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Adenosine Signalling and Function in Glial Cells

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Abstract

Despite major advances in a variety of *neuro*scientific research fields, the majority of *neuro*degenerative and *neuro*logical diseases are poorly controlled by currently available drugs, which are largely based on a *neuro*centric drug design. Research from the past five years has established a central role of *glia* to determine how neurons function and – consequently – glial dysfunction is implicated in almost every neurodegenerative and neurological disease. Glial cells are key regulators of the brain's endogenous neuroprotectant and anticonvulsant adenosine. This review will summarize how glial cells contribute to adenosine homeostasis and how glial adenosine receptors affect glial function. We will then move on to discuss how glial cells interact with neurons and the vasculature and outline new methods to study glial function. We will discuss how glial control of adenosine-function affects neuronal cell death and its implications for epilepsy, traumatic brain injury, ischemia, and Parkinson's disease. Eventually, glial adenosine-modulating drug targets might be an attractive alternative for the treatment of neurodegenerative diseases. There are, however, several major open questions that remain to be tackled.

Keywords

astrocyte; adenosine receptor; adenosine kinase; epilepsy; excitotoxicity; neurodegeneration

1. Introduction

It is becoming increasingly clear that inflammatory processes play an important role in neurodegenerative disease, just as inflammation is becoming increasingly implicated in various systemic diseases elsewhere in the body1. Because the immune responses in brain show uncommon features it was long considered to be "immune privileged". However, cells that are involved in adaptive immune reactions do enter the brain, and this can result in major CNS pathology. Multiple sclerosis is an important example. Furthermore, immune reactions, often attributable to the innate immune system, feature in many if not all neurodegenerative diseases. Microglial cells, astrocytes, endothelial cells, oligodendrocytes, and neurons all produce signals to orchestrate these reactions. Arachidonic acid metabolites, nitric oxide, cytokines, and chemokines all appear to play some role. Recently, the role of ATP and adenosine as critically important signaling molecules has become appreciated2,3. This awareness that a multiplicity of signals other than those principally involved in nerve-nerve communication play a role in neurodegenerative disease has also meant that we have to consider other cells than neurons as critically important players. The present brief review will focus on adenosine

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(and to a lesser extent ATP) signaling, and the role of glial cells in neurodegenerative disease. The review will feature results from the authors' own work more prominently than would be motivated from an objective standpoint.

2. Glial control of adenosine homeostasis

In mammals, purine *de novo* synthesis proceeds via formation of IMP, which is then converted into AMP; however, there is no *de novo* synthesis pathway for adenosine. Physiologically, intracellular adenosine can be formed by either dephosphorylation of AMP by 5′-nucleotidase, or, alternatively, by hydrolysis of *S*-adenosylhomocysteine, whereas extracellular adenosine can be formed from released adenine nucleotides by a cascade of ectonucleotidases[4]. Two metabolic pathways are responsible for the removal of adenosine: deamination into inosine via adenosine deaminase (ADA; EC 3.5.4.4) and phosphorylation into AMP via adenosine kinase (ADK; EC 2.7.1.20). Based on its low K_M for adenosine, ADK is considered to be the primary route of adenosine metabolism[5]. Recent findings indicate that extracellular levels of adenosine, and consequently the levels close to synapses, are largely regulated by astrocytes[6–10], and an astrocyte-based adenosine-cycle has been proposed[11,12].

2.1. Glial release of ATP as source for extracellular adenosine

ATP can be released from neurons and astrocytes, is identified as a neurotransmitter in both CNS and PNS, and exerts a multitude of largely excitatory effects by activation of specific ATP receptors (P2X and P2Y receptors)[13]. Vesicular release has also been clearly demonstrated from endocrine cells and here the release of ATP may differ in several ways from that of the hormone, because of so called kiss-and-run release14. Thus, ATP can be released even in situations when the vesicle fusion is too incomplete and transient to allow release of the stored hormone. Perhaps this can occur also in nerves. However, under physiological conditions the vesicular release of ATP, not from neurons, but from astrocytes, has been identified as a major source of synaptic adenosine15. Transgenic mice that express a dominant-negative SNARE domain selectively in astrocytes were characterized by the loss of the adenosine A_1 receptor mediated tonic inhibition in synaptic slices, indicating that under physiological conditions astrocytic release of ATP (followed by degradation into adenosine via ectonucleotidases) is a major source of adenosine[15] that affects synaptic transmission. A kiss and run like exocytotic release of ATP has been detected in astrocytes16. Another, possibly related, mechanism involves release of ATP from a lysosome pool in astrocytes17. However, astrocytes appear to use also other mechanisms to release ATP as a signal. One of those involve connexin hemichannels18, but recent work provides strong reason to assume that pannexin-1, rather than connexin-43, provides the most responsible channel[19]. Another proposal is that ATP is released via maxi-anion channels, and this may be particularly important in astrocytic swelling[20]. The $P2X_7$ receptor may in some conformations allow ATP to be released. Finally, ATP may be released whenever there are increases in membrane volume via incorporation of intracellular vesicles into the membrane, or when there is a shedding of small vesicles[21]. In addition to the mechanisms described above, astrocytes can directly release adenosine, especially in response to hypoxic stimulation[6,22], even though release of adenosine *per se* is more typical of neurons[23]. In that case the release depends on export of adenosine via the equilibrative nucleoside transporters.

Since astrocytes can contact thousands of synapses and coordinate synaptic networks[8,24], it is conceivable that astrocytic release of ATP and its subsequent degradation into adenosine has a major regulatory function in setting a global adenosine-mediated inhibitory tone within a neuronal network. In addition, other glial cells can also contribute. For example, in retinal tissue stimulation of glial cells leads to an adenosine-mediated inhibition of neuronal activity, but in this case Muller cells rather than astrocytes appear to be most important[25].

The distribution of adenosine formed from breakdown of released ATP will obviously also depend on the distribution of the enzymes that degrade the nucleotide. It was shown long ago that 5′ nucleotidase tended to accumulate in areas of a lesion[26] and we now know that CD73, the ecto- 5′ nucleotidase, is highly expressed in microglial cells, as is the ecto-NTPDase, CD39[27].

