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Neuropeptide Y protects cerebral cortical neurons by regulating microglial immune function

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Abstract

Neuropeptide Y has been shown to inhibit the immunological activity of reactive microglia in the rat cerebral cortex, to reduce N-methyl-D-aspartate current (I_{NMDA}) in cortical neurons, and protect neurons. In this study, after primary cultured microglia from the cerebral cortex of rats were treated with lipopolysaccharide, interleukin-1β and tumor necrosis factor-α levels in the cell culture medium increased, and mRNA expression of these cytokines also increased. After primary cultured cortical neurons were incubated with the lipopolysaccharide-treated microglial conditioned medium, peak I_{NMDA} in neurons increased. These effects of lipopolysaccharide were suppressed by neuropeptide Y. After addition of the neuropeptide Y Y1 receptor antagonist BIBP3226, the effects of neuropeptide Y completely disappeared. These results suggest that neuropeptide Y prevents excessive production of interleukin-1β and tumor necrosis factor-α by inhibiting microglial reactivity. This reduces I_{NMDA} in rat cortical neurons, preventing excitotoxicity, thereby protecting neurons.

Key Words: *nerve regeneration; microglia; immunological activity; neuropeptide Y; interleukin-1β; tumor necrosis factor-α; I_{NMDA}; neural regeneration*

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Introduction

Microglia are a type of macrophage extensively distributed in the central nervous system (Ginhoux et al., 2010), and strongly associated with immunity. Microglia play major roles in nervous system disease and immune responses (Graeber and Streit, 1990; Aloisi, 1999; Wirenfeldt et al., 2011). Moreover, microglia can produce inflammatory factors and mediate inflammation in the nervous system (Rivest, 2009; Kraft and Harry, 2011). Under normal conditions, microglia are in a resting state and play an immune surveillance role in the central nervous system. When stimulated, microglia are activated, and can release interleukin-1β and tumor necrosis factor-α, as well as a large number of bioactive substances, including reactive oxygen, nitrogen and lipid species (Giulian et al., 1994; Liu et al., 2002; Rivest, 2009; Wu et al., 2009; Mayer et al., 2011). Excessive release of these bioactive substances can induce neuronal injury (Auvin et al., 2010; Friedman and Dingledine, 2011; Vezzani et al., 2011b). Microglial activation is a key step in the inflammatory process in the central nervous system (Hanisch, 2002; Kaur et al., 2010; Kraft and Harry, 2011). Animal experiments and clinical studies show that an inflammatory reaction occurs in the focal zone after epileptic seizures (Auvin et al., 2010; Vezzani and Friedman, 2011; Vezzani et al., 2013). Moreover, microglia are noticeably activated in the focal zone, and interleukin-1β and tumor necrosis factor-α expression are increased. Simultaneously, neuronal excitability is elevated, and the number of apoptotic neurons increases (Ravizza et al., 2005; Vezzani and Granata, 2005). In addition, inflammatory factors are strongly associated with epilepsy. Thus, reducing the release of inflammatory factors or blocking their effects could lessen the severity of epilepsy (Maroso et al., 2011; Vezzani et al., 2011a, b; Gao et al., 2012).

Previous studies have shown that some medicines protect neurons by inhibiting microglial reactivity (Tikka et al., 2001; Dheen et al., 2007; Park et al., 2007). For example, one study showed that after microglial activation was suppressed by the cyclooxygenase 2 inhibitor celecoxib in the hippocampus of epileptic rats, spontaneous seizures and neuronal injury were significantly reduced (Zhang et al., 2008), suggesting that inhibiting microglial activation and bioactive substance release is an effective method of treating epilepsy.

It remains poorly understood whether there was a kind of medicine that directly acted on neurons, regulated the immunological activity of microglia and finally protected neurons against injury through nerve-immunity pathway.

Neuropeptide Y is widely expressed in the central and peripheral nervous systems, and is strongly associated with epilepsy, learning and memory (Wettstein et al., 1995; Vezzani et al., 1999). Neuropeptide Y exerts protective effects against neuronal injury and can alleviate epilepsy (Smialowska et al., 2009; Baptista et al., 2012; Goncalves et al., 2012; Gotzsche et al., 2012; Malva et al., 2012). Neuropeptide Y exerts protective effects on neurons mainly through the Y2 receptor (Greber et al., 1994; Woldbye et al., 2010; Decressac et al., 2012; Goncalves et al., 2012). In a recent study, neuropep-

tide Y suppressed the activation of the mouse microglial cell line N9 induced by lipopolysaccharide, and it reduced the generation of interleukin-1β and nitric oxide (Ferreira et al., 2010). Neuropeptide Y was also found to suppress microglial migration and phagocytosis (Ferreira et al., 2011, 2012).

It is unclear whether neuropeptide Y can reduce the release of bioactive substances and protect neurons by regulating microglial immunological activity. Cytokines can activate glutamic acid receptors and induce changes in ion currents, thereby contributing to neuronal injury (Yang et al., 2005). Glutamic acid receptors include ionotropic and metabotropic receptors. The N-methyl-D-aspartate (NMDA) receptor, an ionotropic glutamic acid receptor, is extensively distributed in the central nervous system. The NMDA receptor can be controlled by membrane potential, glutamic acid and NMDA. In the resting state, the NMDA receptor binds to Mg^{2+}, which blocks the opening of the ion channel. When bound to excitatory neurotransmitters such as glutamate and NMDA, the receptor is activated, opening the ion channel and allowing cations such as Ca^{2+}, Na^{+} and K^{+} to enter, resulting in the NMDA current (I_{NMDA}). Excessive cation flux, especially Ca^{2+} influx, results in excitotoxic neuronal injury (Norris et al., 2006; Deshpande et al., 2008; Zhu et al., 2010).

