UNIVERSITAT DE BARCELONA

RESPUESTA DE LAS CÉLULAS GLIALES AL DAÑO NEURONAL *IN VITRO*

Tesis doctoral presentada por
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RESULTADOS

4.1- PRIMER TRABAJO

Glial activation modulates glutamate neurotoxicity in cerebellar granule cell cultures

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RESUMEN

Las células gliales tienen un papel muy importante en el funcionamiento del SNC tanto en condiciones fisiológicas como en condiciones patológicas. Frente a estímulos externos o como consecuencia del daño neuronal, estas células tienen la capacidad de responder con cambios morfológicos y funcionales constituyendo lo que se ha denominado activación glial o glía reactiva. Existe cierta controversia respecto al papel nocivo o beneficioso de la glía reactiva para las neuronas. En este trabajo se ha estudiado cómo la presencia y el estado (quiescencia/activación) de las células gliales pueden modificar la respuesta de las neuronas frente a un estímulo nocivo. Para ello se han utilizando dos tipos de cultivos primarios de células de cerebelo: cultivos enriquecidos en neuronas granulares y cultivos mixtos de neuronas granulares y células gliales. Los cultivos han sido tratados con concentraciones crecientes de glutamato con la finalidad de inducir muerte neuronal por excitotoxicidad y determinar si la presencia de células gliales induce diferencias en esta respuesta. En paralelo, se ha estudiado si el pretratamiento con LPS, agente ampliamente utilizado para inducir activación glial *in vitro*, es capaz de modular la toxicidad del glutamato en estos cultivos. En las diferentes situaciones experimentales se han evaluado los cambios inducidos en las células gliales mediante la detección de cambios morfológicos y la producción de NO, que está incrementada en respuesta a numerosos estímulos que inducen activación glial. Los resultados obtenidos muestran que la presencia de las células gliales protege de la neurotoxicidad del glutamato dentro de un cierto rango de concentraciones, por encima del cual tiende a incrementarla. La activación glial inducida por el LPS anula el efecto neuroprotector de la glía en los cultivos mixtos de neurona-glía e incrementa el efecto neurotóxico del glutamato. Estos resultados ponen de manifiesto que el estado en que se encuentran las células gliales puede jugar un papel muy importante en la respuesta neuronal a un determinado estímulo.

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Glial Activation Modulates Glutamate Neurotoxicity in Cerebellar Granule Cell Cultures

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KEY WORDS excitotoxicity; lipopolysaccharide; reactive glial cells; microglia; astroglia

ABSTRACT We studied the influence of glial cells on the neuronal response to glutamate toxicity in cerebellar granule cell cultures. We compared the effect of glutamate on neuronal viability in neuronal vs. neuronal-glial cultures and determined this effect after pretreating the cultures with the lipopolysaccharide (LPS) of *Escherichia coli*, agent widely used to induce glial activation. Morphological changes in glial cells and nitric oxide (NO) production were evaluated as indicators of glial activation. We observed that glutamate neurotoxicity in neuronal-glial cultures was attenuated in a certain range of glutamate concentration when compared to neuronal cultures, but it was enhanced at higher glutamate concentrations. This enhanced neurotoxicity was associated with morphological changes in astrocytes and microglial cells in the absence of NO production. LPS treatment induced morphological changes in glial cells in neuronal-glial cultures as well as NO production. These effects occurred in the absence of significant neuronal death. However, when LPS-pretreated cultures were treated with glutamate, the sensitivity of neuronal-glial cultures to glutamate neurotoxicity was increased. This was accompanied by additional morphological changes in glial cells in the absence of a further increase in NO production. These results suggest that quiescent glial cells protect neuronal cells from glutamate neurotoxicity, but reactive glial cells increase glutamate neurotoxicity. Therefore, glial cells play a key role in the neuronal response to a negative stimulus, suggesting that this response can be modified through an action on glial cells.

INTRODUCTION

In physiological conditions, glial cells play a key role in the normal function of the central nervous system (CNS) both during development and in the adult. Moreover, they are essential to the response of the CNS to pathological conditions. Astroglial and microglial cells respond to neuronal damage with morphological and functional changes and thus become reactive or activated glial cells. Reactive astroglial and microglial cells produce a variety of growth factors, cytokines, nitric oxide (NO), and neuropeptides with known neurotrophic or neurotoxic properties (Chao et al., 1995b; Raivich et al., 1999; Streit et al., 1999; Vitkovic et al., 2000). In addition, unidentified factors synthesized by glial cells have also been described as neurotrophic and neurotoxic products (Giulian, 1993; Giulian et al., 1993; Amano et al., 1994; Shea, 1994; Yoshida et al., 1995; Zhang and Fedoroff, 1996). Moreover, some of the compounds produced by reactive glial cells induce either neuroprotection or neurodegeneration depending

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on the experimental model used (Chao et al., 1995b; Allan and Rothwell, 2001), suggesting that they have a dual role and that additional factors determine their final effect.

Glial activation is a complex phenomenon and in vitro approaches have provided useful information about the response of glial cells to several stimuli. Various types of cell cultures are used to study glial cell function: enriched cultures of astroglial, microglial or oligodendroglial cells, as well as mixed glial or mixed neuronal-glial cultures from several brain regions can be obtained. In this regard, the behavior of neurons and glial cells in culture depends on the cells they are surrounded by, because of the interactions between the neural cells. Several studies in vitro show that the interaction between neurons and glial cells, either through physical contact or the release of soluble factors, determines the morphology of astrocytes (Matsutani and Yamamoto, 1997), the expression of specific glutamate transporters in astrocytes (Gegelashvili et al., 1997), the neuronal sensitivity to glutamate excitotoxicity (Brown, 1999), the expression of transcription factors in glial cells (Pennypacker et al., 1996) or neurons (Kaltschmidt and Kaltschmidt, 2000), and neuronal viability (Zhang and Fedoroff, 1996). The interaction between astroglial and microglial cells determines microglial morphology (Tanaka and Maeda, 1996; Tanaka et al., 1999). In addition, the interactions between neural cells define their response to external stimuli (Vincent et al., 1996; Chang et al., 2000; Solá et al., 2002).

Here we examined the effect of resting and activated glial cells on the neuronal response to a harmful stimulus. We used two types of cerebellar granule cell cultures, neuronal-enriched cultures and neuronal-glial cultures, and determined the toxicity of glutamate in cerebellar granule neurons in the absence and in the presence of glial cells and after inducing alterations in glial cell function.

MATERIALS AND METHODS

Reagents were from Sigma Chemicals (St. Louis, MO) unless otherwise stated.

Cell Culture

Cultures of cerebellar granule cells were prepared from 7-day-old pups (Wistar; Iffa Credo, Lyon, France) as described elsewhere (Novelli et al., 1988). Briefly, cerebella from 8–10 animals were quickly removed and freed of meninges, followed by manual slicing with a sterile blade. The tissue was then dissociated with 0.25 mg/ml trypsin and passed through a Pasteur pipette about 60 times in the presence of 80 μg/ml DNase and 0.52 mg/ml soybean trypsin inhibitor. Nondissociated material was allowed to settle for 2–3 min, the supernatant was removed, and the pellet was triturated again as described above. Cells in the pooled supernatants were recovered by brief centrifugation and resuspended in basal Eagle's medium without glutamine (Gibco-BRL, Life Technologies, Paisley, U.K.) supplemented with 20 mM KCl (25 mM final concentration), 2 mM glutamine, 100 μg/ml gentamycin, and 10% fetal bovine serum (Gibco-BRL), then seeded in 5 μg/ml poly-L-lysine-coated 24-well plates (Nalge Nunc International, Naperville, IL) at a density of 3×10^5 cells/cm^2 in 0.5 ml of medium. In some cases, cells were seeded over 10 μg/ml poly-L-lysine-coated glass coverslips (12 mm diameter; Marienfeld, Germany) placed on the culture wells. Cells were maintained at 37°C in a 5% CO_2 and 95% humidity atmosphere. Three types of cultures were obtained: neuronal cultures, where 10 μM cytosine B-D-arabinofuranoside (Ara-C) was added 16–18 h after seeding to inhibit nonneuronal cell proliferation; neuronal-glial cultures, which were not treated with Ara-C; and mixed glial cultures, where medium was changed 24 h after seeding to a medium without added KCl. In neuronal and neuronal-glial cultures, the medium was never changed, but 5.6 mM glucose was added 4 days after seeding to enhance survival. The medium of glial cultures was renewed every 3 days. Glia content in neuronal and neuronal-glial cultures was assessed after 8 days in culture using immunocytochemical techniques as described below. Neuronal cultures contained 2% of astrocytes and 3% of microglia and neuronal-glial cultures contained 7% of astrocytes and 5% of microglia.

Treatments

Cell cultures were treated with glutamate 8 days after seeding. The preconditioned growth medium was collected, and cells were washed twice with 1 ml of standard Locke's solution containing 155 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 8.4 mM HEPES, and 5.6 mM glucose, pH 7.4. Primary cultures were incubated for 20 min at room temperature with 0.5 ml of Mg^{2+}-free Locke's solution containing various concentrations of glutamate: 10, 15, 20, 50, and 100 μM. Glutamate exposure was stopped by washing the cells twice with 1 ml of standard Locke's solution. The preconditioned growth medium was then replaced and the cultures were returned to the incubator. Neuronal viability was assessed 24 h later. In some experiments, cell cultures were exposed to the N-methyl-D-aspartate (NMDA) receptor antagonist hydrogen maleate (MK-801, 10 μM) in Mg^{2+}-free Locke's solution for 15 min before and during glutamate treatment.

To evaluate the effect of glial activation on the toxicity of glutamate, cell cultures were pretreated with LPS (1 μg/ml: *Escherichia coli* 026:B6, reconstituted in PBS and kept at −20°C) 7 days after seeding. LPS was added directly to the culture medium. Cultures were then treated with glutamate 8 days after seeding as described above.

Nitrite Assay

The production of NO was assessed by the Griess reaction, a colorimetric assay that detects a stable re-

action product of NO and molecular oxygen, nitrite (NO_2^-), accumulated in the culture medium. Briefly, 100 μl aliquots of culture supernatants were collected 48 h after stimulation with 1 μg/ml LPS and incubated with equal volumes of Griess reagent [1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, and 5% phosphoric acid] for 10 min at room temperature. Absorbance at 540 nm was measured using a microplate reader (iEMS Reader MF, Labsystems, Finland). Nitrite concentration was determined from a sodium nitrite standard curve (0–50 μM).

Single Immunocytochemical Analysis

Astroglial and microglial cells were identified in neuronal, neuronal-glial, and mixed glial cultures by immunostaining with a rabbit anti-GFAP polyclonal antibody (Dako, Glostrup, Denmark) and biotin-labeled lectin from *Lycopersicon esculentum* (tomato), respectively. Cells were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 20 min at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2/methanol for 2 min and cells were then permeated in 0.2% Triton X-100/PBS for 8 min. Nonspecific staining was blocked by incubating the cells with 5% serum in PBS/1% bovine serum albumin for 20 min at room temperature. The cells were then incubated with anti-GFAP antibody (1/500) or lectin (1/400) overnight at 4°C in the presence of 1% serum. After rinsing in PBS, they were incubated with a biotinylated goat antirabbit antibody (1/200) in the presence of 1% serum for 1 h at room temperature. This step was omitted in the case of the biotin-labeled lectin. Following incubation with the avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) for 1 h at room temperature, cells were washed in PBS and color was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB). The reaction was stopped by rinsing the cells in PBS. In some cases, hematoxylin staining was additionally carried out.

Double Immunocytochemical Analysis

To identify NO-producing cells in neuronal-glial and mixed glial cultures, double immunocytochemistry was performed using specific markers for each glial cell type and specific antibodies for iNOS, which is responsible for the synthesis of NO. Rabbit anti-GFAP polyclonal antibody and anti-CD11b (MRC OX42) monoclonal antibody (Serotec, Oxford, U.K.) were used to identify astroglial and microglial cells, respectively. The expression of iNOS was detected either with an anti-iNOS monoclonal antibody (Transduction Laboratories, Lexington, KY) or with an anti-iNOS polyclonal antibody (Chemicon International, Temecula, CA). Cells grown on glass coverslips were fixed, permeated, and nonspecific staining was blocked as described above. Cells were coincubated overnight at 4°C with either rabbit anti-GFAP polyclonal antibody (1/500) and anti-iNOS monoclonal antibody (1/200) or OX-42 monoclonal antibody (1/50) and anti-iNOS polyclonal antibody (1/200). After rinsing in PBS, cells were incubated for 1 h at room temperature with fluorescent secondary antibodies: goat antirabbit tetramethylrhodamine isothiocyanate (TRITC; 1/400) for the rabbit anti-GFAP polyclonal antibody; sheep antimouse fluorescein isothiocyanate (FITC; 1/50) for the anti-iNOS monoclonal antibody; goat antimouse Alexa 488 and goat antimouse ALEXA 546 (1/1,500; Molecular Probes, Eugene, OR) for the OX-42 monoclonal antibody and the anti-iNOS polyclonal antibody, respectively. Immunoreactivity was visualized under a fluorescence microscope (Nikon Eclipse E1000).

Assessment of Cell Viability

Neuronal viability in cerebellar granule cell cultures was routinely estimated by fluorescent staining. Twenty-four hours after glutamate treatment, cells were washed with standard Locke's solution before and after incubation with fluorescein diacetate (FDA; 15 μg/ml) and propidium iodide (PI; 4.6 μg/ml) for 5 min, then examined under a fluorescence microscope (Olympus IX70). Using appropriate filters, FDA-positive live neurons showed bright green small cell bodies and neurites (morphology clearly different from that presented by nonneuronal cells), while the propidium iodide-positive dead cells showed red nuclei. Images of 2–4 microscopic fields in each well were obtained with a digital camera (ColorView 12) using a 20× objective. Three wells per experimental condition were processed and each experimental condition was repeated at least four times. Visual counting of live and dead neurons was performed with the imaging analysis software "analySIS" (Soft Imaging System GmbH). The total number of living neurons was determined using FDA staining only, because a certain amount of dead cells had been lost during the experimental processing of the samples. Neuronal viability was expressed as a percentage of control.

Data Presentation and Statistical Analysis

Results are presented as the mean ± SEM values. Statistical analysis were performed using one- or two-way analysis of variance (ANOVA) to identify overall treatment effects, followed by Student's *t*-test or Dunnett's test for the selective comparison of individual data groups vs. their respective controls. Values of $P < 0.05$ were considered significant.

RESULTS

Cell Culture Morphology

Neurons grown in neuronal cultures showed the same morphology as those grown in neuronal-glial cul-

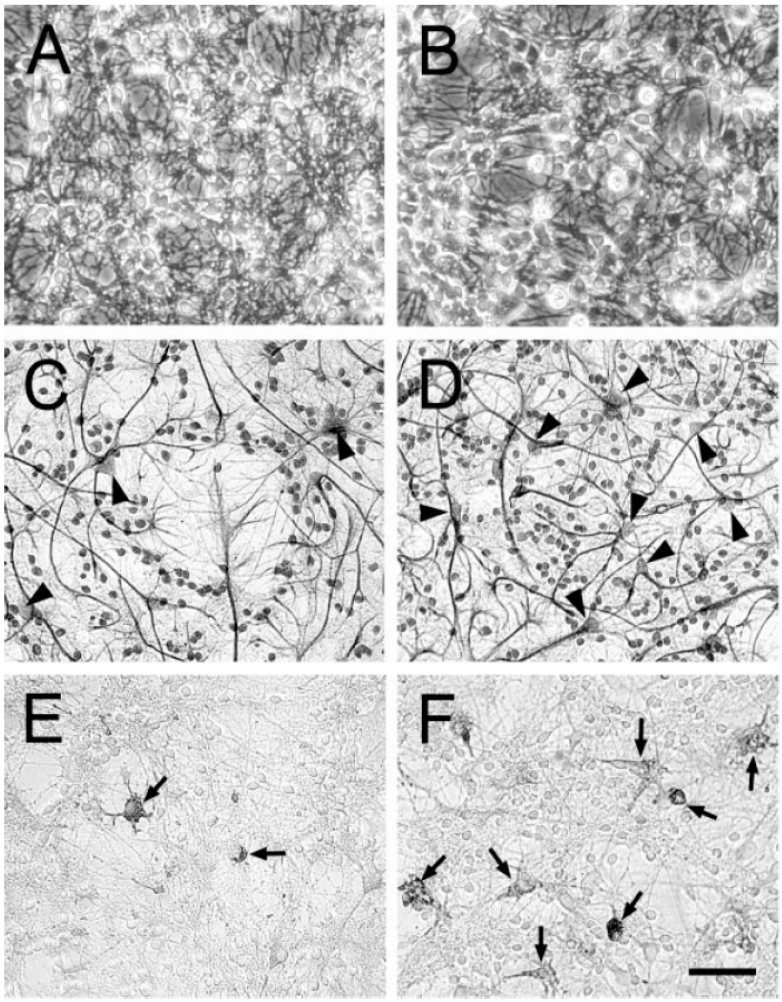

Fig. 1. Presence of glial cells in neuronal and neuronal-glial cultures of cerebellar granule cells. Images in the left column correspond to neuronal cultures and images in the right column correspond to neuronal-glial cultures 8 days after seeding. **A** and **B** are phase contrast images. **C** and **D** correspond to GFAP immunolabeling, showing astrocyte content in the cerebellar cultures and hematoxylin staining of the cellular nuclei. Arrowheads show GFAP and hematoxylin costaining (corresponding to astrocytes). **E** and **F** correspond to lectin staining, a specific marker of microglial cells (small arrows). Bar = 50 μm.

tures (Fig. 1A and B). However, they were more homogeneously distributed in neuronal cultures than in neuronal-glial cultures, where groups of neurons in close contact could be observed. The morphology of astrocytes in neuronal and neuronal-glial cultures was similar, but it differed from that observed in mixed glial cultures: in the presence of neurons, astrocytes showed a stellate morphology (Fig. 1C and D), with a reduced perinuclear soma and several long and thin processes, while in the mixed glial cultures they showed a polygonal morphology (data not shown). Very few astrocytes were observed in neuronal cultures, although their long processes were present in most of the culture well surface. Astrocytes processes constituted a dense and complex network in neuronal-glial cultures. As regards microglial cells, scattered cells of irregular morphology were observed in neuronal cultures (Fig. 1E), while cells with shapes ranging from small and round to stellate were consistently observed in neuronal-glial cultures (Fig. 1F). The stellate morphology was less pronounced in microglial cells growing in mixed glial cell cultures (data not shown).