2.2. Elimination of adenosine via astrocytic adenosine kinase

Several lines of evidence indicate that astrocytic ADK is the key regulator for ambient levels of adenosine: *(i)* In adult brain, ADK is predominantly expressed in astrocytes[10]. *(ii)* Pharmacological inhibition of ADK is sufficient to prevent seizures in various models of epilepsy[28]. *(iii)* Genetic knockout or knockdown of ADK in cultured cells induces the secretion of adenosine into the medium[29–32]. *(iv)* Transgenic overexpression of ADK triggers seizures by reduction of ambient adenosine[33]. *(v)* Inhibition of ADK in hippocampal slices increases endogenous adenosine and depresses neuronal firing, whereas inhibition of ADA had no effect[34]. *(vi)* A substrate cycle between AMP and adenosine, which involves ADK and 5′-nucleotidase, enables minor changes in ADK activity to rapidly translate into major changes in adenosine[35]. *(vii)* ADK activity is regulated in response to brain injury and is subject to developmental regulation[10,36,37]. Based on these considerations, and based on the lack of a classical transporter-regulated-reuptake system for adenosine and the ubiquitous presence of bi-directional equilibrative nucleoside transporters[38], ADK likely fulfills the role of a metabolic reuptake system for adenosine. Thus, tight regulation of ADK expression levels and of its specific activity becomes a necessity. Therefore, it is not surprising that ADK is highly conserved in evolution, that no naturally occurring mutations of the *Adk*-gene are known, and that a genetic disruption of the *Adk*-gene is lethal[5,39].

The fact that astrocytic ADK is of critical importance in regulating extracellular adenosine concentrations implies that the nucleoside transporters that facilitate adenosine uptake into astrocytes are important. Astrocytes express one concentrating (sodium-dependent) and two equilibrative nucleoside transporters[40]. Inhibition of this transport could potentially be used to elevate brain adenosine levels under conditions when extracellular adenosine is derived from extracellular ATP, but they would be less useful under conditions when adenosine is derived from intracellular production41. It is interesting to note that cannabinoids can block the equilibrative transporter and this effect can partly explain the immunosuppressive effects of these compounds42. The central role of astrocytes in regulating extracellular levels of adenosine is demonstrated in Figure 1.

3. Glial adenosine receptors

There are four types of evolutionarily conserved and pharmacologically well-characterized adenosine receptors called A_1, A_{2A}, A_{2B} and A_3[43] (Fig. 2). Adenosine is the endogenous agonist at all these receptors, but at A_1 and A_3 receptors inosine can act as a partial agonist[44,45]. The A_1 and A_3 receptors couple to the G_i family of G proteins and thus stimulate K^+ channels, reduce transient voltage dependent Ca^{2+} channels and inhibit cAMP formation; A_{2A} receptors couple to members of the G_s family (G_{olf} in striatal neurons), whereas A_{2B} receptors couple to many G proteins including G_s, G_q and G_{12}. Adenosine is approximately equipotent on A_1, A_{2A} and A_3 receptors, whereas A_{2B} receptors require higher agonist concentrations, if cAMP changes is the readout[45], but if MAP kinase activation is used to measure receptor activation adenosine is virtually equipotent on all four receptors[46]. All four adenosine receptors are detected in astrocytes[22], and all have been reported to be expressed in microglial cells or microglial cell lines[47–49].

3.1. A_1 receptors

A_1 receptors on astrocytes reduce their proliferation rate in culture[50]. As in many other types of cells activation of A_1 receptors can not only decrease cAMP accumulation but also stimulate phospholipase C, especially if this pathway is simultaneously activated by other stimuli[51–53]. A_1 receptors help protect astrocytes from damage and cell death[22,54,55], partly via activation of PI3K and Erk 1/2 phosphorylation. Nerve activity promotes myelination and it has been shown that this response is dependent on ATP[56], but ATP acts on oligodendrocytes indirectly, because ATP acts on astrocytes to release leukemia inhibitory factor. This response may be modified by adenosine, as A_1 receptors are present in oligodendroglia[57] and stimulate their migration. It has been shown that A_1 receptor activation leads to white matter loss, and that A_1 receptors contribute to hypoxia-induced white matter loss[58]. A_1 receptors on microglial cells are reported to reduce excessive activation of microglial cells upon immune activation[59]. Activation of these microglial receptors may secondarily affect oligodendroglial cells[59] and also astrocyte proliferation[60], which emphasizes the possibility of an extended glial network of signaling. A_1 receptors on neurons (especially at nerve terminals) are critically important in mediating the dampening effect on neuronal activity mediated by adenosine generated from ATP released from astrocytes[6,61]. The highly abundant A_1 receptors at nerve endings may preferentially signal via G_o proteins to inhibit transient calcium channels, whereas the same receptors in nerve cell bodies and dendrites may preferentially regulate potassium channel conductance via G_1 proteins.

3.2. A_{2A} receptors

In brain, A_{2A}Rs are expressed at high levels in striatal neurons and at low levels in neurons outside of the striatum and in glial cells[62,63]. Many functional measurements (such as cAMP levels and cytokine release) coupled with pharmacological tools have clearly demonstrated the presence and function of A_{2A}Rs in glial cells[64]. A_{2A}R binding densities are at the range ~30–60 fmole/mg protein in primary cultured microglial cells or in sorted microglial cells derived from striatum, as estimated by ^{3}H-ligand binding studies[65,66]. Furthermore, the expression of the A_{2A}R in glial elements in both the striatum and the solitary tract is confirmed by electronmicroscopic studies[63,67]. It should be noted that A_{2A}R expression in microglia and astrocytes is usually low under physiological conditions and frequently below the detection limit of histological methods (i.e. immunohistochemistry, autoradiography, or in situ hybridization)[62,64,68].

Importantly, the expression of A_{2A}Rs in glial cells is induced following brain insults. For example, LPS treatment induced A_{2A}R mRNA and protein in primary cultures of (mixed) glial cells (mainly in microglial cells) at 16 hours and peaked at 48 hours after the treatment[65]. Recently, using double immunohistochemistry analysis, we demonstrated that A_{2A}R expression is induced in microglial cells and astrocytes of mouse substantia nigra at 24 hours after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication. The induction of A_{2A}Rs in glial cells by brain insults and inflammatory signals, coupled with a local increase in adenosine and pro-inflammatory cytokines (such as IL-1β, which further induces A_{2A}R expression), may serve as part of an important feed-forward mechanism to locally control neuroinflammatory responses in the brain. It has been shown that adenosine, acting on A_{2A} receptors, can increase extracellular levels of glutamate, both by reducing glutamate uptake via GLT-1 and by direct release[69,70]. Thus, some of the reported effects of A_{2A} receptors on glutamate release may be based on mechanisms mediated by astrocytes rather than by neurons.

A_{2A}Rs in glial cells may exert complex actions on neuronal cell death (both, potentially deleterious as well as neuroprotective) and possibly other functions such as modulation of synaptic transmission. In astrocytes, activation of A_{2A}Rs by extracellular adenosine increases astrocyte proliferation and activation[71,72], but inhibits the expression of iNOS and the

production of NO[73], and regulates glutamate efflux by astrocytes[69]. Thus, modulation of astrogliosis by A_{2A}Rs is likely involved in brain repair processes, possibly via the formation of tissue scar. In microglial cells, activation of A_{2A}Rs has mixed effects on microglial proliferation, but has clear facilitating effects on the release of cytokines including up-regulation of cyclo-oxygenase 2 and the release of prostaglandin E2 (PGE2)[74], and on increases in NOS activity and NO release65 and nerve growth factor expression75.

