



# The role of the ATP-adenosine axis in ischemic stroke

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## Abstract

In ischemic stroke, the primary neuronal injury caused by the disruption of energy supply is further exacerbated by secondary sterile inflammation. The inflammatory cascade is largely initiated by the purine adenosine triphosphate (ATP) which is extensively released to the interstitial space during brain ischemia and functions as an extracellular danger signaling molecule. By engaging P2 receptors, extracellular ATP activates microglia leading to cytokine and chemokine production and subsequent immune cell recruitment from the periphery which further amplifies post-stroke inflammation. The ectonucleotidases CD39 and CD73 shape and balance the inflammatory environment by stepwise degrading extracellular ATP to adenosine which itself has neuroprotective and anti-inflammatory signaling properties. The neuroprotective effects of adenosine are mainly mediated through A<sub>1</sub> receptors and inhibition of glutamatergic excitotoxicity, while the anti-inflammatory capacities of adenosine have been primarily attributed to A<sub>2A</sub> receptor activation on infiltrating immune cells in the subacute phase after stroke. In this review, we summarize the current state of knowledge on the ATP-adenosine axis in ischemic stroke, discuss contradictory results, and point out potential pitfalls towards translating therapeutic approaches from rodent stroke models to human patients.

**Keywords** Stroke · ATP · Adenosine · P2X7 · Ectoenzymes

## Abbreviations

AAV	Adeno-associated virus
ADA	Adenosine deaminase
ADAC	Adenosine amine congener
ADP	Adenosine diphosphate
ADPR	ADP ribose
AMP	Adenosine monophosphate
APEC	2-[(2-Aminoethylamino)-carbonyl ethyl phenylethylamino]-5'-N-ethylcarboxo amidoadenosine
AR	Adenosine receptor
AR132	N <sup>6</sup> -Methyl-2-phenylethynyladenosine

ATP	Adenosine triphosphate
BAY60-6583	2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy) phenyl]-2-pyridinyl] thio]-acetamide
BBB	Blood-brain-barrier
BBG	Brilliant Blue G
5-BDBD	5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one
BzATP	2'-3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate
cAMP	Cyclic AMP
CGS 15943	9-Chloro-2-(2-furanyl)-[1,2,4] triazolo[1,5-c]quinazolin-5-amine
CGS 21680	2-p-(2-Carboxyethyl)-phenethylamino-5'-N-ethylcarboxamidoadenosine
CHA	Cyclohexyladenosine
CI-IB-MECA	Chloro-N <sup>6</sup> -(3-iodo-benzyl)-adenosine-5'-N-methyluronamide
cM1	Contralesional primary motor cortex
CNS	Central nervous system
CPA	N <sup>6</sup> -cyclopentyladenosine
CP 66,713	4-Amino-1-phenyl[1,2,4]-triazolo[4,3-a] quinoxaline

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CSC	8-(3-Chlorostyryl) caffeine	NTPDase1/CD39	Ecto-nucleoside triphosphate diphosphohydrolase 1
DAMPs	Danger-/damage-associated molecular patterns	NT5E/CD73	Ecto-5'-nucleotidase
DCs	Dendritic cells	OGD	Oxygen-glucose deprivation
2'-dCCPA	2-Chloro-2'-deoxy-N <sup>6</sup> -cyclopentyladenosine	PLC	Phospholipase C
3'-dCCPA	2-Chloro-3'-deoxy-N <sup>6</sup> -cyclopentyladenosine	pMCAO	Permanent middle cerebral artery occlusion
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine	pmeLUC	Plasma membrane luciferase
EAE	Experimental autoimmune encephalomyelitis	PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid
ENPP1/CD203a	Ectonucleotide pyrophosphatase/phosphodiesterase 1	SCH 58261	7-(2-Phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine
GSK	GlaxoSmithKline	tMCAO	Transient middle cerebral artery occlusion
h	Hours	TNAP	Tissue non-specific alkaline phosphatase
i.c.v.	Intracerebroventricular	Tregs	Regulatory T cells
IL-2/IL-2Ab	Interleukin-2/interleukin-2 antibody	WT	Wild type
iM1	Ispilesional primary motor cortex	VSMCs	Vascular smooth muscle cells
i.p.	Intraperitoneal	VT72	N <sup>6</sup> -Methoxy-2-phenylethynyl
irAE	Immune-related adverse event	VT158	N <sup>6</sup> -Methoxy-2-phenylethynyl
i.v.	Intravenous	VT160	N <sup>6</sup> -Methoxy-2-(2-pyridinyl)-ethynyl
LJ529	2-Chloro-N <sup>6</sup> -(3-iodobenzyl)-5'-N-methylcarbamoyl-4'-thioadenosine	VT163	N <sup>6</sup> -Methoxy-2-p-acetylphenylethynyl
LJ1251	(2R,3R,4S)-2-(2-chloro-6-(3-iodobenzylamino)-9H-purin-9-yl) tetrahydrothiophene-3,4-diol	ZM 241385	4-(-2-[7-Amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol
MAPK	Mitogen-activated protein kinase		
K <sub>1/2</sub>	Half-maximal response		
min	Minutes		
MRI	Magnetic resonance imaging		
MRS1523	3-Propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate		
MRS1754	8-[4-(((4-Cyanophenyl)carbamoylmethyl)oxy)phenyl]-1,3-di(n-propyl)xanthine hydrate		
MRS2500	(1R*,2S*)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphono-oxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester		
MRS2578	1,4-Di[3-(3-isothiocyanatophenyl)thioureido]butane		
MRS4719	5-(2-(5-Thioxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)pyridin-4-yl)-1,5-dihydro-2H-naphtho[1,2-b][1,4]diazepine-2,4(3H)-dione triethylamine salt		
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide		
NF449	4,4',4''-(Carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))) tetrakis-benzene-1,3-disulfonic acid		
NK cells	Natural killer cells		

## Introduction

Stroke ranks among the leading causes of death and disability worldwide. With 7.6 million new cases in 2019, ischemic stroke accounted for 62% of all strokes, and 3.3 million patients died from this widespread disease [1]. At present, treatment options of acute ischemic stroke are limited to intravenous (i.v.) thrombolysis and mechanical thrombectomy both aiming at reperfusion of the clotted vessel. Due to strict inclusion criteria, these therapies are only available for a subset of patients, and many patients present with persistent neurological deficits despite successful recanalization. Therefore, additional treatment strategies to combat ischemic brain injury are urgently needed. Research over the last three decades identified the substantial contribution of sterile inflammation to secondary neuronal injury following ischemic stroke. Consequently, targeting secondary infarct growth mediated by infiltrating immune cells emerged as a promising therapeutic strategy. The inflammatory cascade is initiated by the excessive release of danger-/damage-associated molecular patterns (DAMPs) which activate pattern recognition receptors on microglia and astrocytes with subsequent cytokine and chemokine production and immune cell attraction from the periphery [2]. Among these DAMPs,

the purine adenosine triphosphate (ATP) plays a pivotal role. Geoffrey Burnstock pioneered the concept that ATP released by cells acts as an extracellular signaling molecule and thus exerts key functions beyond energy supply [3].

