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Deletion of the adenosine A_1 receptor gene does not alter neuronal damage following ischaemia *in vivo* or *in vitro*

Tomas Olsson,[1] Tobias Cronberg,[1] Anna Rytter,[1] Fredrik Asztély,[2] Bertil B. Fredholm,[3] Maj-Lis Smith[1] and Tadeusz Wieloch[1]

[1]Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University, BMC A13, SE-221 84 Lund, Sweden

[2]Department of Physiology, Göteborg University, SE-405 30 Göteborg, Sweden

[3]Department of Physiology and Pharmacology, Karolinska Institute, SE-171 77 Stockholm, Sweden

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Abstract

Extracellular adenosine is dramatically increased during cerebral ischaemia and is considered to be neuroprotective due to its inhibitory effect on synaptic transmission mediated by the adenosine A_1 receptor (A_1R). We investigated the importance of the A_1R in a mouse model of global ischaemia and in a murine hippocampal slice culture model of *in vitro* ischaemia, using mice with the A_1R gene deleted. In brains from mice lacking the A_1R, damage induced by global ischaemia was similar to that in wild-type animals. In contrast, treatment with a selective A_1R antagonist [8-cyclo-pentyl theophylline (8-CPT)], administered before the ischaemic insult in naive wild-type mice, exacerbated the neuronal damage following global ischaemia. Although the inhibitory action of adenosine on excitatory neurotransmission in hippocampal slices was lost in A_1R knockout mice, there was no difference in damage between slices from wild-type and knockout mice after *in vitro* ischaemia. The results suggest that some effects of the A_1R are compensated for in knockout animals.

Introduction

Adenosine is an important inhibitory neuromodulator in the brain. The main source of adenosine is the hydrolysis of adenosine triphosphate (ATP), and it is known that levels of adenosine rise under conditions, like ischaemia, when ATP hydrolysis exceeds its synthesis (Dunwiddie & Fredholm, 1997; Dunwiddie & Masino, 2001). Indeed, using microdialysis, the levels of adenosine were found to increase dramatically during cerebral ischaemia (Hagberg *et al*., 1987; Dux *et al*., 1990). Moreover, several studies have shown that adenosine analogues can protect against cerebral damage (Rudolphi *et al*., 1992). These findings suggest that adenosine might be an endogenous neuroprotective agent (Wieloch *et al*., 1986; Fredholm, 1996).

In the brain there are four different subtypes of adenosine receptors: A_1, A_{2A}, A_{2B} and A_3, which are located on neurons, glial cells, blood vessels, leucocytes and platelets (Fredholm *et al*., 2001). High levels of A_1 receptors (A_1R) are found in hippocampus, cortex and cerebellum, while lower levels are found in striatum (Fastbom *et al*., 1987).

Ischaemia triggers glutamate receptor activation, which allows calcium to enter neurons and cause cell death (Rothman & Olney, 1986). It is believed that the neuroprotective action of adenosine is mediated by presynaptic A_1Rs, which inhibit release of neurotransmitters (especially glutamate), or through postsynaptic A_1R by hyperpolarizing membranes and preventing calcium influx through voltage-dependent calcium channels (Dunwiddie & Masino, 2001). The downstream effects of adenosine thereby include inhibition of glutamate release, and reduced calcium toxicity and a decrease in oxidative stress (Schubert *et al*., 1997).

Studies using *in vivo* and *in vitro* models of cerebral ischaemia have generally demonstrated a neuroprotective effect of A_1R stimulation (Bischofberger *et al*., 1997). In the gerbil global ischaemia model, a selective A_1R agonist (N^6-cyclohexyladenosine) mitigated hippocampal CA1 damage (von Lubitz *et al*., 1988), whereas an A_1R antagonist (theophylline) aggravated damage in the same region (Rudolphi *et al*., 1987), but prolonged treatment actually had the opposite effect (Rudolphi *et al*., 1989). Likewise, adenosine protects against oxygen and glucose deprivation in dissociated cell cultures (Goldberg *et al*., 1988) and organotypic hippocampal tissue cultures (Newman *et al*., 1998). The A_1R antagonist 8-CPT (8-cyclo-pentyl theophylline) has also been found to increase damage after oxygen-glucose deprivation in dissociated cultures (Lobner, 2002).

We have recently established a model of global cerebral ischaemia in mouse, which allows control of body and brain temperature as well as cortical blood flow during the insult (Olsson *et al*., 2003). The aim of the present investigation was to study the contribution of the A_1R for the development of ischaemic neuronal death after global ischaemia in the mouse brain. We used a mouse strain deficient of the A_1R (Johansson *et al*., 2001), as well as pretreatment with the A_1R antagonist (8-CPT). To study the contribution of parenchymal A_1R for the cell death process, a new model of *in vitro* ischaemia in mouse hippocampal organotypic tissue cultures was used (Rytter *et al*., 2003). In both models a brief ischaemic episode of 12 min leads to delayed, glutamate receptor-dependent cell death in the CA1 region of the hippocampus.

Correspondence: Dr T. Olsson, as above.
E-mail: tomas.olsson@expbr.lu.se

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Materials and methods

The global ischaemia model

The surgery procedure for the global ischaemia model has been described in detail elsewhere (Olsson *et al*., 2003). All animal experiments were approved by the Malmoe/Lund animal ethics committee. Mice were initially anaesthetized with 2.5% halothane in N_2O and O_2 (70/30), intubated and connected to a respirator (model SAR-830-P, CWE, Ardmore, PA, USA). Anaesthesia was maintained using 1.0% halothane. Muscle relaxant (vecuronium bromide, Norcuron®), 0.25 mL (0.04 mg/mL) was given i.p. to prevent spontaneous breathing movements during ischaemia. Carbon dioxide partial pressure was measured in gas from the expiratory limb of the anaesthesia circuit with a capnometer (CAP star 100, Columbus Institute, Columbus, OH, USA) and controlled at 35–40 mmHg.