3.3. A_{2B} receptors

It was shown early on that cyclic AMP accumulation in brain slices was due to a different adenosine receptor than that responsible for adenylate cyclase stimulation in striatum, and it is now clear that a major part of the brain slice cAMP response is due to A_{2B} receptor activation on astrocytes[76]. A_{2B} receptors can couple to two different classes of G proteins, G_q and G_s. G_q regulates intracellular calcium and vesicular release, whereas G_s affects a plethora of cAMP dependent signaling pathways. The A_{2B} receptor may also activate phospholipase C[77] and appears to be responsible for the adenosine-induced stimulation of Il-6 from astrocytes[78]. In airways, A_{2B} receptors, via cAMP, regulate chloride channels[79]. Since this occurs also in the intestine, it is fair to predict that cells in the CNS likewise alter chloride flux via A_{2B}R signaling. In particular, the possibility exists that adenosine is an important regulator of astrocytic swelling via modulation of volume-regulated anion channels. A_{2B} receptors may also play an impiortant role in the development of the nervous system; it was intially proposed that the important regulator netrin required signaling via A_{2B} receptors[80], but later studies showed that netrin does not require simulataneous A_{2B}R signaling[81], but that netrin signaling requires prior A_{2B} activation because the receptor is thereby regulated[82].

3.4. A_3 receptors

Astrocytic A_3 receptors appear to regulate chemokine release[83]. Activation of A_3 receptors by endogenous adenosine protects astrocytes from cell death induced, e.g., by hypoxia[22]. Microglial cells have functional A_3 receptors coupled to MSAP-kinase and p38 signaling[47,84]. Although adenosine itself has little effect on microglial migration, it was shown that the migration induced by ATP[85], perhaps predominantly via P2Y12 receptors[86] is lost when ATP hydrolysis via CD39 is eliminated and can be restored by adenosine[27]. Although it is not absolutely certain that the relevant adenosine receptor is the A_3R, it is tempting to speculate that there is a similarity to the situation in neutrophil leucocytes where ATP acting on a P2Y receptor acts in concert with adenosine acting on A_3 receptors to stimulate migration[87]. Microglial cells (and A_3 receptors) may also be of particular importance in the regulation of chemokine release and chemokine actions[88].

4. Glial cells and neurovascular coupling

Glial cells play important roles in coupling neuronal function to the cerebral microvasculature that controls cerebral blood flow (CBF)[89,90] in the sense that increased neuronal activity requires corresponding increases in CBF. Apart from large processes that stain for intermediate filaments and give astrocytes their stellar appearance, astrocytes have a multitude of fine processes that have little overlap with processes from other astrocytes and that define individual astrocytic domains, which each contain 300–600 neuronal dendrites and 10^5 synapses in rodent hippocampus[8,91–93]. Thus, a single astrocyte can sense the activity, and integrate the function, of hundreds of neurons within its domain. In addition, each astrocyte extends at least one process with endfeet surrounding blood vessels of the microvasculature. Therefore, astrocytes are uniquely located to adjust regional CBF to regional energy metabolism.

The vasodilator adenosine has been identified as an important mediator that couples cerebral blood flow to neuronal activation[94]. Thus, adenosine was demonstrated to mediate glutamate-

induced vasodilation in the cerebral cortex[95,96]. Topical application of glutamate dilated pial arterioles, an effect that could be reversed by an $A_{2A}R$ antagonist, but not by an $A_{2A}R$ antagonist[95]. Likewise topical superfusion AMPA on the cortical surface through a closed cranial window resulted in increases in pial arteriolar diameter, an effect that could be reversed by A_{2A} and $A_{2B}R$ blockade, but not by inhibition of NO synthase, cyclooxygenase-2, or cytochrome P-450 epoxygenase[96]. Apart from the activation of vascular adenosine receptors, adenosine can exert important regulatory functions by activation of astrocytic adenosine receptors[97]. Thus, the adenosine-evoked calcium response in acutely isolated astrocytes was found to be coupled to the A_{2B} receptor[77]; based on these findings adenosine could be implicated in promoting the propagation of calcium-increases throughout astrocytic processes. Increased Ca^{2+} in turn is also associated with the release of ATP through connexin hemichannels, a process that is potentiated by $A_{2B}R$ activation[98,99]. Through these mechanisms ATP-release and degradation into adenosine via ectonucleotidases appears to mediate arteriolar dilation in response to neuronal activation[100]. This process was dependent on astrocytes, since the application of the selective gliotoxin L-AAA, led to complete loss of arteriolar dilation in response to neuronal activation[101].

5. Glial control of glutamate and excitotoxicity

Astrocytes play a fundamental role in the pathogenesis of ischemic neuronal death[102]. A large body of evidence indicates that astrocytes are involved in the control of glutamate homeostasis and the susceptibility of the brain to excitotoxic injury[103]. Glutamate transporters are expressed in many different types of brain cells, but astrocytes are primarily responsible for glutamate uptake. Studies using genetic deletion or antisense-oligonucleotide mediated knockdown of the astroglial glutamate transporter GLT-1 have demonstrated that this transporter is the predominant subtype responsible for the clearance of extracellular glutamate in the brain[104,105]. Affected animals were highly susceptible to glutamate-dependent excitotoxicity and developed epileptic seizures[104,105]. After uptake of glutamate into astrocytes the enzyme glutamine synthetase converts glutamate into glutamine, which is then transported into neurons, where it is converted back into glutamate. Interestingly, a loss of glutamine synthetase was found in the sclerotic hippocampus of human patients with temporal lobe epilepsy[106] and the authors of that study concluded that reduced activity of the glutamate-glutamine cycle led to an accumulation of extracellular glutamate.

Apart from the mechanisms described above, astrocytes themselves can be a significant source of extracellular glutamate, which can be released by a variety of mechanisms[107]. It has been demonstrated that Ca^{2+} elevations in astrocytes induce the excitotoxic release of glutamate from these cells[108]. Most importantly, the Ca^{2+}-dependent astrocytic release of glutamate was also dependent on the vesicular glutamate transporters (VGLUT1/2) and the vesicular SNARE protein, cellubrevin, and was consistent with a vesicular release mechanism of glutamate that was similar to synaptic release of glutamate[108]. Finally, it was shown that astrocyte-derived glutamate targets synaptic NMDA receptors109, providing a rational explanation for the astrocyte-based control of neurotoxicity. Given the emerging roles of astrocytes in the control of neuronal excitotoxicity neuroprotective efforts targeting the functional integrity of astrocytes may constitute a superior strategy for future neuroprotection.

