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Interleukin-6-type cytokines in neuroprotection and neuromodulation: oncostatin M, but not leukemia inhibitory factor, requires neuronal adenosine A_1 receptor function

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Abstract

Neuroprotection is one of the prominent functions of the interleukin (IL)-6-type cytokine family, for which the underlying mechanism(s) are not fully understood. We have previously shown that neuroprotection and neuromodulation mediated by IL-6 require neuronal adenosine A_1 receptor (A_1R) function. We now have investigated whether two other IL-6-type cytokines [oncostatin M (OSM) and leukemia inhibitory factor (LIF)] use a similar mechanism. It is presented here that OSM but not LIF, enhanced the expression of A_1Rs (both mRNA and protein levels) in cultured neurons. Whereas the neuroprotective effect of LIF was unchanged in A_1R deficient neurons, OSM failed to protect neurons in the absence of A_1R. In addition, OSM pre-treatment for 4 h potentiated the A_1R-mediated inhibition of electrically evoked excitatory post-synaptic currents recorded from hippocampal slices either under normal or hypoxic conditions. No such effect was observed after LIF pre-treatment. Our findings thus strongly suggest that, despite known structural and functional similarities, OSM and LIF use different mechanisms to achieve neuroprotection and neuromodulation.

Keywords: adenosine A_1 receptor, excitotoxicity, hypoxia, leukemia inhibitory factor, neuroprotection, oncostatin M.

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Glutamate-induced excitotoxicity is a major cause for neuronal loss in many neurodegenerative disorders. This process is mediated by excessive activation of glutamate receptors and has been associated with damage observed after epileptic convulsions, stroke, spinal cord trauma, head injury (Faden *et al.* 1989; Dirnagl *et al.* 1999; Vincent and Mulle 2009), and several neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, Huntington's disease, Amyotrophic lateral sclerosis, and Multiple sclerosis (Dong *et al.* 2009). Excitotoxicity therefore remains a challenging problem in neuropathology ever since it was first described in the 1970s (Olney *et al.* 1972).

Excitotoxicity causes excessive ATP hydrolysis, resulting in pronounced release of adenosine in affected brain tissue (Dunwiddie and Masino 2001). Extracellular adenosine suppresses neuronal activity by at least three cellular mechanisms: (i) pre-synaptic inhibition of neurotransmitter release, (ii) post-synaptic inhibition of calcium influx through voltage dependent calcium channels, and (iii) activation of G-protein dependent inwardly rectifying K^+ channels that mediate

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Abbreviations used: A_1Rs, A_1 receptors; aCSF, artificial CSF; CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EPSCs, excitatory post-synaptic currents; ERK1/2, extracellular signal-regulated kinases 1 and 2; IL-6, interleukin 6; LIF, leukemia inhibitory factor; MAP2, Microtuble-associated protein 2; MK801, dizocilpine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFκB, nuclear transcription factor-kappa B; OSM, oncostatin M; PBS-T, phosphate-buffered saline + 0.1% Tween 20; PI3K, phosphatidylinositol 3-kinase; STAT3, signal transducer and activator of transcription 3.

post-synaptic membrane hyperpolarization (De Mendonca *et al.* 2000; Dunwiddie and Masino 2001). These effects also reduce metabolic demand, thereby preserving ATP stores, suppress glutamatergic transmission, and thus protect neurons from excitotoxicity (Schubert *et al.* 1997). These effects in neurons are predominantly mediated by A_1 receptors (A_1Rs). Although several adenosine receptor agonists, such as N^6-cyclopentyladenosine (CPA) or 2-chloroadenosine are neuroprotective, their therapeutic application in neurodegenerative diseases failed, as they provoke severe peripheral side effects and/or receptor desensitization (De Mendonca *et al.* 2000; Stone 2002). In addition, attempts to rescue neurons using anti-excitotoxic drugs, seriously affected various aspects of synaptic plasticity rendering them useless for therapy (Parsons *et al.* 2007; Mimica and Presecki 2009). This augments the urge for the development of new therapeutic approaches to suppress excitotoxicity.

Other neuroprotective factors include the family of interleukin 6 (IL-6)-type cytokines, which therefore are also called neurokines (Patterson 1992). Members of this family, such as IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor, novel neurotrophin-1, and cardiotropin-1 have been shown to have neuroprotective properties in various neuronal subpopulations (Holtmann *et al.* 2005; Wen *et al.* 2005; Weiss *et al.* 2006; Gurfein *et al.* 2009; Suzuki *et al.* 2009). Despite this large body of evidence, it is currently not very well understood how IL-6-type cytokines mediate their neuroprotective effects. Recently, we demonstrated that IL-6 affords neuroprotection and neuromodulation by enhancing the expression and function of neuronal A_1Rs (Biber *et al.* 2008). All IL-6-type cytokines require gp130 receptor subunits for signaling leading to many shared redundant functions (Heinrich *et al.* 2003; Kamimura *et al.* 2003). We therefore hypothesized that IL-6-type cytokines in general exert their neuroprotective properties via a facilitation of neuronal A_1R function.

Interleukin-6 and IL-11 are the only IL-6-type cytokines that signal via gp130 homodimers, while the remaining members signal via heterodimers of either gp130 and LIFr (LIF, ciliary neurotrophic factor, cardiotropin-1, and novel neurotrophin-1) or gp130 and OSMr (OSM) (see Bauer *et al.* 2007 for review). Using LIF and OSM allows investigation of all major possible receptor signaling combinations that are known for the IL-6-type cytokine family. We thus here compared the neuroprotective properties of OSM and LIF in cultured cortical and hippocampal neurons and investigated the electrophysiological effects of these cytokines in mouse hippocampal slices.

Materials and methods

Chemicals

Neurobasal media, Basal Medium Eagle media, Hank's Balanced Salt Solution, phophate-buffered saline (PBS), sodium pyruvate, L-glutamine, penicillin-streptomycin, HEPES, glutaMAX-1, and B27 supplement were from Gibco (Breda, The Netherlands). Dulbecco's modified Eagle's media and Fetal Calf Serum were from PAA laboratories (Cölbe, Germany). Mito serum extender was from Becton Dickinson Labware (Breda, The Netherlands). Trypsin was from Life Technologies (Breda, The Netherlands) and papain from Worthington Biochemical Corporation (Lakewood, NJ, USA). Other chemicals were from Sigma-Aldrich (Zwijndrecht, The Netherlands). Cytokines, recombinant mouse LIF was from Millipore (Amsterdam, The Netherlands; Cat. No: LIF2005), and recombinant mouse OSM from Sigma-Aldrich. Mouse monoclonal anti-adenosine A_1R antibody supernatant was kindly provided by Prof. Yuko Sekino and mouse monoclonal anti-β-actin was from Abcam (Cambridge, MA, USA). Fluorescence conjugated secondary antibody, donkey anti-mouse (IR Dey680) used separately to detect adenosine A_1R and β-actin bands was from LI-COR biosciences (Cambridge, United Kingdom).

