Neurotox Res (2011) 19:484–495
DOI 10.1007/s12640-010-9215-x

Neuroprotection of Interleukin-6 Against NMDA-Induced Apoptosis and Its Signal-Transduction Mechanisms

Zhan Liu · Yi-Hua Qiu · Bing Li · Song-Hua Ma · Yu-Ping Peng

Received: 26 September 2009 / Revised: 5 August 2010 / Accepted: 5 August 2010 / Published online: 18 August 2010

Abstract We have previously shown that interleukin-6 (IL-6)-protected neurons against the suppression of neuronal vitality and overload of intracellular Ca^{2+} induced by glutamate or *N*-methyl-D-aspartate (NMDA). Herein we provide further evidence for IL-6 neuroprotection against NMDA-induced apoptosis and explore the signal-transduction mechanisms underlying the anti-apoptotic action of IL-6. Cerebellar granule neurons (CGNs) from postnatal 8-day infant rats were chronically exposed to IL-6 (40 or 120 ng/ml) for 8 days, and stimulated with NMDA (100 μM) for 30 min. To observe the signaling pathways, we employed AG490 (5 or 10 μM), an inhibitor of Janus kinases (JAKs), or LY294002 (5 or 10 μM), an inhibitor of phosphatidylinositol 3-kinase (PI3K), to pretreat the CGNS together with IL-6. The levels of phosphorylation for the downstream effectors of JAKs and PI3K, i.e., phosphorylated STAT3 and Akt, were quantified by Western blot assay. In the cultured CGNs with various drug exposures, the expressions of Bcl-2, Bax, and caspase-3 were measured by real-time PCR and Western blot, and the percentage of apoptotic nuclei was tested by Hoechst 33342 staining. After the CGNs were chronically exposed to IL-6, NMDA stimulation led to an increase in the expression of Bcl-2 mRNA and a decrease in the expression of Bax and caspase-3 mRNAs and proteins when compared with those neurons lacking IL-6 exposure. IL-6 pretreatment of the neurons without NMDA stimulation concentration-dependently enhanced the expressions of Bcl-2 mRNA and protein while attenuating the expressions of Bax and caspase-3 mRNAs and proteins in comparison with control lacking any treatment. Furthermore, IL-6 prevented the increase in the percentage of apoptotic neurons induced by NMDA. The combined pretreatment of the CGNs with AG490 and IL-6 or with LY294002 and IL-6 reduced these anti-apoptotic effects of IL-6. Neither AG490 nor LY294002 exposure alone altered the expressions of Bcl-2, Bax, and cleaved caspase-3 proteins. IL-6 up-regulated the levels of phosphorylated STAT3 and Akt, and this was blocked by AG490 and LY294002, respectively. These results suggest that IL-6 protects neurons against NMDA-induced apoptosis, and that the IL-6 neuroprotection is jointly mediated by JAK-STAT3 and PI3K-Akt signaling pathways.

Keywords Interleukin-6 · Neuronal apoptosis · NMDA · Bcl-2 · Bax · Caspase-3 · JAKs · STAT3 · PI3K · Akt

Introduction

Cytokine interleukin-6 (IL-6) and its receptor have been found to be expressed by neurons and glial cells in the brain (Gadient and Otten 1993; 1994; Schöbitz et al. 1993). The expression of brain IL-6 is dramatically increased in some diseases, injuries, and pathological states of the brain (Ali et al. 2000; Baranzini et al. 2000; Baker et al. 2001; Gruol and Nelson 2005). This elevation of IL-6 levels under neuronal disorder conditions must have its functional significance. It may reflect a compensatory neuroprotection or neurodegeneration mechanism (Baker et al. 2001). Cerebral ischemia triggers neuronal IL-6 expression, and intracerebroventricular

Z. Liu · Y.-H. Qiu (✉) · B. Li · S.-H. Ma · Y.-P. Peng (✉)
Department of Physiology, School of Medicine, Nantong University, 19 Qixiu Road, Nantong 226001, China
e-mail: yhqiu@ntu.edu.cn

Y.-P. Peng
e-mail: yppeng@ntu.edu.cn

Y.-H. Qiu
The Key Laboratory of Neuroregeneration of Jiangsu Province, Nantong University, 19 Qixiu Road, Nantong 226001, China

injection of IL-6 significantly reduces ischemic brain damage (Loddick et al. 1998). The large increase in endogenous IL-6 bioactivity in response to ischemia, together with the marked neuroprotection produced by exogenous IL-6 suggest that this cytokine is an important endogenous inhibitor of neuronal death during cerebral ischemia (Loddick et al. 1998). IL-6-deficient mice have a reduced survival, worse neurological status, and larger infarcts of the cerebral ischemia (Herrmann et al. 2003). IL-6 rescues neurons and oligodendrocytes from the *N*-methyl-D-aspartate (NMDA)-induced neuronal death and oligodendrocyte degeneration (Inomata et al. 2003; Pizzi et al. 2004). These reported facts represent a neuroprotective effect of IL-6. Contradictorily, endogenous or exogenous increase in brain IL-6 contributes to the pathogenesis of neurodegenerative disorders (Qiu et al. 1995, 1998; Qiu and Gruol 2003; Conroy et al. 2004; Orellana et al. 2005). Therefore, understanding the dichotomy pathogenesis/neuroprotection of IL-6 may provide a rationale for better therapeutic strategies (Muñoz-Fernández and Fresno 1998). We previously showed that chronic IL-6 exposure of cerebellar granule neurons (CGNs) protected the neurons against glutamate- or NMDA-induced neurotoxicity, including improvement of neuronal vitality and suppression of intracellular Ca^{2+} overload (Peng et al. 2005; Wang et al. 2009). Here, we provide further evidence for the IL-6 neuroprotection at the profile of neuronal apoptosis.

Apoptosis, a process of programmed cell death, is controlled by a series of relevant events. Bcl-2 is the prototype for a family of mammalian genes and the proteins they produce. They can be either pro-apoptotic, such as Bax, Bad, Bak and Bid, or anti-apoptotic, including Bcl-2, Bcl-xl, and Bcl-w (Chao and Korsmeyer 1998). A balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family is important to the homeostasis of cellular life and death. Many pathways and signals lead to apoptosis, but there is only one mechanism that actually causes the death of a cell. After a cell receives stimulus, it undergoes organized degradation of cellular organelles by activated proteolytic caspases. Caspases, or cysteine–aspartic acid proteases, are essential in cells for apoptosis and have been termed "executioner" proteins for their roles in the cells (Kothakota et al. 1997; Yuan and Horvitz 2004). Thus, we measured expressions of Bcl-2 and Bax, a couple of antagonistic regulators of apoptosis, as well as caspase-3, a member of caspase family, at gene and protein levels to determine NMDA neurotoxicity and the effect of IL-6 on NMDA-induced apoptosis. In addition, a cell that is undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst 33342 and fluorescence microscopy (Allen et al. 2001). The observation of apoptotic neurons therefore helps to show the consequences of these changes in the genes, proteins, and enzyme regulating apoptosis.

Although the importance of IL-6 in brain function is extensively investigated, the signaling pathways triggered by the cytokine with respect to neuronal survival or repair are not explored in detail. In general, binding of IL-6 to its receptor triggers the recruitment of gp130, a 130-kDa signal transducing subunit coupled with a membrane-associated 80-kDa ligand-binding subunit (IL-6 receptor), subsequently leading to the activation of the gp130-associated Janus kinases (JAKs) (Murakami et al. 1993; Lutticken et al. 1994; Narazaki et al. 1994). JAKs phosphorylate gp130 on several tyrosine residues and these phospho-tyrosines recruit various Src homology 2 (SH2) domain-containing proteins, such as signal transducer and activator of transcription 3 (STAT3) and SH2 domain-containing protein-tyrosine phosphatase-2 (SHP-2) (Sadowski et al. 1993; Taga and Kishimoto 1997). SHP-2 links cytokine receptor to mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK or MEK) and is essential for mitogenic activity, whereas STAT3 can induce Bcl-2 and is involved in anti-apoptosis (Fukada et al. 1996). In addition to JAK-STAT and MAPK/ERK pathways, phosphatidylinositol 3-kinase (PI3K)-Akt pathway is also activated in many tissues in response to IL-6 (Kamimura et al. 2003).

