Neurotoxicity Research, 2005, VOL. 8(3,4). pp. 295-304

F.P. Graham Publishing Co.

Role of the JAKs/STATs Pathway in the Intracellular Calcium Changes Induced by Interleukin-6 in Hippocampal Neurons

DANIEL I. ORELLANA[a,†], RODRIGO A. QUINTANILLA[a,†], CHRISTIAN GONZALEZ-BILLAULT[a] and RICARDO B. MACCIONI[a,b,*]

[a]*Laboratory of Cellular, Molecular Biology and Neurosciences, Millennium Institute for Advanced Studies in Cell Biology and Biotechnology (CBB), Department of Biology, Faculty of Sciences;* [b]*Department of Neurological Sciences, Faculty of Medicine, Universidad de Chile, Las Encinas 3370, Ñuñoa, Santiago, Chile. rmaccion@uchile.cl*

(Received 10 May 2005; Revised 12 June 2005; In final form 04 August 2005)

Recent studies show that inflammation has an active role in the onset of neurodegenerative diseases. It is known that in response to extracellular insults microglia and/or astrocytes produce inflammatory agents. These contribute to the neuropathological events in the aging process and neuronal degeneration. Interleukin-6 (IL-6) has been involved in the pathogenesis of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. Here, we show that IL-6 treatment of rat hippocampal neurons increases the calcium influx via NMDA-receptor, an effect that is prevented by the specific NMDA receptor antagonist MK-801 (dizocilpine). We also show that this calcium influx is mediated by the JAKs/STATs pathway, since the inhibitor of JAKs/STATs pathway, JAK 3 inhibitor, blocks calcium influx even in the presence of IL-6. This increase in calcium signal was dependent on external sources, since this signal was not observed in the presence of EGTA. Additional studies indicate that the increase in cytosolic calcium induces tau protein hyperphosphorylation, as revealed by using specific antibodies against Alzheimer phosphoepitopes. This anomalous tau hyperphosphorylation was dependent on both the JAKs/STATs pathway and NMDA receptor. These results suggest that IL-6 would induce a cascade of molecular events that produce a calcium influx through NMDA receptors, mediated by the JAKs/STATs pathway, which subsequently modifies the tau hyperphosphorylation patterns.

Keywords: Interleukin-6; Alzheimer's disease; Tau protein; Calcium; NMDA receptor

Abbreviations
Aß, Amyloid beta peptide
AD, Alzheimer's disease
IL-6, interleukin-6
NMDA, *N*-methyl-D-aspartate

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine member of the family of the neuropoietic cytokines, which display a wide variety of biological effects in many tissues and cell types (Kishimoto *et al*., 1992). Under physiological conditions, cellular sources for IL-6 are widely distributed in the nervous system, and its expression is developmentally regulated (Ershler and Keller, 2000). In the peripheral nervous system IL-6 and IL-6 receptor (IL-6R) transcripts are expressed in sympathetic and sensory ganglia of adult rats (Gadient and Otten, 1996), as in the rat adrenal medulla (Gadient and Otten, 1995). In the central nervous system, IL-6 and IL-6R are expressed in neurons and astrocytes from various brain areas, such as the hippocampus, neocortex and the cerebellum (Gadient *et al*., 1995; Gadient and Otten, 1997). It is known that the effects of the cytokines are mainly transduced by a signaling pathway involving JAKs/STATs activation (Jones *et al*., 2001), and by the MAPK-dependent cascades (Gadient and Otten, 1997; Jones *et al*., 2001). IL-6 preferentially activates STAT3 (Sengupta *et al*., 1996), while the calcium ionophore ionomycin was able of to inhibit the IL-6 activation of JAKs/STATs (Sengupta *et al*., 1996). After using specific inhibitors, for MAPKs, the inhibition by ionomycin was reversed (Sengupta *et al*., 1998).

*Corresponding author. Tel.: 562-678 7224; FAX: 562-271 2983; E-mail: rmaccion@uchile.cl; †Contributed equally
ISSN 1029 8428 print/ ISSN 1476-3524 online.

IL-6 has been involved in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease (AD) (Bauer *et al.*, 1991; Campbell *et al.*, 1993), Parkinson's disease (Blum-Degen *et al.*, 1995), multiple sclerosis (Frei *et al.*, 1991), and HIV encephalopathy (Merrill and Chen, 1991). Animal models of neurodegenerative diseases have evidenced elevated levels of IL-6 (Gijbels *et al.*, 1990; Grau *et al.*, 1990; Minami *et al.*, 1991). Furthermore, transgenic mice that chronically overexpress IL-6 in the CNS showed prominent neurodegeneration (Campbell *et al.*, 1993). In AD not only IL-6 but also other cytokines, mainly IL-1β and TNF-α, are secreted by microglial cells, astrocytes and/or neuronal cells. These cytokines can induce synthesis of certain acute-phase proteins including the amyloid precursor protein (APP) (Del Bo *et al.*, 1995; Blasko *et al.*, 2000). On the other hand, APP or the amyloid-β peptide (Aβ-peptide) can induce the expression of IL-1β , TNF-α, and IL-6 in astrocytes and microglial cells in culture (Gitter *et al.*, 1995; Chong, 1997; Mehlhorn *et al.*, 2000; Apelt *et al.*, 2001; Toro *et al.*, 2001).

We have shown that one of the main protein kinases involved in tau hyperphosphorylation in neurodegenerative diseases is the cdk5/p35 complex (Alvarez *et al.*, 1999; 2001). Also, we have shown that both Aβ-peptide and IL-6 treatment of rat hippocampal cell cultures induced an increase in the cdk5 activity, which appears to be responsible for an Alzheimer´s-type phosphorylation of tau protein (Alvarez *et al.*, 1999; Quintanilla *et al.*, 2004). Moreover, both Aβ-peptide and IL-6 have been reported to modify the intracellular calcium levels on neurons (Mattson *et al.*, 1992; Holliday *et al.*, 1995; Qiu *et al.*, 1995; 1998). Aß-peptide treatment of human cortical neurons and rat hippocampal cell cultures induced an increase in the intracellular calcium levels (Mattson *et al.*, 1992; 1993). Chronic IL-6 treatment of culture rat granule neurons enhanced the calcium response to glutamate stimulation (Holliday *et al.*, 1995), and significantly enhanced the response to NMDA (Qiu *et al.*, 1995; 1998). Reducing extracellular calcium and depleting intracellular stores, decreased the amplitude of the response to glutamate and NMDA in chronic IL-6 treated granule neurons (Qiu *et al.*, 1995; 1998).

In the current study, we analyze the molecular events involved in the increase of intracellular calcium influx induced by IL-6 through NMDA receptors. This calcium influx was mediated by JAKs/STATs pathway activation. Moreover, the effect of the cytosolic calcium in the hyperphosphorylation of tau protein was observed in hippocampal cell cultures treated with IL-6.

