Nicotine Activating $\alpha_4\beta_2$ Nicotinic Acetylcholine Receptors to Suppress Neuroinflammation via JAK2-STAT3 Signaling Pathway in Ischemic Rats and Inflammatory Cells

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Abstract
Nicotine plays a role in inhibiting inflammatory factors, which contributes to improving cognitive impairment by activating $\alpha_4\beta_2$ nAChRs in ischemic rats, but the underlying mechanism has not been fully elucidated. Janus tyrosine kinase 2-signal transducer and activator of transcription 3 (JAK2-STAT3) signaling pathway is involved in cognitive improvement, and there seems to be a relationship between nAChRs and JAK2-STAT3 as well. The aim of this study is to explore the role of JAK2-STAT3 signaling pathway in nicotine-mediated anti-inflammatory effect. Nicotine, DHβE (the strongest competitive antagonist of $\alpha_4\beta_2$ nAChRs), and AG490 (a specific JAK2-STAT3 blocker) were used to intervene and treat ischemic rats and HEK-293 T-h$\alpha_4\beta_2$ cells. The Morris water maze (MWM) test and 2-$^{[18F]}$-A-85380 PET imaging were performed to detect the cognitive function and $\alpha_4\beta_2$ nAChRs density in ischemic rats. The results demonstrated that nicotine intervention increased the density of $\alpha_4\beta_2$ nAChRs and improved cognitive impairment, but this effect was blocked by AG490, and the receptors were still upregulated. Essentially, when the JAK2-STAT3 signaling pathway was blocked, nicotine could only upregulate the expression of $\alpha_4\beta_2$ nAChRs, but not improve the cognitive function. PCR and Western blot analysis further confirmed these results. The cell experiments also showed that nicotine could reduce inflammatory factors stimulated by LPS and upregulate the expression of pJAK2 and pSTAT3 in HEK-293 T-h$\alpha_4\beta_2$ cells, while AG490 and DHβE reversed the effect of nicotine. To sum up, our work indicated that JAK2-STAT3 signaling pathway played an important role in nicotine-induced cognitive improvement by upregulating $\alpha_4\beta_2$ nAChRs in ischemic rats.

Keywords Cognitive impairment · $\alpha_4\beta_2$ nicotinic acetylcholine receptor · Nicotine · JAK2-STAT3 · Neuroinflammation

Introduction
Vascular cognitive impairment (VCI) refers to the contribution of vascular pathology of any severity to cognitive impairment of any degree [1], ranging from cognitive impairment to dementia. In Western countries, it is the second leading cause of dementia after Alzheimer’s disease, and it may be the most important cause in East Asia [2]. Cholinesterase inhibitors and the N-methyl D-aspartate antagonist memantine are involved in symptomatic treatment of VCI [2]. However, they showed little benefit on cognition improvement in randomized controlled trials [2]. Recent studies have showed that nicotine could play an important role in protecting cognitive function [3–6]. Nicotine is an agonist of neuronal nicotinic acetylcholine receptors (nAChRs), which are composed of five subunits arranged around a water-filled pore. The most abundant

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subtypes of nAChR in the mammalian brain are hetero-meric α4β2 nAChRs and homomeric α7 nAChRs, among which α4β2 is the most abundant subtype in the CNS and with high affinity for nicotine [7–9]. Histopathological studies showed that inflammation caused by excessive activation of microglia can destroy the blood–brain barrier, cause brain injury, and promote the occurrence and development of VCI under ischemic conditions [10–13]. Our prior studies have confirmed that the chronic ischemic cognitive dysfunction was correlated with the decrease of α4β2 nAChRs [14], and nicotine played a role in inhibiting the inflammatory factors, which contributes to improving cognitive impairment by activating α4β2 nAChRs in ischemic rats [15]. How α4β2 nAChRs activated by nicotine acts on inflammatory factors remains uncertain.

It is known that Janus tyrosine kinase 2/signal transducer and activator of transcription 3 (JAK2-STAT3) signaling pathway is the interaction chain between intracellular proteins, and it is involved in processes of immunity, cell division, cell death, and tumor formation. Abnormal activation of JAK2-STAT3 signaling pathway is often closely related to the occurrence, development, and prognosis of inflammatory diseases, tumors, autoimmune diseases, and so on [16–18]. Studies have shown that JAK2 and STAT3 can regulate the plasticity of hippocampal synapses, which is closely related to learning and memory [19]. Recent studies have also found that JAK2-STAT3 signaling pathway is involved in cognitive improvement [20–23], and JAK2-STAT3 signaling pathway is considered to play a role in the activation of α4-, α5-, and α7- nAChRs [24–28]. There also seems to be a relationship between α4β2 nAChRs and JAK2-STAT3 [29, 30].

