Review Article

Adenosine A_{2A} Receptors Modulate Acute Injury and Neuroinflammation in Brain Ischemia

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The extracellular concentration of adenosine in the brain increases dramatically during ischemia. Adenosine A_{2A} receptor is expressed in neurons and glial cells and in inflammatory cells (lymphocytes and granulocytes). Recently, adenosine A_{2A} receptor emerged as a potential therapeutic attractive target in ischemia. Ischemia is a multifactorial pathology characterized by different events evolving in the time. After ischemia the early massive increase of extracellular glutamate is followed by activation of resident immune cells, that is, microglia, and production or activation of inflammation mediators. Proinflammatory cytokines, which upregulate cell adhesion molecules, exert an important role in promoting recruitment of leukocytes that in turn promote expansion of the inflammatory response in ischemic tissue. Protracted neuroinflammation is now recognized as the predominant mechanism of secondary brain injury progression. A_{2A} receptors present on central cells and on blood cells account for important effects depending on the time-related evolution of the pathological condition. Evidence suggests that A_{2A} receptor antagonists provide early protection via centrally mediated control of excessive excitotoxicity, while A_{2A} receptor agonists provide protracted protection by controlling massive blood cell infiltration in the hours and days after ischemia. Focus on inflammatory responses provides for adenosine A_{2A} receptor agonists a wide therapeutic time-window of hours and even days after stroke.

1. Introduction

Ischemic stroke is the second leading cause of death in major industrialized countries, with a mortality rate of around 30%, and the major cause of long-lasting disabilities [1]. Ischemic stroke results from a transient or permanent reduction in cerebral blood flow which is, in most cases, caused by the occlusion of a major brain artery, either by an embolus or by local thrombosis. Currently, there is no promising pharmacotherapy for acute ischemic stroke aside from intravenous or intra-arterial thrombolysis. Yet, because of the narrow therapeutic time-window involved, thrombolytic application is very restricted in clinical settings [2]. Neuroprotective drugs such as glutamate receptor antagonists have shown therapeutic potential in animal stroke trials but have failed to be efficacious during clinical trials [3, 4].

Death-signaling proteins involved in the progression from N-methyl-D-aspartic acid (NMDA) receptor stimulation to excitotoxic neuronal death emerged as possible novel targets for neuroprotection. In particular, inhibition of activation of transcription factors and related proteins, including p38, JNK, and SREBP1, is neuroprotective in animal models of stroke [5]. On the other hand, ischemia is a multifactorial pathology characterized by different events evolving in the time. After ischemia the early massive increase of extracellular glutamate is followed by activation of resident immune cells, that is, microglia, and production or activation of inflammation mediators [6]. Proinflammatory cytokines, which upregulate cell adhesion molecules, exert an important role in promoting neutrophil infiltration and accumulation in brain parenchyma [7, 8]. Although after ischemia precocious activation of immune cells may be neuroprotective and

supportive for regeneration, protracted neuroinflammation is now recognized as the predominant mechanism of secondary brain injury progression.

The extracellular adenosine concentration increases dramatically during *in vivo* ischemia as demonstrated first by the cortical cup technique [9, 10] and later on by the microdialysis technique [11–15]. The increase of adenosine extracellular level is attributable to different reasons. Early after ischemia, the increase of adenosine is mainly attributable to extracellularly released ATP [16] that is hydrolysed by ectonucleotidases (NTPDases 1, 2, and 3 that convert ATP to ADP and AMP) and ecto-5$'$-nucleotidase that converts AMP to adenosine [17, 18]. Thereafter adenosine *per se* is mainly released from cells likely by the equilibrative nucleoside transporter (ENT) 2 [16]. Inhibition of adenosine-uptake processes due to downregulation of concentrative nucleoside transporters (CNT) 2 and 3 and of the ENT1 also contributes to the extracellular adenosine increase after stroke [19].

Numerous authors have indicated adenosine and its receptors as a target for therapeutic implementation in the treatment of stroke. Extracellular adenosine acts through multiple *G*-protein coupled receptors (adenosine receptor subtypes A_1, A_{2A}, A_{2B}, and A_3) to exert a variety of physiological effects [20]. Adenosine receptors are expressed at significant levels in neurons and glial cells and in inflammatory cells (such as lymphocytes and granulocytes) [21–26] (Figure 1). The wide distribution is consistent with the multifaceted neurochemical and molecular effects of adenosine receptor activation and suggests that the role of adenosine in ischemia is the consequence of an interplay among different receptor activation in neuronal, glial, and inflammatory cells, which changes depending on the time-related development of the pathological condition.

During ischemia, adenosine has long been known to act predominantly as a neuroprotectant endogenous agent [27–32]. Adenosine infusion into the ischemic striatum has been shown to significantly ameliorate neurological outcome and reduce infarct volume after transient focal cerebral ischemia [33]. Protective effects are greatly attributed to A_1 receptor activation due to reduced Ca^{2+} influx, thus lowering presynaptic release of excitatory neurotransmitters [33–38] and in particular of glutamate which exerts an excitotoxic effect during ischemia mainly by overstimulation of NMDA receptors [39]. In addition, by directly increasing the K^+ and Cl^- ion conductances, adenosine stabilises the neuronal membrane potentials, thus reducing neuronal excitability [39]. Consequent reduction in cellular metabolism and energy consumption [40] and moderate lowering of the body/brain temperature [41] protect against ischemia.

Although data demonstrate a neuroprotective effect of adenosine through A_1 receptors during ischemia, the use of selective A_1 agonists is hampered by undesirable effects such as sedation, bradycardia, and hypotension [42, 43]. More recently adenosine A_{2A} receptors emerged as an interesting target in ischemia.