After exposure of the common carotid arteries via a small ventral neck incision, arteries were encircled loosely with a 4/0 silk thread to enable later occlusion. Regional cerebral blood flow (rCBF) in parietal cortex was recorded in both hemispheres with a two-channel laser Doppler flowmeter (Periflux System 5000, Perimed, Stockholm, Sweden), with the help of optical filaments attached to the skull bone 1 mm caudal to bregma and 4 mm lateral to the midline. Cortical blood flow was continually measured before, during and for 5 min after the ischaemia. Only hemispheres with a rCBF reduction to below 10% of baseline within 1 min after carotid occlusion were regarded as ischaemic and included in the study.

A homeothermic blanket was used during the experiment to maintain rectal temperature at 37.2 ± 0.2 °C. The temporal muscle temperature was kept at 37.1 ± 0.1 °C during ischaemia with the help of a continuous flow of humidified warm air, ensuring a normothermic brain during the occlusion time. The ischaemic period, induced by bilateral common carotid artery occlusion with a non-traumatic aneurysm clip, was 12 min. After clip removal, laser Doppler confirmed the recovery of cerebral blood flow. Five minutes after ischaemia, the laser Doppler filaments were removed from the skull bone and sutures were placed to seal the skin over the skull and in the neck.

When the animals were able to breathe, normally after 10–30 min of reperfusion, they were disconnected from the respirator and placed in an incubator at 33.5 °C for 24 h to maintain a normothermic body temperature. The body temperature was measured rectally at 0–2 h, 2–4 h, 4–8 h and 24 h after ischaemia. Animals were given 0.5 mL 5% glucose subcutaneously in the neck at the end of the day.

Perfusion fixation and histological preparation

Three or four days (depending on the experimental group) after the ischaemic insult, animals were anaesthetized with 4% halothane in oxygen/nitrous oxide (30/70) and the animal was transcardially perfused with 4% phosphate-buffered formaldehyde. The brains were taken out and stored for at least 24 h in 4% formaldehyde at 4 °C before dehydration and embedding in paraffin. Coronal sections were cut (5 μm) and stained with celestine blue/acid fuchsin for evaluation in light microscopy.

Evaluation of ischaemic damage

The ischaemia-induced neuronal injury was evaluated by light microscopy. The damage to the hippocampus, cortex and thalamus was evaluated in three different coronal sections: 1.4, 1.7 and 2.0 mm caudal to bregma (Franklin & Paxinos, 1997). In the hippocampus, neurons in the entire CA1, CA3 and dentate gyrus in each chosen section were counted, as were neurons in the barrel field in the neocortex. The total number of neurons that appeared normal and cells showing morphological features of ischaemic cell death (shrunken cell bodies, eosinophilic cytoplasm and triangulated nuclei) were counted in the hippocampus and cortex. The neuronal damage was calculated as a percentage of the total neuronal cell numbers of the structure in that section. Damage to the thalamus was evaluated in the same sections using a scale graded 0–3 (0, no damage; 1, 0–10%; 2, 10–50%; 3, 50–100% of the neurons damaged). Striatal damage was assessed in sections taken at 0.6, 0.9 and 1.2 mm rostral to bregma using a scale graded 0–5 (0, no damage; 1, 0–20%; 2, 20–40%; 3, 40–60%; 4, 60–80%; 5, 80–100% of the neurons damaged).

Assessments of rCBF, expired CO_2 and post-ischaemic temperature

The cortical blood flow, rCBF, was recorded continuously from at least 5 min before ischaemia until 5 min after the end of ischaemia by laser Doppler flowmeter. Changes in rCBF during ischaemia (1 and 2 min after occlusion) and 1, 3 and 5 min after start of reperfusion were compared between groups. The level of rCBF during occlusion and early reperfusion is presented as a percentage of starting value (ischaemia rCBF/pre-ischaemia rCBF) × 100. The pCO_2 from expired air was monitored constantly from 15 min before ischaemia until 5 min after the end of ischaemia by the capnometer.

A_1R-deficient mice

The studies were performed on male adenosine A_1R knockout (ko), heterozygous (hz) and wild-type (wt) littermates. Animals were derived from intercrossed A_1R hz (129/OlaHsd/C57BL), originally generated from mating of male chimeric A_1R ko mice (129/OlaHsd) with normal C57BL females (Johansson *et al*., 2001). The phenotype of the A_1R-deficient mice has previously been described (Johansson *et al*., 2001; Gimenez-Llort *et al*., 2002). In summary, the A_1R ko mice appear normal, but show increased hyperalgesia, aggressiveness, anxiety and a slight increase in mean arterial blood pressure (Brown *et al*., 2001). A_1R ko and wt mice were genotyped with polymerase chain reaction (PCR). Briefly, DNA was extracted from tail biopsies, and amplification by PCR was performed by 'wildband' (about 600 bp) and 'knockband' (361 bp) primers (70405 and 70406, respectively, Cybergene AB, Sweden) and subsequently run on an electrophoresis gel.

For global ischaemia, in the first part of the study (Study 1) male A_1R wt and ko littermate, 6–8 months old, were subjected to the 12-min ischaemic insult as described above. In the second part (Study 2), animals (ko, hz and wt) were generated from mating of hz females with either ko or wt males. Neuronal damage in hz males from both ko and wt breeding strains were compared and no difference in neuronal injury was found, allowing comparison between ko and wt animals even though the animals were generated from different breeding lines. Male mice, 4–8 months old, were subjected to 12-min global ischaemia.