Several reports have implicated the STAT signaling molecule in the effects of IL-6 on neuronal functions. For example, these roles of IL-6 in treating neurodegenerative and demyelinating diseases occur through activating the signal transducer gp130 and are associated with stimulation of transcription factors STAT1 and STAT3 (Pizzi et al. 2004); endogenous IL-6 plays a critical role in preventing damaged neurons from undergoing apoptosis in the acute phase of cerebral ischemia and its role may be mediated by STAT3 activation (Yamashita et al. 2005). We previously showed that gp130, JAK-STAT3, and MAPK/ERK signaling pathways participated in mediating IL-6 neuroprotection against the attenuation of neuronal vitality and the overload of intracellular Ca^{2+} induced by glutamate or NMDA (Peng et al. 2005; Wang et al. 2009). However, the cascades mediating IL-6-induced neuronal survival or repair are complicated, and especially the involvement of PI3K-Akt signaling pathway in the IL-6 neuroprotection has been poorly explored. Thus, before being able to utilize the neuroprotective effect of IL-6, it is required to study the mechanisms and functions of each signaling pathway downstream of IL-6.

Materials and Methods

Cell Culture

Sprague–Dawley infant rats of postnatal day 8 (The Center of Experimental Animals, Nantong University, China) were anesthetized by hypothermia via placing them in an

ice bath for 2–4 min. Cell preparation and culture of rat cerebellum were carried out as previously described (Peng et al. 2005; Wang et al. 2009). Briefly, the cerebella were removed from the rats and minced with sterile surgical blades. The minced cerebella were chemically dissociated in the presence of trypsin and DNase I and plated in a poly-L-lysine-coated (25 mg/l) six-well plate. Cells were seeded at a density of 2.83×10^5 cells/cm^2 in basal Eagle's medium supplemented with 0.1 g/L gentamicin, 2.2 g/L $NaHCO_3$, 2.385 g/L HEPES, 25 mM KCl, and 10% heat-inactivated fetal bovine serum. The plates were incubated at 37°C with a humidified 5% CO_2/95% air atmosphere in a CO_2 incubator. Cytosine arabinoside (10 μM) was added to the medium 24 h after the cells were plated to inhibit glial proliferation.

Drug Exposures

Rat recombinant IL-6 (BD Biosciences, San Jose, CA, USA) of 40 or 120 ng/ml was added to the CGN cultures on days 1, 4, and 7 (every 72 h) to keep the concentration of exogenously added IL-6 relatively constant during the period of chronic treatment. AG490 (Calbiochem, San Diego, CA, USA) and LY294002 (Cell Signaling Technology, Beverly, MA, USA) were injected to the medium 1 h prior to IL-6. NMDA was dissolved in Locke's solution containing 10 μM of glycine, which was applied to the 8-day cultures at a concentration of 100 μM for 30 min at 20°C. The cultures were again incubated with the primary culture medium in the CO_2 incubator for 6–8 h.

Real-Time Quantitative PCR

The expressions of Bcl-2, Bax, and caspase-3 mRNAs were examined by quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Potentially contaminating residual genomic DNA was eliminated with RNAse-free DNAse (Promega, Madison, WI, USA). After the RNA content was determined by spectrophotometric analysis at 260 nm, 2 μg of total RNA was used for cDNA synthesis with murine myelomonocytic lymphoma virus reverse transcriptase (Promega, Madison, WI, USA). The single-stranded cDNA was then amplified by real-time quantitative PCR for evaluation of relative expression levels of three genes of the interest. The PCR reaction was performed in a Rotor-Gene 3000 Real-Time Cycler (Corbett Research, Australia); detection involved measuring the binding of the fluorescence dye SYBR Green I (Molecular Probe, Eugene, OR, USA) to double-stranded DNA. Each 20 μl of reaction mixture contained 1 μl of cDNA synthesized as above, 2 μl PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each pair of oligonucleotide primers (Table 1), and 1 U Taq DNA polymerase. The reaction procedures were as follows: an initial step at 95°C for 5 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The data was collected using the instrument's software (Rotor-Gene software, version 6.0) and relative quantification was performed using the comparative threshold (CT) method after determining the CT values for reference (β-actin) and target genes (Bcl-2, Bax, or caspase-3) in each sample sets according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), as described by the manufacturer (User Bulletin). Changes in mRNA expression levels were calculated after normalization to β-actin. As calibrator sample we used cDNA from control cells without any treatment. The program calculates the ΔCts and the ΔΔCt with the formulas below:

$$\Delta Ct = Ct\_Mean(\beta\text{-}actin) - Ct\_Mean(Target);$$

$$\Delta\Delta Ct = \Delta Ct - \Delta Ct\_Mean; Gene\ expression\ level = 2^{-\Delta\Delta Ct}$$

Values of fold changes in the control sample versus the treated samples represent averages from three- or four-time measurements. Changes in gene expressions were reported as fold changes relative to controls. To verify the specificity of the amplification reaction, melting curve analysis was performed.

Western Blot Analysis

The cultured neurons were grown in a six-well plate (Corning, NY, USA), and harvested following treatment. Cells were homogenized in lysis buffer (62.5 mM Tris-HCl at pH 6.8, 2% sodium dodecyl sulfide, 4% β-mercaptoethanol, 10% glycerol, 50 mM DTT, and 1 mM phenylmethanesulfonyl) and the supernatant was collected by centrifuging at 4°C at 12,000×g for 15 min. The supernatants, containing 20 μg of total cellular protein, were mixed with sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% sodium dodecyl sulfide, 4% β-mercaptoethanol, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) and boiled for 5 min. The cellular protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Pall, Port Washington, NY, USA) using a semi-dry transfer apparatus. After blocking nonspecific binding with 5% (w/v) nonfat dry milk, the membranes were probed with rabbit antibodies specific for Bcl-2 (1:1000, Cell Signaling Technology, Beverly, MA, USA), Bax (1:1000, Cell Signaling Technology, Beverly, MA, USA), cleaved caspase-3 (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phosphorylated STAT3 (1:750, Cell Signaling Technology, Beverly, MA, USA), and phosphorylated Akt (1:1000, Cell Signaling

Table 1 Sequences of PCR primers

Gene	Sense primer	Antisense primer	Product size (bp)	Accession Nos.
Bcl-2	5′-GCAGAGATGTCCAGTCAGC-3′	5′-CCCACCGAACTCAAAGAAGG-3′	129	NM_016993
Bax	5′-ATGGGCTGGACACTGGACTTC-3′	5′-GAGCGAGGCGGTGAGGAC-3′	146	NM_017059
Caspase-3	5′-GGAACGAACGGACCTGTG-3′	5′-GCCTCCACTGGTATCTTCTG-3′	188	NM_012922
β-actin	5′-CGTTGACATCCGTAAAGACC-3′	5′-TAGAGCCACCAATCCACAC-3′	176	NM_031144

Technology, Beverly, MA, USA) at room temperature for 2 h or at 4°C overnight. They were incubated with the IRDye 800-conjugated affinity purified goat anti-rabbit IgG (1:5000, Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) for 1 h at room temperature and visualization by Odyssey laser scanning system (LI-COR Inc, LINCOLN, NE, USA). Blots were re-probed with the monoclonal mouse anti-β-actin antibody (1:5000, Sigma, ST. Louis, MO, USA), followed by reaction with IRDye 800-conjugated affinity purified goat anti-mouse IgG (1:5000, Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) to confirm equal protein loading. The molecular weight and relative quantity of the protein bands were determined by an image analysis system.

Hoechst 33342 Staining

The CGNs were cultured on poly-L-lysine-coated glass coverslips at a density of 1×10^5 cells/cm^2 for 8 days. The cells were fixed in 4% paraformaldehyde for 30 min at room temperature. After stained with 10 μg/ml Hoechst 33342 (Dojindo, Osaka, Japan) for 15 min in a dark, the cells were observed for nuclear changes under a DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany) on ultraviolet illumination. The Hoechst dye was excited at 350 nm, and fluorescence emission was filtered with a 460 nm barrier filter. The nuclei with the apoptotic characteristics, i.e., chromatin condensation and/or nuclear fragmentation (Gschwind and Huber 1995; Lizard et al. 1995), were counted. The experiment was repeated for three times, and for each sample, five visual fields were randomly selected on each slide to quantify the percentage of apoptotic cells in total cells.