We largely limit our overview to the A_{2A} adenosine receptor subtype in brain whose new insights are into control of excitotoxicity and neuroinflammation phenomena in ischemia. In this paper, we summarize recent developments that have contributed to the understanding of how this adenosine receptor subtype modulates tissue damage in brain ischemia models. A list of A_{2A} receptor ligands used in different "*in vitro*" and "*in vivo*" hypoxia/ischemia models is provided in Table 1.

2. Adenosine A_{2A} Receptor Antagonists Protect against Primary Ischemic Injury

2.1. A_{2A} Receptor Antagonists Are Protective against Ischemic Damage. Gao and Phillis [50] demonstrated for the first time that the nonselective A_{2A} receptor antagonist, 9-chloro-2-(2-furanyl)-[1,2,4] triazolo[1,5-c]quinazolin-5-amine (CGS15943), reduced cerebral ischemic injury in the gerbil following global forebrain ischemia. Thereafter many reports have confirmed the neuroprotective role of A_{2A} receptor antagonists in different models of ischemia. The selective A_{2A} receptor antagonist, 8-(3-chlorostyryl) caffeine (CSC), as well as the less selective antagonists, CGS15943 and 4-amino [1,2,4] triazolo [4,3a] quinoxalines (CP66713), both administered before ischemia, protected against hippocampal cell injury during global forebrain ischemia in gerbils [49, 52]. The selective A_{2A} receptor antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4] triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl) phenol (ZM241385), administered before ischemia, reduced hippocampal injury and improved performance in the Morris water maze in hyperglycemic four-vessel occluded rats [54]. In all the mentioned studies, adenosine A_{2A} receptor antagonists were administered before ischemia. Relevantly to a possible clinical use of drugs in stroke, in subsequent studies, A_{2A} antagonists were administered after ischemia. The selective A_{2A} receptor antagonist, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4,triazolo[1,5-c]pyrimidine (SCH58261), acutely administered after hypoxia/ischemia in neonatal rats [57] and soon after focal ischemia in adult rats [58, 59] reduced brain damage 24 hours thereafter. The same antagonist, administered subchronically, was protective against brain damage, neurological deficit [60, 61, 67], and disorganization of myelin [61] 24 hours after focal cerebral ischemia in the adult rat. In the model of global ischemia (i.e., 7 min asphyxic cardiac arrest) in newborn piglets, posttreatment with SCH58261, infused soon after resuscitation and for 6 hours, improved neurologic recovery and protected striatopallidal neurons after 4 days from ischemia [63]. SCH58261 behaves as a significant protective agent at a dose (0.01 mg/kg) that does not have cardiovascular effects. This low dose does not affect motor activity in naive animals but decreases contralateral turning behaviour after monolateral middle cerebral artery occlusion (MCAo) induced by the monofilament technique [59, 60]. At a higher dose, in the range that is effective in different models of Parkinson's Disease (PD), the same drug significantly increases motility and rearing in the rat [68]. A noxious role of A_{2A} receptors during ischemia is supported by the observation that A_{2A} receptor knock-out (KO) mice show significantly decreased infarct volumes after focal cerebral ischemia when compared with their wild-type littermates [69, 70].
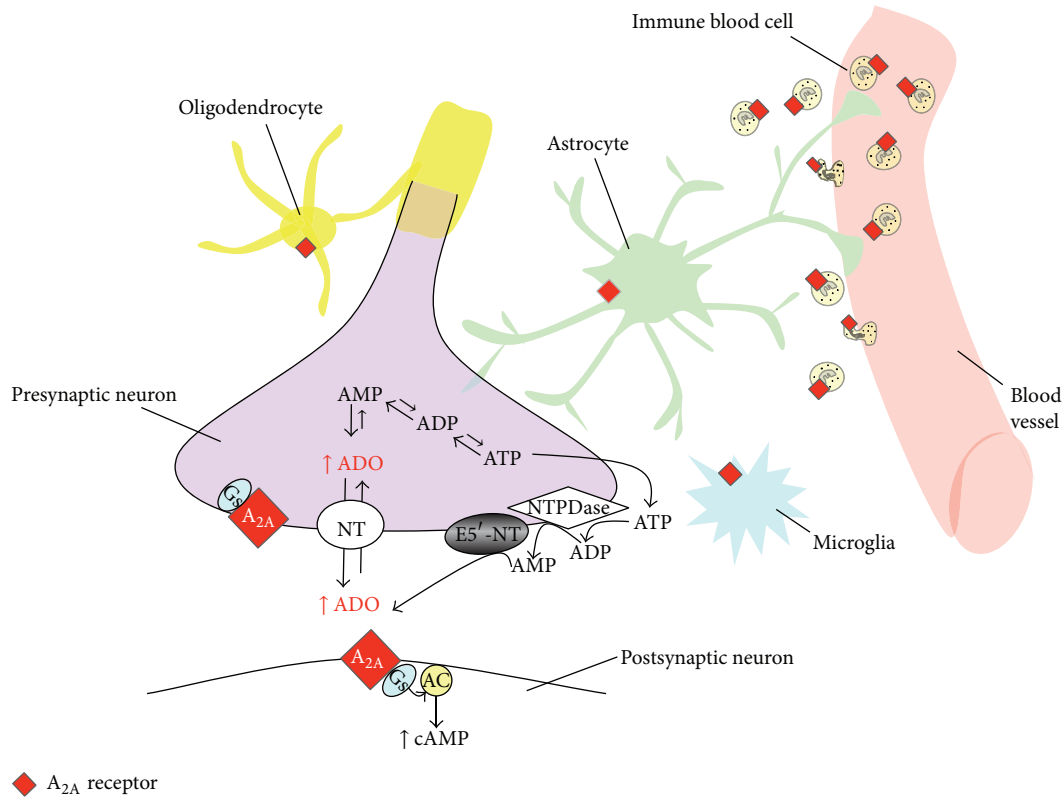
FIGURE 1: Schematic drawing of adenosine A_{2A} receptor localization on different cell types. Adenosine A_{2A} receptors are expressed at central level on presynaptic and postsynaptic neurons, on astrocytes, on microglia, and on oligodendrocytes. A_{2A} receptors are present also at peripheral level on leukocytes and vasculature. After cerebral ischemia, leukocytes infiltrate into ischemic tissue due to increased permeability of blood-brain barrier (BBB). During ischemia, extracellular adenosine level increases mainly due to (i) extracellular ATP degradation by NTPDases; (ii) release of adenosine *per se* from cells likely by the equilibrative nucleoside transporter (ENT); (iii) inhibition of adenosine-uptake processes due to downregulation of concentrative nucleoside transporters (CNT) 2 and 3 and of ENT. AC: adenylate cyclase; ADO: adenosine; ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; E5′-NT: ecto-5′-nucleotidase; NT: nucleoside transporter; Gs: stimulatory *G*-protein; NTDPase: ecto-nucleoside triphosphate diphosphohydrolases. The proportions of the various components of the nervous tissue have not been kept.