Previous studies suggested that interleukin-1β and tumor necrosis factor-α elevate NMDA receptor expression, and increase NMDA activity (Balosso et al., 2008; Zheng et al., 2010; Zhu et al., 2010). Therefore, neuropeptide Y may diminish the release of interleukin-1β and tumor necrosis factor-α derived from microglia by inhibiting microglial activation, thereby suppressing NMDA receptor expression, decreasing NMDA activity, reducing I_{NMDA}, and ultimately protecting neurons.

In the present study, lipopolysaccharide was used to activate primary cultured microglia derived from the rat cerebral cortex. We observed the effects of neuropeptide Y on interleukin-1β and tumor necrosis factor-α in activated microglia. Microglial conditioned medium treated or untreated with neuropeptide Y was used to incubate primary neurons from the rat cerebral cortex. The patch-clamp technique was used to detect changes in I_{NMDA} induced by the different microglial conditioned media.

Materials and methods

Animals

A total of 64 clean Sprague-Dawley rats born within 24 hours were provided by the Experimental Animal Center of Hebei Medical University in China (animal license No. SCXK (Ji) 2013-1-03). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and the protocol was approved by the Institutional Animal Care Committee of Hebei Medical University.

Neuronal and microglial cultures

Mixed microglial cultures, microglial isolation and purification were conducted in accordance with a previous method (Nakajima et al., 1992). The brain was obtained aseptically from neonatal rats after craniotomy. After removal of the meninges and blood vessels, a part of the cerebral cortex was cut into pieces and digested in 0.125% trypsin at 37°C for 15 minutes. Samples were agitated with a pipette, centrifuged at 1,000 r/min for 5 minutes, and filtered. The supernatant was discarded. The pellet was resuspended with microglia medium (Dulbecco's modified Eagle's medium/F12 medium, DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 1 U/mL penicillin and 100 μg/mL streptomycin. The cell suspension was incubated in a 250 mL culture flask at 37°C, 5% CO_2. On the second day, the medium was replaced once with new medium. From then on, half of the medium was replaced every 3 days. At 14 days, when cells were confluent, they were shaken on a rocker at 37°C, 180 r/min for 2 hours. The cell suspension was centrifuged at 1,000 r/min for 5 minutes. After removal of the supernatant, the samples were agitated into a cell suspension using microglia medium. Cells were adjusted to about 1×10^5/mL, and seeded in 6-well plates precoated with polylysine (Sigma, St. Louis, MO, USA), 3 mL in each well, in a constant-temperature incubator (Heraeus, Hanau, Germany) for 30 minutes without stirring. The medium was replaced once by new medium. After removal of oligodendrocytes, the samples were incubated with microglia medium for 3–5 days, resulting in a purified microglial culture.

Isolation and culture of rat cerebral cortical neurons were conducted in accordance with a previous method (Yang et al., 2006). The brain was obtained aseptically from neonatal rats by craniotomy. Brain tissue was placed in a petri dish with precooled DMEM/F12 medium. The petri dish was placed on ice. A median sagittal incision was made. After removal of the cerebellum and subcortical tissue, meninges and blood vessels were dissociated with a dissecting microscope. The cortex was placed in an additional petri dish with precooled DMEM/F12 medium. The petri dish was placed on ice. The cerebral cortex was cut into about 1 mm^3 blocks, digested in 0.125% trypsin at 37°C for 5 minutes, and agitated into a cell suspension. Cell aggregates were removed by filtration to obtain a single-cell suspension. Cells were adjusted to about 1×10^5/mL using microglia medium. Cell suspensions were seeded in a 6-well plate with a polylysine-treated coverslip, 3 mL in each well, in a 5% CO_2 incubator at 37°C for co-culture. Twenty-four hours later, the medium was replaced by neuronal medium (Neurobasal medium) (Gibco), supplemented with 2% B27 (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin. The medium was replaced once every 3 days. After 7–9 days of culture, purified neurons were obtained.

Identification of microglia and neurons

Purity of microglia was assessed using immunofluorescence staining (Schluesener et al., 1999; Yamasaki et al., 2010; Azemi et al., 2011; Savard et al., 2013; Yao et al., 2013). Isolated microglia were incubated in a culture plate with polylysine-treated slides for 3 days. The samples were washed with PBS, fixed in 4% paraformaldehyde for 30 minutes, treated with 0.3% Triton X-100 for 10 minutes, blocked with 3% goat serum for 30 minutes, incubated with mouse

Table 1 Primer sequences for interleukin-1β and tumor necrosis factor-α transcript amplification