MATERIALS AND METHODS

Reagents

Human recombinant IL-6 (Calbiochem, La Jolla) was dissolved in physiological saline at a concentration of 100,000 U/ml, as stock solution and stored at -20ºC. MK-801 maleate (dizocilpine) (Calbiochem, USA), a non-competitive NMDA receptor antagonist, was prepared in sterile water and used at a final concentration of 10 μM. JAK3 inhibitor (Calbiochem), a specific inhibitor of the JAKs/STATs pathway, was prepared in dimethyl sulfoxide (DMSO), and used at a final concentration of 30 μM. BAPTA-AM (Calbiochem) was used at a final concentration of 25 μM, and EGTA (Calbiochem) to a final concentration of 2 mM. Minimum essential medium (MEM), Neurobasal media and N2-supplement (Gibco BRL) were used on primary cultures.

Primary Cultures of Hippocampal Neurons

Primary rat hippocampal neuronal cultures were established from the hippocampus of 18-day-old Sprague-Dawley rat fetuses according to methods described previously (Banker and Cowan, 1977). Briefly, brains were removed and freed from meninges, and the hippocampus was dissected in a Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBBSS) Cells were then dispersed by incubation for 10 min at 37ºC in a 0.5% trypsin solution. Cells were then rinsed 3 times in HBBSS and mechanically dissociated by a fire-polished Pasteur pipette in MEM supplemented with 4.4 mM sodium-bicarbonate plus 10% heat-inactivated horse serum (MEM-10). Cells were initially plated in MEM-10 media and maintained at 37ºC in a humid atmosphere with 5% CO_2/95% air. One hour after plating, media MEM-10 was removed and replaced with serum-free Neurobasal media supplemented with N2. Neurons were seeded onto 0.1% polylysine-coated 35-mm plastic Petri dishes (10^6 cell/35 mm culture dish) for biochemical experiments. IL-6 at a concentration of 5 ng/ml (500 U/ml) was added to hippocampal neurons cultured for 4-days *in vitro* (4-DIV), which continued in culture for an additional 48 h. Sister cultures that did not receive IL-6 treatment were used as controls.

Primary Antibodies

The following primary antibodies were used in this study: AT8, monoclonal antibody (mAb) which recognizes phosphorylated tau epitopes Ser^{202} and Thr^{205} (clone AT8; Innogenetics, Belgium); Tau-1, mAb which recognizes the same unphosphorylated tau epitopes;

PHF1, mAb which recognizes phosphorylated tau epitopes Ser^{396} and Thr^{404}; and Tau5 mAb which recognizes an independent phosphorylation tau epitope.

Immunofluorescence Studies

Hippocampal cell cultures were fixed in 2% paraformaldehyde for 10 min at 37ºC. Subsequently, fixation samples were permeabilized with 0.2% Triton X-100 at room temperature for 2 min. Samples were then washed three times in PBS and blocked with 5% BSA for 1 h at room temperature. Primary antibodies were diluted in 1% BSA, 0.1% Triton X-100, and incubated in a humid chamber overnight at 4ºC. After washing three times with PBS, preparations were incubated with fluorescein-conjugated secondary antibody (Sigma, St. Louis, MO) for 1 h at room temperature. Finally, samples were washed with PBS and mounted with Prolong mounting media (Molecular Probes, Eugene, OR, USA). Images were acquired with a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany) of the Millennium Institute CBB.

Immunodetection Assays

The immunological reactivity of hippocampal cell extracts treated with cytokines and the untreated controls, was assayed by Western blot techniques using antibodies that recognize Alzheimer's phosphoepitopes on tau (AT8, PHF-1), and with the antibody Tau5. The neuronal extracts from IL-6-treated neurons and controls were prepared according with the following steps: The cells were washed with cold PBS solution for two-times and scraped with a rubber policeman. Cells were homogenized and the extract was dissolved in buffer RIPA plus protease inhibitors leupeptin (2 µg/ml), pepstatin (2 µg/ml), aprotinin (1 µg/ml) and PMSF (50 µg/ml). The samples were quantified by the Bradford protein assay using bovine serum albumin as a standard (Bio-Rad), then dissolved in Laemmli SDS-sample buffer, heat-denatured, reduced by addition of 5% β-mercaptoethanol, and run on 10% SDS-PAGE mini-gels. The electrophoresis-separated proteins were then transferred by electroblotting onto nitrocellulose filters for 1 h at 100 V. Protein loading for all the lanes in nitrocellulose was determined by staining the membranes with the reversible dye Ponceau S. After blocking non-specific sites on the membrane by incubation with 5% low-fat milk, expression of hyperphosphorylated proteins was determined by immunoblots using AT8, PHF1 and Tau5 as anti-tau primary antibodies by ECL detection (Amersham). Quantification of blots was carried out by scanning the photographic films of nitrocellulose membranes by using the Kodak digital Science densitometry program from Kodak. Statistical analyses were done by Sigma Plot software Jandel.

Calcium Measurements

Cells grown on glass coverslips were loaded for 30 min (37ºC) with 5 µM Fluo-3 in its acetoxymethyl ester form (Fluo-3 AM) in KRH-glucose (Krebs Ringer Hepes) containing 0.02% pluronic acid. Then, coverslips were washed three times and left in KRH-glucose for 10 min until cell fluorescence had reach plateau. Fluorescence was imaged with a LSM 510 META Zeiss confocal microscope according with methods described previously (Quintanilla *et al.*, 2000). Background was measured in parts of the field devoid of cells and found to be not significantly different from the signal recorded in cells depleted of dye with 100 µM digitonin. This value was subtracted from the cell measurements. The maximal Fluo-3 cell fluorescence was recorded in the presence of 2 µM ionomycin in KRH-glucose. Greater detail has been described previously (Quintanilla *et al.*, 2000). The fluorescence intensity variation was recorded from a set of 15-20 neurons as an average per experiment. Statistical analyses were done by Sigma Plot software Jandel.

RESULTS

IL-6 Induces a Cytoplasmic Calcium Increase in Hippocampal Neurons.

