Activity of Adenosine Receptors Type 1 Is Required for CX_3CL1-Mediated Neuroprotection and Neuromodulation in Hippocampal Neurons

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_J Immunol_ 2008; 180:7590-7596; doi: 10.4049/jimmunol.180.11.7590

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Activity of Adenosine Receptors Type 1 Is Required for CX₃CL1-Mediated Neuroprotection and Neuromodulation in Hippocampal Neurons

Clotilde Lauro,* Silvia Di Angelantonio,* Raffaela Cipriani,* Fabrizia Sobrero,* Letizia Antonilli,* Valentina Brasadin,† Davide Ragozzino,‡ and Cristina Limatola**

The chemokine fractalkine (CX₃CL1) is constitutively expressed by central neurons, regulating microglial responses including chemotaxis, activation, and toxicity. Through the activation of its own specific receptor, CX₃CR1, CX₃CL1 exerts both neuroprotection against glutamate (Glu) toxicity and neuromodulation of the glutamatergic synaptic transmission in hippocampal neurons. Using cultured hippocampal neuronal cell preparations, obtained from CX₃CR1-GFP/GFP mice, we report that these same effects are mimicked by exposing neurons to a medium conditioned with CX₃CL1-treated microcellar cell line BV2 (BV2-st medium). Furthermore, CX₃CL1-induced neuroprotection from Glu toxicity is mediated through the adenosine receptor 1 (AR1), being blocked by neuronal cell preparations treatment with 1,3-dipropyl-8-cyclopentanyxanthine (DPCPX), a specific inhibitor of AR₁, and mimicked by both adenosine and the specific AR₁ agonist 2-chloro-N⁶-cyclopentyladenosine. Similarly, experiments from whole-cell patch-clamped hippocampal neurons in culture, obtained from CX₃CR1ΔGFP/GFP mice, show that CX₃CL1-induced depression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid- (AMPA-) type Glu receptor-mediated current (AMPA-current), is associated with AR₁ activity being blocked by DPCPX and mimicked by adenosine. Furthermore, BV2-st medium induced a similar AMPA-current depression in CX₃CR1ΔGFP/GFP hippocampal neurons and this depression was again blocked by DPCPX. We also report that CX₃CL1 induced a significant release of adenosine from microglial BV2 cells, as measured by HPLC analysis. We demonstrate that (i) CX₃CL1, along with AR₁, are critical players for countering Glu-mediated neurotoxicity in the brain and (ii) AR₁ mediates neuromodulatory action of CX₃CL1 on hippocampal neurons.


The chemokine fractalkine (CX₃CL1) and its specific receptor, CX₃CR1, are constitutively expressed throughout the CNS, (1) where in addition to modulating neurotransmission (2), they play the pathophysiological role of regulating microglial neurotoxicity (3, 4), neuropathic nociception (5), NK cell recruitment (6), and neuron survival (7, 8). CX₃CR1 is expressed by microglia under physiological conditions (1), and its expression can be induced or up-regulated by cytokines both on astrocytes and microglia (9, 10). In contrast, CX₃CR1 expression on neurons is controversial, pointing to microglial cells as privileged target implicated in CX₃CL1-induced activities in the CNS (3, 7, 8, 11, 12). Direct effects of CX₃CL1 on microglia include migration (1, 9, 13, 14), activation (9), proliferation (15), inhibition of Fas-ligand-induced cell death (16), and inhibition of cytokine release (3, 17). In recent years, it has become clear that microglia plays a double role in its contribution to neuronal damage, which occurs in neurodegenerative diseases and ischemic injury; it can either drive neurotoxicity or act favoring proregenerative and neuroprotective strategies (18, 19). Different observations described a protective role of both exogenous and endogenous microglia toward ischemic injury (20, 21). Although microglia-induced neurotoxicity has been, at least in part, explained by the production of cytokines and reactive oxygen species that follows microglia overactivation (19), the mechanisms underlying the neuroprotective activity of microglia likely involve the production of a number of growth factors that possess neurotrophic activity (22); furthermore, the impairment of microglia-neuron communication through the pair CX₃CL1/CX₃CR1 increases the severity of neurodegeneration, which occurs in different diseases (4).