Gene	Primer sequence	Product size (bp)
Tumor necrosis factor-α	Sense: 5′-TGC CTC AGC CTC TTC TCA TT-3′ Antisense: 5′-GCT TGG TGG TTT GCT ACG AC-3′	208
Interleukin-1β	Sense: 5′-CTC CAT GAG CTT TGT ACA AGG-3′ Antisense: 5′-TGC TGA TGT ACC AGT TGG GG-3′	245
GAPDH	Sense: 5′- TGA ACG GGA AGC TCA CTG G-3′ Antisense: 5′-GCT TCA CCA CCT TCT TGA TGT C-3′	120

anti-ionized calcium-binding adapter molecule 1 monoclonal antibody (1:200; Sigma) at 4°C overnight, and then with FITC-labeled goat anti-mouse IgG (1:100; Proteintech, Chicago, IL, USA) at 37°C for 1 hour. Except for goat serum blocking, three PBS washes were performed between each step. After mounting, a fluorescence microscope (DMI3000B and DFC450C, Leica, Wetzlar, Germany) was used to observe the percentage of positive cells at 400 × magnification. Microglial morphology was observed after treatment with lipopolysaccharide (100 ng/mL; Sigma) for 6 hours.

Immunofluorescence staining was used to identify whether the cultured cells were neurons (Andreyeva et al., 2012; Soetedjo et al., 2013). The procedures were identical to that for microglia. The antibody used was rabbit anti-microtubule-associated protein-2 polyclonal antibody (1:100; Proteintech) and TRITC-labeled goat anti-rabbit IgG (1:100; Proteintech). Under a fluorescence microscope, five high-power fields (400 ×) were randomly selected. The percentage of positive cells was calculated with an Image-Pro Plus image analysis system (Media Cybernetics, Silver Spring, ML, USA).

Enzyme linked immunosorbent assay (ELISA) for interleukin-1β and tumor necrosis factor-α protein levels in lipopolysaccharide-treated microglial conditioned medium

Purified microglia were seeded in a 6-well polylysine-treated plate at 1×10^5/mL, 3 mL in each well. Three days later, microglia were cultured with fresh serum-free DMEM/F12 medium for 12 hours, and then incubated with serum-free DMEM/F12 medium containing lipopolysaccharide, 100 ng/mL (Sigma), for 1, 3, 6, 12 and 24 hours. Cell culture medium was collected from each group and centrifuged. Interleukin-1β and tumor necrosis factor-α levels in the medium were assessed with an ELISA kit (Bio-Swamp; Wuhan, China). Microtiter plates were assigned to the positive control well, blank control well and sample well. Standard preparations at different concentrations were added in the positive control well, 50 μL in each well. Blank control well did not contain any sample, reagent, biotinylated anti-tumor necrosis factor-α antibody or anti-interleukin-1β antibody. In the sample well, 40 μL of sample was first added, and then biotinylated anti-tumor necrosis factor-α antibody or anti-interleukin-1β antibody, 10 μL, was added. The plate was sealed with membrane, and then placed at 37°C for 30 minutes. The plate was washed five times, and patted dry. ELISA reagents, 50 μL, were added in each well, except the blank well. After incubation for 30 minutes, the plate was washed. The samples were visualized with developer at 37°C in the dark for 15 minutes. Reaction was terminated by the addition of stop buffer, 50 μL, in each well. Absorbance valus of each well were measured at 450 nm using a microplate reader (Thermo Labsystems, Helsinki, Finland). Blank control well was adjusted to zero. The measurement was performed within 15 mintues after adding stop buffer. Protein levels were calculated according to a standard curve of known concentrations.

Microglial treatment with neuropeptide Y and lipopolysaccharide

Microglia at 1×10^5/mL were seeded in a 6-well polylysine-treated plate, 3 mL in each well, for 3 days. Microglia were incubated with fresh serum-free DMEM/F12 medium for 12 hours to synchronize cells. Subsequently, cells were divided into control, lipopolysaccharide, neuropeptide Y + lipopolysaccharide, neuropeptide Y and BIBP3226 + neuropeptide Y + lipopolysaccharide groups. In the control group, microglia were incubated with serum-free DMEM/F12 medium for 6 hours. In the lipopolysaccharide group, microglia were incubated with serum-free medium containing 100 ng/mL lipopolysaccharide for 6 hours. In the neuropeptide Y + lipopolysaccharide group, microglia were incubated with serum-free DMEM/F12 medium containing neuropeptide Y (final concentration: 1 μmol/L; ENZO, New York, NY, USA) for 0.5 hours, and then treated with lipopolysaccharide (final concentration: 100 ng/mL) for 6 hours. In the neuropeptide Y group, microglia were incubated with serum-free medium containing neuropeptide Y (1 μmol/L) for 6 hours. In the BIBP3226 + neuropeptide Y + lipopolysaccharide group, microglia were first incubated with serum-free DMEM/F12 medium containing the neuropeptide Y1 receptor antagonist BIBP3226 (N-[(1R)]-4-[(Aminoiminomethyl)amino-1-[[[(4-hydroxyphenyl)methyl]amino] carbonyl]butyl-α-phenylbenzeneacetamide trifluoroacetate; molecular weight: 587.59; molecular formula: $C_{27}H_{31}N_5O_3$•C F_3CO_2H; purity > 98%; final concentration: 1 μmol/L; Tocris Bioscience, Ellisville, MO, USA) for 0.5 hours, then with neuropeptide Y (final concentration: 1 μmol/L) for 0.5 hours, and finally with lipopolysaccharide (final concentration: 100 ng/mL) for 6 hours.