Most recently, the question has been raised if A_{2A} receptor continuous blockade over an extended time-window after ischemia is protective. CSC continuously administered over 72 hours, using subcutaneously implanted osmotic minipumps, after permanent MCAo in spontaneously hypertensive rats, did not decrease brain infarct volume determined by magnetic resonance imaging 3 days after induction of ischemia [53]. Authors attributed the lack of protection to high hepatic metabolism and elimination of CSC [53]. Consistently, Melani and coworkers (unpublished observation) found a lack of protection on infarct volume by SCH58261 administered subchronically (three times in the first day) or chronically (twice/day for 7 days) 7 days after 1 hour transient MCAo.

2.2. A_{2A} Receptor Antagonism Protects from the Increase of Glutamate Extracellular Concentrations and NMDA Receptor Function.

A_{2A} receptors are expressed on neurons at high levels in the striatum [71] and at lower levels in all other brain regions as detected by autoradiography [72] and real time PCR [73]. A_{2A} receptors in the striatum are mostly present on GABA-enkephalin neurons [74] but are also located presynaptically [25, 75, 76] on glutamatergic terminals [77] where they can directly regulate glutamate outflow under normoxic [78, 79] and ischemic conditions [65, 66]. Adenosine, by A_{2A} receptor stimulation, promotes glutamate release under normoxic and ischemic conditions *in vivo* [44, 51, 80–82]. Consistently, A_{2A} receptors play an important modulation of synaptic transmission [83, 84] as mostly demonstrated in the hippocampus [85–87]. In the CA1 area of the rat hippocampus, which is the most sensitive region to ischemia, the selective A_{2A} receptor agonist, CGS21680, clearly reduces the depression of synaptic activity brought about by OGD [47]. Following A_{2A} receptor stimulation the increase of extracellular glutamate concentration counteracts depression brought about by adenosine A_1 receptors. In agreement, the selective A_{2A} receptor antagonists, ZM241385 and SCH58261, delay the appearance of anoxic depolarization (AD), a phenomenon strictly related to cell damage and death [88], protect from the synaptic activity depression brought about by a severe (7 min) OGD period, and protect CA1 neuron and astrocyte from injury [55]. Same effects of ZM241385 were observed

TABLE 1: Adenosine A_{2A} receptor ligands used in brain ischemia *"in vivo"* and *"in vitro"* models.

	Brain ischemia model	References
Adenosine A_{2A} receptor agonists		
CGS21680	Global ischemia in rat	[44]
	Global ischemia in gerbil	[45]
	Focal ischemia in rat	[46]
	OGD hippocampal slices	[47, 48]
APEC	Global ischemia in gerbil	[49]
Adenosine A_{2A} receptor antagonists		
CGS15943	Global ischemia in gerbil	[50]
	Global ischemia in rat	[51]
CSC	Global ischemia in gerbil	[49, 52]
	Focal ischemia in hypertensive rat	[53]
CP66713	Global ischemia in gerbil	[52]
ZM241385	Global ischemia in rat	[54]
	OGD hippocampal slices	[55, 56]
	Hypoxia/ischemia in neonatal rat	[57]
SCH58261	Focal ischemia in rat	[58–62]
	Global ischemia in newborn piglet	[63]
	OGD hippocampal slices	[55, 64]
	OGD cerebrocortical slices	[65, 66]
DMPX	OGD hippocampal slices	[48]

APEC: 2-[(2-aminoethylamino)-carbonylethylphenylethylamino]-5′-N-ethylcarboxoamidoadenosine; CGS15943: 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine; CGS21680: 2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine; CP66713: 4-amino[1,2,4]triazolo[4,3a]quinoxalines; CSC: 8-(3-chlorostyryl)caffeine; DMPX: 3,7-dimethyl-1-propargylxanthine; OGD: oxygen and glucose deprivation; SCH58261: 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-[1,2,4]triazolo[1,5-c]pyrimidine; ZM241385: 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol.

after a severe 9 min OGD period in the gyrus dentatus of the hippocampus [56]. The time-window of the protective effects of the A_{2A} receptor antagonists in the hippocampus overlaps with the delay obtained by treating the slices with glutamate receptor antagonists [89, 90], indicating that their effects are attributable to reduced glutamate excitotoxicity.