Treatment with the A_1R blocker and global ischaemia

Male mice of the C57BL/6 strain from Taconic M&B A/S (Copenhagen, Denmark), 8–10 weeks old, were used as the model was established in this mouse strain, which is an appropriate strain for carotid artery-induced global ischaemia due to their high frequency in the absence of the posterior communicating artery. The adenosine

A_1R-selective antagonist, 8-CPT (Sigma-Aldrich, Sweden), > 100 times more specific for A_1R compared with A_2R (Dragunow & Robertson, 1987), was suspended in a solution (2.5 mg/mL) of saline and 0.2% Tween 80. Vehicle (saline and 0.2% Tween 80) or drug (20 mg/kg) was administered i.p. 30 min before the global ischaemic insult. The mortality rate of 8-CPT-treated animals increased at 4 days of reperfusion. Therefore, animals were killed at 3 days of recovery, which is sufficient for maturation of brain damage (Olsson *et al*., 2003).

Hippocampal organotypic tissue cultures

Hippocampal organotypic slice cultures were prepared from 6-day-old adenosine receptor ko and wt littermates (described above). For the pharmacological experiments, slice cultures from Balb/c mice from Møllegaard (Copenhagen) were used as this strain has been used in establishing the model. Slices, 250 μm thick, were grown on Millicell culture inserts, one per insert (0.4 μm Millicell-CM, 12 mm in diameter, Millipore, Bedford, MA, USA). Temperature was maintained at 35 °C throughout culture and experiments. Cultures were grown according to the previously described protocol (Rytter *et al*., 2003), with the modifications that cultures were maintained for 3 weeks, the glucose level was 20 mM, and that after the first week of culture B27 was omitted from the medium, a change that has improved the quality of our cultures (Cronberg *et al*., 2004).

Induction of in vitro *ischaemia (IVI)*

IVI experiments were performed according to Rytter *et al*. (2003) and Cronberg *et al*. (2004). Cultures were washed in glucose-free medium, transferred to the anaerobic incubator (Elektrotek, England), that had an atmosphere of 10% H_2, 5% CO_2 and 85% N_2, and placed in wells with anoxic medium. After 12 min of IVI, cultures were returned to culture medium. The IVI-medium, ischaemic cerebrospinal fluid (iCSF), contained (in mM): $CaCl_2$, 0.3; NaCl, 70; $NaHCO_3$, 5.25; KCl, 70; NaH_2PO_4, 1.25; $MgSO_4$, 2; sucrose, 40; and had a pH of 6.8. 8-CPT was dissolved in sterile water and was present in the medium from 1 h before IVI, during IVI and throughout the recovery period.

Quantification of cell death

Cell death was quantified with the fluorescent cell death marker propidium iodide (PI, 1 μg/mL) present in the medium from 24 h before experiment and throughout the recovery period. Fluorescence images were obtained at 24 and 48 h of recovery, and the mean fluorescence intensity (MFI) was measured in a standardized area covering the CA1 region and in a small hexagon placed in an undamaged area outside the CA2/3 cell band for background value (Fig. 1). In the pharmacological experiments on Balb/c mice the groups of slices were matched so that treated and control groups consisted of slices from the same animals. Values of cell damage were obtained by subtracting MFI in the background area from the MFI measured in the standardized CA1 area on these slices, and one *n* represents one culture. For adenosine A_1R ko and wt animals, every *n* represents the mean of 2–4 slices from one genetically modified animal. The amount of cell death was calculated as a mean for each animal. After 48 h of recovery, these cultures were subjected to an additional 1 h IVI to induce maximal neuronal death. The PI intensity in the CA1 area was measured 24 h later. The amount of cell death (relative neuronal death at time *t*, where $t = 24$ or 48 h) was expressed as a percentage of the maximal amount according to the following formula:

$$100 \times (\mathrm{CA1}^t - \mathrm{CA1}^0)/(\mathrm{CA1}^{\mathrm{MAX}} - \mathrm{CA1}^0).$$

The advantage of this formula is that it compensates for inherent differences between groups such as the number of neurons present in the culture and the thickness of the slice. The major disadvantage is that it does not take into consideration the clearance of PI-positive dead neurons between the first insult and the measurement after the

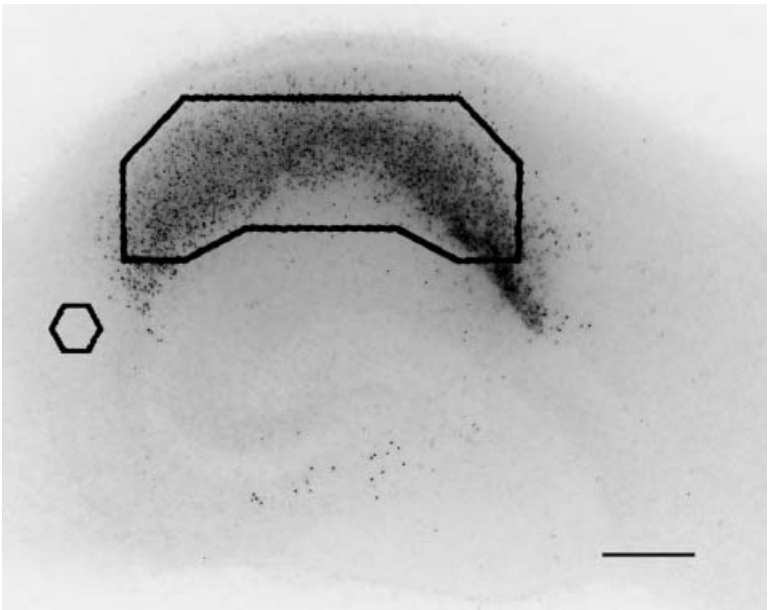

FIG. 1. Inverted fluorescence image of a hippocampal organotypic slice 48 h after *in vitro* ischaemia. Cell death was evaluated with the fluorescent cell death marker PI. MFI of PI is measured in the standardized area in the CA1 region and background MFI is measured in the standardized hexagon outside the pyramidal cell band. Scale bar, 200 μm.

second maximal death insult 72 h later. Compared groups were always run in parallel inside the anaerobic incubator and all statistical groups consist of data obtained from at least three separate experiments.

