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Adenosine A₁ receptors and microglial cells mediate CX3CL1-induced protection of hippocampal neurons against Glu-induced death.

Running title: CX3CL1 is not neuroprotective on A₁R^{-/-} hippocampal neurons.

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Abstract

Fractalkine/CX3CL1 is a neuron-associated chemokine, which modulates microglia-induced neurotoxicity activating the specific and unique receptor CX3CR1. CX3CL1/CX3CR1 interaction modulates the release of cytokines from microglia, reducing the level of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL1- β) and nitric oxide and induces the production of neurotrophic substances, both *in vivo* and *in vitro*. We have recently shown that blocking adenosine A₁ receptors (A₁R) with the specific antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) abolishes CX3CL1-mediated rescue of neuronal excitotoxic death and that CX3CL1 induces the release of adenosine from microglia. In this paper we demonstrate that the presence of extracellular adenosine is mandatory for the neurotrophic effect of CX3CL1 since reducing adenosine levels in hippocampal cultures, by adenosine deaminase (ADA) treatment, strongly impairs CX3CL1-mediated neuroprotection. Furthermore, we confirm the predominant role of microglia in mediating the neuronal effects of CX3CL1, since the selective depletion of microglia from hippocampal cultures treated with clodronate-filled liposomes causes the complete loss of effect of CX3CL1. We also demonstrate that hippocampal neurons obtained from A₁R^{-/-} mice are not protected by CX3CL1 whereas A_{2A}R^{-/-} neurons are. The requirement of functional A₁R for neuroprotection is not unique for CX3CL1 since A₁R^{-/-} hippocampal neurons are not rescued from Glu-induced cell death by other neurotrophins like brain-derived neurotrophic factor (BDNF) and erythropoietin (EPO), which are fully active on *wt* neurons.

Keywords: fractalkine, excitotoxicity, neuroprotection, A₁R, microglia, clodronate

Abbreviations: A₁R, adenosine receptor type 1; A_{2A}R, adenosine receptor type 2; A₃R, adenosine receptor type 3; AOPCP, α - β -Methyleneadenosine 5'-diphosphate; ADA, adenosine deaminase; BDNF, brain-derived neurotrophic factor; DPCPX, 1,3-dipropyl-8-

cyclopentylxanthine; MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle medium; Glu, glutamate; EPO, erythropoietin; FBS, foetal bovine serum; GPCR, G protein coupled receptor; HBSS, Hank's balanced salt solution; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBTI, S-(4-Nitrobenzyl)-6-thioinosine.

Introduction

The chemokine family comprises more than 40 members in 4 different subfamilies, whose expression in the nervous system has been correlated with different pathological conditions (Tran and Miller, 2003). CX3CL1, also called fractalkine, is the unique member of the CX3C (or δ) family, and, together with CXCL16, are the only two transmembrane chemokines described until now. It is converted to a soluble form upon cleavage from the plasma membrane through the action of metalloproteinases, like a disintegrin and metalloproteinase domains (ADAM) 10 and ADAM17 on leukocytes (Hundhausen et al., 2003) or cathepsin S in the spinal cord (Clark et al., 2007). CX3CL1 is constitutively expressed in the nervous system, but levels in the brain can be modulated under diverse pathological conditions (Pan et al., 1997; Hughes et al., 2002; Kastenbauer et al., 2003; Sunnemark et al., 2005; Huang et al., 2006). The presence and the stimulation (Zujovic et al., 2000, 2001; Mizuno et al., 2003; Cardona et al., 2006, Lyons et al., 2009) of the CX3CL1 receptor CX3CR1 has been correlated with a reduced release of interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF α) from microglial cells and a lower rate of neuronal degeneration in different experimental models of neuropathologies like experimental autoimmune encephalomyelitis (EAE), 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine hydrochloride (MPTP) striatal injection, lipopolysaccharide (LPS) administration and superoxide dismutase (SOD1) mutation (Huang et al., 2006; Cardona et al., 2006). These data attest to a role of the pair CX3CL1/CX3CR1 in reducing neuronal degeneration upon several types of brain injury. Exceptions are however reported: in experimental transient brain ischemia the absence of CX3CL1 or CX3CR1 is correlated with reduced IL-1 β and TNF- α production and a better outcome for neurons (Soriano et al., 2002; Dénes et al., 2008).

The neurotrophic activity of exogenously administered CX3CL1 has been related to the simultaneous production of protective factors from microglial cells and in particular with the

activation of adenosine receptors (ARs, Lauro et al., 2008). Adenosine is a cellular metabolite whose intracellular and extracellular levels can be rapidly modulated by variation of cellular metabolic state (see Fredholm 2007). Under physiological conditions, in the brain, ATP can be released by neurons and glial cells (Pascual et al., 2005; Burnstock et al., 2007). The released ATP is rapidly degraded to ADP, AMP and adenosine by the sequential activity of extracellular nucleotidase (Zimmermann et al., 1996). Pathological stimuli, which led to the imbalance of membrane potential, like energy failure due to a reduced tissue perfusion, prolonged activation of glutamate (Glu) receptors (Manzoni et al., 1994), transient oxygen and glucose deprivation (Lloyd et al., 1993), or prolonged electric activity like that observed during seizures (Cunha et al., 1996), have all been associated with an increased release of adenosine in the extracellular space, in some cases due to altered activity of adenosine kinase (Boison 2006).

Adenosine is a pleiotropic agent which, in the nervous system, exerts a wide range of effects (see Fredholm et al., 2005): it has a general inhibitory pre-synaptic activity on glutamatergic transmission (Dolphin and Archer, 1983), modulates the response to noxious stimuli (de Mendonça et al., 2000), regulates pain sensation (Sawynok and Liu, 2003), and has been implicated in pre-conditioning (reviewed in Fredholm, 2007). Adenosine effects are mediated through four G protein coupled receptors (GPCRs). It has long been recognized that adenosine is a modulator and that therefore signaling via its receptors occurs together with signaling via other GPCRs like metabotropic Glu receptors (mGluRs), dopamine, purine and cannabinoid receptors, (Agnati et al., 2003), as well as tyrosine kinase receptors like the fibroblast growth factor receptors (FGFRs, Flajolet et al., 2008). For example, ARs enhance the effect of other substances, like FGF (Flajolet et al., 2008) ATP (Gerwins et al., 1992; Färber et al., 2008), BDNF (Diógenes et al., 2007; Tebano et al., 2008) and GDNF (Gomes et al., 2009).

