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RESEARCH PAPER

The adenosine A_{2A} receptor antagonist ZM241385 enhances neuronal survival after oxygen-glucose deprivation in rat CA1 hippocampal slices

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Background and purpose: Activation of adenosine A_{2A} receptors in the CA1 region of rat hippocampal slices during oxygen-glucose deprivation (OGD), a model of cerebral ischaemia, was investigated.
Experimental approach: We made extracellular recordings of CA1 field excitatory postsynaptic potentials (fepsps) followed by histochemical and immunohistochemical techniques coupled to Western blots.
Key results: OGD (7 or 30 min duration) elicited an irreversible loss of fepsps invariably followed by the appearance of anoxic depolarization (AD), an unambiguous sign of neuronal damage. The application of the selective adenosine A_{2A} receptor antagonist, ZM241385 (4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-ylamino]ethyl)phenol; 100–500 nmol·L^{-1}) prevented or delayed AD appearance induced by 7 or 30 min OGD and protected from the irreversible fepsp depression elicited by 7 min OGD. Two different selective adenosine A_{2A} receptor antagonists, SCH58261 and SCH442416, were less effective than ZM241385 during 7 min OGD. The extent of CA1 cell injury was assessed 3 h after the end of 7 min OGD by propidium iodide. Substantial CA1 pyramidal neuronal damage occurred in untreated slices, exposed to OGD, whereas injury was significantly prevented by 100 nmol·L^{-1} ZM241385. Glial fibrillary acid protein (GFAP) immunostaining showed that 3 h after 7 min OGD, astrogliosis was appreciable. Western blot analysis indicated an increase in GFAP 30 kDa fragment which was significantly reduced by treatment with 100 nmol·L^{-1} ZM241385.
Conclusions and implications: In the CA1 hippocampus, antagonism of A_{2A} adenosine receptors by ZM241385 was protective during OGD (a model of cerebral ischaemia) by delaying AD appearance, decreasing astrocyte activation and improving neuronal survival.
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Keywords: adenosine A_{2A} receptors; synaptic potentials; anoxic depolarization; cerebral ischaemia; propidium iodide; GFAP; reactive astrocytes; OGD; Western blot; immunohistochemistry

Abbreviations: aCSF, artificial cerebrospinal fluid; AD, anoxic depolarization; CGS21680, 2-(4-[2-carboxyethyl]-phenethylamino)adenosine-50-N-ethyluronamide; d.c., direct current; DMSO, dimethyl sulphoxide; GFAP, glial fibrillary acid protein; OGD, oxygen-glucose deprivation; ROI, region of interest; SCH58261, 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine; SCH442416, 5-amino-2-(2-furyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine; ZM241385, 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-ylamino]ethyl)phenol

Introduction

Adenosine acts as a modulator in the brain activating four receptor subtypes, A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 2001), all of which are expressed in the brain (Dixon *et al.*, 1996). One of the early events occurring during oxygen-glucose deprivation (OGD) caused by an ischaemic episode is the release of substantial amounts of adenosine (Latini *et al.*, 1998; Latini and Pedata, 2001) and evidence suggests that A_1, A_{2A} and A_3 receptors play a role in this pathological event (Pedata *et al.*, 2007). The protective role of adenosine is mainly attributed to a well-established inhibitory tone on synaptic transmission through activation of the A_1 receptor subtype (Latini and Pedata, 2001). Unfortunately, the development of A_1 selective agonists as possible anti-ischaemic drugs has been hindered by cardiovascular side effects, including sedation, bradycardia

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and hypotension (White *et al.*, 1996). Therefore, research is now focussing on the contribution of the other adenosine receptors, in order to identify putative targets for therapeutic intervention. Although in cerebral ischaemia the role of A_3 and A_{2A} receptors is still controversial (Jacobson and Gao, 2006), most evidence suggests a deleterious effect of adenosine A_{2A} receptor stimulation (Chen *et al.*, 2007). The selective block of this receptor is protective in different *in vivo* models of cerebral ischaemia (Gao and Phillis, 1994; von Lubitz *et al.*, 1995; Bona *et al.*, 1997; Monopoli *et al.*, 1998; Melani *et al.*, 2003; 2006) and the brain damage induced by transient focal ischaemia is attenuated in A_{2A} knockout mice (Chen *et al.*, 1999). A major protective effect of A_{2A} receptor antagonists in stroke has been attributed to reduced glutamate outflow from neurons (Marcoli *et al.*, 2003; Pedata *et al.*, 2003; 2005) and therefore, to reduced excitotoxic damage.

Adenosine A_{2A} receptors are present in the hippocampus, albeit with a considerably lower density than in the striatum (Lopes *et al.*, 2002; Rebola *et al.*, 2005a), as suggested by *in situ* hybridization and RT-PCR studies (Cunha *et al.*, 1994; Dixon *et al.*, 1996) and further confirmed by immunohistochemistry studies (Rosin *et al.*, 1998). In rat hippocampus, adenosine A_{2A} receptors are localized on nerve terminals (Rebola *et al.*, 2005b) where they facilitate excitatory neurotransmission (Cunha *et al.*, 1997; Lopes *et al.*, 2002) but also at postsynaptic sites (Rebola *et al.*, 2005a,b) in particular at CA3 synapses (Rebola *et al.*, 2008).

In agreement with the permissive role of the adenosine A_{2A} receptor on excitatory transmission, we have already demonstrated that selective stimulation of A_{2A} receptors reduced the synaptic depression brought about by an episode of 2 min OGD, to mimic ischaemia *in vivo*, in rat hippocampal slices (Latini *et al.*, 1999).

In the present study we investigated the effects of an adenosine selective A_{2A} receptor agonist and several selective adenosine A_{2A} receptor antagonists in the CA1 region of acute rat hippocampal slices under severe (7- or 30-min) OGD episodes. These OGD episodes bring about irreversible depression of neurotransmission and the appearance of anoxic depolarization (AD) (Pugliese *et al.*, 2006; 2007). AD is a severe neuronal depolarization, which is an early and critical event that has been demonstrated both *in vivo* (Somjen, 2001) and *in vitro* (Tanaka *et al.*, 1997; Pugliese *et al.*, 2006). AD is believed to trigger a variety of molecular events which contribute to cell death and represents an unequivocal sign of neuronal injury (Somjen, 2001). The amount of time spent by neurons in AD is an important determinant of neuron fate. Propagation of AD from the ischaemic core is one major factor contributing to neuronal death in the area surrounding the ischaemic core (the penumbra) (Koroleva and Bures, 1996). This penumbra constitutes potentially salvageable tissue and hence a pharmacological treatment that delayed the onset of AD would help to protect brain tissue after ischaemia (Jarvis *et al.*, 2001; Somjen, 2001).

In order to characterize the OGD-induced cell injury and any pharmacological protection, the extent of neuronal damage and glial (astrocytic) response was assessed by propidium iodide (PI) staining and immunohistochemical determination coupled to Western blot.

Our results indicate that adenosine A_{2A} receptor antagonism is protective against AD propagation and neuronal damage.

Methods

Slice preparation

All animal procedures were conducted according to the European Community Guidelines for Animal Care, DL 116/92, application of the European Communities Council Directive (86/609/EEC). Experiments were carried out on acute hippocampal slices (Pugliese *et al.*, 2006), prepared from male Wistar rats (Harlan, Udine, Italy, 150–200 g body weight). Animals were killed with a guillotine under ether anaesthesia and their hippocampi were rapidly removed and placed in ice-cold oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (aCSF) of the following composition (mmol·L^{-1}): NaCl 125, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, $NaHCO_3$ 25 and D-glucose 10. Slices of hippocampi (400 μm thick) were prepared using a McIlwain tissue chopper (The Mickle Lab. Engineering, Co. Ltd., Gomshall, UK) and kept in oxygenated aCSF for at least 1 h at room temperature. A single slice was then placed on a nylon mesh, completely submerged in a small chamber (0.8 mL) and superfused with oxygenated aCSF (31–32°C) at a constant flow rate of 1.5–1.8 mL min^{-1}. Changes in superfusing solutions (OGD or drugs) reached the preparation in 60 s and this delay was taken into account in our calculations.