Electrophysiological experiments

For electrophysiological recordings of excitatory postsynaptic currents (EPSC), slices (cultured for 3 weeks as described above) on their membranes were placed in a recording chamber and submerged in artificial cerebrospinal fluid (aCSF) consisting of (in mM): NaCl, 119; KCl, 2.5; $MgSO_4$, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 26.2; NaH_2PO_4, 1; glucose, 11; which was gassed with 95% O_2 and 5% CO_2. All experiments were performed in the presence of 100 μM picrotoxin in order to block fast γ-aminobutyric acid (GABA)ergic synaptic transmission. The temperature of the recording chamber was kept between 21 °C and 23 °C. The electrodes were placed under visual guidance in the stratum radiatum (stimulating electrode) and in the cell layer (whole-cell recording pipette) of the CA1 region. Whole-cell voltage-clamp recordings were made using pipettes (4–6 MΩ) containing (in mM): K gluconate, 122.5; KCl, 17.5; HEPES, 10; EGTA, 0.2; NaCl, 8; MgATP, 2; GTP, 0.3 (pH 7.2; osmolarity 295 mOsm). The holding potential was −70 mV. Membrane currents were amplified and filtered at 2.9 kHz and sampled at 10 kHz with an EPC-9 patch-clamp amplifier. The amplitude of the EPSC at its peak was measured over a period of 1–2 ms. For measurement of the effect of adenosine on A_1Rs, 20 mM adenosine was added to the slices (Mitchell *et al*., 1993; Johansson *et al*., 2001). The Schaffer/collaterals were stimulated every 5 s. In each experiment the measurements are the average of six successive responses.

Data analysis

Individual hemispheres showed no dependency of the contralateral blood flow (Olsson *et al*., 2003). Therefore all hemispheres were counted separately and in all graphs each circle represents one hemisphere. The Mann–Whitney *U*-test was used to compare neuronal damage between two groups at a specific coronal level. Unpaired Student's *t*-test was employed to compare the values for body temperature, rCBF and body weight between the groups. The Chi-2 test was used for testing difference in mortality between groups. For hippocampal organotypic tissue cultures, two-way ANOVA (including date as a factor for compensation of variability between experimental dates) with Scheffé's *post-hoc* test was used to evaluate differences between groups. Data are expressed as mean ± SEM. For statistical analyses the commercial software Statview 4.0 (Abacus Concepts, Berkley, CA, USA) was used. Difference between groups in EPSC was compared using permutation test. A *P*-value of <0.05 was considered statistically significant.

Results

Lack of adenosine inhibition of EPSC in A_1R ko animals

In order to confirm that the animals were functional kos, we examined the ability of adenosine to block excitatory neurotransmission in the organotypic slices. Figure 2 shows the effect of adenosine on EPSC in A_1R ko animals ($n = 4$) and their wt littermates ($n = 3$). As seen, adenosine did not decrease EPSC amplitude in organotypic hippocampal slices from A_1R ko animals, although adenosine inhibited EPSC in slices prepared from wt littermates.

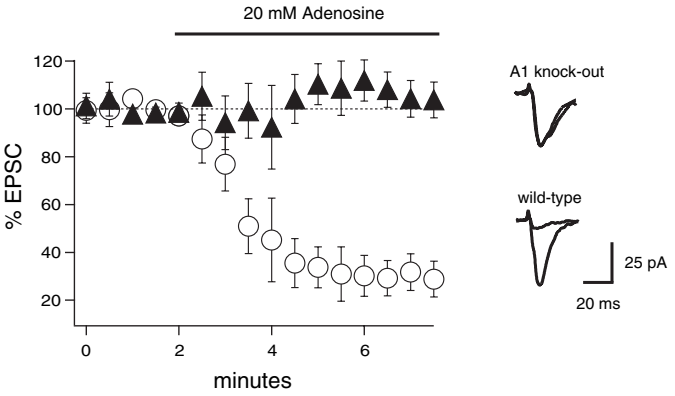

FIG. 2. The effect of adenosine on excitatory postsynaptic current (EPSC) amplitude in organotypic hippocampal slices from adenosine A_1 receptor knockout and wild-type mice. Average EPSC (± SEM), in percentage of the average EPSC value obtained during the 2 min preceding the addition of adenosine, are shown for A_1 knockout (filled triangles, $n = 4$) and wild-type (open circles, $n = 3$) mice. Adenosine was added by switching from 'normal' aCSF to aCSF with 20 mM adenosine. The right panel shows the average of six successive EPSCs recorded before and after adding adenosine to a hippocampal organotypic slice from an A_1 knockout (top) and wild-type (bottom) mouse, respectively. Groups are significantly different (permutation test).

Deletion of the A_1R gene in mouse does not modulate ischaemic brain injury in vivo *and* in vitro

Studies 1 and 2 (different breeding protocols) showed no difference in amount of brain damage, and the studies were pooled together. The A_1R ko ($n = 18$, $n_{\text{hemispheres}} = 27$–$28$) and wt ($n = 15$, $n_{\text{hemispheres}} = 20$) animals were subjected to 12 min global cerebral ischaemia and 4 days of reperfusion. There was no significant difference in ischaemic damage in any brain region in the three different rostral-caudal levels evaluated (Table 1). Figure 3 shows the distribution of neuronal damage in the subregion CA1 of hippocampus, barrel field of cortex and striatum. The body weight was not different between the groups before or after ischaemia; neither were the body temperatures in the early reperfusion phase (Table 2). rCBF was measured before, during ischaemia, and during the first 5 min of reperfusion. No difference in rCBF at any time point was found between ko and wt animals (Table 2). Of the animals subjected to ischaemia, 50% did not meet the inclusion criteria of reduction in rCBF in any hemisphere. The mortality among those included was 27% and did not significantly differ between wt and ko animals.