In the present study we have further examined the role of adenosine in mediating the neurotrophic activity of CX3CL1. We have explored the role of microglial cells, the release of adenosine, and the receptors responsible for the effect.

Materials and methods

Materials

Transwell inserts were from BD Labware (Franklin Lakes, NJ); recombinant rat CX3CL1 and recombinant human BDNF were from Calbiochem/Merck (Nottingham, UK); adenosine deaminase (ADA) from calf intestinal mucosa, recombinant human EPO, S-(4-Nitrobenzyl)-6-thioinosine (NBTI), α - β -Methyleneadenosine 5'-diphosphate sodium salt (AOPCP) and poly-L-lysine were from Sigma-Aldrich (Milan, Italy); all culture media were from Invitrogen Life Technologies (San Giuliano Milanese, Italy); Cl₂MDP (or clodronate) was a gift of Roche Diagnostics GmbH (Mannheim, Germany).

Animals and cell lines

Procedures using laboratory animals were in accordance with the international guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609/EEC). CX3CR1^{GFP/GFP} mice (Jung et al., 2000) were obtained from Jackson Laboratory; A₁R^{-/-} (Johansson et al., 2001) and A_{2A}R^{-/-} (Chen et al. 1999), were backcrossed at least 10 times on a C57BL/6 background; CX3CL1^{-/-} (Cook et al., 2001) were kindly provided by Dr. Richard M. Ransohoff (Cleveland Clinic, OH).

Hippocampal neuronal cultures

Primary hippocampal neuronal cultures were prepared from 0-2 day-old (p0-p2) C57BL/6 (*wt*), A₁R^{-/-}, A_{2A}R^{-/-}, CX3CL1^{-/-} and CX3CR1^{GFP/GFP} mice. Briefly, after careful dissection from diencephalic structures, the meninges were removed and hippocampal tissues were chopped and digested for 15 min at 37°C in 0.025% trypsin and Hank's balanced salt solution (HBSS). Cells were washed twice with HBSS to remove the excess of trypsin, mechanically dissociated in

minimal essential medium (MEM) with Earl's Salts and GLUTAMAX supplemented with 10% dialyzed and heat inactivated foetal bovine serum (FBS), 100 µg/ml gentamycin and 25mM KCl. Cells were plated at a density of 2.5×10^5 in the same medium on poly-L-lysine (100 µg/ml) coated plastic 24-well dishes. After 1-2 h the medium was replaced with serum-free Neurobasal/B27 medium. Cells were kept at 37°C in 5% CO₂ for 11 days with twice a week medium replacement (1:1 ratio). At this time point we have $2.1 \times 10^5 \pm 0.05 \times 10^5$ alive cells (which corresponds to about 85 % of initially plated cells); no significant differences were obtained in the number of alive cells in hippocampal preparations obtained from the brains of *wt* and genetically modified mice after 11 days in culture. With this method we obtained: 60-70% neurons, 30-35% astrocytes, 4-8% microglia, as determined with β-tubulin III, GFAP and IBA-I staining. For details see Supplementary Methods. Cells were used for experiments after 11 days.

Microglia cultures

Cortical mixed glia cultures were obtained from p0-p1 mice. Cerebral cortex were chopped and digested in 20 U/ml papain for 40 min at 37°C. Cells (5.0×10^5 cells/cm²) were plated on dishes coated with poly-L-lysine (100 µg/ml) in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin. After 7-9 days cells were shaken for 2 h at 37°C to detach and collect microglial cells. These procedures gave almost pure (no more than 2% contamination) microglial cell populations, as verified by staining with GFAP and Iba I Abs.

Excitotoxicity experiments

To induce excitotoxicity, 11 day-old hippocampal cultures were washed and stimulated in modified Locke's buffer (CaCl₂ 2.3 mM, glucose 5.6 mM, glycine 10 mM, NaCl 154 mM, KCl 5.6 mM, NaHCO₃ 3.6 mM, Hepes 5 mM pH 7.2) with 100 µM Glu alone or together with 100 nM CX3CL1

or vehicle, for 30 min. Following stimulation, cells were washed in Locke's buffer and re-incubated in the conditioned Neurobasal/B27 medium for additional 18 h. When BDNF (100 nM) and EPO (40 U/ml, 10 nM) were used, hippocampal neurons were pre-treated for 7 h with drugs or vehicle; medium was removed and cells were treated as described above with Glu (in the presence or in the absence of BDNF and EPO), washed and further incubated with the original medium containing BDNF or EPO for additional 18 h, till the end of the experiment. For experiments with conditioned medium, microglial cells (obtained from *wt* mice) were treated with CX3CL1 for 30 min, washed and re-incubated in growth medium. Eight h after CX3CL1 treatment, media were collected and used to stimulate neuronal cultures (obtained from CX3CR1^{GFP/GFP} mice) treated with Glu to induce excitotoxicity. Conditioned media from glia cultures were always diluted 1:1 with the original medium of neuronal cultures. For experiments with ADA, hippocampal cells were pre-incubated for 1 h with 1 U/ml ADA, treated with Glu or Glu/CX3CL1 in the presence of ADA, washed and reincubated in the original conditioned medium for 18 h. For experiments with conditioned media, medium obtained from CX3CL1-stimulated microglia was treated with or without ADA (1 U/ml) for 1 h before administration to neuronal cells treated with Glu. In these protocols, ADA was present till the end of the experiments. To evaluate neuron viability, cells were then treated with detergent-containing buffer (0.05% ethyl hexadecyl dimethylammonium bromide, 0.028% acetic acid, 0.05% Triton X-100, 0.3mM NaCl, 0.2 mM MgCl₂, in PBS pH 7.4) and counted in a hemacytometer as already described (Lauro et al., 2008). Alternatively, cell viability was analyzed by the MTT assay: in detail, 5 mg/ml MTT was added 1:10 to the cell medium and incubated for 2 h; the medium was aspired, cells were treated with DMSO and incubated at 37°C for 10 min. Samples were then analyzed with a microplate reader at 490 nm and 630 nm to subtract background. In all excitotoxicity experiments, results are expressed as % of cell survival, taking as 100% untreated cells in control conditions. Exactly the same procedures (plated cell number,

volumes of reagents) were applied to experiments with cells obtained from mice of different genotypes for comparison of cell viability also under basal conditions.

