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# Genetically Controlled Upregulation of Adenosine $A_1$ Receptor Expression Enhances the Survival of Primary Cortical Neurons

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**Abstract** Adenosine has a key endogenous neuroprotective role in the brain, predominantly mediated by the adenosine $A_1$ receptor ($A_1R$). This has been mainly explored using pharmacological tools and/or receptor knockout mice strains. It has long been suggested that the neuroprotective effects of $A_1R$ are increased following receptor upregulation, thus attenuating neuronal damage in pathological conditions. We have previously shown that the neuroprotective and neuromodulatory actions of the cytokines IL-6 and oncostatin M are mediated by induction of neuronal $A_1R$ expression. In order to investigate the direct effects of $A_1R$ upregulation in neurons, we have generated a tetracycline-regulated expression system with a bidirectional promoter, directing the simultaneous expression of the mouse $A_1R$ and GFP/mCherry reporter genes. In a first step, we tested the efficacy of the system in transiently transfected human embryonic kidney 293 cells. In addition, we confirmed the functional integrity of the expressed $A_1R$ by whole-cell patch clamp recordings. We demonstrated that $A_1R$-transfected primary neurons show enhanced survival against *N*-methyl-D-aspartate-induced excitotoxicity. Pretreatment with an $A_1R$-selective agonist additionally strongly decreased neuronal cell death, while an $A_1R$ antagonist completely abolished the neuroprotective effects of $A_1R$ upregulation. The presented data provide for the first time direct evidence that the upregulation of $A_1R$ enhances neuronal survival.

**Keywords** Adenosine $A_1$ receptor · Overexpression · Excitotoxicity · Neuroprotection · Tetracycline expression system · Transfection

## Introduction

Glutamate-mediated excitotoxicity is a major area of interest because of its involvement in brain trauma, stroke and many neurodegenerative disorders. This process is mediated by excessive activation of glutamate receptors and has been associated with neuronal loss observed after epileptic seizures, spinal cord and head injury [1–3] and many neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, Huntington's disease and multiple sclerosis [4].

Excitotoxicity causes a pronounced increase of adenosine levels in the affected brain tissue (see for review [5]). The extracellular adenosine acts as a major neuroprotective system in the brain, and its effects are predominantly mediated by adenosine $A_1$ receptor ($A_1R$). The $A_1R$ suppresses neuronal activity by presynaptic inhibition of glutamate release [6] and postsynaptic hyperpolarization of the cell membrane, caused by increasing the conductance of $K^+$ channels [7], inhibition of $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels and stabilisation of the $Mg^{2+}$ blockade of *N*-methyl-D-aspartate (NMDA) receptors [8].

The neuroprotective role of $A_1R$ in the brain has been explored mainly by taking advantage of different pharmacological tools, like selective agonists such as $N^6$-cyclopentyladenosine (CPA) and 2-chloroadenosine and antagonists such as 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) in

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combination with several receptor knockout mice strains (see for review [9]). However, the $A_1R$ agonists are not used for therapeutic purposes, mostly due to severe peripheral side effects, short half-life and/or receptor desensitization [10, 11]. It has been suggested that the induction of $A_1R$ expression might efficiently enhance $A_1R$-mediated neuroprotection (see for review [5, 12, 13]). Indeed, we have recently reported that the upregulation of $A_1R$ expression is a key mechanism that mediates the neuroprotective function of IL-6 and oncostatin M cytokines [14, 15].

Thus, in order to study the direct effects and a putative neuroprotective role of $A_1R$ upregulation in neurons, we have generated and functionally tested a tetracycline-regulated expression system for mouse $A_1R$. The results presented here provide direct evidence that the upregulation of $A_1R$ enhances neuronal survival against NMDA-induced excitotoxicity.

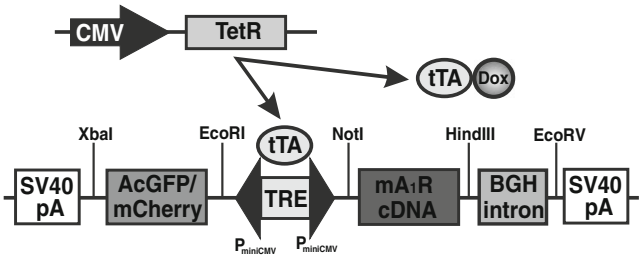


**Fig. 1** Tetracycline-regulated (Tet-Off) expression of the mouse $A_1R$. The full-length mouse adenosine $A_1$ receptor coding region (*$mA_1R$ cDNA*) was ligated into NotI/HindIII digested pTRE-tight-BI-AcGFP-BGH tetracycline-inducible bidirectional expression vector, containing EcoRV subcloned human beta-globin intron (*BGH intron*). Alternatively, red fluorescent protein expressing construct (pTRE-tight-BI-mCherry-$A_1R$-BGH) was created by XbaI/EcoRI substitution of AcGFP with mCherry. The human cytomegalovirus (*CMV*) promoter controls the expression of the *tTA* gene product, which induces the simultaneous transcription of the $A_1R$ and AcGFP/mCherry, by binding to a tetracycline-responsive element (*TRE*) containing a bidirectional promoter ($P_{miniCMV}$). Thus, the gene expression could be blocked by tetracycline or its stable analogue doxycycline (*Dox*). *SV40pA* simian-virus 40 polyadenylation signal

## Materials and Methods

### Materials

Dulbecco's modified Eagle's medium (DMEM), Neuronal Base medium (NB), Hanks' balanced salt solution, phosphate-buffered saline (PBS), sodium pyruvate, L-glutamine, penicillin–streptomycin, HEPES, NeuroMix, fetal bovine serum and trypsin–EDTA were from PAA laboratories (Cölbe, Germany). Lipofectamine 2000, Opti-MEM and Alexa Fluor 647 donkey anti-rabbit IgG were from Invitrogen (Karlsruhe, Germany). CPA and DPCPX were from Tocris Bioscience (Bristol, UK). Adenosine deaminase (ADA) was from Roche Biohemicals (Mannheim, Germany). Anti-adenosine $A_1$ receptor rabbit polyclonal antibody was from Abcam (Cambridge, UK). Anti-actin rabbit polyclonal antibody and all other chemicals were from Sigma-Aldrich (Munich, Germany).

