

Thioperamide Alleviates Lipopolysaccharide-Induced Neuroinflammation and Promotes Neurogenesis by Histamine Dependent Activation of H2R/PKA/CREB Pathway

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Research

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Abstract

Background

Adult neurogenesis in hippocampus dentate gyrus (DG) is associated with numerous neurodegenerative diseases such as aging and Alzheimer's disease (AD). Overactivation of microglia induced neuroinflammation is well acknowledged to contribute to the impaired neurogenesis in pathologies of these diseases and then leading to cognitive dysfunction. Histamine H3 receptor (H3R) is a presynaptic autoreceptor regulating histamine release via negative feedback way. Recently, studies show that H3R are highly expressed not only in neurons but also in microglia to modulate inflammatory response. However, whether inhibition of H3R is responsible for the neurogenesis and cognition in chronic neuroinflammation induced injury and the mechanism remains unclear.

Methods

Microglia activity, inflammation and neurogenesis were assessed in vivo by using lipopolysaccharide (LPS) induced model of inflammation. Mice were treated with thioperamide, pyrilamine or cimetidine to evaluate the effect of thioperamide on inflammation and the involving role of histamine. Protein levels of PKA/CREB and NF- κ B were assessed to investigate the mechanism by which thioperamide regulate inflammatory response and neurogenesis. The cognitive function was tested by novel object recognition, Y maze and morris water maze.

Results

In this study, we found that inhibition of H3R by thioperamide reduced the microglia activity and promoted a phenotypical switch from pro-inflammatory M1 to anti-inflammatory M2 in microglia, and ultimately attenuated LPS induced neuroinflammation in mice. Additionally, thioperamide rescued the neuroinflammation induced impairments of neurogenesis and cognitive function. Mechanically, the neuroprotection of thioperamide was involved in histamine dependent H2 receptor (H2R) activation, because cimetidine, an H2R antagonist but not pyrilamine, an H1R antagonist reversed the above effects of thioperamide. Moreover, thioperamide activated the H2R downstream phosphorylated protein kinase A (PKA)/cyclic AMP response element-binding protein (CREB) pathway but inhibited nuclear factor kappa-B (NF- κ B) signaling. Activation of CREB by thioperamide promoted interaction of CREB-CREB Binding Protein (CBP) to increase anti-inflammatory cytokines (Interleukin-4 and Interleukin-10) and brain-derived neurotrophic factor (BDNF) release but inhibited NF- κ B-CBP interaction to decrease pro-inflammatory cytokines (Interleukin-1 β , Interleukin-6 and Tumor necrosis factor α) release. H89, an inhibitor of PKA/CREB signaling, abolished effects of thioperamide on neuroinflammation and neurogenesis.

Conclusions

Taken together, these results suggested under LPS induced neuroinflammation, the H3R antagonist thioperamide inhibited microglia activity and inflammatory response, and ameliorated impairment of neurogenesis and cognitive dysfunction via enhancing histamine release. Histamine activated H2R and reinforced CREB-CBP interaction but weakened NF- κ B-CBP interaction to exert anti-inflammatory effects. This study uncovered a novel histamine dependent mechanism behind the therapeutic effect of thioperamide on neuroinflammation.

Background

Adult neurogenesis, a process of generating functional neurons from adult neural precursors, occurs throughout life in restricted brain regions in mammals [1, 2]. Within the hippocampal dentate gyrus (DG), newly born neurons in the granule cell layer (GL) send axonal projections to the CA3 subfield of the hippocampus and spineless dendrites to the molecular layer [3]. The new neurons then integrate into the existing hippocampal tri-synaptic circuitry by establishing synapses in the molecular layer with other neurons,[4] and then promote the ability of learning and memory [5]. Dysregulation of hippocampal neurogenesis has been shown to be an important mechanism underlying the cognitive impairment associated with normal aging, as well as the cognitive deficits in Alzheimer's disease (AD) and other neurodegenerative diseases [6]. Chronic neuroinflammation is a common pathological feature in normal aging as well as in these neurodegenerative conditions and has been shown to negatively affect hippocampal neurogenesis and cognitive processes across the lifespan [7]. Conversely, neurotrophic factors, environmental enrichment, learning, and exercise could positively regulate adult hippocampal neurogenesis and associated cognitive function [6, 8, 9]. Therefore, clarify the fundamental mechanisms regulating adult neurogenesis in physiological and pathological conditions will thus provide the basis for cell replacement therapy for treatment of disorders of central nervous system (CNS).

Microglia account for approximately 10% of cells in the CNS, and help shape neural circuits by modulating the strength of synaptic transmissions and sculpting neuronal synapses [10]. Overactivation of glial cells is commonly found in numerous neurodegenerative diseases such as normal aging and AD in CNS [11, 12]. Microglia activation is often categorized as either classical (M1) or alternative (M2). M1 microglia produce proinflammatory cytokines and chemokines, such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor α (TNF α) whereas M2 microglia produce anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10) and neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) [13]. Recent studies show that microglia are involved in neurogenesis [14]. IL4-driven M2 microglia polarization in the hippocampus trigger BDNF-dependent neurogenesis [15]. However, mediators of M1 microglia, including IL-1 β , IL-6 and TNF- α have been shown to have a negative effect on hippocampal neurogenesis by reducing the proliferation and survival of newborn cells [6, 16, 17]. Thus, it is of great importance to study focusing on therapeutic agents capable of inducing M2 or preventing M1 polarization in chronic neurodegenerative diseases.

Histamine is a heterocyclic amine formed by decarboxylation of the amino acid L-histidine. It is involved in the local regulation of physiological processes but also can occur exogenously in the food supply [18]. In the CNS, histamine is an endogenous neurotransmitter regulating many physiological processes including temperature regulation, emesis, food intake, and avoidance behavior [19, 20]. Accumulating evidence suggests that the activating histaminergic system in CNS may regulate brain injury [21, 22]. However, the direct application of histamine is clinically limited due to its poor penetration of the blood-brain barrier and its pro-inflammatory effect in the peripheral system [22]. Histamine H3 receptor (H3R) is a presynaptic autoreceptor that regulates histamine release from histaminergic neurons via negative feedback way [23, 24], as well as a heteroreceptor that regulates the release of other neurotransmitters [25–30]. A number of experiments have also provided evidences that inhibition of H3R could alleviate cognitive deficit in chronic inflammation related neurodegenerative diseases such as normal aging and AD [31–37]. H3R antagonist rescues autistic spectrum disorder-like behaviors through attenuating the proinflammatory cytokines [38]. Thioperamide, a histamine H3R antagonist, also suppresses inflammatory cell recruitment through histamine dependent mechanism [39]. Recent studies indicate that histamine and H3R are also involved in modulation of neurogenesis. Histamine enhances neurogenesis, and promotes neuronal differentiation and dendritic arbor complexity [40, 41]. Inhibition of H3R promotes neurogenesis in preterm white matter injury, traumatic brain injury and aging mice [36, 42–44]. Moreover, H3R is also involved in migration of neural stem cells (NSCs) [45]. Therefore, H3R antagonist may serve to develop new therapeutic approaches to overcome chronic inflammation related neurodegenerative disorders. However, the mechanism underlying the anti-inflammatory effect of H3R antagonist on microglial phenotypes and its effect on inflammation-related impairment of neurogenesis has not been reported.

In this study, we hypothesized that thioperamide, a H3R antagonist has a microglia-dependent anti-inflammatory effect, which is involved with neurogenesis. We investigated the effects of thioperamide on microglial phenotypes and hippocampal neurogenesis in LPS-induced inflammation in mice. We found that thioperamide inhibited inflammation and promoted neurogenesis through histamine-dependent H2R/cAMP/PKA/CREB pathway. Furthermore, this study showed that thioperamide promoted secretion of anti-inflammatory cytokines including IL-4, IL-10 and BDNF from M2 microglia via enhancing CREB-CREB binding protein (CBP) interaction but inhibited secretion of pro-inflammatory cytokines IL-1 β , IL-6 and TNF α from M1 microglia via suppressing CREB- nuclear factor kappa-B (NF- κ B) interaction. We would uncover a novel mechanism that H3R antagonist-mediated neurogenesis in chronic inflammation related neurodegenerative diseases.

Methods

Ethical statement

All animal studies were carried out according to protocols approved by the Institutional Animal Care and Use Committee of Binzhou Medical University Hospital, and conducted in compliance with the National

Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize any pain or discomfort, and the minimum number of animals was used.

Animals

Adult male C57BL/6 mice (Pengyue Laboratory, Jinan, China) weighing 22-25g were used in this study. The mice were housed in a temperature- and humidity-controlled animal facility, which was maintained on a 12 h light/dark cycle, food and water were given *ad libitum*.

Stereotaxic surgery

Stereotaxic surgery was performed as previously described [46]. Mice were anesthetized with the intraperitoneal injection of 1% chloral hydrate and then immobilized on a stereotaxic frame. The gauge guide cannula was implanted into the lateral ventricle (0.2 mm posterior, 1.1 mm lateral and 2.7 mm ventral to the bregma). After surgery, mice were housed individually and allowed to recover for 7 d.

Drug treatments

For *in vivo* study, a stainless-steel injector connected to a 5-ml syringe was inserted into the guide cannula and extended 1 mm beyond the tip. The administration of the chemicals was administered on the basis of previous studies [47]. LPS (i.p., 1 mg/kg) or vehicle was administered daily at 7 days after stereotaxic surgery for 5 days. Thioperamide (i.p., 5 mg/kg) or vehicle was administered daily at 7 days after stereotaxic surgery until the beginning of the behavior tests on day 14. H89 or vehicle (*i.c.v.*, 2 µg of 2 µl) was administered 0.5 h before thioperamide injection. 5-bromo-2'-deoxyuridine (BrdU) (i.p., 50mg/kg) was administered for 4 times every 4 h at 7 days after stereotaxic surgery, and then administered every other day before the behavior experiments [48]. At 14 days after stereotaxic surgery, the novel object recognition (NOR) test was carried on for 4 days. At 18 days after stereotaxic surgery, Y maze (YM) test was carried on for 1 day. At 19 days after stereotaxic surgery, morris water maze (MWM) test was carried on for 6 days. During the period of behavior test, thioperamide or vehicle was administered 0.5 h, whereas H89 or vehicle was administered 1 h before the test at day 14, day 18 and day 19.

Immunohistochemistry

Immunostaining was performed in frozen brain sections. Frozen brain sections were fixed in 4% paraformaldehyde for 2 h, and then incubated in 5% bovine serum albumin (BSA, Solarbio) for 2 h to block nonspecific binding of IgG. Then the cells were reacted with goat antibody against Iba1 (Abcam, 1:300), mouse antibody against NeuN (Millipore, 1:300), MAP2 (Millipore, 1:300) and rabbit monoclonal antibody against DCX (Cell Signaling Technology, 1:100). After repeated washes in PBS buffer, cells were incubated with secondary antibody in 3% BSA for 2 h at 25°C. The secondary antibodies used in this experiment were donkey anti-mouse IgG-AlexaFluo 488 (1:300, 21202, Invitrogen), goat anti-rabbit IgG-AlexaFluo 546 (Invitrogen, 1:300), goat anti-rabbit IgG-AlexaFluo 647 (Invitrogen, 1:300), goat anti-mouse IgG-AlexaFluo 647 (Invitrogen, 1:300), donkey anti-rabbit IgG-AlexaFluo 488 (Invitrogen,

1:300), goat anti-mouse IgG-AlexaFluo 546 (Invitrogen, 1:300), and donkey anti-goat IgG-AlexaFluo 546 (Invitrogen, 1:300). After further washing in PBS, cultures were dried, cover slipped and mounted on glass slides. All the immunohistochemical data represent mean values of 5 brain sections per mouse. The area coverage as well as the number of Iba1-positive, DCX-positive cells were quantified using ImageJ.

