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Neurobiology of Disease

# CXCL16 Orchestrates Adenosine $A_3$ Receptor and MCP-1/CCL2 Activity to Protect Neurons from Excitotoxic Cell Death in the CNS

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**A role for chemokines as molecules mediating neuron-glia cross talk has emerged in recent years, both in physiological and pathological conditions. We demonstrate here for the first time that the chemokine CXCL16 and its unique receptor CXCR6 are functionally expressed in the CNS, and induce neuroprotection against excitotoxic damage due to excessive glutamate (Glu) exposure and oxygen glucose deprivation (OGD). In mice and rats we found that, to exert neuroprotection, CXCL16 requires the presence of extracellular adenosine (ADO), and that pharmacological or genetic inactivation of the ADO $A_3$ receptor, $A_3$R, prevents CXCL16 effect. In experiments with astrocytes cocultured with cxcr6$^{gfp/gfp}$ hippocampal cells, we demonstrate that CXCL16 acts directly on astrocytes to release soluble factors that are essential to mediate neuroprotection. In particular, we report that (1) upon stimulation with CXCL16 astrocytes release monocyte chemoattractant protein-1/CCL2 and (2) the neuroprotective effect of CXCL16 is reduced in the presence of neutralizing CCL2 antibody. In conclusion, we found that chemokine CXCL16 is able to mediate cross talk between astrocytes and neighboring neurons and, in pathological conditions such as excessive Glu or OGD exposure, is able to counteract neuronal cell death through an ADO-dependent chemokine-induced chemokine-release mechanism.**

## Introduction

We are getting increasingly aware that in the CNS chemokines possess pleiotropic properties beyond chemotaxis. Constitutive brain expression of chemokines and their receptors on endothelial cells but also on neurons and glia, suggests a role for such molecules in mediating homeostatic cross talk between cells. Cytokines and chemokines are important neuroinflammatory mediators involved in the pathological processes resulting from brain trauma, ischemia, and chronic neurodegenerative diseases, but may also be involved in brain repair and recovery, playing roles in the modulation of neuroprotection, neurogenesis, and neurotransmission.

CXCL16 is a transmembrane chemokine originally discovered as a scavenger receptor for oxidized LDL (Shimaoka et al., 2000), that can be cleaved to a soluble form by metalloproteinases, such as ADAM10 or ADAM17 (Schulte et al., 2007). CXCL16 and its specific receptor CXCR6 are constitutively expressed in brain parenchyma by endothelial cells, microglia, and astrocytes, which release soluble CXCL16 upon inflammatory conditions and overexpress CXCR6 upon malignant transformation (Ludwig et al., 2005). Indeed, soluble CXCL16 is normally present in the CSF at levels higher than in the serum, and in inflammatory disorders, such as multiple sclerosis, a significant increase is reported in CSF (le Blanc et al., 2006).

Adenosine (ADO) is a cellular metabolite that is formed by the breakdown of intracellular and extracellular adenine nucleotides that are physiologically present at nanomolar concentration inside and outside the cells (Fredholm, 2007). It represents an endogenous modulator of brain functions acting to fine-tune inhibitory and excitatory synaptic transmission (Ribeiro et al., 2010), glial function (Boison et al., 2010), and blood flow (Kusano et al., 2010); moreover, in the nervous system, the level of extracellular ADO rises upon brain damage which follows stroke, ischemia, and epileptic seizures (Ribeiro et al., 2003). Acting through four different G-protein-coupled receptors ($A_1$R, $A_{2A}$R, $A_{2B}$R, and $A_3$R), extracellular ADO determines the outcome of different brain injuries, mediating neuroprotective or neurotoxic effects. In this regard, we have recently shown that ADO plays a significant role in mediating $CX_3CL1$, BDNF, and erythropoietin-mediated neuroprotection through the activation of $A_1$ receptors (Lauro et al., 2008; 2010), and other reports describe that interleukin-6 (IL-6) and oncostatin M (OSM) require $A_1$R functions to exert their neuroprotective effects (Biber et al., 2008; Moidunny et al., 2010).

In this paper we found for the first time that soluble CXCL16 is able to preserve neurons from glutamate (Glu)-induced excitotoxicity, a mechanism that is probably responsible for neuronal

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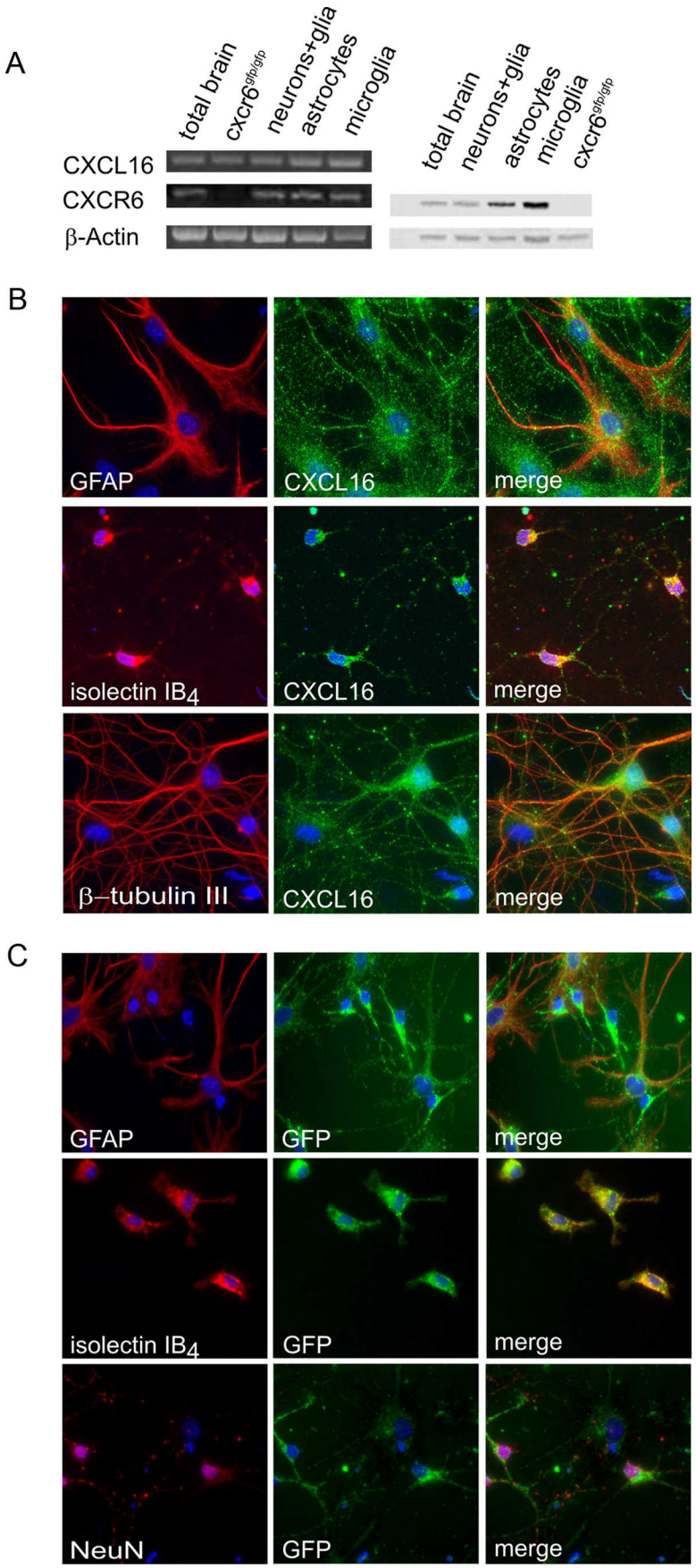


