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Inhibitory role of cholinergic system mediated via α7 nicotinic acetylcholine receptor in LPS-induced neuro-inflammation

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This study investigated the influence of the cholinergic system on neuro-inflammation using nicotinic and muscarinic receptor agonists and antagonists. Intracerebroventricular (ICV) injection of lipopolysaccharide (LPS, 50 μg) was used to induce neuro-inflammation in rats and estimations of pro-inflammatory cytokines, α7 nicotinic acetylcholine receptor (nAChR) mRNA expression were done in striatum, cerebral cortex, hippocampus and hypothalamus at 24 h after LPS injection. Nicotine (0.2, 0.4 and 0.8 mg/kg, i.p.) or oxotremorine (0.2, 0.4 and 0.8 mg/kg, i.p.) were administered 2 h prior to sacrifice. We found that only nicotine was able to block the pro-inflammatory cytokines induced by LPS whereas, oxotremorine was found ineffective. Methyllycaconitine (MLA; 1.25, 2.5 and 5 mg/kg, i.p.), an α7 nAChR antagonist or dihydro-β-erythroidine (DHβE; 1.25, 2.5 and 5 mg/kg, i.p.), an α4β2 nAChR antagonist, was given 20 min prior to nicotine in LPS-treated rats. Methyllycaconitine antagonized the anti-inflammatory effect of nicotine whereas DHβE showed no effect demonstrating that α7 nAChR is responsible for attenuation of LPS-induced pro-inflammatory cytokines. This study suggests that the inhibitory role of the central cholinergic system on neuro-inflammation is mediated via α7 nicotinic acetylcholine receptor and muscarinic receptors are not involved.

Keywords: α7 Nicotinic acetylcholine receptor, α4β2 nicotinic acetylcholine receptor, lipopolysaccharide, nicotine, oxotremorine, pro-inflammatory cytokines

Introduction

Neuro-inflammation has gained increasing relevance in recent times as it is now realized that all major neuropathological states are characterized by significant inflammatory responses and is thought to play a prominent role in neurodegeneration associated with a variety of acute and chronic insults in both the central (CNS) and peripheral nervous system. The contribution of the inflammatory component of diverse disorders to disease progression has led to novel attempts aimed at discovering ways to attenuate inflammation therapeutically.[1] Pro-inflammatory mediators released by activated glial cells during brain inflammation have been proposed to contribute to neuropathology underlying cognitive deficits in Alzheimer's disease.[2] Secretary products of immune-activated glial cells have been shown to disrupt the cholinergic system selectively.[3]

Central or peripheral administration of lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is known to produce a model of neuro-inflammation.[4,5] Lipopolysaccharide activates glial cells in brain releasing inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).[6] The equilibrium between the secretion levels of pro- and anti-inflammatory cytokines and their sequential release may be one of the key determinant factors that accounts for the severity of the inflammatory responses. Any alteration in this equilibrium may convert a beneficial inflammatory response into a severe pathological

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inflammatory outcome. Consequently, great attention is currently devoted to cellular mechanisms which control cytokine levels in neuro-inflammatory conditions.

Among the endogenous mechanisms that regulate the inflammatory response, cross-talk between the immune and nervous systems plays an important role. The cholinergic system has been suggested as a mediator of neuro-immune interactions, or internal regulator of immune responses.[7] The concept of a 'cholinergic anti-inflammatory pathway', by which brain modulates the systemic inflammatory response to endotoxin (LPS), was introduced recently.[8] Anti-inflammatory effects of cholinergic activities have also been shown experimentally by using acetylcholinesterase inhibitors, which prevent acetylcholine (ACh) degradation with resulting inhibition of pro-inflammatory cytokine production in the brain.[5] Acetylcholine is reported to inhibit the release of IL-1β, IL-6, and IL-18 but does not modify IL-10 production in LPS-stimulated macrophages.[9] In addition, it has been reported that acetylcholinesterase (AChE) activity is altered in Alzheimer's disease patients,[10] with the expression and production of the pro-inflammatory and anti-inflammatory cytokines TNF-α, IL-1, IL-6, IL-4.[11] We have reported an increased AChE activity with LPS-induced neuro-inflammatory conditions which shows an inverse relationship between the cholinergic system and neuro-inflammation.[4]

In the peripheral nervous system, studies suggest that the vagus nerve acts as an endogenous 'cholinergic anti-inflammatory pathway' that regulates systemic inflammatory responses by modulating α7 receptors on blood-borne macrophages.[12] To determine whether a similar endogenous 'cholinergic anti-inflammatory pathway' exists in the brain, we have studied the effect of nicotinic and muscarinic receptor agonists and antagonists on intracerebroventricular (ICV) LPS-induced neuro-inflammation.

Materials and Methods

Animals

Adult, male Sprague–Dawley rats weighing 170–200 g were procured from the Laboratory Animal Services Division of Central Drug Research Institute, Lucknow, India. They were kept in polyacrylic cages and maintained under standard housing condition (room temperature 25 ± 1°C and humidity 60–65%) with 12-h light and dark cycle. Food (in the form of dry pellets) and water were available *ad libitum*. Each experimental group consisted of 5 rats. Experiments were performed as per international ethical standards, after obtaining clearance from the animal ethics committee of the Central Drug Research Institute and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Materials

Lipopolysaccharide from *Escherichia coli*, nicotine, oxotremorine M, methyllycaconitine citrate hydrate (MLA), dihydro-β-erythroidine hydrobromide (DHβE), protease inhibitor cocktail, TRIZOL Reagent and bovine serum albumin (BSA) were purchased from Sigma Chemicals (St Louis, MO, USA).