BrdU/DCX/NeuN staining

BrdU was used to label newly born cells, DCX was used to label immature neurons and NeuN was used to label mature neurons. The frozen brain sections (40 μ m) were incubated for 30 min in 2 N HCl at 37°C, and neutralized with 0.1 M borate buffer (Sinopharm Chemical Reagent, PH=8.5) for 10 min. After incubating in 1% H₂O₂ (30% H1009, Sigma) for 10 min, the sections were blocked with PBS containing 1% BSA and 0.3% (w/v) Triton X-100 (T8787, Sigma) for 1 h at room temperature. They were then reacted with rat monoclonal anti-BrdU (1:50, Abcam), rabbit monoclonal anti-DCX (1:50, Cell Signaling Technology) and mouse monoclonal antibody against NeuN (1:300, Millipore) at 4°C overnight. After washing with PBS, they were incubated with secondary antibody for 2 h at room temperature. The stained cells were observed under a laser scanning confocal microscope (Leica TCS SPE, Germany). Image analysis was performed using Image J software.

Stereological cell counting of BrdU+ cells

We used the stereological techniques to count the number of BrdU+ cell as described previously [49]. BrdU+ and BrdU+/DCX+ cells from every eighth section covering the entire rostrocaudal axis of the DG were counted using a high-power (40 \times) microscope. Cells were counted in a blind manner. At least eight sections from both sides of the DG were counted per animal. The number for each group of animals is indicated in figure legends.

ELISA

Mice were anaesthetized by i.p. injection of 1% chloral hydrate, sacrificed and the brain was quickly removed. The separated tissues were lysed in ice-cold RIPA lysis buffer (R0020, Solarbio), then centrifuged at 14,000 \times g at 4°C for 20 min, and the supernatants were measured for soluble IL-1 β , IL-6, TNF- α , IL-4 and BDNF ELISAs (R&D) according to the manufacturer's instructions. The values were expressed as amount per total protein.

RT-PCR

Total RNA from mice hippocampus and cortex was extracted using Trizol reagent (Invitrogen, USA). Reverse transcription was performed with an Exscript RT Reagent Kit (Takara Bio Inc., China). Real-time PCR analysis was undertaken using SYBR Premix Ex Taq (Takara Bio Inc., China). The primer sequences for IL-1 β were 5'-CAAGGCCACAGGTATTTTGT-3' (sense) and 5'-GAAATGCCACCTTTTGACAG-3' (antisense). The primer sequences for IL-6 were 5'-GGCCTTCCCTACTTCACAAG-3' (sense) and 5'-ATTTCCACGATTTCCAGAG-3' (antisense). The primer sequences for TNF- α were 5'-CAGCCTCTTCTCATTCTGCTTGTG-3' (sense) and 5'-CTGGAAGACTCCTCCAGGTATAT-3' (antisense).

The primer sequences for IL-4 were 5'-GGCATTTTGAACGAGGTCAC-3' (sense) and 5'-AAATATGCGAAGCACCTTGG-3' (antisense). The primer sequences for IL-10 were 5'-ATTTGAATTCCTGGGTGAGAAG-3' (sense) and 5'-CACAGGGGAGAAATCGATGACA-3' (antisense). The primer sequences for BDNF I were 5'-GCTTTGCGGATATTGCGAAGGGTT-3' (sense) and 5'-ACCTGGTGG AACATTGTGGCTTTG-3' (antisense). The primer sequences for BDNF II were 5'-TGAAGTTGGCTTCCTAGCGGTGTA-3' (sense) and 5'-TGGTGGAACTTCTTT GCGGCTTAC-3' (antisense). The primer sequences for BDNF III were 5'-CCAGAGCAGCTGCCTTGATGTTTA -3' (sense) and 5'-CCGCCTTCATGCAAC CGAAGTAT-3' (antisense). The primer sequences for BDNF IV were 5'-TGACAA CAATGTGACTCCACTGCC-3' (sense) and 5'-ATGGTCATCACTCTTCTCACCTGG-3' (antisense). Polymerase chain reaction parameters were 95°C for 3 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative expression of the genes was normalized to the mean levels of β -actin.

Western blot

Western blot was performed as previously described [50]. Briefly, mice were anaesthetized by i.p. injection of chloral hydrate (400 mg/kg), sacrificed and the brain was quickly removed. The separated tissues were lysed in ice-cold RIPA lysis buffer (R0020, Solarbio), then centrifuged at 14,000×g at 4°C for 20 min, and the protein concentration in the extracts was determined by the Bradford assay (Thermo, Hercules, CA). The precipitates were denatured with SDS sample loading buffer and separated on 10% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes using a Bio-Rad mini-protein-III wet transfer unit overnight at 4°C. Transfer membranes were then incubated with blocking solution (5% nonfat dried milk dissolved in tris buffered saline tween (TBST) buffer (in mM): 10 Tris-HCl, 150 NaCl, and 0.1% Tween-20) for 2 h at room temperature, and incubated with primary antibody overnight at 4°C. The primary antibodies used in this experiment were phospho-PKA (Cell Signaling Technology, 1:1,000), PKA (Cell Signaling Technology, 1:1,000), phospho-CREB (Cell Signaling Technology, 1:1,000), CREB (Cell Signaling Technology, 1:1,000), phospho-P65 NF- κ B (abcam, 1:1000), P65 NF- κ B (abcam, 1:1000), CBP (abcam, 1:1000) and GAPDH (Boster, 1:3,000). Membranes were washed three times in TBST buffer and incubated with the appropriate secondary antibodies (LI-COR, Odyssey, 1:5,000) for 2 h. Images were acquired with the Odyssey infrared imaging system and analyzed as specified in the Odyssey software manual. The results were expressed as the target protein/GAPDH ratio and then normalized to the values measured in the control groups (presented as 100%).

Novel object recognition (NOR)

The NOR test was performed 14 days after stereotaxic surgery as previously described [46, 51]. Briefly, mice received 2 d of habituation in a 45 × 45 cm square arena, and on the third day, they were allowed to explore two identical objects made from large Lego bricks for 10 min (training trial). They were returned to their home cage, and 24 h later, a different shape and color object replaced one of the objects and the mice were returned to the arena for 10 min (testing trial). The time spent on each object was then calculated as a percentage of total object exploration.

Y maze (YM)

The Y maze test was performed 18 days after stereotaxic surgery as previously described [46, 51]. Briefly, the apparatus for YM was made of gray plastic, with each arm 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top. The three arms were connected at an angle of 120°. Mice were individually placed at the end of an arm and allowed to explore the maze freely for 8 min. The total arm entries and spontaneous alternation percentage (SA%) were measured. SA% was defined as a ratio of the arm choices that differed from the previous two choices ('successful choices') to total choices during the run ('total entry minus two' because the first two entries could not be evaluated). For example, if a mouse made 10 entries, such as 1-2-3-2-3-1-2-3-2-1, there are 5 successful choices in 8 total choices (10 entries minus 2). Therefore, SA% in this case is 62.5%.

Morris water maze (MWM)

The MWM maze test was performed 19 days after stereotaxic surgery as previously described [46, 52]. Briefly, the water maze of 1.50 m in diameter and 0.50 m in height was filled with water ($20 \pm 1^\circ\text{C}$) to maintain the water surface 1.50 cm higher than the platform (10 cm in diameter). Water was dyed white and the tank was divided into four quadrants by four points: North (N), South (S), East (E), and West (W). The platform was placed at the center of either quadrant and video tracking software was used to automatically track the animals. Learning and memory acquisition lasts for five days. Animals were put into the water from four points in random order every day until they found the platform and stayed for 10 s within 1 min. If the mice cannot find the platform within 1 min, they were guided to the platform. Following acquisition test, on the sixth day, learning and memory maintenance test was carried on. The platform was removed, and the mice were placed in water from the opposite quadrant of the platform, and then the times crossing the platform was recorded within 1 min.

Statistical analysis

Results are expressed as mean \pm SEM. Statistical analysis was performed by one-way ANOVAs followed by Tukey's *post hoc* comparisons or two-way ANOVAs followed by Bonferroni *post hoc* comparisons, using prism software. *P* value <0.05 was considered statistically significant.

Results

Thioperamide decreases LPS-induced microglial activation and pro-inflammatory cytokines production

The study of the microglia response in the inflammatory process has been copiously supported by the use of LPS, a gram-negative cell wall component [53]. LPS binds to the CD14/TLR4/MD2 receptor complex, located on the cell membrane, triggering classical microglial responses such as proliferation, migration, phagocytosis and release of inflammatory mediators [54, 55]. Therefore, LPS was used to evaluate the effect of thioperamide, a H3R antagonist on neuroinflammation in mice. We examined the activated microglia in DG of the hippocampus by Iba1 immunostaining. Results showed that the area of

Iba1⁺-cells in the hippocampal DG of LPS treated mice increased dramatically compared with the vehicle treated mice (from 0.9187 ± 0.08736 to 5.200 ± 0.4575 , $P < 0.001$, Figure 1A, B), which was reversed by administration of thioperamide (to 2.258 ± 0.2632 , $P < 0.01$, Figure 1A, B).

The activation of microglia may promote the pathological process of chronic neuroinflammation-related diseases through releasing of pro-inflammatory cytokines which could lead to neuronal damage and cognitive impairments [56, 57]. Moreover, the inflammatory cytokines in neurodegenerative diseases are thought to lead to an impairment of neurogenesis [6]. Therefore, we investigated transcriptional and expressional level of pro-inflammatory factors in LPS treated mice. To confirm the effect of thioperamide on the gene transcription of inflammatory cytokines, we tested the effects of thioperamide on mRNA expression of IL-1 β , IL-6 and TNF- α by RT-PCR. We found that LPS up-regulated the mRNA level of pro-inflammatory cytokines IL-1 β (increased to $363.8 \pm 39.05\%$ of control, $p < 0.01$, Figure 1C), IL-6 (increased to $431.7 \pm 84.46\%$ of control, $p < 0.01$, Figure 1D) and TNF- α (increased to $571.1 \pm 83.20\%$ of control, $p < 0.001$, Figure 1E) in hippocampus. Interestingly, thioperamide decreased the mRNA level of IL-1 β (decreased to $122.0 \pm 37.85\%$ of control, $p < 0.05$, Figure 1C), IL-6 (decreased to $150.0 \pm 15.90\%$ of control, $p < 0.01$, Figure 1D) and TNF- α (decreased to $187.8 \pm 63.97\%$ of control, $p < 0.001$, Figure 1E). We further confirmed the effect of thioperamide on protein levels of inflammatory cytokines by ELISA. Similarly, we found that the upregulated protein level of IL-1 β (from $311.0 \pm 44.29\%$ to $109.5 \pm 23.29\%$ of control, $p < 0.01$, Figure 1F), IL-6 (from $280.8 \pm 42.87\%$ to $110.3 \pm 26.32\%$ of control, $p < 0.05$, Figure 1G) and TNF- α (from $262.8 \pm 18.67\%$ to $116.3 \pm 22.52\%$ of control, $p < 0.001$, Figure 1H) induced by LPS in hippocampus was significantly reversed by thioperamide. Taken together, all these results suggested that thioperamide could effectively suppress both activation of microglia and secretion of pro-inflammatory cytokines in LPS treated mice.