Several mechanisms contribute to the A_{2A} receptor regulation of extracellular glutamate concentrations. A_{2A} receptor stimulation might regulate extracellular glutamate not only by reducing release from glutamatergic terminals but also by modulation of glutamate uptake transporter. In the brain, adenosine A_{2A} receptors are expressed on both neurons and glia [21, 71]. In particular, A_{2A} receptors located on astrocytes mediate inhibition of glutamate uptake by glutamate transporter-1 (GLT-1) [91–93]. Recent data show that while acute exposure to the selective A_{2A} receptor agonist, CGS21680, reduces glutamate uptake, prolonged exposure to the same agonist inhibits GLT-1 and glutamate-aspartate transporter mRNA and protein levels from astrocytes [94]. Such inhibition is exerted through modulation of Na^+/K^+-ATPase [95]. An imbalance of A_1/A_{2A} receptor expression might also contribute to inhibition of excitatory synaptic transmission under ischemia. Short periods of global ischemia decrease A_1 adenosine receptor density in the brain likely due to an internalization of A_1 adenosine receptors in nerve terminals [96]. Moreover tight A_1/A_{2A} receptor interaction exists. In hippocampal and cortical nerve terminals A_{2A} receptors might increase glutamate outflow by a protein kinase C-mediated decrease of the affinity of A_1 receptors [97]. A heteromerization of adenosines A_1 and A_{2A} receptors in striatal glutamatergic nerve terminals might allow adenosine to exert a fine-tuning modulation of glutamatergic neurotransmission. A main biochemical characteristic of the A_1/A_2 receptor heteromer is the ability of A_{2A} receptor activation to reduce the affinity of the A_1 receptor for agonists with an ultimate switch mechanism by which low and high concentrations of adenosine inhibit and stimulate, respectively, glutamate release [98].

Adenosine acting on A_{2A} receptors is such an important modulatory substance by controlling synaptic transmission and also by regulating AMPA [99] and NMDA receptor function [100]. In striatal membranes, the NMDA-mediated excitation, leading to a depolarized plateau potential and spike firing, is regulated by dopamine and adenosine acting at D_2 and A_{2A} receptor heteromers that regulate Ca^{++} channel activity through mechanisms relying upon specific protein-protein interactions [101]. A_{2A} receptor chronic blockade by treatment with SCH58261 induces a remodeling of NR1 and NR2A/NR2B subunit expression of NMDA receptors in the striatum of Huntington transgenic mice [102]. Moreover, given that mGlu5 receptors "set the tone" of NMDA receptor-mediated neurotransmission [103], it appears important that mGlu5 receptors are under the tight control of A_{2A} receptors [100]. In the hippocampus A_{2A} and mGlu5 receptors are colocated and A_{2A} receptors play a permissive role in mGlu5 receptor-mediated potentiation of NMDA effects

[104]. Such modulations by A_{2A} receptors might be relevant in pathological conditions such as ischemia. By the use of SCH58261, it was demonstrated that A_{2A} receptors support the expression and recruitment of calcium-permeable AMPA receptors during LTP induced by OGD in rat hippocampal slices [64]. In a model of global ischemia in newborn piglets (7 min Asphyxic Cardiac Arrest), inhibition of phosphorylation of NMDA receptor NR1 subunit and inhibition of Na^+/K^+-ATPase and of cAMP-regulated phosphoprotein 32 kDa (DARPP32) might also account for protective effect of the selective A_{2A} receptor antagonist SCH58261 [63]. The ability of adenosine A_{2A} receptors in controlling glutamate receptor functions might represent an attractive mechanism in protecting against acute excitoxicity after ischemia. In fact, in a number of *in vitro* and *in vivo* experimental models of ischemia, glutamate receptor antagonists, acting either on NMDA receptor or on group I metabotropic receptors, are effective neuroprotective agents; none of the glutamate receptor antagonists tested in clinical trials showed positive results or had an acceptable benefit/side effects ratio [105].

In vivo, a definite overexpression of A_{2A} receptors was found in neurons of the striatum and cortex 24 hours after focal ischemia [106] and, in *in vivo* experiments, the low dose of SCH58261 that protects against tissue damage induced by MCAo or quinolinic acid (QA) excitotoxicity also reduces glutamate extracellular concentrations estimated by microdialysis [59, 107]. This supports that protective effects of low doses of A_{2A} receptor antagonists administered early after brain ischemia are largely due to reduced excitotoxicity and to the ensuing excitotoxic cascade attributable to stimulation of NMDA receptors [59]. The robust protection by A_{2A} receptor antagonism is consistent with the observation that adenosine A_{2A} receptor KO mice are protected from an excess of striatal glutamate outflow and damage induced by transient MCAo [69, 70].

A further protective effect of A_{2A} receptor antagonism may be attributed to the capability of increasing GABA outflow during ischemia. The major part of excitatory glutamatergic innervation is modulated by inhibitory GABA-releasing interneurons. Potentiation of GABAergic synaptic transmission has neuroprotective effects in several experimental models of cerebral ischemia [108]. GABA is strongly increased in the cortex and striatum during ischemia [15, 109] and evidence shows that selective A_{2A} receptor stimulation decreases ischemia-evoked GABA outflow [109, 110] and enhances GABA transport into nerve terminals by restraining PKC inhibition of GAT-1 [111].

The neuroprotective properties of A_{2A} receptor antagonists largely reside in effects mediated by A_{2A} receptors located on brain cells, in particular in control of excitotoxicity as demonstrated by the observation that the A_{2A} receptor selective antagonist, ZM241385, injected peripherally or directly intra-hippocampus is protective against excitotoxicity induced by kainate [48] and by the combinations of quinolinic acid and IL-1β [112].

