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Neuroprotective potential of adenosine A_1 receptor partial agonists in experimental models of cerebral ischemia

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Abbreviations: A₁R, adenosine A₁ receptor; AC, adenylyl cyclase; ACSF, artificial cerebrospinal fluid; ADAC, adenosine amine congener; AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate; ATP adenosine 5'-triphosphate; BBB, blood brain barrier; cAMP, cyclic AMP; CCPA, 2-chloro- N^6 -cyclopentyladenosine; CHO, chinese hamster ovary; CNS, central nervous system; CPA, N^6 -cyclopentyl-adenosine; DMSO, dimethyl sulfoxide; DPCPX; 8-cyclopentyl-1,3-dipropylxanthine; fEPSP, field excitatory postsynaptic potential; HEMADO, 2-(1-Hexynyl)- N^6 -methyladenosine; i.p., intraperitoneally; min, minute; NECA, 5'-N-Ethylcarboxamidoadenosine; NMDA, N-methyl-D-aspartic acid; OGD, oxygen-glucose deprivation; PKA, protein kinase A; PPF, paired-pulse facilitation; PPR, paired-pulse ratio; PPS, paired-pulse stimulation; R-PIA, (R)-phenyl-isopropyl-adenosine; RRID, Research Resource Identifier; SEM, standard error of mean.

Abstract

Cerebral ischemia is the second most common cause of death and a major cause of disability worldwide. Available therapies are based only on anticoagulants or recombinant tissue plasminogen activator. Extracellular adenosine increases during ischemia and acts as a neuroprotective endogenous agent mainly by activating adenosine A_1 receptors (A_1Rs) which control calcium influx, glutamate release, membrane potential, and metabolism.

Accordingly, in many experimental paradigms it has been already demonstrated that the stimulation of A_1R with full agonists is able to reduce ischemia-related structural and functional brain damage; unfortunately, cardiovascular side effects and desensitization of A₁R induced by these compounds have strongly limited their exploitation in stroke therapy so far. Among the newly emerging compounds, A_1R partial agonists could be almost free of side effects and equally effective. Therefore, we decided to evaluate the neuroprotective potential of two A₁R partial agonists, namely 2'-dCCPA and 3'-dCCPA, in *in vitro* and *ex vivo* experimental models of cerebral ischemia. Within the experimental paradigm of oxygenglucose deprivation (OGD) in vitro in human neuroblastoma (SH-SY5Y) cells both A_1R partial agonists increased cell viability. Considering the high level of expression of A_1R_5 in the hippocampus and the susceptibility of CA1 region to hypoxia, we performed electrophysiological experiments in this subfield. The application of 7 min of OGD constantly produces an irreversible synaptic failure in all the C57Bl/6 mice hippocampal slices evaluated; both tested compounds allowed a significant recovery of synaptic transmission. These findings demonstrate that A_1R and its partial agonists are still of interest for cerebral ischemia therapy.

INTRODUCTION

In the last report of World Health Organization on noncommunicable diseases (WHO GLOBAL STATUS REPORT, 2014) some impressive numbers clearly show that ischemic brain injury is a widespread pathological condition with deleterious consequences both on quality of life for individual patients and on related costs of health care and social services. Cardiovascular disease was the leading cause of noncommunicable diseases deaths in 2012 and was responsible for 17.5 million deaths. Of these deaths, 6.7 million were due to strokes and ischemic stroke represents approximately 80% of all cases. The majority of cerebral strokes are ischemic in nature and result from the occlusion of a major cerebral artery by a thrombus or an embolism, which leads to loss of blood flow, subsequent OGD and, finally, tissue death in the affected region (Gibson, 2013).

Cerebral ischemia, leading to serious CNS damage (Honig and Rosenberg, 2000), is the second most common cause of death and a major cause of disability worldwide (Macrez et al., 2011).

Unfortunately, very few therapeutic options are available to date: 1) acute treatment with recombinant tissue plasminogen activator in the restricted therapeutic time-window (0-6 hours) after the ischemic attack (Beresford et al., 2003; Chen et al., 2014); 2) aspirin and anticoagulants as preventive therapy (Young et al., 2007; Ginsberg, 2009; Moskowitz et al., 2010; Albers et al. 2011; Macrez et al. 2011).

Such a limited panel of therapeutic options demonstrates the difficulties in the translation from basic research to clinical application in this field: during the last years more than 1000 molecules with brain-protective effects have been identified, targeting and correcting different pathophysiological manifestations of brain ischemia. Approximately 250 molecules deserved a dedicated clinical trial, but, unfortunately, none of them has so far successfully completed phase III. Despite such a discouraging score, looking for, and testing new therapeutic agents for cerebral ischemia remains an absolute priority for global health.

Hippocampus is a brain area particularly vulnerable to ischemia (Yue et al., 1997). Hypoxic/ischemic damage, induced by the reduction of the blood flow, causes generally necrosis, but in most cases there is also a process of slower apoptotic neuronal death in the region (*penumbra*) surrounding the core of the damaged area (Honig and Rosenberg, 2000; Yuan and Yankner, 2000; Schaller et al., 2003; Lo, 2008).

A rapid consequence of OGD in neuronal tissues is a failure in the production of ATP and in the support of the energetic state of neurons, which allows to maintain membrane ionic gradients; as a consequence, there is a loss of membrane potential and an uncontrolled depolarization of nervous connections. Voltage-dependent calcium channels are activated and excitatory amino acids are released into the extracellular space. A further cytotoxic overload of calcium is probably responsible for the beginning of other nuclear and cytoplasmic deleterious pathways, including apoptotic events. The glutamate released in excess binds to ionotropic NMDA and AMPA receptors, inducing a further increase in intracellular calcium. Then, the condition known as "excitotoxicity" (Dong et al., 2009) is the first that takes place after the ischemic attack in the brain and plays a central role in its pathogenesis (Choi and Rothman, 1990).

In addition to the increase of glutamate, hypoxic/ischemic conditions trigger also a robust and sustained enhancement of the extracellular levels of adenosine in the brain parenchyma, but also in *in vitro* brain preparations or in cultured neurons (van Wylen et al., 1986; Fredholm et al., 1994). Adenosine is an endogenous neuromodulator involved in the regulation of many functions within the CNS (Phillis and Wu, 1981) and whose effects are mediated by at least four distinct receptors: A1, A2A, A2B, and A3 (Fredholm et al., 2001). Of the four adenosine receptors, the A_1R is the most abundant and widespread in the brain (Fastborn et al., 1987). The increase in extracellular adenosine early after ischemia is considered an endogenous neuroprotective response aimed at stimulating A_1Rs . Indeed, this receptor is crucial to control several events that have been associated with ischemic damage: first of all, the glutamate release, but also the calcium influx, the alteration of the membrane potential and of metabolism (reviewed in Rudolphi et al., 1992; de Mendonça et al., 2000). In contrast, the blockade of the A₁R generally tends to exacerbate ischemic brain damage (reviewed in de Mendonça et al., 2000). A_1R stimulation prevents excitotoxicity both at the presynaptic (inhibition of glutamate release) and at the postsynaptic (inhibition of AMPA/NMDA currents) level. Furthermore, A_1R stimulation is also able to decrease neuronal excitability by activating G-protein-coupled inwardly rectifying potassium channels (Sickmann and Alzheimer, 2003; Kim and Johnston, 2015), which, in turn, mediate hyperpolarization of the membrane. Since A_1R activation has clear protective effects in brain ischemia, A_1R agonists have been historically considered very promising drugs for this condition.

Selective A_1R agonists, like CPA in acute administration, protect neurons against the damage induced both by focal and by global ischemia (Rudolphi et al., 1992; von Lubitz et al., 1995). Unfortunately, after chronic administration full A_1R agonists lead to desensitization of A_1Rs . Furthermore, the activation of A_1Rs generally induces relevant cardiovascular side effects

including bradycardia and hypotension (Park et al., 2012). Finally, puzzling results about the ability of some full A₁R agonists to cross the BBB have been obtained (Gervitz et al., 2002). All these undesirable effects strongly mitigated their clinical implementation (White et al., 1996; Fredholm et al., 2005).
However, it appears reasonable to explore the efficacy of A₁R partial agonists, low efficacy ligands which elicit only a submaximal response of the receptor in contrast to a full agonist: they should have less pronounced cardiovascular effects and seem to act more selectively

(Mathot et al., 1996; IJzerman et al., 1996); they should induce less receptor downregulation/desensitization and, finally, they should be equally effective in acute as well as in chronic administration (Albrecht-Kupper et al., 2012). Given the higher level of expression of A_1Rs in the brain (particularly in the hippocampus and prefrontal cortex) compared to peripheral organs like the heart (Fredholm et al., 2001; Fredholm et al., 2011), partial agonists should elicit a maximal response only in tissues in which A_1Rs are largely expressed, where a submaximal receptor occupancy leads to a maximal functional effect (Srinivas et al., 1997; van Schaick et al., 1998). These drugs have thus the potential to induce limited cardiovascular side effects and to cause less pronounced receptor desensitization than full agonists after chronic exposures (Parsons and Stiles, 1987; Longabaugh et al., 1989). The efficacy of a A_1R partial agonist in angina pectoris treatment has been proven in a clinical trial with capadenoson (Tendera et al., 2007), a non-nucleoside molecule that mediates cardioprotection in the absence of adverse hemodynamic effects (Albrecht-Kupper et al., 2012; Sabbah et al., 2013). Therefore, the aim of the present study was to characterize, for the first time, the possible neuroprotective potential of two A1R partial agonists, namely 2'dCCPA and the newly developed 3'-dCCPA, in *in vitro* and *ex vivo* experimental models of ischemia.

