR-shRNA. The lentivirus transfected microglial cells were added to the four-day-old neuronal cultures, which were then incubated for three days prior to LPS treatment. The expression of $\alpha 7$ nAChR in the microglial cells was tested to evaluate transfection efficiency three days after transfection (data not shown).

2.7 Western blot analysis

Protein extracts (30 μ g/lane) from either microglia or neuron-microglia were separated by 12% SDS-PAGE. The proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were then probed with primary antibodies against iNOS, TNF- α , $\alpha 7$ nAChR, and β -actin (diluted 1:400; Abcam, Cambridge, UK), and either IL-1 β or insulin like growth factor (IGF)-1 (both diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with appropriate infrared fluorophore-tagged secondary antibodies (Rockland, Gilbertsville, PA, USA), membranes were scanned with an Odyssey infrared scanner (LI-COR Inc., Lincoln, NE, USA). Protein band intensities were quantified by densitometric analysis via an image analysis system (Odyssey 3.0 software, LI-COR Inc., Lincoln, NE, USA).

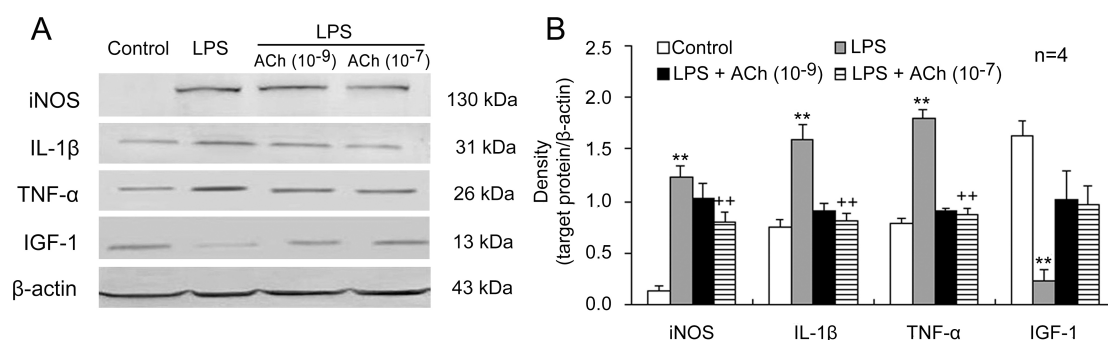


Figure 1. ACh reduces LPS-induced upregulation of pro-inflammatory mediators in neuron-microglia co-cultures. Cultures treated with LPS (100 ng/mL) for two hours followed by ACh addition (10^{-7} or 10^{-9} mol/L) and a 24 hour incubation. Western blot assay is employed to detect the expression of pro-inflammatory and neurotrophic factors ($n = 4$). ** $p < 0.01$ versus control; ++ $p < 0.01$ -versus LPS.

