The Receptors

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The Adenosine Receptors

Humana Press

The Receptors

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Foreword

It is an extraordinary pleasure to introduce the book *The Adenosine Receptors* edited by our friend and colleague Pier Andrea Borea, reporting the history, the pathophysiological roles, and the recent exciting developments of adenosine receptors.

Several decades have passed since purinergic signaling, i.e., the role of nucleotides and nucleosides as extracellular signaling molecules, was originally proposed by one of us (Burnstock G., "Purinergic nerves" *Pharmacological Reviews* 24: 509–581, 1972).

The concept of purinergic transmission was not, however, well accepted by the scientific community until the early 1990s, when evidence of the existence of receptor subtypes for purines and pyrimidines was brought forward by the cloning and characterization of four subtypes of the P1 (adenosine) receptors, seven subtypes of P2X ion channel receptors, and eight subtypes of the P2Y G protein-coupled receptors (Abbracchio M. & Burnstock G., "Nomenclature and classification of purinoceptors" *Pharmacological Reviews* 46:143–156, 1994). Early studies were largely concerned with the physiology, pharmacology, and biochemistry of purinergic signaling and demonstrated the role of adenosine 5′-triphosphate (ATP) (and its breakdown product adenosine) as a co-transmitter with classical transmitters in both the peripheral and central nervous systems. It was then showed that purines are powerful extracellular messengers also to non-neuronal cells, including secretory, exocrine and endocrine, endothelial, immune, musculoskeletal, and inflammatory cells. Purinergic signaling is rapid in neurotransmission, neuromodulation, and secretion, but it is also involved in long-term effects including proliferation, differentiation, migration, and death in development and regeneration.

As beautifully outlined in this very well-conceived and comprehensive book, we are now in the exciting phase when focus in the field primarily concerns the therapeutic potential linked to the pharmacological modulation of these receptors via selective agonists and antagonists.

The 24 chapters focus on the most updated and interesting developments related to the modulation of adenosine receptors in cardiovascular, neurodegenerative, inflammatory, and immune disorders.

We are sure that this work will contribute to diffuse the purinergic approach to the international scientific community and attract more young scientists to the field, to eventually solve the remaining issues related to the translation of basic purinergic research into the cure of human diseases, and possibly unveil further exciting applications.

Melbourne and Milan, April 2018
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Preface

I am pleased to be able to present this fascinating book, which details our current knowledge in the adenosine receptor (AR) field. Adenosine, a ubiquitously distributed endogenous nucleoside, is one of the major essential components of cellular life, and the 24 chapters in this book contain a wide range of up-to-date information, including various aspects of the biochemistry, molecular and cellular pharmacology, and physiology of adenosine and the G-protein-coupled receptors that it interacts with. These ARs, respectively named A_1, A_{2A}, A_{2B} and A_3, are distributed throughout the body and provide a means by which adenosine and its derivatives can modulate several normal and pathological processes, and the latest research into AR signal transduction pathways, new drug discoveries and potential therapeutic research are reported in depth. The chapters in this book cover both basic science, and preclinical and clinical applications, and thereby provide a scientifically excellent reference source.

The first chapter is dedicated to the status of art in adenosine and AR field. It spans the enormous amount of research carried out in the field worldwide since its discovery—which dates back to the early 1980s—and continues to yield new and surprising discoveries today. The vital role played by adenosine in various physiological functions is explored in Chap. 2, as well as the means by which its levels are kept in equilibrium by different enzymes and transporters. Indeed, components of extracellular adenosine homeostasis are implicated in various pathological conditions.

Chapter 3 goes on to analyse the widespread distribution and signalling events of the ARs, together with their molecular structure and signal transduction pathways. Chapters 4, 5, 6, 7 focus on a detailed chemical analysis of the selective agonists, antagonists, partial agonists and allosteric modulators of A_1, A_{2A}, A_{2B} and A_3ARs, in addition to the structure-activity relationships (SARs) of the compounds interacting with ARs. The potential applications of these compounds as both pharmacological tools and therapeutic agents are discussed.

Investigations into the thermodynamic parameters of the compounds interacting with ARs are detailed in Chap. 8. Intriguingly, findings to date suggest that the binding of AR agonists may be entropy-driven, while AR antagonist binding may be enthalpy-driven—a phenomenon known as thermodynamic discrimination.

Adenosine tone and ARs are involved in a number of processes critical for neuronal functions and homeostasis. These include the modulation of synaptic activity and excitotoxicity, the control of neurotrophin levels and functions, and the regulation of protein degradation mechanisms. As reported in the following chapters, ARs play a range of roles in neuroinflammation (Chap. 9); Parkinson's, Alzheimer's and Huntington's diseases (Chaps. 10, 11 and 12); epileptic seizures and in brain ischemia (Chaps. 13 and 14, respectively); as well as the control of cognition and pain (Chaps. 15 and 16). New pathophysiological insights and recent research developments regarding purinergic signalling in the cardiovascular system have opened new therapeutic avenues for the treatment of the infarcted heart (Chap. 17). Moreover, numerous studies indicate that adenosine signal transduction is involved in asthma and chronic obstructive pulmonary diseases (Chap. 18), as well as in renal failure (Chap. 19).

In fact, the engagement of ARs on the surface of several immune cell populations—including neutrophils, macrophages, dendritic cells, mast cells and lymphocytes—shapes a broad array of immune cell functions, which include cytokine production, degranulation, chemotaxis, cytotoxicity, apoptosis and proliferation (Chap. 20). The critical role of adenosine in maintaining cartilage and chondrocyte homeostasis under physiological conditions—and its selective protection against the onset of osteoarthritis—is described (Chap. 21). Interestingly, AR agonists and/or antagonists may also conceivably be employed in the fight against diabetes mellitus and obesity, as they act to normalise lipolysis, insulin sensitivity and thermogenesis (Chap. 22).

Regarding ARs' anticancer applications, Chap. 23 reports how A_{2A}AR antagonists may enhance tumour immunotherapy in cancer treatment protocols. Indeed, the effectiveness of A_3AR agonists in several animal tumour models has already led to preclinical and clinical trials of these molecules. Furthermore, the relevance and action of ARs and pulsed electromagnetic fields (PEMFs) are explored in various inflammatory diseases of both the peripheral and central nervous system; in this context it appears that PEMFs may be a useful, non-invasive anti-inflammatory treatment with only minor impact on daily life (Chap. 24).

I thank all the scientists and young investigators who have made contributions to this book, furthering the status of the art in AR research. I trust that their remarkable achievements are sufficient to highlight the potential of ARs in many health and disease contexts. If one overarching conclusion can be drawn from this book, it is that engagement of the scientific community in multidisciplinary AR research projects will almost certainly lead to discoveries that will translate into the development of better targeted and more efficacious treatments; novel adenosine drugs will undoubtedly have fundamental roles to play in both safeguarding and improving human health.

Last, but certainly not least, I thank the members of my Research Group for their scientific work in the field of adenosine receptors, and Dr. William F. Curtis (Executive Vice-President), Dr. Giuseppe di Giovanni (Series Editor), and Dr. Jayashree Dhakshnamoorthy and Dr. Simina Calin (Neuroscience Editors) at Springer International Publishing.

Ferrara, Italy Pier Andrea Borea

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Chapter 1
Adenosine Receptors: The Status of the Art

Stefania Gessi, Stefania Merighi, and Katia Varani

Abstract Adenosine is an ubiquitous molecule which is involved in the regulation of the function of every tissue and organ. This nucleoside mediates its effects through activation of a family of four G-protein-coupled adenosine receptors, namely, A_1, A_{2A}, A_{2B}, and A_3. Adenosine plays a significant role in the protection against cellular damage in the regions with high metabolism and prevents the subsequent dysfunction of the affected organs. Its levels rise during conditions concerning increased metabolic demand and/or lack of oxygen occurring in several pathological states like ischemia, stress, seizures, pain, diabetes, inflammation, cancer, and trauma, where it may behave like a guardian angel against cellular damage or may show its bad side in conditions deriving from its long-lasting increases responsible for chronic inflammation, fibrosis, and organ damage. The aim of this chapter is to offer an overview on the status of the art of the current drugs, agonists and antagonists, in clinical development.

Keywords Adenosine receptors · Agonists · Antagonists · Clinical trials · Human diseases

1.1 Introduction

The purine nucleoside adenosine, an integral part of adenosine triphosphate (ATP), is commonly recognized as a crucial guardian regulating local tissue function during stress conditions implying a dysregulation of energy support (Borea et al. 2016). When oxygen demand is increased during work or exercise or its supply is reduced due to ischemia/hypoxia, adenosine generated by the degradation of ATP interacts with A_1- and A_3-Gi as well as A_{2A}- and A_{2B}-Gs protein coupled receptors, respectively, to compensate for this imbalance. First of all through activation of A_1 subtype, it reduces oxygen demand in neurons and cardiomyocytes; through A_2 it increases vasodilation and oxygen delivery, gaining the designation of "retaliatory

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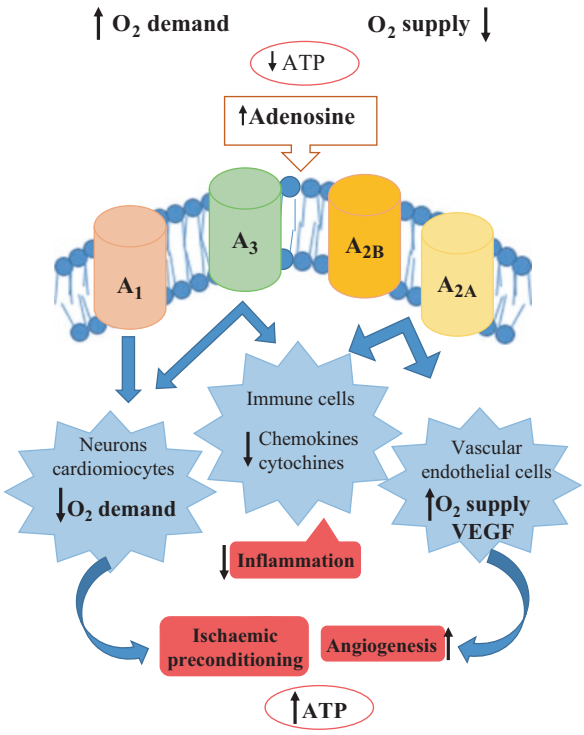

Fig. 1.1 Picture describing the retaliatory role of Adenosine interacting with A_1, A_{2A}, A_{2B}, and A_3 receptors. Adenosine derived from ATP degradation occurring under metabolic stressing conditions triggers different effect to restore physiological conditions and ATP support. It reduces oxygen request in neurons and cardiac cells while it augments oxygen support through vasodilation. Other effects include decrease of inflammation, increase of angiogenesis, and ischemic preconditioning protection

metabolite" (Newby 1984). Other effects of adenosine include reduction of cytokine release and inflammation, angiogenesis, and protection during ischemic preconditioning (Fig. 1.1). Adenosine is present everywhere, always produced in all cells, being generated extracellularly by dephosphorylation of ATP operated by specific ectoenzymes. Its concentration in the interstitial fluid is considered around 30–300 nM, but it dramatically increases up to the micromolar level following adverse metabolic conditions such as hypoxia, causing the ATP degradation. Adenosine being limited by an extremely short half-life, due to its deamination or cellular reuptake, is not dispensed like an hormone but regulates only local adenosine receptor transduction, behaving like an autacoid (Baraldi et al. 2008).

The first reported studies showing evidence of the existence of adenosine receptors date back to 1979 (van Calker et al. 1979). Now, more than 40 years later, the knowledge about their distribution, molecular structure, and physiopathological effects has expanded significantly, and in parallel new progress in medicinal chemistry of these drug targets have led to the development of novel molecules in specific therapeutic field (Peleli et al. 2017). Specifically, the joint efforts of genetics, molecular biology, pharmacology, medicinal chemistry, and crystallography using selective molecules, mutated receptors, and genetically modified mice have allowed to reach important advancement in the comprehension on the structure and role of adenosine receptors in a multitude of pathologies spanning from ischemia,

neurodegenerative diseases, epilepsy, pain, cardiovascular, renal and pulmonary diseases, autoimmune and inflammatory conditions, and diabetes to cancer (Chen et al. 2013). Nevertheless, until now the number of clinically approved drugs on the market, targeting the adenosine system, is limited to few molecules including adenosine itself as Adenocard for paroxysmal supraventricular tachycardia; Regadenoson for myocardial perfusion imaging; Theophylline, Doxofylline, and Bamifylline for asthma; and Istradefylline for Parkinson's disease (Gessi et al. 2011). Currently other new selective adenosine receptors ligands are under evaluation for numerous new indications. This is the topic we will deal with, and we will attend to this aim by describing drugs under active development that selectively interact with the four known adenosine receptor subtypes.

1.2 Adenosine Receptor Agonists

1.2.1 Neladenoson

Neladenoson is a potent and selective partial A_1 receptor agonist, derived by the optimization of Capadenoson structure (Meibom et al. 2017). To optimize the solubility and formulation concerns of Neladenoson, the prodrug Neladenoson bialanate has been developed, presenting adequate solubility after oral administration. This compound has been evaluated in a completed clinical phase II study (NCT02040233) for the treatment of heart failure, but results have not been yet revealed, and it is currently under examination in phase IIb clinical trials (NCT03098979, NCT02992288) again for heart failure, all studies being supported by Bayer. This compound maintains the high potency and cardioprotection concerning Capadenoson with less central side effects. In phase I and IIa studies, it did not induce sedation nor atrioventricular (AV) block and was well tolerated, thus supporting the use of this drug in chronic therapy as requested for heart failure treatment.

The motivation at the basis of A_1 agonist employment in this high-impact pathology has to be found in its signal transduction pathway. Indeed A_1 receptor induces a decrease of adenyl cyclase, thus lowering intracellular concentrations of cyclic adenosine monophosphate (cAMP) (Borea et al. 2016). This effect counteracts cardiac adrenergic overstimulation and increases atrial natriuretic peptide production with consequent cardioprotection (Yuan et al. 2005). In addition, A_1 receptor modulates phospholipase C-triggered signaling which activates PKCε-dependent activation of mitochondrial K_{ATP} channels. This event leads to a decrease of mitochondrial protein transition pore opening, responsible for apoptosis with advancement of hypoxic mitochondrial function (Greene et al. 2016). Such result is useful in heart failure characterized by abnormal mitochondrial structure and activity. In addition A_1 receptor activation protects from calcium overloads modulating calcium currents at the atrioventricular node through nitric oxide-mediated effects (Varani et al. 2017). Finally, A_1 receptor triggers pertussis-toxin-sensitive potassium channel activation

(Kirsch et al. 1990). Overall, these effects exerted by A_1 activation improve mitochondrial electron transport chain efficacy, thus ameliorating the production of ATP and reducing the extreme reactive oxygen species synthesis. Altogether through these mechanisms, A_1 receptor activation promotes cardiac protection. However, stimulation of this subtype by using a full agonist does not induce only beneficial therapeutic effects but unfortunately implies also important drawbacks such as bradycardia, atrioventricular blocks, vasoconstriction, negative inotropy and dromotropy, sedation, and antidiuretic effects (Greene et al. 2016). Therefore a way to overcome such undesirable effects is to activate A_1 receptor with a partial agonist such as Neladenoson.

1.2.2 Regadenoson

Regadenoson is a potent and selective short-acting A_{2A} receptor agonist approved for myocardial perfusion imaging studies as a fast infusion during a heart stress test in people who are unable to exercise enough to put stress on their heart and was launched in 2008 by Astellas Pharma (Gessi et al. 2011). The maximal plasma concentration of Regadenoson is reached within 1–4 min, and its half-life is approximately 2–4 min. Currently, it is monitored in various clinical studies of phase IV here briefly described:

- NCT01446094, NCT02115308 to evaluate the feasibility and diagnostic performance of Regadenoson in stress cardiovascular magnetic resonance scan for detection of coronary artery disease and the changes in cardiac function.
- NCT02130453 to compare Regadenoson nuclear stress testing with echocardiography strain measurements (an ultrasound imaging method that measures heart function) in revealing coronary artery disease.
- NCT02589977 to consider Regadenoson in myocardial perfusion, oxidative metabolism, and fibrosis present in heart failure with preserved ejection fraction (HFpEF) patients. Specifically, the aim of this protocol is to use noninvasive cardiac imaging techniques to describe cardiac structure, function, blood flow, energetics, and fibrosis, in order to better clarify the mechanisms in HFpEF comparing three subject groups (HFpEF vs hypertension vs healthy) both at rest and stress following coronary vasodilation with Regadenoson.
- NCT03249272 to evaluate microvascular function through a cardiovascular magnetic resonance measurement of whole-heart perfusion reserve in the presence of Regadenoson or adenosine. The aim is to assess the prevalence of myocardial vascular dysfunction (MVD) in two common forms of nonischemic cardiomyopathy, hypertrophic cardiomyopathy (HCM) and idiopathic dilated cardiomyopathy (IDCM).

Importantly, Regadenoson is under clinical evaluation in other clinical trials for different therapeutic applications. Specifically, a phase II NCT01788631 trial is ongoing for sickle cell anemia (SCD), an inherited blood disorder that modifies the

red blood cells' shape, changing it from a round to a half-moon/crescent shape. Patients affected by SCD present a particular type of hemoglobin responsible for this red blood cell change that causes blood vessel obstruction, inflammation, and injury. In this condition Regadenoson may avoid this damage caused by the sickle-shaped cells. Other clinical studies concerning this drug are ongoing to evaluate stress and rest perfusion imaging using Regadenoson as the coronary vasodilator (pharmacological stressor) (phase II NCT03103061; phase I NCT01433705).

1.2.3 Piclidenoson, CF101

Piclidenoson is a potent and selective A_3 receptor agonist already positively evaluated being safe and well tolerated in phase II clinical trials on different autoimmune diseases including rheumatoid arthritis (RA) (phase II, NCT00280917; phase II, NCT01034306; phase II, NCT00556894), showing relevant antirheumatic effects and plaque psoriasis (phase II, NCT00428974; phase II/III, NCT01265667). The rationale for its use in these human diseases derives from its effects in immune cells of patients affected by RA, Crohn's disease, and psoriasis, where it additionally appears upregulated, behaving like a novel predictive marker. The overexpression of A_3 receptor is caused by an increase in TNF-α that stimulates the transcription factors NF-κB and CREB mediating A_3 receptor expression (Ochaion et al. 2009). In T cells and synoviocytes derived from RA subjects, A_3 receptor reduced NF-κB transduction cascade and the consequent secretion of inflammatory mediators (Ochaion et al. 2008). Interestingly, basal receptor level corresponded to the response of patients to the drug, indicating that the A_3 receptor could be a biological marker for prognosis (Fishman and Cohen 2016). In particular in plaque psoriasis studies (David et al. 2012, 2016), CF101 demonstrated to be better than apremilast (Otezla), the PDE4 inhibitor. New trials in RA (phase III, NCT code not yet available) and moderate to severe plaque psoriasis (phase III, NCT code not yet available) are in the planning stages (Table 1.1).

1.2.4 Namodenoson, CF102

Namodenoson is a potent and selective A_3 receptor agonist showing a safe and well tolerated profile as observed in clinical trials (phase I and II NCT00790218) for advanced hepatocellular carcinoma. Specifically, the agonist increased the *median overall survival* (OS) by 7.8 months in patients, receiving CF102 in combination with sorafenib (Stemmer et al. 2013). The involvement of A_3 receptor has been deeply investigated in cancerogenesis, and its role as both a predictive marker and a therapeutic target has been widely demonstrated in cancer cell lines, rat and human primary tumors, and in syngeneic, xenograft, orthotopic, and metastatic models of colon, prostate, melanoma, and hepatocellular carcinomas (Merighi et al. 2003;

Table 1.1 Clinical molecules selective for adenosine receptor candidates as new drugs

Candidates	Adenosine receptor involved	Human disease addressed	Phase	Clinical trial identifier code
AGONISTS				
Neladenoson bialanate	A_1	Heart failure	II	NCT03098979
		Heart failure	II	NCT02992288
Regadenoson	A_{2A}	Sickle cell anemia	II	NCT01788631
		Coronary artery disease	IV	NCT01446094
		Coronary artery disease	IV	NCT02115308
		Ischemia	IV	NCT02130453
		Cardiovascular diseases Coronary artery disease	II	NCT03103061
		Heart failure, diastolic Diastolic heart failure Hypertension	IV	NCT02589977
		Retinal artery occlusion	II	NCT03090087
		Hypertrophic cardiomyopathy Nonischemic dilated cardiomyopathy microvascular ischemia of myocardium	IV	NCT03249272
		Heart disease	I	NCT01433705
		Microvascular coronary artery disease	II	NCT03236311
		Coronary microvascular disease	I II	NCT02045459
		Coronary artery disease	I II	NCT03331380
Piclidenoson, CF-101	A_3	Rheumatoid arthritis	III	[a]
		Moderate-to-severe plaque psoriasis	III	[a]
Namodenoson, CF-102	A_3	Hepatocellular carcinoma	II	NCT02128958
		Nonalcoholic fatty liver disease Nonalcoholic steatohepatitis	II	[a]
ANTAGONISTS				
PBF-680	A_1	Asthma	II	NCT02635945
Istradefylline	A_{2A}	Idiopathic Parkinson's disease	III	NCT02610231
Preladenant	A_{2A}	Neoplasm	I	NCT03099161
PBF-509	A_{2A}	Non-small cell lung cancer	I/II	NCT02403193
CPI-444	A_{2A}	Non-small cell lung cancer Malignant melanoma Renal cell cancer Triple-negative breast cancer Colorectal cancer Bladder cancer Metastatic castration-resistant prostate cancer	I	NCT02655822

[a]The Clinical Trial Identifier Code for these studies is not yet available; data come from Can-Fite BioPharma website at www.canfite.com

Gessi et al. 2008; Fishman et al. 2012; Borea et al. 2015). In particular its overexpression has been observed not only in primary solid cancer tissues in comparison to health, but importantly it has been found that this augment is reflected in peripheral blood cells of patients affected by colon and liver cancer (Madi et al. 2004; Gessi et al. 2004; Varani et al. 2006; Bar-Yehuda et al. 2008). Furthermore, the alteration of Wnt pathway playing a role in human cancerogenesis has been demonstrated following A_3 receptor activation (Fishman et al. 2002). A global phase II trial in this patient population is currently underway, and other trials are planned for CF102 in hepatocellular carcinoma treatment (phase II, NCT02128958). Another trial on nonalcoholic fatty liver disease and nonalcoholic steatohepatitis is ongoing, for which NCT number is not yet available (Table 1.1).

1.3 Adenosine Receptor Antagonists

1.3.1 PBF-680

PBF-680 is a potent A_1 receptor antagonist now under evaluation in a clinical study for mild to moderate asthma treatment (phase II NCT02635945). This study aims to evaluate the efficacy of orally administered PBF-680 to attenuate late asthmatic responses (LAR) induced by allergen bronchoprovocation in asthmatic patients treated with corticosteroids and beta-2 agonists.

Many evidences have demonstrated a pro-inflammatory role of A_1 receptor activation in different immune cells as well as a bronchoconstrictory effect in pulmonary tissue. It is also recognized that adenosine provokes bronchoconstriction in patients with asthma and that theophylline, a nonselective adenosine receptor antagonist, is an efficacious drug for its therapy. Indeed this drug inhibits A_1 receptor with a major affinity of that displayed to reduce phosphodiesterases suggesting that selective A_1AR antagonists can be evaluated for asthma therapy (Gao and Jacobson 2017).

1.3.2 Istradefylline

Istradefylline is a potent and selective A_{2A} receptor antagonist approved in Japan for Parkinson's disease (PD) therapy in co-treatment with levodopa (L-DOPA) and currently under clinical evaluation for global approval by Kyowa Hakko Kirin (phase III NCT02610231).

The use of A_{2A} antagonists in the therapy of PD has been studied for several years and is based on their well-recognized effect on the modulation of motor function (Gessi et al. 2011; Preti et al. 2015; Borea et al. 2017). As for the distribution of the A_{2A} receptor in CNS, it is well accepted that this adenosine subtype is the one

prevalent in striatum, where it is co-expressed with dopamine D2 receptors (D2R), to constitute A_{2A}AR/D2R heteromers, having an important effect in the regulation of motor activity (Canals et al. 2003; Fuxe et al. 2003; Borroto-Escuela et al. 2010). PD is a pathology caused by degeneration of dopamine secretion in striatum area actually treated, with severe limitations, by using L-DOPA or dopamine agonists (Fuxe et al. 2015). In this context the relevance of using an A_{2A} antagonist is due to the inhibitory effect of A_{2A} activation on D2R agonists binding affinity (Ferre et al. 1991; Navarro et al. 2016). Therefore, these drugs have been shown to ameliorate motor function in several PD animal models by decreasing the inhibitory effect of A_{2A} receptor on D2R activity in GABAergic neurons of the striatopallidal area (Fuxe et al. 2015).

1.3.3 Preladenant, PBF-509, CPI-444

Preladenant is an A_{2A} receptor antagonist previously developed for treatment of PD that following unsuccessful clinical trials has been discontinued (Navarro et al. 2016). However it is now under evaluation for neoplasm (phase I NCT03099161) to assess the safety and preliminary efficacy of Preladenant alone and in co-treatment with pembrolizumab in patients affected by advanced solid tumors not responding to conventional therapy.

PBF-509 is an A_{2A} receptor antagonist ongoing in a clinical study for the treatment of non-small cell lung cancer (phase I-II NCT02403193). The aim of this trial is to evaluate the safety, tolerability, feasibility, and preliminary efficacy of PBF-509 alone or in combination with PDR001 (programmed cell death 1 receptor antibody).

CPI-444 is an A_{2A} receptor antagonist under evaluation in a clinical study for the treatment of malignant melanoma, non-small cell lung, renal cell, triple-negative breast, colorectal, bladder, and prostate cancers (phase I NCT02655822). This trial will determine the safety, tolerability, and antitumor activity of CPI-444 alone and in combination with atezolizumab, a PD-L1 inhibitor.

The use of an A_{2A} receptor antagonist in treatment of cancer finds its reason in the effects mediated by this receptor subtype in T cells. Specifically, the A_{2A} receptor linked to Gs proteins increases cAMP, thus reducing TCR-triggered intracellular cascade, and decreasing T cell functions, in order to provide protection against exaggerated inflammation (Borea et al 2016). However this immunosuppressive effect is dangerous in solid tumors where both tumor and antitumor T cells are present (Lukashev et al. 2007). Cancer environment presents an increased production of adenosine induced by the hypoxia inducible-factor 1 (HIF-1) present in hypoxic tumors (Borea et al. 2017). As a consequence adenosine activates A_{2A} receptor on T cells, thus favoring the immunoescaping of cancer from immune cells. Such effect explains the importance to block A_{2A} receptor by selective antagonists to potentiate the immunotherapy of cancer.

1.4 Conclusions

The study of adenosine receptors together with their agonists and antagonists is an area of intense research based on the relevance of adenosine signaling in almost all human pathologies. Therefore, it is clear that these ligands have become an important topic for drug research at international level. Indeed, by considering the number of adenosine receptor ligands already existing on the market and available for clinical employment, their number is quite limited to a few drugs: Adenocard for paroxysmal supraventricular tachycardia; Doxofylline, Bamifylline, and Theophylline for asthma; Regadenoson for coronary artery imaging; and lastly Istradefylline for PD. However, 40 years of highly qualified research covering a wide range of knowledge areas and carried out by eminent scientists have led to the discovery of important molecules with novel mechanism of action now under clinical development for the therapy of important human pathologies like heart failure, asthma, PD, autoimmune diseases, as well as cancer (Table 1.1). It is auspicable that these candidates by finishing positively their clinical experimental process development could become in the next future new drugs for a healthy and high-quality world.

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Chapter 2
Regulation of Extracellular Adenosine

Detlev Boison

Abstract Adenosine receptor activation is determined by the availability of extracellular adenosine. The tissue concentration of extracellular adenosine in turn is determined by a combination of transmembrane transport through equilibrative and concentrative nucleoside transporters and intra- and extracellular metabolism. Metabolically, adenosine levels are kept in equilibrium by adenosine-producing reactions, which include ATP-degrading enzymes and S-adenosylhomocysteine hydrolase, and adenosine-consuming enzymes, which include adenosine deaminase and adenosine kinase. The equilibrium of extracellular adenosine is critical for health, but severely compromised in a wide range of pathologies. This chapter will outline key transport- and enzyme-based mechanisms that maintain extracellular adenosine homeostasis and discuss pathological implications of disrupted adenosine homeostasis. The chapter will conclude with considerations how lifestyle choices such as sleep, exercise, and diet can influence the availability of extracellular adenosine.

Keywords Adenosine · ATP · Adenosine transporters · Adenosine metabolism · Adenosine homeostasis

2.1 Introduction

The past decade has brought a wealth of information on how adenosine metabolism affects adenosine receptor activation. Compared to the rich pharmacology and knowledge of adenosine receptor-mediated pathways, the underlying biochemistry that ultimately controls those pathways has received much less attention. From an evolutionary perspective, the regulation of adenosine, which likely played an important role in the origin of life (Oro 1961), is all about energy equilibrium (Boison 2016). The overarching principle is to have a simple regulatory system that links a

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drop in ATP (energy crisis) to a rise in adenosine, which in turn has assumed a role to support mechanisms that conserve energy. Therefore adenosine metabolism is intricately linked to energy homeostasis. The regulation of extracellular adenosine, which ultimately determines adenosine receptor activation, is a complex equilibrium determined by extracellular and intracellular adenosine-producing and adenosine-removing pathways, as well as transmembrane transport systems, which link the extracellular and intracellular compartments of adenosine. Due to the complexity of the system, it is highly likely that there are different "pools" of adenosine, each regulated differently (Boison and Aronica 2015; Cunha 2008). Thus, a homeostatic tissue wide tone of adenosine might be highly suited to provide tonic inhibition, whereas a synaptic pool of adenosine might allow the modulation of specific synaptic functions at a higher temporal resolution within a globally inhibited network and thereby increase salience of the system (Cunha 2008). Thus, the biochemistry of adenosine plays a major role in the control of adenosine receptor-dependent and adenosine receptor-independent pathways. Given the evolutionary ancient roots of this regulatory system, it is not surprising that the biochemistry of adenosine plays major roles in health and disease in multiple organ systems.

