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CX3CL1 protects neurons against excitotoxicity enhancing GLT-1 activity on astrocytes☆

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ABSTRACT

In this paper we show for the first time that: i) astrocytes are required for the neuroprotective activity of CX3CL1 against excitotoxicity; ii) inhibition of the glutamate transporter 1 (GLT-1) prejudices CX3CL1-mediated neuroprotection; iii) CX3CL1 increases GLT-1 activity on astrocytes. The modulation of GLT-1 activity induced by CX3CL1 on astrocytes requires the presence and the activity of A1 adenosine receptor (A_1R), being blocked by the specific antagonist DPCPX and absent in $A_1R^{-/-}$ astrocytes. These data introduce the astrocytes as active players in CX3CL1-mediated signaling between microglia and neurons, identifying GLT-1 as a key mediator of the neuroprotective activity of CX3CL1.

1. Introduction

Under physiological conditions, extracellular glutamate (Glu) level in brain parenchyma is tightly regulated by the activity of Na^+-dependent, high affinity excitatory amino acid transporters (EAATs), whose expression and function are modulated to adjust Glu uptake to local synaptic activity (Benediktsson et al., 2012). Among the five EAATs described in mammalian CNS, glutamate-aspartate transporter (GLAST, EAAT1) and glutamate transporter-1 (GLT-1, EAAT2) are primarily expressed on astrocytes and microglia. Astrocytes are the most important cell type involved in Glu uptake from the synaptic cleft (Rothstein et al., 1996), because of their strategic localization at the so called tripartite synapse (Halassa et al., 2007). Microglia have also been shown to be Glu scavengers (Nakajima et al., 2001; Persson et al., 2009) and recent data highlight the importance of astrocyte-microglia communication in the modulation of Glu clearance. In fact, the activation state of microglia and the corresponding soluble factors released, may modulate EAAT expression and function in astrocytes (Tilleux and Hermans, 2008; Tilleux et al., 2009). In particular adenosine, a nucleoside whose extracellular levels can be modulated by a variety of physiological and pathological stimuli (Latini and Pedata, 2001) regulates astrocytic EAAT2 expression and function *via* the activation of adenosine receptor type 1 (A_1R) and 2A ($A_{2A}R$) (Li et al., 2001; Nishizaki et al., 2002; Pintor et al., 2004; Wu et al., 2010, 2011; Matos et al., 2012; Lee et al., 2013).

Fractalkine (CX3CL1), a chemokine highly expressed by neurons (Harrison et al., 1998), induces neuroprotection from Glu-mediated neurotoxicity acting on its cognate microglial receptor, CX3CR1 (Limatola et al., 2005; Lauro et al., 2008), reduces the production of inflammatory cytokines (Zujovic et al., 2000; Cardona et al., 2006) and promotes the release of neuroprotective soluble factors, including adenosine (Lauro et al., 2008). The central role of microglia-neuron communication through CX3CR1-CX3CL1 signaling in modulating neuron viability has been pointed out in several studies on neurodegenerative and neuroinflammatory disease models, including experimental allergic encephalitis (Huang et al., 2006), superoxide dismutase mutants, lipopolysaccharide-mediated neuroinflammation, toxin-induced Parkinson-like disease (Cardona et al., 2006), stroke (Soriano et al., 2002; Dénes et al., 2008; Cipriani et al., 2011) and Alzheimer disease (Lee et al., 2008; Bhaskar et al., 2010; Fuhrmann et al., 2010). However, the role of astrocytes as possible intermediaries in CX3CL1-mediated communication between microglia and neurons has not been investigated, so far.

☆ Conflict of interest: the authors declare no competing financial interests.

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In this paper we demonstrate for the first time that astrocytes are involved in the neuroprotective activity of CX3CL1 against Glu-induced excitotoxicity, *in vitro*, with a mechanism that requires the A_1R-dependent increase of Glu uptake through GLT-1.

2. Materials and methods

2.1. Materials

Transwell inserts were from BD Labware (Franklin Lakes, NJ); recombinant rat CX3CL1 were from Calbiochem/Merck (Nottingham, UK); dihydrokainic acid (2S,3S,4R)-2-carboxy-4-isopropyl-3-pyrrolidineacetic acid (DHK) and 2-Amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-4*H*-chromene-3-carbonitrile (UCPH-101) were from Abcam (Cambridge, UK); anti-beta III tubulin, anti-glial fibrillary acidic protein (GFAP), anti-ionized calcium binding adaptor molecule I (Iba I) antibodies were from Chemicon (Billerica, MA); goat anti-GLT-1 was from Santa Cruz Biotechnology (Santa Cruz, CA); secondary antibodies were from DAKO (Milan, Italy); L-(-)-threo-3-hydroxyaspartic acid (THA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), adenosine and 2-chloro-N(6)-cyclopentyl-adenosine (CCPA) were from Tocris Bioscience (Avonmouth, UK); D-[2,3-^{3}H] aspartic acid (D-[^{3}H] Asp, 12.8 Ci/mmol) was from GE Healthcare (Milan, Italy); culture media and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) were from Invitrogen Life Technologies (San Giuliano Milanese, Italy); bicinchoninic acid (BCA) protein assay was from Thermo Scientific (Rockford, IL); poly-L-lysine, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), rabbit anti-actin antibody and all the other reagents were from Sigma–Aldrich (Milan, Italy).

2.2. 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). $A_1R^{-/-}$ (Johansson et al., 2001) mice were backcrossed on a C57BL/6 background using the speed congenic procedure of Jackson laboratories and then further backcrossed repeatedly.