2.3. A_{2A} Receptor Antagonists Protect from Ischemia–Induced Activation of Mitogen-Activated Protein Kinases (MAPKs)

and c-fos Expression. Several data indicate that regulation of proteins involved in transcriptional or post-translational mechanisms plays an important role in the neuroprotective effect of A_{2A} receptor antagonism in ischemia.

All members of the MAPKs family are activated up to 24 hours after ischemia [113, 114]. p38 and ERK1/2 are activated in neurons and in microglia [60, 113, 115, 116]. A definite overexpression of A_{2A} receptors was found not only in neurons but also on microglia of the ischemic tissue 24 hours after focal ischemia [106]. Subchronic administration of the A_{2A} receptor antagonist, SCH58261, reduced phospho-p38 in microglia while it did not affect ERK1/2 activation [60]. It is known that soon after excitotoxic phenomena, resident microglial cells initiate a rapid change in their phenotype that is referred to as microglial cell activation [117] and, by producing cytotoxic substances and cytokines, start an inflammatory response that exacerbate brain damage [6]. Since inhibition of p38 activation has direct neuroprotective effects in hippocampal brain slices after OGD [118], a control of p38 activation by A_{2A} receptor antagonism [60] might account for protection after ischemia. Such results are in agreement with the result that intracerebroventricular injection of SCH58261 prevents the recruitment of activated microglial cells and the increase in IL-1β evaluated 4 hours after intraperitoneal administration of lipopolysaccharide (LPS) [119]. It is also important to consider that A_{2A} receptor antagonists are effective in preventing neurotoxicity in isolated glia. A_{2A} receptor stimulation is known in fact to cause activation of microglia [120] and A_{2A} receptor antagonists have been shown to suppress microglia activation in murine N9 microglial cells exposed to an inflammatory stimulus such as LPS [121]. A_{2A} receptor antagonist suppresses the CGS21680-induced potentiation of LPS-induced NO release from mixed glial cultures as well [122]. Overall results indicate that A_{2A} receptors present on microglial cells are pivotal in mediating a secondary damage consisting in neuroinflammation (see later in the paper) after ischemia.

Twenty-four hours after MCAo, subchronic administration of the A_{2A} receptor antagonist, SCH58261, also reduces phospho-JNK, that is expressed in few neurons, but mainly in mature oligodendrocytes and in oligodendrocyte precursors (OPCs) (stained by Olig2 and NG2 antibodies) [61, 123]. Phospho-JNK is a factor involved in oligodendrocyte death [124, 125]. Interestingly activation of JNK has been described in oligodendrocytes in multiple sclerosis lesions where oligodendrocytes are major targets of the disease [126]. A specific peptide inhibitor of JNK protects against cell death induced by OGD *in vitro* [127] and by MCAo *in vivo* [127, 128]. JNK2/3 KO mice are protected from damage following cerebral ischemia [129, 130]. Therefore we must assume that JNK activation in oligodendrocytes and neurons represents a noxious event after ischemia that can damage oligodendrocytes bringing to myelin damage and disorganization [61]. A_{2A} receptor antagonism also reduces Olig2 [61] that is a transcription factor expressed mostly by OPC while mature oligodendrocytes are characterized by lower levels of Olig2 [131]. Data have suggested that A_{2A} antagonism stimulates OPC differentiation to mature cells after ischemia. In agreement we have recently reported

that, in primary OPC culture, selective stimulation of A_{2A} receptors by CGS21680 inhibits maturation of OPC in the firsts 10 days of *in vitro* differentiation [132]. The drug also inhibits K^+ "delayed rectifier" channels (KDR) [132] that are known to inhibit proliferation and differentiation of OPC to mature oligodendrocytes, thus preventing myelin deposition [133, 134].

Besides a direct effect of the A_{2A} receptor antagonists on A_{2A} receptors located on oligodendrocytes or microglia, we must consider that the reduced MAPK activation by SCH58261, in the initial hours after *in vivo* ischemia, is secondary, to overall reduction in the excitotoxic cascade that in turn primes MAPK activation [59]. In fact, oligodendroglia are extremely sensitive to glutamate receptor overactivation and ensuing oxidative stress [135–137] as well as to cytokines [138] and p38 activation is definitely induced by NMDA receptor stimulation in cerebellar granule cells [139] and in spinal cord cultures [140].

It is of note that, twenty-four hours after permanent MCAo, the A_{2A} antagonist, SCH58261, also reduces gene c-fos expression in glial cells [62]. Products of the Fos family are players in inducing inflammatory gene expression in glial cells [141].

3. Adenosine A_{2A} Receptor Agonists Protect against Secondary Injury

3.1. A_{2A} Receptor Agonists Are Protective against Ischemic Damage. While many data support that A_{2A} receptor antagonists protect against central excitotoxicity, the protective effect of A_{2A} receptor agonists appears attributable to different mechanisms. The A_{2A} receptor antagonist ZM241385 administered repeatedly (1 mg/kg i.p.) in the 12 hours after traumatic brain injury was protective 15 min after trauma when cerebro spinal fluid (CSF) glutamate concentration rose; conversely, the A_{2A} receptor agonist, CGS21680, administered repeatedly (0.1 mg/kg i.p.) in the 12 hours after trauma was protective 3 hours after trauma when CSF glutamate concentrations were down [142].