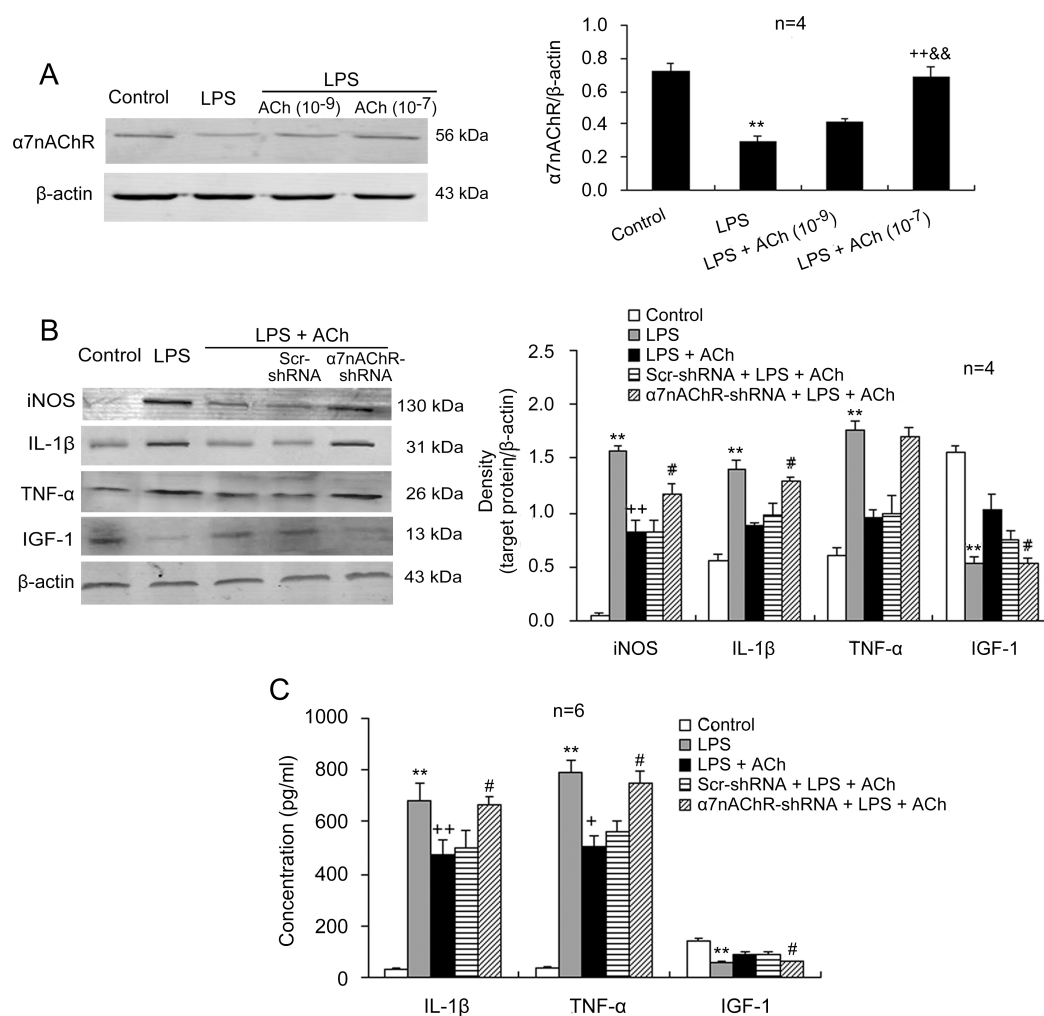


Figure 2. Silencing of $\alpha 7$ nAChR gene in microglia abolishes the ability of ACh to inhibit LPS-induced inflammatory responses. Treatment: Microglia transfected with $\alpha 7$ nAChR-shRNA lentiviral vector co-cultured with hippocampal neurons, Control: Scr-shRNA lentiviral vector transfected microglia. Neuron-microglia co-cultures were treated with LPS (100 ng/mL) for two hours followed by ACh application, then incubated for 24 hours. (A) $\alpha 7$ nAChR expression levels in microglia exposed to various treatments. (B) Representative electrophoretic bands and statistical analysis of protein expression levels. (C) Concentration of TNF- α , IL-1 β , and IGF-1 in neuron-microglia co-culture supernatants. ** $p < 0.01$ versus control; ++ $p < 0.01$ versus LPS; # $p < 0.05$, versus LPS + ACh treatment; && $p < 0.01$ versus LPS + 10⁻⁹ mol/L ACh, $n = 4$ or 6.