Animal experiments

Experiments were performed using wild-type C57BL/6J mice (Harlan, Horst, The Netherlands or Harlan Iberica, Spain) and adenosine A_1R knockout (A_1R−/−) mice with the same genetic background (Universitá di Roma "La sapienza", Rome, Italy). All procedures were in accordance with the regulation of the Ethical Committee for the use of experimental animals of the University of Groningen, The Netherlands (License number DEC 4623), as well as with the Portuguese law on Animal Care and European Union guidelines (86/609/EEC). Mice were housed in standard makrolon cages and maintained on a 12 h light/dark cycle. They received food and water *ad libitum*.

Primary neuron culture from mouse embryo and neonates

Primary neuronal cultures from mouse embryo (~E_{14}) were established as described previously (Biber *et al.* 2008). Primary neuronal cultures from newly born mouse neonates (P_0) were established as described previously (Limatola *et al.* 2005). The neuronal purity, determined by Microtuble-associated protein 2 (MAP2)-staining, was 90–95% (data not shown). Cortical and hippocampal neurons were used for the experiments after 5 and 9 days of *in vitro* culture, respectively.

Induction of excitotoxicity

Following the number of days in culture, cortical and hippocampal neurons were incubated with or without recombinant mouse OSM or LIF (0.1, 1, or 10 ng/mL) for the indicated periods of time. Where indicated, neurons were incubated with a selective A_1R antagonist, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX, 100 nM) or agonist, CPA (100 nM) for 15 min before they were subjected to excitotoxicity with glutamate (50 μM) for 1 h. Following glutamate treatment, the media was refreshed and neuronal cultures were incubated for 18 h before assessing the degree of cytotoxicity.

Determination of neuronal viability

- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: Survival of embryonic (~E_{14}) cortical neurons in culture was measured by the colorimetric MTT assay, as described previously (Mosmann 1983; Biber *et al.* 2008).

• Lysis-buffer assay: 18 h after glutamate treatment, cultures of cortical and hippocampal neurons isolated from neonatal pups (P_0) were treated with detergent-containing lysing solution (0.5% ethylhexadecyldimethylammonium bromide, 0.28% acetic acid, 0.5% Triton X-100, 3 mM NaCl, 2 mM $MgCl_2$, in PBS; pH 7.4; diluted 1 : 10) for 5 min at 21°C and the viable cells were counted on a hemacytometer (Thoma chamber, Brand, Germany), as described previously (Volonté *et al.* 1994; Limatola *et al.* 2005).

Western blot analysis

Equal amounts of protein (30 μg) were loaded on to 15% sodium dodecyl sulfate–polyacrylamide gels and subsequently transferred on to polyvinylidene fluoride membranes. The membranes were blocked using Odyssey blocking buffer (diluted 1 : 1 in PBS) for 1 h and incubated overnight at 4°C on shaker with one of the following primary antibodies diluted in 1 : 1 Odyssey blocking buffer and PBS + 0.1% Tween 20 (PBS-T): mouse monoclonal anti-A_1R antibody supernatant (1 : 50) and mouse monoclonal anti-β-actin antibody (1 : 8000). The membranes were then washed in PBS-T (4 × 5 min), followed by incubation with fluorescent-conjugated secondary antibody, donkey anti-mouse (IR Dey680 LI-COR; 1 : 8000 in PBS-T), for 1 h at 21°C on gentle shaking in the dark. Membranes were washed again in PBS-T (4 × 5 min) and fluorescent bands were detected using LI-COR's Odyssey infrared imaging system. The densiometric analysis of protein bands was performed using Image J program (Abramoff *et al.* 2004).

Real-time PCR (Q-PCR)

Total RNA extracted from cultured cortical neurons were purified and then transcribed into cDNA as described previously (Biber *et al.* 2008). The quality of the cDNA was controlled using GAPDH primers (see Table S1 for primer sequences). Adenosine A_1R mRNA expression of cultured embryonic cortical neurons treated with OSM (1 ng/mL) or LIF (10 ng/mL) for different time periods (1, 2, 4, 8, 12, and 24 h) was analyzed by real-time PCR using the iCycler (Bio-Rad, Veenendaal, The Netherlands) and the iQ SYBR Green supermix (Bio-Rad). Neurons that were not treated with OSM or LIF, served as control. Mouse ribosomal protein L32-A (rpL32A) and GAPDH primers were used for normalization to house-keeping genes and they showed no variations in response to experimental treatments (see Table S1 for primer sequences). The comparative C_t method [amount of target amplicon X in sample S, normalized to a reference R and related to a control sample C, calculated by $2 - ((C_tX, S - C_tR, S) - (C_tX,C - C_tR,C))$] was used to determine the relative expression levels (Livak and Schmittgen 2001; Biber *et al.* 2008). Linear regression analysis of the data was performed to understand the effect of cytokine treatment over time on the neuronal A_1R expression.

Patch-clamp recordings in hippocampal slices

Acute hippocampal slices (300 μm thick) were prepared as previously described (Diógenes *et al.* 2004). Whole-cell patch-clamp recordings of excitatory post-synaptic currents (EPSCs) were obtained from CA1 pyramidal cells upon stimulation of the Schaffer collateral fibers (0.2 ms rectangular pulses delivered each 30 s). EPSCs were acquired at 21°C with an Axopatch 200B amplifier (Axon Instruments; Clampex 10 Software, Sunnyvale, CA, USA) at 10 KHz and were low-pass filtered at 2 KHz. CA1 pyramidal cells were identified by visualization with an upright microscope (Zeiss Axioskop 2FS, Göttingen, Germany) equipped with infrared video microscopy and differential interference contrast optics, and were functionally distinguished from interneurons by their slower firing frequencies, longer action potentials, and for featuring spike-frequency adaptation (Madison and Nicoll 1984). Patch pipettes had resistances of 4–7 MΩ when filled with an internal solution containing (in mM): K-gluconate 125, KCl 11, $CaCl_2$ 0.1, $MgCl_2$ 2, EGTA 1, HEPES 10, MgATP 2, NaGTP 0.3, and phosphocreatine 10, pH 7.3, 280–290 mOsm. Junction potentials were not corrected nor were access resistance compensated for. Whole-cell EPSCs were recorded in voltage-clamp mode ($V_h = -70$ mV) and averages of four consecutive individual responses were determined. Access resistance and holding current were continuously monitored throughout the experiment, and if any varied by more than 20%, the experiment was discarded. Slices were perfused (2–3 mL/min) with artificial CSF (aCSF), which contained (in mM): NaCl 125, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 25, $CaCl_2$ 2, $MgSO_4$ 1, and glucose 10. Drugs were added to the perfusion solution.

