Right Thing at a Wrong Time? Adenosine A_3 Receptors and Cerebroprotection in Stroke

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ABSTRACT: The involvement of adenosine A_3 receptors in normal and pathologic functions of the brain remains to be defined. Previous studies have shown that chronic preischemic administration of the agonist [N^6-(3-iodobenzyl)-5′-*N*-methylcarboxoamidoadenosine or IB-MECA) results in a significant protection of neurons in selectively vulnerable brain regions and in an equally significant reduction of the subsequent mortality. Acute administration of the drug, on the other hand, resulted in a pronounced worsening of these parameters. We now report that the effect of administration of IB-MECA depends on the timing of treatment with respect to the onset of the focal insult, and provide the first data supporting speculation that treatment with adenosine A_3 receptor agonists may decrease the infarct size following focal brain ischemia.[1,2] Treatment with IB-MECA administered 20 min prior to transient middle cerebral ischemia ($MCAO_t$ = 30 min) resulted in a significant increase of the infarct size ($p < 0.01$), whereas administration 20 min after ischemia resulted in statistically significant decrease of the infarct volume. Postischemic treatment results in improved neuronal preservation, decreased intensity of reactive gliosis, and pronounced reduction of microglial infiltration. The data indicate that the effects of adenosine A_3 receptor stimulation depend on the differential impact of these receptors on both neuronal and non-neuronal elements of the cerebral tissue, for example, astrocytes, microglia, and vasculature.

KEYWORDS: Adenosine A_3 Receptors; Stroke; Focal cerebral ischemia; Neuroprotection; Cerebral infarction; Physiology; Pharmacology; Treatment; Mice.

INTRODUCTION

Despite nearly a decade since the discovery of the latest member of the adenosine receptor family,[3,4] the biological role of adenosine A_3 receptors is still ill defined. Studies have shown that stimulation of this receptor results in degranulation of mast cells, histamine release, vasoconstriction, and hypotension.[5–7] Other authors have demonstrated the potent inhibitory effect of adenosine A_3 receptor activation on the processes accompanying inflammation, for example, neutrophil degranulation[8] or

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inhibition of eosinophil migration.[9] Adenosine A_3 receptors also appear to activate certain types of Cl^- channels of the non-pigmented epithelium.[10]

The bulk of experimental work that has attempted to define the role of adenosine A_3 receptors in these pathological processes has concentrated on ischemia of the heart and brain. Several authors have shown that preischemic exposure of either cardiac myocytes or isolated hearts to adenosine A_3 receptor agonists results in protection against ischemic damage,[11–13] indicating that these receptors may be involved in the cardioprotective effects of preconditioning. These results are starkly opposed by the effects of the acute preischemic treatment on the outcome of global cerebral ischemia, where both neuronal damage and postischemic mortality are very significantly aggravated by the agonists of adenosine A_3 receptor.[14] However, protective effects of adenosine A_3 receptor agonist treatment have been reported that are consequent to chronic administration of N^6-(3-iodobenzyl)-5′-*N*-methylcarboxoamidoadenosine (IB-MECA).[14] Involvement of adenosine A_3 receptors in these effects has been confirmed by preliminary reports of the intensely cerebroprotective effect of preischemic exposure to the adenosine A_3 receptor antagonist.[15,16]

Although the damaging, involvement of adenosine A_3 receptors in global ischemia has been demonstrated,[14] nothing is known about the effects of either pre- or postischemic stimulation of these receptors in focal brain ischemia. Based largely on the circumstantial evidence, it has recently been theorized that postischemic stimulation of adenosine A_3 receptors may actually result in cerebroprotection.[1,16] The unquestionable yet rather confusing involvement of adenosine A_3 receptors in ischemic brain damage warrants extension of previous studies to focal ischemia. Stimulation of adenosine A_3 receptors appears to affect at least two transducing systems, and some of the affected pathways appear to be clearly involved in stroke induced pathology.[17,18] Hence, the data obtained during studies of experimental focal ischemia may also shed further light on the pathology of stroke in humans.

MATERIALS AND METHODS

Animals and Drugs

CD-1 ($n = 60$) male mice (35 g; Charles River Laboratories, Wilmington, MA) were used. The animals were randomly separated into three experimental groups ($n = 20$/group). Treated animals were injected i.p. with 1.0 mg/kg N^6-(3-iodobenzyl)-5′-*N*-methylcarboxoamidoadenosine (IB-MECA; RBI, Natick, MA) dissolved in Emulphor (Rhône-Poulenc, Cranbury, NJ) and saline as described elsewhere.[14] The drug was administered either 20 min prior to, or 20 min following middle cerebral artery occlusion. The control group received i.p. injections of the vehicle.