2.8 Immunocytochemistry

At 24 hours following ACh application, cells seeded on the coverslips were fixed at room temperature in 4% paraformaldehyde for 20 minutes, followed by sequential treatment with blocking solution for 30 minutes, primary antibody against NeuN (neuronal marker; 1:400; Millipore, Bedford, MA, USA) overnight at 4°C, and Alexa Fluor 594-conjugated goat secondary antibody (1:200; Jackson, West Grove, PA, USA) for 4 hours at room temperature. After NeuN staining, coverslips were processed for TUNEL assay using an *in situ* cell death detection kit (Roche, Penzberg, Germany) in accordance with the manufacturer's protocol. NeuN-immunoreactive and TUNEL-positive cells were captured and counted using a confocal microscope (TCS-SP2, Leica, Wetzlar, Germany) at 200 \times magnification. TUNEL and NeuN double-positive apoptotic cells and NeuN-positive neurons were counted within five randomly selected visual fields from each coverslip and the average number of each cell type was recorded.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Levels of TNF- α , IL-1 β , and IGF-1 obtained from neuron-microglia co-culture supernatant samples were assayed with ELISA kits (eBioscience, San Diego, CA, USA). All procedures were performed according to the manufacturer's instructions.

2.10 Statistical analysis

All data are expressed as mean \pm standard deviation. The statistical significance of differences among group comparisons was evaluated using one-way ANOVA followed by a Student-Newman-Keul multiple comparison test (SPSS 13.0 Software, SPSS, Chicago, IL, USA). Statistical significance was assumed at $p < 0.05$ for all tests.