The aim of this study is to explore the relationship between JAK2-STAT3 signaling pathway and α4β2 nAChRs activation induced by nicotine in ischemic rats and inflammatory cells. Nicotine, DHβE (the most potent competitive antagonist of α4β2 nAChRs [31, 32]), and AG490 (a specific JAK2-STAT3 blocker [33, 34]) were adopted for intervention treatment in ischemic rats and inflammatory cells, and 2-[^18F]-A-85380 micro-PET imaging was performed for detecting α4β2 nAChRs [35] in ischemic rats.

Methods and Materials

Animal Experiments

All animal experiments were approved by the Ethics Committee of The First Hospital of China Medical University, and all experiments were conducted according to relevant guidelines and regulations.

Laboratory Animals and Groups

Forty-seven male Sprague–Dawley (SD) rats, about 8 weeks old and weighing 250±20 g, were ordered (China Medical University animal laboratory, SYKK (Liao)2008–0005, Shenyang, China) to SPF animal laboratory. Before the experiment, the rats were fed freely and given 12-h–12-h light–dark cycle.

The ischemic model was established by injecting ET-1 (MERCK, USA) into the left thalamus, and the detailed steps were described in the previous study [15]. Establishment of the sham operation group model: the specific method was the same as above, but normal saline is used instead of ET-1. On the first day after operation, nicotine (Sigma-Aldrich), DHβE (ApexBio, USA), AG490 (SELL- ECK, USA), or saline were given in different groups: nicotine group (nicotine, 1.5 mg/kg/d), nicotine+DHβE group (nicotine, 1.5 mg/kg/d; DHβE, 3 mg/kg/d), DHβE group (DHβE, 3 mg/kg/d), nicotine + AG490 group (nicotine, 1.5 mg/kg/d; AG490, 3 mg/kg/d), AG490 group (AG490, 3 mg/kg/d), ischemia group (saline, 0.8 ml/kg/d), and sham operation group (saline, 0.8 ml/kg/d). Each group consisted of 6 rats, and all 42 rats were intervened by intraperitoneal injection for nine consecutive days.

The remaining 3 SD rats did not receive any intervention, neither surgery nor drug treatment, and they were used as a control group for PCR test. Twenty-four hours after operation, two other rats were sacrificed, one from the sham operation group and the other from the ischemia group. Histological analysis was performed only by HE staining to ensure the success of the ischemia model (Fig. 1).

Morris Water Maze Experiment

On the fourth day of drug intervention, the Morris water maze (MWM) (endocrinology laboratory, China Medical University) test was conducted to detect the spatial learning and memory ability of rats. Different groups of rats were tested for six consecutive days, and the experiment was divided into the following two parts:

Directional navigation experiment: the experiment lasted for 5 days. On the first day, a directional navigation program was established. Rats were put into the pool from any two quadrants to get familiar with the water maze environment. The platform was placed in the center of a quadrant of the pool. In the next 4 days, the training test was conducted at the same time every day. The software automatically recorded the swimming track and time of each rat in the pool.

The space exploration experiment: On the sixth day of the experiment, after the establishment of the space exploration scheme, the platform was removed, and the rats were put in any quadrant (except the target quadrant where the
The software automatically recorded and analyzed the number of times that the rats passed through the target quadrant and the activity time in the target quadrant in 120 s.

2-[18F]-A-85380 Micro-PET Imaging

After the MWM test, rats in each group underwent 2-[18F]-A-85380 micro-PET imaging (Shandong Madic Technology Co., Ltd, China) on the 6th day of MWM. 2-[18F]-A-85380 is a selective radioligand of α4β2 nAChR and has a high affinity for this subtype [35]. 2-[18F]-A-85380 was synthesized according to the previous report [36], which included the synthesis procedure, the information of the average yield, specific activity, chemistry, and radiochemistry necessary for the synthesis. The radiochemical purity of the product 2-[18F]-A-85380 was 98.94%, which can meet the imaging requirements of 2-[18F]-A-85380.