Transwell migration assays

Chemotaxis assay was performed on microglia obtained from mice cortex. Cells were re-suspended in serum-free medium and plated on poly-L-lysine-treated 12-mm transwells (8- μ m pore size polycarbonate; 5×10^5 cells/well). The lower chambers contained CX3CL1 100 nM, prepared in the same medium. The chambers were incubated for 2 h at 37°C in a moist 5% CO₂ atmosphere. After incubation, cells were treated with 10% trichloroacetic acid on ice for 10 min and the non migrating cells, adhering to the upper face of the filters, were scraped off, while cells on the lower side were stained with a solution containing 50% isopropanol, 1% formic acid, and 0.5% (w/v) brilliant blue R250 and dried on a glass slide. The number of migrating cells was counted in 20 fields with a 63X objective.

Depletion of microglia with clodronate liposomes

Mixed hippocampal cultures obtained from CX3CR1^{GFP/GFP} mice were treated for different times with liposomes encapsulating clodronate (Cl₂MBP) or as control, with empty liposomes. Clodronate liposomes as well as control liposomes without clodronate were prepared according to the standard method (van Rooijen et al. 1994). The resulting standard suspension of clodronate liposomes is containing 1.2 mg of Clodronate per 1 ml of the suspension. This liposome suspension was diluted 1:10 in the growth medium. At different time points, from 5 h to 72 h, cell cultures were analyzed with a fluorescence microscope to recognize and count the number of EGFP-labeled microglial cells. Cultures were stained with Hoechst to visualize total cell nuclei.

HPLC analysis

Eleven-day old rat hippocampal cultures were pre-treated in Locke's buffer for 10 min with the transporter inhibitor NBTI or with the ectonucleotidase inhibitor AOPCP, and then stimulated 30 min with CX3CL1 100 nM or vehicle, while primary microglial cell cultures were only treated in Locke's buffer for 30 min with CX3CL1 or vehicle. After this time, cells were washed and reincubated in their original conditioned medium, in the presence or in the absence of the inhibitors and, after additional 6 h (or 7.5 h for microglia), the media were collected, added with ice-cold acetonitrile, centrifuged for 5 min at 1.440 x g and the resulting supernatants were analyzed by HPLC. Cells remaining in the dish were analyzed for protein content with a BCA assay.

Chromatographic analyses were conducted using a Merck Hitachi HPLC system equipped with programmable autosampler (model L-7250), pump (model L-7100), and diode array detector (model L-7455). Data were stored and processed using appropriate software (D-7000 HPLC System Manager Ver. 3.1; Hitachi). Separation was achieved by using a column Reprosil-Pur C18-AQ (5 μ m, 250x4 mm) with precolumn Reprosil-Pur C18-AQ 5 μ m, 5 x 4 mm (Dr. Maisch, Ammerbruch, Germany). Elution was performed isocratically with a mobile phase consisting of 10 mM potassium phosphate (pH 6) and acetonitrile (90:10). The pump flow rate was set at 1.0 ml/min, and the injection volume was 40 μ l. Adenosine was monitored by UV diode array detection at 260 nm, and was identified on the basis of its retention time (3.90 min) and spectral data relative to reference standards. All separations were conducted at room temperature. The limit of detection and quantification for adenosine were found to be 18.7 nM and 187 nM, respectively.

Statistical data analysis

For all the experiments shown in the manuscript, significance was evaluated with *t*-test analysis and differences between groups of data were considered highly significant with $P \leq 0.01$ (**) and significant with $P \leq 0.05$ (*).

Results

Microglia depletion with clodronate liposomes impairs the neuroprotective activity of CX3CL1.

To study the neuroprotective role of CX3CL1, we used an injury model involving glutamate (Glu)-induced excitotoxicity in hippocampal cultures obtained from p0-p2 mice. Upon treatment with Glu (100 μ M, 30 min), we consistently obtained about 40-50 % of cell death in comparison with untreated control cultures. This corresponded to about 70 % of total neuronal loss upon Glu treatment, as assessed by immunofluorescence analysis with β -tubulin III staining (data not shown). We confirm, in this manuscript, that CX3CL1 protects hippocampal neurons from Glu-induced excitotoxicity (Fig. 1A) similarly to what already shown in neuronal preparations which contain different ratios of neurons:astrocytes:microglia (Limatola et al., 2005; Lauro et al., 2008). Given that CX3CR1 are predominantly expressed in microglial cells, it is likely that microglial cells mediate the neurotrophic effect of CX3CL1. To substantiate this, hippocampal neuronal cultures were treated with clodronate liposomes to specifically kill microglia (van Rooijen et al., 1996; Marín -Teva et al., 2004). We first performed a kinetic analysis on hippocampal cultures obtained from CX3CR1^{GFP/GFP} mice, where microglial cells are labeled by EGFP (Jung et al., 2000). Data reported in Fig. 1B indicate that the number of EGFP-labeled microglial cells selectively decreases with time in hippocampal cultures treated with clodronate-filled liposomes, while it is not affected in cultures treated with empty liposomes. At the same time points chosen for microglia cell viability, liposome-treated hippocampal cultures were analyzed for CX3CL1 responsiveness in terms of protection from Glu-induced toxicity. To this end, cells were treated with empty or clodronate-filled liposomes, washed, stimulated with Glu and analyzed for viability 18 h later. Results in Fig. 1C demonstrate that, after 72 h of treatment with clodronate liposomes and microglial cells in culture have almost completely disappeared (see Fig. 1B), CX3CL1 is not able to reduce Glu-induced toxicity. Unexpectedly, the same effect is observed as early as 5 h of clodronate

liposome treatment (Fig 1C), when most of microglial cells are still present, suggesting that microglia are strongly affected well before they are eliminated and in such a way that response to CX3CL1 is strongly impaired at this time point. Note that, in basal conditions (C), clodronate-filled liposomes did not significantly modify total cell survival in culture, suggesting that neither neurons nor astrocytes, which together account for more than 95% of total cell population (see methods), are significantly affected by these treatments. Neuronal cell treatment with empty liposomes did not affect CX3CL1-induced neuroprotection (Fig. S1).