6. Novel methods for studying the role of glia

Co-culture of glia and neurons

Many original insights into glio-transmission were discovered using co-culture systems, and then extended to brain slices or the intact brain. For example, the first evidence for gliotransmission came from studies in mixed cultures of astrocytes and neurons demonstrating that experimentally evoked Ca^{2+} elevation in astrocytes evoked the elevation of Ca^{2+} in

adjacent neurons[110,111]. In this method, cultured cells from postnatal day 1–4 rodent brain are first enriched with one population of either glial or neuronal origin using specific culture conditions and then confirmed by immunohistochemistry. Glial or neuronal cells are then plated separately or together onto a coated substrate. The distinct morphology of glial cells and neurons allows the identification of these distinct cell types and permits the direct application of electric field potentials, micropipette tips, or neurochemical substrates to astrocytes in order to evoke a specific elevation of calcium in glial cells (e.g. in astrocytes)[110,111]. The spatiotemporal control over mechanical stimuli has permitted the selective stimulation of single astrocytes in mixed cultures of rat forebrain astrocytes and neurons. Using this strategy it was demonstrated that the elevation of calcium, triggered by focal electric field potentials in single astrocytes, induced a wave of calcium increase that was propagated from astrocyte to astrocyte, and importantly, this wave of calcium increase also evoked large increases in the concentration of cytosolic calcium in neurons depending on those astrocytes[110,111]. More recently, mixed co-cultures in multi-compartment dishes were equipped with the capability to provide electronic stimuli selectively to neurons in mixed cultures of oligodendrocytes and dorsal root ganglion neurons. This approach led to the finding that ATP and adenosine, released from neurons, acts as a potent neuron-glial transmitter that inhibited oligodendrocyte progenitor cell proliferation, stimulated their differentiation, and promoted the formation of myelin[56,112].

Transgenic overexpression and targeted knockout of genes in defined glial populations

Glial cells have been shown to release gliotransmitters, including ATP[9,17], glutamate[111, 113], and D-serine[114] to coordinate synaptic networks. However, neurons and glia share these same chemical signaling molecules, making it difficult to define the role of gliotransmitters. To molecularly dissect the role of glial signaling molecules, genetic approaches have been developed to selectively manipulate the SNARE-dependent release of gliotransmitters using a glia-specific promoter. Pascual and Haydon developed a transgenic mouse line, which uses the tet-off system to allow conditional expression of the cytosolic portion of the SNARE domain of synptobrevin 2 [dominant-negative SNARE (dn-SNARE)] selectively in astrocytes[15]. The selective expression of dn-SNARE was achieved by the use of an astrocyte-specific glial fibrillary acid protein promoter. To confirm the cell type selectivity of astrocytic transgene expression, Pascual et al. used EGFP as a reporter system and showed that EGFP was visually detectable in 97% of the dnSNARE-transgene-expressing cultured astrocytes, and that EGFP-positive cells colocalized specifically with the astrocytic marker, but not with neuronal, NG2-glial, or oligodendroglial markers. This transgenic line has successfully been used to demonstrate the functional significance of gliotransmission on synaptic plasticity in hippocampus[15] and more recently in the sleep-wake cycle[61]. Similarly, transgenic over-expression of a mutant disease-causing gene in a defined glial cell population has been used to study the role of SOD1 in astrocytes in the development of motor neuron death. Nagai et al demonstrated that the astrocyte selective expression of mutant human SOD1 (but not in spinal motor neurons, microglia or fibroblasts) killed spinal primary and embryonic mouse stem cell-derived motor neurons[115].

As a complementary genetic approach, the selective deletion of signaling molecules in defined glial cell populations can be accomplished using the Cre-loxP strategy. For example, Boillee et al generated a transgenic line (LoxSOD1^{G37R}) that carried a mutant human SOD1 gene flanked at both ends by a 34-base pair LoxP sequence[116]. These “floxed” mice were then cross-bred to two transgenic lines with expression of the Cre protein under control of *(i)* the promoter from the Islet-1 transcription factor, which directs the expression exclusively in progenitors of motor and dorsal root ganglion neurons, and *(ii)* the CD11b promoter, which directs the expression exclusively in the myeloid lineage (including macrophages and microglial cells). The establishment of these two novel transgenic lines allowed to demonstrate that the SOD1 mutation in motor neurons and microglial cells contributes distinctly to the onset and

progression of amyotrophic lateral sclerosis: while expression of SOD1 in motor neurons is the primary signal for the initiation of motor neurodegeneration and an early sign of disease progression, the genetic inactivation of SOD in mciroglial cells had little effect on the early disease phase, but markedly attenuated disease progression[116]. Thus, these genetic approaches to selectively manipulate signaling molecules in defined glial (or neuronal) populations provide critical insights into the distinct role of glial cells in the development of neurodegeneration.

Flow cytometric analysis and fluorescence activated cell sorting (FACS)

The structural complexicity of brain tissues hampers the dissection of unique roles of glial cells under various physiological and pathological conditions. Glial cells are characterized by a unique morphology, which permits the distinction of neuronal versus glial cells in intact brain by immunohistochemistry. However, it is difficult to quantify immunohistochemical changes without performing labor-intensive stereological analysis. Furthermore, there is a critical need to isolate large numbers of pure glial cells from intact brain for detailed molecular analysis such as qPCR and microarray analysis. Flow cytometric and fluorescence activated cell sorting can be adapted to partially circumvent these limitations for the study of glial cell functions in brain.

In the first application, defined glial cell populations (such astrocytes, microglia, or oligodendrocytes) are identified by labeling with a fluorescent antibody directed against specific cell surface markers. Quantitative changes of glial populations in normal and injured brains are determined by flow cytometry. For example, we recently utilized this analysis to evaluate the change of CD11b+ (a cell surface marker for microglial cells) and GFAP+ (a maker for astrocytes) cells in mouse striatum after treatment with MPTP and the $A_{2A}R$ antagonist KW6002[117]. This analysis not only provided an improved quantitative assessment of the effect of the $A_{2A}R$ antagonist on microglial activation at the very early phase of MPTP intoxication, but also identified a specific microglial cell population (i.e. CD11b+ cells with a large cell size representing fully activated microglial cells), which are most sensitive to KW6002 treatment in the brain[117].

In the second application, fluorescence activated cell sorting permits isolation and purification of distinct populations of glial cells from neurons from brain tissues using fluorescent antibodies directed against cell surface markers. The sorted glial cell populations can then be used for detailed molecular analyses such as quantitative PCR and microarray analysis. For example, Lovatt et al. successfully performed microarray profiling of sorted astrocytes from mouse cortex using FACS and (surprisingly) demonstrated that most enzymes in the tricarboxylic acid cycle are expressed at higher relative levels in astrocytes than in neurons[118].

7. Adenosine signaling in glial cells, excitotoxicity and cell death

As outlined above, synaptic levels of adenosine are largely controlled by an astrocyte-based adenosine cycle and the activity of the astrocyte-based enzyme ADK. Consequently, adenosine signaling in glial cells effects excitotoxicity and cell death in a variety of experimental paradigms. In addition, several forms of brain insult activate microglial cells. Here, ATP release is critically important[85], but the ATP response in microglial cells is markedly enhanced by adenosine generated from ATP[27].