Micro-PET imaging was performed with a spatial resolution of 1.3 mm in the transverse and axial directions and field of vision (transaxial: 128 mm, axial: 59 mm). Rats were injected with 37 MBq 2-[18F]-A-85380 through tail vein. After 120 min, brain scan was performed for 10 min.

Quantitative analysis: Drawing ROI was based on standard rat brain atlas by Paxinos [37]. The cerebellum was used as the reference area, and the ratio of the average SUV (SUVave) in each area to the SUVave of the cerebellum was calculated. The distribution of 2-[18F]-A-85380 in the left thalamus and the whole brain of each group was analyzed quantitatively.

Real-time PCR Detection of Receptor Subunits of nAChRs

Firstly, RNA was extracted, and then, cDNA was synthesized by the reverse transcription. The mixture was homogenized, and the sample was put on a reverse transcription instrument at 37 °C, 15 min; 85 °C, 5 s; and 4 °C, 10 min. After the reaction, the real-time PCR amplification curve and dissolution curve were determined, and the CT value of the sample was automatically calculated by software.

Western Blot

The tissue of the left thalamus was taken and stored in an Eppendorf tube of the refrigerator at −80 °C. One milliliter of RIPA buffer and PMSF mixture (RIPA:PMSF = 100:1) were added to each group of tissues. After homogenization on ice by tissue homogenizer, the tissues were placed on ice for 30 min, centrifuged at 4 °C, 15,000 rpm for 15 min. Then, the supernatant was then then stored at −80 °C. According to BCA protein quantitative standard curve, different samples were adjusted to the same concentration with lysis buffer (3–5 μg/μl for each sample). All the samples were denatured by heating at 95 °C for 10 min, and stored at −20 °C. Finally, a 15–20-μl sample was added into each lane to ensure the same amount of protein. Equal amounts of proteins were fractionated by SDS–PAGE electrophoresis apparatus, transferred to PVDF membranes. Immunoblotting was carried out with antibodies against α4-nAChR (1:500), β2-nAChR (1:500), IL-1β (1:2000), IL-6 (1:2000), JAK2 (1:1000), STAT3 (1:1000), p-JAK2 (1:1000), p-STAT3 (1:2000), and β-actin (1:1000) at 4 °C for 24 h. The membranes were incubated with corresponding secondary antibodies at room temperature for 1 h. Western blots were developed by enhanced chemiluminescence detection system. Image analysis of gel electrophoresis was carried out.
with Image Lab software. Quantitative analysis is carried out with the gray value of the panel, and the histogram was obtained, which shows the protein expression of each group in each index. Quantitative analysis was carried out based on three separate samples’ preparation following with Western blot.

Primary antibodies (α4-nAChR, ab41172; β2-nAChR, ab189174; IL-1β, ab2105; IL-6, ab9324; p-JAK2, ab32101) were purchased from ABCAM company, USA; primary antibodies (JAK2, D2E12; STAT3, 12H6; p-STAT3, D3A7) were from Cell Signaling Technology, USA; BCA Protein Assay Kit was from Beyotime Biotechnology Research Institute, China; RIPA Lysis Buffer was from Beyotime Biotechnology Research Institute, China; PMSF was from Beijing Solarbio Science & Technology company, China.

Cell Experiment

Cell Culture

HEK-293 T cells were cultured at 37 °C in condition of 5% CO2 and 95% oxygen in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 units/ml). The cells were divided into two groups: (1) the control (Con) group: no cells were infected with lentiviral; (2) the over-expression (OE) group: the cells were infected with two lentiviruses that upregulated the expression of CHRNA4 and CHRNB2, which encode the α4 subunit and β2 subunit of α4β2 nAChR respectively.

Construction of a Stable Cell Line with Upregulated CHRNA4 and CHRNB2 Expression

For lentivirus production, HEK-293 T cells were cotransfected with PGMLV-CMV-H_CHRNA4-EF1-ZsGreen1-T2A-Puro or PGMLV-CMV-H_CHRNB2-EF1-mCheery-T2A-Blasticidin. The plasmid was amplified in E. coli DH5 cells, purified using a Plasmid Maxi Kit (Tiangen, Beijing, China), and transfected into 70% confluent 293 T cells using HG transgene reagent (Genomeditech, Shanghai, China). Seventy-two hours after the transfection, the lentiviral particles were harvested from the supernatant and purified by ultracentrifugation. These particles will be referred to hereinafter as LEN-CHRNA4 and LEN-CHRNB2. Stably infected 293 T cells with CHRNB2 were selected using blasticidin (10 μg/ml; Beyotime Biotechnology, CA), which killed the uninfected cells. Real-time PCR and Western blotting confirmed that the LEN-CHRNB2-infected 293 T cells stably over-expressed CHRNA4.

