NEURAL REGENERATION RESEARCH
Volume 8, Issue 16, June 2013

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2013.16.003 [http://www.nrronline.org; http://www.sjzsyj.org]
Hu YE, Mao JH, Zhang Y, Zhou AL. Role of Toll-like receptor 4 in inflammatory reactions of hippocampal neurons. Neural Regen Res. 2013;8(16):1465-1472.

Role of Toll-like receptor 4 in inflammatory reactions of hippocampal neurons★

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Abstract

Lipopolysaccharide stimulates Toll-like receptor 4 on immune cells to produce immune mediators. Toll-like receptor 4 is also expressed by non-immune cells, which can be stimulated by lipopolysaccharide. However, whether Toll-like receptor 4 is expressed by primary cultured hippocampal neurons and its specific role in lipopolysaccharide-induced neuroinflammation is currently undefined. In this study, Toll-like receptor 4 antibody blocking was used to analyze the Toll-like receptor 4 signaling pathway and changes in inflammation of lipopolysaccharide stimulated hippocampal neurons. Immunofluorescence showed that Toll-like receptor 4 protein was mainly located in the membrane of hippocampal neurons. Quantitative reverse transcription-PCR and western blot assay showed that after stimulation of lipopolysaccharide, the mRNA and protein levels of Toll-like receptor 4 and the mRNA levels of interleukin-1β and tumor necrosis factor-α were significantly increased. In addition, there was increased phosphorylation and degradation of kappa B α inhibitor in the cytosol and increased nuclear factor-κB p65 expression in the nuclei. Pretreatment with Toll-like receptor 4 antibody could almost completely block this increase. These experimental findings indicate that lipopolysaccharide participates in neuroinflammation by stimulating Toll-like receptor 4/nuclear factor-κB pathway in hippocampal neurons, which may be both "passive victims" and "activators" of neuroinflammation.

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Received: 2012-12-11
Accepted: 2013-03-17
(N20120528003)

Key Words

neural regeneration; inflammation; Toll-like receptor 4; lipopolysaccharide; nuclear factor-kappa B; interleukin-1 beta; tumor necrosis factor-alpha; hippocampus; neurons; grants-supported paper; neuroregeneration

Research Highlights

(1) Lipopolysaccharide can activate the signaling pathway of nuclear factor-kappa B through Toll-like receptor 4 in rat hippocampal neurons, thus inducing neuroinflammation.
(2) Neurons may be both "passive victims" and "activators" of neuroinflammation.

INTRODUCTION

Toll-like receptors are type-I transmembrane pattern-recognition receptors that initiate signals in response to diverse pathogen-associated molecular patterns, such as lipopolysaccharide[1]. Roles of Toll-like receptor 2 and Toll-like receptor 4 have recently been established in the pathogenesis of neurodegenerative disease[2]. Major pathways activated by Toll-like receptor 4 include kappa B inhibitor kinase-nuclear factor-kappa B kinase cascades. Nuclear factor-kappa B induces the expression of genes encoding inflammation-associated molecules and cytokines, resulting in the release of

inflammatory factors and tissue injury[3]. Recent findings suggest that Toll-like receptor 4 expressed in the central nervous system, especially in glial cells, plays a vital role in neuroinflammation and neurodegenerative conditions[4].

Traditional theory suggests that neurons are injured by inflammatory factors released from glial cells, and that neurons are the victims of neuroinflammation[5]. However, it has recently been suggested that Toll-like receptor 4 is expressed by cerebral cortical neurons[6]. Here, we investigated whether neurons in the hippocampus, which are closely related to Alzheimer's disease, can express Toll-like receptor 4. A specific role for Toll-like receptor 4 in lipopolysaccharide-responsive hippocampal neurons is currently undefined. In the current study, to investigate the expression pattern of Toll-like receptors in hippocampal neurons and the key role of neuronal Toll-like receptor 4 signaling in neuronal inflammation induced by lipopolysaccharide, primary cultured hippocampal neurons from rats were treated with a ligand for Toll-like receptor 4 (lipopolysaccharide) or anti-Toll-like receptor 4 antibody to stimulate or block Toll-like receptor 4 functions.

RESULTS

Identification of primary cultured hippocampal neurons determined by immunofluorescence

Hippocampal neurons were immunostained with neuronal nuclei (red fluorescence in the nuclei). All cell nuclei were stained with Hoechst (blue fluorescence). Cells stained with red and blue fluorescence represented hippocampal neurons. Approximately 90% of primary cultured cells were positively stained with neuronal nuclei (Figure 1).