Surgery

Anesthesia and Temperature Maintenance

Presurgical anesthesia was initiated with 4% isoflurane carried in the 70:30 mixture of nitrous oxide and oxygen, and maintained at 1.5% isoflurane. The gas was administered through a face mask (Kent Scientific, Litchfield, CT). Body

temperature was maintained at 37.5°C throughout the surgery and during recovery using a rectal temperature probe and a heating pad (Kent Scientific, Litchfield, CT).

Surgical Procedure

The model of filament induced transient middle cerebral artery occlusion was used. Following a ventromedial incision of the neck, the left common carotid artery was exposed under surgical microscope (Vision Engineering, New Milford, CT). The occipital branches of the external carotid and the terminal lingual and maxillary branches were then isolated and coagulated. Subsequently, the internal carotid was exposed and its pterygopalatine branch ligated close to its origin using a 6-0 silk suture. The procedure ensured that only the intracranial ramus of the common carotid artery remained patent. A 2-cm length of 5-0 blunt tipped (through heat application) nylon suture was then introduced into the external carotid and gently advanced into the internal branch of the artery. Resistance and slight bending of the suture indicated that the tip lodged in the proximal segment of the anterior cerebral artery. Typically, the distance traveled by the tip (i.e., between the tip and the bifurcation of the common carotid artery) was 10–11 mm. After ensuring that the tip was firmly lodged in the artery, the suture was anchored for 30 min. After withdrawing the suture, the wound neck was sutured, and the animal was left to recover. To prevent postsurgical infection, a mixture of neomycin, polymycin, B-sulphate, and bacitracin (Triple-Antibiotic Ointment, E. Fougera & Co., Melville, NY) was topically applied to the wound.

Verification of the Occlusion

The completeness of the artery occlusion was verified using a laser-Doppler technique (Perimed, North Royalton, OH). Prior to the occlusion, the cortical blood flow was measured transcranially by means of a 1-mm diameter probe apposed to the skull surface over the area of the expected infarct.[14] Five minutes after the occlusion the probe was advanced to the same place, and the continuous flow measurement was made over the next five minutes. Consistent depression of the postischemic flow by at least 95%, compared to the preischemic values, indicated successful occlusion of the artery.

Determination of the Infarct Size

Seven days after the occlusion, the animals were anesthetized with Nembutal (50 mg/kg) and decapitated. Following removal, the brains were sectioned into 1-mm thick coronal slices that were then immersed in a warm (37°C) 2% solution of 2,3,5-triphenyl-tetrazolium chloride (TTC) in 1X PBS. After 20 min, the slices were gently washed with several rinses of 2% buffered (pH 7.4), 2% solution of paraformaldehyde in PBS, followed by additional fixation in fresh paraformaldehyde solution. The slices were then placed under an automated scanning system (EXPRESSION 636, Epson, Japan) and scanned at 720 d.p.i. The volume of the infarct was determined using NIH Image analyzing software. In the animals that showed signs of secondary cerebral hemorrhages (seen only in the pretreatment group), the area affected was considered part of the total infarct zone.

Histology

Separate groups of animals ($n = 20$) were used for the assessment of histological damage and immunocytochemistry. Seven days after ischemia, the animals were anesthetized and perfused with buffered paraformaldehyde (4.5%, pH 7.4). After fixation, the brains were removed and cut on a freezing microtome into 25 μm slices. Slices from the penumbra zone (determined by means of laser-Doppler measurement during ischemia) were subjected to either to Nissl or GFAP and lectin immunocytochemical[19] staining to indicate the extent of neuronal preservation in the hippocampus, and the extent of astrocyte and microglia activation in the hippocampus and the cortex of the penumbra zone.

Statistics

Infarct volume data were analyzed using GraphPad/Inplot (GraphPazd Software, San Diego, CA) software. Statistical parameters were determined by means of ANOVA followed by the Student–Newman–Keuls test with $p < 0.05$ indicating significant difference.

RESULTS

At seven days postischemia, the differences in the extent of the infarcted brain tissue were easily perceived (see FIGURE 1). Apart from the clearly demarcated infarct, the gross appearance of coronally sectioned brains in controls and posttreated animals was unremarkable. In the pretreated group, several brains (4/7) showed widespread intracerebral hemorrhages and partial collapse of the tissue (FIG. 1). Comparison of infarct volumes (see FIGURE 2) showed significant reduction of infarct volume in the post treated animals.