RNA Extraction and Quantitative RT-PCR

The total RNA from cells was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by RevertAid Reverse Transcriptase (Takara, Tokyo, Japan). Real-time PCR was carried out on StepOnePlus Real Time System (Applied Biosystems, Foster City, CA, USA), and the reaction steps were as follows: 95 °C for 30 s, then 40 cycles at 95 °C for 5 s, and then 60 °C for 30 s. The primers used for RT-PCR to amplify α4-nAChR and β2-nAChR were as described above. The primers for GAPDH (internal standard) are as follows.

GAPDH: F: 5′-GAGTCAACGGATTTGGTCTCG-3′
R: 5′-TTGATTTTTGAGGGATCTCG-3′

The 2–ΔΔCt method was used to quantify mRNA expression relative to GAPDH.

Protein Extraction and Western Blot Analysis

HEK-293 T cells were seeded in 6-well plates. After treatments, the total extracts were extracted according to the protocols supplied by the manufacturer (Sangon, Shanghai, China). Next, identical amounts of proteins were separated by SDS–PAGE and then blotted onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk as described elsewhere. They were then incubated with conjugated goat antirabbit antibodies at 4 °C overnight. Thereafter, the membranes were incubated with the secondary antibody for 1 h at room temperature. Finally, the membranes were detected using the EZ-ECL.

Primary antibodies (GAPDH, AF1186) were purchased from Beyotime Biotechnology Research Institute, China; primary antibodies (TNF-α, ab66579; p-STAT3 ab76315) were purchased from ABCAM company, USA. Other materials were mentioned above.

Statistical Analysis

All statistical analyses were performed using ANOVA of IBM SPSS Statistics. Variance analysis was applied for inter-group comparison and Tukey’s method was used for multiple comparison correction. The Shapiro–Wilk test was used to check the normal distribution. At least three individual trials were performed for each experiment and data was represented as mean ± SD. p < 0.05 was considered statistically significant. Specific p values were indicated in notes of tables and figures.
Results

Animal Experiments

MWM Test Indicated Nicotine Improving Learning and Spatial Memory Was Blocked by DHβE and AG490

Normality was checked using the Shapiro–Wilk test, which showed that the data fit a normal distribution. We assumed the normal distribution, because the sample size was too small to properly test the normal assumption. The latency in directional navigation experiment was analyzed by ANOVA of repeated measurement design data. The escape latency of rats in each group decreased with the increase of experimental days, but the interaction between time and groups was not statistically significant ($F = 0.058$, $p = 0.99 > 0.01$), indicating that the time factors did not change with the grouping. There were significant differences between different groups in escape latency ($F = 4.942$, $p = 0.01 < 0.05$) (Table 1). The escape latency of rats in the nicotine group and the sham operation group was shorter than that of ischemic rats treated with saline only, and the difference was statistically significant ($p < 0.05$) (Table 1, Fig. 2). The escape latency between rats in the nicotine + DHβE group, DHβE group, nicotine + AG490 group, and AG490 group had no significant difference compared with ischemic rats given saline ($p > 0.05$).

The results of the space exploration experiment: the number of times that the rats crossed the target quadrant and the activity time in the target quadrant are listed in Table 1. Compared with the ischemia group, the nicotine group and sham operation group had significant differences in the number of times crossing the target quadrant and the activity time in target quadrant ($p < 0.05$). There was no significant difference in the nicotine + DHβE group, DHβE group, nicotine + AG490 group, and AG490 group compared with ischemia group ($p > 0.05$), which indicated that nicotine improved the cognitive function of ischemic rats, but its effect was blocked by DHβE and AG490.

Micro-PET Imaging Showed Nicotine Upregulating α4β2 nAChRs Was Blocked by DHβE Not AG490

The uptake of 2-[18F]-A-85380 in the left thalamus and whole brain of rats in the nicotine group, nicotine + AG490 group, and sham operation group was significantly higher than that of ischemic rats treated with saline only ($p < 0.05$) (Fig. 3, Table 2). There was no significant difference between the nicotine + DHβE group, the DHβE group, and the AG490 group compared with the ischemia group ($p > 0.05$).