Thioperamide ameliorates LPS-induced impairment of neurogenesis

Normal aging might prime microglia towards the classic M1 phenotype, and increase basal release of the pro-inflammatory cytokines IL-1 β , interleukin-6 and TNF α , which have been shown to reduce hippocampal neurogenesis [58]. Therefore, we next assessed the effect of thioperamide on adult neurogenesis in the DG region of the hippocampus. We examined the newborn cells labeled by BrdU in DG 1 day after the last time of LPS administration. The results indicated that the BrdU⁺ cells in the DG region of hippocampus decreased in the vehicle group compared to the control group (decreased to $51.62 \pm 1.475\%$ of control, $P < 0.001$, Figure 2A, D), and thioperamide rescued the decreased BrdU⁺ cells to $85.84 \pm 4.788\%$ significantly ($P < 0.001$, Figure 2A, D).

To further investigate the effect of thioperamide on neurogenesis in LPS-treated mice, we analyzed the presence of neurogenesis in DG of hippocampus. Firstly, Doublecortin (DCX)⁺ neuroblasts and immature neurons were detected. The results indicated that the number of DCX⁺ cells decreased significantly after LPS treatment (decreased to $63.36 \pm 3.342\%$ of control, $P < 0.001$, Figure 2B, E), which was reversed dramatically by administration of thioperamide (increased to $92.47 \pm 5.029\%$ of control, $P < 0.01$, Figure 2B, E). The number of BrdU⁺/DCX⁺ cells was also analyzed to assess the effect of thioperamide on

immature newborn neurons. Results showed that the number of BrdU⁺/DCX⁺ cells decreased significantly after LPS treatment (decreased to $37.25 \pm 1.307\%$ of control, $P < 0.001$, Figure 2B, F), and thioperamide remarkably rescued the impairment of immature newborn neurons (increased to $85.62 \pm 4.999\%$ of control, $P < 0.001$, Figure 2B, F). Moreover, the percentage of BrdU⁺/DCX⁺ newborn neuronal cells over BrdU⁺ newborn cells was further analyzed, and the results showed that the decreased percentage of BrdU⁺/DCX⁺ cells over BrdU⁺ cells in the LPS treated mice was significantly reversed by administration of thioperamide (from $32.66 \pm 1.37\%$ to $45.09 \pm 1.543\%$, $P < 0.05$, Figure 2B, G).

We further examined the effect of thioperamide on mature newborn neurons in DG of hippocampus by BrdU⁺/NeuN⁺ staining. Results showed that, number of BrdU⁺/NeuN⁺ cells decreased significantly in the LPS vehicle group compared to the control group (decreased to $42.36 \pm 2.778\%$ of control, $P < 0.001$, Figure 2C, H), and thioperamide rescued the impairment of mature newborn neurons significantly (increased to $93.06 \pm 5.534\%$ of control, $P < 0.001$, Figure 2C, H). In addition, the percentage of BrdU⁺/NeuN⁺ newborn mature neurons over BrdU⁺ newborn cells were also analyzed, and the results showed that the decreased percentage of BrdU⁺/NeuN⁺ cells over BrdU⁺ cells in the LPS treated mice was significantly reversed by administration of thioperamide (from $31.44 \pm 0.8138\%$ to $43.39 \pm 1.269\%$, $P < 0.01$, Figure 2B, G).

Taken together, results above showed that thioperamide promoted neurogenesis in LPS induced neuroinflammation.

Thioperamide alleviates LPS-induced cognitive dysfunction

Neuroinflammation plays an important role in the onset and progression of neurodegenerative diseases such as aging and AD. LPS level is higher in the brains of AD patients and is associated with neuroinflammation and cognitive dysfunction [59]. In this research, we studied the effect of thioperamide on LPS-induced cognitive decline. The NOR test indicated that time spending on novel objection decreased significantly in the LPS group compared with the control group (from $70.15 \pm 2.384\%$ to $51.57 \pm 4.737\%$, $P < 0.01$, Figure 3A). Administration of thioperamide significantly increased the time spending on novel object (to $66.07 \pm 2.282\%$, $P < 0.05$, Figure 3A) in LPS treated mice. In the YM test, we observed a decreased spontaneous alternation % (SA%) in the LPS group compared with the control group (from $83.20 \pm 3.751\%$ to $57.08 \pm 7.202\%$, $P < 0.01$, Figure 3B). Administration of thioperamide increased the SA% to $81.40 \pm 4.007\%$ significantly in LPS treated mice ($P < 0.05$, Figure 3A). In Morris water maze (MWM) test, the escape latency increased significantly in the LPS group on day 3 to day 5 ($P < 0.05$, Figure 3C). Administration of thioperamide significantly decreased the escape latency ($P < 0.01$, Figure 3C) in LPS treated mice. Moreover, times crossing the platform decreased in LPS treated mice (from 7.000 ± 0.5669 to 3.500 ± 0.4226 , $P < 0.01$, Figure 3D) on day 6, and administration of thioperamide increased it significantly (to 6.250 ± 0.5901 , $P < 0.05$, Figure 3D) in LPS treated mice. Results above suggested that thioperamide improved the cognitive impairments in LPS treated mice.

The effects of thioperamide on neuroinflammation, neurogenesis and cognition involve histamine dependent H2R activation

Histamine has been shown to counteract LPS-induced glial activation and release of pro-inflammatory cytokines release as well as neurogenesis impairment [40, 53]. Moreover, numerous evidences indicated that central histamine have an important role in cognitive function as it has been shown to enhance memory [60]. As a presynaptic receptor on histaminergic neurons, H3R suppresses histamine synthesis and releases in a negative feedback way. Therefore, inhibition of H3R by thioperamide leads to enhanced synaptic histamine release [47]. In order to confirm whether or not the effects of thioperamide are histamine dependent, pyrilamine or cimetidine, antagonist of H1R or H2R, was applied.

We found that the effect of thioperamide on the area of ionized calcium binding adapter molecule 1 (Iba1)⁺-cells in hippocampal DG region was reversed by administration of H2R antagonist cimetidine (from 2.258 ± 0.2632 to 4.914 ± 0.7837 of control, $P < 0.01$, Figure 1A, B) but not H1R antagonist pyrilamine (from 2.258 ± 0.2632 to 2.288 ± 0.3927 of control, $P > 0.05$, Figure 1A, B). Moreover, the inhibited transcription of pro-inflammatory cytokines was reversed by cimetidine (IL-1 β levels from 109.5 ± 23.29 of control to 298.4 ± 37.33 of control, $P < 0.05$, Figure 1C; IL-6 levels from 110.3 ± 26.32 of control to 260.0 ± 40.27 of control, $P < 0.01$, Figure 1D; TNF α levels from 116.3 ± 22.52 to 240.0 ± 24.06 of control, $P < 0.01$, Figure 1E) but not pyrilamine (IL-1 β , $P > 0.05$, Figure 1C; IL-6, $P > 0.05$, Figure 1D; TNF α $P > 0.05$, Figure 1E). Similarly, decreased protein levels of pro-inflammatory cytokines were also reversed by cimetidine (IL-1 β levels from 122.0 ± 37.85 to 329.0 ± 79.83 of control, $P < 0.01$, Figure 1F; IL-6 levels from 150.0 ± 15.90 to 437.0 ± 63.22 of control, $P < 0.05$, Figure 1G; TNF α levels from 187.8 ± 63.97 to 521.7 ± 45.74 of control, $P < 0.01$, Figure 1H) but not pyrilamine (IL-1 β , $P > 0.05$, Figure 1F; IL-6, $P > 0.05$, Figure 1G; TNF α $P > 0.05$, Figure 1H). Above all, these results showed that thioperamide inhibited LPS induced microglial activation and inflammatory response through histamine dependent H2R activation.

We further examined the role of histamine in the enhanced neurogenesis offered by thioperamide in LPS treated mice. As expect, we found that the BrdU⁺ cells in the DG region of hippocampus were reversed by administration of cimetidine (from 85.84 ± 4.788 to 50.15 ± 3.789 of control, $P < 0.001$, Figure 2A, D) but not pyrilamine (from 85.84 ± 4.788 to 88.20 ± 2.814 of control, $P > 0.05$, Figure 2A, D). Otherwise, the number of BrdU⁺/DCX⁺ cells and BrdU⁺/NeuN⁺ cells were both compromised by administration of cimetidine (BrdU⁺/DCX⁺ cells from 85.62 ± 4.999 to 34.64 ± 2.216 of control, $P < 0.001$, Figure 2B, F; BrdU⁺/NeuN⁺ cells from 93.06 ± 5.534 to 43.06 ± 1.770 of control, $P < 0.001$, Figure 2C, H) but not pyrilamine (BrdU⁺/DCX⁺ cells, $P > 0.05$, Figure 2A, D; BrdU⁺/NeuN⁺ cells from 2.258 ± 0.2632 to 2.288 ± 0.3927 of control, $P > 0.05$, Figure 2A, D). Taken together, these results suggested that thioperamide rescued LPS induced impaired neurogenesis through histamine dependent H2R activation.

Finally, we tested whether histamine was involved in the alleviated cognitive impairment offered by thioperamide in LPS treated mice. The NOR test showed that time spending on novel objection was reversed by administration of cimetidine (from $66.07 \pm 2.282\%$ to $52.62 \pm 3.254\%$ of control, $P < 0.05$, Figure 3A) but not pyrilamine (from $66.07 \pm 2.282\%$ to $65.33 \pm 2.483\%$ of control, $P > 0.05$, Figure

3A). The YM test also showed that SA% was reversed by administration of cimetidine (from $81.40 \pm 4.007\%$ to $58.59 \pm 5.367\%$ of control, $P < 0.05$, Figure 3B) but not pyrilamine (from $81.40 \pm 4.007\%$ to $81.27 \pm 3.499\%$ of control, $P > 0.05$, Figure 3B). In the MWM test, the escape latency was reversed by administration of cimetidine ($P < 0.05$, Figure 3C) but not pyrilamine ($P > 0.05$, Figure 3C) on day 4 to day 5. In addition, increased times crossing the platform offered by thioperamide was reversed by cimetidine (from $6.250 \pm 4.007\%$ to $3.375 \pm 0.8004\%$ of control, $P < 0.05$, Figure 3D) but not pyrilamine ($P > 0.05$, Figure 3D). In all, results above showed that thioperamide alleviated LPS induced cognitive dysfunction through histamine dependent H2R activation.

Thioperamide reverses LPS-induced inactivation of PKA/CREB pathway via histamine dependent H2R activation

Results above showed that histamine dependent H2R activation is involved in the protection against LPS induced inflammatory response. Recent reports indicate that H2R and its downstream activation of cAMP/PKA is also necessary to the inhibited immune response of histamine [61, 62]. Moreover, cAMP/PKA/CREB signaling is considered to play an important role in the suppression of microglia activation and its related neuroinflammation by inhibiting NF- κ B activation [63, 64]. Thus, in order to elucidate the mechanisms of the anti-inflammatory offered by thioperamide in LPS treated mice, we investigated the H2R downstream protein level of PKA and CREB. In consistent with the previous reports, decreased p-PKA and p-CREB level were observed in hippocampus in LPS treated mice (p-PKA decreased to $55.89 \pm 7.068\%$ of control group, $P < 0.01$, Figure 4A, B; p-CREB decreased to $55.87 \pm 3.954\%$ of control group, $P < 0.001$, Figure 4A, C). As expect, thioperamide up-regulated the p-CREB expression (p-PKA increased to $97.94 \pm 18.63\%$ of control group, $P < 0.05$, Figure 4A, B; p-CREB increased to $90.00 \pm 6.892\%$ of control group, $P < 0.01$, Figure 4A, C), which was reversed by cimetidine (p-PKA decreased to $59.38 \pm 8.18\%$ of control group, $P < 0.05$, Figure 4A, B; p-CREB decreased to $59.32 \pm 3.999\%$ of control group, $P < 0.01$, Figure 4A, C) but not pyrilamine, suggesting thioperamide activated the H2R downstream PKA/CREB signaling.