Even in the absence of quantitative determination of neuronal preservation, the extent of the hippocampal damage in the pretreated group was slightly worse than in the controls (see FIGURE 3 A and B). We doubt, however, that neuronal counting would reveal any statistical differences. In the posttreated group, both the thickness and the appearance of the pyramidal neurons in the lateral CA1 segment of the penumbral hippocampus appeared to be normal (FIG. 3 C). The impact of pre- versus postischemic treatment with IB-MECA became fully apparent when GFAP and lectin stains were compared.

The extent of GFAP staining in the controls and pretreated animals (see FIGURE 4 A and B) was fully comparable to that observed in the gerbils exposed to IB-MECA preceding global cerebral ischemia.[19] As in the previous studies, the astrocytes in the pretreated animals were characterized by sturdy processes containing an intense load of GFAP (FIG. 4 B). In the animals treated after ischemia, the appearance of astrocytes changed dramatically. The GFAP containing processes were slender, their elongated ramifications creating a delicate lattice (FIG. 4 C) rather than the coarse network characteristic of the pretreated animals and, to a lesser degree, control mice as well (FIG. 4 A, B, and C).

Microglial infiltration of the penumbral cortex showed similar pattern to that of reactive gliosis. As expected, a significant presence of activated microglia was

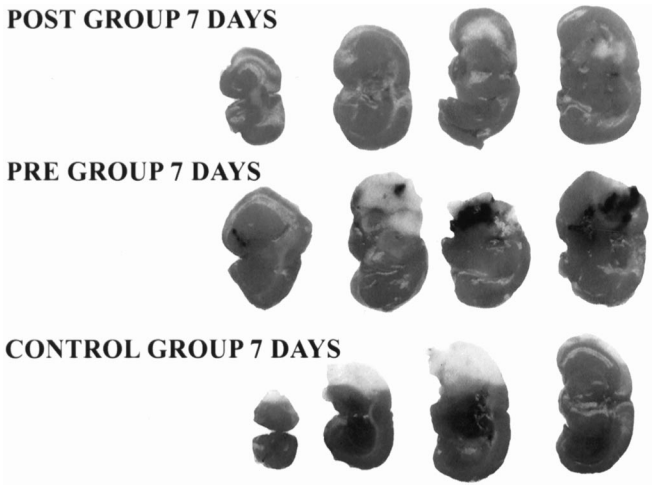

FIGURE 1. Slices of brains removed seven days after ischemia. Note signs of extensive intracerebral hemorrhaging in the striatum and the temporal cortex of the animals treated with IB-MECA prior to the occlusion.

observed in the control animals (FIG. 4 D). In the pretreated animals, the number of microglia reacting to the lectin stain was much higher than in the controls (FIG. 4 E). The surprising finding was the rarity of activated microglia in the penumbra of the posttreated group (FIG. 4 F).

Another unexpected finding was the effect of exposure to IB-MECA on the hillar region of the dentate gyrus in the contralateral hemisphere, where posttreatment with the drug (see FIG. 5 A and C) resulted in a reduction of the changes that were observed following preischemic administration of the agonist (FIG. 5 B and D).

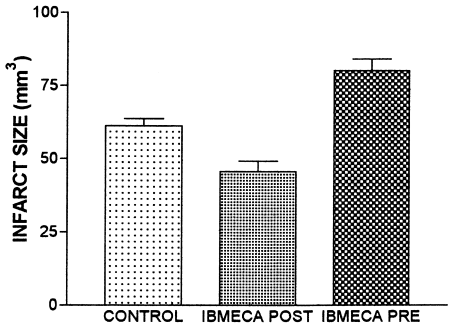

FIGURE 2. Administration of IB-MECA prior or post temporary MCAO. Infarct size in the controls (CTRL), animals pre-treated with IB-MECA (PRE) and post-treated (POST) with the drug. ⊡, control; ▤, IBMECA post; ▩, IBMECA pre.