Real-time PCR Indicated Nicotine Upregulating α4β2 nAChRs Was Blocked by DHβE Not AG490

The relative expression of receptor subunits α4- and β2-nAChR in the left thalamus of rats was detected by real-time PCR. Three samples were detected in each group, and three technical replications for each sample were performed. The average CT values were obtained by amplifying β-actin and α4- and β2-nAChR respectively. The results (Table 3, Fig. 4) showed that the expression of α4- and β2-nAChR mRNA in ischemic rats given saline (ischemia group) was lower than that in the sham operation group (0.51 ± 0.04, 0.68 ± 0.04). The expression of α4- and β2-nAChR mRNA in the left thalamus of rats in the nicotine group was significantly higher than that of rats in the ischemia group (0.75 ± 0.16, $p < 0.05$; 0.86 ± 0.11, $p < 0.05$). The expression levels of α4- and β2-nAChR mRNA in the left thalamus of rats in the nicotine + AG490 group were still significantly higher than those of ischemic rats given saline (0.74 ± 0.07, $p < 0.05$; 0.82 ± 0.03, $p < 0.05$). There was no significant difference between the nicotine + AG490 group and nicotine group, suggesting that nicotine could increase the number

Table 1 Results of MWM test in all groups

| Groups                  | Number | Escape latency (seconds) | Times crossing target quadrant | Time in target quadrant(s) |
|-------------------------|--------|--------------------------|-------------------------------|---------------------------|
|                         |        | 2nd day | 3rd day | 4th day | 5th day |                                      |                                      |
| Ischemia group          | 6      | 84.04 ± 44.10          | 78.96 ± 44.93                | 73.46 ± 42.00            | 60.50 ± 42.88            | 2.33 ± 0.94                          | 27.85 ± 13.32                       |
| Nicotine group          | 6      | 50.67 ± 37.91*         | 41.46 ± 37.38*               | 35.38 ± 30.11*           | 26.46 ± 24.93*           | 5.50 ± 2.06*                         | 52.51 ± 7.56*                       |
| Nicotine + DHβE group   | 6      | 83.67 ± 44.69          | 72.17 ± 45.98                | 61.17 ± 42.62            | 48.83 ± 37.19            | 3.17 ± 2.41                          | 30.18 ± 8.34                        |
| DHβE group              | 6      | 82.67 ± 39.84          | 76.51 ± 39.40                | 65.59 ± 40.90            | 53.78 ± 33.44            | 3.21 ± 2.54                          | 33.23 ± 5.09                        |
| Nicotine + AG490 group  | 6      | 73.04 ± 41.07          | 69.33 ± 47.04                | 52.75 ± 39.69            | 46.92 ± 39.08            | 2.67 ± 1.70                          | 36.57 ± 11.22                       |
| AG490 group             | 6      | 77.38 ± 41.24          | 72.42 ± 43.53                | 61.54 ± 44.01            | 49.83 ± 33.14            | 2.83 ± 1.34                          | 33.71 ± 6.55                        |
| Sham operation group    | 6      | 29.21 ± 17.85*         | 25.67 ± 24.05*               | 19.74 ± 14.53*           | 11.20 ± 9.21*            | 5.90 ± 0.98*                         | 55.35 ± 11.72*                      |

Each group was compared in pairs; only the nicotine group and the sham operation group were statistically significant compared with the ischemia group. *$p < 0.05$ versus the ischemia group
of α4- and β2-nAChR, but AG490 could not block this function. The expression of α4- and β2-nAChR mRNA in the left thalamus of rats in the nicotine + DHβE group, DHβE group, and AG490 group was significantly lower than that in the nicotine group and nicotine + AG490 group, but there was no significant difference compared with the ischemia group.