Extracellular recording

Test pulses (80 μs, 0.066 Hz) were delivered through a bipolar nichrome electrode positioned in the stratum radiatum. Evoked extracellular potentials were recorded with glass microelectrodes (2–10 MΩ, Harvard Apparatus LTD, Edenbridge, UK) filled with 150 mmol·L^{-1} NaCl and placed in the CA1 region of the stratum radiatum. Responses were amplified (BM 622, Mangoni, Pisa, Italy), digitized (sample rate, 33.33 kHz), and stored for later analysis with LTP (version 2.30D) program (Anderson and Collingridge, 2001). Stimulus-response curves were obtained by gradual increases in stimulus strength at the beginning of each experiment, when a stable baseline of evoked response was reached. The test stimulus pulse was then adjusted to produce a field excitatory postsynaptic potential (fepsp) whose slope and amplitude was 40–50% of the maximum and was kept constant throughout the experiment. The fepsp amplitude was routinely measured and expressed as the percentage of the average amplitude of the potentials measured during the 5 min preceding exposure of the hippocampal slices to OGD. In some experiments, both the amplitude and initial fepsp slope were quantified, but because no appreciable differences between these two parameters were observed in drug effect and OGD, only the amplitude measurement is expressed in the figures. Simultaneously with fepsp amplitude, AD was recorded as negative extracellular direct current (d.c.) shifts induced by 7 or 30 min of OGD. The d.c. potential is an extracellular recording considered to provide an index of the polarization of cells surrounding the tip of the glass electrode (Farkas *et al.*, 2008).

Application of OGD and drugs

Conditions of OGD were obtained by superfusing the slice with aCSF without glucose and gassed with nitrogen (95% N_2–5% CO_2) (Pedata *et al.*, 1993). This caused a drop in pO_2 in the recording chamber from ~500 mmHg (normoxia) to a range of 35–75 mmHg (after 7 min OGD) (Pugliese *et al.*, 2003). At the end of the ischaemic period, the slice was again superfused with normal, glucose-containing, oxygenated aCSF. Throughout this paper, the terms 'untreated OGD slices' or 'treated OGD slices' refer to hippocampal slices in which OGD episodes of different duration were applied in the absence or in the presence of drugs, respectively. Control slices were not subjected to OGD or drug treatment, but were incubated in oxygenated aCSF for identical time intervals.

All the selective adenosine A_{2A} receptors antagonists, ZM241385, SCH58261 and SCH442416 were applied 15 min before, during and 5 min after OGD for 7 or 30 min. A concentration of 100 nmol·L^{-1} ZM241385 was used and was chosen according to published data (Latini *et al.*, 1999; Lopes *et al.*, 2002; Pugliese *et al.*, 2003). At this concentration ZM241385 was still selective for rat A_{2A} adenosine receptors, but at a concentration which exceeded its K_i value as determined by binding experiments (Fredholm *et al.*, 2001). Likewise, the concentrations of 50 or 100 nmol·L^{-1} SCH58261 were also chosen according to published data (Fontinha *et al.*, 2008; Rebola *et al.*, 2008). At both these concentrations the compound was still selective for rat A_{2A} adenosine receptors, but at concentrations which exceeded their K_i values as determined by binding experiments in rat brain tissue (Zocchi *et al.*, 1996). Similarly, the concentration of 5 nmol·L^{-1} of SCH442416 used in our experiments is selective for rat A_{2A} adenosine receptors, but again exceeds its K_i value as determined by binding experiments in rat striatal membranes (Todde *et al.*, 2000). The selective adenosine A_{2A} receptor agonist, CGS21680, was applied during OGD. The concentration of CGS21680 (50 nmol·L^{-1}) used in our experiment was chosen on the basis of Ki values obtained in the rat (Fredholm *et al.*, 2001) and according to published data (Latini *et al.*, 1999; Rebola *et al.*, 2003; Tebano *et al.*, 2005). The nomenclature of all drugs used in this study conforms to the BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Propidium iodide staining

Propidium iodide (PI) is a fluorescent marker of cell death: it only enters cells with disrupted plasma membranes. Once inside the cells, PI binds to nucleic acids and induces intense red fluorescence (620 nm) when excited by green light (543 nm).

After the extracellular recording period, each slice (untreated or treated OGD) was maintained in a separate chamber in oxygenated aCSF at room temperature for 3 h from the end of OGD as described by Yin *et al.* (2002). PI (5 μg·mL^{-1}) was added directly in the aCSF during the last 60 min of incubation. Control slices underwent the same staining protocol. After PI staining, each slice was washed for several minutes with oxygenated aCSF and fixed overnight in 1 mL of 4% paraformaldehyde at 4°C. After extensive washings with phosphate buffered saline (PBS) solution, each slice was mounted onto a gelatin-coated slide for microscopic examination using Vectashield (Vectastain, Vector Laboratories, Burlingame, CA, USA) as a mounting medium. In order to avoid the pressure on the slice by the coverslips and the consequent flattening of the slice we used specific (400 μm thick) adhesive cover-wells as glass separators (Grace Bio-labs, Bend, OR, USA). All these experimental procedures were carried out in the dark. The fluorescent images of CA1 pyramidal neurons by PI staining were captured with a Leica SP2-AOBS confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany), through a 20× NA 0.5 objective. Images were acquired as Z-stacks of the entire slice depth (400 μm), sampling at 2 μm intervals (voxel size: 0.7 × 0.7 × 2 μm). Two acquisitions were averaged for each optical section. For quantitative analysis of CA1 fluorescence, the intensity was always estimated in the central portion of the slice (from about 190/210 μm depth where injury resulting from the trauma of slicing is minimized). Each section examined (named region of interest, ROI) had the following dimension: 600 × 200 × 20 μm. In these slices, the more external layers exhibited intense PI staining as a result of neuronal damage produced during slice cutting and incubation. Densitometric analysis was conducted measuring the average gray value in each ROI using Image-J software (NIH, http://rsb.info.nih.gov/ij/). Data were normalized to the average value found in control slices in each experiment. The images were then assembled into montages using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA).

Immunohistochemical assay

After the extracellular recording period, slices were maintained in separate chambers for 3 h from the end of OGD in oxygenated aCSF at room temperature. After this time, we studied the astrocytic response to OGD using immunohistochemical staining. Slices (400 μm) were fixed overnight using 1 mL of 4% ice-cold paraformaldehyde and cryoprotected in an 18% sucrose solution for at least 48 h. Then, the slices were included in a 4% agar solution prepared in 18% sucrose solution, chilled and preserved at 4°C overnight. The following day, slices in agar were frozen and maintained at –80°C for at least 4 h and finally were re-sliced into 20-μm thick slices with a cryostat. The more superficial slices were excluded while those obtained from the inner part were stored in 1 mL of antifreeze solution at –20°C until immunohistochemical assay.

For staining the 20-μm thick slices were mounted on to microscope slides and washed with PBS solution. Then, slices were incubated with PBS-0.3% Triton X-100 (PBS-TX) for 30 min, washed with PBS solution and incubated overnight at room temperature with the primary antibody [rabbit polyclonal antibody against glial fibrillary acid protein (GFAP), 1:700, DakoCytomation, Glostrup Denmark or mouse monoclonal antibody against microglia CD11b/c epitope, OX-42, 1:400, PharMingen International, Franklin Lakes, USA] dissolved in blocking buffer I (5 mg·mL^{-1} of Bovine Serum Albumin/PBS-TX).

The following day, the primary antibody was removed, slices were washed several times with PBS solution and incubated in the dark at room temperature with the fluorescent secondary antibody [fluorescein (FITC)-conjugated goat anti-rabbit IgG,

1:400, Vector laboratories, Burlingame, CA, USA or texas red-coniugated goat anti-mouse IgG, 1:400, Vector laboratories, Burlingame, CA, USA] dissolved in blocking buffer II (1 mg·mL^{-1} of bovine serum albumin/PBS-TX). From this step, all procedures were carried out in the dark. After 2 h of incubation, the secondary antibody was removed and slices were washed with PBS solution. Finally, slices were dried and coverslips were mounted using Vectshield mounting medium.

Images were then acquired (40×, NA: 1.25) at 0.28-μm interval for the entire slice depth (20 μm) by a confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany), using laser excitation at 488 nm.