Under physiological conditions, cells within a tissue, including the brain, release ATP and nicotinamide adenine dinucleotide (NAD<sup>+</sup>) into the interstitial space in an activity-dependent and regulated manner. Release mechanisms include exocytosis, connexin or pannexin hemichannels, ATP-binding cassette (ABC) transporters, calcium homeostasis modulator (CALHM) channels, a macropore after P2X7 receptor overstimulation, maxi-anion channels (MACs), and volume-regulated ion channels (VRACs) as comprehensively reviewed by Giuliani et al. [4]. At sites of inflammation or ischemia, ATP is further liberated in an uncontrolled fashion via cell damage and death [4]. Indeed, an increase in extracellular ATP concentrations within the brain parenchyma during in vivo ischemia was measured by microdialysis [5, 6]. To improve the accuracy of extracellular ATP sensing and to circumvent methodological challenges of purine measurements within intact tissues, the group of Francesco Di Virgilio engineered a chimeric plasma membrane luciferase (pmeLUC) [7]. PmeLUC emits a measurable bioluminescent signal upon D-luciferin administration in the presence of extracellular ATP. Using this in vivo ATP biosensor, ATP release could be visualized as early as 30 min and up to 24 h after transient middle cerebral artery occlusion (tMCAO) [8]. After being released, ATP can either signal through its ionotropic P2X and metabotropic P2Y receptors or experience degradation to adenosine by ectoenzymes. The present review will give an overview about the different players of purinergic signaling, their expression in mice and humans (Fig. 1), and their contribution to tissue damage and regeneration in ischemic stroke. Details on the cited experimental stroke studies using mice with global genetic deficiency for the different players of purinergic signaling can be found in STab. 1.

### Signaling of ATP via P2X receptors in stroke

Extracellular ATP serves as ligand for all purinergic receptors of the ionotropic P2X family and for several P2Y receptors, while its metabolite ADP binds exclusively to P2Y receptors. The P2X family comprises seven members (P2X1–P2X7). Three P2X receptor subunits form an ion channel that upon ATP-mediated gating conducts the rapid influx of calcium and sodium ions and efflux of potassium ions. The expression of P2X receptors has been ascribed to various brain-resident cells as well as to cells of the immune system [9] with P2X1, P2X4, and P2X7 being associated with the outcome of cerebral ischemic events. In the central nervous system (CNS) of both mice and humans, microglia show the highest expression of these three receptors [10–13] (Fig. 1).

Of importance in the context of ischemic stroke, P2X1 is expressed on platelets. ATP (10 μM) increases platelet aggregation in vitro, which can be prevented by the P2X1 antagonist NF449 [14]. P2X1 is also expressed by neutrophil granulocytes of the mouse, where its activation promotes neutrophil chemotaxis [15]. Pharmacological blockade of P2X1 and genetic deletion of *P2rx1* in mice diminished the activation and recruitment of neutrophils to inflamed arteriolar endothelial cells, accompanied by impaired platelet aggregation and fibrin generation [16]. Therefore, targeting of P2X1 could be a promising new approach to ameliorate neutrophil driven post-stroke inflammation as well as stroke-associated thromboinflammation [17]. Apart from immune cells, P2X1 is also expressed on vascular smooth muscle cells (VSMCs) of rat middle cerebral arteries [18]. It has been shown that P2X1 and P2X4 form heterotrimers in VSMCs, which, in contrast to P2X1 homotrimers, contribute to ATP-mediated vasoconstriction. The impact of P2X1 signaling on VSMCs in the context of stroke still needs to be investigated.

ATP released during cerebral ischemia triggers P2X4 opening on brain innate immune cells such as microglia or infiltrating monocytes/macrophages and sustained P2X4 activation contributes to the ischemic injury [19, 20]. Among immune cells, P2X4 is expressed on T cells promoting early T cell activation in concert with P2X7 [21] as well as chemotaxis [22]. Indeed, *P2rx4* deficiency in mice led to smaller infarcts after tMCAO [23]. Interestingly, when specifically deleting *P2rx4* in myeloid cells using *LysM-Cre* mice, only female mice showed a reduction in infarct size, suggesting a sex difference in the P2X4 response after stroke [23]. Another experimental stroke study by the same group verified the detrimental role of P2X4 in brain ischemia by using the P2X4 antagonist 5-BDBD: When applied 4 hours (h) after tMCAO, 5-BDBD reduced infarct size, number of infiltrating pro-inflammatory myeloid cells, and blood-brain-barrier (BBB) disruption [24]. 5-BDBD was also shown to attenuate brain injury in a mouse model of intracerebral hemorrhage, significantly reducing brain edema, BBB disruption, neuronal cell death, and neurological deficits [25]. Another study demonstrated that P2X4 is also expressed by pyramidal neurons of the rat hippocampal CA1 region [26]. In line with tMCAO data, intracerebroventricular (i.c.v.) 5-BDBD treatment immediately after cerebral ischemia (four-vessel occlusion model) and once 24 h later was also protective by blocking neuronal apoptosis, thereby promoting neuronal survival. Similar results were obtained with the P2X4 antagonist PSB-12062 [26]. Given the central role of P2X4 in inflammation, cell migration, and also pain, new P2X4 antagonists are continuously developed. The group of Kenneth A. Jacobson recently described the generation and evaluation of MRS4719 and MRS4596, two potent new P2X4 antagonists (IC<sub>50</sub> 0.503 and 1.38 μM for human P2X4, respectively) with neuroprotective effects in murine ischemic



potent trigger for the NLRP3 inflammasome formation in macrophages and microglia, leading to the release of pro-inflammatory interleukin-1 $\beta$  (IL-1 $\beta$ ), a powerful driver of post-stroke inflammation [32–34]. Yet, deciphering the complex role of P2X7 during the interlinked processes of post-stroke inflammation, BBB breakdown and neuronal recovery remains challenging.

First studies by Nancy Rothwell's group did not show a difference in stroke size when comparing *P2rx7*-deficient and WT mice [35], and i.c.v. treatment with P2X antagonists oxidized ATP and PPADS immediately before and again 30 minutes (min) after tMCAO did not affect lesion size after tMCAO either [36]. However, other studies demonstrated that intraperitoneal treatment with the broad P2 antagonist Reactive Blue 2 5 min after intraluminal permanent MCAO (pMCAO) in rats reduced brain damage and that MCAO led to an upregulation of P2X7 on microglia [37]. P2X7 upregulation had already been observed by earlier studies with hypertensive rats subjected to pMCAO by electrocoagulation [38]. Other studies suggested a protective role of P2X7 in stroke: I.c.v. injection of the P2X7-specific agonist BzATP into rats 1 h after tMCAO improved their motor functions when compared to mock-injected controls [39]. Studies using Brilliant Blue G (BBG) as P2X7 antagonist reported different outcomes regarding the role of P2X7 in rodent stroke models: The group of Carlos Matute reported that intraperitoneal BBG treatment 30 min after ischemia onset reduced the lesion size in rats subjected to tMCAO [40] and later reproduced their findings in mice [41]. The study by Caglayan and colleagues in 2017 again confirmed that i.c.v. injection of BBG reduced lesion size in mice when injected 30 min before tMCAO [42]. Similarly, intraperitoneal BBG administration also turned out to be protective in murine photothrombotic stroke when applied 1, 3, and 5 days after ischemia [43]. In contrast, a study, in which BBG was injected intraperitoneally (i.p.) daily for 3 days immediately after a short (15 min) tMCAO in mice, did not demonstrate a difference in lesion size between treatment and control group [44].