Hippocampal neurons after 4-days *in vitro* (4-DIV) were loaded with Fluo-3 AM (5 µM) to register the intracellular fluorescence changes in a confocal microscope. The intensity of the fluorescence change is representative of variations in the cytoplasmic calcium levels in hippocampal neurons (La Ferla, 2002). IL-6 induced a significant increase of cytoplasmic calcium levels in hippocampal neurons, as observed in the recordings of the calcium response (FIG. 1A). The shape of the response was rapidly enhanced after IL-6 addition (arrow in Fig. 1A). After reaching peak fluorescence amplitude, the entry of calcium was regulated by the neurons until it attained the original baseline level (FIG. 1A). In control neurons, no mayor calcium changes were observed. The fluorescence levels in the neurons exposed to IL-6 increased by 100% above the levels observed in the IL-6-untreated neurons (FIG. 1A, right plot). The increase in the cytosolic calcium was significant ($p<0.05$) as observed from data obtained from 6 culture experiments. Our data are in agreements with previous studies that registered a

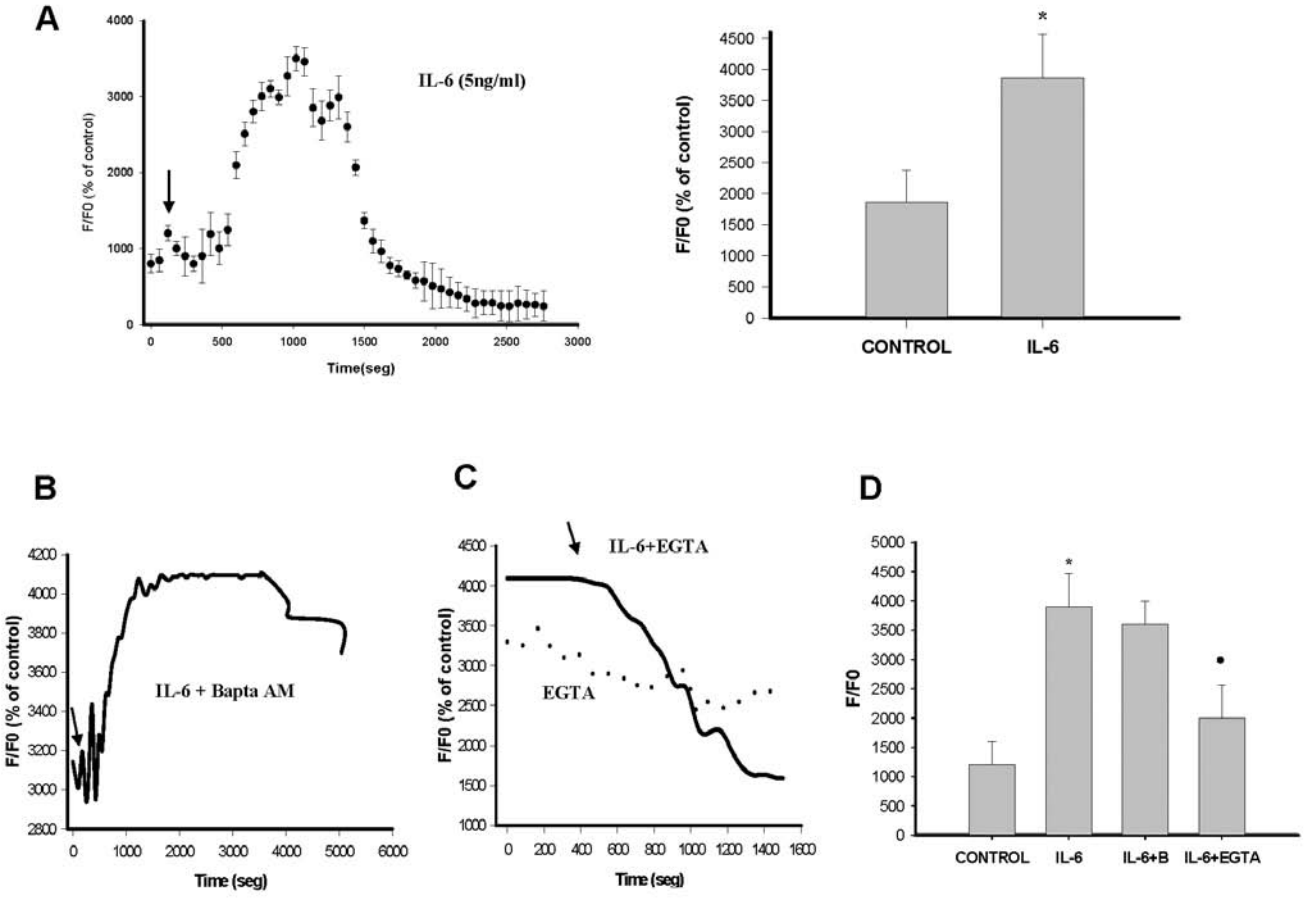

FIGURE 1 IL-6 induces a cytoplasmic calcium increase, which is dependent on an external source in hippocampal neurons. The cytoplasmic calcium level was evaluated in hippocampal neurons (at 4-DIV) loaded with Fluo3 AM. The fluorescence variations were detected by confocal microscopy. (**A**) Representative recordings (n=6) of calcium responses produced by addition of IL-6 (5 ng/ml) (applied at the arrow) to hippocampal neurons. Means ± SEM values for the peak amplitude of the intracellular calcium signals are showed in the plot at the right. (**B**) Representative recordings (n=4) of calcium responses produced by addition of IL-6 (5 ng/ml) (applied at the arrow) in the presence of BAPTA-AM (25 μM), an intracellular calcium chelator. (**C**) Representative recordings (n=4) of calcium responses produced by addition of IL-6 (5 ng/ml) alone (dotted line) and IL-6 (5 ng/ml) plus EGTA (2 mM) (solid line), an extracellular calcium chelator. (**D**) Figure 1D shows a graph with the mean ± SEM values (n=4) for peak amplitude of the intracellular calcium signals in control, IL-6, IL-6 plus BAPTA-AM (IL-6+B) and IL-6 plus EGTA (IL-6+EGTA) Asterisk (*) indicates significant differences (p<0.005) between control and IL-6. Solid circle indicates a significant differences (p<0.005) between IL-6 addition alone and IL-6+EGTA.

cytosolic calcium increase in cerebellar neurons using the same confocal fluorescence techniques (Holliday *et al.*, 1995).

IL-6 Calcium-mediated Influx is Dependent of an External Calcium Source.

To determinate the source of the cytoplasmic calcium influx, we carried out the same experiments described above, but in the presence of BAPTA-AM (25 μM), an intracellular calcium chelator, and in the presence of EGTA (2 mM), an extracellular calcium chelator. Hippocampal neurons were incubated 30 min with BAPTA-AM before IL-6 addition (5 ng/ml) (FIG. 1B, arrow). IL-6 caused an enhancement in the calcium influx. This calcium influx would be at least partially due to a release of calcium from intracellular stores or from extracellular calcium sources. The necessary time to reach the peak of fluorescence was not affected and it was similar to fig 1A. The oscillations in the shape of fluorescence signal at the beginning of the recording were due to IL-6 addition with pipette; therefore, these must be disregarded (FIG. 1B, arrow). The fluorescence change data were representatives of 4 culture sets.