**Figure 1.** CXCL16 and CXCR6 are expressed by astrocytes, microglia, and neurons. ***A***, RT-PCR analysis for CXCL16 and CXCR6 mRNA in adult brain tissues of *wt* and cxcr6 $^{gfp/gfp}$ mice and *wt* primary cell cultures as indicated (left), Western blot analysis for CXCR6 protein in lysates derived from *wt* and cxcr6 $^{gfp/gfp}$ brain tissues, and *wt* primary cell cultures as indicated (right). mRNAs and protein lysates from cxcr6 $^{gfp/gfp}$ mice adult brain were used as negative controls. ***B***, Immunohistochemical analysis of CXCL16 expression. In primary hippocampal cultures (top and bottom) staining for CXCL16 was observed in cells identified as astrocytes (GFAP positive) and neurons (β-tubulin III positive); in primary pure microglia cultures (isolectin $IB_4$-positive cells) CXCL16 staining was also observed (middle). ***C***, Immunohistochemical analysis of GFP expression in primary cultures derived from cxcr6 $^{gfp/gfp}$ mice. In hippocampal cultures (top and bottom) GFP was expressed in astrocytes and neurons as identified by costaining with GFAP and NeuN (neuronal marker); isolectin $IB_4$ and GFP costaining was also identified in pure microglia cultures (middle). Nuclei are stained with Hoechst (blue); images acquired with 60× objective.

cell death upon traumatic brain injury and in several inflammatory and chronic neurodegenerative brain disorders (Shaw et al., 1995; Castillo et al., 1996; Hallett et al., 2004; Lipton et al., 2005). CXCL16-mediated neuroprotective effect requires the functional cross talk between astrocytes and neurons, the activation of $A_3R$, and the astrocytic release of at least one intermediate modulator, the chemokine CCL2/ monocyte chemoattractant protein-1(MCP-1).

## Materials and Methods

*Animals.* Procedures using laboratory animals were in accordance with the international guidelines on the ethical use of animals from the European Communities Council Directive of November 24, 1986 (86/609/EEC). Homozygous cxcr6 $^{gfp/gfp}$ knock-in mice (Unutmaz et al., 2000), in which the coding region of the receptor has been substituted with the coding region of the green fluorescent protein (GFP), were used. Such mice lack CXCR6 and express the GFP under the CXCR6 promoter, thus GFP cells represent cells that potentially express CXCR6. C57BL/6J [wild-type (*wt*)], and Wistar rats (Charles River Laboratory), and $A_3R$ knock-out mice ($A_3R^{-/-}$) (Salvatore et al., 2000) were also used. Animals of either sex were used.

*Hippocampal neuronal cultures.* Primary hippocampal neuronal cultures were prepared from the brain of 0–2-d-old *wt*, cxcr6 $^{gfp/gfp}$, $A_3R^{-/-}$ mice and Wistar rats. In brief, after careful dissection from diencephalic structures, the meninges were removed and the hippocampi chopped and digested in 0.025% trypsin (for mice) or 1.25 mg/ml papain (for rats), in HBSS for 20 min at 37°C. Cells were mechanically dissociated and plated at a density of $2.5 \times 10^5$ in poly-L-lysine-coated plastic 24-well dishes, in serum-free Neurobasal medium supplemented with B27, 0.5 mM L-glutamine, and 100 μg/ml gentamicin. Successively, cells were kept at 37°C in 5% $CO_2$ for 10–11 days *in vitro* (DIV) with a twice a week medium replacement (1:1 ratio). With this method we obtained 60–70% neurons, 30–35% astrocytes, and 4–5% mi-

croglia, as determined with β-tubulin III, glial fibrillary acidic protein (GFAP), and isolectin $IB_4$ staining (Lauro et al., 2010).

*Glial primary cultures.* Primary cortical glial cells were prepared from 0- to 2-d-old mice. Cerebral cortices were chopped and digested in 30 U/ml papain for 40 min at 37°C followed by gentle trituration. The dissociated cells were washed, suspended in DMEM with 10% FBS (Invitrogen) and 2 mM L-glutamine and plated at a density of $9–10 \times 10^5$ in 175 $cm^2$ cell culture flasks. At confluence (10–14 DIV), glial cells were shaken for 2 h at 37°C to detach and collect microglial cells. These procedures gave almost pure (<2% astrocyte contamination) microglial cell population, and astrocytes cell population (4–6% of microglia contamination), as verified by staining with GFAP and isolectin $IB_4$.

*Glia-neuron cocultures.* After 10–14 DIV, *wt* astrocytes/microglia cells prepared as above, were replated by seeding $2 \times 10^5$ viable cells onto 0.33 $cm^2$ transwell cell-culture inserts (pore size 0.4 μm; Corning Life Sciences) which allows traffic of small diffusible substances, but prevents cell contact. A transient coculture was initiated 1 d after replating by transferring inserts into 24-well culture plates containing 10–11 DIV primary hippocampal cells from cxcr6$^{gfp/gfp}$ mice.

*mRNA analysis.* Total RNA was extracted from adult mouse brain, primary hippocampal mixed cell cultures, primary pure astrocytes, and microglia cells using commercially available RNA kit extraction (Absolutely RNA Miniprep Kit; Stratagene). One microgram of total RNA was reverse-transcribed using ThermoScript RT-PCR System (Invitrogen) and 150 ng of the reverse transcription products were used as a template for PCR amplification. Primer sequences targeted against CXCL16 (BC019961.1, GenBank) and CXCR6 (NM_030712.4, GenBank) transcript were as follows: forward 5′-AAAGAGTGTGGAACTGGTCA TG-3′ and reverse 5′-AGCTGGTGTGCTAGCTCCAG-3′ for CXCL16 and forward: 5′-TCGTTTCATTGTAGTGGTCCAG-3′ and reverse: 5′-CATAAGTTT CCAGACGTTCTTC-3′ for CXCR6. The PCR was as follows: 95°C for 5 min, 30 cycles 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. An MJ Mini Thermal Cycler (Bio-Rad) was used for all reactions and amplification products were analyzed on 1.8% agarose gel stained with ethidium bromide.

*Western blot analysis.* Adult mouse brain, hippocampal neuronal cultures, pure astrocyte, and microglial cell cultures were washed with PBS, scraped and lysed in buffer (Tris-HCl 50 mM pH 7.5, NaCl 150 mM, EGTA 1 mM, EDTA 1 mM, 1%, Triton X-100, 0.1% SDS, phosphatase and protease inhibitor mixture). Protein concentration was determined by BCA assay (Pierce), and the same amounts of proteins (30 μg) were separated on 10% SDS-PAGE and analyzed by Western immunoblot using rabbit polyclonal anti-CXCR6 antibody (1:1000 sc-8527; Santa Cruz Biotechnology) and HRP-tagged rabbit anti-goat IgG secondary antibody (1:3000; Dako), and subsequently detected using a commercial chemiluminescent assay (Immun-Star WesternC Kit; Bio-Rad).

*Immunofluorescence.* Primary hippocampal cultures were extensively washed with PBS, fixed with paraformaldehyde 4%, sucrose 4% for 15 min, and permeabilized with 0.1% Triton X-100 for 3 min. The cells were kept for 60 min at room temperature with blocking solution (PBS containing 1% BSA), then incubated with primary antibody overnight at 4°C. Primary antibodies were as follows: polyclonal mouse CXCL16 (1:100 AF503; R&D Systems), monoclonal mouse β-tubulin III (1:1000 MCA2047; Serotec), monoclonal mouse NeuN (1:100 MAB377; Millipore Bioscience Research Reagents), and monoclonal mouse GFAP (1:1000 MAB360; Millipore Bioscience Research Reagents). Microglia cells were stained using isolectin $IB_4$ (1:300 Alexa Fluor 594 dye; Invitrogen). Nuclei were stained with Hoechst 33342 trihydrochloride, trihydrate (Invitrogen). Images were acquired by a fluorescent microscope (Nikon) using the OptiGrid structured illumination system (Quioptiq) and analyzed with MetaMorph software.