GFAP Western blot

After the extracellular recording period, slices were maintained in separate chambers for 3 h from the end of OGD in oxygenated aCSF at room temperature. After this time, each slice was placed on a glass slide kept on dry ice and quickly frozen. Thereafter, using a small scalpel the CA1 region was dissected and then stored in an Eppendorf tube at –80°C until required. Care was taken to ensure that the slices remained frozen throughout the procedure. Each sample was homogenized in 60 μL of ice-cold RIPA buffer on ice directly into the Eppendorf tube. RIPA buffer had the following composition: Igepal 1:100; Na-deoxycholate 0.5%; sodium dodecyl sulphate (SDS, Sigma, St Louis, Missouri) 0.1%; one tablet of protein inhibitors (Sigma, St Louis, Missouri); sodium pyrophosphate 2 mmol·L^{-1}; paranitrophenylphosphate 4 mmol·L^{-1}; sodium orthovanadate 1 mmol·L^{-1}. Proteins were quantified in 4 μL of each sample using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). Samples were boiled for 5 min in an appropriate volume of 6× loading buffer and loaded (30 μg of proteins per well) into a 10% SDS-PAGE gel and run at 120 mV for 90 min at 4°C. The proteins were then transferred electrophoretically onto nitrocellulose membranes (Hybond-C extra; Amersham, Arlington Heights, IL, USA) using a transfer tank kept at 4°C, with typical parameters being overnight with a constant current of 10 mA. Membranes were blocked for 1 h at room temperature with blocking buffer (5% non-fat dry milk in tris buffered saline, 0.1%TWEEN-20, TBS-T), then probed using a primary antibody anti-GFAP (1:60 000; DakoCytomation, Glostrup, Denmark) overnight at 4°C. After washing in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:10 000; Pierce, Biotechnology, Rockford, IL, USA) 2 h at room temperature and proteins were visualized using chemiluminescence (ECL, Pierce Biotechnology, Rockford, IL, USA). In order to normalize the values of GFAP we detected β-actin in the same Western blot run. Membranes were stripped by strong agitation with 0.2 N NaOH (10 min, room temperature), blocked in blocking buffer for 1 h at room temperature and probed for 2 h at room temperature with anti-β-actin antibody (1:5000; Sigma, St Louis, MO). Membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:7500; Pierce, Biotechnology, Rockford, IL, USA) and developed as above. The bands were acquired as TIFF files and the densitometric analysis of the bands was quantified by Image J software. GFAP values were expressed as percentage of β-actin run in the same Western blot analysis.

Statistics

Data were analysed using Prism 3.02 software (Graphpad Software, San Diego, CA, USA). All numerical data are expressed as the mean ± SEM. Data were tested for statistical significance with the unpaired two-tailed Student's *t*-test or by analysis of variance (one-way ANOVA), as appropriate. When significant differences were observed, the Newman-Keuls multiple comparison test was done. A value of $P < 0.05$ was considered significant.

Materials

ZM241385 (4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-ylamino] ethyl)phenol), SCH58261 (5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-e]-1,2,4-triazolo[1,5c]pyrimidine), SCH442416 (5-amino-2-(2-furyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine) and CGS21680 (2-(4-[2-carboxyethyl]-phenethylamino)adenosine-50-N-ethyluronamide) were purchased from Tocris (Bristol, UK). All the compounds were made up to a 10 mmol·L^{-1} stock solution in dimethyl sulphoxide (DMSO). Aliquots of these stock solutions were stored at –20° and diluted again in DMSO up to 10–100 μmol·L^{-1} before the last dilution in aCSF. The final concentration of DMSO (0.05% and 0.1% in aCSF) used in our experiments did not affect either fepsp amplitude or the depression of synaptic potentials induced by OGD (not shown). PI was purchased from Sigma (Sigma-Aldrich, Italy).

Results

ZM241385, a selective adenosine A_{2A} receptor antagonist, prevented the appearance of AD and the irreversible loss of neurotransmission induced by 7-min OGD

Evoked field potentials were recorded from 197 slices taken from 71 rats to test the role of A_{2A} adenosine receptors under OGD and to record simultaneously the negative voltage shift, that is an electrophysiological signature of AD (Somjen, 2001).

In agreement with our previous results (Pugliese *et al*., 2006; 2007), a d.c. shift was always present when the OGD was 7 min long. The d.c. shift presented a mean latency of 6.5 ± 0.1 min (calculated from the beginning of OGD) and a mean peak amplitude of –7.1 ± 0.4 mV (n = 31) (Figure 1A). In addition, 7 min OGD completely abolished fepsps which did not recover their amplitude after return to oxygenated aCSF (Figure 1C). OGD was then applied in the presence of the selective adenosine A_{2A} receptor antagonist ZM241385 (100 nmol·L^{-1}, n = 25). During 7 min OGD, the A_{2A} receptor antagonist prevented the appearance of AD in 23 out of 25 slices tested (Figure 1B). In the remaining two slices a delay in AD latency was recorded (AD latency: 8.25 and 7.75 min; peak amplitude: –6.32 and –12.58 mV respectively, Figure 1B). In addition, there was a significant recovery of fepsps in the presence of ZM241385 (94.4 ± 5.0%, n = 25, $P < 0.001$, one-way ANOVA, Newman-Keuls, Figure 1C) compared with that found in untreated OGD slices (5.1 ± 2.0%, n = 31, Figure 1C). These effects of ZM241385 were evident in 81% of the slices. In six out of 31 slices treated with

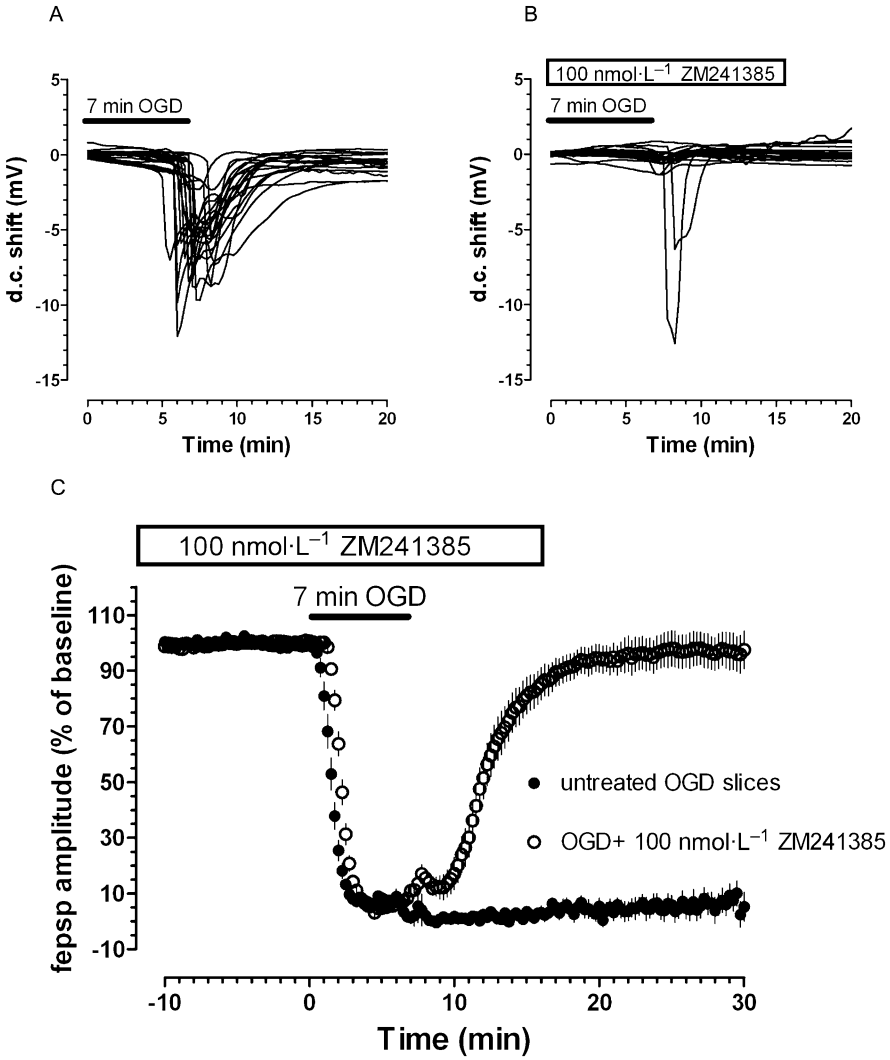

Figure 1 The selective adenosine A_{2A} receptor antagonist, ZM241385, prevented the appearance of AD and the irreversible loss of neurotransmission induced by 7 min OGD in rat hippocampal slices. AD was recorded as the negative d.c. shift in response to 7 min OGD in the absence (A, $n = 31$) and in the presence (B, $n = 25$) of 100 nmol·L^{-1} ZM241385. Note that the A_{2A} adenosine receptor antagonist prevented the appearance of AD in 23 out of 25 slices and delayed it in the remaining two slices. (C) Graph shows the time course of 7 min OGD effects on fepsp amplitude in untreated (mean ± SEM, $n = 31$) OGD slices and in 100 nmol·L^{-1} ZM241385 (mean ± SEM, $n = 25$) treated OGD slices. Amplitude of fepsps is expressed as per cent of baseline. Note that, after reperfusion in normal oxygenated standard solution, a significant recovery of fepsp was found only in ZM241385 treated OGD slices.