MATERIALS AND METHODS

Animals

All animal procedures were carried out according to the principles and procedures outlined in the European Community Guidelines for Animal Care, DL 26/2014, application of the European Communities Council Directive, 2010/63/EU, FELASA and ARRIVE guidelines. All animal procedures were approved by the Italian Ministry of Health and by the local Institutional Animal Care and Use Committee (IACUC) at Istituto Superiore di Sanità (Rome, Italy; protocol number n. 7496) and at University of Camerino (Camerino, Italy; protocol in agreement with the Article 3, paragraph 1, of the DL 26/2014). All functional assays experiments were conducted on male BALB/c mice (CAT# JAX:000651, RRID:IMSR_JAX:000651; 25.5 ± 0.5 g weight, 8 weeks old) bred at the University of Camerino. Electrophysiology and behavioral experiments were performed on male C57Bl/6 mice (CAT# JAX:000664, RRID:IMSR_JAX:000664; 25.5 ± 2 g weight, 8 to 16 weeks-old) purchased from Charles River (Calco, Italy). No sample calculation was performed to estimate the number of mice used in each experimental group. No randomization was performed to allocate subjects in the study. Anyway, we allocated arbitrarily the animals to the different experimental groups on which to perform the euthanasia and the withdrawn of organs (ileum, heart, brain) for ex vivo evaluations, or the i.p. injection of A₁ agonists and the following behavioral test. Additionally, no blinding was performed. The animals were kept in standard cages (48 x 26 x 20 cm, 4 mice per cage) under standardized temperature (22°C), humidity (55%) and lighting conditions (12:12 h light:dark cycle, with lights on at 6 am and euthanasia/withdrawn of organs and in vivo experiments conducted as soon as possible after the start of the light phase; usually, behavioral experiments were conducted between 7:30 am and 10:00 am) with free access to water and food. For animals used in the present study, proper treatment, care, and humane conditions have been provided. All efforts were made to reduce the number of animals used and to minimize their pain and discomfort. Permanent veterinary surveillance and animal welfare evaluation have been provided by the host Institutions. As for behavioral experiments, we did not apply any inclusion or exclusion criteria, and no animals died during experiments. A time-line diagram of animal experiments is provided in Figure 1.

Drugs

The A₁R partial agonists 2'-dCCPA and 3'-dCCPA are indicated below as compounds **4** and **7**. CPA (A₁R agonist, Cat# 1702), CCPA (A₁R agonist, Cat# 1705), DPCPX (A₁R antagonist, Cat# 0439), forskolin (adenylyl cyclase, AC, activator, Cat# 1099) were purchased from Tocris Biosciences (Bristol, UK). All pharmacological agents (CPA, DPCPX, compounds **4** and **7**) were dissolved in DMSO or in distilled water to obtain a stock solution of 5 mM (CPA and DPCPX) and 50 mM (**4** and **7**). Stock solutions were made to obtain concentrations of DMSO lower than 0.001% in the superfusing ACSF and in cell cultures. This DMSO concentration did not affect cell viability of SH-SY5Y or basal synaptic transmission in

hippocampal slices. For behavioral experiments, compound 7, CPA, and CCPA were dissolved in DMSO and then diluted in saline (0.9% NaCl), to obtain a solution for i.p. injection with a 5% maximal concentration of DMSO. This DMSO concentration induced a slight and non-significant reduction of mouse locomotor activity. The synthesis of compound 4 has been previously reported, hence, it will not be described here (Fig. 2, scheme 1, Vittori et al., 2000). The custom-made compounds used in this study will be shared upon reasonable request.

Synthesis of compound 7

General chemistry. Melting points were determined with a Büchi apparatus and are uncorrected. ¹H NMR spectra were obtained with Varian Mercury 400 MHz spectrometer; δ in ppm, *J* in Hz. All exchangeable protons were confirmed by addition of D₂O. Mass spectra were recorded on an HP 1100-MSD series instrument. All measurements were performed using electrospray ionization (ESI-MS) on a single quadrupole analyzer. Thin Layer Chromatography (TLC) was carried out on precoated TLC plates with silica gel 60 F-254 (Fluka). For column chromatography, silica gel 60 (Merck) were used. Elemental analyses were determined on Fisons Instruments Model EA 1108 CHNS-O model analyzer and are within 0.4% of theoretical values. The purity of the compounds was \geq 95% according to elemental analysis data.

(2*S*,4*R*,5*R*)-4-acetoxy-5-(2,6-dichloro-9*H*-purine-9-yl)tetrahydrofuran-2-yl)methyl benzoate (6):

A solution of 1,2-di-*O*-acetyl-5-*O*-benzoyl-3-deoxy-ribofuranose (Rizzo et al., 1992) (**5**; 0.28 g; 0.96 mmol) in 5 mL of anhydrous CH₃CN was added of desiccated 2,6-dichloropurine (**1**; 0.20 g; 1.03 mmol) and cold in an ice-bath. When the mixture is at 0 °C, SnCl₄ (1.1 mL) is added and the reaction is left at r.t. under stirring for 16 h. After this time, cold saturated solution NaHCO₃ is added to neutralize the reaction pH and the crude extracted with CH₂Cl₂ (3x 100 mL). Organic extracts were collected dried over Na₂SO₄, filtered and concentrated to dryness under vacuum. The residue was chromatographed on silica gel gravity column, eluting with CHCl₃ (100%), to obtain **6** as pure vitreous solid with 84% yield. ¹H NMR (DMSO-d₆) δ 2.10 (s, 3H, COCH₃), 2.32 (dd, 1H, *J* = 6.3 and 13.5 Hz, H-3'a), 2.70 (m, 1H, H-3'b), 4.45 (m, 1H, H-CH-5'), 4.61 (m, 1H, H-CH-5'), 4.70 (m, 1H, H-4'), 5.76 (d, 1H, *J* =

5.7 Hz, H-2'), 6.25 (d, 1H, *J* = 1.2 Hz, H-1'), 7.45 (t, 2H, *J* = 7.6 Hz, H-Ph), 7.63 (t, 1H, *J* = 7.5 Hz, H-Ph), 7.81 (m, 2H, H-Ph), 8.84 (s, 1H, H-8).

(2*R*,3*R*,5*S*)-2-(2-chloro-6-(cyclopentylamino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3-ol (**7**):

To 0.25 grams (0.55 mmol) of **6**, cyclopentylamine (5 ml) was added and the mixture let to react at r.t. for 1 h. Then the residual amine was removed under vacuum and, to the crude mixture, methanolic ammonia (25 mL) was added. The reaction was left at 75°C for 20 h. Volatiles were removed under vacuum and the residue was chromatographed on silica gel flash column, eluting with CHCl₃-cC₆H₁₂-MeOH (70:27.5:2.5), to obtain **7** as a white solid after crystallization from CH₃OH-Et₂O with 93% yield. M.p.: 161-164 °C. ¹H NMR (DMSO-d₆): δ 1.56 (m, 4H, H-cyclohexyl), 1.72 (m, 1H, H-cyclohexyl), 1.91 (m, 3H, H-3' and H-cyclohexyl), 3.19 (m, 1H, H-3'), 3.54 (m, 1H, H-CH-5'), 3.68 (m, 1H, H-CH-5'), 4.36 (m, 1H, H-4'), 4.40 (m, 1H, CH-NH), 4.52 (m, 1H, H-2'), 5.02 (m, 1H, OH), 5.69 (d, 1H, *J* = 5.0 Hz, OH), 5.82 (bs, 1H, H-1'), 8.30 (1H, m, NH), 8.40 (s, 1H, H-8); ¹³C NMR (DMSO-d₆): δ 23.9; 34.3; 52.1; 62.8; 75.3; 81.5; 91.1; 118.5; 136.5; 139.5; 153.6; 155.1.

Binding assay of compounds 4 and 7

Dissociation constants of unlabeled compounds (*K*i values) were determined in competition experiments in 96-well microplates as described earlier (Klotz et al., 1998).

Membrane preparation

Membranes for radioligand binding were prepared from CHO cell line stably transfected with the four human adenosine receptor subtypes. The cell line used was provided by Dr. Klotz from Wurzburg University (among the authors; details available in the reference Klotz et al., 1998) and was not further authenticated. Anyway, the original CHO cell line is not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee (ICLAC; http://iclac.org/databases/cross-contaminations/). Crude membranes were prepared by collecting cells in ice-cold hypotonic buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized on ice (Ultra-Turrax, 2 x 20 sec at full speed) and the homogenate was spun for 10 min (4 °C) at 1,000 x g in order to remove cell fragments and nuclei. The supernatant was then centrifuged for 50 min at 100,000 x g at 4°C. The

membrane pellet was resuspended in the specific binding buffer used for the binding experiments, frozen in liquid nitrogen and stored at -80 °C (ref Klotz et al., 1998). The preparation of a membrane fraction for the measurement of AC activity followed a simplified protocol with only one high-speed centrifugation of the homogenate. The resulting crude membrane pellet was resuspended in 50 mM Tris/HCl, pH 7.4 and immediately used for the cyclase assay without freezing.

Radioligand binding

For competition binding the following radioligands and binding buffers were used: 1 nM [³H]CCPA for A₁Rs (50 mM TRIS/HCl, pH 7.4), 10 nM [³H]NECA for A_{2A}Rs (50 mM Tris, pH 7.4, 10 mM MgCl₂), 1 nM [³H]HEMADO for A₃Rs (50 mM TRIS/HCl, pH 8.25, 10 mM MgCl₂, 1 mM EDTA) (Klotz et al., 2007). Binding assay was not performed at A_{2B} receptors since in our hands the available radioligands to target these receptors did not gave reproducible results. Nonspecific binding of [³H]CCPA was determined in the presence of 1 mM theophylline, 100 μ M R-PIA was used in the case of [³H]NECA and [³H]HEMADO binding. Dissociation constants (K_i-values) were calculated from competition experiments utilizing the program SCTFIT (De Lean et al., 1982) and are given as geometric means with 95% confidence intervals (n \geq 3).

Functional assays (in vitro and ex vivo) of compounds 4 and 7

Adenylyl Cyclase Activity.

The functional activity of new derivatives was determined in AC activity evaluation experiments. Therefore, receptor-mediated stimulation of AC activity was measured to determine agonist potency given in EC_{50} -values. The stimulation of AC via $hA_{2B}Rs$ was measured as described earlier (Klotz et al., 1998).

Functional interaction with A_1Rs was studied in receptor-mediated inhibition of cyclase activity. AC was inhibited with the receptor agonists after stimulation with 10 μ M forskolin. Inhibition with selected compounds was compared to CCPA-induced cyclase inhibition as a full agonist. For details see Klotz et al., 1985, 1998.