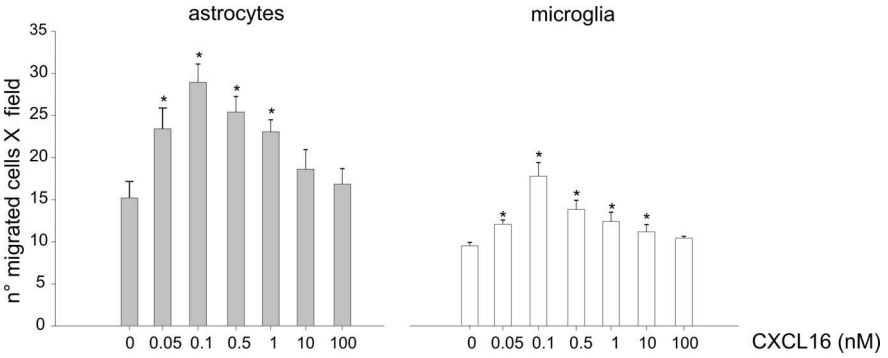


**Figure 2.** Primary astrocytes and microglia migrate in response to CXCL16. Bar histograms of concentration-dependent astrocytes and microglia migration: primary astrocytes or microglia were plated on transwell filters and exposed to different concentrations of chemokine (0–100 nM) as indicated. After 3 h of incubation, migrated cells were counted in at least 20 fields with a 40× objective. Results represent the mean ± SEM ($n = 5–6$). Statistical analysis: one-way ANOVA followed by Holm–Sidak *post hoc* test; *$p < 0.05$.

*Chemotaxis assay.* CXCL16-induced chemotaxis was investigated in mouse astrocyte and microglia primary cultures. To assess migration of glial cells *in vitro*, the established transwell migration assay using Costar transwell inserts (8 μm pore filter of 24-well cell clusters) was used (Saadoun et al., 2005; Xu et al., 2007; Ryu et al., 2009; Huang et al., 2010). In detail after 10–14 DIV cells were resuspended in chemotaxis medium (DMEM containing 0.1% BSA and 25 mM HEPES). The transwell inserts were incubated for 1 h at 37°C with chemotaxis medium and glial cells (astrocytes or microglia) were added to the upper chamber ($2.5 \times 10^5$/insert). In the lower chamber hCXCL16 was added at different concentrations (ranging from 0.01 to 100 nM) in the same medium. After 3 h of incubation at 37°C, the cells on the upper side of the membrane were removed with a cotton swab. The cells on the lower filter surface were fixed with ice-cold 10% trichloroacetic acid for 10 min and stained with a solution containing 50% isopropanol, 1% formic acid, and 0.5% (w/v) brilliant blue R250. Stained cells were counted in >20 randomly selected fields with a 40× objective. Each insert was counted and the average value was taken as the final result. For chemotaxis experiments with HEK 293 cells, cells were transfected with CCR2- or empty-vector (pcDNA 3.1). Conditioned media (for 18 h) derived from untreated astrocytes, CXCL16-treated astrocytes (100 nM, 30 min), or 2-chloro-N6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine (2-CL-IB-MECA)-treated astrocytes (100 nM, 18 h) were used as chemoattractant agents. After 3 h, cells migrating through 12 μm pore polycarbonate filters (Corning Life Sciences) were counted in >20 fields with a 40× objective. Conditioned medium (c.m.) from astrocytes treated with 2-Cl-IB-MECA (100 nM, 18 h; Tocris Bioscience) was used as positive control for migration of CCR2-HEK cells.

*Glu-induced excitotoxicity.* Primary hippocampal cultures were plated on poly-L-lysine-coated dishes. After 10–11 DIV the c.m. was removed and stored for later usage; neurons were washed and stimulated with Glu (100 μM, 30 min) in modified Locke's buffer (without $MgCl_2$ plus 1 μM glycine to stimulate all types of Glu receptors) in the presence or in the absence of CXCL16, $hCX_3CL1$, or hCCL2 (100 nM) (Peprotech). Under these experimental conditions, only neurons die (Chen et al., 2000; Lauro et al., 2010). After treatment, cells were reincubated in the original c.m. for 18–20 h, treated with lysis buffer, and counted in a hemocytometer for viability, as described previously (Volonté et al., 1994). Data were expressed as a percentage of viable cells taking as 100% the number of viable cells in control cultures. Variability in the number of viable cells in control conditions never exceeded 10%. For concentration–response analysis, CXCL16 concentrations ranging from 0.01 to 200 nM were used. When necessary, cells were pretreated with minocycline hydrochloride (200 nM, 30 min; Sigma), ADO deaminase (ADA; 1 U/ml, 1 h; Roche), monoclonal mouse αCCL2 Ab (3 μg/ml, 30 min; R&D System MAB479), rat IgG (3 μg/ml, 30 min; Santa Cruz Biotechnology sc-2032), or the following ADO receptor antagonists (15 min; Tocris Bioscience): 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS15943; 100 nM), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 50 nM), 2-(2-furanyl)-7-