Hypoxia was induced by substituting the aCSF with an identical aCSF pre-equilibrated with 95% N_2 and 5% CO_2 for 4 min. This manipulation reduces bath oxygen tension in the recording chamber from ∼600 to ∼250 mmHg (Sebastião *et al.* 2001). Each slice was subjected to a single period of hypoxia, since the effects of hypoxia may be modified by subsequent episodes in the same slice (Pérez-Pinzón 1999).

Statistical analysis

The absolute data values were converted to percentage of control to allow multiple comparisons and statistical analysis performed by one-way ANOVA followed by the Bonferroni correction, using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA). Analysis of the electrophysiology data was carried out using paired *t*-test and that of the western blot data, by non-parametric Mann–Whitney *U*-test. In all cases, values of $p < 0.05$ were considered statistically significant.

Results

OSM, but not LIF-induced neuroprotection against excitotoxicity is dependent on neuronal A_1Rs

Treatment with various concentrations of glutamate (20 up to 200 μM) for 1 h caused a concentration-dependent cell death in cultured embryonic cortical neurons that was abolished by the NMDA receptor antagonist dizocilpine, as described earlier (Figure S1; Biber *et al.* 2008). Pre-treatment with OSM or LIF (0.1, 1, and 10 ng/mL for 24 h) alone had no effect on neuronal viability but significantly protected neurons against toxicity induced by glutamate (50 μM for 1 h) (Figure S2a). Subsequently, the effect of OSM (1 ng/mL) and LIF (10 ng/mL) treatment on neuronal survival was tested over various time periods (1, 2, 4, 8, 12, and 24 h) (Figures S2b and c). In addition, pre-treatment with OSM (1 ng/mL) or LIF (10 ng/mL) for 24 h showed a reduced caspase 3 activation and reduced propidium iodide uptake in MAP2-positive neurons subjected to glutamate toxicity (50 μM for 1 h) (Figure S3a and b).

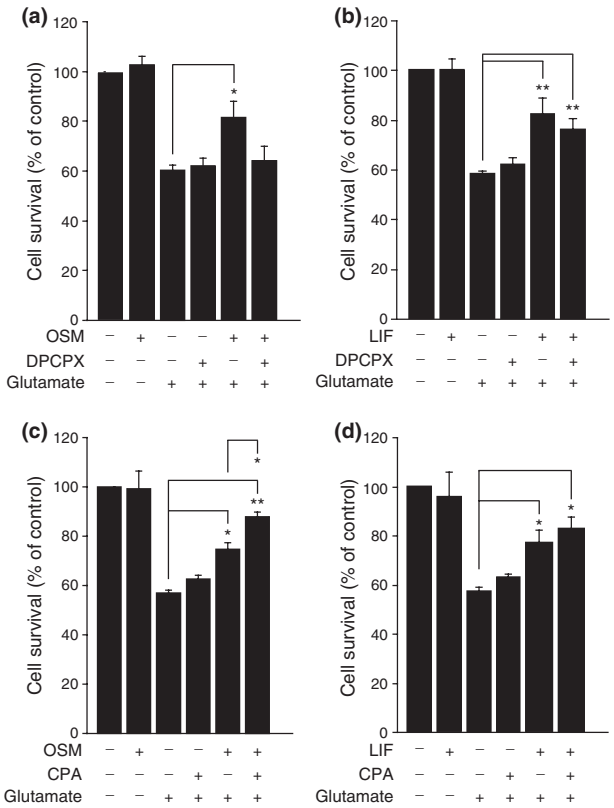

Fig. 1 Effect of A_1R antagonist and agonist on oncostatin M (OSM)- and leukemia inhibitory factor (LIF)-induced neuroprotection against glutamate toxicity. Primary cortical neurons from wild-type (C57BL/6J) mice embryo ($\sim E_{14}$) were treated with or without OSM (1 ng/mL) (a, c) and LIF (10 ng/mL) (b, d) for 24 h after 5 days in culture and were subsequently challenged with glutamate (50 μM) for 1 h. Where indicated, cultures were treated with A_1R antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 100 nM) or agonist, N^6-cyclopentyladenosine (CPA; 100 nM) for 15 min prior to glutamate insult. Neuronal survival was assessed 18 h following the glutamate insult by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The optical densities were measured at 570 nm with a 630 nm and blank correction. The bars represent mean ± SEM of three independent experiments; $^{**}p < 0.001$, $^{*}p < 0.05$.

Inhibition of A_1R activity in embryonic cortical neurons by DPCPX abolishes the neuroprotective function of IL-6 (Biber *et al.* 2008). Indeed, pre-incubation with DPCPX (100 nM for 15 min before glutamate) completely abolished the neuroprotective effects of OSM (1 ng/mL for 24 h) (Fig. 1a), but left neuroprotection by LIF unaffected (10 ng/mL for 24 h) (Fig. 1b). DPCPX alone, however, did not influence glutamate-induced neurotoxicity (Fig. 1a and b).

Neuroprotection against glutamate toxicity induced by OSM, but not LIF, is enhanced by the A_1R agonist, CPA

We have previously shown that treatment of embryonic cortical neurons with the A_1R agonist, CPA alone did not affect glutamate toxicity, but that neuroprotection induced by IL-6 was further enhanced by CPA (Biber *et al.* 2008). Similarly, we now describe that CPA (100 nM for 15 min before glutamate) significantly enhanced the neuroprotective effect of OSM against glutamate (50 μM for 1 h), but it did not affect neuronal survival in the absence of OSM treatment (Fig. 1c). In contrast, LIF-induced neuroprotection was not affected by CPA (Fig. 1d). In accordance with the survival experiments, CPA (100 nM for 30 min) alone did not affect the glutamate mediated calcium transients in cultured cortical neurons (Figure S4c). Whereas, in neurons pre-treated with OSM (1 ng/mL for 24 h), CPA not only delayed the influx of calcium, but also decreased the rate of calcium entry (Figure S4d).