Hippocampal neurons from CX3CL1^{-/-} mice are not more vulnerable to Glu injury in comparison with wt neurons. To investigate the role of endogenous CX3CL1 as neuroprotective agent upon Glu-induced excitotoxicity, hippocampal cultures were obtained from CX3CL1^{-/-} mice, treated with different Glu concentrations (from 1 μ M to 1 mM) and analyzed for cell viability. No significant differences in neuron death were observed between *wt* and CX3CL1^{-/-} mice at all tested Glu concentrations (Fig. S2). This suggests that endogenous levels of CX3CL1, neither before nor after Glu treatment (Chapman et al., 2000; Erichsen et al., 2003; Limatola et al., 2005), are sufficient to protect neurons by excitotoxicity under our *in vitro* conditions. To analyze if the effect of the administration of the soluble form of CX3CL1 could be different in *wt* vs CX3CL1^{-/-} mice, evidencing a possible cooperative role of the endogenous CX3CL1, excitotoxicity experiments were performed as shown in Fig. 2. Data obtained indicate that in the absence of endogenous (membrane bound and shed forms) CX3CL1, exogenous administration of soluble CX3CL1 is still able to reduce Glu-induced cell death (CX3CL1^{-/-} mice: Glu 48.6% \pm 3.5% vs Glu/CX3CL1 73.5 % \pm 3.0% $p \leq 0.001$) albeit at lower levels. No difference in cell viability were observed, in the absence of Glu, between *wt* and CX3CL1^{-/-} cultures (data shown in the legend to Fig. 2).

Role and origin of extracellular adenosine. We have previously shown that CX3CL1 induces the release of adenosine from the murine microglial cell line BV2 and from mixed hippocampal cultures (Lauro et al., 2008). However, since immortalized cell lines may differ from primary cells, we wanted to investigate: i) if primary microglia also release adenosine upon CX3CL1 treatment and ii) if the reduction of extracellular adenosine levels, by treating cultured cells with adenosine deaminase (ADA, the enzyme which degrades adenosine to inosine), is sufficient to prevent CX3CL1 neuroprotection against Glu-induced excitotoxicity. Figure S3 shows that CX3CL1 treatment of primary cultures of murine microglia induces adenosine release, as previously shown with BV2 cells (Lauro et al., 2008). Results in Fig. 3A demonstrate that ADA treatment (1 U/ml, 1 h, 37°C) of Glu-treated hippocampal cultures completely abolished CX3CL1-mediated neuroprotection. Interestingly, ADA treatment *per se* already results in some cell toxicity (19.5 % ± 0.9 % reduction of cell viability), suggesting that basal adenosine levels contribute to keep cells healthy.

We next used the medium conditioned by CX3CL1-stimulated (*st*) primary *wt* microglia (at the same time point shown in Fig. S3), to reduce Glu-induced cell death of CX3CR1^{GFP/GFP} neurons (confirming previous data with the microglia cell line BV2, Lauro et al., 2008); in the absence of CX3CL1 (not-stimulated cells, *ns*), this medium is not able to prevent Glu-induced cell death (Fig. 3B). When *st* medium was pre-treated with ADA (1 U/ml, 1 h, 37°C) and then given to CX3CR1^{GFP/GFP} hippocampal neurons, the neuroprotective properties were completely lost (Fig. 3B). Extracellular adenosine, which accumulates upon CX3CL1 stimulation of hippocampal cultures (Lauro et al., 2008 and Fig. 4), likely derived from released ATP since, in the presence of the specific ectonucleotidase inhibitor, AOPCP (1 µM, 10 min pre-incubation), the level of extracellular adenosine was not increased by CX3CL1 treatment (Fig. 4). When higher levels of AOPCP were used (5µM), the same results were obtained upon CX3CL1 treatment (data not

shown). However, in those conditions, the basal extracellular levels of adenosine were reduced at the limit of method detection. In contrast, the presence of the equilibrative transporter inhibitor NBTI did not significantly alter extracellular adenosine accumulation upon CX3CL1 treatment (Fig. 4).

Hippocampal neurons obtained from $A_1R^{-/-}$ mice are not protected by CX3CL1 against Glu excitotoxicity. We recently showed that the protective effect of CX3CL1 against Glu-induced hippocampal neuron injury could be eliminated by the A_1R antagonist DPCPX (Lauro et al., 2008). Although selective, this antagonist is not absolutely selective and, to prove the involvement of A_1R in the neurotrophic activity of CX3CL1, hippocampal cultures obtained from $A_1R^{-/-}$ mice were treated with Glu (100 μ M, 30 min) to induce excitotoxicity in the presence or in the absence of CX3CL1. We demonstrated that, in contrast with data obtained in *wt* mice, $A_1R^{-/-}$ cultures were not protected from Glu-induced cell death by CX3CL1 treatment (100 nM, Fig. 5) thus providing further evidence that A_1R s are required for the neuroprotective effect of the chemokine (Lauro et al., 2008). To exclude that the lack of neuroprotective effects of CX3CL1 on $A_1R^{-/-}$ neurons was due to an impairment of CX3CR1 functional properties on these specific genetically modified mice, experiments were addressed to investigate CX3CL1-induced chemotaxis on microglial cells obtained from $A_1R^{-/-}$ mice. Data reported in Fig. S4 demonstrate that $A_1R^{-/-}$ microglia responds to CX3CL1 similarly to *wt* microglia in terms of transwell migration. Similarly, $A_1R^{-/-}$ and *wt* cultured hippocampal neurons responded to CX3CL1 treatment with comparable levels of ERK phosphorylation (data not shown). These data indicate that there is no gross functional impairment of CX3CR1 pathway when A_1R are absent. Considering the physical and functional interaction described for A_1R/A_2AR pair (Ciruela et al., 2008), we wanted to analyze the possible involvement of A_2AR in the neurotrophic effect of CX3CL1 using $A_2AR^{-/-}$ mice. Data, reported in Fig. 5,

