Activation of the α_{2A} adrenoceptor in microglia promotes LPS-induced TNF-α production and cognitive impairment in mice

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Research

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Abstract

Background: a_{2A} adrenoceptor receptor (a_{2A}-AR) plays an important role in inflammatory response in Kupffer cells in sepsis. Blockage of a_{2A}-AR inhibits lipopolysaccharide (LPS)-induced tumor necrosis factor a (TNF-a) production and protects the target organ functions in sepsis animal models; however, its expression and function in microglia have remained obscure. This study aimed to determine whether a_{2A}-AR was expressed in microglia and whether its activation would exacerbate microglial inflammation and sepsis-related neurological dysfunction.

Methods: Western blotting and immunofluorescence were used to detect a_{2A}-AR expression in BV-2 microglia. Enzyme-linked immunosorbent assay (ELISA) was used to assess the TNF-a production in the supernatant after LPS induced BV-2 cells were pretreated with a_{2A}-AR agonist BHT933, and/or a_{2A}-AR antagonist BRL44408, and also in the supernatants derived from BV-2 cells treated with BHT933 and/or PKC inhibitor. Signaling pathways including JNK、P38、ERK、IκBa, CREB and PCK were detected by western blotting. a_{2A}-AR gene knock-out (KO) and wild type (WT) mice were prepared by intraperitoneal injection of LPS. Lectin /TNF-a labeled microglia and synaptophysin/NeuN expression in the hippocampus were localized by immunofluorescence. Morris water maze test, Rotating-stick test, Elevated plus maze test and Open-field test were conducted over 4 weeks.

Results: a_{2A}-AR was constitutively expressed in BV-2 microglia, which was enhanced by LPS. Pretreatment with BHT933 promoted LPS-induced IκB and JNK phosphorylation, and TNF-a secretion in BV-2 microglia which were abrogated by BRL44408. Activation of a_{2A}-AR by BHT933 also increased PKC phosphorylation in LPS-treated BV-2 microglia. PKC inhibitor significantly reversed the promoting effects of BHT933 on IκB and JNK phosphorylation as well as TNF-a secretion in LPS-treated BV-2 microglia. Furthermore, LPS treatment significantly increased hippocampal microglia activation and TNF-a expression, decreased hippocampal synaptophysin expression, and impaired cognitive and motor functions in WT mice, all of which were markedly reversed by a_{2A}-AR gene knockout.

Conclusion: a_{2A}-AR activation promotes LPS-induced IκB and JNK phosphorylation as well as TNF-a production in microglia through the PKC signaling pathway. Knockout of a_{2A}-AR gene significantly improves LPS-induced cognitive and motor impairments in mice, indicating that a_{2A}-AR is a potential therapeutic target for sepsis-associated encephalopathy.

Background

Sepsis-associated encephalopathy (SAE) is a diffuse cerebral dysfunction during sepsis in the absence of direct central nervous system infection. The incidence of SAE is as high as 70% in the intensive care units [1], manifesting as diffuse neuropsychiatric disorders such as attention deficit, inappropriate behavior, confusion, orientation disorder and especially consciousness disorder[2]. It was reported that SAE not only increased the mortality rate of sepsis patients[1], but also resulted in long-term dysfunction

of cognition and emotion as well as that of motor function in survivors [3]. It is therefore important to elucidate the pathogenesis and identify appropriate therapeutic targets for the prevention and treatment of SAE.

Microglia are innate immune cells in the brain. They are readily activated after sepsis and highly expressed pro-inflammatory mediators thus triggering an inflammatory cascade [4] to remove the pathogens and participate in tissue repair. On the one hand, they produce neurotrophic factors which are neuroprotective; on the other hand, they release large amounts of proinflammatory mediators that are detrimental to neurons. Therefore, activated microglia have been reported to play like a "double-edged sword" [5]. It has been reported that chronic inflammation induced by long-term excessive microglia activation in rat SAE model can cause neurodegenerative changes or even loss of neurons and affect neurological function [6]. At 2–4 weeks after intraperitoneal injection of IL-1β into the abdominal cavity of newborn mice for 5 consecutive days, hypomyelinated white matter axons, reduced mature oligodendrocytes and abnormal axonal development were common features [7, 8]. Immunofluorescence showed that microglia aggregated abnormally in the hippocampus of sepsis animal, indicating that microglia activation may be associated with sepsis related recognition dysfunction [9]. Additionally, microglia-derived IL-1β was found to suppress axon development through in LPS treated rats [10]. All these had pointed to the possibility that excessive release of cytokines by activated microglia in sepsis can lead to neurotoxicity, neuronal damage, synaptic structure and plasticity damage, thus hindering the formation and function of axons. It is therefore justified to argue that inhibiting microglia overactivation may prove to be an important preventive and therapeutic strategy for SAE.

Norepinephrine (NE) is an important catecholamine hormone and neurotransmitter of noradrenergic neurons, whose level rises sharply in sepsis to maintain the steady state of cardiovascular system or maintain the state of awakening [11]. NE is also an important immune regulatory substance, which is involved in the inflammatory reaction caused by sepsis [12]. It has been reported that NE promoted the expression and release of TNF-α in mouse primary Kupffer cells. Concomitant to this was increased expression of α_{2A} adrenergic receptor (α_{2A}-AR). Interestingly, α_{2B}-AR and α_{2C}-AR expression remained unaltered [13]. Treatment with the specific α_{2A}-AR blocker BLR44408 reduced the expression of TNF-α in the presence of NE in both LPS-treated primary mouse Kupffer cells and septic mice with cecal ligation and perforation (CLP) [14]. All these findings have strongly indicated that NE can promote inflammation in macrophages by activating α_{2A}-AR and that blocking α_{2A}-AR may be a potential strategy for treating sepsis. In rat model with CLP sepsis, blockade of α_{2A}-AR with BRL-44408 reduced the levels of pro-inflammatory mediators such as TNF-α, IL-6, iNOS and chemokines, along with that of the liver enzymes (AST and ALT) and lactate. More significantly, the survival rate of rats after CLP was improved [15]. Furthermore, blocking α_{2A}-AR also alleviated lung injury in sepsis by ameliorating lung water and histological score [16].

In consideration of the fact that microglia and macrophages are both of mononuclear origin [17–19], we surmised here that microglia might also express α_{2A}-AR that partakes in the inflammatory response in

septic brain and SAE. We reported here that indeed microglia exhibited α_{2A}-AR whose expression was upregulated by LPS and that it was involved in production of the proinflammatory cytokine, TNF-α that is well documented to be detrimental to neurons during sepsis. Blocking the α_{2A}-AR of microglia may therefore help quell the inflammatory response and ultimately proven to be a therapeutic strategy for treatment of SAE.

Methods

Cell culture

BV-2 microglia (CHI Scientific, Wuxi, China, Cat. No. 7-1502) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco by Thermo Fisher Scientific, United States, C11995500BT) supplemented with 20% fetal bovine serum (FBS, Gibco by Life Technologies, United States, 10099-141) at 37 ℃ in humidified 5% CO_2. The medium was partially changed every 48 h for 3–4 days until the cells grew to confluence. For Western blotting and immunofluorescence labeling, cells were plated in six-well plates (3.5×10^5 cells/well) and grown to confluence for experimental treatments.

Cell viability assay of BV-2 cells

Viability of BV-2 microglia was assessed by Cell Counting kit-8 (CCK8; BestBio, Shanghai, China, BB-4202-2). To determine the cytotoxic effect of BHT933 (α_{2A}-AR agonist, Sigma-Aldrich, USA) and BRL44408 (α_{2A}-AR antagonist, Sigma-Aldrich, USA; Cat. No. #B4559), BV-2 microglia were plated into 96-well microplates (10^4 cells/well) and cultured overnight. After this, they were treated with BHT933 (ranging from 0.1 to 10.0 μM) and BRL44408 (ranging from 0.2 to 20.0 μM), for 2, 4, 6 and 8 h in triplicates. After removal of medium, 90 ∝l of new basic medium and 10 ∝l of CCK8 were added to each well and the plate was incubated for additional 3 h (final concentration 10%). The optical density (OD) was then read at 450 nm using a microplate reader. The assays were repeated for 5 times. No statistical difference was found in the viability of BV-2 microglia when incubated with BHT993 (range from 0.1–10.0 μM) or BRL44408 (range from 0.2–20.0 μM) within 8 h. Based on the above results, we have used both BHT933 and BRL44408 at concentration of 10 μM for 15–30 min for subsequent in vitro analysis.