Western Blot Showed Nicotine Inhibiting Inflammation by α4β2 nAChRs Upregulation and Activation of JAK2-STAT3 Signaling Pathway

The relative expression of α4- and β2-nAChR, IL-1β, IL-6, JAK2, STAT3, p-JAK2, and p-STAT3 proteins in the left thalamus of rats was detected by Western blot. The internal reference is β-actin. Three samples were collected from each group, and the expression of each group was obtained by gray-scale detection. Western blot (Table 4, Fig. 5) showed that the expression of α4-nAChR and β2-nAChR proteins in the nicotine group and nicotine + AG490 group was significantly higher than that in ischemic rats treated with saline only (1.91 ± 0.18, p < 0.05; 2.05 ± 0.12, p < 0.05; 1.88 ± 0.12, p < 0.05; 1.91 ± 0.03, p < 0.05). The expression of p-JAK2 and p-STAT3 proteins in the nicotine group was significantly higher than that in ischemic rats given saline (0.95 ± 0.03, p < 0.05; 1.12 ± 0.02, p < 0.05). The expression of p-JAK2 and p-STAT3 protein in the nicotine + DHβE group, nicotine + AG490 group, and AG490 group was not significantly different from rats in the ischemia group. The expression of IL-1β and IL-6 protein in the left thalamus of rats in the nicotine group was lower than that in the ischemia group (0.67 ± 0.02, p < 0.05; 1.17 ± 0.03, p < 0.05), but the relative expression of these inflammatory factors in the nicotine + DHβE group, nicotine + AG490 group, and AG490 group had no significant difference with that in the ischemia group (p > 0.05). These results indicated that the inflammatory response in rats’ brain was enhanced after thalamic ischemia. After nicotine intervention, α4β2 nAChRs in the brain were upregulated, and JAK2-STAT3 signaling pathway was activated by α4β2 nAChRs, which reduced the expression of inflammatory factors in the brain.

Cell Experiment

Over-expression of CHRNA4 and CHRN82 in HEK-293 T Cells

In order to ensure our cell model, the gene and protein levels of HEK-293 T cells were reported. Initial experiments confirmed that the HEK-293 T-hα4β2 cells were expressing the α4β2 subunit proteins and mRNA. The over-expression of the human α4 subunit and β2 subunit in HEK-293 T-hα4β2 cells...
Fig. 3 2-[^18F]-A-85380 micro-PET imaging in 7 groups. A is the ischemia group. The lower uptake of tracer was observed in the left thalamus; B is the nicotine group and E is the nicotine + AG490 group. Increased uptake of tracer was observed in the left thalamus; C is the nicotine + DHβE group and D is the DHβE group. The uptake of tracer in bilateral thalamus and whole brain was poor; F is the AG490 group. There was a decreased uptake of tracer in the left thalamus, similar as A; G is the sham operation group. The distribution of tracer in bilateral thalamus was uniform and symmetrical.
were stimulated by bacterial endotoxin LPS, the expression of inflammatory factors such as IL-6 and TNF-α was increased, which indicates that the inflammatory cells have been successfully established. In order to explore the role of JAK2-STAT3 in α4β2-mediated anti-inflammatory, AG490 and DHβE were used for intervention treatment. HEK-293 T-hα4β2 cells were stimulated with 10 μg/ml LPS for 24 h at 37 °C, and then incubated with nicotine, nicotine + AG490, and nicotine + DHβE. Nicotine can reduce the inflammatory factors because of its anti-inflammatory effect, while AG490 and DHβE restore LPS-induced inflammation in Western blots. Nicotine upregulated the expression of p-JAK2, p-STAT3, and β2-nAChR, but AG490 and DHβE reversed the effect of nicotine. According to these results, we can infer that JAK2-STAT3 mediates α4β2-mediated anti-inflammatory effect.

### Discussion

Neuroinflammation is an important part of ischemic stroke [38]. Ischemia quickly induces primary irreversible tissue damage, and following the acute focal brain injury is a secondary immune response characterized by glial activation, recruitment of peripheral immune cells, and release of cytokines and chemokines [40]. Now researches show that neuroinflammation is closely related to cognitive impairment [39–41], especially the activation of microglia is the key point leading to neuroinflammation. Anti-inflammatory therapy has also been used to treat cognitive impairment. Guan Y.Z. et al. [42] found that nicotine inhibited the proliferation of microglia after nicotine intervention, thus inhibiting the release of inflammatory factors.