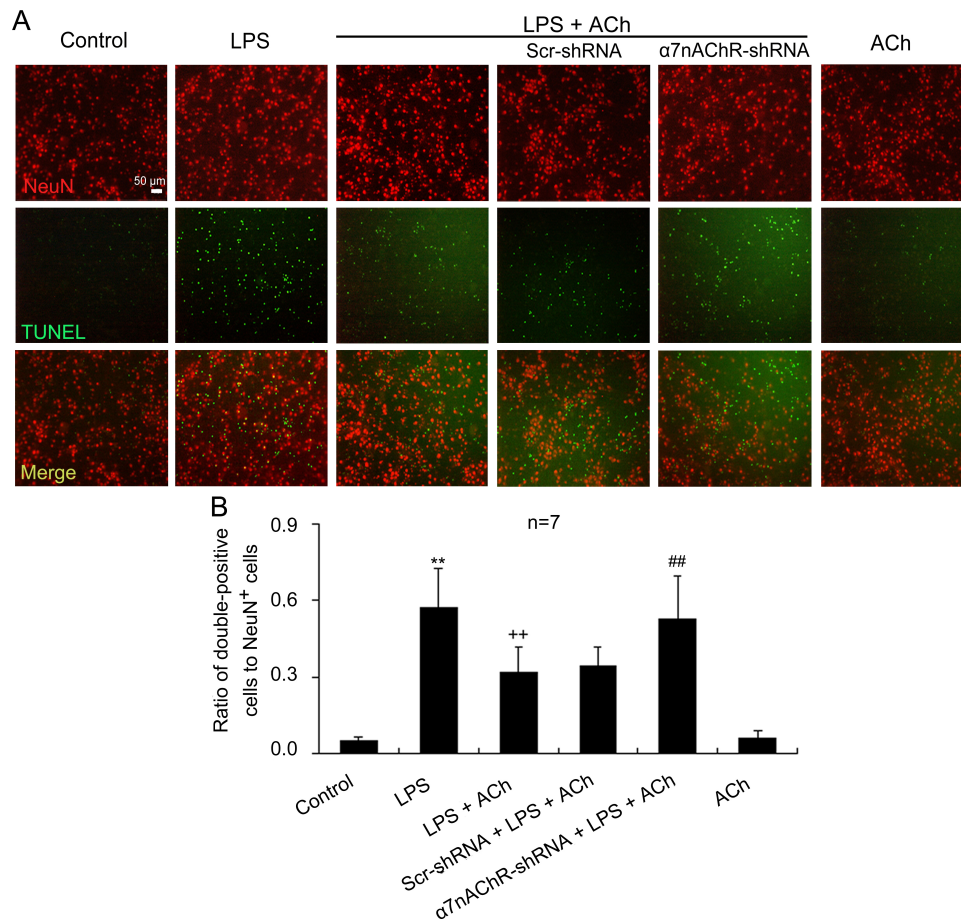


Figure 3. Silencing of $\alpha 7$ nAChR gene in microglia abolishes the ability of ACh to inhibit LPS-induced neuronal apoptosis. Neuron-microglia co-cultures were treated as described in preceding figure legends. Cells grown on coverslips inside 24-well plates were subjected to NeuN immunostaining and TUNEL assay. (A) Representative photomicrographs are given. (B) Quantitative summary and statistical analysis. Histogram shows the relative proportion of TUNEL and NeuN double-positive cells in the NeuN-positive cell population ($n = 7$). ** $p < 0.01$ versus control; ++ $p < 0.01$ versus LPS; ## $p < 0.01$ versus LPS + ACh treatment.

3. Results

3.1 ACh inhibits LPS-elicited pro-inflammatory increase and anti-inflammatory decrease in neuron-microglia co-cultures

Protein expression of pro-inflammatory factors, including iNOS, TNF- α and IL-1 β , increased and the neurotrophic factor, IGF-1, decreased when LPS was applied to neuron-microglia co-cultures (Fig. 1). A higher concentration of ACh (10^{-7} mol/L) significantly suppressed LPS-elicited elevation of pro-inflammatory factor expression but had no significant effect on IGF-1 expression (Fig. 1).

3.2 Silencing of the $\alpha 7$ nAChR gene in microglia counteracts the effect of ACh on suppression of LPS-elicited pro-inflammatory properties

Initially, expression of $\alpha 7$ nAChR in microglia was assessed. LPS significantly downregulated $\alpha 7$ nAChR expression with respect to control, and ACh upregulated $\alpha 7$ nAChR expression (Fig. 2A). Alternatively, a sufficient concentration of ACh (10^{-7} mol/L) significantly altered the reduction of $\alpha 7$ nAChR expression elicited by LPS, whereas, at the lower dose (10^{-9} mol/L) there was no evident effect (Fig. 2A).

Subsequently, $\alpha 7$ nAChR-shRNA transfected microglia were

co-cultured with neurons. The treatment counteracted the effect of ACh on inhibiting upregulation of pro-inflammatory factor expression (including iNOS, TNF- α , and IL-1 β) and downregulation of the neurotrophic factor (IGF-1) expression elicited by LPS in the co-cultures (Fig. 2B). Pro-inflammatory and neurotrophic factor (TNF- α , IL-1 β , and IGF-1) levels in the supernatants of neuron-microglia co-cultures were also determined. As determined by protein expression, $\alpha 7$ nAChR gene silencing in microglia removes the ACh anti-inflammatory ability to suppress elevated TNF- α and IL-1 β production as well as attenuating LPS elicited IGF-1 release (Fig. 2C).

3.3 Silencing of $\alpha 7$ nAChR gene in microglia counteracts the effect of ACh on inhibition of LPS-elicited neuronal apoptosis

In neuron-microglia co-cultures, LPS-elicited neuronal apoptosis and ACh prevented the effect of LPS via $\alpha 7$ nAChR. After co-culturing of $\alpha 7$ nAChR-shRNA transfected microglia with neurons, the silencing of the $\alpha 7$ nAChR gene in microglia abolished the ability of ACh to inhibit LPS-elicited neuronal apoptosis (Fig. 3).