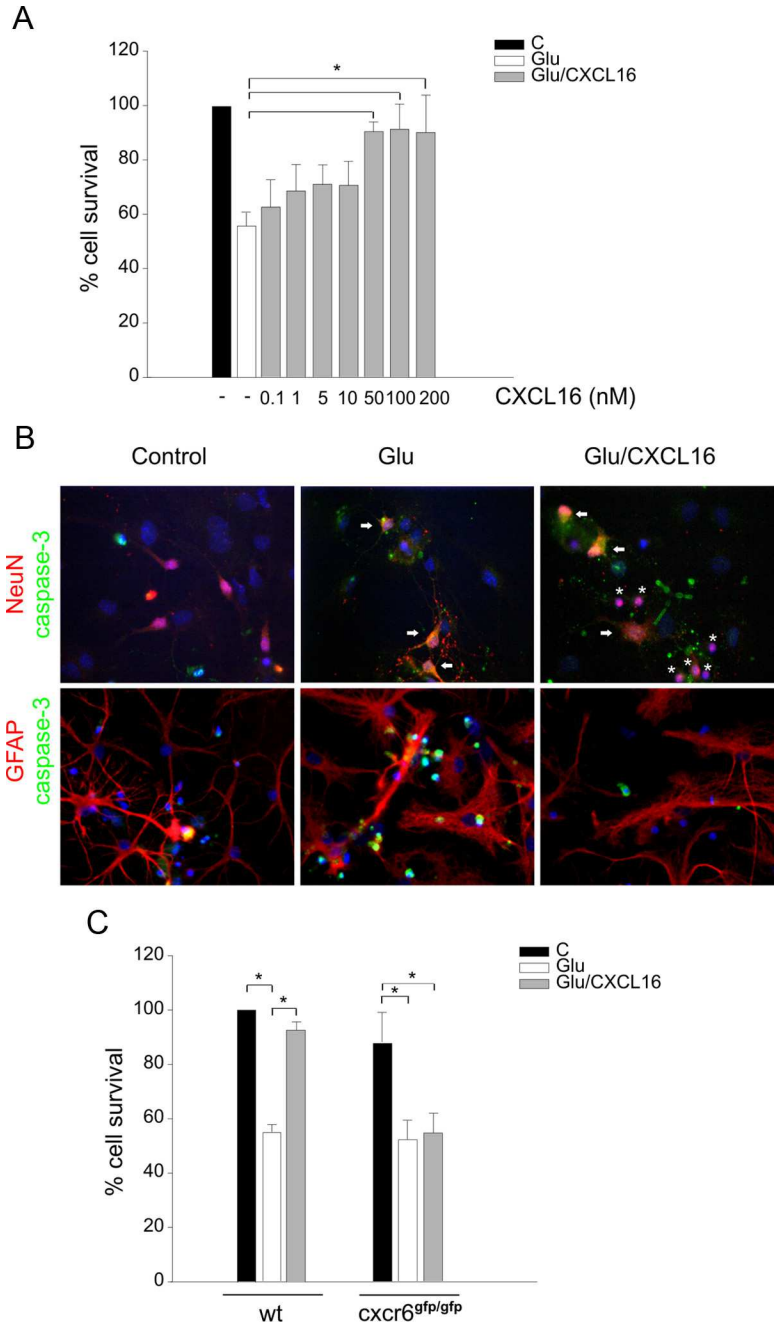


**Figure 3.** CXCL16 protects hippocampal neurons from excitotoxic cell death. ***A***, CXCL16 concentration–survival response relationship. Primary hippocampal neurons were exposed to Glu (100 $\mu$M, 30 min) in the presence or in the absence of CXCL16 (at indicated concentrations), and after 18 h analyzed for cell survival (see Materials and Methods). Results represent the mean ± SEM ($n = 5–9$). ***B***, Example of immunohistochemical analysis of cleaved caspase-3 (green), NeuN or GFAP (red) expression in control, Glu-challenged and Glu/CXCL16-treated mixed hippocampal cultures. Upon Glu challenge there is an increase in the number of caspase-3 positive cells that are also positive for NeuN with respect to control or Glu/CXCL16-treated cultures. Nuclei are stained with Hoechst (blue); white arrows indicate caspase-3-positive neurons; white stars indicate caspase-3-negative neurons; images acquired with 40× objective. ***C***, Histogram bar of cell survival upon Glu insult in *wt* and cxcr6 $^{gfp/gfp}$ mice. In cxcr6 $^{gfp/gfp}$ hippocampal cultures, CXCL16 (100 nM) is not able to increase neuronal survival upon toxic insult. Results represent the mean ± SEM ($n = 4$). Data are expressed as the percentage of viable cells in treated cultures taking as 100% the number of viable cells in *wt* control condition. Statistical analysis: one-way ANOVA followed by Holm–Sidak *post hoc* test $^*p < 0.05$ (***A***); two-way ANOVA followed by Holm–Sidak *post hoc* test $^*p < 0.05$ (***C***).

(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH58261; 5 nM), *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]acetamide (MRS1706; 20nM), and 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2phenyl-4-propyl-3-pyrinide carboxylate (MRS1523; 100 nM, Sigma) directly in culture medium; drugs were present also during and after Glu challenge.

*Analysis of cleaved caspase-3.* Immunofluorescence by using polyclonal mouse cleaved caspase-3 (1:600 Ab 9661; Cell Signaling Technology), monoclonal mouse NeuN (1:100 MAB377; Millipore Bioscience Research Reagents), monoclonal mouse GFAP (1:1000 MAB360; Millipore Bioscience Research Reagents), and Hoechst 33342 (Invitrogen) for nuclei was performed on primary hippocampal cultures untreated (control) or treated with Glu (100 $\mu$M 30 min) in the absence or in the presence of CXCL16 (100 nM). In each experimental condition we randomly analyzed a total of 400 cells, and counted those that were caspase-3 positive. We further identified cells that were both positive for casapse-3 and NeuN, or caspase-3 and GFAP. Images were acquired by a fluorescent microscope (Nikon) and analyzed with MetaMorph software.

*MTT Cell Viability.* Cell viability was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide) assay. After excitotoxic experiments, MTT solution (1.25 mg/ml; Sigma) was added to cells and after 2 h incubation cells were lysed in DMSO for 5 min. Reduced MTT staining was determined by measuring the absorbance with an automated ELISA reader (EL800 Universal Microplate Reader; Bio-Tek Instruments) at 590 nm with a background correction at 620 nm. Data were expressed as the percentage of cell viability in treated cells [Glu without/with CXCL16, or oxygen glucose deprivation (OGD) without/with CXCL16] considering as 100% the number of viable cells in control or normoxic conditions, respectively.

*ELISA.* Pure astrocytes were selected from glia cultures (10–11 DIV) by removing microglia, and $2.5 \times 10^5$ cells were seeded onto a 24-well culture plate, after 72 h cells were starved with serum-free DMEM for 2 h and then stimulated with CXCL16 (100 nM, 30 min). Medium was than replaced, collected after 6 h, centrifuged at 10,000 × *g* for 2 min, and supernatant was stored at − 80°C. Control cells were stimulated only with vehicle. CCL2 present in the supernatant was measured using a specific ELISA for mouse CCL2 (R&D Systems) as described by the manufacturer. For each sample, cells were detached and proteins were quantified (BCA assay).

*OGD.* Ten to eleven DIV primary hippocampal neurons were preincubated with different concentrations of CXCL16 (10–100 nM, 30 min) and then exposed to OGD, in the presence or in the absence of CXCL16. Briefly, culture medium was replaced with modified Locke's buffer (without glucose), bubbled with 95% $N_2$/5% $CO_2$, and transferred into an anaerobic chamber (Billups-Rothenberg MIC-101) containing a mixture of 95% $N_2$/5% $CO_2$, and humidified at 37°C for 180 min. OGD was terminated by replacing the OGD medium with the original c.m. in the presence or in the absence of CXCL16. For comparative purposes, control cultures were treated under normoxic conditions (95% $O_2$/5% $CO_2$) in complete Locke's

buffer. Cell viability was analyzed as described (see above, Glu-induced excitotoxicity).

*Statistical analysis.* The data are expressed as the means ± SEM. Where appropriate *t* test or ANOVA was used. Since in all experiments data passed the normality and the equal variance tests, we performed the parametric one-way ANOVA followed by Holm–Sidak multiple-comparison test or two-way ANOVA followed by multiple-comparison Holm–Sidak test. A value of $p < 0.05$ was considered significant. All statistical analysis was done using SigmaPlot 11.0 Software.

## Results

### CXCL16/CXCR6 pair is expressed on astrocytes, microglia, and primary neurons

We examined the expression of CXCL16 and its receptor, CXCR6, in mouse brain. mRNAs and protein lysates from adult mouse brain, from primary hippocampal cell cultures, pure astrocytes, and microglia cultures were analyzed by PCR and Western blot (Fig. 1*A*); samples from the brain of adult cxcr6$^{gfp/gfp}$ mice were used as negative control for CXCR6 expression (Unutmaz et al., 2000). Both chemokine and receptor were expressed in adult mouse brain, in primary hippocampal cultures, in astrocytes, and in microglial cells, in agreement with what was already reported (Ludwig et al., 2005; McKimmie et al., 2010). To determine CXCL16 and CXCR6 expression by neurons, we performed immunofluorescence analysis on cultured primary hippocampal cells and primary microglia cells derived from *wt* or cxcr6$^{gfp/gfp}$ mice (Unutmaz et al., 2000). In *wt* animals, immunofluorescence analysis revealed CXCL16 expression on both astrocytes and microglia, as revealed by costaining with GFAP and isolectin $IB_4$ (Fig. 1*B*), but also a neuronal distribution, as shown by colocalization of CXCL16 and β-tubulin III signals (Fig. 1*B*). In primary cell cultures derived from cxcr6$^{gfp/gfp}$ mice, GFP fluorescence is present not only on astrocytes and microglia but also on neuronal cells (as revealed by costaining with NeuN), thus suggesting a neuronal expression also for CXCR6 (Fig. 1*C*).

### Astrocytes and microglia express functional CXCR6

Since soluble CXCL16 has chemoattractive properties on CXCR6-expressing lymphocytes (Matloubian et al., 2000; Wilbanks et al., 2001; Nakayama et al., 2003), we decided to assess the functionality of CXCR6 expressed on glia analyzing, in a chemotactic assay, astrocytes and microglia migration upon stimulation with CXCL16. As reported in Figure 2, both primary astrocytes and microglia were able to migrate in response to exogenously administrated CXCL16 in a concentration-dependent way, with an effect that is maximal at ~0.1 nM ($n = 5–6$; $p < 0.05$).

### CXCL16 protects hippocampal neurons from excitotoxic cell death

CXCL16 is a proinflammatory chemokine, whose extracellular levels increase upon TNFα and IFNγ treatment of murine astrocytes (Ludwig et al., 2005; McKimmie et al., 2010). We decided to investigate the possible role of CXCL16 in protecting neurons from cell death following Glu-excitotoxic insult (100 μM, 30 min). In this experimental condition Glu induces specific neuronal cell death without affecting glial cells (Chen et al., 2000; Lauro et al., 2010). When CXCL16 was administrated during Glu challenge, neuronal cell death was reduced in a concentration-dependent way, as shown in Figure 3*A* ($n = 5–9$; $p < 0.05$). At all the tested concentrations, CXCL16 per se was not toxic (data not shown). We decided to use, in all future experiments, 100 nM of CXCL16 since, at this concentration, neuronal cell death was efficiently prevented. To further confirm that CXCL16 exerts a protective effect on neurons, 6 h after Glu challenge of hippocampal cultures in the presence or in the absence of CXCL16, cells were analyzed for cleaved caspase-3, NeuN, and GFAP staining. We observed that CXCL16 treatment significantly reduced the number of caspase-3-positive cells from 28.3 ± 4.2% (Glu) to 15.3 ± 2.9% (Glu/CXCL16) ($p < 0.05$), similar to what was observed in control untreated cells (11.5 ± 0.7%). Costaining for NeuN/caspase-3 was observed in 5.2 ± 0.9% of cells in control conditions, in 15.1 ± 2.4% upon Glu ($p < 0.05$), and in 7.0 ± 1.1% in Glu/CXCL16, while GFAP/caspase-3 costaining did not significantly differ between experimental groups (being 1.6 ± 0.3%, 2.8 ± 0.7%, and 1.8 ± 0.2%, respectively in control, Glu, and Glu/CXCL16 conditions) ($n = 3$; one-way ANOVA followed by Holm–Sidak test *post hoc* test).