2.2 Extracellular Adenosine Metabolism

2.2.1 Adenosine-Producing Enzymes

The major source of extracellular adenosine is ATP. Extracellular levels of ATP can rise as a result of tissue injury implying a role as "danger signal" mediated by its degradation product adenosine (Fredholm 2007). In the brain ATP can also actively be released as a largely excitatory neurotransmitter (Burnstock 1972) or as a gliotransmitter (Pascual et al. 2005). Therefore, the termination of ATP signaling via adenosine-producing enzymes is critical in transforming a largely excitatory signal (ATP) into a largely inhibitory signal (adenosine). The two major extracellular adenosine-producing enzymes are CD39 (EC 3.6.1.5, apyrase), which converts ATP or ADP into AMP, and CD73 (EC 3.1.3.5, ecto-5′-nucleotidase), which converts AMP into adenosine. The enzymatic activities of CD39 and CD73 play important roles in defining the duration, magnitude, and chemical nature of immune responses. Both enzymes promote a shift from an ATP-driven pro-inflammatory environment to an anti-inflammatory milieu induced by adenosine (Antonioli et al. 2013). They have been described as "immune checkpoint mediators" which interfere with antitumor immune responses (Allard et al. 2017). Therefore, the CD39/CD73 pathway plays important dynamic roles in a wide range of medical conditions with an immunological component. Because both enzymes are also expressed in microglia, they play an important role in defining the inflammatory environment of the brain (Matyash et al. 2017). An additional adenosine producing extracellular pathway converts cyclic AMP (cAMP) into AMP and via CD73 into adenosine. This reaction is catalyzed by ecto-phosphodiesterase (ecto-PDE, CD203c, nucleotide

diphosphatase, EC 3.6.1.9) and involved in a mechanism that via cAMP efflux through multidrug resistance proteins transforms an intracellular adenylyl cyclase signal into an extracellular adenosine signal (Godinho et al. 2015).

2.2.2 Adenosine-Degrading Enzymes

The only route for the direct extracellular degradation of adenosine is catalyzed by adenosine deaminase (ADA, CD26, EC 3.5.4.4), which not only exists in the intracellular compartment but exists also as an extracellular form (Franco et al. 1988). ADA catalyzes the deamination of adenosine into inosine and is a low-affinity, high-capacity enzyme, which assumes a role in the extracellular clearance of excessive levels of adenosine. However, extracellular ADA is not the major route of adenosine removal and the subsequent termination of adenosine receptor signaling because the enzyme has a rather high K_m for adenosine (in the range of 40–60 μM) and therefore might play a more important role for the removal of 2′-deoxyadenosine (Fredholm and Lerner 1982; Smith and Henderson 1982). For those reasons the transport of adenosine into the cell and the subsequent intracellular metabolism of adenosine play dominant roles in the termination of the extracellular adenosine signal.

2.3 Adenosine Transporters

Whereas ATP and cAMP can be released from a cell via channels, pores, or vesicles, the reuptake of adenosine is mediated via specific nucleoside transporters, which fall into the families of equilibrative and concentrative nucleoside transporters (Cass et al. 1999; Parkinson et al. 2011; Young et al. 2013).

2.3.1 Equilibrative Nucleoside Transporters

The human SLC29 family of proteins contains four energy-independent equilibrative nucleoside transporters ENT1, ENT2, ENT3, and ENT4 (Baldwin et al. 2004; Boswell-Casteel and Hays 2017). All four isoforms are widely distributed in mammalian tissues and considered ubiquitous, although with distinct variations in abundance according to tissue (Anderson et al. 1999; Jennings et al. 2001). ENT1 and ENT2 are both prevalent in brain and are expressed in all cell types including neurons and astrocytes. Their apparent K_m values for adenosine are 40 and 100 μM, respectively (Molina-Arcas et al. 2009). Whereas ENT1 is more prominently expressed in rostral brain areas, ENT2 expression appears to be more restricted to caudal brain regions. Interestingly, ENT1 appears to be co-localized with adenosine A_1 receptor expression, suggesting a functional role in the termination of A_1R

signaling (Anderson et al. 1999; Jennings et al. 2001). The transporters play important roles in nucleoside and nucleobase uptake for salvage pathways implicated in nucleotide synthesis and are also responsible for the cellular uptake of nucleoside analogues used in the treatment of cancers and viral diseases. In addition, by regulating the concentration of adenosine available to cell surface receptors, they influence many physiological processes ranging from cardiovascular activity to neurotransmission (Baldwin et al. 2004). In general, if intracellular adenosine metabolism keeps intracellular adenosine levels low, there is a directed flux of adenosine through ENTs into the cell. In contrast, under conditions of compromised intracellular adenosine metabolism, there is a flux of adenosine into the extracellular space (Boison 2013). Thereby, ENTs effectively equilibrate intra- and extracellular levels of adenosine. ENTs are also targets for ethanol and cannabidiol (Carrier et al. 2006; Choi et al. 2004). Thus, it has been shown that the inhibition of ENT1 through cannabidiol leads to an increase in extracellular adenosine, a likely mechanism for the immunosuppressive activities of cannabidiol (Carrier et al. 2006). Through the same mechanism, ENT1 blockade also contributes to the anticonvulsant activities of cannabidiol (Devinsky et al. 2014). ENT1 is inhibited by acute ethanol and downregulated after chronic exposure to ethanol (Choi et al. 2004). Both mechanisms increase extracellular adenosine and thereby contribute to the cardioprotective activity of moderate ethanol consumption (Ramadan et al. 2014).

2.3.2 Concentrative Nucleoside Transporters

The human SLC28 family of proteins contains three sodium-dependent concentrative nucleoside transporters CNT1, CNT2, and CNT3, of which CNT2 and CNT3 are involved in adenosine transport (Gray et al. 2004). CNTs are cotransporters of sodium and nucleosides using energy from sodium gradients across plasma membranes. When extracellular sodium concentrations are high and intracellular levels are low, nucleosides are transported into the cell, a mechanism initially thought to be essential for nucleoside salvage. However, recent findings suggest more complex interactions. In particular, CNT2, the major concentrative transporter for adenosine, is under the control of the adenosine 1 receptor, and thereby contributes to the termination of A1 receptor signaling (Aymerich et al. 2005). CNT2 plays likely an additional role in energy metabolism because its activation depends on the opening of ATP-sensitive K^+ channels (Aymerich et al. 2005). Whereas ENTs are thought to be ubiquitously expressed, the expression of CNTs is more restrictive. Thus, CNTs have been found in specialized epithelial cells in the small intestine, kidney, and liver (Felipe et al. 1998; Pennycooke et al. 2001; Valdes et al. 2000) as well as in immune cells (Pennycooke et al. 2001; Soler et al. 1998) and the brain (Guillen-Gomez et al. 2004). CNT2 and CNT3 have a higher affinity for adenosine compared to ENTs with apparent K_m values of 8 and 15 μM, respectively (Molina-Arcas et al. 2009). Because mammalian tissues express several nucleoside transporters in a single cell type, often combining equilibrative and concentrative transporters, the

regulation of extracellular adenosine is a complex process. The termination of extracellular adenosine signaling is therefore the result of a concerted effort between these nucleoside transport systems as well as intracellular adenosine metabolism.

2.4 Intracellular Adenosine Metabolism

Due to the existence of ubiquitous transport systems for adenosine, the intracellular metabolism of adenosine plays a major role for the regulation of extracellular levels of adenosine. Three enzymes contribute to the regulation of intracellular adenosine: S-adenosylhomocysteine hydrolase, adenosine deaminase, and adenosine kinase.

2.4.1 S-Adenosylhomocysteine Hydrolase

S-Adenosylhomocysteine hydrolase (SAHH, EC 3.3.1.1) is a key enzyme of the S-adenosylmethionine-dependent transmethylation pathway. SAHH cleaves S-adenosylhomocysteine, the ubiquitous product of transmethylation, into adenosine and homocysteine. Interestingly, the thermodynamic equilibrium favors the formation of SAH from adenosine and homocysteine. High levels of SAH block transmethylation. Transmethylation can therefore only proceed if adenosine is effectively removed metabolically through adenosine kinase (Bjursell et al. 2011; Boison et al. 2002; Moffatt et al. 2002; Williams-Karnesky et al. 2013). Importantly those mechanistic implications are supported by findings that both SAHH as well as adenosine kinase are expressed at high levels in organs such as liver, which have a high methylation demand (Finkelstein 1998). Due to its role in a major metabolic pathway, SAHH can contribute both to the formation (under methylating conditions) or removal (under conditions of elevated adenosine) of adenosine. In the heart SAH cleavage through SAHH appears to contribute to a majority of basal adenosine under conditions of normoxia; however the role of SAHH in the control of intracellular adenosine levels in other organs such as the brain appears to be more limited (Dulla et al. 2005; Frenguelli et al. 2007; Pascual et al. 2005).

2.4.2 Adenosine Deaminase

Adenosine deaminase (ADA, E.C 3.5.4.4) converts adenosine into inosine through hydrolytic deamination. Inhibition of ADA therefore increases adenosine-mediated effects such as sedation (Major et al. 1981; Virus et al. 1983) and neuroprotection in animal models of global forebrain ischemia or focal ischemia (Lin and Phillis 1992; Phillis and O'Regan 1989). However, neuroprotection conferred by inhibitors of ADA during hypoxia or ischemia (Lin and Phillis 1992) mostly results from

potentiation of the stress-induced increase in intracellular adenosine, which leads to enhanced adenosine release through transport reversal (Phillis and O'Regan 1989). This is because ADA, with an apparent K_m for adenosine in the 40–60 μM range, is a low-affinity but high-capacity enzyme for effective adenosine removal under conditions of stress during which adenosine accumulates to excessive levels. ADA is expressed at high levels in placenta and seems to play a crucial role for fetal and perinatal development (Blackburn et al. 1995; Gao et al. 1994; Knudsen et al. 1992). During early brain development, transient ADA expression was found in specific subsets of neurons (Senba et al. 1987). In adulthood, the highest expression levels of ADA are found in the tongue and in cells lining the intestinal tract (Chinsky et al. 1990; Mohamedali et al. 1993). In the adult brain, ADA is associated with neurons and exhibits an uneven expression pattern (Geiger and Nagy 1986). Whereas many brain areas such as hippocampus are characterized by very low levels of ADA, higher ADA levels are only found in specialized nuclei such as the posterior hypothalamic magnocellular nuclei (Geiger and Nagy 1986). Importantly, ADA activity decreases during brain maturation in several brain areas such as superior colliculus, cortex, hippocampus, cerebellum, olfactory bulb, and olfactory nucleus indicating further that ADA plays important roles during brain development rather than in mature brain (Geiger and Nagy 1987). In contrast, ADK expression in astrocytes increases during brain maturation (Studer et al. 2006) indicating that the brain undergoes a developmental switch from ADA to ADK as the key regulators of ambient brain adenosine. Thus, in slices from adult brain, pharmacological inhibition of ADK, but not of ADA, leads to adenosine-induced inhibition of neuronal activity (Pak et al. 1994).

2.4.3 Adenosine Kinase

The major metabolic route of adenosine clearance is mediated by phosphorylation to AMP via adenosine kinase (ADK, EC: 2.7.1.20). Importantly, 5′-nucleotidase and ADK are part of a highly active substrate cycle between adenosine and AMP, which enables a cell to rapidly respond to changes in the energy status; it has been shown that minor changes in ADK activity rapidly translate into major changes in the concentration of adenosine (Bontemps et al. 1983, 1993a, b). Because levels of intracellular AMP, ADP, and ATP are high (millimolar range), and levels of adenosine low (nanomolar range), any changes in the adenosine/AMP substrate cycle flow selectively effect the adenosine concentration without having major impact on the equilibrium of the phosphorylated compounds (Boison et al. 2010; Fredholm et al. 2005). Because ADK is a low-capacity and low-K_m enzyme, it is the primary enzyme for metabolic adenosine clearance under baseline conditions, with the goal to keep adenosine levels low (Boison et al. 2010). Therefore, ADK expression is highest in organs, in particular the liver and placenta (Andres and Fox 1979), which have the highest needs for metabolic adenosine clearance (Finkelstein and Martin 1986). As outlined above, ADA is a high-capacity and high-K_m enzyme, which assists in

metabolic adenosine clearance under conditions in which adenosine levels become excessive (e.g., due to pathological activity) and the capacity of ADK is exceeded (Boison et al. 2010). Of note, ADK is an evolutionary ancient and highly conserved enzyme, which is directly related to bacterial ribokinases and fructokinases (Park and Gupta 2008; Spychala et al. 1996). Based on those early evolutionary roots, it is not surprising that ADK has been identified in almost all living organisms that have been analyzed genetically, including microorganisms, yeasts, plants and animals, and in every tissue assayed (Boison 2013).

2.5 Pathologies with Disrupted Adenosine Metabolism

Temporal lobe epilepsy (TLE), Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) share a wide range of comorbid symptoms, which involve increased neuronal excitability and a wide range of cognitive and psychiatric symptoms. This remarkable overlap suggests the existence of common pathogenic mechanisms, which include synaptic dysfunction and synaptotoxicity (Jensen 2011; Noebels 2011; Swann and Rho 2014; Zhou and Roper 2012), inflammatory processes (Kobow et al. 2012; Miller and Spencer 2014; Perry 2012), and glial activation (Ravizza et al. 2013; Stanimirovic and Friedman 2012; Suvisaari and Mantere 2013). Synaptotoxicity, immune activation, inflammatory processes, and glial activation play a major role in the pathogenesis of all four conditions (Aronica et al. 2012; Jensen et al. 2013; Lucin and Wyss-Coray 2009), leading ultimately to astrogliosis, overexpression of ADK, and a deficiency in the availability of adenosine – a sequence, which has been identified as characteristic pathological hallmark of human TLE (Aronica et al. 2013). Through the tripartite synapse, astrocytes interact with neurons (Araque et al. 1999; Halassa and Haydon 2010), and adenosine itself affects synaptic function (Duarte et al. 2012; Matos et al. 2012a; Silva et al. 2007) with a gain of function of synaptic A_{2A}Rs contributing to synaptotoxicity and adaptive processes of astrocytes affecting glutamate homeostasis and thereby synaptic function (Matos et al. 2012a, b, 2015). Thus, a self-reinforcing triad of astrocyte activation, adenosine dysfunction, and synaptotoxicity may contribute to the development of comorbid symptomatology. The apparent common overlap of maladaptive changes in adenosine homeostasis suggests common pathogenic mechanisms, which might be tied to common triggers of disease initiation. For example, the ADK hypothesis of epileptogenesis suggests that a precipitating injury triggers an acute surge in adenosine, which facilitates inflammatory processes and glial activation, resulting in astrogliosis, overexpression of ADK, and adenosine deficiency, which in turn drives hypermethylation of DNA (Boison 2008; Li et al. 2008; Williams-Karnesky et al. 2013). Similar mechanisms might also play a role in neurodegenerative conditions. If adenosine deficiency is a common pathological hallmark in a wider range of neurological conditions, then therapeutic adenosine augmentation might have the potential to treat comorbid conditions, such as those discussed here, in a holistic manner. Adenosine deficiency can explain a wide range

of comorbid symptoms including seizures (Boison 2012b; Dragunow 1991; Dunwiddie 1980; During and Spencer 1992), cognition (Costenla et al. 2011; d'Alcantara et al. 2001; Rebola et al. 2008), psychosis (Cunha et al. 2008; Gomes et al. 2011; Lucas et al. 2011), depression (Hines et al. 2013), motor control (Shen et al. 2012), and sleep (Basheer et al. 2004; Bjorness et al. 2009; Blutstein and Haydon 2012; Halassa et al. 2009; Porkka-Heiskanen et al. 1997). To provide a causal link between ADK overexpression-induced adenosine deficiency and the emergence of comorbid pathophysiological conditions, a "comorbidity model" was created based on the transgenic overexpression of ADK in the brain of mice. Those *Adk-tg* mice were characterized by spontaneous seizures, sleep alterations, cognitive impairment, psychiatric features, and loss of dopamine function (Boison and Aronica 2015; Fedele et al. 2005; Klein et al. 2018; Li et al. 2008; Palchykova et al. 2010; Shen et al. 2012; Yee et al. 2007).

2.6 Adenosine Augmentation Therapies

The findings outlined above provide a neurochemical rationale to use therapeutic adenosine augmentation for the treatment of seizures and associated comorbidities. Several approaches demonstrate a potent antiictogenic and antiepileptogenic role of adenosine therapy.

2.6.1 Pharmacology

Adenosine augmentation therapies (AATs) are based on the rational therapeutic use of an endogenous anticonvulsant and neuroprotectant with the potential to not only suppress seizures but also to prevent the development of epilepsy and its progression (Boison 2009, 2012a). The most efficient strategy to raise extracellular levels of adenosine and hence adenosine receptor activation is the use of ADK inhibitors (Boison 2013), which reduce the metabolic clearance of adenosine and thereby can potentiate an endogenous adenosine response in a site- and event-specific manner (Kowaluk et al. 1998; Kowaluk and Jarvis 2000; McGaraughty et al. 2001, 2005). Because ADK is pathologically overexpressed in seizure-generating brain areas (Aronica et al. 2011; Boison 2012b; Gouder et al. 2004; Li et al. 2008) and because overexpression of ADK is sufficient to promote seizures (Etherington et al. 2009; Li et al. 2012, 2008; Theofilas et al. 2011), the use of ADK inhibitors for the treatment of seizures is based on a strong neurochemical rationale. Importantly, the ADK inhibitor 5-iodotubercidin (5-ITU) effectively suppressed seizures in a mouse model of pharmacoresistant temporal lobe epilepsy (Gouder et al. 2004) suggesting that ADK inhibitors might be superior to conventional antiepileptic drugs. Because ADK, rather than ADA, is the major adenosine-metabolizing enzyme in the brain, the ADK inhibitor 5′-amino-5′-deoxyadenosine or 5-ITU, but not the ADA

inhibitor 2′-deoxycoformycin, suppressed bicuculline-induced seizures in rats, suggesting that the antiictogenic activity of ADK inhibition is superior to ADA inhibition (Zhang et al. 1993). Several ADK inhibitors have been developed for seizure control (Ugarkar et al. 2000a, b). However, despite an improved side effect profile of newer inhibitors, the chronic, systemic use of ADK inhibitors for epilepsy therapy might not be an option due to liver toxicity (Boison et al. 2002) and the occurrence of cognitive and sedative side effects (Boison 2013).

2.6.2 Cell-Based Adenosine Delivery

Focal drug delivery to the brain is an alternative approach to avoid systemic side effects (Nilsen and Cock 2004). One strategy for focal adenosine augmentation is an ex vivo gene therapy approach to first delete the *Adk* gene in cultured cells to induce therapeutic adenosine release (Fedele et al. 2004) and then to transplant the resulting adenosine-releasing cells into the host brain. The first successful cell therapy approach was achieved with baby hamster kidney (BHK) cells that were engineered to lack the *Adk* gene (Huber et al. 2001). These ADK-deficient BHK cells released about 40 ng adenosine per 10^5 cells per day. After encapsulation into semipermeable polymer membranes and transplantation into the brain ventricles of kindled epileptic rats, the implants induced almost complete seizure suppression in an A_1R-dependent manner (Huber et al. 2001). Seizure suppression however was limited to 2 weeks due to the reduced life expectancy of the encapsulated cells. A more versatile cell-based system for seizure control was developed by disrupting both alleles of the *Adk* gene in mouse embryonic stem (ES) cells. When differentiated into neural precursor cells and grafted into the intrahippocampal fissure of rats, the adenosine-releasing cells suppressed kindling epileptogenesis (Li et al. 2007). Likewise, when grafted into the intrahippocampal fissure of mice 24 h after a status epilepticus, the $Adk^{-/-}$ cells prevented the development of epilepsy (Li et al. 2008). Specifically, the cell transplants attenuated astrogliosis, prevented overexpression of ADK, and led to a complete lack of any seizures (Li et al. 2008). These findings show that disruption of ADK expression in cells is a promising therapeutic strategy to boost adenosine signaling focally within a seizure-generating brain area with potent therapeutic effects resulting in seizure suppression and prevention of epileptogenesis.

2.6.3 Gene Therapy

Conventional gene therapies overexpress a transgene to produce a therapeutic agent. However, for the development of an adenosine augmentation therapy, the therapeutic goal is the reduction of the expression of the *endogenous Adk* gene. This can be

achieved with antisense approaches (Boison 2010) designed to knock down gene expression. To this end an adeno-associated virus (AAV)-based vector was constructed to express an *Adk* cDNA in antisense orientation under the control of an astrocyte-specific gfaABC$_1$D promoter (Lee et al. 2008). Intrahippocampal injection of this virus into transgenic mice with spontaneous electrographic seizures resulted in a substantial unilateral decrease in seizure activity ipsilateral to the virus injection site with 0.6 ± 0.6 seizures/h, compared to 5.8 ± 0.5 seizures/h on the contralateral (non-injected) side (Theofilas et al. 2011). This proof of feasibility study shows that a gene therapy targeting ADK restricted to astrocytes can have a potent therapeutic effect based on enhancing the anticonvulsive properties of adenosine. More work is needed to evaluate whether anti-ADK gene therapies are effective in clinically relevant models of TLE.

2.6.4 Antiepileptogenesis

Several lines of evidence demonstrate that adenosine not only prevents seizures but also prevents epileptogenesis. Transgenic mice with an engineered reduction of ADK expression in forebrain did not develop epilepsy, even when an epileptogenesis-triggering status epilepticus was coupled with transient blockade of the A_1R (Li et al. 2008). In addition, adenosine-releasing stem cells – implanted into the hippocampal formation *after* triggering epileptogenesis – dose-dependently attenuated astrogliosis, suppressed ADK increases, and attenuated the development of spontaneous seizures (Li et al. 2008). Likewise, the transient delivery of adenosine for 10 days by intraventricular silk provided long-lasting antiepileptogenic effects in the rat kindling model (Szybala et al. 2009). Those data suggest the existence of a specific mechanism through which adenosine interferes with the epileptogenic process. Maladaptive changes in DNA methylation are now widely recognized to play a key role in epileptogenesis (Kobow and Blumcke 2011, 2012; Kobow et al. 2013; Miller-Delaney et al. 2015; Williams-Karnesky et al. 2013). A novel antiepileptogenic mechanism of adenosine was recently described in a model system, where a transient dose of adenosine administered to epileptic rats after the onset of epilepsy not only suppressed seizures during active adenosine release but also prevented further disease progression in the long term even after the therapy was suspended. Adenosine treatment restored normal DNA methylation in the otherwise hypermethylated hippocampus of the epileptic rat. More specifically, genome-wide analysis using a methylated DNA immunoprecipitation array revealed that out of the 125 genes which showed increased DNA methylation in epilepsy, 66 also showed reduced DNA methylation after adenosine therapy in treated epileptic rats. Interestingly, multiple targets that function to either interact with DNA or play a role in gene transcription and translation (*PolD1, Polr1e, Rps6kl1, Snrpn, Znf524, Znf541, and Znf710*) responded to adenosine therapy. Those targets are therefore of interest as likely candidates to mediate adenosine-dependent changes in major homeostatic functions of the epigenome (Williams-Karnesky et al. 2013).

2.7 Lifestyle Choices and Extracellular Adenosine

There is now ample evidence that certain lifestyle choices and external stimuli influence adenosine metabolism and function. The interactions between adenosine and sleep are well known; however factors such as diet and exercise receive increased attention.

2.7.1 Sleep

In line with the early evolutionary role of adenosine to couple energy expenditure to energy supplies, the adenosine regulation of sleep has assumed a major function to restore energy during sleep. Adenosine levels increase in the brain during prolonged wakefulness, most prominently in the basal forebrain cholinergic area and in cortex (Porkka-Heiskanen and Kalinchuk 2011; Porkka-Heiskanen et al. 1997). In line with the energy hypothesis, the disruption of mitochondrial electron transport in the basal forebrain to induce an energy crisis increased the levels of adenosine and induced sleep (Kalinchuk et al. 2003). It is now well established that sleep is significantly regulated by adenosine metabolism (Kalinchuk et al. 2003; Porkka-Heiskanen et al. 1997; Shaw et al. 2000). Although sleep deprivation does not affect the activity of ADA (Mackiewicz et al. 2003), the enzyme shows significant circadian variation (Chagoya de Sanchez et al. 1993; Mackiewicz et al. 2003). A human gene variant of ADA (G > A polymorphism), which reduces the enzyme's efficacy in turning adenosine into inosine and thereby compromises adenosine metabolism, was shown to promote sleep (Retey et al. 2005). A subsequent epidemiological study confirmed that individuals with the G > A variant had higher sleep efficacy and more rapid eye movement (REM) sleep compared to G > G carriers (Mazzotti et al. 2012, 2011). Like ADA, ADK also shows a clear circadian variation (Alanko et al. 2003; Chagoya de Sanchez et al. 1993); however ADK activity and expression do not seem to be influenced by sleep deprivation (Alanko et al. 2003; Mackiewicz et al. 2003). An animal study using transgenic Adk-tg mice with overexpression of cytoplasmic ADK-S in the brain, but lack of nuclear ADK-L, has shown a reduction in EEG low-frequency power in all vigilance states, and the animals spent more time in waking. Sleep homeostasis was also affected as evidenced by a more modest increase in slow wave activity during recovery sleep. Together, all data demonstrate that lower levels of adenosine or any increase in adenosine metabolism promote an increase in wakefulness.

2.7.2 Diet

The high-fat low-carbohydrate ketogenic diet is a metabolic intervention, which provides effective seizure control in many forms of pharmacoresistant epilepsy (Freeman 2009; Kossoff and Rho 2009; Kossoff et al. 2009; Neal et al. 2008; Yellen

2008). Although it has been used clinically for almost 100 years, the underlying mechanisms of its activity have not been fully explored. A ketogenic diet forces the brain to use ketones as primary energy source instead of glucose, and it is those metabolic changes that are thought to mobilize the therapeutic effects of this type of metabolic intervention (Bough 2008; Bough et al. 2006; Kalapos 2007; Ma et al. 2007; Yellen 2008). Although several mechanisms of ketogenic diet therapy may synergistically contribute to antiepileptic outcome, a large body of evidence supports the notion that a ketogenic diet increases adenosine signaling in the brain (Masino and Geiger 2008, 2009; Masino et al. 2009, 2012). In a seminal study, we demonstrated that a ketogenic diet reduced the expression of ADK in mice (Masino et al. 2011). In line with this finding, the ketogenic diet suppressed seizures in adenosine-deficient Adk-tg mice, but not in A_1R-deficient mice, demonstrating that functional A_1R activation is necessary for the antiepileptic effects of the diet (Masino et al. 2011). Subsequent studies demonstrated an antiepileptogenic and disease-modifying activity of ketogenic diet therapy (Kobow et al. 2013; Lusardi et al. 2015). Ketogenic diet therapy suppressed pentylenetetrazole (PTZ) kindling in mice and suppressed epileptogenesis in the rat pilocarpine model of TLE, an effect that was maintained even after discontinuation of the diet (Lusardi et al. 2015). As a potential mechanism of action, it was shown that ketogenic diet therapy restored normal adenosine levels in otherwise adenosine-deficient epileptic rats. This restoration of normal adenosine homeostasis was linked to the period of active ketogenic diet feeding, whereas reversal to normal diet abolished those effects. Importantly, the transient use of a ketogenic diet lead to lasting reductions in the global DNA methylation status in epileptic rats. This effect was maintained even after reversal to a normal diet. Together those findings suggest that ketogenic diet therapy may exert potent antiepileptogenic and disease modifying effects by adenosine-induced epigenetic mechanisms (Lusardi et al. 2015).

2.7.3 Exercise

Regular physical activity has well-known benefits on physical and mental performance. During intense neuronal activation as occurs during intense physical activity, the central command and sensory input to the brain is high, whereas metabolic demand exceeds metabolic availability resulting in an increased breakdown of ATP and an associated increase in adenosine (Basheer et al. 2004). Adenosine concentrations in rat neostriatum and hippocampus were not only found to be higher during the active period (as opposed to sleep) but also depended on exercise intensity (Huston et al. 1996). Whereas moderate exercise did not affect brain adenosine levels, intense exercise increased the ratio of metabolite demand to metabolite availability with an associated production of adenosine from ATP breakdown (Dworak et al. 2007). These findings might indicate a state of bioenergetic stress, possibly resulting from the enhanced breakdown of high-energy phosphates. Changes in the ATP/adenosine equilibrium during exercise can directly affect behavior. The net

effect of increased adenosine is a presynaptic reduction in transmitter release in wakefulness-promoting networks, including the cholinergic and monoaminergic systems, as well as postsynaptic hyperpolarization and EEG desynchronization (Latini and Pedata 2001; Rainnie et al. 1994). In particular, the basal forebrain and mesopontine cholinergic neurons, whose discharge activity plays an integral role in EEG arousal and maintenance of wakefulness, are postsynaptically inhibited by endogenous adenosine (Arrigoni and Rosenberg 2006; Latini and Pedata 2001). Exercise induced adenosine increases have also been linked to the phenomenon of central fatigue due to the inhibitory actions of adenosine on excitatory neurotransmission (Arrigoni et al. 2006; Rainnie et al. 1994). Through those same mechanisms, exercise is thought to improve sleep quality (Driver and Taylor 2000; O'Connor and Youngstedt 1995).

2.8 Conclusions and Outlook

Adenosine metabolism plays a key role in linking energy homeostasis to extracellular functions of adenosine that are mediated by the activation of adenosine receptors. Importantly, adenosine metabolism is tightly linked to a variety of lifestyle choices and external triggers, which thereby can directly influence adenosine receptor-mediated signaling pathways discussed in subsequent chapters of this volume. The realization that adenosine metabolism links metabolic and bioenergetic functions with adenosine receptor-mediated pathways offers new opportunities for therapeutic intervention. Rather than blocking (or activating) adenosine receptors selectively through specific ligands, the therapeutic manipulation of adenosine metabolism offers unique opportunities to reset the adenosinergic network on a more holistic level. Thereby restoration of network homeostasis becomes a unique therapeutic opportunity for a variety of pathological conditions.

