Stimulation of Adenosine A_3 Receptors in Cerebral Ischemia

Neuronal Death, Recovery, or Both?

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ABSTRACT: The role of the adenosine A_3 receptor continues to baffle, and, despite an increasing number of studies, the currently available data add to, rather than alleviate, the existing confusion. The reported effects of adenosine A_3 receptor stimulation appear to depend on the pattern of drug administration (acute vs. chronic), dose, and type of the target tissue. Thus, while acute exposure to A_3 receptor agonists protects against myocardial ischemia, it is severely damaging when these agents are given shortly prior to cerebral ischemia. Mast cells degranulate when their A_3 receptors are stimulated. Degranulation of neutrophils is, on the other hand, impaired. While reduced production of reactive nitrogen species has been reported following activation of A_3 receptors in collagen-induced arthritis, the process appears to be enhanced in cerebral ischemia. Indeed, immunocytochemical studies indicate that both pre- and postischemic treatment with A_3 receptor antagonist dramatically reduces nitric oxide synthase in the affected hippocampus. Even more surprisingly, low doses of A_3 receptor agonists seem to enhance astrocyte proliferation, while high doses induce their apoptosis. This review concentrates on the studies of cerebral A_3 receptors and, based on the available evidence, discusses the possibility of adenosine A_3 receptor serving as an integral element of the endogenous cerebral neuroprotective complex consisting of adenosine and its receptors.

THE NATURE OF THE ADENOSINE A_3 RECEPTOR

In the very beginning of the present decade, the family of adenosine receptors (A_1, A_{2A}, and A_{2B}) increased by the addition of another distinct type—the A_3 receptor. Cloning of the cDNA from the rat testis library revealed approximately 40% identity with A_1 and A_{2A} types.[1] Further cloning studies[2–4] revealed intriguing differences in the degree of homologies among different species, indicating a possibility that A_3 receptors may, in fact, exist as a range of subtypes.[5]

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The A_3 receptor is a G-protein-coupled entity and, like all other known receptors of this type, consists of a long polypeptide chain with seven α-helices residing within the cellular membrane ending in an extracellular N- and an intracellular C-terminus.[6] The region between the central and the extracellular region of V–VII helices appears to constitute the active site of the receptor.[6] Signal transduction at the A_3 receptor is mediated by G_i/G_o proteins,[7,8] and the receptor appears to regulate their expression. The latter process is, however, somewhat heterogenous, and the responses of different subunits to the receptor's prolonged exposure to an agonist differ.[7] Long-lasting stimulation of A_3 receptors *in vitro* also results in their functional desensitization[9] and a noticeable reduction of high-affinity binding sites.[7] A detailed discussion can be found in the reviews by Palmer and Stiles[8] and Olah and Stiles.[10] In the context of the present paper, the finding that A_3 receptors desensitize rapidly may provide at least partial explanation for rather striking outcome differences observed following acute or chronic exposure to A_3 receptor agonists *in vivo* (see below).

Adenyl cyclase and phospholipase C serve as the second messenger systems of the A_3 receptor. Its stimulation results in depression of cyclic adenosine-5′,3′-monophosphate (cAMP) synthesis[2,3] or activation of phospholipase C[11] and subsequent stimulation of inositol-1,4,5-triphosphate synthesis.[12] Although it is unlikely that both second messengers are triggered into action at the same time, there are presently no studies analyzing these processes in detail. However, recent observations on the dependence of astrocytic response on the concentration of the stimulating A_3 agonist[13] (see below) indicate the possibility that the intensity and/or duration of receptor activation may have a possibly preferential impact on the selection of the second messenger system.