A protective role of adenosine A_{2A} receptor in hypoxia/ ischemia was demonstrated in newborn rodents. A_{2A} receptor KO neonatal mice show aggravated hypoxic/ischemic injury in comparison to wild-type mice [143] and, in immature brain forebrain slices, it was demonstrated that cannabinoids induce robust neuroprotection through both CB(2) and A_{2A} adenosine receptors [144]. Most recently it was demonstrated that A_{2A} receptor KO mice subjected to chronic cerebral hypoperfusion by permanent stenosis of bilateral common carotid artery show impairment in working memory, increased demyelination, proliferation of glia, and increased levels of proinflammatory cytokines [145]. In adult gerbil, a protective effect of adenosine A_{2A} receptor agonists was reported by Von Lubitz et al. [49] who demonstrated that the A_{2A} receptor agonist, APEC, administered systemically before a global 10 min ischemia, ameliorated recovery of blood flow and animal and neuron survival. Moreover Sheardown and Knutsen [45] demonstrated that a high dose of the selective A_{2A} receptor agonist, CGS21680

(10 mg/kg i.p.), administered after 5 min of global ischemia in gerbil, exhibited highly significant protection against neuronal loss, but was inactive at 3 mg/kg. In these two works in adult gerbils, adenosine agonists were administered before ischemia or at a high dose. In considering translation to clinic, a main problem of A_{2A} receptor agonists is their cardiovascular effect: adenosine A_{2A} receptors located on vase smooth muscle and endotelial cells exert a vasodilatory effect [146]. Consistently A_{2A} receptor agonists might induce hypotension and increase hearth rate. Schindler and coworkers [147, 148] reported that the decrease of blood pressure induced by 0.5 mg/kg i.p. CGS21680 in conscious rats is most probably mediated in the periphery, while the increase of heart rate is mediated at central level. We recently demonstrated that the selective A_{2A} receptor agonist, CGS21680, at dose of 0.1 mg/kg i.p., increased heart rate only in the first hour after administration, but no effect on blood pressure or on heart rate was observed at the lower dose of 0.01 mg/kg [46]. Relevantly our recent experiments have demonstrated that the A_{2A} receptor agonist, CGS21680, administered twice/day for 7 days (chronic protocol) at dose of 0.01 and 0.1 mg/kg, starting 4 hours after transient (1 hour) MCAo, induced protection from neurological deficit, weight loss, cortical infarct volume, myelin disorganization and glial activation [46]. Protective effect is exerted only when CGS21680 is chronically administered. In fact the A_{2A} receptor agonist administered at the same dose (0.1 mg/kg) but in a shorter therapeutic window (4 and 20 hours after induction of MCAo, subchronic protocol) has not reduced the infarct volume 24 hours after permanent MCAo nor 7 days after transient MCAo (unpublished data; see Table 2). The protective effects of chronic administration of CGS21680 at dose of 0.01 and 0.1 mg/kg neither can be attributed to changes in the cardiovascular parameters either at peripheral or central level nor can be attributed to direct effects on motility because CGS21680 at these low doses does not affect motor behavior of rats [149].

Several mechanisms might account for protection by A_{2A} receptor stimulation by direct effects on brain cells. In a rat model of intracerebral hemorrhage, CGS21680 administered directly into the striatum immediately prior to the induction of intracerebral hemorrhage reduces parenchymal neutrophile infiltration and tissue damage: an effect that might be mediated by inhibition of TNF-α expression [150]. Moreover, activation of central A_{2A} receptors is known to increase expression and release of neurotrophic factors [151] as NGF in microglia [152], BDNF in mice hippocampus [153], in rat cortical neurons [154], and in primary cultures of microglia [121], and GDNF in striatal neurons [155]. Consistently it was recently demonstrated that *in vivo* chronic oral administration of the A_{2A} receptor antagonist, KW-6002, decreases both mRNA and protein levels of BDNF receptor (TrkB-FL) and its signaling in the hippocampal CA1 area [156]. The increase in neurotrophic factor expression by adenosine A_{2A} receptor stimulation may contribute to restore neurological functions and cerebral damage after brain ischemia. We must also remember that adenosine is implicated in cerebral blood flow regulation as a vasodilator

TABLE 2: Effect of A_{2A} receptor agonist, CGS21680, in acute and delayed phase of stroke.

Drug	24 h after pMCAo		7 days after tMCAo		
	Infarct volume (mm^3)		Treatment	Infarct volume (mm^3)	
	Cortex	Striatum		Cortex	Striatum
Vehicle	69.43 ± 1.87	46.03 ± 2.7	Subchronic	75.1 ± 5.1	28.4 ± 2.2
			Chronic	76.2 ± 4.3	31.3 ± 2.6
CGS21680	61.37 ± 8.26	45.68 ± 2.6	Subchronic 0.1	62.7 ± 5.8	30.5 ± 2.5
			Chronic 0.1	$48.6 \pm 9.5^{\#}$	27.3 ± 1.7
			Chronic 0.01	$51.9 \pm 10.4^{\#}$	20.9 ± 3.8

Data are the mean ± S.E.M. of n = 6–8 animals. In the model of permanent MCAo (pMCAo), CGS21680 was administered at the dose of 0.1 mg/kg (i.p.) after 4 h and 20 h from ischemia induction. The infarct volume was evaluated 24 h thereafter.

In the model of transient MCAo (tMCAo), CGS21680 was administered *in subchronic protocol* 4 h and 20 h after ischemia at the dose of 0.1 mg/kg (i.p.) and *in chronic protocol* starting 4 h after ischemia, at the dose of 0.01 or 0.1 mg/kg (i.p.), twice/day for 7 days. The infarct volume was evaluated 7 days after MCAo.

One-way ANOVA: $^{\#}P < 0.05$ versus chronic vehicle-treated rats.

Effects of CGS21680 chronically administered are published [46].

agent acting on A_{2A} receptors on endothelial cells of brain vessels, thus favouring brain perfusion [146].

