The Journal of Neuroscience, November 1, 1999, *19*(21):9192–9200

A_{2A} Adenosine Receptor Deficiency Attenuates Brain Injury Induced by Transient Focal Ischemia in Mice

Jiang-Fan Chen,[1] Zhihong Huang,[2] Jianya Ma,[2] JinMin Zhu,[2] Rosario Moratalla,[3] David Standaert,[3] Michael A. Moskowitz,[2] J. Stephen Fink,[1] and Michael A. Schwarzschild[1]

[1]*Molecular Neurobiology Laboratory,* [2]*Stroke and Neurovascular Regulation Laboratory, and* [3]*Neurology Research Laboratory, Department of Neurology and Neurosurgery, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114*

Extracellular adenosine critically modulates ischemic brain injury, at least in part through activation of the A_1 adenosine receptor. However, the role played by the A_{2A} receptor has been obscured by intrinsic limitations of A_{2A} adenosinergic agents. To overcome these pharmacological limitations, we explored the consequences of deleting the A_{2A} adenosine receptor on brain damage after transient focal ischemia. Cerebral morphology, as well as vascular and physiological measures (before, during, and after ischemia) did not differ between A_{2A} receptor knock-out and wild-type littermates. The volume of cerebral infarction, as well as the associated neurological deficit induced by transient filament occlusion of the middle cerebral artery, were significantly attenuated in A_{2A} receptor knock-out mice. This neuroprotective phenotype of A_{2A} receptor-deficient mice was observed in different genetic backgrounds, confirming A_{2A} receptor disruption as its cause. Together with complimentary pharmacological studies, these data suggest that A_{2A} receptors play a prominent role in the development of ischemic injury within brain and demonstrate the potential for anatomical and functional neuroprotection against stroke by A_{2A} receptor antagonists.

Key words: A_{2A} adenosine receptor; ischemia; stroke; purine receptor; knock-out; neuroprotection

The ubiquitous metabolic intermediary and nucleoside adenosine also serves as a neuromodulator under physiological conditions (Fredholm. et al., 1994). Growing evidence supports an important role for adenosine in modulating ischemic neuronal injury as well (Rudolphi et al., 1992; Deckert and Gleiter, 1994; Phillis, 1997; von Lubitz, 1997). First, adenosine levels markedly increase in response to cerebral ischemia and hypoxia as ATP breakdown dramatically increases the formation of adenosine. The extracellular levels of adenosine often rise faster (within minutes), higher (by more than 50-fold), and after smaller reductions in cerebral blood flow (CBF) compared with the levels of neurotransmitters such as glutamate (Hagberg et al., 1987; Matsumoto et al., 1992). Second, elevating extracellular adenosine levels by inhibiting adenosine degradation or uptake reduces ischemia-induced brain damage (Rudolphi et al., 1992). Third, adenosine analogs can attenuate hypoxic–ischemic neuronal injury, whereas certain adenosine antagonists exacerbate it (Rudolphi et al., 1992; Phillis, 1997; von Lubitz, 1997).

Although early studies suggested that adenosine acts predominantly as a neuroprotectant during cerebral ischemia (Deckert and Gleiter, 1994; Rudolphi et al., 1992), the complexity of the role of adenosine has been increasingly appreciated with the identification of four major adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3), each having a unique distribution among brain regions and their neuronal, glial, and vascular elements (Fredholm et al., 1994). Furthermore, these receptors are differentially coupled through G-protein receptors to second messengers, including cAMP and calcium (Fredholm et al., 1994, 1997). Nevertheless, the neuroprotective effects of adenosine can be attributed at least in part to A_1 receptor stimulation, as A_1-specific agonists and antagonists consistently attenuate and potentiate ischemic brain injury, respectively (Rudolphi et al., 1992). A_1 receptor-mediated neuroprotection may be a result of the inhibitory action of A_1 receptors on the release of excitatory amino acids such as glutamate (Rudolphi et al., 1992).

Much less is known about the role of A_{2A} receptors in ischemic damage (Phillis, 1997; von Lubitz, 1997). A_{2A} receptors are expressed at high levels in the striatum (Shiffmann et al., 1991; Fink et al., 1992; Svenningsson et al., 1998) but are also present in other brain regions, such as the cortex and hippocampus (Johansson et al., 1993; Weaver, 1993; Rosin et al., 1998), and on the endothelial and smooth muscle cells of the cerebral vasculature (Kalaria and Hank, 1986). Efforts to clarify the role of A_{2A} receptors in ischemic injury have produced mixed results. The relatively specific A_{2A} agonist CGS 21680 reduces ischemic or excitotoxic hippocampal damage (Scheardown and Knutsen, 1996; Jones et al., 1998). A_{2A} receptor-mediated vasodilation (Phillis, 1989; Ibayashi et al., 1991), inhibition of platelet aggregation (Sandoli et al., 1994; Ledent et al., 1997), and suppression of neutrophil superoxide generation (Cronstein, 1994; Jordan et al., 1997) may account for A_{2A} receptor-mediated protection. These beneficial vascular effects of A_{2A} receptor activation have been suggested as a partial explanation for the exacerbation of ischemic brain damage induced by the nonselective adenosine antagonists theophylline and caffeine (Rudolphi et al., 1992). On

Received April 30, 1999; revised Aug. 6, 1999; accepted Aug. 12, 1999.

This work was supported by National Institutes of Health Grants DA07496, 5P50 NS10828, NS01729, and NS31579 and grants from the National Alliance for Research on Schizophrenia and Depression, the Scottish-Rite, and the National Parkinson Foundation. We thank Dr. E. Li for assistance in the generation of the A_{2A} knock-out mice, and Yuehang Xu and Mark Beilstein for excellent technical assistance.

Correspondence should be addressed to Dr. Jiang-Fan Chen, Molecular Neurobiology Laboratory, 149 Massachusetts General Hospital East, 13th Street, Charlestown, MA 02129. E-mail: chenjf@helix.mgh.harvard.edu.