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Chapter 3
Adenosine Receptors: Structure, Distribution, and Signal Transduction

Stefania Merighi, Stefania Gessi, and Pier Andrea Borea

Abstract Adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 are effector proteins triggered by the endogenous nucleoside adenosine to exert its numerous vital physiological effects, behaving like a guardian angel. This chapter offers an overview of the updated knowledge concerning the structure, distribution, and signal transduction of adenosine receptors. They are a family of G protein-coupled receptors widely distributed through the body, from central nervous system to peripheral organs, important and ubiquitous regulators of numerous cellular signaling. Their presence on every cell renders them an attractive opportunity for the pharmacological research and development of new drugs but also a challenge in the difficulty to produce tissue-selective ligands avoided of side effects. To aid this process, several efforts have been invested to reveal the molecular structure and the consequent mechanism of ligand binding of these receptors, and until now more than 30 structures have been published for the human A_{2A} subtype. Finally, the principal adenosine receptor signaling pathways including adenylyl cyclase, phospholipase C, inositol triphosphate, diacylglycerol, phosphatidylinositol 3-kinase, and mitogen-activated protein kinases determining their effects on several transcription factors, such as hypoxia-inducible factor 1, cyclic AMP (cAMP)-responsive elements, nuclear factor-kB, and exchange protein directly activated by cAMP as the most relevant, are presented.

Keywords Adenosine receptors · Signal transduction · cAMP · Distribution · Kinases

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3.1 Introduction

Adenosine is a purine nucleoside released by almost all cells mediating its effects through activation of four G protein-coupled adenosine receptors, classified as A_1, A_{2A}, A_{2B}, and A_3 (Borea et al. 2016). The first demonstration of their existence has been offered more than 40 years ago by the observation that methylxanthines such as caffeine and theophylline were able to antagonize the cardiac and cerebral effects of adenosine. These receptors are characterized by different affinity for adenosine, G protein coupling, as well as intracellular signal transduction inside cells. In general adenosine interacts with A_1, A_{2A}, or A_3 subtypes with an EC_{50} in the range 10 nM–1 μM, while activation of the A_{2B} subtype needs concentrations higher than 10 μM, rarely obtained in physiological conditions but present in hypoxic/injured tissues (Eltzschig 2009). Anyway, the affinity of adenosine to its receptors may also depend on the effect investigated, e.g., cAMP level determination versus MAPK activation or the number of receptors expressed (Chen et al. 2013). Specifically, on the one hand, A_1 and A_3 adenosine receptors show high and low affinity for adenosine, respectively, and are able to reduce adenylyl cyclase activity. On the other hand, A_{2A} and A_{2B} subtypes display high and low affinity for the nucleoside, respectively, and activate adenylyl cyclase, thus stimulating cyclic AMP (cAMP) levels (Fredholm et al. 2011; Borea et al. 2017). Adenosine receptors are present in every organ, tissue, and cell of the body rendering them attractive targets for the research and development of new drugs in many pathological conditions related with raised adenosine levels (Gessi et al. 2011). Anyway this wide distribution implies the lack of specificity of a given receptor subtype that may be present in both tissues involved in disease but also in healthy organs with consequent side effects, rendering difficult the development of drugs for specific medical needs. In this chapter updated informations concerning the molecular structure, distribution, and signal transduction of adenosine receptors are provided.

3.2 Molecular Structures of Adenosine Receptors

Adenosine receptors have been cloned in the beginning of the 1990s and deeply pharmacologically characterized and consist of a similar structure represented by a core domain crossing the plasma membrane seven times, with an extracellular N-terminus, an intracellular C-terminus, and three intracellular and three extracellular loops (IL and EL, respectively) of different lengths and functions among the four adenosine receptor subtypes (Fredholm et al. 2000). These domains give specific characteristics important for receptor-ligand interactions. Specifically, the EL1, EL2, and EL3 of GPCRs contribute significantly to receptor function as evidenced by crystal structures, and cysteine amino acids forming disulfide bonds in the EL domains of GPCRs are important not only in ligand binding but also in receptor stability and function (Avlani et al. 2007; Schiedel et al. 2011). The N-terminus presents one or more glycosylation sites, while the C-terminus

possesses phosphorylation and palmitoylation loci, which are important for receptor desensitization and internalization. Specifically, mutation studies revealed that glycosylation is relevant for the recruitment of receptors to the plasma membrane, while palmitoylation sites, located at the end of helix 8 and absent in A_{2A} adenosine receptors, influence receptor degradation. Depalmitoylation of A_3 adenosine receptors, in contrast to what happens for A_1 subtype, induces a fast receptor desensitization through GPCR kinase phosphorylation induction (Piirainen et al. 2011). Adenosine receptors are characterized by a high homology sequence among them, ranging from 41% to 58% of sequence identity for the human species, with the most conserved region being in the extracellular region of the receptor reaching 71%.

3.2.1 A_1 Adenosine Receptors

A_1 adenosine receptors are 326 amino acid long distributed among 7 transmembrane domains (TM) of which TM3 and TM7 result strictly conserved sequences for ligand interaction with the receptor, as reported from mutagenesis studies (Jespers et al. 2018). The A_1AR orthosteric site is found inside the TM packet, but also EL2 has been implicated in the ligand affinity and signal transduction (Peeters et al. 2012; Nguyen et al. 2016a, b). In addition, in the A_1 adenosine receptor EL2, the presence of an allosteric site has been reported through molecular modeling characterization (Narlawar et al. 2010). Recently, the crystal structure of A_1 adenosine receptors bound to a selective covalent antagonist has been revealed (Glukhova et al. 2017). Interestingly, significant differences with respect to already presented A_{2A} adenosine receptor structure indicate a different conformation of EL2 and a bigger extracellular cavity presenting an alternative binding pocket accepting both orthosteric and allosteric molecules. It has been suggested that this configuration confers ligand selectivity instead of the simple amino acid sequence. From this knowledge more selective drugs could be projected with both agonist and allosteric properties, useful for the therapy of neuropathic pain, ischemia-reperfusion damage, and renal pathologies (Glukhova et al. 2017; Cheng et al. 2017).

As for allosteric sites located on the EL region, a crystal structure with an allosteric modulator has not been provided but through mutagenesis studies the amino acid sequence responsible for these ligands involved in the binding site of A_1AR allosteric modulators (Jespers et al. 2018) has been reported.

3.2.2 A_{2A} Adenosine Receptors

A_{2A} adenosine receptors in human species are 412 amino acid long, but this number may slightly change from 409 to 412 in other species (de Lera Ruiz et al. 2014). At variance with other adenosine subtypes, it presents a long carboxy-terminal

domain, responsible for a major molecular weight (45 kDa) with respect to the other adenosine subtypes (Preti et al. 2015). A_{2A} adenosine receptors are formed by 7 TM of 20–27 amino acids with TM3 and EL2 containing cysteine residues giving a disulfide bond. In addition an extra short TM8 domain is present toward the membrane cytoplasmic surface (Jaakola and IJzerman 2010; de Lera Ruiz et al. 2014). Interestingly, two new cholesterol-binding sites have been described on it, one of which interacts with cholesterol only when bound to an inverse agonist, as demonstrated through numerous high-resolution crystal structure studies (Rouviere et al. 2017). Indeed, the last 10 years have seen a huge development of novel crystallization strategies that have introduced enormous changes in the knowledge of structural biology of GPCRs. Specifically, the A_{2A} adenosine receptor has been one of the best studied and characterized by a structural point of view, having more than 30 structures been described (Carpenter and Lebon 2017). In particular, crystal structures of A_{2A} adenosine receptors have been solved in complex with both agonists and antagonists, which provide informations concerning the binding sites and the conformational changes occurring following ligand-receptor interactions (Jaakola et al. 2008; Xu et al. 2011; Lebon et al. 2011, 2015; Doré et al. 2011; Hino et al. 2012; Congreve et al. 2012; Liu et al. 2012; Carpenter et al. 2016; Jazayeri et al. 2017; Carpenter and Lebon 2017). Specifically, the most observed phenomenon taking place after binding of the agonist is a contraction of the binding site due to TM3, 5, 6, and 7 rearrangements (Jespers et al. 2018). In addition an outward rotation of TM6 on the cytoplasmic side, consequent to receptor activation, allows G protein activation and signal transduction propagation. In addition it has been revealed that the ribose moiety is a key component of A_{2A} receptor agonists that helps to stabilize the intermediate-active state before the occurrence of the fully active receptor conformation, following G protein coupling (Carpenter and Lebon 2017). Numerous mutagenesis studies investigating the ligand binding of A_{2A} adenosine receptors have been performed. Interestingly, from them, the relevance of a glutamic acid and a histidine in TM1 and TM7, respectively, has been found taking part into the agonist binding process. In addition a relevant role for H bonds in ligand binding affinity has been revealed following the observation that loss of interactions between ligand and water is reflected in worsen affinity of both agonists and antagonists (Jespers et al. 2018). Overall from the data emerging by complementary techniques such as crystal structures as well as X-rays and mutagenesis studies, it is possible today to address a structure-based rationale design of new ligands interacting with A_{2A} adenosine receptors (Jespers et al. 2017).

3.2.3 *A_{2B} Adenosine Receptors*

A_{2B} adenosine receptors in human species are 328 amino acid long, organized following the typical GPCR architecture consisting of 7 TM domains presenting the highest homology between A_{2B} and the other adenosine receptors. This core is formed by hydrophobic amino acids linked by three EL and three IL and terminates

with an extracellular N-terminus and an intracellular C-terminus. Combination of homology modeling of rhodopsin GPCR structure and mutational studies of the A_{2B} adenosine receptors leads to the knowledge of its binding site, where TM regions 3, 5, 6, and 7 are involved in agonist and antagonist recognition (Beukers et al. 2000, 2004; Aherne et al. 2011). Interestingly, the EL2 of A_{2B} receptor, the longest of all the other adenosine receptor subtypes, presents four cysteine amino acids (C154, C166, C167, C171) responsible for disulfide bonds connecting EL and TM domains. Interestingly, only disulfide bond occurring between C171 in EL2 and C78 present in TM3 is essential for A_{2B} adenosine receptor-ligand binding and function, and it may also play a role in the transport of the receptors toward the membrane. As for the other cysteine residues in the ECL2 of the A_{2B} receptor, they may have different functions in comparison to the role that they play in the A_{2A} receptor (Schiedel et al. 2011). In addition subsequent site-directed mutagenesis studies have reported that introducing ECL2 of A_{2A} adenosine receptors in the structure of A_{2B} adenosine receptors provides a mutant A_{2B} receptor that displays higher affinity for both agonist and antagonists, thus suggesting that ECL2 is crucial for ligand binding. Therefore the major length of ECL2 in the A_{2B} adenosine subtype is responsible for the lower affinity of ligands to it in comparison to A_{2A} receptors, because it may hamper the ligand interaction to the binding site (Schiedel et al. 2011; Seibt et al. 2013; da Rocha Lapa et al. 2014). Other mutational studies have discovered the amino acids involved in ligand binding of three different classes of molecules including xanthine, adenosine, and aminopyridine derivatives. In particular, the amino acids Asn282 and His280 by forming H bond stabilize the binding site as occurs in the A_{2A} adenosine receptor. Trp247, Val250, and especially Ser279 are crucial for adenosine binding. Leu81, Asn186, and Val250 are important for binding of the xanthine antagonists (Thimm et al. 2013).

3.2.4 A_3 Adenosine Receptors

A_3 adenosine receptors in human species are 318 amino acid long. As with the other adenosine receptors, the A_3 is constituted by seven TM domains with an intracellular C-terminal sequence containing six Ser and Thr amino acids undergoing phosphorylation by GPCR kinases during rapid receptor desensitization occurring in the order of minutes. Specifically, this process triggered following agonist binding to the A_3 adenosine receptors causes subsequent internalization through clathrin-coated pits in rat A_3 adenosine receptors (Palmer and Stiles 2000; Trincavelli et al. 2002a, b; Madi et al. 2003; Pugliese et al. 2007; Jacobson et al. 2018). However, the fast desensitization has not been observed in A_1, A_{2A}, and A_{2B} receptor subtypes where this process takes place after hours. The reason for this discrepance has been attributed to the lack of Ser and Thr residues in the C-terminus, for example, of the A_1 subtype. Another reason explaining the rapid desensitization of A_3 receptors resides in the presence of Cys amino acids in its C-terminus tail, crucial for GRK activation. As the sequence identity between rat and human A_3 receptors is only

72%, this point has been recently addressed. Specifically, it has been shown that the C-terminus of the human subtype is not involved in βarr2 recruitment, receptor desensitization, and internalization, suggesting that other different regions of the human A_3 adenosine receptors, either cytosolic or exposed upon receptor activation, are involved in this process. It has been observed that C-terminal truncation, in combination with mutation of the "DRY" motif located at the boundary between TM3 and IL-2, significantly decreased βarr2 recruitment (Storme et al. 2018). Interestingly, mutational studies demonstrated that the active shape of the human A_3 receptor needs the highly conserved Trp (W6.48) in TM6, important to activate signal transduction pathways, to interact with β-arrestin2, and to undergo receptor internalization (Gao et al. 2002; Stoddart et al. 2014). Furthermore, use of a novel fluorescent A_3 agonist has allowed for the observation of co-localization with internalized receptor βarr3 complexes (Stoddart et al. 2015).

3.2.5 Adenosine Receptor Heteromers

Homomer, oligomer, and heteromer formation has been recently recognized as a common phenomenon affecting numerous GPCRs including adenosine receptors (Ferré et al. 2010a, b; Navarro et al. 2010a, b, 2016b; Brugarolas et al. 2014). The possibility of homo- or hetero-oligomer formation lies on, at least in part, high receptor levels (Fredholm et al. 2011). Specifically, GPCR heteromers are new entities for signal transduction with different functions if compared to homomers. In the field of adenosine receptors, A_1-A_{2A} oligomers are present in neural tissue, comprising two different receptors coupled to two different G proteins (Brugarolas et al. 2014; Navarro et al. 2016b). In particular the A_1 component, through Gi and the A_{2A} part via Gs, confers to the heteromer the possibility to signal in an opposite way on cyclic adenosine monophosphate (cAMP) intracellular pathway. Therefore, this complex constitutes a cell surface sensor of adenosine concentration, distinguishing between low and high nucleoside concentration (Navarro et al. 2016b). Indeed the A_1 unit of this complex interacts with Gi/o protein, thus decreasing cAMP levels, PKA, and GABA uptake, when adenosine levels are low. The A_{2A} monomer of the heteromer takes place in cAMP signaling when adenosine levels increase, due to its inhibition of A_1 component and activation of Gs proteins, thus obtaining GABA uptake increase (Cristóvão-Ferreira et al. 2013). In addition various physiological process, such as glutamate release, may be regulated on the basis of adenosine concentration (Ciruela et al. 2006). Heteromerization has been described as a general process involving other receptors inside adenosine receptor family including A_3ARs, forming homodimers and A_1-A_3 heterodimers (Kim and Jacobson 2006; Hill et al. 2014). In addition, heteromerization involves also the interaction of adenosine receptors with other GPCRs. For example, A_1 may form oligomers with P2Y1 (Yoshioka et al. 2001), D1 dopamine (Ginés et al. 2000), and mGlu1αR receptors (Ciruela et al. 2001). As for A_{2A} receptors, the most studied combination in this field is represented by the A_{2A}-D_2 dopamine complex, detected in the striatum, and a viable therapeutic target in PD (Fuxe et al. 2005, 2007; Ferré et al. 2010b; Navarro

et al. 2016a). In addition they may oligomerize with mGlu5 (Ferré et al. 2002), P2Y1 (Arellano et al. 2009), and cannabinoid CB1 receptors (Carriba et al. 2007).

3.3 Distribution of Adenosine Receptors

Adenosine receptors are widely distributed throughout the body spanning from the central nervous system, cardiovascular apparatus, respiratory tract, gastrointestinal tissue, and immune system to different organs or tissues including the kidney, bone, joints, eyes, and skin, suggesting a wide influence of adenosine in almost all physiological processes (Peleli et al. 2017). This distribution reflects a significative function of adenosine in the neurons, heart, and kidney.

3.3.1 A_1 Adenosine Receptors

In the brain A_1 adenosine receptors are highly distributed in different regions, including the cortex, hippocampus, cerebellum and spinal cord, autonomic nerve terminals, and glial cells (Chen et al. 2013; Ballesteros-Yáñez et al. 2018). In the heart, A_1 adenosine receptor expression has been detected with higher levels in atria and less in the ventricular myocardium (Varani et al. 2017). At vascular level A_1 adenosine receptors are present on coronary smooth muscle arteries and endothelial cells (Headrick et al. 2013). Furthermore, A_1 adenosine receptors are found in the lung endothelial cells, in smooth muscle cells of airway, in alveolar epithelial cells, and in macrophages (Sun et al. 2005). In the kidney, A_1 adenosine receptors are located in the collecting ducts of the papilla, inner medulla, and cells of the juxtaglomerular apparatus (Varani et al. 2017; Soni et al. 2017). A_1 adenosine receptors are expressed in pancreas tissues and adipocytes (Meriño et al. 2017). As for immune system, A_1 adenosine receptors are present on different immune cells, such as neutrophils, eosinophils, macrophages, and monocytes (Sachdeva and Gupta 2013; Boros et al. 2016). A_1 adenosine receptors have also been localized in the retina, intestine, skeletal muscle, and vascular cells of skeletal muscle (Varani et al. 2017).

3.3.2 A_{2A} Adenosine Receptors

A_{2A} adenosine receptors are mostly expressed in selected areas of the central nervous system as well as in peripheral immune cells. Specifically, concerning brain regions A_{2A} adenosine receptors are expressed at high level in striatal neurons, while lower presence has been detected in extra-striatal and in glial cells (Fredholm et al. 2011; Boison et al. 2012; Borea et al. 2017). In particular, they are numerous in the caudate and putamen, in the nucleus accumbens, as well as in the olfactory tubercle.

The presence of A_{2A} adenosine receptors has been demonstrated in the heart, in both atria and ventricle and in coronary vessels, but also in the lung and liver. Finally, high expression of A_{2A} adenosine receptors has been reported in platelets, lymphocytes, neutrophils, monocytes, macrophages, dendritic cells, vascular smooth muscle, and endothelial cells (Gessi et al. 2000).

3.3.3 A_{2B} Adenosine Receptors

At the central level, the A_{2B} adenosine receptors are expressed in astrocytes, neurons, and microglia (Koupenova et al. 2012; Merighi et al. 2015; Pedata et al. 2016). As for the periphery, they are found in the bowel, bladder, lung, vas deferens, and different cell types including fibroblasts; smooth muscle, endothelial, alveolar epithelial, chromaffin, and taste cells; platelets; myocardial cells; and retinal, intestinal and pulmonary epithelial, and endothelial cells. A_{2B} adenosine receptors are expressed in several immune cells including mast cells, macrophages, lymphocytes, neutrophils, and dendritic cells (Aherne et al. 2011).

3.3.4 A_3 Adenosine Receptors

A_3 adenosine receptors are present in several cells and tissues with a different degree of expression at central and peripheral level. In the brain tissue, they are present in low amount in the thalamus, hypothalamus, and hippocampus. At cellular level they are expressed in motor nerve terminals, microglia, astrocytes, cortex, and retinal ganglion cells while at cerebral vascular level in the pial and intercerebral arteries (Janes et al. 2014; Borea et al. 2016). A_3 adenosine receptors are present in the coronary and carotid artery and in the heart but only at low level. At the periphery A_3 adenosine receptors have been demonstrated in lung parenchyma and bronchi, enteric neurons and colonic mucosa, and epithelial cells. Finally, A_3 adenosine receptors have a wide distribution in immune and inflammatory cells including lymphocytes, neutrophils, eosinophils, monocytes, macrophages, dendritic cells, foam cells, mast cells, splenocytes, bone marrow cells, lymph nodes, synoviocytes, chondrocytes, and osteoblasts. Interestingly, A_3 adenosine receptors are overexpressed in different cancer tissues such as the colon, liver, lung, melanoma, and glioblastoma (Borea et al. 2015).

3.4 Signal Transduction of Adenosine Receptors

All adenosine receptors are coupled to G proteins and trigger several transduction pathways that may differ depending on the specific cell activated (Fredholm et al. 2001).

3.4.1 A_1 Adenosine Receptors

The Gi-coupled A_1 adenosine receptor inhibits adenylyl cyclase (AC) activity thus decreasing cAMP levels. This leads to the inhibition of cAMP-dependent protein kinase A (PKA) activation and cAMP-responsive element-binding protein 1 (CREB-1) phosphorylation, resulting in the reduction of CREB transcriptional activation. In addition it also induces phospholipase C (PLC)-β stimulation, by link to Gq proteins, thus rising diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) that, through interaction with its cytoplasmic receptor, rises intracellular Ca^{2+} concentrations, which activate calcium-dependent protein kinases (PKC) and/or other calcium-binding proteins. PKC may be phosphorylated also by DAG. In addition, βγ subunits of Gi/o protein are involved by A_1 adenosine receptor to induce PLC activation (Biber et al. 1997). In addition A_1 adenosine receptor enrolls pertussis-toxin-sensitive potassium (K) and K_{ATP} channels, expressed in neurons and myocardium, while reduces Ca^{2+} channels of Q, P, and N type. Recently, it has been reported that it increases PC12 cell damage following intermittent hypoxia through PKC and K_{ATP} mediators (Mei et al. 2018). Furthermore, the first report describing the link between A_1 adenosine receptor and the family of mitogen-activated protein kinase (MAPK) indicated the stimulation by it of extracellular signal-regulated kinase (ERK) (Schulte and Fredholm 2000) (Fig. 3.1). Since then many studies have found different effects on MAPK modulation depending on the cell investigated. For example, it has been reported that A_1 adenosine receptor in brain neurons increases p38 to reduce apoptosis in a rat model of brain injury (Zhai et al. 2016). Accordingly, it activates p38 and also c-Jun N-terminal kinase (JNK) in hippocampal neurons, thus inducing clathrin-mediated internalization of GluA2 and GluA1 subunits responsible for synaptic depression that caused hippocampal neurodegeneration after hypoxia/cerebral ischemia (Brust et al. 2006; Liang et al. 2008; Chen et al. 2014). Previous data in the hippocampus demonstrated that the increase in p38 phosphorylation induced by A_1 receptor was involved in brain-derived neurotrophic factor (BDNF) generation (Katoh-Semba et al. 2009). In astrocytes, A_1 adenosine receptor reduces ERK and AKT, thus provoking the inhibition of LPS-induced hypoxia-inducible factor (HIF)-1α activation with reduction of genes involved in inflammation and hypoxic injury (Gessi et al. 2013). In ear cochlea, it inhibits p38, ERK, and JNK activation and decreases cisplatin-induced signal transducer and activator of transcription (STAT-1) phosphorylation, thus reducing apoptosis and inflammation. This mechanism may be relevant to provide otoprotection against ototoxicity induced by this chemotherapeutic drug (Kaur et al. 2016). In cardiomyocytes, A_1 adenosine receptor phosphorylates p38, present downstream the mitochondrial K(ATP) channel, protecting cells from hypoxia injury (Leshem-Lev et al. 2010). Accordingly, in these cells it activates p38, ERK, and JNK phosphorylation, producing an increase of tissue transglutaminase (TG2) and cytoprotection (Vyas et al. 2016). An increase of p38 was also discovered in the reduction by A_1 adenosine receptor of beta-adrenergic-induced contractile function as a mechanism of adenoprotection (Fenton et al. 2010). In mouse coronary artery smooth muscle cells, it activates the PKC-alpha transduction pathway, causing ERK phosphorylation (Ansari et al. 2009). In foam cells, A_1 adenosine

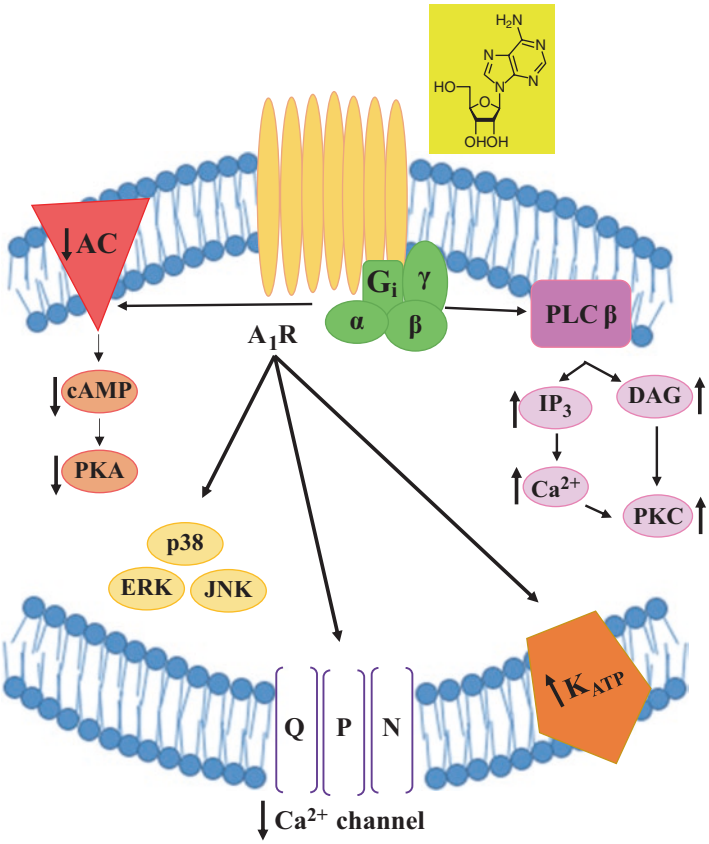

Fig. 3.1 Schematic picture of A_1 adenosine receptor signaling cascade. Adenosine activates A_1R to reduce AC activity and cAMP levels thus blocking PKA and CREB while stimulates PLC-β and Ca^{2+}. In addition adenosine triggers K^+ channels and inhibits Q, P, N, and Ca^{2+} channels. p38, ERK1/2, and JNK1/2 phosphorylation is determined by A_1R stimulation

receptor contributed to atherosclerosis by inducing HIF-1 accumulation through an increase of p38 and AKT phosphorylation (Gessi et al. 2010a). In contrast in neutrophils, it reduces p38 thus decreasing chemotaxis (Xu et al. 2017). Together, these data indicate that modulation of MAPK signaling, especially the one related to p38 phosphorylation, by A_1 adenosine receptor occurs in different organs and tissues thus affecting numerous pathological processes.

3.4.2 A_{2A} Adenosine Receptors

The Gs-coupled A_{2A} adenosine receptor stimulates AC activity, thereby increasing cAMP levels, with consequent PKA phosphorylation that causes activation of numerous proteins, including receptors, phosphodiesterases, cAMP-responsive

element-binding protein (CREB), and dopamine- and cAMP-regulated phosphoprotein (DARPP-32) (Preti et al. 2015). Interestingly, in hepatocyte membranes two different cAMP-responsive macrocomplexes activated by adenosine have been demonstrated that contain their own sequestered cAMP pools to generate their selective effects. One of these complexes responds to A_{2A} adenosine receptor that activates AC6, linked to A-kinase-anchoring proteins (AKAP)79/150, to produce cAMP available for AKAP79/150-tethered proteins, named protein kinase A (PKA) and phosphodiesterase 3A (PDE3A). The other complex responds to A_{2B} adenosine receptor, and the novel generated cAMP does not diffuse between these "signalosomes," thus suggesting that a spatiotemporal regulation of cAMP exists in the cell to obtain receptor-specific responses (Guinzberg et al. 2017). In addition, in the brain, A_{2A} adenosine receptor regulates a specific neuron type of Gs protein named Golf, which is also related to AC (Kull et al. 2000). In the rat tail artery, it promotes noradrenaline release through both PKC and PKA recruitment (Fresco et al. 2004). A_{2A} adenosine receptor may also bind, through its long C-terminus, to various accessory proteins including D_2 dopamine receptors, α-actinin, ADP ribosylation factor nucleotide site opener (ARNO), ubiquitin-specific protease (USP4), and translin-associated protein X (TRAX) (Baraldi et al. 2008). Importantly, A_{2A} adenosine receptor plays a role in the regulation of MAPK affecting the transduction pathway of several cells from different organs and tissues (Baraldi et al. 2008; Chen et al. 2013) (Fig. 3.2). In neutrophils, A_{2A} adenosine receptor by increasing cAMP decreases phosphorylation of p38, ERK, PI3K/AKT, Hck, and Syk, thus inducing inhibition of their functions (Giambelluca and Pouliot 2017). Accordingly, in the same cells, the agonist ATL313 was able to suppress selectin-mediated activation of Src kinases (SFKs) and p38, thus reducing cell adhesion (Yago et al. 2015). In contrast, an increase in ERK, nuclear factor (NF)-κB, and pSTAT was involved in the reduction of inflammatory cytokines produced by methotrexate through A_{2A} receptor activation in T cells (Ma et al. 2018). In dermal fibroblasts the A_{2A} receptor increases collagen (col) 1 and 3 production via cAMP, PKA, ERK, p38, and AKT pathways, confirming data obtained in hepatic stellate cells where collagen 1 production was influenced also by A_{2A} receptor-mediated ERK activity (Chan et al. 2006; Che et al. 2007; Shaikh and Cronstein 2016). It is known that also Wnt signaling is important in fibrosis where cAMP and Wnt pathways may converge. In this context it has been found that A_{2A} receptor increases synthesis of collagen 3 through the activation of β-catenin, suggesting a role for this subtype in dermal fibrosis and scarring (Shaikh and Cronstein 2016). In normal skin col1 is more expressed than col3 that increases in immature scars and is then replaced by col1 in mature scars, a process regulated by A_{2A} receptor and Epac2. At nanomolar levels of adenosine, the receptor via PKA induces col1 and reduces col3 production, respectively. At higher levels, the raised cAMP levels promote Epac2 signaling producing col3 (Perez-Aso et al. 2012, 2014). In mice adipose tissue, A_{2A} receptor stimulation induces an increase in p38 phosphorylation, thus resulting in improvements in glucose homeostasis and adipose tissue inflammation (DeOliveira et al. 2017). In the brain, following ischemia-reperfusion (IR) damage, a huge increase of adenosine stimulates A_{2A} receptor to potentiate neuronal injury by increasing ERK and consequently

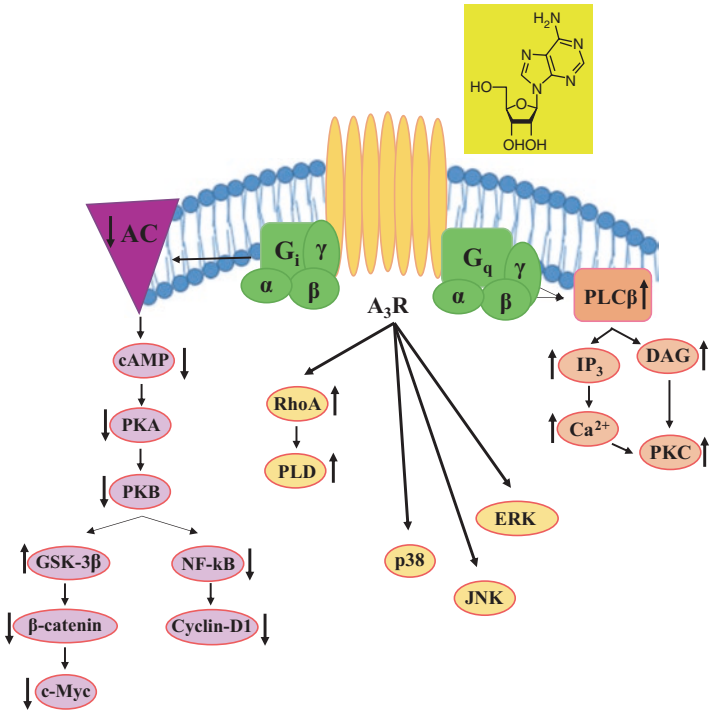

Fig. 3.2 Schematic picture of A_{2A} adenosine receptor signaling cascade. Stimulation of $A_{2A}R$ by adenosine rises AC activity and increases cAMP levels, PKA, and CREB phosphorylation. In addition its stimulation enrolls AKT, p38, ERK1/2, and JNK1/2

stimulating microglial activation, glial tumor necrosis factor-alpha (TNF-α) and BDNF, glutamate, inducible nitric oxide synthase (iNOS), as well as apoptosis (Mohamed et al. 2016). In an vitro model of osteoclast, differentiation occurs through activation of A_{2A} receptor activation of PKA and ERK1/2, thus inhibiting NF-κB nuclear translocation (Mediero et al. 2013). In cancer cells, A_{2A} receptor activation stimulates proliferation phospholipase C (PLC), protein kinase C-delta (PKC-δ), ERK, JNK, and AKT (Gessi et al. 2017). Accordingly, the same effect was reached by combination of TLR2 and adenosine receptor agonists, through ERK stimulation, in oral squamous carcinoma cells (Palani et al. 2018).