OSM, but not LIF, enhances A_1R expression in cultured cortical neurons

Interleukin-6 treatment enhances A_1R expression in neurons (Biber *et al.* 2008). We here investigated whether treatment with OSM (1 ng/mL) or LIF (10 ng/mL) for various time periods (1, 2, 4, 8, 12, and 24 h) has a similar effect in cultured embryonic cortical neurons. Linear regression analysis of RT-PCR experiments shows a time-dependent increase in the A_1R mRNA expression, when normalized to GAPDH (Fig. 2a; $R^2 = 0.402$, $p < 0.001$, $n = 3$) and to rpL32A (Figure S5; $R^2 = 0.284$, $p = 0.005$, $n = 3$), in neurons treated with OSM, with an effect already apparent after 1 h of incubation. In addition, western blot analysis confirms this since treatment with OSM (1 ng/mL for 24 h) significantly ($p < 0.05$; $n = 3$) increased A_1R protein expression compared with the non-treated control (Fig. 2b). LIF treatment, however, did not influence A_1R mRNA or protein expression in cortical neurons (Fig. 2 and Figure S5).

The protective effect of OSM, but not of LIF, against glutamate-induced excitotoxicity is lost in $A_1R-/-$ neurons

To further evaluate the involvement of A_1Rs in OSM-induced neuroprotection, neuronal cultures were prepared from $A_1R-/-$ mice and data were compared with that obtained from wild-type mice. Neuronal cultures were prepared from newborn mice (P_0) to allow genotype confirmation, as well as evaluation of neuroprotection in distinct brain areas (cortex and hippocampus). Pre-incubation of wild-type cortical and hippocampal neurons with OSM or LIF (1 or 10 ng/mL for 24 h) significantly increased their survival against glutamate toxicity (Fig. 3a and b). As shown for embryonic neurons, the protective effects of OSM in neonatal neurons were also abolished by DPCPX (100 nM, for 15 min before glutamate challenge) (Fig. 3a). Pre-incubation with OSM did not protect against glutamate toxicity in cultured cortical and hippocampal neurons from $A_1R-/-$ mice (Fig. 3c). In contrast to OSM, DPCPX treatment (100 nM, added 15 min prior to glutamate challenge) did not change the protective effect of LIF (Fig. 3b), thus confirming the findings in embryonic neurons. Consistently, cortical and

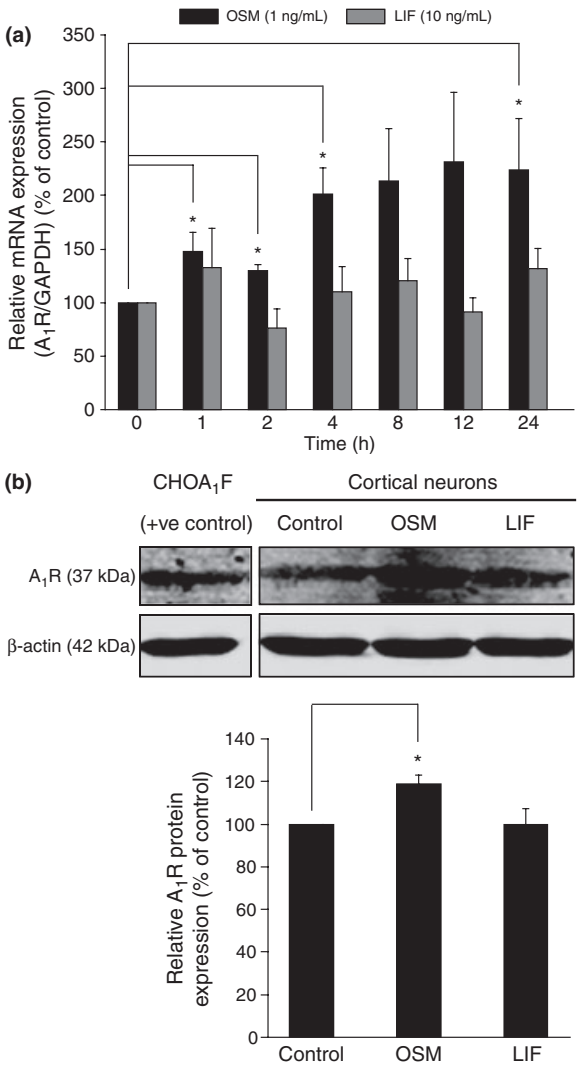

Fig. 2 Oncostatin M (OSM), but not leukemia inhibitory factor (LIF), enhances A_1R expression in wild-type (C57BL/6J) cortical neurons *in vitro*. (a) Real-time PCR analysis of A_1R mRNA expression in cultured embryonic ($\sim E_{14}$) cortical neurons. Graph shows the relative expression level of A_1R mRNA (normalized to GAPDH) in cultures incubated with OSM (1 ng/mL) or LIF (10 ng/mL) for 1–24 h, compared with the non-treated controls. Data presented as mean ± SEM of three separate experiments, expressed as percentage of the control; $^*p < 0.05$ (compared with the control); independent samples *t*-test. (b) Western blot analysis of A_1R protein expression in cultured embryonic cortical neurons incubated with OSM (1 ng/mL) or LIF (10 ng/mL) for 24 h, compared with the control. Relative optical densiometric values of A_1R proteins are shown in the lower panel. Data is presented as percentage of each respective ratio between optical density value of A_1R band intensity and optical density value of the matched β-actin band intensity ($n = 3$); $CHOA_1F$ – Chinese hamster ovary cells transfected with full-length A_1R gene (positive control for A_1R); $^*p < 0.05$.

hippocampal neurons from $A_1R-/-$ mice were still protected by LIF pre-treatment (10 ng/mL, for 24 h before glutamate) (Fig. 3d).