Animals were sacrificed by cervical dislocation; the ileum was isolated, cleaned of the connective tissue and dipped into Krebs solution of the following mM composition: NaCl 119, KCl 4.5, MgSO₄ 2.5, NaHCO₃ 25, KH₂PO₄ 1.2, CaCl₂ 2.5, glucose 11.1. The 20 mm ileum segments were suspended in organ baths containing 10 ml of Krebs solution kept at 37 $^{\circ}$ C and aerated with 5% CO₂ : 95% O₂. The proximal end was connected with a silk thread to an isometric force transducer (Cat# 7003/4/5/10, Ugo Basile, Biological Research Apparatus, VA, Italy). Mechanical activity was digitized by an A/D converter, visualized, recorded, and analyzed on a personal computer using the PowerLab/400 system.

Longitudinal preparations were subjected to an initial tension of 200 mg and were allowed to equilibrate for at least 30 min. Rhythmic spontaneous contractions of varying amplitude developed in all preparations. After the equilibration time, preparations were challenged with the known agonist CCPA, which triggers a gradual spontaneous contraction reduction in a concentration-dependent manner. Concentration–response curve for CCPA was constructed by cumulative addition of the drug. In order to validate the assay, the well-known A_1R antagonist DPCPX in the presence of CCPA was studied and the shift on the right of CCPA concentration-response curve confirmed the involvement of A_1R in mouse ileum rhythmic spontaneous contractions. Time control experiments showed that a second curve of the agonist was reproducible. A_1R ligands under study were evaluated as for CCPA and the curve registered. The agonist potency was expressed as ED_{50} as previously reported (Lambertucci et al., 2011).

Mouse heart rate

Animals were sacrificed by cervical dislocation, the heart was rapidly removed and mounted in Krebs solution at 30°C and aerated with 5% CO₂ : 95% O₂. Chronotropic activity was recorded isometrically. Tissues were equilibrated for at least 30 min and a cumulative concentration-response curve to CCPA was constructed. The concentration of agonist in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Following 30 min of washing, tissues were incubated with the antagonist DPCPX (30 nM) for 60 min, and a new concentration-response curve to the agonist was obtained. When studying the activation of the receptor, after 30 min washing, a cumulative concentration-

response curve of the agonist under study was constructed. Responses were expressed as a percentage of the maximal response obtained in the control curve. Contractions were recorded by means of a force displacement transducer connected to the MacLab system PowerLab/800. In addition, parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity. In all cases, parallel experiments in which tissues received only the reference agonist were run in order to check any variation in sensitivity. In order to check any variation in sensitivity. The agonist potency was expressed as ED_{50} and represents the dose which triggers 50% of the maximum effect.

Cell cultures

SH-SY5Y human neuroblastoma cells (RRID: CVCL_0019; Sigma-Aldrich from The European Collection of Authenticated Cell Cultures, ECACC, Public Health England) are not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee (ICLAC; http://iclac.org/databases/cross-contaminations/) and were not further authenticated. Cells were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μ g/ml) and L-glutamine (2 mM). The culture was maintained at 37°C in 95% air- 5% CO₂ in a humidified incubator. Experiments were performed next day after seeding with 80% of confluence.

In vitro OGD

The in vitro model of ischemia was achieved maintaining the cells under OGD for 4h followed by 24 h of recovery. Briefly, SH-SY5Y neuroblastoma cells were washed once with pre-warmed (37 °C) OGD medium (in mM, 154 NaCl, 5.4 KCl, 0.8 MgSO₄, 1 NaHPO₄, 1.8 CaCl₂, 26.2 NaHCO₃) that has been bubbled with an anaerobic gas mixture (95% N₂, 5% CO₂) for 30 min to remove residual oxygen. Cells were incubated with this medium at 37 °C for 4 h in an anoxia chamber [Modular incubator chamber (Cat# MIC-101) Billups-Rothenberg Inc] to produce lethal oxygen deprivation. Then, the OGD medium was replaced by an oxygenated minimum essential medium with Earle salts (L-glutamine-free) supplemented with 11 mM glucose and cells were further incubated in a humidified aerobic incubator at 37 °C for 24 h of recovery. Control cells were incubated with OGD medium with glucose (11 mM) for 4 h at normoxia.

In vitro treatment

 A_1R full (CPA) and partial (4 and 7) agonists were added to the cultures in a similar manner, 1 h prior, during OGD exposure and also during the subsequent 24 h (recovery period). The treatment with the selective A_1R antagonist DPCPX (100 nM) or the AC activator forskolin (1 μ M) was performed by adding the drug 30 min before full or partial agonists and keeping it in the culture medium until the cell survival evaluation.

Cell viability measurement

Trypan blue staining was carried out to assess the extent of cell death occurring after 24 h of recovery from OGD exposition. Cells were trypsinized and incubated with 0.1% trypan blue. A hemocytometer was used to count stained and unstained cells.

Electrophysiological experiments

Slice preparation and recordings

Hippocampal slices were prepared as follows. After sacrifice of animals by cervical dislocation, the hippocampus was removed and 450-um slices cut with a McIlwain tissue slicer (Cat # TC752, The Mickle Lab). Slices were maintained at room temperature (22-24°C) in ACSF containing (in mM): 126 NaCl, 3.5 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2 CaCl₂, 25 NaHCO₃, 11 glucose (pH 7.3) saturated with 95% O₂ and 5% CO₂. After incubation in ACSF for at least 1 h, a single slice was transferred to a submerged recording chamber and continuously superfused at 32-33°C with ACSF at a rate of 2.6 ml/min. The drugs were added to this superfusion medium. The perfusion apparatus was made of chemically inert materials (i.e., silicone tubing). Extracellular fEPSPs were recorded in stratum radiatum of the hippocampus with a glass microelectrode filled with 2 M NaCl solution (pipette resistance $2-5 \text{ M}\Omega$) upon stimulation of Schaffer collaterals with an insulated bipolar twisted NiCr electrode (50 μ m OD). Each pulse was delivered every 20s (square pulses of 100 μ s duration at a frequency of 0.05 Hz), and three consecutive responses were averaged. The stimulation intensity used in the fEPSP recordings was always adjusted to obtain a submaximal fEPSP slope (~60% of maximum) with minimum population spike contamination. Signals were acquired with a DAM-80 AC differential amplifier (Cat# 3484, WPI) and analyzed with the LTP program (Anderson and Collingridge, 2001). At least

10 min of stable baseline recording preceded drug application. Data were expressed as mean \pm SEM of n experiments (one slice tested per experiment. Slices were obtained from at least two animals for each set of the experiment). To allow for comparisons between different experiments, slope values were normalized, taking the average of the baseline values to be 100%. The drug effect was expressed as the mean percentage variation of the slope from baseline over the last 5 min of drug perfusion. The washout period lasted at least 30 min.

Paired-pulse stimulation

In PPS protocol, two consecutive pulses are applied 50-msec apart. In control condition, this protocol elicits a PPF, in which the response elicited by the second stimulus (R2) is greater than that elicited by the first stimulus (R1). The degree of PPF is quantified by the R2/R1 ratio and a modification of this ratio is an indication of a presynaptic action on neurotransmitter release (Schulz et al. 1994).

OGD in hippocampal slices

In hippocampal slices, OGD was obtained by superfusing the slice with ACSF with sucrose instead of glucose and gassed with nitrogen (95% $N_2 - 5\%$ CO₂). After 7 min of OGD, the slice was again superfused with normal, glucose-containing, oxygenated ACSF (Pugliese et al., 2003). The effects of drugs on OGD were expressed as the mean percentage variation of the slope from baseline over the last 5 min of washout.

In vivo evaluation of compound 7

In behavioral experiments we decided to evaluate how much compound **7**, the most promising of the two A_1 partial agonists tested, affect mouse locomotor activity *in vivo*, in comparison to the well-known A_1 full agonists CPA and CCPA. To this end we performed the *open field test* on C57Bl/6 mice (25.5 ± 2 g weight, 16 weeks-old) injected i.p. with different doses (0.1, 1, and 10 mg/kg) of compound **7**. The effects induced by compound **7** were compared to the decrease of locomotor activity induced by CPA and CCPA (0.1 and 1 mg/kg). We failed to compare the effects at 10 mg/kg because, given their high potency, the full agonists gave already the maximal response at 1 mg/kg.

After acclimation to the behavioral testing room for at least 30 min, mice were administered with vehicle, CCPA, CPA or compound **7** and, after 5 minutes from injection of drugs, placed

into the center of a Plexiglas chamber (43x43x20cm) for 30 minutes. During the first 5 minutes after the administration of compound 7 mice were awake and maintained a normal posture while CPA and CCPA seemed to affect mice wakefulness and posture soon after their administration (personal observation). Mouse movement was monitored with an overhead camera and total distance traveled (measure of locomotion) was measured by using the Anymaze video tracking system (Cat# 60000, UGO BASILE).

Statistics

Results from *in vitro* experiments were expressed as a percentage of control, which was considered as 100%, and as mean \pm SEM values of at least 3 independently performed experiments (each independent experiment corresponds to an independent cell culture preparation). In vitro data were first subjected to normality test (D'Agostino and Pearson Omnibus Normality test); since we found that parameters did not regularly follow a normal distribution, we applied Mann-Whitney test. Results from electrophysiological experiments were expressed as mean \pm SEM from n slices. In the Legends the number of animals from which the slices have been obtained are reported for each data set. Results from behavioral experiments were presented as mean \pm SEM from n mice. A p-value <0.05 was considered to indicate a significant difference. Statistical analysis of the data was performed using Mann-Whitney or Wilcoxon tests for electrophysiology experiments and for behavioral experiments. Statistical analyses and curve fittings were obtained by using GraphPad Prism software (version 6.05, GraphPad Software, San Diego, CA, US). Test for outliers (i.e. Grubbs test) were not applied.

Results

Synthesis of deoxynucleosides 4 and 7

The 2'- and 3'-deoxy derivatives were synthesized using a convergent approach: the suitable sugars were synthesized and then they were reacted with the appropriate purine base. The obtained products were further modified to obtain the final desired nucleosides (Fig. 2, schemes 1 and 2).

The synthesis of 2-chloro-2'-deoxy- N^6 -cyclopentyladenosine (4) has been previously reported, hence, it will not be described in detail here (Fig. 2, scheme 1, Vittori et al., 2000).