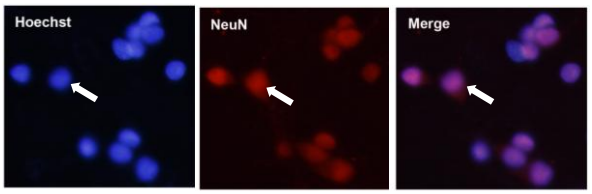

Figure 1 Morphology of primary cultured hippocampal neurons determined by fluorescence microscopy (× 400).

Cultured cells were stained with neuron-specific nuclear protein (neuronal nuclei; NeuN) to label neurons (red fluorescence) and Hoechst to label all nuclei (blue fluorescence). Costaining of red and blue fluorescence in the same field was determined and merged. Approximately 90% of cultured cells were costained with red and blue fluorescence.

Expression of Toll-like receptor 1–9 mRNA in normal hippocampal neurons detected by reverse transcription-PCR

The relative levels of Toll-like receptor 1–9 mRNA in normal hippocampal neurons were determined by reverse transcription-PCR (n = 3; Figure 2A). Primary cultured hippocampal neurons generally expressed mRNA for Toll-like receptor 1–9. Among these Toll-like receptors, the level of Toll-like receptor 4 was the highest. Toll-like receptor 4 mRNA levels were 3.3, 1.4, 3.0, 2.1, 2.4, 1.3, 1.6 and 1.9 folds greater than Toll-like receptor 1–9 mRNA, respectively (Figure 2B).

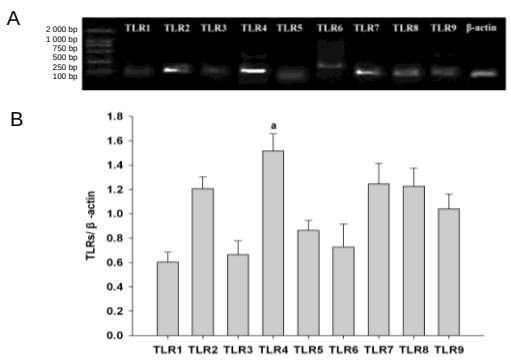

Figure 2 Toll-like receptor (TLR) 1–9 mRNA expression patterns in rat normal primary cultured hippocampal neurons by reverse transcription-PCR.

(A) Expression of TLR 1–9 mRNA in normal hippocampal neurons. (B) Relative quantification of TLR mRNA expression.

TLR mRNA/β-actin mRNA absorbance ratios in various groups were analyzed and expressed as a bar chart to represent the relative expression of TLR 1–9 mRNA. Data are expressed as mean ± SD and n = 3 per group. Among the TLRs, TLR4 expression levels were highest. $^{a}P < 0.05$, *vs*. other TLRs (one-way analysis of variance followed by least significant difference and Student-Newman-Keuls *post-hoc* test).

Cellular localization of Toll-like receptor 4 protein in hippocampal neurons determined by immunofluorescence double staining

The expression of Toll-like receptor 4 in the hippocampal neurons was observed by Toll-like receptor 4 (green) and neuronal nuclei (red) immunofluorescence double staining (Figure 3). Almost all the cultured cells were neuronal nuclei/Toll-like receptor 4-double positive, and Toll-like receptor 4 staining was mostly found on the cell surface with limited staining in the cytoplasm.

Effect of lipopolysaccharide on the expression of Toll-like receptor 4 mRNA and protein in hippocampal neurons

To determine whether Toll-like receptor 4 signaling is involved in hippocampal neuron responses to

lipopolysaccharide, we evaluated the expression of Toll-like receptor 4 mRNA and protein in cultured hippocampal neurons exposed to lipopolysaccharide (10 μg/mL) at 2, 6, 12 and 24 hours by quantitative reverse transcription-PCR and western blot assay (Figures 4A, B). Compared with the control group (0 hour), Toll-like receptor 4 mRNA was significantly increased by approximately 6.10, 4.38, 3.64 and 2.86 folds ($P < 0.01$) after lipopolysaccharide stimulation at 2, 6, 12 and 24 hours, respectively. In addition, lipopolysaccharide enhanced the expression of Toll-like receptor 4 protein by 2.05, 1.64 and 1.26 folds ($P < 0.01$) at 2, 6 and 12 hours, respectively (Figure 4C).