Probably the most striking aspect of A_3 receptor pharmacology is its very low affinity for adenosine. Although more recent studies reduced the original estimates of K_i from 30 μM[2] to 1 μM,[5] the value is still approximately two orders of the magnitude higher than that characterizing either A_1 or A_2 receptors (10 and 30 nM, respectively). The low affinity of A_3 receptors for adenosine poses one of many difficulties in envisaging the biological role of the receptor since, under the normal conditions, the extracellular concentration of adenosine in the brain does not exceed 300 nM (for review, see Ref. 14). However, none of the currently available methods of measuring adenosine concentration in the normal brain offers any indication of its value in the "operational" areas of neurons, i.e., perisynaptic space. Hence, intense synaptic activity, even under the normal conditions, may elevate local adenosine concentration to the level that is sufficient for A_3 receptor activation. Recent physiological studies of Dunwiddie *et al.*[15] indicate such a possibility. While interspecies differences in the affinity of A_3 receptors for antagonists are also striking, it is more likely that they mirror differences of receptor structure rather than function as such.[16] Much less pronounced, the variability in agonist affinity has been demonstrated as well.[16]

The progress in understanding of the 3-dimensional structure of A_3 receptors that derived from introduction of new techniques[17] is paralleled by a continuously increasing availability of selective A_3 receptor agonists and antagonists.[18–20] Regrettably, the development of these agents is not matched by the corresponding effort to synthesize equally selective radio-labeled ligands. Hence, our knowledge of A_3 receptor distribution is comparatively meagre, although considerable variation both in

its distribution and density among several nonhuman mammalian species has been reported.[5,16] The exact cellular location of A_3 receptors is still unknown as much in the brain as elsewhere.

THE ACTIONS OF ADENOSINE A_3 RECEPTOR

Nonneural Tissues

Most of the hitherto known effects elicited by the stimulation of A_3 receptors have been reviewed in the volume of *The Annals of the New York Academy of Sciences* devoted to the preceding Conference on Neuroprotective Agents held at Lake Como, Italy in 1996.[14] Since that time, experimental work concentrated predominantly (and unsurprisingly in view of the clinical uses of adenosine) on cardiovascular involvement of A_3 receptors shown to participate in hypotensive and vasoconstrictive responses,[21–24] and in cardioprotection both in myocytes[25–27] and in perfused rabbit hearts.[28] While there are indications that A_3 receptors may also be involved in ischemic preconditioning (IP) of the heart, the exact nature of their contribution to the phenomenon appears to need further clarification. Thus, while showing that the agonists of both A_1 and A_3 receptors mimic cardiac IP, Hill *et al*.[29] also indicated that A_1 rather than A_3 receptors are the principal source of the adenosine-mediated component of preconditioning. Other authors[30] concluded, however, that both receptors seem to play a role, while others described a complex interaction of adenosine A_1/A_3 and bradykinin B_2 receptors.[31]

Several recent studies focused their attention on the antiinflammatory effect of adenosine A_3 receptor agonists, e.g., prevention of neutrophil degranulation,[24] attenuation of inflammatory mediator,[33] and chemo- and cytokine release.[33–35] Moreover, since stimulation of A_3 receptors markedly decreases eosinophil chemotaxis,[35,36] it has been suggested that A_3 receptor agonists may be potentially useful in treatment of the pulmonary (asthma) and rheumatoid disorders.[32,33]

Nervous System

Astrocytes and hippocampal neurons have been the primary target of *in vitro* studies of the effects elicited by A_3 receptors. Fleming and Mogul[37] showed that exposure to the nonselective A_3 receptor agonist N^6-2-(4-aminophenyl)ethyl-adenosine (APNEA) potentiated high threshold Ca^{2+} current. The potentiating effect was sustained in the presence of A_1 and A_2 receptor antagonists and of protein kinase C (PKC) peptide inhibitor. The inhibitor of protein kinase A, on the other hand, blocked the potentiation. Since activation of A_3 receptors also results in the depression of adenylyl cyclase,[2,3] the authors concluded that PKA is negatively coupled to the Ca^{2+} current. The latter finding may have a significance in explaining at least some of the consequences following preischemic exposure of A_3 to selective agonists (see below; also the review of Domanska-Janik[38] on the importance of various protein kinases in the generation of ischemic damage). Of similar importance are the studies of Dunwiddie *et al*.[15] showing that stimulation of A_3 receptors with a selective agonist 2-chloro-N^6-(iodobenzyl)-adenosine-5′-*N*-methyluronamide (Cl-IB-MECA) antagonizes adenosine A_1 receptor-mediated inhibition of excitatory neu-

rotransmission. Similar functional disinhibition results from A_3 receptor inhibitory influence on the function of presynaptic group III metabotropic glutamate receptors (metGluRs)[39] that are responsible for the reduction of glutamatergic neurotransmission at a wide variety of synapses.[40]