ZM241385, no effect on AD development and fepsp recovery after 7 min OGD was observed. The compound did not change fepsp amplitude under normoxic conditions (from 1.1 ± 0.03 mV to 1.0 ± 0.13 mV, $n = 31$).

A further series of experiments was conducted using other two different selective A_{2A} adenosine receptor antagonists: SCH58261 and SCH442416. Both compounds were effective in preventing AD appearance and the irreversible loss of synaptic activity induced by 7 min OGD in 25% of the respective slices tested. In particular, SCH58261 (50 nmol·L^{-1}, $n = 8$) was able to prevent appearance of AD in two out of eight slices tested. In these two slices, a complete fepsp recovery after 7 min OGD was found (not shown). In the remaining six slices the AD latency in the presence of 50 nmol·L^{-1} SCH58261 was of 6.5 ± 0.2 min, a value not different from that observed in respective untreated OGD slices (AD latency: 5.7 ± 0.3 min, $n = 5$). No difference in the amplitude of AD was found between the OGD-untreated slices and SCH58261 treated slices (from –7.6 ± 1.4 mV to –8.2 ± 0.7 mV respectively).

In the presence of SCH442416 (5 nmol·L^{-1}, $n = 4$) AD, elicited by 7 min OGD, was recorded in three out of four slices tested. In these three slices the AD latency was 7.1 ± 0.1 min, a value not different from that observed in respective untreated OGD slices (6.3 ± 0.4 min, $n = 5$). No differences in the amplitude of AD was found between the OGD-untreated slices and SCH442416-treated slices (from –7.7 ± 1.3 mV to –8.6 ± 1.4 mV respectively). In the remaining slice no AD was detected and a complete fepsp recovery was recorded. Similar to effects previously observed with ZM241385, both SCH58261 and SCH442416, *per se*, did not change fepsps amplitude before OGD application (data not shown). Thus, all the A_{2A} adenosine receptor antagonists tested did not modify low frequency-induced synaptic transmission under

normoxia, in agreement with previous observations (Latini *et al.*, 1999; Sperlagh *et al.*, 2007; Cunha, 2008; Fontinha *et al.*, 2008).

ZM241385 significantly delayed the appearance of AD induced by 30-min OGD

In order to characterize the role of adenosine A_{2A} receptors on AD development, we prolonged the duration of the OGD from 7 to 30 min; the longer duration of OGD is invariably associated with tissue damage (Pearson *et al.*, 2006; Pugliese *et al.*, 2006). We compared the time of the appearance and the magnitude of depolarizing d.c. shift, in the absence or in the presence of different A_{2A} adenosine receptor antagonists (Figure 2). As illustrated in Figure 2A, 30 min OGD elicited the appearance of AD in untreated OGD slices, with a mean peak amplitude of –8.4 ± 0.4 mV ($n = 11$) and a mean latency shown in Figure 2C. When OGD was applied in the presence of 100 nmol·L^{-1} ZM241385, the d.c. shifts were always delayed (Figure 2B; mean values in Figure 2C) although the AD amplitude (–7.0 ± 0.5 mV, $n = 11$) was not significantly changed. The effect of ZM241385 on AD development induced by 30 min OGD was concentration-dependent in the range of 0.1–500 nmol·L^{-1} (Figure 2C and D). The A_{2A} adenosine receptor antagonist was ineffective at a concentrations of 0.1 and 1 nmol·L^{-1}, while a significant effect started from 100 nmol·L^{-1}. No significant further increase in the effect of ZM241385 was observed at 500 nmol·L^{-1} (Figure 2C and D). The apparent calculated EC_{50} value was 115 nmol·L^{-1} (95% confidence limit: 17–914 nmol·L^{-1}). Concentrations of ZM241385 higher than 500 nmol·L^{-1} were not used as this compound can exert non-specific effects not associated with interaction with the adenosine A_{2A} receptor subtype (Fredholm *et al.*, 2001). As shown in Figure 2C, no significant changes in AD latency was found when 30 min OGD was applied either in the presence of SCH58261 (50 nmol·L^{-1}, 100 nmol·L^{-1}, 500 nmol·L^{-1}) or 5 nmol·L^{-1} SCH442416, compared with that obtained in OGD-untreated slices. Finally, no difference in AD amplitude among all experimental groups was found (data not shown).

The selective adenosine A_{2A} receptor agonist, CGS21680, did not modify the AD latency during OGD

In order to elucidate the role of A_{2A} adenosine receptors in AD development and synaptic activity under OGD we performed

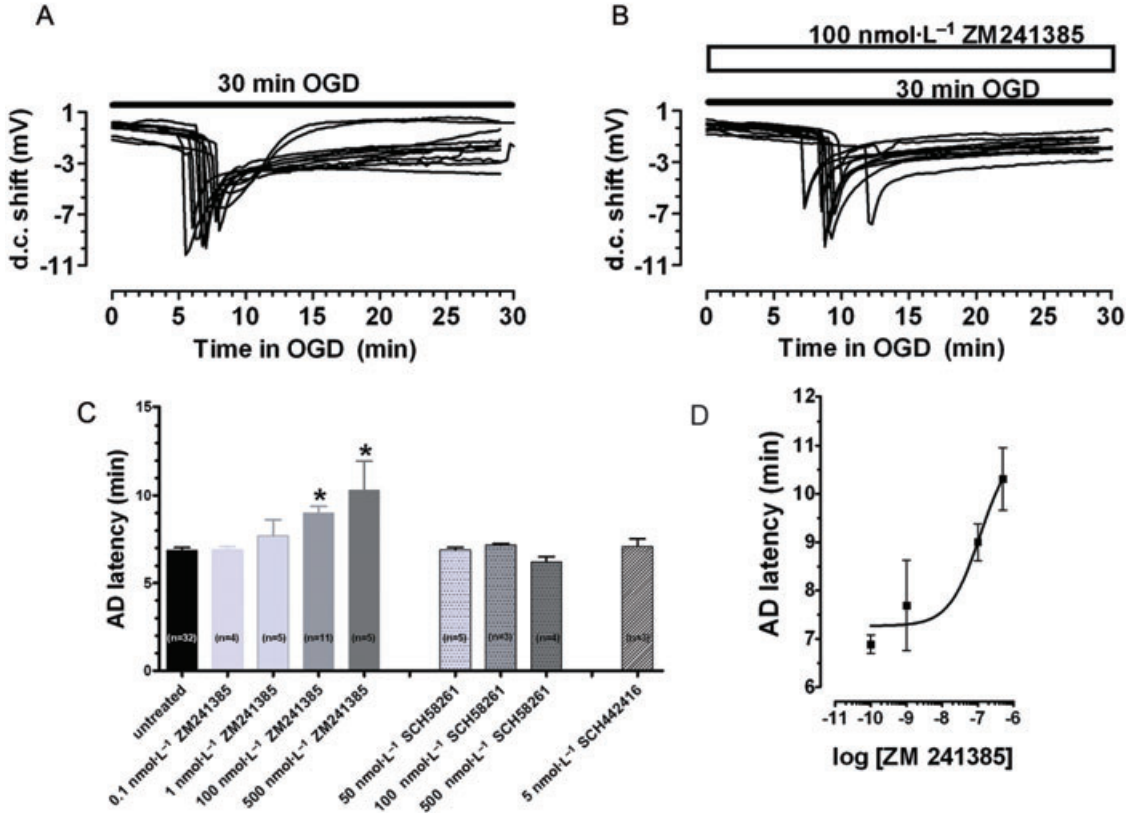

Figure 2 ZM241385 delayed the appearance of AD induced by 30 min OGD in rat hippocampal slices. (A) The graph shows the d.c. shift traces during 30 min OGD in untreated slices ($n = 11$). (B) The graph shows the d.c. shift traces during 30 min OGD carried out in the presence of 100 nmol·L^{-1} ZM241385 ($n = 11$). (C) Each column represents the mean ± SEM of AD latency recorded in hippocampal slices during 30 min OGD in different experimental groups. AD was measured from the beginning of OGD insult. Note that only ZM241385, among all the A_{2A} adenosine receptor antagonists, significantly delayed AD development. *$P < 0.01$, unpaired two-tailed Student's *t*-test in comparison to untreated OGD slices. The number (n) of slices tested is reported inside columns. (D) Concentration-response curve of ZM241385. The graph shows the curve of concentration-dependent effects of ZM241385 on AD latency during 30 min OGD recorded in hippocampal slices. Each value represents the mean ± SEM ($n = 5$ in 0.1 nmol·L^{-1}, $n = 5$ in 1 nmol·L^{-1}, $n = 11$ in 100 nmol·L^{-1}, $n = 5$ in 500 nmol·L^{-1}).