Modulation of A_1R-mediated actions on synaptic transmission by OSM, but not LIF

Since receptors for OSM and LIF are strongly and specifically expressed in hippocampal principal layers (Rosell *et al.* 2003), we investigated whether sustained exposure of hippocampal slices to OSM or LIF could modify the neuromodulatory actions of A_1Rs on synaptic transmission, using whole-cell recordings of electrically evoked EPSCs from CA1 pyramidal cells. To prevent pre-conditioned responses, the effects of the selective A_1R agonist (CPA, 30 nM) were investigated in only one neuron per slice, from control and OSM or LIF(10 ng/mL, for 4 h) treated slices, prepared from the same hippocampus. Activation of A_1Rs is well known to decrease synaptic transmission in the hippocampus (Sebastião *et al.* 1990). Accordingly, CPA (30 nM) inhibited EPSC peak amplitude by 48 ± 7.7% ($n = 4$), in control conditions (Fig. 4). When recording from slices pre-incubated with OSM, EPSC inhibition by CPA was significantly increased to 73 ± 6.7% ($n = 4$, $p < 0.05$, compared with the effect of CPA in the absence of OSM). In contrast to OSM, EPSC inhibition by CPA was not significantly affected ($p > 0.05$, $n = 4$ for each group, comparisons within slices from the same hippocampus) by pre-exposure to LIF (Fig. 5). Furthermore, the experiments with LIF incubation served as control for the possibility that prolonged pre-incubation time (4 h) *per se* would be responsible for the potentiation of the inhibition caused by CPA, observed in slices incubated with OSM but not in slices incubated with LIF for similar time periods.

OSM potentiates A_1R-mediated depression of synaptic transmission during hypoxia

The consistent increase in the extracellular concentration of endogenous adenosine that follows hypoxia and the subsequent A_1R-dependent inhibition of synaptic transmission (Sebastião *et al.* 2001) are main neuroprotective mechanisms in ischemic brain damage and hypoxia (Rudolphi *et al.* 1992). We therefore investigated if the OSM-induced modulation of A_1R function could have any functional impact in hypoxic conditions in pyramidal cells.

In control slices, brief (4 min) hypoxic insult caused a 42 ± 4.8% maximum inhibition of EPSC amplitude ($n = 7$). In hippocampal slices previously exposed to OSM (10 ng/mL, for 4 h), the same hypoxic insult caused a 69 ± 3.4% EPSC inhibition (Fig. 6a), which was significantly higher ($p < 0.05$) than that observed in control slices prepared from the same hippocampus. The hypoxia-induced depression of EPSC amplitude and its potentiation by OSM were abolished upon A_1R blockade by DPCPX (50 nM, applied 30 min before hypoxia induction) (Fig. 7).

Discussion

We have recently provided a possible explanation for IL-6-mediated neuroprotection. IL-6 treatment (thus gp130

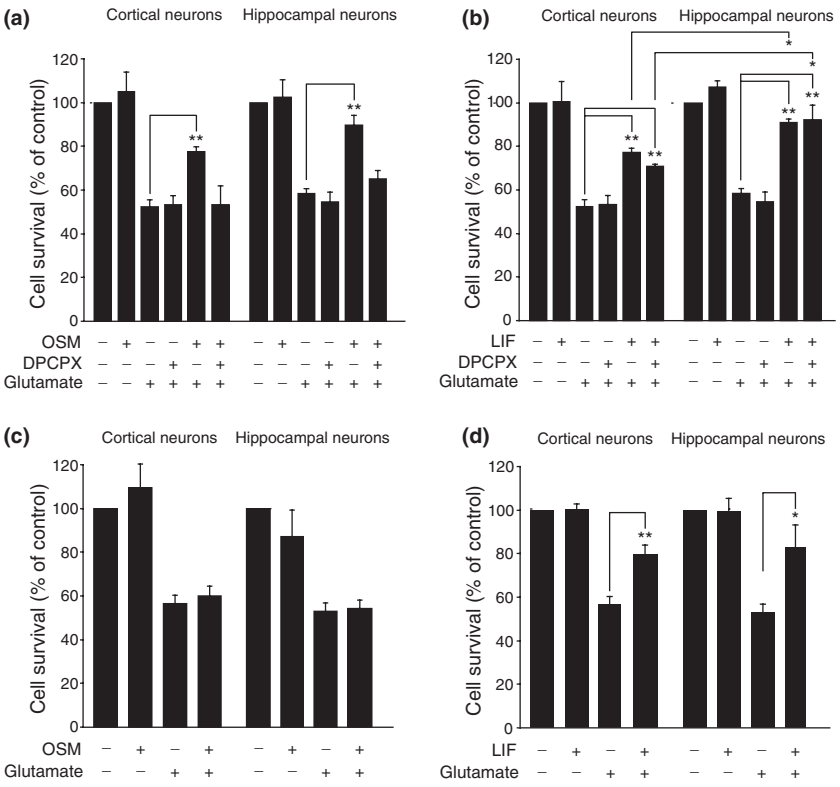

Fig. 3 Oncostatin M (OSM)- and leukemia inhibitory factor (LIF)-induced neuroprotection against glutamate toxicity in wild-type (C57BL/6J) and $A_1R-/-$ neurons. Primary cortical and hippocampal neurons from (a, b) C57BL/6J and (c, d) $A_1R-/-$ mice neonates (P_0) were pre-incubated with or without OSM (10 ng/mL for 24 h) (a, c) or LIF (10 ng/mL for 24 h) (b, d). Where indicated, the neurons were also pre-incubated with the A_1R antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 100 nM) for 15 min and were subsequently challenged with glutamate (100 μM) for 1 h. Neuronal viability was measured 18 h after the glutamate challenge by lysis-buffer assay. The bars represent mean ± SEM of three independent experiments; $^{**}p < 0.001$, $^{*}p < 0.05$.

homodimer activity) caused an up-regulation of neuronal A_1R expression and function *in vitro* and *in vivo*, which was mandatory for IL-6-dependent neuroprotection (Biber *et al.* 2008). This hypothesis is corroborated by our findings that IL-6 does not affect glutamate-induced excitotoxicity in A_1R-deficient neurons (unpublished observations). Notably, the members of IL-6-type cytokine family often show overlapping biological properties, because of the shared usage of gp130 receptor subunits in their signaling cascade (Taga and Kishimoto 1997; Bauer *et al.* 2007). We here investigated the possible role of A_1R function in neuroprotection and neuromodulation by OSM (OSMr/gp130 heterodimer) and LIF (LIFr/gp130 heterodimer) in hippocampal slices and in cultured neurons from wild-type and $A_1R-/-$ mice.