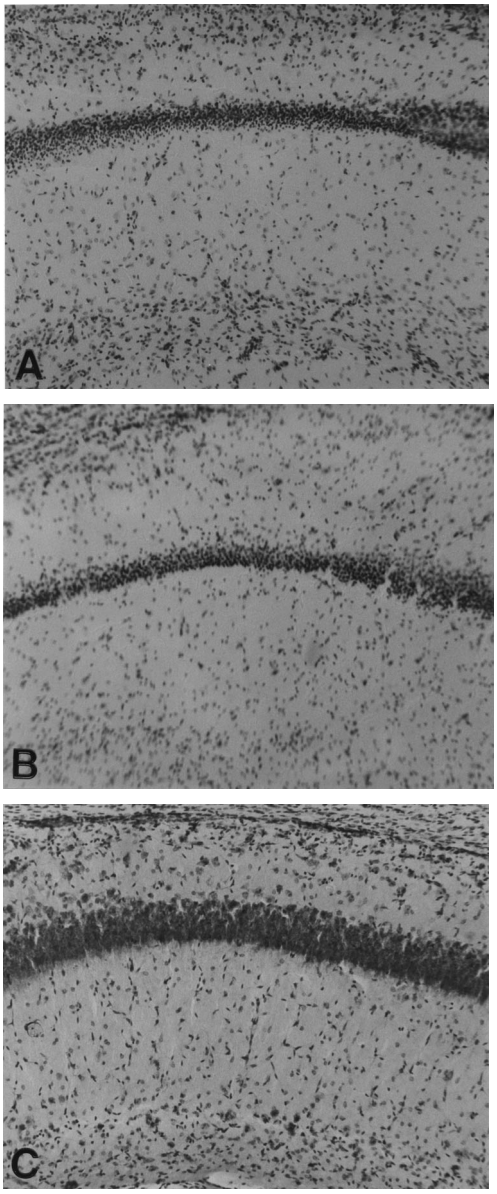

FIGURE 3. The effect of focal ischemia (**A**), IB-MECA prior to focal ischemia (**B**), and IB-MECA after focal ischemia (**C**). Treatment following MCAO occlusion results in a pronounced sparing of the hippocampal neurons in the hippocampus adjacent to the lesion. The damage in the two other groups is, essentially, identical.