Cell groups and treatments

To investigate the expression of α_{2A}-AR in microglia, the following cell groups and treatments were carried out: 1) in immunofluorescence, 3×10^5/well BV-2 microglia were divided into control and 100 ng/mL LPS (Sigma-Aldrich, USA; Cat. No. L2880) groups; 2) in western blotting, 6×10^5/well BV-2 microglia were divided into control and LPS groups; the LPS group was further divided into 3 sub-groups based on the LPS dosage used (10 ng/mL, 100 ng/mL and 1000 ng/mL) for 6 h incubation, and then western blot was conducted. In ELISA for the investigation of the effect of α_{2A}-AR activation on inflammatory response in LPS-treated microglia, a total of 8 cell experimental groups were prepared: control, LPS (100 ng/mL for 6 h), BHT933 + LPS (pretreated with α_{2A}-AR agonist BHT933 at 10 μM for

15 min and then incubated with 100 ng/mL LPS for 6 h), BRL44408 + BHT933 + LPS (pretreated with α_{2A}-AR antagonist BRL44408 at 10 μM for 30 min and followed by 10 μM BHT933 for 15 min, then incubated with 100 ng/mL LPS for 6 h), BRL44408 + LPS (pretreated with 10 μM BRL44408 for 30 min and then incubated with 100 ng/mL LPS for 6 h), BHT933 (10 μM BHT933 for 15 min), BRL44408 (10 μM BRL44408 for 30 min), and BRL44408 + BHT933 (10 μM BRL44408 for 30 min followed by 10 μM BHT933 for 15 min). To explore the inflammatory signaling pathways involved in α_{2A}-AR activation, the same 8 groups of cells were prepared, except that the duration of LPS incubation was for 30 min. For the purpose of investigation of the signaling pathways involved in α_{2A}-AR activation, 4 groups of BV-2 cells were used: control, LPS (100 ng/mL for 6 h), BHT933 + LPS (10 μM BHT933 for 30 min + 100 ng/mL LPS for 6 h) and BHT933 (10 μM BHT933 for 15 min). To test the role of PKC in promoting inflammation after α_{2A}-AR activation, 8 groups of BV-2 cells were prepared for detection of TNF-α by ELISA: control, LPS (100 ng/mL for 6 h), BHT933 + LPS (BHT933 for 15 min + 100 ng/mL LPS for 6 h), Bisindolylmaleimide-1 + BHT933 + LPS [pretreated with PKC inhibitor, Bisindolylmaleimide-1(Abcam, UK; Cat. No. S7208), 1.0 μM for 3 0 min, followed by 10 μM BHT933 for 15 min, and then incubated with 100 ng/mL LPS for 6 h], Bisindolylmaleimide-1 + LPS (1.0 μM Bisindolylmaleimide-1 for 30 min + LPS for 6 h), BHT933 (10 μM BHT933 for 15 min), Bisindolylmaleimide-1 (1.0 μM Bisindolylmaleimide-1 for 30 min) and Bisindolylmaleimide-1 + BHT933 (1.0 μM Bisindolylmaleimide-1 for 30 min + 10 μM BHT933 for 15 min). Additionally, to detect the related signaling pathways, the same 8 groups of cells were prepared, except that the duration of LPS incubation was for 30 min.

Western blotting

For BV-2 cells of different groups, after the medium was discarded, the cell samples were rinsed more than twice with 1 × PBS at 4 ℃ and then lysed with the lysis buffer. After mechanically scraping off with a rubber scraper and centrifuged at 13,000 rpm for 25 min, the supernatant was collected. Protein concentrations were determined by using Pierce TM BCA Protein Assay Kit (Thermo Fisher Scientific, United States, Prod # 23227). Samples of supernatant containing 40 mg protein of BV-2 cells were heated to 95℃for 5 min and then separated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis in 10% or 12% gels. Protein bands were electroblotted onto polyvinylidene difluoride membrane and blocked with 5% BSA for 1 h. The membranes were incubated with primary antibodies (Table 1) which were diluted in 5% BSA overnight at 4℃ before the membranes were incubated with the secondary antibodies, either with anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, United States, 7074S) or anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, United States, 7076S). The immunoblots were detected by a SuperLumia ECL Kit (Abbkine, United States, K22020). The band intensity was quantified in Image J software (National Institutes of Health, NIH, United States). All experiments were repeated at least in triplicate.

Double immunofluorescence labeling of BV-2 microglia

About 3×10^5/well BV-2 microglia cells were seeded on poly-L-lysine (Sigma, Cat. NO. P4707) pre-coated coverslips in a 24-well plate and incubated for 24 h to ensure their full adherence to the coverslip surface before treatment. After treatment, the cells were fixed in 4% paraformaldehyde for 15 min at room

temperature followed by blocking with 5% goat serum for 1 h after washing with 1 × PBS for 3 times at 5 min each. Subsequently the cells were incubated with the respective primary antibodies (Table 1) diluted in 1 × PBS (1:100–500) at 4 ℃ overnight. Antibodies were detected with the relevant FITC/Cy3-conjugated secondary antibodies (1:200, diluted by 1 × PBS) for 1 h at room temperature. The coverslips were then mounted in DAPI containing the mounting medium (Sigma, Cat. No. F6057) after rinsing in 1 × PBS. Images were captured by a confocal microscope (FLUOVIEW FV1000; Olympus, Japan).

Table 1

Antibodies used for western blotting and immuno-staining

Antibody	Host	Source	Catalog number	Dilution for staining	Dilution for western blot
Lectin	Lycopersiconesculentum	Sigma-Aldrich	L0401	1:500	
GAPDH	Rabbit polyclonal	Affinity Biosciences	AF0911		1:3000
α_{2A}-AR	Rabbit polyclonal	Abcam	ab65833	1:100	1:1000
JNK	Rabbit polyclonal	Cell Signaling Technology	#9252		1:1000
p-JNK	Rabbit monoclonal	Cell Signaling Technology	#4671		1:1000
P38	Rabbit monoclonal	Cell Signaling Technology	8690S		1:1000
p-P38	Rabbit monoclonal	Cell Signaling Technology	4511S		1:1000
IκBα	Rabbit monoclonal	Abcam	ab32518		1:1000
p-IκBα	Goat polyclonal	Santa Cruz Biotechnology	Sc-7977		1:1000
ERK	Rabbit monoclonal	Cell Signaling Technology	4370S		1:1000
p-ERK	Rabbit monoclonal	Cell Signaling Technology	4695S		1:1000
CREB	Rabbit polyclonal	Affinity Biosciences	AF6188		1:1000
p-CREB	Rabbit polyclonal	Affinity Biosciences	AF3189		1:1000
PKCε	Rabbit Monoclonal	Cell Signaling Technology	#2683		1:1000
p-PKCε	Rabbit polyclonal	Abcam	ab63387		1:1000
TNF-α	Rabbit polyclonal	Abcam	Ab6671	1:100	1:1000
Synaptophysin	Rabbit Monoclonal	Abcam	ab32127	1:100	
NeuN	Rabbit Monoclonal	Millipore	ab177487	1:100	

ELISA

TNF-α concentration of supernatant derived from different experimental groups was measured by TNF-α ELISA kit (R&D Systems, Inc, Minneapolis, MN, United States) according to the manufacturer's protocol. The reaction plates were read within 30 min in an ELISA plate reader (Molecular Devices®, Eugene, OR, United States) at 450 nm.

Animals and treatments

Male wild-type (WT) littermates and α_{2A}-AR knockout (α_{2A}-AR KO) mice,SPF clean grade, 7–9 weeks old and weighed 25 ± 2 g were prepared for the following test. They were generated by Adra2a^{tm1Bkk} mice (Strain Name: B6.129-Adra2a^{tm1Bkk}/J, #004367), which were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The quality certificate number of overseas laboratory animals is 99410000209 (7–9 weeks old, 4 males and 7 females). Animal colony expansion, rearing and identification were carried out at the Medical Laboratory Animal Management Center of Jinan University. Animals were reared in separate cages and fed freely with drinking water. Before the experiment, they were placed in the experimental environment for acclimatization for a week.

A total of 158 male mice (Table 2) were divided into 4 groups: WT-sham, WT-LPS, KO-sham and KO-LPS group. LPS mouse model was performed by a single intraperitoneal injection of LPS at 15 mg/kg; the equal volume of normal saline was intraperitoneally injected in sham. After treatment with LPS, the mice were mentally depressed, less active, slowly moving, viscous eyelid secretions, turbid urine and erect back hair, indicating that the model was successfully established. The overall mortality rate of LPS-treated mice was 33.3%. There was no significant difference in mortality rate between wild type and α_{2A}–AR gene knockout group.

Double immunofluorescence in mice

A total of 24 mice from different experimental groups were used for double immunofluorescence labeling. Following deep anesthesia with 6% sodium pentobarbital, the rats were sacrificed by perfusion with 2% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed, embedded with paraffin and sectioned at 7 μm thickness on a microtome (Model: 2165; Leica, Bensheim, Germany). For blocking of non-specific binding proteins, tissue sections were incubated in 5% normal goat serum diluted in PBS for 1 h at room temperature (22 to 24℃). The sections were then incubated in a humidified chamber with the primary antibodies targeting against TNF-α, synaptophysin and Neuronal nuclei (NeuN). The remaining protocols followed the same as that in cells experiment. Each experiment was done in triplicate.

Neurobehavioral assessments

Morris water maze test was conducted to record latency, the fourth quadrant dwelling time and the frequency of crossing the platform. A circular pool (D = 1.25 m) full of water was divided into four quadrants, and a platform was placed in the fourth quadrant; the water depth was about 1-1.2 cm higher than the platform; the indoor and the water temperature were kept at 22–24 ℃ and the humidity was 62–65%. WMT-100 s Morris water maze experimental system was used to track for 60 s each time. The mice (n = 62) were placed into each quadrants of the pool respectively from the pool side and the duration

taken by them to locate the platform (latency) were recorded. If the mice could not find the platform in 60 s, they were forced to stay on the platform for 20 s. The above steps were repeated at the same time daily for 4 consecutive weeks. The mean value of the latency at 1-5th day in each week was recorded. On the 6th day of each week, the platform was removed. The same groups of mice were then placed in the second quadrant and the movement track was trailed for 60 s. The fourth quadrant dwelling time and the frequency of crossing the platform were recorded for 4 consecutive weeks.

Rotating-stick test was used to determine the duration of staying on the stick before the mice fell down. The mice (N = 24) were placed on the rotating stick with a uniform acceleration mode (uniform acceleration from 4 rpm to 50 rpm in 5 min; thereafter, the uniform rotation was maintained at 50 rpm). Duration of stay on the stick was recorded before the mice fell off for 4 consecutive weeks. The experiment was conducted for 3 times with an interval of at least 30 min between each round at the 7th day in each week. The results of the last round were used for statistical analysis.

Elevated plus maze and Open-field test were used to measure anxiety related behaviors. The same groups of mice (n = 48) were assessed for this two behavioral tests for 4 consecutive weeks. Through Elevated plus maze test, the frequency of entering the open arms and the duration of staying on the open arms were measured. Ethovison XT behavior track analysis system was used and the open arms, closed arms and central area were set. The length of the elevated plus maze was 100 cm and the tracking time was 5 min. The mice were placed in the central area for habituation at the 3rd day, and then the frequency of entering the open arms and the duration of staying on the open arm were recorded at the 4th day in each week. In Open-field test, the length and width of the calibration box were 50 cm and the tracking time was 10 min. The mice were placed in the center of the bottom of the box at the 5th day for habituation followed by the measurement of the frequency of entering the center field and the duration of activity in the center field at the 7th day in each week for 4 consecutive weeks by Ethovison XT behavior track analysis system.