Several lines of evidence in excitotoxicity and spinal cord trauma *in vivo* models do not support, however, that protection by A_{2A} receptor agonists is exerted at A_{2A} receptors located on CNS cells. Jones and coworkers [157] showed that peripheral administration of the A_{2A} receptor agonist, CGS21680, protected the hippocampus against kainate-induced excitotoxicity while the direct injection of CGS21680 into the hippocampus failed to afford protection [157]. Similar results were obtained after spinal cord trauma where CGS21680 protected from damage when injected systemically but not when centrally injected into the injured spinal cord [158].

3.2. A_{2A} Receptor and Neuroinflammation. Minutes to hours after onset of cerebral ischemia, a cascade of inflammatory events is initiated through activation of resident cells [159]. The early massive increase in extracellular glutamate after ischemia has a main role in activating resident immune cells and producing mediators of inflammation [6]. Immunity and inflammation are key elements of the pathology of stroke. Recent developments have revealed that stroke engaged both innate and adaptative immunity. Molecules generated by cerebral ischemic tissue activate components of innate immunity, promote inflammatory signaling, and contribute to tissue damage. The A_{2A} adenosine receptors are expressed both on cells of innate (microglia, macrophages, mast cells, monocytes, dendritic cells, and neutrophils) and on cells of adaptive (lymphocytes) immunity [160, 161]. Soon after excitotoxic phenomena, microglial cells initiate a rapid change in their phenotype [60, 119] that is referred to as microglial cell activation [117]. Microglia typically respond with proliferation, migration, and production of inflammatory substances to viral or bacterial stimuli or to cell damage and degeneration [121, 162] and, by producing cytotoxic substances, cytokines (TNF-α, IL-1β) [119, 120, 163], and chemokines, contribute to the inflammatory response that follows ischemic insult, further exacerbating brain damage [6]. Proinflammatory mediators and oxidative stress contribute to the endothelial expression of cellular adhesion molecules [7, 8] and to

an altered permeability of the blood-brain barrier (BBB) that promotes the infiltration of leukocytes (neutrophils, lymphocytes, and monocytes) [164] in the brain ischemic tissue.

In a model of transient focal cerebral ischemia induced by MCAo, definite microglial activation is present after 12 hours [165]. After ischemia, although reperfusion is necessary for tissue survival, it also contributes to additional tissue damage. Under reperfusion, there is an initial increase of BBB permeability (see [166]) followed by a biphasic increase at 5 and 72 hours [167]. Changes in BBB permeability are responsible for cell infiltration. The nature of BBB permeability is dependent on the duration of ischemia, the degree of reperfusion, and the animal stroke model. Studies in the human brain after ischemic stroke confirm that neutrophils intensively accumulate in the regions of cerebral infarction [6, 168]. Selective immunostaining for granulocytes, by anti-HIS-48 antibody, shows numerous infiltrated cells in ischemic striatal and cortical core two days after tMCAo, while seven days thereafter infiltrated blood cells were not anymore observed [46]. Three days after tMCAo the majority of immune cells are neutrophils and at less extent lymphocytes [165, 169]. After tMCAo, a peak of neutrophil infiltration occurs at 6 and 48 hours thereafter [169]. Infiltrated neutrophils expressing cytokines and chemotactic factors promote expansion of the inflammatory response in ischemic tissue [160]. Correlations among neutrophil accumulation, severity of brain tissue damage, and neurological outcome have been reported by Akopov et al. [168]. Neuroinflammation is now recognized as a predominant mechanism of secondary progression of brain injury after ischemia.

Two days after MCAo, chronic treatment with the A_{2A} adenosine receptor agonist, CGS21680, has definitely reduced the number of infiltrated blood cells in the ischemic areas [46]. These results are in agreement with previous observations that A_{2A} receptor agonists systemically administered after spinal cord injury in mice protect from neurological and tissue damage, reduce inflammation parameters and blood cell infiltration [170–172]. An unequivocal role of A_{2A} receptor in controlling blood cell infiltration was demonstrated also in a model of autoimmune encephalomyelitis:

A_{2A} receptor KO mice displayed increased inflammatory cell infiltration, higher neurological deficit scores and increase of different neuroinflammation parameters [173].

A bulk of evidence indicate that bone marrow-derived cells (BMDCs) are targets of A_{2A} receptor agonist protective effects. Li et al. [174] demonstrated that the protective effect against motor deficits of A_{2A} receptor agonists, systemically administered after spinal trauma, is lost in mice lacking A_{2A} receptors on BMDCs, but is restored in A_{2A} receptor KO mice reconstituted with A_{2A} receptors on BMDCs. Many studies have reported that selective activation of A_{2A} receptors directly on blood cells, including platelets, monocytes, some mast cells, neutrophils, and T cells, inhibits proinflammatory responses [175–177], reduces production of adhesion cell factors, and reduces neutrophil activation, thereby exerting antioxidant and anti-inflammatory effects [178]. A_{2A} receptor activation is known to reduce ischemia-induced rolling, adhesion, and transmigration of various peripheral inflammatory cells (such as lymphocytes, neutrophils) [160]. Overall results suggest that protection due to A_{2A} receptor agonists systemically and repeatedly administered after brain ischemia is strongly exerted at peripheral BMDCs resulting ultimately in reduced leukocyte infiltration and reduced inflammatory cascade at the central level. Consistent with its anti-inflammatory and immunosuppressive role, the protective effect of adenosine A_{2A} receptor stimulation has been observed in different pathologies where inflammatory process has an important role in tissue damage [124, 172, 179, 180] such as ischemia/reperfusion liver injury [181], spinal cord trauma [158], rheumatoid arthritis (RA) [182], acute lung inflammation [183], intestine ischemia/reperfusion injury [184], and experimental autoimmune encephalomyelitis [185].