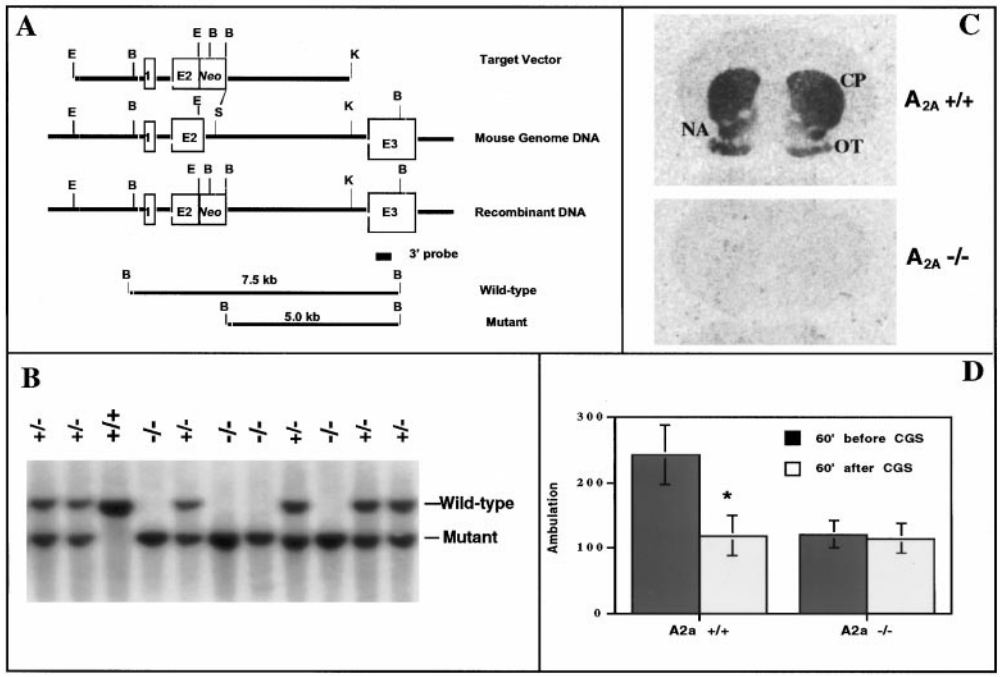

Figure 1. Generation of A_{2A} KO mice with target inactivation of the A_{2A} receptor. *A*, Schematic diagram of the A_{2A} receptor targeting vector; a standard replacement-type vector was constructed with 5 and 4.5 kb A_{2A} receptor genomic fragments split by a positive selection marker (*Neo* cassette), which replaced the 3′ end of exon 2 (*E2*) and the adjacent 5′ splice junction and intron sequences. Digestion of wild-type and mutant A_{2A} receptor genes with *Bam*HI (at sites labeled *B*) generates 7.5 and 5.0 kb fragments, respectively, that can be distinguished using a nonoverlapping 3′ probe (as in *B*). *B*, Genomic Southern analysis of WT (+/+), heterozygous (+/−), and homozygous (−/−) mice with respect to the A_{2A} receptor gene was performed as described in Materials and Methods, using the 3′ nonoverlapping probe illustrated in *A*. WT mice displayed a single 7.5 kb band, whereas homozygous A_{2A} KO mice showed a single 5.0 kb band corresponding to the restriction fragments for WT and mutant alleles, respectively. Heterozygous mice showed both 7.5 and 5.0 kb bands. *C*, Homozygous A_{2A} receptor KO mice are deficient in A_{2A} receptors detected by receptor autoradiography; A_{2A} receptor binding was determined using ^{3}H-CGS 21680 as a ligand. A representative coronal brain section from a WT mouse shows specific labeling of A_{2A} receptors in striatum (caudate putamen, *CP*; nucleus accumbens, *NA*) and olfactory tubercle (*OT*), whereas that from a homozygous mouse shows no ^{3}H-CGS 21680 binding. *D*, Behavioral responses to the A_{2A} agonist CGS 21680 in WT and A_{2A} KO mice; ambulation was measured in WT and A_{2A} KO mice (n = 14–16) before and after challenge with CGS 21680 (0.2 mg/kg, i.p.) by recording contiguous photobeam interruptions (ambulation) for 60 min. Error bars represent the mean ± SEM. $^*p < 0.05$ (Student's t test) when compared with ambulation in the WT mice before treatment.

the other hand, several relatively specific A_{2A} antagonists have been found to reduce ischemic damage in animal models of global or permanent ischemia, as well as in excitotoxic neuronal damage (Gao et al., 1994; Phillis, 1995; von Lubitz et al., 1995; Jones et al., 1998; Monopoli et al., 1998). A_{2A} receptor-mediated facilitation of glutamate release observed in ischemic cortex (O'Regan et al., 1992; Simpson et al., 1992) and striatum (Popoli et al., 1995; Corsi et al., 1997) may explain a protective effect of A_{2A} antagonists. Thus, the contradictory data on A_{2A} receptors in cerebral ischemia may reflect their potential to produce opposing effects through different (vascular and neuronal) mechanisms.

In addition, our understanding of how A_{2A} receptors influence ischemic injury has been confounded by the poor specificity and solubility of adenosine drugs. Almost all A_{2A} adenosine receptor agonists and antagonists also have some effects on A_1 or A_3 receptors (Jacobson et al., 1992; Ongini and Fredholm, 1996; Ongini et al., 1999). To help clarify the role that A_{2A} receptors play in neurological disorders such as stroke, we generated A_{2A} receptor knock-out (A_{2A} KO) mouse strains (which are distinct from a previously reported A2a KO strain) (Ledent et al., 1997) and examined the susceptibility of these mice to ischemic brain injury. We demonstrate that A_{2A} receptor inactivation attenuates brain damage and preserves neurological function after transient middle cerebral arterial (MCA) occlusion. These results strongly support the prospect that A_{2A} receptor blockade may offer neuroprotection against brain damage induced by transient focal ischemia.

MATERIALS AND METHODS

Generation of A_{2A} KO mice. Three independent genomic clones (~20 kb genomic DNA fragment) encoding a putative A_{2A} receptor gene from a mouse 129-Steel genomic library were isolated using the rat A_{2A} receptor cDNA as a probe. Characterization of the mouse A_{2A} receptor gene revealed an additional (previously unknown) exon in the 5′ untranslated region (Chen and Fink, 1996) (Fig. 1*A*). Based on this A_{2A} receptor genomic map, a standard replacement-type vector was constructed to inactivate the A_{2A} receptor gene. It consists of 5 and 4.5 kb of A_{2A} receptor genomic fragments (as the *left* and *right arms* of the *inset*, respectively) flanking a positive selection marker (PGK-*Neo* cassette). This target vector disrupts the A_{2A} receptor gene by replacing the 3′ end of exon 2 (12 bp from the splice junction site) and the adjacent intron sequences (0.9 kb from 5′ end of the splice junction site) with the PGK-*Neo* cassette. A dysfunctional mutant gene product was expected because the deleted 3′ end of exon 2 in the mouse A_{2A} receptor gene corresponds to a highly conserved region of the mouse A_{2A} receptor between the third and fourth transmembrane domains (Peterfreund et al., 1996).

Embryonic stem (ES) cells [129/SvJae, "Steel substrain" (Simpson et al., 1997)] were obtained from Dr. E. Li (Li et al., 1992) in the Knockout Core Facility at Massachusetts General Hospital. The A_{2A} receptor targeting vector was transferred into ES cells by electroporation. Targeted ES clones were selected and expanded in medium containing the aminoglycoside antibiotic G418. Mutant clones with the desired recombinant allele were identified by Southern blotting using a nonoverlapping 3′ probe after digestion with *Bam*HI (at sites designated by *B* in Fig. 1*A*). One of the ES cell clones (#50) containing the recombinant allele was injected into blastocysts and transferred to a host in the Knockout Core Facility. Viable chimeric mice were maintained until weaning, and these chimeric mice (F0) were bred to C57BL/6 (Taconic, Germantown, NY) or 129/SvEvTac mice [Steel substrain (Simpson et al., 1997), Taconic]. The germ line-transmitting mice with the A_{2A} receptor mutation were identified by Southern blot (F1). Heterozygous female and male mice from different founder mice were interbred to generate homozygous, heterozygous, and wild-type (WT) littermates mice, which were delivered at gestation day 21 in a normal mendelian distribution of A_{2A} receptor genotypes. The F2–F4 generations of A_{2A} homozygous, heterozygous, and wild-type littermates were used here. The hybrid (C57BL/6 × 129-Steel) mice were used for anatomical, immunohistochemical, and behavioral characterization of A_{2A} KO mice. Ischemic injury studies (including hemodynamic and other physiological measure-

ments) were performed in both the hybrid (C57BL/6 × 129-Steel) and pure 129-Steel strains.