Here we describe that pre-treatment for 24 hours with OSM or LIF attenuates excitotoxicity in cultured neurons from cortex and hippocampus. These findings reinforce the idea of a principal neuroprotective effect of IL-6-type cytokines (see for review: Ransohoff *et al.* 2002). Since both cytokines display a comparable efficacy, similar mechanisms of action might be expected. However, blocking neuronal A_1R function with a selective antagonist (DPCPX, 100 nM) completely abolishes the neuroprotective effects of OSM, but leaves LIF-induced neuroprotection unaffected. In addition, activation of A_1R function with CPA (100 nM; 15 min before glutamate) is effective in OSM-treated, but not in LIF-treated neurons. The difference between OSM and LIF was also found in cultured cortical and hippocampal neurons from $A_1R-/-$ mice. While the neuroprotective effect of LIF is preserved in $A_1R-/-$ neurons, OSM pre-treatment does not affect neuronal survival against glutamate toxicity in the absence of functional adenosine A_1Rs. The lack of effects of CPA and DPCPX in untreated neurons most likely reflects an insufficient A_1R expression level in these cells *in vitro*. Whether or not this is because of the culture conditions is not clear at the moment. However, since in untreated acute slices both CPA and DPCPX showed effects, it is indicated that in brain tissue basal A_1R expression level is sufficient for neuroprotection and can be increased by IL-6-type cytokines.

When evaluating consequences for synaptic transmission, we found that a 4-h pre-treatment with IL-6 potentiates the A_1R-mediated inhibition of synaptic transmission in hippocampal slices, an effect that was of particular importance during short periods of hypoxia (Biber *et al.* 2008). Here, we present that OSM, but not LIF, significantly increases A_1R-mediated inhibition of EPSCs. Similarly to IL-6, OSM pre-treatment also causes a significantly higher drop in EPSCs in response to a 4-min hypoxic period, which is dependent on A_1R function. Thus, OSM, but not LIF, sensitizes neuronal A_1R-mediated responses, equally to the effects described earlier for IL-6. It may therefore be concluded that OSM treatment potentiates the functioning of neuronal A_1R, thereby increasing adenosinergic effects required for inhibi-

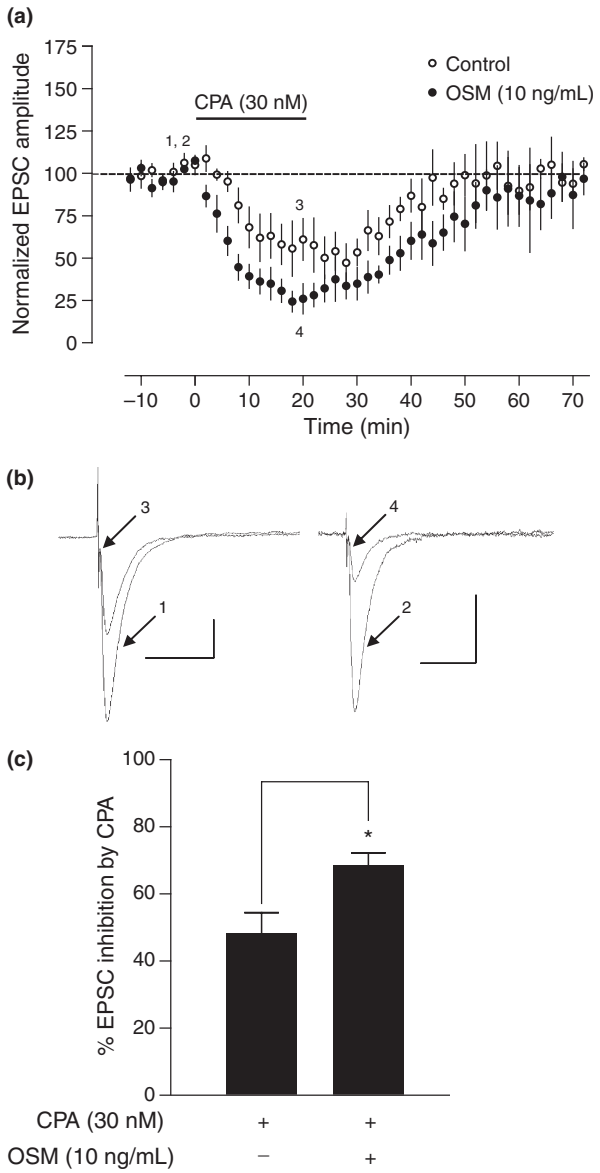

Fig. 4 Oncostatin M (OSM) potentiates inhibition of synaptic transmission caused by A_1R activation. (a) Averaged time-course of afferent evoked excitatory post-synaptic current (EPSC) peak amplitude changes caused by a 20 min application of the A_1R agonist N^6-cyclopentyladenosine (CPA; 30 nM), in control versus OSM (10 ng/mL for 4 h) treated slices, from the same hippocampus ($n = 4$). Each point represents average amplitude of four EPSCs evoked once every 30 s by electrical stimulation of the Schaffer collaterals; 100% corresponds to the averaged amplitude calculated for 5–10 EPSCs recorded just before adding CPA. (b) Superimposed current tracings of EPSCs recorded before (1, 2) and 20–30 min after (3, 4) introduction of CPA in the superfusion medium, in representative control (1, 3) and OSM-treated (2, 4) cells. Percent of EPSC inhibition corresponds to the average EPSC decrease 20–30 min after starting CPA application. Histogram (c) shows percent inhibition of synaptic transmission (EPSC) caused by the activation of A_1Rs was significantly higher in OSM-treated slices compared with the control; $^*p < 0.05$, paired *t*-test. Calibration: 30 ms, 50 pA.