Glutamate Neurotoxicity in Absence and Presence of Glial Cells

Neuronal and neuronal-glial cultures treated with increasing concentrations of glutamate showed a con-

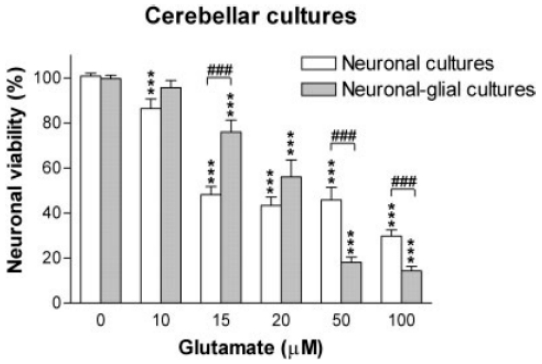

Fig. 2. Neuronal viability in neuronal and neuronal-glial cultures of cerebellar granule cells treated with glutamate. Neuronal and neuronal-glial cultures were treated with increasing glutamate concentrations. Bars represent the mean ± SEM of at least four cultures. Glutamate treatment decreased neuronal viability vs. control untreated cultures in neuronal and neuronal-glial cultures in a concentration-dependent manner, triple asterisk, $P < 0.001$, Dunnett's test. The only exception occurred in neuronal-glial cultures treated with 10 μM glutamate, where no alterations in neuronal viability were observed. Glutamate-induced decrease in neuronal viability in neuronal-glial cultures differed from that observed in neuronal cultures (two-way ANOVA, $P < 0.01$). Triple number sign, $P < 0.001$ represents differences between glutamate-induced decrease in neuronal viability in neuronal cultures vs. neuronal-glial cultures at the concentration of glutamate tested, Student's t-test.

centration-dependent effect on neuronal viability (Fig. 2). Glutamate treatment significantly altered neuronal viability in both types of cultures (ANOVA, $P < 0.001$). Glutamate neurotoxicity was inhibited when cultures were treated with 10 μM MK-801 (data not shown). The magnitude of the effect of glutamate treatment on neuronal viability differed according to the culture tested (two-way ANOVA, $P < 0.01$). In neuronal cultures, neuronal viability decreased at 10 μM glutamate compared with control untreated cultures (Dunnett's test), while in neuronal-glial cultures, neuronal viability was significantly reduced from 15 μM glutamate (Dunnett's test; Fig. 2). In addition, 10, 15, and 20 μM glutamate decreased neuronal viability in neuronal cultures to a higher extent than in neuronal-glial cultures, but the opposite occurred at 50 and 100 μM glutamate (Fig. 2). Toxicity was not significant in mixed glial cell cultures treated with the same concentrations of glutamate (data not shown).

Glial Cell Activation After Glutamate and/or LPS Treatment

Changes in the cell morphology or NO production induced in glial cells by glutamate and/or LPS treatment were studied as signals of glial activation. Changes in cell morphology, as determined by GFAP immunostaining and lectin staining, were evaluated at 15 μM glutamate, as the lowest concentration tested decreasing neuronal viability in both neuronal and neuronal-glial cultures, and at 100 μM glutamate, as the concentration inducing the maximal decrease in neuronal viability in both types of cell cultures. Glutamate treatment at 15 μM did not significantly modify astroglial or microglial cell morphology in neuronal-glial cultures (data not shown). However, treatment with 100 μM glutamate induced morphological changes in both astroglial and microglial cells in neuronal-glial cultures: astrocytes predominantly showed shorter and thicker processes than in control cultures (Fig. 3A and B), while a large proportion of microglial cells showed increased size, round morphology, numerous refringent vacuoles, and lighter lectin staining in glutamate-treated cultures (Fig. 4A and B). No significant morphological changes were observed in glial cells in mixed glial cultures treated with 15 or 100 μM glutamate (data not shown). Glutamate treatment did not alter NO production in neuronal-glial or mixed glial cultures at any of the concentrations tested (data not shown).

Neuronal-glial cultures treated with 1 μg/ml LPS showed no evident morphological changes in astrocytes (Fig. 3C), but a higher proportion of microglial cells showed a rounded morphology after LPS treatment than in control cultures (Figs. 4C and 5A and B). This was also observed in mixed glial cell cultures (Fig. 5E and F), where changes in astrocytes were not detected either. Lectin staining (Fig. 4) and OX-42 immunolabeling (Fig. 5) showed microglial cells with very distinct appearance. This may be due to the fact that each marker labels specific molecules of the cell surface and that microglial cells growing on plastic culture plates or over glass coverslips change shape. In addition, LPS treatment significantly increased NO production in both cultures, which was not observed in neuronal cultures (asterisk, $P < 0.05$, and triple asterisk, $P < 0.001$, Dunnett's test vs. control values in neuronal-glial and glial cultures, respectively; Fig. 6). Double immunostaining for OX-42 and iNOS revealed that the cells responsible for iNOS induction were microglial cells (Fig. 5, right column).

The morphological changes induced by LPS in microglial cells in neuronal-glial and mixed glial cultures were also detected after glutamate treatment (Fig. 4D). In addition, morphological changes in astrocytes were also observed at both 15 and 100 μM (Fig. 3D) glutamate in LPS-pretreated neuronal-glial cultures: astrocytes mainly showed thicker and shorter processes than in control conditions. However, glutamate treatment of LPS-pretreated cultures did not further modify NO production in neuronal-glial and glial cultures ($P > 0.05$, Dunnett's test vs. LPS-treated samples; Fig. 6).

Modulation of Glutamate Neurotoxicity in Presence of Glial Activation

LPS treatment for 48 h did not cause significant death in mixed glial cell cultures (data not shown) and did not alter neuronal viability in neuronal and neuronal-glial cultures (Fig. 7A). Glutamate treatment had the same effect on neuronal viability in neuronal cul-

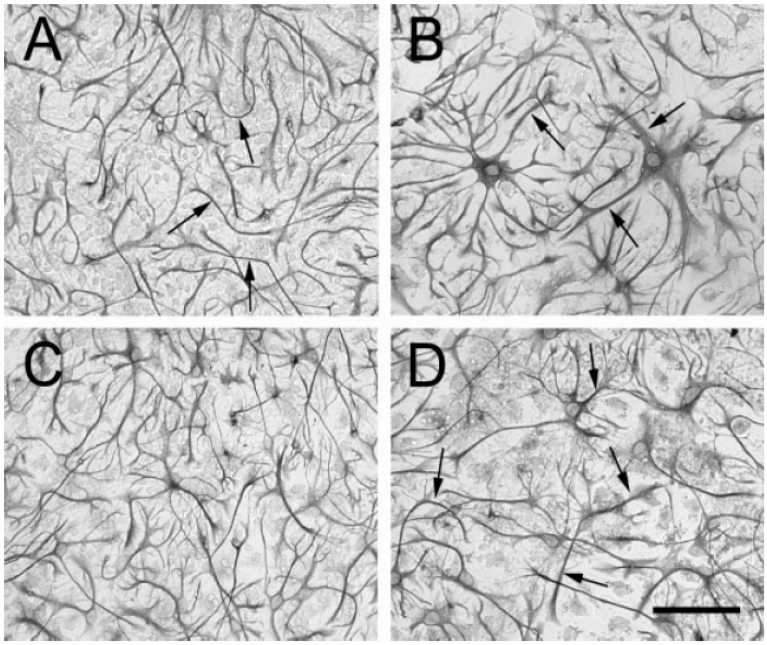

Fig. 3. Morphological changes in astrocytes in neuronal-glial cultures after LPS and LPS plus glutamate treatment. GFAP immunoreactivity in control cultures (**A**) and 100 μM glutamate (**B**), LPS (**C**), and LPS plus 100 μM glutamate (**D**) treated cultures. Arrows point out astrocyte processes, which show evident changes in the treated cultures as regards thickness, length, and spatial distribution. Bar = 100 μm.

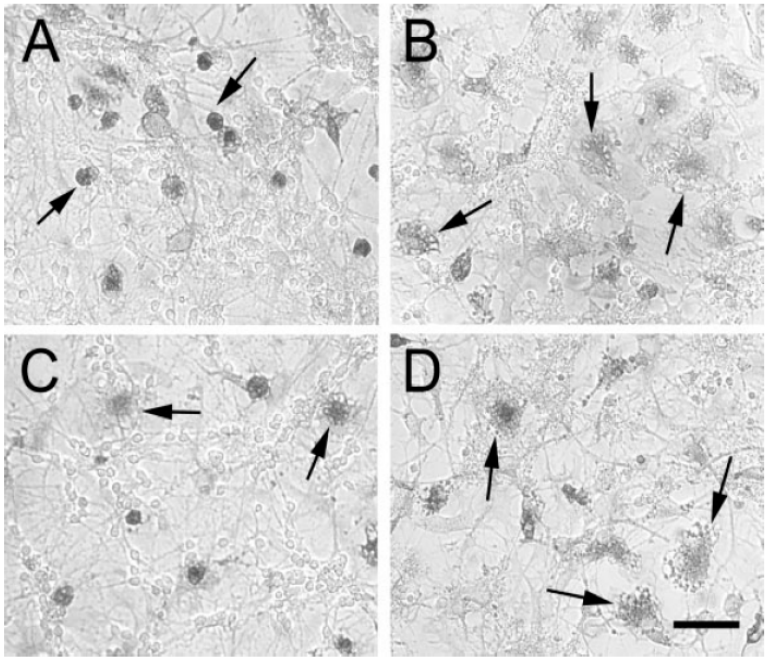

Fig. 4. Morphological changes in microglial cells in neuronal-glial cultures after LPS and LPS plus glutamate treatment. Lectin staining in control cultures (**A**) and 100 μM glutamate (**B**), LPS (**C**), and LPS plus 100 μM glutamate (**D**) treated cultures. Arrows point out lectin-positive cells, which show evident morphological changes and plenty of vesicles in the treated cultures. Bar = 50 μm.

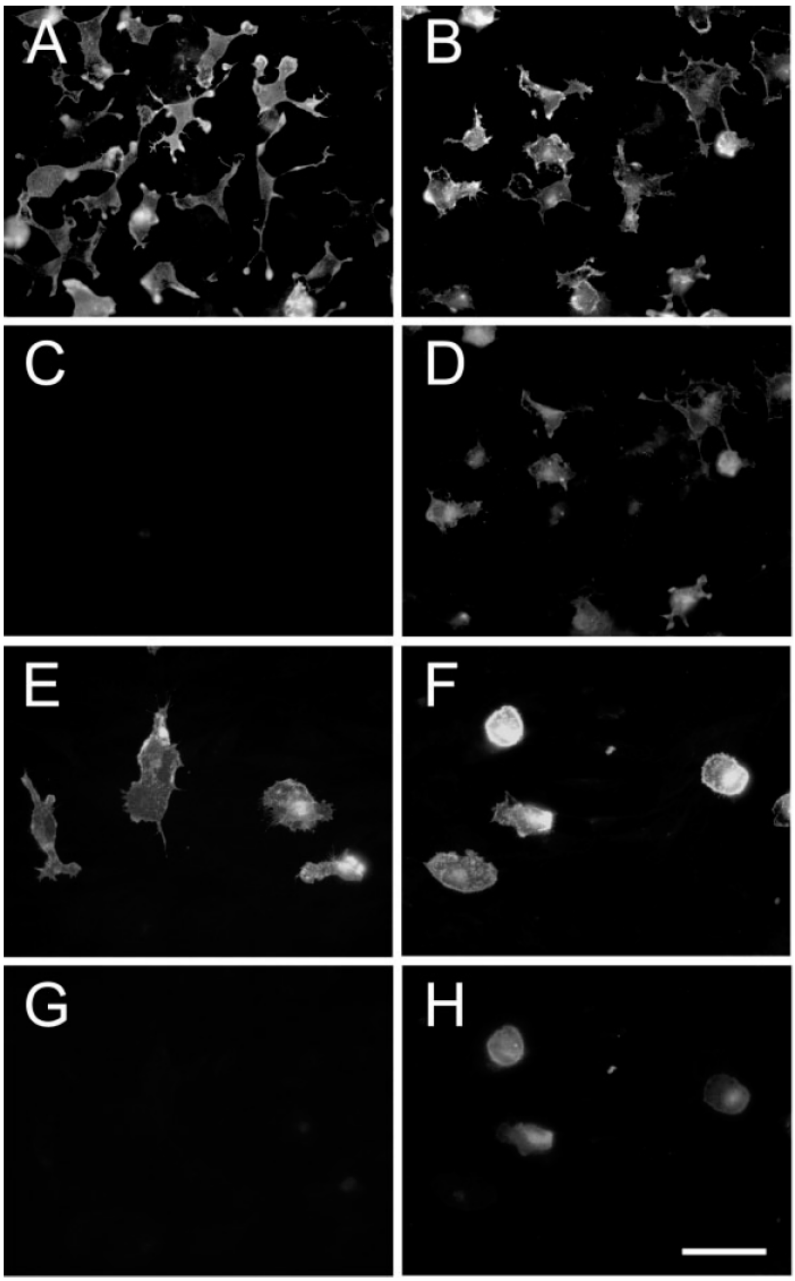

Fig. 5. Identification of NO-producing cells in cerebellar cell cultures. OX-42 (**A**, **B**, **E**, and **F**) and iNOS (**C**, **D**, **G**, and **H**) immunostaining in control (left column) and LPS-treated (right column) neuronal-glial (A–D) and mixed glial cultures (E–H). Bar = 50 μm.

tures in the presence and in the absence of LPS pretreatment (two-way ANOVA, $P > 0.05$; Fig. 7B). However, the glutamate-induced decrease in neuronal viability was modified in LPS-pretreated neuronal-glial cultures (two-way ANOVA, $P < 0.001$; Fig. 7C): neuronal viability was further decreased after glutamate treatment at the concentrations of 10, 15, and 20 μM, although the effect of 100 μM glutamate was similar in the presence and in the absence of LPS pretreatment.

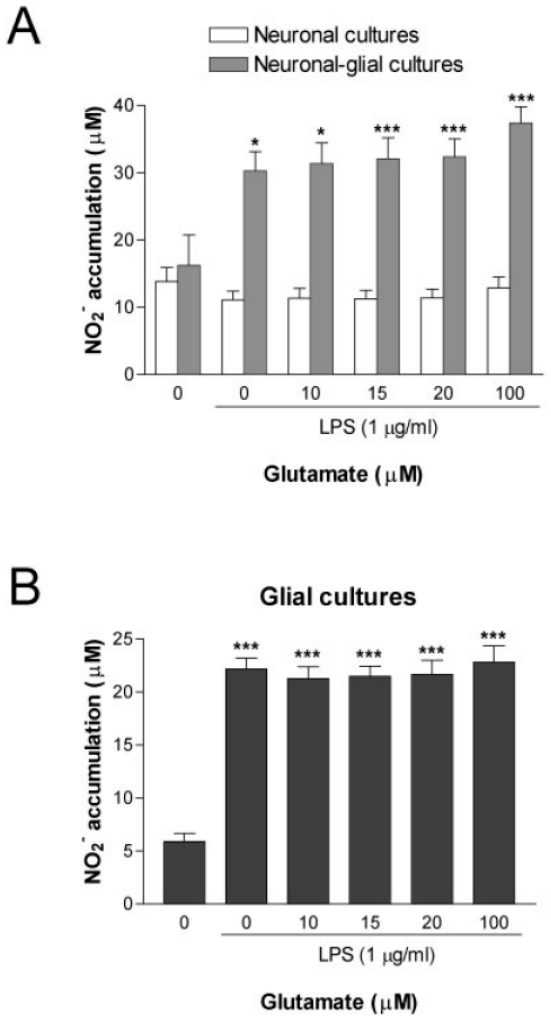

Fig. 6. Nitrite production in cerebellar cell cultures after LPS and LPS plus glutamate treatment. NO production in (**A**) neuronal and neuronal-glial cultures and (**B**) mixed glial cultures of cerebellar cells after LPS treatment in the absence or in the presence of several concentrations of glutamate. Bars represent the mean ± SEM of five neuronal and neuronal-glial cultures and three mixed glial cultures. Asterisk, $P < 0.05$, and triple asterisk, $P < 0.001$, represent differences between nitrite accumulation in the culture medium in LPS-treated cultures vs. control cultures at 48 h, Dunnett's test.

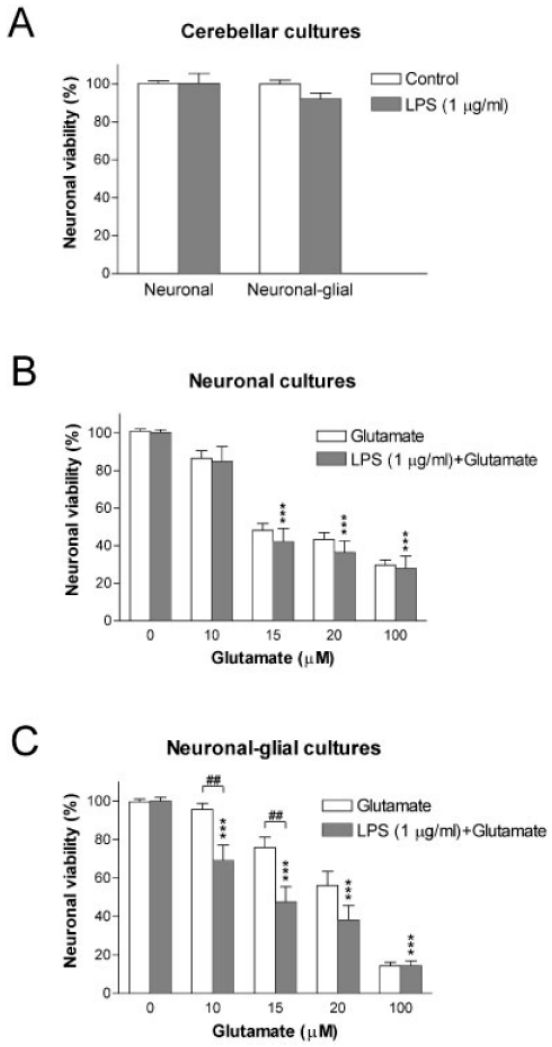

Fig. 7. Neuronal viability in neuronal and neuronal-glial cultures of cerebellar granule cells treated with LPS and LPS plus glutamate. **A:** Effect of LPS treatment on neuronal viability. Effect of glutamate on neuronal viability in neuronal cultures (**B**) and in neuronal-glial cultures (**C**) after LPS pretreatment. Bars represent the mean ± SEM of six cultures. White bars represent the same data as Figure 2, but are presented again to facilitate the comparison of glutamate neurotoxicity in the presence and in the absence of LPS pretreatment. LPS plus glutamate treatment significantly decreased neuronal viability vs. control untreated cultures in neuronal and neuronal-glial cultures, triple asterisk, $P < 0.001$, Dunnett's test. Glutamate-induced decrease in neuronal viability in neuronal cultures was similar in the absence and in the presence of LPS pretreatment (two-way ANOVA, $P > 0.05$). Nevertheless, glutamate-induced decrease in neuronal viability in neuronal-glial cultures was modified in the presence of LPS pretreatment ($P < 0.001$, two-way ANOVA). Double number sign, $P < 0.01$, represents differences between glutamate-induced decrease in neuronal viability in LPS-pretreated vs. LPS-untreated cultures at the concentration of glutamate tested, Student's t-test.