Statistical Analysis

Data were expressed as means ± standard deviation ($M \pm$ SD). Statistical analyses were performed with the Statistics Package for Social Science (SPSS, 12.0). The data were subjected to the one-way analysis of variance (ANOVA), followed by Student–Newman–Keul's test to compare the data of all groups relative to each other. Differences were considered statistically significant at $P < 0.05$.

Results

IL-6 Protects Neurons Against NMDA-Induced Changes in Expressions of Bcl-2, Bax and Caspase-3 mRNAs and Proteins

Stimulation of cultured CGNs with NMDA (100 μM) induced a decrease in expressions of Bcl-2 mRNA and protein. CGNs that had been chronically exposed to IL-6 (40 or 120 ng/ml) for 8 days had higher levels of Bcl-2 mRNA and protein after NMDA stimulation when compared with neurons lacking IL-6 exposure (Fig. 1). Further statistical analysis revealed that in the expressions of Bcl-2 mRNA and protein, only the difference in Bcl-2 mRNA expression between 40 ng/ml of IL-6 alone and IL-6 plus NMDA was significantly smaller than that between untreated control and NMDA (Table 2). It shows that IL-6 of 40 ng/ml significantly altered the reduction of Bcl-2 mRNA expression by NMDA, but at 120 ng/ml dose there was no evident effect of IL-6 on NMDA produced reduction of Bcl-2 mRNA and protein. The chronic exposure of CGNs to IL-6 alone up-regulated the expressions of Bcl-2 mRNA and protein relative to non-IL-6-exposed control neurons depending on dose (Fig. 1).

To the contrary, the NMDA stimulation up-regulated expressions of Bax mRNA and protein in the cultured CGNs. The chronic exposure of IL-6 (40 or 120 ng/ml) reduced the up-regulation of the expressions of Bax mRNA and protein induced by NMDA (Fig. 1). The IL-6 exposure alone down-regulated the expressions of Bax mRNA and protein of the cultured CGNs, and this effect of IL-6 depended on its concentration (Fig. 1). The difference in the expression of Bax mRNA or protein between IL-6 alone and IL-6 plus NMDA-treated CGNs was statistically smaller than that between control and NMDA-treated neurons (Table 2), showing a counteractive effect of IL-6 on NMDA-enhanced Bax expression. The further statistical analysis indicated that at the mRNA level, 40 ng/ml of IL-6 was more effective than the higher dose (120 ng/ml), and at the protein level both concentrations of IL-6 were equally effective (Table 2).

The expressions of caspase-3 mRNA and protein in the cultured CGNs were elevated when the neurons were stimulated by NMDA. The pretreatment of IL-6 (40 or

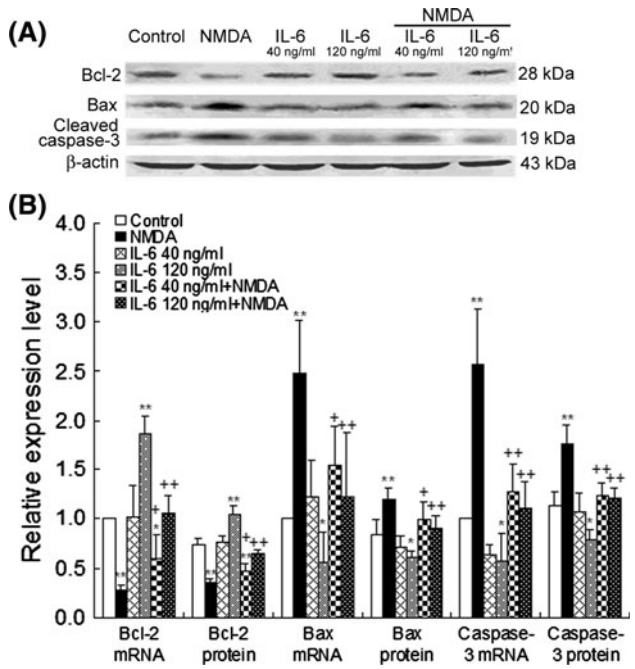

Fig. 1 IL-6 counteracts NMDA-induced changes in the expressions of Bcl-2 mRNA, Bax and caspase-3 mRNAs and proteins. CGNs from postnatal 8-day infant rats were chronically exposed to IL-6 (40 or 120 ng/ml) for 8 days and then stimulated with NMDA (100 μM) for 30 min. Total RNA and proteins in the cells were extracted and analyzed using SYBR Green real-time PCR and Western blot assay for the expressions of Bcl-2, Bax and caspase-3 mRNAs and proteins, respectively. **a** Electrophoretic bands showing the protein expressions in the cultured CGNs with various treatments. β-actin is an internal control. **b** Statistic graph for four repeated experiments. The quantitative analysis of the immunoblots was normalized to β-actin expression. * $P < 0.05$, ** $P < 0.01$, compared with control without any treatment; $^{+}$ $P<0.05$, $^{++}$ $P < 0.01$, compared with NMDA group

120 ng/ml) reduced the elevated expressions of caspase-3 mRNA and protein induced by NMDA (Fig. 1). IL-6 itself had an inhibitory effect on the caspase-3 expression at the gene and protein levels in a dose-dependent manner (Fig. 1). Similarly, the difference in the expression of caspase-3 mRNA or protein between IL-6 alone and IL-6 plus NMDA-treated CGNs was significantly smaller than that between control and NMDA-treated neurons (Table 2).

JAK Inhibitor AG490 Reduces the Counteractive Effects of IL-6 on NMDA-Induced Changes in the Expressions of Bcl-2, Bax and Caspase-3 mRNAs and Proteins

As shown in Fig. 1, NMDA down-regulated the expressions of Bcl-2 gene and protein, and IL-6 counteracted the down-regulated Bcl-2 mRNA expression by NMDA. After the co-exposure of CGNs to AG490 (5 or 10 μM) and IL-6 (120 ng/ml), NMDA stimulation resulted in the reduction of the expressions of Bcl-2 gene and protein when compared with the NMDA stimulation of the neurons exposed to IL-6 alone (Fig. 2). The pretreatment of CGNs with AG490 (5 or 10 μM) alone did not alter the expression of Bcl-2 protein (Fig. 4).

NMDA up-regulated the expressions of Bax gene and protein, and this was counteracted by IL-6 exposure. AG490 reduced the counteractive effect of IL-6 on NMDA-enhanced Bax expressions in a dose-dependent pattern (Fig. 2). The exposure of CGNs to AG490 (5 or 10 μM) alone did not affect the expression of Bax protein (Fig. 4).

Similarly, the NMDA-induced elevation of the expressions of caspase-3 mRNA and protein was counteracted by chronic IL-6 treatment. AG490 reduced the effects of IL-6 in a concentration-dependent manner (Fig. 2). The treatment of CGNs with AG490 (5 or 10 μM) alone did not influence the neuronal expression of cleaved caspase-3 protein (Fig. 4).

PI3K Inhibitor LY294002 Reduces the Counteractive Effects of IL-6 on NMDA-Induced Changes in the Expressions of Bcl-2, Bax and Caspase-3 mRNAs and Proteins

NMDA attenuated the expressions of Bcl-2 mRNA and protein in the cultured CGNs, and IL-6 counteracted the

Table 2 Statistical analysis of the differences in expression of genes and proteins of the apoptotic regulators between the different treatments

Regulators of apoptosis	Difference between control and NMDA	Difference between IL-6 (40 ng/ml) alone and IL-6 plus NMDA	Difference between IL-6 (120 ng/ml) alone and IL-6 plus NMDA
Bcl-2 mRNA	0.72 ± 0.06	0.42 ± 0.16*	0.80 ± 0.02
Bcl-2 protein	0.38 ± 0.04	0.30 ± 0.05	0.39 ± 0.06
Bax mRNA	1.47 ± 0.34	0.32 ± 0.08**	0.67 ± 0.19*
Bax protein	0.33 ± 0.07	0.16 ± 0.06*	0.18 ± 0.03*
Caspase-3 mRNA	1.56 ± 0.46	0.64 ± 0.21**	0.54 ± 0.25**
Caspase-3 protein	0.63 ± 0.09	0.16 ± 0.07**	0.43 ± 0.07*

The data are from Fig. 1

* $P < 0.05$, ** $P < 0.01$, compared with the difference between control and NMDA