Receptor autoradiography and immunohistochemistry. Receptor autoradiography for detecting A_{2A} and NMDA receptors using the specific ligands ^{3}H-CGS 21680 (46.0 Ci/mmol; NEN, Boston, MA) and ^{3}H-MK-801 (22.5 Ci/mmol; NEN), respectively, was performed as described previously (Johansson et al., 1993). For A_{2A} receptor binding, coronal brain sections were preincubated at room temperate with 50 mM Tris-HCl buffer, pH 7.7, and 1 U of adenosine deaminase for 20 min and then incubated with the Tris buffer containing 2.5 nM ^{3}H-CGS 21680 for 60 min. For NMDA receptor binding, the slides were preincubated in 50 mM Tris-acetate buffer twice at 4°C for 15 min each time and were then incubated with 5.0 nM ^{3}H-MK-801 in the presence of 30 μM glutamate and 10 μM glycine. To define nonspecific binding for the A_{2A} and NMDA receptors, 20 μM of 2-chloroadenosine or 5.0 μM MK-801, respectively, was coincubated in adjacent sections.

For immunohistochemistry, mice were anesthetized with Avertin (2% tribromoethanol, 1% tertiary amylalcohol) and fixed by transcardial perfusion with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The brains were post-fixed in the same solution for 2 hr and then cryoprotected in 20% glycerol. Brains were cut coronally in 25 μm sections with a sliding microtome. Immunostaining was performed in free-floating sections following standard avidin–biotin procedures described previously (Moratalla et al., 1996).

Hemodynamic and other physiological measurements. All procedures, measurements, and analyses were performed in a manner blinded to A_{2A} receptor genotype. Adult littermate mice (male and female, weighing 18–25 gm) were housed in the Massachusetts General Hospital Knockout Core facility under conditions of diurnal light cycling with access to food and water *ad libitum*. Anesthesia was induced by 2% halothane and maintained with 1% halothane in 70% N_2O and 30% O_2 using a Fluotec 3 vaporizer (Colonial Medical). In randomly selected mice ($n = 6$ for each group), the right femoral artery was cannulated with PE-10 polyethylene tubes for arterial blood pressure and heart rate measurement (ETH 400 transducer and MacLab/8 data acquisition system; AD Instruments) and blood gas determination using a pH/Blood Gas Analyzer (Corning 178; Ciba Corning Diagnostics, Medfield, MA). Core temperature was measured using a BAT-12 thermometer (Physitemp, Clifton, NJ). Core temperature was maintained at ~36.5–37.0°C with a thermostat (Frederick Haer Company, Bowdoinham, ME). Because hypothermia is a well known complication of prolonged ischemia, mice were kept in an incubator (ThermoCare Systems) at 32°C and 45% humidity for 6 hr after ischemia.

Focal transient ischemia (MCA occlusion) model. Focal cerebral ischemia was induced by occlusion of the left MCA with an 8–0 nylon monofilament (Ethicon, New Brunswick, NJ) coated with a mixture of silicone resin (Xantopren, Osaka, Japan) and a hardener (Elastomer Activator; Bayer, Etobicoke, Ontario, Canada) as described previously (Huang et al., 1994; Hara et al., 1996; Bonventre et al., 1997). This coated filament was introduced into the internal carotid artery through the external carotid artery, up to the origin of the anterior cerebral artery to occlude the MCA and anterior cerebral artery for 2 hr. For filament withdrawal, mice were briefly reanesthetized with halothane. In randomly selected mice ($n = 6$ for each group), cortical CBF was determined by a PF2B laser–Doppler flowmetry (Perimed, Stockholm, Sweden) and recorded on a MacLab/8 data acquisition system (AD Instruments). The tip of the probe was fixed 2 mm posterior and 6 mm lateral to bregma on the ipsilateral hemisphere. These coordinates identified the site on the convex brain surface within the vascular territory supplied by proximal segments of the MCA, and they corresponded to brain ischemic core area (Huang et al., 1994). Steady-state baseline values were recorded before MCA occlusion. Cortical CBF was recorded continuously before, during, and after ischemia and reperfusion and was expressed as percentage relative to the baseline value.

Measurement of neurological deficits and locomotion. For scoring neurological deficits, mice were ranked as described previously (Hara et al., 1996): 0, no observable neurological deficit (normal); 1, failure to extend right forepaw (mild); 2, circling to the contralateral side (moderate); and 3, loss of walking or righting reflex (severe). The animals were rated by an observer blinded to the genotypes.

Horizontal locomotor activity was assessed in polypropylene cages that were placed into adjustable frames equipped with seven infrared photocell beams, recorded, and analyzed on a computer (San Diego Instruments, San Diego, CA). Ambulation was quantified as the number of sequential breaks in adjacent beams. Mice were habituated in the test cages for at least 120 min before recording basal locomotion for 60 min. In assessing the motor-depressant effect of CGS 21680, locomotor activity was measured during the dark phase of the light cycle to obtain high basal locomotion. CGS 21680 was administered intraperitoneally (0.1 ml/10 gm), and locomotion was recorded for an additional 60 min.

Infarct volume measurement. Twenty-two hours after reperfusion, animals were decapitated under deep halothane anesthesia, and the brains were removed. For 2,3,5-triphenyltetrazolium chloride (TTC)-stained sections, brains were sectioned coronally into five 2 mm slices in a mouse brain matrix (RBM-2000C; Activational Systems). Slices were stained with 2% TTC (Sigma, St. Louis, MO) in PBS, followed by 10% formalin overnight. For hematoxylin–eosin stained cryostat sections, the brains were first immediately frozen in 2-methylbutane on dry ice and then sectioned coronally into 10 20-μm-thick slices (from +2.80 to −4.84 mm relative to bregma) in a microtome. Coronal sections were stained with hematoxylin and eosin. The infarct area (in square millimeters) of each TTC-stained section or hematoxylin–eosin-stained cryostat section was measured using an image analysis system (M4; Imaging Research, St. Catharine's, Ontario, Canada) on the posterior surface of each section. The total infarct volume was calculated by summing the volumes of the sections as described previously (Huang et al., 1994).

RESULTS

Targeted inactivation of the A_{2A} receptor in homozygous mutant mice

To generate mice lacking the A_{2A} receptor, a gene targeting vector was constructed with 10 kb of the murine A_{2A} receptor gene disrupted by a positive selection marker, *Neo* (Fig. 1*A*). The replacement of a critical stretch of nucleotides at the junction of exon 2 and its 3′ intron with the *Neo* cassette was designed to ensure that the resulting mutant gene does not encode a functional A_{2A} receptor. The A_{2A} receptor genotypes of mice generated with this vector were determined by Southern blot analysis, yielding the expected 7.5 and 5.0 kb labeled restriction fragments for wild-type and mutant alleles, respectively (Fig. 1*A*,*B*).

The absence of functional A_{2A} receptors in A_{2A} KO mice was demonstrated by receptor autoradiography with the A_{2A} receptor agonist ^{3}H-CGS 21680 (Fig. 1*C*). WT mice show specific labeling of A_{2A} receptors in the striatum and olfactory bulb, whereas homozygous A_{2A} KO mice show no ^{3}H-CGS 21680 binding in these regions. Finally, we examined the behavioral response to the A_{2A} receptor agonist CGS 21680 in A_{2A} KO mice to confirm the functional inactivation of A_{2A} receptors in the CNS. CGS 21680 (0.2 mg/kg) significantly decreased locomotor activity ($p < 0.05$) in WT mice. Although spontaneous locomotion in A_{2A} KO mice was lower than in WT mice, it was not decreased further by treatment with CGS 21680 (Fig. 1*D*). Similarly, the A_{2A} antagonist 8-(3-chlorostyryl)caffeine (CSC) induced motor stimulation in WT mice but not in A_{2A} KO mice (data not shown). Together, these genetic, neurochemical, and behavioral data demonstrate the functional disruption of A_{2A} receptors in homozygous mutant mice.