DISCUSSION

The present results show that cerebellar granule neurons are protected from the toxicity of glutamate when they are cultured in the presence of glia, but this protective effect is lost if glial cells are activated. Glial cells protect neurons from glutamate toxicity in a certain range of glutamate concentration, but at higher concentrations of glutamate, glial cells enhance its neurotoxic effect. In addition, when neuronal-glial cultures are pretreated with LPS, which induces glial cell activation, the protective effect of glial cells disappears and neurons become more sensitive to glutamate toxicity.

We observed that glutamate neurotoxicity in neuronal-glial cultures was lower than in neuronal cultures at glutamate concentrations of 10–20 μM, showing that glial cells protect neurons from glutamate toxicity in these conditions. Several authors show the protective effect of astrocytes on glutamate-induced neuronal death in various experimental models using rat or mouse cell cultures (Rosenberg and Aizenman, 1989; Rosenberg et al., 1992; Dugan et al., 1995; Amin and Pierce, 1997; Beaman-Hall et al., 1998). Various factors may be responsible for this protection: the presence of astrocytes surrounding neurites and synaptic connections, which provide a physical barrier to glutamate availability at neuronal receptors, astrocytic glutamate uptake, which reduces the extracellular concentration of glutamate, and the release of protective factors by astrocytes. There is evidence supporting that several mechanisms mediate astrocyte protection from glutamate neurotoxicity. The protective effect of astrocytes is reverted or attenuated when glial glutamate uptake is inhibited (Rosenberg et al., 1992; Dugan et al., 1995; Amin and Pierce, 1997; Kawahara et al., 2002). bFGF has been suggested to mediate astroglial protection (Mattson and Rychlik, 1990). Glial-conditioned medium protects neuronal cells from glutamate neurotoxicity (Amano et al., 1994). Brown (2000) have shown that the neuronal release of vasoactive intestinal peptide (VIP) is necessary for the neuroprotective effect of astrocytes on glutamate neurotoxicity, inducing changes in astrocytes that provide protection for neurons, such as an increase in glutamate uptake and the release of protective factors such as IL-6. As reported by Hailer et al. (2001), astrocyte-conditioned media reduce NMDA-induced neuronal injury in organotypic hippocampal slice cultures, which may be due to the presence of unidentified neuroprotective factors or to the inhibition of microglial activation. However, we observed that the protective role of glial cells was not present over a certain glutamate concentration, at which the neurotoxic effect of glutamate in neuronal-glial cultures was stronger than in neuronal cultures. In this case, glial cells turned from neuroprotective to neurotoxic. This may be due to the fact that, although glutamate treatment did not cause glial cell death at the concentrations tested, it altered the mechanisms responsible for the neuroprotection observed. In this regard, Kawahara et al. (2002) have described that astrocytes use their glutamate transporter GLT-1 to protect neurons from glutamate toxicity but also to kill neurons through glutamate toxicity depending on their status. The increased neuronal death resulting from the loss of neuroprotection by glial cells probably induced glial activation, either by the loss of contact between neurons and glial cells or the release of factors by the damaged neurons. Glial activation involves the production of a series of compounds with known neurotoxic actions, such as cytokines and NO, which may play a role in the increased neuronal death observed in our neuronal-glial cultures. Although we did not detect NO production in neuronal-glial cultures at any of the concentrations of glutamate used, the production of other neurotoxic compounds cannot be discarded. The morphological changes in both astroglial and microglial cells treated with 100 μM glutamate suggest that glial activation is triggered by glutamate-induced neuronal damage.

Several studies in vitro show that the induction of glial activation has a neurotoxic effect and that NO and glutamate are key mediators of this effect (Boje and Arora, 1992; Chao et al., 1992, 1995a; Bronstein et al., 1995; Meda et al., 1995; Bal-Price and Brown, 2001). As reported by Bal-Price and Brown (2001), NO production by glial cells inhibits neuronal respiration, leading to glutamate release and excitotoxicity. NO also induces glutamate release from astrocytes (Bal-Price et al., 2002). In addition, cytokines can inhibit astrocytic glutamate uptake through a pathway involving NO (Ye and Sontheimer, 1996). However, NO production induced by cytokines in neuronal-glial cultures is not always associated with neuronal death (Jehon et al., 1998). We treated neuronal and neuronal-glial cerebellar granule cell cultures with LPS to induce glial activation. NO production increased in neuronal-glial cultures after LPS treatment owing to the induction of iNOS in microglial cells, but we did not detect significant neuronal death at the concentration of LPS used. However, LPS pretreatment rendered neurons more sensitive to glutamate in our neuronal-glial cultures. Indeed, the neurotoxicity of 15 and 20 μM glutamate in neuronal-glial cultures after LPS treatment was similar to that observed in neuronal cultures treated with glutamate alone, and the neurotoxicity of glutamate was higher at 10 and 100 μM in the LPS-pretreated neuronal-glial than in glutamate-treated neuronal cultures ($P < 0.05$ and $P < 0.001$, respectively, Student's t-test). These results suggest that LPS treatment resulted in the loss of the protective properties of glial cells from glutamate neurotoxicity and/or in the production of factors that directly or indirectly modulate glutamate neurotoxicity. Although LPS-induced NO production was not linked to significant neuronal death and NO production in LPS-treated cultures did not further increase after glutamate treatment, the observed NO production by microglial cells may be involved in the increased sensitivity to glutamate neurotoxicity through the above-mentioned effects on the inhibition of glutamate uptake by astrocytes (Ye and Sontheimer, 1996), the inhibition of neuronal respiration (Bal-Price and Brown, 2001), or the induction of the release of vesicular glutamate by astrocytes (Bal-Price et al., 2002). However, as LPS treatment did not modify glutamate neurotoxicity in our neuronal cultures, the increased glutamate neurotoxicity observed after LPS pretreatment in neuronal-glial cultures is probably mediated by glial cells. Nevertheless, factors other than NO may participate in this effect. Several reports show that treatment of neuronal cultures in the presence of glial cells with substances inducing glial activation enhances the effect of additional insults, such as oxygen and/or glucose deprivation (Hewett et al., 1996; Kim et al., 1999a, 1999b), 1-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxydopamine (Mc-

Naught and Jenner, 1999), or NMDA treatment (Hewett et al., 1994; Kim and Ko, 1998). In addition, glial cells indirectly increase the neurotoxic effect of trimethyltin through the production of neurotoxic substances (Viviani et al., 1998). NO, glutamate, Ca^{2+}, reactive oxygen species, and cytokines contribute to the enhancement observed in these situations.

Our results and those cited in the present discussion show that the response of neurons to a certain stimulus depends on the presence of glial cells, and that the status of glial cells has a critical role in the final response of neurons. The induction of glial activation may enhance the negative effects of neurotoxic stimuli mostly because of microglial activation and partially through the action of the induced NO production. Glial activation associated with neuronal damage plays a crucial role in the progression and the magnitude of the neural damage and probably in neurodegenerative diseases. Activated glial cells expressing cytokines and inflammatory mediators are concomitant to neuronal damage in several neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, in which inflammation mediated by glial activation may be critical for the progression of neuropathology (Benveniste et al., 2001; Teismann et al., 2003). Strategies to control this inflammatory response have been proposed as new therapeutic approaches for the treatment of certain neurodegenerative diseases. In addition, as the neuronal response to a negative stimulus can be modulated through an action on glial cells, strategies aimed not only to inhibit the negative effects of glial activation but also at enhancing the protective properties of glial cells as mechanisms of neuroprotection may offer great potential.

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REFERENCES

Allan SM, Rothwell NJ. 2001. Cytokines and acute neurodegeneration. Nat Rev 2:734–744.

Amano S, Ohashi M, Ishiko T, Hazama F. 1994. Humoral factors derived from glial cells protect cultured neuroblastoma cells against glutamate toxicity. Brain Res 645:347–350.

Amin N, Pearce B. 1997. Glutamate toxicity in neuron-enriched and neuron-astrocyte co-cultures: effect of the glutamate uptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylate. Neurochem Int 30:271–276.

Bal-Price A, Brown GC. 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci 21:6480–6491.

Bal-Price A, Moneer Z, Brown GC. 2002. Nitric oxide induces rapid, calcium-dependent release of vesicular glutamate and ATP from cultured rat astrocytes. Glia 40:312–323.

Beaman-Hall CM, Leahy CL, Benmansour S, Vallano ML. 1998. Glia modulate NMDA-mediated signalling in primary cultures of cerebellar granule cells. J Neurochem 71:1993–2005.

Benveniste ET, Nguyen VT, O'Keefe GM. 2001. Immunological aspects of microglia: relevance to Alzheimer's disease. Neurochem Int 39:381–391.

Boje KM, Arora PK. 1992. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. Brain Res 587: 250–256.

Bronstein DM, Perez-Otano I, Sun V, Mullis Sawin SB, Chan J, Wu G-C, Hudson PM, Kong L-Y, Hong J-S, McMillian MK. 1995. Glia-dependent neurotoxicity and neuroprotection in mesencephalic cultures. Brain Res 704:112–116.

Brown DR. 1999. Neurons depend on astrocytes in a coculture system for protection from glutamate toxicity. Mol Cell Neurosci 13:379–389.

Brown DR. 2000. Neuronal release of vasoactive intestinal peptide is important to astrocytic protection of neurons from glutamate toxicity. Mol Cell Neurosci 15:465–475.

Chang RCC, Hudson P, Wilson B, Haddon L, Hong J-S. 2000. Influence of neurons on lipopolysaccharide-stimulated production of nitric oxide and tumor necrosis factor-α by cultured glia. Brain Res 853:236–244.

Chao CC, Hu S, Molitor TW, Shaskan, EG, Peterson PK. 1992. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. J Immunol 149:2736–2741.

Chao CC, Hu S, Ehrlich L, Peterson PK. 1995a. Interleukin-1 and tumor necrosis factor-α synergistically mediate neurotoxicity: involvement of nitric oxide and of N-methyl-D-aspartate receptors. Brain Behav Immun 9:355–365.

Chao CC, Hu S, Peterson PK. 1995b. Glia, cytokines and neurotoxicity. Crit Rev Neurobiol 9:189–205.

Dugan LL, Bruno,VMG, Amagasu SM, Giffard RG. 1995. Glia modulate the response of murine cortical neurons to excitotoxicity: glia exacerbate AMPA neurotoxicity. J Neurosci 15:4545–4555.

Gegelashvili G, Danbolt NC, Schousboe A. 1997. Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. J Neurochem 69:2612–2615.

Giulian D. 1993. Reactive glia as rivals in regulating neuronal survival. Glia 7:102–110.

Giulian D, Vaca K, Corpuz M. 1993. Brain glia release factors with opposing actions upon neuronal survival. J Neurosci 13:29–37.

Hailer NP, Wirjatijasa F, Roser N, Hischebeth GTR, Korf H-W, Dehghani F. 2001. Astrocytic factors protect neuronal integrity and reduce microglial activation in an in vitro model of N-methyl-D-aspartate-induced excitotoxic injury in organotypic hippocampal slice cultures. Eur J Neurosci 14:315–326.

Hewett SJ, Csernansky CA, Choi DW. 1994. Selective potentiation of NMDA-induced neuronal injury following induction of astrocytic iNOS. Neuron 13:487–494.

Hewett SJ, Muir JK, Lobner D, Symons A, Choi DW. 1996. Potentiation of oxygen-glucose deprivation–induced neuronal death after induction of iNOS. Stroke 27:1586–1591.

Jehon G-H, Kong L-Y, Wilson B, Hudson P, Hong J-S. 1998. Synergistic neurotoxic effects of combined treatments with cytokines in murine primary mixed neuron/glia cultures. J Neuroimmunol 85: 1–10.

Kaltschmidt B, Kaltschmidt C. 2000. Constitutive NF-κB activity is modulated via neuron-astroglia interaction. Exp Brain Res 130: 100–104.

Kawahara K, Hosoya R, Sato H, Tanaka M, Nakajima T, Iwabuchi S. 2002. Selective blockade of astrocytic glutamate transporter GLT-1 with dihydrokainate prevents neuronal death during ouabain treatment of astrocyte/neuron cocultures. Glia 40:337–349.

Kim W-K, Ko KH. 1998. Potentiation of N-methyl-D-aspartate-mediated neurotoxicity by immunostimulated murine microglia. J Neurosci Res 54:17–26.

Kim W-K, Chung J-H, Kim H-C, Ko KH. 1999a. Nitric-oxide-enhanced excitotoxicity-independent apoptosis of glucose-deprived neurons. Neurosci Res 33:281–289.

Kim WK, Seo DO, Choi JJ, Ko KH. 1999b. Immunostimulated glial cells potentiate glucose deprivation-induced death of cultured rat cerebellar granule cells. J Neurotrauma 16:415–424.

Matsutani S, Yamamoto N. 1997. Neuronal regulation of astrocyte morphology in vitro is mediated by GABAergic signalling. Glia 20:1–9.

Mattson MP, Rychlik B. 1990. Glia protect hippocampal neurons against excitatory amino acid-induced degeneration: involvement of fibroblast growth factor. Int J Dev Neurosci 8:399–415.

Meda L, Cassatella MA, Szendrei GI, Otvos L Jr, Baron P, Villalba M, Ferrari D, Rossi F. 1995. Activation of microglial cells by β-amyloid protein and interferon-γ. Nature 374:647–650.

McNaught KSP, Jenner P. 1999. Altered glial function causes neuronal death and increases neuronal susceptibility to 1-methyl-4-phenylpyridinium- and 6-hydroxydopamine-induced toxicity in astro-

cytic/ventral mesencephalic co-cultures. J Neurochem 73:2469–2476.
Novelli A, Reilly JA, Lysko PG, Henneberry RC. 1988. Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. Brain Res 451:205–212.
Pennypacker KR, Hong JS, Mullis SB, Hudson PM, McMillian MK. 1996. Transcription factors in primary glial cultures: changes with neuronal interactions. Mol Brain Res 37:224–230.
Raivich G, Bohatschek M, Kloss CUA, Werner A, Jones LL, Kreutzberg GW. 1999. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res Rev 30:77–105.
Rosenberg PA, Aizenman E. 1989. Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. Neurosci Lett 103:162–168.
Rosenberg PA, Amin S, Leitner M. 1992. Glutamate uptake disguises neurotoxic potency of glutamate agonists in cerebral cortex in dissociated cell culture. J Neurosci 12:56–61.
Shea TB. 1994. Toxic and trophic effects of glial-derived factors on neuronal cultures. Neuroreport 5:797–800.
Solà C, Casal C, Tusell JM, Serratosa J. 2002. Astrocytes enhance lipopolysaccharide-induced nitric oxide production by microglial cells. Eur J Neurosci 16:1275–1283.
Streit WJ, Walter SA, Pennell NA. 1999. Reactive microgliosis. Prog Neurobiol 57:563–581.
Vitkovic L, Bockaert J, Jacque C. 2000. "Inflammatory" cytokines: neuromodulators in normal brain? J Neurochem 74:457–471.
Tanaka J, Maeda N. 1996. Microglial ramification requires nondiffusible factors derived from astrocytes. Exp Neurol 137:367–375.
Tanaka J, Toku K, Sakanaka M, Maeda N. 1999. Morphological differentiation of microglial cells in culture: involvement of insoluble factors derived from astrocytes. Neurosci Res 34:207–215.
Teismann P, Tieu K, Cohen O, Choi D-K, Wu DC, Marks D, Vila M, Jackson-Lewis V, Przedborski S. 2002. Pathogenic role of glial cells in Parkinson's disease. Mov Disord 18:121–129.
Vincent VAM, Van Dam A-M, Persoons JHA, Schotanus K, Steinbusch HWN, Schoffelmeer, ANM, Berkenbosch F. 1996. Gradual inhibition of inducible nitric oxide synthase but not interleukin-1β production in rat microglial cells of endotoxin-treated mixed glial cell cultures. Glia 17:94–102.
Vitkovic L, Bockaert J, Jacque C. 2000. "Inflammatory" cytokines: neuromodulators in normal brain? J Neurochem 74:457–471.
Viviani B, Corsini E, Galli CL, Marinovich M. 1998. Glia increase degeneration of hippocampal neurons through release of tumor necrosis factor-α. Toxicol Appl Pharmacol 150:271–276.
Ye Z-C, Sontheimer H. 1996. Cytokine modulation of glial glutamate uptake: a possible involvement of nitric oxide. Neuroreport 7:2181–2185.
Yoshida M, Saito H, Katsuki H. 1995. Neurotrophic effects of conditioned media of astrocytes isolated from different brain regions on hippocampal and cortical neurons. Experientia 51:133–136.
Zhang S-C, Fedoroff S. 1996. Neuron-microglia interactions in vitro. Acta Neuropathol 91:385–395.