As for P2X7 targeting compounds, contrasting results have also been reported in studies using *P2rx7*-deficient mice. As mentioned above, first stroke experiments with P2X7ko mice did not reveal differences in stroke size [35]. Later in 2015, the group of Carlos Matute reported smaller stroke lesions (tMCAO) in P2X7ko mice when compared to WT [41]. In contrast, the group of Michael Schäfer reported reduced edema formation in P2X7ko mice 3 days after tMCAO, while stroke lesion size did not differ between P2X7ko and WT mice [45]. A recent study published by our group again suggested a detrimental role of P2X7 in stroke: Mice overexpressing P2X7 in cells that naturally express P2X7 [46] exhibited larger infarcts compared to WT controls. Further, i.c.v. injection of P2X7-blocking nanobodies [47] in mice directly before tMCAO significantly reduced

lesion size compared to the control group [8]. This study represents the first approach to use highly specific biologicals to block P2X7 in the context of stroke. As mentioned above, the BBB constitutes a major obstacle for i.v. application of antibody-based drugs targeting brain-resident cells like microglia. Even for the small P2X7-specific nanobodies only high doses beyond 1 mg lead to detectable amounts of the nanobodies on brain microglia [48]. Interestingly, an alternative approach based on adeno-associated virus (AAV)-mediated muscle cell transduction followed by in vivo production of P2X7 antagonistic nanobodies led to the successful diffusion of the nanobodies into the brain where they could be detected on microglia for up to 120 days after transduction [48]. Since the role of P2X7 in long-term recovery after stroke still remains elusive, the AAV approach could be utilized to investigate this technically challenging question of high translational relevance.

When using antibody or nanobody-based therapeutics, one has to monitor for potential immune-related adverse events (irAE). Antibody-induced inflammation via complement activation or activation of natural killer (NK) cells and macrophages can be prevented by modifying the Fc-region of antibodies. Nanobody monomers lack Fc regions in principle but can be tailored as Fc-fusion proteins with desired or no Fc-related functions [49, 50]. To date, no adverse effects of P2X7 blockade by antibodies or nanobodies have been described. However, one has to keep in mind that P2X7 blockade can also potentially alter the susceptibility towards infections. Indeed, the P2X7 receptor antagonism can have both beneficial and deleterious effects depending on the type of pathogen, its virulence, and the severity of infection as reviewed in [51].

In general, though various studies suggest a role of P2X7 in stroke, many P2X7-related pathways and mechanisms that participate in post-stroke inflammation, thromboinflammation, BBB breakdown, neuroprotection and recovery remain to be revealed and connected. Contrasting results may be the consequence of (1) variations in the specificity of applied P2X7 antagonists, (2) different stroke models that do or do not allow reperfusion or that are based on photothrombosis, (3) differences related to administration routes or pre- versus post-treatment, or (4) the use of *P2rx7*-deficient mice of different origin. For the latter aspect, differences between the GlaxoSmithKline (GSK) and the Pfizer P2X7ko mice have already been demonstrated [52]: GSK P2X7ko mice lack P2X7 expression on innate immune cells but exhibit robust expression of P2X7 on T cells due to an escape splice variant. In contrast, the Pfizer P2X7ko mice lack P2X7 in both immune cell compartments. These differences might explain the opposite outcome of experimental autoimmune encephalomyelitis (EAE) studies: GSK P2X7ko mice showed suppressed EAE development compared to WT mice [53], whereas Pfizer P2X7ko mice exhibited an exacerbated



disease course in EAE when compared to WT mice [54]. GSK P2X7ko mice have not been investigated in murine stroke models yet. Additionally, one has to keep in mind that the Pfizer P2X7ko mice are congenic and therefore might contain passenger mutations from 129 embryonic stem cells used for *P2rx7* gene targeting. Indeed, our group could identify a *P2rx7* passenger mutation in the commonly used P2X4ko mouse [55, 56]. Since *P2rx4* and *P2rx7* are direct neighboring genes, differentially expressed genes in congenic P2X4ko might also occur in congenic P2X7ko mice. If such passenger mutations also exist in the widely used Pfizer P2X7ko mouse [57] still needs to be investigated, in particular if they affect the outcome of stroke.

Of note, the ATP-gated P2X receptors expressed in the brain differ greatly in their sensitivity to ATP. As measured in transfected *X. laevis* oocytes or HEK293 cells by electrophysiology, the ATP concentration to induce the half-maximal response ( $K_{1/2}$ ) is about ten times lower for rat P2X1 (0.82  $\mu\text{M}$ ) than for rat P2X4 (11  $\mu\text{M}$ ). Rat P2X7, in turn, has a  $K_{1/2}$  of 130  $\mu\text{M}$  and is accordingly about ten times less sensitive to ATP than P2X4 [58]. Thus, the activation of P2X receptors in the brain does not only depend on the expression pattern of the receptors, but also on the availability of extracellular ATP.

### Role of P2Y receptors in stroke

P2Y receptors are G-protein-coupled receptors activated by ATP, ADP, and other naturally occurring nucleoside phosphates. The most abundant P2Y receptors in the brain are P2Y12 and P2Y13, expressed at high levels in microglial cells (Fig. 1). The natural ligand for these two receptors is ADP, which upon engagement inhibits adenylate cyclase leading to a decrease in cyclic AMP (cAMP) and subsequent activation of microglia. P2Y12 receptor inhibitors, such as clopidogrel and ticagrelor, are commonly used for the secondary prevention of atherosclerotic ischemic stroke because of their effective reduction of P2Y12-mediated platelet aggregation. However, the beneficial effects of these inhibitors are not limited to this anti-thrombotic effect, since they are also neuroprotective in murine tMCAO (oral administration from 24 h before to 8 h after occlusion) [59], anti-atherosclerotic [60], and can reduce microglial activation and chemotaxis after pMCAO in rats (oral treatment 10 min, 22 and 36 h after occlusion) [61]. Interestingly, physiological concentrations of ADP facilitate the contact of microglia processes with neuronal cell bodies. After neuronal injury, higher concentrations of ADP triggered microglial-mediated neuroprotection in a P2Y12 receptor-dependent manner, sparing viable neurons from cell death and maintaining functional connectivity [62]. These data pose a warning to the treatment with P2Y12 inhibitors for stroke prevention and prompt the analysis of

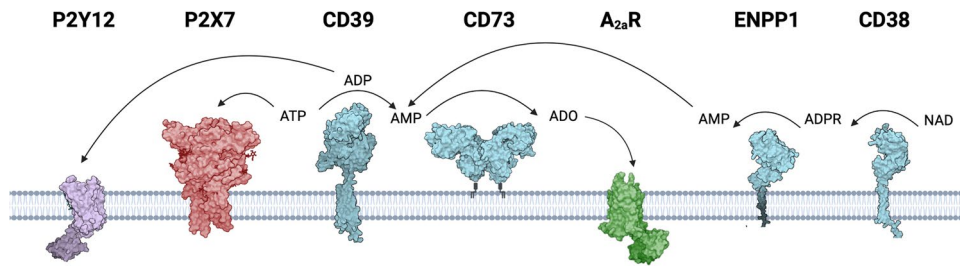
neuronal function in MCAO models under P2Y12 inhibitor treatment.