Intracerebroventricular (ICV) injection of LPS

Intracerebroventricular injection of LPS was given using stereotaxic co-ordinates described by Tyagi *et al*.[4] in rats anaesthetized with chloral hydrate (300 mg/kg. i.p.). Bilateral ICV injection of LPS (50 μg/20 μl), dissolved in artificial CSF, was slowly injected 10 μl in each site, using a Hamilton microsyringe. In the control group, artificial CSF (147 mM NaCl, 2.9 mM KCl, 1.6 mM $MgCl_2$, 1.7 mM $CaCl_2$ and 2.2 mM dextrose) was injected in the same volume.

Drug administration

Nicotine (0.2, 0.4 and 0.8 mg/kg) or oxotremorine (0.2, 0.4 and 0.8 mg/kg) were injected intraperitoneally (i.p.) in LPS-treated groups and rats were sacrificed 2 h after drug administration. In LPS pre-treated groups, methyllycaconitine (MLA; 1.25, 2.5 and 5 mg/kg, i.p.), an α7 nicotinic acetylcholine receptor antagonist or dihydro-β-erythroidine (DHβE; 1.25, 2.5 and 5 mg/kg, i.p.), an α4β2 nicotinic acetylcholine receptor antagonist, were given 20 min prior to nicotine administration. All the drugs were dissolved in normal saline.

Tissue collection

Rats were perfused through the heart with ice-cold normal saline at 24 h of LPS injection,[4] under ether anesthesia. The brain was carefully removed from each rat, kept in a Petri dish placed on ice for 15 min and then dissected into different regions – striatum, cerebral cortex, hippocampus and hypothalamus according to Glowinski and Iversen.[13]

Sample preparation and estimation of cytokines

Brain tissues were homogenized in 1 ml of ice-cold Tris buffer (pH 7.2, 4°C) containing 50 mM Tris, 1 mM

EDTA, 6 mM $MgCl_2$ and 5% (w/v) protease inhibitor cocktail. After homogenization, samples were sonicated for 10 s using an ultrasonic processor (Heat Systems-Ultrasonics Inc.) at a setting of 5, and then centrifuged at 20,800 *g* for 20 min at 4°C. Supernatants were collected and cytokines were estimated using ELISA kits (R&D Systems). Cytokines level are expressed as picograms of cytokines per milligram total protein.[14]

Protein estimation

Protein was estimated in all the brain samples by the method of Lowry *et al*.[15] Bovine serum albumin (BSA) at 0.01–0.1 mg/ml was used as standard.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from brain tissues using TRIZOL reagent by a single-step RNA isolation method. Following precipitation, RNA was resuspended in RNase-free water and its concentration was estimated by absorbance at 260 nm. RNA samples were stored at −80°C until analysis.

Single-stranded cDNA was generated from 5 µg of total cellular RNA using oligo-(dT)-primers and RevertAid cDNA synthesis kit (Fermentas). Briefly, 5 µg of total RNA was uncoiled by heating (70°C for 5 min) and then reverse transcribed into complementary DNA (cDNA) in a 20-µl reaction mixture that contained 200 U Moloney murine leukemia virus reverse transcriptase (M-MuLV-RT), 0.5 µg oligo-(dT)-primer, 1 µl Ribolock RNase Inhibitor (20 U/µl), 2 µl of a 10-mM (each) dNTP mix, 4 µl 5 × reaction buffer.

The resultant cDNA was amplified in a 20-µl reaction volume containing 1 U Taq polymerase, 200 µM (each) dNTP mix, 2 mM $MgCl_2$, and 2 µl 10 × Taq buffer with specific primers. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Bioer XP cycler) through 35-cycles at the specifications described in Table 1.

Polymerase chain reaction products were detected by electrophoresis on a 1% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using 100-bp ladder (Fermentas) as a standard size marker. The intensity of polymerase chain reaction products was measured using the Alpha Imager gel documentation system. The signals for examined mRNAs were normalized against that of the actin mRNAs from each sample and the results were expressed as investigated PCR-product/actin mRNA ratio.

Statistical analysis

Results are expressed as mean ± SEM ($n = 5$). The data were analysed by one-way analysis of variance (ANOVA) followed by Newman–Keuls test to determine the significance of difference. The data for gene expression were analysed by Student's *t*-test (unpaired). A P-value < 0.05 was considered as statistically significant.

RESULTS

Effect of nicotine and oxotremorine on LPS-induced changes in TNF-α

Intracerebroventricular administration of LPS (50 µg) showed a significant increase in TNF-α in all the brain regions as compared to the CSF-treated group. Treatment with nicotine (0.2, 0.4 and 0.8 mg/kg, i.p.) showed a significant decrease in LPS-induced TNF-α in all the brain regions except striatum with a 0.2 mg/kg dose and hypothalamus with 0.8 mg/kg dose (Fig. 1A). *Per se*, nicotine did not produce any significant change on TNF-α in any brain region as compared to the CSF-treated group (data not shown). Whereas oxotremorine did not produce any significant change in TNF-α at any of the doses used (0.2, 0.4 and 0.8 mg/kg, i.p.) in any of the brain regions as compared to the LPS-treated group (Fig. 1B).

Effect of nicotine and oxotremorine on LPS-induced changes in IL-1β

A significant increase was found in IL-1β after LPS (50 µg, ICV) administration in all the brain regions as

Table 1. Sequences of primers used in experiments and products size

Primer	Sequence	Annealing temperature (°C)	bp
α7 nAChR	5′-GTGGAACATGTCTGAGTACCCCGGAGTGAA-3′ 5′-GAGTCTGCAGGCAGCAAGAATACCAGCA-3′	60	510
β-Actin	5′-GGCTGTGTTGTCCCTGTAT-3′ 5′-CCGCTCATTGCCGATAGTG-3′	55.7	352