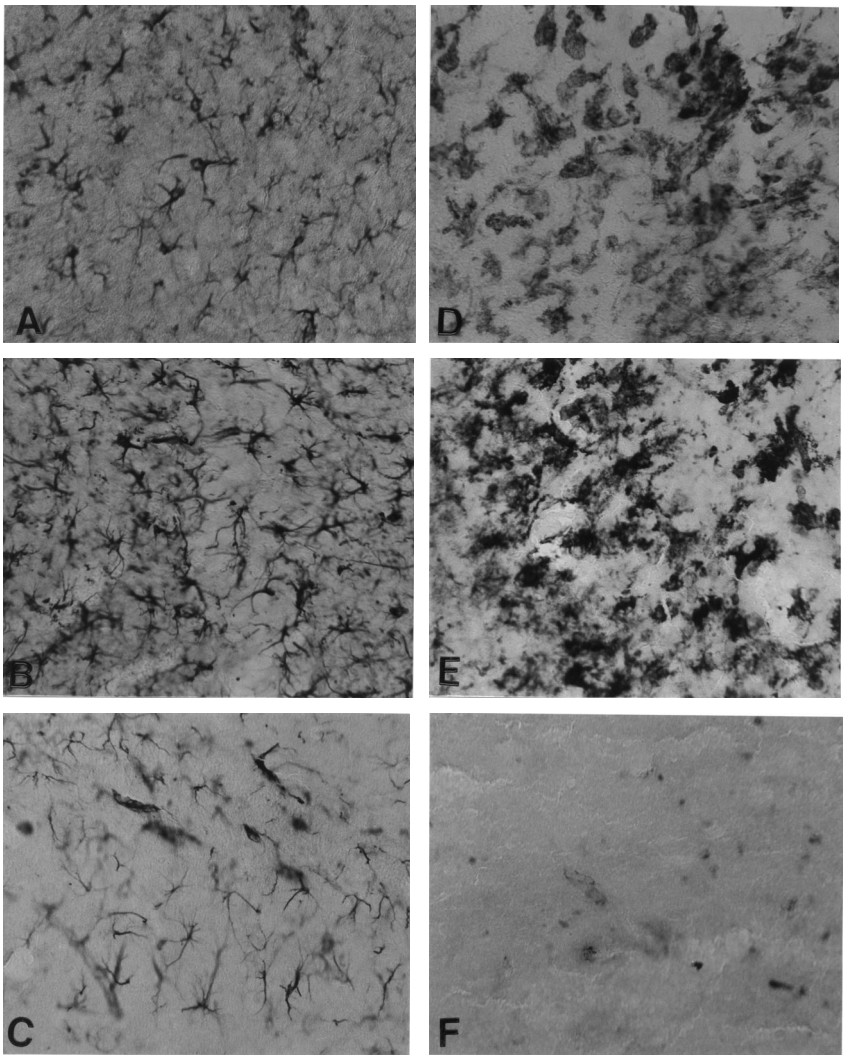

FIGURE 4. Focal ischemia (**A**) and either pretreatment with IB-MECA (**B**) or administration of the drug after removal of the occluding filament (**C**) and their effect on GFAP immunostaining (**A–C**) or microglial infiltration (**D**, ischemia alone; **E**, pretreatment; **F**, posttreatment) within the penumbral zone of the cortex. Note that posttreatment with IB-MECA markedly changes the appearance of astrocytic processes (compare with **A** and **B**) and practically eliminates microglial elements from the affected volume of the brain.

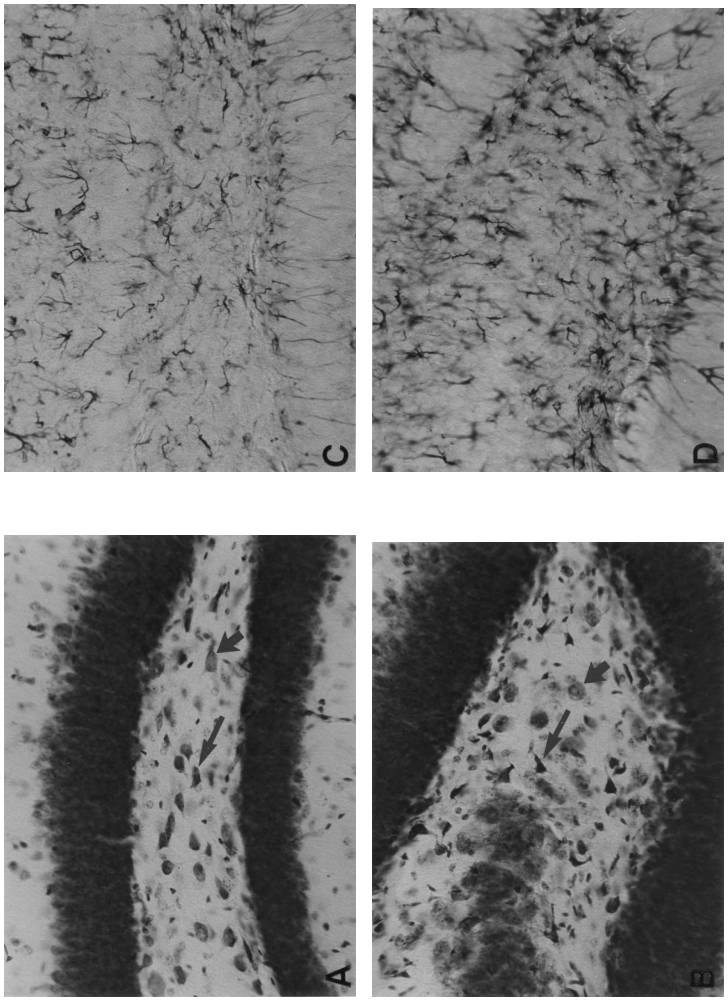

FIGURE 5. Hilus of gyrus dentatus on the contralateral side. **A**, brain of one of the animals posttreated with IB-MECA (Nissl stain). The *thin arrow* points at one of the few damaged neurons, the *thick arrow* at an astrocyte. **C**, GFAP stain from another brain slice in the same animal. Note that the appearance of astrocytes is very similar to those in FIGURE 4 C. **B** represents the same region in the animal pretreated with IB-MECA. The intensity of the collateral damage is far more intense and the astrocytes resemble (**D**) those seen in the ipsilateral cortex (compare to FIG. 4 B).

DISCUSSION

Although the relative importance of the adenosine A_3 receptor in the operation of several cell, tissue, and organ types is the subject of intense speculation,[1,20,21] none of the existing data allow definitive conclusions. To the contrary, many of the recently published studies reveal consistencies within one system, merely to be contradicted by the results obtained in another (for recent reviews see Refs. 16, 20, and 21). The complex intracellular effects evoked by the stimulation of adenosine A_3 receptors (e.g., see Refs. 21–24) are unquestionably among the underlying sources of the ongoing mystery to which the present paper adds, rather than subtracts.