a set of experiments in which 7 or 30 min OGD were applied in the presence of the selective A_{2A} adenosine receptor agonist CGS21680 (50 nmol·L^{-1}). Surprisingly, the A_{2A} adenosine receptor agonist did not shorten AD latency during the application of 7 min OGD (6.12 ± 0.2 min in untreated OGD slices, n = 8 and 6.08 ± 0.2 min in 50 nmol·L^{-1} CGS21680-treated OGD slices, n = 9, Figure 3A). Similar results were obtained also when OGD was prolonged to 30 min (Figure 3C). After 7 min OGD carried out either in the absence or in the presence of CGS21680, no recovery of fepsp amplitude was observed when the slices were reperfused in oxygenated aCSF (Figure 3B).

ZM241385 allowed recovery of synaptic potentials up to 24 h after 7 min OGD

So far, there is no evidence that the loss of neurotransmission induced by the severe 7 min OGD in acutely isolated hippocampal slices can recover after incubation in oxygenated aCSF for longer than 80 min. Examples of fepsps recorded from typical experiments are shown in Figure 4. Hippocampal slices as thick as 400 μm remained viable for several hours after slicing and a steady fepsp could be monitored for up to 6 h (Figure 4A) or more (data not shown). However, even maintaining the slice in adequate experimental conditions, in most cases the slice showed a seizure-like activity with time. This phenomenon may be due to the formation of tissue-destructive oxygen radicals, which are unavoidably produced after prolonged exposition of the slices to a medium containing 95% O_2 (Pomper *et al.*, 2001; D'Agostino *et al.*, 2007). We found that a fepsp was still present at 24 h after slice cutting, although reduced in amplitude in comparison with that recorded at earlier time points (Figure 4A shows a representative fepsp; similar results were obtained in 14 other slices). In slices subjected to OGD no recovery of fepsp was recorded up to 24 h from the end of the insult (Figure 4B; similar results were obtained from 15 other slices). On the contrary, in the presence of ZM241385 a fepsp, similar to those observed in control slices, was recorded up to 24 h from the end of OGD (Figure 4C). Similar results were obtained in four out of the seven slices recorded; in the remaining three slices, fepsp potential was recorded up to 6–12 h. In the presence of 50 nmol·L^{-1} CGS21680 (Figure 4D), no recovery of fepsp after OGD was found at all recording times. Similar results were obtained from five other slices.

ZM241385 attenuated OGD-induced increase in PI uptake in the CA1 region of hippocampal slices

The protective effect of ZM241385 observed with the electrophysiological measurements was confirmed by histochemical experiments using the fluorescent cell-death marker PI, which preferentially accumulates in the nucleus of dying cells. Very little PI fluorescence was seen in the CA1 pyramidal cell layer under normoxic conditions (Figure 5A). Conversely, 7 min OGD induced an increase of PI fluorescence (Figure 5B) which was significantly attenuated in the presence of 100 nmol·L^{-1} ZM241385 (Figure 5C). Quantitative analysis of PI fluorescence values measured in the ROI (framed area of CA1 in Figure 5D) indicated that the treatment with ZM241385

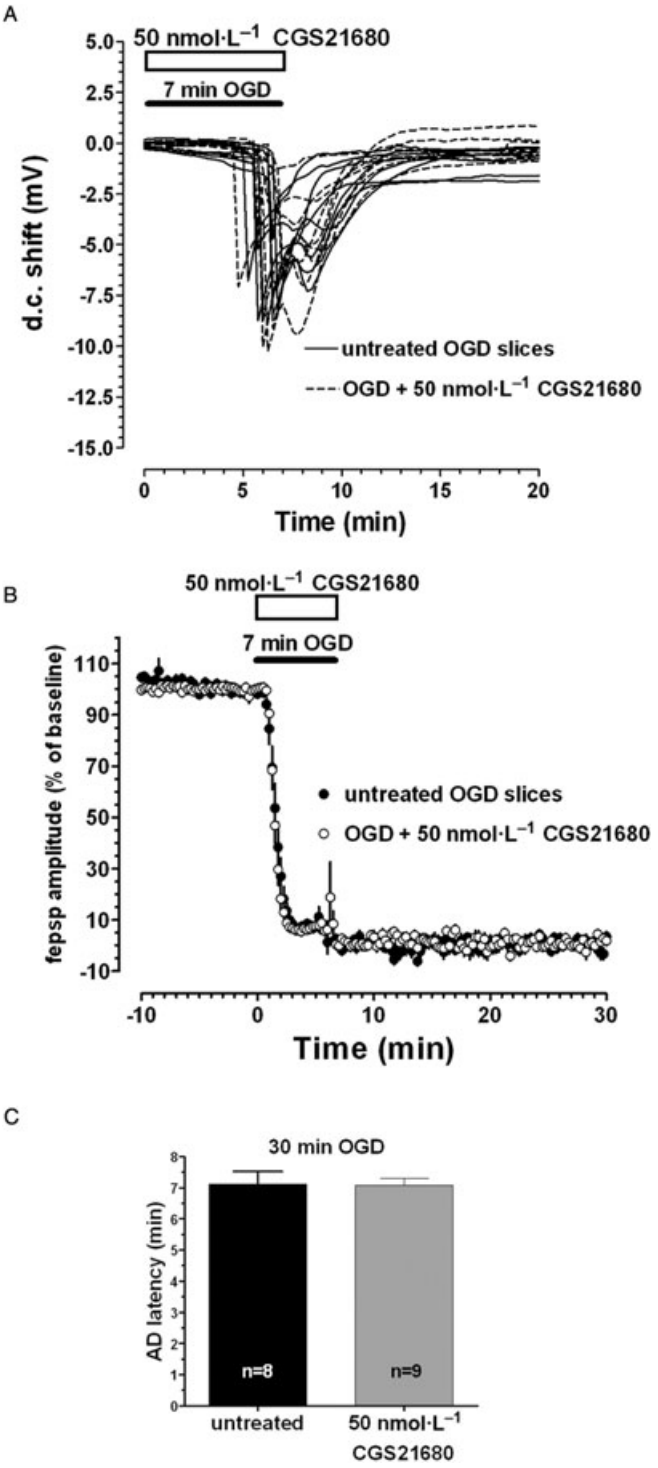

Figure 3 The selective A_{2A} receptor agonist, CGS21680, did not modify the AD latency during OGD. (A) Recordings of AD traces during 7 min OGD in the absence (n = 8) and in the presence of 50 nmol·L^{-1} CGS21680 (n = 9). (B) Time course of 7-min OGD effects on fepsp amplitude in untreated (mean ± SEM, n = 8) and in the presence of 50 nmol·L^{-1} CGS21680 (mean ± SEM, n = 9). (C) Each column represents the mean ± SEM of AD latency recorded during 30 min OGD application in untreated OGD slices and 50 nmol·L^{-1} CGS21680 treated OGD slices. The number (n) of slices tested is shown inside the columns.