demonstrate the selective involvement of A₁R, with no participation of A_{2A}R in CX3CL1-mediated neuroprotection from Glu-excitotoxicity. Furthermore, the number of cells that survived in the controls (C) was not significantly different between *wt*, A₁R^{-/-} and A_{2A}R^{-/-} mice (respectively 41 ± 4.3; 41 ± 2.9; 42 ± 1.1 cells per microscopic field (10 x)).

Glu-injured hippocampal A₁R^{-/-} cultures are not rescued by other neurotrophins. To investigate if A₁R presence was a specific requirement for the neurotrophic activity of CX3CL1 or if it was shared by other neurotrophins, hippocampal A₁R^{-/-} cultures were treated with Glu to induce excitotoxicity in the presence of BDNF (100 nM) or EPO (40 U/ml). Data shown in Fig. 6 indicate that, under these conditions, both these substances protect *wt* neurons but fail to preserve A₁R^{-/-} neurons, thus suggesting that the presence of functional A₁R on neuronal cells is permissive for the activity of different neurotrophic factors. These results further underline that the elimination of the neuroprotective effect of the chemokine cannot be simply explained by a specific loss of ARs and indicate that the mediation of neuroprotective effect by adenosine acting at A₁Rs is quite a general phenomenon.

DISCUSSION

In this paper we demonstrate that microglial cells are required for the neuroprotective activity of CX3CL1, that they release adenosine which activates A₁R and that A₁R presence is necessary for the neurotrophic activity of CX3CL1 on Glu-injured neurons. We also describe for the first time that the expression of A₁R is necessary for the neurotrophic activity against excitotoxicity of other neurotrophins, like BDNF and EPO. These findings will be discussed in turn.

The role of adenosine in neuroprotection is very well established: experimental evidence indicates that activation of A₁R or inhibition of A_{2A}R improves neuronal recovery upon brain injury (Cunha, 2005), while the role played by A₃R and A_{2B}R in neuroprotection is less clear-cut (Michel et al., 1999; Fedorova et al., 2003; Chen et al., 2006; Pugliese et al., 2007). In the present experimental conditions we did not observe any clear effect of eliminating A_{2A} receptors. It has been reported that, in the brain, the level of adenosine and A₁R strongly increases upon trauma like brain ischemia (Pearson et al., 2006), where adenosine is mainly released by astrocytes (Martín et al., 2007) and participates in the protective effect of ischemic preconditioning (Heurteaux et al., 1995) or upon repetitive seizures, where the rapid modulation of adenosine kinase has been reported (reviewed by Boison, 2006). Even the basal level of extracellular adenosine, and the corresponding tonic activation of ARs, can be responsible of the modulatory activity on synaptic transmission (see Fredholm et al., 2005). Furthermore, AR activation is necessary, as co-receptor requirement, either to permit or to enhance neuronal and glial response to purines (ATP, Gerwins and Fredholm, 1992; Färber et al., 2008), neuropeptides (VIP, Cunha-Reis et al., 2007; CGRP, Sebastião et al., 2000; GDNF, Gomes et al., 2009), cytokines (IL6, Biber et al., 2008), growth and trophic factors (FGF, Flajolet et al., 2008; BDNF, Diógenes et al., 2004) and chemokines (CX3CL1, Lauro et al., 2008). Data reported in this paper demonstrate that adenosine, in addition to its well known direct neuroprotective effect on neurons (see above) and indirect protective effects via CCL2, IL-6 and S-

100b release by astrocytes (Schwaninger et al., 1997; Ciccarelli et al., 1999; Wittendorp et al., 2004), appears to enable the neurotrophic activity of different neurotrophins to occur, thereby extending the repertoire of actions for adenosine in brain homeostatic control.

We previously demonstrated that A₁R are probably involved in the neurotrophic activity of CX3CL1 as it was blocked by a relatively selective antagonist (Lauro et al. 2008). We now strongly support this conclusion in experiments where the protective effect is absent in A₁R^{-/-} mice. It might be argued that this is due to some functional impairment of CX3CR1 activation in A₁R^{-/-} mice. However, another effect of CX3CL1, namely direct induced by receptor stimulation, like microglia migration, is similarly activated by CX3CL1 both in *wt* and A₁R^{-/-} mice. Furthermore, the neuroprotection induced by other agents was also reduced. Together these observations make it very unlikely that the reason why mice that lack A₁Rs are not protected by CX3CL1 is that they are unable to respond to the chemokine.

One might argue that the present data are compatible with A₁Rs modulating the neuroprotective effect of endogenous CX3CL1. However, we report that lack of endogenous CX3CL1 (in CX3CL1^{-/-} mice) does not change hippocampal neuron response to Glu but reduces the protective effects induced by exogenous CX3CL1, suggesting a protective effect of the endogenous protein. We also show that this is not due to some major adaptive response to the targeted deletion of CX3CL1.