Hippocampal organotypic tissue cultures of adenosine A_1R ko animals ($n = 13$) and wt littermate controls ($n = 13$) showed no significant difference in the amount of cell death in the CA1 region after 24 or 48 h of recovery following IVI (Fig. 4A). Twelve minutes of IVI resulted in cell death mainly restricted to the CA1 area of ko and wt animals. The 1-h maximal insult led to a dense PI uptake also in the CA2/3 region of the neuronal band and in the dentate gyrus. Control cultures were exposed to the maximal 1-h insult only and the mean fluorescence intensity in the CA1 region was measured 24 h later. There was no difference in the amount of maximal cell death between ko and wt animals, indicating that the number of neurons in the wt and ko cultures were similar.

Blockade of the A_1R by 8-CPT enhanced global ischaemic damage in vivo *but not* in vitro

Pretreatment with the A_1R antagonist 8-CPT ($n = 10$, $n_{\text{hemispheres}} = 18$–$19$) resulted in more extensive neuronal injury in many brain

TABLE 1. Global ischaemia-induced neuronal damage in A_1 receptor (A_1R) knockout/wild-type and animals treated with A_1R antagonist/vehicle

Brain region and rostro-caudal level (mm in relation to bregma)	A_1R ko mice			8-CPT -treated mice		
	wt	ko	*P*-value*	Vehicle	8-CPT	*P*-value*
Neuronal damage (median, %)						
CA1						
−1.4	18	32.5	n.s.	50	85	n.s.
−1.7	9.5	13	n.s.	9.5	66	0.037
−2	5	11.5	n.s.	3	38	0.026
CA3						
−1.4	1	2	n.s.	11	25	0.035
−1.7	2	1	n.s.	2.5	9	n.s.
−2	1	1	n.s.	3	3	n.s.
DG						
−1.4	1	0	n.s.	1.5	8	n.s.
−1.7	1	0	n.s.	1	3	n.s.
−2	0	0	n.s.	1.5	3	n.s.
Cortex						
−1.4	1	1	n.s.	4	19	0.009
−1.7	1	1	n.s.	6	21	0.041
−2	1	1	n.s.	2.5	14	n.s.
Neuronal damage score†						
Thalamus						
−1.4	1	1	n.s.	1	2	0.022
−1.7	1	1	n.s.	1	1.5	n.s.
−2	1	1	n.s.	1	2	n.s.
Striatum						
1.2	0	0	n.s.	3	5	0.011
0.9	0.5	1	n.s.	4	5	0.042
0.6	1	1	n.s.	5	5	n.s.

†See Materials and methods. *Mann–Whitney *U*-test, ko, knockout; n.s., not significant; wt, wild-type.

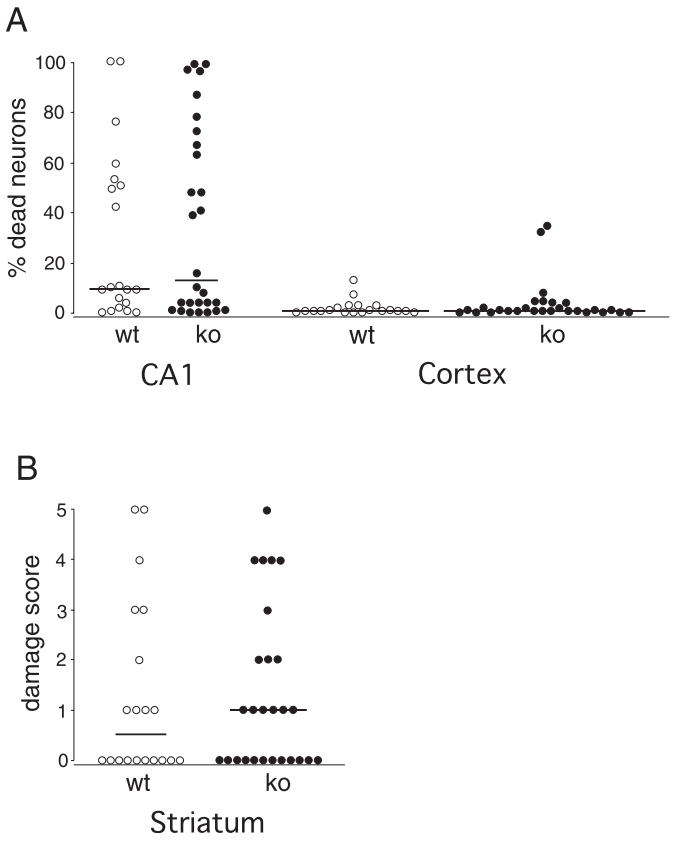

FIG. 3. Adenosine A_1 receptor knockout (ko) and wild-type (wt) animals exposed to 12 min of global ischaemia and 4 days of reperfusion showed no difference in neuronal damage. (A) Ischaemic damage to the hippocampal CA1 sectors, and the barrel field of cortex at the middle level of evaluation, 1.7 mm caudal to bregma. (B) Neuronal injury in striatum represented as damage score at the middle level of evaluation, 0.9 mm rostral to bregma. Bar represents median value. A_1 receptor ko animals ($n = 18$) and wt animals ($n = 15$). Symbols represent ischaemic hemispheres; circles for wt ($n = 20$) and filled circles for ko ($n = 27$–28). Mann–Whitney *U*-test.