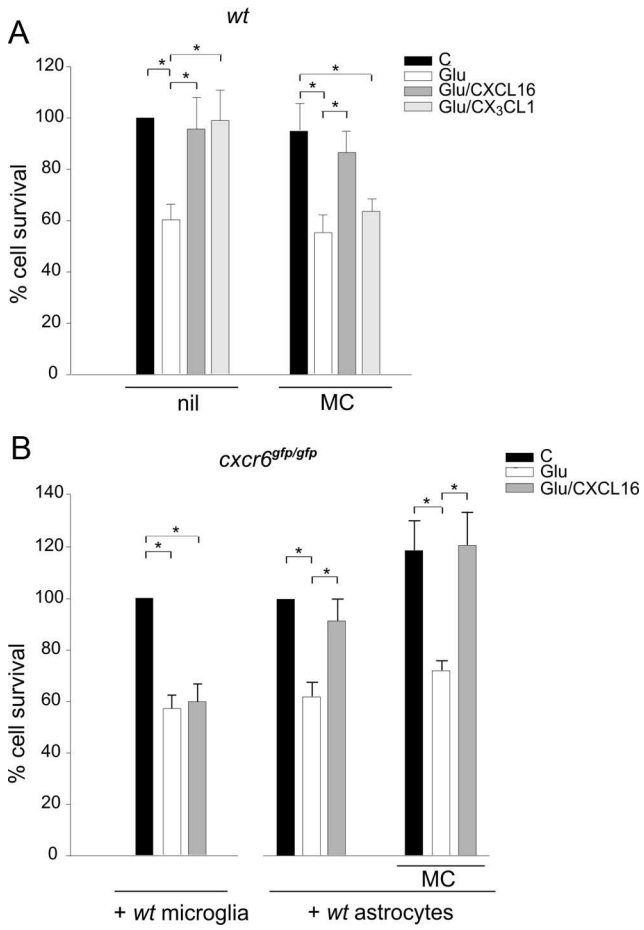


**Figure 4.** CXCL16 acts on astrocytes to promote neuroprotection. ***A***, Minocycline (MC) prevented $CX_3CL1$- but not CXCL16-induced neuroprotection upon Glu insult. Hippocampal cultures were preincubated or not with MC (200 nM, 30 min) and then stimulated for 30 min with Glu, Glu/CXCL16 (100 nM), or Glu/$CX_3CL1$ (100 nM) in the presence or in the absence of MC, as indicated. In normal condition (nil) both CXCL16 and $CX_3CL1$ increased neuronal survival upon Glu insult; in the presence of MC only $CX_3CL1$ failed to protect neurons. Results represent the mean ± SEM ($n = 4–5$). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in control nil condition. ***B***, Astrocytes mediate CXCL16 neuroprotection. Left, When hippocampal cells from cxcr6$^{gfp/gfp}$ mice were cocultured with *wt* microglia and stimulated with Glu, CXCL16 was ineffective on cell survival. Results represent the mean ± SEM ($n = 4$). Data are expressed as the percentage of viable cells in treated cultures taking as 100% the number of viable cells in microglia control condition. Right, When hippocampal cells from cxcr6$^{gfp/gfp}$ mice were cocultured with *wt* astrocytes, in presence or absence of MC, CXCL16 determined increased neuronal survival. Results represent the mean ± SEM ($n = 3–7$). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in astrocytes control condition. Statistical analysis: one-way ANOVA followed by Holm–Sidak *post hoc* test *$p < 0.05$ (***A***, ***B***).

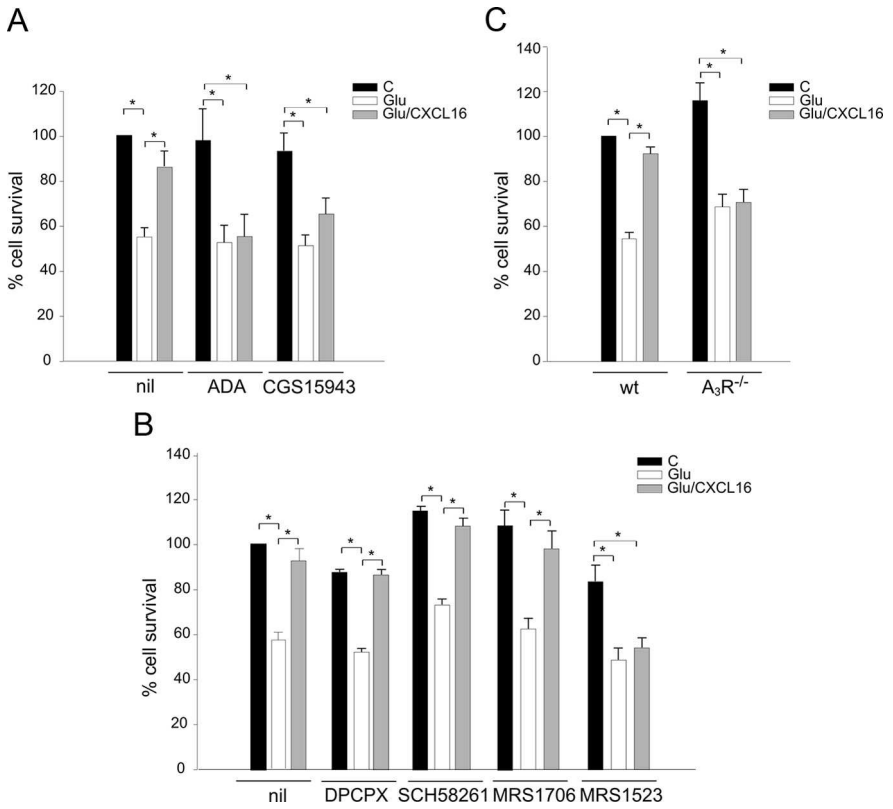


**Figure 5.** CXCL16 neuroprotection requires $A_3R$ activity. ***A***, CXCL16 neuroprotection requires ADO activity. To remove extracellular ADO, primary rat hippocampal cultures were incubated with ADA (1 U/ml, 1 h before, during, and following Glu insult). While in the presence of ADO (nil treatment) CXCL16 prevented neuronal cell death; in absence of ADO (ADA treatment) CXCL16 was not able to promote neuronal survival. To prevent ADO activity hippocampal cultures were incubated with the generic AR antagonist CGS15943 (100 nM). Upon this treatment the protective effect of CXCL16 was significantly reduced compared with control condition (nil treatment). Results represent the mean ± SEM ($n = 4–11$). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in control nil condition. ***B***, Effects of pharmacological inhibition of ARs. Rat hippocampal neurons were treated with specific antagonists (DPCPX, 50 nM; SCH58261, 5 nM; MRS1706, 20 nM; MRS1523, 100 nM) and used for Glu-excitotoxic experiments, as indicated. Only in the presence of MRS1523 was CXCL16 neuroprotection significantly reduced compared with the control condition (nil). Results represent the mean ± SEM ($n = 5–9$). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in control nil condition. ***C***, Genetic deletion of $A_3R$ prevented CXCL16 effect. Hippocampal neurons obtained from *wt* or $A_3R^{-/-}$ mice were stimulated with Glu in the presence or in the absence of CXCL16. In $A_3R^{-/-}$ cultures, CXCL16 failed to increase cell survival upon Glu insult. Results represent the mean ± SEM ($n = 4$). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in wt control condition. Statistical analysis: one-way ANOVA followed by Holm–Sidak *post hoc* test; $^*p < 0.05$ (***A–B***); two-way ANOVA followed by Holm–Sidak *post hoc* test $^*p < 0.05$ (***C***).

our experimental conditions Glu challenge induces selective neuronal death which is prevented by CXCL16 treatment. An example of costaining for caspase-3, NeuN, and GFAP is shown in Figure 3*B*.