7.1. Epilepsy

The adenosine kinase hypothesis of epileptogenesis implies that dysregulation of ADK is a major contributing factor to the epileptogenic cascade[12] (Fig. 3). Consequently, ADK expression levels (that determine levels of ambient adenosine) determine the brain's

susceptibility to acute seizure-induced cell death. Mice with only moderate transgenic overexpression of ADK in brain (141% of normal) were highly susceptible to acute seizure-induced cell death and did not survive beyond 3 days following status epilepticus[37], whereas engineered mice with reduced levels of ADK in forebrain (62% of normal) were completely resistant to seizure-induced cell death37. Resistance to seizure-induced excitotoxic cell death in ADK-deficient mice was dependent on adenosine and increased adenosine A_1R activation, since blockade of A_1Rs with its selective antagonist DPCPX restored wild-type like seizure-induced excitotoxic cell death[37].

Astrogliosis is a pathological hallmark of the epileptic brain and contributes to seizure generation by a variety of mechanisms[12,119,120]. A recent study from our lab has identified the enzyme ADK in astrocytes as a molecular link between astrogliosis and neuronal dysfunction in epilepsy[37]. In a mouse model of CA3-selective epileptogenesis we found spatio-temporal co-localization of astrogliosis, upregulated ADK, and focal spontaneous electrographic seizures that were all restricted to the CA3-region, the site of the epileptogenesis precipitating acute injury; importantly, seizures could be suppressed pharmacologically by ADK inhibition[37]. In this model, the seizures remained highly localized and restricted to the astrogliotic scar, presumably due to normal adenosinergic control of the surrounding brain tissue. Transgenic overexpression of ADK, as well as genetic disruption of the A_1R were sufficient to trigger spontaneous seizures, indicating that adenosine dysfunction rather than astrogliosis *per se* was responsible for seizure generation[121]. Conversely, mice with a genetically induced reduction of ADK in forebrain were completely resistant to the development of spontaneous seizures[37]. *In vitro* studies performed on hippocampal slices have subsequently demonstrated that reduction of the basal tone of adenosine by ADK is permissive to seizure generation, whereas ADK did not limit activity-dependant adenosine-release[122]. Together, these findings provide a neurochemical rationale for adenosine augmentation therapies (AATs). Consequently, several focal AAT-approaches – based on intracerebral adenosine-releasing implants – have demonstrated robust anticonvulsive and possibly antiepileptogenic efficacy in a variety of experimental paradigms that have been reviewed elsewhere[123,124].

7.2. Traumatic brain injury

Traumatic brain injury triggers an acute surge in adenosine, presumably as a consequence of ATP release, and this may represent an endogenous neuroprotective mechanism. In one study, adenosine levels increased 61-fold following controlled cortical impact (CCI) in rats and peaked at 20 min following the impact[125]. The existence of an endogenous protective action of adenosine at A_1 receptors early after experimental TBI was further corroborated by the finding of lethal status epilepticus in A_1R knockout mice subjected to either controlled cortical impact[126] or to kainic acid induced hippocampal injury[127]. In contrast to A_1R knockout mice, A_{2A}R knockout mice were largely protected from the adverse effects of CCI[128]. In line with these findings, increases in cerebrospinal fluid caffeine concentration were associated with favorable outcome after severe traumatic brain injury in humans, likely due to caffeine-mediated inhibition of A_{2A}Rs[129]. Likewise, chronic, but not acute caffeine attenuated the consequences of TBI in the mouse CCI model[130]. Although it probably does not contribute to the surge in adenosine following brain injury, acute downregulation of ADK in astrocytes has been described as a consequence of stroke[36] or acute seizures[131], and this may prolong the adenosine increase.

7.3. Parkinson's Disease

The adenosine A_{2A} receptor is a leading non-dopaminergic therapeutic target in Parkinson's disease (PD) (Fig. 4). Interest in this receptor within the context of PD derives primarily from two lines of experimental and clinical investigations: First, decade-long preclinical studies

demonstrated a unique co-localization of A_{2A}Rs and dopamine D_2Rs in striatopallidal neurons. Antagonistic interactions between A_{2A}Rs and D_2Rs at the molecular, neurochemical and behavioral level explain the motor stimulant effects of A_{2A}R blockade [132–134]. Thus, A_{2A}R antagonists such as KW-6002 (istradefylline) and SCH420814 have now completed clinical phase IIB-III trials. Despite some limitations of these clinical trials and admittedly modest effects ("OFF" time reduced by one hour), these studies confirm that selective A_{2A}R antagonists can stimulate motor activity by potentiating the L-dopa effect in advanced PD patients[135,136]. Second, in addition to symptomatic relief, A_{2A}R antagonists appear to more directly attenuate dopaminergic neurodegeneration, as suggested by convergent epidemiological and experimental evidence. Following an initial report from the Honolulu Heart Program[137] by Ross and colleagues, several large-cohort prospective studies have confirmed a similar inverse relationship between the consumption of caffeinated coffee and the risk of developing PD. Including the Health Professionals' Follow-Up Study and the Nurses' Health Study these studies involved a total of 47,351 men and 88,565 women[138], whereas a more recent study conducted by the Finnish Mobile Clinic Health Examination Survey included 19518 men and women[139]. These studies firmly established a relationship between increased caffeine consumption and decreased risk of developing PD in males. In addition, studies with animal models of PD provide a compelling clue about the potentially protective effects of caffeine, by demonstrating that pharmacological blockade (by caffeine or selective A_{2A}R antagonists) or genetic depletion of the A_{2A}R attenuates dopaminergic neurotoxicity and neurodegeneration[140–142]. These studies provide a neurobiological basis for the inverse relationship between increased caffeine consumption and reduced risk of developing PD.

Despite consistent demonstration that A_{2A}R antagonists afford neuroprotection against MPTP or 6-hydroxydopamine -induced dopaminergic neurotoxicity, the mechanism by which A_{2A}R inactivation protects against the loss of dopaminergic neurons remains unknown. The particular challenge lies in explaining the apparent dichotomy between restricted expression of the A_{2A}R in striatopallidal neurons and neuroprotection against degeneration of dopaminergic neurons in the substantia nigra where only a scattered expression of A_{2A}Rs is detected. An additional challenge is to identify the cellular mechanism, which allows A_{2A}R inactivation to protect neurons against a broad spectrum of brain insults, from ischemia to excitotoxicity to mitochondrial toxicity[143]. In this context, the involvement of the glial A_{2A}R becomes an attractive possibility, since glial function and neuroinflammation is commonly associated with diverse pathological conditions as mentioned above. Indeed, we recently demonstrated that MPTP treatment markedly upregulates A_{2A}R expression in microglial cells at 24–48 hours after treatment[65,66], which may result in further amplification of the A_{2A}R-mediated modulation of neuroinflammation in PD models. Consistent with this notion, an immunohistochemical study showed that KW-6002 reduced the loss of striatal dopamine contents and nigral cell bodies, and this coincided with inhibition of microglial activation[144]. Furthermore, we demonstrated by flow cytometery that KW-6002 attenuated MPTP-induced microglial activation at 48 hours after MPTP treatment[117]. In conclusion, A_{2A}R antagonists may confer neuroprotection by acting at A_{2A}Rs in glial cells, at least in the MPTP model of PD.