The phosphorylated CREB exerts a dual function in inflammatory response. First, by forming a complex with CBP to activate transcription of anti-inflammatory cytokines such as IL-4, IL-10 as well as BDNF. Secondly, activated CREB blocks the nuclear factor kappa-B (NF- κ B)/CBP interaction, which is responsible for the transcription of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF α [65, 66]. Therefore, we examined whether the activated CREB offered by thioperamide might regulate the activity of NF- κ B. As expect, increased p-NF- κ B level was observed in hippocampus in LPS treated mice (increased to $197.5 \pm 17.96\%$ of control group, $P < 0.001$, Figure 4A, D). interestingly, thioperamide decreased the p-NF- κ B expression significantly (decreased to $92.72 \pm 11.99\%$ of control group, $P < 0.001$, Figure 4A, D), which was reversed by cimetidine (increased to $177.0 \pm 15.81\%$ of control group, $P < 0.01$, Figure 4A, D) but not pyrilamine, suggesting that thioperamide inhibited the activation of NF- κ B signaling in LPS treated mice via activating H2R.

Furthermore, we also examined the effects of thioperamide on the interaction of both CREB/CBP and NF- κ B/CBP. Interestingly, we found that the LPS induces decreased CREB/CBP (decreased to $56.64 \pm 3.195\%$ of control group, $P < 0.05$, Figure 4E, F) but increased NF- κ B/CBP (increased to $599.0 \pm 47.92\%$ of control group, $P < 0.001$, Figure 4E, G) interaction. However, thioperamide significantly increased the interaction of CREB/CBP (increased to $113.2 \pm 5.767\%$ of control group, $P < 0.01$, Figure 4E, F) but decreased NF- κ B/CBP (decreased to $111.8 \pm 23.41\%$ of control group, $P < 0.001$, Figure 4E, G). The effects of thioperamide on the regulation of CREB/NF- κ B/CBP interaction was reversed by cimetidine (CREB/CBP: decreased to $55.57 \pm 8.421\%$ of control group, $P < 0.01$, Figure 4E, F; NF- κ B/CBP: increased to $504.6 \pm 53.00\%$ of control group, $P < 0.001$, Figure 4E, G) but not pyrilamine, indicating an H2R dependent effects offered by thioperamide.

Thioperamide promotes polarization of M2 microglia from M1 microglia via activating PKA/CREB pathway in LPS-treated mice

In order to further investigate the involvement of PKA/CREB signaling in the effects of thioperamide on the activation of microglia, H89, the inhibitor of PKA/CREB was administrated to inhibit p-CREB. The results showed that the area of Iba1⁺-cells in hippocampus DG markedly increased in the thioperamide + H89 group compared with the thioperamide group in LPS treated mice (from 2.542 ± 0.3964 to 5.114 ± 0.4003 , $P < 0.001$, Figure 5A, B).

Reports have shown that CREB is involved in polarizing microglia from M1 to M2 phenotype. M1 microglia produce pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α , whereas M2 microglia produce anti-inflammatory cytokines such as IL-4, IL-10 and BDNF [13]. Thus, we further investigated the involvement of PKA/CREB signaling in the effect of thioperamide on microglia phenotypes. Firstly, we examined the role of CREB activation in the secretion of M1 microglia related pro-inflammatory cytokines, including IL-1 β , IL-6 and TNF- α . Results showed that the decreased mRNA level of all the three pro-inflammatory cytokines offered by thioperamide in hippocampus were reversed significantly after H89 treatment (IL-1 β : from 40.28 ± 11.50 to 86.62 ± 1.721 of vehicle, $P < 0.01$; IL-6: from 37.12 ± 4.813 to 88.47 ± 4.688 of vehicle, $P < 0.01$; TNF α : from 43.27 ± 13.97 to 91.37 ± 7.308 of vehicle, $P < 0.05$; Figure 5C) in LPS treated mice. Otherwise, a reversed protein level of three pro-inflammatory cytokines were also observed in the Thio+H89 group compared with the thio group in hippocampus (IL-1 β : from 40.76 ± 8.946 to 95.35 ± 5.991 of vehicle, $P < 0.01$; IL-6: from 38.55 ± 9.505 to 88.15 ± 4.179 of vehicle, $P < 0.05$; TNF α : from 48.36 ± 9.778 to 98.20 ± 4.629 of vehicle, $P < 0.01$; Figure 5D) in LPS treated mice.

Secondary, we investigated the role of CREB activation in the secretion of M2 microglia related anti-inflammatory cytokines and neurotrophic factors, including IL-4, IL-10 and BDNF. Interestingly, we found that administration of thioperamide up-regulated the transcription of anti-inflammatory cytokines IL-4 (increased to 254.1 ± 54.91 of vehicle, $P < 0.001$, Figure 5E), IL-10 (increased to 258.9 ± 47.13 of vehicle, $P < 0.05$, Figure 5E) and neurotrophic factors BDNF (BDNF I: increased to 286.8 ± 66.33 of vehicle, $P < 0.05$; BDNF II: increased to 342.2 ± 40.23 of vehicle, $P < 0.01$; BDNF III: increased to 281.1 ± 42.78 of vehicle, $P < 0.01$; BDNF IV: increased to 246.2 ± 36.64 of vehicle, $P < 0.01$, Figure 5G), which were all reversed by H89

to suppress CREB activation (IL-4: to 110.4 ± 9.017 of vehicle, $P < 0.001$; IL-10: to 110.9 ± 26.56 of vehicle, $P < 0.05$, Figure 5E; BDNF I: to 111.0 ± 10.91 of vehicle, $P < 0.05$; BDNF II: to 152.0 ± 43.82 of vehicle, $P < 0.01$; BDNF III: to 133.6 ± 17.09 of vehicle, $P < 0.05$; BDNF IV: to 109.9 ± 23.48 of vehicle, $P < 0.05$, Figure 5G) in LPS treated mice. In addition, results also showed that administration of H89 compromised the up-regulated expression of IL-4 (from 270.2 ± 15.93 to 128.7 ± 19.82 of vehicle, $P < 0.001$, Figure 5F), IL-10 (from 203.7 ± 9.452 to 118.8 ± 15.97 of vehicle, $P < 0.01$, Figure 5F) and BDNF (from 199.9 ± 16.67 to 109.2 ± 12.62 of vehicle, $P < 0.01$, Figure 5H) offered by thioperamide in LPS treated mice.

Taken together, these results indicated that thioperamide promoted switch of microglia from M1 to M2 phenotype through activating H2R downstream PKA/CREB signaling.

Thioperamide enhances neurogenesis via activating PKA/CREB pathway in LPS-treated mice

Studies have shown that activation of CREB promoted neurogenesis [46, 67-70], and enhanced neurite outgrowth and dendritic branching [71]. Therefore, we investigated whether the PKA/CREB signaling was involved in thioperamide induced neurogenesis in LPS treated mice by using H89. We found that the increased BrdU⁺ cells in the DG region of hippocampus were reversed by administration of H89 (from 247.7 ± 14.02 to 134.6 ± 9.957 of vehicle, $P < 0.001$, Figure 6A, D). The increased number of DCX⁺ cells was reversed dramatically by H89 (from $147.1 \pm 7.805\%$ to $107.3 \pm 8.417\%$ of vehicle, $P < 0.05$, Figure 6B, E). Moreover, the number of BrdU⁺/DCX⁺ cells and BrdU⁺/NeuN⁺ cells were also both compromised by H89 (BrdU⁺/DCX⁺ cells from 270.4 ± 21.79 to 113.0 ± 10.72 of vehicle, $P < 0.001$, Figure 6B, F; BrdU⁺/NeuN⁺ cells from 221.3 ± 6.858 to 104.9 ± 5.437 of vehicle, $P < 0.001$, Figure 6C, H). Moreover, administration of H89 reversed thioperamide mediated increased percentage of both BrdU⁺/DCX⁺ (from 50.20 ± 1.925 to 35.41 ± 3.633 , $P < 0.05$, Figure 6B, F) and BrdU⁺/NeuN⁺ (from 46.89 ± 2.322 to 35.11 ± 2.352 , $P < 0.01$, Figure 6C, F) newborn neuronal cells in LPS treated mice. Above all, these results suggested that thioperamide promoted hippocampal neurogenesis by activating H2R downstream PKA/CREB signaling.

Thioperamide increases the dendritic complexity via activating PKA/CREB pathway in LPS-treated mice

The previous studies have shown that the pro-inflammatory cytokines can induce abnormal neuronal morphology and promote the loss of synapses in AD [40, 72-74]. Therefore, we explored the effects of thioperamide on morphologies of neurons and further analyzed the involvement of PKA/CREB signaling. We examined both DCX⁺ immature neurons and MAP2⁺ mature neurons in LPS treated mice. We found that thioperamide significantly increased either total dendritic length (from 354.4 ± 33.70 to 500.5 ± 34.16 , $P < 0.05$, Figure 7A, C) or total branches (from 4.6 ± 0.7483 to 8.400 ± 0.5099 , $P < 0.01$, Figure 7A, D) in LPS treated mice by DCX staining, which was reversed by H89 (total dendritic length: to 361.2 ± 34.03 , $P < 0.05$, Figure 7A, C; total branches: to 5.400 ± 0.6782 , $P < 0.05$, Figure 7A, D). Moreover, thioperamide also alleviated the abnormal morphologies of mature neurons (total dendritic length: from 729.9 ± 38.29 to 1025 ± 78.92 , $P < 0.01$, Figure 7B, E; total branches: from 9.600 ± 0.5099 to 12.60 ± 0.600 , $P < 0.05$, Figure 7B, F), and was compromised by H89 (total dendritic length: to 743.2 ± 25.98 , $P < 0.01$, Figure 7B, E;

total branches: to 10.00 ± 0.7071 , $P < 0.05$, Figure 7B, F). Above all, these results showed that thioperamide rescued the abnormal morphologies of neurons through activating H2R dependent PKA/CREB signaling.

Discussion

In the present study, we have shown that inhibition of H3R by thioperamide inhibited microglial activation, suppressed inflammatory response and further enhanced neurogenesis in LPS induced neuroinflammation in mice. Importantly, the alleviated effects of thioperamide on reactivity of microglia, neuroinflammation, neurogenesis and cognitive function were all compromised by cimetidine but not pyrilamine, suggesting a mechanism involvement of histamine dependent H2R activation. Moreover, H89 reversed the decreased phosphorylation of NF- κ B, and the increased interaction of CREB-CBP offered by thioperamide, suggesting an underlying mechanism involving H2R-cAMP/PKA/CREB signaling.

Up to now, the population of aged individuals is increasing worldwide with significant health and socio-economic implications. Studies on aging have discovered myriad changes in the brain, including reduced neurogenesis, increased synaptic aberrations, higher metabolic stress associating with cognitive decline [11]. Accumulating evidence suggests that the pathological changes occurs in part because the environment is affected during disease in a cascade of processes collectively termed neuroinflammation [75]. Microglia are the resident immune cells in CNS and play a pivotal role in maintaining brain homeostasis. In the aging brain, microglia lose their homeostatic molecular signature and show profound functional impairments, such as increased production of pro-inflammatory cytokines and buildup of dysfunctional lysosomal deposits indicative of impaired phagocytosis [76]. In aging pathogenesis, microglia activation plays a dual role: on one side, activation of pro-inflammatory M1 microglia contributes to neurotoxicity and synapse loss by triggering several proinflammatory cascades. In contrary, acute activation of anti-inflammatory M2 microglia induces increased phagocytosis or clearance [77].