ADP can also activate P2Y1 receptors, expressed in astrocytes, and is a partial agonist for the P2Y6 receptor, expressed by microglial cells. While pre- and post-stroke blockade of P2Y1 by MRS2500 i.c.v. proved beneficial for stroke outcome in transient and permanent MCAO models [63, 64], the role of P2Y6 in stroke is still controversial: Pharmacological inhibition of P2Y6-mediated phagocytosis with MRS2578 on 3 consecutive days after tMCAO aggravated infarct size, brain atrophy, and neurological deficits [65]. However, in a model of brain ischemia induced by endothelin-1 injection, knockdown of the P2Y6 receptor had a beneficial effect by delaying phagocytosis of stressed neurons in the peri-infarct region [66]. P2Y2 and P2Y11 receptors are not expressed on brain-resident cells but are present on immune cells that can be attracted to the brain after ischemia-reperfusion injury. Indeed, ATP signaling through P2Y2 and P2Y11 promotes neutrophil and monocyte migration [67–69]. Therefore, a transient increase of systemic ATP after stroke may contribute to brain immune cell infiltration. Of note, ATP has antagonistic effects on P2Y12 [70], which may constitute an intrinsic regulatory mechanism to prevent extended inflammation.

### Ectoenzymes convert extracellular ATP via ADP and AMP to adenosine

Extracellular ATP has a half-life of less than 5 min. Ecto-nucleotidases sequentially degrade extracellular ATP to dampen the pro-inflammatory effects of ATP signaling and to generate adenosine as a metabolite with signaling properties itself [4]. Once produced, adenosine signals through four different G-protein-coupled P1 receptors and has neuroprotective [71] and anti-inflammatory [72] properties. Modulation of ischemic brain injury by adenosine signaling will be elucidated in the next section.

On the canonical pathway of adenosine production (Fig. 2), ecto-nucleoside triphosphate diphosphohydrolase 1 (NTPDase1/CD39) hydrolyzes ATP to adenosine diphosphate (ADP) and then to adenosine monophosphate (AMP). AMP is metabolized to adenosine by ecto-5'-nucleotidase (NT5E/CD73) which is the dominant adenosine-generating enzyme. CD73 activity can be inhibited by high concentrations of ATP and ADP [73]. The alternative non-canonical pathway to generate AMP for subsequent adenosine formation independently of CD39 involves NAD glycohydrolase/CD38 and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1/CD203a): CD38 hydrolyzes extracellular  $\text{NAD}^+$  to ADP ribose (ADPR) which is further converted to AMP by ENPP1 [74]. On top of that, tissue non-specific alkaline phosphatase (TNAP) mainly expressed at the vasculature contributes to adenosine formation in the rodent and



**Fig. 2** Nucleotide-metabolizing enzymes. Extracellular ATP serves as ligand for ionotropic P2X receptors, e.g., P2X7. On the canonical pathway, ATP is degraded to ADP and AMP by the ectonucleotidase CD39. ADP is a natural ligand for P2Y12. CD73 finally dephosphorylates AMP to generate adenosine (ADO). On the alternative non-

canonical pathway, AMP is generated from NAD<sup>+</sup>: CD38 hydrolyzes NAD<sup>+</sup> to generate ADPR, which is further converted to AMP by ENPP1. Extracellular adenosine serves as ligand for adenosine receptors, e.g., A<sub>2A</sub>R

human brain by catalyzing all steps in the degradation of nucleoside 5'-tri-, -di-, and -monophosphates to adenosine [75, 76].

In mouse and human brain, CD39 expression is highest on microglia followed by endothelial cells [10–13, 76] (Fig. 1). In the immune cell compartment it is expressed by neutrophils, monocytes, dendritic cells (DCs), B cells, regulatory T cells (Tregs), and NK cells (Immunological Genome Project). In the mouse brain, CD73 expression is highest on oligodendrocytes whereas both astrocytes and oligodendrocytes show high expression in humans [10–13] (Fig. 1). It has to be noted that, in contrast to human brain endothelial cells [77], CD73 could not be detected on mouse brain endothelial cells *in vivo* but shows high expression in the olfactory bulb and in the caudoputamen [76, 78, 79]. In the immune cell compartment CD73 is expressed by T and B cells, neutrophils, and macrophages (Immunological Genome Project). CD38 expression is highest on astrocytes followed by endothelial cells in both mice and humans, whereas ENPP1 is mainly expressed by microglia in both species and by oligodendrocytes in mice [10–13] (Fig. 1). The enzyme adenosine deaminase (ADA) completes the purine-inactivating machinery and catalyzes the deamination of adenosine to inosine. The highest tissue ADA enzyme activity in humans was found in lymphoid tissues, particularly the thymus, the brain, and the gastrointestinal tract [80]. In the rodent brain, immunohistochemistry revealed an extensive plexus of ADA-containing neurons in the hypothalamus [81]. Genetic deficiency of ADA2 results in an autoimmune syndrome characterized by systemic vasculitis and inflammation. Ischemic strokes occur early in childhood and are often a consequence of systemic vasculitis affecting the CNS, but stroke can also represent the initial clinical symptom without any signs of cerebral vasculitis [82, 83].

Taken together, ectonucleotidases shape the inflammatory microenvironment by tightly controlling and balancing the extracellular concentration of pro-inflammatory ATP and anti-inflammatory adenosine.

In the late 1990s, CD39 was identified as a potent thromboregulator by rapidly degrading ADP released from activated platelets at sites of vascular injury, thus inhibiting ADP-induced platelet activation, recruitment, and aggregation [84–86]. Consistent with the notion that platelet and fibrin accumulation occurs distal to the occluded vessel leading to microvascular thrombosis [87], CD39 knockout mice exhibited increased cerebral infarct volumes and reduced post-ischemic reperfusion after tMCAO [88], whereas treatment of WT mice with recombinant soluble human CD39 up to 3 h after induction of stroke reduced infarct sizes at 24 h and promoted an increase in post-ischemic blood flow [88]. Global transgenic overexpression of CD39 in mice was likewise protective in photothrombotic stroke [89]. Selective CD39 overexpression in myeloid lineage cells also conferred a reduction in infarct volume indicating a contribution of the ATP-degrading machinery on infiltrating immune cells to the protective phenotype [89]. However, these experiments were only conducted in a small number of animals.

Regarding the role of CD73 in ischemic stroke, contradictory results can be found in the literature. While mice deficient for CD73 exhibited larger infarcts and increased immune cell infiltration into the ischemic hemisphere than wild-type mice after photothrombotic stroke [79], global CD73 deficiency did not affect infarct volume, immune cell infiltration, and glia cell activation profiles following ischemia-reperfusion injury induced by tMCAO in our laboratory [90]. It seems most plausible that these conflicting findings can be attributed to the different experimental stroke models used: In the photothrombotic stroke model, focal activation of the photosensitive dye induces severe endothelial cell injury, local thrombus formation, and rapid blood-brain barrier breakdown leading to pronounced vascular (extracellular) edema. The irreversibly injured infarct core is not surrounded by functionally impaired tissue predisposed for infarction but capable of surviving, the so-called penumbra, and the vessel is permanently occluded not allowing reperfusion. On the contrary, ischemic stroke

induced by tMCAO is characterized by a leading cytotoxic (intracellular) edema, a penumbra with delayed neuronal cell death due to secondary mechanisms such as inflammation and reperfusion [91].