3.4.3 A_{2B} Adenosine Receptors

The Gs-coupled A_{2B} adenosine receptor activates AC, causing phosphorylation of PKA and recruitment of various effectors like guanine nucleotide exchange factor 2 (Epac), directly stimulated by cAMP. However it has been recently reported that a complex constituted by A_{2B} receptor stimulates AC5 bound to D-AKAP2 to generate cAMP, activating two other tethered proteins named Epac2 and PDE3B

(Guinzberg et al. 2017). Epac activation by A_{2B} receptor stimulation has been previously reported to affect cell proliferation in human umbilical vascular endothelial cells and to induce early gene expression decreasing cell proliferation in human coronary artery smooth muscle cells (Fang and Olah 2007; Mayer et al. 2011). In addition, by enrolling Gq proteins, it triggers PLC activation, thus determining Ca^{2+} increase, and through βγ subunits modulates ion channels. Furthermore, A_{2B} adenosine receptor presents numerous binding actors that influence its responses and effects such as netrin-1, E3KARP-ezrin-PKA, SNARE, NF-κB 1/P105, and α-actinin-1. Specifically, netrin-1 is a neuron protein hypoxia-dependent, which by binding to A_{2B} adenosine receptor reduces neutrophil migration and consequent inflammation (Rosenberger et al. 2009). SNARE protein is responsible for the translocation of the receptor from the cytoplasm to the cell membrane in the presence of an agonist through a mechanism involving (Wang et al. 2004) a structure composed by E3KARP (NHERF2) and ezrin which fixes A_{2B} adenosine receptor at cell surface (Sitaraman et al. 2002). In particular, A_{2A} and A_{2B} receptor dimerization is induced by α-actinin-1 promoting expression of the A_{2B} subtype on the cell surface (Moriyama and Sitkovsky 2010). In addition, it interacts with P105 then blocking NF-kB inflammatory effects (Sun et al. 2012). MAPK and AKT are target also for A_{2B} receptor in different cells thus regulating numerous pathophysiological functions (Sun and Huang 2016) (Fig. 3.3). In cardiac fibroblasts its stimulation reduced fibroblast proliferation and α-SMA expression generated by endothelin or angiotensin II, through a pathway dependent on cAMP, Epac, PI3K, and AKT signaling, thus contrasting cardiac fibrosis (Phosri et al. 2017, 2018). In bone A_{2B} subtype stimulation decreases ERK1/2, p38, and NF-κB induced by RANKL, thus contributing to the reduction of osteoclastogenesis (Kim et al. 2017). In human coronary artery smooth muscle cells, the A_{2B} adenosine receptor, cAMP, and PKA signaling decrease cell growth by inhibiting ERK1/2, AKT, and Skp2 stimulators of the cell cycle regulator cyclin D (Dubey et al. 2015). In the placenta A_{2B} receptor activation depresses trophoblast migration through MAPK signaling inhibition and lower proMMP-2 levels, suggesting a role for it in placenta formation and preeclampsia (Darashchonak et al. 2014). In glioblastoma cells prostatic acid phosphatase increases proliferation in a HIF-2α-dependent manner, requiring activation of A_{2B} receptors AKT and ERK pathways, suggesting this receptor subtype as a target for antiglioblastoma therapies (Liu et al. 2014). In microglia A_{2B} receptor increases IL-10 through p38 phosphorylation as well as IL-6 secretion and cell proliferation, through PLC, PKC-ε, PKC-δ, and p38 pathways, thus indicating their role in microglial activation and neuroinflammation (Koscso et al. 2012; Merighi et al. 2017). In enterochromaffin cells this subtype increases serotonin hypoxic synthesis and release through MAPK, CREB, and tryptophan hydroxylase-1 stimulation, a signaling having relevance in inflammatory bowel disease (Chin et al. 2012; Dammen et al. 2013). In HEK293 cells and in cardiomyocytes, A_{2B} receptor inhibited superoxide generation from mitochondrial complex I via Gi/o protein, ERK, PI3K, and NOS signaling having a role in ischemic preconditioning (Yang et al. 2011). In foam cells it accumulates HIF-1α through involvement of ERK, p38, and AKT and induces VEGF and IL-8 secretion, playing a role in atherosclerosis development (Gessi et al. 2010a).

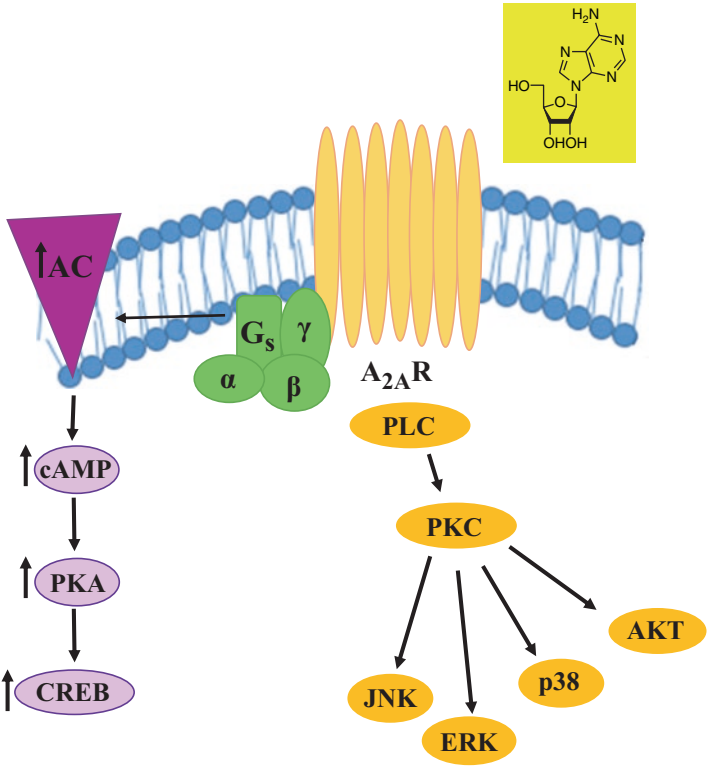

Fig. 3.3 Schematic picture of A_{2B} adenosine receptor signaling cascade. Activation of $A_{2B}R$ stimulates AC activity, increase of cAMP, and PKA phosphorylation. $A_{2B}R$ triggers PLC-β and increases Ca^{2+}. Other effectors activated by $A_{2B}R$ include p38, ERK1/2, and JNK1/2

3.4.4 A_3 Adenosine Receptors

The Gi-coupled A_3 adenosine receptor inhibits AC, thus reducing cAMP accumulation, while through Gq coupling stimulates PLC, thereby increasing Ca^{2+} release from intracellular stores in different cellular models (Gessi et al. 2008; Borea et al. 2015). Other signal transductors coupled to this receptor subtype include the monomeric G protein RhoA and phospholipase D as well as sarcolemmal K_{ATP} channels, to produce cardioprotection (Borea et al. 2015). In addition a role for PKC has been reported in both early and delayed preconditioning (Borea et al. 2016). Specifically, in cardiac mast cells, A_{2B}/A_3 receptor stimulation leads to activation of aldehyde dehydrogenase type 2, via PKC-ε, thus reducing renin release and the activation of renin-angiotensin system (Koda et al. 2010). Concerning delayed preconditioning, A_3 receptor activation exerts a protective role through PKC-δ (Zhao and Kukreja 2003). A pro-survival intracellular cascade involving ERK, PI3K, and AKT is enrolled by it to decrease caspase-3 activity and apoptosis (Hussain et al. 2014). Another relevant effect induced by A_3 receptor activation is neuroprotection through

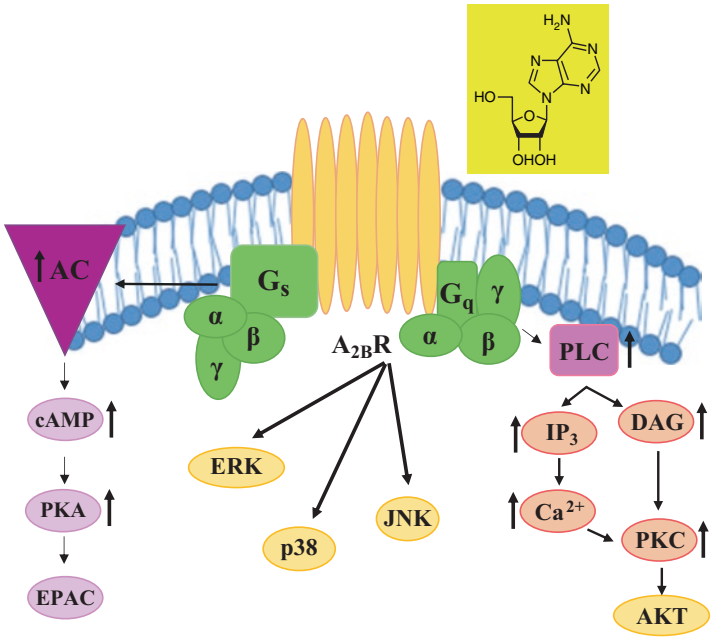

Fig. 3.4 Schematic picture of A_3 adenosine receptor signaling cascade. Interaction of adenosine with A_3R reduces AC activity and cAMP levels and stimulates GSK-3β with consequent reduction of β-catenin, cyclin D1, and c-Myc. Stimulation of A_3R activates PLC-β and Ca^{2+}, RhoA, and PLD. p38, ERK1/2, and JNK1/2 are phosphorylated through A_3R activation

PLC, PKC, or intracellular Ca^{2+} sequestration giving synaptic depression following oxygen-glucose deprivation as well as a reduction in AMPA receptors on hippocampal neurons (Dennis et al. 2011). It is well known that A_3 receptor intracellular transduction occurs through modulation of MAPKs in numerous cellular models (Merighi et al. 2010; Jacobson et al. 2018) (Fig. 3.4). This receptor induced ERK1/2 and cell proliferation in human fetal astrocytes, CHO cells expressing the human A_3AR (CHO-hA_3), microglia, colon carcinoma, glioblastoma, melanoma, and foam cells (Neary et al. 1998; Schulte and Fredholm 2000, 2002; Hammarberg et al. 2003; Merighi et al. 2006, 2007a, b, Gessi et al. 2010a, b; Soares et al. 2014). In contrast, a decrease of ERK activation has been reported in melanoma, prostate cancer, and glioma cells, reducing proliferation as well as decreasing TNF-α release in RAW 264.7 cells (Madi et al. 2003; Martin et al. 2006; Jajoo et al. 2009; Kim et al. 2012). A_3 receptor activation modulates also p38 in several cell types such as CHO-hA_3, human synoviocytes, melanoma, glioblastoma, and colon carcinoma (Merighi et al. 2005b, 2006, 2007b; Varani et al. 2010). In addition it regulates JNK, in microglia and glioblastoma cells, to increase cell migration and matrix metalloproteinase-9 (MMP-9) secretion, respectively (Gessi et al. 2010b; Ohsawa et al. 2012). Interestingly, A_3 receptor increases chemoresistance induced by multiple resistance-associated protein-1 (MRP1) transporter through a pathway involving PI3K/AKT and MEK/ERK1/2 (Torres et al. 2016; Uribe et al. 2017). Another

effect induced by this subtype through AKT phosphorylation was protection from apoptosis in RBL-2H3 and stimulation of MMP-9 in glioblastoma cells (Gao et al. 2001; Merighi et al. 2005a, 2007a; Gessi et al. 2010b). In melanoma the same pathway modulated by A_3 receptor decreased proliferation and increased pigmentation (Madi et al. 2013). Anti-inflammatory effects are produced by its modulation of PI3K/AKT and NF-κB transduction systems in BV2 microglial cells, monocytes, arthritis, and mesothelioma (Haskó et al. 1998; la Sala et al. 2005; Fishman et al. 2006; Lee et al. 2006, 2011; Madi et al. 2007; Varani et al. 2011). Instead reduction of AKT has been reported in murine astrocytes to decrease HIF-1α accumulation (Gessi et al. 2013). Accordingly, A_3 receptor inhibits angiogenesis in endothelial cells through PI3K/AKT/mammalian target of rapamycin (mTOR) signaling decrease (Kim et al. 2013). Finally, the inhibition of PKA mediated by A_3 receptor stimulation raised glycogen synthase kinase-3β (GSK-3β), inducing beta-catenin reduction; decrease of its transcriptional gene products, such as cyclin D1 and c-Myc; as well as reduction of NF-κB DNA-binding capacity in melanoma, hepatocellular carcinoma, and synoviocytes from RA patients and in adjuvant-induced arthritis rats (Fishman et al. 2002, 2004; Bar-Yehuda et al. 2008; Ochaion et al. 2008). Accordingly, following a reduction of A_3 receptor expression in colon cells after ulcerative colitis due to miR-206 activity, increased NF-κB, and related cytokines in the mouse colon, has been observed resulting in a proinflammatory event (Wu et al. 2016).

3.5 Conclusion

Adenosine receptors are important targets for drug development in several pathologies spanning from ischemic brain and heart injury, pain, neurodegenerative diseases, cancer, and inflammation, and for this reason there is a big interest in the development of novel selective and potent molecules targeting this system. This issue today may be better afforded, thanks to the improvement in the knowledge about the structure of receptor subtypes, which are the targets of new drugs. During the last 10 years, the crystallization approach has dramatically revealed the biological structure of GPCRs, and the A_{2A} receptor has been the pioneer in this process, followed by the A_1 subtype. In the next future, continued energy to reveal the structures of all four adenosine receptor subtypes in the three distinct activation states is fundamental to better improve the rational drug design process to develop novel molecules. From the extensive literature mentioned in this chapter, it is evident that adenosine modulates different intracellular signaling pathways involving MAPK and AKT to produce its pathophysiological effects. The regulation of these cascades is not univocal meaning that stimulation or inhibition of specific kinases may occur differentially depending on the receptor subtype involved and the cell system investigated. It is auspicable that future drugs coming from the adenosinergic field could exploit separated signaling pathway linked to a specific adenosine subtype, thus avoiding or limiting side effects.

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Chapter 4
A_1 Adenosine Receptor Agonists, Antagonists, and Allosteric Modulators

Zhan-Guo Gao, Dilip K. Tosh, Shanu Jain, Jinha Yu, Rama R. Suresh, and Kenneth A. Jacobson

Abstract One of the four G protein-coupled receptors for adenosine, the A_1 adenosine receptors (A_1AR), is widely distributed in the body and modulates numerous normal and pathological processes, through signaling pathways including those downstream from its coupled G_i protein. It is an attractive drug target for heart failure, arrhythmias, angina, asthma, stroke, seizure, pain, depression, and diabetes. In this chapter, we describe the A_1AR structure, function, signaling pathways, and therapeutic applications. We detail numerous structure-activity features of A_1AR agonists, antagonists, and allosteric modulators, introduced as pharmacological tools and molecules for clinical development.

Keywords A_1 adenosine receptors · A_1AR agonists and antagonists · A_1AR allosteric modulators · Structure-activity relationships · A_1AR structural characterization

4.1 Introduction

Adenosine exerts physiological actions by binding at the cell surface to four G protein-coupled receptors (GPCRs), namely, A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (ARs). The abundance, wide distribution, and signaling events of the A_1AR suggest a modulatory role in the body due to its effects in many organs and tissues. Among these effects are bradycardia, inhibition of neurotransmitter release, inhibition of lipolysis in adipocytes, inhibition of renal excretion, and induction of smooth muscle contraction. Due to the role of the A_1AR in the heart, agonists and partial

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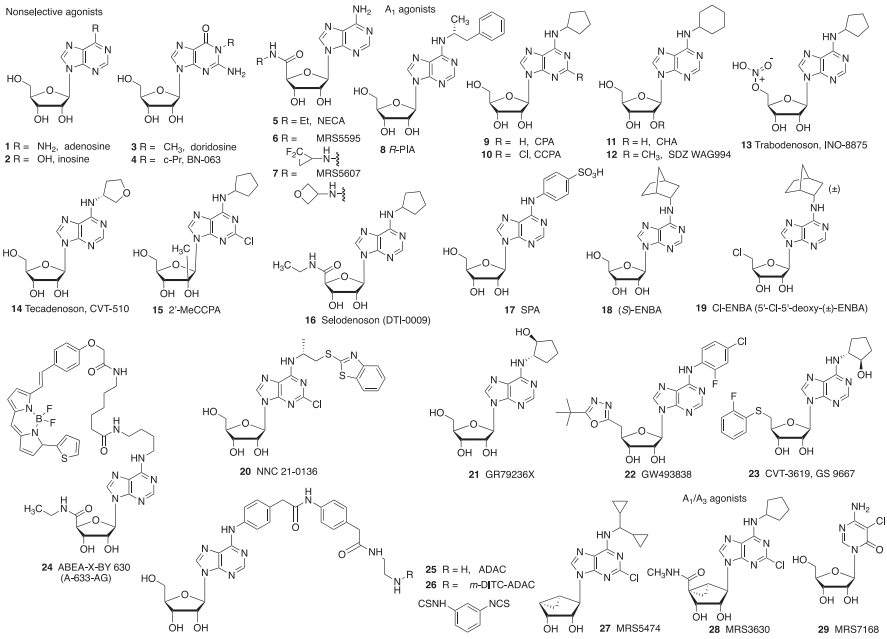

Fig. 4.1 Nucleoside A_1AR agonists

agonists have been pursued for the treatment of heart failure, arrhythmias, and angina (Elzein and Zablocki 2008; Kiesman et al. 2009; Gao and Jacobson 2007; Dinh et al. 2017; Varani et al. 2017). Given adenosine's role in cerebral protection, neurotransmitter release, and cell membrane potential, A_1AR agonists have been proposed for therapeutic use in the nervous system based on their antiseizure, antiischemic, and antidepressant activity (Fedele et al. 2006; Tosh et al. 2012b; Serchov et al. 2015). The role of A_1AR in lipolysis led to approaches for treating obesity and type 2 diabetes (T2D) (Antonioli et al. 2015). The abundance in tracheal smooth muscle cells and its role in mediating bronchial contraction (especially in asthmatics) led to the development of A_1AR antagonists for asthma. The abundance of the A_1AR in the kidney indicated a role for antagonists as potassium-sparing diuretics (Massie et al. 2010).

We summarize here the structure-activity relationships (SAR) of A_1AR agonists and partial agonists (Figs. 4.1 and 4.2), allosteric modulators (Fig. 4.3), and antagonists (Fig. 4.4), both as pharmacological tools and as potential therapeutic agents. Additional details on SAR are provided in earlier review papers (Fredholm et al. 2001; Kiesman et al. 2009; Moro et al. 2006; Romagnoli et al. 2015; Müller and Jacobson 2011; Schenone et al. 2010).

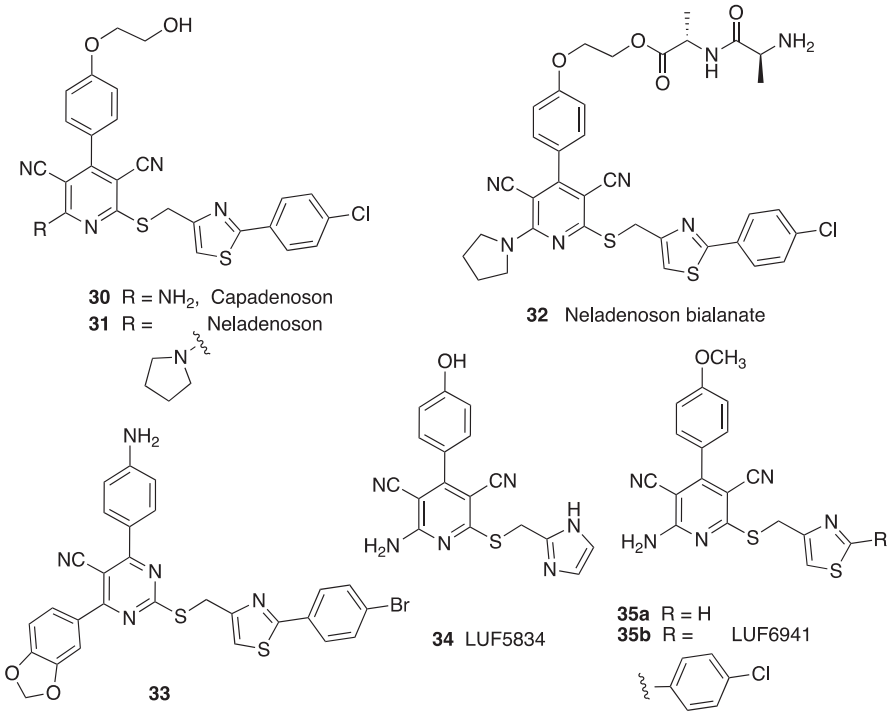

Fig. 4.2 Nonnucleoside A_1AR agonists and partial agonists

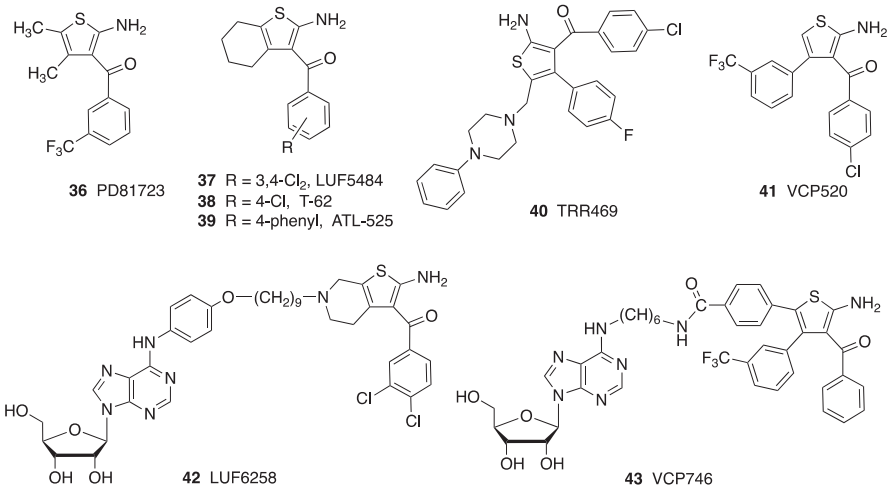

Fig. 4.3 Representative A_1AR allosteric enhancers

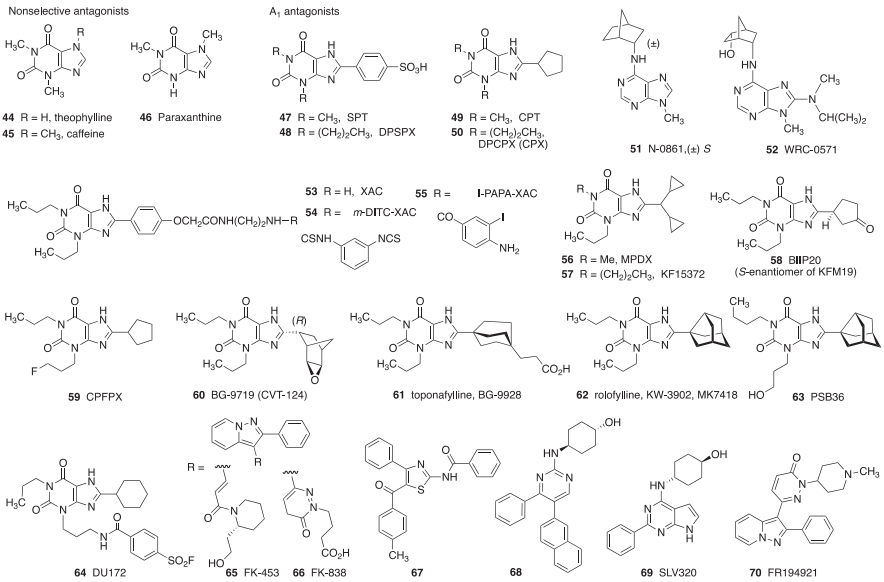

Fig. 4.4 A_1 AR antagonists

4.2 A_1AR Distribution, Endogenous Activation, and Signaling

The A_1AR is widely expressed in the brain and in peripheral tissues: the heart, liver, kidney, and eye and skeletal muscle, smooth muscle, and fat cells (Dixon et al. 1996; Reppert et al. 1991; Lynge and Hellsten 2000). The abundance of the A_1AR in the brain and other organs and tissues is well demonstrated with radioligand-receptor binding studies and imaging (Elmenhorst et al. 2012; Hayashi et al. 2017), in addition to RNA expression, Western blot, and functional characterization. The abundance and wide distribution of the A_1AR suggest its important physiological role in the body.

Adenosine is produced intracellularly from degradation of ATP and hydrolysis of S-adenosyl-homocysteine and extracellularly from ATP released under stress conditions. Since ATP degradation is the main source of local adenosine, a description of the role of adenosine and the A_1AR necessarily involves the role of ATP (Burnstock 2017) and its degradation product AMP, an immediate precursor of adenosine. 5′-Nucleotidase (CD73) is the principal enzyme responsible for local and rapid production of adenosine, and CD73 inhibitors are being considered for conditions in which blockade of ARs is desirable. Other nucleotidases, adenosine deaminases, adenosine kinases, and adenosine transporters also regulate fluctuations of the adenosine levels and indirectly influence the degree of A_1AR activation. Adenosine is considered a neuromodulator, rather than a neurotransmitter-like ATP, because there is no direct evidence that it is stored in granules in its original form and released

from nerve endings upon nerve impulse. Some of ATPs' roles are now demonstrated to be via its degradation product adenosine acting at the A_1AR (Goldman et al. 2010; Fujita et al. 2017; Yen et al. 2017).

The A_1AR couples with many signaling molecules in various tissues, mainly via the pertussis-sensitive $G_{i/o}$ class of G proteins. Historically, Sattin and Rall (1970) found that in addition to stimulatory biogenic amines, adenosine also modulated cAMP levels in cerebral cortex slices. The concentration-response curve for adenosine-induced cAMP production was bell-shaped and inhibited by theophylline, which also potentiated biogenic amine effects. The bell-shaped response was later shown to be presumably due to adenosine's effect on both A_1AR-coupled G_i and A_{2A}AR-coupled Gs proteins (Londos et al. 1980; van Calker et al. 1979).

Downstream mediators of A_1AR action include adenylate cyclases; phospholipase C; protein kinase C; K^+ channels, L-type Ca^{2+} channels and Ca^{2+} influx, Cl^- channels; and many kinases (Brundege and Dunwiddie 1997; Jacobson and Gao 2006; Fredholm et al. 2001), although other G proteins may have a minor role (Hill 2006). Of particular importance, the A_1AR-mediated regulation of the mitochondrial ATP-sensitive K^+ channel plays a crucial role in cerebral and cardiac preconditioning and many other important functions (Jacobson and Gao 2006). Biased signaling is not a widespread phenomenon for A_1AR orthosteric agonists (Langemeijer et al. 2013; Verzijl and IJzerman 2011; Baltos et al. 2016).

4.3 Nucleosides as A_1AR Agonists

Adenosine **1** is the principal endogenous agonist of the A_1AR (estimated K_i at human (h) A_1AR = 100 nM, Müller and Jacobson 2011), while its endogenous deamination product inosine **2** is a much weaker A_1AR agonist (K_i at rat (r) A_1AR = 17 μM, van Galen et al. 1994). An isolated report suggested that AMP itself is also an A_1AR agonist (Rittiner et al. 2012), although its rA_1AR binding affinity was reported as ~50 μM (van Galen et al. 1994).

Adenosine was first introduced as a therapeutic agent in the 1989 (Kiesman et al. 2009) and has been applied clinically for arrhythmias and in diagnostics. Under the name Adenocard, adenosine is used for the treatment of paroxysmal supraventricular tachycardia (SVT) through its A_1AR-mediated negative chronotropic effect. Current development of A_1AR agonists for the treatment of various conditions in the cardiovascular system is still, at least in part, related to the early discovery that adenosine has a significant impact on the cardiac function, including heart rhythm (Drury and Szent-Györgyi 1929).

Numerous A_1AR agonists with modifications at the ribose 5′ position and the adenine N^6 and C2 positions have been reported. In general, adenine displays more flexibility of substitution, consistent with a smaller and more polar pocket on the ARs accessed by the ribose moiety. Nevertheless, both AR affinity and efficacy may be modulated by structural modification of ribose, which is a required moiety for AR activation by nucleosides.