In a previous paper we demonstrated that CX3CL1 induces adenosine release from hippocampal cultures and from a murine microglia cell line (Lauro et al., 2008). Since the mechanisms underlying adenosine release might vary between different cell types of brain parenchyma, being mostly due to equilibrative transporters in neurons and to extracellularly released ATP, subsequently hydrolyzed by ectonucleotidases (Parkinson et al., 2005), in glia cells, we were interested in defining the potential mechanisms implicated in adenosine release by CX3CL1 in hippocampal mixed cultures. Our evidence that only the specific inhibitor of ectonucleotidases is

able to strongly reduce CX3CL1-mediated adenosine release, whereas the inhibitor of equilibrative transport was not, could suggest a predominant involvement of glial cell dependent nucleotide release in this process. The conclusion that glial cells are particularly important is also corroborated by our observations that the simultaneous treatment of hippocampal cultures with ADA and CX3CL1 completely abolished CX3CL1-mediated neurotrophic effect, and that the same result is obtained when ADA treatment is performed on medium collected from CX3CL1-stimulated primary microglia, 1 h before administration to Glu-treated hippocampal cultures.

It is proposed that microglia has a prominent role in mediating the neuroprotective effects of CX3CL1 (Mizuno et al., 2003; Huang et al., 2006; Cardona et al., 2006) and we have recently demonstrated that CX3CL1-stimulated microglia releases neuroprotective substances which reduce Glu-induced cell death (Lauro et al., 2008). However CX3CL1 does not protect against all types of neuronal damage because in transient brain ischemia (Soriano et al., 2002; Denés et al., 2008) and in a rat model of Parkinson's disease, intra-striatal CX3CL1 injection induced both microglia-dependent depletion of dopaminergic cells and motor dysfunction (Shan et al., 2009). In this paper we demonstrate that the selective ablation of microglia from hippocampal cultures, using clodronate-encapsulating liposomes, has the effect to fully abolish the neuroprotective activity of CX3CL1 toward excitotoxic death of hippocampal neurons, confirming that these cells represent the first target which primes the functional effects of CX3CL1. It is interesting to note how a few percentage of microglial cells (such as that present in our hippocampal neuronal preparation) can massively influence neuronal response to CX3CL1. This could explain the reported neuroprotective effect of CX3CL1 in almost pure neuronal cultures (Limatola et al., 2005).

Thus, we hypothesized that soluble factors released by microglia, like adenosine, could also activate astrocytes to release neurotrophic substances (Schwaninger et al., 1997; Ciccarelli et al., 1999; Wittendorp et al., 2004) which contribute to neuroprotection.

In conclusion, these data support the notion that CX3CL1 has neurotrophic activity on hippocampal and cortical neurons through its activity on microglia, which release soluble factors, among which adenosine, determining for neuron protection. CX3CL1-mediated neuroprotection is only possible in the presence of functional A₁R, whose activity is also required for the neuroprotective effect of other neuroactive factors, like BDNF and EPO, thus demonstrating that A₁R co-activation is necessary as permissive signaling which might reinforces or consent to the accomplishment of survival responses. The relevance of these conclusions remain to be confirmed in more physiological systems, like neurons obtained from mature brains, or by *in vivo* studies.

Disclosure/Conflict of Interest

The authors do not have conflicts of interest.

ACKNOWLEDGEMENTS

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Titles and legends to figures

Figure 1 *Effect of microglia depletion with clodronate liposomes on CX3CL1-mediated neurotrophic activity.* (A) Hippocampal cultures were treated with Glu (100 μ M, 30 min) or vehicle in the presence or in the absence of CX3CL1 (100 nM) and analyzed for viability 18 h later as described in the text. Results are expressed as % of cell survival in treated (Glu and Glu/ CX3CL1) vs untreated (C) cells (taken as 100%) and are the mean \pm SE of twelve duplicate experiments. (B) Hippocampal cultures from CX3CR1^{GFP/GFP} mice were treated with empty or clodronate filled liposomes for the indicated times and analyzed for the presence of EGFP-positive cells under fluorescence microscopy. Results are expressed as percentage of EGFP-positive cells (microglia) in the liposome-treated cultures vs control (C) untreated cells, and represent the mean of two duplicate independent experiments. (C) Alternatively hippocampal cultures, treated with clodronate-filled liposomes, were analyzed for Glu-induced excitotoxicity in the presence or in the absence of CX3CL1. Results are expressed as percentage of cell survival, taking 100% as untreated cells at time 0. Data represent the mean \pm SE of four duplicate experiments. For each time point, statistical significance was analyzed in treated (Glu and Glu/ CX3CL1) vs untreated (C) samples. *, $p \leq 0.05$; **, $p \leq 0.01$.

Figure 2 *Endogenous levels of CX3CL1 are not sufficient to protect neurons by excitotoxicity.* Eleven day-old hippocampal cultures obtained from *wt* or CX3CL1^{-/-} mice were treated with Glu (100 μ M, 30 min) or Glu/CX3CL1 and analyzed for viability 18 h later. Results are expressed as % of cell survival taking as 100% the control (C), untreated cells, for each mouse strain and are the mean \pm SE of five duplicate experiments. Statistical significance is analyzed between treated and untreated cells for each mouse strain, unless differently indicated. The number of cells in *wt* and

CX3CL1^{-/-} mice was not significantly different in untreated samples (41 ± 4.3 and 45 ± 2.4 , respectively, per microscopic field, 10 x). *, $p \leq 0.05$; **, $p \leq 0.01$.

Figure 3 ADA treatment abolishes the neuroprotective effect of CX3CL1. (A) Eleven day-old hippocampal cultures were pre-incubated or not with ADA (1U/ml) for 1 h and then co-stimulated with Glu or Glu/CX3CL1. Results represent the mean \pm SE of five independent duplicate experiments and are expressed as percentage of cell survival taking as 100% untreated cells in the absence of ADA. For each experimental condition, statistical significance was analyzed in treated (Glu and Glu/ CX3CL1) vs untreated (C) samples. *, $p \leq 0.05$; **, $p \leq 0.01$. (B) Glu-injured CX3CR1^{GFP/GFP} hippocampal neurons were treated with the medium conditioned by primary microglia not stimulated (*ns*) or stimulated with CX3CL1 (*st*). Alternatively, the conditioned (*st*) medium was collected, incubated for 1 h with ADA (1 U/ml; *st*/ADA) and given to CX3CR1^{GFP/GFP} hippocampal neurons treated with Glu (100 μ M) as described in the experimental section. Cell survival was analyzed after 18 h. Results represent the mean \pm SE of three independent duplicate experiments, and are expressed as percentage of cell survival taking, as 100%, untreated cells in control condition. For each experimental condition, statistical significance is analyzed between treated (Glu) and untreated (C) cells. *, $p \leq 0.05$; **, $p \leq 0.01$