When interpreting and extrapolating results on CD73 from experimental stroke studies conducted in mice, one has to keep in mind that CD73 is absent on murine brain endothelial cells but highly expressed on peripheral endothelial cells and human brain endothelial cells [76, 77, 92] (Fig. 1). These expression differences account for the finding that CD73 knockout mice exhibited increased vascular leakage in peripheral organs where CD73-derived adenosine signaling is implicated in endothelial barrier function via  $A_{2A}$  and  $A_{2B}$  adenosine receptors (AR), whereas vascular permeability in the brain was not affected [93].

Beyond brain-resident cells, infiltrating immune cells play a central role in secondary neuronal injury, and their CD73 expression can also influence stroke outcome. Murine  $CD4^+CD25^+Foxp3^+$  Tregs co-express CD39 and CD73, and adenosine generated by their coordinated activity mediates both their immunosuppressive effects on activated T effector cells and their own expansion and functionality through  $A_{2A}$ R signaling [94, 95]. In vivo expansion of Tregs with interleukin-2/interleukin-2 antibody (IL-2/IL-2Ab) complex reduced infarct volume and infiltration of T cells and macrophages into the ischemic hemisphere after tMCAO. Mechanistically, CD73 expression on Tregs played a crucial role in IL-2/IL-2Ab-afforded protection as Tregs prepared from CD73 knockout mice with or without IL-2/IL-2Ab treatment failed to protect WT-recipient mice in an adoptive transfer experiment [96]. Analogous to Treg functions, Th17 responses are also shaped by CD73 activity [4]. On  $\gamma\delta$  T cells, low CD73 expression correlated with enhanced Th17-response-promoting activity [97]. Given that IL-17A-producing  $\gamma\delta$  T cells promote early detrimental neutrophil infiltration after stroke [98], modulating their CD73 expression may attenuate post-stroke inflammation.

The ectoenzyme CD38 catalyzes, inter alia, the conversion of  $NAD^+$  to ADPR on the alternative pathway of adenosine formation and controls  $NAD^+$  bioavailability and activity of  $NAD$ -dependent enzymes in the brain as reviewed by Guerreiro et al. [99]. Besides, chemotaxis of myeloid cells in response to different chemoattractants depends on CD38 and its products ADPR and cyclic ADPR [100, 101]. Several studies demonstrated protective effects of CD38 deficiency in animal models of neurodegeneration and neuroinflammation [99]. Regarding ischemic stroke, CD38 knockout mice showed smaller infarcts after tMCAO [102] and reduced hippocampal neuronal cell death after transient forebrain ischemia due to occlusion of both common carotid arteries and concomitant reduction of mean arterial blood pressure [103]. CD38 knockout mice also showed a drastic reduction of macrophage recruitment to the ischemic hemisphere

which probably contributed to the protective phenotype [102]. Beneficial effects of CD38 deletion in brain pathologies were additionally attributed to increased  $NAD^+$  levels itself [99, 104] as it has strong neuroprotective and anti-inflammatory properties [105].  $NAD^+$  depletion, on the other hand, leads to mitochondrial dysfunction and oxidative stress and is a common finding in neurodegenerative disorders [99, 106]. CD38 expression and enzymatic activity were recently shown to increase with age in the rat brain [107] complementing previous findings on an age-dependent decrease in brain  $NAD^+$  levels in healthy humans [108]. Spontaneously hypertensive stroke-prone rats, the most frequently used animal model for cerebral small vessel disease leading to lacunar infarction and cognitive decline, also exhibited higher CD38 expression and activity in the brain than age-matched normotensive controls [107]. Taken together, there is growing indirect evidence that CD38 might link aging with neuroinflammation and neurodegeneration and thus remains an interesting target in stroke.

### Role of adenosine receptors in cerebral ischemia

The concentration of extracellular adenosine increases substantially during in vivo ischemia. By microdialysis technique, Melani et al. measured a basal concentration of 130 nmol/l in the rat striatum which increased 10-fold within the first hour after intraluminal pMCAO and then decreased again but remained elevated until the end of the observation period 4 h after stroke induction [6, 109]. The half-life of extracellular adenosine is very short (4–10 s), as it is swiftly degraded by adenosine deaminase to its metabolite inosine. Therefore, to maintain an increased level of adenosine implies a continuous production. The increase of extracellular adenosine during hypoxia mainly relies on two mechanisms: Within the first 20 min of ischemia, adenosine derives mostly from hydrolysis of extracellular ATP involving CD73 leading to a higher extracellular than intracellular adenosine concentration [6]. Thereafter, extracellular adenosine originates mainly from parenchymal cells where it accumulates in a  $pO_2$ -dependent manner [110]. Consequently, the gradient between extracellular and intracellular adenosine concentrations is reversed, and adenosine is released from the cells to the extracellular space [6, 111]. Under hypoxic conditions, mitochondria consume ATP, and accumulating AMP cannot be reconverted to ATP due to the lack of oxygen and glucose. Hence, ATP and ADP levels decrease below the threshold to inhibit cytosolic 5'-nucleotidase [112], and adenosine is excessively formed from AMP. Intracellular adenosine concentrations further rise due to the hypoxia-induced inhibition of adenosine kinase which removes adenosine by phosphorylation into AMP and represents another key enzyme for the regulation of ambient adenosine levels [113–115]. Given that all these cellular



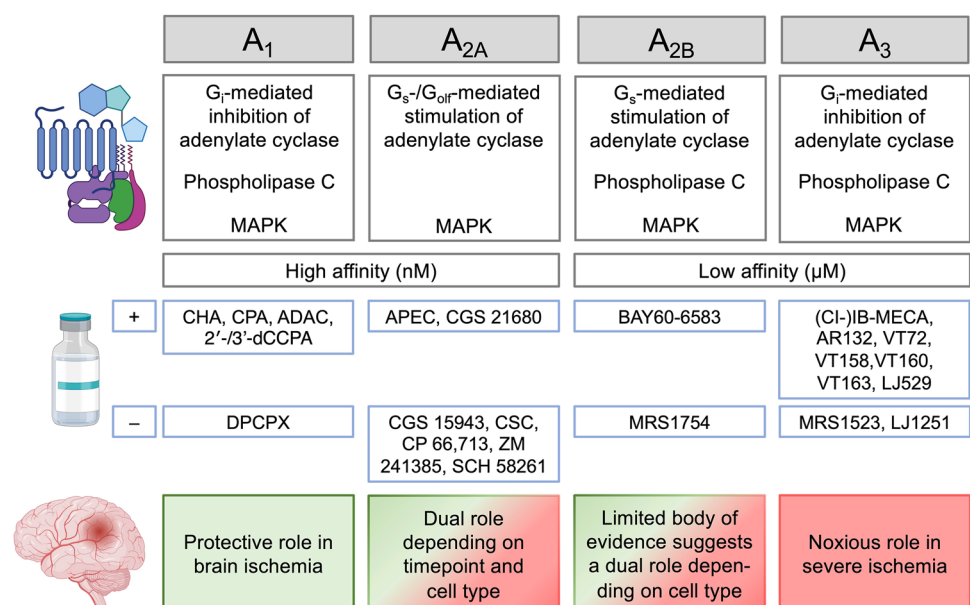
and enzymatic processes determine adenosine concentration, caution should be taken in extrapolating tissue adenosine levels from measurements of CD73 mRNA or protein expression only [111].