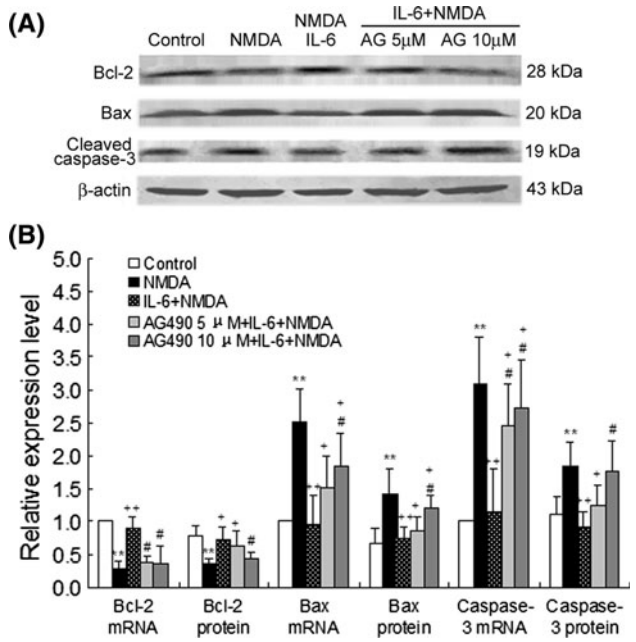

Fig. 2 JAK inhibitor AG490 reduces the counteractive effects of IL-6 on NMDA-induced changes in expressions of Bcl-2 mRNA, Bax and caspase-3 mRNAs and proteins. CGNs from postnatal 8-day infant rats were chronically co-exposed to AG490 (5 or 10 μM) and IL-6 (120 ng/ml) for 8 days and then stimulated with NMDA (100 μM) for 30 min. Total RNA and protein in the cells were extracted and subjected to real-time PCR quantification and Western blot assay for the expressions of the genes and proteins, respectively. **a** Representative Western blot image. **b** Statistic graph for four independent experiments. The values for protein expressions represent the ratio of immunoreactivity of the proteins to *β*-actin. * $P < 0.05$, ** $P < 0.01$, compared with control without any treatment; $^{+}$ $P<0.05$, $^{++}$ $P < 0.01$, compared with NMDA group; $^{\#}$ $P < 0.05$, compared with IL-6 plus NMDA group. AG = AG490

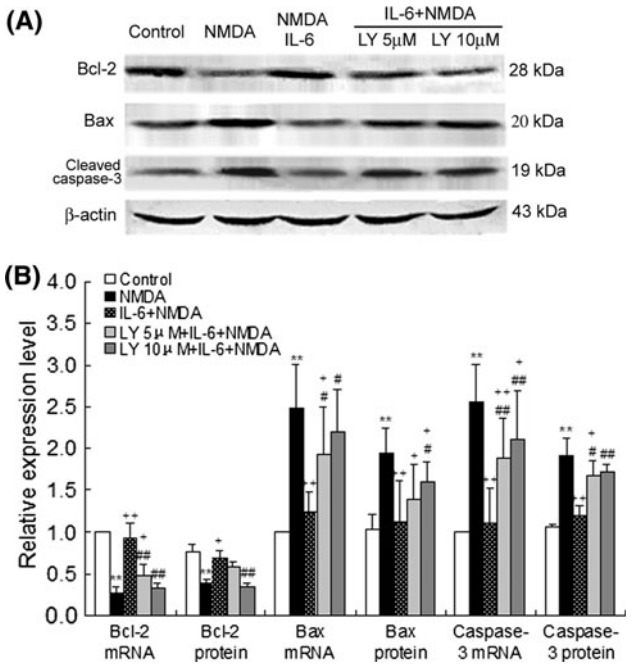

Fig. 3 PI3K inhibitor LY294002 reduces the counteractive effects of IL-6 on NMDA-induced changes in expressions of Bcl-2 mRNA, Bax and caspase-3 mRNAs and proteins. The expressions of the genes and proteins of the anti-apoptotic and pro-apoptotic markers in CGNs exposed to LY294002 (5 or 10 μM) and IL-6 (120 ng/ml) were measured by real-time PCR and Western blot assay, respectively. **a** Immunoreactive bands of the proteins in the cultured CGNs with various treatments. **b** Statistical diagram for mRNA and protein expressions of Bcl-2, Bax and caspase-3. Data are mean ± SD of four separate experiments. ** $P < 0.01$, compared with control without any treatment; $^{+}$ $P<0.05$, $^{++}$ $P < 0.01$, compared with NMDA group; $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, compared with IL-6 plus NMDA group. LY = LY294002

NMDA-depressed Bcl-2 mRNA expression. After the neurons were co-exposed to LY294002 (5 or 10 μM) and IL-6 (120 ng/ml), the NMDA stimulation still caused the expressions of Bcl-2 mRNA and protein to decrease (Fig. 3), indicating that LY294002 reduced IL-6 effects. The reduction by LY294002 depended on its concentration (Fig. 3). The pretreatment of CGNs with LY294002 (5 or 10 μM) alone did not alter the expression of Bcl-2 protein (Fig. 4).

The expressions of Bax mRNA and protein in the cultured CGNs were enhanced by NMDA stimulation. The high levels of expressions induced by NMDA were counteracted by chronic IL-6 exposure. The combined exposure of CGNs to LY294002 (5 or 10 μM) and IL-6 (120 ng/ml) reduced the counteractive effects of IL-6 on NMDA-enhanced expressions of Bax gene and protein (Fig. 3). The exposure of LY294002 (5 or 10 μM) alone to CGNs did not affect the expression of Bax protein (Fig. 4).

Similarly, the expressions of caspase-3 mRNA and protein in the cultured CGNs were increased by NMDA stimulation, and IL-6 (120 ng/ml) counteracted the NMDA-elevated expressions of caspase-3. LY294002 (5 and 10 μM) reduced the effects of IL-6 (Fig. 3). The exposure of LY294002 (5 or 10 μM) alone to CGNs did not influence the neuronal expression of cleaved caspase-3 protein (Fig. 4).

IL-6 Prevents NMDA-Induced Apoptosis, and AG490 and LY294002 Block This Effect

As illustrated in Fig. 5, CGNs stimulated with NMDA exhibited numerous cells with fragmented nuclei. When we counted the cells, we observed an increased percentage of apoptotic cells when compared with non-stimulated control neurons. In the IL-6-pretreated CGNs, NMDA stimulation did not trigger the increase in apoptotic cells, with a remarkable decrease in the percentage of apoptotic nuclei relative to that in IL-6-unexposed CGNs. Either JAK inhibitor AG490 (10 μM) or PI3K inhibitor LY294002 (10 μM) blocked the preventive effect of IL-6 (120 ng/ml)

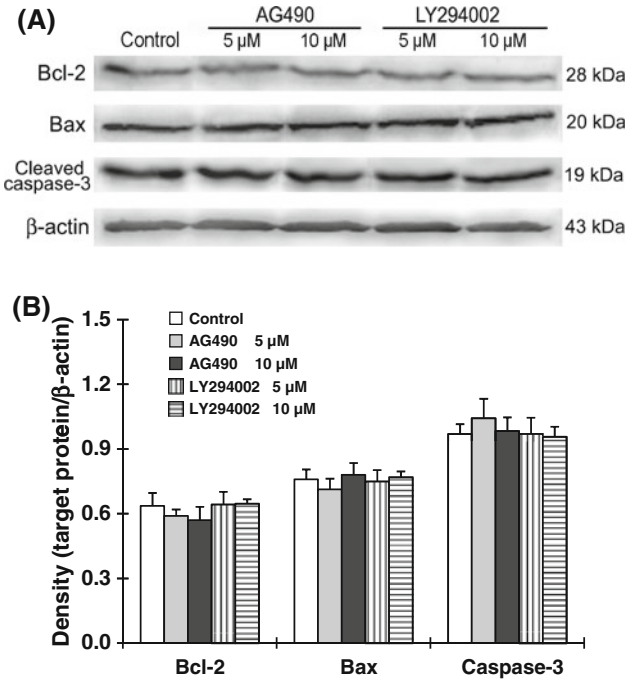

Fig. 4 Effects of AG490 or LY294002 on expressions of Bcl-2, Bax, and cleaved caspase-3 in the cultured CGNs. The expressions of the anti-apoptotic and pro-apoptotic proteins in CGNs exposed to AG490 or LY294002 (5 or 10 μM) for 8 days were measured by Western blot assay. **a** Representative Western blot image. **b** Statistic graph for three independent experiments

on NMDA-induced apoptosis (Fig. 5). IL-6 (120 ng/ml) itself did not significantly alter the apoptosis. The difference in the percentage of apoptotic cells between IL-6 alone and IL-6 plus NMDA-treated CGNs was significantly smaller than that between control and NMDA-treated neurons (4.27 ± 1.16 vs. 20.50 ± 1.92, $P < 0.01$).