A series of papers by Abbracchio *et al.*[13,41] and Yao *et al.*[42] indicated that the response of astrocytes to A_3 receptor stimulation is clearly dependent on its intensity, confirming theoretical arguments of von Lubitz *et al.*[43] based on their studies of acute and chronic administration of A_3 receptor agonists. Thus, Abbracchio *et al.*[13,41] reported that exposure of human astrocytoma ADF cells to nanomolar concentration of the selective A_3 receptor agonist Cl-IB-MECA results in increased ramification of astrocytes accompanied by reorganization of cytoskeleton, accompanied by Bcl-XL protein expression within the newly appeared astrocyte protrusions. Higher (micromolar) concentrations of the drug resulted in apoptosis.[41] Similar, concentration-dependent results were also obtained in the studies of human leukemia (HL-6) and lymphoma (U-937) cells.[42] Finally, a very high concentration of Cl-IB-MECA (>10 μM) is required to induce necrosis of cerebellar granule cells in culture.[44] However, when the cells were exposed to 1 μM Cl-IB-MECA in the presence of nontoxic glutamate concentration (50 μM), the necrosis was very rapid and virtually complete. Glutamate alone had no effect.

The data on the effect of adenosine A_3 receptor stimulation obtained in isolated cell systems may explain, at least in part, the outcome of *in vivo* acute exposure to A_3 agonist IB-MECA prior to global and focal cerebral ischemia[43] (see also this conference). Significant delay in the normalization of the postischemic cortical blood flow was the most immediate sign of the effect of the drug. Postischemic mortality

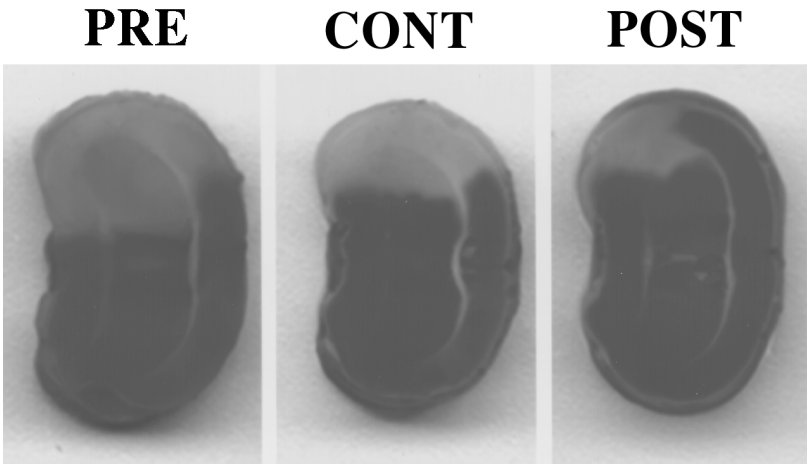

FIGURE 1. Acute administration of IB-MECA (100 μg/kg) 20 min prior to (PRE) or 20 min after (POST) initiation of permanent middle cerebral artery occlusion (MCAO) in mice. Following removal, brain slices were stained with the 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) method. Preischemic administration results in a larger infarct (approximately 40% increase compared to control animals (CONT), $p > 0.01$, $n = 15$/group). Compared to saline injected controls, administration of the drug after initiation of MCAO shrunk the infarcted volume by approximately 20% ($p > 0.05$, $n = 15$/group).