Up to now, no therapy is available to block or slow down aging related diseases such as AD, and the involved mechanisms are still not fully understood [78]. In the CNS, the histaminergic system is involved in regulating many basic physiological functions including cognition [79]. H3R is a presynaptic autoreceptor negatively regulating histamine release from histaminergic neurons [23, 24], which suggesting inhibition of H3R induces increased histamine release. Therefore, we studied the effect of H3R antagonist in neuroinflammation induced pathology in mice. We found that under LPS induced neuroinflammatory condition, inhibition of H3R by thioperamide significantly inhibited activation of microglia. Meanwhile, thioperamide also inhibited the pro-inflammatory cytokines transcription and expression. The mechanism might be related to its upregulated release of histamine and subsequent activation of H2R, because inhibiting histaminergic neurons by H2R antagonist cimetidine but not H1R antagonist pyrilamine reversed the microglia activation and anti-inflammatory effects of thioperamide. In accordance with these results, our previous study showed that thioperamide offered anti-inflammatory effects in AD [37]. Moreover, other study also shows that H3R antagonist JNJ10181457 reduces the

upregulation of microglial pro-inflammatory cytokines and improved depression-like behavior, which is consistent with our results [80]. Conversely, there is also report shows activation but not inhibition of microglial H3R suppresses acute LPS-induced proinflammatory activities in primary cultured microglia [81]. Concerning that acute moderate activation of microglia promotes anti-inflammation M2 microglia phenotypes, whereas chronic microglial activation exerts pro-inflammation M1 phenotypes [82]. The differences might be attributed to the regulation of two different polarization of microglia by different factors.

Based on these studies, we found for the first time that the mechanism by which thioperamide offered anti-inflammatory effects was related to up-regulated histamine release and subsequent H2R activation. This is in accordance with other reports showing that histamine was able to counteract LPS-induced glial activation and release of pro-inflammatory molecules [40]. Depletion of histaminergic neurons in the hypothalamus induces potentiated microglial response to challenge with LPS in histidine decarboxylase (HDC) knockout mice [83]. In contrast, other reports show that histamine promotes microglia activation and induces phagocytosis [84, 85]. These contradictory studies may be due to the different regulation of two microglia polarization under different circumstance through activating different receptors. Although histamine is acknowledged to be involved in promoting inflammatory effects in the peripheral system, accumulating evidences show that in the CNS, histamine has a dual role in the modulation of microglial inflammatory responses.⁸⁵ Histamine per se triggers microglia activation and inflammation, whereas exerts anti-inflammatory effects under stress induced pathological conditions [40, 53, 86]. Moreover, activation of H1R induces activation of microglia and pro-inflammatory effects [84, 85] but H2R induces inhibition of microglia and anti-inflammatory effects [39, 62, 87].

Neuroinflammation is a significant pathological feature affecting cognition in aging, and recent evidence demonstrates that it also negatively affects hippocampal neurogenesis [6]. We observed that under LPS induced physiological conditions, thioperamide promoted hippocampal neurogenesis significantly through a histamine dependent mechanism. In concomitant with our results, either H3R antagonist or histamine has been shown to upregulate neurogenesis [40, 41, 43], showing that activating histaminergic neurons may represent a new tool for brain repair strategies in CNS. Interestingly, we found that the effects of thioperamide on neurogenesis involves activating histamine H2R, because they were reversed by cimetidine but not pyrilamine. In support with our results, other studies also show that histamine play a vital role in cerebral neurogenesis by activation of H2R to promote proliferation of neural precursors [88]. However, some reports also show that H1R is involved in the promoted neurogenesis offered by histamine in traumatic brain injury [43]. Moreover, deficiency of H1R leads to a reduced neurogenesis and cognition [89]. The different mechanism offered by histamine on neurogenesis might be related to different pathologies of diseases. It is possible that under chronic neuroinflammation, inflammatory blockade by histamine rescues impaired neurogenesis via activating H2R. Because a majority of studies indicate that anti-inflammatory drug restores neurogenesis [17, 90, 91]. Moreover, we also observed a relationship of histamine dependent H2R activating and neuroinflammation induced cognition. Although reports show that both H1R and H2R mediates effects of histamine on cognition. In

chronic neuroinflammation related diseases such as aging, AD, schizophrenia, and autism spectrum disorder, histamine promotes cognition mainly through modulating microglial function [92]. Meanwhile, histamine exerts its effects on microglia and neuroinflammation mostly through activating H2R [64, 89]. Therefore, the alleviated cognitive impairment by H2R activating might be related to its suppression on microglia activation and neuroinflammation.

To further understand the molecular mechanism underlying the effect mediated by H2R, we examined the H2R downstream signaling PKA/CREB. Reduced p-CREB level has been observed in neurodegenerative diseases [93, 94]. In addition, the decreased p-CREB expression is also thought to be important for regulation of microglia activation and neuroinflammation in CNS [95-97]. Stimulating CREB pathway enhances M2 microglia polarization [98] and inhibits microglia-mediated neuroinflammation [63, 99]. Therefore, CREB might play an important role in mediating the effect of H2R activation on neuroinflammation by histamine. Moreover, It is well acknowledged that activation of H2R induces activating cAMP/PKA/CREB pathway [100]. Therefore, we investigated the effect of thioperamide on PKA/CREB phosphorylation in LPS induced neuroinflammation. In agreement with previous studies, we found that LPS induced neuroinflammation was accomplished by decreased levels of p-PKA and p-CREB, which were both reversed by thioperamide treatment, showing thioperamide activated the PKA/CREB pathway in LPS induced neuroinflammation. Specially, both cimetidine, an H2R antagonist and H89, an inhibitor of PKA/CREB compromised the effects offered by thioperamide, including decreased microglia activity, decreased inflammatory cytokines levels and neurogenesis, further suggesting that the anti-inflammatory effects and promoted neurogenesis offered by thioperamide involves activation of H2R-PKA/CREB pathway.

The expression of pro-inflammatory cytokines requires NF- κ B activation and its nuclear translocation to interact with DNA [101]. Studies indicate that activation of CREB induces reduced expression of p-NF- κ B and decreased production of pro-inflammatory cytokines in brain injury [102]. We found that LPS induced activation of NF- κ B, and thioperamide mitigated this effect, showing thioperamide inhibited LPS induced activation of NF- κ B. A critical step in the transcriptional regulation mediated by NF- κ B or CREB is the interaction of each of these transcription factors with the co-activator CBP [103]. Interaction of NF- κ B-CBP mediates pro-inflammatory cytokines release whereas CREB-CBP mediates anti-inflammatory cytokines release [64]. Interestingly, we found that LPS induced an increased interaction of NF- κ B-CBP but decreased CREB-CBP. Thioperamide promoted CREB-CBP but inhibited NF- κ B-CBP interaction, which were both reversed by cimetidine, suggesting that thioperamide promoted CREB-CBP combination via histamine dependent activating H2R. Studies indicate that activation of CREB induces reduced expression of p-NF- κ B and decreased production of pro-inflammatory cytokines in brain injury [102]. Consistent with these results, we found that thioperamide inhibited transcriptional and protein levels of pro-inflammatory cytokines, including IL-1, IL-6 and TNF α from M1 microglia but promoted anti-inflammatory cytokines and neurotrophic factor including IL-4, IL-10 and BDNF from M2 microglia, which were both reversed by H89, suggesting that thioperamide regulated inflammatory cytokines transcription through activating CREB. Phosphorylation of CREB induced by thioperamide might promoted CREB-CBP

interaction but inhibited NF- κ B-CBP interaction to transcriptional regulate the inflammatory cytokines levels.

Conclusions

In conclusion, the present study indicates that H3R antagonist thioperamide improved cognitive impairment in LPS treated mice via histamine dependent H2R downstream up-regulating CREB-CBP interaction mediated inhibited M1 microglia related pro-inflammatory cytokines and M2 microglia related anti-inflammatory cytokines transcription, which contributed to neurogenesis (Figure 9). These results uncovered a novel mechanism behind the therapeutic effect of thioperamide in neuroinflammation and further provided an experimental basis for starting a clinical trial for H3R antagonists as a treatment for chronic neuroinflammation related diseases such aging and AD.

Abbreviations

DG, dentate gyrus; AD, alzheimer's disease; H3R, histamine H3 receptor; LPS, lipopolysaccharide; H2R, histamine H2 receptor; PKA, protein kinase A; CREB, cyclic AMP response element-binding protein; NF- κ B, nuclear factor kappa-B; CBP, CREB binding protein; IL-4, interleukin-4; IL-10, interleukin-10; BDNF, brain-derived neurotrophic factor; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; TNF α , tumor necrosis factor α ; GL, granule cell layer; CNS, central nervous system; IGF-1, insulin-like growth factor-1; AC, adenylyl cyclase; NSCs, neural stem cells; BrdU, 5-bromo-2'-deoxyuridine; NOR, novel object recognition; YM, Y maze; MWM, morris water maze; BSA, bovine serum albumin; DCX, Doublecortin; spontaneous alternation% SA%; Iba1, ionized calcium binding adapter molecule 1; HDC, histidine decarboxylase

Declarations

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Availability of data and materials

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

Authors' contributions

HY conceived and designed the project. HY, JW, BL, FS, YX, DZ, HL, MY, CW, JL, WW, DL and LS performed the experiments. HY and JW analyzed the data and drafted the manuscript. All authors reviewed the

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Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Figures