For all behavior tests, mice were habituated to the testing room by placing home-cages for 2 h before testing. All apparatus were cleaned with 75% ethanol after each test to avoid the influence of excrement and smell on the next round of experiment. All outputs were measured by a researcher blinded to the experimental groups.

Table 2
Number of mice used in various treatments

	WT-sham	WT-LPS	KO-sham	KO-LPS
Immunofluorescence	6	6	6	6
Morris water maze test	18	16	14	14
Rotating-stick test	6	6	6	6
Elevated plus maze test	12	12	12	12
Open-field test				
Total	42	40	38	38

Statistical analysis

Statistical analysis was performed by SPSS 22.0 statistical software. The data were expressed as mean ± SEM. One-way Analysis of Variance (ANOVA) was used for comparison between groups: if the variances between groups were homogeneous, comparisons between groups were tested by LSD method, and Tamhane's T2 method was used for comparisons between groups if the variances were not homogeneous. All experiments from different cell and tissue samples were conducted in triplicate. The difference was considered statistically significant when $P < 0.05$.

Results

α_{2A}-AR expression in BV-2 microglia and its roles in LPS-induced inflammation

By immunofluorescence (Fig. 1A), α_{2A}-AR (red) was constitutively expressed in BV-2 microglia in the control group, which was markedly augmented by 100 ng/mL LPS. α_{2A}-AR protein expression was confirmed by Western blotting (Fig. 1B), which showed an increase by about 50% with 100 ng/mL LPS incubation, and by more than two folds with 1000 ng/mL LPS in comparison with the control. ELISA showed that TNF-α secretion in LPS-activated BV-2 microglia was drastically increased regardless of whether α_{2A}-AR was activated with its agonist BHT933 or not. As shown in Fig. 1C, TNF-α secretion in LPS group was significantly higher than that in the control group ($P < 0.05$); however, in BHT933 + LPS group, TNF-α secretion was further elevated by about 40% ($P < 0.05$). TNF-α secretion in LPS group pretreated with α_{2A}-AR antagonist BRL44408 was comparable to that of the LPS group. Compared with the control group, TNF-α secretion remained relatively unchanged in BHT933 group, BRL44408 group and BRL44408 + BHT933 group not treated with LPS. The results showed that TNF-α secretion in α_{2A}-AR stimulated LPS-activated BV-2 microglia was significantly suppressed by α_{2A}-AR antagonist.

α_{2A}-AR activation increased inflammation in LPS-activated BV-2 microglia via JNK and IκBα pathways

Western blotting was used to explore the signaling pathways involved when α_{2A}-AR is activated. The relative density of p-IκBα/IκBα (Fig. 2A) and p-JNK/JNK (Fig. 2B) in LPS group was significantly higher than that in control ($P < 0.05$); it was further increased when BHT933 was added in A + LPS group ($P < 0.05$). In BRL44408 + BHT933 + LPS group, p-IκBα/IκBα and p-JNK/JNK protein expression level was decreased to level comparable to that of the LPS group, which was significantly lower than that in BHT933 + LPS group ($P < 0.05$). The results indicated that activation of α_{2A}-AR associated with activated microglia increased LPS-induced phosphorylation of IκB; in other words, these signaling pathways participate in increased inflammation upon α_{2A}-AR activation. As a corollary, it is suggested that NF-κB as well as JNK signaling pathway is involved in LPS-induced TNF-α production by activation of α_{2A}-AR in BV-2 microglia.

Compared with the control group, the relative density of p-p38/p38 (Fig. 2D) in LPS group, BHT933 + LPS group, BRL44408 + LPS group and BRL44408 + BHT933 + LPS group increased significantly ($P < 0.05$), but there was no statistical difference between the groups. Similarly, there was no significant difference in the relative density of p-ERK/ERK (Fig. 2C) between each group. The results indicated that neither ERK nor p38 MAPK signaling pathway participated in the increased of inflammatory response after α_{2A}-AR activation.

PKC was the key pathway of intracellular signal transduction for α_{2A}-AR activation in BV-2 microglia

Western blotting was used to identify the intracellular signal transduction for α_{2A}-AR activation in BV-2 microglia. p-CREB/CREB protein expression level was first evaluated to determine whether PKA pathway was involved. As shown in Fig. 3A, there was no significant difference in p-CREB/CREB ratio between each group. Next, p-PKC/PKC protein expression was assessed which showed that (Fig. 3B) the expression between the control and LPS group was comparable. However, p-PKC/PKC expression level was increased significantly when α_{2A}-AR agonist was added in BHT933 + LPS group and BHT933 group ($P < 0.05$). These results suggest that PKC pathway may be involved in signal transduction after α_{2A}-AR activation. To further confirm the role of PKC in intracellular signal transduction after α_{2A}-AR activation, Bisindolylmaleimide-1, a classic PKC inhibitor, was used for determination of TNF-α secretion by ELISA. The results (Fig. 3C) showed that TNF-α secretion in LPS was significantly increased compared with control, and that it was further enhanced in α_{2A}-AR agonist-treated microglia. When Bisindolylmaleimide-1 was administered followed by LPS treatment, TNF-α secretion was markedly decreased to level comparable to that of LPS group. The results indicated that PKC inhibitor had neutralized the effect of agonist of α_{2A}-AR. In addition, the relative density of p-IκBα/IκBα and p-JNK/JNK_in BHT933 + LPS group (Fig. 4A and Fig. 4B) was significantly higher than that in LPS group ($P < 0.05$), but it was markedly

decreased when Bisindolylmaleimide-1 was added in Bisindolylmaleimide-1 + BHT933 + LPS group compared with the BHT933 + LPS group ($P < 0.05$).

Decreased microglia activation in LPS treated α_{2A}-AR gene knock-out mice

Lectin labeled microglia were widely distributed in the hippocampal CA2-3 regions in all groups (Fig. 5, white arrows). In WT-sham group, microglia exhibited moderate lectin (green) labeling, while TNF-α (red) was hardly detected. In WT-LPS group, lectin labeling colocalized with TNF-α immunofluorescence was markedly increased. Lectin labeled microglia were devoid of TNF-α immunofluorescence in KO-sham group. In KO-LPS group, moderate TNF-α expression was detected in lectin labeled microglia, but whose immunofluorescence was evidently lower than that in WT-LPS group. The results suggested that α_{2A}-AR gene knockout reduced microglia activation in the hippocampus of mice in sepsis.

Decreased synaptophysin staining in the hippocampus in LPS-treated α_{2A}-AR knock-out mice

There was no significant difference in NeuN staining (red) in the pyramidal cell layer (blue nucleus aggregation area) in the CA2-3 regions (Fig. 6A, 6B) between each group. The radiation area (class 2 synaptic dense area), indicated by the white arrow, was enriched in synaptophysin staining with green fluorescence in WT-sham group; however, in WT-LPS group, synaptophysin expression was significantly decreased. As distinct from this, synaptophysin expression was slightly decreased in KO-LPS group in comparison with KO-sham group.

α_{2A}-AR knock-out increased cognition and motor function in LPS-treated mice

Morris water maze test was conducted to evaluate the learning and memory function of mice during 1–4 weeks after LPS treatment. As shown in Fig. 7A, the latency of mice in WT-LPS group was significantly longer than that of WT-sham group in 1–4 weeks ($P < 0.05$); in KO-LPS group, the latency was longer than that of KO-sham group in 1–3 weeks ($P < 0.05$), but there was no statistical difference between them in the fourth week ($P < 0.05$); furthermore, at each time point of 1–4 weeks, the latency of mice in KO-LPS group was significantly longer than that in WT-LPS group ($P < 0.05$). Similarly, both the fourth quadrant detention time (Fig. 7B) and the frequency of platform crossings (Fig. 7C) of mice in WT-LPS group were significantly shorter than that of WT-sham group during 1–4 weeks ($P < 0.05$). In KO-LPS group, the detention time and the platform crossing frequency were sharply lower than those of KO-sham group in 1–2 weeks ($P < 0.05$), but no difference was found between the two groups in 3–4 weeks. Besides, the fourth quadrant detention time of KO-LPS group was significantly longer than that of WT-LPS group at each time point of 1–4 weeks ($P < 0.05$). Rotating-stick test was used to evaluate motor function with the

duration of staying on the stick before falling down (Fig. 7D). The duration of staying on the stick in WT-LPS group was significantly shorter than that in WT-sham at week 1–4 ($P < 0.05$), when compared with KO-sham group, the duration in KO-LPS group was shorter only at week 1–2 ($P < 0.05$), but there was no statistical difference between them at week 3–4. In the third week, the duration of KO-LPS group was significantly longer than that of WT-LPS group ($P < 0.05$). These results suggested that sepsis significantly decreased the function of cognition and motor, while α_{2A}-AR gene knockout may help abolish such effects.

α_{2A}-AR gene knock-out had no effect on emotional behavior, autonomous behavior, inquiry behavior and tension in LPS-treated mice

Elevated plus maze test was used to evaluate the changes of anxiety and depression to represent the emotional behavior, and Open-field test was used to evaluate the autonomous behavior, inquiry behavior and tension when mouse was placed in a new environment as well. Figure 8A and 8B showed no difference in the frequency of entering or the duration of staying on the open arms in both wild type mice and α_{2A}-AR knock-out mice, no matter the mice were treated with LPS or not. Similarly, as shown in Fig. 8C and 8D, the frequency of entering or the duration of activity in the center field in Open-field test was not significantly different between the sham and LPS group.