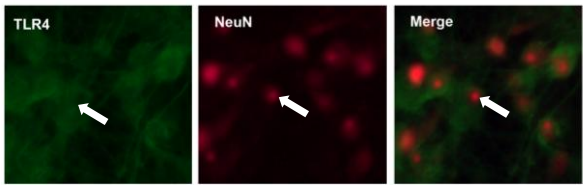

Figure 3 Localization of Toll-like receptor 4 (TLR4) in primary cultured hippocampal neurons by fluorescence microscopy (× 400).

Cultured cells were stained with neuron-specific nuclear protein (neuronal nuclei; NeuN) to label neurons (red) or TLR4 (green). Almost all the cultured cells are NeuN/TLR4-double positive. TLR4 was mostly expressed on the cell surface with limited staining in the cytoplasm.

Effect of lipopolysaccharide on nuclear factor-kappa B signaling pathway in rat hippocampal neurons

The cytosolic levels of phospho-inhibitor of kappa B α and nuclear levels of nuclear factor-kappa B p65 subunit were analyzed by western blot analysis (Figures 5A, B). Lipopolysaccharide induced the phosphorylation of inhibitor of kappa B α in the cytosol, resulting in a significant increase of phospho-inhibitor of kappa B α protein at 0.5 and 2 hours after lipopolysaccharide stimulation ($P < 0.01$). Meanwhile, lipopolysaccharide significantly increased nuclear factor-kappa B p65 protein levels in the nucleus at 2, 6 and 12 hours ($P < 0.01$; Figure 5C).

Effect of lipopolysaccharide on interleukin-1β and tumor necrosis factor-α mRNA expression in hippocampal neurons and the production of interleukin-1β and tumor necrosis factor-α in cultured supernatant

We observed that the levels of interleukin-1β and tumor necrosis factor-α mRNA increased after lipopolysaccharide stimulation. Interleukin-1β mRNA was increased by 157.89, 127.99 and 110.58 folds compared with the control group at 6, 12 and 24 hours ($P < 0.01$, $P < 0.05$). Tumor necrosis factor-α mRNA increased by 346.90, 153.31 and 104.25 folds at 6, 12 and 24 hours ($P < 0.01$) after lipopolysaccharide administration, respectively (Figure 6A). The concentration of interleukin-1β and tumor necrosis factor-α in culture supernatant increased from 64.21 and 58.79 pg/mL to 150.89 and 164.45 pg/mL ($P < 0.05$) at 24 hours after lipopolysaccharide stimulation, respectively (Figure 6B).

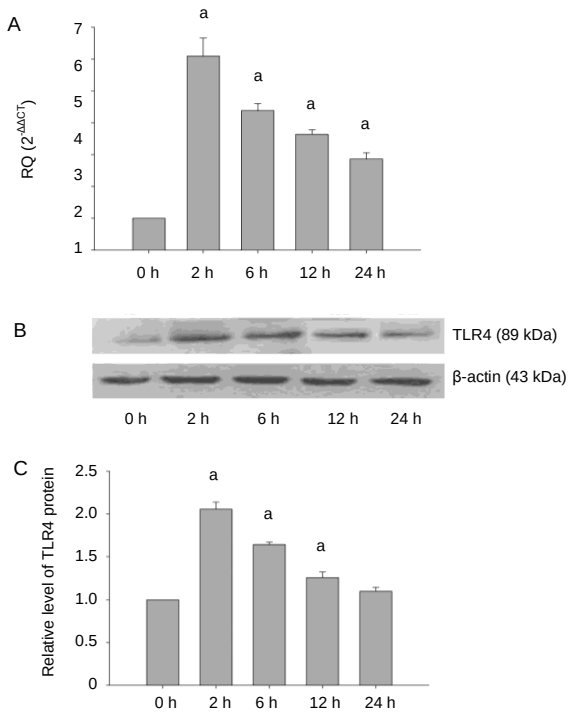

Figure 4 Effect of lipopolysaccharide (LPS) on the expression of Toll-like receptor 4 (TLR4) mRNA and protein in hippocampal neurons after LPS (10 μg/mL) stimulation for 0, 2, 6, 12 and 24 hours, detected by quantitative reverse transcription-PCR and western blot analysis.

(A) The relative quantitation of TLR4 mRNA induced by LPS was detected by real-time PCR. β-actin was used as an internal control. Data were plotted as RQ (relative quantitation, RQ = $2^{-\Delta\Delta CT}$) of target gene cDNA on the Y-axis and as various time points after LPS stimulation on the X-axis. The expression of target gene in the control group (0 hour) was denoted by 1, and the RQ numbers of other groups represented the fold changes compared with the control group (reference sample). $^{a}P < 0.01$, *vs.* control group (0 h).