Effects of neuropeptide Y on interleukin-1β and tumor necrosis factor-α mRNA levels in lipopolysaccharide-treated microglia, as detected by real-time PCR

Culture medium was discarded and Trizol (1 mL/well) was added in each well. Samples were agitated and placed in an RNAse-free centrifuge tube for 5 minutes. Into each tube, 0.2 mL chloroform was added. After shaking for 15 seconds and incubating for 5 minutes, samples were centrifuged at 12,000 r/min for 15 mintues. The colorless liquid in the

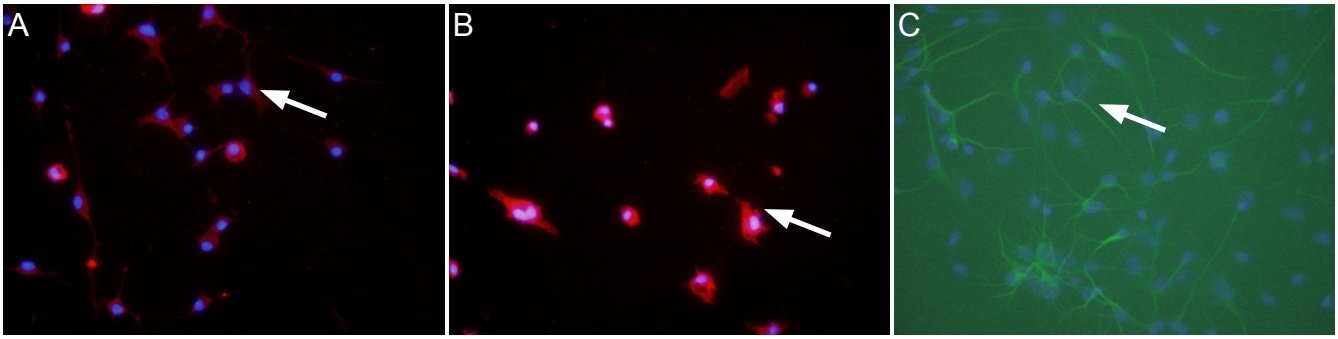

Figure 1 Primary cultured microglia and neurons (immunofluorescence, × 400).
(A) Microglia in the control (lipopolysaccharide-untreated) group had a ramified appearance, a small body, thin long processes, and expressed ionized calcium-binding adapter molecule 1 (IBA-1) (arrow); (B) after treatment with lipopolysaccharide, microglia were in an activated state, with a round amoeboid body, retracted processes, and darkly stained with IBA-1 (arrow); (C) neurons extended axons and long dendrites and expressed microtubule-associated protein 2 (arrow).

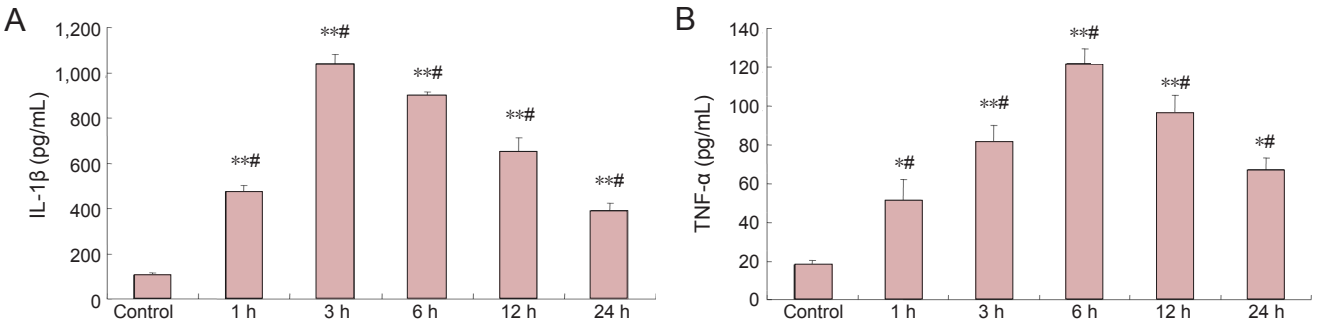

Figure 2 Effects of lipopolysaccharide on IL-1β and TNF-α secretion by microglia (enzyme linked immunosorbent assay).
(A) IL-1β levels in medium after incubation with 100 ng/mL lipopolysaccharide. (B) TNF-α levels in medium after incubation with 100 ng/mL lipopolysaccharide. Data are expressed as mean ± SD. The experiment was performed in triplicate. Completely random design was employed in analysis of variance. Paired comparison was done using least significant difference test. $*P < 0.05$, $**P < 0.01$, *vs.* control group; $\#P < 0.05$, *vs.* the previous time point. 1, 3, 6, 12, 24 h: 1, 3, 6, 12, 24 hours after incubation with lipopolysaccharide. IL-1β: Interleukin-1β; TNF-α: tumor necrosis factor-α.