Adenosine is an endogenous compound with different physiological activities: it is produced both inside and outside cells, and is a metabolic product intermediate of different pathways (23). There are different sources of extracellular adenosine in the nervous system; it can derive from the degradation of the released adenine nucleotides through the activity of extracellular nucleotidases, from equilibrative transporters, or as result of cell damage (24). Moreover, a recent report described adenosine release from the parallel fibers of cerebellum with an activity-dependent mechanism (25). In the CNS, adenosine functions mainly involve modulation of neurotransmission and neuroprotection, and its extracellular concentration rapidly increases upon brain damage, which follows stroke, ischemia, and epileptic seizures (26). Adenosine-induced activities are mediated through the activation of four different G-protein coupled receptors (adenosine receptor 1 (AR₁), AR₂A, AR₂B, and AR₃), which are widely distributed.

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Received for publication November 15, 2007. Accepted for publication March 26, 2008. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 Abbreviations used in this paper: AR, adenosine receptor; Glu, glutamate; DPCPX, 1,3-dipropyl-8-cyclopentanyxanthine; CCPA, 2-chloro-N⁶-cyclopentyladenosine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EGFP, enhanced green fluorescent protein; NES, normal external solution.

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throughout the brain and the spinal cord (26). Activation of AR1 has been often correlated with neuroprotection in cell culture and brain slice experiments (27), but also using knock-out animals for AR1 (28).

In contrast, AR1−/− mice show no differences in the brain damage observed after ischemia in comparison with wild-type animals, probably due to compensatory mechanisms (29).

Using primary hippocampal neuronal cultures from either wild-type or CX3CR1GFPCFP mice, which express the gene reporter enhanced green fluorescent protein (EGFP) in place of CX3CR1, and the mouse microglial cell line BV2, we addressed the experiments to see whether the neuroprotective and neurmodulatory role of CX3CL1 could be mediated through a cross-talk between glia and neurons. We demonstrate that CX3CL1 exerts its neurotrophic and neurmodulatory actions with mechanisms that involve AR1 activity, and that it is able to induce adenosine release from microglial cells. Our report provides new findings, involving two very well known pairs like adenosine/AR1 and CX3CL1/CX3CR1, on the cross-talk between glia and neurons.

**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 24 November 1986 (86/609/EEC). Homozygous CX3CR1GFP/GFP knock-in mice (The Jackson Laboratory) were obtained from Charles River.

**Microglia culture and conditioned media preparation**

BV2 cells were routinely maintained in culture in DMEM containing 10% FBS. Cells were used up to passage 20. CX3CR1 expression was verified by RT-PCR (data not shown). For medium conditions, cells were plated at 2 × 10^5 in a 24-multwell plate at day 0, shifted to vehicle- or 100 nM CX3CL1-containing 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, and HEPES 5 mM (pH 7.2)) for 30 min at day 1 and then to Neurobasal B27 medium for different times, from 1 to 18 h. Media were collected, centrifuged at 3,000 × g for 5 min, and added to the medium of cultured hippocampal neurons in a 1:1 ratio.

**Primary hippocampal neuronal cultures**

Hippocampal neuronal cultures were prepared from 1- or 2-day-old (P1-P2) Wistar rats, and from C57BL/6 or CX3CR1GFPCFP mice. In brief, after careful dissection from diencephalic structures, the meninges were removed and the hippocampi were chopped and digested in 1.25 mg/ml papain for 20 min at 37°C. Cells were mechanically dissociated and plated at a density of 2.5 × 10^5 in poly-l-lysine coated plastic 24-well dishes in serum-free Neurobasal medium, supplemented with B27 and 100 μg/ml gentamicin. Successively, cells were kept at 37°C in 5% CO2 for 11 days with a twice a week medium replacement (1:1 ratio). The percentage of neuronal cells obtained with this method is around 60%, as determined by β-tubulin III staining.

**Glutamate- (Glu-) induced excitotoxic experiments**

The 11-day-old rat hippocampal cultures were washed and stimulated in Locke’s buffer with Glu (100 μM) alone or together with CX3CL1 (100 nM) for 30 min. Following stimulation, cells were washed and re-incubated in Neurobasal medium supplemented with B27 containing 100 μg/ml gentamicin for an additional 18 h. In some experiments, cells were both pretreated (for 15 min) and re-incubated with different agonists and antagonists of ARs. Neuronal cell preparation was then treated with detergent-containing buffer (0.5% ethylenediaminetetraacetic acid, 0.28% acetic acid, 0.5% Triton X-100, 3 mM NaCl, and 2 mM MgCl2, in PBS (pH 7.4) diluted 1/10) and counted in a hemacytometer for viability (8) or was treated for 30 min with CX3CL1, Glu, or CX3CL1/Glu, while murine BV2 cells were treated for 30 min with CX3CL1. After this time, cells were washed, re-incubated in their original conditioned medium, and, after additional 7.5 h, were collected, centrifuged at 3,000 × g for 5 min, and added to the medium of cultured hippocampal neurons in a 1:1 ratio.