(B) TLR4 protein levels in each group were detected by western blot analysis. β-actin was used as an internal control.

(C) The mean densitometry values of TLR4/β-actin in various groups were analyzed and expressed as bar charts to represent the relative expression of TLR4 protein. $^{a}P < 0.01$, *vs.* control group (0 h).

Data are expressed as mean ± SD and $n = 3$ per group. One-way analysis of variance and comparisons between two groups were made with the least significant difference and Student-Newman-Keuls *post-hoc* test. h: Hour.

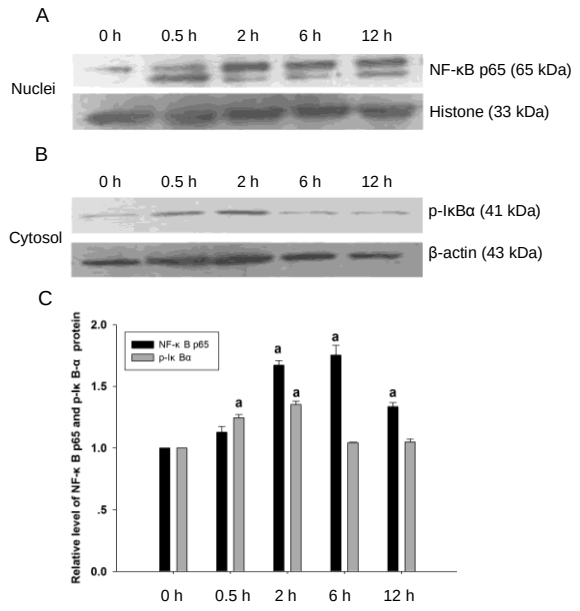

Figure 5 Effect of lipopolysaccharide (LPS) on the expression of nuclear factor-kappa B (NF-κB) p65 and phospho-inhibitor of kappa B α (p-IκBα) protein at 0, 0.5, 2, 6 and 12 hours after LPS (10 μg/mL) stimulation determined by western blot analysis.

(A) NF-κB p65 protein level in the nuclei of hippocampal neurons was detected by western blot analysis. Histone was used as an internal control in the nuclei.

(B) p-IκBα protein level in the cytosol of each group was detected by western blot analysis. β-actin was used as an internal control.

(C) Quantification of NF-κB p65 and p-IκBα protein expression. The mean densitometry values of NF-κB p65/histone and p-IκBα/β-actin in various groups were analyzed and expressed as bar charts to represent the relative expression of NF-κB p65 protein in the nuclei and p-IκBα protein in cytosol. $^{a}P < 0.01$, *vs*. control group (0 h). Data are expressed as mean ± SD and $n = 3$ per group. One-way analysis of variance and comparisons between two groups were made with the least significant difference and Student-Newman-Keuls *post-hoc* test. h: Hour.

Effect of Toll-like receptor 4 antibody on the expression of nuclear factor-kappa B p65, phospho-inhibitor of kappa B α and the production of interleukin-1β and tumor necrosis factor-α

Lipopolysaccharide stimulation induced a significant increase in nuclear factor-kappa B p65 and inhibitor of kappa B α phosphorylation at the 2-hour time point. Cells were then pretreated with Toll-like receptor 4 antibody for 1 hour before lipopolysaccharide stimulation for 2 hours to observe the effect of Toll-like receptor 4 antibody on the nuclear factor-kappa B signal by western blot analysis (Figure 7A). Nuclear factor-kappa B p65 in the nuclei and phospho-inhibitor of kappa B α in the cytosol were significantly increased to 1.58 and 1.85 folds, respectively ($P < 0.01$) after lipopolysaccharide stimulation for 2 hours, and were almost completely blocked by Toll-like receptor 4 antibody ($P < 0.05$; Figure 7B). Our previous results also showed that lipopolysaccharide stimulation induced a secretion peak of interleukin-1β and tumor necrosis factor-α at the 24-hour time point. Cells were then pretreated with Toll-like receptor 4 antibody for 1 hour before lipopolysaccharide stimulation for 24 hours to observe the effect of Toll-like receptor 4 antibody on the production of inflammatory factors. We found that the concentration of interleukin-1β and tumor necrosis factor-α in culture supernatant increased by 3.24 and 4.36 folds ($P < 0.01$) after lipopolysaccharide stimulation for 24 hours, respectively, which was almost completely blocked by Toll-like receptor 4 antibody incubation ($P < 0.01$; Figure 7C).