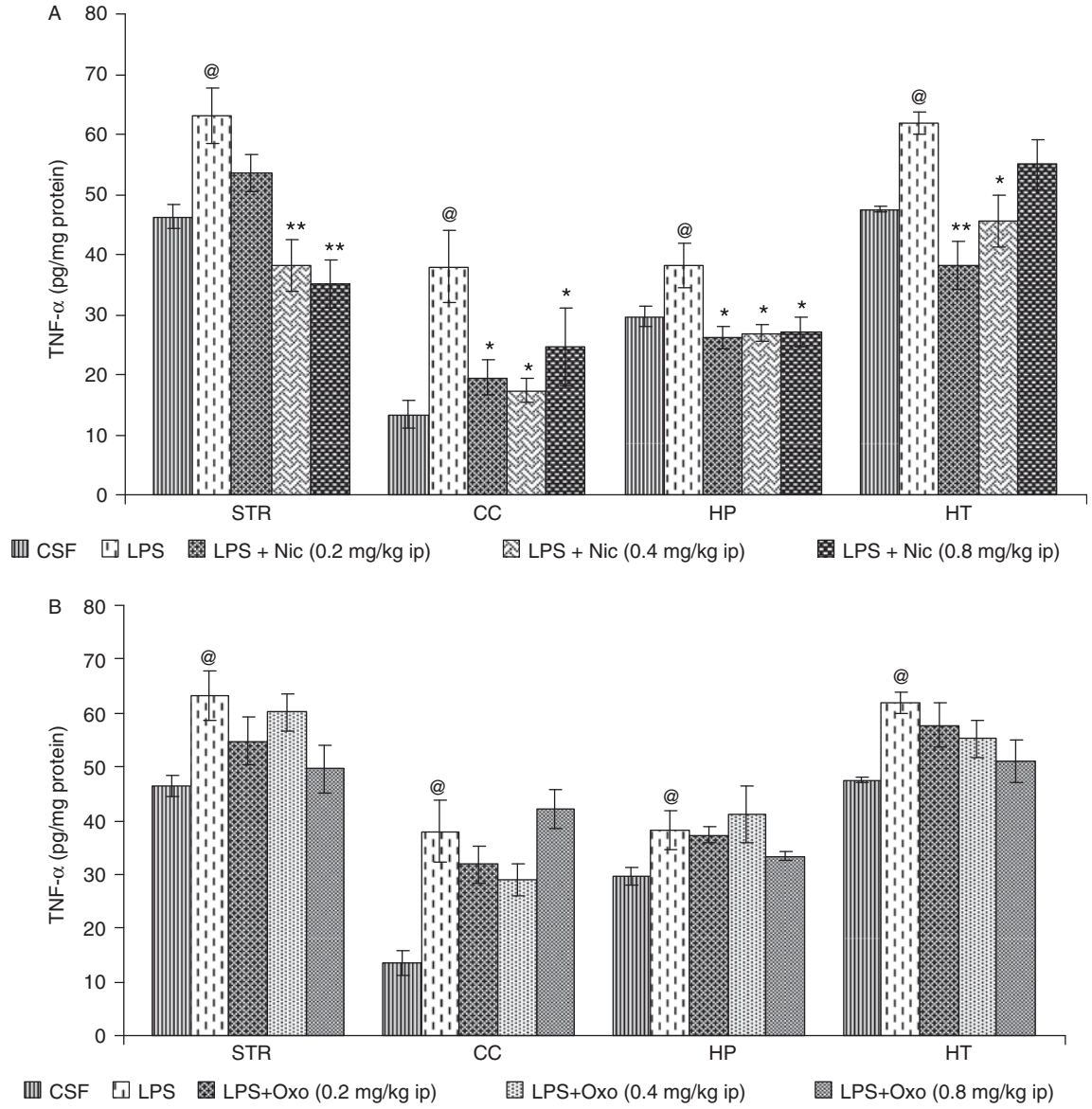

Fig. 1. Effect of (A) nicotine (0.2, 0.4 and 0.8 mg/kg, i.p.) and (B) oxotremorine (0.2, 0.4 and 0.8 mg/kg, i.p.) on LPS (50 μg, ICV) induced changes in TNF-α in different brain regions – striatum (STR), cerebral cortex (CC), hippocampus (HP) and hypothalamus (HT). Values are expressed as mean ± SEM of each group $n = 5$. $^{@}P < 0.05$ CSF- versus LPS-treated group; $^{*}P < 0.05$, $^{**}P < 0.01$ LPS- versus LPS + drug-treated groups.

compared to the CSF-treated group. Treatment with nicotine (0.4 and 0.8 mg/kg, i.p.) showed a significant decrease in elevated level of IL-1β in all the brain regions except hypothalamus whereas the lower dose (0.2 mg/kg, i.p.) of nicotine showed a significant decrease in IL-1β only in striatum and hippocampus (Fig. 2A). *Per se*, nicotine did not show any significant change on IL-1β in any brain region as compared to the CSF-treated group (data not shown). Oxotremorine (0.2, 0.4 and 0.8 mg/kg, i.p.) treatment did not show any significant change in IL-1β in any of the brain regions as compared to the LPS-treated group (Fig. 2B).

Only nicotine was found effective in inhibiting TNF-α and IL-1β induced by LPS. Therefore, we used MLA and DHβE, specific antagonists for α7 and α4β2 nicotinic acetylcholine receptors (nAChRs), respectively, for further studies with nicotine at the dose of 0.4 mg/kg, as this dose of nicotine was effective in regulating LPS-induced pro-inflammatory cytokines in all the brain regions.