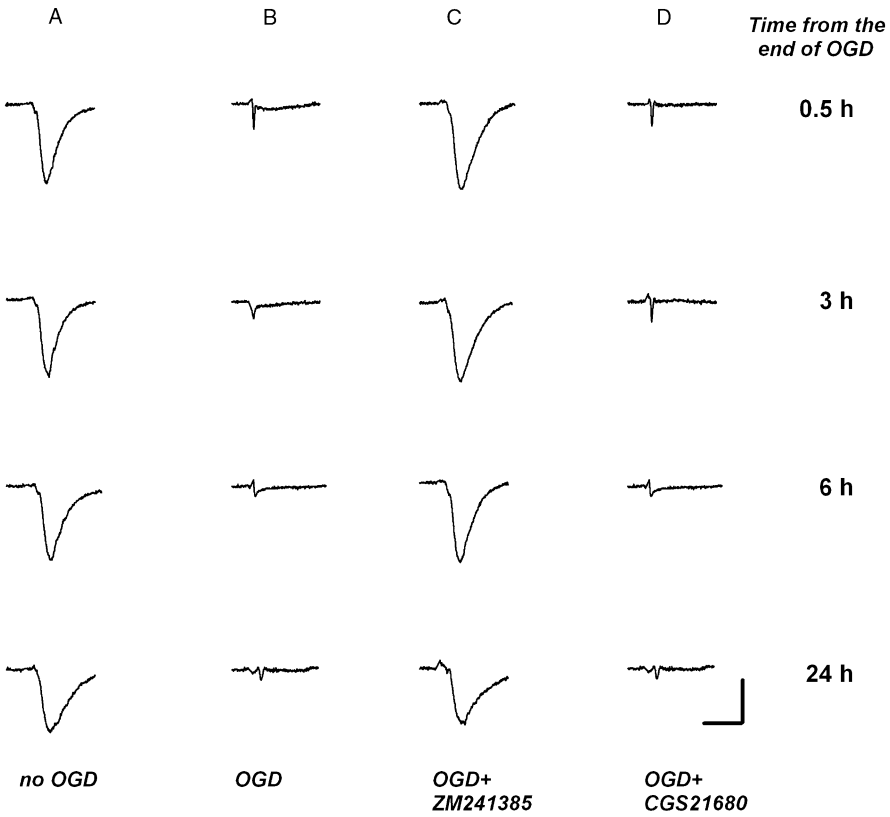

Figure 4 ZM241385 treated slices allowed recovery of synaptic potentials up to 24 h after 7-min OGD. (A) In control conditions a stable fepsp can be recorded for up to 24 h after cutting. After the end of OGD fepsps were recorded at different times in untreated OGD slice (B), in 100 nmol·L^{-1} ZM241385 treated OGD slice (C) and in 50 nmol·L^{-1} CGS21680 treated OGD slice (D). Each trace, taken from a typical experiment, represents the average of three fepsps recorded. Note that after OGD in untreated and CGS21680 treated slices only the afferent volley was recorded. Calibration: 0.5 mV, 10 ms.

significantly decreased the OGD-induced increase of PI fluorescence in CA1 pyramidal cells, indicative of reduced cell death, thus confirming the neuroprotective role of ZM241385 under OGD conditions (Figure 5E).

ZM241385 reduced activation of astrocytes in the stratum radiatum of CA1 after 7 min OGD

Data obtained with PI staining demonstrate that CA1 neurons are susceptible to 7 min OGD application. To investigate whether other cellular types, such as astrocytes, abundantly expressed in this brain area, were affected by OGD we conducted a series of experiments on GFAP immunoreactivity, as a marker of astrocytes. Representative microphotographs of GFAP immunostaining in the pyramidal cell layer and stratum radiatum of CA1 are shown in Figure 6. Data from a control slice, not subjected to OGD, are shown in Figure 6A. After 3 h from the end of 7 min OGD, a consistent increase in the size and, apparently, in the number of GFAP-immunopositive cells was observed (Figure 6B). Astrocyte processes appeared thicker and longer and cell bodies appeared hypertrophic. These phenotypic changes suggest that astrocytes were in an active state. ZM241385 (100 nmol·L^{-1}) modified the OGD-induced GFAP immunostaining (Figure 6C). No change in microglia immunostaining after 3 h from the end of 7 min OGD was found (data not shown). As the immunohistochemistry experiments indicated an increase in GFAP fluorescence after the 7 min OGD, further experiments were conducted in order to quantify the GFAP protein expression by Western blot in the CA1 pyramidal layer and adjacent area. As shown in Figure 6E, the Western blots for GFAP resulted in three different molecular weight bands at apparent molecular weight of 55, 50 and 38 kDa. Statistical analysis showed that total GFAP (sum of the 55, 50 and 38 kDa bands) did not change among treatments (Figure 6F). The sum of the 55 and 50 kDa bands decreased (although not significantly) in untreated OGD slices. Interestingly, the 38 kDa GFAP fragment was significantly increased by about 210% ($P < 0.05$, one-way ANOVA, Neuman-Keuls's test) in untreated OGD slices. Treatment with 100 nmol·L^{-1} ZM241385 significantly reduced this lower molecular weight band to control levels ($P < 0.05$, one-way ANOVA Neuman-Keuls's test) (Figure 6F).

Discussion and conclusions

Hippocampal pyramidal neurons in the CA1 region are susceptible to ischaemia and fepsps are definitely depressed even after a short period (2–5 min) of OGD (Latini *et al.*, 1999; Pugliese *et al.*, 2003). However, depending on duration of OGD, fepsps can or cannot restore their amplitude (Pugliese *et al.*, 2003; Pearson *et al.*, 2006; Frenguelli *et al.*, 2007) when