regions at several rostral-caudal levels compared with vehicle-treated mice ($n = 9$, $n_{\text{hemispheres}} = 16$) after 12 min global ischaemia and 3 days of reperfusion (Fig. 5, Table 1). Figure 6 illustrates hippocampal damage in the CA1 subregion in a drug-treated animal (Fig. 6B) and in a vehicle-treated animal (Fig. 6A). The mortality of 34% was not significantly different between vehicle- and drug-treated animals (Chi-2 test). All operated animals

TABLE 2. Body weight, body temperature and cortical blood flow (% of baseline) in A1 receptor (R) knockout/wild-type animals and animals given an A_1R antagonist/vehicle

Parameter	A_1R ko mice		8-CPT -treated mice	
	wt	ko	Vehicle	8-CPT
Body weight (g)				
Before ischemia	37.5 ± 4.3	35.0 ± 5.1	24.4 ± 0.9	24.5 ± 0.8
End of reperfusion	34.0 ± 4.9	31.6 ± 5.7	20.9 ± 3.1	18.7 ± 3.0
Body temperature during reperfusion (°C)				
0–2 h	36.5 ± 0.8	36.7 ± 0.4	36.2 ± 0.3	36.4 ± 0.3
2–4 h	36.5 ± 0.9	36.8 ± 0.3	36.2 ± 0.5	36.6 ± 0.6
4–8 h	36.7 ± 1.0	37.0 ± 0.6	36.5 ± 0.4	36.4 ± 0.6
rCBF				
Ischemia 1 min	7.2 ± 3.3	6.4 ± 2.8	4.6 ± 2.9	3.6 ± 2.0
Ischemia 2 min	3.7 ± 2.1	4.2 ± 2.1	4.3 ± 2.8	3.0 ± 2.0
Recirculation 1 min	47.4 ± 27.4	45.4 ± 33.4	31.0 ± 16.1	41.1 ± 16.4
Recirculation 3 min	75.0 ± 16.9	86.0 ± 30.7	61.2 ± 23.1	78.8 ± 32.0
Recirculation 5 min	80.1 ± 15.4	100.6 ± 52.8	75.9 ± 23.6	90.8 ± 20.7

Data are means ± SD. No statistically significant differences were found between knockout (ko) and wild-type (wt) animals (Student's *t*-test).

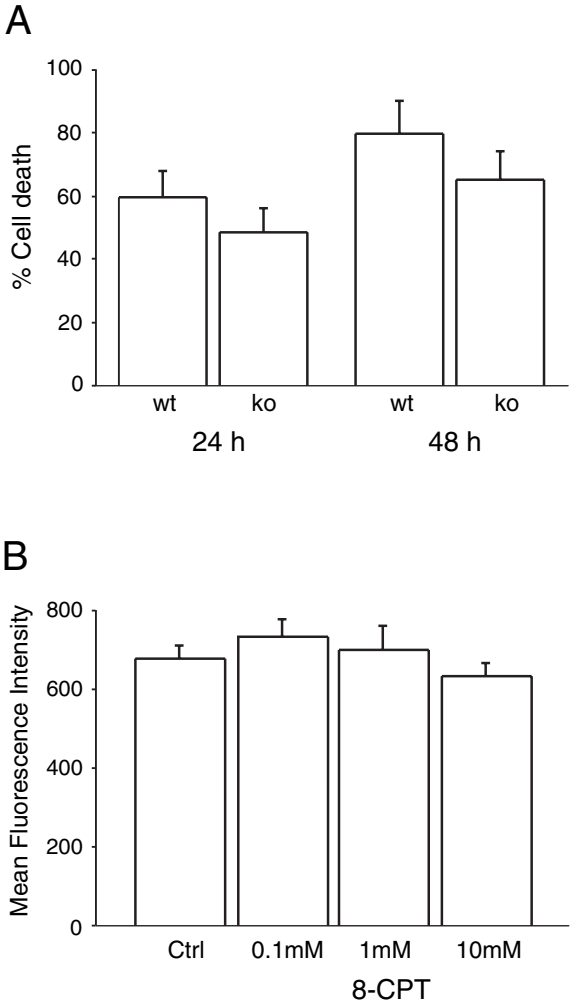

FIG. 4. (A) Relative amount of cell death in the CA1 region of slice cultures from adenosine A_1 receptor knockout (ko, $n = 13$) or wild-type (wt) animals ($n = 13$) after a 12-min IVI. No significant difference between the groups was found at 24 or 48 h recovery. (B) Amount of cell death in the CA1 region of slice cultures subjected to a 12-min IVI with 48 h of recovery in iCSF ($n = 15$) or iCSF with 8-cyclo-pentyl theophylline (8-CPT), 0.1 ($n = 15$), 1 ($n = 15$) and 10 mM ($n = 15$). No significant effect of the drug was seen. Two-way ANOVA.

fulfilled the criteria of 10% reduction to less than 10% of control in rCBF during ischaemia in at least one hemisphere after 1 min occlusion. The reduction in rCBF during ischaemia was similar in vehicle- and drug-treated animals (Table 2). Body temperature during the first period of reperfusion, as well as body weight was similar in both groups (Table 2).

Hippocampal organotypic cultures from Balb/c mice were subjected to 12 min IVI in iCSF alone or iCSF with 0.1, 1 and 10 μM of the adenosine A_1R antagonist 8-CPT ($n = 15$ in all groups). The drug had no effect on the amount of cell death, expressed as background-subtracted MFI of the CA1 region at 48 h after IVI (Fig. 4B). Likewise, 24 h after IVI, no effect of 8-CPT was found (data not shown).