Effect of MLA and DHβE on nicotine-induced attenuation of LPS-induced TNF-α

The attenuating effect of nicotine on LPS-induced TNF-α was blocked by MLA at a dose of 5 mg/kg (i.p.) in all the brain regions, as TNF-α was found

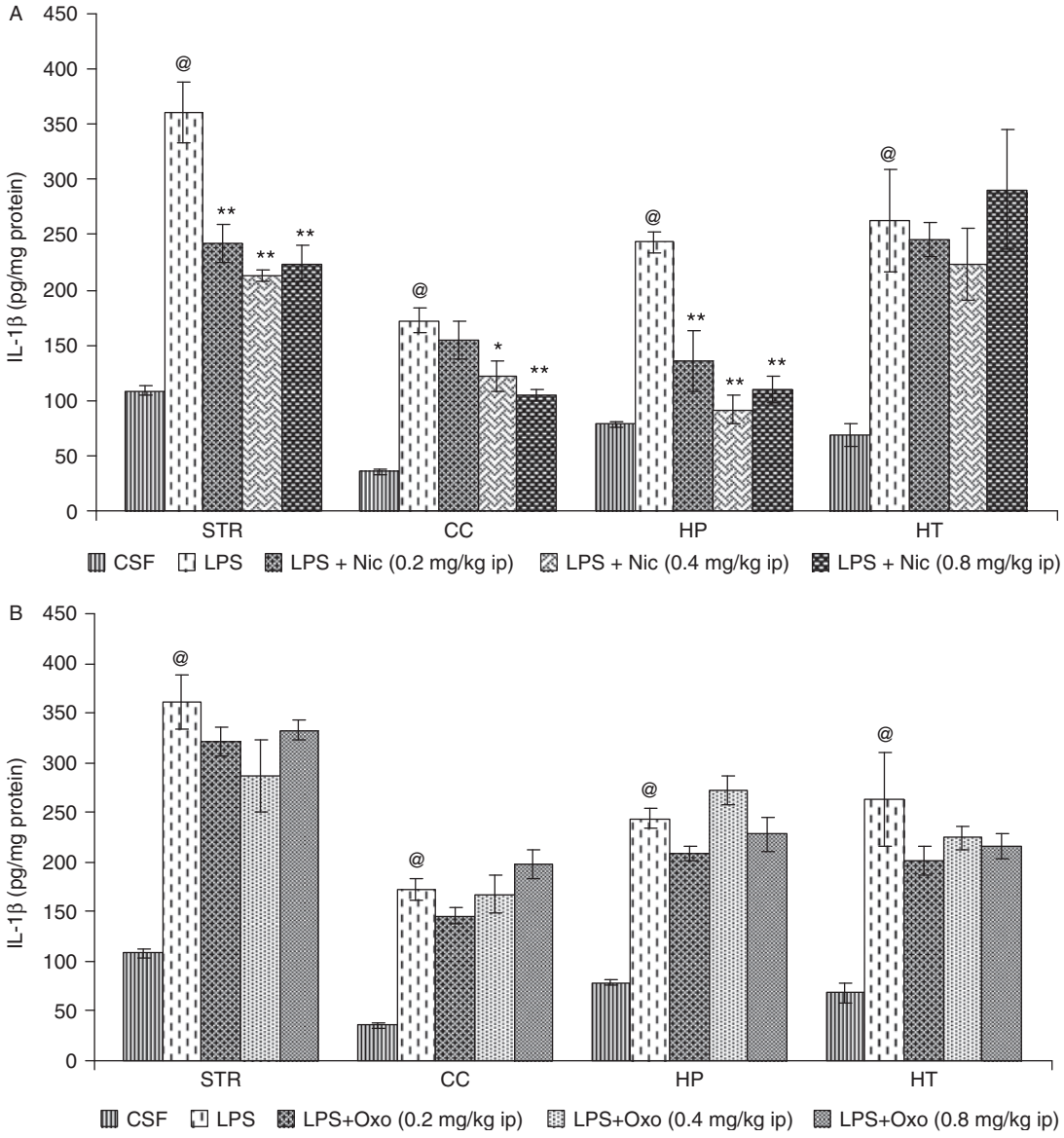

Fig. 2. Effect of (A) nicotine (0.2, 0.4 and 0.8 mg/kg, i.p.) and (B) oxotremorine (0.2, 0.4 and 0.8 mg/kg, i.p.) on LPS (50 μg, ICV) induced changes in IL-1β in different brain regions – striatum (STR), cerebral cortex (CC), hippocampus (HP) and hypothalamus (HT). Values are expressed as mean ± SEM of each group $n = 5$. $^{@}P < 0.05$ CSF- versus LPS-treated group; $^{*}P < 0.05$, $^{**}P < 0.01$ LPS- versus LPS + drug-treated groups.

to be significantly increased in the MLA-treated group as compared to the nicotine-treated group. The lower dose (1.25 mg/kg, i.p.) of MLA was effective in antagonizing the effect of nicotine only in striatum and cerebral cortex whereas with the 2.5 mg/kg dose, MLA showed its effect only in cerebral cortex (Fig. 3A).

Dihydro-β-erythroidine at any dose (1.25, 2.5 and 5 mg/kg, i.p.) did not show the antagonizing effect on nicotine-mediated inhibition of LPS-induced TNF-α in any of the brain regions as there was no significant difference found in TNF-α in the DHβE-treated group as compared to the nicotine-treated group (Fig. 3B).

Effect of MLA and DHβE on nicotine-induced attenuation of LPS-induced IL-1β

Treatment with MLA (5 mg/kg, i.p.) significantly antagonized the nicotine-mediated inhibition of LPS-induced IL-1β in all the brain regions except hypothalamus whereas with 1.25 and 2.5 mg/kg dose, MLA showed its effect only in striatum and hippocampus as there was significant increase found in IL-1β after MLA treatment when compared with the nicotine-treated group (Fig. 4A).