Discussion

It is well documented that α_{2A}-AR is expressed in monocytes and macrophages (Kupffer cells, Langerhans cells, etc.), and that it plays a pivotal role in the inflammatory response of sepsis [15, 16, 20, 21]. Blood monocytes have been proposed as a source of microglia [22], it was therefore speculated that like tissue macrophages, microglia might also express α_{2A}-AR. Although microglia in the spinal cord had been reported to express α_{2A}-AR [23], it has remained uncertain whether brain microglia would also express α_{2A}-AR. The present results have demonstrated for the fist time that α_{2A}-AR was constitutively expressed in resting microglia and that it was upregulated in activated microglia induced by LPS. In the latter, α_{2A}-AR expression was enhanced in LPS treated microglia in a dose-dependent manner, indicating that it plays specific roles in microglia functions.

Further analysis by ELISA in BV-2 microglia treated with α_{2A}-AR agonist BHT933 and antagonist BRL44408 followed by LPS was then carried out to detect TNF-α secretion. It was previously reported that the LPS concentration in animal blood under sepsis condition was about 10^{-8} g/mL[24]. In the present study, the final LPS concentration was 100 ng/mL to simulate the condition in vivo. As shown in Fig. 1C, BHT933 or BRL44408 did not elicit microglia activation; however, TNF-α secretion was further increased and substantially by about 50% in LPS group exposed to BHT933 when compared with LPS group. TNF-α secretion was decreased to a level comparable to that of LPS group when pretreated with BRL44408,

suggesting that α_{2A}-AR activation promotes TNF-α secretion in LPS-activated BV-2 microglia; in other words, α_{2A}-AR is instrumental to secretion of inflammatory mediators in LPS-activated BV-2. This would be highly consistent with the role of α_{2A}-AR in peripheral macrophages.

To further demonstrate the role of α_{2A}-AR in inflammation, the signaling pathways involved in TNF-α production, namely, inhibitor of nuclear factor kappa B (IκB), mitogen-activated protein kinases (MAPKs) of p38, c-Jun N-terminal kinases (JNK), extracellular signal-regulated kinase (ERK) and the phosphorylated forms were explored. We confirmed that nuclear factor-kappa B (NF-κB) and JNK, but not ERK and P38 MAPKs are involved in promoting inflammatory response in activated microglia and that it is mediated by α_{2A}-AR.

A pertinent question arose from this would be how activated α_{2A}-AR might transmit intracellular signals and ultimately promote the inflammation-related pathways. α_{2A}-AR belongs to G-protein-coupled receptors family, the cell signaling pathways of which include cAMP and phosphatidylinositol signaling pathway [24]. The former depends on adenylate cyclase to guide the expression of various substrates and genes through cAMP-dependent protein kinase A (PKA) (cAMP-PKA pathway). The latter relies on phospholipase C to hydrolyze 4,5-diphosphate phosphatidylinositol (PIP2) to 1,4,5-triphosphate inositol (IP3) and diacylglycerol (DG). Among them, DG-specific activated protein kinase C (PKC) causes phosphorylation of various proteins or enzymes (DG-PKC pathway), and then regulates the biological effects of cells. Renal tubular cells treated with BHT933 showed no effect on intracellular cAMP, while DG increased 46% which were blocked by BRL44408 treatment [26], suggesting that α_{2A}-AR signal in renal tubular cells may involve PKC pathway. It is relevant to note that α_{2A}-AR in the prefrontal cortex was reported to increase testosterone-treated impulsivity through the PKA pathway [27].

Against the above background, we have focused our study on the role of PKA and PKC when α_{2A}-AR is activated. When activated PKA enters the nucleus which activated cAMP-response element binding protein (CREB) and whose phosphorylation is indicative of PKA activation. The present results showed that the PKA pathway did not participate in the signal transduction of microglia α_{2A}-AR. PKC binding to the second messenger is through phosphorylation of Thr566, C-terminal hydrophobic sites Ser729 and Thr710 in its catalytic region [28–30]. There is compelling evidence indicating that PKCε was indispensable in macrophage for the phosphorylation of I-κB and activation of MAPKs [31]. Macrophages knocked out of PKCε had serious defects, and the host could not tolerate bacterial infection and the mortality increased [32]. In view of this, we also detected the phosphorylation of PKCε on Ser729 in this study. The results showed that p-PKCε was increased significantly in BV-2 cells treated with BHT933, suggesting that PKC was involved in the signal transduction following α_{2A}-AR activation. Furthermore, bisindolylmaleimide-1 interference ELISA showed that specific inhibition of PKC significantly reduced TNF-α secretion, confirming that PKC pathway is one of the main signal transduction pathways of α_{2A}-AR activation. This was further substantiated by JNK and I-κB pathways that were detected in microglia incubated with bisindolylmaleimide-1.

We next investigated the effect of α_{2A}-AR on sepsis brain damage. For this, we have used α_{2A}-AR knockout mice. It was argued that α_{2A}-AR would be activated in WT-LPS mice because sepsis induced the release of large amounts of NE [11]. On the other hand, in KO mice α_{2A}-AR was considered blocked because its gene was knocked out. It has been reported that peripheral inflammation induced by intraperitoneal injection of LPS can cause the inflammation in periventricular brain tissues and also the brain parenchyma. It is conceivable that this would activate microglia, cause neuroinflammation, and lead to cognitive impairment [33, 34]. We have used the sepsis model by intraperitoneal injection of LPS in mice. In order to mimic the actual clinical situation and observe the cognitive and motor functions in the middle and late stages of sepsis, we had opted a lower dose of LPS (15 mg/kg) to minimize the mortality rate of mice. The results showed that the mortality rate of sepsis mice induced by LPS was 33% which we considered to be acceptable.

Immunofluorescence showed that microglia lacking TNF-α expression were widely distributed in WT and KO-sham groups. When challenged with LPS, however, robust microglia activation occurred in WT-LPS group as manifested by the vigorous expression of TNF-α compared with KO-LPS mice. This strongly argued that excess release of NE in WT-LPS mice had activated and increased TNF-α production in activated microglia. This would be consistent with the results in vitro.

Additionally, double immunofluorescence of NeuN and synaptophysin showed that NeuN immunofluorescence in the pyramidal layer of the hippocampal CA2-3 regions in septic mice with α_{2A}-AR gene knockout was comparable to the WT-LPS mice. However, synaptophysin, a synaptic marker in the radiation layer (second synaptic connection area) was more obvious in KO-LPS mice than that in WT-LPS mice, indicating that α_{2A}-AR activation in sepsis had caused synaptic damage or synaptic dysfunction. This may offer an explanation for the decline in clinical cognitive impairment of learning and memory. Synaptic connections in the hippocampus are key units of learning and memory in the brain and convert short-term memory into long-term memory [35–37]. It has been reported that inflammatory factors can affect the function of synaptic connections. In a mouse model of sleep deprivation, an upsurge in inflammatory factors such as TNF-α, IL-1β and IL-6 derived from activated microglia was found in the dentate gyrus and CA2-3 areas of the hippocampus, which was coupled by a significant decline in spatial memory function [38]. IL-1β can reduce the expression of synaptophysin in presynaptic membrane and PSD-95 in postsynaptic of septic rats model, thereby reducing LTP [39, 40] through MAPK pathway. TNF-α can directly inhibit the production of LTP [41], change excitatory protrusion transmission and reduce the occurrence of LTP [42].

Finally, we evaluated the learning and memory function, motor function and emotional response of mice [43] in different experimental groups. The latency of mice in WT-LPS was significantly longer than that of WT-sham at all time points within 4 weeks, while the latency of KO group showed no difference between the sepsis group and the sham group at 3–4 weeks. It is noteworthy that the latency of KO-LPS mice was significantly lower than that of WT-LPS mice during 1–4 weeks. A similar phenomenon was observed in the experiment of the targeting quadrant detention time and the frequency of platform crossings. The

present Morris water maze experiment confirmed that sepsis can significantly reduce the learning and memory ability of mice as reported by many studies [44, 45]. Of note, blocking α_{2A}-AR can improve the learning and memory function of sepsis mice, especially in the later sepsis stage. In terms of motor function, rotating-stick test was used whereby mice were placed and keep balance from falling down [46]. If the mice had dyskinesia or poor coordination, they would fall off the stick very quickly. We showed here that sepsis could significantly reduce the motor function of mice, suggesting that this may be partly attributed to activation of α_{2A}-AR.

Behavior includes adaptation to the natural and social environment, and this involves psychology and emotion. Anxiety, depression or post-traumatic stress disorder often occur in sepsis patients [47]. Anxiety-like behavior was reported 10 days after operation in sepsis rats, accompanied by increased levels of inflammatory factors TNF-α, IL-1β and IL-6 in serum and brain tissues, suggesting that CNS inflammatory mediators are involved in anxiety-like symptoms in sepsis-associated encephalopathy [48]. In light of above, we have used the elevated plus maze and open-field test to evaluate the emotional and psychological state of sepsis mice. The results showed that there was no difference in both wild type mice and α_{2A}-AR knock-out mice, and that this was independent of sepsis. It stands to reason therefore that sepsis did not increase the anxiety and depression behavior, autonomous behavior, exploratory behavior and tension of animals and of animals in new and different environments; more importantly, α_{2A}-AR was not involved in these situations.

In conclusion, the present results have shown unequivocally that microglia express α_{2A}-AR. Remarkably, α_{2A}-AR activation can amplify LPS-induced microglia activation and enhance the expression and secretion of inflammatory factor TNF-α. It was further demonstrated that this was via PKC transmission which activates intracellular JNK and NF-κB pathways. In vivo experiments confirmed that α_{2A}-AR activation promotes the early activation of microglia and the expression of TNF-α in the hippocampus of LPS-treated mice. It is suggested that excess production of TNF-α would inhibit synaptic function, causing dysfunction of learning, memory and motor as demonstrated in the present neurobehavioral score study. On the basis of the present results, it is concluded that blocking α_{2A}-AR may be a potential therapeutic strategy for amelioration of brain function-related damage and neurological dysfunctions caused by sepsis.