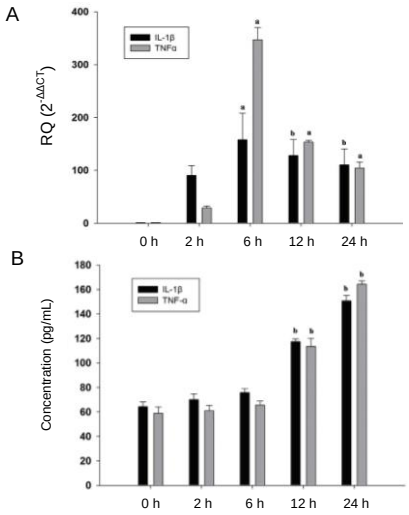

Figure 6 Effect of lipopolysaccharide (LPS) on interleukin-1 beta (IL-1β) mRNA and tumor necrosis factor-alpha (TNF-α) mRNA expression in hippocampal neurons and the production of IL-1β and TNF-α in culture supernatant at 0, 2, 6, 12, 24 hours after LPS stimulation.

(A) The relative quantitation of IL-1β mRNA and TNF-α mRNA induced by LPS was determined by quantitative reverse transcription-PCR. β-actin was used as an internal control. Data were plotted as relative quantitation (RQ = $2^{-\Delta\Delta C_T}$) of target gene cDNA on the Y-axis and as various time points after LPS stimulation on the X-axis. The expression of target gene in the control group (0 h) was denoted by 1, and the RQ numbers of other groups represented the fold changes compared with the control group (reference sample). $^{a}P < 0.01$, $^{b}P < 0.05$, *vs*. control group (0 h). Data are expressed as mean ± SD and $n = 3$ per group.

(B) The concentration of IL-1β and TNF-α in culture media. Culture media were collected at 0, 2, 6, 12 and 24 hours to measure IL-1β and TNF-α concentrations by enzyme-linked immunosorbent assay. $^{b}P < 0.05$, *vs*. control group (0 h).

Data are expressed as mean ± SD and $n = 6$ per condition. One-way analysis of variance and comparisons between two groups were made with the least significant difference and Student-Newman-Keuls *post-hoc* test. h: Hour.

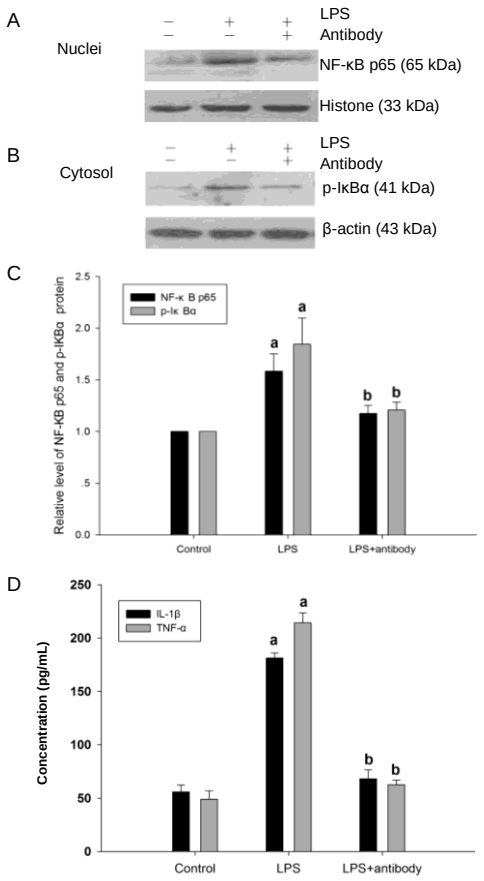

Figure 7 Effect of Toll-like receptor 4 (TLR4) antibody on the expression of nuclear factor-kappa B (NF-κB) p65, phospho-inhibitor of kappa B α (p-IκBα) and the production of interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α).

(A) Cells were pretreated with TLR4 antibody (10 μg/mL) for 1 hour and were then stimulated with lipopolysaccharide (LPS; 10 μg/mL) for 2 hours. NF-κB p65 protein (65 kDa) levels in the nucleus of hippocampal neurons of different groups were detected by western blot analysis. Histone (33 kDa) was used as an internal control in the nuclei.

(B) p-IκBα protein (41 kDa) level in the cytosol of each group was detected by western blot analysis. β-actin (43 kDa) was used as an internal control.

(C) Relative levels of NF-κB p65 in the nucleus and p-IκBα in the cytosol. The mean densitometry values of NF-κB p65/histone and p-IκBα/β-actin in various groups were analyzed and expressed as bar charts to represent the relative expression of NF-κB p65 protein in the nucleus and p-IκBα protein in the cytosol. $^{a}P < 0.01$, *vs*. control group; $^{b}P < 0.05$, *vs*. LPS group.