was substantially elevated, especially during the initial 3 days of postischemic recovery, while neuronal damage (cortex, hippocampus, striatum) was intensified in the surviving drug-treated animals at 7 days postischemia.[14,43] These results are similar to those obtained in the model of permanent middle cerebral artery occlusion in mice, where the initial mortality among IB-MECA-treated (100 μg/kg) mice increased by almost 30% (von Lubitz *et al.*, this conference, and in preparation). Increase in the infarct size was also very significant ($p > 0.01$, FIG. 1). Moreover, acute preischemic treatment with IB-MECA elevated the presence of nitric oxide synthase (NOS) and accelerated deterioration of cytoskeletal protein MAP-2 as shown using quantitative and semiquantitative immunocytochemical methods.[14,45] Chronic treatment with daily IB-MECA doses as low as 5 μg/kg (daily for 60 days) had, on the other hand, a diametrically opposite effect, i.e., improved postischemic blood flow, reduced mortality, and a significantly better neuropathological and neurological (e.g., improved postischemic memory and learning ability[14]) outcome. Finally, immunocytochemical expression of NOS was fully suppressed, and there was an excellent preservation of MAP 2, accompanied by a striking increase in the density of glial fibrillary acidic protein (GFAP, i.e., activated) astrocytes.[45] Importantly, acute preischemic treatment with the selective adenosine A_3 receptor MRS 1191 reproduced the effects of chronic exposure to IB-MECA, indicating that the observed phenomena were indeed A_3 receptor-induced (Ref. 46, and in preparation).

It is unclear what mechanism is directly responsible for the *in vivo* effects of A_3 receptor activation. As indicated in the preceding section, stimulation of A_3 receptors elicits a broad range of effects. Some of these, e.g., degranulation of mast cells and vasoconstriction (which may be the mechanism behind retarded normalization of the postischemic blood flow), attenuation of the inhibitory effects of A_1 and IIImetGLU receptors on glutamatergic neurotransmission, activation of NOS, or liberation of intracellular Ca^{2+} stores and facilitation of extracellular Ca^{2+} influx[47] are highly damaging in the context of cerebral ischemia and the subsequent recovery. Others, such as depression of eosinophil chemotaxis, reduction of neutrophil recruitment, or inhibition of tumor necrosis factor-α (TNF-α) and macrophage inflammatory protein (MIP)1-α release are quite beneficial, since studies have shown that reduction of inflammatory phenomena results in improvement of postischemic outcome in the brain.[48–50] Astrocyte activation appears to be equally recovery-enhancing.[51,52] One is thus presented with a complex mix of A_3 receptor-induced effects, some of which clearly promote, while others retard, the demise of ischemia-injured neurons, and make the interpretation of A_3 receptor involvement in brain pathology a seemingly hopeless task. Yet, the studies that have emerged recently offer a number of alluring clues which, taken together, indicate that adenosine A_3 receptor may be a very important constituent of a very efficient endogenous system defending brain against injury.

ADENOSINE A_3 RECEPTOR AND BRAIN INJURY: A SPECULATION ON FACTS

All known, and often contradictory, aspects of adenosine A_3 receptor-mediated actions notwithstanding, two facts are strikingly prominent: their very wide interspe-

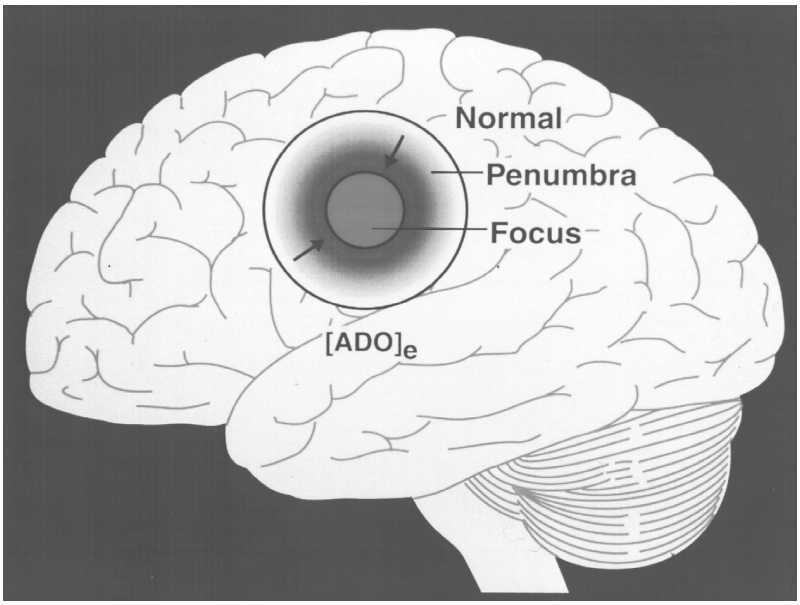

FIGURE 2. Schematic representation of the ischemic focus and the penumbra zone. It is very likely that the concentration of interstitial adenosine released during ischemia ($[ADO]_e$) increases progressively in the direction of the ischemic focus, attaining its highest value within the focus itself.

