JAK2/STAT3 Signaling Pathway Plays a Key Role in Nicotine Mediated Neuroinflammation Suppression in an Ischemic Rat Model

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Research Article

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Abstract

Background

To explore the mechanism of nicotine mediated improvement of cognitive impairment in an established ischemic rat model.

Methods

Endothelin-1 (ET-1) was injected into the left thalamic region in adult male Sprague-Dawley (SD) rats to establish ischemia model. 6 groups of rats (6 rats in each group) were then treated with nicotine, nicotine+DHβE, DHβE, AG490, nicotine +AG490 and saline respectively via intraperitoneal injection for 9 days. Another sham operation group was treated with saline as above. Morris Water Maze (MWM) test was performed for 6 consecutive days starting on the 4th day after operation to detect the cognitive function of rats in each group. 2-[18F]-A-85380 microPET imaging was performed on day 10 to evaluate the changes of α4β2 nAChRs in different brain regions of rats. Real-time PCR and Western blot were used to detect the amount of α4β2 nAChRs, JAK2, STAT3 and inflammatory factors in thalamus of rats in each group.

Results

The results of MWM test showed the spatial learning and memory abilities of rats in the nicotine and sham operation groups were significantly better than the saline treating group in this ischemic rat model (p<0.05). There was no significant difference in other groups (p>0.05). MicroPET imaging showed more uptake of 2-[18F]-A-85380 in the nicotine, nicotine+AG490 and sham operation groups than in saline treating group, while there was no significant difference found in other groups (p>0.05). The expression of α4- and β2-nAChR in nicotine, nicotine+AG490 and sham operation groups was significantly higher than the saline treating group (p<0.05). In the nicotine group, the expression of p-JAK2 and p-STAT3 in left thalamus of rats was significantly higher than the saline treating group (p<0.05), and the expression of IL-1β and IL-6 protein was found to be lower than the saline treating group (p<0.05). While the expression of p-JAK2, p-STAT3 and inflammatory factors was not significantly different in all the other groups (p>0.05).

Conclusion

The study suggests nicotine inhibits the expression of inflammatory factors by activating α4β2 nAChRs through the activation of JAK2-STAT3 signaling pathway to improve cognitive impairment in ischemic rats.

Introduction

Vascular cognitive impairment (VCI) is a kind of syndrome mainly caused by cerebral vascular disease, ranging from mild cognitive impairment to dementia. It is the second leading cause of dementia after Alzheimer’s disease[1]. Studies showed that nicotine can play an important role in the protection of cognitive function[2–5]. 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 nAChR subtypes in the mammalian brain are heteromeric α4β2 nAChRs and homomeric α7 nAChRs, and α4β2 is the most abundant subtype in the CNS and with high affinity for nicotine [6–8]. Histopathological studies have shown that under ischemic conditions, inflammation caused by microglia overactivation can destruct the blood-brain barrier, cause brain damage and prompt the occurrence and development of VCI[9–12]. Our prior studies have confirmed that the chronic ischemic cognitive dysfunction was correlated with the decrease of α4β2 nAChRs[13], and nicotine played a role in inhibiting the inflammatory factors, which contributes to improving cognitive impairment by activating α4β2 nAChRs in ischemic rats[14]. How α4β2 nAChRs activated by nicotine acts on inflammatory factors remains uncertain.

The JAK-STAT signalling pathway is known to be a chain of interactions between proteins in a cell, and is involved in processes such as 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, etc.[15–17]. Studies have shown that JAK2 and STAT3 can regulate the plasticity of hippocampal synapses and are closely related to learning and memory[18]. In this study, we aimed to explore the relationship between JAK2-STAT3 signaling pathway and α4β2 nAChRs in ischemic rats.
nAChRs activation induced by nicotine in ischemic rats. Nicotine, DHβE (the most potent competitive antagonist of α₄β₂
nAChRs[19, 20]) and AG490 (a specific JAK2/STAT3 blocker[21, 22]) were adopted for intervention treatment, and 2-[¹⁸F]-A-85380 micro-PET imaging was performed for detecting α₄β₂ nAChRs[23] in ischemic rats in this study.