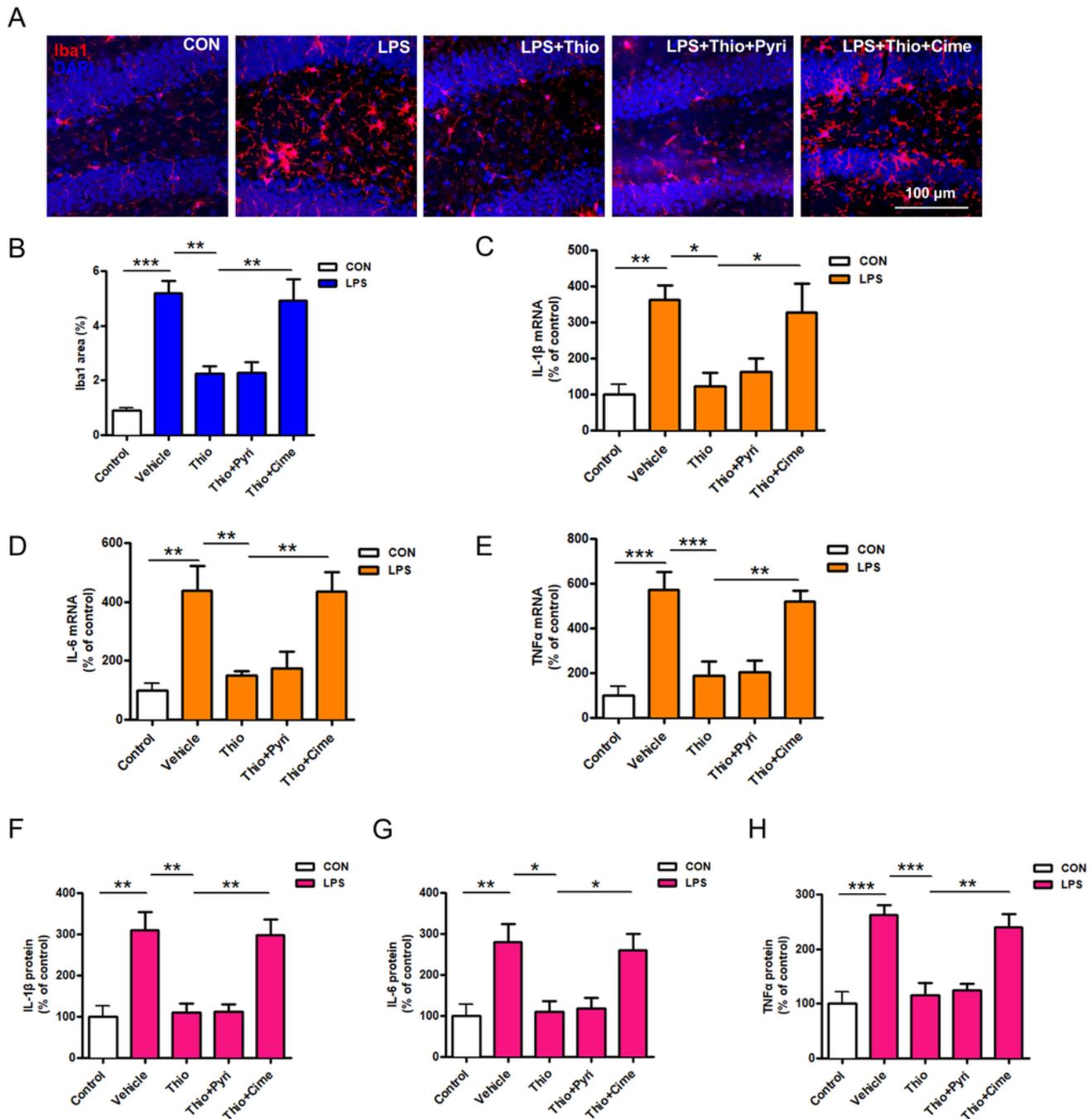


Figure 1

Effects of thioperamide and histamine receptor antagonists on microglial reactivity and proinflammatory cytokines production in LPS treated mice. (A-B) Representative immunohistochemical staining of Iba1 (red) (A) and bar graph (B) in hippocampal DG showing the Iba1+ microglial reactivity in those receiving vehicle, thioperamide, pyrilamine and cimetidine in LPS treated mice. Scale bar: 100 μ m. (C-E) Transcriptional levels of IL-1 β (C), IL-6 (D) and TNF- α (E) in the hippocampal DG were measured via RT-

PCR in those receiving vehicle, thioperamide, pyrilamine and cimetidine in LPS treated mice. (F-H) Protein levels of IL-1 β (F), IL-6 (G) and TNF- α (H) in the hippocampal DG were measured via ELISA in those receiving vehicle, thioperamide, pyrilamine and cimetidine in LPS treated mice. n = 5 per group. *P <0.05, **P <0.01, ***P <0.001. Mean \pm SEM. One-way ANOVA followed by Tukey's post hoc test.

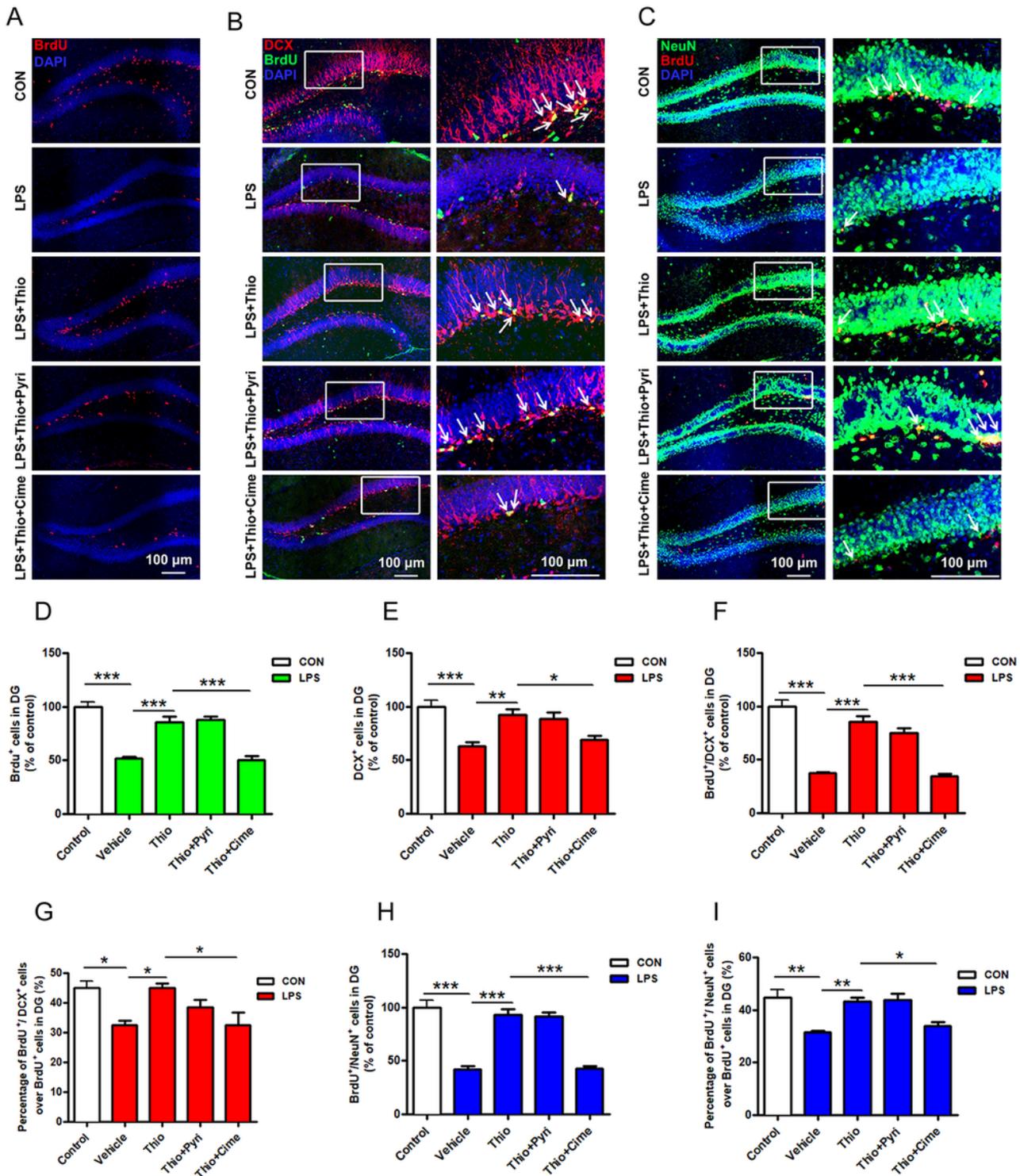


Figure 2

Effects of thioperamide and histamine receptor antagonists on neurogenesis in LPS treated mice. (A, D) The immunohistochemical staining of BrdU (Red) (A) and bar graph (D) in hippocampus DG region showing the effects of thioperamide, pyrilamine and cimetidine on BrdU-positive newborn cells in DG in LPS treated mice. Scale bar: 100 μ m. (B, E-G) The co-immunohistochemical staining of BrdU (Green) and DCX (Red) (B) and bar graph (E-G) in hippocampus DG region showing the effects of thioperamide, pyrilamine and cimetidine on DCX- (E) and BrdU/DCX- (F, G) positive newborn cells in DG in LPS treated mice. Scale bar: 100 μ m. (C, H, I) The co-immunohistochemical staining of BrdU (Red) and NeuN (Green) (C) and bar graph (H, I) in hippocampus DG region showing the effects of thioperamide, pyrilamine and cimetidine on BrdU/DCX-positive newborn cells (H, I) in DG in LPS treated mice. n = 5 per group. *P < 0.05, **P < 0.01, ***P < 0.001. Mean \pm SEM. One-way ANOVA followed by Tukey's post hoc test.

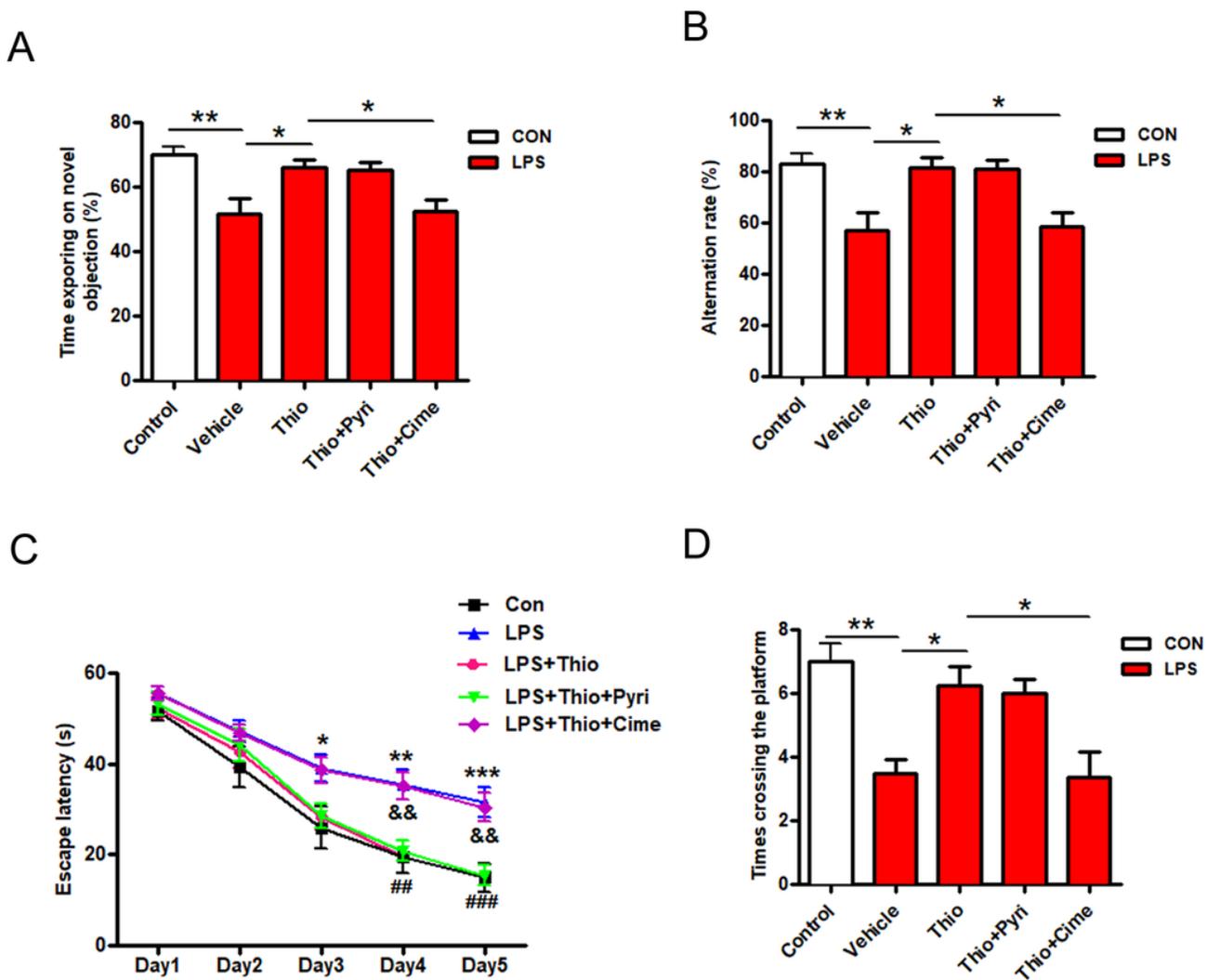


Figure 3

Effects of thioperamide and histamine receptor antagonists on cognitive function in LPS treated mice. (A) The NOR test showing effects of thioperamide, pyrilamine and cimetidine on the exploring time on new object in LPS treated mice. (B) The Y maze test showing the alternation rate in those receiving vehicle, thioperamide, pyrilamine and cimetidine in LPS treated mice. (C, D) The MWM test showing the escape latency on training days (C) and crossing times on testing day (D) in those receiving vehicle, thioperamide, pyrilamine and cimetidine in LPS treated mice. n = 8 per group. *P <0.05, **P <0.01 in A, B and D. *P <0.05, **P <0.01, ***P <0.001 vs. the control group; ##P <0.01, ###P <0.001 vs. the LPS treated group; &&P <0.01 vs. the thioperamide treated group in group in C. Mean \pm SEM. One-way ANOVA followed by Tukey's post hoc (A, B and D) and two-way ANOVA followed by Bonferroni post hoc test (C).