To prove that the neuroprotective effect of CXCL16 was specifically mediated by CXCR6, excitotoxic experiments were performed not only in primary cultures obtained from *wt*, but also from cxcr6$^{gfp/gfp}$ mice (that lack CXCR6), as shown in Figure 3*C* ($n = 4$). Two-way ANOVA analysis indicated a significant interaction between genotypes and treatments ($p = 0.035$). *Post hoc* evaluation revealed that CXCL16 was ineffective in neuroprotection in cxcr6$^{gfp/gfp}$ mice, since significant differences between Glu and Glu/CXCL16 treatments occurred only in *wt* animals ($p < 0.05$). However, the lack of CXCL16/CXCR6 signaling did not render hippocampal neurons more susceptible to Glu excitotoxicity since cell death obtained in *wt* and cxcr6$^{gfp/gfp}$ neurons was comparable (54.6 ± 2.7% of cell survival in *wt* vs 59.5.7 ± 7.9% of cell survival in cxcr6$^{gfp/gfp}$). CXCL16 neuroprotective effect was also confirmed by MTT assay: cell viability, 100% in untreated cells; 76.7 ± 3.3% in Glu-treated cells ($p < 0.05$); 94.4 ± 3.6% in Glu/CXCL16-treated cells ($n = 4$; one-way ANOVA followed by Holm–Sidak test *post hoc* test).

### CXCL16 acts on astrocytes to promote hippocampal neuroprotection

We have previously shown that another transmembrane chemokine, $CX_3CL1$, induces protection of hippocampal neurons from Glu-induced excitotoxicity with a mechanism mediated by microglia (Lauro et al., 2008, 2010). Since we found that CXCR6 is functionally expressed on microglia (see Fig. 2), we wanted to investigate the possible involvement of microglia in mediating CXCL16-induced neuroprotective effect. For this reason we pretreated hippocampal neuronal cultures with minocycline, a tetracycline-like antibiotic known to inhibit microglia activation, before Glu insult. As shown in Figure 4*A*, the inhibition of microglia activation selectively prevented $CX_3CL1$-induced neuroprotection, while CXCL16 was still able to rescue neurons from cell death (Fig. 4*A*, $n = 4–5$; $p < 0.05$) indicating that microglia activation is not required for CXCL16-mediated neuroprotection. To further exclude the involvement of microglia in the neuroprotective effect of CXCL16, we did coculture experiments with pure *wt* microglia and mixed cxcr6$^{gfp/gfp}$ hippocampal cultures (that lack CXCR6 receptor). Hippocampal cells were then treated with Glu, while microglia was stimulated with CXCL16. Under these conditions we found that the percentage of cell death upon Glu insult was not significantly altered by chemokine stimulation (Fig. 4*B*, left) ($n = 4$). This result showed that signaling of CXCR6 on microglia cells is not important, or not sufficient per se, to promote neuronal survival.

To analyze the involvement of astrocytes in CXCL16 neuroprotection, cxcr6$^{gfp/gfp}$ hippocampal cells were challenged with Glu while coculturing with *wt* astrocytes treated or not with CXCL16. Results, shown in Figure 4*B* (right), demonstrate that neuronal cell death was strongly reduced by substance(s) present in the medium conditioned by CXCL16-treated astrocytes ($n = 7$; $p < 0.05$), thus suggesting that astrocytes are active players in CXCL16-mediated neuroprotection. Since in the coculture experiments astrocytes were not completely devoid of microglia cells (representing 4–6% of total cells), we could not rule out that astrocytes-microglia cross talk was necessary for CXCL16-mediated neuroprotection. To exclude this possibility, minocycline was used in astrocyte-neuron coculture experiments: as shown in Figure 4*B* (right), CXCL16 was still effective in neuroprotection ($n = 3$; $p < 0.05$), thus confirming that the chemokine directly acts on astrocytes to promote cell survival.

### ADO, acting on $A_3R$, is a mediator of CXCL16-induced neuroprotective effect

In the brain, astrocytes represent the main cellular source of extracellular ADO and express all the four known types of adenosine receptors (ARs): $A_1R$, $A_{2A}R$, $A_{2B}R$, $A_3R$ (Verkhratsky et al., 2009). We therefore decided to study if ADO was able to contribute to the CXCL16-mediated neuroprotective effect. To remove extracellular ADO we treated rat primary hippocampal cultures with ADA, an enzyme that converts ADO to inosine (1 U/ml, 1 h), and performed Glu-excitotoxic experiments in the presence of ADA. As shown in Figure 5*A*, upon ADA treatment, CXCL16 failed to protect neurons from Glu insult ($n = 4$). Similarly, when experiments were performed in the presence of the nonspecific AR antagonist CGS15943 (100 nM) (Fig. 5*A*), CXCL16 was ineffective in neuroprotection ($n = 7$).

To determine which AR was involved in mediating CXCL16 neuroprotection, we performed excitotoxic experiments in the presence of specific AR antagonists. Results shown in Figure 5*B* demonstrated that only the $A_3R$ antagonist, MRS1523, was capable of blocking CXCL16 neuroprotection against Glu excitotoxicity. MRS1523 per se was not toxic (83.3 ± 7.1% cell survival compared with nil control; $n = 5$). In the presence of all the other antagonists, DPCPX for $A_1R$, SCH58261 for $A_{2A}R$, and MRS1706 for $A_{2B}R$, CXCL16 was still able to preserve cells at the concentration used ($n = 5$–9; $p < 0.05$). To confirm the role of $A_3R$, we performed experiments in hippocampal cultures obtained from $A_3R^{-/-}$ mice. As shown in Figure 5*C*, at difference with *wt*, in $A_3R^{-/-}$ cultures CXCL16 was not able to prevent Glu-induced cell death, thus confirming that ADO acts via $A_3R$s to mediate CXCL16 neuroprotective effect.