Adenosine release during ischemia is considered an endogenous neuroprotective response as adenosine infusion through a microdialysis probe into the rat striatum reduced neurological deficits after tMCAO [116]. Pre- and post-ischemic (2 h) i.v. delivery of adenosine-containing nanoparticles allowing prolonged circulation of the nucleoside also resulted in a significant improvement of infarct volume and neurologic deficit score after transient and permanent intraluminal MCAO [117]. Administration of an adenosine kinase inhibitor likewise attenuated ischemic damage in rats after tMCAO [118], whereas transgenic overexpression of adenosine kinase in the murine brain leads to increased infarct volumes after tMCAO [119]. Given that targeted delivery of adenosine to the CNS is hampered by a very short half-life in plasma, low blood-brain barrier permeability [120], and major systemic side effects such as bradycardia via cardiac A<sub>1</sub>R [121], therapeutic approaches focused on the modulation of adenosine receptor signaling. The A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors are the four known AR subtypes, which all belong to the superfamily of G-protein-coupled receptors and differ regarding their affinity for adenosine and tissue distribution. Apart from inhibition or stimulation of adenylate cyclase, signaling occurs through phospholipase C (PLC), calcium, and mitogen-activated protein kinases (MAPK) [122] (Fig. 3). At the concentration range during physiologic conditions, adenosine can stimulate the high affinity adenosine receptors A<sub>1</sub> and A<sub>2A</sub>, but during ischemia, adenosine reaches concentrations in the low micromolar range sufficient to also activate low affinity A<sub>2B</sub>

and A<sub>3</sub>R [123]. A<sub>1</sub>R shows an overall higher expression in mice than in humans with a quite even distribution across neurons in cortex, hippocampus, and cerebellum, oligodendrocytes, and astrocytes. At the cellular level, A<sub>2A</sub>R expression is highest on endothelial cells followed by astrocytes in both species, and at the spatial level, A<sub>2A</sub>R density is highest at post-synaptic neurons in the striatum. In the striatum, A<sub>2A</sub>Rs mainly signal through activation of stimulatory G<sub>oif</sub> [124]. While astrocytes followed by murine OPCs show the highest expression of A<sub>2B</sub>R in the CNS, A<sub>3</sub>R expression at the cellular level is highest on microglia in both mice and humans and at the spatial level most dense on hippocampal neurons [10–13, 125] (Fig. 1). The main approach in the development of AR agonists has been the chemical modification of adenosine itself, whereas AR antagonists are mainly derived from xanthines such as caffeine and theophylline [122]. A review by Pedata et al. comprehensively summarized our current knowledge on the role of all four adenosine receptors in brain ischemia both in vitro and in vivo [126]. In the present review, we will therefore only give a condensed overview with focus on data from in vivo stroke studies. STab. 2 provides detailed information for all the cited studies using pharmacological modulation of AR signaling with focus on common quantitative outcome measures of translational relevance, e.g., infarct volume and neurological outcome.

A<sub>1</sub>R signaling in response to ischemia exerts protective effects by inhibiting excitatory synaptic transmission [127–129], in particular glutamate, which is crucial for the functional recovery of hippocampal circuits upon reoxygenation [130–132]. Acute systemic administration of different A<sub>1</sub>R agonists (CHA, CPA, ADAC) was protective in a model of global cerebral ischemia in gerbils [133–135], whereas

**Fig. 3** Overview on the signaling pathways and affinity of the four known subtypes of adenosine receptors (ARs), stimulatory (+) and inhibitory (–) compounds used in the cited studies on OGD or in vivo ischemic stroke and their role in brain ischemia. For details, see Supplementary Table 2. Images from [BioRender.com](https://www.biorender.com)



acute treatment with the antagonist DPCPX exacerbated ischemic brain injury [134]. Of note, the effects of chronic administration, i.e., pre-treatment for several days before the induction of stroke, are opposite [134]. It has been suggested that ischemic protection by chronic administration of adenosine antagonists may be a consequence of A<sub>1</sub>R upregulation and that detrimental effects of chronic A<sub>1</sub>R agonism are due to the phenomenon of A<sub>1</sub>R desensitization [136] which has been described for rat hippocampal slices upon hypoxia [137]. A<sub>1</sub>R signaling also plays a central role in mediating the beneficial effects of ischemic preconditioning in the brain [138, 139], a phenomenon in which the previous exposure to a short period of ischemia confers resistance to a following prolonged ischemic insult. To overcome the limitations of full A<sub>1</sub>R agonists for clinical application, mainly receptor desensitization and cardiovascular side effects such as bradycardia and hypotension, the neuroprotective potential of partial agonists (2'-dCCPA, 3'-dCCPA) was recently investigated and proven in oxygen-glucose deprivation (OGD) as the most frequently used in vitro model of cerebral ischemia [140].

Protective effects of A<sub>2A</sub>R antagonism (CGS 15943) on ischemic injury were first demonstrated in 1994 by Gao and Phillis after global forebrain ischemia in gerbils [141]. In the following years, many studies using different models of rodent experimental stroke (global forebrain ischemia, four-vessel occlusion, intraluminal pMCAO, pMCAO by electrocoagulation) confirmed the beneficial role of various A<sub>2A</sub>R antagonists—namely, CSC, CP 66,713, ZM 241385, SCH 58261—when being administered before ischemia [142, 143] or acutely after stroke [144–146]. The latter three studies on SCH 58261 evaluated infarct volume at 24 h after stroke. Later, Melani et al. demonstrated that ischemic protection by SCH 58261 was limited to outcome parameters on day 1, whereas prolonged treatment for 7 days did not confer sustained reduction in infarct volume [147]. In line with the findings on drug-mediated antagonism, mice genetically deficient for A<sub>2A</sub>R exhibited smaller infarcts 2 h, 24 h, and 48 h after tMCAO [148, 149] and 24 h after embolic MCAO [150]. Interestingly, RNA-sequencing analysis of the ipsilesional and contralesional primary motor cortices (iM1 and cM1) 15 days after tMCAO revealed that downregulation of *Adora2a* encoding A<sub>2A</sub>R in cM1 correlated with improved recovery [151]. Detrimental effects of A<sub>2A</sub>R signaling are largely attributed to an increase of extracellular glutamate concentrations leading to excitotoxicity, thus counteracting the depression of excitatory transmission mediated by A<sub>1</sub>R [126]. Several mechanisms contribute to glutamatergic excitatory transmission upon A<sub>2A</sub>R stimulation: First, it increases neuronal glutamate release during ischemia in vitro [152, 153], and genetic A<sub>2A</sub>R deficiency was shown to reduce striatal glutamate outflow measured by microdialysis during tMCAO and reperfusion [148]. Second, A<sub>2A</sub>R activation

on astrocytes inhibits glutamate uptake [154–157]. Third, A<sub>1</sub> and A<sub>2A</sub>R can form heteromers on striatal glutamatergic nerve terminals in which A<sub>2A</sub>R activation reduces the affinity of the A<sub>1</sub>R for agonists shifting the balance towards A<sub>2A</sub>R effects in an adenosine-rich milieu such as ischemia [158].