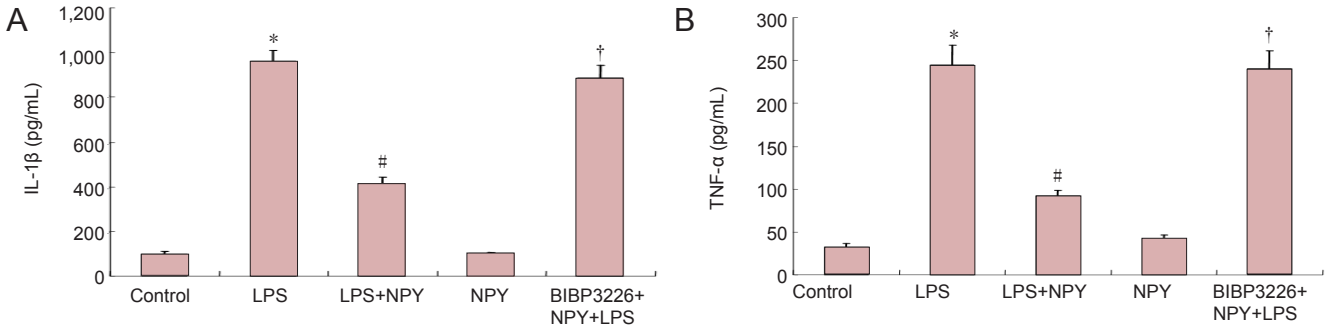

Figure 3 Inhibitory effects of NPY on LPS-induced production of IL-1β and TNF-α by microglia (enzyme linked immunosorbent assay).
IL-1β (A) and TNF-α (B) levels in microglial conditioned medium after culture in 1 μmol/L BIBP3226, 1 μmol/L NPY and 100 ng/mL LPS. Data are expressed as mean ± SD. The experiment was performed in triplicate. Completely random design was employed in analysis of variance. Paired comparison was done using least significant difference test. $*P < 0.01$, *vs.* control group; $\#P < 0.01$, *vs.* LPS group; $\dagger P < 0.01$, *vs.* LPS + NPY group. NPY: Neuropeptide Y; LPS: lipopolysaccharide; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α.

upper layer was removed to a new centrifuge tube. An equal volume of isopropanol was added, and mixed by inversion, followed by centrifugation at 12,000 r/min for 10 minutes. A white precipitate was visible at the bottom of the tube. Supernatant was completely discarded. The precipitate, following addition of 1 mL 75% alcohol (in DEPC-treated water), was washed. After centrifugation at 7,500 r/min at 4°C for 5 minutes, the supernatant was discarded. The samples were air-dried for 3–5 minutes. 20–30 μL of DEPC-treated water was added to thoroughly dissolve RNA. To verify RNA integrity and purity, 1 % agarose gel electrophoresis was used. A UV spectrophotometer was used to determine RNA

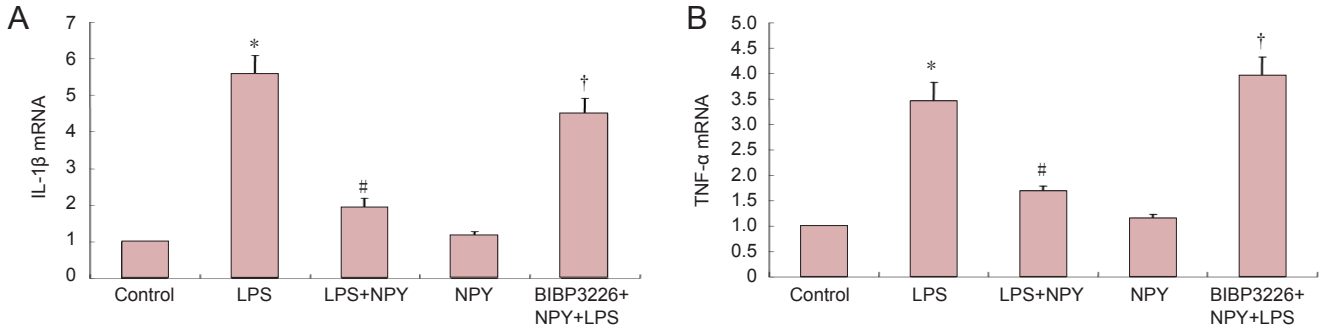

Figure 4 Inhibitory effects of NPY on LPS-induced expression of IL-1β and TNF-α by microglia (real-time PCR).
(A) IL-1β mRNA levels in microglia cultured in 1 μmol/L BIBP3226, 1 μmol/L NPY and 100 ng/mL LPS. (B) TNF-α mRNA levels in microglia cultured in 1 μmol/L BIBP3226, 1 μmol/L NPY and 100 ng/mL LPS. The ratio of target gene expression in the control group to the internal reference GAPDH was 1. Data are expressed as mean ± SD. The experiment was performed in triplicate. Completely random design was employed in analysis of variance. Paired comparison was done using least significant difference test. ∗$P < 0.01$, *vs.* control group; #$P < 0.01$, *vs.* LPS group; †$P < 0.01$, *vs.* LPS + NPY group. NPY: Neuropeptide Y; LPS: lipopolysaccharide; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α.