Abbreviations

α_{2A}-AR	adrenergic receptor
CLP	cecal ligation and puncture
CREB	cAMP-response element binding protein
DG	diacylglycerol
ELISA	Enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinase
IκB	inhibitor of nuclear factor kappa B
JNK	c-Jun N-terminal kinases
KO	gene knock-out
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinases
NE	norepinephrine
NF-κB	nuclear factor-kappa B
PKA	protein kinase A
PKC	protein kinase C
SAE	Septic associated encephalopathy
TNF-α	tumor necrosis factor-α
WT	wild type

Declarations

Ethics approval and consent to participate

Ethical guidelines as stated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals were followed. The experiment was carried out under the supervision and authorization of the Experimental Animal Care and Use Committee at Jinan University (No. 20180225016).

Consent for publication

All authors have read and approved the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

H-DW and MF conceived the project and designed the experimental project; L-BZ designed the behavior experiments; MF, W-LS, X-MD, R-JW, D-MY performed the *in vivo* and *in vitro* experiments; HX, X-XT, YX and Y-QX analyzed the results; H-KZ contributed reagents and analytical tools; MF prepared the first draft of the manuscript. All authors participated in discussion and editing, and approved the final manuscript.

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Figures

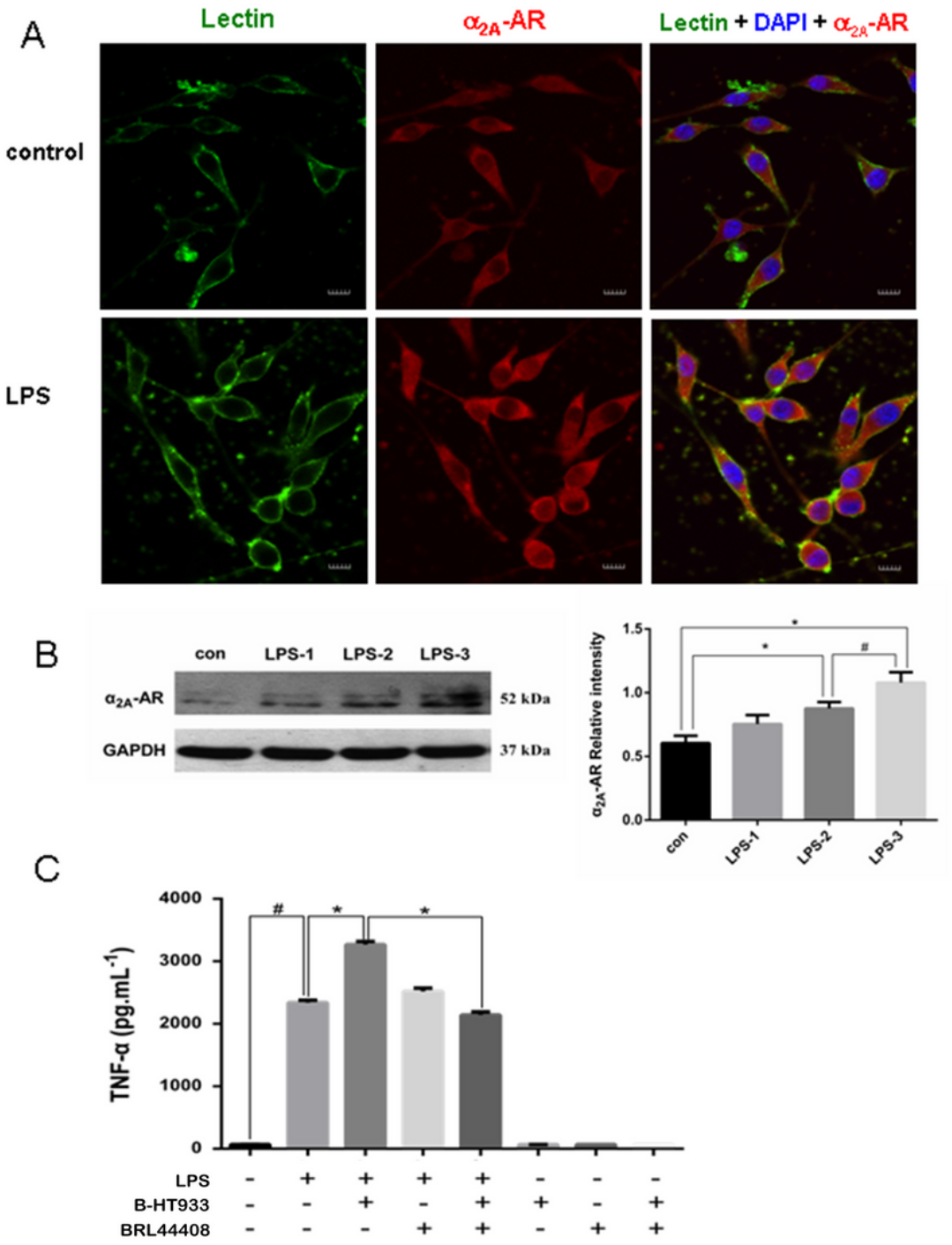

Figure 1

a2A-AR expression and the effect of its activation on TNF-α production in LPS-treated BV-2 microglia. (A) Confocal images showing a2A-AR (red) was expressed in control and LPS-treated BV-2 microglia labeled with lectin (green). The expression of a2A-AR was enhanced in activated microglia treated with 1000 ng/mL LPS for 6 h. Scale bar: 20 μm. (B) Western blotting showed that a2A-AR was expressed in BV-2 microglia that was dose-dependently augmented by LPS treatment for 6 h. LPS-1: 10 ng/mL; LPS-2: 100

ng/mL; LPS-3: 1000 ng/mL. (C) ELISA showed that BV-2 microglia hardly secreted TNF-α in control. TNF-α secretion was drastically increased in LPS group with 100 ng/mL LPS for 6 h, which was promoted by pretreatment with 1.0 μM BHT933, an a2A-AR agonist, for 15 min. The effect of BHT933 on TNF-α secretion was abolished by pretreatment with 1.0 μM BRL44408, an a2A-AR antagonist, for 30 min in LPS-treated BV-2 microglia. Compared with control group, #P<0.05. Compared with BHT933+LPS group, *P<0.05.

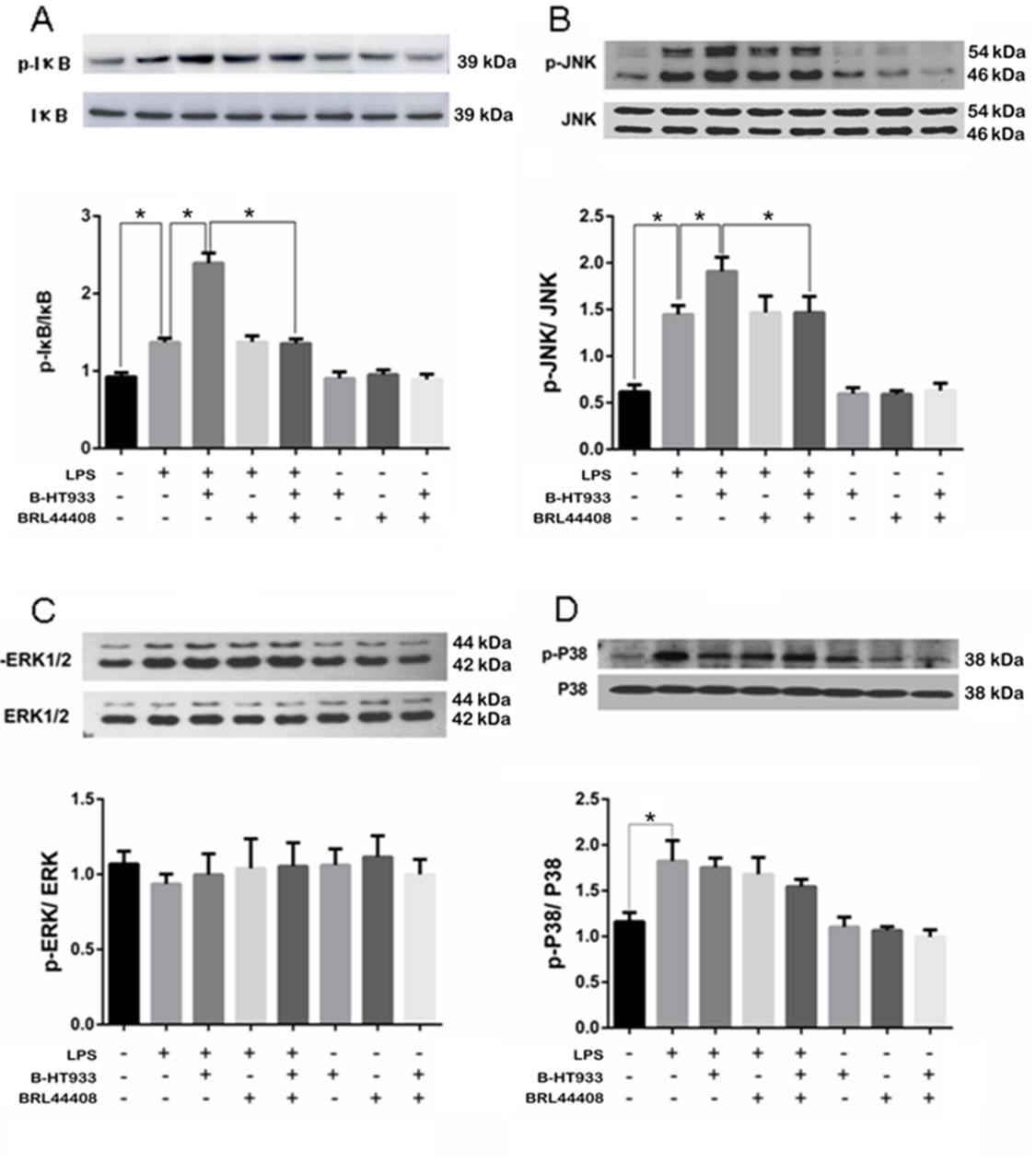

Figure 2

a2A-AR activation increased p-JNK and p-IκBa in LPS-activated BV-2 microglia. BHT933, an a2A-AR agonist, markedly enhanced LPS-induced IκBα (A) and JNK (B) phosphorylation in BV-2 microglia, whereas pretreatment with BRL44408, an a2A-AR antagonist, reversed the effect of BHT933. No changes were found in phosphorylation of ERK1/2 (C) and p38 (D). BV-2 microglia cells were pretreated with 1.0 μM BRL44408 or vehicle for 30 min, followed by 1.0 μM BHT933 or vehicle for 15 min, then incubated with 100 ng/mL LPS or vehicle for 30 min. *P<0.05.