Effects of AG490 on IL-6-Induced Phosphorylation of STAT3 and Akt

The CGNs exposed to IL-6 increased phosphorylation of STAT3 relative to control without IL-6 exposure, no matter whether these neurons were stimulated by NMDA or not (Fig. 6). The combined pretreatment of CGNs with AG490 and IL-6 no more produced the increased phosphorylation of STAT3, regardless of with or without the NMDA stimulation. The AG490 exposure alone did not significantly alter the phosphorylation of STAT3 under the NMDA stimulation or not (Fig. 6). Further statistical analysis for the data revealed that the difference in phosphorylation of STAT3 between IL-6 and AG490 plus IL-6-treated CGNs was significantly larger than that between untreated control and AG490-treated CGNs. More statistical comparison is listed in Table 3.

Similarly, IL-6 enhanced the phosphorylation of Akt in the conditions with or without NMDA stimulation. However, the co-exposure of CGNs to AG490 and IL-6 still had the higher levels of phosphorylation of Akt, like those of IL-6 exposure alone (Fig. 6). Compared with control, the neurons with AG490 pretreatment alone had the same levels of phosphorylation of Akt in the conditions with or without NMDA stimulation. Only stimulation of the neurons with NMDA did not alter the phosphorylation of STAT3 or Akt (Fig. 6).

Effects of LY294002 on IL-6-Induced Phosphorylation of Akt and STAT3

Likewise, IL-6 evoked an increase in the phosphorylation of Akt in the cultured CGNs, and LY294002 notably suppressed the increased phosphorylation of Akt by IL-6 (Fig. 7). These effects occurred similarly in the conditions with and without NMDA stimulation. The LY294002 treatment of CGNs alone did not significantly alter the levels of phosphorylation of Akt, no matter whether the neurons were stimulated by NMDA or not (Fig. 7). Further statistical analysis for the data revealed that the difference in the levels of Akt phosphorylation between IL-6 and LY294002 plus IL-6-treated CGNs was significantly larger than that between untreated control and LY294002-treated CGNs and more comparison appeared in Table 3.

However, the increased phosphorylation of STAT3 by IL-6 was not blocked by LY294002 (Fig. 7). LY294002 itself did not alter the phosphorylation of STAT3. These effects were the same in the conditions with and without the NMDA stimulation (Fig. 7).

Discussion

Sustained progression of neuronal apoptosis causes brain tissue loss and subsequent functional deficits following stroke or brain trauma and in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Bredesen et al. 2006; Culmsee and Plesnila 2006). NMDA down-regulated the expression of Bcl-2 and up-regulated the expressions of Bax and caspase-3 at gene and protein levels in the cultured CGNs. These changes of anti-apoptotic and pro-apoptotic markers verify the excitatory neurotoxicity of NMDA, facilitating neuronal apoptosis. The chronic exposure of CGNs to IL-6 reduced the down-regulation of Bcl-2 and the up-regulation of Bax and caspase-3 expressions induced by NMDA at both the transcriptional and the translational levels. Furthermore, the increased number of apoptotic neurons by NMDA was also prevented by IL-6 chronic exposure. These data show that the exogenously added IL-6 protects neurons against NMDA-induced apoptosis. Although IL-6 itself had an effect of up-regulation of Bcl-2 expression or down-regulation of Bax and

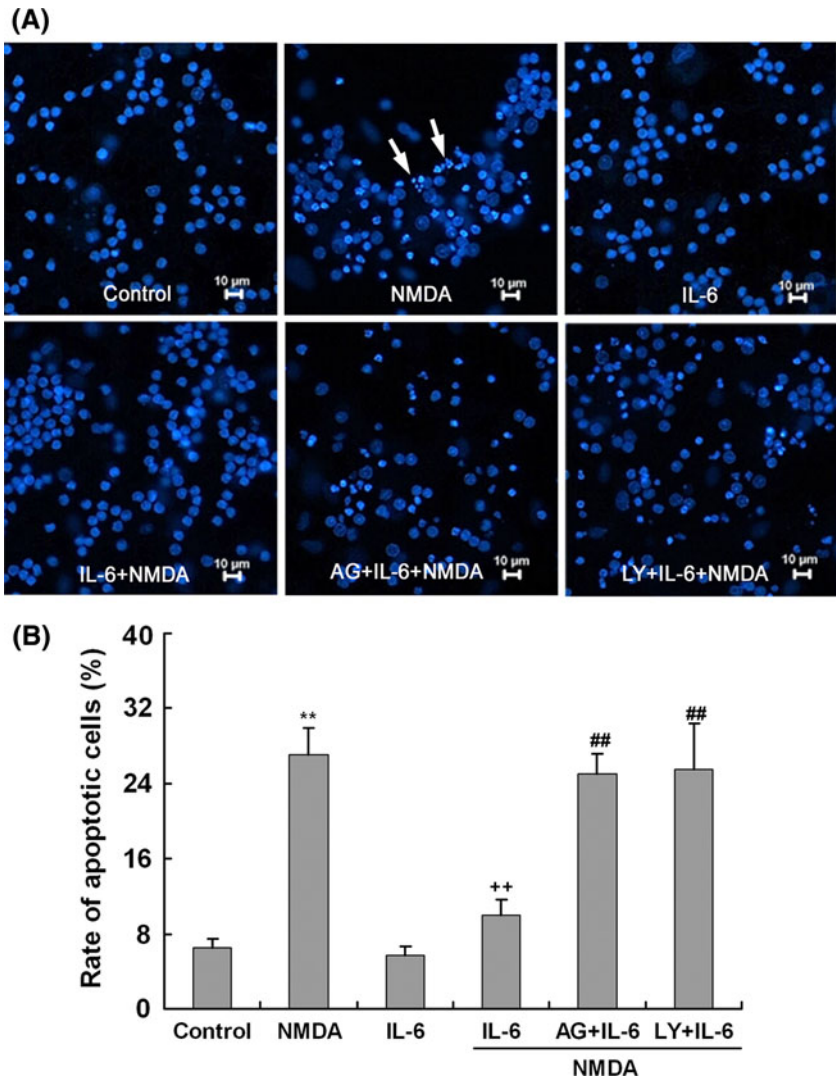

Fig. 5 AG490 or LY294002 compromises the protective effect of IL-6 against NMDA-induced increase in apoptotic cells. The CGNs were stained with fluorescent dye Hoechst 33342 for 15 min after NMDA stimulation. The slides were then examined by fluorescence microscopy and photographed. **a** Photomicrograph showing apoptotic changes for the cells with the various treatments. Cells with signs of apoptosis (fragmented nuclei) are like those denoted by *arrows*. (**b**) Statistic histogram indicating percentage of apoptotic cells in total cells with these treatments. The data are from three separate experiments, and for each sample, five visual fields were randomly selected on each slide to count the percentage of apoptotic nuclei. ** $P < 0.01$, compared with control lacking any treatment; $^{++}$ $P < 0.01$, compared with NMDA group; $^{\#\#}$ $P < 0.01$, compared with IL-6 plus NMDA group. AG = AG490; LY = LY294002

caspase-3 expressions, the difference between IL-6 alone and IL-6 plus NMDA-treated neurons was significantly smaller than that between control without any treatment and NMDA stimulation alone. This testifies the neuroprotective action of IL-6 against NMDA-induced apoptosis. We previously reported that the chronic IL-6 exposure prevented neurons from the inhibited neuronal vitality and the elevated intracellular Ca^{2+} levels induced by glutamate and NMDA (Peng et al. 2005; Wang et al. 2009). Here, we provide further evidence of neuroprotection by IL-6 against NMDA neurotoxicity at the profile of pro-apoptotic and anti-apoptotic events. Several studies in vivo and in vitro support our present data. IL-6 reduces ischemic brain damage (Loddick et al. 1998; Ali et al. 2000; Herrmann et al. 2003) and rescues neurons from NMDA-induced neuronal death and degeneration (Inomata et al. 2003; Pizzi et al. 2004). Expression of IL-6 is enhanced in various chronic or acute brain disorders, e.g., brain injury, infections, ischemia, and multiple sclerosis, as well as Alzheimer's and Parkinson's diseases (Ali et al. 2000; Baker et al. 2001; Gruol and Nelson 2005). Based on the findings that IL-6 prevents neuronal damage and death, we hypothesized that the enhanced IL-6 expression in brain disorders reflects a compensatory neuroprotection.