### Plasmid and DNA Construct Cloning

We have previously isolated and subcloned the mouse adenosine $A_1$ receptor cDNA into pCRII cloning vector [16]. A NotI/HindIII fragment, containing the full-length adenosine $A_1$ receptor coding region, was excised from a pCRII vector and ligated into a NotI/HindIII digested pTRE-tight-BI-AcGFP-BGH tet-inducible bidirectional expression vector. Alternatively, a red fluorescent protein-expressing construct (pTRE-tight-BI-mCherry-$A_1R$-BGH) was created by XbaI/EcoRI substitution of the AcGFP with mCherry (Fig. 1). pTRE-tight-BI-AcGFP-BGH plasmid containing EcoRV subcloned human beta-globin intron and pTet-Off transactivator-expressing plasmid were a kind gift from K.-P. Knobeloch (Department of Neuropathology, University Freiburg).

### Cell Culture and Transfection

The human embryonic kidney 293 (HEK293) cells were maintained in DMEM 1 g/l glucose, 10 % fetal calf serum, penicillin 100 U/ml and streptomycin 100 μg/ml at 5 % $CO_2$ and 37 °C and were subcultured twice a week in order to prevent from reaching confluence. The HEK293 cells were transfected with Lipofectamine 2000 according to the manufacturer's instructions. Primary neuronal cultures from mouse embryo (~E16.5) were established as described previously [14]. The cells were grown on poly-L-lysine-coated glass cover slips in 24-well plates. After 4 days in vitro, the cultures were transfected using the $Ca^{2+}$-phosphate transfection method described by Jiang et al. [17], except that the cells were maintained only in NB medium supplemented with NeuroMix, 1 mM sodium pyruvate, 0.5 mM L-glutamine, penicillin 100 U/ml and streptomycin 100 μg/ml. The neuronal purity, determined by neuronal nuclei (NeuN) staining, was 90–95 % (data not shown). The HEK293 cells were transfected with pcDNA-$A_1R$ or co-transfected with pTRE-tight-BI-AcGFP/mCherry-$A_1R$-BGH and pTet-Off transactivator-expressing plasmid in a 1:5 ratio and treated with 100 ng/ml doxycycline for 48 h before the experiments. The primary neuronal cultures were co-transfected with pTRE-tight-BI-AcGFP/mCherry-$A_1R$-BGH or pTRE-tight-BI-AcGFP/mCherry and pTet-Off transactivator-expressing plasmid in a 1:2 ratio and used for experiments after 48 h in vitro.

### Western Blot Analysis

The cells were washed twice with ice-cold PBS and then lysed with Laemmli sample buffer (50 mM Tris–HCl (pH

6.8), 2 % SDS, 5 % 2-mercaptoethanol, 0.2 M DTT, 10 % glycerin, 0.04 % Pyronin Y) and boiled at 95 °C. The extracts were separated on 10 % SDS-PAGE and electro-transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5 % nonfat dry milk in PBS-T (1 % Tween 20 in PBS) for 1 h at room temperature (RT) and then incubated with the respective primary antibody (rabbit polyclonal anti-adenosine $A_1$ receptor 1:1,000 and rabbit polyclonal anti-actin 1:5,000) overnight at 4 °C. The membranes were washed three times for 5 min in PBS-T and incubated for 1 h at RT with secondary horseradish peroxidase-conjugated antibody, diluted in PBS-T and then washed again three times for 5 min in PBS-T. The signal was detected using an enhanced chemiluminescent detection reagent kit (GE Healthcare; München, Germany) and a Fusion-SL imaging system (peQlab; Erlangen, Germany). The immunoreactive bands along with their respective loading controls were quantified densitometrically by using the TINA 2.09 software (ISBM; Oxford, UK).

### Whole-Cell Patch Clamp Recordings

The cells were transferred to the submerged-type recording chamber and continuously perfused with extracellular recording solution containing 135 mM NaCl, 2 mM TEA, 2 mM $MgCl_2$, 10 mM HEPES and 20 mM $BaCl_2$ (pH 7.35) at a flow rate of 5–10 ml/min (chamber volume approximately 2 ml); 10 μl of stock solutions of CPA (10 μM) and DPCPX (1 μM) was directly applied to the recording chamber that had a volume of 1 ml. The recordings were performed on cells identified by using infrared differential interference contrast video microscopy (Axioskop 2 FS plus, Zeiss Germany, IMAGO-VGA, Till Photonics, Germany), and the transfected GFP cells were visualised at 475-nm wavelength. The recording pipettes were pulled from borosilicate glass tubing (2.0-mm outer diameter, 0.5-mm wall thickness; Hilgenberg, Germany). The patch pipettes with resistances 4–7 MΩ were filled with a solution containing 140 mM CsCl, 2 mM $MgCl_2$, 2 mM $Na_2ATP$, 0.3 mM NaGTP, 10 mM EGTA and 10 mM HEPES (pH 7.35). Patch clamp recordings were performed with an EPC-9 amplifier (HEKA, Lambrecht, Germany), and the signals were filtered at 5 and 10 kHz and stored online. For data acquisition and analysis, Pulse and PulseFit softwares (HEKA, Lambrecht, Germany) were used. Data represent the mean values ± SEM of the averages of 10–15 consecutive sweeps of current amplitudes of five to seven cells per condition.

### Induction of Excitotoxicity

Neurons maintained in doxycycline-free medium were used after 7 days in culture. Cells were incubated for 1 h with 1 U/ml ADA and then either stimulated for 1 h with NMDA at various concentrations or incubated for 15 min with 100 nM CPA or 100 nM DPCPX before they were subjected to NMDA challenge for 1 h. Following the NMDA treatment, the media were refreshed and the neuronal cultures were incubated with propidium iodide (PI, 1:1,000) for 18 h before immunocytochemistry.