(D) Effect of TLR4 antibody on the concentration of IL-1β and TNF-α in culture supernatant. Cells were pretreated with TLR4 antibody (10 μg/mL) for 1 hour and were then stimulated with LPS (10 μg/mL) for 24 hours. The concentration of IL-1β and TNF-α in culture supernatant was detected by enzyme-linked immunosorbent assay. $^{a}P < 0.01$, *vs*. control group; $^{b}P < 0.01$, *vs*. LPS group.

(C–D) Data are expressed as mean ± SD and $n = 3$ per group. One-way analysis of variance and comparisons between two groups were made with the least significant difference and Student-Newman-Keuls *post-hoc* test.

DISCUSSION

Recently, evidence for the neuronal expression of Toll-like receptors has increased. Tang *et al* [6] demonstrated that Toll-like receptors 1, 3, 6, 7, and 8 are expressed at relatively low levels, Toll-like receptor 2 and 4 at intermediate levels, and Toll-like receptor 5 and 9 at higher levels in rat primary cortical neurons. In this study, we found that hippocampal neurons generally expressed Toll-like receptors 1–9 mRNA. Among these Toll-like receptors, the expression level of Toll-like receptor 4 was highest. This suggested that Toll-like receptor 4 might have important roles in neuroinflammation. Previous studies showed that Toll-like receptor 4 was located in the membrane and cytoplasm, and that localization varied from cell to cell. In our study, we found that Toll-like receptor 4 was mostly expressed on the cell surface with a limited presence in the cytoplasm. Activation of Toll-like receptor 4 on immune cells by lipopolysaccharide initiates signaling cascades that result in the activation of transcription factors, leading to the induction of cytokines and other inflammatory mediators[7]. We investigated whether Toll-like receptor 4 in hippocampal neurons can also be activated by lipopolysaccharide, similar to immune cells. We found that Toll-like receptor 4 was significantly up regulated by lipopolysaccharide stimulation, which indicated that Toll-like receptor 4 in hippocampal neurons could be activated by lipopolysaccharide. We then investigated the downstream signaling pathway of Toll-like receptor 4 and its functions following the activation of Toll-like receptor 4 in hippocampal neurons by lipopolysaccharide.

Lipopolysaccharide is an effective activator of nuclear factor-kappa B, which plays a critical role in controlling most inflammatory responses[8]. Neurons injured by inflammatory factors released from glial cells were traditionally thought as the “victims” of neuroinflammation[9]. A recent study reported that unlike other central nervous system resident cells, Toll-like receptor 4 activation of cortical neurons by lipopolysaccharide does not induce nuclear factor-kappa B activation[6]. In the present study, however, we found that lipopolysaccharide significantly increased the expression of phospho-inhibitor of kappa B α in the cytoplasm and nuclear factor-kappa B p65 in the nucleus and accordingly enhanced the release of interleukin-1β and tumor necrosis factor-α. These results indicate that lipopolysaccharide also has a potent pro-inflammatory effect on hippocampal neurons by activating nuclear

factor-kappa B. It has been demonstrated that both Toll-like receptor 2 and Toll-like receptor 4 participate in immune responses induced by lipopolysaccharide[10]. To assess the role of Toll-like receptor 4 in lipopolysaccharide-induced nuclear factor-kappa B activation in hippocampal neurons, we used Toll-like receptor 4 antibody blocking technology. Toll-like receptor 4 antibody is a macromolecule and cannot enter the cytoplasm. However, when combined with Toll-like receptor 4 on the cell membrane, it can block the function of membrane Toll-like receptor 4. In our study, the lipopolysaccharide-induced increase of nuclear nuclear factor-kappa B, cytosolic phospho-inhibitor of kappa B α, interleukin-1β and tumor necrosis factor-α in hippocampal neurons was almost completely blocked by incubation with the Toll-like receptor 4 antibody. These results suggest that lipopolysaccharide activates nuclear factor-kappa B mainly through Toll-like receptor 4 in hippocampal neurons and further proves that Toll-like receptor 4 is mainly located on the cell membrane. Therefore, blocking membrane Toll-like receptor 4 by Toll-like receptor 4 antibody can suppress nuclear factor-kappa B activation in lipopolysaccharide-stimulated hippocampal neurons and the production of inflammatory mediators. Toll-like receptor 4 antibody showed a potent anti-inflammatory effect and significantly counteracted the effects of Toll-like receptor 4 stimulation by lipopolysaccharide.