The synthesis has been carried out by reacting 2,6-dichloropurine (1) with 1-chloro-2-deoxy-3,6-ditolylribose (2) in anhydrous conditions in the presence of sodium hydride to get the protected nucleoside **3** (Fig. 2, scheme 1). This intermediate was then reacted with cyclopentylamine and then with ammonia saturated in methanol to remove the protecting groups at the sugar moiety. The suitable nucleoside **4** (2'-dCCPA) has been obtained in good yield (Fig. 2, scheme 1). The 2-chloro-3'-deoxy- N^6 -cyclopentyladenosine (7) was prepared by reaction of the protected 3-deoxy-ribofuranose **5**, prepared by the yet reported procedure, (Rizzo et al., 1992) with the commercially available 2,6-dichloropurine (**1**, Fig. 2, scheme 2). The glycosylation reaction was performed using tin tetrachloride in anhydrous acetonitrile. The protected 2,6-dichloropurine glycoside **6** is obtained in 84% yield after silica gel chromatography. The latter compound **6** was first reacted with cyclopentylamine and then with methanolic ammonia to completely remove protecting groups at the sugar moiety of the obtained nucleoside. The desired 3'-deoxynucleoside **7** (3'-dCCPA) was obtained in 93% yield after silica gel chromatography and crystallization (Fig. 2, scheme 2).

Binding assay of compounds 4 and 7

The deoxy nucleosides **4** and **7** were evaluated at the human recombinant ARs, stably transfected into CHO cells, using radioligand binding studies (A₁R, A_{2A}R, A₃R, Table 1 in *Supporting information*) or AC activity assay (A_{2B}R) to assess their affinity for A₁R and their binding selectivity for the other adenosine receptor subtypes. Receptor binding affinity was determined using [³H]CCPA as a radioligand for A₁R, whereas [³H]NECA was used for A_{2A}R and [³H]HEMADO for A₃R subtype (Table 1 in *Supporting information*; Klotz et al., 1998; Klotz et al., 2007). In the case of A_{2B}R, potential stimulation or inhibition (EC₅₀ and IC₅₀ values) was determined by measurement of AC activity or of inhibition of NECA-stimulated AC activity, respectively. Both deoxy nucleosides showed no measurable interaction with the A_{2B} subtype (EC₅₀ and IC₅₀ >30,000 nM), hence, these data are not reported in the table 1. The full A₁R agonist CCPA was reported as a reference compound (Cristalli et al., 1998).

The reference A_1R agonist CCPA is endowed with high affinity for A_1Rs , with Ki = 0.83 nM, and high selectivity versus the $A_{2A}R$ subtype of about 2,770 fold, while it presents a moderate selectivity versus the A_3Rs (51 fold). Removal of the 2' or 3'-hydroxyl group of the sugar moiety reduced the affinity for the A_1Rs . In particular, the 2'-hydroxyl group seemed to be

essential for the receptor binding, in fact, its lack caused a reduction of affinity by more than 600 fold of affinity (2'-dCCPA, **4**, *K*i $A_1 = 550$ nM). On the contrary, the lack of the 3'hydroxyl group of the sugar moiety seems to be better tolerated leading only to a 12 fold decrease in A_1 affinity (3'-dCCPA, **7**, *K*i $A_1 = 10$ nM). The lack of the hydroxyl groups caused also a consistent reduction in affinity at the two other adenosine receptor subtypes, which causes, in general, a reduction of the A_1R selectivity. An exception is represented by the 3'-deoxy derivative **7**, which showed an increased selectivity versus the A_3R sub type compared to the reference compound CCPA (select. $A_3/A_1 = 530$ vs 51, respectively). Hence, the 3'-dCCPA showed the best balance of A_3 affinity and selectivity (**7**, *K*i = 10 nM, sel $A_{2A}/A_1 = 1,040$, select. $A_3/A_1 = 530$).

Functional assays (in vitro and ex vivo) of compounds 4 and 7

Functional activity of compounds **4** and **7** was assessed both *in vitro*, on CHO cells stably transfected with human A_1 adenosine receptors, and in *ex vivo* assays on mouse ileum and on mouse heart (Table 2 in *Supporting information*; Fig. 3).

Functional assays at human A_1R stably transfected in CHO cells

Firstly, functional activity of compounds **4** and **7** at A_1Rs was studied at human adenosine receptors stably transfected in CHO cells by evaluating the inhibition of AC activity. AC was first stimulated by forskolin at the concentration of 10 μ M and the inhibition of the enzyme activity caused by agonists was evaluated (Klotz et al., 1985, 1998). Inhibition of AC by compounds **4** and **7** was compared to that induced by the full agonist CCPA whose intrinsic activity (α) was set equal to 1 (Table 2 in *Supporting information*; Fig. 3, panel a).

In this assay, as expected, CCPA was able to induce the maximal response with an EC₅₀ of 15 nM. Compounds **4** and **7** showed a different behavior. In fact, while **4** was not able to induce the maximal effect ($\alpha = 0.70$, EC₅₀ = 832), compound **7** behaved as a full agonist with an EC₅₀ = 269 nM. It is worthwhile to note that in this *in vitro* assay at hA₁Rs the 3'-deoxy derivative resulted to be a full agonist while the 2'-deoxy analog presented as a partial agonist.

Mouse Ileum Assay

This assay was performed on a segment of the intestine where the A_1R subtype is highly expressed, and it allows the monitoring of the spontaneous variation of intestinal contractions after agonist stimulation of the A_1R (Lambertucci et al., 2011). After the equilibration time, preparations were challenged with the full agonist CCPA, which triggers a gradual spontaneous reduction of contraction in a concentration-dependent manner (Fig. 3, panel b). Furthermore, the A_1R antagonist DPCPX was studied in the presence of CCPA and the rightshift of the CCPA concentration-response curve confirmed the involvement of A_1R in mouse ileum rhythmic spontaneous contractions (data not shown).

The two deoxy derivatives **4** and **7**, tested at increasing concentrations, were not able to reach the reduction of intestinal contraction induced by CCPA, hence, they acted as A₁R partial agonists. It is worthwhile to note that both their intrinsic activity and potency were very similar (**4**, $\alpha = 0.75$, EC₅₀ = 1,380 nM vs **7**, $\alpha = 0.78$, EC₅₀ = 1,318 nM).

Heart rate

To assess the effect induced by the administration of the compounds on a cardiovascular parameter (heart rate), compounds **4** and **7** were evaluated in an *ex vivo* model represented by the mouse isolated heart. The effect of the two deoxy nucleosides has been investigated in comparison with the A₁R full agonist CCPA which caused a decrease of heart rate as a consequence of its potent inhibitory activity. CCPA, at 10 μ M, reduced the normal mouse heart rate by 80%, while compounds **4** and **7** reduced the heart rate by 32 and 42%, respectively (Fig. 3, panel c). Both the deoxy nucleosides **4** and **7** behaved as partial A₁R agonists at mouse receptors, showing potency in the micromolar range on heart rate (Table 2; **4**: EC₅₀ = 1,698 nM; **7**: EC₅₀ = 1,548 nM).

Nucleosides 4 and 7 protect SH-SY5Y from OGD injury

The SH-SY5Y cell line is extensively used as an *in vitro* model to study neuronal injury or death because of its similarity to neurons in morphological, neurochemical and electrophysiological properties (Dal-Cim et al., 2012; Jantas et al., 2015; Zhang et al., 2011, Hermann et al., 2013). Moreover, these cells constitutively express moderate levels of A_1Rs (Salim et al., 2000; Canals et al., 2005).

SH-SY5Y cells were exposed to OGD for 4 h, followed by 24 h of reoxygenation; under this experimental condition, their viability was reduced to $\sim 60\%$ (third column in every panel, $63.2\pm3.7\%$ in Fig. 4a, $59.1\pm1.4\%$ in 4b, $60.4\pm2.1\%$ in 4c) in comparison to control-normoxia (99.7±1.1%, first column of each panel in Fig. 4a, 4b, 4c). To evaluate a possible neuroprotective effect, the A₁R partial agonists were applied to SH-SY5Y culture 1h before, during OGD treatment and during the recovery period (24 hours), until the assessment of cell viability. To compare the effects of our drugs to a full A_1R agonist, CPA was used at concentrations already known as selective (Vissiennon et al., 2006). Compound 4 exerted a protective effect toward OGD exposure at concentration of 0.5, 1, 10 µM (78.3±4.5%, $85.8\pm 3\%$, $82.6\pm 4\%$ respectively, as compared to the control; Fig. 4a); compound 7 showed a protective effect only at 0.5 and 1 μ M (76.7±6.4%, 73.6±3.5 of the control; Fig 4b). Both effects were blocked by the A₁R antagonist DPCPX (0.1 μ M, Fig. 4a, 4b). CPA showed a significant protective effect toward OGD damage at concentrations of 0.5 and 1 μ M (94.2±8.2% and 86.7±5.9% of the control, respectively; Fig. 4c), whereas at a concentration of 0.1 µM it was ineffective. DPCPX completely blocked CPA neuroprotection (Fig. 4c). DPCPX alone did not affect cell viability. Compound 4 (1 μ M), compound 7 (0.5 μ M), and CPA (0.5 μ M), did not show any effect on normoxia cell viability. Further data about the effect of forskolin versus A₁R partial agonists are reported in *Supporting information* (Fig. S1).

Effects of nucleosides 4 and 7 on synaptic transmission in hippocampal slices and on presynaptic neurotransmitter release

A₁Rs are largely distributed in the brain, especially in the hippocampal area (Fastbom et al., 1987; Wakabayashi et al., 2000; Fukumitsu et al., 2003); furthermore, given the well-known inhibitory action of A₁R full agonists on synaptic transmission (Dunwiddie and Fredholm, 1989; Wu and Saggau, 1994; Johansson et al., 2001), we started to explore the effects of **4** and **7** on fEPSPs recorded in extracellular electrophysiology performed in hippocampal CA1 area. Slices were perfused with **4** or **7** at a concentration of 2 μ M over 20 min, since lower (1 μ M) or higher (10 μ M) concentrations of the two compounds failed to clearly affect fEPSP (data not shown). Treatment with **4** induced a brief but significant reduction of the fEPSP slope (62.01±10.51% of the basal at the end of the 20 min application, n = 6, *p < 0.05 vs basal, according to Wilcoxon test, Fig. 5a, 5b). Perfusion with **7** provoked a more pronounced

reduction of fEPSP slope (50.65±6.12% of the basal at the end of the 20 min application, n = 5, *p < 0.05 vs basal, according to Wilcoxon test, Fig. 5d, 5e) lasting throughout the washout period (30 min). The effects of both A₁R partial agonists were fully prevented by DPCPX (500 nM, 30 min; 100.60±1.83%, n = 4, °p < 0.05 vs **4** and 107.9±5.28%, n = 4, °p < 0.05 vs **7** respectively, according to Mann-Whitney test, Fig. 5b, 5e). In our experimental conditions, DPCPX, when given alone, induced a slight and non-significant increase of the fEPSP slope (data not shown).