Beyond the control of glutamatergic excitotoxicity, A<sub>2A</sub>R antagonists exert their protective effects through inhibition of phospho-p38 MAPK [145] and by reducing JNK MAPK activation in oligodendrocytes [159]. Moreover, A<sub>2A</sub>R activation was shown to drive microglia process retraction into an amoeboid shape upon activation which represents a hallmark of neuroinflammation and modulates their phagocytotic capacities [160]. A<sub>2A</sub>R signaling on brain endothelial cells contributes to ischemic brain injury as endothelium-specific A<sub>2A</sub>R-deficient mice also showed reduced infarct volumes 24 h after stroke [150].

When given systemically and chronically 13 days before global brain ischemia or until day 7 after tMCAO, A<sub>2A</sub>R agonists (APEC, CGS 21680) also conferred protection [161, 162]. These findings seem paradoxically but rely on different mechanisms than protection by antagonists. While central effects like regulation of cerebral blood flow [161] and control of neurotrophic factors [163] might account in part for this phenomenon, protection is mainly attributed to immunomodulatory effects of A<sub>2A</sub>R signaling. The substantial contribution of sterile inflammation to secondary neuronal injury is nowadays widely accepted and summarized elsewhere [2, 164, 165]. A<sub>2A</sub>R is abundantly expressed on innate and adaptive immune cells, and its activation serves as a strong anti-inflammatory signal [166, 167]. A<sub>2A</sub>Rs even increase in density and affinity on lymphocytes from ischemic stroke patients indicating the translational relevance of this pathway [168]. It has to be noted that the A<sub>2A</sub>R agonist CGS 21680 only conferred protection when being chronically administered, whereas delivery limited to the first 24 h after stroke did not reduce infarct volume [169] as it probably missed the peak of immune cell infiltration at day 3 after tMCAO [170].

Taken together, research over the last decades demonstrated a dual role of A<sub>2A</sub>R signaling in brain ischemia depending on the timepoint and the leading mechanism of injury: A<sub>2A</sub>R antagonists reduce ischemic injury early after stroke by reducing excitotoxicity, whereas A<sub>2A</sub>R agonists confer protection when being administered for several days post-ischemia by attenuating secondary sterile inflammation. From a translational perspective, therapeutic strategies with antagonists or agonists at the A<sub>2A</sub>R should therefore be carefully evaluated in terms of timing.

Compared to A<sub>2A</sub>R signaling, far less literature is available on the role of A<sub>2B</sub>R signaling in ischemic stroke. Though ubiquitously expressed [171], the low affinity of A<sub>2B</sub>R for its endogenous ligand adenosine and the lack of potent and selective agonists and antagonists has hampered

investigations into its specific function in health and disease for a long time. Moreover, the role of  $A_{2B}R$  is often masked by co-expression of  $A_{2A}R$ , which especially holds true for monocytes and macrophages, where  $A_{2A}R$  remains the predominant adenosine receptor. Due to recent innovations regarding pharmacological tools and molecular approaches,  $A_{2B}R$ s are increasingly recognized as orchestrators of immunity and inflammation as reviewed by Haskó and colleagues [172]. In the immune cell compartment,  $A_{2B}R$  activation increases pro-inflammatory cytokine production by mast cells, whereas it shapes the function and cytokine profile of antigen-presenting cells such as DCs and macrophages towards an anti-inflammatory and tolerance-inducing phenotype [172].  $A_{2B}R$  knockout mice displayed a mild but significant increase in pro-inflammatory cytokines under basal conditions [171].

On endothelial cells,  $A_{2B}R$  signaling plays a central role in controlling barrier function. The protective effect is even potentiated under hypoxic conditions because of adenosine accumulation and  $A_{2B}R$  upregulation [173, 174]. Consequently,  $A_{2B}R$  knockout mice exhibited increased vascular leakage measured by Evans's blue extravasation in multiple organs including the brain during hypoxia, whereas  $A_1R$ ,  $A_{2A}R$ , and  $A_3R$  deficiency did not accentuate hypoxia-induced vascular leakage compared to wild-type littermate controls [175].  $A_{2B}R$  knockout mice expressed higher levels of adhesion molecules at the peripheral vasculature resulting in higher numbers of leukocytes adhering at the endothelium under basal conditions [171] and increased infiltration of polymorphonuclear leukocytes in various organs including the brain after hypoxia [175]. Bone marrow transplantation experiments demonstrated that  $A_{2B}R$  signaling on hematopoietic cells mainly accounted for  $A_{2B}R$ -mediated attenuation of hypoxia-associated neutrophil transmigration, while vascular  $A_{2B}R$  conferred protection to fluid leakage [171, 175].

A recent *in vivo* study in rats demonstrated that systemic and chronic treatment with the  $A_{2B}R$  agonist BAY60-6583 after tMCAO diminished immune cell infiltration at day 2 and reduced infarct volume at day 7 after stroke [176]. These protective effects of  $A_{2B}R$  signaling are contrasted by a study indicating a rather detrimental role of central  $A_{2B}R$  signaling: In transient global brain ischemia induced by four-vessel occlusion in rats, the selective  $A_{2B}R$  antagonist MRS1754 reduced the phosphorylation of p38 MAPK and subsequent activation of neutral sphingomyelinase 2 which otherwise leads to pro-inflammatory ceramide accumulation in hippocampal astrocytes [177]. Taken together, central  $A_{2B}R$  signaling on astrocytes seems to exert a pro-inflammatory role, while  $A_{2B}R$  activation on immune cells and endothelial cells dampens inflammation and vascular leakage.

Several *in vivo* stroke studies demonstrated a protective effect of  $A_3R$  agonists: Pre-treatment with the  $A_3R$  agonist

CI-IB-MECA either once *i.c.v.* 15 min or twice *i.v.* 165 min and 15 min before transient MCA ligation reduced infarct volume at day 2 in rats and mice [178]. In the same study,  $A_3R$  knockout mice exhibited larger infarcts after tMCAO than WT controls [178]. With higher relevance from a translational perspective, post-treatment with agonists also proved effective: Administration of the  $A_3R$  agonist LJ529 twice within the first seven hours after tMCAO reduced infarct volume at day 1 in rats. This effect was only reversed by application of the  $A_3R$  antagonist MRS1523 but not by co-administration of an  $A_{2A}R$  antagonist (SCH 58261) indicating that ischemic protection was indeed mediated by  $A_3R$  [179]. Recently, delivery of the  $A_1/A_3R$  agonist AST-004 via bolus and continuous infusion starting 2 h after tMCAO limited the lesion growth rate measured by repeated magnetic resonance imaging (MRI) scans and overall stroke volume at day 5 in macaques as an example of non-human primates [180]. *In vitro* data from various OGD experiments seemed to be contradictory regarding the effects of  $A_3R$  signaling on synaptic transmission and excitotoxicity during ischemia with both agonists and antagonists exerting ischemic protection under different experimental conditions [126]. In 2007, Pugliese and colleagues deciphered the divergent contribution of  $A_3R$  signaling depending on the severity of OGD in rat hippocampal slices: While  $A_3R$  agonists had an acute  $A_1R$ -like inhibitory effect on synaptic transmission during a short period of OGD (2–5 min), both antagonists (LJ1251, MRS1523) and prolonged exposure to agonists (CI-IB-MECA, VT72, VT158, VT160, VT163, AR132) prevented or delayed anoxic depolarization and were protective during severe OGD (7 min) [181]. It is assumed that severe ischemia switches  $A_3R$ -mediated effects from beneficial to detrimental via sustained activation of PLC and protein kinase C through  $G_q$  proteins and subsequent calcium mobilization. The yet protective effects of chronic  $A_3R$  agonist treatment are a consequence of receptor desensitization in an *per se* adenosine-rich microenvironment [181], which has been shown for both rat and human  $A_3R$  [182, 183].