### Immunocytochemistry

Immunocytochemistry was performed at RT unless mentioned otherwise. Cultures were washed twice for 5 min in PBS and then fixed with 4 % paraformaldehyde in PBS for 20 min. Subsequently, the cultures were washed twice with PBT (PBS/A (1 % bovine serum albumin in PBS) and 0.1 % Tritone X-100) and further incubated with PBT containing 5 % goat serum for 1 h. Cultures were incubated with the primary antibody (rabbit polyclonal anti-adenosine $A_1$ receptor—1:1,000) diluted in PBS/A overnight at 4 °C. After three washes with PBS/A, the cells were incubated with specific dye-conjugated secondary antibody (Alexa Fluor 647 donkey anti-rabbit—1:1,000) diluted in PBS/A for 1 h. Cultures were washed twice with PBS before incubation with 4′-6-diamidino-2-phenylindole (DAPI, 1:1,000) in PBS to label cell nuclei for 10 min. After a final washing in PBS, the cover slips were mounted on slides using Mowiol-DABCO. All immunofluorescence images were detected and photographed using LSM-U-2 laser scanning confocal microscope and documented using ZEN 2009 software (Carl Zeiss, Germany).

### Counting and Statistical Analysis

The number of PI- and GFP-positive cells was determined by counting the number of cells in three randomly chosen microscopic observation fields per cover slip. Data represent the mean ± SEM of experiments performed at least three times on independent culture preparations, each performed in triplicate. Statistical analysis of the data was performed using one-way ANOVA, and statistical significance between the two groups was determined by applying Student's *t* test. Analysis of the electrophysiology and Western blot data was carried out using Student's *t* test performed on SPSS 17.0 software (SPSS Inc., Chicago, USA). In all cases, values of $p<0.05$ were considered statistically significant.

## Results

### Generation of the Tetracycline-Regulated $A_1R$ Expression System

In order to create a conditionally regulated gene expression system with simultaneous expression of $A_1R$ and a fluorescent

protein reporter gene, we cloned the full-length mouse adenosine $A_1$ receptor coding region ($mA_1R$ cDNA) into a pTRE-tight-BI-AcGFP-BGH (pTRE-GFP) tetracycline-inducible bidirectional expression vector, containing an EcoRV subcloned human beta-globin intron (BGH intron). Alternatively, a red fluorescent protein reporter gene-expressing construct (pTRE-tight-BI-mCherry-$A_1$R-BGH) was created by substitution of AcGFP with mCherry. The system requires co-transfection of two different plasmids: pTet-Off, encoding the tetracycline transactivator (tTA) gene product, which induces the transcription of $A_1R$ and AcGFP/mCherry by binding to a tetracycline-responsive element (TRE) containing a bidirectional promoter ($P_{miniCMV}$) located on the second plasmid—pTRE-tight-BI-AcGFP/mCherry-$A_1$R-BGH (pTRE-GFP/mCherry-$A_1R$). The gene expression can be blocked by tetracycline or its stable analogue doxycycline, which inhibits the tTA binding to the TRE promoter (Fig. 1). In order to enhance the expression of the $A_1R$, we inserted the human beta-globin intron sequence (BGH intron) after the $A_1R$ coding region [18].

## Tetracycline-Regulated Expression of Mouse $A_1R$ in HEK293 Cells

The tetracycline-regulated $A_1R$ expression system was first tested in cultured cortical neurons by Western blotting or immunohistochemistry. However, a significant effect of doxycycline treatment on $A_1R$ expression was not found. This most likely reason was the low transfection efficiency of primary neurons, which was less than 10 % (see below) and the fact that neurons already endogenously express $A_1R$. We assumed that an increase caused by our construct was not pronounced enough to be reliably detected. We therefore used HEK293 cells for further characterization of our system, since these cells are easily transfected and do not express endogenous $A_1R$ (see for review [19]). Initially, we compared the $A_1R$ protein expression of pTRE-GFP/mCherry-$A_1R$ and pTet-Off co-transfected (pTRE-$A_1R$) HEK293 cells in the absence of doxycycline with those transfected with the pcDNA3-$A_1R$ expression plasmid, where high levels of constitutive $A_1R$ expression, directed by the CMV promoter, are achieved. The tetracycline-regulated $A_1R$ expression system showed a slightly less expression compared to the widely used pcDNA3 vector. Addition of 100 ng/ml doxycycline strongly inhibited the $A_1R$ protein expression down to basal endogenous levels in HEK293 cells (Fig. 2a).

The doxycycline-regulated $A_1R$ expression in HEK293 cells was further analysed by immunocytochemistry. We did not observe a specific $A_1R$ staining in the non-transfected HEK293 cells. However, in the pTRE-$A_1R$ cultures, we found a high number of $A_1R$-positive cells, similar to that observed in pcDNA3-$A_1R$-transfected HEK293 cells (Fig. 2b). Though only 40–60 % of the $A_1R$ stained cells were dual positive for GFP and $A_1R$ (Fig. 2c), we did not find any cell positive only for GFP. Addition of 100 ng/ml doxycycline strongly inhibited both $A_1R$ and GFP expression to undetectable levels (Fig. 2b).

## $A_1R$-Mediated Inhibition of Voltage-Gated $Ca^{2+}$ Channels in $A_1R$-Transfected HEK293 Cells

It has been previously reported that the $A_1R$ inhibits $Ca^{2+}$ influx by modulation of voltage-gated $Ca^{2+}$ channels [20, 21]. Accordingly, to test the functionality of expressed $A_1R$, we performed whole-cell patch clamp recordings. First, the effect of the selective $A_1R$ agonist CPA on voltage-gated $Ca^{2+}$ channels of pTet-Off and pTRE-GFP (mock transfected) or pTRE-GFP-$A_1R$ ($A_1R$ transfected) co-transfected primary neurons was tested. The GFP reporter gene expression allowed us to select the transfected cells, and indeed an increased inhibitory effect of CPA on $Ca^{2+}$ currents was detected occasionally (Suppl. Fig. 3). However, patching transfected neurons was extremely difficult and most of the time a stable recording was not possible in transfected cells. This most likely was caused by the transfection procedure, because non-transfected neurons were patched with no such problems (data not shown). Therefore, it was decided to continue this approach in HEK293 cells that often are used for electrophysiological experiments (see for review [19]) and are known to express endogenous voltage-dependent $Ca^{2+}$ channels that slightly differ in their pharmacological properties from classical N- and L-type channels but are functionally regulated by $G_{i/o}$ proteins [22, 23].