Development of striatum, cortex, and cerebral vasculature in the absence of A_{2A} receptors

A_{2A} KO mice appeared healthy and displayed no gross anatomical or behavioral abnormalities. The average body weight of WT and A_{2A} KO mice between postnatal days 76 and 90 did not differ (30.3 ± 1.0 and 30.1 ± 1.0 gm, respectively, for male mice; 25.7 ± 1.2 and 27.3 ± 0.8 gm, respectively, for female mice; $n = 23$–24).

The neuropeptide enkephalin is highly colocalized with A_{2A} receptors in the striatopallidal projection neurons (Shiffmann et al., 1991; Fink et al., 1992; Svenningsson et al., 1998), and thus its pattern of expression may be most sensitive to the absence of these receptors during development. The extent and distribution of enkephalin immunoreactivity appeared normal in KO mice (Fig. 2). Enkephalin immunostaining is concentrated in the ma-

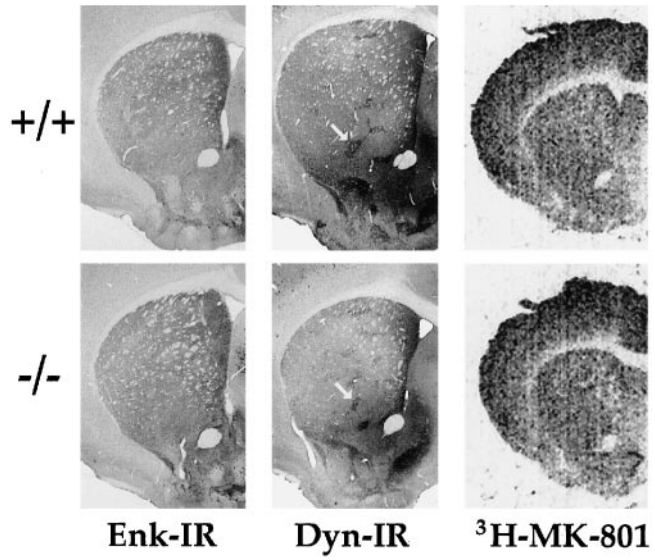

Figure 2. Neurochemical markers of striatal and cortical development in the absence of A_{2A} receptor. Representative coronal sections through corresponding levels of cortex and striatum are shown for A_{2A} WT (+/+, *top*) and KO (−/−, *bottom*) adult mice. Immunohistochemistry for enkephalin (*Enk-IR*) or dynorphin (*Dyn-IR*) and receptor autoradiography for NMDA receptor (*^{3}H-MK-801*) in striatum and cortex were performed on brain sections as described in Materials and Methods. The characteristic striosomal pattern of dynorphin in striatum was indistinguishable between A_{2A} WT and KO mice (*arrows*).

trix compartment of the striatum, and this pattern is preserved in A_{2A} KO mice. Conversely, dynorphin is a relatively specific marker for the striosomal compartment (Graybiel, 1990). Characteristic clusters of dynorphin-immunoreactive neurons in A_{2A} KO striatum were indistinguishable from those in WT mice (Fig. 2). In addition, cortical lamination assessed by Nissl staining was indistinguishable between A_{2A} WT and KO mice (data not shown). These results indicate that striatal and cortical architecture appears to have developed normally in the absence of A_{2A} receptors.

The possibility of neurochemical adaptations to the absence of A_{2A} receptors in the KO mice was also considered. Because NMDA receptors play a critical role in focal ischemia-induced neuronal cell death, we measured their binding density in cortex and striatum by receptor autoradiography using ^{3}H-MK-801 (dizocilpine). The levels for MK-801 binding sites were relatively high in cortex and moderate in striatum, and there was no significant difference between A_{2A} KO mice and their WT littermate ($n = 5–6$). The MK-801 binding densities in cortex were 856 ± 84 and 747 ± 37 fmol/mg tissue, and in striatum were 840 ± 89 and 777 ± 59 fmol/mg tissue, for A_{2A} KO and WT mice, respectively (Fig. 2).

Cerebrovascular and systemic physiology are indistinguishable between A_{2A} KO and WT mice in the MCA occlusion ischemia model

In the A_{2A} KO and WT mice with hybrid genetic background (C57BL/6 × 129-Steel; $n = 6$) (Table 1), there were no significant differences in basal mean arterial blood pressure (MABP) and heart rate. MABP in unanesthetized free-moving mice (pure 129-steel strain) also did not differ between the two groups (115 ± 5 and 110 ± 3.7 mmHg for A_{2A} WT and KO mice, respectively). Furthermore, preliminary study showed no significant difference in absolute blood flow between the two groups (data not shown). To exclude the potential contribution of strain-specific genes to the phenotypes, we also measured these physiological parameters in anesthetized A_{2A} KO and WT mice of a pure 129-Steel substrain (Simpson et al., 1997). Although we detected higher basal MABP in the pure 129-Steel mice (96 ± 12 and 98 ± 3 mmHg for A_{2A} KO and WT mice, respectively) compared with the hybrid C57BL/6 × 129-Steel mice (77 ± 4 and 74 ± 5 mmHg for A_{2A} KO and WT mice, respectively), there was no significant difference in MABP between A_{2A} KO and WT mice in either genetic background. Of note, Ledent et al. (1997) reported a hypertensive phenotype of A_{2A} KO mice, in contrast to the normal MABP we observed. The discrepancy in MABP may caused by the different strains of A_{2A} KO mice generated by Ledent et al. (1997) (hybrid CD-1 × 129/Sv strain) and our group (a hybrid C57BL/6 × 129-Steel strain, as well as a pure 129-Steel substrain).

Other physiological parameters that may influence ischemic injury did not differ between the two groups at any time point during the experiment. Immediately after MCA occlusion, cortical CBF decreased to ~20% of baseline and remained at this level during the 2 hr of ischemia ($n = 6$) (Table 1). After reperfusion, cortical CBF increased to 98–100% in both groups within 5 min. There were no significant differences in cortical CBF before, during, and after MCA occlusion between A_{2A} WT and KO mice with hybrid genetic background (C57BL/6 × 129-Steel) (Table 1) or with pure 129-Steel genetic background (data not shown). Finally, before and after ischemia, there was also no difference in core body temperature, arterial pH, or blood gas (PaO_2 and $PaCO_2$) values of mice with a hybrid C57BL/6 × 129-Steel strain (Table 1) or a pure 129-Steel substrain (data not shown) between A_{2A} KO and WT littermates.

A_{2A} receptor inactivation attenuates transient MCA occlusion-induced cerebral infarction

Twenty-two hours after reperfusion (i.e., 24 hr after onset of ischemia), total and regional (cortical and striatal) infarction was assessed by hematoxylin and eosin staining with volumetric analysis. Total infarct volume was reduced by 26% in A_{2A} KO (C57BL/6 × 129-Steel) mice compared with their WT littermates (61.2 ± 9.1 compared with 87.2 ± 3.2 mm^3, respectively; $n = 6$; $p < 0.05$) (Fig. 3*A*). Similarly, in pure 129-Steel mice, infarct volume was reduced by 30% in A_{2A} KO mice (51.0 ± 4.9 mm^3) compared with that of their WT littermates (72.8 ± 3.5 mm^3; $p < 0.05$). In a separate set of experiments, ischemic lesion volume was also determined by staining with TTC, a marker of intact cellular metabolism. A_{2A} KO mice (C57BL/6 × 129-Steel) showed an even more pronounced (77%) reduction of total lesion volume compared with their WT littermate control (18.0 ± 4.7 compared with 77.6 ± 13.4 mm^3, respectively; $n = 8–9$; $p < 0.05$). Lesion volumes appeared intermediate in size for A_{2A} heterozygous mice (56.1 ± 20.2 mm^3; $n = 6$; $p > 0.05$ compared with WT, and $p < 0.05$ compared KO mice).