Since IL-6 is a pleiotropic cytokine, it exerts neurotrophic and neuroprotective effects, and yet can also function as a mediator of inflammation, demyelination, and astrogliosis, depending on the cellular context (Van Wagoner and Benveniste 1999). Therefore, dosage of IL-6, degree of neuronal damage, type and environment of neurons, and existence of soluble IL-6 receptors can influence IL-6 effects (Toulmond et al. 1992; Thier et al. 1999). In this study, it seems that the higher dosage of IL-6 (120 ng/ml) was more effective in reducing the effects of NMDA on Bcl-2, Bax, and caspase-3 expressions than the lower concentration of IL-6 (40 ng/ml), as the higher dosage of IL-6 caused the NMDA-induced changes in the anti-apoptotic and pro-apoptotic markers to return to the control levels more than the lower

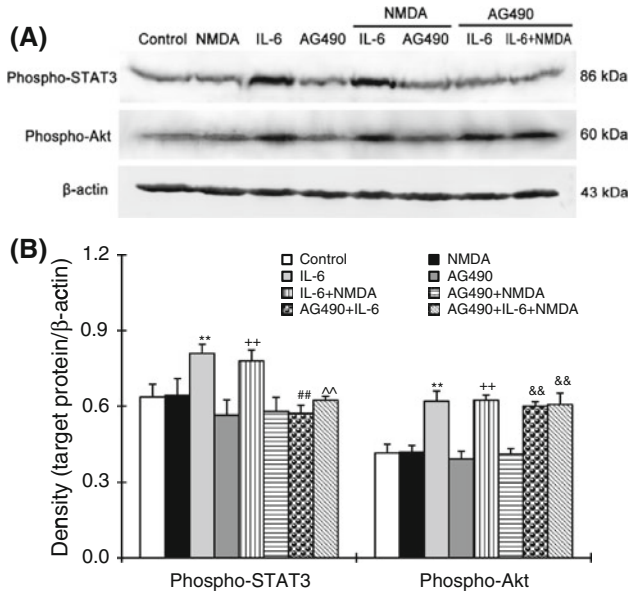

Fig. 6 Action of AG490 on IL-6-induced activation of STAT3 and Akt. CGNs from postnatal 8-day infant rats were chronically co-exposed to AG490 and IL-6 for 8 days and then stimulated with NMDA for 30 min. Total protein in the cells were extracted and subjected to SDS-PAGE. The blots were probed with antibody to phosphorylated STAT3 or to phosphorylated Akt, which reflect the activated levels of the two proteins. The same blots were reprobed with a β-actin antibody. Densitometry was performed to quantify each lane. Values represent mean ± SD of three separate experiments. ** $P < 0.01$, compared with control without any treatment; $^{++}$ $P < 0.01$, compared with NMDA group; $^{\#\#}$ $P < 0.01$, compared with IL-6 group; ^^ $P < 0.01$, compared with IL-6 + NMDA group; $^{\&\&}$ $P < 0.01$, compared with AG490 group

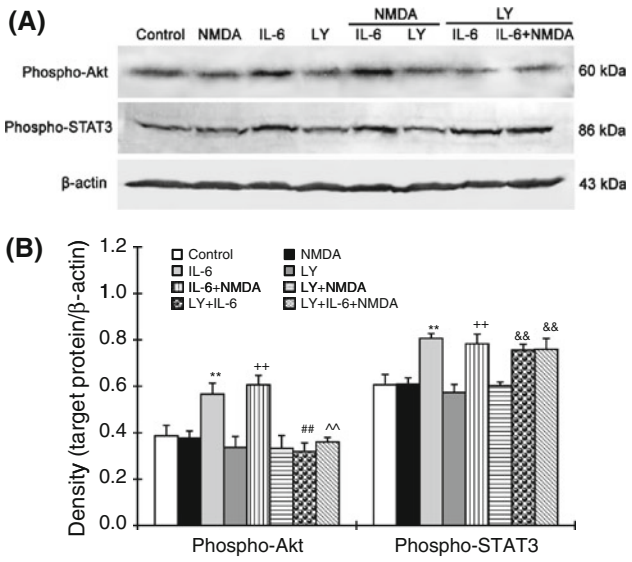

Fig. 7 Action of LY294002 on IL-6-induced activation of Akt and STAT3. Design of the experiment and layout of the diagram are the same as Fig. 6, except that AG490 was replaced by LY294002. LY = LY294002

concentration of IL-6. However, because the higher dosage of IL-6 itself had the much greater influences on the expressions of mRNAs and proteins related to apoptosis than the lower concentration of IL-6, compositive effects were different between the two concentrations of IL-6. As shown in Table 2, the further statistical analysis indicates that the reduction in Bcl-2 mRNA produced by NMDA is significantly less in cultures treated with 40 ng/ml of IL-6 than in control cultures, but the reduction produced by NMDA in cultures treated with 120 ng/ml of IL-6 is not significantly different from the reduction produced by NMDA in control cultures. Therefore, for the Bcl-2 mRNA expression, it is more effective for the lower dose of IL-6 than for its higher dose in reducing the effect of NMDA. Similarly, the phenomenon also occurs in the Bax mRNA and caspase-3 protein expressions. This suggests that the counteractive effects of IL-6 on NMDA-induced changes in Bcl-2, Bax, and caspase-3 expressions lack dose-dependency. This suggestion is supported by our previous work, in which we found that 40 ng/ml of IL-6 was the most effective in counteracting the effect of NMDA-suppressed neuronal vitality between these concentrations of 10, 40, and 160 ng/ml of IL-6 used (Wang et al. 2009). It was like a bell-shaped dose-dependency. As we have known, for any regulatory factors, their effects need an optimal concentration. Beyond the concentration, the effects will decrease because of some

Table 3 Statistical analysis of the differences in levels of STAT3 and Akt phosphorylation between the different treatments

Items	Phospho-STAT3	Phospho-Akt
Difference between control and IL-6	0.173 ± 0.025	0.180 ± 0.036
Difference between AG (for phospho-STAT3)/LY (for phospho-Akt) and AG/LY plus IL-6	0.007 ± 0.029**	0.017 ± 0.023**
Difference between AG/LY plus NMDA and AG/LY plus IL-6 plus NMDA	0.043 ± 0.042*	0.027 ± 0.035**
Difference between IL-6 and AG/LY plus IL-6	0.237 ± 0.035	0.247 ± 0.023
Difference between control and AG/LY	0.070 ± 0.026^{++}	0.050 ± 0.036^{++}
Difference between NMDA and AG/LY plus NMDA	0.063 ± 0.038^{++}	0.043 ± 0.040^{++}

The data are from Figs. 6 and 7

* $P < 0.05$, ** $P < 0.01$, compared with the difference between control and IL-6; $^{++}$ $P < 0.01$, compared with the difference between IL-6 and AG/LY plus IL-6. AG = AG490; LY = LY294002

mechanisms related to negative feedback regulation. This may also explain the phenomenon in this study that 40 ng/ml of IL-6 is more effective than 120 ng/ml of IL-6 in reducing NMDA-induced changes of anti-apoptotic and pro-apoptotic markers. On the other hand, the neuroprotective effect of IL-6 also depends on the degree of damage to the neurons (Peng et al. 2005; Wang et al. 2009). In addition, NMDA infusion into the rat striatum results in a decrease in striatal cholinergic and GABAergic neurons, and co-infusion of IL-6 and NMDA reduces the loss of cholinergic neurons but fails to prevent the loss of GABAergic neurons (Toulmond et al. 1992). The difference of response to IL-6 between different types of neurons explains the distinct effects of IL-6, neuroprotective, or noneffective. Furthermore, soluble IL-6 receptors are necessary to achieve the neurotrophic effect of IL-6. For example, sensory dorsal root ganglion neurons express and secrete bioactive IL-6, but the endogenously secreted IL-6 does not enhance survival of these neurons in vitro, indicating that dorsal root ganglion neurons do not sufficiently express cell surface IL-6 receptors, and therefore the exogenously added soluble IL-6 receptors render these neurons responsive to secreted IL-6 (Thier et al. 1999). Similarly, pretreatment with either IL-6 or soluble IL-6 receptors alone does not bring about any neuroprotective effect, while pretreatment with a combined administration of IL-6 and soluble IL-6 receptors leads to a significant neuroprotective effect against NMDA-induced retinal damage and apoptosis (Inomata et al. 2003). These reported facts explain the importance of the factors, from neuronal interior and exterior, in determining IL-6 effects. CGNs, used in the study, have been reported to express IL-6 and IL-6 receptors (Schöbitz et al. 1993; Gadient and Otten 1994). Thus, our present data that IL-6 protected the cultured CGNs against NMDA-induced apoptosis, on the one hand, support the existence of IL-6 receptors on the CGNs at the functional profile, and on the other hand, demonstrate that the IL-6 neuroprotection is indeed mediated by IL-6 receptors.