7.4. Ischemia

During ischemia, an imbalance between ATP degradation and resynthesis brings about a rapid and marked increase in extracellular level of adenosine in the brain during ischemia [145–154]. In addition hypoxia will increase ATP release, resulting in further adenosine production. While no clinical reports with purinergic compounds in human stroke exist, it is widely believed that adenosine and its receptors function as an endogenous neuroprotectant under these conditions[155–161]. Indeed, adenosine[162] or adenosine-potentiating agents (such as inhibitors

of ADA or ADK[163–167] or of adenosine transport[151,166,168–173]) offer protection against ischemic neuronal damage in different *in vivo* ischemia models. Furthermore, transgenic overexpression of ADK aggravates cell death, while reduction of ADK in the hippocampus increases protection after transient focal ischaemia[36,174].

Such a protective effect is attributed to stimulation of adenosine A_1 receptors that exert a protective role in ischemia by presynaptic reduction of Ca^{2+} influx, by inhibition of the release of excitatory neurotransmitters[175,176], and by postsynaptic hyper-polarization and reduction of neuronal activity through increases in K^+ and Cl^- ion conductances[177]. The efficacy of A_1 receptors stimulation on neuroprotection depends on the model used and no protective effect was observed in a global ischemia model[178]. Since adenosine does influence glutamate release it is suggested that this is not critically important in some ischemic models. However, adenosine (probably acting at the A_{2A} receptor) may in fact contribute to neurotoxicity, neuronal damage, and cell death. The potential neuroprotection by A_{2A}R antagonists was first reported in a global ischemia model with the less selective antagonist CGS 15943[179,180]. Further studies substantiated this finding in different models of ischemia with the selective A_{2A} receptor antagonist 8-(3-chlorostyryl)caffeine (CSC) and SCH 58261[181,147,182] in various animal models of stroke[183,184]. Studies in genetically manipulated mice confirmed the neuroprotective role of A_{2A} receptor antagonists on ischemic brain damage[185]. Major protective effects of A_{2A} receptor antagonists in stroke have been attributed to reduced glutamate outflow[183,186,187]. It should be considered however, that in several studies, A_{2A} receptor agonists have been found protective in the global ischaemia model in the gerbil[180] and that A_{2A} knockout mice show aggravated hypoxic ischaemic injury in neonatal mice[188]. Possible mechanisms are not clear yet, but include A_{2A}R-mediated protection via inhibition of platelet aggregation, vasodilation[163,189], or anti-inflammatory actions. Lastly, activation of A_3Rs produced mixed results and the exact contribution of A_3Rs to ischemic brain injury is not clear[190].

So far, support for a role for glia in the neuroprotective effect of A_{2A}R antagonism in ischemia comes from the observation that the A_{2A} receptor antagonist SCH 58261 reduces p38 MAPK activation in microglial cells[184] and phospho-JNK in neurons and oligodendrocytes[191] in the ischaemic hemisphere 24 hours after permanent MCAO. Since p38 MAPK and JNK are activated up to 24 hours after ischemia[192,193] and are involved in neuronal death[194,195], this correlation indicates that A_{2A}R antagonists may confer neuroprotection against ischemic brain injury through modulation of glial function and neuroinflammation. However, reduced MAPK activation might be secondary to a reduction in the excitotoxic cascade that primes p38 and JNK activation, since reduction glutamate outflow in the ischemic brain by A_{2A}R blockade is believed to be one of the main underlying mechanisms[196]. Further studies with selective manipulation of glial adenosine receptors or glial function *in vivo* are critical to our understanding to what extend adenosine regulation of glial signalling and function is responsible for ischemic brain injury.

8. Conclusions and major open questions

The discussion above has shown that much is now known about a role of glial cells in mediating the effects of adenosine (and other purines) in different neurodegenerative states. It is clear that many of the actions of endogenous or exogenous adenosine (and ATP) are in fact due to actions on glial cells. This makes it much more complicated to understand precisely how adenosine acts, and it has become apparent that the often diverse actions reported are due to the fact that several different receptors, located on many cell types are involved. In order to get a better understanding, there are several major questions that require an answer. Among the questions that need to be addressed in future research we find:

- Is the acute adenosine surge that follows brain injury a trigger for subsequent astrocyte activation?
- Do all the proposed mechanisms of ATP release from glial cells in fact occur, and are the triggers for astrocytic ATP release via these mechanisms different?
- To what extent does regional variation in the expression of ecto-nucleotidases control the distribution of adenosine in brain?
- Can adenosine-signals propagate within the brain via astrocyte-astrocyte communication?
- Given the major increase in glial cells in humans, how far can we extrapolate from rodents to man?
- When several adenosine receptors, with at least partly opposing signaling, appear to regulate a single biological response, are the receptors located on different cells or on very different parts of the same cell?
- Given that two GPCR molecules can form dimers, but apparently only one of them can actually signal, is there any functional significance of heterodimers between two types of purine receptors?
- Can activation of endothelial cells at the vascular interphase signal to synapses?
- Are microglial cells directly involved in synaptic transmission or do they mainly help prune synaptic contacts?
- Why are effects of A2ARs on inflammation different in CNS and peripheral organs?