In summary, Toll-like receptor 4 expressed by hippocampal neurons has an important role in neuroinflammation. Neurons may not only be “passive victims” but also the “activators” of neuroinflammation.

MATERIALS AND METHODS

Design

A randomized, controlled, *in vitro* study.

Time and setting

The experiment was performed at the Pathophysiology Laboratory, Medical School of Nantong University, China from October 2010 to April 2012.

Materials

Sprague-Dawley female rats, specific pathogen-free grade, were supplied by the Experimental Animal Center of Nantong University, China (license No. SYXK (Su) 2002-0022). The protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[11].

Methods

Primary culture of hippocampal neurons

Primary cultures of hippocampal neurons were performed as described in Brinton[12]. Briefly, hippocampi were dissected from the brains of Sprague-Dawley rat fetuses at embryonic day 18. After treatment with 0.13% trypsin for 15 minutes at 37°C, the hippocampi were dissociated by trituration in Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 with 10% inactivated fetal bovine serum. After 2 days *in vitro*, non-neuronal cell growth was inhibited with 10 μmol/L cytosine arabinoside for 2 days.

Primary cultured hippocampal neurons were treated with lipopolysaccharide or anti-Toll-like receptor 4 antibody to stimulate or block the functions of Toll-like receptor 4

The control group (0 hour group) was untreated. To detect the expression of Toll-like receptor 4, interleukin-1 beta and tumor necrosis factor-alpha, primary cultured hippocampal neurons were treated with lipopolysaccharide (from *Escherichia coli* 0111:B4, Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China; 10 μg/mL) for 2, 6, 12 and 24 hours, respectively. To determine the effect of lipopolysaccharide on the expression of nuclear factor-kappa B p65 and phospho-inhibitor of kappa B α protein, cells were treated with lipopolysaccharide (10 μg/mL) for 0.5, 2, 6 and 12 hours, respectively. In another experiment, three groups were used: control group, lipopolysaccharide group (cells treated with 10 μg/mL lipopolysaccharide for 2 or 24 hours), and lipopolysaccharide + antibody group (cells pretreated with 10 μg/mL anti-Toll like receptor 4 antibody for 1 hour, followed by 10 μg/mL lipopolysaccharide for 2 hours to investigate the expression of nuclear factor-kappa B p65 mRNA and phospho-inhibitor of kappa B α, or followed by lipopolysaccharide stimulation for 24 hours to detect the secretion of interleukin-1 beta and tumor necrosis factor-alpha protein).

Immunofluoresence

Neurons grown on coverslips in 24-well plates were fixed in 4% paraformaldehyde in PBS (0.01 mol/L, pH 7.4) for 30 minutes at room temperature and washed three times with PBS. After blocking for 1 hour in PBS with 10% goat serum, cultures were incubated overnight at 4°C with mouse anti-neuronal nuclei monoclonal antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-Toll-like receptor 4 polyclonal antibody (1:200;

Santa Cruz Biotechnology). This was followed by three washes with PBS, and then cultures were incubated with TRITC-conjugated goat anti-mouse IgG (1:400; Santa Cruz Biotechnology) or FITC-conjugated goat anti-rabbit IgG (1:400; Santa Cruz Biotechnology), respectively, for 2 hours. Nuclei were stained with Hoechst (Dojindo Laboratories, Kumamoto, Japan; 1 μg/mL) for 10 minutes at room temperature. After three washes with ddH_2O, images of cells were acquired using a fluorescence microscope (Olympus, Tokyo, Japan).

Reverse transcription-PCR and real-time quantitative reverse transcription-PCR analysis

Total RNA was isolated using Trizol™ and 3 μg of total RNA was reverse transcribed into cDNA following the reverse transcription protocol. Primers were designed by Primer Premier 5.0 software (Premier Co., Canada). To detect Toll-like receptor 1–9 mRNA expression in normal hippocampal neurons, reverse transcription-PCR was performed. cDNA (2 μL) was replicated in a 20 μL PCR reaction system. The cycle profile was as follows: denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 45 seconds, 54°C for 30 seconds, and 72°C for 50 seconds, followed by 5 minutes at 72°C. Beta-actin expression was amplified as an internal, housekeeping gene control. Following electrophoresis (100 V for 30 minutes) in 1% agarose gel, the PCR products were scanned and quantified by Scion Image software (Scion, Frederick, MD, USA) and the amount was normalized with beta-actin values in the same group.