Analysis of discrete infarct areas shows significant reductions in both cerebral cortex (33%) and striatum (27%) of A_{2A} KO mice (pure 129-Steel) when compared with the WT littermates (Fig. 3*B*). Cortical infarct volumes were 51.9 ± 3.1 and 35.0 ± 3.6 mm^3 for A_{2A} WT and KO mice, respectively ($n = 6$; $p < 0.05$). Striatal infarct volumes were 21.2 ± 1.3 and 15.5 ± 1.8 mm^3 for A_{2A} WT

Table 1. Cerebrovascular and systemic physiology before, during, and after MCA occlusion-induced ischemia in A_{2A} wild-type and knock-out mice

	Mean arterial BP (mmHg)		Cortical blood flow (%)		Heart rate (beats/min)	
Hemodynamics	A_{2A} +/+	A2a −/−	A_{2A} +/+	A2a −/−	A_{2A} +/+	A2a −/−
Before ischemia						
5′	74 ± 5	77 ± 4	100 ± 0	100 ± 0	486 ± 45	466 ± 38
During ischemia						
5′	80 ± 10	78 ± 13	20 ± 7	18 ± 3	494 ± 101	483 ± 39
10′	79 ± 10	76 ± 13	22 ± 8	19 ± 4	529 ± 70	495 ± 46
20′	80 ± 11	74 ± 8	21 ± 6	19 ± 4	542 ± 51	504 ± 63
30′	82 ± 10	75 ± 8	22 ± 5	20 ± 3	557 ± 75	506 ± 64
Before reperfusion						
5′	81 ± 7	84 ± 5	22 ± 7	20 ± 3	480 ± 21	471 ± 79
During reperfusion						
5′	81 ± 5	83 ± 7	66 ± 32	63 ± 21	474 ± 24	473 ± 70
10′	86 ± 9	83 ± 8	86 ± 19	82 ± 20	476 ± 23	461 ± 62
20′	85 ± 11	87 ± 12	104 ± 20	96 ± 21	498 ± 62	473 ± 108
30′	87 ± 9	85 ± 13	110 ± 11	108 ± 20	485 ± 80	475 ± 108

	pH		PaCO2 (mmHg)		PaO2 (mmHg)	
Blood gas analysis	A_{2A} +/+	A2a −/−	A_{2A} +/+	A2a −/−	A_{2A} +/+	A2a −/−
Before ischemia	7.34 ± 0.08	7.25 ± 0.05	40 ± 7	48 ± 4	140 ± 25	133 ± 26
After ischemia	7.30 ± 0.05	7.28 ± 0.05	46 ± 6	50 ± 7	166 ± 31	163 ± 52

Body temperature (°C)	A_{2A} +/+	A2a −/−
Before ischemia	36.9 ± 0.31	36.9 ± 0.23
After ischemia		
1 hr	36.9 ± 0.39	36.8 ± 0.30
3 hr	36.6 ± 0.32	36.7 ± 0.28
6 hr	36.5 ± 0.26	36.6 ± 0.16
20 hr	36.4 ± 0.20	36.4 ± 0.15

and KO mice, respectively ($p < 0.05$). Furthermore, there was no significant induction of A_{2A} receptors in cortex or striatum 24 hr after ischemia in WT mice. In fact, receptor autoradiography showed that focal ischemia significantly reduced binding density in the ipsilateral striatum (data not shown).

A_{2A} receptor inactivation preserves behavioral function after ischemic injury

We also evaluated functional outcome after MCA occlusion. A_{2A} KO mice displayed significantly fewer signs of neurological deficit compared with their WT littermates 24 hr after ischemia. Neurological deficit scores, assigned by an observer blinded to genotype, were reduced by 50–60% in A_{2A} KO compared with WT mice, in both hybrid C57BL/6 × 129-Steel ($n = 6$; $p < 0.05$) and pure 129-Steel ($n = 11$–12; $p < 0.05$) genetic backgrounds (Fig. 4).

DISCUSSION

The present study using an A_{2A} KO model clearly demonstrates that inactivation of the A_{2A} receptor protects the brain from transient focal ischemia. MCA occlusion followed by reperfusion produces significantly smaller infarct volumes and fewer neurological deficits in mice lacking the A_{2A} receptor. These data establish an important role for the A_{2A} receptor in neuroprotection against ischemic injury and advance the prospects for A_{2A} receptor blockade as a pharmacological strategy to reduce ischemic brain injury.

Transgenic inactivation of A_{2A} receptors attenuates ischemic injury

Pharmacological analyses of A_{2A} receptor involvement in cerebral ischemia have produced conflicting results. Nonselective antagonists caffeine and theophylline have been shown to either potentiate or attenuate ischemia-induced brain damage depending on treatment paradigm (Rudolphi et al., 1992; Jacobson et al., 1996). Furthermore, both agonists (Scheardown and Knutsen, 1996; Jones et al., 1998) and antagonists (Phillis, 1995; Jones et al., 1998; Monopoli et al., 1998) with relative selectivity for A_{2A} receptors have been shown to protect against brain damage in animal models of ischemic and excitotoxic neuronal injury. These mixed results may reflect complex actions of A_{2A} receptor activation during ischemia, as well as the intrinsic pharmacokinetic limitations of A_{2A} adenosine agents. For example, CGS 21680, one of the most selective and widely used A_{2A} receptor agonists, displays only a 140-fold selectivity for A_{2A} over A_1 receptors (Jacobson et al., 1992; Ongini and Fredholm, 1996). However, the effective concentrations of CGS 21680 used in different studies to modify neuronal death have differed by as much as 5000-fold (Scheardown and Knutsen, 1996; Jones et al., 1998). Similarly, CSC, an A_{2A} receptor antagonist frequently used to explore the

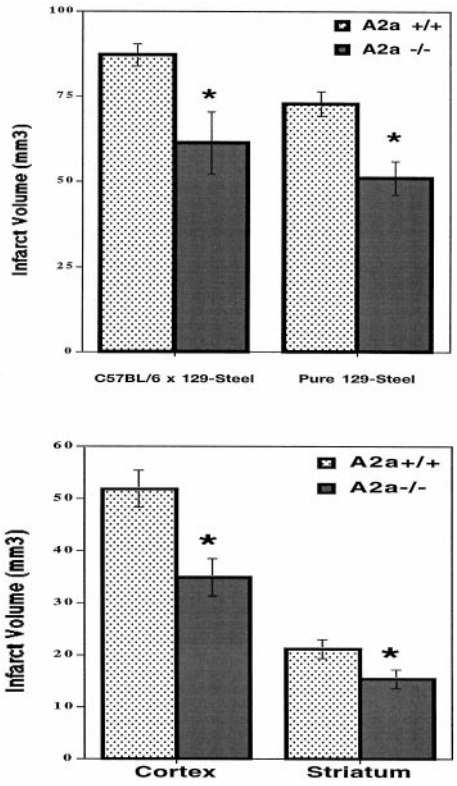

Figure 3. Inactivation of A_{2A} receptors attenuated MCA occlusion-induced infarction. *A*, Twenty-two hours after reperfusion, infarct volumes were determined using hematoxylin and eosin staining as described in Materials and Methods for A_{2A} KO and WT mice with hybrid C57BL/6 × 129-Steel genetic background ($n = 6$) as well as with pure 129-Steel genetic background ($n = 11$–12). *B*, Regional infarct volume was analyzed with respect to cerebral cortex and striatum of pure 129-Steel substrain mice ($n = 11$–12). $^*p < 0.05$ when comparing infarct volumes of A_{2A} KO mice with those of WT littermates (Student's *t* test).