Since the A₁R-mediated inhibitory effect on neurotransmission predominantly results from a presynaptic reduction of neurotransmitter release (Manabe et al., 1993; Calabresi et al., 1997; Lupica et al., 1992; Prince and Stevens, 1992) accompanied by changes of the PPR (Schulz et al., 1994), we analyzed the fEPSP slope ratios of paired pulses delivered with a 50-ms interval. Perfusion with **4** (2 μ M, 20 min) induced a slight and non-significant increase of mean PPR (Fig. 5c), suggesting a low effect on presynaptic release, at least in our experimental conditions. Conversely, the application of compound **7** (2 μ M, 20 min) clearly increased the mean PPR (Fig. 5f), suggesting a strong effect of this drug on presynaptic release.

CPA was used as a positive control. The drug (30 nM, given over 20 min) induced a significant reduction of neurotransmission ($52.92 \pm 13.70\%$ of the basal at the end of the 20 min application, n = 5, *p < 0.05 vs basal, according to Wilcoxon test, Fig. 5g, 5h). The effect of CPA was fully prevented by DPCPX (500 nM, 30 min; 93.81 ± 3.14\%, n = 4, °p < 0.05 vs CPA, according to Mann-Whitney test, Fig. 5h). As expected, the application of CPA clearly increased the mean PPR (Fig. 5i), confirming the strong effect of this drug on presynaptic release.

Effects of compounds 4 and 7 on OGD in hippocampal slices

Hippocampus, and particularly the CA1 area, is more sensitive to ischemic insult than other areas of the brain (Schmidt-Kastner, 2015). We applied an OGD protocol of 7 min to hippocampal slices to produce an irreversible depression of synaptic transmission (Pugliese et al., 2003), eliminating transient reappearance of synaptic potentials linked to hyperexcitability phenomena (Zhu and Krnjevic, 1999; Fowler, 1990; Luhmann and Heinemann, 1992; Doolette and Kerr, 1995) in order to point out the possible protective effect of A_1R partial agonists. CPA was used as a control to confirm, in our experimental

conditions, the well-known protective effect of the A₁R full agonists on OGD treatment. The drug (30 nM, given over 10 min before OGD) allowed a significant recovery of neurotransmission ($62.95 \pm 10.61\%$, n = 5, *p < 0.05 vs OGD, according to Mann–Whitney test, Fig. 6a, 6b). In the presence of CPA, recovery was observed in 5 out of 7 slices tested.

Compound **4** (2 μ M, given over 10 min before OGD) induced a partial but significant recovery of neurotransmission (38.82±13.53%, n = 6, *p < 0.05 vs OGD according to Mann–Whitney test, Fig. 6c, 6d).

Analogously, we tested compound **7** in similar experiments in which the drug seemed to be very promising in terms of protection against OGD. Indeed, it allowed, in 4 out of 8 slices tested, a strong and clear recovery of synaptic activity after OGD, in a range between 27 and 160% of basal slope ($86.62\pm29.05\%$, n = 4, *p < 0.01 vs OGD according to Mann–Whitney test, Fig. 6e, 6f). Despite the huge variability observed in the effect of compound **7**, which prompted us to show the time course of single experiments, the neuroprotective potential of the drug in ischemia seems to be clear and to deserve further experimental evaluation.

Effects of nucleoside 7 on mouse locomotor activity

In behavioral experiments we decided to evaluate the potency of compound **7**, the most promising of the two A_1 partial agonists tested, in terms of effect on mouse locomotor activity *in vivo*, in comparison to the well-known inhibitory action of A_1 full agonists CPA and CCPA on the same parameter. To this end we performed the *open field test* in C57BI/6 mice injected i.p. with different doses (0.1, 1, and 10 mg/kg) of compound **7**. The effects induced by compound **7** were compared to the decreased locomotor activity induced by CPA and CCPA (0.1 and 1 mg/kg). We failed to compare the effects of the different compounds at 10 mg/kg because, given their high potency, the full agonists gave already the maximal response at 1 mg/kg.

In more detail, as can be seen in Figure 7, panel a, the compound 7 at the highest doses tested (i.e 1 and 10 mg/kg) induced a slight and non-significant reduction in locomotor activity, in terms of total distance traveled by mice, with respect to vehicle (Compound 7 at 10 mg/kg: 34.55 ± 4.04 m, n=4; Vehicle 42.09 ± 4.96 m, n=3; p>0.05 according to Mann–Whitney test); on the contrary, at the dose of 1 mg/kg either CCPA and CPA significantly reduced mice activity (CCPA: 2.96 ± 0.51 m, n=3; CPA: 6.49 ± 2.74 m, n=3; p<0.05 vs. Vehicle in both cases

according to Mann–Whitney test). When activity was analyzed over time, it was even more evident that none of the tested drugs at the dose of 0.1mg/kg was able to significantly affect locomotor activity of mice, although a trend to a reduction was induced by the full agonist CPA (Fig. 7 panel b). Oppositely, both CCPA and CPA administered at 1 mg/kg completely inhibited mice locomotor activity soon after ten minutes from the start of the test (Fig. 7 panel c, p<0.05 vs. Vehicle according to Mann–Whitney test); at the same dose, compound **7** induced a slight reduction of mice locomotor activity, quite evident after 20 minutes spent in the field. The weaker effect exerted by the compound **7** on locomotor activity, in comparison with CPA or CCPA given at the same dose, is in agreement with its effect on synaptic transmission in hippocampal slices; in these experiments a comparable depression of the fEPSP slope was obtained with the compound **7** at 2 μ M (Fig. 5d) and with CPA at 30 nM (Fig. 5g).

DISCUSSION

The neuroprotective effects of A_1R agonists have been widely demonstrated in several cerebral ischemia models, both *in vitro* (Goldberg et al., 1988) and *in vivo* (Rudolphi et al., 1992; von Lubitz et al., 1995). These effects are mostly attributable to the ability of A_1Rs to reduce, once activated, the presynaptic intake of calcium and, as a consequence, to inhibit the release of glutamate, the neurotransmitter responsible for excitotoxicity (Choi and Rothman, 1990). Unfortunately, the clinical exploitation of such protective effects has been strongly limited by several disadvantages typically associated with A_1R full agonists, first of all, their serious cardiovascular side effects (Belardinelli et al., 1995; Olsson et al., 1990).

Furthermore, other therapeutic tools currently available so far did not meet the expectations. Firstly, ADAC, very promising at the beginning, gave controversial results in terms of ability to cross the BBB (Gervitz et al., 2002). Secondly, the inhibition of adenosine kinase, and the consequent increase of adenosine, despite its protective action shown in a model of *in vivo* focal ischemia (Jiang et al., 1997), would expose to a risky non-selective stimulation of other P1 purinergic receptors. Finally, the allosteric modulators of A₁Rs were already successfully tested *in vitro* (Romagnoli et al., 2014), but are still under evaluation and their application in therapy is yet to come.

Based on their pharmacological properties, A_1R partial agonists should induce fewer side effects than full agonists and show comparable efficacy both in acute and chronic treatments. Even though such an issue remains to be fully elucidated, in the present study we evaluated,

for the first time, the neuroprotective potential of two A_1R partial agonists, namely compounds 4 and 7, in experimental models of cerebral ischemia, *in vitro* and *ex vivo*.

In a first screening, these compounds showed interesting features in terms of A_1R selectivity (Table 1) and intrinsic activity, which resulted to be low in mouse cardiac tissue and higher in mouse ileum (Table 2). Such an observation could encourage a possible use of such compounds in ischemia therapy, eventually in chronic treatments and, hopefully, with reduced cardiovascular side effects. First experimental results on partial agonists of the present study have been achieved in an *in vitro* experimental model of ischemia obtained in human neuroblastoma cells (SH-SY5Y) and support such a notion.

Once we got convincing protective effects in *in vitro* ischemia model, the efficacy of partial agonists 4 and 7 was verified in extracellular electrophysiological experiments performed in mouse hippocampal slices subjected to OGD; this ex vivo model is highly informative because it is characterized by a level of integration between pre- and postsynaptic neuronal, and also neuronal/glial, components, that is not reproducible in cell cultures. Furthermore, given the high level of A_1R expression in the hippocampus, and the well-known susceptibility of CA1 region to hypoxia, the OGD ex vivo model is particularly suitable for a preclinical evaluation of protective properties of new candidate compounds for therapy in cerebral ischemia. Moreover, such a model has been extensively developed and validated in previous extracellular electrophysiological studies performed by Pugliese and collaborators (Pugliese et al., 2003, 2009, Traini et al., 2011), in which the exposure of the hippocampal slice to seven-minutes OGD induces a huge, reproducible, and irreversible depression of synaptic transmission. The choice of such a drastic protocol has the advantage to confidently reveal any possible protective effect of tested drugs against OGD. Conversely, these effects may not be observed in the totality of slices challenged, and then it is a good rule to specify, as we also did in the results section, the number of slices that positively responded to the treatment. First of all, we started by confirming the protective effect of the A_1R full agonist CPA on OGD in hippocampal slices under our experimental conditions. As expected, this drug significantly increased the recovery of fEPSP after OGD, in addition to having clearly reduced the basal synaptic transmission in the very first minutes of the application at a very low concentration (30 nM, Fig. 6a). Such a high responsivity of the hippocampus to A_1R stimulation confirms both the high expression of the receptor in this specific brain area and its inhibitory properties on synaptic activity. After the refinement of the protocol obtained by using CPA, we moved to the evaluation of the effects of A_1R partial agonists 4 and 7 in similar extracellular electrophysiology experiments. The two compounds were able to induce, as expected after A_1R stimulation, the reduction of both basal synaptic transmission and presynaptic neurotransmitter release; these effects were particularly evident and long lasting in the case of compound 7 (Fig. 5d, 5e, 5f), in agreement with its higher affinity and selectivity for A_1R compared to 4 (Tables 1 and 2 in Supporting information). The ability to reduce the presynaptic neurotransmitter release is the main mechanism by which the A_1R exerts its control on synaptic transmission (O'Kane and Stone, 1998), and it is a fundamental requirement for a potential therapy of cerebral ischemia, in which the excessive glutamate release induces most of the tissue damage (Choi and Rothman, 1990). Finally, the more relevant results of the present study regard the protective effects shown by 4 and 7 in the OGD experimental paradigm. Both compounds induced a significant recovery of hippocampal synaptic transmission after the irreversible disappearance of fEPSP due to OGD. In particular compound 7, in addition to the clear effect of reduction of presynaptic neurotransmitter release (Fig. 5f), allowed a very good recovery of hippocampal synaptic transmission, partial or even full depending on the single experiments (Fig. 6e, 6f); such a result is very promising given the severity of the OGD protocol applied to hippocampal slices.