Our previous studies [14, 15] showed that the cognitive impairment in ischemic rats induced by ET-1 was related with the damage of α4β2 nAChRs. An appropriate dose of nicotine could improve the learning and memory ability of ischemic rats. This improvement of cognition was related to the increase of α4β2 nAChR density in the thalamus and whole brain after nicotine intervention [15]. When thalamic ischemia occurs, the inflammatory response in the brain of rats increases. Nicotine can further inhibit the expression of inflammatory factors by activating the α4β2 nAChRs, thus improving cognitive function [15]. How nicotine activating α4β2 nAChRs suppress inflammation is the main work of our study.

The JAK-STAT signaling pathway is an important target of a variety of human diseases. In the CNS, this signaling pathway is closely related to brain inflammation and the survival and development of neurons and glial cells [43]. It has been found that JAK2 and STAT3 can regulate synaptic plasticity in the hippocampus, which is closely related to learning and memory, and JAK2-STAT3 signaling pathway is involved in cognitive improvement [20–23]. Therefore, the role of JAK2-STAT3 signaling pathway in central nervous system...
system diseases, including Alzheimer’s disease, depression, and anxiety disorders, has attracted more and more attention. AG490 is a specific antagonist of JAK2 and one of the PTK inhibitors [33]. As a specific blocker of JAK2-STAT3 signaling pathway, AG490 inhibits the phosphorylation of JAK2 and STAT3 downstream of JAK2, thus blocking cell signal transduction [44].

In this study, the rat model of ET-1-induced ischemic cognitive impairment and a HEK-293 T cell line with upregulated expression of CHRNA4 and CHRNB2 transduced by lentivirus were established, and the relationship among neuroprotective mechanism of nicotine, α4β2 nAChRs, inflammation, and JAK2-STAT3 signaling pathway was discussed. In animal experiments, the MWM test showed that nicotine intervention significantly improved the learning and memory ability, but no significant improvement was observed when nicotine was administered together with DHβE or AG490. 2-[18F]-A-85380 PET imaging showed that α4β2 nAChRs in rats’ brain were significantly increased when nicotine was given alone, or combined with AG490. The study showed that nicotine intervention increased the density of α4β2 nAChRs and improved cognitive impairment, but this effect would be blocked by AG490, and the receptors were still upregulated. Essentially, when the JAK2-STAT3 signaling pathway was blocked, nicotine could only upregulate the expression of α4β2 nAChRs, but cannot improve the cognitive function. PCR and Western blot analysis further confirmed these results. In addition, in cell experiment, nicotine reduced the inflammatory factor such as TNF-α and IL-6 stimulated by LPS in HEK-293 T-hα4β2 cells, while AG490 and DHβE reversed the effect of nicotine. Nicotine upregulated the expression of pJAK2, pSTAT3, and β2-nAChR, but AG490 and DHβE also reversed the effect of nicotine. Our findings indicate

![Fig. 4](image)

The relative expression of α4- and β2-nAChR mRNA in the left thalamus of each group. Note: Relative expression was the value of each index in the groups relative to the normal rats which was the control group in RT-PCR. *p < 0.05 versus the ischemia group.

### Table 4 Western blot results: the relative expression of protein α4- and β2-nAChR, IL-1β, IL-6, JAK2, STAT3, p-JAK2, and p-STAT3 in ischemic thalamus of each group (x ± s) (n = 3)

| Groups          | α4-nAChR   | β2-nAChR   | IL-1β      | IL-6       | JAK2      | p-JAK2     | STAT3     | p-STAT3   |
|-----------------|------------|------------|------------|------------|-----------|------------|-----------|-----------|
| Ischemia group  | 1.44 ± 0.05| 1.53 ± 0.10| 0.85 ± 0.03| 1.65 ± 0.08| 1.44 ± 0.04| 0.57 ± 0.01| 1.32 ± 0.01| 0.68 ± 0.02|
| Nicotine group  | 1.91 ± 0.18*| 2.05 ± 0.12*| 0.67 ± 0.02*| 1.17 ± 0.03*| 1.42 ± 0.05| 0.95 ± 0.03*| 1.30 ± 0.01| 1.12 ± 0.02*|
| Nicotine + DHβE | 1.39 ± 0.09| 1.47 ± 0.11| 0.82 ± 0.02| 1.64 ± 0.05| 1.40 ± 0.07| 0.61 ± 0.07| 1.32 ± 0.01| 0.68 ± 0.05|
| Nicotine + AG490| 1.88 ± 0.12*| 1.91 ± 0.03*| 0.81 ± 0.01| 1.58 ± 0.02| 1.41 ± 0.06| 0.62 ± 0.04| 1.35 ± 0.05| 0.66 ± 0.02|
| AG490 group     | 1.42 ± 0.18| 1.51 ± 0.10| 0.82 ± 0.01| 1.61 ± 0.03| 1.42 ± 0.06| 0.60 ± 0.01| 1.31 ± 0.02| 0.63 ± 0.02|