**Patch-clamp recordings**

In brief, hippocampal cultured neurons, bathed in standard external medium, containing (normal external solution (NES): 140 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES-NaOH, and 10 mM glucose (pH 7.3), were visualized using Nomarski optics with an upright microscope (Zeiss Axioptic). Patch-clamp whole cell recordings were performed at room temperature (23–25°C) using borosilicate glass electrodes (3–5 MΩ) filled with (in mM): 140 mM Cs-methanesulfonate, 2 mM MgCl2, 10 mM HEPES, 2 mM MgATP, and 0.5 mM EGTA (pH 7.3) with CsOH. Tetrodotoxin (0.2 μM), unless otherwise indicated, was routinely added to the bath solution. Neurons were clamped to −70 mV. The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiment, and recordings were discarded when any of these parameters changed by >10%. Membrane currents, recorded with a patch-clamp amplifier (Axopatch 200A; Axon Instruments), were acquired with Clampex 10 software at 2 kHz (Axon Instruments).

**Drugs and application procedures**

Neuronal cell preparations were continuously superfused using a gravity-driven perfusion system consisting of independent tubes for standard and agonist-containing solutions, positioned 50–100 μm from the recording pipette and connected to a fast exchanger system (RSC-100; Bio-Logic). To activate postsynaptic GluR, the ionotropic Glu receptor agonist N-methyl-D-aspartate-5-hydroxy-5-methylisoxazole-4-propionate (AMPA, 100 μM; Tocris) was delivered together with cyclohexadiole (50 μM; Tocris). CX3CL1 (chemo-kine domain, human; PeproTech), tetrodotoxin (Ascent Scientifics), and 1,3-dipropyl-8-cyclopyrpanylthine (DPCPX; stock solution 5 mM in DMSO) were similarly applied to neurons by gravity-driven perfusion. For experiments with microglia conditioned medium, BV2 cells, plated at 2 × 10^5 in a 100-mm plate, were shifted to NES or 5 mM CX3CL1-containing NES for 30 min. Media were collected, centrifuged at 3,000 × g for 5 min, and continuously superfused to the recorded cell using the gravity-driven perfusion system. Adenosine, the adenosine agonists, and antagonists 2-chloro-N’-cyclopentyladenosine (CCPA), DPCPX, and triazoloquinazoline (CGS15943), were also purchased from Tocris. All other reagents were of analytical grade and purchased from Sigma-Aldrich.

**HPLC analysis**

The 11-day-old rat hippocampal cultures were treated in Locke’s buffer for 30 min with CX3CL1, Glu, or CX3CL1/Glu, while murine BV2 cells were treated for 30 min with CX3CL1. After this time, cells were washed, re-incubated in their original conditioned medium, and, after additional 7.5 h, the media were collected, added with ice-cold acetonitrile, centrifuged for 5 min at 1,440 × g, and the resulting supernatants were analyzed by HPLC. For HPLC analysis of medium obtained from cocultures of BV2 and hippocampal neuronal preparations, 2 × 10^5 BV2 cells were plated on 11-day-old hippocampal cultures and, after 8 h, the media were collected and processed as above. Cells were then washed with NaOH 0.1 M, and analyzed for protein content with a BCA assay (Pierce). Chromatographic analyses were conducted using a Merck Hitachi HPLC system equipped with programmable autosampler (model L-7250), pump (model L-7100), diode array detector (model L-7455), and fluorescence detector (model L-7480). 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 μm, 250 × 4 mm) with precolumn Reprosil-Pur C18-AQ 5 μm, 5 × 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 μl. Adenosine was monitored by UV diode array detection at 250 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.