4.2- SEGUNDO TRABAJO

Excitotoxic and apoptotic neuronal death induce different patterns of glial activation in vitro

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RESUMEN

La activación glial asociada al daño neuronal puede ser determinante en la progresión y la magnitud del mismo. Se ha sugerido que la glía reactiva contribuye a la disfunción y a la consecuente muerte neuronal en enfermedades neurodegenerativas o como consecuencia de lesiones o agentes neurotóxicos. La naturaleza de la señal que desencadena la activación glial se desconoce, pero se ha propuesto que tanto el contacto directo entre las neuronas y las células gliales y/o los factores solubles secretados por las neuronas dañadas juegan un papel relevante. Dado que según el tipo de muerte neuronal inducida por un determinado estímulo estos parámetros pueden diferir, la respuesta de las células gliales también puede ser diferente. En este trabajo hemos estudiado la activación glial inducida por dos tipos de muerte neuronal *in vitro*. Para ello se han utilizado cultivos mixtos de neurona-glía de cerebelo de rata, los cuales han sido expuestos a: a) una concentración elevada de glutamato, que induce muerte neuronal por excitotoxicidad y b) la deprivación de K^+ en el medio de cultivo, un modelo ampliamente utilizado para inducir muerte por apoptosis de las neuronas granulares de cerebelo. Hemos comparado el patrón de activación glial en estos dos modelos de muerte neuronal mediante la evaluación de los siguientes parámetros: activación de NFκB, producción de NO y de TNF-α, proliferación y fagocitosis. Aunque en los dos modelos se produce la muerte de la mayoría de las neuronas después de 24 h de los tratamientos, se observaron diferencias en distintos parámetros evaluados. Cuando las neuronas mueren por excitotoxicidad las células gliales responden con cambios morfológicos, producen moléculas relacionadas con una respuesta pro-inflamatoria, proliferan y fagocitan. En contraste, la respuesta pro-inflamatoria y la proliferación no tienen lugar en presencia de muerte neuronal por apoptosis, mientras que la fagocitosis es rápidamente inducida. Estos resultados sugieren que el mecanismo por el cual mueren las neuronas determina el patrón de activación de las células gliales, lo cual puede influir en el daño final inducido. También muestran que diferentes aspectos de la activación glial pueden ser regulados de manera independiente.

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Excitotoxic and apoptotic neuronal death induce different patterns of glial activation *in vitro*

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Abstract

We have studied glial activation in rat cerebellar neuronal-glial cultures after inducing neuronal death using various stimuli. Cultures were exposed to 100 μM glutamate for 20 min, which induces excitotoxic neuronal death, or to potassium/serum deprivation, which induces apoptosis of granule neurons. We evaluated alterations in several parameters related to glial activation: nuclear factor-κB activation, nitric oxide and tumour necrosis factor-α production, which are associated with a pro-inflammatory response, glial proliferation and phagocytic activity. Although the two experimental models of neuronal damage resulted in the death of most neuronal cells within 24 h, differences were observed in the response of the various glial parameters evaluated. While nitric oxide production was not detected in any case, tumour necrosis factor-α production, nuclear factor-κB activation and glial proliferation were only induced in the presence of excitotoxic neuronal death. However, phagocytosis was induced in both cases, although earlier in the case of apoptotic neuronal death. These results show that glial cells respond to excitotoxic neuronal death with an inflammatory response associated with proliferation and phagocytosis. In contrast, whilst glial cells do not produce pro-inflammatory molecules in the presence of apoptotic neuronal death, phagocytic activity is rapidly induced.

Keywords: astroglia, microglia, nuclear factor-κB, phagocytosis, proliferation, tumour necrosis factor-α.

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In the mammalian CNS, neuronal and glial cells establish a close relationship that is responsible for the normal function of the brain. Consequently, alterations in the normal behaviour of either neuronal or glial cells affect normal cerebral function (reviewed in Aschner *et al*. 2002; Polazzi and Contestabile 2002; Chen and Swanson 2003). Although various stimuli can selectively affect glial or neuronal function, the final outcome may be an alteration in the function of both cell types. Most studies of neuron–glia interaction have made use of *in vitro* approaches, where the role of different cell populations can be more easily discriminated. Thus, mixed neuronal-glial cultures, co-cultures of neurons and glial cells with or without direct contact between the different cell populations, or the use of conditioned media from neuronal or glial-enriched cultures have been successfully used to study neuron–glia interactions. Using these approaches, various authors have shown that stimulation of glial cells using inflammatory agents or cytokines results in neuronal death, although these agents do not have any direct effect on neuronal viability (Meda *et al*. 1995; Jeohn *et al*. 1998; Bal-Price and Brown 2001). In addition, glial activation enhances the effects of additional insults, such as oxygen and/or glucose deprivation (Hewett *et al*. 1996; Kim *et al*. 1999a,b), exposure to MPP^+ and 6-hydroxydopamine (McNaught and Jenner 1999), NMDA (Hewett *et al*. 1994; Kim and Ko 1998) or glutamate (Pérez-Capote *et al*. 2004). Similarly, the presence of glial cells has been shown to play a role in the induction of neurotoxicity by molecules involved in certain neurodegenerative diseases (Brown 1998; Malchiodi-Albedi *et al*. 2001).

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Abbreviations used: ABC, avidin-biotin-peroxidase complex; BME, Eagle's basal medium; BrdU, 5′-bromo-2′deoxyuridine-5′-monophosphate; DAB, 3,3′-diaminobenzidine tetrahydrochloride; FBS, fetal bovine serum; FDA, fluorescein diacetate; GFAP, glial fibrillary acidic protein; LPS, lipopolysaccharide; NeuN, neuronal nuclei protein; NF-κB, nuclear factor-κB; NO, nitric oxide; PB, phosphate buffer; PBS, phosphate-buffered saline; PBST, PBS-0.1% Triton X-100; RT, room temperature; TBS, Tris-buffered saline; TBST, TBS-0.1% Triton X-100; TNF-α, tumour necrosis factor-α.

Neuronal damage induced by different stimuli usually results in morphological and functional changes in glial cells, constituting what is known as glial activation or formation of reactive glial cells. Activation of glial cells has been proposed to contribute to neuronal dysfunction and neuronal cell death in neurodegenerative diseases (González-Scarano and Baltuch 1999; Benveniste *et al.* 2001; Brown 2001; Teismann and Schulz 2004). Astroglial and microglial cells are able to respond in this way, producing growth factors, cytokines, nitric oxide (NO) and neuropeptides, among others; glial activation can also be accompanied by glial cell proliferation and phagocytosis (reviewed in Raivich *et al.* 1999; Streit *et al.* 1999). The factors produced by reactive glial cells can be involved in either neuroprotection or neurotoxicity, playing an important role in the neuronal damage initiated by a given stimulus. The role played by reactive glial cells in the development of neuronal damage appears to depend on the stimulus that induces neuronal damage and the severity of the damage itself; this determines which molecules are produced by glial cells, as well as the presence or absence of glial cell proliferation and phagocytosis. The nature of the signal that induces glial activation in response to neuronal damage remains unknown, although direct contact between neuronal and glial cells as well as soluble factors delivered by the damaged neurons probably play an important role (Neumann 2001). In addition, when neuronal damage results in neuronal death, the factors released by the damaged neurons will differ depending on whether death occurs by necrosis, in which the integrity of the cell membrane is compromised and the cellular components reach the extracellular compartment, or apoptosis, whereby cell membranes keep their integrity. Consequently, the glial response to neuronal death may also differ according to the way in which it occurs.

Given the complexity of studying glial activation *in vivo*, numerous authors have approached the subject using *in vitro* experimental models. Various *in vitro* studies show that glial activation is associated with the production of cytokines, reactive oxygen species and NO, among others. However, most experimental approaches use glial cell cultures, and/or the stimulus used to induce glial activation is an inflammatory agent or a mixture of cytokines that have a direct effect and cause significant modifications of glial cells (Meda *et al.* 1995; Forloni *et al.* 1997; Yao and Johnson 1997; Murphy *et al.* 1998; Conti *et al.* 1999; Johnstone *et al.* 1999; Possel *et al.* 2000; Golde *et al.* 2002). In contrast, comparatively few studies have addressed glial activation in the presence of neurons employing neuronal damage as the inducing stimulus (Viviani *et al.* 1998, 2000). In the present work, we have studied glial activation occurring in cerebellar neuronal-glial cell cultures in response to different forms of neuronal death. First, these neuronal-glial cultures were exposed to a high concentration of glutamate, which induces excitotoxic neuronal death. Second, we exposed cerebellar neuronal-glial cultures, which need to be cultured in a medium containing a high concentration of K^+ (25 mM K^+), to serum-free medium containing a normal concentration of K^+ (5 mM K^+), a well established and widely used model of apoptosis in cerebellar granule neurons first described by D'Mello *et al.* (1993). When medium is changed in mature cerebellar granule neurons in culture (from 4 to 5 days *in vitro*), serum has to be omitted because addition of medium containing fresh serum has a toxic effect on these cells, resulting in rapid and extensive neuronal death (Schramm *et al.* 1990). In parallel, we induced glial activation in neuronal-glial cultures with lipopolysaccharide (LPS) from *E. coli*, an inflammatory agent widely used to induce glial activation *in vitro*. The aim of this study was (i) to assess the presence of glial activation *in vitro* in response to neuronal death and (ii) to investigate possible differences in glial activation in response to excitotoxic or apoptotic neuronal death.

Materials and methods

Materials

Reagents were from Sigma Chemicals Co. (St Louis, MO, USA) unless otherwise stated.

Cell cultures

Cultures of cerebellar granule cells were prepared from 7-day-old rats (Wistar; Iffa Credo, Lyon, France) as previously described (Pérez-Capote *et al.* 2004). Cells were seeded in 5 μg/mL poly-L-lysine-coated 24-well plates (Nalge Nunc International, Naperville, IL, USA) at a density of 3×10^5 cells/cm^2 in 0.5 mL medium [Eagle's basal medium (BME; Gibco-BRL, Life Technologies, Paisley, UK) supplemented with 20 mM KCl (25 mM final concentration), 2 mM glutamine, 100 μg/mL gentamicin and 10% fetal bovine serum (FBS; Gibco-BRL)]. Cells were maintained at 37°C in a 5% CO_2 and 95% humidity atmosphere. Two types of cultures were prepared: (i) neuronal-glial cultures and (ii) mixed glial cultures. Cultures were routinely used at 8 days *in vitro*. In neuronal-glial cultures, the medium was never changed, but 5.6 mM glucose was added 4 days after seeding to enhance survival. Glial content in neuronal-glial cultures was assessed after 8 days in culture using immunocytochemical techniques as described below. Neuronal-glial cultures contained 6.3 ± 0.7% astrocytes and 7.6 ± 1.3% microglia. In mixed glial cultures, medium was changed 24 h after seeding and replaced with medium without added KCl, then renewed every 3 days. After 8 days in culture, these cultures contained mainly astroglial and microglial cells in similar proportions, as assessed using immunocytochemical techniques as described below.

Treatments

Glutamate treatment

Cell cultures were treated with glutamate 8 days after seeding. The preconditioned growth medium was collected, and cells were washed twice with 1 mL Mg^{2+}-free Locke's solution containing 155 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 8.4 mM HEPES and 5.6 mM glucose, pH 7.4. Primary cultures were incubated for

20 min at room temperature with 0.5 mL Mg^{2+}-free Locke's solution containing 100 μM glutamate. Glutamate exposure was stopped by washing the cells twice with 1 mL standard Locke's solution (containing 1 mM $MgCl_2$). The preconditioned growth medium was then replaced and the cultures were returned to the incubator. Control cells were processed in parallel and exposed to Mg^{2+}-free Locke's solution without glutamate.

Potassium deprivation

Cerebellar neuronal-glial cultures were allowed to develop for 8 days in culture medium with 10% fetal bovine serum (FBS) and 25 mM KCl; this ensured the differentiation and survival of cerebellar granule neurones in culture. Eight days after seeding, cells were washed once with standard Locke's solution and exposed to potassium deprivation by maintaining them in serum-free BME (containing 5 mM KCl) supplemented with 2 mM glutamine and 100 μg/mL gentamicin. Control cells were processed in parallel and maintained in the same medium supplemented with 20 mM KCl (25 mM KCl final concentration).

Staurosporine treatment

Staurosporine treatment was used as a positive control for apoptotic neuronal death. Neuronal-glial cultures were treated with 1 μM staurosporine for 3 h, 8 days after seeding. Staurosporine was added directly to the culture medium.

Lipopolysaccharide (LPS) treatment

In some groups of experiments, LPS treatment was used as a positive control for glial activation. In these cases, neuronal-glial cultures were treated with LPS (1 μg/mL: *E. coli* 026:B6) 8 days after seeding. LPS was added directly to the culture medium. In preliminary experiments, we observed that LPS treatment of mixed glial cultures induced NO and tumour necrosis factor-α (TNF-α) production, as well as nuclear factor-κB (NF-κB) nuclear translocation (data not shown).

Assessment of cell viability

Neuronal viability in neuronal-glial cell cultures was routinely estimated by fluorescent staining. Twenty-four hours after glutamate treatment or potassium deprivation, cells were washed with standard Locke's solution before and after incubation with fluorescein diacetate (FDA, 15 μg/mL) for 5 min, and then examined under a fluorescence microscope. Images of two to four microscopic fields were obtained per well using a 20× objective. Two wells per experimental condition were processed and each experimental condition was repeated three times. Visual counting of live neurons was performed and neuronal viability was expressed as a percentage of control.

Assessment of nuclear morphology

Cells were fixed with 4% paraformaldehyde in 0.1 phosphate buffer (PB, pH 7.4) for 20 min at room temperature (RT), permeated in chilled methanol for 8 min and then labelled with Hoechst 33258 (1.25 μg/mL) for 15 min. Labelled nuclei were viewed on a microscope with ultraviolet light illumination.

Caspase 3 activation

We used a rabbit polyclonal antibody [cleaved caspase 3 (Asp175) antibody, Cell Signalling Technology, Beverly, MA, USA] to detect endogenous levels of activated caspase 3 in order to study apoptotic cell death. Immunocytochemistry was performed according to the manufacturer's instructions. Briefly, cells were washed with standard Locke's solution and fixed with 3% paraformaldehyde in 0.1 M PB (pH 7.4) for 20 min at 4°C. Non-specific staining was blocked by incubating with 5% serum in Tris-buffered saline (TBS)-0.1% Triton X-100 (TBST) for 60 min at RT. The cells were then incubated with the antibody (1/100) for 24 h at 4°C in TBST containing 5% serum, and with biotinylated goat anti-rabbit secondary antibody (1/500) in TBST containing 5% serum for 1 h at RT. Endogenous peroxidase activity was blocked by incubation with 0.6% H_2O_2 in TBS. The cells were then incubated with avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories, Peterborough, UK) for 1 h at RT, and colour was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Activation of caspase 3 was analysed 4 and 8 h after glutamate treatment or potassium deprivation.

To identify the caspase 3-positive cells, sequential double immunofluorescence was performed using anti-caspase 3 antibody and anti-neuronal nuclei protein (NeuN, MAB377; Chemicon International, Temecula, CA, USA; 1/150 dilution), anti-glial fibrillary acidic protein (GFAP) and anti-CD68 antibodies.

NF-κB activation

We detected translocation of the NF-κB subunit p65 in glial cells by immunocytochemistry, as a marker of glial activation, using the protocol described in the Immunocytochemical analysis section below. The p65 translocation was analysed 1, 4, 8, 10 or 12 h after glutamate treatment or potassium deprivation.

Nitrite assay

The production of NO was assessed using the Griess reaction. Briefly, 100 μL aliquots of culture supernatant fluids were collected 24 h after glutamate treatment, potassium deprivation or LPS treatment and incubated with equal volumes of Griess reagent for 10 min at RT. Absorbance at 540 nm was measured using a microplate reader (iEMS Reader MF, Labsystems, Espoo, Finland). Nitrite concentration was determined from a sodium nitrite standard curve (0–50 μM).

Immunocytochemical analysis

Single immunocytochemistry using peroxidase

Astroglial and microglial cells were identified in cerebellar cultures with a rabbit anti-GFAP polyclonal antibody (Dako, Glostrup, Denmark) and an anti-CD68 (or ED1) monoclonal antibody (Serotec, Oxford, UK), respectively. Cells were fixed with 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 20 min at RT. Cells were permeated and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 10 min. Non-specific staining was blocked by incubating the cells with 5% serum in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 20 min at RT. The cells were then incubated with anti-GFAP anti-GFAP (1/500) or anti-CD68 (1/200) antibodies overnight at 4°C in 1% serum, and with biotinylated goat anti-rabbit (1/200) or horse anti-mouse (1/200) secondary antibodies in 1% serum for 1 h at RT. Following incubation with ABC reagent for 1 h at RT, colour was developed with DAB. In some cases, counterstaining with haematoxylin was also carried out.

Double immunocytochemistry using fluorescence labelling

To identify NF-κB nuclear translocation in glial cells, sequential double immunocytochemistry was performed using a monoclonal antibody for the p65 subunit of NF-κB (Santa Cruz, Heidelberg, Germany), together with a rabbit anti-GFAP polyclonal antibody (Dako) or an anti-CD68 monoclonal antibody (Serotec) to label astroglial and microglial cells, respectively. Cells were fixed with chilled methanol for 8 min and non-specific staining was blocked as described above. Cells were incubated overnight at 4°C with anti-p65 monoclonal antibody (1/50) and for 1 h at RT with the secondary antibody goat anti-mouse ALEXA 488 (1/1000) (Molecular Probes Europe BV, Breda, the Netherlands). Immunoreactivity was visualized under a fluorescence microscope and images were obtained using a 40× objective. Then, non-specific staining was blocked again as described above, and the cells were incubated overnight at 4°C with either rabbit anti-GFAP polyclonal antibody (1/2000) or anti-CD68 monoclonal antibody (1/500), and for 1 h at RT with the corresponding fluorescent secondary antibodies: goat anti-rabbit ALEXA 546 (1/1500) for rabbit anti-GFAP and goat anti-mouse ALEXA 546 (1/1500) for mouse anti-CD68 (Molecular Probes). Images from the fields that were photographed previously were obtained using a 40× objective.

TNF-α ELISA

The production of TNF-α was assessed using an ELISA kit specific for rat TNF-α (Diaclone, Besançon, France). Briefly, 100 μL aliquots of culture medium were collected 6 h after glutamate treatment, potassium deprivation or LPS treatment and stored at − 80°C until assayed for TNF-α content. The ELISA was performed using the standard and instructions supplied by the manufacturer.