In general, signaling by the IL-6 receptors is mediated through the signal transducing subunit gp130 and involves the activation of JAK-STAT, Ras-MAPK, or/and PI3K-Akt pathways. STAT3, a member of STAT family, is continuously expressed in neurons in the brain. Once STAT3 is activated by tyrosine phosphorylation in response to cytokines, including IL-6, it becomes capable of translocating into the nucleus and modulating the expression of target genes (Zhong et al. 1994). IL-6 binding to its receptors induces phosphorylation of the receptor-associated JAKs, and the down-stream STAT family of transcription factors, which amplify the IL-6 signal transduction (Satriotomo et al. 2006). In this study, IL-6 evoked an increase in the phosphorylation of STAT3 in the cultured CGNs. This effect of IL-6 was blocked by AG490, a JAK phosphorylation inhibitor, but not by LY294002, a PI3K inhibitor. Neither AG490 nor NMDA affected the baseline levels of STAT3 phosphorylation. These data not only prove that AG490 is a specific inhibitor for the JAK-STAT3 pathway but also show that CGNs employ JAK-STAT3 signaling pathway to transduce IL-6 regulatory message. The blockage of JAK-STAT3 pathway in CGNs by AG490 reduced the counteractive effects of IL-6 on NMDA-induced up-regulation of Bax and caspase-3 gene and protein expressions. And also the blockage of JAK-STAT3 pathway in CGNs by AG490 prevented the IL-6 protection against the NMDA-increased number of apoptotic cells. AG490 itself did not alter the expressions of Bcl-2, Bax, and cleaved caspase-3 proteins. These findings reveal that the blocking effect of AG490 is attributable to decreased signaling in the JAK-STAT3 pathway, and suggest that the JAK-STAT3 signal-transduction pathway is implicated in the IL-6 neuroprotection against NMDA-induced neuronal apoptosis. In our previous work, blockage of JAK-STAT3 pathway in CGNs by AG490 reversed IL-6 neuroprotection against decreased neuronal vitality and increased intracellular Ca^{2+} levels induced by glutamate or NMDA (Peng et al. 2005; Wang et al. 2009). The present results provide further evidence for the participation of JAK-STAT3 pathway in IL-6 neuroprotective mechanisms. Several studies in vivo support our findings. After middle cerebral artery occlusion, blockage of IL-6 signaling STAT3 leads to an increase in number of apoptotic cells in the peri-infarct area and enlargement of the size of the infarct, and it has adversely affected neurological function (Yamashita et al. 2005). These findings demonstrate that the activation of the signaling pathway of JAK-STAT3 in the brain by IL-6 is important for its neuroprotection from brain injury and diseases. However, AG490 did not completely block the neuroprotective effect of IL-6 against NMDA-induced apoptosis in this study. It implies that other signaling pathways may also be involved in transducing the anti-apoptotic activity of IL-6.

The PI3K-Akt signaling pathway has been reported to play a critical role in mediating survival signals in a wide range of neuronal cell types (Brunet et al. 2001). Phosphorylation of Akt may be involved in determining cell survival or cell death after transient focal cerebral ischemia (Noshita et al. 2001). Inhibition of Akt pathway hastens hypoglossal axotomy-induced neuronal death in the neonate, and the dominant active Akt-overexpressing adult hypoglossal neurons show accelerated axonal regeneration after axotomy (Namikawa et al. 2000). These reports present that PI3K-Akt pathway is associated with neuronal survival or regeneration. However, whether IL-6 uses the signaling pathway to transduce its anti-apoptotic activity in neurons is not well known. In this study, IL-6 increased the phosphorylation of Akt in cultured CGNs, and LY294002

prevented the increased phosphorylation of Akt by IL-6. Since the serine–threonine kinase Akt is a key downstream effector of PI3K, the results demonstrate that PI3K-Akt pathway is also involved in neuronal response to IL-6. Moreover, the fact that AG490 did not block the Akt activation induced by IL-6 proves LY294002 is specific for the blockage of PI3K-Akt pathway. Neither LY294002 nor NMDA affected the baseline levels of Akt phosphorylation. This verifies that the PI3K-Akt pathway is also actively implicated in IL-6 signaling transduction. Importantly, the blockage of PI3K-Akt pathway by LY294002 reduced the counteractive effects of IL-6 on NMDA-induced up-regulation of Bax and caspase-3 at transcriptional and translational levels. Simultaneously, the blockage of PI3K-Akt pathway by LY294002 also abolished the IL-6 prevention of NMDA-induced increase in apoptotic neurons. LY294002 itself did not alter the expressions of Bcl-2, Bax, and cleaved caspase-3 proteins. These findings suggest that PI3K-Akt signaling pathway also participates in mediating the neuroprotective actions of IL-6. In PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla, the signaling pathway for the anti-apoptotic effect of IL-6 is mediated in major part by activation of the PI3K/Akt pathway and thus is different from that in hematopoietic cells (Kunioku et al. 2001). In basal cell carcinoma cells, the up-regulation of the anti-apoptotic Mcl-1 protein by IL-6 is mainly through the PI3K/Akt, but not the STAT3 pathway; however, both the PI3K/Akt and the STAT3 pathways are potentially involved in IL-6-mediated cell survival activity (Jee et al. 2002). These reported facts explain that the cascades mediating IL-6-induced growth, survival, or repair are complicated and vary depending upon the cell type and function. In CGNs, we propose that IL-6 activates both the JAK-STAT3 and PI3K-Akt signal pathways to implement its anti-apoptotic effect. Since the blockage of either the pathway did not fully abolish the IL-6 anti-apoptotic effect, we suggest that a crosstalk between JAK-STAT3 and PI3K-Akt signaling pathways may be required for IL-6 neuroprotection. On the other hand, our recent results that blockage of Ras-MAPK signal pathway in CGNs can block the preventive effect of IL-6 from NMDA-induced decrease in neuronal vitality (Wang et al. 2009) present that other IL-6-mediated signaling pathways besides JAK-STAT3 and PI3K-Akt are also involved in the IL-6 neuroprotection.

Taken together, the current results reveal that IL-6 protects neurons against the down-regulated expression of Bcl-2 mRNA, the up-regulated expressions of Bax and caspase-3, and the increased number of apoptotic neurons induced by NMDA, and therefore suggest that IL-6 is a neuroprotectant against apoptosis. JAK inhibitor AG490 or PI3K inhibitor LY294002 suppresses IL-6-induced activation of downstream signal STAT3 or Akt, respectively, and importantly, either AG490 or LY294002 reduces the anti-apoptotic activity of IL-6. The findings imply that JAK-STAT3 and PI3K-Akt signal-transduction pathways jointly mediate the neuroprotective action of IL-6.

Acknowledgments This study was supported by Grants 2007-5 for Excellent Sci-Tech Innovation Teams in Universities from the Educational Department of Jiangsu Province of China, H200761 from the Health Department of Jiangsu Province of China, 30870819 and 30870929 from the National Natural Science Foundation of China, and K2008006 and K2008019 from the Nantong Applied Research Program of China.