The attenuating effect of nicotine on LPS-induced IL-1β was not affected by DHβE in any of the brain regions, at any dose (1.25, 2.5 and 5 mg/kg, i.p.), as there was no

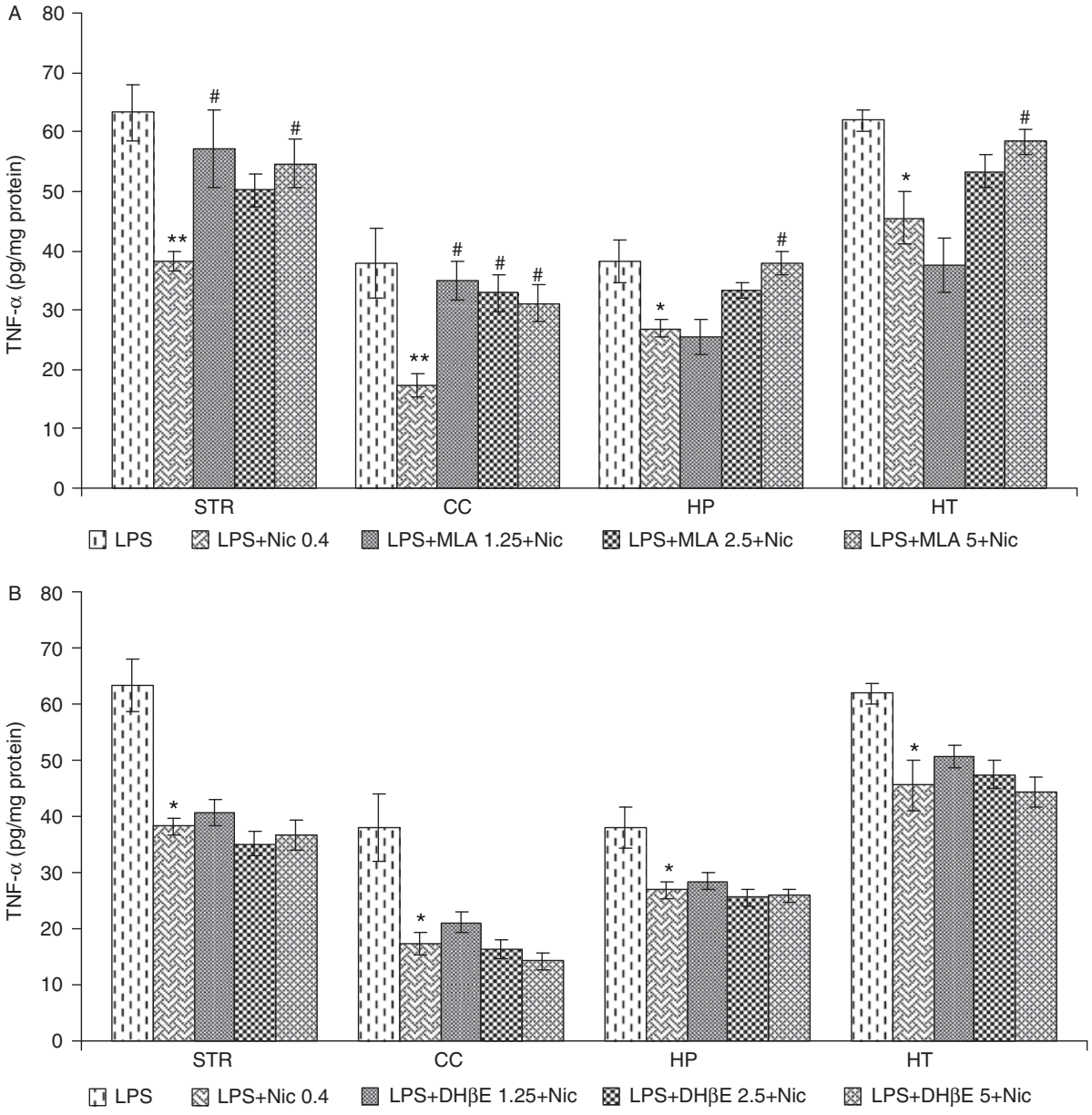

Fig. 3. Effect of (A) MLA (1.25, 2.5 and 5 mg/kg, i.p.), an α7 nAChR antagonist and (B) DHβE, an α4β2 nAChR antagonist, on nicotine (0.4 mg/kg, i.p.) induced attenuation of LPS (50 μg, ICV) induced TNF-α in different brain regions – striatum (STR), cerebral cortex (CC), hippocampus (HP) and hypothalamus (HT). Values are expressed as mean ± SEM of each group $n = 5$. $^{*}P < 0.05$ LPS versus LPS + Nic; $^{\#}P < 0.05$ LPS + Nic versus LPS + MLA + Nic and LPS + DhβE + Nic treated group.

significant difference found in IL-1β in the DHβE-treated group in comparison to the nicotine-treated group (Fig. 4B).

Effect of MLA on LPS-induced TNF-α and IL-1β

Per se treatment of MLA (5 mg/kg, i.p.) did not show any significant changes in LPS-induced TNF-α and IL-1β, when compared with the LPS-treated group (Fig. 5A,B).

Effect of LPS on α7 nAChR mRNA expression by RT-PCR

Expression of α7 nAChR mRNA was examined by RT-PCR in different brain regions. Treatment with LPS