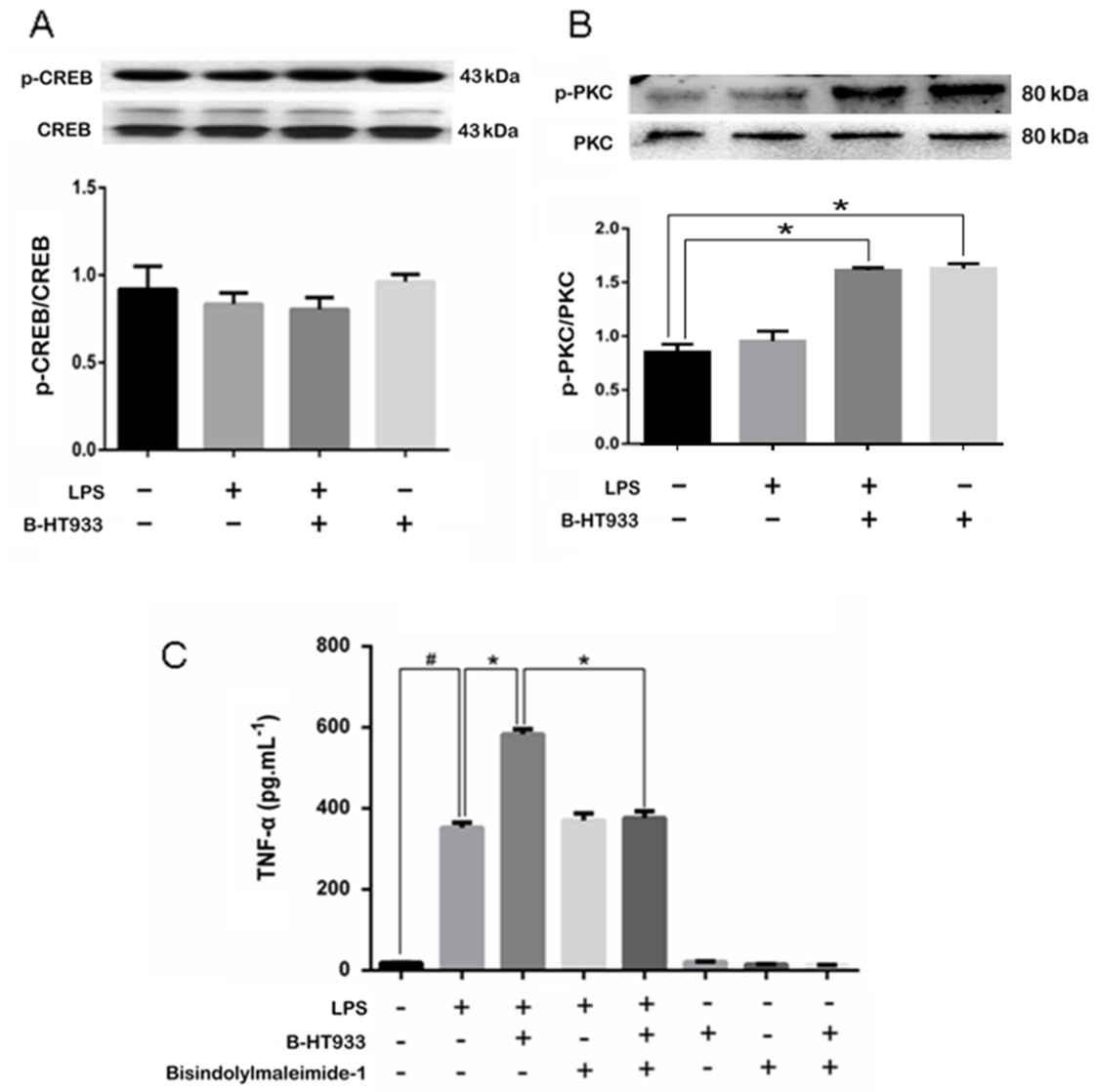

Figure 3

The role of PKC in a2A-AR activation in BV-2 microglia. (A, B) BV-2 microglia were incubated with 100 ng/mL LPS for 6 h with or without pretreatment with 1.0 μM BHT933 for 15 min. Western blotting showed that BHT933 and LPS did not affect CREB phosphorylation, whereas BHT933 increased PKC phosphorylation in BV-2 microglia with or without LPS treatment. (C) ELISA showed that BHT933 (1.0 μM for 15 min) enhanced TNF-α secretion and PKC inhibitor bisindolylmaleimide-1 (1.0 μM for 30 min) abolished the effect of BHT933 on TNF-α secretion in LPS-treated BV-2 microglia (100 ng/mL LPS for 6 h). Compared with control, #P<0.05; compared with BHT933+LPS, *P<0.05.

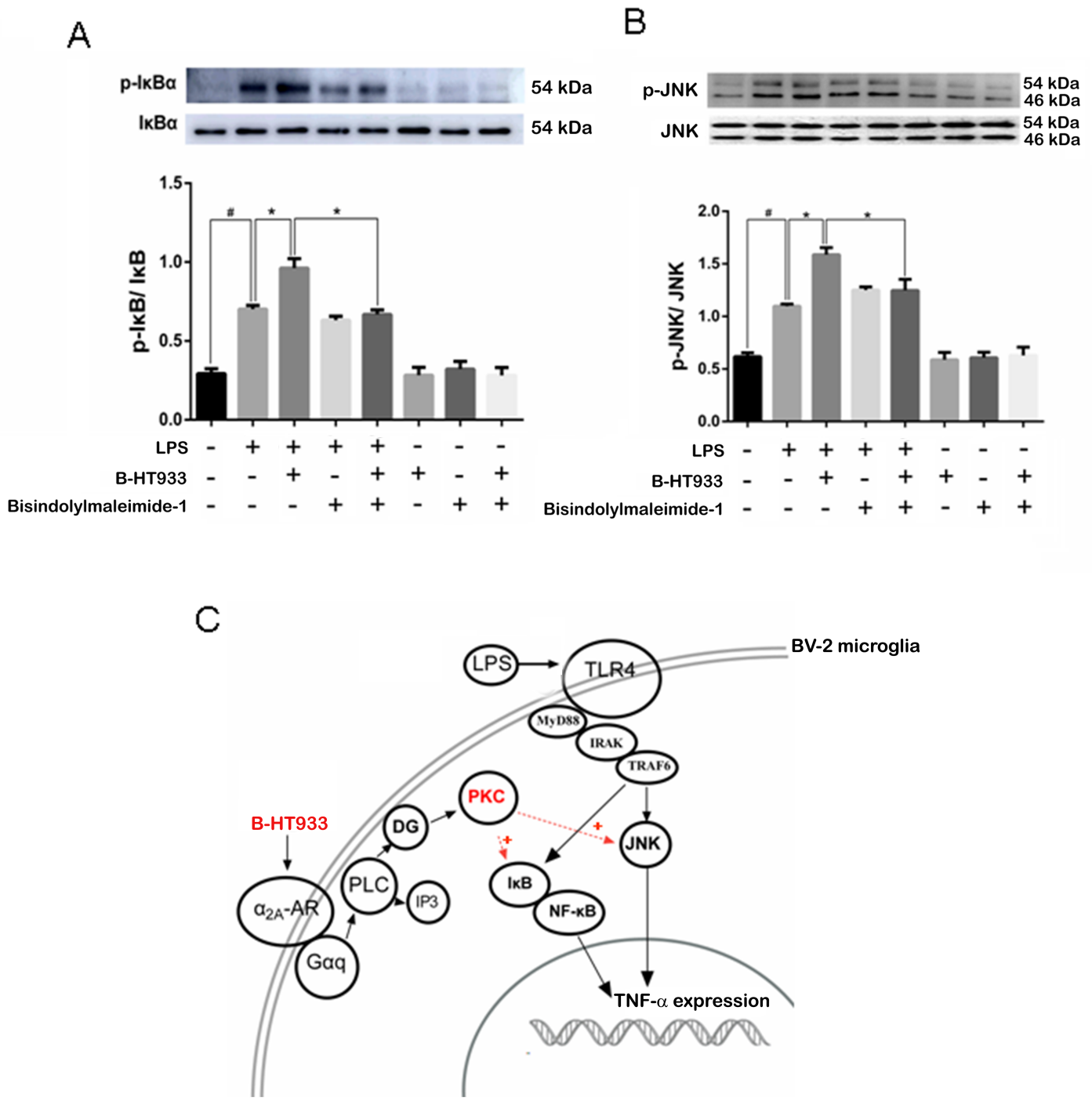

Figure 4

PKC-inhibition reversed the increase of p-IκBα and p-JNK caused by BHT933 in LPS-treated BV-2 microglia. (A, B) BHT933 (1.0 μM for 15 min) enhanced LPS-induced (100 ng/mL for 30 min) IκBα and JNK phosphorylation in BV-2 microglia, which was reversed by pretreatment with PKC inhibitor bisindolylmaleimide-1(1.0 μM for 30 min). Compared with control, #P<0.05; compared with A+LPS, *P<0.05. (C) A schematic diagram depicting the mechanisms, by which microglial a2A-AR activation by BHT933 promoted JNK and IκBα phosphorylation as well as TNF-α secretion through PKC pathway in LPS-treated BV-2 microglia.