Materials And Methods

All animal experiments were approved by the Ethics Committee of The First Hospital of China Medical University, and all experiments were performed in accordance with relevant guidelines and regulations.

Laboratory Animals and Groups

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

Ischemic models were established by injecting ET-1 (MERCK, USA) into the left thalamus, the steps in detail were described in the previous study[14]. The model of sham operation group was established: the specific method was the same as above, but ET-1 was replaced by normal saline. On the first day after operation, nicotine (Sigma Aldrich), DHβE (ApexBio, USA), AG490 (SELECK, USA) or saline were administered according to different grouping: nicotine group (nicotine, 1.5 mg/kg/d), nicotine + DHβE group (nicotine, 1.5mg/kg/d; DHβE, 3mg/kg/d), DHβE group (DHβE, 3mg/kg/d), nicotine + AG490 group (nicotine, 1.5mg/kg/d; AG490, 3mg/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 received the intervention by intraperitoneal injection for nine consecutive days.

The other 3 SD rats did not receive any intervention neither operation nor drugs and they were used as a control group for PCR test. Another two rats were sacrificed 24 hours after surgery only for histological analysis with HE staining to make sure the success of ischemic model (Fig. 1), one from the sham operation group, the other from ischemia group.

Morris Water maze experiment

On the fourth day of drug intervention, Morris water maze (MWM) (endocrinology laboratory, China Medical University) test was performed to test the spatial learning and memory ability of rats. Rats of different groups were subjected to six consecutive days of experiments, which were divided into the following two parts:

Directional navigation experiment: the experiment lasted for 5 days. On the first day, a directional navigation program was set up. Rats were put into the pool from any two quadrants to familiarize themselves with the water maze environment. Platform was placed in the center of a quadrant of the pool. The training tests were conducted at the same time every day for the next four days. The software automatically recorded the swimming trajectory and swimming time of each rat in the pool.

The space exploration experiment: on the sixth day of the experiment, after setting up the space exploration program, the platform was removed, and the rats were put into any quadrant (except the target quadrant where the original platform was located). The software automatically recorded and analyzed the times that the rats crossed the target quadrant and the activity time in target quadrant in 120 seconds.

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 was synthesized according to the previous report [24]. 37 MBq 2-[¹⁸F]-A-85380 was injected by tail vein of rats. Head scan was performed for 10 minutes after 120 minutes.

Quantitative analysis: Drawing ROI was based on standard rat brain atlas by Paxinos[25]. The cerebellum was used as the reference area, and the ratio of the average SUV (SUV_{ave}) in each area to the SUV_{ave} of the cerebellum was calculated. The distribution of 2-[¹⁸F]-A-85380 in the left thalamus and the whole brain of each group was quantitatively analyzed.
Real-Time PCR Detection of Receptor Subunits of nAChRs

The RNA extraction was performed firstly, followed by the reverse transcription synthesis of cDNA. The mixture was homogenized and the sample was placed on a reverse transcription instrument at 37°C, 15 min; 85°C, 5 s; 4°C, 10 min. Real-time PCR amplification curve and dissolution curve were confirmed after the reaction, and the CT value of the sample was calculated automatically by software. RNAiso plus (NO.9108/9109), SYBR Premix Ex Taq II (RR820A), Prime Script RT Reagent Kit (#RR037A) were purchased from TaKaRa company, Japan. Primers used for the study were as follows:

α4 nAChR: F: 5’-ATGGATGAAACCTACCTGAGCA-3’
R: 5’-GCTGGGGGTTGTAGCAGGCAC-3’

β2 nAChR: F: 5’-CGGGAAGCAGTGGATGGCGTA-3’
R: 5’-GTCCTCCCTCACACTCTGGCATCA-3’

β-Actin: F: 5’-CATCCTCGTCTGGACCTGG-3’
R: 5’-TAATGCACGCGACTTTCC-3’.