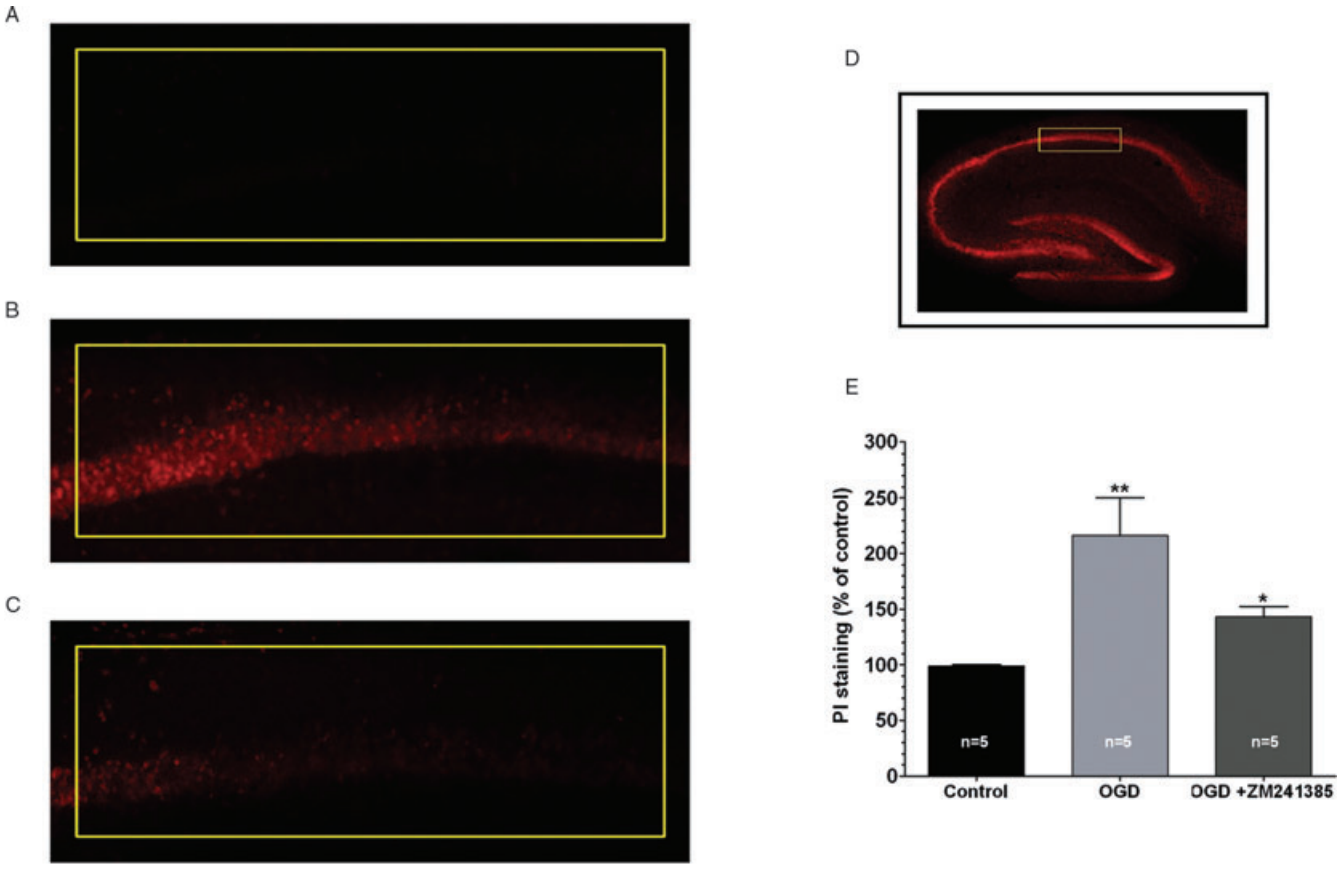

Figure 5 ZM241385 attenuated OGD-induced increase in PI uptake in the CA1 region of acutely isolated hippocampal slices. Example of PI fluorescence in the ROI region of CA1 pyramidal layer of a control slice (A) and of a slice subjected to 7 min OGD in the absence (B) or in the presence of 100 nmol·L^{-1} ZM241385 (C). Death of pyramidal CA1 neurons was assessed 3 h post-OGD using PI fluorescence. The rectangular drawings showed in each panel represents ROI (600 × 200 × 20 μm) used for PI quantification. Images shown were taken at the same depth within the slice, between 190–210 μm, where cell death due to trauma of slicing is minimized. (D) A hippocampal slice to show the localization of ROI in CA1. (E) Quantification of PI staining in different experimental conditions. Results are expressed as percent of control values and are means ± SEM. Statistical analysis was carried out by one-way ANOVA followed by Neuman-Keuls's *post hoc* test (*P < 0.05 vs. untreated OGD group, **P < 0.001 vs. control group).

reperfused with oxygenated aCSF. After short OGDs (2–5 min), periods that never elicit the appearance of AD, synaptic transmission fully recovers (Fowler, 1992; Latini *et al.*, 1999; Pugliese *et al.*, 2003). However, when an OGD of 7 min or longer is applied, it elicits the appearance of an AD, a clear sign of cell damage (Somjen, 2001), seen as a negative d.c. shift of membrane potential. These data are in agreement with previous results obtained in the same preparation *in vitro* (Pugliese *et al.*, 2006; 2007; Frenguelli *et al.*, 2007). After the appearance of AD, no recovery of neurotransmission is observed. We have shown for the first time that the loss of neurotransmission induced by 7 min OGD was maintained for up to 24 h after the end of the insult and was accompanied by a definite increase in PI staining (determined 3 h after OGD) on CA1 pyramidal neurons. These results indicate that this cell type was irreversibly damaged by the insult, data which are in agreement with previous studies conducted in acutely isolated or in organotypic hippocampal slices (Yin *et al.*, 2002; Noraberg *et al.*, 2005). Control slices that did not undergo OGD were viable for several hours after cutting. PI staining after 3 h was faint and a stable neurotransmission could be recorded up to 6 h and more, showing that, at least up to this time point, slices were fully viable. 24 h after preparation acutely isolated hippocampal slices were always viable, although in most cases they showed a seizure-like activity.

We report here that the selective adenosine A_{2A} receptor antagonist, ZM241385, delays the occurrence of AD and improves neuronal survival, following severe OGD in the CA1 hippocampus as demonstrated by the significant recovery of an otherwise disrupted neurotransmission and by the significant attenuation of the increase of PI fluorescence induced by 7 min OGD. The effects of ZM241385 were not attributable to adenosine A_1 or A_3 receptor-mediated effects since, at the concentrations used, this compound is about 6000 times more selective for A_{2A} versus A_1 receptors and it does not bind to A_3 receptors (Fredholm *et al.*, 2001).

In our experiments with 7 min OGD, two other A_{2A} adenosine receptor antagonists, SCH58261 and SCH442416 [used at concentrations selective for rat A_{2A} receptors but higher than their respective Ki values for these receptors determined in rat striatal membranes (Zocchi *et al.*, 1996; Todde *et al.*, 2000)] were effective only in 25%, while ZM241385 was effective in 81%, of the treated slices. These data are in accord with previous observations demonstrating that SCH58261 is

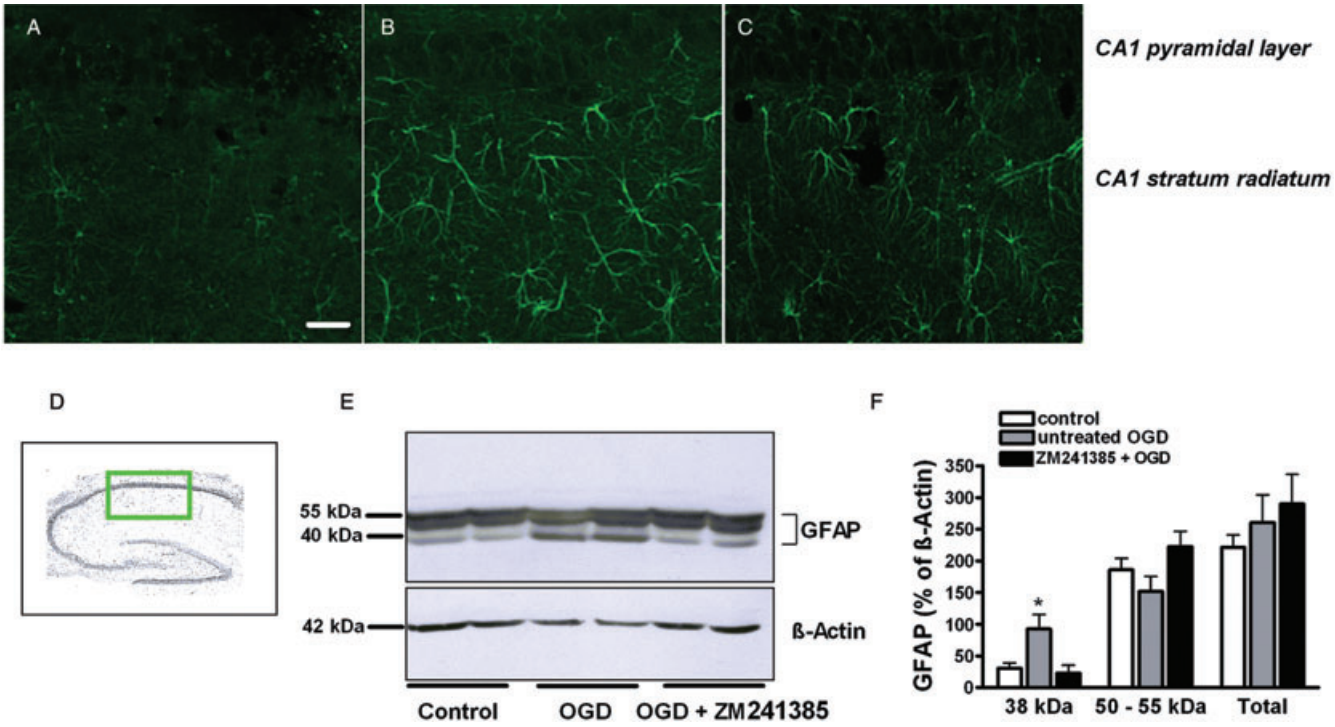

Figure 6 OGD for 7 min increased immunoreactivity for GFAP in astrocytes located in the stratum radiatum and induces GFAP fragmentation, an effect prevented by ZM241385. GFAP immunoreactivity in a control slice (A), in an untreated OGD slice (B) and in a 100 nmol·L^{-1} ZM241385 treated OGD slice (C). A faint GFAP immunoreactivity was detected in the control slice in comparison with that found in the untreated OGD slice. Hypertrophy of cellular processes was particularly evident in untreated 7 min OGD slices. Images are representative of three different determinations. Scale bar: 30 μm. (D) A representative hippocampal slice which shows in the framed region the area used for immunohistochemistry studies. (E) A representative Western blot analysis of GFAP (upper panel) and of actin (lower panel) as control. The three bands resolved are at the apparent molecular weight of 55, 50 and 38 kDa and represent fragmentation of GFAP protein. Note that the 38 kDa GFAP band was stronger in untreated OGD slices. Quantitative analysis was performed on the bands in comparison with actin in the same gel and results are shown in panel F. The fragment at 38 kDa was significantly increased by 7 min OGD and ZM241385 treatment prevents this effect. (F) Bar graph of total, 55–50 kDa and 38 kDa GFAP expression in control slices ($n = 7$), in untreated OGD slices ($n = 5$) and 100 nmol·L^{-1} ZM241385 OGD treated slices ($n = 5$). Each column represents the level of GFAP expressed as mean ± SEM, normalized versus the respective actin. Note that 7 min OGD induced a significant increase in the 38 kDa fragment (*$P < 0.05$, one-way Anova followed by Neuman-Keuls *post hoc* test vs. control and 100 nmol·L^{-1} ZM241385 groups), but total GFAP expression and the sum of 55 and 50 kDa protein were unchanged.