The effect of EGTA was also analyzed. The increase in the cytoplasmic calcium levels due to IL-6 was inhibited in the presence of 2 mM of EGTA (FIG. 1C, solid line). As a control, EGTA was applied in the absence of IL-6, and it did not induce any significant variation on

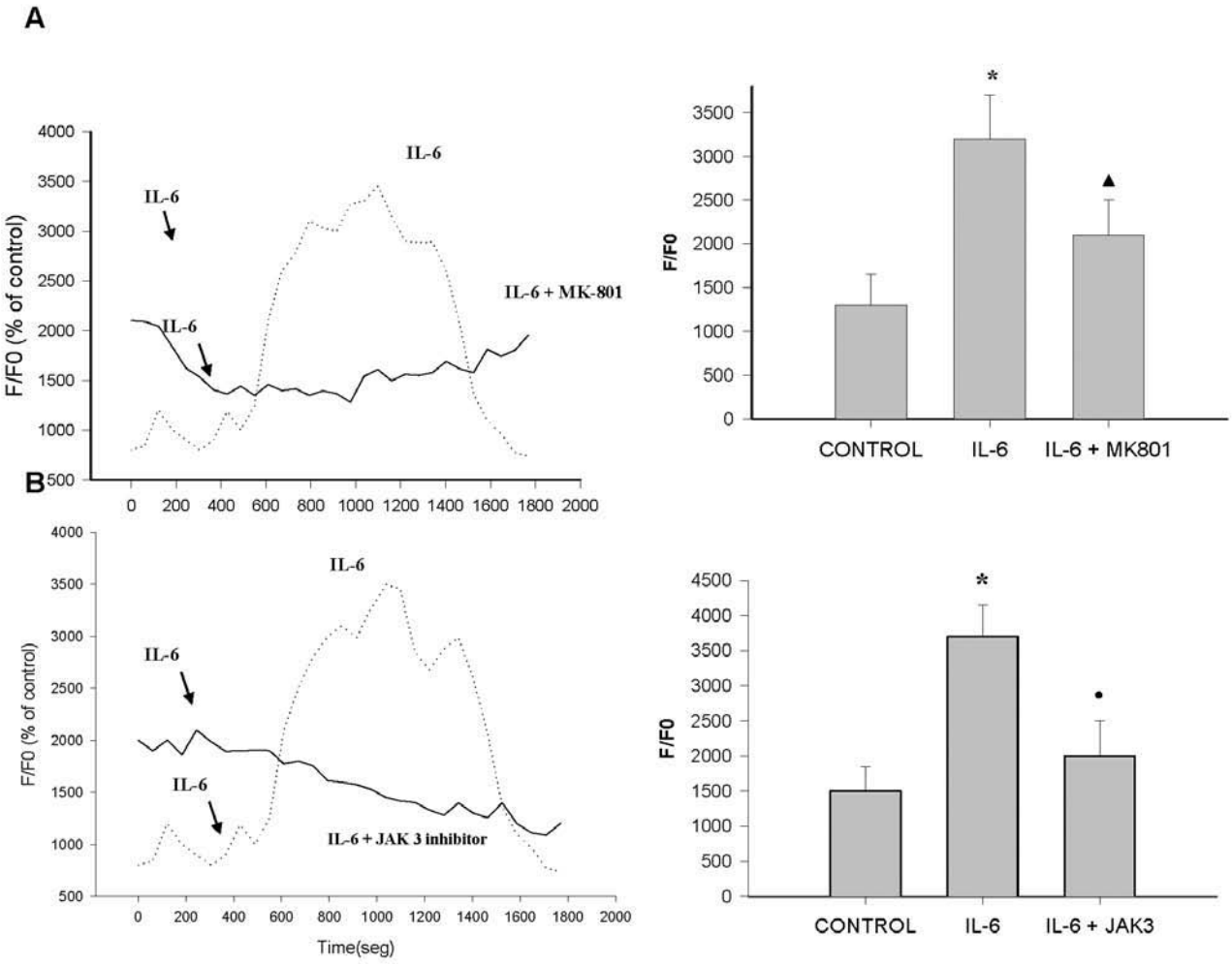

FIGURE 2 Signal transductions involved in the cytoplasmic calcium influx induced by IL-6 treatment on hippocampal neurons. Hippocampal neurons (4- DIV) were loaded with Fluo3 AM, and the fluorescence variations were detected by confocal microscopy. (**A**) Representative recordings (n=4) of calcium responses produced by addition of IL-6 (5 ng/ml) (applied at the arrow) to hippocampal neurons (dotted line), and IL-6 addition in the presence of 10 μM of MK-801 (IL-6 + MK-801) to hippocampal neurons (solid line). Means ± SEM values for the peak amplitude of the intracellular calcium signals are shown in the plot at the right. (**B**) Representative recordings (n=5) of calcium responses produced by IL-6 addition (5 ng/ml) to hippocampal neurons (dotted line) and IL-6 addition in presence of 30 μM of inhibitor of JAKs/STATs pathway JAK 3 (IL-6 + JAK 3) to hippocampal neurons (solid line). Means ± SEM values for the peak amplitude of the intracellular calcium signals are shown in the plot at the right. Asterisk (*) indicates significant differences (p<0.005) between control and IL-6. Solid circle (") indicates significant differences (p<0.005) between IL-6 addition alone and IL-6+JAK3 inhibitor III. Triangle (Δ) indicates significant differences (p<0.005) between IL-6 addition alone and IL-6 + MK-801.

calcium signals on hippocampal neurons (FIG. 1C, dotted line). The shape and duration of the response was relatively unaffected. Reducing extracellular calcium sources with EGTA, significantly decreased the amplitude of the IL-6 induced calcium influx. Moreover, the incubation with BAPTA-AM, before IL-6 addition, did not influence the effects of IL-6 on the increase of intracellular calcium. BAPTA-AM was apparently not capable of complexing all the calcium that entered to the cells from extracellular sources, as induced by IL-6. Therefore, altogether these results suggest the participation of an external calcium source in the calcium influx mediated by IL-6.

These results are in agreement with previous experiments, in which chronic IL-6 treatment produces an enhancement on the calcium signal in response to NMDA. This increase in calcium influx was through voltage-sensitive calcium channels (VSCCs) (Qiu *et al.*, 1998). FIG. 1D shows a summary plot of the cytoplasmic calcium behavior observed in each condition. Data are representatives of the quantification of 4 independent experiments. Non-significant differences were observed between IL-6 and IL-6 + BAPTA-AM. However, the fluorescence levels in the neurons exposed to IL-6 + EGTA decreased significantly, 50% below the levels observed in IL-6-treated neurons without EGTA (FIG. 1D).