cies distribution,[16] and their very low affinity for adenosine.[5] In the evolutionary sense, its retention across the spectrum of several species, and its development into a functionally identical entity of a significantly varying molecular structure indicate that A_3 receptor must have an important survival value. On the other hand, the very low affinity of A_3 receptor for its endogenous agonist, adenosine, and a very low extracellular concentration of the latter under normal conditions,[53] also indicate that the receptor is, most likely, dormant under most but the most extreme conditions. Conditions such as encountered during ischemia, traumatic brain injury, or seizures, where the brain is exposed to extreme metabolic stress, where the bulk of adenosine released into the extracellular space originates from a furious pace of adenosine triphosphate (ATP) catabolism,[54] and where its extracellular level rapidly reaches and exceeds the threshold necessary for stimulation of A_3 receptors.[55–57] But, even before that level is reached, enough adenosine is already present to activate the neuroprotective A_1 receptors strategically located in the perisynaptic regions of neurons (see Ref. 58 for the most recent review). An important aspect of this process is the fact that enhancement of adenosine release is not a global, indiscriminate process but rather a localized "adenosine pulse" that is both event and site specific event.[59] While it has not been demonstrated due to the substantial technical difficulties in measurement of its interstitial concentration,[60] it may be expected that the level of adenosine released into the extracellular space diminishes with the distance from the center of the metabolic disturbance (FIG. 2). Thus, in focal ischemia, the territory of

the intensely hypoxic/ischemic tissue enveloping the immediate proximity of the occluded vessel will be rapidly surrounded by a zone of elevated extracellular adenosine in which the concentration of the latter is high enough to sustain continuous stimulation of A_1 receptors. Since the main effects induced by such stimulation manifest as the reduction of glutamate release, depression of *N*-methyl-D-aspartate (NMDA) receptor hyperstimulation, lowered influx of Ca^{2+}, etc., the net result shows as diminished electrical activity, reduced metabolic pace, and hypothermia (for reviews, see Refs. 58 and 60–62). Consequently, the progression of the infarct enlargement may be slowed down and, in cases of very mild ischemia, arrested entirely. There is, indeed, indirect evidence for such a possibility. Thus, studies of Matsumoto *et al.*[63] have shown that depression of the local cerebral blood flow (CBF_L) to 25 ml/100 g/min (i.e., above the level of bona-fide ischemia) is sufficient to raise adenosine 5–15-fold above its resting concentration, i.e., enough to activate A_1 receptors. Extracellular concentration of glutamate, on the other hand, begins to increase when CBF_L decreases to 20 ml/100g/min. Since activation of A_1 receptors results in hyperpolarization of the presynaptic terminals and attenuation of glutamate release, the latter process will be operational at CBF_L values that are depressed below normal but are still above the level necessary to induce neuronal damage.[64] There are thus good reasons to believe that adenosine A_1 receptors may be an important element sustaining the existence of the "penumbra zone,"[64] i.e., metabolically depressed but still viable volume of cerebral tissue surrounding the infarct core. Eventually, since the stimulation of A_1 receptors effectively increases the threshold of critical NMDA receptor input frequencies (i.e., depresses receptor excitability[65]), and since activity-dependent release of adenosine is, in turn, directly related to the intensity of NMDA and AMPA receptor stimulation,[66–68] the local extracellular concentration of adenosine will decrease due to the attenuation of glutamate receptor excitability, and due to the rapid metabolic removal of extracellular adenosine. In consequence, and providing the original insult is mild (e.g., occlusion of a minor arteriole), the tissue within the penumbra zone will regain its normal (or almost normal) function. Anti-inflammatory actions elicited by stimulation of A_{2A} receptors (reviewed in Ref. 69) will unquestionably assist in this process. Thus, but for the existence of A_3 receptors, the endogenous mechanism aimed at the reduction of neuronal injury would be an ideal example of interaction among several receptor systems whose joint task is to maintain functional integrity of the brain. However, many hitherto described effects elicited by the stimulation of A_3 receptors appear to contradict that goal entirely.