Proliferation assay

To evaluate proliferation in neuronal-glial cultures, 5′-bromo-2′deoxyuridine-5′-monophosphate (BrdU) was added to the medium, at a concentration of 5 μM, 22 h after treating the cultures with 100 μM glutamate for 20 min or exposing them to potassium deprivation. BrdU was allowed to incorporate into proliferating cells for 2 h and the cultures were then fixed with 4% paraformaldehyde in 0.1 M PB, pH 7.4, at RT for 20 min. BrdU incorporation was assessed using a biotinylated anti-BrdU monoclonal antibody (Developmental Studies Hybridoma Bank-University of Iowa, IA, USA). Briefly, cells were permeated with cold methanol for 30 min. Then, they were incubated in 2 M HCl for 30 min, followed by rinsing in 200 mM borate (pH 8) for 15 min. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 10 min. Non-specific staining was blocked with 10% serum in PBS-0.1% Triton X-100 (PBST) for 30 min at RT. The cells were then incubated with anti-BrdU (1/1000) overnight at 4°C and with biotinylated horse anti-mouse antibody (1/200) in PBST for 1 h at RT. Following incubation with ABC reagent in PBST for 1 h at RT, colour was developed with DAB. Images of nine microscopic fields were obtained per well using a 10× objective. Two wells per experimental condition were processed and each experimental condition was repeated at least five times. The number of proliferating cells per microscopic field was referred to the number of glial cells in an equivalent field obtained by calculating the average number of astroglial cells (GFAP-positive cells) plus the number of microglial cells (CD68-positive cells) in five equivalent microscopic fields. Results were expressed as percentage of proliferating glial cells.

To identify the BrdU-positive cells, sequential double immunofluorescence was performed using anti-BrdU antibody and anti-NeuN, anti-GFAP and anti-CD68 antibodies.

Phagocytosis assay

Phagocytic activity was determined in neuronal-glial cultures 4 and 8 h after glutamate treatment or potassium deprivation. Briefly, the cells were incubated for 1 or 2 h at 37°C in the presence of fluorescent microspheres (1/300) [FluoSpheres, carboxylate-modified microspheres, 2.0 μm, red fluorescent (580/605), 2% solids; Molecular Probes, Eugene, OR, USA]. Then, the cells were washed several times with PBS and fixed in chilled methanol for 8 min. Immunocytochemistry was performed using monoclonal anti-CD68 antibody (1/200), a specific marker for microglial cells, according to the protocol described in the Immunocytochemical analysis section above. ALEXA 488 goat anti-mouse secondary antibody was used at 1/1000. Images of nine microscopic fields were obtained per well under a fluorescence microscope using a 40× objective. Two wells per experimental condition were processed and each experimental condition was repeated at least four times. Visual counting of FluoSpheres was performed and results were expressed as average number of fluorescent microspheres per microglial cell.

Imaging and analysis

Microscopy images were obtained with an Olympus IX70 microscope (Okoya, Japan) and a digital camera (CC-12, Soft Imaging System GmbH, Munich, Germany). Visual counting of labelled cells (cell viability, proliferation and phagocytosis assays) or FluoSpheres (phagocytosis assay) was performed with analySIS analysis imaging software (Soft Imaging System GmbH, Germany).

Data presentation and statistical analysis

Results are presented as the mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) to identify overall treatment effects, followed by unpaired or paired Student's *t*-test for comparison of individual data groups with their respective controls. Values of $p < 0.05$ were considered significant.

Results

Induction of neuronal death in cerebellar granule neurons

Rat cerebellar granule neurons were cultured for 8 days in depolarizing levels of K^+ (25 mM KCl) in the presence of serum. Treatment of cerebellar neuronal-glial cultures with 100 μM glutamate for 20 min or lowering the K^+ concentration to 5 mM KCl and removing serum (potassium deprivation) resulted in neuronal death. Morphological changes in the cell cultures were assessed by phase-contrast microscopy at different times following the treatments. At 24 h, neuronal viability was assessed using FDA staining. Glutamate treatment induced rapid alterations in the morphology of neuronal-glial cultures (Fig. 1). Significant alterations in neuronal morphology were observed immediately after glutamate treatment and neuronal death was apparent at

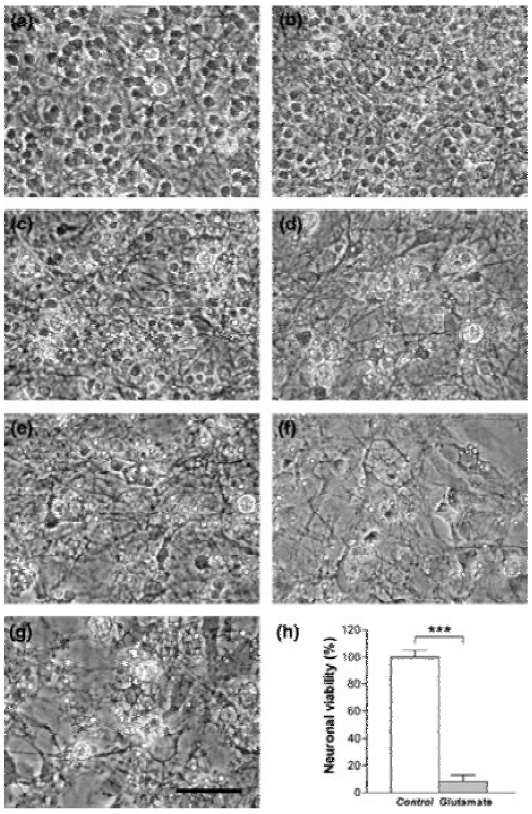

Fig. 1 Effect of glutamate treatment on neuronal-glial cultures. Phase-contrast images showing the appearance of cells in a control culture (a), or following treatment with 100 μM glutamate for 20 min and incubation for 1 h (b), 2 h (c), 4 h (d), 8 h (e), 12 h (f) and 24 h (g). Bar = 50 μm. (h) Neuronal viability 24 h after glutamate treatment. Bars represent the mean ± SEM of three cultures; ***$p < 0.001$ represents differences between neuronal viability in treated versus control cultures; Student's unpaired *t*-test.

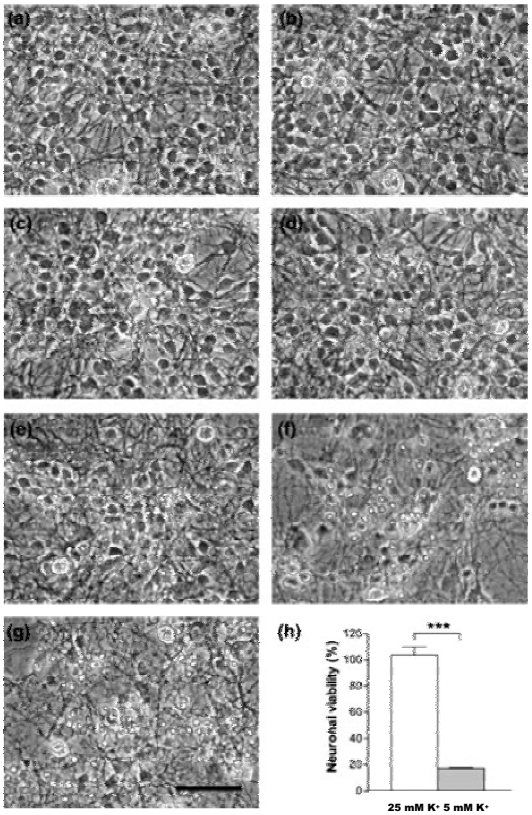

Fig. 2 Effect of potassium deprivation on neuronal-glial cultures. Phase-contrast images showing the appearance of a control culture (a), or the effect of potassium/serum withdrawal after 1 h (b), 2 h (c), 4 h (d), 8 h (e), 12 h (f) and 24 h (g). Bar = 50 μm. (h) Neuronal viability 24 h after potassium deprivation. Bars represent the mean ± SEM of three cultures; ***$p < 0.001$ represents differences between neuronal viability in treated versus control cultures; Student's unpaired *t*-test.

4 h, an effect that increased rapidly with time. Twenty-four hours after treatment with 100 μM glutamate, 92% of the cerebellar granule neurons were dead (Fig. 1). In contrast, most of the neurons remained normal in appearance until 4 h after potassium deprivation. At 4–6 h, some neuronal death was observed and this effect became gradually more evident with time (Fig. 2). Potassium deprivation for 24 h resulted in the death of 83% of granule neurons (Fig. 2).

Changes in nuclear morphology induced in cerebellar granule neurons by glutamate treatment and potassium deprivation

Evident changes in nuclear morphology were observed in granule neurons treated with glutamate. All neuronal nuclei adopted a spherical and condensed appearance immediately after glutamate treatment, an effect that became more evident with time (Figs 3a–e). This effect was not accompanied by nuclear fragmentation. In contrast, granule neurons did not show changes in nuclear morphology shortly after potassium deprivation, but neurons showing both nuclear condensation and apoptotic bodies were observed from 4 h (Figs 3f–l).

Caspase 3 is activated in cerebellar granule neurons in response to potassium deprivation but not to glutamate treatment

Activation of caspase 3, as determined by immunostaining of cleaved caspase 3, was evaluated in neuronal-glial cultures 4 or 8 h after 100 μM glutamate treatment, or after exposure to potassium deprivation for 4 or 8 h. Only scattered neurons were labelled in control and glutamate-treated cultures, at

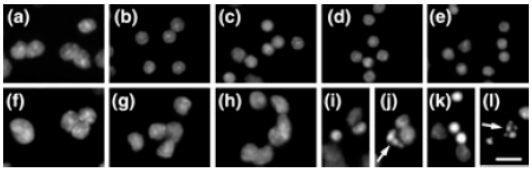

Fig. 3 Changes in nuclear morphology of granule neurons after glutamate treatment or potassium deprivation. Hoechst 33258 fluorescence microscopy showing nuclear morphology of granule neurons in a control culture (a) and following treatment with 100 μM glutamate for 20 min and incubation for 1 h (b), 2 h (c), 4 h (d) and 8 h (e). Note that neuronal nuclei become rounded and smaller after glutamate treatment, showing a condensed appearance that becomes more evident with time. Hoechst 33258 fluorescence microscopy showing nuclear morphology of granule neurons in cultures exposed to fresh serum-free medium (containing 25 mM KCl) (f) or to potassium deprivation (fresh serum-free medium containing 5 mM KCl) for 1 h (g), 2 h (h), 4 h (i, j) and 8 h (k, l). Note that changes in nuclear morphology are evident from 4 h, when both a condensed appearance and apoptotic bodies (arrows) are observed. Bar = 10 μm.

either 4 or 8 h post-treatment (Figs 4a–d). In contrast, numerous neurons showed cleaved caspase 3 immunolabelling in cultures exposed to potassium deprivation for 4 or 8 h, revealing the presence of apoptotic neuronal death (Figs 4e–h). No caspase 3 immunolabelling was detected in glial cells as assessed by double immunolabelling using anti-GFAP and anti-CD68 antibodies. Neurons showing activation of caspase 3 were also observed 3 h after 1 μM staurosporine treatment, which was used as a positive control for apoptosis (data not shown).

Selective induction of NF-κB activation in glial cells in response to neuronal death

NF-κB activation was observed in glial cells in cerebellar neuronal-glial cultures treated with glutamate, as shown by immunolabelling to reveal nuclear translocation of p65. Control cultures generally showed a faint labelling in the cytoplasm of glial cells (Fig. 5a). Some glial cells showing nuclear immunolabelling were also detected in control cultures 1 and 4 h after handling, but not at 8 h. Although similar results were obtained shortly after glutamate treatment (1 h), labelled glial nuclei were consistently observed 4 and 8 h (Fig. 5b) after treatment. Nuclear translocation of p65 was localized in both astrocytes (Fig. 5c) and microglial cells (Fig. 5d). This effect was not observed at later times (10 or 12 h). To test whether the effect observed was a direct response of glial cells to glutamate, the same treatment was applied to glial cell cultures. In this case, only scattered labelled nuclei were observed in both control and glutamate-treated cells 1 and 4 h after handling, but not at later times (data not shown).

NF-κB activation was not observed in neuronal-glial cultures in response to potassium deprivation. The majority

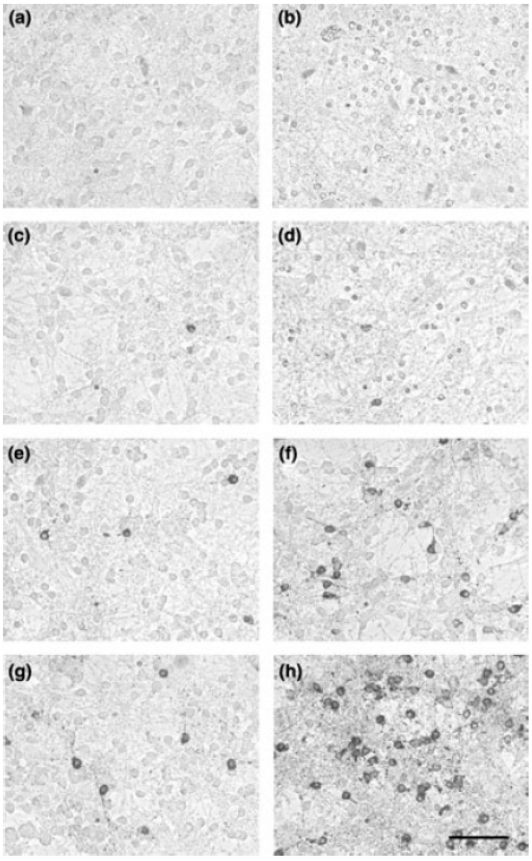

Fig. 4 Caspase 3 activation in neuronal-glial cultures after glutamate treatment or potassium deprivation. Cleaved caspase 3 immunocytochemistry in control (left column) and glutamate-treated cultures (right column, 100 μM glutamate for 20 min), 4 h (a and b) and 8 h (c and d) after treatment, and in cultures exposed to fresh serum-free medium (containing 25 mM KCl, left column) or to potassium deprivation (fresh serum-free medium containing 5 mM KCl, right column) for 4 h (e and f) or 8 h (g and h). Note the presence of a significant number of labelled cells only in the cultures exposed to potassium deprivation; these numbers increase with time. Bar = 50 μm.

of glial cells in control cultures showed faint cytoplasmic immunolabelling for p65 (Fig. 5e), although some scattered glial cells showing nuclear immunolabelling were occasionally detected, mainly soon (1 h) after medium change. Similar results were obtained in cultures submitted to potassium deprivation (Fig. 5f).

When neuronal-glial cell cultures were treated with LPS, nuclear translocation of p65 was consistently observed in the majority of glial cells 1 h after treatment (Figs 5g and h). The effect was attenuated at later times (data not shown). The intensity of p65 immunolabelling attained in LPS-treated

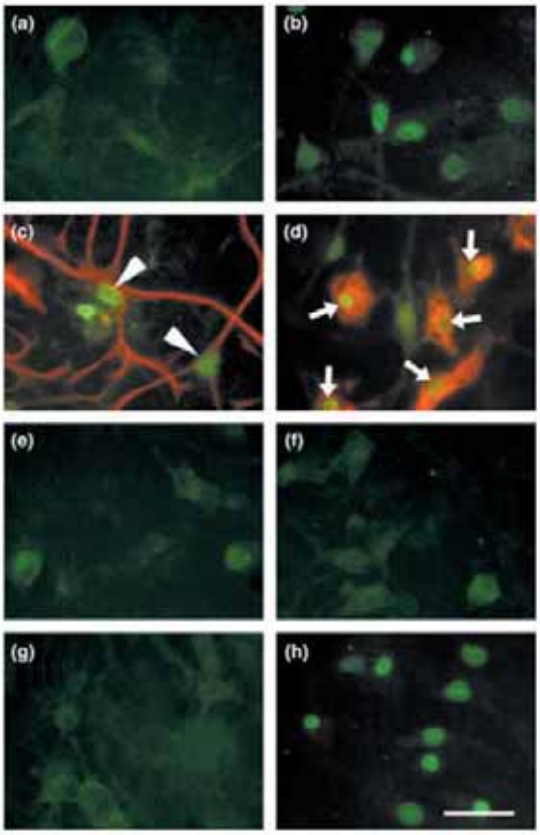

Fig. 5 NF-κB activation in neuronal-glial cultures exposed to glutamate, potassium deprivation or LPS. p65 immunocytochemistry in control (a) and glutamate-treated (100 μM glutamate for 20 min) cultures (b) 8 h after treatment. (c) p65 (green) and GFAP (red) double immunocytochemistry in a glutamate-treated culture showing nuclear staining of p65 in astrocytes (arrowheads). (d) p65 (green) and CD68 (red) double immunocytochemistry in a glutamate-treated culture showing nuclear staining of p65 in microglial cells (arrows). p65 immunolabelling in cultures exposed to (e) fresh serum-free medium (containing 25 mM KCl) or (f) potassium deprivation (fresh serum-free medium containing 5 mM KCl) for 8 h, and in control (g) and LPS-treated (1 μg/mL) cultures (h) 1 h after treatment. Significant translocation of p65 into the nucleus is only observed in glutamate- and LPS-treated cultures. Bar = 30 μm.

cultures was stronger than that observed in glutamate-treated cultures.

NO production is not detected in neuronal-glial cultures after inducing neuronal death by glutamate treatment or potassium deprivation

No increase in NO production was detected in neuronal-glial cell cultures exposed to glutamate or potassium deprivation, because no differences in NO_2^- accumulation

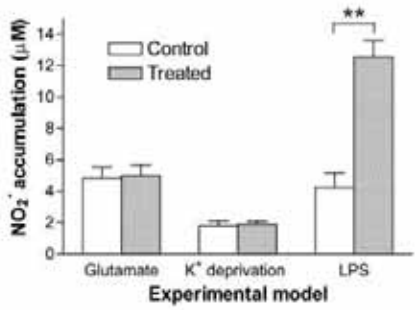

Fig. 6 NO production in neuronal-glial cultures exposed to glutamate, potassium deprivation or LPS. NO production was estimated through the quantification of nitrite accumulated in the culture medium 24 h after treating the cultures with 100 μM glutamate for 20 min, or exposing them to potassium deprivation (5 mM KCl) or to 1 μg/mL LPS. Bars represent the mean ± SEM of three cultures; $^{**}p < 0.01$ represents differences between nitrite accumulation in the culture medium of treated versus control cultures; Student's unpaired *t*-test.

in the culture medium were observed between control and treated cultures 24 h after treatment (Fig. 6). Control values in the glutamate model were higher than in the potassium deprivation model due to the different experimental conditions; in the former, NO_2^- determination was performed in culture medium in contact with the cells for 9 days and in the latter, in culture medium in contact with the cells only for 24 h. Treatment with LPS, however, did cause NO to be produced, indicating that the ability of glial cells to produce NO is not perturbed by the presence of cerebellar granule neurons (Fig. 6).

TNF-α production

Glutamate treatment resulted in an increase in TNF-α production in neuronal-glial cultures, as measured by TNF-α release into the culture medium 6 h after treatment (Fig. 7). No modification of TNF-α production was observed in cultures exposed to potassium deprivation for 6 h (Fig. 7). In contrast, treatment of neuronal-glial cultures with LPS resulted in a drastic increase in TNF-α production 6 h later (Fig. 7). This increase was two orders of magnitude higher than that observed in response to glutamate treatment. In parallel, glial cell cultures were treated with glutamate to test whether the effect observed in neuronal-glial cultures was due to a direct action of glutamate on TNF-α production in glial cells. In this case, no increase in TNF-α production was detected (data not shown).