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Abbreviations

AAT	adenosine augmentation therapy
ADA	adenosine deaminase
ADK	adenosine kinase
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AR	adenosine receptor
CBF	cerebral blood flow
CCI	controlled cortical impact
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
EGFP	enhanced green fluorescent protein
DR	dopamine receptor
FACS	fluorescence activated cell sorting
MAP	microtubule associated protein
NMDA	N-methyl-D-aspartic acid

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NOS	nitric oxide synthase
NTPDase	nucleoside triphosphate diphosphohydrolase
PD	Parkinson's disease
SNARE	soluble NSF attachment protein
SOD	superoxide dismutase

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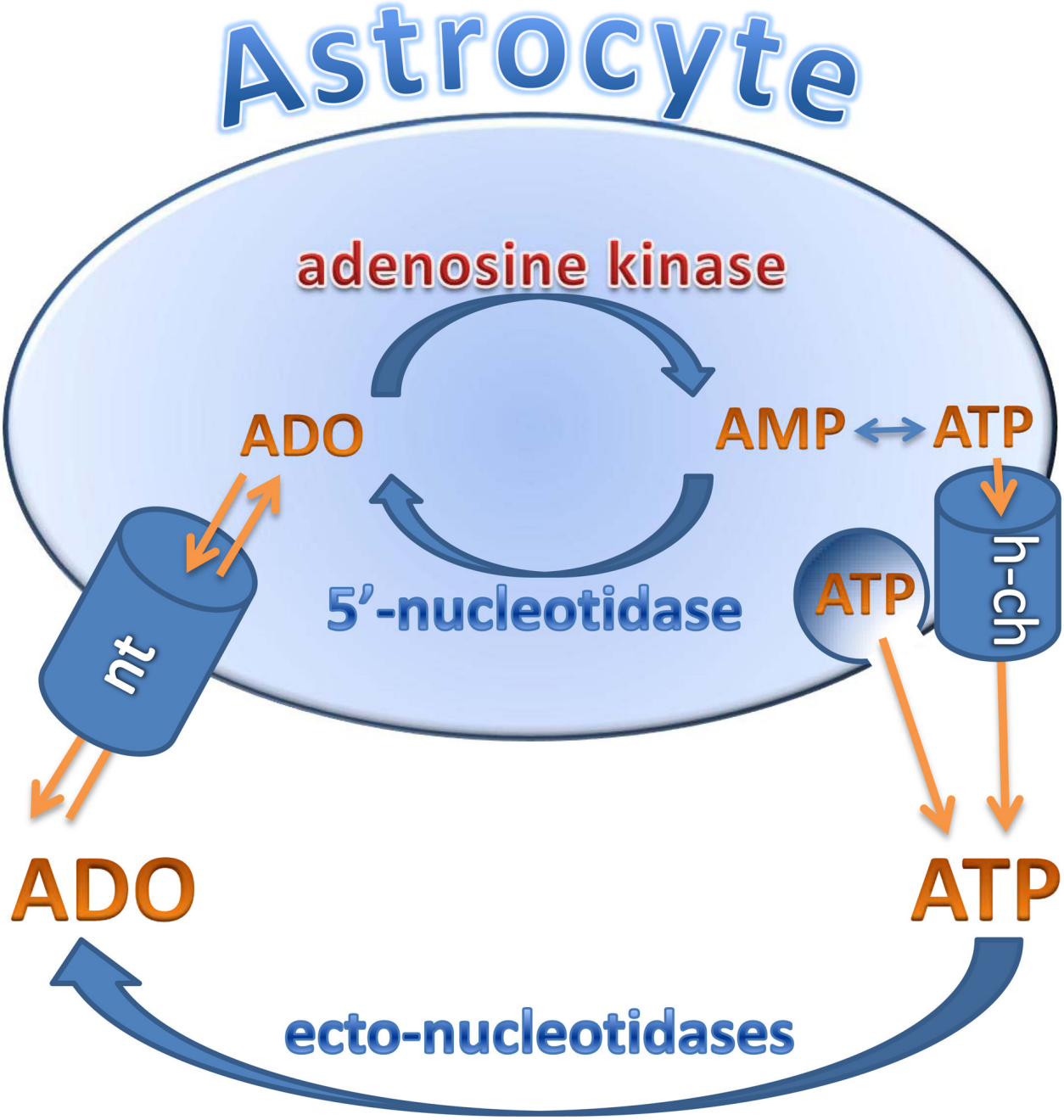

Figure 1.
Extracellular adenosine levels are thought to be regulated by an astrocyte-based adenosine-cycle. Astrocytes can release ATP via vesicular release and/or by direct release through hemichannels (h-ch). Extracellular ATP is rapidly degraded into adenosine (ADO) by a series of ectonucleotidases. Adenosine can also be released directly via equilibrative nucleoside transporters (nt). Intracellularly adenosine levels are largely controlled by adenosine kinase, which is part of a substrate cycle between adenosine and AMP. Small changes in adenosine kinase activity rapidly translate into major changes in adenosine. Intracellular adenosine kinase is considered to be a metabolic reuptake system for adenosine. Only selected mechanisms and pathways are shown; for details please refer to main text.

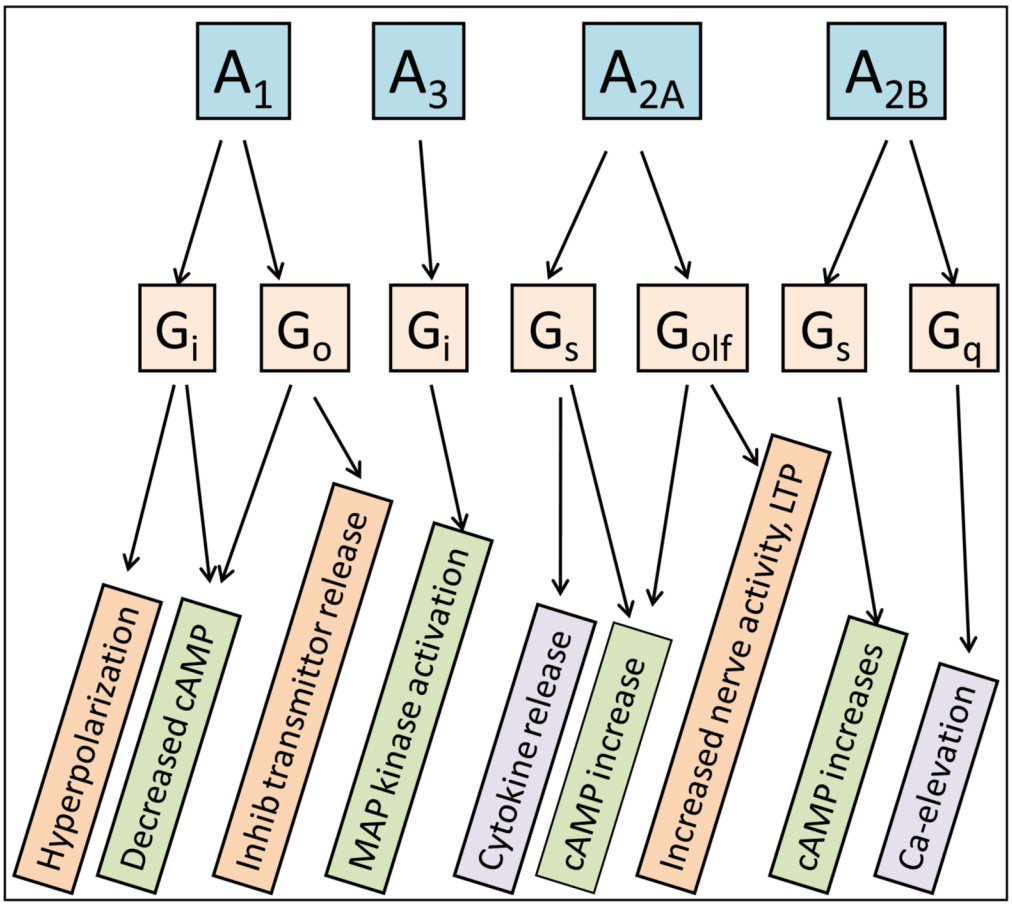

Figure 2.
Adenosine receptors, their coupling to G-proteins and some of the down-stream consequences of receptor activation.