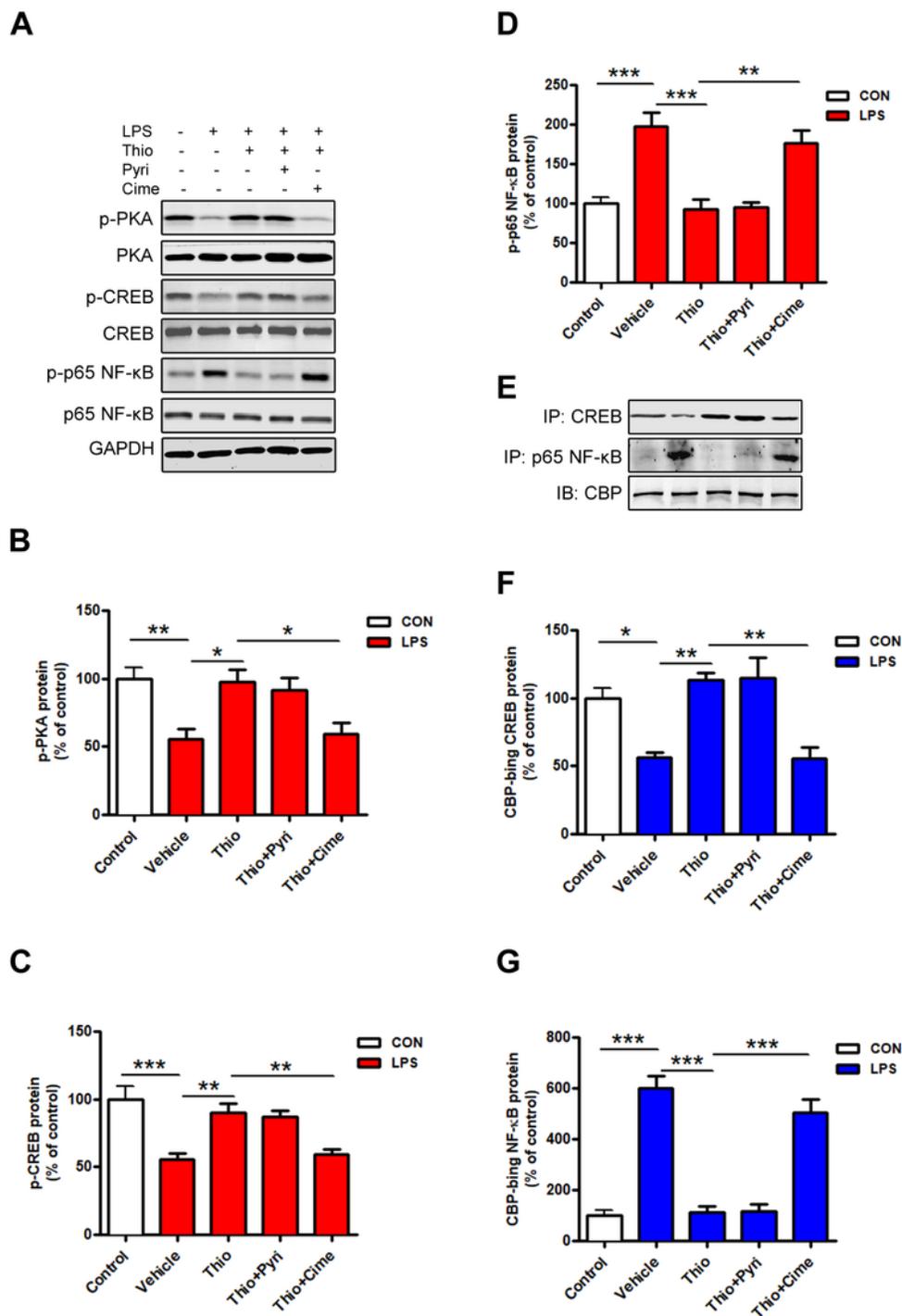


Figure 4

Effects of thioperamide and histamine receptor antagonists on PKA/CREB and NF-κB pathway in LPS treated mice. (A-D) Representative Western blots (A) and bar graph (B-D) showing the effect of thioperamide, pyrilamine and cimetidine on expression of p-PKA (B), p-CREB (C) and p-P65 NF-κB (D) in hippocampus in LPS treated mice. (E-G) Representative co-immunoprecipitation results (E) showing the effect of thioperamide, pyrilamine and cimetidine on interaction of CREB-CBP (F) and NF-κB-CBP (G) in

hippocampus in LPS treated mice. n = 5 per group. *P <0.05, **P <0.01, ***P <0.001. Mean ± SEM. One-way ANOVA followed by Tukey's post hoc test.

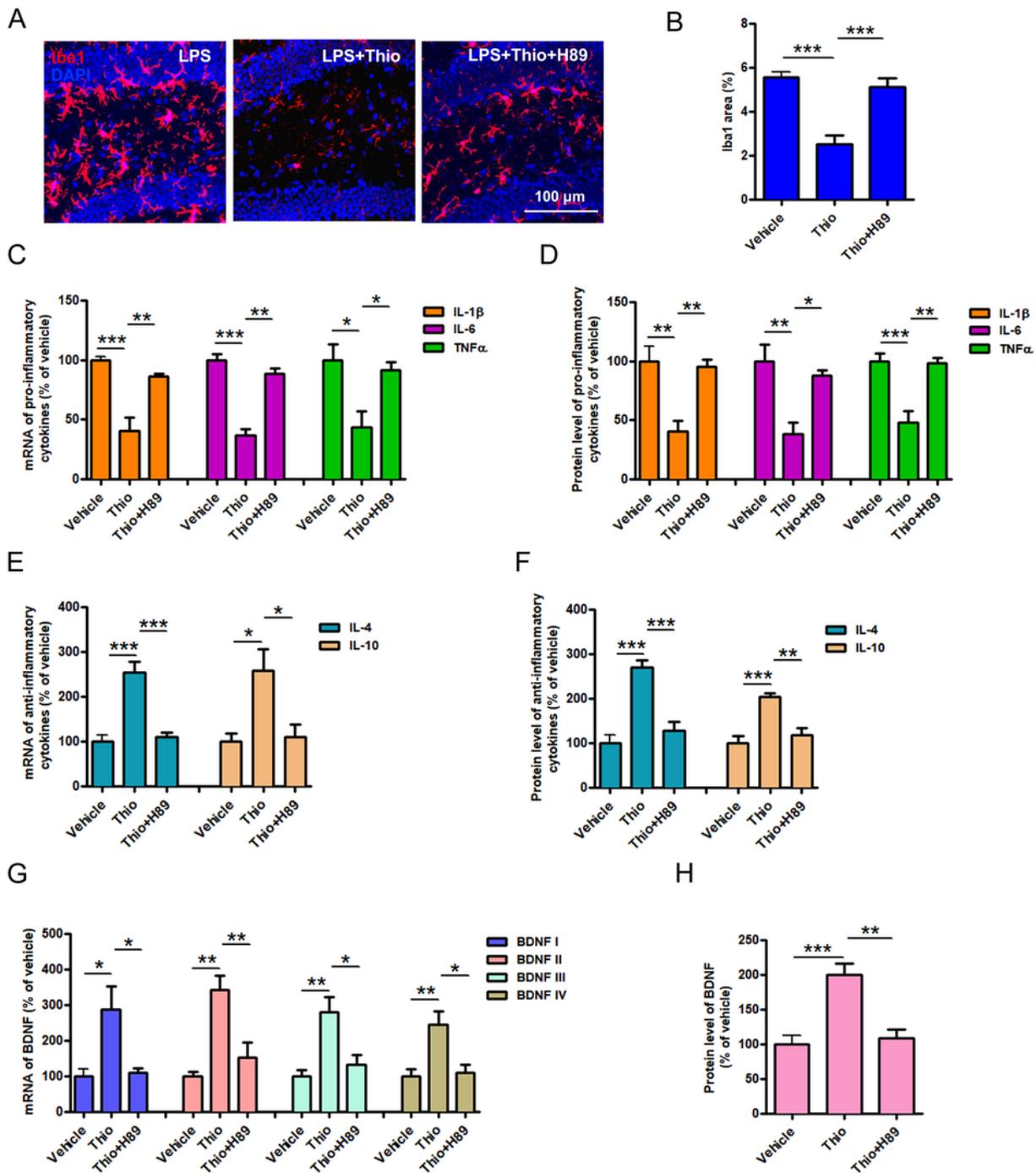


Figure 5

H89 reverses the decreased microglia reactivity and anti-inflammatory response by thioperamide in LPS treated mice. (A, B) Representative immunohistochemical staining of Iba1 (red) (A) and bar graph (B) in hippocampal DG showing the Iba1+ microglial reactivity in those receiving vehicle, thioperamide, and H89

in LPS treated mice. Scale bar: 100 μ m. (C, D) mRNA levels (C) and protein levels (D) of pro-inflammatory cytokines including IL-1 β , IL-6 and TNF- α in the hippocampal DG were measured by RT-PCR (C) and ELISA (D) in those receiving vehicle, thioperamide and H89 in LPS treated mice. (E, F) mRNA levels (E) and protein levels (F) of anti-inflammatory cytokines including IL-14 and IL-10 in the hippocampal DG were measured by RT-PCR (E) and ELISA (F) in those receiving vehicle, thioperamide and H89 in LPS treated mice. (G, H) mRNA levels (G) and protein levels (H) of neurotrophic factor BDNF in the hippocampal DG were measured by RT-PCR (G) and ELISA (H) in those receiving vehicle, thioperamide and H89 in LPS treated mice. n = 5 per group. *P <0.05, **P <0.01, ***P <0.001. Mean \pm SEM. One-way ANOVA followed by Tukey's post hoc test.