Glial cell proliferation

A significant increase in cell proliferation was observed in neuronal-glial cultures 24 h after glutamate treatment, as indicated by the increased percentage of cells showing nuclear BrdU incorporation in treated cultures. No labelled

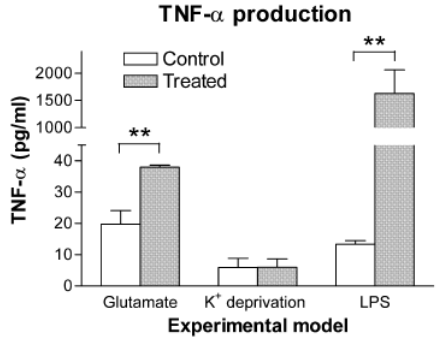

Fig. 7 TNF-α production in neuronal-glial cultures exposed to glutamate, potassium deprivation or LPS. TNF-α levels were determined by ELISA in the culture medium 6 h after treating the cultures with 100 μM glutamate for 20 min, or exposing them to potassium deprivation (5 mM KCl) or to 1 μg/mL LPS. Bars represent the mean ± SEM of three cultures; $^{**}p < 0.01$ represents differences between TNF-α levels in the culture medium of treated versus control cultures; Student's unpaired *t*-test.

neuronal cells were detected as assessed by double immunocytochemistry using anti-NeuN antibody (data not shown). The percentage of proliferating glial cells (Fig. 8) was calculated as explained in Materials and methods. Glial cell proliferation was not significantly increased in neuronal-glial cultures exposed to potassium deprivation for 24 h (Fig. 8).

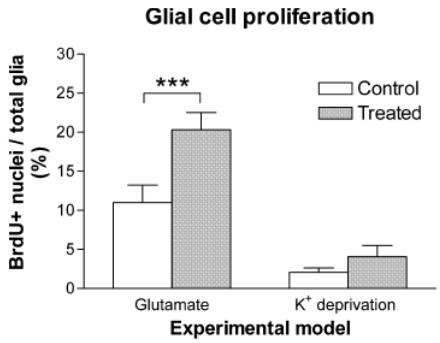

Fig. 8 Glial cell proliferation in neuronal-glial cultures exposed to glutamate or to potassium deprivation. Proliferating cells were identified by BrdU incorporation and subsequent detection by immunocytochemistry using a monoclonal anti-BrdU antibody 24 h after treating the cultures with 100 μM glutamate for 20 min or exposing them to potassium deprivation (5 mM KCl). Data are expressed as percentage of proliferating glial cells in each experimental condition at 24 h, calculated as explained in Materials and methods. Bars represent the mean ± SEM of seven cultures for the glutamate model and five cultures for the potassium deprivation model; $^{***}p < 0.001$ represents differences between glial cell proliferation in treated cultures versus spontaneous proliferation in control cultures; Student's paired *t*-test.

Microglial cell phagocytosis

Glutamate treatment increased the phagocytic activity of microglial cells in neuronal-glial cultures. Although the percentage of microglial cells containing microspheres was not modified after glutamate treatment (Fig. 9a), a significant increase in the number of microspheres per microglial cell was observed in microglial cells taking up microspheres 8 h after glutamate treatment, but not at 4 h (Fig. 9b). Potassium deprivation also induced a significant increase in the phagocytic activity of microglial cells in neuronal-glial cultures. However, under these conditions, the percentage of microglial cells without microspheres was significantly decreased both at 4 and 8 h (Fig. 9a). In addition, the number of microspheres per phagocytic cell increased at 8 h (Fig. 9c).

Discussion

The results presented here show that glial cells in neuronal-glial cultures respond to neuronal damage with functional changes associated with glial activation, as occurs *in vivo*. However, the pattern of response differs depending on the kind of damage induced in the neurons. Although the two experimental models of neuronal damage used (excitotoxicity and apoptosis) resulted in the death of most neuronal cells after 24 h, differences were observed in the response of the various glial parameters evaluated.

Transcription factors such as NF-κB provide a link between extracellular stimuli and the resulting changes in gene expression. Various stimuli activate NF-κB in glial cells. Pro-inflammatory cytokines, such as TNF-α and interleukin-1, result in activation of this transcription factor, and target genes for NF-κB include genes associated with inflammatory responses in the CNS. In the present work, we observed NF-κB activation in astroglial and microglial cells in neuronal-glial cultures treated with glutamate. This effect was transient; it was first detected 4 h after glutamate treatment and was evident at 8 h, but it disappeared subsequently. In contrast, astroglial and microglial cells in mixed glial cultures did not show NF-κB activation in response to glutamate treatment. These results suggest that the NF-κB activation observed in glial cells in neuronal-glial cultures treated with glutamate is indirect, occurring as a consequence of the neuronal alterations induced by the neurotoxic action of glutamate. NF-κB activation was not observed in glial cells after inducing neuronal damage in neuronal-glial cultures by potassium deprivation. This observation suggests that the specific nature of neuronal death determines the response of the surrounding glial cells.

NO production is usually associated with glial activation and appears to play a role in the induction of neuronal damage by activated glial cells. However, NO production by glial cells is not always associated with neuronal damage (Jeohn *et al.* 1998; Pérez-Capote *et al.* 2004), and glial activation and neuronal damage can occur in the absence of

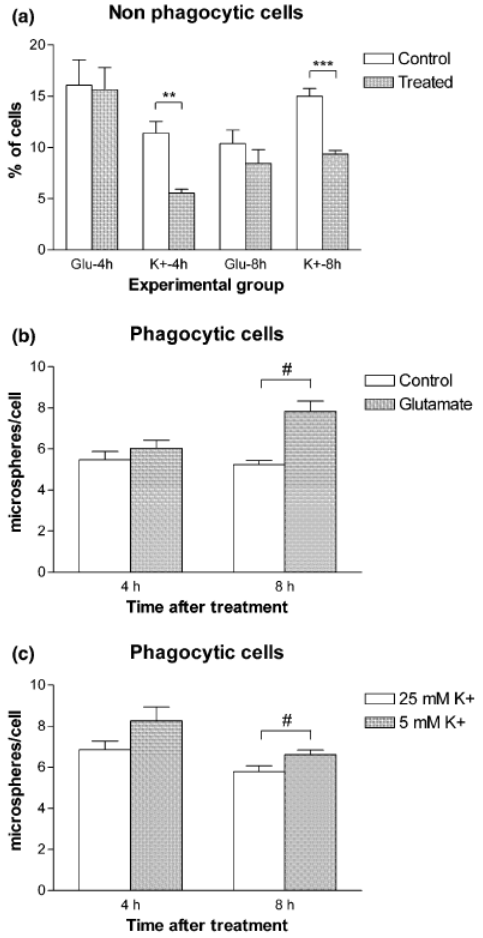

Fig. 9 Glial cell phagocytosis in neuronal-glial cultures exposed to glutamate or to potassium deprivation. Phagocytic activity in glial cells was evaluated through the ingestion of fluorescent microspheres after treating the cultures with 100 μM glutamate for 20 min or exposing them to potassium deprivation (5 mM KCl). Internalization of microspheres was determined after immunofluorescence labelling of microglia using an anti-CD68 monoclonal antibody. (a) Percentage of microglial cells without microspheres in the different experimental conditions. Number of microspheres per phagocytic microglial cell after glutamate treatment (b) or potassium deprivation (c). Bars represent the mean ± SEM of five cultures for the glutamate model and four cultures for the potassium deprivation model; $^{**}p < 0.01$ and $^{***}p < 0.001$ represent differences between the percentage of microglial cells without microspheres in treated versus control cultures; Student's unpaired *t*-test; $\#p < 0.05$ represents differences between phagocytic activity of microglial cells in treated versus control cultures; Student's paired *t*-test.

NO production (Araki *et al.* 2001; Pérez-Capote *et al.* 2004). In the present work, we did not detect NO production in cerebellar neuronal-glial cultures after inducing neuronal death with glutamate treatment or potassium deprivation, although it was observed in response to LPS treatment. In a previous study, we showed that LPS but not glutamate treatment induced NO production in mixed glial cell cultures (Pérez-Capote *et al.* 2004). The present results confirm our previous observation that the induction of excitotoxic neuronal death by glutamate does not result in NO production by glial cells (Pérez-Capote *et al.* 2004), and extend this observation to apoptotic neuronal death induced by potassium deprivation.

TNF-α is induced in both microglia and astroglia in response to several stimuli. We observed an induction of TNF-α production in neuronal-glial cultures treated with glutamate but not in cultures exposed to potassium deprivation. Different authors show induction of TNF-α production in glial cells *in vitro* in response to neuronal death (Viviani *et al.* 1998, 2000; Eskes *et al.* 2003), homogenates of damaged neurons or cerebrospinal fluid of Alzheimer's disease patients (Eskes *et al.* 2003). They suggest that neuronal death induces TNF-α production in glial cells and that this effect is mediated by some soluble factor released by the damaged neurons. However, we did not detect TNF-α production in neuronal-glial cultures exposed to potassium deprivation, although most neuronal cells died by apoptosis as a consequence of this treatment. This observation suggests that TNF-α production by glial cells is induced by changes occurring in neuronal cells when excitotoxic, but not apoptotic, neuronal death occurs. One of the targets of NF-κB is the TNF-α gene. Consequently, the presence of NF-κB activation in glial cells in response to neuronal death induced by glutamate, but not potassium deprivation, is consistent with TNF-α production in glutamate-treated but not potassium-deprived cultures.

In the adult CNS, glial cells proliferate in response to appropriate stimulation, such as inflammation or injury. Glial cells isolated from the brain also proliferate *in vitro*. Various factors regulate glial cell proliferation *in vitro*, ranging from soluble factors present in the culture medium or released by glial cells to non-soluble factors present in the cell membrane of adjacent cells (Hatten 1985, 1987; Giulian *et al.* 1988; Aloisi *et al.* 1992; Thery *et al.* 1992; Nakatsuji and Miller 1998). In the present study, we evaluated whether the presence of neuronal death in neuronal-glial cultures induced glial cell proliferation, and whether neuronal death occurring by excitotoxicity or apoptosis could have some differential effect on this parameter. We observed a significant increase in glial cell proliferation in response to glutamate-induced neuronal death. The effect was not observed after inducing neuronal death by potassium deprivation. These results suggest that soluble factors released by either neurons or glial cells as a result of excitotoxic, but not apoptotic, neuronal death play a more significant role in the induction of glial cell proliferation than the loss of contact between neuronal and glial cells.

Neuronal death *in vivo* induces phagocytic activity in glial cells, mainly in microglia, to remove dying cells and neuronal debris from the extracellular space and maintain normal tissue homeostasis. Beyer *et al*. (2000) showed that microglial cells in culture maintain their capacity to acquire an activated phenotype and become phagocytic. However, they also showed that although various stimuli can cause the transformation of microglia into phagocytes, it does not necessarily mean that microglial cells undergo the same changes when acquiring an activated phenotype. Witting *et al*. (2000) studied the effect of soluble factors released by dying neurons on the phagocytic activity of microglial cells in culture using conditioned medium from neuronal cultures undergoing apoptotic or necrotic neuronal cell death. They observed that phagocytic activity in microglial cells is only detected in the case of necrotic neuronal death. In addition, they observed that in the case of apoptotic death, cell–cell contact is necessary to induce phagocytosis. Adayev *et al*. (1998) showed that phosphatidylserine externalization is a necessary signal to induce phagocytosis of apoptotic cells by microglia. This observation is consistent with the need for cell–cell contact to induce phagocytosis in microglial cells in response to apoptotic neuronal death. We detected an increase in the phagocytic activity of microglial cells in neuronal-glial cultures treated with glutamate or exposed to potassium deprivation, suggesting that the presence of neuronal death is enough to induce phagocytosis, irrespective of whether it occurs by excitotoxicity or apoptosis. However, this effect was observed earlier in the case of apoptotic neuronal death, before significant neuronal death is observed. This observation is in agreement with the suggestion that externalization of phosphatidylserine is an early marker in apoptotic death (Martin *et al*. 1995; Rimon *et al*. 1997) and, as mentioned above, is the signal that induces phagocytosis of apoptotic cells by microglia. In addition, the fact that an increase in phagocytic activity in microglial cells is initially observed at later times in glutamate-treated cultures is consistent with the requirement for accumulation of soluble factors released by neurons undergoing excitotoxic neuronal death. Various authors have shown that the interaction between apoptotic cells and macrophages results in the induction of anti-inflammatory or suppressive properties in macrophages (Fadok *et al*. 1998; De Simone *et al*. 2002, 2003). This observation could explain the absence of NF-κB activation and TNF-α production in neuronal-glial cultures in which apoptotic neuronal death was induced by potassium deprivation.

In conclusion, the results presented here show that although glial cells undergo functional changes in response to neuronal death, different aspects of glial activation may be induced depending on whether it is caused by excitotoxicity or apoptosis. In the presence of excitotoxic neuronal death, glial cells increase their production of agents associated with an inflammatory response, as well as proliferate and become phagocytic. This appears to be a drastic reactive response of glial cells that, on the one hand, clears the extracellular milieu of any neuronal debris or factors released by the dying neurons that may negatively affect the cellular homeostasis, and on the other, results in the production of factors that in turn can be deleterious for the remaining live cells. In contrast, glial cells do not produce pro-inflammatory molecules in the presence of apoptotic neuronal death, but phagocytic activity is quickly induced. In this case, glial activation appears to constitute a preventative response resulting from a crosstalk between dying neuronal cells and glial cells, aimed at preventing alterations in the extracellular milieu through the release of injuring factors by dying neurons, as well as limiting the glial response as much as possible. Our results also show that different aspects of glial activation are independently regulated in response to neuronal damage. It would be of interest to identify the different signal transduction pathways involved in each case. In this way, it may be possible to specifically interfere with individual aspects of glial activation in order to favour actions of reactive glial cells that could help neurons to overcome a negative stimulus and, likewise, to inhibit activities that enhance neuronal damage.

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References

Adayev T., Estephan R., Meserole S., Mazza B., Yurkow E. J. and Banerjee P. (1998) Externalization of phosphatidylserine may not be an early signal of apoptosis in neuronal cells, but only the phosphatidylserine-displaying apoptotic cells are phagocytosed by microglia. *J. Neurochem.* **71**, 1854–1864.

Aloisi F., Care A., Borsellino G., Gallo P., Rosa S., Bassani A., Cabibbo A., Testa U., Levi G. and Peschle C. (1992) Production of hemolymphopoietic cytokines (IL-6, IL-8, colony-stimulating factors) by normal human astrocytes in response to IL-6 beta and tumor necrosis factor-alpha. *J. Immunol.* **149**, 2358–2366.

Araki E., Forster C., Dubinsky J. M., Ross M. E. and Iadecola C. (2001) Cyclooxygenase-2 inhibitor NS-398 protects neuronal cultures from lipopolysaccharide-induced neurotoxicity. *Stroke* **32**, 2370–2375.

Aschner M., Sonnewald U. and Tan K. H. (2002) Astrocyte modulation of neurotoxic injury. *Brain Pathol.* **12**, 475–481.

Bal-Price A. and Brown G. C. (2001) Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *J. Neurosci.* **21**, 6480–6491.

Benveniste E. N., Nguyen V. T. and O'Keefe G. M. (2001) Immunological aspects of microglia: relevance to Alzheimer's disease. *Neurochem. Int.* **39**, 381–391.

Beyer M., Gimsa U., Eyüpoglu I. Y., Hailer N. P. and Nitsch R. (2000) Phagocytosis of neuronal or glial debris by microglial cells: upregulation of MHC class II expression and multinuclear giant cell formation *in vitro*. *Glia* **31**, 262–266.

Brown D. R. (1998) Prion protein-overexpressing cells show altered response to a neurotoxic prion protein peptide. *J. Neurosci. Res.* **54**, 331–340.

Brown D. R. (2001) Microglia in prion disease. *Microsc. Res. Techn.* **54**, 71–80.

Chen Y. and Swanson R. A. (2003) Astrocytes and brain injury. *J. Cereb. Blood Flow Metab.* **23**, 137–149.

Conti B., Park L. C. H., Calingasan N. Y., Kim Y., Kim H., Bae Y., Gibson G. E. and Joh T. H. (1999) Cultures of astrocytes and microglia express interleukin-18. *Mol. Brain Res.* **67**, 46–52.

D'Mello S. R., Galli C., Ciotti T. and Calissano P. (1993) Induction of apoptosis in cerebellar granule neurons by low potassium: Inhibition of death by insulin-like growth factor I and cAMP. *Proc. Natl Acad. Sci. USA* **90**, 10 989–10 993.

De Simone R., Ajmone-Cat A., Nicolini A. and Minghetti L. (2002) Expression of phosphatidylserine receptor and down-regulation of pro-inflammatory molecule production by its natural ligand in rat microglial cultures. *J. Neuropathol. Exp. Neurol.* **61**, 237–244.

De Simone R., Ajmone-Cat A., Tiraza P. and Minghetti L. (2003) Apoptotic PC12 cells exposing phosphatidylserine promote the production of anti-inflammatory and neuroprotective molecules by microglial cells. *J. Neuropathol. Exp. Neurol.* **62**, 208–216.

Eskes C., Juillerat-Jeanneret L., Leuba G., Honegger P. and Monnet-Tschudi F. (2003) Involvement of microglia–neuron interactions in the tumor necrosis factor-α release, microglial activation, and neurodegeneration induced by trimethyltin. *J. Neurosci. Res.* **71**, 583–590.

Fadok V. A., Bratton D. L., Konowal A., Freed P. W., Westcott J. Y. and Henson P. M. (1998) Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TFG-beta, PGE2, and PAF. *J. Clin. Invest.* **101**, 890–898.

Forloni G., Mangiarotti F., Angeretti N., Lucca E. and De Simoni M. G. (1997) Beta-amyloid fragment potentiates IL-6 and TNF-alpha secretion by LPS in astrocytes but not in microglia. *Cytokine* **9**, 759–762.

Giulian D., Young D. G., Woodward J., Brown D. C. and Lachman L. B. (1988) Interleukin-1 is an astroglial growth factor in the developing brain. *J. Neurosci.* **8**, 709–714.