References

Ali C, Nicole O, Docagne F, Lesne S, MacKenzie ET, Nouvelot A, Buisson A, Vivien D (2000) Ischemia-induced interleukin-6 as a potential endogenous neuroprotective cytokine against NMDA receptor-mediated excitotoxicity in the brain. J Cereb Blood Flow Metab 20:956–966

Allen S, Sotos J, Sylte MJ, Czuprynski CJ (2001) Use of Hoechst 33342 staining to detect apoptotic changes in bovine mononuclear phagocytes infected with *Mycobacterium avium* subsp. paratuberculosis. Clin Diagn Lab Immunol 8:460–464

Baker DG, Ekhator NN, Kasckow JW, Hill KK, Zoumakis E, Dashevsky BA, Chrousos GP Jr, Geracioti TD (2001) Plasma and cerebrospinal fluid interleukin-6 concentrations in posttraumatic stress disorder. Neuroimmunomodulation 9:209–217

Baranzini SE, Elfstrom C, Chang SY, Butunoi C, Murray R, Higuchi R, Oksenberg JR (2000) Transcriptional analysis of multiple sclerosis brain lesions reveals a complex pattern of cytokine expression. J Immunol 165:6576–6582

Bredesen DE, Rao RV, Mehlen P (2006) Cell death in the nervous system. Nature 443:796–802

Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11:297–305

Chao DT, Korsmeyer SJ (1998) BCL-2 family: regulators of cell death. Annu Rev Immunol 16:395–419

Conroy SM, Nguyen V, Quina LA, Blakely-Gonzales P, Ur C, Netzeband JG, Prieto AL, Gruol DL (2004) Interleukin-6 produces neuronal loss in developing cerebellar granule neuron cultures. J Neuroimmunol 155:43–54

Culmsee C, Plesnila N (2006) Targeting Bid to prevent programmed cell death in neurons. Biochem Soc Trans 34:1334–1340

Fukada T, Hibi M, Yamanaka Y, Takahashi-Tezuka M, Fujitani Y, Yamaguchi T, Nakajima K, Hirano T (1996) Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. Immunity 5:449–460

Gadient RA, Otten U (1993) Differential expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) mRNAs in rat hypothalamus. Neurosci Lett 153:13–16

Gadient RA, Otten U (1994) Identification of interleukin-6 (IL-6)-expressing neurons in the cerebellum and hippocampus of normal adult rats. Neurosci Lett 182:243–246

Gruol DL, Nelson TE (2005) Purkinje neuron physiology is altered by the inflammatory factor interleukin-6. Cerebellum 4:198–205

Gschwind M, Huber G (1995) Apoptotic cell death induced by beta-amyloid 1–42 peptide is cell type dependent. J Neurochem 65:292–300

Herrmann O, Tarabin V, Suzuki S, Attigah N, Coserea I, Schneider A, Vogel J, Prinz S, Schwab S, Monyer H, Brombacher F,

Schwaninger M (2003) Regulation of body temperature and neuroprotection by endogenous interleukin-6 in cerebral ischemia. J Cereb Blood Flow Metab 23:406–415

Inomata Y, Hirata A, Yonemura N, Koga T, Kido N, Tanihara H (2003) Neuroprotective effects of interleukin-6 on NMDA-induced rat retinal damage. Biochem Biophys Res Commun 302:226–232

Jee SH, Chiu HC, Tsai TF, Tsai WL, Liao YH, Chu CY, Kuo ML (2002) The phosphotidyl inositol 3-kinase/Akt signal pathway is involved in interleukin-6-mediated Mcl-1 upregulation and anti-apoptosis activity in basal cell carcinoma cells. J Invest Dermatol 119:1121–1127

Kamimura D, Ishihara K, Hirano T (2003) IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev Physiol Biochem Pharmacol 149:1–38

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ, Williams LT (1997) Caspase 3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science 278:294–298

Kunioku H, Inoue K, Tomida M (2001) Interleukin-6 protects rat PC12 cells from serum deprivation or chemotherapeutic agents through the phosphatidylinositol 3-kinase and STAT3 pathways. Neurosci Lett 309:13–16

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using Real-Time quantitative PCR and the $2^{-\triangle\triangle Ct}$ method. Methods 25:402–408

Lizard G, Fournel S, Genestier L, Dhedin N, Chaput C, Flacher M, Mutin M, Panaye G, Revillard JP (1995) Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. Cytometry 21:275–283

Loddick SA, Turnbull AV, Rothwell NJ (1998) Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. J Cereb Blood Flow Metab 18:176–179

Lutticken C, Wegenka UM, Yuan J (1994) Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. Science 263:89–92

Muñoz-Fernández MA, Fresno M (1998) The role of tumour necrosis factor, interleukin 6, interferon-gamma and inducible nitric oxide synthase in the development and pathology of the nervous system. Prog Neurobiol 56:307–340

Murakami M, Hibi M, Nakagawa N (1993) IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science 260:1808–1810

Namikawa K, Honma M, Abe K, Takeda M, Mansur K, Obata T, Miwa A, Okado H, Kiyama H (2000) Akt/protein kinase B prevents injury-induced motoneuron death and accelerates axonal regeneration. J Neurosci 20:2875–2886

Narazaki M, Witthuhn BA, Yoshida K (1994) Activation of JAK2 kinase mediated by the interleukin-6 signal transducer gp130. Proc Natl Acad Sci USA 91:2285–2289

Noshita N, Lewén A, Sugawara T, Chan PH (2001) Evidence of phosphorylation of Akt and neuronal survival after transient focal cerebral ischemia in mice. J Cereb Blood Flow Metab 21:1442–1450

Orellana DI, Quintanilla RA, Gonzalez-Billault C, Maccioni RB (2005) Role of the JAKs/STATs pathway in the intracellular calcium changes induced by interleukin-6 in hippocampal neurons. Neurotox Res 8:295–304

Peng YP, Qiu YH, Lu JH, Wang JJ (2005) Interleukin-6 protects cultured cerebellar granule neurons against glutamate-induced neurotoxicity. Neurosci Lett 374:192–196

Pizzi M, Sarnico I, Boroni F, Benarese M, Dreano M, Garotta G, Valerio A, Spano P (2004) Prevention of neuron and oligodendrocyte degeneration by interleukin-6 (IL-6) and IL-6 receptor/IL-6 fusion protein in organotypic hippocampal slices. Mol Cell Neurosci 25:301–311

Qiu Z, Gruol DL (2003) Interleukin-6, β-amyloid peptide and NMDA interactions in rat cortical neurons. J Neuroimmunol 139:51–57

Qiu Z, Parsons KL, Gruol DL (1995) Interleukin-6 selectively enhances the intracellular calcium response to NMDA in developing CNS neurons. J Neurosci 15:6688–6699

Qiu Z, Sweeney DD, Netzeband JG, Gruol DL (1998) Chronic interleukin-6 alters NMDA receptor-mediated membrane responses and enhances neurotoxicity in developing CNS neurons. J Neurosci 18:10445–10456

Sadowski HB, Shuai K, Darnell JE Jr, Gilman MZ (1993) A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science 261:1739–1744

Satriotomo I, Bowen KK, Vemuganti R (2006) JAK2 and STAT3 activation contributes to neuronal damage following transient focal cerebral ischemia. J Neurochem 98:1353–1368

Schöbitz B, de Kloet ER, Sutanto W, Holsboer F (1993) Cellular localization of interleukin 6 mRNA and interleukin 6 receptor mRNA in rat brain. Eur J Neurosci 5:1426–1435

Taga T, Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. Annu Rev Immunol 15:797–819

Thier M, März P, Otten U, Weis J, Rose-John S (1999) Interleukin-6 (IL-6) and its soluble receptor support survival of sensory neurons. J Neurosci Res 55:411–422

Toulmond S, Vige X, Fage D, Benavides J (1992) Local infusion of interleukin-6 attenuates the neurotoxic effects of NMDA on rat striatal cholinergic neurons. Neurosci Lett 144:49–52

Van Wagoner NJ, Benveniste EN (1999) Interleukin-6 expression and regulation in astrocytes. J Neuroimmunol 100:124–139

Wang XQ, Peng YP, Lua JH, Cao BB, Qiu YH (2009) Neuroprotection of interleukin-6 against NMDA attack and its signal transduction by JAK and MAPK. Neurosci Lett 450:122–126

Yamashita T, Sawamoto K, Suzuki S, Suzuki N, Adachi K, Kawase T, Mihara M, Ohsugi Y, Abe K, Okano H (2005) Blockade of interleukin-6 signaling aggravates ischemic cerebral damage in mice: possible involvement of STAT3 activation in the protection of neurons. J Neurochem 94:459–468

Yuan J, Horvitz HR (2004) A first insight into the molecular mechanisms of apoptosis. Cell 116:53–56

Zhong Z, Wen Z, Darnell JE Jr (1994) STAT3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 264:95–98