Accordingly, we tested the effect of the selective $A_1R$ agonist CPA on endogenous voltage-gated $Ca^{2+}$ channels of pTet-Off and pTRE-GFP (mock transfected) or pTRE-GFP-$A_1R$ ($A_1R$ transfected) co-transfected HEK293T cells. The holding potential was −70 mV, and the whole-cell $Ba^{2+}$ currents through $Ca^{2+}$ channels were measured by 50-ms steps to a recording potential of +10 mV. The currents reached typical peak amplitudes of −50 to −400 pA at 0 mV (Fig. 3a, Suppl. Fig. 2) and were completely blocked by 25 μM CdCl ($n$=2, data not shown). We found that 10 μM CPA strongly inhibited voltage-dependent $Ba^{2+}$ currents in $A_1R$-transfected cells, while it did not significantly affect current amplitudes of mock-transfected cells (Fig. 3a, b). The effect of 10 μM CPA was completely antagonised in the presence of the adenosine $A_1R$ antagonist DPCPX (1 μM), suggesting that the observed current inhibition in $A_1R$-transfected cells was specifically mediated by the expressed $A_1R$ (Fig. 3c); 1 μM DPCPX alone had no significant effect both in $A_1R$-transfected cells (Fig. 3c) and in mock-transfected cultures (data not shown). Neither CPA nor DPCPX significantly changed the kinetics of the recorded currents (data not shown).

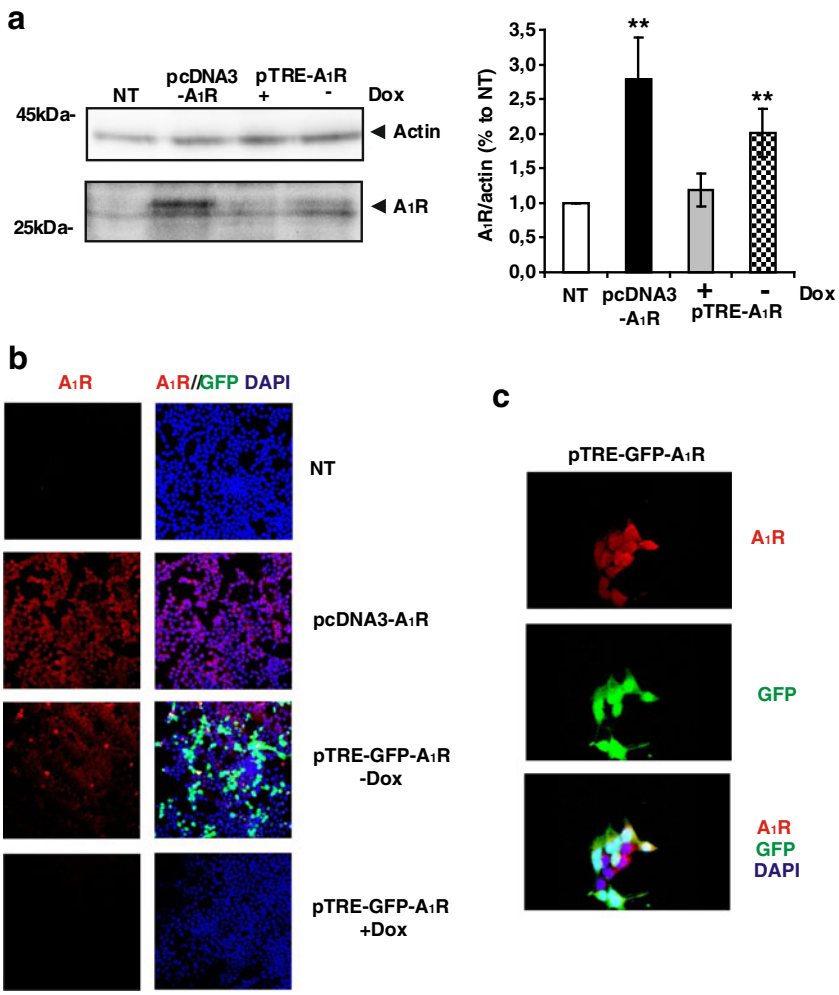


**Fig. 2** Tetracycline-regulated expression of mouse $A_1R$ in HEK293 cells. **a** Representative Western blot and densitometric quantification of $A_1R$ protein levels in control non-transfected (*NT*), *pcDNA3-A1R* expression plasmid transfected and pTRE-tight-BI-AcGFP/mCherry-$A_1R$-BGH and pTet-Off transactivator-expressing plasmid co-transfected (*pTRE-A1R*) HEK293T cells in the absence (−) or presence (+) of 100 ng/ml doxycycline. The mouse whole brain lysate was used as a positive control for $A_1R$. Data represent a densitometric quantification of relative mean levels of $A_1R$ protein normalised to actin ($n$=3, $^{**}p<0.01$ in comparison to NT, Student's $t$ test). **b** Representative fluorescence photomicrographs for three independent experiments depicting the $A_1R$ (*red*), GFP (*green*) and nuclei counterstained with DAPI (*blue*) in transfected HEK293T cells; 100 ng/ml doxycycline treatment strongly inhibits $A_1R$/GFP expression (*pTRE-GFP-A1R+ Dox*). **c** $A_1R$ staining and GFP fluorescence co-localises in pTRE-$A_1R$-transfected HEK293T cells

## Upregulated $A_1R$ Expression Enhances the Survival of Transfected Primary Cortical Neurons

The neuronal $A_1R$ has a key neuroprotective function, and it has long been suggested that upregulation of $A_1R$ expression increases its neuroprotective capacities (see for review [5, 13]). In order to investigate the role of $A_1R$ overexpression on neuronal survival, primary neuronal cultures were co-transfected with pTet-Off transactivator-expressing plasmid and pTRE-GFP/mCherry-$A_1R$ ($A_1R$ transfected) or pTRE-GFP/mCherry (mock transfected) plasmids in a ratio of 2:1 by the $Ca^{2+}$-phosphate transfection method described by Jiang et al. [17]. Using this transfection protocol, we were able to achieve up to 10 % transfection efficacy while maintaining more than 60 % survival of the transfected neurons (Fig. 4b), determined by counting of GFP/mCherry-positive cells and PI staining of the dead cell nuclei (Suppl. Fig. 1), respectively.