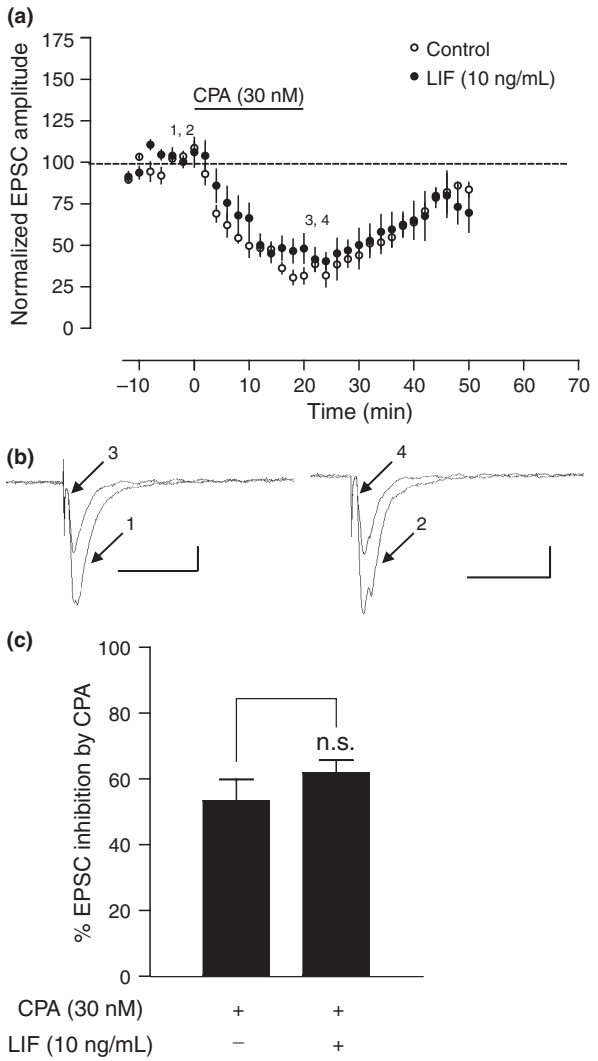

Fig. 5 Leukemia inhibitory factor (LIF) does not alter inhibition of synaptic transmission caused by A_1R activation. (a) Averaged time-course of afferent evoked excitatory post-synaptic current (EPSC) peak amplitude changes caused by a 20 min application of the A_1R agonist N^6-cyclopentyladenosine (CPA; 30 nM), in control versus LIF (10 ng/mL for 4 h) treated slices, from the same hippocampus ($n = 4$). Each point represents average amplitude of four EPSCs evoked once every 30 s by electrical stimulation of the Schaffer collaterals; 100% corresponds to the averaged amplitude calculated for 5–10 EPSCs recorded just before adding CPA. (b) Superimposed current tracings of EPSCs recorded before (1, 2) and 20–30 min after (3, 4) introduction of CPA in the superfusion medium, in representative control (1, 3) and LIF-treated (2, 4) cells. Histogram (c) shows percent inhibition of synaptic transmission (EPSC) caused by activation of A_1Rs is not significantly different between control and LIF-treated slices; n.s. (not significant), $p > 0.05$, paired *t*-test. Calibration: 30 ms, 50 pA.

tion of synaptic transmission and neuroprotection. Moreover, real-time PCR and western blot analysis strongly support this hypothesis as A_1R mRNA and protein expression levels are increased in OSM-treated, but not in LIF-treated neurons.

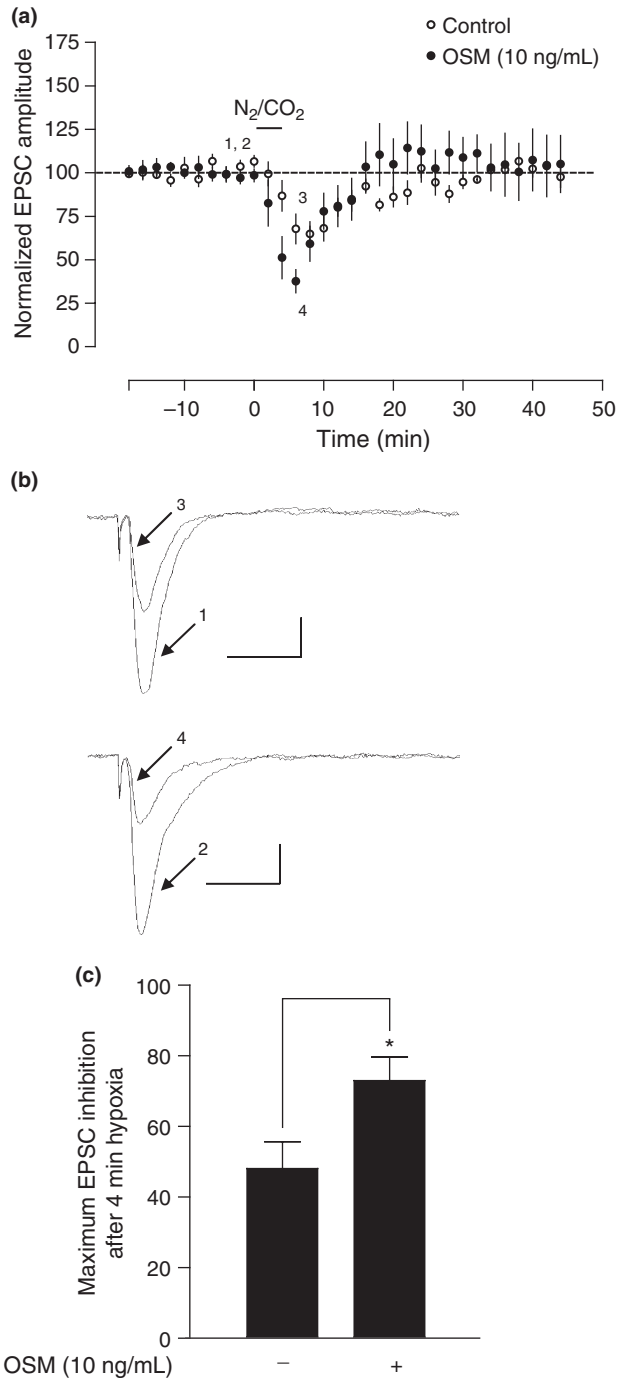

Fig. 6 Oncostatin M (OSM) potentiates hypoxia-induced inhibition of synaptic transmission. (a) Averaged time-course of afferent-evoked EPSC peak amplitude changes caused by a 4 min hypoxic insult, recorded from control [artificial CSF (aCSF)-treated] and OSM (10 ng/mL for 4 h) treated slices prepared from the same hippocampus. Each point represents average amplitude of four EPSCs evoked once every 30 s by electrical stimulation of the Schaffer collaterals; 100% corresponds to the averaged amplitude calculated for 5–10 EPSCs recorded just before hypoxia. The hypoxic insult consisted of replacing oxygenated aCSF (95% O_2 and 5% CO_2) by aCSF saturated with 95% N_2 and 5% CO_2 for 4 min. Maximum inhibition was determined as the lowest EPSC amplitude recorded for each experiment, at either 6 or 8 min after hypoxia onset. (b) Superimposed current tracings of EPSCs recorded before (1, 2) and 8 min after hypoxia (3, 4) was induced, in representative control (1, 3) and OSM-treated (2, 4) cells. Histogram (c) shows maximum inhibition of synaptic transmission induced by hypoxia is significantly higher when recording from slices that were pre-treated with OSM ($n = 7$); $^*p < 0.05$, paired *t*-test. Calibration: 30 ms, 50 pA.

Taken together, our findings strongly suggest that two members of the IL-6-type cytokine family (IL-6 and OSM) depend on A_1R function for their neuroprotective properties, whereas LIF induces neuroprotection via a different, unknown, mechanism. However, since both cytokines are neuroprotective *in vitro* and may reduce ischemic damage *in vivo* (see Suzuki *et al.* 2009 for review), a further understanding of their mechanisms of action may provide new therapeutic possibilities in stroke patients.