ineffective in antagonizing CGS21680 in hippocampal slices during OGD (Latini *et al*., 1999) and that it displays a lower potency in blocking OGD evoked glutamate outflow in the hippocampus in comparison with ZM241385 (Sperlagh *et al*., 2007). The A_{2A} adenosine receptor agonist CGS21680 binds to two pharmacologically different binding sites in the rat hippocampus: a prototypical (striatal-like) binding site and an atypical binding site, which comprises the majority (88%) of [^{3}H]-CGS21680 binding sites of the hippocampus and the cortex and it is suggested to mediate the facilitation of synaptic transmission in Shaffer fibre/collateral pyramidal synapses (Cunha *et al*., 1996; 1997). This A_{2A} receptor-like atypical site present in cortex and hippocampus is not identical to the prototypical A_{2A} adenosine receptor and may represent a novel binding site (Halldner *et al*., 2004; Lopes *et al*., 2004).

Our observations that the effects induced by severe OGD in the CA1 hippocampus were strongly antagonized by ZM241385 but only poorly antagonized by SCH58261 at a concentration selective for A_{2A} receptors, may be attributed to the higher abundance of atypical binding sites than prototypical A_{2A} receptors in this brain area and suggest the possibility that the prototypical A_{2A} receptors are less involved, compared with atypical binding sites, in the mechanisms of OGD. Also of note is the observation that prototypical A_{2A} adenosine receptors are involved in the mechanisms of synaptic plasticity in the CA1 and CA3 hippocampus (Fontinha *et al*., 2008; Rebola *et al*., 2008).

The time window of A_{2A} receptor-mediated effects found in the present studies overlaps with the delay that can be obtained by treating the slices with glutamate receptor antagonists (Tanaka *et al*., 1997; Yamamoto *et al*., 1997). In particular, NMDA receptors are essential to AD initiation and propagation (Somjen, 2001). Pharmacological activation of adenosine A_{2A} receptors modulates synaptic transmission and glutamate release/outflow (Lopes *et al*., 2002; Diógenes *et al*., 2004; Tebano *et al*., 2005). Endogenous adenosine released by prolonged OGD, acting through A_{2A} receptors exerts a facilitatory effect on glutamate outflow from hippocampal slices, as demonstrated by the decrease of glutamate outflow induced by the A_{2A} adenosine receptor antagonist ZM241385 (Sperlagh *et al*., 2007). In the rat hippocampus, adenosine A_{2A} receptors have been localized on glutamatergic terminals (Rebola *et al*., 2005b) but also at postsynaptic sites (Rebola

et al., 2005a). Recent evidence indicates that A_{2A} receptors localized postsynaptically at CA3 synapses are essential for long-term potentiation induced by short bursts of mossy fibre stimulation (Rebola *et al.*, 2008). Such a phenomenon requires coactivation of A_{2A}, NMDA and mGluR5 receptors (Rebola *et al.*, 2008). It seems likely that A_{2A} receptors are able to potentiate NMDA responses in the CA1 hippocampus (Tebano *et al.*, 2005). Therefore, selective A_{2A} adenosine receptor antagonism, by removing A_{2A} receptor potentiation of glutamate transmission both at pre- and/or postsynaptic sites, may reduce the participation of glutamate in triggering the AD. Such an effect may arise either from a direct stimulation of A_{2A} adenosine receptors or may require A_1 receptor activation (Lopes *et al.*, 2002) which inhibits glutamate outflow in CA1 hippocampus (Sebastião *et al.*, 2001; Lopes *et al.*, 2002). A functional interaction between A_1 and A_{2A} receptors is supported by the evidence that they are co-expressed in a subset (25%) of hippocampal pyramidal glutamatergic terminals (Cunha *et al.*, 1994; Rebola *et al.*, 2005b). Indeed, in different experiments under normoxic or hypoxic-ischaemic conditions, it has been demonstrated that A_{2A} adenosine receptor stimulation reduces the inhibitory A_1 receptor activity (Cunha *et al.*, 1994; Dixon *et al.*, 1997; O'Kane and Stone, 1998; Latini *et al.*, 1999; Lopes *et al.*, 1999; Pugliese *et al.*, 2003; Coelho *et al.*, 2006).

In our experiments we found that the selective A_{2A} adenosine receptor agonist CGS21680 applied during 7 min OGD does not modify AD latency. Similar effects were also reported by Tanaka *et al.* (2002) in hippocampal slices. The absence of the effect of CGS21680 cannot be attributed to A_{2A} adenosine receptor desensitization because the binding characteristics of the A_{2A} ligand [^{3}H]-CGS21680 to hippocampal membranes do not significantly change after prolonged OGD (Sperlagh *et al.*, 2007). During prolonged severe OGD, adenosine is released in large amounts from hippocampal slices reaching extracellular concentrations of between 5 (3 min after OGD) and 25–30 $\mu mol \cdot L^{-1}$ (5 min after OGD) (Latini *et al.*, 1999; Pearson *et al.*, 2006). These concentrations can maximally stimulate A_{2A} adenosine receptors which have affinity in the range of 1–4 $nmol \cdot L^{-1}$ (Fredholm *et al.*, 2001), thus explaining the lack of effect of CGS21680 in our experiments.

In this paper, we demonstrate for the first time that in acutely isolated hippocampal slices 3 h after 7 min OGD there is a definite reactive astrogliosis adjacent to the CA1 damaged area. Indeed, we found that these cells were characterized by stellate morphology, hypertrophic body and increased GFAP staining, indicating that they are reactive astrocytes. Our results are consistent with a study by Xie *et al.* (2008) who showed that in rat hippocampal slices, a 30 min OGD induced a fully, but reversible, astrocyte membrane depolarization. Adenosine A_{2A} receptors are located not only on neurons but also on microglia (Fiebich *et al.*, 1996; Saura *et al.*, 2005; Yu *et al.*, 2008) and astrocytes (Lee *et al.*, 2003). A *de novo* expression of A_{2A} adenosine receptor immunoreactivity was detected both on neurons and glial cells of the rat ischaemic cortex and striatum (Chen *et al.*, 2007; Trincavelli *et al.*, 2008). Non-neuronal A_{2A} adenosine receptors may be involved in protection against ischaemia (Yu *et al.*, 2004; Chen *et al.*, 2007).

A_{2A} adenosine receptor antagonists prevent the increase in striatal glutamate levels induced by glutamate uptake inhibitors, suggesting that a glial mechanism is important for their capability of constraining glutamate excitoxicity (Pintor *et al.*, 2004). Therefore, it is likely that the delay of AD and neuron survival induced by ZM241385 is also due to reduced glutamate outflow from this cell type.

ZM241385 prevented the increase in 38 kDa GFAP protein induced by 7-min OGD, thus preventing the fragmentation of GFAP. There is controversy about the significance of the lower molecular weight band (Gray *et al.*, 2006) and of GFAP fragmentation. It has been suggested that the lower molecular fragment of GFAP may be linked to cytoskeletal architecture damage which then results in GFAP fragmentation which is seen early in recovery after hypoxia or ischaemia (Norenberg, 1994). GFAP cleavage is induced by energy failure or environmental acidification (Lee *et al.*, 2000; Vannucci *et al.*, 2004; Acarin *et al.*, 2007). We hypothesize that the increased GFAP immunoreactivity seen after 3 h from the end of OGD may be caused by increased accessibility of the GFAP small fragment to the antibody or to an increase in antigenic epitopes elicited by post-translational modification (Goldmuntz *et al.*, 1986).

Our results indicate that ZM241385-induced prevention of AD, of the irreversible loss of neurotransmission, of astrocytic activation and GFAP fragmentation may account for its neuroprotective effects in hippocampal CA1 area during prolonged OGD. The mechanism(s) through which adenosine A_{2A} receptors or atypical A_{2A} binding sites control cellular damage following OGD require further elucidation.

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Conflict of interest

The authors state no conflict of interest.

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