Figure 4 Adenosine produced by CX3CL1-stimulated hippocampal cultures is reduced by ectonucleotidases inhibition with AOPCP. Hippocampal neurons were treated with 100 nM CX3CL1 or vehicle for 30 min, in the presence or in the absence of NBTI (1 μ M) and AOPCP (1 μ M), washed and re-incubated in growth medium. After 6 h medium was analyzed by HPLC for adenosine content. Results are expressed as nmoles of adenosine produced for mg of cellular proteins and significance is analyzed, for each condition, between CX3CL1-treated vs

corresponding control samples. Data are the mean \pm SE from 5 independent quadruplicate experiments.

Figure 5 *Excitotoxic cell death of hippocampal neurons is not inhibited by CX3CL1 in A₁R^{-/-} mice.* Hippocampal neurons obtained from *wt*, A₁R^{-/-} or A_{2A}R^{-/-} mice were cultured for 11 days and then stimulated with Glu (100 μ M, 30 min) in the presence or in the absence of CX3CL1 (100 nM). Cell death was analyzed after 18 h. Results represent the mean \pm SE of four independent duplicate experiments and are expressed as % of cell survival taking as 100% the control (C), untreated cells, for each mouse strain. Statistical significance is analyzed between treated and untreated cells for each mouse strain. *, $p \leq 0.05$; **, $p \leq 0.01$. The number of untreated cells in the control (C) are not significantly different between *wt*, A₁R^{-/-} and A_{2A}R^{-/-} mice (see data in the text).

Figure 6 *The neuroprotective effect of BDNF and EPO is abolished in A₁R^{-/-} mice.* Hippocampal neurons obtained from *wt* or A₁R^{-/-} mice were cultured for 11 days and then stimulated with Glu (100 μ M, 30 min) in the presence or in the absence of BDNF (100 nM) or EPO (10 nM). Results represent the mean \pm SE of four independent duplicate experiments and are expressed as in Fig. 5.

Supplementary Figure 1 *Neuroprotective effect of CX3CL1 is unaffected by liposome treatment.* CX3CR1^{GFP/GFP} hippocampal cultures, treated with empty liposomes for the indicated times, were analyzed for Glu-induced excitotoxicity in the presence or in the absence of CX3CL1. Results are expressed as percentage of cell survival, taking 100% as untreated cells at time 0. Data represent the mean \pm SE of four duplicate experiments. For each time point, statistical significance was analyzed in treated (Glu and Glu/ CX3CL1) vs untreated (C) samples. *, $p \leq 0.05$; **, $p \leq 0.01$.

Supplementary Figure 2 *Wt and CX3CL1^{-/-} hippocampal cultures are similarly vulnerable to Glu-induced injury.* Eleven day-old hippocampal cultures obtained from *wt* or *CX3CL1^{-/-}* mice were treated with different Glu concentrations (from 1 μ M to 1 mM) or vehicle and analyzed for viability 18 h later. Results are the mean \pm SE of four duplicate experiments and are expressed as % of cell survival taking as 100% the untreated cells in the absence of Glu. Statistical analysis is performed, for each Glu dose, between *wt* and *CX3CL1^{-/-}* cultures.

Supplementary Figure 3 *CX3CL1 induces extracellular adenosine accumulation in primary microglia cultures.* Media obtained from microglia treated with vehicle (C) or CX3CL1 (100 nM, 30 min), as described in the Method section, were analyzed by HPLC for adenosine content. Results are expressed as nmoles of extracellular adenosine accumulated per mg of cellular proteins and are the mean \pm SE of four independent triplicate experiments.

Supplementary Figure 4 *Wt and A₁R^{-/-} microglia respond similarly to CX3CL1-induced chemotaxis.* Transwell chemotaxis assay toward CX3CL1 (100 nM, 2 h) was performed on purified microglia obtained from the cerebral cortex of *wt* and *A₁R^{-/-}* mice. For each mice strain, results are expressed as percentage of cell migration taking as 100% control (C), untreated cells. Data represent the mean \pm SE of four duplicate experiments. Statistical significance is analyzed for each mice strain between CX3CL1- and vehicle-treated samples. **, $p \leq 0.01$.