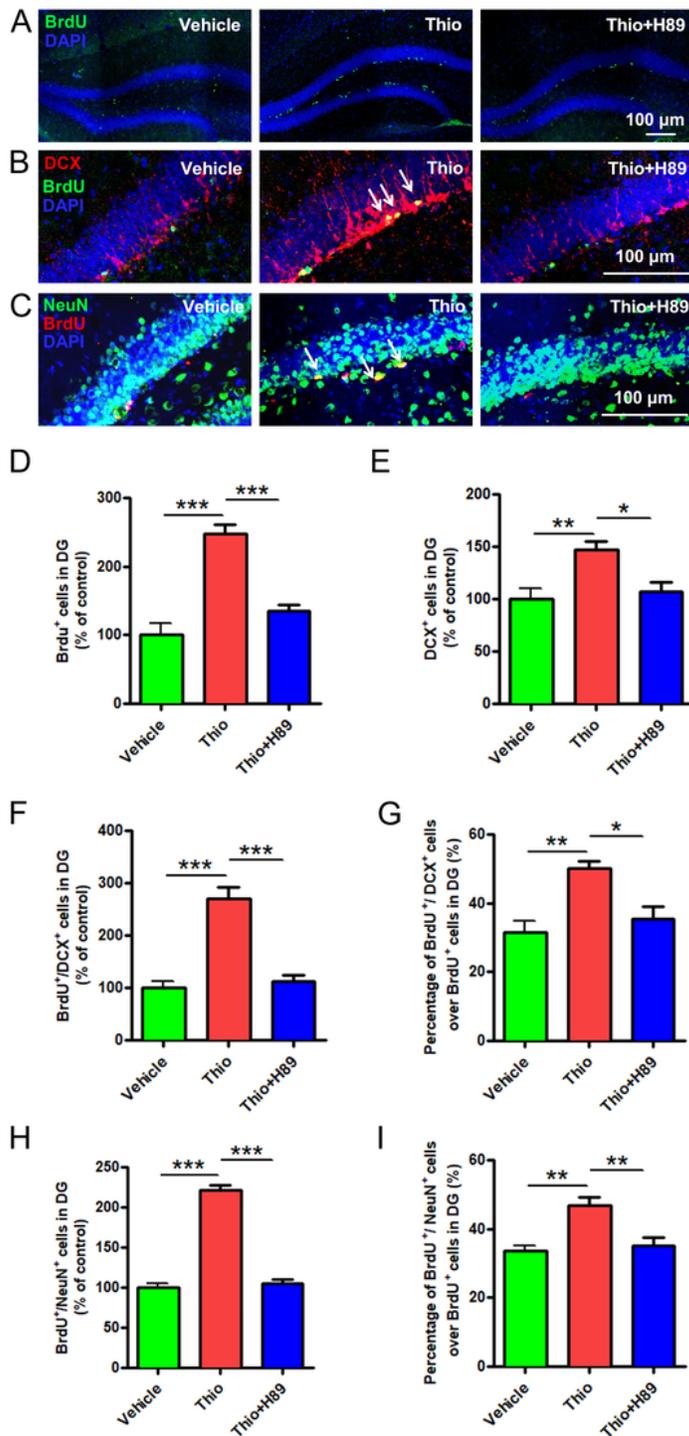


Figure 6

H89 reverses the enhanced neurogenesis offered by thioperamide in LPS treated mice. (A, D) The immunohistochemical staining of BrdU (Green) (A) and bar graph (D) in hippocampus DG region showing the effects of thioperamide and H89 on BrdU-positive newborn cells in DG of hippocampus in LPS treated mice. Scale bar: 100 μ m. (B, E-G) The co-immunohistochemical staining of BrdU (Green) and DCX (Red) (B) and bar graph (E-G) in hippocampus DG region showing the effects of thioperamide and H89 on DCX-

(E) and BrdU/DCX (F, G)-positive newborn cells in DG in LPS treated mice. Scale bar: 100 μ m. (C, H, I) The co-immunohistochemical staining of BrdU (Red) and NeuN (Green) (C) and bar graph (H, I) in hippocampus DG region showing the effects of thioperamide and H89 on BrdU/DCX-positive newborn cells (H, I) in DG in LPS treated mice. n = 5 per group. *P < 0.05, **P < 0.01, ***P < 0.001. Mean \pm SEM. One-way ANOVA followed by Tukey's post hoc test.

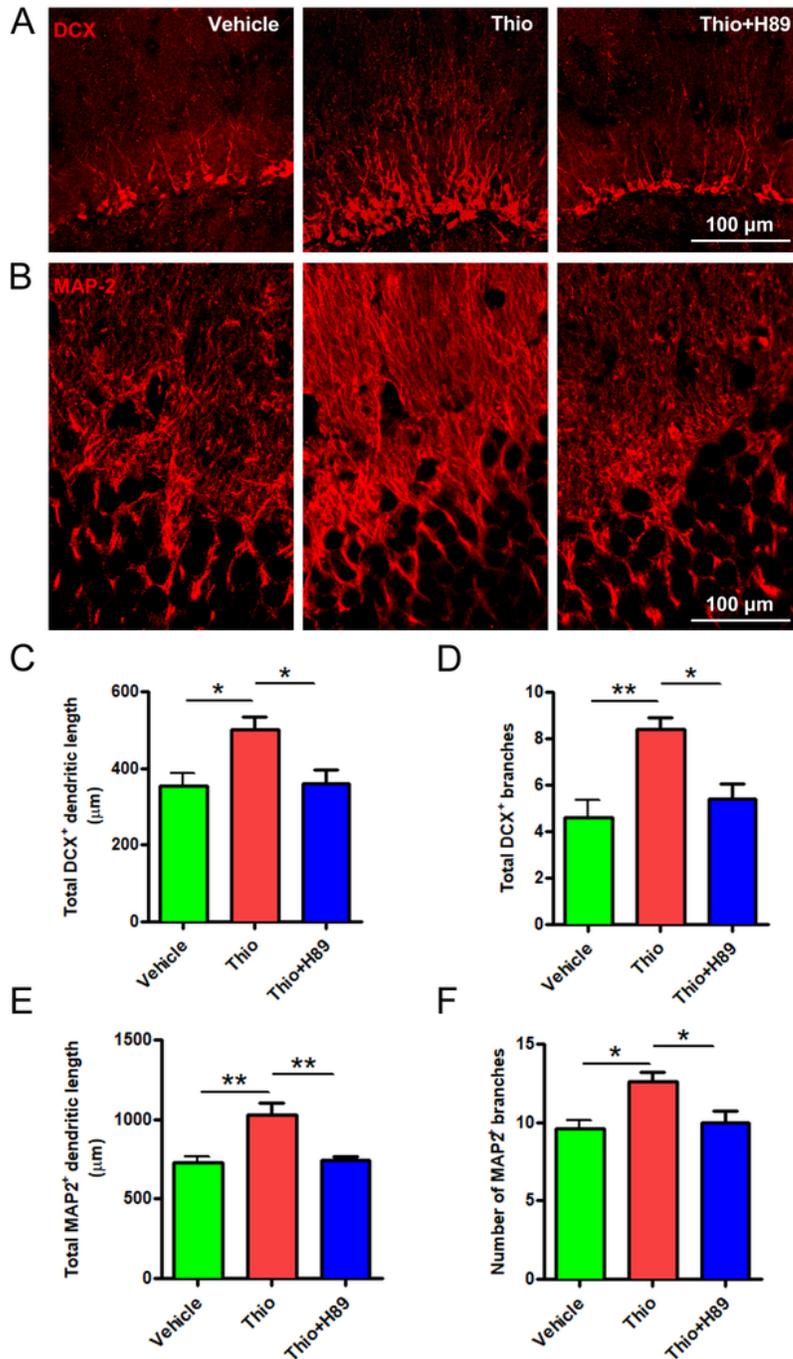


Figure 7

H89 reverses the effect of thioperamide on dendritic complexity in LPS-treated mice. (A, B) The immunohistochemical staining showing the effects of thioperamide and H89 on morphology of DCX-positive immature neurons (red) (A) and MAP-2-positive mature neurons (red) (B) in LPS treated mice. Scale bar: 100 μ m. (C-F) The bar graph indicated the effects of thioperamide and H89 on the total dendritic length (C, E) and total branches (D, F) of DCX- positive immature neurons (C, D) and MAP-2- positive mature neurons (E, F) in LPS treated mice. n = 5 per group. *P <0.05, **P <0.01. Mean \pm SEM. One-way ANOVA followed by Tukey's post hoc test.

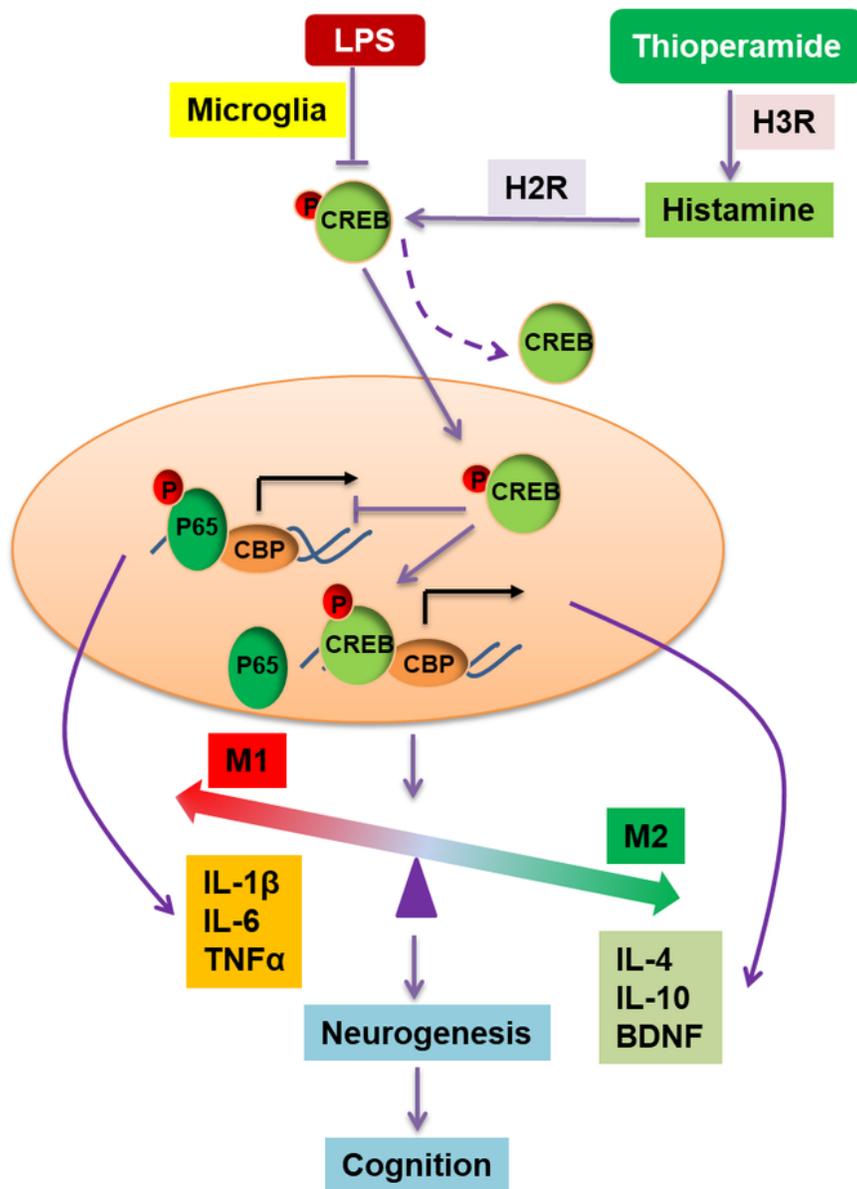


Figure 8

A proposed model for the neuroprotective effects of thioperamide against LPS induced neuroinflammation. Inhibition of H3R by thioperamide promotes release of histamine, then activates H2R and its downstream CREB signaling, upregulates anti-inflammatory cytokines from M2 microglia and downregulates pro-inflammatory cytokines from M1 microglia, and ultimately enhances neurogenesis and improves cognitive function.