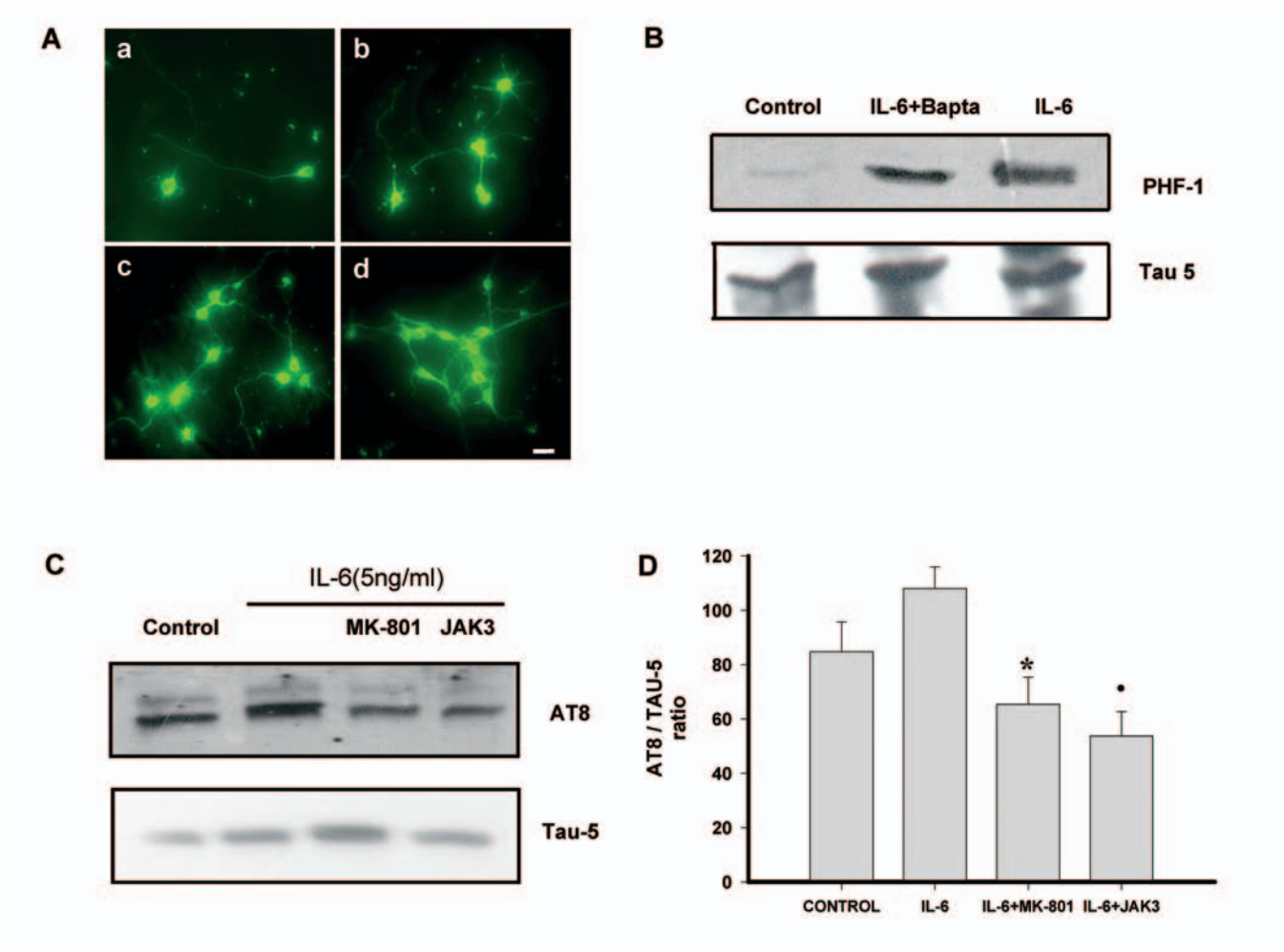

FIGURE 3 Role of cytosolic calcium and JAKs/STATs pathway in the tau hyperphosphorylation induced by IL-6 in hippocampal neurons. (**A**) Immunofluorescence of hippocampal neurons treated and untreated with IL-6 (5 ng/ml), showing the variations on tau hyperphosphorylation. **A, panel a**: Hippocampal neurons in untreated controls, stained with AT8 antibody (1:200); **A, panel b**: untreated control neurons, stained with Tau1 antibody (1:200); **A, panel c**: IL-6 treated neurons (48 h), stained with AT8 antibody; **A, panel d**: IL-6 treated neurons (48 h), stained with TAU-1 antibody (Bar=50 μm). Clearly, IL-6 induces an increase in the phosphorylated forms of tau protein recognized by AT8 antibody and a decrease in the unphosphorylated forms of tau protein recognized by Tau 1 antibody. (**B**) Western blots (n=3) of hippocampal neurons treated with IL-6 (48 h) and treated with IL-6 plus 25 μM of BAPTA-AM (48 h). Western blot to PHF-1 and Tau5 are shown. (**C**) Representative Western blot of hippocampal neurons treated with IL-6 (48 h) and treated with IL-6 plus MK-801 or JAK 3 inhibitor. Western blot to AT8 and Tau 5 are show. (**D**) Means ± SEM values for a representative summary plot of AT8/Tau5 ratios from three independent experiments.

Cytosolic Calcium Increase Induced by IL-6 is Through NMDA Receptor and it is Dependent on the JAKs/STATs Pathway.

It has been reported that IL-6 alters neuronal calcium homeostasis associated with NMDA receptors (Holliday *et al.*, 1995; Qiu *et al.*, 1995; 1998) Moreover, it has been documented that the signaling transduction pathway used by many cytokines to carry their function on cells is mainly transduced by the Janus kinase-signal transducer and activator of the transcription (JAKs/STATs) signaling pathway (Sengupta *et al.*, 1996; 1998; Jones *et al.*, 2001; Sriram *et al.*, 2004). It is known that IL-6 preferentially activates STAT3 (Sengupta *et al.*, 1996). On the other hand, we have demonstrated that IL-6 increases phosphorylated STAT3 and ERK1/2 levels in hippocampal neurons (Quintanilla *et al.*, 2004).

In this way, in order to elucidate the signal transduction involved in the cytosolic calcium increase mediated by IL-6, we decided to investigate the potential role of the NMDA receptor in the cytosolic calcium increase mediated by IL-6. For this purpose we carried out experiments in the presence of MK-801, a specific and non-competitive NMDA receptor antagonist (Wong *et al.*, 1986). The incubation of hippocampal neurons with 10 μM MK-801 induced a significant decrease in the fluorescence levels in neurons treated with IL-6 (FIG. 2A, solid line). IL-6 treatment in the absence of MK-801, produced the same shape and

duration in the fluorescence observed in our previously recording (FIG. 2A, dotted line). These observations are representative of 4 experiments. Data are plotted at the right of figure 2A. Altogether, these results indicated that NMDA receptor activation was involved in the cytosolic calcium influx induced by IL-6 in hippocampal neurons.

In order to evaluate the possible effect of the JAKs/STATs pathway on the cytosolic calcium levels induced by IL-6 treatment, we performed experiments in hippocampal neurons incubated with 30 μM of the JAK 3 inhibitor, a specific inhibitor of JAKs/STATs pathway (Sudbeck *et al.*, 1999). The incubation with the inhibitor of JAKs/STATs prevented the calcium influx in neurons exposed to IL-6. It can be observed in the representative recordings of calcium fluorescence (FIG. 2B, solid line). The fluorescence levels in the neurons exposed to JAK 3 inhibitor decreased by 40% below levels observed in IL-6-treated neurons (FIG. 2B, right plot). The findings are representative of 5 experiments. In these experiments, the quantifications were made by recording the fluorescence changes, on average, in approximately 10 neurons. Altogether, these results suggest that the JAKs/STATs signaling pathway transduced part of the effects produced by IL-6 in this neuronal system. These results are in agreement with previous results that reported that IL-6 effects were abolished in the presence of inhibitors of the JAKs/STATs pathway, such as AG-490 and JAK 3 inhibitor (Jee *et al.*, 2004; Quintanilla *et al.*, 2004).