Although LIF and OSM are highly related members of the IL-6 type cytokine family (Jeffery *et al.* 1993; Nicola *et al.* 1993) sharing most properties, distinct effects upon activation of LIF and OSM receptor complexes have been reported. For example, only OSMr/gp130 heterodimer activation is able to promote osteoblast differentiation, whereas activation of both OSMr/gp130 and LIFr/gp130 heterodimers in these cells inhibited the expression of osteocalcin, a protein required for bone-building (Malaval *et al.* 2005). Furthermore, selective roles of OSM and LIF during haematopoiesis and their effects on the regulation of certain target genes have been reported (Tanaka *et al.* 2003; Weiss *et al.* 2005), reinforcing the idea that different IL-6-type cytokine receptor complexes may activate specific signaling cascades.

It is at the moment unclear which signaling pathways are important for the neuroprotective effects of LIF, OSM, and IL-6. In our preliminary observations, using blockers for any of the possibly involved signaling pathways [ERK1/2, STAT3, PI3K, or nuclear transcription factor-kappa B (NFκB)], we were not able to identify the different signaling pathways for the neuroprotective effects of LIF, OSM, and IL-6 (data not shown). In fact, most of the blockers (toward ERK1/2, PI3K, or NFκB) completely abolished neuroprotection by all cytokines; blocking STAT3 partially diminished cytokine effects, but did not discriminate between OSM and LIF (data not shown). The reasons for these unexpected results are not clear, but it became evident that to elucidate which signaling cascade is related to each cytokine neuroprotective action, more selective approaches, e.g. RNAi, will be required. In this context, it would be particularly relevant to unravel the cellular mechanism(s) by which IL-6 and OSM regulate A_1R expression. It has been shown that the basal as well as induced expressions of A1Rs are regulated by NFκB (Jhaveri *et al.* 2007). Noteworthy, OSM regulates protein

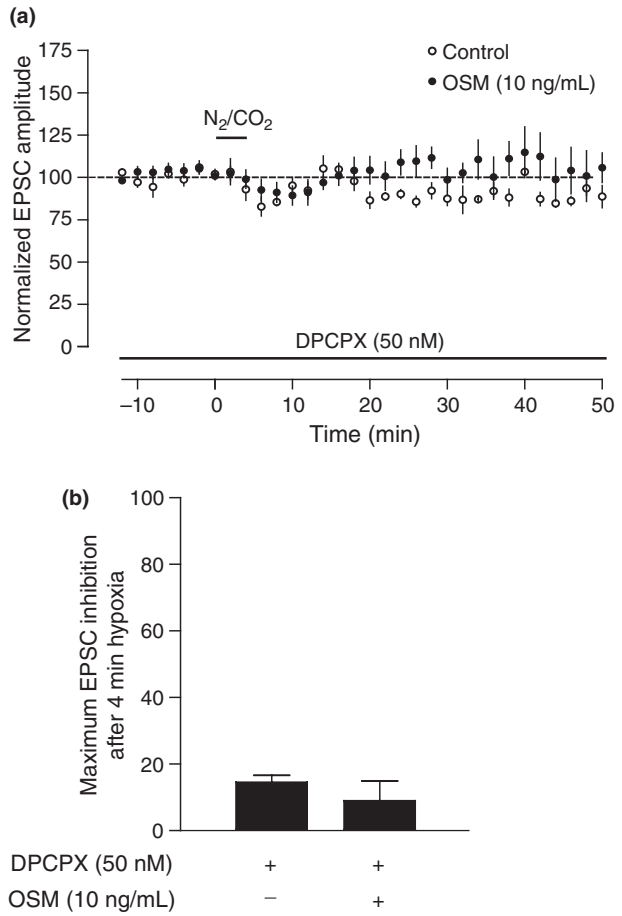

Fig. 7 Hypoxia-induced inhibition of synaptic transmission and its potentiation by oncostatin M (OSM) is dependent on A_1R activation. Averaged time-course of afferent-evoked EPSC peak amplitude changes caused by a 4 min hypoxic insult, delivered under conditions of A_1R blockade by the selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 50 nM); EPSCs were recorded from control (○) and OSM-treated (•) slices from the same hippocampus ($n = 5$); DPCPX was applied 30 min before induction of hypoxia. Further details as in Figure 6.

synthesis, through NFκB activation, in smooth muscle cells (Nishibe *et al.* 2001). When subjected to oxidative stress, these cells show an increase in A_1R mRNA that is prevented by inhibitors of the NFκB (Nie *et al.* 1998). Incidentally, IL-6 has also been shown to activate NFκB in intestinal cells (Wang *et al.* 2003). Therefore, it is likely that increased expression of A_1Rs by OSM and IL-6 is also, at least partially, regulated by NFκB. Nevertheless, it is irrefutable that adenosine A_1Rs are key players in neuroprotection against excitotoxicity induced by OSM and IL-6.

It has long been known that neuronal A_1R suppress neuronal activity and glutamatergic transmission, reduce oxidative stress, minimize metabolic demand thereby preserving ATP stores, and protect neurons from excitotoxicity (Schubert *et al.* 1997). The concept now emerges that cytokines from various families may utilize A_1R to exert their neuroprotective functions. In the absence of functional adenosine A_1Rs, numerous cytokines, such as IL-6 (Biber *et al.* 2008), OSM (present work), chemokine (C–X3–C motif) ligand 1 (CX3CL1) (Lauro *et al.* 2008), fail to protect neurons under excitotoxic conditions. Given the urgent need for new therapies in neurodegenerative diseases, it is concluded that a detailed analysis of the intracellular signaling cascade activated by IL-6 and OSM is not only important for our molecular understanding of cytokine biology, but might furthermore provide ideas of cellular mechanisms by which the expression and function of neuronal A_1Rs can be therapeutically increased.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Glutamate-induced excitotoxicity in neurons is mediated through NMDA receptors.

Figure S2. LIF and OSM protect cortical neurons from glutamate-induced excitotoxicity.

Figure S3. LIF and OSM reduces caspase 3 activation and protect cultured cortical neurons against glutamate toxicity.

Figure S4. Calcium transients in response to glutamate in cultured cortical neurons.

Figure S5. OSM, but not LIF, enhances A1R expression in wild-type (C57BL/6J) cortical neurons *in vitro*.

Table S1. Sequence of the primers used for reverse transcriptase and real-time PCR.

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