Golde S., Chandran S., Brown G. C. and Compston A. (2002) Different pathways for iNOS-mediated toxicity *in vitro* dependent on neuronal maturation and NMDA receptor expression. *J. Neurochem.* **82**, 269–282.

González-Scarano F. and Baltuch G. (1999) Microglia as mediators of inflammatory and degenerative diseases. *Ann. Rev. Neurosci.* **22**, 219–240.

Hatten M. E. (1985) Neuronal regulation of astroglial morphology and proliferation *in vitro*. *J. Cell Biol.* **100**, 384–396.

Hatten M. E. (1987) Neuronal inhibition of astroglial cell proliferation is membrane mediated. *J. Cell Biol.* **104**, 1353–1360.

Hewett S. J., Csernansky C. A. and Choi D. W. (1994) Selective potentiation of NMDA-induced neuronal injury following induction of astrocytic iNOS. *Neuron* **13**, 487–494.

Hewett S. J., Muir J. K., Lobner D., Symons A. and Choi D. W. (1996) Potentiation of oxygen-glucose deprivation-induced neuronal death after induction of iNOS. *Stroke* **27**, 1586–1591.

Jeohn G. H., Kong L. Y., Wilson B., Hudson P. and Hong J. S. (1998) Synergistic neurotoxic effects of combined treatments with cytokines in murine primary mixed neuron/glia cultures. *J. Neuroimmunol.* **85**, 1–10.

Johnstone M., Gearing A. J. and Miller K. M. (1999) A central role for astrocytes in the inflammatory response to beta-amyloid; chemokines, cytokines and reactive oxygen species are produced. *J. Neuroimmunol.* **93**, 182–193.

Kim W. K. and Ko K. H. (1998) Potentiation of *N*-methyl-D-aspartate-mediated neurotoxicity by immunostimulated murine microglia. *J. Neurosci. Res.* **54**, 17–26.

Kim W. K., Chung J. H., Kim H. C. and Ko K. H. (1999a) Nitric-oxide-enhanced excitotoxicity-independent apoptosis of glucose-deprived neurons. *Neurosci. Res.* **33**, 281–289.

Kim W. K., Seo D. O., Choi J. J. and Ko K. H. (1999b) Immunostimulated glial cells potentiate glucose deprivation-induced death of cultured rat cerebellar granule cells. *J. Neurotrauma* **16**, 415–424.

Malchiodi-Albedi F., Domenici M. R., Paradisi S., Bernardo A., Ajmone-Cat M. A. and Minghetti L. (2001) Astrocytes contribute to neuronal impairment in βA toxicity increasing apoptosis in rat hippocampal neurons. *Glia* **34**, 68–72.

Martin S. J., Reutelingsperger C. P., McGahon A. J., Rader J. A., van Schie R. C., LaFace D. M. and Green D. R. (1995) Early distribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus. *J. Exp. Med.* **182**, 1545–1556.

McNaught K. St P. and Jenner P. (1999) Altered glial function causes neuronal death and increases neuronal susceptibility to 1-methyl-4-phenylpyridinium- and 6-hydroxydopamine-induced toxicity in astrocytic/ventral mesencephalic co-cultures. *J. Neurochem.* **73**, 2469–2476.

Meda L., Cassatella M. A., Szendrei G. I., Otvos L., Jr, Baron P., Villalba M., Ferrari D. and Rossi F. (1995) Activation of microglial cells by β-amyloid protein and interferon-γ. *Nature* **374**, 647–650.

Murphy G. M., Jr, Yang L. and Cordell B. (1998) Macrophage colony-stimulating factor augments beta-amyloid induced interleukin-1, interleukin-6, and nitric oxide production by microglial cells. *J. Biol. Chem.* **273**, 20 967–20 971.

Nakatsuji Y. and Miller R. H. (1998) Homotypic cell contact-dependent inhibition of astrocyte proliferation. *Glia* **22**, 379–389.

Neumann H. (2001) Control of glial immune function by neurons. *Glia* **36**, 191–199.

Pérez-Capote K., Serratosa J. and Solà C. (2004) Glial activation modulates glutamate neurotoxicity in cerebellar granule cell cultures. *Glia* **45**, 258–268.

Polazzi E. and Contestabile A. (2002) Reciprocal interactions between microglia and neurons: from survival to neuropathology. *Rev. Neurosci.* **13**, 221–242.

Possel H., Noack H., Putzke J., Wolf G. and Sies H. (2000) Selective upregulation of inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS) and cytokines in microglia: *in vitro* and *in vivo* studies. *Glia* **32**, 51–59.

Raivich G., Bohatschek M., Kloss C. U. A., Werner A., Jones L. L. and Kreutzberg G. W. (1999) Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res. Rev.* **30**, 77–105.

Rimon G., Bazenet C. E., Philpott K. L. and Rubin L. L. (1997) Increased surface phosphatidylserine is an early marker of neuronal apoptosis. *J. Neurosci. Res.* **48**, 563–570.

Schramm M., Eimerl S. and Costa E. (1990) Serum and depolarizing agents cause acute neurotoxicity in cultured cerebellar granule cells: role of the glutamate receptor responsive to *N*-methyl-D-aspartate. *Proc. Natl Acad. Sci. USA* **87**, 1193–1197.

Streit W. J., Walter S. A. and Pennell N. A. (1999) Reactive microgliosis. *Prog. Neurobiol.* **57**, 563–581.

Teismann P. and Schulz J. B. (2004) Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. *Cell Tissue Res.* **318**, 149–161.

Thery C., Stanley E. R. and Mallat M. (1992) Interleukin 1 and tumor necrosis factor-alpha stimulate the production of colony-stimulating factor 1 by murine astrocytes. *J. Neurochem.* **59**, 1183–1186.

Viviani B., Corsini E., Galli C. L. and Marinovich M. (1998) Glia increase degeneration of hippocampal neurons through release of tumor necrosis factor-α. *Toxicol. Appl. Pharmacol.* **150**, 271–276.

Viviani B., Corsini E., Galli C. L., Padovani A., Ciusani E. and Marinovich M. (2000) Dying neural cells activate glia through the release of a protease product. *Glia* **32**, 84–90.

Witting A., Müller P., Herrmann A., Kettenmann H. and Nolte C. (2000) Phagocytic clearance of apoptotic neurons by microglia/brain macrophages *in vitro*: involvement of lectin-, integrin-, and phosphatidylserine-mediated recognition. *J. Neurochem.* **75**, 1060–1070.

Yao J. and Johnson R. W. (1997) Induction of interleukin-1 beta converting enzyme (ICE) in murine microglia by lipopolysaccharide. *Mol. Brain Res.* **51**, 170–178.

4.3- TERCER TRABAJO

Expresión de C/EBPs en las células gliales activadas

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Pendiente de publicación

Introducción

Como se ha comentado a lo largo de esta tesis, la activación glial que se da en el SNC como consecuencia del daño neuronal inducido por lesiones o enfermedades neurodegenerativas va asociado a la expresión modificada o *de novo* de un número importante de moléculas de diversa índole. Esta expresión viene regulada por numerosos factores de transcripción. Recientemente se ha sugerido que los factores de transcripción de la familia de las C/EBPs pueden tener un papel primordial en la activación glial que se da en procesos inflamatorios del SNC. Esta familia está constituida por seis miembros: C/EBPα, -β, -δ, -γ, -ε y -ζ (Cao y cols., 1991), que proceden de la expresión de seis genes distintos. Los distintos C/EBPs pueden formar homo y heterodímeros que se unen con varias afinidades a elementos reguladores de distintos genes que participan en la diferenciación celular, en el control de respuestas inflamatorias e immunes, en el metabolismo y en la proliferación celular (revisado en Poli, 1998; Ramji y Foka, 2002). En el cerebro, C/EBPα, C/EBPβ y C/EBPδ son los que más ampliamente se expresan, aunque se conoce poco sobre su función en el SNC. Sin embargo, dado que se han descrito lugares de unión de estos factores de transcripción en numerosos genes involucrados en la respuesta inflamatoria, como los que codifican las citocinas proinflamatorias IL-6, IL-1β y TNF-α y enzimas como la iNOS y la COX2, es posible que los C/EBPs jueguen un papel en la respuesta inflamatoria cerebral asociada al daño neuronal que se observa como consecuencia de lesiones o enfermedades neurodegenerativas. En este sentido, Walton y cols. (1998) detectan la expresión de C/EBPα en microglía activada en un modelo experimental de daño neuronal por hipoxia-isquemia en rata. En trabajos recientes se ha sugerido un posible papel de los C/EBPs en la enfermedad de Alzheimer. Por una parte, distintos genes relacionados con una reacción inflamatoria y que están regulados por C/EBPs se encuentran alterados en la enfermedad de Alzheimer (Akiyama y cols. 2000). Por otra parte, se ha mostrado la existencia de un incremento en la expresión de C/EBPβ en el cerebro de pacientes que padecen esta enfermedad (Colangelo y cols., 2000). Además, se ha observado un incremento en la expresión de C/EBPδ en astrocitos activados que

rodean los depósitos de β-amiloide en las placas seniles del cerebro de afectados por la enfermedad de Alzheimer (Li y cols., 2004).

Dado el papel protagonista que tienen las células gliales en la reacción inflamatoria cerebral, el objetivo de este trabajo consistió en estudiar la expresión de los factores de transcripción C/EBPα y C/EBPβ después de inducir activación glial *in vitro.* Para este estudio utilizamos dos tipos de cultivos de cerebelo, uno mixto neurona-glía y otro mixto de células gliales. Por una parte, inducimos activación glial con LPS, agente ampliamente utilizado para inducir activación glial *in vitro*. Por otra parte, estudiamos la expresión de estos factores de transcripción en las células gliales después de inducir muerte neuronal mediante dos mecanismos distintos: por excitotoxicidad, utilizando elevadas concentraciones de glutamato, y por apoptosis, al cultivar las neuronas granulares de cerebelo en bajas concentraciones de K^+, un modelo ampliamente utilizado *in vitro* para inducir muerte por apoptosis en este tipo de células (D'Mello y cols. 1993).

Materiales y Métodos

A menos que se indique lo contrario los reactivos utilizados proceden de Sigma (St. Louis, MO, USA)

Cultivo celular

Los cultivos de células de cerebelo fueron obtenidos a partir de ratas de 8 días (Wistar, Iffa Credo, Lyon, France) según el protocolo descrito por Pérez-Capote y cols. (2004). Las células fueron sembradas a una densidad de 3 x 10^5 céls/cm^2 en placas de 24 pozos previamente recubiertos con poli-L-lisina (5 μg/ml), en 0.5 ml de medio basal EAGLE (BME; GIBCO-BRL, Life Technologies, Paisley, UK) suplementado con 20 mM de KCl, 100 μg/ml de gentamicina, 2 mM de L-glutamina y 10% de suero fetal bovino (FBS; GIBCO-BRL, Life Technologies, Paisley, UK). Las células se mantuvieron a 37ºC en una atmósfera de CO_2 al 5% y una humedad del 100%. Se obtuvieron dos tipos de cultivos: un cultivo de neuronas y células gliales, constituido principalmente por neuronas granulares, astroglía y microglía, y un cultivo mixto de células gliales, constituido principalmente por astroglía y microglía. Los cultivos fueron

utilizados a los 8 DIV. Al cultivo neurona-glía no se le cambió nunca el medio, pero a los 4 DIV se le añadió 5.6 mM de glucosa para favorecer la supervivencia y compensar las pérdidas de agua. En los cultivos mixtos de células gliales el medio se sustituyó a las 24 h por uno que no contenía KCl añadido y cada 3 días fue renovado.

Tratamientos

Tratamiento con glutamato

Los cultivos fueron tratados con glutamato a los 8 DIV. El medio condicionado fue recogido y guardado a 37°C. Las células se lavaron dos veces, primero con solución de Locke completa (155 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 8.4 mM HEPES, 5.6 mM Glucosa y 1 mM $MgCl_2$, pH 7.4) y después con solución de Locke sin Mg^{2+}. Seguidamente, los cultivos fueron incubados en solución de Locke sin Mg^{2+} conteniendo 100 μM de glutamato a TA durante 20 min, tras los que las células se lavaron dos veces con solución de Locke completa. Finalmente, se devolvió a los cultivos su medio condicionado y se incubaron nuevamente a 37°C en una atmósfera de CO_2 al 5% y una humedad del 100% durante 24 h. En paralelo se procesaron las células controles, que se trataron con solución de Locke sin Mg^{2+} y sin adición de glutamato.

Deprivación de potasio

Los cultivos mixtos de neurona-glía fueron mantenidos durante 8 días en un medio conteniendo 25 mM de KCl y 10% de FBS, que asegura la supervivencia y diferenciación de las neuronas granulares de cerebelo en cultivo. A los 8 DIV las células se lavaron con solución de Locke completa y fueron expuestas a deprivación de K^+ al mantenerlas durante 24 horas en BME sin suero (conteniendo 5 mM de KCl) suplementado con 100 μg/ml de gentamicina y 2 mM de L-glutamina. En paralelo se procesaron las células controles, que se mantuvieron en el mismo medio suplementado con 20 mM de KCl (concentración final 25 mM KCl).

Tratamiento con LPS

Los cultivos neurona-glía y los cultivos mixtos de glía fueron tratados a los 8 DIV con 1 μg/ml de LPS (lipopolisacárido de *E. coli* 026:B6). El LPS fue añadido

directamente al medio en los cultivos neurona-glía, mientras que en el caso de los cultivos mixtos de células gliales se realizó un cambio de medio antes de añadir el LPS.

Análisis inmunocitoquímico

La presencia de los factores de transcripción C/EBPα y C/EBPβ en los cultivos se detectó por imunocitoquímica utilizando anticuerpos policlonales de conejo (Santa Cruz, Heidelberg, Germany), a las 24 h del inicio de los distintos tratamientos. Las células fueron fijadas con 4% PFA durante 20 min a TA. Tras lavar con PBS, la actividad peroxidasa endogéna fue bloqueada mediante una incubación con 3% de H_2O_2 en metanol durante 10 min a TA. Después de varios lavados con PBS, se bloquearon las uniones inespecíficas con PBS-1% BSA conteniendo un 5% de suero de cabra durante 20 min a TA. Seguidamente las células fueron incubadas con los anticuerpos primarios anti-C/EBPα o anti-C/EBPβ (1/1000 en PBS-1% BSA conteniendo un 1% de suero de cabra) durante 24 h a 4°C. Después de 3 lavados con PBS, se incubaron las células con un anticuerpo secundario biotinilado anti-conejo obtenido en cabra (Vector Laboratories, Peterborough, UK; 1/200 en PBS-1% BSA conteniendo un 1% de suero de cabra) durante 1 h a TA. Tras lavar con PBS, se incubaron las células con ABC (1/100) o extravidina (1/500) en PBS-1% BSA conteniendo un 1% de suero de cabra 1 h a TA. La reacción de color se obtuvo con DAB y se detuvo añadiendo PBS. Las placas se guardaron a 4°C en 0.4 mg/ml de PBS-Timerosal hasta su visualización al microscopio.

Captura de imágenes y análisis

Las imágenes fueron obtenidas con un microscopio Olympus IX70 (Okoya, Japan) y una cámara digital (CC-12, Soft Imaging System GmbH, Munich, Germany).

Resultados

Expresión glial de C/EBPs tras el tratamiento con LPS.

La expresión de C/EBPα y C/EBPβ fue evaluada mediante inmunocitoquímica después de 24 h del tratamiento de los cultivos neurona-glía y los cultivos mixtos de células gliales. En los cultivos neurona-glía, detectamos una expresión basal de C/EBPα

principalmente en las células microgliales, generalmente nuclear y ocasionalmente también citoplasmática (Fig. 1A). En el caso del C/EBPβ, observamos una expresión basal tanto en los núcleos de los astrocitos como en los de la microglía (Fig. 1B). El tratamiento con LPS provocó modificaciones en la expresión de C/EBPα y C/EBPβ. Por una parte detectamos una disminución en el marcaje de C/EBPα en el núcleo de la microglía (Fig. 1C) y, por otra parte, observamos un incremento de la expresión de C/EBPβ en los núcleos de las células gliales, principalmente en los astrocitos (Fig. 1D). En los cultivos mixtos de células gliales control observamos un patrón similar de expresión de C/EBPα y C/EBPβ al de los cultivos neurona-glía. Sin embargo, aunque el tratamiento con LPS provocó un incremento en la expresión de C/EBPβ, no detectamos alteraciones en la expresión de C/EBPα (Fig. 2).

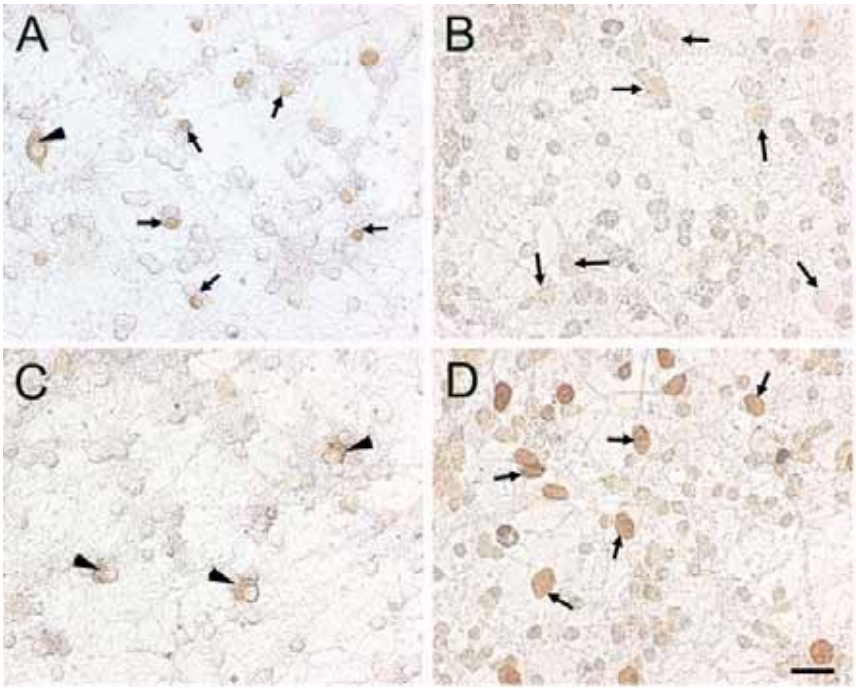

Figura 1. Efecto del tratamiento con LPS en la expresión de C/EBPα y C/EBPβ en cultivos neurona-glía. La microglía presenta una expresión basal de C/EBPα, principalmente nuclear, (A) que disminuye después del tratamiento con LPS (B). La expresión de C/EBPβ es tenue en las células gliales controles (C) y el tratamiento con LPS provoca un incremento de su expresión nuclear principalmente en los astrocitos (D). Las flechas indican los núcleos marcados, mientras que las puntas de flecha indican el marcaje citoplasmático. Barra=20 μm.