Role of the Cytosolic Calcium Increases in the IL-6 Dependent Hyperphosphorylation of Tau

We have shown previously that IL-6 induces tau hyperphosphorylation in hippocampal neurons, by deregulating the cdk5/p35 complex (Quintanilla *et al.*, 2004). In these experiments, IL-6 increased the tau hyperphosphorylation detected by the AT8 antibody that recognized the phosphorylation in Alzheimer's epitopes (Quintanilla *et al.*, 2004). These results were reconfirmed by using immunofluorescence techniques (FIG. 3A). Untreated hippocampal neurons are show in figure 3A (panel a), as stained with AT8 antibody; and figure 3A (panel b), as stained with Tau1 antibody. The treatment of hippocampal neurons during 48 hours with IL-6 (5 ng/ml) exhibited enhanced levels of hyperphosphorylated tau after detection with AT8 antibody (FIG. 3A, panel c). The fluorescence label patterns were quite homogeneous, concentrating tau hyperphosphorylation in the neuronal cell body (FIG. 3A, panel c). Consistent with these findings, a decrease in the non-phosphorylated tau levels, recognized with Tau1 antibody that tag unphosphorylated epitopes, was observed in hippocampal cells treated with IL-6 (FIG. 3A, panel d).

In order to evaluate the effect of the cytosolic calcium influx induced by IL-6 in the patterns of phosphorylation of tau protein, we analyzed the effect of BAPTA-AM. Hippocampal neurons were incubated during 48 hours with IL-6 in the presence of BAPTA-AM (25 μM), and the phosphorylation of tau protein was analyzed by Western blot. The incubation with IL-6 plus BAPTA-AM decreases partially the tau hyperphosphorylation levels induced by IL-6 (FIG. 3B, second lane), detected by PHF-1 antibody as compared with IL-6 treated neurons without the calcium chelator (third lane). Tau5 antibody recognizes conformational epitopes on total tau protein in a phosphorylation-independent way. Therefore, the variations in the relative abundance of phosphorylated tau isoforms were not due to differences in the amount of total protein (FIG. 3B below).

Inhibition of the NMDA Receptor and AKs/STATs Pathway Prevent the Tau Hyperphosphorylation Induced by IL-6 in Hippocampal Neurons.

It is known that IL-6 can modulate cytosolic calcium levels by activation of the NMDA receptor (Qiu *et al.*, 1995; 1998). As was indicated above, this increase in the level of calcium caused an increase in hyperphosphorylated tau levels. In this way, we decided to evaluate the role of the NMDA receptor in the IL-6-induced tau hyperphosphorylation. Therefore, we treated hippocampal neurons during 48 hours with IL-6 in the presence of MK-801. This study showed that incubation with the NMDA receptor inhibitor MK-801 prevents tau hyperphosphorylation induced by IL-6 (FIG. 3C, third lane). After 48 hour treatment, MK-801 reduced the hyperphosphorylated tau level, as determined by AT8 antibody, and this effect was not dependent on changes in total tau levels, as detected with the Tau5 antibody (FIG. 3C).

It was also important to evaluate the effects of the JAKs/STATs pathway in the cytosolic calcium increase induced by IL-6. Thus, we performed experiments in hippocampal neurons incubated during 48 hours with IL-6 in the presence of JAK 3 inhibitor, an inhibitor of JAKs/STATs pathway. FIG. 3C (fourth lane) shows that JAK 3 inhibitor reduced hyperphosphorylated tau levels, previously elevated with the IL-6 treatment. FIG. 3D shows a representative summary plot of AT8/Tau5 ratios from three independent experiments of each condition. These results are in total agreement with evidence that shows the dependence of the tau hyperphosphorylation induced by IL-6 on the signaling JAKs/STATs pathway (Quintanilla *et al.*, 2004).

Altogether, these results indicate the dependence of

tau hyperphosphorylation induced by IL-6 on NMDA receptor activation. Data also indicate that changes in the cytosolic intracellular calcium could be in direct relation with the hyperphosphorylation of tau of the Alzheimer's type, as induced by IL-6. Besides, these studies indicate that the cytosolic calcium influx induced by IL-6 has a partial role in tau hyperphosphorylation, because the incubation with an intracellular calcium chelator reduced partially the hyperphosphorylation of tau protein triggered by IL-6.

DISCUSSION

Inflammation is implicated in neuronal damage attending many neurodegenerative disorders (Bauer *et al.*, 1991; Campbell *et al.*, 1993; Blum-Degen *et al.*, 1995; Quintanilla *et al.*, 2004). In this study, we found that IL-6 induced a cytosolic calcium influx in primary rat hippocampal neuronal cultures. Further studies showed that this increase in the cytosolic calcium levels is dependent on external calcium sources and not derived from intracellular stores. Since, the use of extracellular calcium chelators reduced the calcium signal induced by IL-6, this is in agreement with previous studies (Holliday *et al.*, 1995; Qiu *et al.*, 1998).

Additional studies showed that this enhancement in cytosolic calcium levels induced by IL-6 stimulation on hippocampal neurons is most likely to be through NMDA receptors, and it could be mediated by JAKs /STATs pathway, since the use of an antagonist of NMDA receptors and specific inhibitors of JAKs /STATs pathway were able to block the calcium entry. However, it was relevant not to rely only on the changes of calcium transients, but also to evaluate the changes in tau phosphorylation patterns as indicated above. Thus, one of the main effects of enhancement in the intracellular calcium induced by IL-6 on hippocampal neurons was an increase in tau phosphorylation in Alzheimer's type epitopes, as denoted by an increase of phosphospecific epitopes recognized by AT8 and PHF-1. In support of these findings, the increase in hyperphosphorylation of tau protein was partially diminished by the calcium chelator BAPTA-AM, and inhibited by MK-801 and JAK 3 inhibitor reagents. In this context, IL-6 effects on tau phosphorylations could be mediated by a dual mechanism: (i) through NMDA receptors being responsible for the increase in extracellular calcium-dependent phosphorylation, and (ii) by increasing the release of intracellular deposits of calcium sensitive to BAPTA effects. It is likely that the increase in calcium entry could trigger mobilization from intracellular deposits, thus increasing cytosolic calcium even more. The inhibition in the hyperphosphorylation of tau protein by JAK 3 inhibitor has been reported previously (Quintanilla *et al.*, 2004), and suggests possible implications in the neurodegenerative pathway of neurons. Survival of neurons treated with IL-6 (5 ng/ml) was not compromised (data not shown), which is in agreement with previous studies (Holliday *et al.*, 1995; Qiu *et al.*, 1995; 1998; Quintanilla *et al.*, 2004).