4.3.1 Nucleobase Substitutions

4.3.1.1 Purine 6 Position Substitutions

N^6-Modified *R*-PIA **8** was one of the first AR agonists to be considered in the 1970s for use as an antihypertensive agent, deriving from its A_{2A}AR activity, and it is moderately A_1AR-selective (Table 4.1). Most of the subsequently identified and still commonly used highly A_1AR-selective agonists, including CPA **9**, CCPA **10**, CHA **11**, and (2*S*)-ENBA (PD 126,280) **18**, are modified with N^6-cycloalkyl groups. A sulfonated A_1AR agonist, SPA **17**, was introduced as an N^6-phenyl derivative that would not cross the blood-brain barrier. The A_1AR selectivity of these compounds was initially defined only based on their affinity at the rat brain A_1AR vs. the A_{2A}AR (Daly et al. 1986; Trivedi et al. 1989). However, a comparison of their binding affinities at the four hAR subtypes indicated only limited A_1AR selectivity for CHA, *R*-PIA, and CPA compared to the hA_3AR of typically 10- to 30-fold (Klotz et al. 1998; Gao et al. 2003b; Alnouri et al. 2015). Nevertheless, *S*-ENBA shows exceptional selectivity, >1000-fold for the A_1AR vs. r and hA_3AR (Gao et al. 2003b). Franchetti et al. (2009) later synthesized a series of N^6, 5′-disubstituted adenosine analogues and found that 5′-chloro-5′-deoxy-(±)-ENBA (Cl-ENBA **19**), originally reported by Trivedi et al. (1989), had exceptionally high hA_1AR affinity and selectivity versus the other AR subtypes. Recently, hetero-bicycloalkyl substituents were found to provide high A_1AR selectivity (Knight et al. 2016).

Carlin et al. (2017) compared hypothermic activity induced by peripherally administered AR agonists at a central A_1AR vs. a peripheral A_3AR. CHA, CPA, and SPA all activated the peripheral mast cell A_3AR at similar or lower doses than needed for the central A_1AR. Thus, in vivo data with many N^6-substituted A_1AR agonists should be interpreted cautiously. Cl-ENBA was the only agonist that had a dose window of selective A_1AR action in this in vivo model. In mouse (m) AR binding assays, the mA_1AR selectivities (fold vs. mA_3AR) were CHA (284), CPA (2430), CCPA (59), and Cl-ENBA (12,100). However, caveats in this study are (1) most nucleoside AR agonists do not readily cross the blood-brain barrier (Schaddelee et al. 2005); and (2) the A_3AR agonist efficacy is not to be assumed to be full for all analogues. The maximal efficacy (E_{max}) at mA_3AR of Cl-ENBA was not determined, but various A_1AR agonists with bulky N^6 substitutions are of reduced efficacy or possibly antagonists at the hA_3AR (Gao et al. 2003b).

The N^6 position of adenosine was utilized as a relatively insensitive site for the design of A_1AR agonist functionalized congeners, containing an N^6-*p*-(carbonylmethyl)-phenyl linker, which promoted rA_1AR affinity (Jacobson et al. 1989). A terminal amino group in ADAC **25** (adenosine amine congener) served as an attachment point for bulky moieties, including biotin, which was used in conjunction with avidin complexation to estimate the depth of the rA_1AR binding site (Jacobson et al. 1985). *m*-DITC-ADAC **26** and its *p*-isomer were shown to be potent irreversible inhibitors of the rA_1AR. Similarly, an N^6-*p*-(aminomethyl)-phenyl

Table 4.1 Affinity of selected A_1AR agonists and partial agonists

Compound	pK_i value or % inhibition at 1 μM			Ref
	A_1AR	A_{2A}AR	A_3AR	
6	8.09 (h)	6.77 (h)	5.75 (h)	Tosh et al. (2012a)
7	7.92 (h)	6.07 (h)	6.52 (h)	Tosh et al. (2012a)
8, *R*-PIA	8.92 (r)	6.66 (r)	6.80 (r)	Müller and Jacobson (2011)
9, CPA	8.64 (h)	6.10 (h)	7.37 (h)	Carlin et al. (2017)
	9.66 (m)	6.09 (m)	6.27 (m)	Carlin et al. (2017)
	9.23 (r)	6.33 (r)	6.62 (r)	van Galen et al. (1994)
10, CCPA	9.08 (h)	5.64 (h)	7.37 (h)	Carlin et al. (2017)
	9.57 (m)	6.01 (m)	7.80 (m)	Carlin et al. (2017)
	8.89 (r)	6.02 (r)	6.63 (r)	Müller and Jacobson (2011)
11, CHA	8.62 (h)	5.86 (h)	7.14 (h)	Carlin et al. (2017)
	8.67 (m)	5.77 (m)	6.21 (m)	Carlin et al. (2017)
12, SDZ WAG 994	7.64 (p)	4.64 (p)	ND	Müller and Jacobson (2011)
	7.12 (r)	5.24 (r)	ND	Jacobson and Knutsen (2001)
13, INO-8875	5.79 (h)	5.89 (h)	8.63 (h)	Müller and Jacobson (2011)
14, Tecadenoson	8.19 (p)	5.64 (h)	ND	Müller and Jacobson (2011)
15, 2′Me-CCPA	8.48 (h)	5.02 (h)	5.94 (h)	Cappellacci et al. (2005)
17, SPA	8.10 (h)	5.42 (h)	6.61 (h)	Carlin et al. (2017)
	8.97 (m)	<5 (m)	5.51 (m)	Carlin et al. (2017)
18, *S*-ENBA	9.47 (r)	6.32 (r)	6.04 (h)	Gao et al. (2003b)
19, Cl-ENBA	9.29 (h)	5.87 (h)	5.89 (h)	Carlin et al. (2017)
	9.70 (m)	5.40 (m)	5.62 (m)	Carlin et al. (2017)
	9.38 (r)	5.68 (r)	ND	Trivedi et al. (1989)
21, GR79236X	8.52 (r)	5.89 (h)	ND	Müller and Jacobson (2011)
23, GS 9667	7.26 (h)	<5 (h)	>6 (h)	Müller and Jacobson (2011)
25, ADAC	9.07 (r)	6.68 (r)	6.55 (r)	Jacobson and Knutsen (2001)
27, MRS5474	7.30 (h)	5.40 (h)	6.33 (h)	Carlin et al. (2017)
	8.49 (m)	<5 (m)	5.98 (m)	Carlin et al. (2017)
28, MRS3630	7.74 (h)	5.49 (h)	8.43 (h)	Jacobson et al. (2005)
29, MRS7168	6.44 (h)	<5 (h)	6.25 (h)	Rodriguez et al. (2016)
30, Capadenoson	8.85 (h)	0%	1%	Louvel et al. (2015)
33	8.28 (h)	ND	ND	Louvel et al. (2014)
34, LUF5834	8.59 (h)	~7.6 (h)	~6.6 (h)	Lane et al. (2010)
35a	8.89 (h)	1%	50%	Louvel et al. (2015)
35b, LUF6941	8.30 (h)	6%	19%	Louvel et al. (2015)

h human, *r* rat, *p* pig, *m* mouse

linker was used in new chemically reactive, agonist affinity labels (Jörg et al. 2016). ADAC and its related antagonist functionalized congener XAC **53** (xanthine amine congener, Fig. 4.4) were conjugated with peptides, reactive groups (**54**), and reporter groups for fluorescent, spin label, NMR, and radioactive (e.g., **55**) detection that retained high A_1AR affinity (Jacobson et al. 1987). **24** is a fluorescent ligand derived from **5** for A_1AR characterization (Middleton et al. 2007).

The N^6-substituted agonist [^{3}H]CHA was the first high-affinity A_1AR agonist radioligand and contributed to the definitive study showing that theophylline **44** and caffeine **45** (Fig. 4.4) are AR antagonists (Bruns et al. 1980). Several other agonist radioligands displayed high A_1AR affinity: [^{3}H]*R*-PIA, [^{3}H]CCPA, [^{3}H]ADAC, and [^{3}H]NECA (Jacobson and Gao 2006; Jacobson et al. 1987). Various N^6-substituted A_1AR agonists, e.g., **12–14** and **20–23**, have been developed as clinical candidates (Kiesman et al. 2009).

4.3.1.2 Purine C2 and C8 Position Substitutions

C8-Alkylamino groups were identified as a means of reducing the E_{max} at the rA_1AR (van der Wenden et al. 1998), although affinity was also diminished. A 2-chloro substitution is present in many selective A_1AR agonists, such as **10** and **15**. Adding steric bulk at the C2 position often reduces A_1AR but maintains A_{2A}AR affinity. However, there are example A_1AR-selective adenosine derivatives containing bulky C2 substitution, such as 2-pyrazolyl (Elzein et al. 2007).

4.3.1.3 Nucleosides with Deazaadenines and Alternative Nucleobases

1-Deazaadenosine derivatives often retain high affinity at the A_1AR and are compatible with N^6 modifications that enhance affinity (Vittori et al. 2000). However, simple 3-deazaadenosines only weakly interacted with A_1AR, and 7-deaza- and 1,3-dideazaadenosine derivatives were inactive.

Marine natural product doridosine (1-methylisoguanosine, **3**), an analogue of inosine, was previously found to be an AR agonist (Kim et al. 1981), without binding affinity reported, although its functional effect resembled A_1AR agonism. Isoguanosine was recently confirmed as a fairly potent hA_1AR agonist in radioligand binding (K_i = 0.47 μM, nonselective) and functional studies (Rodríguez et al. 2016). Other isoguanosine derivatives (1-cyclopropyl **4** and 1-allyl) were found to be more A_1AR selective than doridosine (Tao et al. 1993). Isoguanosine derivatives with high A_1AR affinity and excellent agonist selectivity vs. A_{2A}AR were reported (Rodríguez et al. 2016; Nair and Fasbender, 1991), but their selectivity needs to be further evaluated at the A_{2B} and A_3ARs.

Anticancer drug 5-azacytidine (structure not shown) was identified as a novel A_1AR partial agonist (K_i at hA_1AR 2.0 μM) through in silico screening to discover new nucleoside AR agonists (Rodríguez et al. 2016). By this approach, **29** was synthesized and demonstrated to be a mixed full agonist at hA_1AR (K_i ~0.3 μM) and hA_3AR.

4.3.2 Ribose Group Modifications

4.3.2.1 5′ Position

5′-Carboxamide derivative NECA **5** is a potent nonselective AR agonist and has been used in many studies of the A_1AR especially as a standard for A_1AR agonist efficacy comparison (Middleton et al. 2007; Gao et al. 2003b). The 5′-ethyl amide analogue **5** has been the focus of many studies due to its potent full agonism, but a later comparison of small 5′-alkyluronamides of adenosine found the 5′-cyclopropyl amide to be at least as potent at the hA_1AR and hA_{2A}AR (Tosh et al. 2012a). A difluorocyclopropyl amide **6** and an oxetane 2-amide **7** were identified as potent and A_1AR-selective (partial) agonists with E_{max} values of 81% and 57%, respectively. The combination of 5′-*N*-alkyl carboxamide and N^6-cycloalkyl groups preserved A_1AR selectivity, e.g., in **16**.

A 5′-chloro-5′-deoxyadenosine substitution is present in some selective A_1AR agonists, e.g., **19**. 5′-Deoxyadenosine derivatives containing 5′-alkylthio- and alkylseleno-ether groups and 5′-alkylamines displayed reduced the E_{max} at the rA_1AR, which, along with C8 substitutions, provided a general means to produce partial agonists (Roelen et al. 1996; van der Wenden et al. 1998). 5′-Arylthio ethers were later identified to be partial agonists, leading to a clinical candidate for cardiac arrhythmias, GS9667 **23** (Zablocki et al. 2004). LUF5589 (2-chloro-5′-deoxyadenosine-5′-ethyloxy-N^6-(3-iodobenzyl)adenosine, structure not shown) appeared to be a G_i protein-biased A_1AR agonist (Langemeijer et al. 2013).

Agonists for PET (positron emission tomography), 5-*N*-(2-[^{18}F]fluoroethyl)-carboxamidoadenosine (not A_1AR selective) and 5-(methyl[^{75}Se]seleno)-N^6-cyclopentyladenosine have been reported (structures not shown, Lehel et al. 2000; Blum et al. 2004). However, these agonists might be more suitable for imaging ARs peripherally than in the brain.

Aromatic or phenylthio groups have been appended to the 5′-CH_2 group of adenosine with retention of high A_1AR affinity, e.g., **22** and **23**. Petrelli et al. (2017) found that a 5′-C-ethyl-tetrazolyl group combined with various N^6 substituents could provide either a potent mixed A_1AR/A_3AR agonist or mixed A_1AR agonist/A_3AR antagonist.

4.3.2.2 Ribose 2′ and 3′ Hydroxyl Group Modifications

Signaling through the ARs and other purine GPCRs can be considered a vestige of the RNA world, and consequently, 2′-deoxynucleosides often display weak affinity (van Galen et al. 1994). Although the 2′ and 3′ hydroxyl groups are generally required for high AR affinity, there are some notable exceptions, e.g., 2′-methyl ether **12** of CHA, SDZ WAG994, a clinical candidate for T2D in the 1990s, and 2′-deoxy partial A_1AR agonists (Jacobson and Knutsen 2001; Vittori et al. 2000). Deletion of a 2′ or 3′ hydroxyl group is known to generally reduce the A_1AR E_{max} (Zablocki et al. 2004). Deletion of both 2′ and 3′ hydroxyl groups produces weak A_1AR antagonists (Lohse et al. 1988).

4.3.2.3 Carbocyclic Pseudoribose Substitutions

Carbocyclic nucleosides that selectively activate A_1AR, such as ARA (structure not shown), are known (Kiesman et al. 2009). The (N)-methanocarba substitution of ribose (with a [3.1.0]bicyclohexyl ring system) reduces affinity consistently at the A_{2A}AR compared to ribose and has been extensively developed for A_3AR agonists (Jacobson et al. 2000). Nevertheless, it is also relatively well tolerated in A_1AR agonists, as indicated by mixed A_1AR/A_3AR full agonist **28**.

Tosh et al. (2012b) reported 5′-truncated MRS5474 **27**, which is a methanocarba rather than a ribose-based A_1AR agonist with moderate A_1AR selectivity. MRS5474 was the only member of this homologous series to display that profile. The A_1AR maximal efficacy (E_{max}) of MRS5474 across species and under various conditions remains to be explored, although agonist affinity at various species has been compared (Carlin et al. 2017). Unlike other A_1AR agonists, MRS5474 was well tolerated in mouse seizure models and did not cause rotarod "toxicity" at doses that had antiseizure activity (Tosh et al. 2012b).

4.3.3 Nonnucleoside Agonists and Partial Agonists

The class of 6-amino-3,5-dicyano-4-phenyl-2-thiopyridines was discovered at Bayer Pharmaceuticals to be the first nonnucleoside class of AR agonists (Rosentreter et al. 2004). However, unlike nucleoside agonists, there is a wide range of AR subtype selectivities and E_{max} for members of the same 3,5-dicyanopyridine family (Fig. 4.2, **30**–**32**, **34**, **35**). 3,5-Dicyanopyridines and related 4-amino-6-aryl-5-cyano-2-thiopyrimidines (e.g., **33**, E_{max} hA_1AR 51%) have been identified as hA_1AR full agonists, partial agonists, and antagonists (Beukers et al. 2004; Chang et al. 2005; Louvel et al. 2015). Some of these non-adenosine agonists remarkably showed a maximum A_1AR agonist effect. Compound **33** and **35b** displayed long hA_1AR residence times of 59 and 132 min, respectively (Louvel et al. 2015). A radioligand in this chemical series, [^{3}H]LUF5834 **34**, bound specifically to the A_1AR in both the G protein-coupled and uncoupled states (Lane et al. 2010). Considering the need for partial A_1AR agonists with good physical properties for a variety of conditions, such as angina and diabetes, especially those locations in which there is an A_1AR reserve, this pyridine class of compounds may represent a unique approach (Albrecht-Küpper et al. 2012).

Capadenoson (BAY68–4986, **30**), neladenoson **31**, and its prodrug neladenoson bialanate (BAY 1067197, **32**) were potent and selective partial A_1AR agonists. However, capadenoson was recently found to also potently activate the hA_{2B}AR (Baltos et al. 2017), although the affinities at A_1AR and A_{2B}AR were not strictly compared. Capadenoson **30** was less efficacious in calcium mobilization in comparison with other signaling pathways (Baltos et al. 2016).

The effects of the phytochemical paeoniflorin (structure not shown) to reduce pain and increase sleep have been interpreted in the context of observed A_1AR activation (Tang et al. 2003; Yin et al. 2016; Andoh et al. 2017). Paeoniflorin-induced functional effects disappeared in A_1AR knockout mice (Chen et al. 2016; Yin et al. 2016), but A_1AR antagonists, e.g., **49** and **50**, effectively blocked in some (Liu et al. 2005), but not all studies (Liu et al. 2006). Paeoniflorin is suggested to activate the A_1AR by potentiation, possibly at an allosteric site, which is different from the known orthosteric A_1AR agonist CPA **9** or the allosteric agonists PD81723 **36** and T62 **38**. Consistent with allostery, paeoniflorin inhibited A_1AR agonist, but not antagonist radioligand binding (Tang et al. 2003; Liu et al. 2005, 2006). However, the allosteric action of paeoniflorin remains to be carefully and extensively characterized.

4.4 Allosteric Modulators of the A_1AR

Amiloride analogues act as A_1AR negative allosteric modulators (NAMs) to increase the dissociation rate of antagonist [^{3}H]DPCPX without affecting the dissociation rate of an agonist radioligand (Garritsen et al. 1990; Gao et al. 2003a).

A class of 2-amino-3-benzoylthiophenes, represented initially by PD81723 **36**, was identified as modest enhancers of agonist radioligand binding and function at the rA_1AR (Bruns and Fergus 1990). These were among the first positive allosteric modulators (PAMs) identified for any GPCR. PD81723 inhibits antagonist binding but increases agonist binding, in addition to displaying its own agonist activity (Gao et al. 2005).

The SAR of A_1AR PAMs has been extensively explored (van der Klein et al. 1999; Baraldi et al. 2007; Romagnoli et al. 2015), resulting in enhancers that display more potent allosteric effects, e.g., representative PAMs **37**–**41** (Baraldi et al. 2007; Obata et al. 2004; Pan et al. 2001). T62 **38** was tested clinically for pain, and three 2-amino-3-benzoylthiophenes, i.e. PD81723, T62 and optimized PAM TRR469 **40,** showed unique binding and activation patterns with both direct agonist activity and allosteric enhancing activity and decreased the agonist, but not antagonist dissociation rate (Bruns and Fergus 1990). They increased both agonist binding and function, i.e., inhibited forskolin-stimulated cAMP accumulation directly and stimulated GTPγS binding with a lower E_{max} compared to full A_1AR agonists (Bruns and Fergus 1990; van der Klein et al. 1999; Childers et al. 2005; Vincenzi et al. 2014). PD81723 and T62 also enhanced the effect of A_1AR agonists CPA or *R*-PIA in a cAMP accumulation assay and a GTPγS binding assay.

Bitopic conjugates of A_1AR PAMs and nucleoside agonists, i.e., **42** and **43**, were intended to bridge the two respective sites on the A_1AR (Narlawar et al. 2010; Baltos et al. 2016). VCP746 **43** displayed biased A_1AR signaling, although more pathways need to be tested to draw a clear conclusion.

4.5 A_1AR Antagonists

4.5.1 Xanthine Antagonists

Methylxanthines (1,3,7-trimethyl, i.e., caffeine **45** and 3,7-dimethyl, i.e., theobromine) are abundant in the human diet (Fredholm 2011). Methylxanthines, particularly theophylline (1,3-dimethyl) **44**, has long been used for the inhibition of phosphodiesterases (Butcher and Sutherland 1962), but their major relevant actions at concentrations achieved following ingestion are related to AR antagonism (Huang et al. 1972; Fredholm et al. 2001). Theobromine was shown to suppress adipogenesis via A_1AR inhibition (Mitani et al. 2017). In a rat brain Alzheimer's disease model, the A_1AR expression is decreased, and theobromine restored A_1AR expression and improved cognitive functions (Mendiola-Precoma et al. 2017). The major metabolite of caffeine in man, i.e., the central stimulant paraxanthine (1,7-dimethylxanthine **46**), is approximately equipotent to caffeine at the A_1AR (Table 4.2, van Galen et al. 1994).

8-Aryl and later 8-cycloalkyl-xanthines were found to display considerably enhanced A_1AR affinity. Also, the extension of 1,3-dimethyl groups of theophylline

Table 4.2 Affinity of selected A_1AR antagonists

Compound	pK_i value or % inhibition at 1 μM			Ref
	A_1AR	A_{2A}AR	A_3AR	
44, Theophylline[a]	5.07 (r)	4.60 (r)	<4 (r)	van Galen et al. (1994)
	5.17 (h)	5.77 (h)	4.65 (h)	Müller and Jacobson (2011)
45, Caffeine[a]	4.54 (r)	4.32 (r)	<4 (r)	van Galen et al. (1994)
	4.97 (h)	4.63 (h)	4.88 (h)	Müller and Jacobson (2011)
46, Paraxanthine	4.52 (r)	4.71 (r)	<4 (r)	van Galen et al. (1994)
48, DPSPX	6.85 (r)	6.10 (r)	<4 (r)	van Galen et al. (1994)
49, CPT	7.96 (r)	5.85 (r)	<4 (r)	van Galen et al. (1994)
50, DPCPX[a]	8.52 (h)	6.89 (h)	6.10 (h)	Carlin et al. (2017)
	8.82 (m)	6.22 (m)	<<5 (m)	Carlin et al. (2017)
	9.34 (r)	6.47 (r)	<5 (r)	van Galen et al. (1994)
52, WRC-0571[a]	8.48 (h)	6.80 (h)	5.19 s (h)	Robeva et al. (1996)
53, XAC	8.92 (r)	7.20 (r)	<4 (r)	van Galen et al. (1994)
60, BG-9719	9.35 (h)	5.96 (h)	5.32 (h)	Müller and Jacobson (2011)
61, BG-9928[a]	8.13 (h)	5.19 (h)	<5 (h)	Hocher (2010)
62, KW-3902	9.14 (h)	6.79 (h)	5.36 (h)	Müller and Jacobson (2011)
63, PSB36[a]	9.15 (h)	6.01 (h)	5.64 (h)	Müller and Jacobson (2011)
65, FK-453	7.74 (h)	5.89 (h)	<5 (h)	Müller and Jacobson (2011)
66, FK-838[b]	6.92 (r)	5.23 (r)	ND	Kuroda et al. (2000)
67	7.24 (h)	5.20 (h)	5.67 (h)	Müller and Jacobson (2011)
68	8.11 (h)	5.86 (h)	9%	Alachouzos et al. (2017)
69, SLV320	9.00 (h)	6.40 (h)	6.70 (h)	Müller and Jacobson (2011)

h human, *r* rat, *m* mouse

[a]Binding pK_i at hA_{2B}AR: 5.04 (**44**), 4.47 (**45**), 6.73 (**63**) (Müller and Jacobson 2011); 7.40 (**50**), 5.19 (**52**) (Robeva et al. 1996); 7.05 (**61**) (Hocher 2010)

[b]Binding, pIC_{50}

to 1,3-dipropyl had a ~40-fold affinity-enhancing effect. Although it suffered from relatively low affinity and high nonspecific binding, the early xanthine A_1AR radioligand [^{3}H]DPX (1,3-dipropyl-8-phenylxanthine, structure not shown) helped demonstrate definitively that the effect of naturally occurring methylxanthines was through a specific receptor site (Bruns et al. 1980).

In order to enhance the physical properties and A_1AR affinity of the 8-phenyl xanthines, a functionalized congener approach was applied, leading to a terminal amino derivative XAC **53** (Jacobson et al. 1986). The *p*-phenyl position in XAC, derivatized as a carboxymethoxyl ether, assured high A_1AR affinity and allowed the conjugation to a wide variety of carrier moieties with the retention of affinity. Thus, this freedom of substitution established that the 8 position of xanthines was directed toward the solvent-exposed opening of AR binding site, much like the N^6 position of agonists, as confirmed in later AR X-ray structures (Jespers et al. 2018; Cheng et al. 2017). Low aqueous solubility is characteristic of many xanthine A_1AR-selective antagonists. In addition to appending distal polar groups at insensitive sites on the pharmacophore, phosphate ester-containing xanthine prodrugs have been introduced for in vivo applications (Weyler et al. 2006).

[^{3}H]XAC was introduced as an antagonist radioligand with high affinity (~1 nM) and moderate selectivity at the rA_1AR (Jacobson et al. 1986). Subsequently, it was found that the hA_{2A}AR affinity of XAC was enhanced compared to the rA_{2A}AR, and therefore it was nonselective in human. This allowed [^{3}H]XAC to be used in human platelet membranes as the first hA_{2A}AR antagonist radioligand (Ukena et al. 1986).

Among 8-cycloalkylxanthine homologues, the A_1AR-selective 8-cyclopentyl analogue DPCPX **50** notably displayed ~1 nM affinity and was more water soluble than DPX (Shamim et al. 1988). [^{3}H]DPCPX later became a standard antagonist radioligand (Bruns et al. 1987). From this original finding of highly potent and selective 8-cycloalkylxanthines, A_1AR antagonist clinical candidates were introduced: as cognitive enhancers KF15372 **57** and BIIP20 **58** and for kidney treatment KW-3902 **62** (Suzuki et al. 1992), BG-9719 (naxifylline) **60,** and BG-9928 **61** (Gottlieb et al. 2002, 2011; Zablocki et al. 2004; Kiesman et al. 2006a, b). All were 1,3-dipropyl derivatives, but later a tuning of these substituents achieved higher A_1AR selectivity with PSB36 **63**, which was cocrystallized with the A_1AR (Cheng et al. 2017). Irreversibly binding A_1AR antagonists include isothiocyanate **54** and sulfonyl fluoride **64** that was cocrystallized with the A_1AR (Stiles and Jacobson 1988; Glukhova et al. 2017).

Many antagonist PET ligands have been introduced for quantitative imaging of the human brain A_1AR as a means of establishing the receptor occupancy by CNS-active A_1AR drugs (Bauer and Ishiwata 2009; Paul et al. 2011). These include xanthines [^{11}C]MPDX **56** (Hayashi et al. 2017), [^{11}C]KF15372 **57** (Ishiwata et al. 2007), [^{18}F]CPFPX **59** (Elmenhorst et al. 2017), and the [^{11}C] form of nonxanthine antagonist FR194921 **70** that displays antianxiety activity (Matsuya et al. 2005; Maemoto et al. 2004). [^{18}F]CPFPX showed good A_1AR affinity and selectivity in vitro and high specific binding in vivo. Its A_1AR binding in human brain was displaced by up to half after caffeine ingestion but was elevated after sleep deprivation (Elmenhorst et al. 2017).

4.5.2 Antagonists with Xanthine-Related Scaffolds

By varying the substitution on the purine scaffold, various xanthine-related antagonists of the A_1AR have been reported. 9-Deaza- and 7-deazaxanthines were reported by Grahner et al. (1994) as potent AR antagonists with increased A_1AR selectivity observed for 9-deaza analogues (pyrrolo[3,2-*d*]pyrimidine-2,4-diones). By analogy to potent xanthines, Weyler et al. (2006) introduced various alternatively cyclized pyrimido[1,2,3-*cd*]purine-8,10-diones as A_1AR antagonists that display enhanced aqueous solubility. Recently, potent and A_1AR-selective hypoxanthine derivatives based on **50** were described (Koul et al. 2017), with a wide range of amino, aryl, and ether substitutions at the C2 position.

4.5.3 Adenine Antagonists

Adenine derivatives were explored as antagonists, particularly containing a 9-methyl group (Thompson et al. 1991). Adenine derivative **52** is more A_1AR selective compared to **51** with respect to A_{2B}AR. A series of 7-deazaadenines (1*H*-pyrrolo[2,3-*d*] pyrimidin-4-amines) included A_1AR-selective SLV320 **69** (Kalk et al. 2007; Kiesman et al. 2009). Other 7-deazaadenine derivatives, including ADPEP (structure not shown), containing a chiral phenylethyl group at N^9, are selective A_1AR antagonists (Müller 1997). Fusion of the pyrrole ring with an additional ring, i.e., benzene formed pyrimido[4,5-*b*]indoles that were also antagonists with similar affinity (Müller 1997). In addition to replacement of adenine nitrogens with CH, substitution was also fruitful in the search for A_1AR antagonists, e.g., substituted 8-azadenines, such as *N*-(9-benzyl-2-phenyl-8-azapurin-6-yl)-amides (Müller 1997; Giorgi et al. 2009). Pyrazolo[3,4-d]pyrimidines, i.e., 7-deaza-8-azapurines, and triazolo[1,5-a]quinoxalines, which are more distantly related to adenine, have also been reported to be A_1AR antagonists (Müller 1997).

4.5.4 Nonpurine Scaffolds as A_1AR Antagonists

4.5.4.1 Diverse Di- and Tricyclic Scaffolds

Early screening of chemical libraries identified various novel heterocycles, e.g., pyrazolopyridines (including antianxiety drugs etazolate and tracazolate), triazolopyridazines, thiazolopyrimidines, benzimidazoles, imidazopyrimidines, pteridines, cytochalasins, carbolines, flavins, amfonelic acid, doxepin, and other heterocycles as weak rA_1AR ligands (Daly et al. 1988; Siddiqi et al. 1996). Tetrahydrobenzothiophenones displayed selectivity as rA_1AR antagonists

(van Rhee et al. 1996). A 2-phenylpyrazolo[1,5-*a*]pyridine scaffold has provided a series of selective A_1AR antagonists including **65**, **66,** and **70** (Müller 1997).

The virtual screening of diverse chemical libraries, using computational approaches using recently revealed receptor structures, has continued the process of discovering novel A_1AR antagonist (Kolb et al. 2012). Virtual screening for A_{2A}AR ligands based on its X-ray structure often results in the discovery of new chemotypes for other ARs, including the A_1AR (Katritch et al. 2010). For example, a 7-amino-5-phenylimidazo-[1,5-b]pyridazine derivative, MRS5942, (structure not shown) displayed a K_i of 63 nM at the hA_1AR (Rodríguez et al. 2015).

Many naturally occurring and synthetic flavonoids are A_1AR antagonists, even though mostly they lack nitrogen atoms (Jacobson et al. 2002; Alexander 2006). Swertisin, a flavone from *Swertia japonica*, has been shown to improve cognitive dysfunction in a mouse model via the inhibition of the A_1AR (Lee et al. 2016). Methoxy flavonoids from *Orthosiphon stamineus* were shown to be A_1AR antagonists and have diuretic activity (Yuliana et al. 2009). The phytochemicals oxogalanthine lactam, hematoxylin, and arborinine are rA_1AR antagonists with K_i values of 3–13 μM. α-Naphthoflavone displayed higher rA_1AR affinity (0.79 μM) than the β-isomer (Ji et al. 1996).