It should be noted that the performance of the compound 7 in terms of protection against OGD in slices (Fig. 6e, 6f) seems to be proportional, analogously to the full A_1 agonist CPA (Fig. 6a, 6b), to the magnitude and duration of the reduction of the basal synaptic transmission and presynaptic release (Fig. 5d, 5e, 5f), which are both maintained all along the washout period.

Anyway, it is likely that the neuromodulation exerted by A₁R on presynaptic release is not sufficient to explain the neuroprotective effects of adenosine or partial agonists. Indeed, immediately after few minutes of hypoxia, a large and long-lasting neuronal depolarization occurs (Zhang and Krnjevic, 1993; Kaminogo et al., 1998); this seems to depend not only on the adenosine-induced inhibition of presynaptic release but also on the failing energy potential in cells, which represents an extreme attempt to protect neurons from a harmful condition (Fowler and Li, 1998; Balestrino et al., 1999). Furthermore, A₁R stimulation is also able to activate G-protein-coupled inwardly rectifying potassium channels (Sickmann and Alzheimer, 2003), which, in turn, mediate hyperpolarization of the membrane and decrease neuronal excitability; such an effect is particularly evident in CA1 dorsal hippocampal pyramidal cells (Kim and Johnston, 2015). Differently from adenosine which exerts its

protective function after the ischemic episode, in a time window of hours, in our experimental condition the efficacy of partial agonists depends mainly on the fact that they are applied before the OGD. It will be necessary, in future studies, to evaluate the therapeutic potential of these compounds when administered after the ischemic attack. In any case, even if their efficacy is confirmed only in pretreatment, these compounds may still be useful alternatively to anticoagulant drugs currently used to prevent relapse in patients already affected by mild stroke (Young et al., 2007; Ginsberg, 2009; Moskowitz et al., 2010; Albers et al., 2011; Macrez et al., 2011).

In the present work we started to explore, to a limited extent, the behavioral profile in mice of the compound **7**, the most promising of the two A_1 partial agonists tested, by using the *open field* test. In such a model, it has been previously demonstrated that A_1 agonists (i.e. CPA) are able to reduce spontaneous motor activity by acting at central, and not peripheral, level (Marston et al., 1998). Similarly, the reduction in locomotor activity induced by the compound **7** (Fig. 7) should be due to a central effect, and suggests an ability of the drug to cross the BBB.

By the way, a further evaluation of the effects of compounds **4** and **7** in *in vivo* models of cerebral ischemia is mandatory in order to confirm their ability to cross the BBB and to induce central effects, like the full agonist CCPA (from which they are derived) does; it will be necessary to have confirmation, both *ex vivo* and *in vivo*, of a reduced receptor desensitization induced by compounds **4** and **7**, a key requirement for chronically administering these substances; finally, and above all, it will be necessary to get confirmation of a reduced cardiovascular effect caused by these partial agonists, only suggested, at the moment, by the lowest intrinsic activity they showed in mouse heart tissue compared to other isolated organs.

Although further studies are needed to fully elucidate their neuroprotective potential, our results indicate that A_1R partial agonists **4** and **7** may represent a potential alternative to the treatment of cerebral ischemia, given alone or in combination with other substances such as antagonists of the $A_{2A}R$, which in hippocampus inhibits the modulation of synaptic transmission mediated by A_1Rs (Cunha et al., 1994).

Pre-registration of the study

The present study has not been pre-registered.

--Human subjects -Involves human subjects:
If yes: Informed consent & ethics approval achieved:
=> if yes, please ensure that the info "Informed consent was achieved for all
subjects, and the experiments were approved by the local ethics committee." is
included in the Methods.

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ARRIVE guidelines have been followed:
Yes
=> if it is a Review or Editorial, skip complete sentence => if No, include a
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Conflict of interest

The authors declare no issues regarding conflicts of interest.

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Legends

Figure 1

Time-line diagram of animal experiments and analysis.

Figure 2

Synthetic schemes for the synthesis of nucleosides 4 and 7.

The new nucleosides were obtained through a convergent approach by reaction of 1 (2,6dichloropurine) with the suitable sugar 2 (1-chloro-2-deoxy-3,6-ditolylribose) or 5 (3-deoxyribofuranose). The two intermediates 3 or 6 were, then, treated, in turn, with cyclopentylamine and methanolic ammonia to furnish final compounds 4 (2-chloro-2'-deoxy- N^6 -cyclopentyladenosine) and 7 (2-chloro-3'-deoxy- N^6 -cyclopentyladenosine).

Scheme 1, reagents and conditions: (a) NaH, CH₃CN anhydrous; (b) i. $cC_5H_9NH_2$, Et₃N, Δ , ii. NH₃/CH₃OH.

Scheme 2, reagents and conditions: (a) $SnCl_4$, CH_3CN anhydrous; (b) i. $cC_5H_9NH_2$, Et_3N , Δ , ii. NH_3/CH_3OH .

Figure 3

Functional activity of compounds 4 and 7 versus the activity of the reference agonist CCPA. Panel a: concentration-response curve of compounds 4 and 7 in comparison with that of the reference agonist CCPA at CHO cells stably expressing the hA_1AR ; changes in cAMP levels are expressed as % decrease of luminescence intensity.

Panel b: concentration-response curves of compounds **4**, **7**, and CCPA obtained by inhibition of mouse ileum rhythmic spontaneous contractions.

Panel c: concentration-response curves of compounds 4, 7, and CCPA obtained by inhibition of mouse heart rate. Each point represents the mean of four or five experiments with a maximum SE lower than ± 10 .

Effect of A₁R partial (4 and 7) and full (CPA) agonists on SH-SY5Y cell death induced by OGD.

Compound 4 (a), compound 7 (b), and CPA (c) were applied to SH-SY5Y culture 1h prior, during OGD treatment, and during the recovery period. DPCPX was added 30 min before the drugs and kept throughout the OGD time and recovery time. Cell viability was evaluated by using trypan blue dye exclusion. In the dot-plot graphs, single values are indicated; the mean±SEM of at least 3 independent experiments (each one performed with an independent cell culture preparation) is expressed as a percentage of control which was considered to be 100%. [§]p < 0.01 vs CTR-normoxia group; *p < 0.05 vs OGD group; °p < 0.05 vs corresponding drug/OGD group (in every case according to Mann-Whitney test). Compound 4 (1 μ M), compound 7 (0.5 μ M), and CPA (0.5 μ M), did not show any effect on normoxia cell viability.

Figure 5

Effects of compounds 4 and 7 on synaptic transmission and on presynaptic neurotransmitter release in hippocampal slices

The time course of the fEPSP slope during the perfusion with compounds 4 (n = 4 animals, 6 slices), 7 (n = 5 animals, 5 slices), and CPA (n= 3 animals, 5 slices) is shown respectively in panels a, d, and g. The related dot-plot graphs (panels b, e, and h) represent the mean values of the fEPSP slope (taken at the end of the 20 min application) in slices perfused with compounds 4, 7, and CPA, in the presence (n = 4 animals, 4 slices for compound 4; n = 3 animals, 4 slices for compound 7; n = 4 animals, 4 slices for CPA) or absence of DPCPX. *p < 0.05 versus basal; °p < 0.05 versus 4, 7, and CPA.

The time course of the PPR (R2/R1) during the application of compounds 4 (n = 6), 7 (n = 5), and CPA (n=5) is shown respectively in panels c, f, and i. PPR has been evaluated with the same animals/slices used for the time course of the fEPSP slope.

Figure 6

Effects of compounds 4 and 7 on hippocampal slices subjected to OGD damage

The time course of the fEPSP slope before, during and after reperfusion in oxygenated ACSF (panels a, c, and e, black dots, n = 4 animals, 7 slices) shows the irreversible synaptic failure induced by OGD protocol.

CPA (panel a, white dots, and panel b) was used as a control to confirm the neuroprotection induced by A_1R full agonists toward the OGD effect (n = 5 animals, 5 slices, *p < 0.05 vs OGD). Insets in panel a show representative fEPSP recordings in correspondence of baseline (a), OGD (b), and washout (c). Calibration bars: 1 mV, 10 ms.

Compounds 4 and 7 were added 10 min before OGD. The time course of the fEPSP slope during the perfusion with compounds 4 (n = 3 animals, 6 slices, white dots) and 7 (n = 4 animals, 4 slices) is shown respectively in panels c and e.

Insets in panels c and e show representative fEPSP recordings in correspondence of baseline (a), OGD (b), and washout (c). Calibration bars: 0.5 mV, 10 ms in panel c and 1 mV, 10 ms in panel e (in which the representative fEPSPs are referred to the time course of experiment 4).

The dot-plot graphs show the mean values of the fEPSP slope obtained in slices subjected to OGD in presence of compounds 4 (panel d, *p < 0.05 vs OGD) and 7 (panel f, *p < 0.01 vs OGD).

Figure 7

Effects of compounds 7, CPA, and CCPA on locomotor activity in C57Bl/6 mice

The compound **7** at the highest doses tested (i.e 1 and 10 mg/kg) induced a slight and nonsignificant reduction in locomotor activity, in terms of total distance traveled by mice (n = 3 animals at 1 mg/kg, n = 4 animals at 10 mg/kg; panel a), with respect to vehicle (n = 3 animals, panel a); on the contrary, at the dose of 1 mg/kg either CCPA and CPA significantly reduced mice activity (CCPA: n=3, p<0.05 vs. vehicle; CPA: n=3, p<0.05 vs. vehicle). In panel b activity was analyzed over time; none of the tested drugs at the dose of 0.1 mg/kg (n = 6 animals for compound **7**; n = 3 animals for CCPA; n = 3 animals for CPA) was able to significantly affect locomotor activity of mice. CCPA and CPA administered at 1 mg/kg completely inhibited mice locomotor activity soon after ten minutes from the start of the test (panel c, p<0.05 vs. Vehicle); at the same dose, compound **7** induced a slight reduction of mice locomotor activity, quite evident after 20 minutes spent in the field (panel c).