Further explanation of these findings is analyzed below. Activation of NMDA receptors increases intracellular calcium via several pathways: (i) calcium influx through receptor-gated channels, (ii) calcium influx through voltage-sensitive calcium channels (VSCCs) activated by membrane depolarization, and (iii) release of calcium from intracellular stores. The mechanism underlying the possible contribution of VSCCs to calcium signal was not assessed in the current study. However, previous studies (Qiu *et al.*, 1998) showed that the VSCCs contribution to the intracellular calcium signal to NMDA in chronic IL-6 treated neurons declines with development. Our treatments were at 4-DIV and previous studies showed that VSCCs, in cerebellar granule neuron of rat at 5-DIV, contribute to the intracellular calcium signal (Qiu *et al.*, 1998). In previous studies, chronic treatment of IL-6 on cerebellar granule neurons and the calcium influx in response to NMDA were investigated (Qiu *et al.*, 1995; 1998). Our experimental approach differs from previous ones, since we observed a direct effect of IL-6 in the enhancement of cytosolic calcium signal on hippocampal neurons in the absence of glutamate receptor agonist, such as domoate and NMDA.

The transduction pathway mediating the effects of IL-6 on NMDA responses has not been elucidated yet. Recent evidence implicates the Janus Kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Sengupta *et al.*, 1998; Sriram *et al.*, 2004). Other cytokines such as IL-1β can induce cytosolic calcium influx by NMDA receptor activation mediated by activation of Src tyrosine kinase and MAPK-p38 in cortical neurons (Viviani *et al.*, 2003; Li *et al.*, 2003).

Changes in cdk5 regulation by varying the neuronal specific activator p35 and/or its soluble cytosolic fragment p25 have correlated with increased tau phosphorylation (Tsai *et al.*, 1994; Patrick *et al.*, 1999; Lee *et al.*, 2000; Quintanilla *et al.*, 2004). We have previously shown that IL-6 induces an Alzheimer's type phosphorylation of tau protein by deregulating the cdk5/p35 pathway. IL-6 induced an increase in the protein levels of the cdk5 activator p35 (Quintanilla *et al.*, 2004). The increase in the levels of p35 was mediated by an increase in the transcription factor Egr-1, which has been demonstrated to control the expression of

p35 (Harada *et al.*, 2001). The increase in intracellular calcium levels induced by IL-6 seems to be important to cdk5 activity. As analyzed, this intracellular calcium increase may be related with a concomitant release of calcium from intracellular deposits and the increase in extracellular calcium entry. It has been shown that calcium influx induces the cleavage of p35 in its active cytosolic fragment p25 (Patrick *et al.*, 1999; Lee *et al.*, 2000). Our results suggest that variations in the influx of extracellular calcium levels due to IL-6 could modify calpain activity, and then increase the activity of the kinase. Besides, it has been reported that cdk5 can activate the NMDA receptor (Li *et al.*, 2001; Wang *et al.*, 2003) producing an increase in the calcium influx. Therefore, the cdk5/p35 pathway appears to be involved in the mechanism on how IL-6 could activate the NMDA receptor. IL-6 induces an increase in the cdk5/p35 activity in hippocampal neurons. This deregulation of cdk5 activity seems to produce the phosphorylation of NMDA receptors and their activation, which could activate VSCCs to result in an enhancement in the cytoplasmic calcium influx. This calcium entry also could affect cdk5 activity through cleavage of p35 to p25 by calpain. Thus, IL-6 appears to be a critical factor in the pathological process occurring during neurodegenerative disorders, such as Alzheimer's disease.

Acknowledgements

We thank Dr. Lester Binder (Northwestern University, IL) for a generous gift of Tau1 and Tau5 antibodies, and Dr. Peter Davis (Albert Einstein College, NY) for the valuable PHF1 antibody. This research was supported by grants 1050198 from FONDECYT, the Millennium Institute for Advanced Studies (CBB) and project P99-031F from the Millennium Science Initiative.

References

Alvarez A, R Toro, A Caceres and RB Maccioni (1999) Inhibition of tau phosphorylating protein kinase cdk5 prevents beta-amyloid-induced neuronal death. *FEBS Lett.* **459**, 421-426.

Alvarez A, JP Munoz and RB Maccioni (2001) A Cdk5-p35 stable complex is involved in the beta-amyloid-induced deregulation of Cdk5 activity in hippocampal neurons. *Exp. Cell. Res.* **264**, 266-274.

Apelt J and R Schliebs (2001) Beta-amyloid-induced glial expression of both pro- and anti-inflammatory cytokines in cerebral cortex of aged transgenic Tg2576 mice with Alzheimer plaque pathology. *Brain Res.* **894**, 21-30.

Banker GA and WM Cowan (1977) Rat hippocampal neurons in dispersed cell culture. *Brain Res.* **126**, 397-402.

Bauer J, S Strauss, B Volk and M Berger (1991) IL-6-mediated events in Alzheimer's disease pathology. *Immunol. Today* **12**, 422.

Blasko I, R Veerhuis, M Stampfer-Kountchev, M Saurwein-Teissl, P Eikelenboom and B Grubeck-Loebenstein (2000) Costimulatory effects of interferon-gamma and interleukin-1beta or tumor necrosis factor alpha on the synthesis of Abeta1-40 and Abeta1-42 by human astrocytes. *Neurobiol. Dis.* **7**, 682-689.

Blum-Degen D, T Muller, W Kuhn, M Gerlach, H Przuntek and P Riederer (1995) Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and *de novo* Parkinson's disease patients. *Neurosci. Lett.* **202**, 17-20.

Campbell IL, CR Abraham, E Masliah, P Kemper, JD Inglis, MB Oldstone and L Mucke (1993) Neurologic disease induced in transgenic mice by cerebral overexpression of interleukin 6. *Proc. Natl. Acad. Sci. USA* **90**, 10061-10065.

Chong Y (1997) Effect of a carboxy-terminal fragment of the Alzheimer's amyloid precursor protein on expression of proinflammatory cytokines in rat glial cells. *Life Sci.* **61**, 2323-2333.

Del Bo R, N Angeretti, E Lucca, MG De Simoni and G Forloni (1995) Reciprocal control of inflammatory cytokines, IL-1 and IL-6, and beta-amyloid production in cultures. *Neurosci. Lett.* **188**, 70-74.

Ershler WB and ET Keller (2000) Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. *Annu. Rev. Med.* **51**, 245-270.

Frei K, S Fredrikson, A Fontana and H Link (1991) Interleukin-6 is elevated in plasma in multiple sclerosis. *J. Neuroimmunol.* **31**, 147-153.

Gadient RA and U Otten (1995) Interleukin-6 and interleukin-6 receptor mRNA expression in rat central nervous system. *Ann. NY Acad. Sci.* **762**, 403-406.