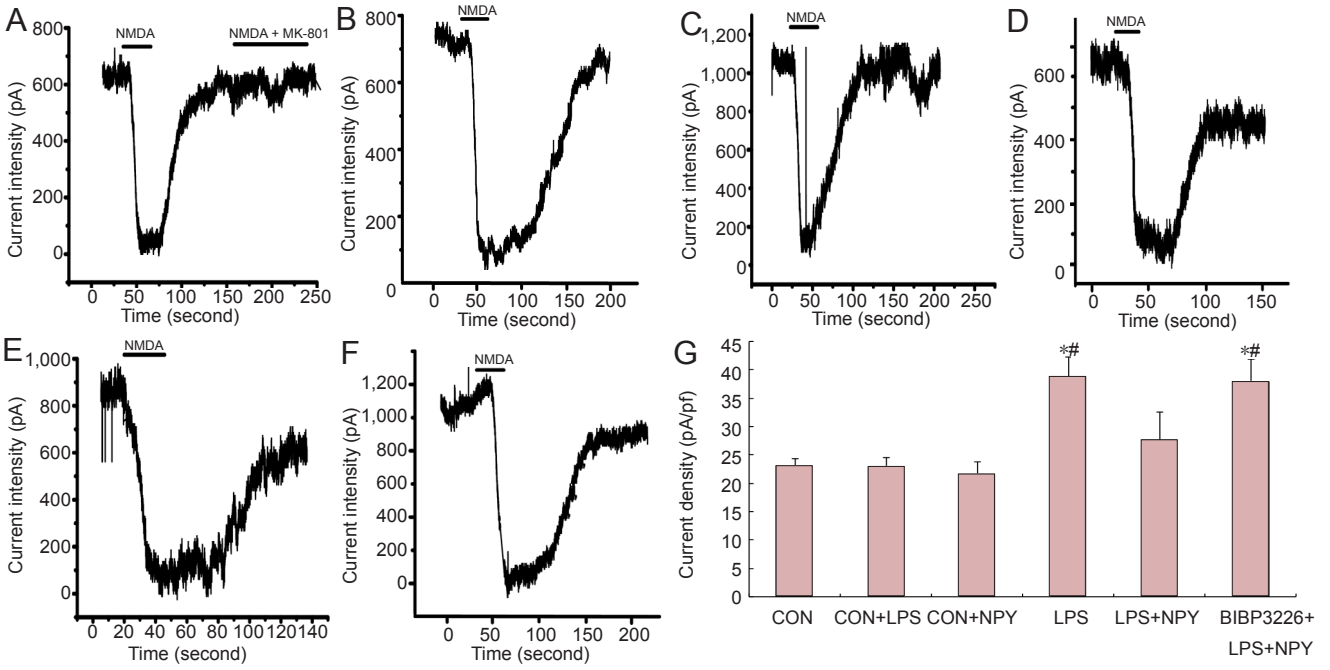

Figure 5 Effects of NPY on I_{NMDA} in neurons incubated with LPS-treated microglial conditioned medium.
(A) CON group; (B) CON + LPS group; (C) LPS group; (D) CON + NPY group; (E) LPS + NPY group; (F) BIBP3226 + NPY + LPS group. NMDA and glycine were simultaneously added to the perfusate to activate the NMDA receptor. After whole-cell recording for 5 minutes, intracellular fluid was thoroughly replaced by interelectrode solution. At this time, 100 μmol/L NMDA and 10 μmol/L glycine were added extracellularly to induce inward current changes. I_{NMDA} was measured. After eluting NMDA and glycine, the membrane current was restored to normal. After treatment with 100 μmol/L NMDA, 10 μmol/L glycine and 10 μmol/L NMDA receptor antagonist MK-801, this inward current disappeared. Therefore, the recorded current was I_{NMDA}. (G) Peak I_{NMDA} after incubation with microglial conditioned medium. Data are expressed as mean ± SD. The experiment was performed in triplicate. Completely random design was employed in analysis of variance. Paired comparison was done using least significant difference test. ∗$P < 0.01$, *vs.* CON group; #$P < 0.01$, *vs.* LPS + NPY group. NPY: Neuropeptide Y; LPS: lipopolysaccharide; I_{NMDA}: N-methyl-D-aspartate current; NMDA: N-methyl-D-aspartate; CON: control.

concentration. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China.

Primer sequences were shown in **Table 1**. Reverse transcription reaction: Total RNA 8 μL, random primer 1 μL, 2 × ES reaction mix 10 μL, RT/RI enzyme mix 1 μL; total volume: 20 μL. Amplification system: 2 × UltraSYBR mixture (with ROX) 10 μL, forward primer (10 μmol/L) 1 μL, reverse primer (10 μmol/L) 1 μL, cDNA 8 μL, total volume: 20 μL. RT-PCR parameters: predenaturation at 95°C for 10 minutes; 40 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 20 seconds, extension at 72°C for 27 seconds. An ABI 7300 Real-Time PCR System (Applied Biosystems, Foster, CA, USA) was used to detect and calculate the relative quantity of the target gene to the internal reference (GAPDH).