Discussion

The aim of this study was to take advantage of the gene deletion technology, to prove the important role of the adenosine A_1R in global cerebral ischaemia, which appeared to be quite firmly established from previous studies (see Introduction). However, much to our surprise we found that the deletion of the adenosine A_1R gene did not change the density of the ischaemic brain injury in a model of global cerebral ischaemia or in hippocampal brain slices subjected to IVI.

As the A_1R gene deletion had no effect on ischaemic brain damage, we inhibited the A_1R using drug treatment to assess whether a pharmacological intervention at the A_1R level would modulate ischaemic damage following global cerebral ischaemia in the mouse. We used the selective adenosine A_1R antagonist, 8-CPT, which readily crosses the blood–brain barrier (Dragunow & Robertson, 1987), and found that pretreatment with 8-CPT aggravated damage, which is in line with other reports using A_1R antagonists in models of global cerebral ischaemia in the gerbil or rat (Rudolphi *et al*., 1987; Von Lubitz *et al*., 1994). The increase in damage was not due to a more severe brain ischaemia, as intra-ischaemic blood flow, measured cortically by laser Doppler, showed no difference between 8-CPT and vehicle-treated animals (Table 2). This was expected because the regulatory effect of adenosine on cerebral blood flow has been shown to be mediated mainly by the adenosine A_{2A}R (Phillis, 1989). Also, other reports have shown that 8-CPT does not influence blood pressure (Gervitz *et al*., 2003).

Hypothermia is known to confer a robust neuroprotection when applied during or after global ischaemia in rats (Boris-Moller *et al*., 1989) and mice (Olsson *et al*., 2003). Body temperature is lowered by adenosine via actions on the A_1R (Johansson *et al*., 2001), and 8-CPT is known to affect body temperature (Anderson *et al*., 1994). Nevertheless, the effect of 8-CPT on ischaemic brain damage in our study is probably not due to alterations in body temperature, as the animals were housed in a heated incubator after the ischaemic insult and the post-ischaemic body temperature was carefully monitored (Table 2).

Similarly, it is difficult to explain the lack of an aggravating effect of the A_1R deletion by effects on cerebral blood flow or body temperature. Cortical blood flows during and up to 5 min after the end of ischaemia were not different between A_1R wt and ko mice (Table 2). In this study, head and body temperature was rigorously controlled during ischaemia, and we found no difference in body temperature between A_1R ko and wt animals for up to 8 h after ischaemia (Table 2).

The reason for this lack of an effect of the A_1R deletion on brain damage can only be speculated upon, but the deletion may have induced processes in the brain preventing the detrimental consequences of A_1R loss. There is another example of an adaptation that may have a similar background. We recently found that sleep is clearly affected by 8-CPT, but is unaltered in A_1R ko mice, and it was speculated that sleep regulation is such an important process that any loss of a regulatory mechanism must be compensated for by some adaptation (Stenberg *et al*., 2003). The nature of these adaptive changes remains unknown. It is clear that adaptive changes in the expression of other adenosine receptors do not appear to play a major role (Johansson *et al*., 2001; Lopes *et al*., 2003). Similarly, there do not appear to be any major changes in $GABA_B$ receptor-mediated responses (Johansson *et al*., 2001).

The neuroprotective effect of adenosine is generally attributed to a depression of excitatory synaptic transmission by a rise in the extracellular adenosine concentration (Fowler, 1990) and stimulation of the A_1R (Latini *et al*., 1999). Stimulation of the presynaptic A_1Rs inhibits the vesicular release of glutamate (Corradetti *et al*., 1984). Here we show that exogenous adenosine depresses EPSCs in organotypic tissue cultures from wt but not A_1R ko mice, thus verifying a lack of electrophysiological A_1R response in ko mice. This

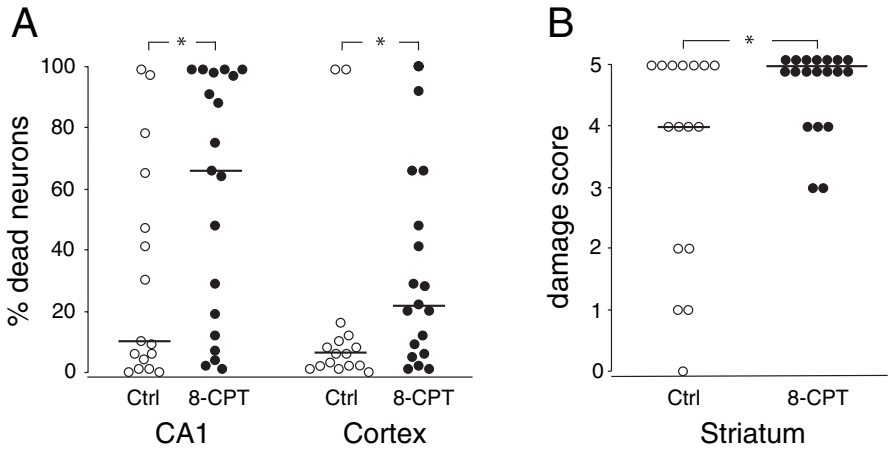

FIG. 5. Neuronal damage in C57BL/6 animals treated with an adenosine A_1R-selective antagonist, 8-cyclo-pentyl theophylline (8-CPT), given i.p. 30 min before ischaemia (8-CPT, $n = 10$), and control animals (Ctrl, $n = 9$) treated with an equivalent vehicle protocol. Both groups were exposed to 12 min of global ischaemia. Animals treated with A_1R antagonist demonstrated more widespread neuronal damage compared with vehicle-treated animals. (A) Ischaemic damage to the hippocampal sectors CA1 and the barrel field of cortex at the middle level of evaluation, 1.7 mm caudal to bregma. (B) Neuronal injury in striatum represented as damage score at the middle level of evaluation, 0.9 mm rostral to bregma. Bar represents median value. Hemispheres, $n_{Ctrl} = 16$; $n_{A1Rantag} = 18–19$. Mann–Whitney *U*-test, *Statistical significance ($P < 0.05$).