Gadient RA and U Otten (1996) Postnatal expression of interleukin-6 (IL-6) and IL-6 receptor (IL-6R) mRNAs in rat sympathetic and sensory ganglia. *Brain Res.* **724**, 41-46.

Gadient RA and UH Otten (1997) Interleukin-6 (IL-6)--a molecule with both beneficial and destructive potentials. *Prog. Neurobiol.* **52**, 379-390.

Gadient RA, A Lachmund, K Unsicker and U Otten (1995) Expression of interleukin-6 (IL-6) and IL-6 receptor mRNAs in rat adrenal medulla. *Neurosci. Lett.* **194**, 17-20.

Gijbels K, J Van Damme, P Proost, W Put, H Carton and A Billiau (1990) Interleukin 6 production in the central nervous system during experimental autoimmune encephalomyelitis. *Eur. J. Immunol.* **20**, 233-235.

Gitter BD, LM Cox, RE Rydel and PC May (1995) Amyloid beta peptide potentiates cytokine secretion by interleukin-1 beta-activated human astrocytoma cells. *Proc. Natl. Acad. Sci. USA* **92**, 10738-10741.

Grau GE, K Frei, PF Piguet, A Fontana, H Heremans, A Billiau, P Vassalli and P Lambert (1990) Interleukin 6 production in experimental cerebral malaria: modulation by anticytokine antibodies and possible role in hypergammaglobulinemia. *J. Exp. Med.* **172**, 1505-1508.

Harada T, T Morooka, S Ogawa and E Nishida (2001) ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat. Cell. Biol.* **3**, 453-459.

Holliday J, K Parsons, J Curry, SY Lee and DL Gruol (1995) Cerebellar granule neurons develop elevated calcium responses when treated with interleukin-6 in culture. *Brain Res.* **673**, 141-148.

Jee SH, SY Chu, HC Chiu, YL Huang, WL Tsai, YH Liao and ML Kuo (2004) Interleukin-6 induced basic fibroblast growth factor-dependent angiogenesis in basal cell carcinoma cell line via JAK/STAT3 and PI3-kinase/Akt pathways. *J. Invest. Dermatol.* **123**, 1169-1175.

Jones SA, S Horiuchi, N Topley, N Yamamoto and GM Fuller (2001) The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *FASEB J.* **15**, 43-58.

Kishimoto T, S Akira and T Taga (1992) Interleukin-6 and its receptor: a paradigm for cytokines. *Science* **258**, 593-597.

LaFerla FM (2002) Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease. *Nat. Rev. Neurosci.* **3**, 862-872.

Lee MS, YT Kwon, M Li, J Peng, RM Friedlander and LH Tsai (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature* **405**, 360-364.

Li BS, MK Sun, L Zhang, S Takahashi, W Ma, L Vinade, AB Kulkarni, RO Brady and HC Pant (2001) Regulation of NMDA receptors by cyclin-dependent kinase-5. *Proc. Natl. Acad. Sci. USA* **98**, 12742-12747.

Li Y, L Liu, SW Barger and WS Griffin (2003) Interleukin-1 mediates pathological effects of microglia on tau phosphorylation and on synaptophysin synthesis in cortical neurons through a p38-MAPK pathway. *J. Neurosci.* **23**, 1605-1611.

Mattson MP, B Cheng, D Davis, K Bryant, I Lieberburg and RE Rydel (1992) beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.* **12**, 376-389.

Mattson MP, KJ Tomaselli and RE Rydel (1993) Calcium-destabilizing and neurodegenerative effects of aggregated beta-amyloid peptide are attenuated by basic FGF. *Brain Res.* **621**, 35-49.

Mehlhorn G, M Hollborn and R Schliebs (2000) Induction of cytokines in glial cells surrounding cortical beta-amyloid plaques in transgenic Tg2576 mice with Alzheimer pathology. *Int. J. Dev. Neurosci.* **18**, 423-431.

Merrill JE and IS Chen (1991) HIV-1, macrophages, glial cells, and cytokines in AIDS nervous system disease. *FASEB J.* **5**, 2391-2397.

Minami M, Y Kuraishi and M Satoh (1991) Effects of kainic acid on messenger RNA levels of IL-1 beta, IL-6, TNF alpha and LIF in the rat brain. *Biochem. Biophys. Res. Commun.* **176**, 593-598.

Patrick GN, L Zukerberg, M Nikolic, S de la Monte, P Dikkes and LH Tsai (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* **402**, 615-622.

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

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

Quintanilla RA, OH Porras, J Castro and LF Barros (2000) Cytosolic [Ca(2+)] modulates basal GLUT1 activity and plays a permissive role in its activation by metabolic stress and insulin in rat epithelial cells. *Cell Calcium* **28**, 97-106.

Quintanilla RA, DI Orellana, C Gonzalez-Billault and RB Maccioni (2004) Interleukin-6 induces Alzheimer-type phosphorylation of tau protein by deregulating the cdk5/p35 pathway. *Exp. Cell. Res.* **295**, 245-257.

Sengupta TK, EM Schmitt and LB Ivashkiv (1996) Inhibition of cytokines and JAK-STAT activation by distinct signaling pathways. *Proc. Natl. Acad. Sci. USA* **93**, 9499-9504.

Sengupta TK, ES Talbot, PA Scherle and LB Ivashkiv (1998) Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. *Proc. Natl. Acad. Sci. USA* **95**, 11107-11112.

Sriram K, SA Benkovic, MA Hebert, DB Miller and JP O'Callaghan (2004) Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astrogliosis *in vivo*? *J. Biol. Chem.* **279**, 19936-19947.

Sudbeck EA, XP Liu, RK Narla, S Mahajan, S Ghosh, C Mao and FM Uckun (1999) Structure-based design of specific inhibitors of Janus kinase 3 as apoptosis-inducing antileukemic agents. *Clin. Cancer Res.* **5**, 1569-1582.

Toro VC, R Tehranian, M Zetterstrom, G Eriksson, U Langel, T Bartfai and K Iverfeld (2001) Increased gene expression of interleukin-1alpha and interleukin-6 in rat primary glial cells induced by beta-amyloid fragment. *J. Mol. Neurosci.* **17**, 341-350.

Tsai LH, I Delalle, VS Caviness Jr, T Chae and E Harlow (1994) p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* **371**, 419-423.

Viviani B, S Bartesaghi, F Gardoni, A Vezzani, MM Behrens, T Bartfai, M Binaglia, E Corsini, M Di Luca, CL Galli *et al.* (2003) Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J. Neurosci.* **23**, 8692-8700.

Wang J, S Liu, Y Fu, JH Wang and Y Lu (2003) Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. *Nat. Neurosci.* **6**, 1039-1047.

Wong EH, JA Kemp, T Priestley, AR Knight, GN Woodruff and LL Iversen (1986) The anticonvulsant MK-801 is a potent *N*-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. USA* 83, 7104-7108.