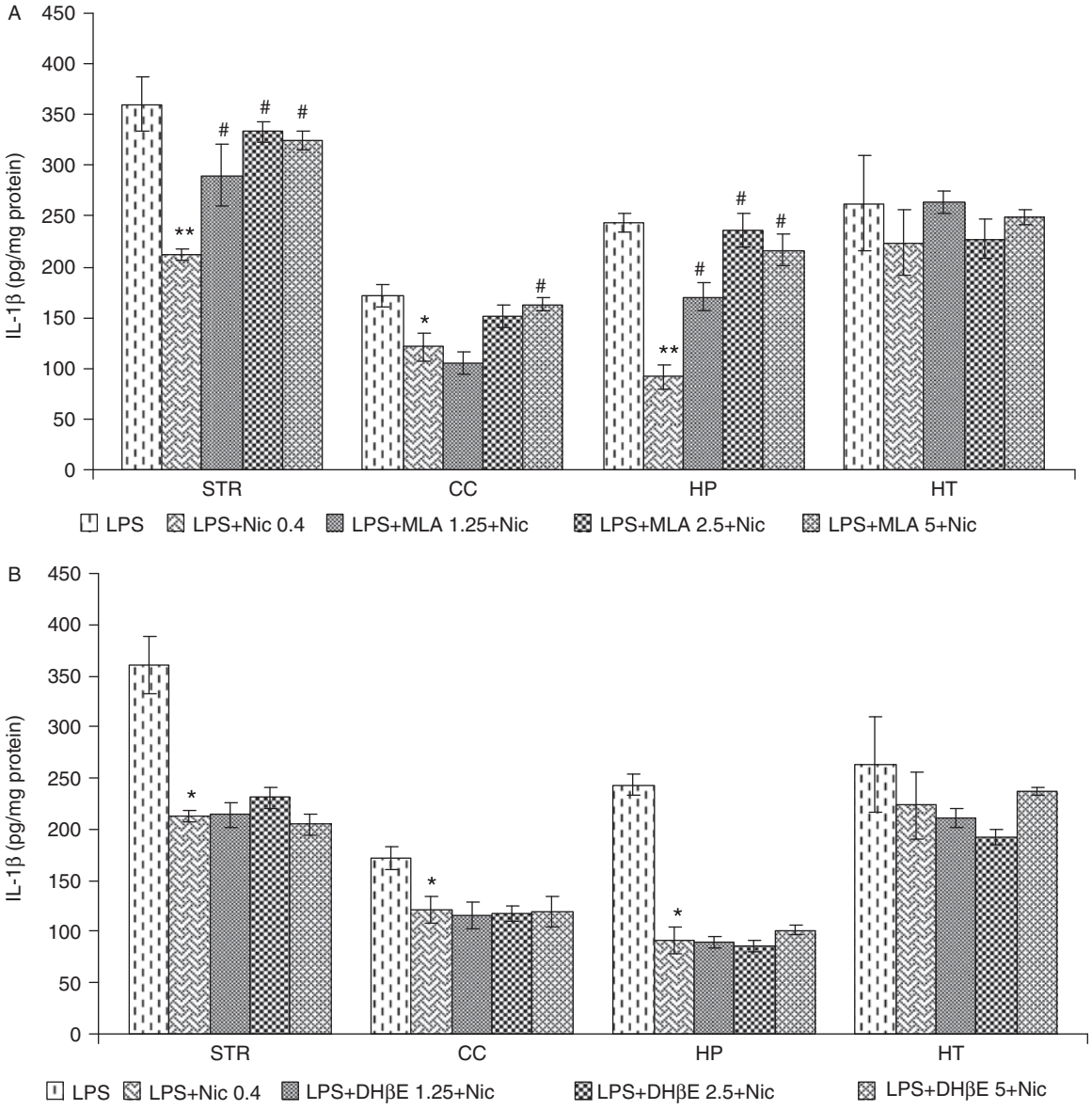

Fig. 4. Effect of (A) MLA (1.25, 2.5 and 5 mg/kg, i.p.), an α7 nAChR antagonist and (B) DHβE, an α4β2 nAChR antagonist, on nicotine (0.4 mg/kg, i.p.) induced attenuation of LPS (50 μg, ICV) induced IL-1β in different brain regions – striatum (STR), cerebral cortex (CC), hippocampus (HP) and hypothalamus (HT). Values are expressed as mean ± SEM of each group $n = 5$. $^{*}P < 0.05$ LPS versus LPS + Nic; $^{\#}P < 0.05$ LPS + Nic versus LPS + MLA + Nic and LPS + DhβE + Nic treated group.

(ICV, 50 μg) did not show any significant change in gene expression of α7 nAChR as compared to the CSF-treated group in any of the brain regions (Fig. 6).

Discussion

Recent findings indicate that neural mechanisms are involved in limiting inflammatory responses. Acetylcholine is a major parasympathetic neurotransmitter and inhibits LPS-induced production of pro-inflammatory cytokines, including IL-1, TNF-α from macrophages,[16] and microglia.[17] Recent reports suggest that electrical or pharmacological activation of the efferent vagus nerve, which activates the release of acetylcholine, inhibits the release of TNF-α and attenuates the development of endotoxin-induced shock in rodents.[9] Immune cells possess a complete cholinergic system consisting of acetylcholine muscarinic and nicotinic receptors (mAChR and nAChR), choline acetyl-transferase (ChAT) and acetylcholinesterase (AChE).[18] Nicotinic acetylcholine receptors are