Gütschow et al. (2012) reported 2-(acyl)amino-4*H*-3,1-benzothiazin-4-ones and related thienothiazinone derivatives, which are unrelated to xanthines, as novel bicyclic antagonists of the A_1AR and other AR subtypes.

4.5.4.2 Monocyclic Scaffolds

Various monocyclic derivatives have been reported as selective A_1AR antagonists, including pyrazole derivatives (Müller 1997). 2,4,6-Trisubstituted pyrimidines were identified as a new class of selective A_1AR antagonists, which were conceptually derived from the xanthine scaffold (Chang et al. 2004, 2005; van Veldhoven et al. 2008). Compound **68**, an *N*-substituted 2-amino-4,5-diarylpyrimidines, was a potent, selective A_1AR antagonist (Alachouzos et al. 2017). 2-Amino-5-benzoyl-4-phenylthiazoles were reported as selective A_1AR antagonists (Scheiff et al. 2010).

4.6 A_1AR Structural Characterization

The canine, rat, human, and bovine A_1ARs were cloned in 1991 and 1992 (van Galen et al. 1992; Mahan et al. 1991; Reppert et al. 1991; Libert et al. 1992; Olah et al. 1992). Extensive molecular modeling and mutagenesis work has been done before the AR crystal structures were revealed (Barrington et al. 1989; Olah et al. 1994). Prior to its X-ray structural determination, ligand recognition at the A_1AR was modeled computationally based on its homology to rhodopsin and later to the A_{2A}AR structure (Tosh et al. 2012b).

4.6.1 X-Ray Structural Determination

The crystal structure of an A_1AR complex with a covalent xanthine antagonist DU172 **64** was revealed in 2017 (Glukhova et al. 2017). Although the structure is overall similar to the previously crystalized A_{2A}AR structure, a distinct conformation was found for the second extracellular loop (EL2). A wider extracellular cavity, or "vestibule," was proposed to be a possible allosteric binding pocket.

Several other A_1AR antagonist complexes with PSB36 **63** and a range of simple xanthines were subsequently reported by Heptares (Cheng et al. 2017). These structures of the mutation-stabilized A_1AR revealed that the same scaffold or ligand is able to assume different poses in the binding site.

4.6.2 Computer Modeling

Pharmacophore models for the interaction of the N^6 groups of adenosine analogues with the A_1AR were proposed (van Galen et al. 1989; Daly et al. 1986), with much emphasis on the importance of cycloalkyl substituents. An A_1AR antagonist pharmacophore model was also proposed (van Galen et al. 1990).

The first receptor-based ligand A_1AR binding model was based on the low-resolution cryo-EM structure of bacteriorhodopsin, but it proposed features that were later validated by homology to X-ray A_{2A}AR structures: a vertical orientation of the agonist and antagonist ligands, a similar position of N^6-phenyl (agonist) and C8-phenyl (antagonist) groups pointing toward the binding site opening (IJzerman et al. 1992). More recent modeling of the A_1AR has been mainly based on homology to the crystal structures of the A_{2A}R (Tosh et al. 2012b; Kennedy et al. 2014; Nguyen et al. 2016a).

The N^6-dicyclopropylmethyl group of MRS5474 **27** is also present on the 8 position of A_1AR selective antagonist KF15372 **57**, further illustrating the parallel in SAR between the xanthines and adenosines in A_1AR recognition. The two cyclopropyl groups of MRS5474, when docked in an A_1AR model, were found to fit two small subpockets previously proposed for the N^6 group in a pharmacophore model of *R*-PIA **8** (Daly et al. 1986).

4.6.3 Mutagenesis

Extensive site-directed mutagenesis (SDM) studies of the A_1AR have been performed (Olah et al. 1992; Rivkees et al. 1999; Glukhova et al. 2017), and SDM is critical for guiding and interpreting molecular modeling of GPCRs. Both His278 and His251 are important for ligand binding, with H278, which is conserved in all

ARs, being more crucial. Thr277 was suggested to mediate agonist, but not antagonist binding (Townsend-Nicholson and Schofield 1994). By using chimeric A_1/A_{2A}ARs, Rivkees et al. (1995) emphasized the importance of transmembrane helices (TMs) 1–4 for the specificity of ligand interactions with the A_1AR.

Rivkees et al. (1999) proposed that Thr91 and Gln92 in TM3 interact with the adenine moiety, and Leu88 and Pro86 are important for A_1AR selectivity. Xie et al. (2006) suggested that Leu65 and Ile69 in TM2 are important for the recognition by the ribose moiety. Both ELs 2 and 3, as well as TM3, have been proposed to be involved in the binding and function of allosteric enhancers (Peeters et al. 2012; Kennedy et al. 2014; Nguyen et al. 2016b).

Sodium ions, but not lithium or potassium, selectively regulate the binding of A_1AR agonists, but not antagonists (Goodman et al. 1982). The conserved Na^+-binding residue Asp55 in TM2 was found responsible for this allosteric modulation, while Ser94 is critical for ligand binding (Barbhaiya et al. 1996).

4.7 Therapeutic Application of A_1AR Modulators

4.7.1 Arrhythmias

Many pathophysiological conditions including hypoxia and ischemia may cause cardiac arrhythmias. Adenosine is considered as an endogenous anti-arrhythmic agent partly due to its endogenous anti-ischemic property (Szentmiklósi et al. 2011, 2015; Kiesman et al. 2009). As described above, adenosine (or its precursor ATP) has long been used for the treatment SVT, originally based on the pioneering work of Berne and Belardinelli (Kiesman et al. 2009; Szentmiklósi et al. 2015; Pelleg et al. 2012). The anti-arrhythmic action of adenosine is via A_1AR activation in the sinoatrial and atrioventricular (AV) nodes to induce negative chronotropic and dromotropic effects.

Despite its demonstrated anti-arrhythmic effect, adenosine is known to cause atrial fibrillation in about 15% of patients by decreasing the atrial refractory period and to cause other adverse effects related to activating other AR subtypes (Glatter et al. 1999). Thus, efforts have been made to develop selective A_1AR agonists as anti-arrhythmic agents and for treating atrial fibrillation (Mason and DiMarco 2009). A_1AR full agonists trabodenoson (INO-8875, PJ-875) **13**, tecadenoson **14** (Corino et al. 2014), and selodenoson **16** had been under development for these indications (Mason and DiMarco 2009; Kiesman et al. 2009). However, full agonists are known to cause tachyphylaxis presumably due to A_1AR desensitization. Tecadenoson was in advanced clinical trials for termination of SVT and treatment of atrial fibrillation (ClinicalTrials.gov Identifier: NCT00713401) (Elzein and Zablocki 2008; Mason and DiMarco 2009), which were discontinued.

4.7.2 A_1AR Agonists for Angina

Anti-ischemic effects of A_1AR agonists have been demonstrated in animal studies (Borea et al. 2016). An adenosine uptake inhibitor, dipyridamole, which is used in stress testing, has been assessed in patients as an antianginal agent (Picano and Michelassi 1997). The nonnucleoside capadenoson **30** was evaluated in patients with stable angina (Kiesman et al. 2009; Tendera et al. 2012), but later withdrawn (ClinicalTrials.gov Identifier: NCT00518921).

4.7.3 A_1AR Antagonists and Partial Agonists for Heart Failure

Worsening kidney function is common in heart failure patients. Theophylline is known to have a diuretic effect via A_1AR inhibition (Osswald and Schnermann 2011). A_1AR antagonists, such as nonxanthine derivative **66**, increased sodium excretion in kidneys, enhanced diuretic responsiveness, and maintained the glomerular filtration rate (Schnackenberg et al. 2003; Massie et al. 2010). BG-9928 **61** increased sodium excretion without worsening renal function (Gottlieb et al. 2011). Xanthine A_1AR antagonists BG-9719 **60**, BG-9928 **61**, and KW-3902 **62** have been in clinical trials for patients with congestive heart failure (Massie et al. 2010; Hocher 2010; ClinicalTrials.gov Identifiers: NCT00709865, NCT00328692 and NCT00354458). However, the trial of **62** was discontinued due to the lack of a clear therapeutic effect and the presence of side effects, especially seizures, a known side effect of A_1AR antagonists (Massie et al. 2010).

Rather than antagonists, an A_1AR partial agonist Neladenoson **31** is now being tested as its prodrug BAY 1067197 **32** in patients with chronic heart failure (Dinh et al. 2017; Voors et al. 2017; Meibom et al. 2017; Greene et al. 2016). Compared with capadenoson (Baltos et al. 2017), neladenoson is a more selective partial agonist for A_1AR. It has been shown that neladenoson improves cardiac function without producing bradycardia, atrioventricular blocks, or undesirable effect on blood pressure (Voors et al. 2017; Meibom et al. 2017). The rationale for using partial A_1AR agonists is based on the observation that the activation of myocardial A_1ARs by partial agonists protects cardiac function related to ischemia and reperfusion injury without producing severe side effects (Voors et al. 2017; Albrecht-Küpper et al. 2012). As of Dec. 2017, a multiple dose study of **32** in heart failure (PARSiFAL) is ongoing (ClinicalTrials.gov Identifier: NCT02040233).

4.7.4 Asthma

It has long been known that adenosine induces tracheal contraction in asthmatic patients, and methylxanthines, such as theophylline, are effective for asthma treatment (Gao and Jacobson 2017). Although theophylline is a PDE inhibitor, it is more potent as a nonselective A_1AR antagonist. Also, it has been shown that theophylline

more potently inhibits adenosine-induced than histamine-induced bronchoconstriction, which suggests that AR antagonism is involved. As the A_1AR is involved in bronchoconstriction (Hua et al. 2007; McNamara et al. 2004; Ponnoth et al. 2010; Gao and Jacobson 2017), selective A_1AR antagonists can be considered for the treatment of asthma. Indeed, the A_1AR antagonist PBF-680 (structure not disclosed) is now in clinical testing for mild-to-moderate asthma (ClinicalTrials.gov Identifier: NCT02635945). The efficacy of a single oral dose of PBF-680 was evaluated for alleviating AMP-induced airway hyperresponsiveness in mild-to-moderate asthma (ClinicalTrials.gov Identifier: NCT01939587).

4.7.5 A_1AR Agonists for Type 2 Diabetes (T2D)

Dysregulation of insulin secretion by pancreatic β cells and enhanced insulin resistance in metabolically active organs (adipose tissue, liver, skeletal muscle) are manifestations of T2D (Tuomi et al. 2014). Adenosine acts on widely expressed ARs and regulates the functioning of these metabolically active organs (Antonioli et al. 2015; Peleli and Carlstrom 2017). Adipocytes express high levels of A_1AR, and its activation inhibits lipid breakdown into free fatty acids (Dhalla et al. 2007, 2009; Fredholm et al. 2011). Thus, A_1AR agonists, GR79236X **21** and GS-9667 **23** and ARA and RPR-749 (structures not shown), have been clinical candidates for T2D due to their ability to increase insulin sensitivity (Kiesman et al. 2009). However, development of full agonists, such as GR79236X and ARA, was not successful due to cardiovascular side effects (Elzein and Zablocki 2008). Although both full and partial agonists may lower nonesterified fatty acid levels, it is suggested that partial agonists may improve insulin sensitivity without producing severe cardiovascular side effects (Elzein and Zablocki 2008). The A_1AR partial agonist GS-9667 has been reported to lower free fatty acids in both healthy and obese subjects without showing evidence of A_1AR desensitization (Staehr et al. 2013).

However, few recent studies indicate toward improvement of metabolism due to abrogation of A_1AR signaling. Genetic ablation of A_1AR in mice leads to enhanced insulin secretion from pancreatic β cells (Johansson et al. 2007; Yang et al. 2015). In liver, A_1AR activation was shown to promote ethanol-induced lipogenesis and development of liver steatosis (Peng et al. 2009). A study by Yang et al. showed that A_1AR deletion reduced oxidative stress and inflammatory responses and hence prevents development of metabolic disorder associated with aging and obesity (Yang et al. 2015). Chronic treatment of Zucker obese mice with A_1AR antagonist BW-1433 (structure not shown) led to improved glucose tolerance (Xu et al. 1998).

4.7.6 A_1AR Agonists for Epilepsy and Other CNS Disorders

As a neuromodulator, one of the important functions of adenosine is to coordinate some neurotransmitter functions in the brain. The A_1AR is involved in many physiological and pathophysiological roles of adenosine in the brain, including sleep,

seizure, stroke, and convulsion. Thus, A_1AR has been suggested to be involved in epilepsy and several other neurological disorders. Both agonists and allosteric enhancers have been proposed for those conditions. Dipyridamole has been in clinical testing for treating anxiety disorder that was unsuccessful (Stein et al. 1993), possibly due to the indirect and nonselective activation of other AR subtypes, to peripheral A_1AR activation leading to severe side effects, or to limited CNS bioavailability. More recently, Vincenzi et al. (2016) suggested that an A_1AR PAM, such as TRR469 **40**, is an attractive approach for the treatment of anxiety-related disorders. A_1AR activation was demonstrated to have an anticonvulsant action (Amorim et al. 2016; Klaft et al. 2016). Amorim et al. (2016) showed that A_1AR activation reduced hippocampal excitability, while A_1AR antagonists increased it. A_1AR activation has a protective role during asphyxia in the fetal sheep (Hunter et al. 2003).

About 30% of epilepsy patients do not respond satisfactorily with current seizure control therapies. A_1AR activation inhibited seizures in over 70% of the temporal cortex slices from treatment-resistant epilepsy patients (Klaft et al. 2016), supporting A_1AR as an attractive antiepileptic drug target. Wagner et al. (2010) found a correlation of posttraumatic seizures with genetic variability in the A_1AR. Adenosine has sedative and anticonvulsive properties, possibly by inhibiting the release of several neurotransmitters (Boison 2007).

Other CNS conditions that have suggested benefit from A_1AR agonists are depression (Serchov et al. 2015) and Huntington's disease (Ferrante et al. 2014) and from antagonists are dementia (Paul et al. 2011; Mendiola-Precoma et al. 2017) and anxiety (Maemoto et al. 2004). However, there was no correlation between A_1AR gene polymorphisms and methamphetamine dependence and psychosis (Kobayashi et al. 2011). Prolonged A_1AR activation might promote neurodegeneration (Stockwell et al. 2017).

4.7.7 A_1AR Agonists and Allosteric Modulators for Pain Treatment

The antinociceptive effects of activating A_1AR and A_{2A}AR was identified several decades ago, and more recently A_3AR (Sawynok 2016). Chronic pain patients dosed with dipyridamole reported subjective benefit (Merskey and Hamilton 1989). The A_1AR was identified as a main target of antinociception, although other ARs may also play a role. There are peripheral, spinal, and supraspinal mechanisms implicated in the antinociceptive actions of the A_1AR (Sawynok 2016). In the spinal cord, Luongo et al. (2012) found microglial cells to be involved, using highly A_1AR-selective Cl-ENBA **19**. Analgesia is lost in mice with the A_1AR genetically knocked out. Adenosine and ATP have been administered intravenously in patients for perioperative and chronic pain. Selective A_1AR agonists, including SDZ WAG

994 **12** and GW493838 **22,** have been developed (Elzein and Zablocki 2008; Jacobson and Gao 2006). However, systemically administered A_1AR agonists have been limited by cardiovascular side effects. Thus, more recently, it has been proposed that selective partial A_1AR agonists and event- and site-specific allosteric modulators may be better options (Kiesman et al. 2009; Sawynok 2016). A_1AR PAM T62 **38** was in clinical trials for neuropathic pain treatment, following its ability to reduce hypersensitivity in neuropathic pain models (Li et al. 2004, 2003; ClinicalTrials.gov Identifiers: NCT005066102, NCT00506610). However, it was due to the lack of efficacy (Giorgi and Nieri 2013). A more potent allosteric modulator, TRR469, was also shown to produce antinociception in neuropathic pain models (Vincenzi et al. 2014).

It has been suggested that chronic exposure to A_1AR agonist CPA **9** can induce type II hyperalgesic priming, similar to μ-opioid agonists. However, its prolongation is dependent on the Gαi subunit, rather than the β,γ subunit involved in the opioid effect (Araldi et al. 2016).

Acupuncture is commonly used to relieve pain for over 2000 years in Asia. However, the molecular basis of its pain-relieving effect is still not fully understood. Goldman et al. (2010) found that adenosine was released during acupuncture in mice, and its antinociceptive action is A_1AR dependent. Fujita et al. (2017) found that acupuncture-induced analgesia via the A_1AR was antagonized by the AR antagonist caffeine. Adenosine, acting via the A_1AR, has also been suggested to play a role in electroacupuncture-alleviated pain in mice (Yen et al. 2017; Liao et al. 2017).

4.7.8 A_1AR Agonists for Glaucoma

A Phase 3 clinical trial of INO-8875 **13** (PJ-875; ClinicalTrials.gov Identifier: NCT02565173) for glaucoma (Myers et al. 2013) ended after failing to reach the primary end point.

4.8 Conclusions

A vast range of agonists, partial agonists, antagonists, and allosteric modulators of the A_1AR have derived from purines and nonpurines and are studied in disease models. Selective agonists, antagonists, and an allosteric enhancer have been used in patients or in clinical trials for arrhythmias, angina, heart failure, pain, and diabetes, although most of these trials were discontinued. Antagonists are in clinical trials for the potential treatment of asthmatic patients and diagnostic use. In conclusion, given the large body of research in this area suggesting benefit, there is reason to consider the clinical development of newer A_1AR modulators.

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Chapter 5
A_{2A} Adenosine Receptor: Structures, Modeling, and Medicinal Chemistry

Stefania Baraldi, Pier Giovanni Baraldi, Paola Oliva, Kiran S. Toti, Antonella Ciancetta, and Kenneth A. Jacobson

Abstract Many selective agonists and antagonists of the A_{2A} adenosine receptor (AR) have been reported, while allosteric modulators specific for this receptor are still needed. Many heterocyclic chemotypes have been discovered as A_{2A}AR antagonists, while most of the known AR agonists are nucleosides or 3,5-dicyanopyridine derivatives. A few A_{2A}AR ligands have been in clinical trials as antihypertensives, anti-inflammatory or diagnostic compounds (agonists), and as drugs for treating Parkinson's disease and cancer (antagonists). The A_{2A}AR has become one of the most widely investigated G protein-coupled receptor (GPCR) structures using X-ray crystallography and also biophysical techniques such as NMR. Thus, the design of agonists, antagonists, and allosteric modulators has become structure-based, with numerous examples of in silico approaches, including virtual ligand screening (VLS), leading to the discovery of both novel agonists and antagonists.

Keywords A_{2A} adenosine receptors · A_{2A} agonists · A_{2A} antagonists · A_{2A} allosteric modulators · X-ray structures

5.1 A_{2A}AR Structures and Their Use in Ligand Design

The medicinal chemistry and clinical advances associated with small-molecule modulators of the A_{2A}AR as a drug discovery target have been recently reviewed (Baraldi et al. 2008; Shook and Jackson 2011; Müller and Jacobson 2011; Armentero et al. 2011; Chen et al. 2013; de Lera et al. 2014; Pinna 2014; Yuan and Jones 2014). The design of A_{2A}AR ligands is now based on many available high-resolution X-ray structures, as described in the next section (Fig. 5.1). The A_{2A}AR has become one of

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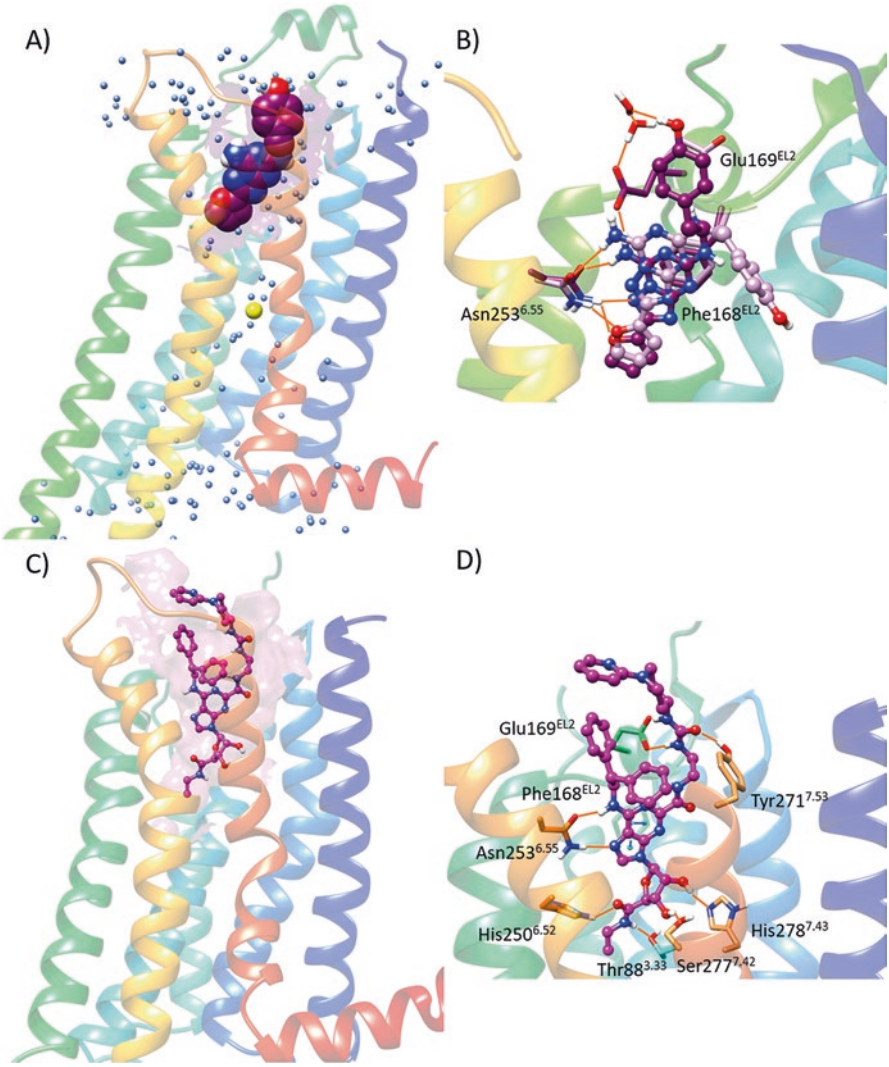


Fig. 5.1 (**a**) Overlay of the $hA_{2A}AR$ X-ray structure in complex with the antagonist ZM241385 (**84**): the ligand is in space-filling representation with magenta carbon atoms, co-crystallized water molecules and the sodium ion are depicted as cyan and yellow spheres, respectively. (**b**) Comparison between the **84**-$hA_{2A}AR$ interactions established in two different X-ray complexes: dark magenta carbon atoms (PDB ID: 4EIY), pink carbon atoms (PDB ID: 3PWH). (**c**) Overlay of the $hA_{2A}AR$ X-ray structure in complex with the agonist UK-432097 (**28**). (**d**) Detail of the **28**-$hA_{2A}AR$ interactions established in the X-ray complex (PDB ID: 3QAK). TMs are color coded sequentially from blue (TM1) to orange (TM7)

the most commonly studied G protein-coupled receptors (GPCRs) in structural probing using biophysical techniques, which will be discussed first.

Most of the A_{2A}AR agonists, partial agonists and allosteric modulators (Figs. 5.2 and 5.4), and antagonists (Figs. 5.5 and 5.15), described later in Sects. 5.2 and 5.3, were discovered by empirical probing of the structure activity relationships (SARs). However, the current trend is to use rational approaches based on the 3-dimensional knowledge of the receptor to guide modification of known ligands and to discover novel chemotypes by virtual ligand screening (VLS) approaches (Katritch et al. 2010; Carlsson et al. 2010). Thus, the design of A_{2A}AR agonists and antagonists has become structure-based. Allosteric modulators specific for this receptor are still needed.

5.1.1 *A_{2A}AR X-Ray Structures Determined*

More than 33 X-ray crystallographic structures have been determined for the human (h) A_{2A}AR in complex with >16 different antagonists and agonists (Fig. 5.1) in the inactive, active-intermediate, and fully active states (Carpenter and Lebon 2017; Jespers et al. 2018). With new methods for high-throughput GPCR X-ray crystallography, that number promises to rise rapidly in coming years (Rucktooa et al. 2018).

Prior to the A_{2A}AR X-ray structures, many 3D homology models were proposed and supported with site-directed mutagenesis (SDM) and SAR analysis. Some of the ligand interactions in both agonist-bound and antagonist-bound A_{2A}AR complexes were shown to be consistent with previous predictions based on modeling and mutagenesis (Kim et al. 1995; Jespers et al. 2018). The availability of the same receptor captured in different conformational states bound to structurally and functionally diverse ligands prompted the application of structure-based drug design (SBDD) strategies to discover new AR binders. In addition to X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy using transverse relaxation optimized spectroscopy (TROSY) correlation has also been used to characterize A_{2A}AR complexes (Eddy et al. 2018). NMR signals assigned to individual Trp and Gly residues throughout the receptor can be followed in response to ligand-induced conformational changes. The A_{2A}AR activation pathway that passes through a conserved Na^+ binding site, i.e., D52 in transmembrane helical domain (TM)2, was followed through conformational changes of specific amino acid residues.

5.1.2 *Use of X-Ray Structures for Ligand Design*

A library of 4 million compounds was screened in silico for novel AR antagonists using the X-ray structure of the inactive antagonist ZM241385 **84**-hA_{2A}AR complex (PDB ID: 3EML, Figs. 5.1a, b) (Katritch et al. 2010). Three highly structured

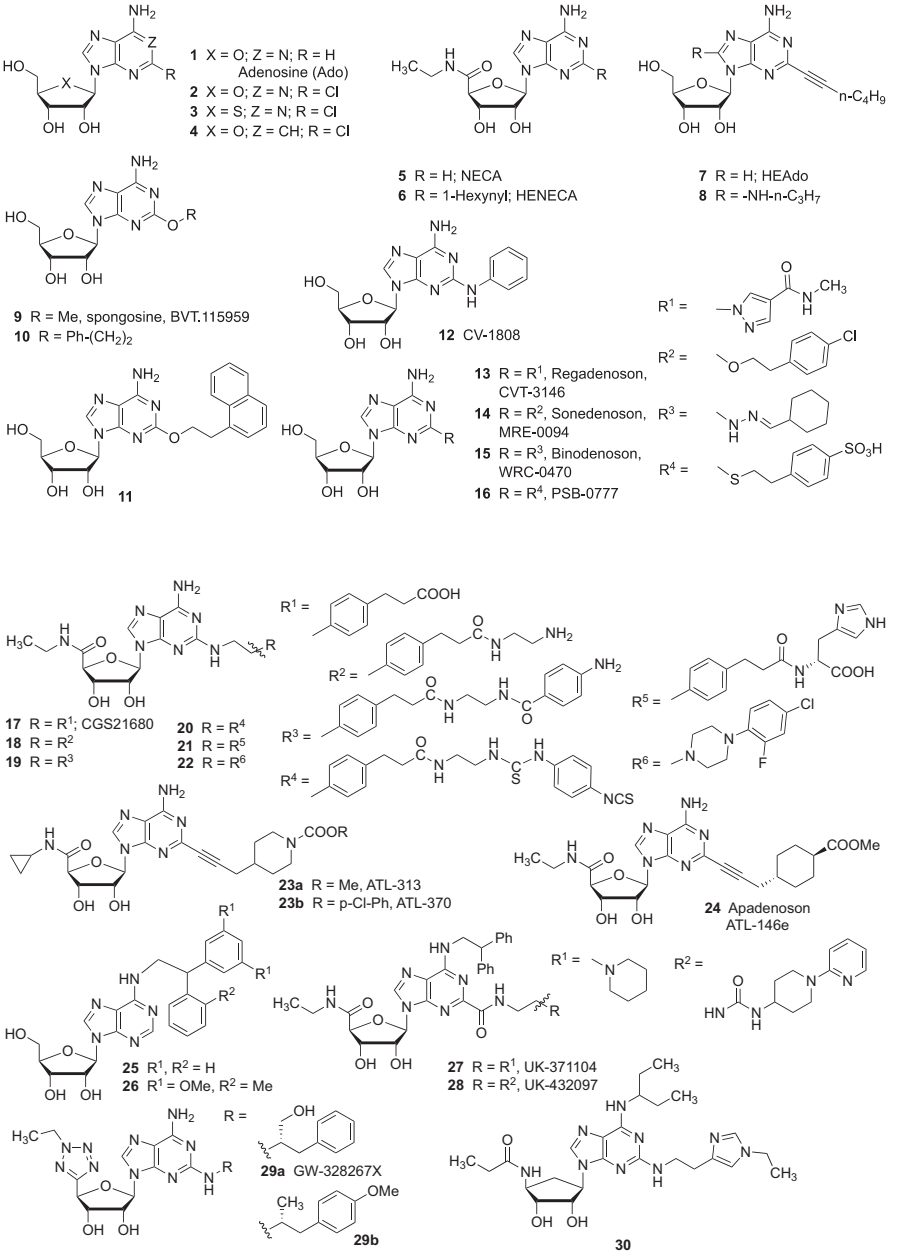


Fig. 5.2 (**a**) Ribose-modified adenosine analogs as $A_{2A}AR$ agonists, (**b**) Combined sugar- and base-modified nucleoside analogs as $A_{2A}AR$ agonists

water molecules establishing an extended network of H-bonds with residues in the orthosteric binding site observed in the X-ray complex were retained during the VLS procedure. The campaign led to the identification of 11 hits with sub-micromolar affinity belonging to nine different novel chemotypes (41% success rate).

The ribose moiety was predicted to be bound in a small subpocket of the $hA_{2A}AR$ involving TMs 3 and 7 (Jacobson et al. 2005), which was later confirmed in the first crystallographic structure of an agonist-bound $hA_{2A}AR$ (Xu et al. 2011). This ribose-binding region was the subject of a VLS study in which a focused library of 2000 adenosine-linked fragments was screened against the active-intermediate UK-432097 **28**-$hA_{2A}AR$ X-ray structure (PDB ID: 3QAK, Figs. 5.1c, d) to search for novel adenosine 5′-carboxamide derivatives as receptor agonists (Tosh et al. 2012). The hydrophilic 5′ region of the $A_{2A}AR$ binding site is sterically limited, and subtle structural changes of an agonist were expected to have large effects on affinity, selectivity, and efficacy. Thus, the library was compiled by linking small amino fragments (MW <150) extracted from commercial building block databases to 5′-carboxy-adenosine via an amide bond. The campaign led to the identification of 15 functional agonists with $A_{2A}R$ K_i values of 10 nM to 1 μM. In both cases, subtype selectivity was not explicitly addressed, and some of the identified hits turned out to be mixed A_{2A} and A_1AR ligands.