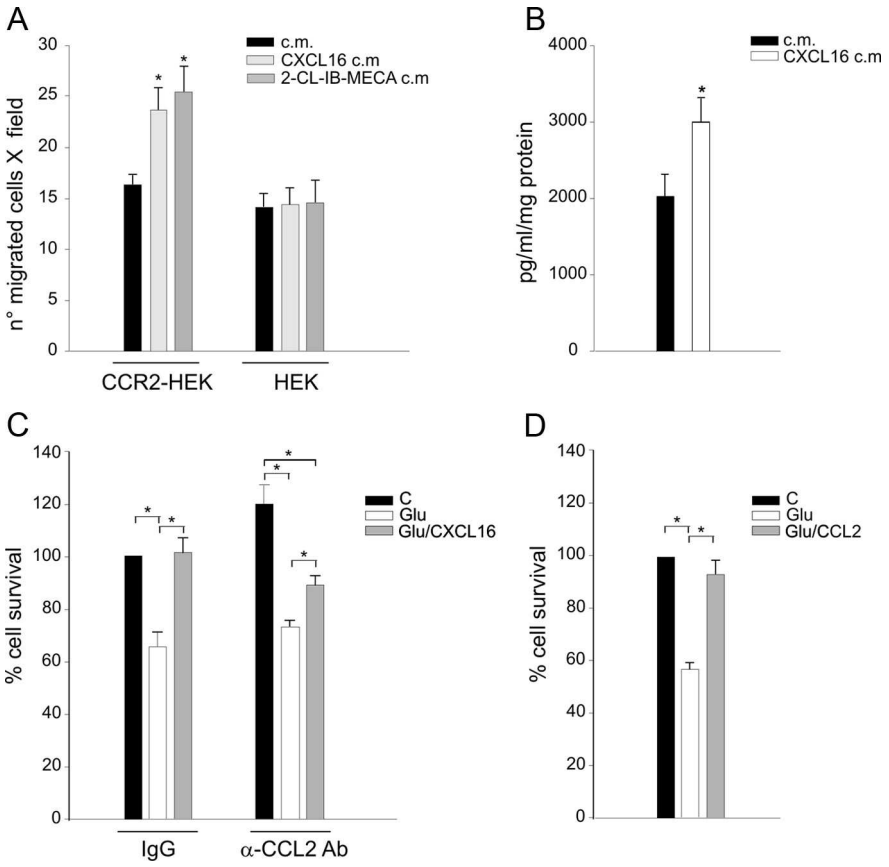


**Figure 6.** The release of CCL2 by astrocytes upon CXCL16 stimulation is determinant for CXCL16 neuroprotection. ***A***, HEK cells that express CCR2 migrate in response to conditioned medium (c.m.) derived from CXCL16 stimulated astrocytes. The c.m. derived from astrocytes treated with vehicle, CXCL16 (100 nM) or 2-CL-IB-MECA (100 nM), was collected after 18 h. The c.m. of CXCL16- and 2-CL-IB-MECA-treated astrocytes specifically increased the chemotaxis of HEK-expressing CCR2 (CCR2-HEK). Results represent the mean ± SEM ($n = 8$–10). ***B***, CXCL16 stimulation induces CCL2 release from astrocytes. Astrocytes were incubated with CXCL16 (100 nM, 30 min) or vehicle and the media were replaced and collected after 6 h. CCL2 levels in the media were measured by ELISA. Results represent the mean ± SEM ($n = 7$). ***C***, Neutralization of CCL2 activity significantly prevented CXCL16 neuroprotection. In primary hippocampal cultures treated with neutralizing α-CCL2 Ab (3 μg/ml; 30 min before, during, and after Glu challenge), neuronal survival following CXCL16 stimulation was reduced compared with survival obtained in control condition (IgG treatment; 3 μg/ml). Results represent the mean ± SEM ($n = 5$–8). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in IgG control condition. ***D***, CCL2 is able to reduce Glu-excitotoxic cell death. Primary hippocampal cultures were exposed to Glu (100 μM; 30 min) in the presence or in the absence of CCL2 (100 nM) as indicated, and analyzed for cell survival. Results represent the mean ± SEM ($n = 5$–6). Statistical analysis: one-way ANOVA followed by Holm–Sidak *post hoc* test, $^*p < 0.05$ (***A***, ***C***, ***D***); Student's *t* test $^*p < 0.05$ (***B***).

In particular, two-way ANOVA indicated a significant interaction between genotypes and treatment ($p = 0.003$) with a main effect of treatments. *Post hoc* evaluation revealed a significant difference between Glu and Glu/CXCL16 treatments only in *wt* animals ($n = 4$; $p < 0.05$).

### CCL2 is released by astrocytes upon CXCL16 stimulation

In the coculture experiments, mixed hippocampal cultures from $cxcr6^{gfp/gfp}$ mice were incubated with *wt* astrocytes and separated by a transwell membrane that allowed for transfer of soluble factors while preventing direct contact. Data obtained with this approach lead to the hypothesis that soluble factors released from astrocytes are essential mediators of CXCL16 neuroprotection. It is known that astrocytes might exert a neuroprotective role upon brain insult by buffering extracellular Glu and releasing neuroprotective molecules (Bélanger and Magistretti, 2009). Moreover, upon ADO treatment, cultured astrocytes release neuroprotective molecules (Schwaninger et al., 1997; Ciccarelli et al., 1999), and stimulation of primary mouse astrocytes with the specific $A_3R$ agonist 2-CL-IB-MECA induces the release of the chemokine CCL2 (Wittendorp et al., 2004). We therefore decided to investigate the ability of CXCL16 to induce CCL2 release from astrocytes. The c.m. from *wt* astrocytes, treated with CXCL16 (100 nM) for 30 min, was collected after 18 h and used in a chemotaxis assay on HEK cells transfected with CCR2 (CCR2-HEK). As shown in Figure 6*A*, CXCL16 c.m. was able to increase the rate of CCR2-HEK cell migration ($n = 8$–10; $p < 0.05$), while it was ineffective on mock-transfected HEK cells ($n = 4$–5). 2-CL-IB-MECA astrocytic c.m. was used as positive controls for migration of CCR2-HEK.

To assess the role of $A_3R$ in CXCL16-mediated CCL2 release, chemotaxis experiments were performed on astrocytes derived from $A_3R^{-/-}$ mice. We found that the medium conditioned by CXCL16 treatment of these cells was not able to induce CCR2-HEK cell migration, as for the medium conditioned by 2-CL-IB-MECA (c.m.

22.3 ± 3.6, CXCL16 c.m. 23.9 ± 2.4, 2-CL-IB-MECA c.m. 24 ± 2.6 migrated cells × field; $n = 4$, one-way ANOVA followed by Holm–Sidak *post hoc* test).

To confirm the ability of CXCL16 to cause CCL2 release from astrocytes, we measured the level of CCL2 in the c.m. of unstimulated astrocytes or astrocytes stimulated with CXCL16 (100 nM, 30 min). Six hours after stimulation, astrocyte culture media were collected and analyzed by ELISA. We found a basal release of CCL2 from astrocytes, in agreement with previous data (Giullemin et al., 2003; Wittendorp et al., 2004; Madrigal et al., 2009), that increases upon CXCL16 stimulation, as shown in Figure 6*B* ($n = 7$; $p < 0.05$). When experiments were performed with astrocytes derived from $A_3R^{-/-}$ mice, CXCL16 was not able to increase the basal release of CCL2 (1660 pg/ml/mg vs 1410 pg/ml/mg).

### Release of CCL2 from astrocytes is important to mediate CXCL16 neuroprotection

It has been recently found that astrocyte-derived CCL2 represents an intermediate player in the neuroprotective effect exerted by noradrenaline against excitotoxic or OGD damage (Madrigal et al., 2009). Since we found that CXCL16 induces the release of CCL2 from astrocytes, we wanted to determine whether astrocyte-derived CCL2 is a mediator of CXCL16 neuroprotection. For this reason hippocampal cultures were challenged with Glu and CXCL16 in the presence of CCL2 neutralizing antibody (Ab). Data, reported in Figure 6*C*, show that in the presence of α-CCL2 Ab (3 μg/ml), the neuroprotective effect of CXCL16 was significantly and specifically reduced, being unaffected by control IgG ($n = 5–8$; $p < 0.05$). We then assessed the ability of exogenous CCL2 to reduce Glu-excitotoxic cell death. Indeed, as reported in Figure 6*D*, administration of CCL2 (100 nM, 30 min) to hippocampal cultures, during Glu insult, prevented neuronal cell death ($n = 5–6$; $p < 0.05$).

The inability of α-CCL2 Ab to totally prevent CXCL16 neuroprotection could be due to a nonsaturating affect of the Ab at the concentration used, or to the existence of other soluble factors that, together with CCL2, contribute to CXCL16 neuroprotection.

### CXCL16 protects from OGD

Glu excitotoxicity plays an important role in triggering neuronal death upon ischemia (Choi et al., 1990). Hypoxic/ischemic events contribute to neuronal degeneration in many acute CNS disorders including stroke, traumatic brain injury, and epilepsy, playing also a role in chronic diseases like amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD; Liou et al., 2003). We therefore decided to verify the ability of CXCL16 to protect from cell death upon OGD insult. Exposure of primary hippocampal cells to OGD caused significant cell death (59.63 ± 6.38% of cell survival compared with normoxic control conditions), which was significantly reduced when cells were incubated with CXCL16 (100 nM), as shown in Figure 7 ($n = 5–6$; $p < 0.05$). Similar results were obtained by MTT assay: cell viability, 100% in normoxia; 72.3 ± 4.0% in OGD ($p < 0.05$); 70.7 ± 3.5% in OGD/CXCL16 10 nM ($p < 0.05$); 88.2 ± 2.3% in OGD/CXCL16 100 nM ($n = 5$; one-way ANOVA followed by Holm–Sidak *post hoc* test).