References

Albers G. W., Goldstein L. B., Hess D. C., Wechsler L. R., Furie K. L., Gorelick P. B., Hurn P., Liebeskind D. S., Nogueira R. G., Saver J. L. and STAIR VII Consortium (2011) Stroke Treatment Academic Industry Roundtable (STAIR) recommendations for maximizing the use of intravenous thrombolytics and expanding treatment options with intra-arterial and neuroprotective therapies. Stroke. 42, 2645-2650.

Albrecht-Kupper B. E., Leineweber K. and Nell P. G. (2012) Partial adenosine A1 receptor agonists for cardiovascular therapies. Purinergic Signal. 8, 91-99.

Anderson W.W., Collingridge G.L. (2001) The LTP Program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events, J Neurosci Methods. 108, 71-83.

Balestrino M., Rebaudo R., Lunardi G. (1999) Exogenous creatine delays anoxic depolarization and protects from hypoxic damage: dose-effect relationship. Brain Res. 816, 124-130.

Belardinelli, L., Shryock, J.C., Song, Y., Wang, D., Srinivas, M. (1995) Ionic basis of the electrophysiological actions of adenosine on cardiomyocytes. FASEB J. 9, 359–365.

Beresford I. J., Parsons A. A. and Hunter A. J. (2003) Treatments for stroke. Expert Opin. Emerg. Drugs. 8, 103-122.

Calabresi P., Centonze D., Pisani A. and Bernardi G. (1997) Endogenous adenosine mediates the presynaptic inhibition induced by aglycemia at corticostriatal synapses. J. Neurosci. 17, 4509-4516.

Canals M., Angulo E., Casado V., Canela E. I., Mallol J., Vinals F., Staines W., Tinner B., Hillion J., Agnati L., Fuxe K., Ferre S., Lluis C. and Franco R. (2005) Molecular mechanisms involved in the adenosine A and A receptor-induced neuronal differentiation in neuroblastoma cells and striatal primary cultures. J. Neurochem. 92, 337-348.

Chen F., Qi Z., Luo Y., Hinchliffe T., Ding G., Xia Y. and Ji X. (2014) Non-pharmaceutical therapies for stroke: mechanisms and clinical implications. Prog. Neurobiol. 115, 246-269.

Choi D. W. and Rothman S. M. (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu. Rev. Neurosci. 13, 171-182.

Cunha, R.A., Johansson, B., van der Ploeg, I., Sebastião, A.M., Ribeiro, J.A., Fredholm, B.B. (1994) Evidence for functionally important adenosine A2a receptors in the rat hippocampus. Brain Res. 649, 208-216.

Cristalli G., Camaioni E., Costanzi S., Vittori S., Volpini R., Klotz K.-N. Characterization of Potent Ligands at Human Recombinant Adenosine Receptors (1998) Drug Develop. Res. 45:176–181.

Dal-Cim T., Molz S., Egea J., Parada E., Romero A., Budni J., Martin de Saavedra M. D., del Barrio L., Tasca C. I. and Lopez M. G. (2012) Guanosine protects human neuroblastoma SH-SY5Y cells against mitochondrial oxidative stress by inducing heme oxigenase-1 via PI3K/Akt/GSK-3beta pathway. Neurochem. Int. 61, 397-404.

De Lean A., Hancock A. A. and Lefkowitz R. J. (1982) Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. Mol. Pharmacol. 21, 5-16.

de Mendonça A., Sebastiao A. M. and Ribeiro J. A. (2000) Adenosine: does it have a neuroprotective role after all? Brain Res. Brain Res. Rev. 33, 258-274.

Dong X. X., Wang Y. and Qin Z. H. (2009) Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharmacol. Sin. 30, 379-387.

Doolette D. J. and Kerr D. I. (1995) Hyperexcitability in CA1 of the rat hippocampal slice following hypoxia or adenosine. Brain Res. 677, 127-137.

Dunwiddie T. V. and Fredholm B. B. (1989) Adenosine A1 receptors inhibit adenylate cyclase activity and neurotransmitter release and hyperpolarize pyramidal neurons in rat hippocampus. J. Pharmacol. Exp. Ther. 249, 31-37.

Fastbom J., Pazos A. and Palacios J. M. (1987) The distribution of adenosine A1 receptors and 5'-nucleotidase in the brain of some commonly used experimental animals. Neuroscience. 22, 813-826.

Fowler J. C. (1990) Adenosine antagonists alter the synaptic response to in vitro ischemia in the rat hippocampus. Brain Res. 509, 331-334.

Fowler J.C., Li Y. (1998) Contributions of Na+ flux and the anoxic depolarization to adenosine 5'-triphosphate levels in hypoxic/hypoglycemic rat hippocampal slices. Neuroscience 83, 717-722.

Fredholm B. B., Abbracchio M. P., Burnstock G., Daly J. W., Harden T. K., Jacobson K. A., Leff P. and Williams M. (1994) Nomenclature and classification of purinoceptors. Pharmacol. Rev. 46, 143-156.

Fredholm B. B., Chen J. F., Masino S. A. and Vaugeois J. M. (2005) Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. Annu. Rev. Pharmacol. Toxicol. 45, 385-412.

Fredholm B. B., IJzerman A. P., Jacobson K. A., Linden J. and Muller C. E. (2011) International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. Pharmacol. Rev. 63, 1-34.

Fredholm B. B., Irenius E., Kull B. and Schulte G. (2001) Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. Biochem. Pharmacol. 61, 443-448.

Fukumitsu N., Ishii K., Kimura Y., Oda K., Sasaki T., Mori Y. and Ishiwata K. (2003) Imaging of adenosine A1 receptors in the human brain by positron emission tomography with [11C]MPDX. Ann. Nucl. Med. 17, 511-515.

Gervitz L.M., Lutherer L.O., Hamilton M.E., Fowler J.C. (2002) Lack of central effects of peripherally administered adenosine A(1) agonists on synaptic transmission in the rat hippocampus. Brain Res. 951, 141-145.

Gibson C. L. (2013) Cerebral ischemic stroke: is gender important? J. Cereb. Blood Flow Metab. 33, 1355-1361.

Ginsberg M. D. (2009) Current status of neuroprotection for cerebral ischemia: synoptic overview. Stroke. 40, S111-4.

Goldberg, M.P., Monyer, H., Weiss, J.H., Choi, D.W. (1988) Adenosine reduces cortical neuronal injury induced by oxygen or glucose deprivation in vitro. Neurosci. Lett. 89, 323-327.

Hermann D. M., Buga A. M. and Popa-Wagner A. (2013) Enabling brain plasticity and neurological recovery in the ischemic brain: effect of age and vascular risk factors as confounders. Rom. J. Morphol. Embryol. 54, 687-693.

Honig L. S. and Rosenberg R. N. (2000) Apoptosis and neurologic disease. Am. J. Med. 108, 317-330.

IJzerman A. P., Von Frijtag Drabbe Kunzel J. K., Kim J., Jiang Q. and Jacobson K. A. (1996) Site-directed mutagenesis of the human adenosine A2A receptor. Critical involvement of Glu13 in agonist recognition. Eur. J. Pharmacol. 310, 269-272.

Jantas D., Greda A., Leskiewicz M., Grygier B., Pilc A. and Lason W. (2015) Neuroprotective effects of mGluR II and III activators against staurosporineand doxorubicin-induced cellular injury in SH-SY5Y cells: New evidence for a mechanism involving inhibition of AIF translocation. Neurochem. Int. 88, 124-137.

Jiang N., Kowaluk E.A., Lee C.H., Mazdiyasni H., Chopp M. (1997) Adenosine kinase inhibition protects brain against transient focal ischemia in rats. Eur J Pharmacol. 320, 131–137.

Johansson B., Halldner L., Dunwiddie T. V., Masino S. A., Poelchen W., Gimenez-Llort L., Escorihuela R. M., Fernandez-Teruel A., Wiesenfeld-Hallin Z., Xu X. J., Hardemark A., Betsholtz C., Herlenius E. and Fredholm B. B. (2001)

Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. Proc. Natl. Acad. Sci. U. S. A. 98, 9407-9412.

Kaminogo M., Suyama K., Ichikura A., Onizuka M., Shibata S. (1998) Anoxic depolarization determines ischemic brain injury. Neurol. Res. 20, 343-348.

Kim C.S., Johnston D. (2015) A1 adenosine receptor-mediated GIRK channels contribute to the resting conductance of CA1 neurons in the dorsal hippocampus. J Neurophysiol. 113, 2511-2523.

Klotz K. N., Cristalli G., Grifantini M., Vittori S. and Lohse M. J. (1985) Photoaffinity labeling of A1-adenosine receptors. J. Biol. Chem. 260, 14659-14664.

Klotz K. N., Falgner N., Kachler S., Lambertucci C., Vittori S., Volpini R. and Cristalli G. (2007) 3H]HEMADO--a novel tritiated agonist selective for the human adenosine A3 receptor. Eur. J. Pharmacol. 556, 14-18.

Klotz K. N., Hessling J., Hegler J., Owman C., Kull B., Fredholm B. B. and Lohse M. J. (1998) Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. Naunyn Schmiedebergs Arch. Pharmacol. 357, 1-9.

Lambertucci C., Buccioni M., Cacciari B, Dal Ben D., Federico S., Klotz K.-N., Marucci G., Volpini R., Spalluto G., Cristalli G. (2011) New 9-methyl-8-(4-hydroxyphenyl)adenine derivatives as A₁ adenosine receptor antagonists. Collect. Czech. Chem. Commun. 76(11), 1379–1393.

Lo E. H. (2008) A new penumbra: transitioning from injury into repair after stroke. Nat. Med. 14, 497-500.

Longabaugh JP, Didsbury J, Spiegel A, Stiles GL. (1989) Modification of the rat adipocyte A1 adenosine receptor-adenylate cyclase system during chronic exposure to an A1 adenosine receptor agonist: alterations in the quantity of GS alpha and Gi alpha are not associated with changes in their mRNAs. Mol Pharmacol. 36, 681-688.