**Dose-response relations**

Drug concentration/cell survival relations were obtained by pretreating hippocampal neuronal cell preparations with the antagonists, for −15 min, and inducing excitotoxicity as already described in the presence of the agonist. Cells were then left in the presence of the antagonist until analyzed for survival. The IC50 of the AR antagonist DPCPX was estimated by fitting the data to the Hill equation, using least-square routines: \( I_{max} = IC_{50}^{H} \times \text{[Antagonist]}^{H} \), where \( IC_{50}^{H} \) is the Hill coefficient, \( I \) is the inhibition of CX3CL1-induced survival to excitotoxic insult at increasing doses of the agonist, and \( I_{max} \) is the maximum inhibition of CX3CL1-induced survival to excitotoxic insult at increasing doses of the agonist.
Results

CX3CL1-stimulated microglial cells release neurotrophic factors

We have previously demonstrated (8), and here confirmed, that CX3CL1 protects hippocampal neurons against Glu-induced excitotoxicity. Since the hippocampal neuronal cultures we used in the current experiments were composed by a mixed cellular population containing, in addition to neurons, also astroglial and microglial cells (see below), we wanted to analyze the role of microglial cells in CX3CL1-mediated neuroprotective effect. For this reason, we decided to use hippocampal neuronal cultures obtained from CX3CR1<sup>GFP/GFP</sup> mice, which completely lack the CX3CL1 receptor, CX3CR1 (11). A preliminary analysis of hippocampal neuronal cultures obtained from CX3CR1<sup>GFP/GFP</sup> mice revealed that (at 11 days in cultures) neither neurons nor astrocytes, evidenced by MAP2 or GFAP staining, were labeled by EGFP, whereas microglial cells (stained by CD11b) were EGFP positive (data not shown). Similar indications of a lack of EGFP expression in neurons and astrocytes from these mice were obtained when CX3CR1<sup>GFP/GFP</sup> hippocampal slices (obtained from 1- to 2-mo-old mice) were analyzed using the patch clamp technique; all the EGFP-expressing cells had the membrane current properties typical (30) of microglial cells (n = 30, data not shown). First, we confirmed that CX3CL1 protects mouse CX3CR1<sup>1/+/</sup> neurons (8), whereas it fails to protect CX3CR1<sup>1/−</sup> (CX3CR1<sup>GFP/GFP</sup>) neurons exposed to excitotoxic Glu (Fig. 1A). Second, we exposed CX3CR1<sup>GFP/GFP</sup> hippocampal neuronal cell preparations to media conditioned with microglial BV2 cells treated either with vehicle (not stimulated, BV2-ns) or with CX3CL1 (stimulated, BV2-st), demonstrating that only BV2-st medium-protected CX3CR1<sup>GFP/GFP</sup> neurons against Glu-mediated excitotoxicity (Fig. 1B), likely through microglia released factor(s). The activity of BV2-st medium was time-dependent, starting to significantly protect neurons at ~8 h (medium collected 8 h after CX3CL1 stimulation) and lasting up to 18 h (Fig. 1C). Following this experimental protocol, a complete reversal of events leading to Glu-induced cell death was obtained, indicating that at least part of the neuroprotective activity of CX3CL1 accounted for an indirect microglial contribution.

The specific AR<sub>1</sub> antagonist DPCPX abolishes CX3CL1-induced neuroprotective activity

Trying to identify the soluble factor(s) released from microglial cells upon CX3CL1 stimulation, we looked at adenosine as a possible candidate, because its neurotrophic activities are very well known (26). We report that the AR<sub>1</sub> antagonist, DPCPX (100 nM) and the non-specific AR antagonist triazoloquinazoline (CGS15943, 100 nM), effectively blocked neuroprotection against Glu-mediated excitotoxicity induced either by BV2-st medium in CX3CR1<sup>1/+GFP/GFP</sup> neuronal cell preparations (Fig. 2A) or by CX3CL1 in CX3CR1<sup>1/−</sup> neuronal cell preparations (Fig. 2B and C, respectively, mouse and rat). Comparable results were obtained with a TUNEL assay to monitor cell death on rat hippocampal neurons (Fig. 2D). The dose-dependency of the inhibitory effect of DPCPX in rat hippocampal neurons disclosed an IC<sub>50</sub> value of 31.5 ± 2.5 nM (Fig. 3, n = 4), which is far below the IC<sub>50</sub> reported for receptor subtypes other than AR<sub>1</sub> (31). Furthermore, the specific antagonists for AR<sub>2A</sub> (SCH58261, 5 nM), AR<sub>2B</sub> (MRS1706, 20 nM), and AR<sub>3</sub> (MRS3777, 50 nM) were ineffective on CX3CL1-mediated neuroprotection at the indicated concentrations (Table I). Considered together, these findings show an involvement of AR<sub>1</sub> on the CX3CL1-induced neuroprotective activity.