2.3. Primary hippocampal and cortical cultures

2.3.1. Hippocampal neuronal cultures

Primary hippocampal neuronal cultures were prepared from postnatal day 0–2 (P0–P2) Wistar rats. 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 papain (1.25 mg/mL) solution in Hank's balanced salt solution (HBSS). Cells were washed twice with HBSS to remove the excess of papain, mechanically dissociated in minimal essential medium (MEM) with Earl's salts and plated in Neurobasal/B27 with 100 μg/mL gentamycin at a density of 13×10^4/cm^2 on poly-L-lysine (100 μg/mL) coated plastic dishes or 5×10^4/cm^2 on cover glass for intracellular pH (pH_i) measurements. Cells were kept at 37 °C in 5% CO_2 for 11 days with twice a week medium replacement (1:1 ratio). The percentage of neuronal cells obtained with this method is around 60% (Lauro et al., 2010). Cultures were used after 10–11 days.

2.3.2. Cortical neuronal cultures

Primary cortical neuronal cultures were obtained from P0–P1 Wistar rats or C57/BL6 mice. Cerebral cortices were chopped and digested in 20 U/ml papain for 40 min at 37 °C. Cells (13×10^4 cells/cm^2) were plated on dishes coated with poly-L-lysine (100 μg/mL) in basal medium Eagle (BME) supplemented with 1 mM sodium pyruvate, 30 mM glucose, 0.1% Mito™ serum extender, 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 10 mM HEPES-NaOH (pH 7.4). After 4 h the medium was changed with Neurobasal medium supplemented with 1 mM glutamine, 0.1% Mito™ serum extender, 2.5% B27, 100 U/ml penicillin and 0.1 mg/mL streptomycin. After 2 days, arabinofuranosyl cytidine (AraC, 5 μM) was added to avoid the growth of glial cells. The percentage of neuronal cells obtained with this method is 80–90% (Cipriani et al., 2011). Cultures were used after 10–11 days.

2.3.3. Mixed glia cultures, microglial and astrocytic pure cultures

Cortical cultures from P0–P1 Wistar rats, C57BL/6 or A_1R deficient ($A_1R^{-/-}$) mice were obtained as above, plated on flasks at a density of 5×10^4/cm^2 and grown in DMEM (for murine cultures) or BME (for rat cultures) with 10% FBS and after 9–14 days used as mixed glia cultures or shaken for 2 h at 37 °C to detach and collect microglia. Astrocytes which remained attached on the bottom of the flask were treated with trypsin and collected. These procedures gave almost pure (no more than 2% contamination) microglial and astrocytic cell populations (Lauro et al., 2010). For all the different primary cultures described above, the relative percentage of neurons, astrocytes and microglia in total cell population was calculated by immunostaining with antibodies specific for neurons (beta III tubulin), astrocytes (GFAP) or microglia (Iba I), and staining cell nuclei with Hoechst. In some cases, CX3CR1$^{+/GFP}$ mice (where only microglia cells are GFP positive, see Jung et al., 2000) were used to verify the reliability of Iba I staining.

2.4. Co-cultures

Cortical neuronal cultures (9 days *in vitro*) were co-cultured with glia (cortex/mixed glia co-cultures) or microglia (cortex/microglia co-cultures) plated (at the density of 13×10^4 cells/cm^2) on 0.4 μm pore size polycarbonate membrane of transwell for 48 h. In some experiments, chimera of glia population was obtained mixing $A_1R^{-/-}$ microglia with *wt* astrocytes in 1:9 ratio (as observed in mixed glia cultures, see above). The transwell system has been used also for Asp uptake experiments on glial co-cultures (astrocytes/microglia co-cultures) in which microglia was plated on the bottom of the dish and astrocytes on the polycarbonate membrane, both at a density of 8×10^4/cm^2, for 48 h.

2.5. Excitotoxicity experiments

Before stimulation, medium conditioned by cultured cells was removed and stored for later use. To induce excitotoxicity, hippocampal or cortical neuronal cultures grown for 11 days *in vitro* were washed and stimulated in modified Locke's buffer (in mM: 154 NaCl, 5.6 KCl, 3.6 $NaHCO_3$, 5 HEPES, 2.3 $CaCl_2$, 5.6 glucose, 10 glycine pH 7.4) 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 previously stored conditioned medium for additional 16 h. Under these experimental conditions, we have previously demonstrated that Glu consistently induced about 40–50% of cell death in comparison with untreated control cultures. This corresponded to about 70% of total neuronal loss (Lauro et al., 2010). In the co-culture experiments, cortical neurons were treated with Glu (100 μM, 30 min) and glia or microglia were treated with CX3CL1 simultaneously. Excitotoxicity experiments with transporter inhibitors were performed treating hippocampal cultures simultaneously with Glu (100 μM) and THA (200 μM), DHK (500 μM) or UCPH-101 (25 μM), in the presence or in the absence of CX3CL1. To evaluate neuron viability, cells were analyzed by the MTT assay: in detail, 5 mg/mL MTT was added 1:10 to the cell medium and incubated for 2 h at 37 °C; 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. Cell viability was also measured treating cells with detergent-containing buffer (0.05% ethyl hexadecyl dimethylammonium bromide, 0.028% acetic acid, 0.05% Triton X-100, 0.3 mM NaCl, 0.2 mM $MgCl_2$, in PBS pH 7.4) and counting them in a