We found that in control conditions, the $A_1R$-transfected neurons had a significantly higher survival than mock-transfected neurons, expressing only the reporter gene GFP/mCherry (Fig. 4b). In all experiments, non-neuronal cells, negative for neuronal nuclei marker NeuN, were excluded from the analysis.

It is well known that treatment with high concentration of glutamate for 1 h causes cell death in cultured embryonic cortical neurons that can be abolished by NMDA receptor antagonists [14]. Thus, 48 h after the transfection, we stimulated mock-transfected and $A_1R$-transfected neuronal cultures, simultaneously with non-transfected neurons, with various concentrations of NMDA for 1 h. Neuronal survival was assessed 18 h later. Treatment with 10, 50 and 100 μM NMDA caused a concentration-dependant increase of PI-positive cells in non-transfected and mock-transfected neuronal cultures (Fig. 4a, b). In contrast, stimulation with 10 μM NMDA was not able to significantly increase the number of PI-labelled $A_1R$-transfected neurons, compared to control, non-NMDA-treated $A_1R$-transfected cultures. In addition, we found significantly reduced number of PI-stained GFP/mCherry-positive neurons in all NMDA-treated $A_1R$-transfected cultures, compared to the corresponding mock-transfected neurons (Fig. 4b). Interestingly, the efficacy (slope

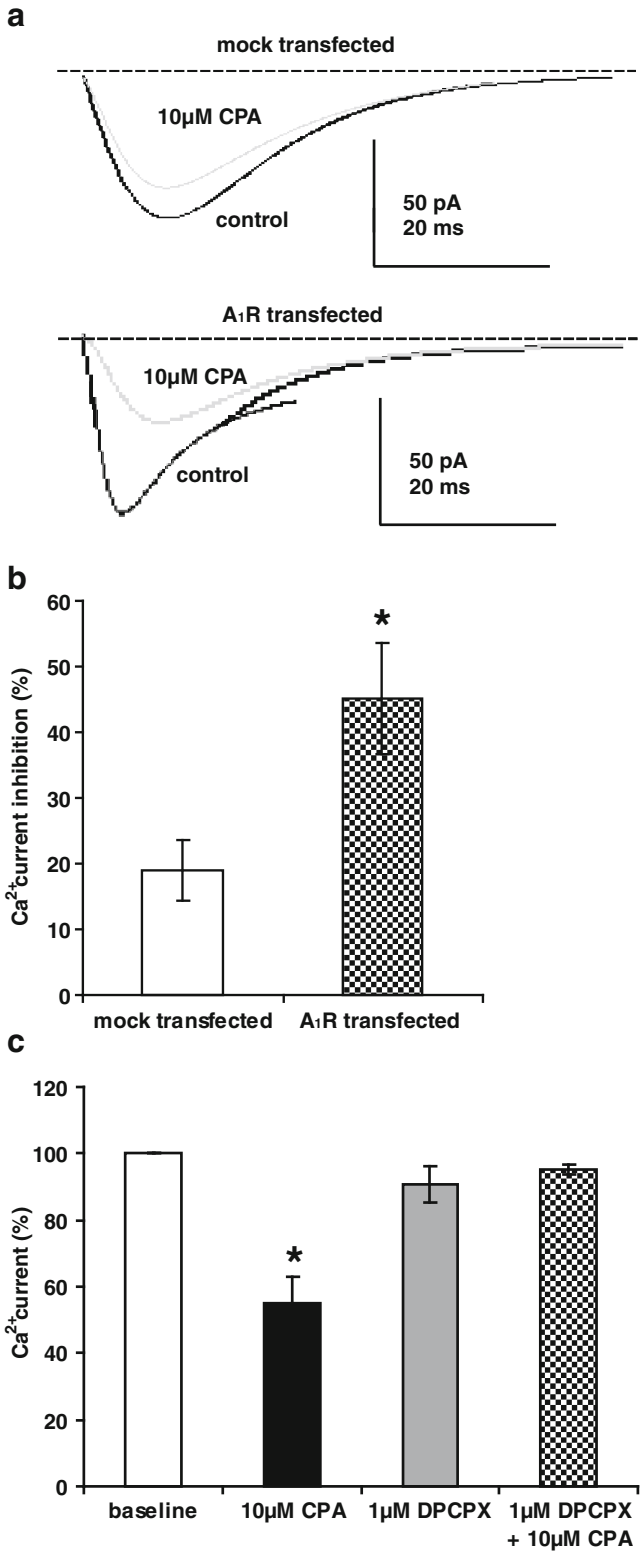


**Fig. 3** $A_1R$-mediated inhibition of voltage-gated $Ca^{2+}$ channels in transfected HEK293 cells expressing upregulated $A_1R$. The holding potential of cells was −70 mV and whole-cell $Ba^{2+}$ currents were measured by 50-ms steps to a recording potential of +10 mV; 10 μl of stock solutions of CPA (10 μM) and DPCPX (1 μM) was directly applied to the recording chamber that had a volume of 1 ml. **a** Representative whole-cell voltage-gated $Ba^{2+}$ currents of the pTet-Off and pTRE-tight-BI-AcGFP (mock transfected) or pTRE-tight-BI-AcGFP-$A_1R$-BGH ($A_1R$ transfected) co-transfected HEK293T cells, baseline in *black* and 10 μM CPA effect in *grey*. **b** Current inhibition by 10 μM CPA in mock ($n$=7) and $A_1R$ ($n$=5) transfected HEK293T cells (averaged peak current amplitude in percent ± SEM of baseline currents; *$p$<0.01, Student's $t$ test). **c** Comparison of the effects of 10 μM CPA, 1 μM DPCPX or both together on $A_1R$-transfected HEK293T cells. Data represent the amplitude of the voltage-gated $Ba^{2+}$ currents normalised to baseline (±SEM of the averaged peak amplitudes of 10–15 consecutive sweeps; $n$=5–7 cells per condition; *$p$<0.01 in comparison to baseline, Student's $t$ test)

of concentration dependency) of NMDA to induce neuronal death was not changed between non-transfected and mock-transfected cells. In neurons, where $A_1R$ was upregulated, the neurotoxic effect of NMDA was significantly impaired (Fig. 4c). We did not observe any significant differences in cell viability between mCherry and GFP-transfected neurons (data not shown).