CX3CL1 induces adenosine release from both hippocampal cultures and microglial cell line BV2

To analyze whether adenosine was produced upon CX3CL1 stimulation, HPLC analysis was performed on the media conditioned with rat hippocampal cultures treated for the excitotoxic assay. Fig. 4A shows that, after 8 h, CX3CL1 doubled adenosine accumulation in the extracellular medium (44.3 ± 3.3 vs 23.6 ± 2.8 nmoles adenosine/mg protein) in unstimulated cells, p < 0.01, n = 8; adenosine concentration achieved in the culture medium upon CX3CL1 treatment was 6.9 ± 2.4 μM, and that significant increases occurred with Glu (to 35.5 ± 2.9 nmoles adenosine/mg protein, p < 0.05, n = 8) and with Glu/CX3CL1 (to 45.8 ± 6.3 nmoles adenosine/mg protein, p < 0.01,
n = 8). To determine whether adenosine was produced by the microglial cells that, in the neuronal cell culture from CX3CR1GFP/GFP mice, were present at the percentage of 7.8 ± 1.3% of total cell population, HPLC analysis was performed on the media obtained from BV2-st and BV2-st cells. Results shown in Fig. 4B indicate that, after 8 h, CX3CL1 significantly (p < 0.001, n = 5) enhanced adenosine accumulation to 28.5 ± 3.5 vs 6.7 ± 1.3 nmoles adenosine/mg protein in control, BV2-st cells (adenosine concentration achieved in the culture medium upon CX3CL1 treatment was 7.5 ± 2.0 μM).

To analyze a potential role of neuronal expressed endogenous CX3CL1 for its ability to induce adenosine release upon CX3CR1 engagement, the medium conditioned by a mixed microglial-neuronal enriched cell population was analyzed by HPLC for adenosine content. In these cocultures, extracellular adenosine, after 8 h, was 9.5 ± 3.1 nmoles adenosine/mg protein (four independent quadruplicate experiments). This value was about three times less than the algebraic sum of extracellular adenosine measured for sibling separate cultures (30.1 ± 2.7 nmoles adenosine/mg protein), indicating that the direct cell-to-cell (and likely neuronal CX3CL1-microglial CX3CR1) contact did not mimic the effect of exogenous CX3CL1 on adenosine release. Nevertheless, we cannot exclude an effect of endogenous CX3CL1 on extracellular adenosine accumulation since the coculture likely activates additional interactions which may have opposite effect.

Furthermore, we demonstrated that both adenosine and the specific AR1 agonist CCPA significantly protected neurons from Glu-mediated excitotoxicity, their effect being maximal at 1 nM (adenosine, 105 ± 0.6%; adenosine/Glu, 101 ± 8.1%, p < 0.005; CCPA, 93.8 ± 0.3%; and CCPA/Glu, 93.3 ± 0.3% of control, p < 0.005, n = 4. The data reported represent cell survival as % of control).

**DPCPX inhibits CX3CL1- and BV2-st medium-mediated AMPA-current depression**

Since we have found that the neuroprotective action of CX3CL1 on hippocampal neurons is dependent on AR1 activation, we were interested to investigate whether AR1 could be also involved in the previously described CX3CL1-induced neuromodulation (2, 8). We report that the CX3CL1-mediated inhibition of the amplitude of AMPA-currents in cultured hippocampal neurons was abolished by DPCPX treatment. As illustrated in Fig. 5, A and B, AR1 activity was required for CX3CL1-induced depression of AMPA-...

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<th>AR Antagonists</th>
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<th>MRS1706 (20 nM, A2b)</th>
<th>MRS3777 (50 nM, A3)</th>
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<td>Glu</td>
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<td>80.2 ± 4.5</td>
<td>85.3 ± 5.7</td>
<td>91.6 ± 3.2</td>
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*Results shown as percentage ± SEM of cell survival in comparison with control cells for each treatment. Variations among different control conditions never exceeded 8%. For details see Materials and Methods and text.
CX₃CL1 activities on hippocampal neurons mediated by AR₁