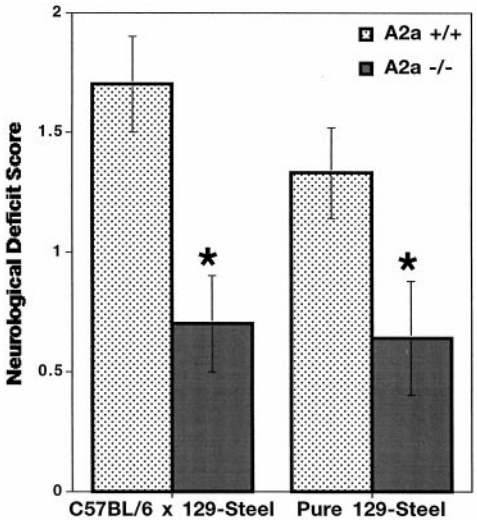

Figure 4. Inactivation of A_{2A} receptors enhances neurological function after MCA occlusion. Neurological deficit behavioral scores were assessed by a trained observer in a blinded manner as described in Materials and Methods. Neurological deficits were determined for A_{2A} KO and WT mice with the hybrid C57BL/6 × 129-Steel strain ($n = 6$) and with the pure 129-Steel strain ($n = 11$–12). $^*p < 0.05$ when comparing neurological deficit scores of A_{2A} KO mice with those of their WT littermates (Mann–Whitney *U* test).

effects of A_{2A} receptor blockade on ischemic damage, possesses high A_{2A} over A_1 receptor selectivity but displays poor solubility and CNS permeability, and it rapidly photoisomerizes to an inactive form (Ongini and Fredholm, 1996). These pharmacological limitations are overcome by genetic deletion of the A_{2A} receptor, leading to complete and selective inactivation of the A_{2A} receptor in A_{2A} KO mice. Thus, attenuation of MCA occlusion-induced cerebral infarction and neurological disability in A_{2A} KO mice provides the strongest evidence to date that blockade of the A_{2A} receptor reduces ischemic damage. Together with previous demonstrations of A_{2A} antagonist-induced protection from global or permanent ischemia, the neuroprotection observed in a transient focal ischemia model in A_{2A} KO mice advances the prospects for pharmacological intervention in ischemic stroke by blocking A_{2A} receptors.

Despite their important advantages, transgenic models raise unique considerations that must be addressed to properly interpret the data they generate (Silva et al., 1997). Most critically, a potential contribution of genetic background to a KO phenotype must be ruled out before the phenotype can be definitely attributed to the disruption of the "knocked-out" gene (Banbury Conference on Genetic Background in Mice, 1997). Transgenic studies of neuroprotection may be particularly susceptible to misinterpretation because of differences in genetic background (Schauwecker and Steward, 1997). In the present study, the attenuation of ischemic brain damage observed in A_{2A} KO mice on a standard hybrid genetic background (C57BL/6 × 129-Steel) was confirmed in separately derived A_{2A} KO mice of a pure 129-Steel substrain. This result verifies A_{2A} receptor inactivation as the basis for neuroprotection from cerebral ischemia in A_{2A} KO mice.

Also in contrast to pharmacological approaches, an A_{2A} receptor KO model does not readily distinguish between developmental, chronic, and acute effects of receptor inactivation. The detection of A_{2A} receptor mRNA in the CNS as early as embryonic day 15 in rats (Weaver, 1993) raises the possibility that brain development could be altered in A_{2A} KO mice such that their predisposition to ischemic injury is reduced. Similarly, chronic A_{2A} receptor antagonism may lead to upregulation of other receptors such as the A_1 receptor, which can itself attenuate ischemic injury. Indeed, neuroprotection offered by chronic treatment with the nonselective adenosine antagonist caffeine has been attributed to upregulation of A_1 receptors (Jacobson et al., 1996).

We found no evidence for contributing developmental or chronic changes in relevant anatomical and neurochemical systems. Vascular and parenchymal brain structures that we assessed were indistinguishable between A_{2A} KO and WT mice. Nissl staining and neuropeptide immunohistochemistry demonstrate normal laminar and compartmental patterns of cortical and striatal organization, respectively. Focal ischemic injury can be reduced by the NMDA receptor antagonist MK-801 (Pulsinelli et al., 1993) and the A_1 receptor agonist CHA (Rudolphi et al., 1992; von Lubitz, 1997). However, we demonstrated normal tritiated MK-801 binding density (Fig. 2*C*) and found no evidence for upregulation of A_1 receptor binding site density using the tritiated A_1 receptor ligands N^6 cyclohexyladenosine (CHA) and

1,3-dipropyl-8-cyclopentylxanthine (J.-F. Chen and M. A. Schwarzschild, unpublished observations). Thus, neurochemical assessment of these receptor binding sites showed no alteration in A_{2A} KO brains to account for their resistance to ischemia.

Alternatively, neuroprotection from cerebral ischemia in A_{2A} KO mice may reflect an effect of A_{2A} receptor inactivation during ischemic injury. Recent studies with a new generation of more specific A_{2A} receptor antagonists have suggested that blocking A_{2A} receptors can directly contribute to neuroprotection in a model of kainate-induced hippocampal damage (Jones et al., 1998), neonatal hypoxia–ischemia (Bona et al., 1997), and cerebral ischemia (Monopoli et al., 1998). Monopoli et al. (1998), for example, found that low doses of the A_{2A} receptor antagonist SCH 58261 (which binds A_{2A} receptors with 500-fold greater affinity than A_1 receptors) significantly reduces cortical infarction volume, even when administered 10 min after MCA occlusion. These emerging data are consistent with an acute effect of A_{2A} receptor deficiency in the neuroprotection observed in A_{2A} KO mice.

Multiple mechanisms may underlie the neuroprotection offered by A_{2A} KO mice

The apparent contradictions of published data on the A_{2A} receptor in neuroprotection point not only to the shortcomings of A_{2A} receptor pharmacology but also to the complexity of A_{2A} receptor biology. Indeed, multiple mechanisms, involving neuronal, vascular, and microglial elements, may underlie protection from cerebral ischemia offered by A_{2A} receptor deficiency in A_{2A} KO mice.

A neuronal basis for A_{2A} receptor modulation of ischemic injury has been suggested by studies showing adenosinergic regulation of glutamate and aspartate release. A massive release of these excitatory amino acids during brain ischemia plays a critical role in subsequent neuronal death. A_1 receptor stimulation attenuates this release and in this way likely attenuates ischemic damage (Rudolphi et al., 1992; von Lubitz, 1997). Conversely, A_{2A} receptor agonists enhance the release of glutamate under ischemic and nonischemic conditions (O'Regan et al., 1992; Simpson et al., 1992; Popoli et al., 1995), as well as the release of other neurotransmitters such as acetylcholine (Sebastiao and Ribeiro, 1996; Dunwiddie and Fredholm, 1997). To the extent that the inhibition of release by the A_1 receptor can be attributed to its negative coupling to calcium influx and/or cAMP production (Fredholm et al., 1994, 1997), the enhancement of neurotransmitter release by the A_{2A} receptor may be caused by its positive coupling to these second messenger systems (Gubitz et al., 1996; Fredholm et al., 1997). Thus, a pharmacological blockade or transgenic deficiency of the A_{2A} receptor may afford neuroprotection after ischemia because of reduced glutamate release and excitotoxicity.