## Modulation of $A_1R$ Upregulation-Mediated Neuroprotection by Pretreatment with ADA, CPA and DPCPX

We show here that upregulation of $A_1R$ expression enhances the survival of transfected primary neurons without specific $A_1R$ agonist stimulation, suggesting for the presence of adenosine in the culture media. In order to test that hypothesis, the extracellular adenosine was removed by pre-incubating the transfected neuronal cultures with 1 U/ml ADA for 1 h, which is sufficient to degrade the adenosine present in the media. Thereafter, neurons were challenged with 50 μM NMDA for 1 h and cell viability was assessed. Our results show that ADA treatment had no significant effect on the survival of the mock-transfected neuronal cultures; however, it completely abolished the neuroprotective effect of upregulated $A_1R$ expression in both the control and 50 μM NMDA-treated $A_1R$-transfected neurons (Fig. 5a).

To further confirm the involvement of $A_1R$ in the observed neuroprotective effect, we pre-incubated cultures with the selective $A_1R$ agonist CPA for 15 min prior to the NMDA stimulation. Pretreatment with 100 nM CPA of mock-transfected neurons had no significant effect on neuronal survival at NMDA concentration of 10 and 50 μM. We detected a slight neuroprotective effect of CPA in 100 μM NMDA-challenged mock-transfected neurons (Fig. 5b). In contrast, treatment with CPA strongly enhanced survival in $A_1R$-transfected neurons; 100 nM CPA treatment completely abolished neurotoxicity of 10 and 50 μM NMDA and strongly reduced the cell death even in response to 100 μM NMDA in $A_1R$-transfected neurons (Fig. 5b).

In order to further investigate the specificity of the $A_1R$ upregulation-mediated enhancement of the survival of

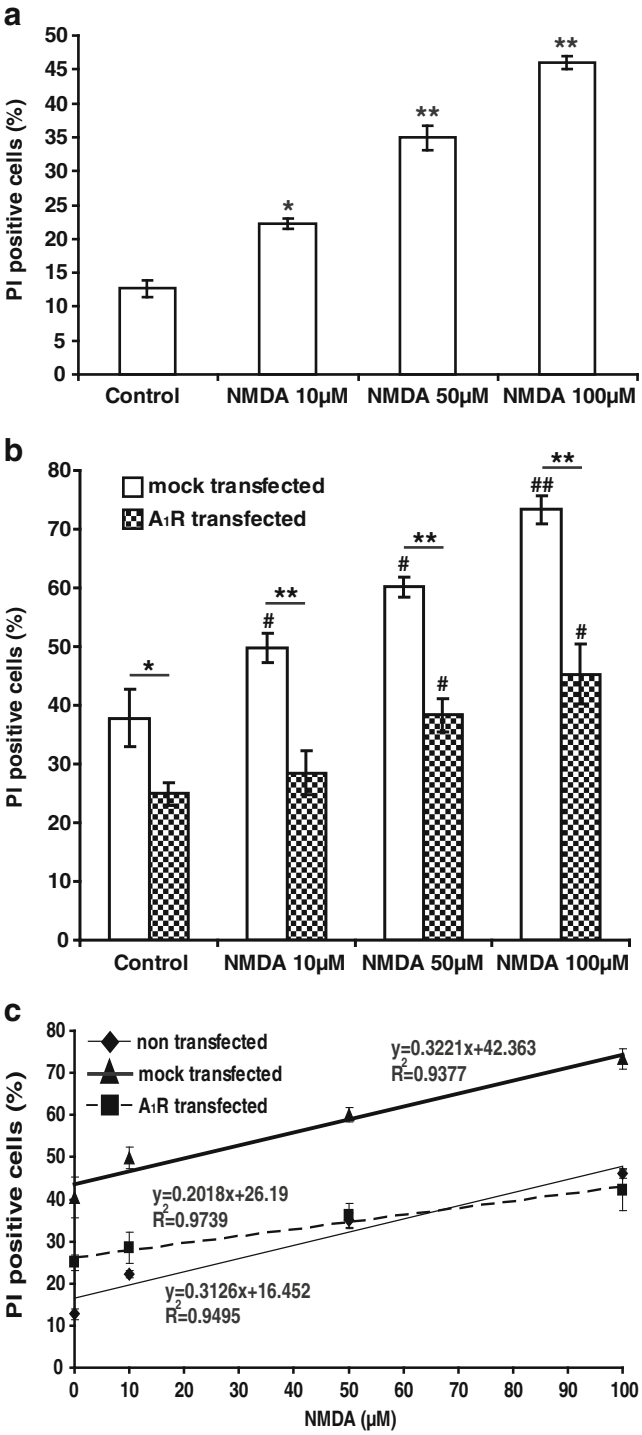


**Fig. 4** Effect of upregulated $A_1R$ expression on the survival of primary cortical neurons after NMDA-induced excitotoxicity. Primary neuronal cultures were directly challenged with NMDA (10, 50 and 100 μM) for 1 h (non-transfected) (**a**) or after co-transfection with pTRE-GFP/mCherry-$A_1R$ ($A_1R$ transfected) or pTRE-GFP/mCherry (mock transfected) and pTet-Off plasmid in a 1:2 ratio (**b**). Neuronal survival was assessed 18 h following the NMDA treatment by immunocytochemistry using PI staining of the dead cell nuclei. Percentage of PI-stained nuclei vs. the total number of DAPI-stained nuclei for the non-transfected (**a**) or the PI-stained nuclei of the GFP/mCherry-expressing cells vs. the total number of GFP-positive cells for transfected cultures (**b**) (±SEM; $n=5$; *$p<0.05$, **$p<0.01$, #$p<0.05$, ##$p<0.01$ in comparison to the respective control, Student's $t$ test). **c** The slope comparison of the linear regression lines representing the NMDA- (10, 50 and 100 μM) induced concentration-dependent cell death in non-transfected, GFP(mCherry)-expressing (mock transfected) and $A_1R$/GFP(mCherry)-expressing ($A_1R$ transfected) neuronal cultures. Data represent the mean ± SEM of five independent experiments

transfected primary cortical neurons, the specific $A_1R$ antagonist DPCPX was used. Blockade of $A_1R$ by pre-incubation with 100 nM DPCPX completely abolished the neuroprotective effect of $A_1R$ upregulation in both control and 50 μM NMDA-treated $A_1R$-transfected neurons, but it did not affect the neuronal survival of mock-transfected cultures (Fig. 5c). Similarly, pre-incubation with 100 nM DPCPX for 15 min followed by 15 min 100 nM CPA stimulation abolished both the effects of $A_1R$ upregulation and CPA treatment on neuronal survival in $A_1R$-transfected cultures and it did not affect the number of the PI-positive GFP/mCherry-expressing mock-transfected neurons (Fig. 5d).