Currents, a mechanism counteracting Glu-mediated excitotoxicity (8). Specifically, Fig. 5A shows that AR₁ antagonist DPCPX (100 nM) blocked the depression of AMPA-currents induced by CX₃CL1 in CX₃CR₁⁺/⁺ mouse neurons (average depression to 50 ± 5%; n = 5, control medium; and p < 0.01; to 101 ± 3%; n = 5, DPCPX). Fig. 5B shows that adenosine (50 μM), in the same neurons, mimicked the CX₃CL1-induced AMPA-currents depression that was again sensitive to DPCPX (average depression to 69 ± 8%; n = 5; p < 0.05, control medium; and 97 ± 2%; n = 4; DPCPX), indicating that adenosine itself modulates AMPA-receptors function. Furthermore, using neurons from CX₃CR₁⁺GFP/GFP mice, we found that the BV2-ns medium (Fig. 6A) induced a reduction of the AMPA-currents (fall to 85 ± 5%, n = 8) insensitive to DPCPX (fall to 83 ± 4%; n = 8; p > 0.1 in the presence of DPCPX). The additional reduction of the AMPA-currents induced by BV2-st medium (fall to 68 ± 4%; n = 9 in the absence of DPCPX; Fig. 6B) compared with BV2-ns medium, was instead suppressed by DPCPX, the AMPA-currents being recovered to

**FIGURE 5.** CX₃CL1-induced depression of AMPA-currents is suppressed by the adenosine receptor antagonist DPCPX. A, Time course of the effect of CX₃CL1 at indicated dose (horizontal bar) on the amplitude of AMPA-currents (AMPAR 100 μM; CTZ 50 μM; 250 ms application; AMPA signal, black arrow) recorded in hippocampal cultured neurons obtained from CX₃CR₁⁺/⁺ mouse neurons (average depression to 50 ± 5%; n = 5, control medium; and p < 0.01; to 101 ± 3%; n = 5, DPCPX). B, Time course of the effect adenosine (50 μM) on AMPA-currents as A (control medium, n = 5; DPCPX 100 nM, n = 4). Right, as A before (control) and at maximum adenosine effect (adenosine) (top, 100 nM DPCPX; bottom, control medium).

**FIGURE 6.** Depression of AMPA-currents induced by BV2-st medium is suppressed by the specific AR₁ antagonist DPCPX. A, Time course of the effect of BV2-ns medium (horizontal bar) on the amplitude of AMPA-evoked currents (as Fig. 5) in cultured hippocampal neurons from CX₃CR₁⁺GFP/GFP mice; control (n = 8), DPCPX (n = 8). Superimposed current traces at right, recorded in the presence of 100 nM DPCPX (top, control) or in control medium (bottom, before) and at maximum BV2-ns medium effect. Note AMPA-current depression, induced by unknown microglia released factors, not influenced by DPCPX. B, Time course of the effect BV2-st medium on AMPAR-currents as A (control, n = 9; DPCPX 100 nM, n = 7). Right, as A before (control) and at maximum BV2-st medium effect as indicated (top, 100 nM DPCPX; bottom, control). Note additional AMPA-current depression induced by BV2-st medium, abolished by DPCPX.
values observed with BV2-ns medium (85 ± 4%, n = 7 in the presence of DPCPX). These findings point to an indirect effect on AMPA-currents by CX3CL1 again mediated by AR1 activity.

Discussion
A large body of evidence indicates that CX3CL1 exerts neuroprotective activities in cultured neurons (8, 17, 32–34) and in microglia cells (16). A recent report also demonstrates that CX3CR1-expressing microglial cells are neuroprotective in different in vivo models of neurodegenerative diseases (4). We demonstrate that, in an excitotoxic model of neuronal death, the neurotrophic activity of CX3CL1 can be mediated by soluble, microglial-derived factor(s) and by the activation of AR1.

The pathophysiological roles of microglial cells and the effect of their activation on neurons is an intensely debated issue (35–37). Our observation that CX3CL1 induces the release of soluble neurotrophic factor(s) from microglia cells, together with the known constitutive expression of the pair CX3CL1/CX3CR1 in the brain, are consistent with the current view of the constant surveying activity exerted by microglia in the brain (36). Furthermore, the description of AR1 involvement in the neurotrophic activity of CX3CL1 is in accordance with several reports where DPCPX or other AR1 antagonists aggrivate, while AR1 agonists ameliorate, the neurotoxicity resulting from different in vitro and in vivo experimental models (reviewed in Ref. 27). Similarly, other reports show that the expression and the activation of AR1 are involved in the severity of demyelinating pathologies (28), in the induction of cerebral preconditioning (38), and that the neuroprotective activity of the cytokine IL-6 could be dependent on AR1 up-regulation (39, 40).