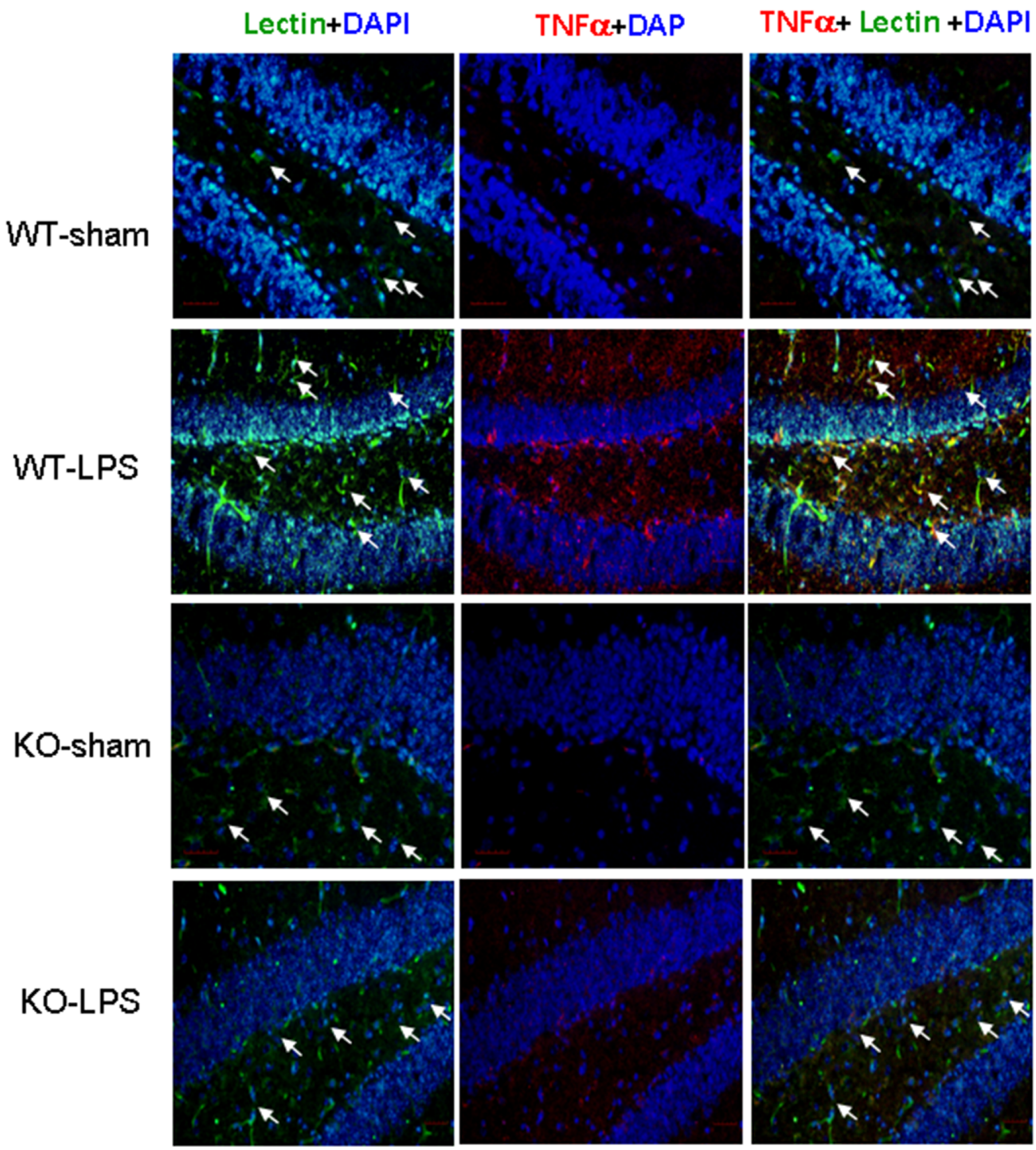

Figure 5

α2A-AR KO inhibited microglia activation in the hippocampal CA2/3 area in LPS-treated mice. Brain sections were prepared at 8 h after the mice were intraperitoneally injected with LPS at 15 mg/kg or with the same volumes of normal saline. After treatment with LPS, lectin-labeled microglia (green) were widely distributed in the hippocampal CA2/3 area in all groups (indicated by white arrows). In the WT-sham mice, lectin-labeled microglia did not express TNF-a (red). Lectin (green) and TNF-a (red) colocalization

was commonly observed in LPS-treated WT mice. In contrast, lectin-labeled microglia exhibited weak TNF-a expression in LPS-treated KO mice. Nucleus was stained with 4’,6-diamidino-2-phenylindole (DAPI, blue). Scale bars represent 50 μm.

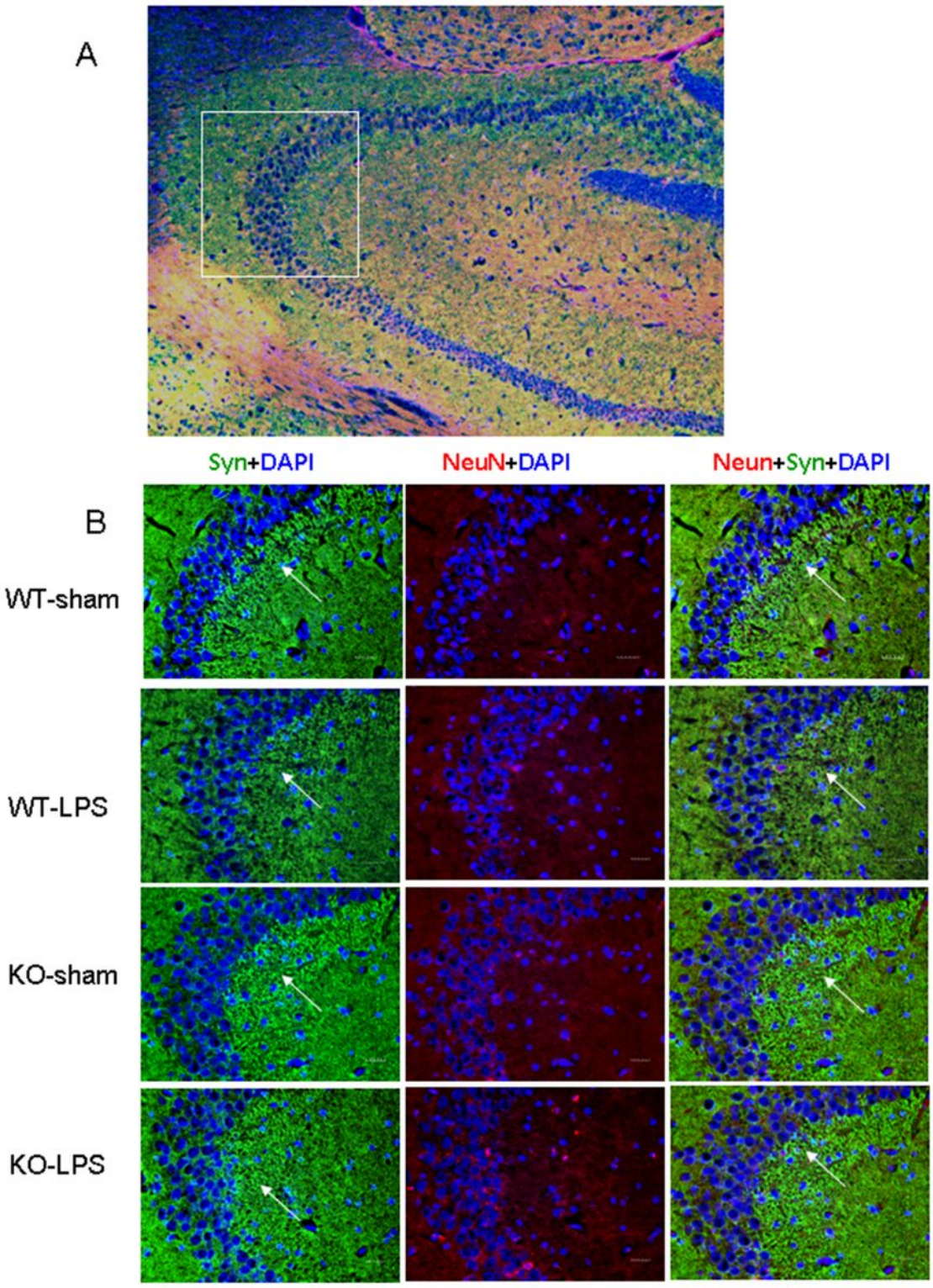

Figure 6

α2A-AR KO decreased synaptophysin staining in the CA2/3 hippocampal region in LPS-treated mice. Brain sections were prepared at 7 d after the mice were intraperitoneally injected with LPS 15mg/kg or

with equal volume of normal saline. (A) Hippocampal CA2/3 areas. Scale bar: 100 μm. (B) There was no significant difference in NeuN staining (red) in the pyramidal cell layer as evident by the closely packed cell nuclei (DAPI, blue) among the groups. A noticeable difference was observed in synaptophysin staining (green) in the radiation area. As indicated by the white arrow, the green synaptophysin fluorescence in the radiation area of the WT-sham mice was dense and discrete, while the green fluorescence in the corresponding area of LPS-treated WT mice was evidently decreased. However, compared with LPS-treated WT mice, synaptophysin immunofluorescence in the corresponding areas of LPS-treated KO mice was moderately decreased. Scale bar: 20 μm.