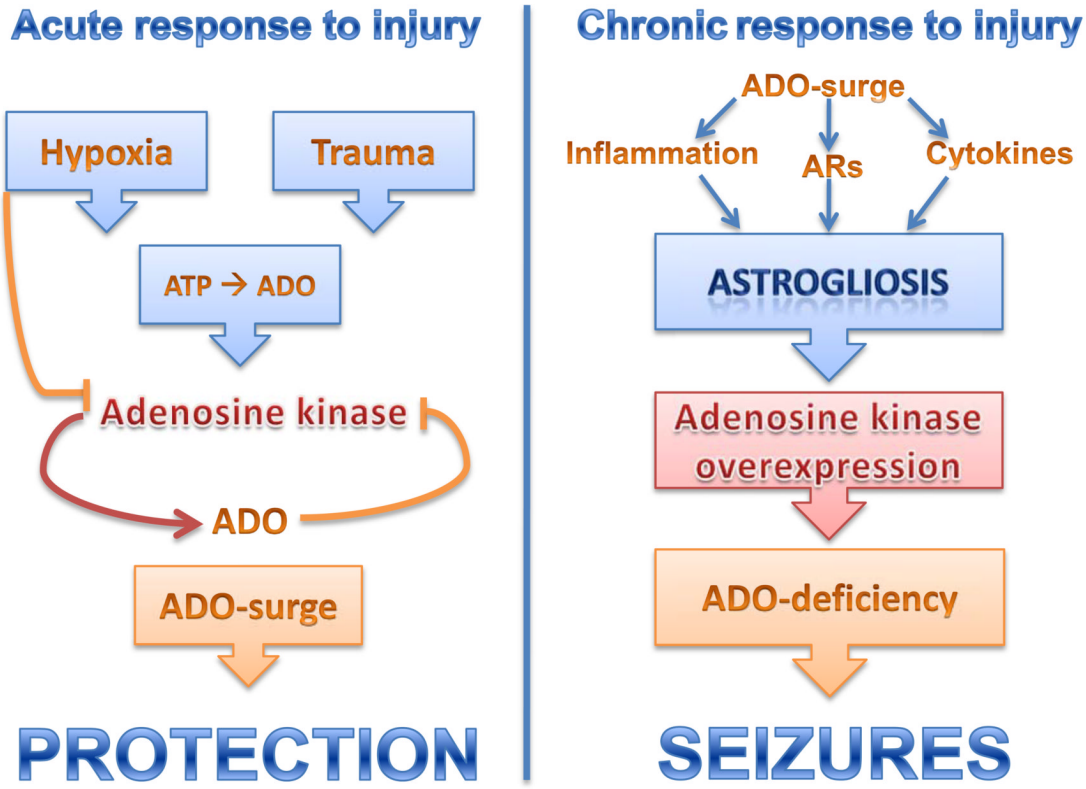

Figure 3.

Role of the adenosine (ADO) / adenosine kinase (ADK) system in regulating acute and chronic responses to injury. Left: Within hours after brain injury (e.g. stroke, trauma, prolonged seizures) a surge in micromolar levels of ADO results that protects the brain from further injury and from seizures. Hypoxia and trauma can directly lead to a rise in extracellular ATP that is rapidly degraded into adenosine. High levels of adenosine are known to inhibit ADK, further amplifying the adenosine surge. Right: The acute adenosine surge contributes to trigger astrogliosis via a variety of mechanisms that include modulation of astrocytic adenosine receptors (ARs), modulation of inflammatory processes and the release of cytokines. Astrogliosis leads to overexpression of ADK resulting in adenosine-deficiency, which contributes to seizure generation.

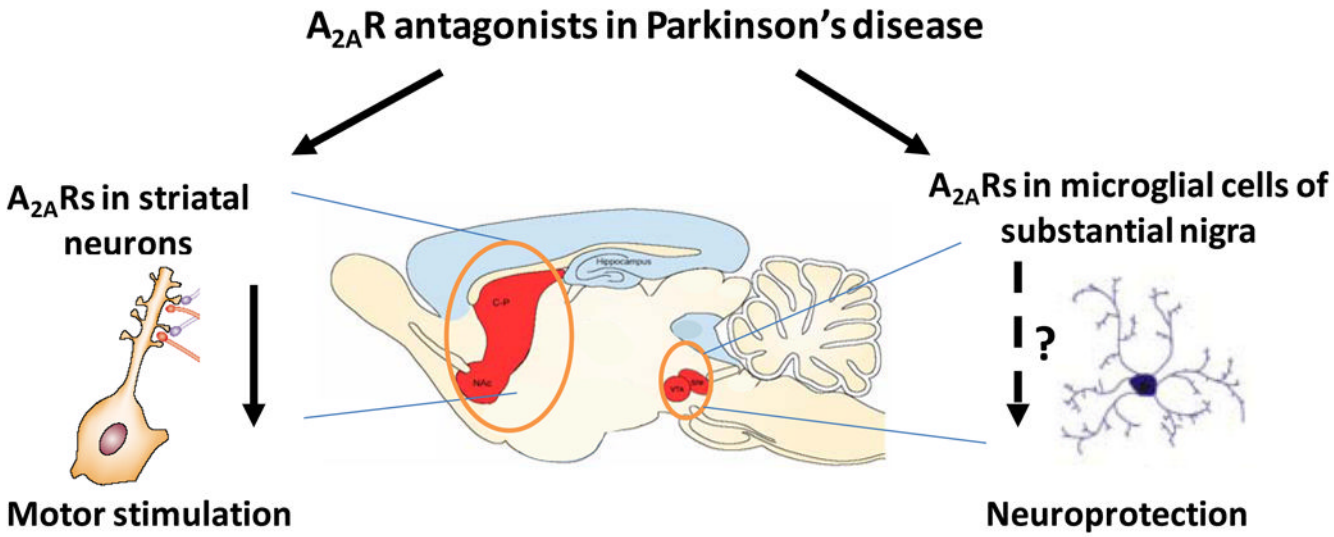

Figure 4.

The dual functions of A_{2A} receptor antagonists in Parkinson's disease models: $A_{2A}R$ antagonists act at the $A_{2A}R$ in striatal neurons to stimulate motor activity. Furthermore, it is postulated that $A_{2A}R$ antagonists may modulate microglial activation in substantia nigra to exert a possible neuroprotective effect in an animal model of Parkinson's disease.