Interestingly, the prominence of cortical as well as subcortical (striatal) protection from ischemia in A_{2A} KO mice belies the intense localization of brain A_{2A} receptors to the striatum (Fig. 1*C*). This apparent mismatch of regional protection and receptor density may be explained by the ability of the relatively sparse but well documented cortical A_{2A} receptors to markedly enhance glutamate release (Johansson et al., 1993; Sebastiao and Ribeiro, 1996; Dunwiddie and Fredholm, 1997). Evidence that these relatively low levels of A_{2A} receptor are in fact sufficient for potentiating neurotransmitter release is provided by demonstrations of A_{2A} receptors agonist-induced release in cortical (synaptosomal and slice) preparations (Sebastiao and Ribeiro, 1996; Dunwiddie and Fredholm, 1997). Monopoli et al. (1998) also noted the discrepancy between cortical protection by A_{2A} receptor inactivation and the dearth of cortical A_{2A} receptors. Although they raise the interesting possibility that ischemia may induce A_{2A} receptors in cortical glia, we found no autoradiographic evidence for cortical A_{2A} receptor induction 24 hr after transient ischemia. Alternatively, A_{2A} receptors may act trans-synaptically (at a neuronal network level) to modify cortical ischemic damage. For example, the extensive feedback projection from striatum to cortex via glutamatergic thalamocortical neurons potentially links striatal A_{2A} receptors with excitatory nerve terminals in cortex.

A vascular basis for the anti-ischemic phenotype of A_{2A} KO mice might also explain the widespread cerebral protection (i.e., far beyond the high density of neuronal A_{2A} receptors in striatum). A_{2A} adenosine agents are well known for their vasoactive properties (Phillis, 1989; Ibayashi et al., 1991), which result from the functions of A_{2A} receptors located on cerebral, as well as systemic, vasculature (Kalaria and Hank, 1986). However, activation of A_{2A} receptors on cerebral vascular smooth muscle and endothelial cells produces vasodilatation and thus may increase cerebral blood flow (Phillis, 1989; Ibayashi et al., 1991). Indeed, under hypoxic conditions, cortical blood flow is enhanced by the A_{2A} receptor agonist CGS 21680 and is reduced by the specific A_{2A} receptor antagonist ZM 241385 (Coney and Marshall, 1988). Hence, the pharmacological data would not predict a vascular mechanism of attenuated ischemic damage in A_{2A} KO mice. Moreover, direct comparison of cerebral blood flow and systemic cardiovascular parameters showed no difference between A_{2A} KO and WT mice before, during, or after MCA occlusion.

A_{2A} receptor activation also regulates the aggregation of platelets and the generation of reactive oxygen species, which may participate in the development of ischemic injury. Again however, A_{2A} receptor pharmacology would suggest that these functions do not contribute to the cerebroprotective phenotype of A_{2A} KO mice. A_{2A} receptor agonists have been shown to inhibit platelet aggregation (Sandoli et al., 1994; Ledent et al., 1997) and free radical generation by neutrophils (Cronstein, 1994; Jordan et al., 1997). Thus, A_{2A} receptor inactivation may be expected to enhance platelet initiation of vascular occlusion and neutrophil-triggered oxidative damage, neither of which would account for the observed reduction in infarct size in mice lacking the A_{2A} receptor. Furthermore, in our study, all other physiological parameters assessed for their potential contribution to ischemic injury (body temperature and blood pH, oxygenation and CO_2 content) were indistinguishable between KO and WT mice, both before and after ischemia. Together with previously published A_{2A} receptor pharmacology, the physiological and anatomical data reported here argue against a vascular mechanism underlying the neuroprotection seen in A_{2A} KO mice. Because of offsetting vascular actions of A_{2A} receptors, the potential for neuroprotection by A_{2A} receptor inactivation may be significantly underestimated. Selective blockade of neuronal A_{2A} receptors may therefore provide further protection against transient focal ischemia in brain than was observed in A_{2A} KO mice.

In conclusion, the A_{2A} KO model presented here demonstrates that A_{2A} receptor deficiency attenuates cerebral damage and dysfunction induced by transient focal ischemia and suggests that A_{2A} receptor stimulation may normally exacerbate cerebral infarction. Together with the well established protective effect of A_1 receptor stimulation, our data support a more refined view of adenosine signaling in ischemic brain injury. The high levels of extracellular adenosine in ischemic brain tissues may trigger

offsetting A_1 and A_{2A} receptor effects on neurotoxicity, possibly through opposing influences on glutamate release. The marked preservation of neurological function associated with attenuated cerebral infarction in A_{2A} KO mice highlights the potential benefit of A_{2A} receptor antagonists in the treatment of ischemic stroke. Moreover, the proposed model encourages the rational development of neuroprotective strategies involving potentially additive or synergistic effects of A_{2A} receptor blockade combined with A_1 receptor stimulation.

REFERENCES

Banbury conference on genetic background in mice (1997) Mutant mice and neuroscience: recommendations concerning genetic background. Neuron 19:755–759.

Bona E, Aden U, Gilland E, Fredholm BB, Hagberg H (1997) Neonatal cerebral hypoxia ischemia: the effect of adenosine receptor antagonists. Neuropharmacology 36:1327–1338.

Bonventre JV, Huang Z, Taheri MR, O'Leary E, Li E, Moskowitz MA, Sapirstein A (1997) Reduced fertility and postischemic brain injury in mice deficient in cytosolic phospholipase A2. Nature 390:622–625.

Chen J-F, Fink JS (1996) Characterization of the genomic structure of mouse A2aR adenosine receptor gene. Soc Neurosci Abstr 22:1569.

Coney AM, Marshall JM (1988) Role of adenosine and its receptors in the vasodilation induced in the cerebral cortex of the rat by systemic hypoxia. J Physiol (Lond) 509:507–518.

Corsi C, Pazzagli M, Bianchi L, Della Corte L, Pepeu G, Pedata F (1997) *In vivo* amino acid release from the striatum of aging rats: adenosine modulation. Neurobiol Aging 18:243–250.

Cronstein BN (1994) Adenosine, an endogenous anti-inflammatory agent. J Appl Physiol 76:5–13.

Deckert J, Gleiter CH (1994) Adenosine-an endogenous neuroprotective metabolite and neuromodulator. J Neural Transm 43:23–31.

Dunwiddie TV, Fredholm BB (1997) Adenosine neuromodulation. In: Purinergic approaches in experimental therapeutics (Jacobson KA, Jarvis MF, eds), pp 359–382. New York: Wiley-Liss.

Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM, Reppert SM (1992) Molecular cloning of the rat A2 adenosine receptor: selective coexpression with D2 dopamine receptors in rat striatum. Mol Brain Res 14:186–195.

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M (1994) Nomenclature and classification of purinoceptors. Pharmacol Rev 46:143–156.

Fredholm BB, Arslan G, Kull B, Kontny E, Svenningsson P (1997) Adenosine (P1) receptor signalling. Drug Dev Res 39:262–268.

Gao Y, Phillis JW (1994) CGS 15943, an adenosine A2 receptor antagonist, reduces cerebral ischaemic injury in the Mongolian gerbil. Life Sci 55:PL61–PL65.

Graybiel AM (1990) Neurotransmitters and neuromodulators in the basal ganglia. Trends Neurosci 13:244–254.