As in global ischemia,[14] acute exposure to IB-MECA prior to middle cerebral artery occlusion (focal ischemia) results in a significant increase of the infarcted brain volume. Since, contrary to our studies of global ischemia,[14] we did not measure postischemic blood flow during the experiments described here, it is unknown whether drug related amplification of the regional disturbances of cerebral blood perfusion contribute to the aggravation of damage in the animals pretreated with IB-MECA. It has been shown, however, that a 200 μg/kg dose of a more selective and potent adenosine A_3 receptor agonist 2-Cl-IB-MECA (2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methylcarboxamide) administered i.v. in conscious rats results in the complete release of vascular histamine stores.[6] Thus, since histamine plays a critical role in the formation of brain oedema following focal cerebral ischemia,[25,26] we can not exclude the possibility that the increased infarct volume in animals pretreated with IB-MECA is, at least in part, influenced by the stimulation of the adenosine A_3 receptors located on mast cells.[5,27] Histamine induced oedema involves a nitric oxide component,[28,29] and our previous studies[15,16] demonstrated increased immunocytochemical reactivity of nitric oxide synthase in the pyramidal and radiatum strata of the hippocampus following preischemic injection of IB-MECA in gerbils. However, activation of nitric oxide synthase was entirely independent of the interaction between adenosine A_3 receptors and histamine releasing mechanisms,[2,16] and the immunocytochemical reaction product appeared to concentrate at the neuronal rather than vascular components of the brain.[16,19] Hence, it is quite likely that the destructive impact of preischemically administered IB-MECA involves both circulatory and neuronal components. Finally, astrocytes and microglia are also affected by IB-MECA as shown by our latest studies,[30] indicating that the degree of astro- and microglial activation depends on the timing of treatment with respect to the insult itself.

The multiplicity of cellular and organs systems affected by stimulation of adenosine A_3 receptors, and the powerful effects elicited by such stimulation,[1,16,18,20,21] stand in contrast to the relative paucity of these receptors in all studied cell/organ systems.[31] Yet, although an ever increasing number of adenosine A_3 receptor mediated effects is being reported, their mechanistic aspects remain unclear.[1,16,21] In view of these uncertainties, any attempt at an explanation of the different outcomes between pre- and posttreated animals is also highly tentative and needs extensive experimental corroboration.

It has been suggested[14] that worsening of the outcome of cerebral ischemia induced by acute preischemic administration of adenosine A_3 agonist may represent the cumulative effect of several adverse events triggered by the drug immediately

prior to the occlusion ("priming effect"). Events such as release of inflammatory mediators and concomitant degradation of the blood brain barrier integrity, and deleterious attenuation of the cerebral blood flow,[6,25–27,32] may be of primary importance. Combined with the increased influx of Ca^{2+} (both passive and through the voltage regulated calcium channels) and the liberation of internal calcium stores elicited by adenosine A_3 receptor stimulation,[22] the overall impact of these events would, indeed, predispose the brain to a significantly increased susceptibility to ischemia. On the other hand, when administered following a focal insult, IB-MECA induces cerebroportection in focal ischemia when the drug is given following transient occlusion of the middle cerebral artery.[30] Whether this cerebroprotective impact of adenosine A_3 receptor agonists is related to astrocyte activation, direct neuroprotective effect, or both[16,18,19,33,34] is unclear. The results of immunocytochemical studies presented in this paper confirm the presence of complex neuronal and glial effects induced by postischemic stimulation of adenosine A_3 receptors. Moreover, the latter observations offer strong support for the recently published hypothesis on the role of cerebral adenosine A_3 receptors as a part of the "adenosine based cerebroprotective complex".[1,16] This paper, and also our previous studies of adenosine A_3 receptor impact on the outcome of focal and global ischemia,[14,16,30] indicate significant involvement of these receptors in the pathology resulting from the arrested cerebral blood supply. However, the relationship between adenosine A_3 receptor stimulation and the outcome of an ischemic event is not straightforward. The presented data have confirmed initial hypothetical assumptions that the extent of the subsequent damage depends on the timing of adenosine A_3 receptor activation versus the onset of ischemia.[30] Most likely, the degree of receptor activation[18,21,33] may be an important factor as well. In summary, although our results provide further illumination of the complexity of A_3 receptor elicited effects, they do not provide a definitive solution to the baffling role of these receptors in the generation of stroke damage. As in many other studies of this still untreatable disease, we too must meekly conclude that "further extensive experiments are necessary" in order to understand the exact nature of the participating mechanisms and the factors determining the dual role of adenosine A_3 receptors in the brain.

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