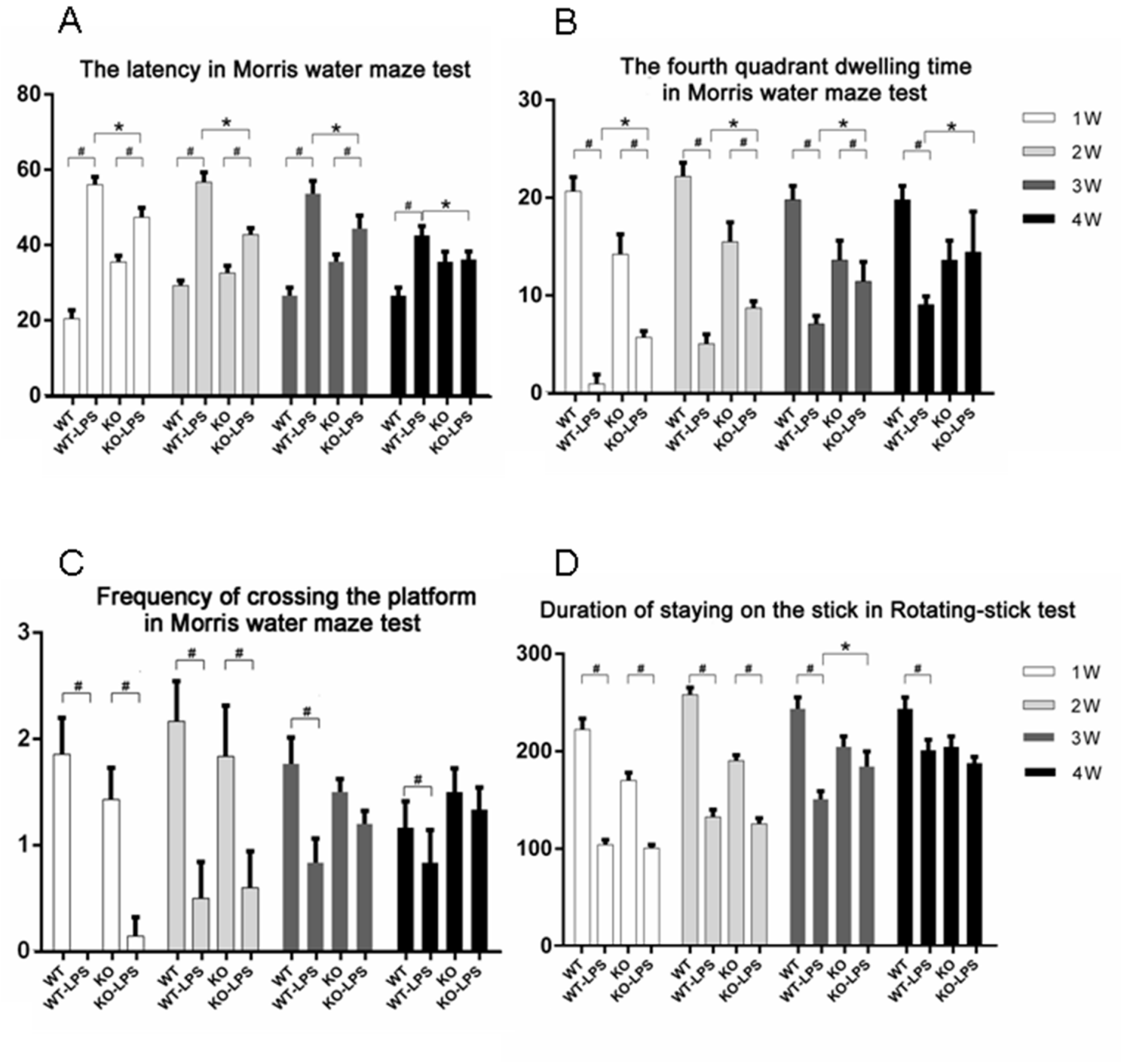

Figure 7

α2A-AR KO increased the outcomes of Morris water maze and Rotating-stick test in LPS-treated mice. In Morris water maze test (n=62), (A) the latency of WT-LPS group was significantly longer in 1-4 weeks in comparison with WT-sham group, while the latency of KO-LPS group was significantly longer, respectively, in 1-3 weeks compared with KO-sham group. In addition, the latency of KO-LPS group was significantly longer than that of WT-LPS group at each time of 1-4 weeks. (B) Compared with WT-sham, the fourth quadrant detention time in WT-LPS group was significantly shorter in 1-4 weeks; while in KO-LPS group, the detention time was significantly shorter than that in KO-sham group in 1-2 weeks, but no significant difference in 3-4 weeks. The fourth quadrant detention time of KO-LPS group was significantly longer than that of WT-LPS group at each time of 1-4 weeks. (C) Compared with WT-sham, the number of platform crossings in WT-LPS group decreased significantly in 1-4 weeks; while in KO-LPS group, the number of platform crossings in 1-2 weeks was significantly lower than that in KO-sham group, but no significant difference was found in 3-4 weeks. In Rotating-stick test (n=24), (D) the residence time of WT-LPS group was significantly reduced in the 1-4 week compared with WT-sham; while in KO-LPS group, the residence time of KO-LPS group was significantly longer than that of KO-sham group in the 1-2 weeks, but no statistical difference was found in 3-4 weeks. In the third week, the residence time of crossbar in KO-LPS group was significantly longer than that in WT-LPS group. #P<0.05, *P<0.05.

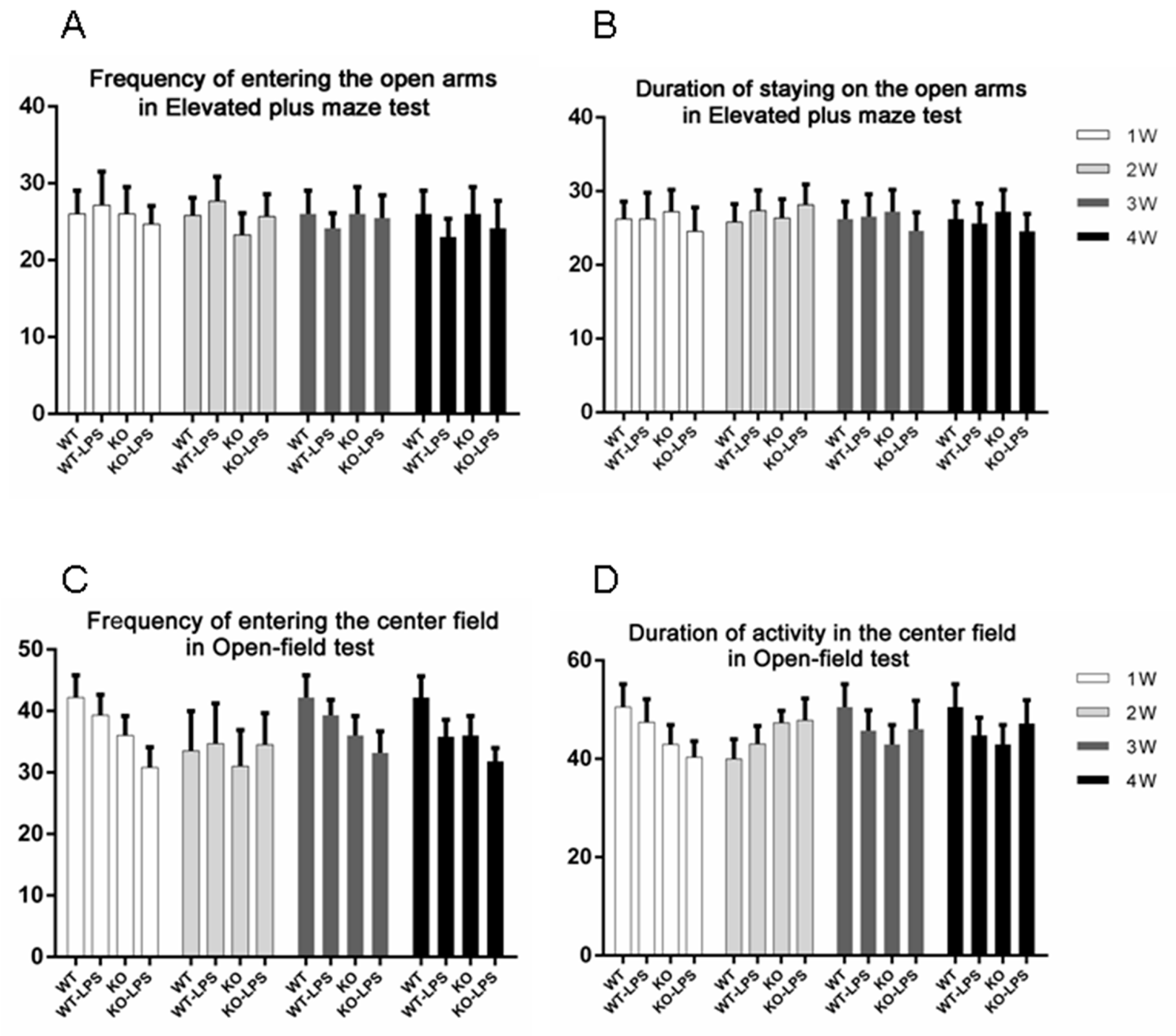

Figure 8

α2A-AR KO did not change the outcomes of Elevated plus maze and Open field test. The same groups of mice (n=48) were assessed across Elevated plus maze and Open-field test for 4 consecutive weeks. Through Elevated plus maze test, mice were placed in the central area for habituation at the 3rd day, and then the frequency of entering the open arms and the duration of staying on the open arm were record at the 4th day in each week. In Open-field test, mice were placed in the center of the bottom of the box at the 5th day for habituation followed by the measurement of the frequency of entering the center field and the duration of activity in the center field at the 7th day in each week. At 1-4 weeks, there was no significant difference in either the frequency of mice entering the open arms (A) or the length of stay in the open

arms (B) in Elevated plus maze test between each group. Similarly, at 1-4 weeks, there was no significant difference in either the number of times of mice entering the central region (C) or the time of central area activity (D) in Open-field test among the groups.