Western blot

The left thalamic tissue was taken and stored in an Eppendorf tube in a refrigerator at −80°C. The tissues of each group were added with 1 ml RIPA buffer and PMSF mixture (RIPA: PMSF = 100:1). After homogenizing on ice by tissue homogenizer, the tissues were placed on ice for 30 minutes, centrifuged, 4°C, 15,000 rpm for 15 min. The supernatant was then taken and stored at −80°C. According to BCA protein quantitative standard curve, different samples were adjusted to the same concentration with lysis buffer (3–5µg/µl for each sample). All the samples were denatured by heating at 95°C for 10 mins, and stored at -20°C. Finally, 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-nAChRs(1:500), β2-nAChRs(1:500), IL-1β(1:2000), IL-6(1:2000), JAK2(1:1000), STAT3(1:1000), p-JAK2(1:1000), p-STAT3(1:2000), β-actin (1:1000) at 4°C for 24h. The membranes were incubated with corresponding secondary antibodies at room temperature for 1h. Western blots were developed by enhanced chemiluminescence detection system. Image Lab software was used for gel electrophoresis image analysis. The gray value of the panel was used for quantitative analysis, and the histogram was obtained to show the protein expression of each group in each index. Quantitative analysis was performed basing on three individual samples preparation following with western blotting.

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; STAT3,124H6; 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.

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 in Multiple Comparison Correction. 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

MWM test indicated nicotine improving cognition but blocked by DHβE and AG490

Normality was checked using the Shapiro-Wilk test, which showed that the data fit a normal distribution. We assumed normal distribution since the sample size was too small to test the normality assumption properly. Using variance analysis of repeated
These results suggested that the inflammatory response in the brain was enhanced after thalamic ischemia in rats. After nicotine β in nicotine+DH group was lower than ischemia group (0.67±0.02, significantly different from rats in ischemia group. The expression of IL-1β and IL-6 protein in left thalamus of rats in nicotine group were significantly higher than ischemic rats given saline (0.95±0.03, 2.05±0.12, β2 nAChR mRNA was lower in ischemic rats given saline (ischemia group) than in the sham operation group (0.51±0.04, 0.68±0.04). The expression of α4 nAChR and β2 nAChR mRNA in left thalamus of rats in nicotine group was significantly higher than rats in ischemia group (0.75±0.16, p<0.05; 0.86±0.11, p<0.05). The expression levels of α4 nAChR and β2 nAChR mRNA in left thalamus of rats in nicotine+AG490 group were still significantly higher than ischemic rats given saline (0.74±0.07, p<0.05; 0.82±0.03, p<0.05), and there was no significant difference between nicotine+AG490 and nicotine groups, suggesting that nicotine could increase the number of α4 nAChR and β2 nAChR, while AG490 could not block this function. The expression of α4 nAChR and β2 nAChR mRNA in left thalamus of nicotine+DHβE group, DHβE group and AG490 group was significantly lower than nicotine and nicotine+AG490 groups, but there was no significant difference compared with ischemia group.

Western blot showed nicotine inhibiting inflammation by α4β2 nAChRs upregulation and activation of JAK2-STAT3 signaling pathway

Western blot was performed to detect the relative expression of protein α4 and β2 nAChR, IL-1β, IL-6, JAK2, STAT3, p-JAK2 and p-STAT3 in the left thalamus of rats. The internal reference was β-actin. Three samples were collected from each group, and the expression of each group was obtained by gray level detection. Western blot results (Table 4, Fig 5) showed that the expression of α4 nAChR and β2 nAChR protein in nicotine and nicotine+AG490 groups was significantly higher than 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 levels of p-JAK2 and p-STAT3 protein in nicotine group were significantly higher than 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 nicotine+DHβE group, nicotine+AG490 group and AG490 group was not significantly different from rats in ischemia group. The expression of IL-1β and IL-6 protein in left thalamus of rats in nicotine group was lower than ischemia group (0.67±0.02, p<0.05; 1.17±0.03, p<0.05), while the relative expression of inflammatory factors in nicotine+DHβE group, nicotine+AG490 group and AG490 group was not significantly different from ischemia group (p>0.05).

These results suggested that the inflammatory response in the brain was enhanced after thalamic ischemia in rats. After nicotine
intervention, $\alpha_4\beta_2$ nAChRs in the brain was up-regulated, and JAK2-STAT3 signaling pathway was then activated by $\alpha_4\beta_2$ nAChRs, thus reducing the expression of inflammatory factors in the brain.