## Discussion

Glu-induced excitotoxicity is likely a major cause of neuronal cell death upon different brain injuries and in several pathological conditions. High levels of Glu in the brain occur following traumatic brain injuries (Zauner et al., 1996), in AD (Lipton et al., 2005), in Parkinson's disease (Hallett et al., 2004), in ischemia (Castillo et al., 1996), and in ALS (Shaw et al. 1995). In the present paper we demonstrate for the first time that soluble CXCL16 is able to prevent neuronal cell death upon insults like Glu-induced excitotoxicity and OGD. We found that, to exert neuroprotection, CXCL16 requires the presence of extracellular ADO and the activation of $A_3R$. Acting on astrocytes, CXCL16 induces the release of soluble mediators that contribute to neuroprotection; in particular, astrocyte release of CCL2 is a major factor in reducing neuronal cell death.

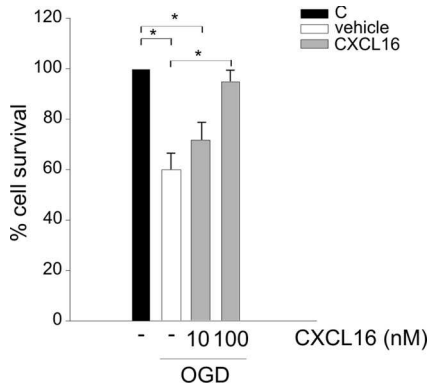


**Figure 7.** CXCL16 protects hippocampal cells form OGD damage. CXCL16 was able to increase cell survival upon OGD. Primary hippocampal cultures were exposed to OGD for 180 min in the absence or presence of different concentrations of CXCL16 (as indicated), and analyzed for cell survival (see Materials and Methods). At higher concentration (100 nM) CXCL16 reduced OGD damage. Results represent the mean ± SEM ($n = 5–6$). Data are expressed as the percentage of viable cells in OGD conditions taking as 100% the number of viable cells in normoxic control conditions (C). Statistical analysis: one-way ANOVA followed by Holm–Sidak *post hoc* test, *$p < 0.05$.

The concept that ADO acts as an endogenous modulator able to exert neuroprotective effects through to the control of glutamatergic transmission is well established (Fredholm et al., 2005). In addition, its role in modulation of cell metabolism (Cunha, 2001), neuron-glia communication (Boison et al., 2010), and the tuning of growth factor signaling (Gomes et al., 2011) is becoming evident. Indeed, we and others have recently shown that the chemokine $CX_3CL1$ and the cytokines IL-6 and OSM are able to drive neuroprotection only in the presence of functional $A_1R$ (Biber et al., 2008; Lauro et al., 2008, 2010; Moidunny et al., 2010; Cipriani et al., 2011). In particular, IL-6 and OSM potentiate the expression and the function of neuronal $A_1R$ and therefore the ADO effects on neurotransmission and neuroprotection (Moidunny et al., 2010); on the other hand, $CX_3CL1$ neuroprotection is due to ADO release from microglia, activation of $A_1R$, and, possibly, the subsequent release of glial-soluble factors that contribute to neuroprotection (Lauro et al., 2010). In contrast, in the present work we found that CXCL16 exerts neuroprotection against Glu insult only in the presence of functional $A_3R$, the inactivation of all of the other ARs being unable to limit the CXCL16 effect. In rodents, among ARs, $A_3R$ has the lower affinity for ADO (Jacobson et al., 1995) and is expressed at low levels in the brain (Ji et al., 1994), including the hippocampus (Dunwiddie et al., 1997; Macek et al., 1998) and the cerebral cortex (Brand et al., 2001). The ability of $A_3R$ to contribute to neuroprotection is controversial: *in vivo* studies suggest that differences in timing of intraperitoneal $A_3R$ agonist administration may alter the outcome of ischemic brain injury (Von Lubitz et al., 1995, 2001). *In vitro* studies showed that, depending on brief or prolonged periods of ischemia, $A_3R$ mediates effects on neurotransmission that can shift from protection to injury (Pugliese et al., 2007). In the present study we report data supporting the hypothesis that $A_3R$ drives neuroprotective effects, in agreement with the finding that upon repeated episodes of hypoxia, $A_3R^{-/-}$ mice have a wider neurodegeneration compared with *wt* (Fedorova et al., 2003), and that central administration of selective $A_3R$ agonist reduces ischemic brain injury in mice (Chen et al., 2006).

Even if CXCR6 is expressed both on glia and neurons, we found that astrocytes are the essential players in CXCL16-induced effect, releasing soluble factor(s), such as CCL2, that mediate protection on neighboring neurons. Our data are in accordance with previous evidence that $A_3R$ is functionally expressed on astrocytes (Di Iorio et al., 2002), and that $A_3R$ modulates CCL2 release form astrocytes (Wittendorp et al., 2004), thus confirming that the modulation of glial function by ADO is an important issue in neuroprotection, and that ARs expressed on astrocytes control the release of neuroactive substances (Daré et al., 2007). Indeed, we have shown that $\alpha$CCL2 neutralizing antibody significantly reduced CXCL16 neuroprotection. The ability of CCL2 to preserve neuronal cell death by other insults, such as HIV-1 *trans*-activator protein (Tat) toxicity, has been documented (Yao et al., 2009). Moreover the hypothesis that CCL2 can act as a critical intermediate factor driving neuroprotection is consistent with the findings that, in human cortical neuronal cultures, CCL2 released by astrocytes, upon RANTES stimulation, reduces the toxic effect of NMDA and Tat (Eugenin et al., 2003), and with recent data showing that astrocyte-derived CCL2 mediates the neuroprotective effect of noradrenaline against excitotoxic and OGD damages (Madrigal et al., 2009).

The target of CCL2, involved in mediating the neuroprotective activity of the chemokine, has not been addressed in the present study: previous reports showed that the main receptor for CCL2 is CCR2, but this chemokine can also bind to other receptors like CCR1, CCR4, and L-CCR, albeit with lower affinity (Biber et al., 2003; Savarin-Vuaillat et al., 2007; White et al., 2007; Quinones et al., 2008). In addition, CCR2 expression in brain parenchyma is controversial. Studies using immunohistochemistry and autoradiography techniques demonstrated either constitutive or inducible expression of CCR2 both in neurons and astrocytes (Meucci et al., 1998; Zuurman et al., 2003; Torres-Muñoz et al., 2004; Rosténe et al., 2007), and several reports described physiological effects of CCL2 on synaptic transmission (Guyon et al., 2009; Zhou et al., 2011). However, recent analyses of CNS tissues of CCR2-RFP mice provided no evidence of CCR2 expression either on neurons, astrocytes, microglia, or oligodendrocytes (Saederup et al., 2010), apparently limiting CCR2 expression in the brain to pathological conditions with infiltration of Ly6C(hi) monocytes. This last piece of evidence suggests that, at least under physiological conditions, other receptors might be involved in transducing CCL2-mediated signaling in the brain, as observed in astrocytes (Quinones et al., 2008).

In conclusion, the activity of CXCR6 and $A_3R$ on astrocytes is the initial event of CXCL16 neuroprotection, followed by release of CCL2 from astrocytes. We speculate that the activation of $A_3R$ is an event downstream of the release of ADO from astrocytes upon CXCL16 treatment; alternatively, also in light of the emerging data on the determinant role of ADO in cytokine neuroprotection (Biber et al., 2008; Lauro et al., 2008, 2010; Moidunny et al., 2010), it can be hypothesized that ADO acts as a synergistic modulator that senses neuronal environment and, together with specific triggering events driven from single cell types, concurs to neuroprotection acting on specific ARs. However, we cannot exclude that other soluble factors might represent key mediators of CXCL16 neuroprotection, since impairing CCL2 activity dramatically reduces, but did not fully abolish, the ability of the chemokine to preserve neurons.

Together these data demonstrate that CXCL16 is constitutively expressed in CNS and that, upon pathological conditions, it is able to reduce neuronal cell death through a mechanism that requires cross talk between astrocytes and neurons, representing an example of how chemokine-induced chemokine-release from astrocytes might be a phenomenon that drives beneficial effects.

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