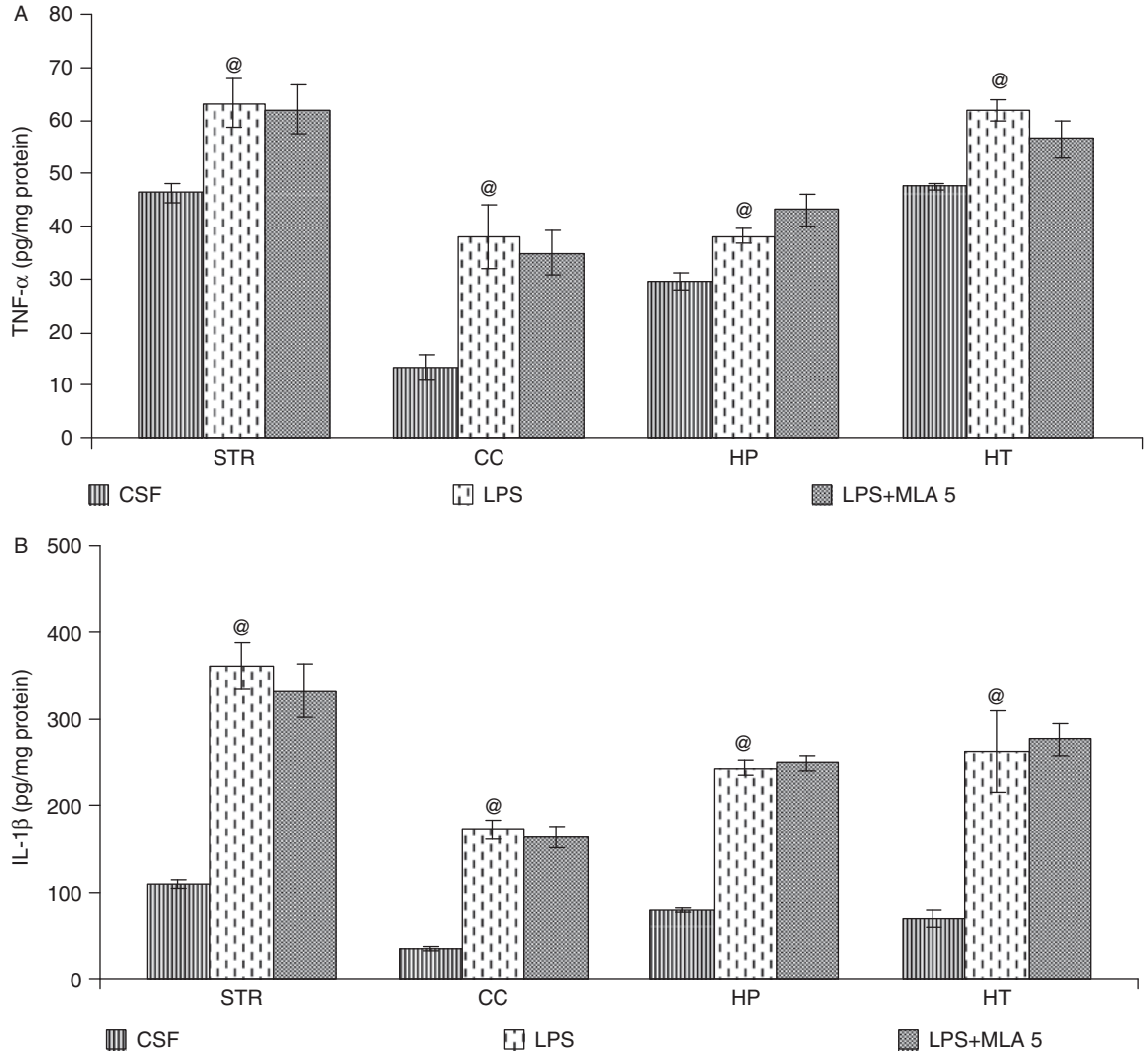

Fig. 5. *Per se* effect of MLA (5 mg/kg, i.p.), an α7 nAChR antagonist, on LPS (50 μg, ICV) induced changes in (A) TNF-α and (B) IL-1β in different brain regions – striatum (STR), cerebral cortex (CC), hippocampus (HP) and hypothalamus (HT). Values are expressed as mean ± SEM. of each group $n = 5$. $^{@}P < 0.05$ CSF- versus LPS-treated group.

ligand-gated pentameric ion channels which consist of homologous or heterologous subunits whose opening is controlled by ACh, nicotine and other receptor agonists. Muscarinic receptors are a family of G protein-coupled receptors that have a primary role in central cholinergic neurotransmission. Both muscarinic and nicotinic receptors are distributed in the CNS and periphery with different synaptic locations and functions in cholinergic transmission. We studied the abilities of nicotine and oxotremorine, agonists of nicotinic and muscarinic acetylcholine receptors respectively, to their effect on LPS-induced neuro-inflammation.

Pavlov *et al.*[19] implicated muscarinic receptor-dependent mechanisms in the central modulation and integration of peripheral inflammation. Central muscarinic acetylcholine receptors have been shown to activate the cholinergic anti-inflammatory pathway and inhibit systemic TNF-α in endotoxemic rats. In contrast, peripheral muscarinic receptors do not have a major role in mediating the inflammatory response to endotoxin and its inhibition by the cholinergic anti-inflammatory pathway.[19] We also observed that the activation of muscarinic receptor via oxotremorine failed to regulate TNF-α and IL-1β in LPS-induced neuro-inflammation.

Nicotine has been recently been reported to inhibit TNF-α production in human macrophages,[9] as well as in rat microglial cultures.[20] Furthermore, epidemiological studies have consistently shown that the incidence of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease is lower in cigarette smokers than in age-matched controls.[21] These findings have prompted speculation that brain nicotinic receptors could be important therapeutic targets for