I_{NMDA} in neurons as determined by patch-clamp technique

Microglial conditioned media obtained from the various

groups were individually mixed with neuronal medium at a ratio of 1:1. Control medium after mixing with neuronal medium at 1:1 was divided into three groups: control (CON) group, CON + lipopolysaccharide group and CON + neuropeptide Y group. Lipopolysaccharide (final concentration of 50 ng/mL) and neuropeptide Y (final concentration of 0.5 μmol/L) were added in the CON + lipopolysaccharide group and CON + neuropeptide Y group, respectively. Purified neuronal cells (3×10^5/well) in each group were incubated with their corresponding treatment for 6 hours. I_{NMDA} in neurons was recorded using the patch-clamp technique (Yang et al., 2005; Zhang et al., 2011). Glass slides with neuronal cells were placed in a 0.3 mL-bath. Cells were continuously perfused with extracellular fluid at 2 mL/min to ensure that fluid exchange was finished within 2 minutes. An inverted microscope (TE2000-S; Nikon, Tokyo, Japan) was used to observe cells. Neurons with a clear outline, a defined three-dimensional structure and a smooth surface were selected for recording. Using a three-dimensional manipulator (Sutter Instruments Company, Novato, CA), a glass microelectrode at 1–3 MΩ impedance and filled with pipette solution (including KCl 10 mmol/L, K-D-gluconate 120 mmol/L, HEPES 10 mmol/L, EGTA 11 mmol/L, NaCl 5 mmol/L, $MgCl_2$ 2 mmol/L, $CaCl_2$ 1 mmol/L, Mg-ATP 2 mmol/L and Li-GTP 1 mmol/L; pH 7.2 adjusted with NaOH) was sealed onto the cell surface. Negative pressure was applied until the patch was formed. Capacitive current and series resistance of the electrode were compensated, and whole-cell recording was commenced. Clamping voltage was set at –60 mV. After whole-cell recording for 5 minutes, the intracellular fluid was thoroughly replaced with electrode solution. At this time, 100 μmol/L NMDA (Sigma) and 10 μmol/L glycine (Sigma) were added extracellularly to induce inward current changes. I_{NMDA} was recorded.

Statistical analysis

The data were analyzed using SPSS 10.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Test of normality and homogeneity test for variance showed that all data obeyed a normal distribution and had homogeneous variance. Completely random design was employed in analysis of variance. Paired comparison was done using least significant difference test. A value of $P < 0.05$ was considered statistically significant.

Results

Isolated culture of microglia and neurons

Ionized calcium-binding adapter molecule and microtubule-associated protein 2 were used as markers of microglia and neurons, respectively (Izant and McIntosh, 1980; Dehmelt and Halpain, 2005; Nakamura et al., 2013). The purities of microglia and neurons were > 95%, which met the requirements of the experiments (**Figure 1**).

Lipopolysaccharide promoted the secretion of interleukin-1β and tumor necrosis factor-α by microglia

ELISA results revealed that interleukin-1β and tumor necrosis factor-α levels in the medium of each group were significantly greater after incubation with lipopolysaccharide compared with the control group ($P < 0.01$ or $P < 0.05$). As incubation proceded, interleukin-1β content gradually increased ($P < 0.05$), peaked at 3 hours, and then gradually decreased ($P < 0.05$; **Figure 2A**). Moreover, tumor necrosis factor-α content gradually increased over the incubation period ($P < 0.05$), peaked at 6 hours, and then gradually diminished ($P < 0.05$; **Figure 2B**).

Neuropeptide Y reduced lipopolysaccharide-induced secretion of interleukin-1β and tumor necrosis factor-α by microglia

ELISA results revealed that interleukin-1β and tumor necrosis factor-α levels were significantly lower in the neuropeptide Y + lipopolysaccharide group than in the lipopolysaccharide group ($P < 0.01$). Interleukin-1β and tumor necrosis factor-α levels were significantly increased in the BIBP3226 + neuropeptide Y + lipopolysaccharide group ($P < 0.01$). Interleukin-1β and tumor necrosis factor-α levels did not significantly change in the medium after normal cells were treated with neuropeptide Y ($P > 0.05$; **Figure 3**).

Neuropeptide Y suppressed interleukin-1β and tumor necrosis factor-α mRNA expression in microglia treated with lipopolysaccharide

At 6 hours after incubation, interleukin-1β and tumor necrosis factor-α mRNA levels were significantly higher in the lipopolysaccharide group and in the BIBP3226 + neuropeptide Y + lipopolysaccharide group than in the control group ($P < 0.01$). No significant difference in interleukin-1β or tumor necrosis factor-α mRNA levels was detected between the lipopolysaccharide and BIBP3226 + neuropeptide Y + lipopolysaccharide groups. Interleukin-1β and tumor necrosis factor-α mRNA levels were significantly lower in the lipopolysaccharide + neuropeptide Y group compared with the lipopolysaccharide group ($P < 0.01$). Interleukin-1β and tumor necrosis factor-α mRNA levels were significantly higher in the BIBP3226 + neuropeptide Y + lipopolysaccharide group than in the lipopolysaccharide + neuropeptide Y group ($P < 0.01$). Interleukin-1β and tumor necrosis factor-α mRNA levels were similar in the neuropeptide Y and control groups ($P > 0.05$). These results suggest that lipopolysaccharide elevates interleukin-1β and tumor necrosis factor-α mRNA levels in microglia and that neuropeptide Y inhibits the effects of lipopolysaccharide. When BIBP3226 was used to block the neuropeptide Y1 receptor, neuropeptide Y had no effect. Neuropeptide Y did not impact interleukin-1β or tumor necrosis factor-α mRNA levels in microglia (**Figure 4**).