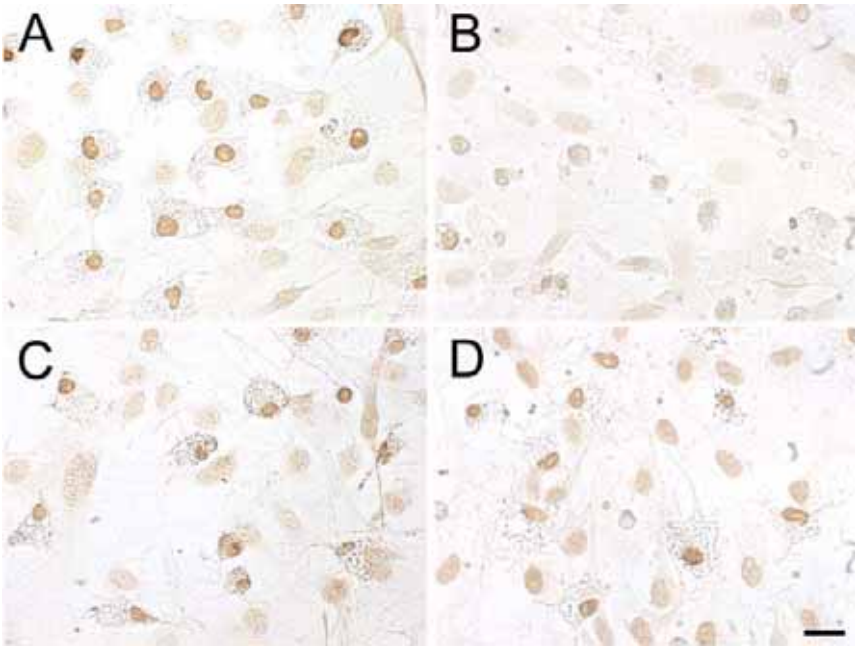

Figura 2. Efecto del tratamiento con LPS en la expresión de C/EBPα y C/EBPβ en cultivos mixtos de células gliales. Se observa una expresión basal de C/EBPα tanto en los núcleos de los astrocitos como de la microglía (A), que no se modifica con el tratamiento con LPS (C). Existe una expresión nuclear basal tenue de C/EBPβ tanto en astrocitos como en microglía (B), que incrementa en presencia de LPS (D). Barra=20 μm.

Expresión glial de C/EBPs tras la inducción de muerte neuronal

Tras inducir muerte neuronal con 100 μM glutamato en los cultivos neurona-glía no se observaron alteraciones en la expresión de C/EBPα (Fig. 3A y B), pero sí detectamos un incremento en la expresión de C/EBPβ, principalmente en el núcleo de los astrocitos (Fig. 3C y D). Este incremento fue menos pronunciando que en el caso del LPS. Sin embargo, en cultivos mixtos de células gliales tratados con 100 μM glutamato no se observaron alteraciones en la expresión de C/EBPα ni C/EBPβ (Fig. 4). Tampoco observamos alteraciones en la expresión de estos factores de transcripción en las células gliales tras inducir muerte neuronal por deprivación de K^+ (Fig. 5).

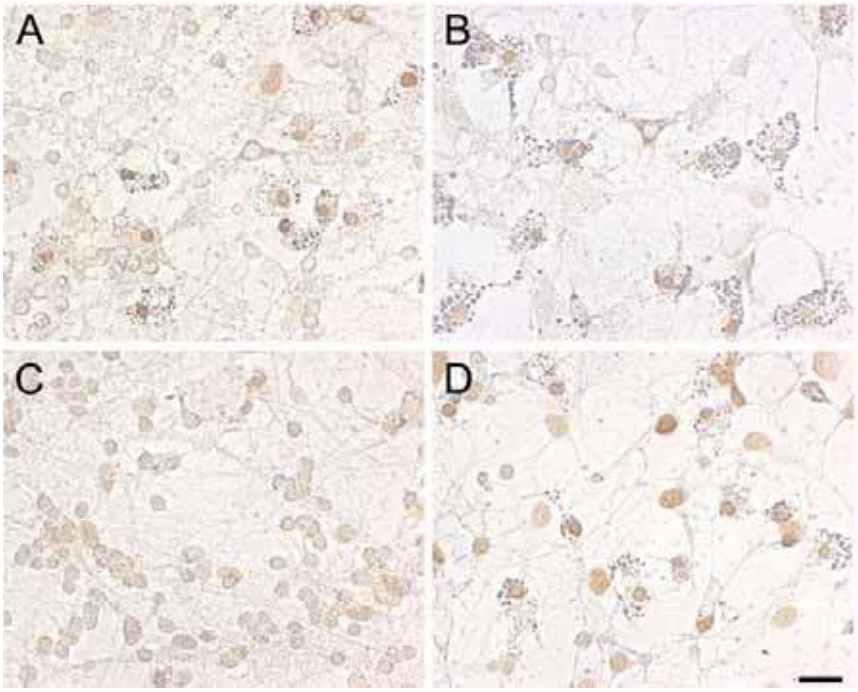

Figura 3. Expresión de C/EBPα y C/EBPβ en cultivos neurona-glía tras inducir muerte neuronal por excitotoxicidad. La expresión basal de C/EBPα en los núcleos de las células gliales (A) no se modifica por el tratamiento con 100 μM glutamato (B). Por el contrario, la expresión basal de C/EBPβ en los núcleos de las células gliales (C) sí incrementa tras el tratamiento, principalmente en los astrocitos (D). Barra=20 μm.

Discusión

En este trabajo hemos observado que la expresión de los factores de transcripción C/EBPα y C/EBPβ se modifica al producir activación glial *in vitro* en respuesta a un determinado tipo de estímulo.

Como se ha comentado en la Introducción de este capítulo, aunque se sabe que los C/EBPs regulan la transcripción de varios genes involucrados en respuestas de fase aguda así como en respuestas inmunes e inflamatorias (Ramji y Foka, 2002), existe poca información respecto a su papel en la activación glial y la respuesta inflamatoria presentes en el cerebro como consecuencia de lesiones o en determinadas enfermedades neurodegenerativas. En cuanto a la expresión de C/EBPα en las células gliales, Walton

y cols. (1998) observan que este factor no se expresa en condiciones basales, pero que se induce en la microglía en presencia de activación glial. Por otra parte, diferentes autores describen que C/EBPα no se expresa en cultivos de astrocitos de roedores (Cardinaux y Magistretti, 1996; Yano y cols., 1996). Respecto a la expresión de C/EBPβ en las células gliales, se ha detectado una expresión basal en cultivos de astrocitos de ratón (Cardinaux y Magistretti, 1996; Cardinaux y cols., 2000) y de rata (Yano y cols., 1996) que incrementa después del tratamiento con diferentes citocinas inflamatorias, tales como IL-6, IL-1β y TNF-α (Cardinaux y cols., 2000). También se ha observado la inducción de C/EBPβ en astrocitos humanos tratados con IL-1β/IFN-γ (Pahan y cols., 2002).

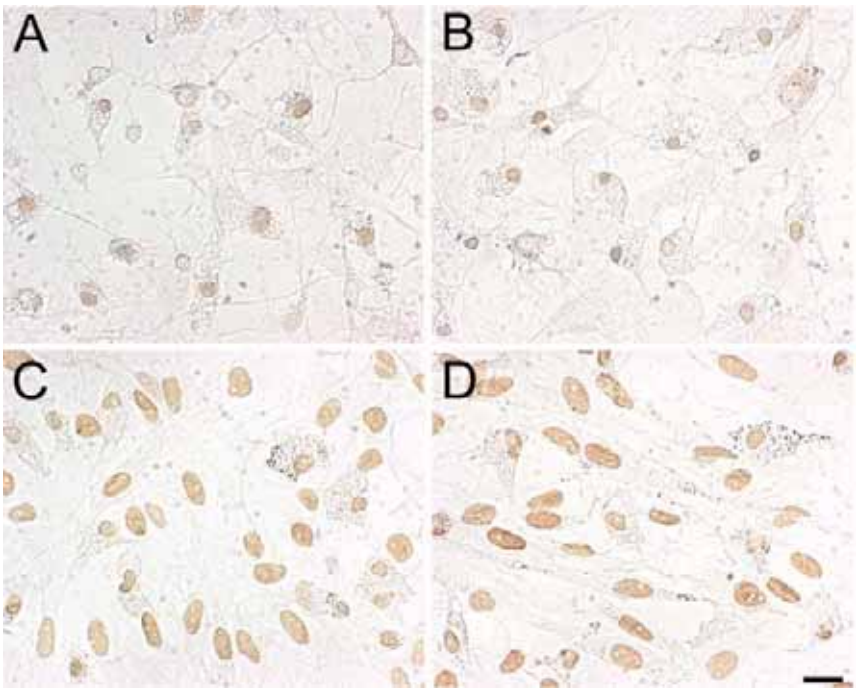

Figura 4. Efecto del tratamiento con glutamato en la expresión de C/EBPα y C/EBPβ en cultivos mixtos de células gliales. A y C, cultivos controles; B y D, cultivos tratados con 100 μM de glutamato. El tratamiento con glutamato no modifica la expresión basal de C/EBPα (A, B) ni de C/EBPβ (C, D) en los núcleos de las células gliales. Barra=20 μm.

Nosotros observamos una clara expresión basal de C/EBPα en la microglía de nuestros cultivos, mientras que su expresión era prácticamente indetectable en los

astrocitos. Este patrón de expresión se observaba tanto en cultivos neurona-glía como en cultivos mixtos de glía. Al inducir activación glial con LPS detectamos una disminución en la expresión de C/EBPα en las células de microglía, aunque sólo en los cultivos neurona-glía. Estos efectos contrastan con el incremento de C/EBPα observado por Walton y cols. (1998) en la microglía activada *in vivo*. Sin embargo, Tengku-Muhammad y cols. (2000) también observan una disminución de la expresión de C/EBPα en respuesta al LPS en una línea de macrófagos de ratón. Por otra parte, observamos que el tratamiento con LPS provocó un incremento en la expresión de C/EBPβ tanto en la microglía como en los astrocitos. Estos resultados estarían de acuerdo con la implicación de este factor en la activación glial asociada a procesos inflamatorios.

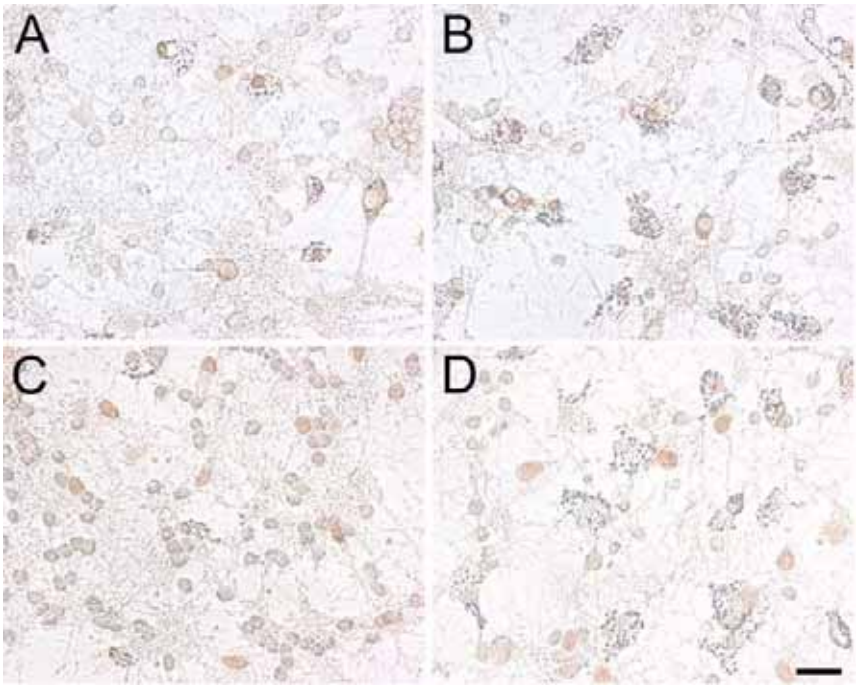

Figura 5. Expresión de C/EBPα y C/EBPβ en cultivos neurona-glía tras inducir muerte neuronal por deprivación de K^+. A y C, cultivos controles (25 mM KCl); B y D, cultivos expuestos a deprivación de K^+ (5 mM KCl). La deprivación de K^+ no modifica la expresión de C/EBPα (A, B) ni de C/EBPβ (C, D) en las células gliales. Barra=20 μm.

La inducción de muerte neuronal por excitotoxicidad no resultó en alteraciones en la expresión glial de C/EBPα en nuestros cultivos neurona-glía, aunque sí observamos un incremento en la expresión glial de C/EBPβ a las 24 del tratamiento con glutamato. En un estudio previo, Yano y cols. (1996) describen un incremento rápido y transitorio en la expresión de C/EBPβ en cultivos de astrocitos tratados con elevadas concentraciones de glutamato, sugiriendo que el glutamato puede modular la expresión de este factor de transcripción en los astrocitos. Sin embargo, el efecto que hemos observado en los cultivos neurona-glía no se debe a una acción directa del glutamato sobre las células gliales, pues en cultivos mixtos de glía tratados de la misma manera no observamos ninguna alteración en la expresión de estos factores de transcripción a las 24 h del tratamiento. Estos resultados sugieren que el incremento observado es consecuencia de la muerte neuronal inducida por el glutamato, aunque no podemos descartar un posible efecto adicional del glutamato sobre la expresión de C/EBPβ en astroglía a tiempos cortos. Por el contrario, la inducción de muerte neuronal por apoptosis no dio lugar a alteraciones en la expresión de C/EBPs. En un trabajo anterior (capítulo anterior de Resultados), hemos descrito que la muerte neuronal por excitotoxicidad induce un patrón de activación glial *in vitro* distinto al inducido por la muerte neuronal por apoptosis. En el primer caso, la activación glial está asociada a la producción de factores proinflamatorios como el TNF-α y el NF-κB, mientras que en el segundo caso no (Pérez-Capote y cols., 2005). Por lo tanto, teniendo en cuenta el papel que se ha sugerido para C/EBPβ en la reacción inflamatoria cerebral, los resultados obtenidos en el presente trabajo concuerdan con los del trabajo anterior.

Globalmente, los resultados obtenidos en el presente estudio estarían de acuerdo con un papel del factor de transcripción C/EBPβ, pero no de C/EBPα, en la activación glial asociada a una reacción de tipo proinflamatorio.

Bibliografía

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G.M., Cooper, N.R., Eikelenboom, P., Emmerling, M., Fiebich, B.L., Finch, C.E., Frautschy, S., Griffin, W.S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I.R., McGeer, P.L., O'Banion, M.K., Pachter, J., Pasinetti, G., Plata-Salaman, C., Rogers, J.,

Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F.L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G., Wyss-Coray, T. (2000) Inflammation and Alzheimer's disease. **Neurobiol. Aging 21**,383-421.

Cao, Z., Umek, R.M., McKnight, S.L (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. **Genes Dev. 5**, 1538-1552.

Cardinaux, J.R., Allaman, I., Magistretti, P.J. (2000) Pro-inflammatory cytokines induce the transcription factors C/EBPβ y C/EBPδ in astrocytes. **Glia 29**, 91-97.

Cardinaux, J.R., Magistretti, P.J (1996) Vasoactive Intestinal Peptide, Pituitary Adenylate Cyclase-Activating Peptide, and Noradrenaline induce the transcription factors CCAAT/enhancer binding protein (C/EBP)-β and C/EBPδ in mouse cortical astrocytes: involvement in cAMP-regulated glycogen metabolism. **J. Neurosci. 16 (3)**, 919-929.

Colangelo, V., Schurr, J., Ball, M.J., Bazan, G., Palacios Pelaez, R., Lukiw, W.J. (2000) Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. **J. Neurosci. Res. 70**, 462-473.

D'Mello, S.R., Galli, C., Ciotti, T., Calissano, P., (1993) Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. **Proc. Natl. Acad. Sci. U.S.A. 90**, 10989-10993.

Li, R., Strohmeyer, R., Liang, Z., Lih-Fen, L., Rogers, J. (2004) CCAAT/enhancer binding protein δ (C/EBPδ) expression and elevation in Alzheimer's disease. **Neurobiol. Aging 25**, 991-999.

Pahan, K., Jana, M., Liu, X., Taylor, B.S., Wood, C., Fischer, S.M. (2002) Gemfibrozil, a lipid-lowering drug, inhibits the induction of nitric-oxide synthase in human astrocytes. **J. Biol. Chem. 277 (48)**, 45984-45991.

Pérez-Capote, K., Serratosa, J., Solà, C. (2004) Glial activation modulates glutamate neurotoxicity in cerebellar granule cell cultures. **Glia 45**, 258-268.

Pérez-Capote, K., Serratosa, J., Solà, C. (2005) Excitotoxic and apoptotic neuronal death induce different patterns of glial activation *in vitro*. **J. Neurochem. 94 (1)**, 226-237.

Poli, V. (1998) The role of C/EBPs isoforms in the control of inflammatory and native immunity functions. **J. Biol. Chem. 273 (45)**, 29279-29282.

Ramji, D.P., Foka, P. (2002) CCAAT/enhancer-binding proteins: structure, function and regulation. **Biochem. J. 365**, 561-575.

Tengku-Muhammad, T.S., Hughes, T.R., Ranki, H., Cryer, A., Ramji, D.P. (2000) Differential regulation of macrophage CCAAT-enhancer binding protein isoforms by lipopolysaccharide and cytokines. **Cytokine 12 (9)**, 1430-1436.

Walton, M., Saura, J., Young, D., MacGibbon, G., Hansen, W., Lawlor, P., Sirimanne, E., Gluckman P., Dragunow, M. (1998) CCAAT-enhancer binding proteinα is expressed in activated microglial cells after brain injury. **Mol. Brain Res. 61**, 11-22.

Yano, S., Fukunaga, K., Takiguchi, M., Ushio, Y. (1996) Regulation of CCAAT/enhancer-binding protein family members by stimulation of glutamate receptors in cultured rat cortical astrocytes. **J. Biol. Chem. 271 (38)**, 23520-23527.