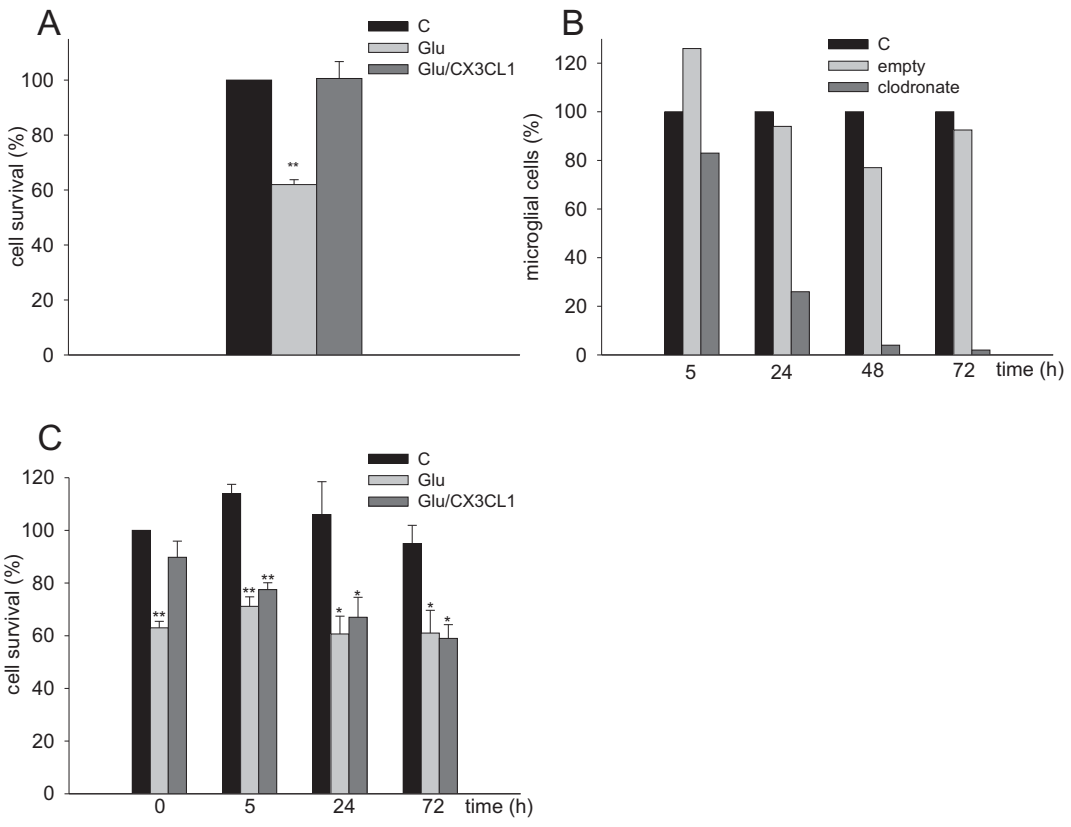


Figure 1

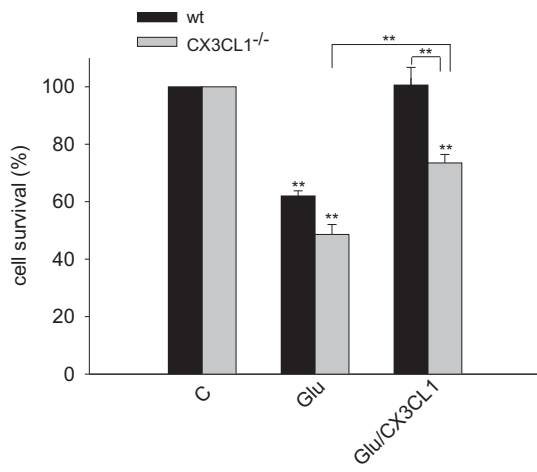


Figure 2

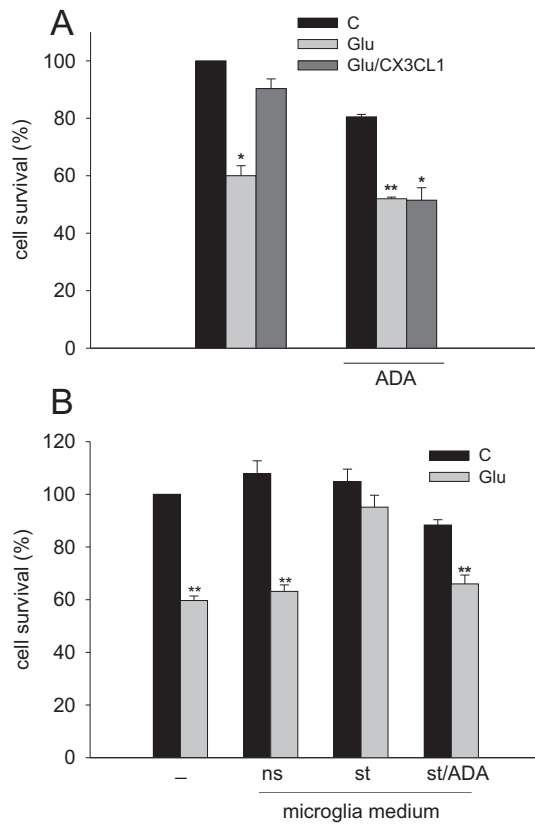


Figure 3

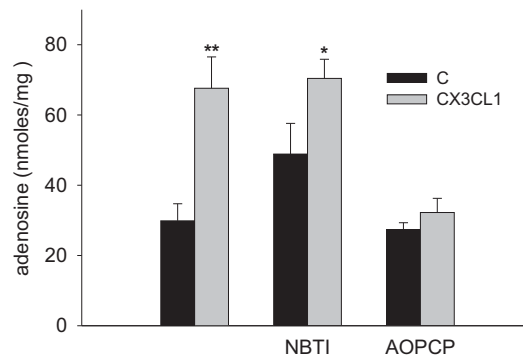


Figure 4

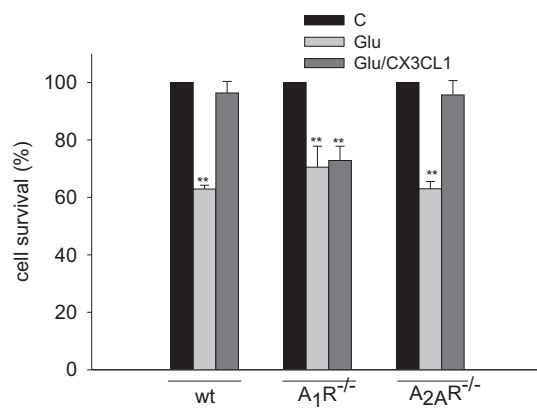


Figure 5

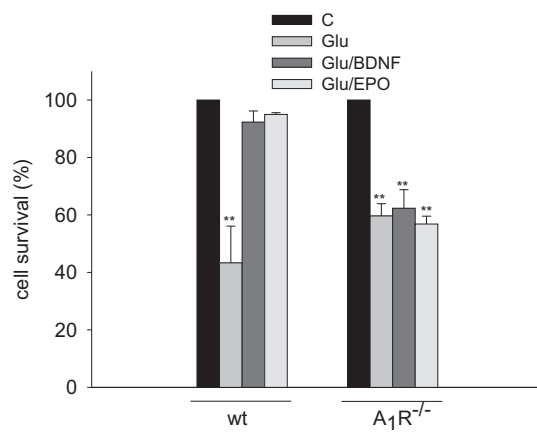


Figure 6