**Discussion**

Our previous studies[13, 14] showed that the cognitive impairment in ischemic rats induced by ET-1 was correlated with the impairment of $\alpha_4\beta_2$ nAChRs. Appropriate dose of nicotine could improve the learning and memory ability of ischemic rats. This improvement of cognition was related to the increased density of $\alpha_4\beta_2$ nAChRs in the thalamus and whole brain after nicotine intervention[14]. When thalamic ischemia happened, the inflammatory response in rat brain was elevated. Nicotine further inhibited the expression of inflammatory factors by activating the $\alpha_4\beta_2$ nAChRs, thereby improving cognitive function[14]. Inflammatory reaction after cerebral ischemia injury is mainly manifested by infiltration of peripheral blood leukocytes in brain parenchyma and up-regulation of the intrinsic glial cells activation (microglia and astrocytes)[26]. In the early stage of cerebral infarction, it can trigger a series of inflammatory cascade reactions and prompt the excessive secretion of local inflammatory cytokines and chemokines, such as selectin, TNF-α, IL-1, IL-6, IL-1β, nitric oxide (NO), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), etc.[26]. The leukocytes were attracted to vascular endothelium and aggregated in ischemic area, thus activating the secondary inflammatory reaction downstream and forming a vicious circle[27, 28]. At the same time, 2–4 hours after the early stage of cerebral ischemia, microglia were activated by inflammatory cytokines, which further aggravated brain injury[29].

Nowadays, studies have shown that neuroinflammation is closely related to cognitive impairment [30–32], 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. [33] et al. found that nicotine could inhibit the release of inflammatory factors by inhibiting the increment of microglia after nicotine intervention.

JAK-STAT signaling pathway is an important target of a variety of human diseases. In the central nervous system, this signaling pathway is closely related to brain inflammation and the survival and development of neurons/glial cells[34]. It has been found that JAK2 and STAT3 can regulate synaptic plasticity in hippocampus, which is closely related to learning and memory. Therefore, more and more attention has been paid to the role of JAK2-STAT3 signaling pathway in central nervous system diseases, including Alzheimer's disease, depression and anxiety disorders. AG490 is a specific antagonist of JAK2 and one of the PTK inhibitors[21]. As a specific blocker of JAK2-STAT3 signaling pathway, AG490 inhibits the phosphorylation of JAK2 and STAT3 downstream of JAK2, thereby blocking cell signal transduction [35, 36].

In this study, we established a rat model of ischemic cognitive impairment induced by ET-1, and explored the relationship between neuroprotective mechanism of nicotine, $\alpha_4\beta_2$ nAChRs, inflammation and JAK2-STAT3 signaling pathway. MWM test showed that the learning and memory ability was significantly improved by nicotine intervention, but no significant improvement was observed when nicotine was given with DHβE together, or with AG490 together. $2^{[18]}$F-A-85380 PET imaging showed that $\alpha_4\beta_2$ nAChRs increased significantly in rats brain when nicotine was given alone, or with AG490 together. The study demonstrated that nicotine intervention increased the density of $\alpha_4\beta_2$ nAChRs and improved cognitive impairment, but this effect would be blocked by AG490, while receptors were still upregulated. Essentially, when JAK2-STAT3 signaling pathway was blocked, nicotine could only upregulate the expression of $\alpha_4\beta_2$ nAChRs, but not improve the cognitive function. The results were further confirmed by PCR and Western blot analysis. Our findings suggest that nicotine can improve ischemic cognitive impairment by up-regulating $\alpha_4\beta_2$ nAChRs, thereby activating JAK2-STAT3 signaling pathway to reduce inflammatory factors. Therefore, $\alpha_4\beta_2$ nAChRs-JAK2/STAT3 signaling pathway plays an important role in inhibiting neuroinflammation in ischemic rats.