4. Discussion

There is evidence to indicate LPS promotes rat hippocampal neuronal apoptosis by increasing cytosolic $[Ca^{2+}]$ when neurons are cultured long-term (> 18 days, regarded as aged neurons), but does not exert an effect on short-term cultured neurons (< 9 days, regarded as young or mature neurone) (Calvo-Rodríguez et al., 2017). The preliminary data reported here shows that LPS has no significant detrimental effect on enriched seven-day-old hippocampal neurons in the absence of microglia, while it demonstrates that apoptosis of hippocampal neurons was elicited when LPS was applied to neuron-microglia co-cultures. Similarly, several reports have suggested that LPS exerts injurious effects on dopaminergic neurons only in the presence of microglial cells (Gao et al., 2002; Block et al., 2004). Accordingly, microglial cells may be key mediators involved in LPS neurotoxicity. As is well known, LPS endotoxins are general activators of immune cells, including microglial cells, which can lead to serious inflammatory responses in the CNS (Paetau et al., 2017). Since microglia can release neurotoxic molecules under many conditions, excess microglial activation provides a background for neuropathology (Block et al., 2007). In this study, it was shown that 100 ng/ml LPS treatment led to an increase in pro-inflammatory factors (including iNOS, TNF- α , and IL-1 β) as well as a decrease in neurotrophic factor, IGF-1, in hippocampal neuron/microglia co-cultures. Thus, further data is provided showing that LPS induces microglia-derived neuroinflammation, which in turn promotes hippocampal neuronal damage.

Previous research has demonstrated that ACh is the principal neurotransmitter of the cholinergic anti-inflammatory pathway. It is derived from splenic T lymphocytes in response to activation of the efferent vagus nerve, and acts with specific $\alpha 7$ nAChR on activated macrophages to suppress their pro-inflammatory factor release (Andersson and Tracey, 2012). Similarly, a higher concentration of ACh notably inhibited the LPS-elicited microglial inflammatory response via reduction of pro-inflammatory factors, thereby inhibiting hippocampal neuronal apoptosis in this neuron-microglia co-culture paradigm. These results show ACh has both anti-inflammation and neuroprotection properties in the LPS-elicited AD inflammatory cell model. Meanwhile, the effectiveness of *in vitro* ACh application on attenuation of LPS neurotoxicity post-injury rather than pre-treatment indicates a potential therapeutic approach for patients suffering AD.

nAChRs consisting of a family of subunits, such as $\alpha 2$ - $\alpha 10$ and $\beta 2$ - $\beta 4$, belong to the class of pentameric ligand gated ion channels (St John, 2009). Among them, $\alpha 4\beta 2$, $\alpha 3\beta 4$ heteromers, and $\alpha 7$ homomers show as the primary subtypes in the CNS (Pym et al., 2005). These kinds of receptors, especially those located on glial cells, play an essential role in AD pathogenesis and therapy (Tuppo and Arias, 2005; Sadigh-Eteghad et al., 2015a,c). Moreover, this study revealed that LPS treatment reduced the level of $\alpha 7$ nAChR in microglia and that conversely ACh increased $\alpha 7$ nAChR expression. Consistent with this, LPS not only reduces TGF- β receptors that are involved in signaling of anti-inflammatory cytokine TGF- $\beta 1$, but also impairs ability of TGF- $\beta 1$ to regulate expression of inflammatory mediators (Mitchell et al., 2014). Collectively, these results suggest ACh inhibition of neuroinflammation may require the presence of a key downstream receptor, such as $\alpha 7$ nAChR, on

microglia. Observations made via $\alpha 7$ nAChR gene knockdown in microglia demonstrate that both the anti-inflammatory and neuro-protective abilities of ACh rely on microglial $\alpha 7$ nAChR signaling. It was found that knockdown of the $\alpha 7$ nAChR gene in microglia suppressed the effect of ACh inhibition of LPS-elicited inflammatory responses and neuronal apoptosis. These data are consistent with previous findings that demonstrated that ACh modulation of the shifting microglial phenotype towards to M1 subtype elicited by LPS is mediated via the $\alpha 7$ nAChR pathway (Zhang et al., 2017).

Collectively, the findings reported here support the hypothesis that the anti-inflammation mediator ACh effectively remits neuronal damage in AD dependent upon limitation of the microglial inflammatory response via $\alpha 7$ nAChR action on these cells. This establishes a significant role for microglia in the pathogenesis of this neurodegenerative disorder.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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