The use of the active-intermediate structures of agonist-$A_{2A}AR$ complexes for VLS tended to identify only novel antagonists (Rodríguez et al. 2015b). However, alternative SBDD strategies to discover atypical AR agonists have been recently proposed by separating the roles of the adenine and the ribose ring in AR activation and by screening for their bioisosteric replacements. The ribose is responsible for AR activation, while adenine substitution tends to direct the molecule to a particular AR subtype. Alternatives to the adenine nucleobase in AR agonists were selected in silico by filtering the ZINC library nucleobase-sized heterocycles (MW < 200) containing a nitrogen atom amenable for chemical condensation to the ribose ring through a β-glycosidic bond (Rodríguez et al. 2016). The resulting focused library of 7000 synthetically feasible ribosides was screened at the active-intermediate $hA_{2A}AR$ X-ray structure (PDB ID: 2YDO) and at A_1AR and A_3AR homology models. During the VLS procedure, the ribose ring was constrained in the conformation observed in the X-ray complex, and a co-crystallized water molecule bridging the adenine core to the ribose moiety of the agonist was retained. Thirteen nucleobase hits were able to be synthesized, and screening for binding activity at A_1AR, $A_{2A}AR$ and A_3AR identified nine compounds with significant activity at one or more AR (69% success rate). The receptor environment around the nucleobase in adenine antagonists was probed through molecular dynamics free energy calculations, leading to novel $A_{2A}AR$-binding fragments (Matricon et al. 2017).

Recently, 7-prolinol-substituted thiazolo[5,4-*d*]pyrimidines were reported as novel non-nucleosides $A_{2A}AR$ partial agonists (Bharate et al. 2016). Docking studies, performed using both active-intermediate and inactive $hA_{2A}AR$ X-ray structures (PDB IDs: 2YDO and 4EIY, respectively), suggested the introduction of 2-hydroxymethyl-pyrrolidine moiety as a ribose bioisostere at the C7-position of

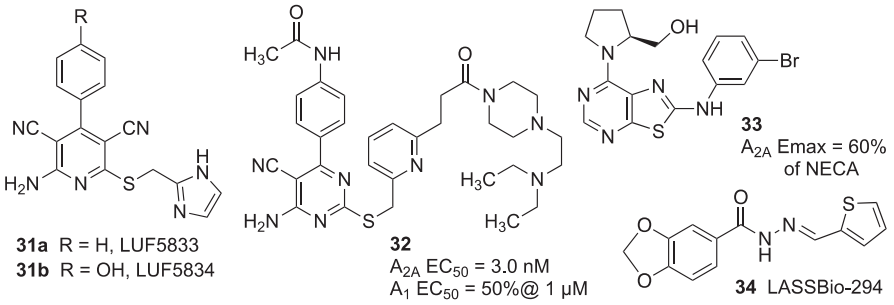


Fig. 5.3 Non-nucleoside A_{2A}AR agonists and partial agonists

the thiazolo[5,4*d*]pyrimidine scaffold mimicking the adenine core as a strategy to achieve atypical A_{2A}AR agonists, e.g., **33** (Fig. 5.3), through H-bond interaction with conserved $His^{7.43}$ (using standard notation for TM residues of rhodopsin-like GPCRs). The new derivatives were shown to be partial agonists in hA_{2A}AR functional assays and exhibited affinity in the high nanomolar range and with moderate selectivity. The comparison between pairs of analogs, having different 2-hydroxymethyl substituents on the pyrrolidine ring, confirmed the hypothesis that this moiety contributes to receptor activation.

Overall, the abovementioned studies demonstrated that SBDD approaches were successful in retrieving and guiding the design of ligands with pharmacological profiles matching the selected receptor conformational state (inactive for antagonists and active-intermediate for agonists). The notion that ligand recognition at the A_{2A}AR receptor occurs through conformational selection has been recently supported by nuclear magnetic resonance experiments (Ye et al. 2016) that captured the A_{2A}AR in an ensemble of conformations. Among these conformations, were two inactive and two active states whose relative populations were determined by the added ligand. This implies that, at least in the case of A_{2A}AR, different types of ligands (agonist vs. antagonist) preferentially bind to diverse pre-existing receptor states, as experimentally demonstrated (Bennett et al. 2013). Taken together, this evidence supports the concept of "reverse pharmacology," i.e., the ligand selects a particular receptor conformational state to define ligand selectivity and signaling profile. As a consequence, the selection of a proper X-ray structure in SBDD is crucial to ensure that newly discovered ligands match the target pharmacological profile. Notably, the recent determination of the fully active state of an agonist-bound hA_{2A}AR (Carpenter et al. 2016) in complex with an engineered G_s protein fragment (PDB ID: 5G53) revealed major rearrangements with respect to the active-intermediate state (PDB IDs: 2YDO and 3QAK). These changes occurred mainly in the cytoplasmic half of the receptor core, especially in TM6, while no substantial changes were detected in the extracellular half of the receptor surrounding the agonist binding site. This evidence further justified the use of active-intermediate structures in SBDD studies aimed at discovering novel AR agonists.

Along with the selection of a proper receptor X-ray structure, another aspect that contributed to the success of SBDD studies is the role played by water molecules in ligand binding. A couple of the above discussed examples (Katritch et al. 2010; Rodríguez et al. 2016), along with a retrospective study focused on the screening of $hA_{2A}AR$ high-affinity antagonists (Lenselink et al. 2014), have emphasized how the retention of carefully selected water molecules improves the success rate of VLS campaigns. Unfortunately, the resolution of most of the X-ray structures does not provide the necessary detail to locate highly structured water molecules, and this task is even more challenging when dealing with homology models. Aiming to overcome these limitations, several molecular dynamics (MD)-based methodologies have been developed using the $hA_{2A}AR$ as a case study, which focused on the prediction of the distribution and the energetic properties of water molecules in the binding site and the impact of their displacement upon ligand binding (Higgs et al. 2010; Bortolato et al. 2013; Sabbadin et al. 2014). As an example, the entropic gain generated by displacement of an "unhappy" water molecule that was otherwise trapped between the ligand and the protein explained the "magic methyl" effect observed in a series of $A_{2A}AR$ antagonists based on a chromone scaffold, where the addition of a methyl group led to a 33-fold affinity increase (Mason et al. 2013).

5.1.3 Biophysical Mapping and Other Advanced Techniques

Biophysical Mapping (BPM) is an experimental approach that is used in combination with molecular modeling techniques (mainly homology modeling and molecular docking) to map binding site interactions with a set of ligands of interest (Jazayeri et al. 2017; Zhukov et al. 2011). BPM was developed and applied for the first time to $hA_{2A}ARs$ that are thermostabilized by mutagensis, which led to the discovery and subsequent optimization of 1,2,4-triazines, such as T4E **125c** (Fig. 5.15, K_i 1.4 nM) (Congreve et al. 2012) and chromones (Andrews et al. 2014) as antagonists. By this approach, several point mutations are introduced in amino acid side chains surrounding the ligand in the orthosteric binding site, as identified by molecular docking, and their effect on ligand binding affinity and kinetics are experimentally determined. To this aim, surface plasmon resonance (SPR) was used to measure ligand binding at the thermostabilized and further mutated $A_{2A}ARs$ immobilized on an SPR chip. The data matrix so gathered describing the effect of several mutations on the binding of a set of ligands can be used to validate and refine receptor homology models, as well as to improve the performance of VLS campaigns. As an example, 1,3,5-triazines, such as **123** and **124** (Fig. 5.15), were identified by VLS using a $hA_{2A}AR$ homology model based on the β_1-adrenergic receptor that was refined and validated using SDM and BPM data (Langmead et al. 2012). In a subsequent study, the scaffold was optimized to 5,6-biaryl-1,2,4-triazine-3-amines, such as T4E **125c**, according to modeling prediction (Congreve et al. 2012, Fig. 5.15). Indeed, docking studies suggested that the introduction of a H-bond acceptor at the 5-aryl group's

para-position would have established a favorable H-bond interaction with $His^{7.43}$, thus driving the scaffold deeper in the binding pocket in a region usually occupied by the ribose ring of nucleoside agonists. Notably, X-ray structures of the $hA_{2A}AR$ in complex with two members of the 1,2,4-triazine series (PDB IDs:3UZA and 3UZC) exhibited ligand binding modes consistent with the docking predictions and the BPM data.

5.1.4 Enhancing the Profile of Known Ligands and Increasing Residence Time

Recently, it has been increasingly acknowledged that the binding affinity of a compound might not be the best predictor of its efficacy in vivo. Instead, the ability to assess, predict, and optimize lead compounds' binding kinetics is a crucial, yet poorly explored, aspect in drug discovery (Guo et al. 2017). To this aim, the structure kinetic relationships (SKRs) of $A_{2A}AR$ agonists together with MD-based techniques have revealed that the functional efficacy of the ten compounds assayed is modulated by their $A_{2A}AR$ residence time (Guo et al. 2012). In another study, 24 triazolotriazine $hA_{2A}AR$ antagonists structurally related to ZM241385 **84** (Fig. 5.10), though exhibiting very minor differences in their binding affinities, showed considerably different $A_{2A}AR$ dissociation rates (Guo et al. 2014). Thus, specific ligand interactions with residues in the extracellular vestibule might modulate the binding kinetics of the ligands during the egress pathway. Segala et al. (2016) performed temperature-accelerated MD simulations on an X-ray structure of the ZM241385 **84**-$hA_{2A}AR$ complex (PDB ID: 4EIY, Fig. 5.1a). The analysis of the MD results suggested 13 residues located in the extracellular tips of TM2, TM6, and TM7 and in the second and third extracellular loops (ECL2 and ECL3, respectively) contacted the ligand during its dissociation route, and their effect on the binding and dissociation kinetics of **84** were experimentally verified. In particular, the study highlighted the role of a salt bridge between Glu169 in the ECL2 and His264 in ECL3, whose disruption accelerated the ligand dissociation. In line with these findings, another study investigated the role of this salt bridge in controlling the binding kinetics at the $hA_{2A}AR$ of a series of four derivatives of **84** (Guo et al. 2016). After initially breaking H-bonds during the antagonist dissociation, a transient contact is made with a hydrophobic region involving residues of TM2 and TM7 of $A_{2A}AR$. Metadynamics investigations revealed that the salt bridge was readily broken in the simulations for ligands exhibiting short residence times, whereas it is maintained for ligands with longer residence times. X-ray structures of the ligand-receptor complexes highlighted differences in the interactions between the ligands and the residues involved in the salt bridge. In particular, long-residence time ligands established stabilizing interactions with His264, which were not detected for ligands having shorter residence times.

5.2 Medicinal Chemistry of A_{2A}AR Agonists, Partial Agonists, and Allosteric Modulators

5.2.1 A_{2A}AR Agonists

Most synthetic A_{2A}AR agonists, like the native agonist adenosine (**1**; Ado; Fig. 5.1), are nucleoside derivatives (de Lera et al. 2014). However, atypical non-nucleoside A_{2A}AR agonists in the structural classes of 3,5-dicyanopyridines and cyanopyrimidines (e.g., **31**, **32**) that lack an attached ribose or ribose mimic have been reported (Lane et al. 2012; Kato et al. 2005). Ado is a nonselective AR agonist (See Table 5.1. for affinity values), and structural modifications were necessary to provide A_{2A}AR selectivity. These modifications were introduced on the ribose and the nucleobase moieties, independently or in combination (Müller and Jacobson 2011; Cristalli et al. 2007, 2008).

5.2.1.1 Ribose-Modified Adenosine Analogs as A_{2A}AR Agonists

The 5′-*N*-ethylcarboxamide derivative NECA **5** displays increased potency, compared to adenosine, by more than an order of magnitude across all the AR subtypes (Prasad et al. 1980; Müller and Jacobson 2011). 5′-*N*-ethyl was found to be among the most potent alkyl groups for A_{2A}AR affinity (Rieger et al. 2001), but 5′-*N*-cyclopropyl is also present in some potent A_{2A}AR agonists, e.g., **23** (Day et al. 2005).

Other modifications at the 5′-position known to be tolerated at the A_{2A}AR include a variety of alkyl-bearing five-membered azoles and ether analogs, often with substitutions at the C2-position combined with N^6–2,2-diphenylethyl substitution of the purine ring (Jacobson 2002). Substitution of the 5′-carboxamide of NECA with bioisosteric alkyl tetrazoles has been introduced in combination with adenine C2 modifications (e.g., **29a**, Cox et al. 1998). The monosubstituted 5′-*C*-ethyltetrazolyladenosine (structure not shown) displayed single-digit nanomolar affinities at three AR subtypes, except A_{2B}AR (Petrelli et al. 2015). The 5′-*C*-ethyltetrazoles typically display increased A_{2A}AR agonist affinity but also A_3AR antagonist activity (Rodríguez et al. 2015a).

Adenine congeners with acyclic sugar mimics, pyranoses, and furanoses other than ribose were inactive at the rat (r) A_{2A}AR (Siddiqi et al. 1995). The 2′,3′-hydroxyl groups are necessary for ligand recognition, and modification to deoxy and ether functionalities are not well tolerated at the A_{2A}AR.

Substituting the furanose oxygen by sulfur as in **3** increased A_{2A}AR affinity compared to **2** as well as A_{2A}AR selectivity (Siddiqi et al. 1995), but this modification is also present in A_3AR-selective ligands (Hou et al. 2012). Conformational locking of a ribose or ribose mimic to the *North* form using a bicyclic methanocarba ring (4′,6′-α-cyclopropyl ring fusion in the carbocycle) or introducing 2′-β-methyl (in ribose) decreased or abolished the A_{2A}AR affinity and increased affinity at A_1 and/or A_3ARs (Jacobson et al. 2000: Franchetti et al. 1998).

Table 5.1 A comparison of affinity values of nucleoside derivatives at all AR subtypes

Compound no. name	A_1 (K_i)	A_{2A} (K_i)	A_{2B} (EC_{50})	A_3 (K_i)
1 adenosine	100 (h) 73 (r)	310 (h) 150 (r)	15,000 (h) 5100 (r)	290 (h) 6500 (r)
2	1039 (h) 1890 (r)	180 (h) 63 (r)	– 2400 (r)	19 (h) 9.3 (r)
3	300 (r)	20 (r)	–	1090 (r)
4	226 (r)	163 (r)	–	2480 (r)
5 NECA	14 (h)	20 (h)	330 (h)	6.2 (h)
6 HENECA	60 (h) 160 (r)	6.4 (h) 1.0 (r)	6100 (h) –	2.4 (h) 18 (r)
7 HEAdo	18 (h) 111 (r)	5.7 (h) 5.2 (r)	>99,000 (h) –	4.7 (h) 24 (r)
8	48% (r)[a]	82 (r)	–	2160 (h)
11 CV-1808	400 (r)	100 (r)	–	–
12 Regadenoson; CVT-3146	>10,000 (h)	290 (h)	>10,000 (h)	>10,000 (h)
13 Sonedenoson; MRE-0094	>10,000 (h)	490 (h)	>10,000 (h)	–
14 Binodenoson; WRC-0470	48,000 (h)	270 (h)	430,000 (h)	903 (h)
15 PSB-0777	541 (h) >10,000 (r)	360 (h) 44.4 (r)	>10,000 (h) –	>10,000 (h) –
17 CGS21680	289 (h) 1400 (r)	27 (h) 19 (r)	361,000 (h) >10,000 (r)	67 (h) 584 (r)
21	350 (h)	40 (h)	–	320 (h)
22	>10,000 (h)	4.78 (h)	>10,000 (h)	1487 (h)
23 ATL-313	57 (h)	0.7 (h)	>1000 (h)	250 (h)
24 Apadenoson; ATL-146e	77 (h)	0.5 (h)	>1000 (h)	45 (h)
28 UK-432097	–	4 (h)	–	–
29a GW-328267X	882 (h)	2.3 (h)	51 (h)	4.2 (h)[b]
30	>10,000 (h)	5.4 (h)	9866 (h)	1640 (h)
33	530 (h)	153 (h)	–	1070 (h)

Affinity values are expressed in nM
"–" data not available, *h* human, *r* rat
[a]At 10 μM
[b]Antagonist

5.2.1.2 Nucleobase-Modified Adenosine Analogs as A_{2A}AR Agonists

Ado is metabolized rapidly in vivo ($t_{1/2}$ ~10 s) by the action of adenosine deaminase to inosine, which is a relatively weak AR agonist (K_i 50 μM at $rA_{2A}AR$, van van Galen et al. 1994). In early studies of the $A_{2B}AR$, introduction of a nitrogen in place of CH at the 2-position of the adenine core increased affinity but decreased efficacy, while an 8-aza modification decreased potency (Bruns 1980). A 1-deaza modification (**4**) reduced $A_{2A}AR$ affinity with respect to simple adenosine derivatives such as

2 (Cristalli et al. 2008). In general, the absence of nitrogen atoms at C3-, C7-, and C6-positions is detrimental to the AR activity (Cristalli et al. 2008).

Substitution at C8 increases a steric clash between the 5′-methylene group and the ribose ring oxygen, which forces the glycosidic bond axis to adopt a more stable *syn* conformation. This conformation disrupts some of the conserved interactions in the binding site, which reduces the affinity toward all ARs. Several exceptions to this pattern have been reported, for example, compound **8** (with C2-hexynyl), which lost affinity and intrinsic activity compared to **7** making it a partial A_{2A}AR agonist (van Tilburg et al. 2003). Compound **8** gained hA_{2A}AR selectivity when compared to hA_1 and hA_3ARs, both of which are known to display species differences in ligand affinities.

No substitution at the N^6-position is preferred over sterically small alkyl groups, and many large groups at the N^6-position alone tend to reduce A_{2A}AR affinity (Gao et al. 2003). However, N^6-(2-phenylethyl)-adenosine analogs maintain considerable A_{2A}AR affinity. This was evident in the early report of N^6-(2,2-diphenylethyl)-adenosine **25** and its substituted analog DPMA **26** as potent A_{2A}AR agonists (Bridges et al. 1988). This former group is also present in the series of agonists that culminated in UK-432097 **28** (Mantell et al. 2009). Hence, certain N^6 substitutions accompanying a larger C2-group with appropriate 5′-sugar modifications provided high A_{2A}AR affinity and selectivity (see Sect. 5.3.).

C2-alkynyladenosine derivatives, such as **7**, were found to be potent A_{2A}AR agonists with hypotensive and antiplatelet aggregatory activity (Homma et al. 1992; Cristalli et al. 1994). Hou et al. (2012) explored combinations of C2- or C8-alkynyl (and other) substitution with 4′-ribose truncation of thioadenosine derivatives to identify a dual acting A_{2A}AR agonist and A_3AR antagonist (structure not shown).

CV-1808 **12** was the earliest C2-functionalized adenosine that demonstrated potent in vivo coronary dilatation as an A_{2A}AR agonist, as well as slight selectivity in receptor binding. This aniline congener of adenosine and a 2-alkynyl derivative, HEAdo **7**, demonstrated that an extended C2-moiety could increase A_{2A}AR potency. Bulky substitution with ethers, e.g., **10** and **11**, or secondary amines, based on the earlier lead of CV-1808, at the C2-position was explored in early SAR studies by Olsson and colleagues (Ueeda et al. 1991) to increase A_{2A}AR potency along with selectivity, which led to subsequently discovered C2-substituted adenosine derivatives with high A_{2A}AR selectivity. Elaboration of this finding in CGS21680 **17** increased selectivity (note that CGS21680 is moderately selective for the rA_{2A}AR but less selective for the hA_{2A}AR). Functionalized congeners of CGS21680, derivatized through the terminal carboxylic acid, provide useful probes for receptor characterization by radioiodination, affinity labeling, photoaffinity labeling, and immobilization on nanocarriers (Jacobson 2013). A radioiodinated form of PAPA-APEC **19** was used for the first molecular characterization of the A_{2A}AR (Barrington et al. 1989). *p*-DITC-APEC **20** was found to be a highly potent irreversibly binding A_{2A}AR agonist (Jacobson et al. 1992). Recently, fluorescent agonists derived from APEC **18** were used to follow the physical association of the A_{2A}AR and D_2 dopamine receptor in real time (Fernández-Dueñas et al. 2012).

Following the lead of C2 derivatization to achieve moderate potency at the A_{2A}AR, regadenoson **13** became the first FDA-approved (in 2008) synthetic AR agonist for clinical use in myocardial perfusion imaging (Al Jaroudi and Iskandrian 2009). Regadenoson features a C2-pyrazolocarboxamide to provide moderate selectivity, and it has a short duration of action (2–3 min) with an in vivo terminal half-life of 33–108 minutes (Al Jaroudi and Iskandrian 2009). Its subnanomolar potency for vasodilation made it suitable for diagnostic imaging. Regadenoson also served as the basis for immobilization of A_{2A}AR agonists on nanocarriers directed toward penetrating the blood-brain barrier (Gao et al. 2014). The other notable A_{2A}AR agonists in this series are aralkyl ethers (sonedenoson, **14**) and alicyclic hydrazinimines (binodenoson, **15**), which were developed for human trials (Müller and Jacobson 2011).

With the A_{2A}AR present at many sites in the body, the side effects of A_{2A}AR agonists, even if subtype selective, are a concern for human use. Consequently, various drug delivery approaches were applied to limited A_{2A}AR activation. A prodrug 5′-phosphate ester of a potent A_{2A}AR agonist was designed to be released as its active form by 5′-nucleotidase (CD73) in vivo at the site of inflammation (El-Tayeb et al. 2009; Flögel et al. 2012). The sulfonic acid analog **16** was envisioned as a targeted delivery for treatment of inflammatory bowel syndrome (IBS) and to restrict absorption into systemic circulation to avoid side effects, e.g., hypotension (El-Tayeb et al. 2011). The C2-position tolerates a range of other chemical variations like alkynes, amide, urea, and heterocycles in assorted combinations. Thus, certain C2-modifications provide A_{2A}AR selectivity, alone or in combination with ribose modifications, as discussed below.

5.2.1.3 Combined Ribose and Base Modifications

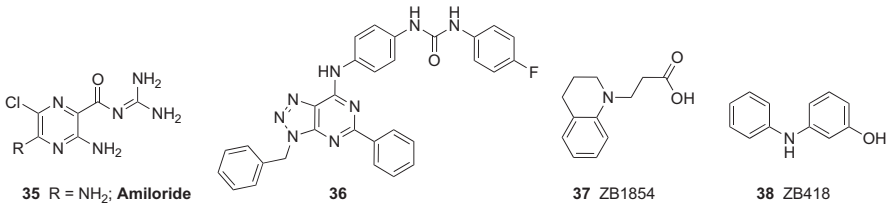
In early 1990s, HENECA **6**, one of the 5′,C2-modified adenosine derivatives, was reported as a selective A_{2A}AR agonist in rat, but in human, it is a mixed A_{2A}/A_3AR agonist of nanomolar affinity but with low selectivity vs. A_1AR (Cristalli et al. 1998). CGS21680 (**17**) was the first A_{2A}AR agonist reported with high potency and moderate selectivity in rats. It features an important combination of adenosine modifications in A_{2A}AR agonists, i.e., 5′-alkylcarboxamide and a large C2-substituent (Hutchison et al. 1990). The ability to extend the C2-substituent indefinitely without losing the ability to bind to the A_{2A}AR was reported soon thereafter (Jacobson et al. 1989). Coupling of D-histidine with CGS21680 resulted in compound **21**, which was more A_{2A}AR selective than its parent compound in comparison to the hA_3AR but with slightly reduced affinity (Deflorian et al. 2012). Apadenoson (**24**; ATL-146e) and ATL-313 (**23a**) with 5′-uronamides and alkyl-alicyclic-carboxylic groups at C2 displayed subnanomolar affinity as A_{2A}AR agonists. Although moderately A_{2A}AR selective (>80-fold), these molecules still have significant affinities at other subtypes. The methyl ester in **24** is readily hydrolyzed to the corresponding acid,

which is equipotent at A_{2A}AR and A_3AR (K_i = 30 nM), while ATL-313 instead contains a relatively stable carbamate group (Day et al. 2005). Compound **28** (UK-432097), a NECA variant with bulky N^6 and C2-groups, was developed to achieve high potency. Although discontinued for COPD trials, **28** was successfully used to determine an agonist-bound A_{2A}AR X-ray structure, stabilized by its high affinity, large size, and many H-bonding interactions. The 4′-alkyltetrazole- and C2-aralkylaminol-bearing compound **29a** is potent but nonselective and an antagonist at A_3AR with comparable affinity (Cox et al. 1998). Bosch and co-workers further optimized compound **29a** by substituting different groups on the C2-phenyl ring and deoxygenated the C2-aminol. Among them, the C2-(*R*) isomer **29b** is the most potent (K_i at A_{2A}AR 1 nM) with >100-fold selectivity against other ARs (Bosch et al. 2004). Interestingly, affinity of the corresponding C2-(*S*) isomer shifted toward A_3AR (K_i = 26 nM, antagonist), and it was 50-fold less potent than **29b** at A_{2A}AR (Rodríguez et al. 2015a).

Compound **30** is a potent and selective molecule across all AR subtypes and has interesting structural characteristics, a carbocycle with transposed amide, smaller N^6-group (compared to UK-432097) with a C2-(*N*-alkyl-histamine) group (Beattie et al. 2010). Recently, molecular modeling of A_{2A}AR crystal structures co-crystallized with agonists and antagonists revealed that the *N*7 side chain of A_{2A}AR antagonist preladenant **51a** (SCH412348, Sect. 5.6.) was seen to share the adenine C2-region of UK-432097 (Preti et al. 2015). This information along with structural optimization resulted in 2-((4-arylpiperazine-1-yl)ethylamino) derivative **22**, one of the most highly potent and selective A_{2A}AR agonists reported so far.

5.2.1.4 Nonadenine Nucleosides and Non-nucleosides as A_{2A}AR Agonists

Several classes of non-nucleoside agonists of the A_{2A}AR have been reported, including 6-amino-3,5-dicyano-4-phenyl-2-thiopyridines of variable selectivity, e.g., partial A_{2A}AR agonist LUF5834 **31b** (Lane et al. 2012), and a class of substituted 4-amino-5-cyanopyrimidines, represented by potent and selective agonist **32** (Fig. 5.4) (Kato et al. 2005). Another class, consisting of C6-prolinyl-9-thio-8-anilino-adenines (**14**), displays partial agonism with relatively low affinity and selectivity as compared to other series (Bharate et al. 2016). *N*-acylhydrazone **34**



35 R = NH_2; **Amiloride** **36** **37** ZB1854 **38** ZB418

Fig. 5.4 Non-nucleoside putative allosteric modulators of the A_{2A}AR

and its congeners are presented as A_{2A}AR agonists (Alencar et al. 2017), but additional functional data indicative of their specific action on the A_{2A}AR are needed.

Thus, the range of heterocyclic templates acting as AR agonists is limited, and virtual (in silico) screening of molecular libraries to discovery novel A_{2A}AR agonists has identified few additional classes of non-nucleoside agonists (Rodríguez et al. 2015b).

In summary, many agonists mentioned in this section are only moderately selective (>10-fold), and some are truly selective A_{2A}AR agonists available for in vivo studies. Historically, A_{2A}AR agonists were considered as antihypertensive agents, with adenosine as the only template available for design, which is a nonspecific AR agonist (although with low A_{2B}AR affinity). Extensive studies in recent times revealed the importance of the A_{2A}AR as a drug target. A few new chemical entities (NCEs) that entered clinical trials were discontinued due to cardiovascular side effects, a common problem associated with A_{2A}AR agonists in systemic circulation. A targeted delivery technique may be necessary to mitigate this effect (e.g., UK-432097 **28** and PSB-0777 **16**). Hence, selective and target optimized agonists are sought. Recent developments, including elucidation of receptor structures, modeling techniques, and newer pharmacological approaches such as allosteric modulation, promise to accelerate drug development for this receptor.

5.2.2 A_{2A}AR Allosteric Modulators

Allosteric modulators are those which bind at a site other than the binding pocket of the respective endogenous ligand (orthosteric site) to enhance or reduce the effect of orthosteric ligands. Molecules increasing agonist effects (or increasing residence time) are positive allosteric modulators (PAMs) and those reducing are negative allosteric modulators (NAMs). Allosteric modulators may or may not exert any effect of their own, and there may be a diverse array of functional modulation by allosteric ligands. Unlike A_1 and A_3ARs, there are few reports on A_{2A}AR allosteric modulators. At physiological concentrations, sodium ion itself is a NAM for agonists. Amiloride **35** (Fig. 5.4) and its derivatives induce allosteric effects on several GPCRs by binding to the sodium ion pocket. Sodium ions and amilorides were shown to enhance A_{2A}AR antagonist (ZM241385) binding and to compete for the same allosteric site (Massink et al. 2016; Gao and IJzerman 2000). Giorgi et al. (2008) reported that 2-phenyl-9-benzyl-8-azaadenine derivative **36** is a selective enhancer of both A_{2A}AR agonists and antagonists. A fragment screening effort resulted in several potential A_{2A}/A_1AR allosteric modulators, notably among them are PAM **37** and NAM **38** (Chen et al. 2012). In addition, AEA061 (structure not disclosed) was shown to be a PAM of A_{2A}AR agonists (Welihinda and Amento 2014).