Lupica C. R., Proctor W. R. and Dunwiddie T. V. (1992) Presynaptic inhibition of excitatory synaptic transmission by adenosine in rat hippocampus:

analysis of unitary EPSP variance measured by whole-cell recording. J. Neurosci. 12, 3753-3764.

Macrez R., Ali C., Toutirais O., Le Mauff B., Defer G., Dirnagl U. and Vivien D. (2011) Stroke and the immune system: from pathophysiology to new therapeutic strategies. Lancet Neurol. 10, 471-480.

Manabe T., Yoshimori T., Henomatsu N. and Tashiro Y. (1993) Inhibitors of vacuolar-type H(+)-ATPase suppresses proliferation of cultured cells. J. Cell. Physiol. 157, 445-452.

Marston H. M., Finlayson K., Maemoto T., Olverman H. J., Akahane A., Sharkey J., Butcher S. P. (1998) Pharmacological characterization of a simple behavioral response mediated selectively by central adenosine A1 receptors, using in vivo and in vitro techniques. J. Pharmacol. Exp. Ther. 285, 1023-1030.

Mathot R. A., Wenden E. M., Soudijn W., Breimer D. D., Ijzerman A. P. and Danhof M. (1996) Partial agonism of the nonselective adenosine receptor agonist 8-butylaminoadenosine at the A1 receptor in vivo. J. Pharmacol. Exp. Ther. 279, 1439-1446.

Moskowitz M. A., Lo E. H. and Iadecola C. (2010) The science of stroke: mechanisms in search of treatments. Neuron. 67, 181-198.

O'Kane, E.M., Stone, T.W. (1998) Interaction between adenosine A1 and A2 receptor-mediated responses in the rat hippocampus in vitro. Eur J Pharmacol. 362, 17-25.

Olsson, R.A., Pearson, J.D. (1990) Cardiovascular purinoceptors. Physiol Rev. 70, 761-845.

Park SW, Kim JY, Ham A, Brown KM, Kim M, D'Agati VD, Lee HT (2012) A1 adenosine receptor allosteric enhancer PD-81723 protects against renal ischemia-reperfusion injury. Am J Physiol Renal Physiol. 303, F721-F732.

Parsons WJ, Stiles GL (1987) Heterologous desensitization of the inhibitory A1 adenosine receptor-adenylate cyclase system in rat adipocytes. Regulation of both Ns and Ni. J Biol Chem 262, 841–847.

Phillis J.W., Wu P.H. (1981) Phenothiazines inhibit adenosine uptake by rat brain synaptosomes. Can J Physiol Pharmacol. 59, 1108-1110.

Prince D. A. and Stevens C. F. (1992) Adenosine decreases neurotransmitter release at central synapses. Proc. Natl. Acad. Sci. U. S. A. 89, 8586-8590.

Pugliese A. M., Latini S., Corradetti R. and Pedata F. (2003) Brief, repeated, oxygen-glucose deprivation episodes protect neurotransmission from a longer ischemic episode in the in vitro hippocampus: role of adenosine receptors. Br. J. Pharmacol. 140, 305-314.

Pugliese A. M., Traini C., Cipriani S., Gianfriddo M., Mello T., Giovannini M.G., Galli A., Pedata F. (2009) The adenosine A2A receptor antagonist ZM241385 enhances neuronal survival after oxygen-glucose deprivation in rat CA1 hippocampal slices. Br J Pharmacol. 157: 818-830.

Rizzo C. J., Dougherty J. P., Breslow R. (1992) 3'-Deoxy-2'phosphoramidites of adenosine and 5-methyluridine used for the solid phase synthesis of unnatural 3'-deoxy-2'-5"-Oligonucleotides. Tetrahedron Lett. 33, 4129-4132.

Romagnoli R., Baraldi P.G., IJzerman A.P., Massink A., Cruz-Lopez O., Lopez-Cara L.C., Saponaro G., Preti D., Aghazadeh Tabrizi M., Baraldi S., Moorman A.R., Vincenzi F., Borea P.A., Varani K. (2014) Synthesis and biological evaluation of novel allosteric enhancers of the A1 adenosine receptor based on 2amino-3-(4'-chlorobenzoyl)-4-substituted-5-arylethynyl thiophene. J Med Chem. 57, 7673-7686.

Rudolphi K. A., Schubert P., Parkinson F. E. and Fredholm B. B. (1992) Neuroprotective role of adenosine in cerebral ischaemia. Trends Pharmacol. Sci. 13, 439-445.

Sabbah HN, Gupta RC, Kohli S, Wang M, Rastogi S, Zhang K, Zimmermann K, Diedrichs N, Albrecht-Küpper BE. (2013) Chronic therapy with a partial adenosine A1-receptor agonist improves left ventricular function and remodeling in dogs with advanced heart failure. Circ Heart Fail. 6: 563-571.

Salim H., Ferre S., Dalal A., Peterfreund R. A., Fuxe K., Vincent J. D. and Lledo P. M. (2000) Activation of adenosine A1 and A2A receptors modulates dopamine D2 receptor-induced responses in stably transfected human neuroblastoma cells. J. Neurochem. 74, 432-439.

Schaller B., Graf R. and Jacobs A. H. (2003) Ischaemic tolerance: a window to endogenous neuroprotection? Lancet. 362, 1007-1008.

Schmidt-Kastner R. (2015) Genomic approach to selective vulnerability of the hippocampus in brain ischemia-hypoxia. Neuroscience. 309, 259-279.

Schulz P. E., Cook E. P. and Johnston D. (1994) Changes in paired-pulse facilitation suggest presynaptic involvement in long-term potentiation. J. Neurosci. 14, 5325-5337.

Sickmann T., Alzheimer C. (2003) Short-term desensitization of G-proteinactivated, inwardly rectifying K+ (GIRK) currents in pyramidal neurons of rat neocortex. J Neurophysiol. 90, 2494-2503.

Srinivas M., Shryock J. C., Dennis D. M., Baker S. P. and Belardinelli L. (1997) Differential A1 adenosine receptor reserve for two actions of adenosine on guinea pig atrial myocytes. Mol. Pharmacol. 52, 683-691.

Tendera M., Sosnowski M., Gaszewska-Zurek E., Parma Z., Neuser D., Seman L. (2007) The oral adenosine A1 receptor agonist, BAY 68-4986, reduces heart rate at peak exercise in patients with chronic stable angina. J Am Coll Cardiol 49 (suppl A): 232A.

Traini C, Pedata F, Cipriani S, Mello T, Galli A, Giovannini MG, Cerbai F, Volpini R, Cristalli G, Pugliese AM. (2011) P2 receptor antagonists prevent synaptic failure and extracellular signal-regulated kinase 1/2 activation induced by oxygen and glucose deprivation in rat CA1 hippocampus in vitro. Eur J Neurosci. 33: 2203-2215.

van Schaick E. A., Zuideveld K. P., Tukker H. E., Langemeijer M. W., Ijzerman A. P. and Danhof M. (1998) Metabolic and cardiovascular effects of the adenosine A1 receptor agonist N6-(p-sulfophenyl)adenosine in diabetic Zucker rats: influence of the disease on the selectivity of action. J. Pharmacol. Exp. Ther. 287, 21-30.

Van Wylen D. G., Park T. S., Rubio R. and Berne R. M. (1986) Increases in cerebral interstitial fluid adenosine concentration during hypoxia, local potassium infusion, and ischemia. J. Cereb. Blood Flow Metab. 6, 522-528.

Vissiennon Z., Sichardt K., Koetter U., Brattstrom A. and Nieber K. (2006) Valerian extract Ze 911 inhibits postsynaptic potentials by activation of adenosine A1 receptors in rat cortical neurons. Planta Med. 72, 579-583.

Vittori S., Lorenzen A., Stannek C., Costanzi S., Volpini R., IJzerman A. P., Kunzel J. K. and Cristalli G. (2000) N-cycloalkyl derivatives of adenosine and 1-deazaadenosine as agonists and partial agonists of the A(1) adenosine receptor. J. Med. Chem. 43, 250-260.

Von Lubitz D. K., Lin R. C. and Jacobson K. A. (1995) Cerebral ischemia in gerbils: effects of acute and chronic treatment with adenosine A2A receptor agonist and antagonist. Eur. J. Pharmacol. 287, 295-302.

Wakabayashi S., Nariai T., Ishiwata K., Nagaoka T., Hirakawa K., Oda K., Sakiyama Y., Shumiya S., Toyama H., Suzuki F. and Senda M. (2000) A PET study of adenosine A1 receptor in anesthetized monkey brain. Nucl. Med. Biol. 27, 401-406.

White P. J., Rose'Meyer R. B. and Hope W. (1996) Functional characterization of adenosine receptors in the nucleus tractus solitarius mediating hypotensive responses in the rat. Br. J. Pharmacol. 117, 305-308.

Wu L. G. and Saggau P. (1994) Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus. Neuron. 12, 1139-1148.

Young A. R., Ali C., Duretete A. and Vivien D. (2007) Neuroprotection and stroke: time for a compromise. J. Neurochem. 103, 1302-1309.

Yuan J. and Yankner B. A. (2000) Apoptosis in the nervous system. Nature. 407, 802-809.

Yue X., Mehmet H., Penrice J., Cooper C., Cady E., Wyatt J. S., Reynolds E. O., Edwards A. D. and Squier M. V. (1997) Apoptosis and necrosis in the newborn piglet brain following transient cerebral hypoxia-ischaemia. Neuropathol. Appl. Neurobiol. 23, 16-25.

Zhang L., Krnjevic K. (1993) Whole-cell recording of anoxic effects on hippocampal neurons in slices. J. Neurophysiol. 69, 118-127.

Zhang H., Wang Z. Q., Zhao D. Y., Zheng D. M., Feng J., Song L. C. and Luo Y. (2011) AIF-mediated mitochondrial pathway is critical for the protective effect of diazoxide against SH-SY5Y cell apoptosis. Brain Res. 1370, 89-98.

Zhu P. J. and Krnjevic K. (1999) Persistent block of CA1 synaptic function by prolonged hypoxia. Neuroscience. 90, 759-770.





Scheme 2







-7 -6 log [Agonist] M

-5

-8

□ CCPA △ 4

-3

-4

0 7







d