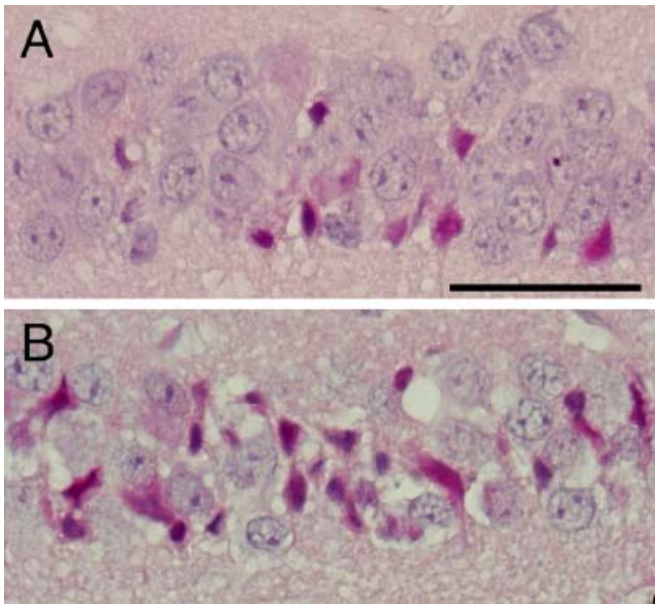

FIG. 6. Histological sections, stained by celestine blue/acid fuchsin, demonstrating typical morphological features of ischaemic cell death (pyknotic nuclei and red cytoplasm) in hippocampal subregion CA1 in C57BL/6 animals after 12 min of global ischaemia. Pretreatment with A_1 receptor antagonist (8-CPT, 20 mg/kg, i.p.) 30 min before ischaemia (B) showing aggravated neuronal damage, compared with vehicle-treated animals (A). Scale bar, 40 μm.

finding is in accordance with Johansson *et al*. (2001), who found that acutely prepared hippocampal slices from A_1R ko mice lacked depression of field excitatory postsynaptic potential (fEPSP) by adenosine. Interestingly, these authors describe a decrease in fEPSP after a hypoxic challenge in slices from A_1R ko mice relative to wt littermates. This post-hypoxic depression in fEPSP, only found in A_1R ko mice, could be the result of a compensatory protective mechanism, that could ameliorate the effect of glutamate toxicity in the reperfusion phase. We have previously shown that post-ischaemic inhibition of glutamate toxicity, by MK-801, is neuroprotective in our global ischaemia model.

The lack of effect on neuronal damage of A_1R ko was also seen in our organotypic hippocampal slices, and may be explained similarly as above for the *in vivo* results. However, in contrast to ischaemia *in vivo*, treatment with 8-CPT prior to IVI did not aggravate cell death, suggesting a fundamental difference in the *in vivo* and *in vitro* systems. The inhibitory effect of A_1R is thought to occur mainly at the presynaptic site (Wu & Saggau, 1997), and A_1R blockade by 8-CPT enhances synaptic transmission (Bauman *et al*., 1992). This is supported by the fact that *N*-methyl-D-aspartate toxicity (acting on postsynaptic membranes) is unaffected by adenosine (Goldberg *et al*., 1988) or 8-CPT (Lobner, 2002), while the latter compound increased damage induced by oxygen and glucose deprivation (OGD), by facilitating glutamate release from presynaptic sites (Lobner, 2002). These authors utilized the commonly used aCSF, with a potassium concentration of 5.4 mM KCl during OGD. In that medium synaptic activity can persist for a long time during OGD. In our model of IVI, the medium used during OGD is designed to mimic the ion concentrations found in the extracellular space during ischaemia *in vivo*. Our IVI medium combines a high level of potassium (70 mM) with a low level of calcium (0.3 mM) and acidosis (pH 6.8). In our model, as in conventional OGD, blockade of ionotropic glutamate receptors during IVI is highly protective. A possible explanation for the lack of effect of A_1R blockade in our IVI paradigm could be that the synaptic increase in glutamate during IVI is dominated by a reversal of the sodium-coupled glutamate uptake carrier (Rossi *et al*., 2000) and that the presynaptic glutamate release is less important for excitotoxicity in this model. Alternatively, forceful depolarization by the high potassium might bypass the effect of the A_1R on presynaptic glutamate release.

The use of genetically modified mice offers new possibilities to study the importance of specific gene products for particular body functions or disease processes. Our results show that gene deletions may lead to the activation of compensatory processes and highlight the difficulties that can be encountered using genetically modified animals. On the other hand, the lack of aggravating effects of ischaemia in the A_1R ko mice suggests that the deletion of the A_1R upregulates protective mechanisms that may prove beneficial for recovery after stroke. The compensatory mechanisms in A_1R ko mice may influence particularly the processes after ischaemia. It appears important to

examine the nature of these post-ischaemic adaptive processes, as therapeutic interventions for treatment of ischaemic stroke in patients would also primarily target post-ischaemic processes.

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Abbreviations

8-CPT, 8-cyclo-pentyl theophylline; A_1R, A_1 receptor; aCSF, artificial cerebrospinal fluid; ATP, adenosine triphosphate; EPSC, excitatory postsynaptic currents; fEPSP, field excitatory postsynaptic potential; GABA, γ-aminobutyric acid; hz, heterozygote; iCSF, ischaemic cerebrospinal fluid; IVI, *in vitro* ischaemia; MFI, mean fluorescence intensity; OGD, oxygen and glucose deprivation; PCR, polymerase chain reaction; PI, propidium iodide; ko, knockout; rCBF, regional cerebral blood flow; wt, wild-type.

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