As discussed above, activation of A_3 receptors results in the desensitization of A_1 receptor-mediated inhibition of excitatory synaptic transmission.[15] With the inhibitory effect of A_3 receptors on IIImetGluR, whose stimulation modulates glutamate signaling,[39] the net effect induced by the A_3 receptors may be the enhancement of glutamate release, invigoration of ischemic NMDA receptor activity, and actual amplification of the subsequent neurotoxic phenomena. Since enhanced activity of NMDA/AMPA receptors increases concentration of interstitial adenosine, the process of "egotropic inhibition" sustained by the interaction of A_1 and NMDA receptors transforms rapidly into a self-fueling "egotropic excitation," in which the continuously increasing production of adenosine caused by intraischemic catabolismm of ATP activates A_3 receptors. A_3 receptors interact, in turn, with A_1 and IIImetGluRs and decrease their inhibitory impact on glutamate release. The constant

presence of the latter sustains activation of NMDA and non-NMDA receptors contributing to further release of adenosine, etc. The intensity of neurodestructive processes initiated by the initial intraischemic "glutamate burst" increases rapidly and sustains propagation of the neuronal destruction. Stimulation of A_3 receptors with micromolar concentration of their agonists results in apoptotic death of astrocytes.[13,41] While it is unknown whether A_3 receptors participate directly in the ischemia-evoked failure of glutamate uptake,[70] it is plausible that the process of A_3 receptor-induced apoptosis may be the source of such failure, contributing even further to the continuous presence of extracellular glutamate. The fairly rapid process of ischemia-induced apoptosis of astrocytes[52,71] is, most likely, hastened by A_3 receptor-mediated induction of NOS,[14,45] since nitric oxide pathway has been implicated in both glial and neuronal apoptosis.[72–74] Thus, activation of adenosine A_3 receptors by maximal intraischemic concentration of extracellular adenosine appears to trigger a massive destruction of cellular components within the entire volume of the tissue exposed to the most severe ischemia. This intensely "suicidal" role of A_3 receptors has been confirmed by the studies of global and focal ischemia, where preischemic administration of the selective A_3 receptor IB-MECA vastly amplified the subsequent brain damage[43] (see FIG. 1). While such paradoxical function of a receptor may have a remote evolutionary value in the context of removing from the population its members handicapped by the intense brain damage incurred, for example, during a mating fight, there are other, and unquestionably more efficient, mechanisms to achieve this goal. More importantly, from the point of *individual survival*, the "killer" role of cerebral A_3 receptor offers no biological advantage at all, especially that in the other tissues (e.g., heart, see Refs. 29, 30) stimulation of A_3 receptors is clearly protective. Very recent studies of von Lubitz *et al*. (this conference, and in preparation), in which administration of A_3 receptor agonist (IB-MECA) shortly after induction of permanent middle cerebral artery in mice resulted in a highly significant reduction of the infarct size (FIG. 1), may offer some indications on the likely function of A_3 receptors in the context of brain injury.

As indicated, it is very likely that the concentration of interstitial adenosine decreases with the distance from the ischemic core (FIG. 2). Hence, within the core itself, adenosine will reach a micromolar level adequate to fully stimulate A_3 receptors, and resulting in the full range of their destructive effects (FIG. 3). Further away, where the concentration decreases to high nanomolar values, more "benign" effects of A_3 receptors will predominate, i.e., activation and proliferation of astrocytes[13,41] (see also Ref. 52), antiinflammatory effects.[24,32–34] Combining their effects with the inhibitory and antiinflammatory effects mediated by A_1 and A_2 receptors, respectively, the A_3 receptors will enhance the chances of survival of the tissue within the volume that, most likely, constitutes the center of the penumbra zone. Finally, at the periphery of the penumbra, the concentration of adenosine will be, most likely, high enough to sustain prolonged activity of A_1 and A_2 but not A_3 receptors, resulting in "prophylactic" effects, i.e., reduction of the intensity of glutamatergic neurotransmission (and hence metabolism) by A_1 and vasodilation by A_{2A} to assure adequate blood supply to the outer rim of penumbra. Viewed in such light, adenosine A_3 receptor becomes an "*in situ* surgeon" rather than a "killer:" metabolic and physical excision of the ischemic core is afforded by the isolation of the affected volume from further blood supply through areteriolar vasoconstriction,[21]