To assess the expression of mRNA for Toll-like receptor 4, interleukin-1 beta and tumor necrosis factor-alpha, hippocampal neurons were treated with lipopolysaccharide (10 μg/mL) for 0, 2, 6, 12 and 24 hours, respectively, and quantitative reverse transcription-PCR was performed. cDNA was amplified using a StepOne™ real-time PCR detection system (ABI, Foster City, CA, USA) using SYBR® Green Supermix (Fermentas, Glen Burnie, MD, USA). All samples were prepared more than three times and each PCR reaction of each sample was set up in triplicate with a negative control. The reactions were run with the following parameters: initial denaturation for one cycle at 95°C for 10 minutes, 40 cycles of 95°C denaturation for 15 seconds, 54°C annealing for 30 seconds, and 72°C extension for 30 seconds. After obtaining real-time fluorescence measurements, cycle threshold (C_T) values were determined. $\triangle C_T$ = (C_T value of target gene – C_T value of internal gene control), $\triangle\triangle C_T$ = (C_T value of target gene – C_T value of internal gene control) $_{\text{sample A}}$ – (C_T value of target gene – C_T value of internal gene control) $_{\text{sample B}}$. Fold change relative quantification (RQ) = $2^{-\Delta\Delta CT}$.

Enzyme-linked immunosorbent assay

The effects of lipopolysaccharide (10 μg/mL) on the production of interleukin-1β and tumor necrosis factor-α in hippocampal neurons were measured by highly sensitive enzyme-linked immunosorbent assay kits (GBD, Santiago, CA, USA) (n = 6) using culture supernatants collected from treated cells. Samples were analyzed according to the manufacturer's recommendations. The absorbance value at 450 nm wavelength was measured using a 96-well plate by an ELX-800 microplate assay reader (BIO-TEK, Winooski, VT, USA) within 30 minutes.

Western blot analysis

Hippocampal neurons were washed twice with ice-cold PBS and lysed in buffer on ice. Twenty micrograms of protein was separated on a 10% sodium dodecyl sulfate polyacrylamide gel and electrotransferred onto polyvinylidene fluoride membranes. Each membrane was incubated in fresh blocking buffer (containing 5% nonfat dry milk) at room temperature for 30 minutes, and was then incubated with rabbit anti-Toll-like receptor 4 polyclonal antibody (1:200; Santa Cruz Biotechnology), mouse anti-β-actin monoclonal antibody (1:2 000; Santa Cruz Biotechnology), mouse anti-histone H1 monoclonal antibody (1:500; Millipore, Billerica, MA, USA), rabbit anti-nuclear factor-kappa B p65 polyclonal antibody (1:1 000; Kangchen BIO-TECH, Shanghai, China) and mouse anti-phospho-inhibitor of kappa B α monoclonal antibody (1:1 000, Kangchen BIO-TECH) at 4°C overnight. The membrane was rinsed three times for 5 minutes each using PBS and 0.1% Tween-20. Afterwards, it was incubated in horseradish peroxidase-conjugated goat anti-mouse IgG or horseradish peroxidase-conjugated goat anti-mouse IgG (1:1 000; Santa Cruz Biotechnology) at room temperature for 2 hours. Enhanced chemiluminescence was used for detection. Immunoreactive bands were scanned and quantified by Scion Image software (Scion), and the amount was normalized with β-actin or histone values in the same lane.

Statistical analysis

All results were expressed as mean ± SD. Statistical analysis was performed using SPSS 15.0 software (SPSS, Chicago, IL, USA). Differences among multiple groups were detected by one-way analysis of variance, and comparisons between two groups were made with the least significant difference and Student-Newman-Keuls *post-hoc* test. A value of $P < 0.05$ was considered

statistically significant.

Acknowledgments: This work was performed at the Pathophysiology Laboratory, Medical School of Nantong University, China. We received help and support from teachers in the laboratory and appreciated their hard work and enthusiasm.

Funding: This work was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions; the Nantong Applied Research Program, No. k2010036; the 2011 Jiangsu Graduated Students' Research and Innovation Program, No. CX2211-0640; the Nantong University Graduated Students' Technological and Innovative Program, No. YKC11033.

Author contributions: Yae Hu and Jiahui Mao participated in the experimental operation. Yu Zhang participated in the data analysis, statistical processing and manuscript writing. Ailing Zhou gave experimental guidance. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: Experimental protocols were permitted by the Animal Ethics Committee of the Shandong University of Traditional Chinese Medicine, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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(Reviewed by Croxford L, Raye Z, Ye M, Gao DM)
(Edited by Wang J, Yang Y, Li CH, Song LP)