Relative expression was the protein expression of each index relative to β-actin. Each group was compared in pairs; only the nicotine group and nicotine + AG490 group were statistically significant compared with the ischemia group. *p < 0.05 versus the ischemia group.
that nicotine can upregulate α_4β_2 nAChRs, thus activating JAK2-STAT3 signaling pathway to reduce inflammatory factors, thereby playing an anti-inflammatory role and improving ischemic cognitive impairment. Therefore, α_4β_2 nAChRs-JAK2-STAT3 signaling pathway plays an important role in inhibiting inflammation in ischemic rats and inflammatory cells.

Neuroinflammation is also a feature of many other neurodegenerative diseases, such as Alzheimer’s disease. Reducing neuroinflammation has aroused widespread concern in the treatment of dementia. Recent study has shown the role of nAChRs in Alzheimer’s disease [45], which provides a new idea for the treatment of dementia.

One limitation of this study is that there is no immunofluorescence co-staining analysis to provide more direct evidence to prove whether nicotine acts through α_4β_2 nAChRs in immune cells (microglia and astrocytes) or α_4β_2 nAChRs in neurons, because some studies show that α_4β_2 nAChRs are over-expressed in both microglia and astrocytes from the 7th to 28th days after experimental ischemic stroke [46]. The reaction of JAK2-STAT3 in immune cells from the ischemic region also needs to be further study. Another field that needs further exploration is that this study could not confirm whether the effect of JAK2-STAT3 activated by α_4β_2 nAChRs was direct or indirect. For example, nicotine affects the expression of another protein, and then activates the JAK2-STAT3 pathway.

### Conclusion

In ischemic rats, nicotine could activate JAK2-STAT3 signaling pathway by upregulating α_4β_2 nAChRs, and inhibit the expression of inflammatory factors, thus improving cognitive function. Nicotine can reduce inflammation by upregulating α_4β_2 nAChRs and activating the JAK2-STAT3 pathway, which has also been confirmed in HEK-293 T-hα_4β_2 cells.

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**Fig. 5** The results of Western blot in the left thalamus of each group. A The protein panel of α_4- and β_2-nAChR, IL-1β, IL-6, JAK2, p-JAK2, STAT3, p-STAT3, and β-actin in the left thalamus of each group. B The relative expression of protein α_4- and β_2-nAChR, IL-1β, IL-6, p-JAK2, and p-STAT3 in the left thalamus of each group. Note: Relative expression was the protein expression of each index relative to β-actin. *p < 0.05 versus the ischemia group.

**Fig. 6** Over-expression of α_4β_2-nAChRs in HEK-293 cell line. A, D Protein levels of α_4 subunit (A) and β_2 subunit (D) in HEK-293 T-hα_4β_2 cells and HEK-293 T cells not infected with lentivirus were measured in Western blot. B, E Immunofluorescence staining of α_4-nAChR (green) and β_2-nAChR (red) on HEK-293 T-hα_4β_2 cells. mRNA of α_4-nAChR (C) and β_2-nAChR (F) in HEK-293 T-hα_4β_2 cells stable strain and HEK-293 T cells not infected with lentivirus were analyzed by real-time PCR.
Y.Y.: conceived and designed the experiments; W.Q.: performed the animal experiments, the analysis and interpretation of data, and drafted the main manuscript text; G.J.: performed the cell experiments; G.S.: assisted in experiments and data analysis, and drafted the manuscript text; W.F., H.T., and L.R.: assisted in experiments and data analysis; Z.D.: synthesized the tracer of 2-[18F]-A-85380; D.Y.: guided the specific implementation of the experiment.

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Data Availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics Approval and Consent to Participate Animal care and all experimental procedures were performed in accordance with the Guideline for the Care and Use of Laboratory Animals. All animals received humane care. Study protocols complied with the institution’s guidelines and were approved by the Ethics Committee of The First Hospital of China Medical University.

Consent for Publication All authors have read and agreed to the published version of the manuscript.

Competing Interests The authors declare no competing interests.

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