## Discussion

Extracellular adenosine acting on neuronal $A_1R$ functions as a major endogenous neuroprotective system in the brain (see for review [5, 12, 13, 24]). Although the precise mechanism by which neuroprotection is achieved is not yet clear (see for review [9]), it seems that $A_1R$, to a large extent, controls the threshold of onset of neuronal damage. Accordingly, it has long been suggested that the neuroprotective effects of $A_1R$ are increased following receptor upregulation; thus, the enhancement of neuronal $A_1R$ expression attenuates neuronal damage in pathological conditions (see for review [5, 13]). Indeed, we recently reported that the cytokines IL-6 and oncostatin M upregulate neuronal $A_1R$ expression and thereby boost neuroprotection in vitro and in vivo [14, 15]. Interestingly, an upregulation of $A_1R$ expression has been observed in mice and rats after chemically induced seizures and in post-mortem tissue of humans suffering from epilepsy [25–27]. This seizure-induced upregulation of $A_1R$ was not observed in IL-6 deficient animals, which was correlated with higher seizure scores and increased mortality, again suggesting that an increased $A_1R$ expression is neuroprotective [14]. An increase of neuronal $A_1R$ expression has yet only been achieved by chronic antagonist treatment [28–31] or stimulation with cytokines [14, 15]. Since these pharmacological interventions may also have effects other than the upregulation of $A_1R$ expression, we here aimed to analyse the direct effects of $A_1R$ upregulation.

In order to investigate the direct effects of upregulation of $A_1R$ expression in neurons, we have generated a tetracycline-