Gubitz AK, Widdowson L, Kurokawa M, Kirkpatrick KA, Richardson PJ (1996) Dual signalling by the adenosine A_{2A} receptor involves activation of both N- and P-type calcium channels by different G proteins and protein kinases in the same striatal nerve terminals. J Neurochem 67:374–381.

Hagberg H, Andersson P, Lacarewicz J, Jacobson I, Butcher S, Sandberg M (1987) Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. J Neurochem 49:227–231.

Hara H, Huang PL, Panahian N, Fishman MC, Moskowitz MA (1996) Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. J Cereb Blood Flow Metab 16:605–611.

Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA (1994) Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science 265:1883–1885.

Ibayashi S, Ngai AC, Meno JR, Winn HR (1991) Effects of topical adenosine analogs and forskolin on rat pial arterioles *in vivo*. J Cereb Blood Flow Metab 11:72–76.

Jacobson KA, van Galen, PJ, Williams M (1992) Adenosine receptors –pharmacology, structure activity relationships, and therapeutic potential. J Med Chem 35:407–422.

Jacobson KA, von Lubitz DK, Daly JW, Fredholm BB (1996) Adenosine receptor ligands: differences with acute versus chronic treatment. Trend Pharmacol Sci 17:108–113.

Johansson B, Georgiev V, Parkinson FE, Fredholm BB (1993) The binding of the adenosine A_{2A} receptor selective agonist [^{3}H]CGS 21680 to rat cortex differs from its binding to rat striatum. Eur J Pharmacol 247:103–110.

Jones PA, Smith RA, Stone TW (1998) Protection against kainate-induced excitotoxicity by adenosine A_{2A} receptor agonists and antagonists. Neuroscience 85:229–237.

Jordan JE, Zhao Z-Q, Sato H, Taft S, Vinten-Johansen J (1997) Adenosine A_2receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary endothelial adherence. J Pharmacol Exp Ther 280:301–309.

Kalaria RN, Harik SI (1986) Adenosine receptors of cerebral microvessels and choroid plexus. J Cereb Blood Flow Metab 6:463–470.

Ledent C, Vaugeois J-M, Schiffmann SN, Pedrazzini T, Yacoubi M, Vanderhaeghen J-J, Costentin J, Heath JK, Vassart G, Parmentier M (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A_{2A} receptor. Nature 388:674–678.

Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926.

Matsumoto K, Graf R, Rosner G, Shimada N, Heiss W-D (1992) Flow thresholds for extracellular purine catabolite elevation in cat focal ischemia. Brain Res 579:309–314.

Monopoli A, Lozza G, Forlani A, Mattavelli A, Ongini E (1998) Blockade of A_{2A} adenosine receptors by SCH 58261 results in neuroprotective effects in cerebral ischaemia in rats. NeuroReport 9:3955–3959.

Moratalla R, Elibol B, Vallejo M, Graybiel AM (1996) Network-level changes in inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. Neuron 17:147–156.

Ongini, E, Fredholm BB (1996) Pharmacology of adenosine A_{2A} receptors. Trend Pharmacol Sci 17:364–372.

Ongini E, Dionisotti S, Gessi S, Irenius E, Fredholm BB (1999) Comparison of CGS 15943, ZM 241385 and SCH 58261 as antagonists at human adenosine receptors. Naunyn Schmiedebergs Arch Pharmacol 359:7–10.

O'Regan MH, Simpson RE, Perkins LM, Phillis JW (1992) The selective A_2 agonist CGS 21680 enhances excitatory transmitter amino acid release from the ischemic rat cerebral cortex. Neurosci Lett 138:169–172.

Peterfreund RA, MacCollin M, Gusella J, Fink JS (1996) Characterization and expression of the human A2aR adenosine receptor gene. J Neurochem 66:362–368.

Phillis JW (1989) Adenosine in the control of the cerebral circulation. Cerebrovasc Brain Metab Rev 1:26–54.

Phillis JW (1995) The effects of selective A_1 and A2a adenosine receptor antagonists on cerebral ischemic injury in the gerbil. Brain Res 705:79–84.

Phillis JW (1997) Adenosine agonists and antagonists. In: Primer on cerebrovascular disease (Welch KMA, Caplan LR, Reis DJ, Siesjo BK, Weir B, eds), pp 250–253. San Diego: Academic.

Popoli P, Betto P, Reggio R, Ricciarello G (1995) Adenosine A_{2A} receptor stimulation enhances striatal extracellular glutamate levels in rats. Eur J Pharmacol 287:215–217.

Pulsinelli W, Sarokin A, Buchan A (1993) Antagonism of the NMDA and non-NMDA receptors in global versus focal brain ischemia. Prog Brain Res 96:125–135.

Rosin DL, Robeva A, Woodard RL, Guyenet PG, Linden J (1998) Immunohistochemical localization of adenosine A_{2A} receptors in the rat central nervous system. J Comp Neurol 401:163–186.

Rudolphi K, Schubert P, Parkinson FE Fredholm BB (1992) Neuroprotective role of adenosine in cerebral ischaemia. Trends Pharmacol Sci 13:439–445.

Sandoli D, Chiu PJS, Chintala M, Dionisotti S, Ongini E (1994) *In vivo* and *ex vivo* effects of adenosine A_1 and A2 receptor agonists on platelet aggregation in the rabbit. Eur J Pharmacol 259:43–49.

Schauwecker PE, Steward O (1997) Genetic determinants of susceptibility to excitotoxic cell death: Implications for gene targeting approaches. Proc Natl Acad Sci USA 94:4103–4108.

Scheardown MJ, Knutsen LJS (1996) Unexpected neuroprotection ob-

served with the adenosine A_{2A} receptor agonist CGS 21680. Drug Dev Res 39:108–114.

Schiffmann SN, Jacobs O, Vanderhaeghen J-J (1991) Striatal restricted adenosine A2 receptor (RDC8) is expressed by enkephalin but not by substance P neurons: an *in situ* hybridization histochemistry study. J Neurochem 57:1062–1067.

Sebastiao AM, Ribeiro JA (1996) Adenosine A2 receptor-mediated excitatory actions on the nervous system. Prog Neurobiol 48:167–189.

Silva AJ, Smith AM, Gieses KP (1997) Gene targeting and the biology of learning and memory. Annu Rev Genet 31:527–546.

Simpson EM, Linder CC, Sargent EE, Davisson MT, Mobraaten LE, Sharp JJ (1997) Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nat Genet 16:19–27.

Simpson RE, O'Regan MH, Perkins LM, Phillis JW (1992) Excitatory transmitter amino acid release from the ischemic rat cerebral cortex: effects of adenosine receptor agonists and antagonists. J Neurochem 58:1683–1690.

Svenningsson P, Le Moine C, Aubert I, Burbaud P, Fredholm BB, Bloch B (1998) Cellular distribution of adenosine A_{2A} receptor mRNA in the primate striatum. J Comp Neurol 399:229–240.

von Lubitz DJKE (1997) Adenosine and acute treatment of cerebral ischemia and stroke—"put out more flags." In: Purinergic approaches in experimental therapeutics (Jacobson KA, Jarvis MF, eds), pp 449–470. New York. Wiley-Liss.

von Lubitz DJKE, Lin, RCS, Jacobson KA (1995) Cerebral ischemia in gerbils: effects of acute and chronic treatment with adenosine A_{2A} receptor agonist and antagonist. Eur J Pharmacol 287:295–302.

Weaver DR (1993) A_{2A} adenosine receptor gene expression in developing rat brain. Brain Res Mol Brain Res 20:313–327.