By controlling brain neuroinflammation and BDNF signalling [186, 187], A_{2A} receptors might also have a potential for synaptic plasticity and neurogenetic processes after ischemia. Neuroinflammation in fact is known to result in inhibition of adult neurogenesis [188].

The notion that A_{2A} receptors on BMDCs are the target of the protective effects of A_{2A} receptor agonists should be reconciled with the information that selective inactivation of A_{2A} receptors on BMDCs (wild-type mice transplanted with A_{2A} receptor KO bone marrow cells) attenuates ischemic brain injury, inhibits inflammatory cytokines production, and increases the expression of anti-inflammatory cytokines in the ischemic brain 22 hours after 2 hours of focal ischemia induced by MCAo. This neuroprotection however cannot be explained by altered infiltration of the major inflammatory cells, neutrophils and microglial cells, in the ischemic brain and remains to be clarified [189].

4. Caffeine Consumption and Stroke Incidence

It has been reported that acute coffee consumption is associated with increased risk of ischemic stroke in the subsequent hour in infrequent coffee drinkers (<1 cup) [190]. The increased risk might be related within hours after consumption to acute deleterious effects of the unselective A_1/A_{2A} receptor antagonist, caffeine, that increases circulating norepinephrine [191], rises mean blood pressure [192], increases arterial stiffness [193], and impairs endothelium-dependent vasodilation [194].

More studies have instead investigated the effect of habitual consumption of caffeine on the risk of stroke. Controversial results, mainly in relation to the dose intake, were obtained [195]. A study showed that the long-term moderate consumption of coffee can provide protective effects (reducing the risk of both coronary heart disease and stroke by 10%–20%) in healthy individuals yet detrimental effects when intake was high [196]. In agreement, Larsson and Orsini [197] reported that it is the moderate coffee consumption (3-4 cups/day) that reduces the risk of stroke. Additionally, one study showed that coffee consumption (more than 4 cups/day) in men was not associated with increased risk of stroke [198] while studies performed in Swedish and USA women have indicated that habitual intake of coffee (from 1 to 5 or more cups/day) was associated with a statistically significant lower risk of total stroke [199], cerebral infarction, and subarachnoid hemorrhage but not intracerebral hemorrhage [200]. In contrast, an epidemiological study showed that neither the high (more than 4 cups/day) nor the low doses (less than 2 cups/day) have the most dangerous effect but it is the intermediate consumption (2–4 cups/day) of coffee which can be the most harmful [201]. Thus the effect of different consumption of caffeine in reducing the risk of ischemic stroke still demand further study.

5. A_{2A} Receptor Based Therapies in Cerebral Ischemia

Evidence reported up to now indicate that antagonism or stimulation of A_{2A} receptors might be a protective strategy secondary to the time-related development of phenomena typical of ischemia. After ischemia, extracellular glutamate concentrations remain elevated at least up to 4 hours after permanent MCAo [15, 59] and up to 12 hours after brain trauma [142]. The massive increase of glutamate excitotoxicity triggers acute tissue injury and the start of an inflammatory cascade that is stressed by blood cell infiltration. While central A_{2A} receptors in the first hours after ischemia are critical in increasing glutamate extracellular concentrations, A_{2A} receptors on blood cells are critical hours and days after ischemia in decreasing activation, adhesion, and infiltration of blood cells in brain parenchyma. Altogether, evidence suggests that A_{2A} receptor antagonists provide protection centrally by reducing excitotoxicity, while A_{2A} receptor agonists provide protection by acting on blood cells controlling massive infiltration and neuroinflammation in the hours after brain ischemia. In agreement the lack of detecting a protection by A_{2A} receptor antagonism at later time after stroke [53, our unpublished observation] might be attributable to the fact that protection is overwhelmed by subsequent damage brought about by blood cell infiltration that starts 6 hours after ischemia and peaks at 2 days thereafter [46, 165, 169].

These observations highlight that a therapeutic strategy with adenosine A_{2A} receptor antagonists/agonists should be

carefully evaluated in terms of time after ischemia. When considering use of adenosine A_{2A} receptor active drugs to protect against brain ischemia, attention should be given to administration time after injury and to the dose used. In fact A_{2A} receptors located on endothelial cells mediate important effect on systemic blood pressure and heart frequency. However both A_{2A} receptor antagonists [58, 59, 61] and agonists [46] are protective in ischemia models at doses that do not modify blood pressure nor the heart frequency.

The design and development of new adenosine A_{2A} receptor ligands is an area of intense research activity [202, 203].

6. Conclusions

Under neurodegenerative conditions involving ischemia, excitotoxicity is a first phenomenon. Thereafter, the interplay of resident glial cells with infiltrating peripheral BMDCs produces neuroinflammation. On the light that the role of adenosine A_{2A} receptors in ischemia is not univocal, it is important to clarify the windows in which A_{2A} receptors play a noxious or protective role after ischemia. This will be important to devise a correct therapeutic strategy with antagonists and/or agonists at this receptor. Considering translation to clinical practice, a very short time-window of minutes/few hours would be available for A_{2A} receptor antagonists after stroke, while a focus on inflammatory responses to stroke provides a wide therapeutic time-window of hours and even days after stroke for adenosine A_{2A} receptor agonists. A novel therapeutic strategy could involve, when possible, early treatment with A_{2A} receptor antagonists to reduce excitotoxicity followed by adenosine A_{2A} receptor agonist treatment for the control of later secondary injury.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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