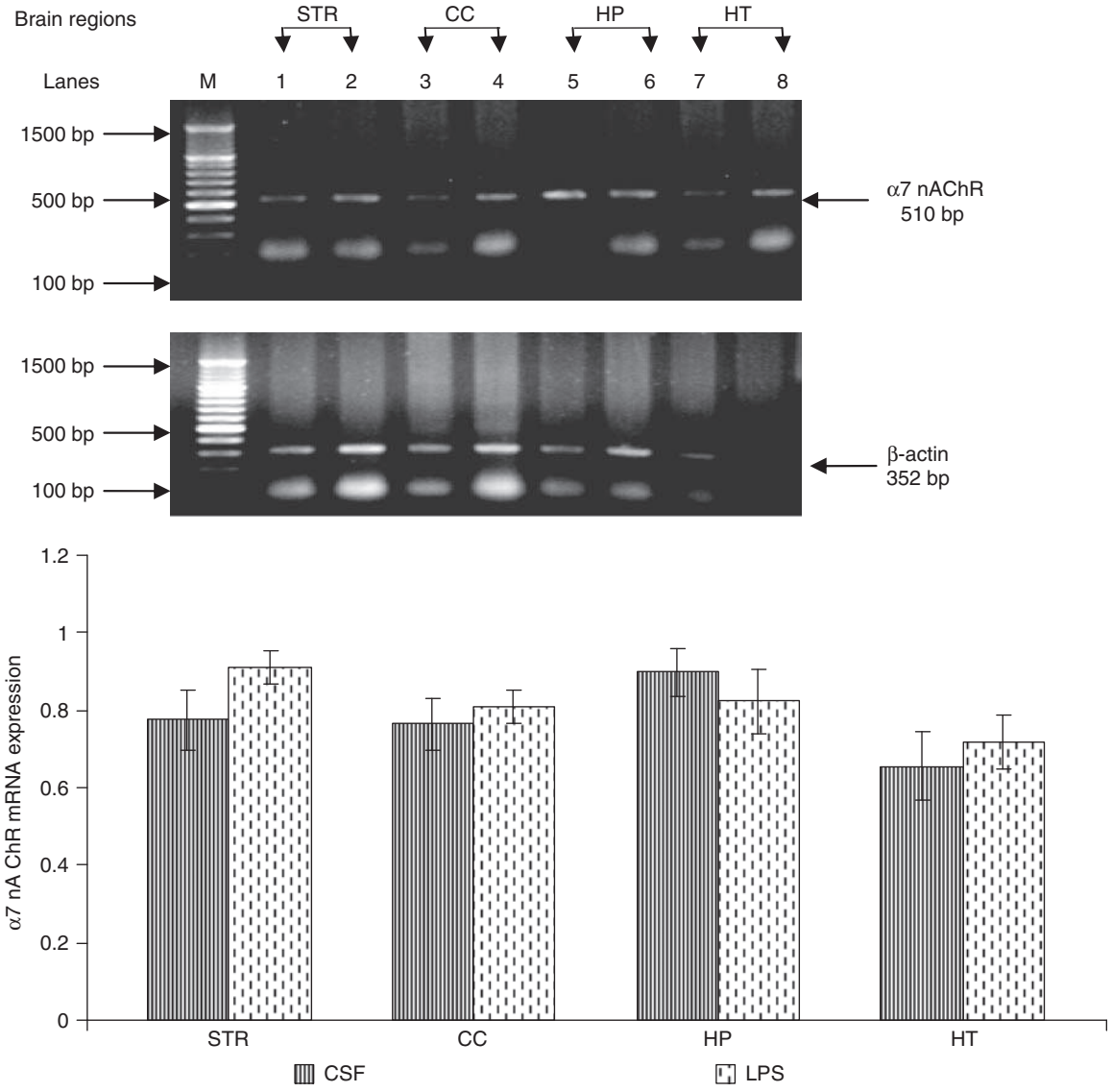

Fig. 6. Expression of α7 nAChR mRNA examined by RT-PCR in different brain regions – striatum (STR), cerebral cortex (CC), hippocampus (HP) and hypothalamus (HT). Lanes 1, 3, 5, and 7 represent CSF treatment; lanes 2, 4, 6, and 8 represent LPS treatment. Lane M is a 100 bp-DNA ladder indicating the size of PCR products. α7 nAChR mRNA levels were normalized with the levels of β-actin.

Alzheimer's disease. The beneficial effects of nicotine have been attributed to an up-regulation of nicotine receptors that are deficient in the Alzheimer's disease brain, or possibly a protection from the Aβ-induced neurotoxicity,[22,23] or inhibiting β-amyloidosis in transgenic mice.[24,25] Loss or down-regulation of the neuronal nAChRs has also been observed,[26,27] in Alzheimer's disease brains.

In the present study, we found that nicotine exerted inhibition of LPS-induced TNF-α and IL-1β whereas there was no protection with oxotremorine. Results obtained from this study suggest that nicotinic receptors are more involved than muscarinic receptors in regulating the inflammatory cascade in brain. However, *per se*, nicotine did not show any effect. While measuring the impact of the nAChR system on pro-inflammatory function, it is important to identify the role of different subunits of nAChR responsible for exerting anti-inflammatory potential by inhibiting LPS-induced central pro-inflammatory cytokines.

Among nAChRs, α7 and α4β2 are the most abundant subunits in the brain.[28] Reports suggest the involvement of α4β2 and α7 nAChRs in the neuroprotective effect of donepezil and of galanthamine.[29] Therefore, we studied the effect of MLA, an α7 nAChR antagonist or DHβE, an α4β2 nAChR antagonist, only MLA was able to block the effect of nicotine on pro-inflammatory cytokine induced by LPS. There was no change in the effect of nicotine with α4β2 treatment, which indicates that the anti-inflammatory effect of nicotine

is mediated through the α7 subunit of the nAChR. Although we have not observed a clear dose response with antagonist treatment, that may be due to the altered sensitivity of receptors in the inflammatory condition. Moreover, ACh-dependent de-activation of peripheral macrophages is mediated by the α7 subunit of the nAChR, which has been suggested important for the 'cholinergic anti-inflammatory pathway'.[12,16] In Alzheimer's disease brains, higher expression levels of α7 nAChRs in the hippocampus,[30] and frontal cortex,[31] were also reported. Further, we have studied whether *per se* MLA would be able to regulate the release of pro-inflammatory cytokines in response to LPS. Results showed that MLA *per se* was unable to affect the LPS-induced release of pro-inflammatory cytokines suggesting that blockade of α7 nAChR does not influence LPS-mediated effects; this indicates that there is no significant role of endogenous acetylcholine through α7 nAChR. We also observed that LPS had no significant effect on mRNA expression of α7 nAChR indicating that exogenous stimulation of α7 nAChR with nicotine is inhibitory for LPS-induced neuro-inflammation.

CONCLUSIONS

The results of the present study suggest that the α7 nAChR subtype may have an important role in attenuating the effect of nicotine on LPS-induced neuro-inflammation. Thus, the inhibitory role of the cholinergic system on neuro-inflammation via modulating α7 nAChR indicates that a similar cholinergic anti-inflammatory pathway exists in the brain as in the periphery and α7 nAChR may have a potential therapeutic target to regulate neuro-inflammation.

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