One of the limitations in this study is that immunofluorescence co-staining assay was not performed to provide more direct evidence to confirm if nicotine acts via $\alpha_4\beta_2$ nAChRs in immune cells (microglia, astrocyte) or in neurons, for some study demonstrated the overexpression of $\alpha_4\beta_2$ nAChRs in both microglia and astrocytes from days 7–28 after experimental ischemic stroke[37]. The reaction of JAK2-STAT3 in immune cells taken from the ischemic region also needs to be investigated further. Another area needs to be further explored is that this study could not confirm the effect of JAK2-STAT3 activating by $\alpha_4\beta_2$ nAChRs was direct or indirect, for example, where nicotine affects the expression of another protein that then activates the JAK2/STAT3 pathway.
Conclusion

In an established ischemic rats model, nicotine could activate JAK2-STAT3 signaling pathway by up-regulating $\alpha_4\beta_2$ nAChRs to inhibit the expression of inflammatory factors, thereby improving rats cognitive function.

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.

Availability of data and material

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

Competing interests

The authors declare no competing interests.

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Authors' contributions

Y. Y.: Conceived and designed the experiments; W.Q.: Performed the experiments, the analysis and interpretation of data, and drafted the main manuscript text; H.T. and L.R.: Assisted in experiments and data analysis; Z.D.: Synthesized the tracer of 2-[18F]-A-85380; D.Y. (Yao Diao): Guided the specific implementation of the experiment; D.Y. (Yong Du): Critical revision of the manuscript.

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### Tables

#### 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 | target quadrant (s) |                          |
| 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* |

Note: 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.

#### Table 2 Quantitative analysis of micro-PET imaging
| Groups                 | \(\text{SUV}_\text{ave left thalamus} / \text{SUV}_\text{ave cerebellum}\) | \(\text{SUV}_\text{ave whole brain} / \text{SUV}_\text{ave cerebellum}\) |
|-----------------------|-------------------------------------------------|-------------------------------------------------|
| ischemic group        | 2.38±0.08                                       | 1.60±0.06                                       |
| nicotine group        | 2.82±0.13*                                      | 1.73±0.09*                                      |
| nicotine + DH\(\beta\)E | 2.43±0.11                                       | 1.59±0.06                                       |
| DH\(\beta\)E group    | 2.39±0.15                                       | 1.57±0.08                                       |
| nicotine + AG490      | 2.80±0.06*                                      | 1.76±0.04*                                      |
| AG490 group           | 2.31±0.04                                       | 1.60±0.04                                       |
| sham operation group  | 2.75±0.19*                                      | 1.73±0.18*                                      |

Note: Each group was compared in pairs, only the nicotine group, nicotine+AG490 group and the sham operation group were statistically significant compared with the ischemia group. *: \(p<0.05\) versus the ischemia group.

### Table 3 RT-PCR results: the relative expression of \(\alpha_4\) and \(\beta_2\) nAChR mRNA in left thalamus of each group\(n=3\)

| Groups                 | \(\alpha_4\) nAChR | \(\beta_2\) nAChR |
|-----------------------|---------------------|-------------------|
| ischemia group        | 0.51±0.04           | 0.68±0.04         |
| nicotine group        | 0.75±0.16*          | 0.86±0.11*        |
| nicotine + DH\(\beta\)E | 0.56±0.14           | 0.57±0.08         |
| DH\(\beta\)E group    | 0.50±0.02           | 0.56±0.02         |
| nicotine + AG490      | 0.74±0.07*          | 0.83±0.02*        |
| AG490 group           | 0.51±0.08           | 0.67±0.05         |
| sham operation group  | 1.00±0.12*          | 0.98±0.18*        |

Note: Relative expression means the mRNA expression of each index in different group relative to the normal rats which was the control group in RT-PCR. Each group was compared in pairs, only the nicotine, nicotine+AG490 and sham operation groups were statistically significant compared with the ischemia group. *: \(p<0.05\) versus the ischemia group.

### Table 4 Western blot results: the relative expression of protein \(\alpha_4\) and \(\beta_2\) nAChR, IL-1\(\beta\), IL-6, JAK2, STAT3, p-JAK2, p-STAT3 in ischemic thalamus of each group\(n=3\)

| Groups                 | \(\alpha_4\) nAChR | \(\beta_2\) nAChR | IL-1\(\beta\) | 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 + DH\(\beta\)E | 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 |

Note: Relative expression was the protein expression of each index relative to \(\beta\)-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.
ischemia group.