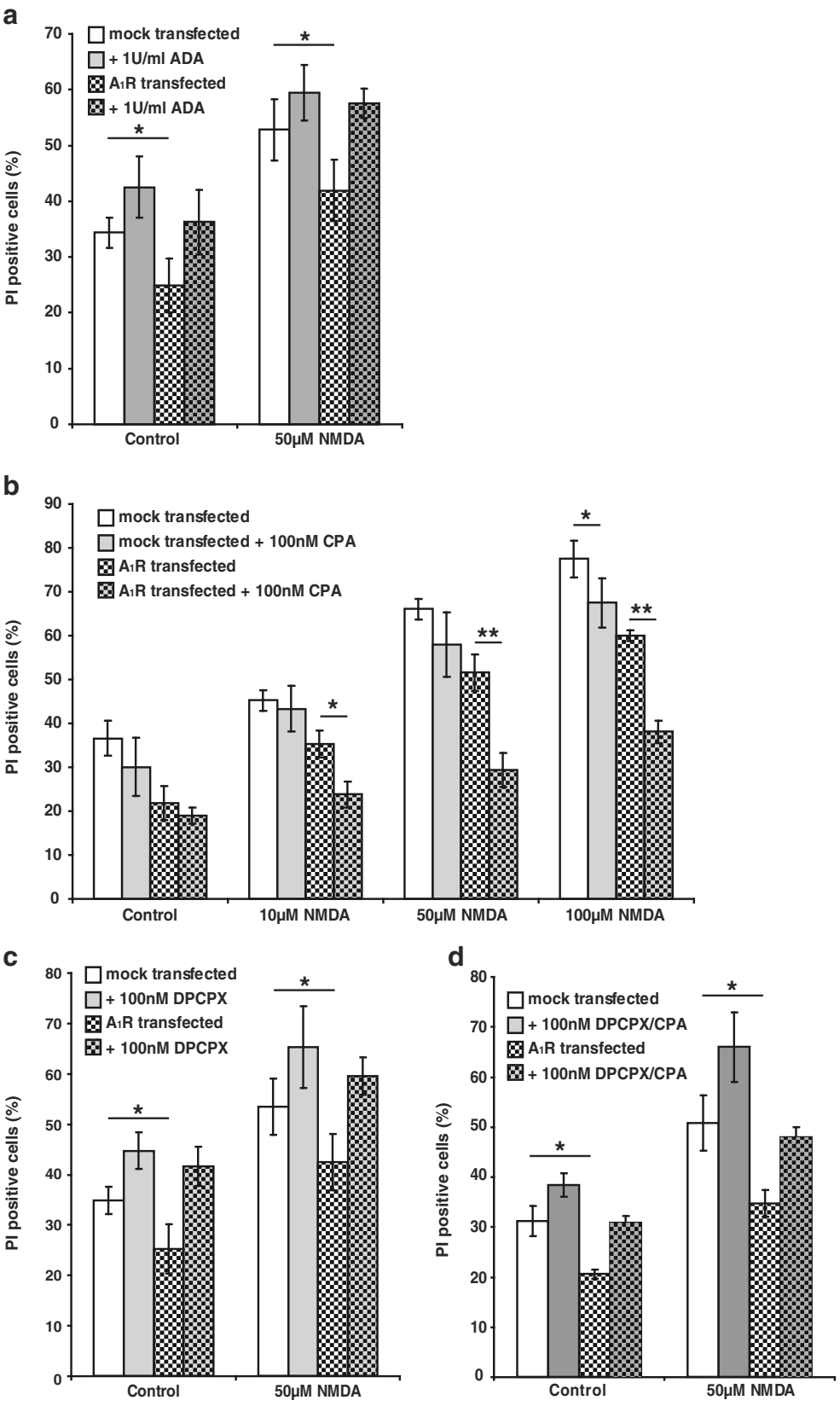


**Fig. 5** Effect of ADA, CPA and DPCPX treatment on the $A_1R$ upregulation-mediated enhanced survival of transfected primary cortical neurons and after NMDA-induced excitotoxicity. The primary neuronal cultures were co-transfected with pTRE-GFP/mCherry-$A_1R$ ($A_1R$ transfected) or pTRE-GFP/mCherry (mock transfected) and pTet-Off transactivator-expressing plasmid in a 1:2 ratio. After 48 h in vitro, the neurons were pre-incubated with 1 U/ml ADA for 1 h (**a**), 100 nM CPA for 15 min (**b**), 100 nM DPCPX for 15 min (**c**) or 100 nM DPCPX for 15 min, followed by 100 nM CPA for 15 min (**d**), and were subsequently challenged with NMDA (10, 50 and 100 μM) for 1 h. Neuronal survival was assessed 18 h following the NMDA treatment by immunocytochemistry using PI staining of the dead cell nuclei. Percentage of the PI-stained nuclei of the GFP/mCherry-expressing cells vs. the total number of GFP/mCherry-positive cells (±SEM; $n=3$; *$p<0.05$, **$p<0.01$, Student's $t$ test)

regulated expression system with a bidirectional promoter, directing the simultaneous expression of mouse $A_1R$ and GFP/mCherry reporter genes. The system was initially tested in transiently transfected HEK293 cells, which were chosen

due to their high transfection efficacy and low expression of endogenous $A_1R$. We have previously cloned the mouse $A_1R$ [16]. In the present study, we subcloned the mouse $A_1R$ cDNA into the Clontech pTRE-tight-BI-AcGFP/mCherry tetracycline-inducible bidirectional expression vector. Thus, the expression of the reporter gene AcGFP/mCherry, which encodes the green/red fluorescent protein, simultaneously with $A_1R$ is a suitable control for the transfection and expression efficacy and its TRE promoter allows conditional regulation. It is shown here that this system allowed both $A_1R$ and reporter gene expression to be tightly regulated by the tetracycline analogue doxycycline. The fact that only 50 % of $A_1R$-positive cells also showed a fluorescent signal for GFP or mCherry (data not shown) is most likely due to the fact that the fluorescent signals are quenched by fixation. Compared to the widely used pcDNA3 vector with its CMV promoter that drives $A_1R$ expression about threefold higher in HEK293 cells, our tetracycline-regulated expression system reached lower levels of about twofold upregulation of $A_1R$ expression, which was more comparable to what has been achieved by pharmacological means in cultured neurons or in vivo [14].

The mouse $A_1R$, encoded by the cDNA used in the present study, has been characterized previously using functional studies and radioligand binding assays [16]. Since several lines of evidence indicate that the $A_1R$ inhibits multiple voltage-gated $Ca^{2+}$ channel subtypes in neurons [20, 21, 32, 33], we performed whole-cell patch clamp recordings to assess the function of the transfected $A_1R$ construct. HEK293 cells express endogenous voltage-dependent $Ca^{2+}$ channels which differ in their pharmacological properties from classical N- and L-type channels and are functionally regulated by $G_{i/o}$ proteins [22, 23]. We demonstrated that upon selective agonist activation, the expressed $A_1R$ inhibits endogenous voltage-gated $Ca^{2+}$ channels specifically in $A_1R$-transfected HEK293 cells showing that the expressed $A_1R$ is functional and corroborating earlier findings [16].

To investigate the role of $A_1R$ upregulation in neuronal survival, we transfected primary cortical cultures with our system, maintained them in the absence of doxycycline and challenge them with various concentration of NMDA. We show here that primary neurons with upregulated $A_1R$ expression display significantly enhanced survival against NMDA-induced excitotoxicity. Further pharmacological studies, using the selective $A_1R$ agonist CPA and antagonist DPCPX, confirmed that the observed enhanced neuroprotection is specifically mediated by the upregulated expression of the $A_1R$.

The $Ca^{2+}$-phosphate transfection method [17], which we used in primary neurons, is a stressful event that caused significant neuronal loss compared to non-transfected cultures. Thus, mock-transfected neurons exhibited more cell death than non-transfected cultures. However, the sensitivity of the neurons against NMDA was unchanged, since the concentration-dependent increase in NMDA-dependent neuronal death had the same slope in non-transfected neurons as compared to mock-transfected cells. In contrast to the mock-transfected neurons, those with upregulated $A_1R$ expression did not differ in neuronal death from non-transfected cells. Furthermore, the impact of NMDA challenge in these neurons was less severe since the concentration-dependent increase of NMDA-induced neuronal death was less steep than in neurons without upregulated $A_1R$ expression. Thus, the presented results clearly show that an increased $A_1R$ expression is protective for neurons, not only under excitotoxic conditions but also in other neuronal stressors, like the transfection and culture procedures.

It is long known that energy imbalance causes a rapid release of adenosine in brain tissue (see for review [9]). The protective function of $A_1R$ upregulation is completely abolished in the presence of ADA or DPCPX, suggesting that also cultured cortical neurons release sufficient amounts of adenosine to stimulate protective $A_1R$. Since there was no effect of either ADA or DPCPX treatment in mock-transfected cells, it is indicated that the receptor expression level in mock-transfected neurons is not high enough for the released adenosine to be neuroprotective. These results are therefore in agreement with the long standing notion that the agonistic potency of adenosine strongly depends on the density of receptors in the cellular membrane (see for review [9, 12]). Thus, two basal properties of adenosine (release by cellular stress and receptor density-dependent effects) are also reflected in our cell culture system. However, the released adenosine most likely did not reach high enough concentrations to fully activate the $A_1R$ response, an assumption based on the observation that an additional treatment with the $A_1R$-specific agonist CPA strongly enhanced neuroprotection in neurons with upregulated $A_1R$ expression. It should be noted here that even at very high concentrations of NMDA, the stimulation with 100 nM CPA almost completely blocked excitotoxicity when $A_1R$ is upregulated, confirming the powerful protection that can be achieved by $A_1R$ upregulation.

In summary, we have generated and functionally tested a tetracycline-regulated expression system, which allows the simultaneous expression of the mouse $A_1R$ and GFP/mCherry reporter genes. The presented data provide for the first time direct evidence that the upregulation of $A_1R$ expression enhances the neuronal survival. The conditionally regulated $A_1R$ expression system is a useful tool for the generation of transgenic animals, where the role of $A_1R$ upregulation can be further investigated in vivo.

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