This noxious role of  $A_3R$  signaling in conditions of severe ischemia explains early findings by von Lubitz and colleagues, who showed that acute treatment with the  $A_3R$  agonist IB-MECA right before global forebrain ischemia increased mortality until day 7 after ischemia in gerbils, whereas chronic pre-treatment over 10 days, probably leading to  $A_3R$  desensitization, enhanced neuronal survival in the hippocampus [184].

Beyond receptor desensitization, the protective effects of  $A_3R$  agonists are also mediated by immune cells as demonstrated by Choi et al. [179]. The  $A_3R$  agonist LJ529 when given twice at 2 h and 7 h after tMCAO reduced the migration of activated microglia and infiltration of monocytes into ischemic cortical and striatal lesions. In a microglial cell culture, LJ529 inhibited chemotaxis of microglia and monocytes,

and co-treatment with  $A_3$  and  $A_{2A}$ R antagonists indicated that this effect was selectively mediated by  $A_3$ R [179].  $A_3$ R was found to be overexpressed in inflammatory tissue and on peripheral blood mononuclear cells from patients with different autoimmune inflammatory diseases such as rheumatoid arthritis, psoriasis, and Crohn's disease [185, 186]. Various  $A_3$ R agonists exerted anti-inflammatory effects across different experimental models of inflammation [187] which is mediated by de-regulation of the NF- $\kappa$ B signaling pathway inhibiting cytokine production and enhancing apoptosis of inflammatory cells [186]. The highly selective  $A_3$ R agonist CF101 even proved effective and safe in a phase II clinical trial in rheumatoid arthritis [188]. If the evidence for a protective role of  $A_3$ R agonists in ischemic stroke mounts, CF101 might indeed represent an interesting therapeutic perspective.

Taken together,  $A_3$ R signaling takes on a noxious role in conditions of severe ischemia such as long OGD and in vivo stroke, and the yet protective effects of chronic  $A_3$ R agonist treatment are mainly attributed to receptor desensitization.

### Differences between murine and human purinergic signaling pathways

Mice and humans differ in their expression and regulation of P2 receptors, particularly with respect to P2X7. P2X7 is expressed at lower levels in humans (at least on circulating T cells) than in mice, as shown, for example, for Tregs, which have a very high P2X7 expression in mice, but low expression in humans [189, 190]. Compared to rodent P2X7, human P2X7 is around ten times less sensitive to ATP [58] and requires ATP concentrations in the high micromolar to low millimolar range for activation [191]. Additionally, only murine P2X7 is activated by low  $NAD^+$  concentrations through ARTC2.2-mediated ADP ribosylation, as there is no human orthologue of ARTC2.2 [192, 193]. P2Y11, an ATP-gated G-protein-coupled receptor, is expressed in humans, but not in mice. P2Y11 interacts with P2X7 and inhibits P2X7-mediated pore formation and cell death [194]. Overall, the hurdle to activate P2X7 appears to be significantly higher in humans than in mice.

Major differences between mice and humans also exist in the ectonucleotidases degrading ATP to adenosine. As mentioned earlier, CD73 is highly expressed on human, but not on murine brain endothelial cells [76, 77, 92]. This could strongly influence brain adenosine concentrations at the site of ischemic stroke. Unlike hypoxia-induced upregulation of CD73 on human microvascular endothelial cells in vitro [173], our group did not detect an upregulation of CD73 on murine cerebral microvessels after ischemic stroke [90]. The expression of CD73 on infiltrating immune cells also differs between the two species. Murine Tregs express both CD39 and CD73 on the cell surface, allowing the generation of adenosine from ATP by the concerted action of both

ectonucleotidases [94]. In contrast, human Tregs exhibit very low CD73 expression [195]. Similarly, almost all conventional T cells in mice express CD73, whereas human CD4 and CD8 cells (except for naïve CD8 T cells and a subset of memory cells) have low CD73 expression [195, 196]. A reason for the differential expression of CD73 in mice and humans may be that adenosine receptor signaling has different effects in the two species. In contrast to mice, where adenosine promotes Treg expansion, adenosine signaling in humans inhibits the activation of Tregs [95, 197–199]. The absence of CD73 on Tregs could prevent adenosine-mediated auto-inhibition. Adenosine is degraded by ADA, which in humans, but not in mice, is “anchored” to the plasma membrane by the ectonucleotidase CD26. ADA in close proximity to the cell surface could reduce the auto-crine effect of adenosine in humans.

Due to these differences in purinergic signaling between mice and humans, results from mouse models need to be carefully evaluated and are unlikely to be directly applicable to humans.

### Concluding remark

A large number of preclinical studies clearly demonstrated the involvement of the ATP-adenosine axis in stroke pathophysiology reaching from excitotoxicity over post-stroke inflammation to recovery. From today's perspective, the translation of drugs targeting key players of purinergic signaling from experimental stroke research into clinical routine is mainly hampered by (i) the BBB that constitutes a major obstacle for the i.v. application of antibody-based therapeutic constructs, (ii) a potentially dual role of certain targets depending on the timepoint and leading mechanism of injury (e.g.,  $A_{2A}$ R), (iii) major cardiovascular side effects (e.g.,  $A_1$ R agonists), (iv) gene expression differences between mice and humans (e.g., CD73), and (v) futility of pre-treatment strategies in human stroke. Especially when aiming at reducing post-stroke inflammation, e.g., through P2X7 blockade, the rapid onset of P2X7 signaling after stroke will probably leave a narrow time window for treatment. To our opinion, the engineering of P2X7-blocking nanobodies with improved ability to cross the BBB after i.v. injection could be a huge step towards translation. Further, the application of nanobodies via the intranasal route could be a promising therapeutic approach. A high dose of P2X7-blocking nanobodies could be given as nasal spray at the first signs of stroke. The successful distribution of nanobodies via the nasal route targeting, e.g., transthyretin in the CNS has already been demonstrated in mice [200]. Apart from targeting P2X7, the use of partial AR agonists offers the greatest therapeutic potential. Nevertheless, any therapeutic approach must be carefully evaluated in terms of timing, efficiency irrespective of age and gender, specificity, and effects on long-term outcome.



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**Authors' contributions** ISS and BR assembled the figures; all authors participated in the writing process and approved the final version of the manuscript. JS provided the results of the human/mouse purinergic key player comparative expression analyses shown in Fig. 1.

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## Declarations

**Conflict of interest** TM and BR are co-inventors on patent applications for CD73- and CD39-specific nanobodies.

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