Neuropeptide Y inhibits I_{NMDA} in neurons after incubation with lipopolysaccharide-treated microglial conditioned medium

Patch-clamp recording showed that, compared with the CON group, I_{NMDA} was higher in the lipopolysaccharide group ($P < 0.01$). I_{NMDA} was significantly lower in the neuropeptide Y + lipopolysaccharide group compared with the lipopolysaccharide group. I_{NMDA} was significantly higher in

the BIBP3226 + neuropeptide Y + lipopolysaccharide group compared with the neuropeptide Y + lipopolysaccharide group ($P < 0.01$). I_{NMDA} was not substantially altered in neurons cultured in normal microglia medium (**Figure 5**).

Discussion

Microglia play an important role in regulating immune function and are strongly associated with the occurrence and development of central nervous system diseases (Harry and Kraft, 2012; Zhao et al., 2013). In central nervous system pathologies, microglia rapidly respond and switch from a resting to an activated state, from a ramified to an amoeboid morphology, with an enlarged body and retracted processes (Harry and Kraft, 2012). Microglia undergo a series of changes, and function as sensors in the central nervous system (Kreutzberg, 1996). Microglias are the macrophages of the central nervous system, and are strongly associated with immune functions in brain tissue. The accumulation of numerous cytokines and bioactive substances after microglial activation is a key event in neuronal injury (Vezzani et al., 2011b). Among these cytokines, interleukin-1β and tumor necrosis factor-α play important roles in physiological and pathological processes in the central nervous system (Lee et al., 2004; Maroso et al., 2011; Savard et al., 2013). The increased concentrations of interleukin-1β and tumor necrosis factor-α in brain tissues exert toxic effects on surrounding neurons (Zheng et al., 2010; Zhu et al., 2010). Lipopolysaccharide-activated microglia produce various factors implicated in pathologies (Lee et al., 2004).

Lipopolysaccharide was used as an activator of microglia in the present study. After 6 hours of intervention with lipopolysaccharide, the immunological activity of microglia increased, and interleukin-1β and tumor necrosis factor-α levels in microglial conditioned medium substantially increased. Neuropeptide Y suppressed the lipopolysaccharide-induced activation of microglia, and it reduced interleukin-1β and tumor necrosis factor-α protein and mRNA levels in these cells. These inhibitory effects of neuropeptide Y were completely suppressed by BIBP3226, indicating that neuropeptide Y diminishes the immunological activity of microglia through the Y1 receptor, thereby reducing interleukin-1β and tumor necrosis factor-α generation, consistent with a previous study (Ferreira et al., 2010). Many studies on the effects of cytokines on neurons suggest that these factors modulate the glutamic acid receptor (Vezzani et al., 2008). For example, interleukin-1β and tumor necrosis factor-α elevate NMDA receptor expression and activity. A previous study demonstrated that interleukin-1β can phosphorylate the NMDA NR2B subunit and activate the receptor (Balosso et al., 2008). Tumor necrosis factor-α increases phosphorylation of the NR1 subunit, and increases its expression as well (Wheeler et al., 2009). In a previous study, after rat hippocampal neurons were incubated with microglial conditioned medium containing tumor necrosis factor-α, NMDARI expression rose in neurons, and this effect was inhibited by addition of an anti-tumor necrosis factor-α antibody (Zhu et al., 2010). Interleukin-1β and tumor necrosis factor-α increase the probability and duration of channel opening, thereby increasing I_{NMDA}. The resulting Ca^{2+} influx into neurons causes calcium overload, resulting in cytotoxicity (Viviani et al., 2003). I_{NMDA} is a direct index of changes in the NMDA receptor. An increase in I_{NMDA} indicates increased activity of the NMDA receptor and an increased number of cations (mainly Ca^{2+}) passing through the ion channel (Malenka and Nicoll, 1999; Gardoni et al., 2001; Yang et al., 2005).

Patch-clamping showed that I_{NMDA} was enhanced after treatment with lipopolysaccharide compared with the control group. Neuropeptide Y suppressed microglial activation and this lipopolysaccharide-mediated increase in I_{NMDA}. When BIBP3226 was added to block the neuropeptide Y Y1 receptor, this effect of neuropeptide Y disappeared. I_{NMDA} did not remarkably change after incubation with lipopolysaccharide and neuropeptide Y conditioned medium, suggesting that changes in I_{NMDA} in neurons were not caused by lipopolysaccharide or neuropeptide Y, similar to a previous study (Bronstein et al., 1995), but were induced by factors in the conditioned medium of activated microglia.

In summary, neuropeptide Y suppresses microglial reactivity, reduces the release of microglial bioactive factors, diminishes I_{NMDA}, and prevents excitotoxicity induced by excessive Ca^{2+} entry into neurons. These effects of neuropeptide Y were fully blocked by the Y1 receptor antagonist BIBP3226. We demonstrated that neuropeptide Y can reduce I_{NMDA} in neurons by regulating the immune function of microglia. Thus, neuropeptide Y plays a major role in regulating immune function in the central nervous system. Neuropeptide Y reduces the generation of interleukin-1β and tumor necrosis factor-α, preventing intracellular Ca^{2+} overload induced by NMDA receptor hyperactivation, thereby protecting neurons.

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