It is known that upon different types of brain injuries, like ischemia, hypoxia, epileptic seizures, and neuroinflammation, adenosine is rapidly released in the extracellular space (41) and that its neuroprotective properties mainly rely on its ability to reduce excitatory neurotransmission (27). This is the first report demonstrating that adenosine can be released upon chemokine stimulation in the nervous system while the contrary event, e.g., adenosine-stimulated chemokine release, has been reported in mouse astrocytes for CCL2, which also exerts neuroprotective activity (42). In different cellular systems, functional cross-talk between adenosine and chemokine receptors has been described in terms of heterologous desensitization (43).

Extracellular adenosine release is known to occur from both glial and neuronal cells (44). A comparison of adenosine accumulation between neuronal cell preparations that, in our experimental conditions, contain neurons among with a significant proportion (~40%) of astrocytes together with microglial cells, vs pure microglial cells indicates that, in the latter, the extracellular basal level of adenosine was lower. However, we report here that microglial cells displayed a stronger increase of adenosine accumulation in response to CX3CL1. From these data, we can conclude that microglia is involved on adenosine accumulation upon CX3CL1 treatment but the contribution of other cell types cannot be excluded. Previous reports in fact demonstrate that astrocytes stimulated with CX3CL1 do not induce the release of substances like TGFβ, NO, or PGF2α (34), do not migrate (9, 14), but do release factors that promote microglial cell proliferation (9), leaving the astrocyte contribution possible. Yet, our findings do not exclude the neuronal participation in releasing adenosine, thus acting in an autocrine manner, or the adenosine-mediated release of additional neuroprotective factors from glial cells.

Glu has been reported to mediate the ischemia-induced adenosine release from glial cells (45); adenosine could, in turn, be important to reduce additional Glu release from neurons, thus acting as a negative feedback signal aimed at the reduction of neuronal loss (46). Our observation that CX3CL1 and Glu induce comparable levels of extracellular adenosine accumulation in hippocampal neuronal cell cultures is suggestive of an additional contribution of CX3CL1 in this mechanism, further amplified by the previously described Glu-induced CX3CL1 cleavage from neurons (8, 13).

We and others have previously shown that (i) CX3CL1 modulates glutamatergic AMPA-currents revealed as a reduction of the amplitude of both synaptic and agonist-evoked currents; (ii) CX3CL1 action on AMPA-currents is stringently and specifically related to the activation of CX3CR1 (2, 8, 32). In this study, we provide evidence that CX3CL1-induced depression of AMPA-currents in hippocampal neurons is suppressed by a specific antagonist of AR1, DPCPX. Furthermore, we report that a similar current depression is induced on hippocampal neurons treated with a medium conditioned by CX3CL1-stimulated microglia cells and is again suppressed by DPCPX. We speculate that CX3CL1 acts on its own receptor CX3CR1, expressed on glial cells, inducing the release of adenosine that, in turn, interacts with neuronal AR1, influencing the functional properties of AMPA receptors depressing their channel gating with mechanisms to be ascertained. The role of adenosine as modulator of synaptic transmission is very well investigated; its main effect on synaptic transmission is presynaptic and consists of the inhibition of neurotransmitter release (47), but postsynaptic membrane hyperpolarization is reported (48). Our findings provide evidence for another inhibitory effect of adenosine, mediated through AR1, and involving the postsynaptic modulation of AMPA receptor function.

In conclusion, our findings demonstrate that the activity of AR1, possibly regulated by adenosine released from glial cells, is involved in CX3CL1-induced neuroprotection and modulation of glutamatergic neurotransmission in hippocampal neurons. The identification of AR1 as downstream element responsible for beneficial effects of CX3CL1 on neuron survival against Glu neurotoxicity may represent an important challenge for management of neurological disorders including acute brain ischemia.

Acknowledgments
We thank Giuseppina Chece for skillful technical expertise, Dr. Myriam Catalano for help with coculture experiments, Drs. Fabrizio Eusebi, Knut Biber, and Flavia Trettel for helpful discussions, and Dr. Sergio Visentin (Istituto Superiore Sanità, Roma) for providing BV2 cells.

Disclosures
The authors have no financial conflict of interest.

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