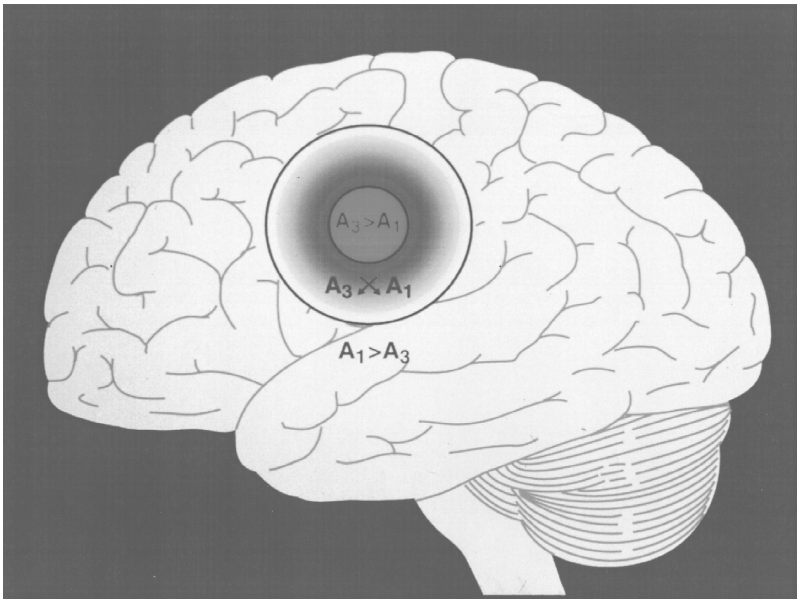

FIGURE 3. Schematic representation of the possible interactions between adenosine receptors and their effects during stroke/brain injury. In the focal volume, the actions of A_3 receptors predominate, resulting in enhanced excitotoxic phenomena and diminished astrocytic efficacy. Within the penumbra, there is a gradual transition from A_3 receptor-mediated effects (close to the focus) to weak A_3 receptor stimulation accompanied by the intense stimulation of A_1 receptors induced by endogenously released adenosine. As a result, at the periphery of the penumbra, one may expect enhanced A_1 receptor-mediated inhibition of glutamate release, and lowered intensity of stimulation accompanied by astrocyte activation resulting from activation of A_3 receptors. In the unaffected volume of the brain, A_1 receptor-mediated effects predominate. See text for further discussion.

and through A_3 receptor-mediated intensification of tissue damage (astrocytes and neurons). Activation of astrocytes within the inner rim of the penumbra promotes rapid scar development,[52] while, at the same time, enhancing neuronal protection through neurotrophin synthesis, potassium buffering, and neurotransmitter uptake[50] within the rest of penumbra volume. Hence, the damage may be contained more easily, and the continuing survival with less than debilitating neurological deficit becomes more likely. Thus, the adenosine A_3 receptor becomes an indispensable component of the "graded injury response complex," and finds a place in Newby's[75] concept of adenosine as a "retaliatory metabolite" that is as important as that of the other two adenosine types—A_1 and A_2,

Much of the preceding discussion is speculative, and the evidence is missing, although the emerging data point compellingly in the direction outlined above. The overriding question of the A_3 receptor biological role is still awaiting a plausible answer. Likely, there may be several roles, their nature depending on the studied tissue, and the context of function within the continuum of functions. But, indisputably, the A_3 receptor is more than just a variation on the "adenosine theme."

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