5.3 Medicinal Chemistry of A_{2A}AR Antagonists

5.3.1 Tricyclic Systems

5.3.1.1 Pyrazolo[4,3-*e*][1,2,4]Triazolo[1,5-*c*]Pyrimidine (PTP) Derivatives

CGS15943 (**39**) (Ghai et al. 1987; Francis et al. 1988), featuring a [1,2,4]triazolo[1,5-*c*]quinazoline scaffold, can be considered the parent compound of PTPs (Fig. 5.5). Displaying subnanomolar affinity for the A_{2A}AR but low selectivity toward the remaining AR subtypes (see Table 5.2.), CGS15943 was evaluated as an interesting starting point for further optimization. Gatta and co-workers described the first example of a PTP antagonist of the A_{2A}AR (**40**, 8FB-PTP), obtained from the bioisosteric replacement of the phenyl ring in CGS15943 with a substituted pyrazole (Gatta et al. 1993). Subsequently, a large library of PTPs resulted from the

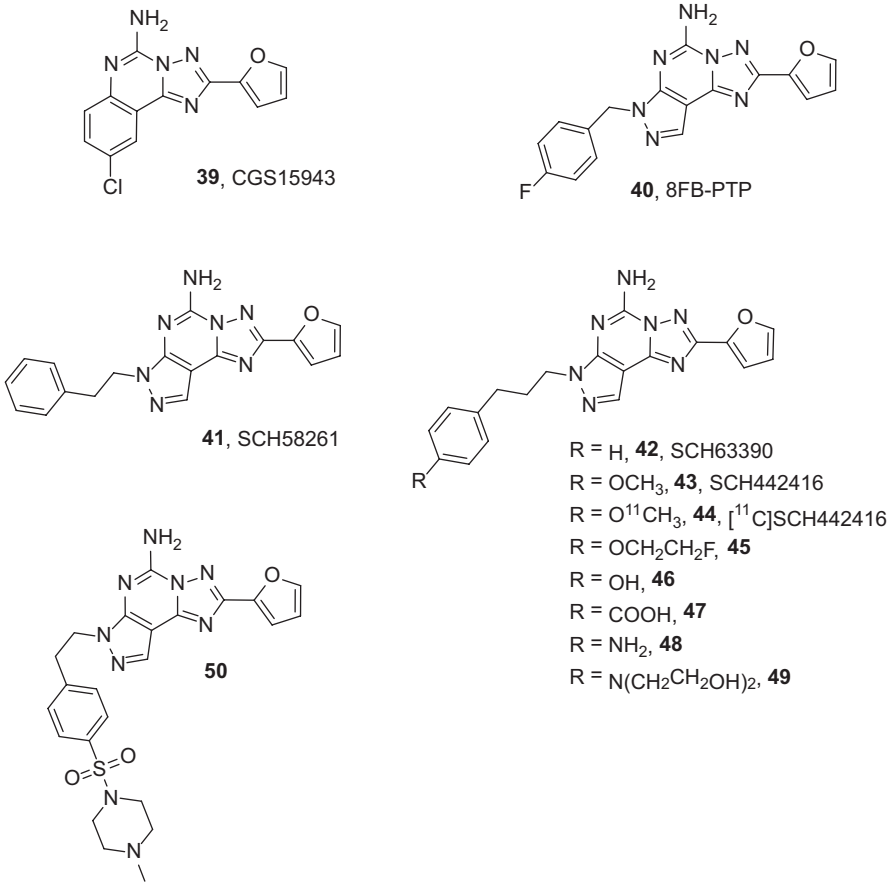


Fig. 5.5 Pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidine (PTP) derivatives as A_{2A}AR antagonists

Table 5.2 Affinity and selectivity of representative $A_{2A}AR$ antagonists

	K_i (nM)[a]							
	A_1AR	$A_{2A}AR$	$A_{2B}AR$	A_3AR	A_1/A_{2A}	A_{2B}/A_{2A}	A_3/A_{2A}	Ref
39 CGS15943	3.5	0.15	71	51	23	473	340	Armentero et al. (2011), de Lera et al. (2014)
	6 (r)[b]	1.2 (r)			5.0 (r)			
40 8FBPTP	3.3 (r)	1.2 (r)			2.8			Armentero et al. (2011)
41 SCH58261	594	1.1	>10,000	>10,000	540	>9091	>9091	Armentero et al. (2011)
42 SCH63390	350	1.2	>10,000	>10,000	292	>8333	>8333	Armentero et al. (2011)
43 SCH442416	1111	0.048	>10,000	>10,000	23,146	>208,333	>208,333	Todde et al. (2000)
	1815 (r)	0.50 (r)		>10,000 (r)	3630 (r)		>20,000	
45	43	12		60	3.4		5	Shinkre et al. (2010)
46	253	1.5		>10,000	169		>6670	Baraldi et al. (1998)
	741 (r)	0.94 (r)			788 (r)			
47	4927	4.6	>10,000	>10,000	1064	>2160	>2160	Baraldi et al. (2002)
48	2160	0.22	>10,000	>10,000	9818	>45,454	>45,454	Baraldi et al. (2002)
49	558	1.1	>10,000	>10,000	507	>9091	>9091	Baraldi et al. (2002)
50	369	3.8	>10,000	>10,000	97	>2631	>2631	Baraldi et al. (2002)
51a Preladenant	1474	1.1	>1700	>1000	1340	>1545	>909	Neustadt et al. (2007), Pinna (2014)
52 SCH412348	>960	0.6			>1600			Neustadt et al. (2007)
53	1062	0.5			2124			Shah et al. (2008a)
54	406	2.4			169			Shah et al. (2008a)
55	680	5.4			126			Shah et al. (2008b)
56	192	3.2			60			Shah et al. (2008b)
57	880	1.9			463			Shah et al. (2008b)
58	358	2.0			179			Shah et al. (2008b)
59	40	24.6		33				Duroux et al. (2017)
	0 (m)[b]	2.096 (m)		2 (m)				

60	1680	30.3		32				Duroux et al. (2017)
	0 (m)	585 (m)		5 (m)				
61	602	0.9			669			Silverman et al. (2007)
62a		0.1–10			7.1			Yang et al. (2014, 2016)
62b		1.5			1100	>3000	> 3000	Basu et al. (2017)
64		5						Moorman (2008)
65		1						(Moorman 2008)
66 PTTP	29,000	6.3			4603			Kumari et al. (2014)
67	1404	23			60			Harris et al. (2011b)
68		0.4						Harris et al. (2011a)
69 KF17837	62 (r)	1.0 (r)			62 (r)			Schulte and Fredholm (2003)
	1500(gp)[b]							
70 KW6002	2830	36	1800	>3000	79	50	>83	Weiss et al. (2003), Armentero et al. (2011)
	230 (r)	2.2 (r)			104 (r)			
71 CSC	>10,000	38	8200	>10,000	>263	216	>263	Armentero et al. (2011), Brunschweiger et al. (2014)
	28,200 (r)	54 (r)			522 (r)			
72 MSX2	2500	5.4	>10,000[c]	>10,000	463	>1852	>1852	Sauer et al. (2000)
	900 (r)	8.0 (r)			112 (r)			
75	>10,000	45	>10,000[c]	>30,000	>222	>222	>667	Yadav et al. (2014)
76a ST1535	72	6.6	352	>1000	11	53	>152	Minetti et al. (2005)
77	80	4.7	2330		17	496		Minetti et al. (2005)
78	3288	6			548			Kiselgof et al. (2005)
79	1780	3.1			574			Kiselgof et al. (2005)
80 VER6947	17	1.1	112	1472	15	102	1338	Weiss et al. (2003), Gillespie et al. (2008b)
81 VER7835	170	1.7	141	1931	100	83	1136	Weiss et al. (2003), Gillespie et al. (2008b)
82 Vipadenant	68	1.3	63	1005	52	48	773	Gillespie et al. (2009a)
84 ZM241385	255	0.8	50	>10,000	319	63	>12,500	de Lera et al. (2014)

(continued)

Table 5.2 (continued)

	K_i (nM)[a]							
	A_1AR	$A_{2A}AR$	$A_{2B}AR$	A_3AR	A_1/A_{2A}	A_{2B}/A_{2A}	A_3/A_{2A}	Ref
85a	1300 (r)	3 (r)			433 (r)			Vu et al. (2004b)
85b	820 (r)	4 (r)			205 (r)			Vu et al. (2004c)
86a	3300 (r)	0.20 (r)			16,500 (r)			Peng et al. (2004)
86b	1350	17	10,700[c]	10,000	80	630	592	Federico et al. (2011)
87	1447	1.5			965			Neustadt et al. (2009)
88	>30,000	7.2	>30,000	>30,000				Falsini et al. (2017)
91a	1680[c]	29[c]						Shook et al. (2011)
91b	1050	32.8						Shook et al. (2011)
92	1010[c]	36[c]						Shook et al. (2011)
93	222[c]	11[c]						Shook et al. (2013)
94	1733	25			69			Gillespie et al. (2009c)
95 VER6623	208 913 (r)	1.4 17 (r)	865	476 5974 (r)	148 54 (r)	618	340 351 (r)	Yang et al. (2007), Gillespie et al. (2008a)
96		100 (58%)[d]						Saku et al. (2010a)
97		(39/81/103)%[e]						Saku et al. (2010a)
98		100 (100%)[d]						Saku et al. (2010a)
99	1000 (4%)[f]	100 (73%)[d]	1000 (21%)[f]					Saku et al. (2010a)
100		0.5						Nakamura et al. (2005)
101		1						Shiohara et al. (2006)
102		0.50						Alanine et al. (2001)
103		0.50						Alanine et al. (2001)
104 SYN115	1350	5.0	700	1570	270	140	314	de Lera et al. (2014)

105	>7000	4.1			>1707			Norcross (2005)
106	2.8	0.0038			737			Luthra et al. (2010)
107	>1500	15			>100			Langmead et al. (2012)
108 LUF6080	12	1.0	34	>1000	12	34	>1000	Mantri et al. (2008)
109	34	41	>1000	>1000	0.8	>24	>24	Mantri et al. (2008)
110	266	2.7 26 (r)[b]			98			Slee et al. (2008c)
111	1730	2.0 14 (r)			265			Slee et al. (2008c)
112	850	135			6.3			Slee et al. (2008a)
113	850	12 131 (r)			71			Slee et al. (2008a, b)
114	850	17			50			Slee et al. (2008b)
115	2000	9			222			Slee et al. (2008a)
116 TC-G 1004	85	0.44 1.50 (r)			193			Zhang et al. (2008)
117	162	4.7 48 (r)	145	19	34	31	4	Lanier et al. (2009)
118		0.22						Zheng et al. (2014)
119	43	1.7	460	1740	25	270	1023	Gillespie et al. (2009c)
120	133	2.5	3185	366	53	1274	146	Gillespie et al. (2009b)
121		1.0						Camacho Gomez and Castro-Palomino Laria (2014)
122		1.0						Yang et al. (2016)
123	59	1.0			59			Langmead et al. (2012)
124	30	1.6			19			Langmead et al. (2012)
125b	32	3.5			9.1			Congreve et al. (2012)
126	>10,000	16			>625			Langmead et al. (2012)

(continued)

Table 5.2 (continued)

	K_i (nM)[a]							
	A_1AR	$A_{2A}AR$	$A_{2B}AR$	A_3AR	A_1/A_{2A}	A_{2B}/A_{2A}	A_3/A_{2A}	Ref
127 ASP5854	>10,000	68			>147			Langmead et al. (2012)
128	500	200	>10,000	600	2.5	>50	3	Carlsson et al. (2012)
129	>10,000	200	>10,000	300	>50	>50	1.5	Carlsson et al. (2012)
130	410	5.9	260	>10,000	69	44	>1695	Sams et al. (2011)

[a]K_i values from competition binding assays to human (h) ARs unless otherwise specified
[b]*r* rat, *m* mouse, *gp* guinea pig
[c]IC50 values from cAMP functional assays
[d]Percentage of inhibition at 100 nM
[e]Percentage of inhibition at $10^{-6}/10^{-7}/10^{-8}$ mol/L
[f]Percentage of inhibition at 1 μM

systematic substitution of the C^2-, C^5-, C^9-, N^7-, and N^8-positions (Baraldi et al. 2003, 2006, 2008, 2012a, c). As reported from Baraldi's group, it emerged from the binding profiles of SCH58261 (**41**) (Baraldi et al. 1994, 1996), SCH63390 (**42**), and SCH442416 (**43**) (Baraldi et al. 1996, Fig. 5.5) that the selectivity for the $hA_{2A}AR$ subtype was promoted by the introduction of an appropriate arylalkyl chain (i.e., phenylethyl and phenylpropyl) at the N^7-position.

The corresponding radioligand [^{3}H]SCH58261 has been widely employed for the pharmacological characterization of the $A_{2A}AR$. In addition, [^{11}C]SCH442416 **44** and the fluoroethoxy ^{18}F-labeled PTP derivative **45** (Todde et al. 2000; Shinkre et al. 2010; Khanapur et al. 2014) have been investigated as positron emission tomography (PET) ligands for the in vivo imaging of the receptor. Even after showing strong in vivo activity when dosed intraperitoneally in animal models of Parkinson's disease (PD), SCH58261 was devoid of efficacy if administered orally and was characterized by poor A_{2A} versus A_1AR selectivity in association with low water solubility.

The first attempt of Baraldi and co-workers to increase aqueous solubility of PTP-related $A_{2A}AR$ antagonists was the introduction of hydrophilic or salifiable functions on the phenyl ring of the N^7 side chain (see compounds **46–49**), which also led to increased potency and selectivity (Baraldi et al. 1998, 2002). As shown with compound **50**, which was employed in in vivo models of PD, the introduction of the methylpiperazine-sulfonylamide chain increased water solubility (Simola et al. 2004, 2008).

Subsequently, the SAR optimization work focused on the manipulation of the N^7 side chain of the PTP scaffold by Schering-Plough Laboratories leading to the identification of preladenant (**51a**, SCH420814, Fig. 5.6, Neustadt et al. 2007). It was also selected as a candidate in Phase 1–3 clinical trials for treating PD (Neustadt et al. 2001, 2007; Zúñiga-Ramírez and Micheli 2013; Pinna 2014; Kuo et al. 2005). [^{18}F]MNI-444 **51b** was evaluated as a PET ligand in healthy human subjects (Barret et al. 2015). Various substitutions of the distal phenyl ring were evaluated, and the 2,4-difluoro derivative SCH412348 **52** showed good A_{2A} versus A_1AR selectivity (K_i A_1AR/K_i $A_{2A}AR > 1600$) (Neustadt et al. 2007). The replacement of the arylpiperazine moiety with (hetero)biaryl functions, as in **53**, and fused heteroaryl bicycles, as in quinoline derivative **54**, generally improved the in vitro and/or in vivo profile compared to SCH58261 but was associated with poor water solubility (Shah et al. 2008a). In order to enhance hydrophilicity, an additional basic nitrogen was introduced as in the isoindoline **55**, tetrahydroisoquinoline **56**, benzazepine **57**, and tetrahydronaphtiridine **58** analogs.

Recently, Jacobson, and colleagues described fluorescent PTP antagonists that were useful for drug screening at the $A_{2A}AR$ (Kecskés et al. 2010; Duroux et al. 2017). A series of pyrazolo[4,3-*e*[1,2,4]triazolo[1,5-*c*]pyrimidine-5-amine $A_{2A}AR$ antagonists was functionalized as amine congeners and fluorescent conjugates. Fluorescent antagonists **59** and **60** (Fig. 5.6) were identified by systematically varying the chain length of the previous PTP analogs. Conjugates **59** and **60** were potent and selective antagonist probes for the $A_{2A}AR$, while **60** was promising for characterization of $hA_{2A}AR$ binding in whole cells by flow cytometry.

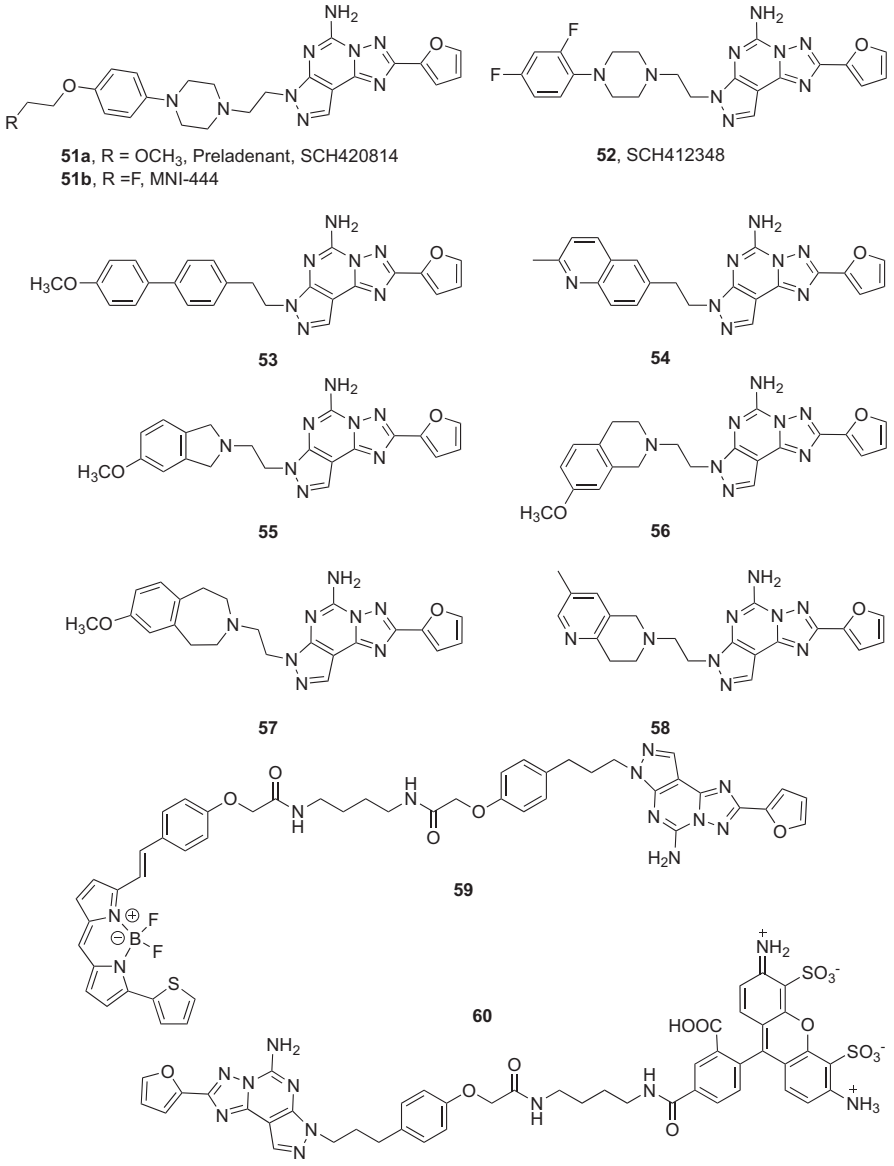


Fig. 5.6 SAR optimization of PTP derivatives leading to preladenant **51a**

The pyrazole ring of the PTP core was also subject of bioisosteric manipulation. The replacement of the pyrazole ring of preladenant with an imidazole ring, as in derivative **61** (Fig. 5.7), produced a significant loss of selectivity against the A_1AR subtype but with a good in vivo profile and pharmacokinetic properties (Silverman et al. 2007). Moreover, in competitive A_{2A}AR radioligand binding assays, the closely related imidazolone derivative **62a** is reported to have a K_i value between 0.1 and 10 nM (Barawkar et al. 2012).

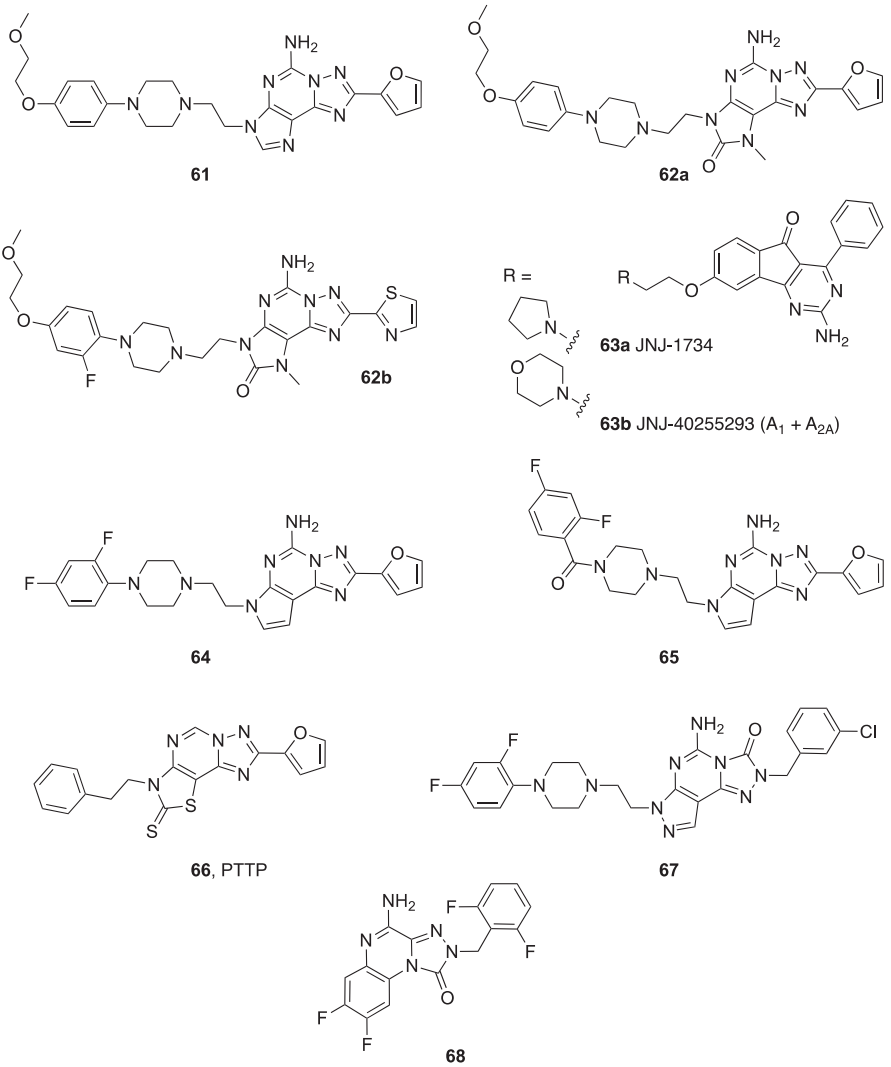


Fig. 5.7 PTP derivatives and indeno[1,2-d]pyrimidine-5-ones as A_{2A}AR antagonists

The [1,2,4]triazolo[5,1-*f*]purin-2-one scaffold is another tricyclic system that was investigated as A_{2A}AR antagonists. The introduction of a fluorine atom at the 2-position of the phenyl ring combined with a 2-thiazole moiety at the 8-position of the tricyclic structure led to **62b** (Fig. 5.7), which displayed both functional antagonism and selectivity at A_{2A}AR. This compound showed satisfactory in vitro and in vivo pharmacokinetic properties (Basu et al. 2017). 5*H*-indeno[1,2-*d*]pyrimidine-5-ones were reported by J&J as A_{2A}AR antagonists (e.g., **63a**) mixed A_1AR/ A_{2A}AR antagonists (e.g., **63b**) (Atack et al. 2014).

King Pharmaceuticals claimed a series of pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*c*] pyrimidine-5-amines as A_{2A}AR antagonists, such as derivatives **64** and **65**, which showed K_i values of 5 nM and 1 nM, respectively (Moorman 2008).

The in vitro potency of compounds **61–65** suggested that the nitrogen at the 8-position of the PTP nucleus is not involved in energetically stabilizing interactions with the A_{2A}AR. Baraldi's group additionally demonstrated the importance of the pyrazole portion of the PTP nucleus in modulating selectivity (Baraldi et al. 2012b).

From the substitution of the pyrazole ring with a thiazole (Mishra et al. 2010), a new series of thiazole[5,4-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine-labeled PTTP was evaluated as PTP-related A_{2A}AR antagonists. Among these, compound **66** showed a good binding profile (K_i hA_{2A} = 6.3 nM, K_i hA_1 = 29 μM), concomitant with its high potency in a cAMP functional assay and efficacy in an in vivo model of PD without significant neurotoxicity (Kumari et al. 2014).

In order to obtain metabolically stable A_{2A}AR antagonists, several attempts to switch the 2-furanyl ring of tricyclic or bicyclic SCH58261 analogs with substituted aryl groups were described by Schering-Plough (Silverman et al. 2007; Neustadt et al. 2009). Nevertheless, the 2-phenyl/heteroaryl derivatives were generally less potent and/or selective. In this series, benzyl substitution was also investigated, and a series of pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine-3-one derivative structurally related to PTPs was synthesized (compound **67**). The furan ring substitution increased A_{2A}AR affinity, but dramatically reduced in vivo efficacy (Harris et al. 2011b). Finally, the [1,2,4]triazolo[4,3-*a*]quinoxalinone **68**, bearing a benzyltriazolone nucleus, was found to be a potent A_{2A}AR antagonist with a K_i value of 0.4 nM (Harris et al. 2011a).

5.3.2 Bicyclic Systems

5.3.2.1 Xanthine-Based Derivatives

Caffeine and theophylline appeared among the first AR antagonists to be discovered but were characterized by a relatively low affinity (K_i values in micromolar range) and selectivity (equally potent toward A_1, A_{2A}, A_{2B}ARs). The attempts of medicinal chemistry researchers to develop more potent and selective compounds were focused on methodical functionalization of 1-, 3-, 7-, and 8-positions of the xanthine core. XAC (xanthine amine congener) was first introduced as a high-affinity rA_1AR-selective antagonist, but further studies comparing affinity in different species found high affinity at the hA_{2A}AR, leading to its use in the first antagonist radioligand binding studies of the hA_{2A}AR in platelets and striatum (Ukena et al. 1986; Ji et al. 1992).

Kyowa Hakko published a series of xanthine A_{2A}AR antagonists with the introduction of an 8-(*E*)-styryl substitution (Fig. 5.8). The progress in 8-styrylxanthines has been undoubtedly hampered by its physicochemical liabilities such as poor water solubility and light sensitivity (Ghai et al. 1987). In fact, representative

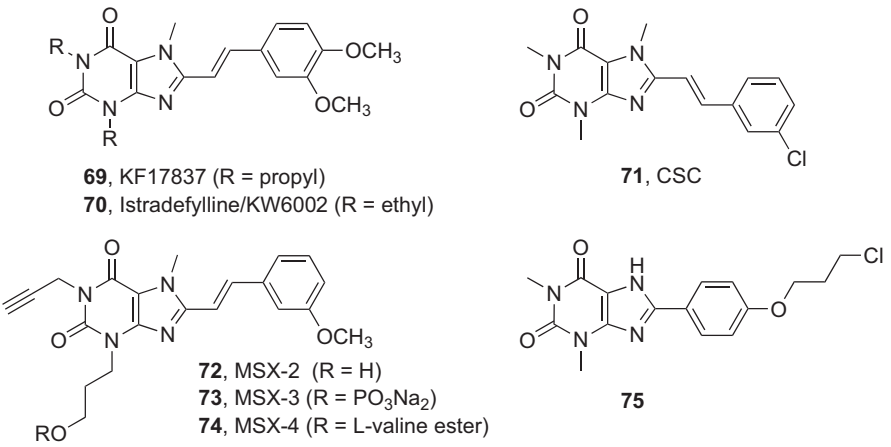


Fig. 5.8 Xanthine-based heterocyclic derivatives identified as A_{2A}AR antagonists

(*E*)-configurated stryrylxanthines have been shown to isomerize in dilute solution to the corresponding (*Z*)-isomers, which possess low or no A_{2A}AR affinity. Nevertheless, the presence of two or three methoxy groups in the 8-styryl phenyl ring of (*E*)-1,3-dipropyl-7-methyl-derivatives proved to increase A_{2A}AR affinity and selectivity, resulting in compounds such as KF17837 (**69**), which is one of the first A_{2A}AR selective xanthine-based antagonists (Shimada et al. 1992; Harada et al. 2001). Consequently, 8-(*m*-chlorostyryl)caffeine (CSC, **71**) was reported by Jacobson et al. who extensively explored the SAR of 8-styrylxanthines (Jacobson et al. 1993). Substitution at the 1- and 3-positions of the bicyclic scaffold was exploited to improve aqueous solubility by introducing polar groups such as in MSX-2 (**72**) or to improve drug likeness of synthesized prodrugs, such as in MSX-3 (**73**) and MSX-4 **(74)** (Sauer et al. 2000; Vollmann et al. 2008). Istradefylline (KW6002, **70**) was the only compound among the 8-styrylxanthines that was successful in clinical trials, and it was approved as an anti-Parkinsonian drug in Japan (Pinna 2014). Some PEGylated analogs of KW6002 with improved water solubility and photostability have been recently synthesized (Pinna 2014).

A novel series of 8-(substituted)phenyl/benzyl-xanthines has been widely investigated, and the chloropropyloxy derivative **75** showed good affinity and selectivity for the A_{2A}AR subtype along with a potent bronchospasmolytic effect in guinea pigs (Yadav et al. 2014).

5.3.2.2 Nonxanthine Purines

From a molecular modeling study by the Sigma-Tau research group, a new series of purine analogs was identified as AR antagonists with different selectivity profiles against the hA_1 and A_{2A}AR subtypes (Minetti et al. 2005; Cabri et al. 2010). Starting with the adenine core, which clearly mimics the endogenous ligand lacking the

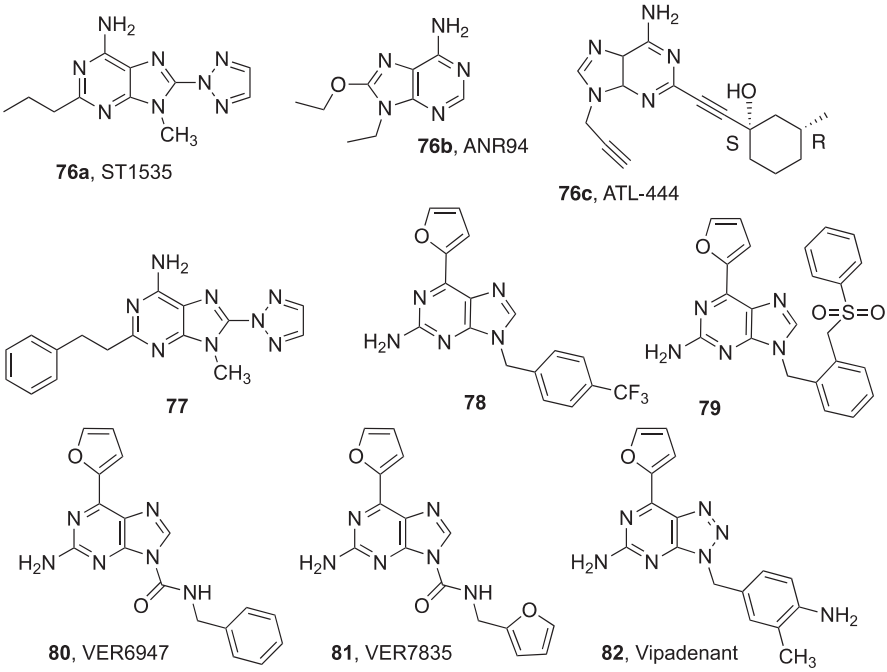


Fig. 5.9 Purine (nonxanthine) derivatives as A_{2A}AR antagonists

intact ribose moiety that is responsible for AR agonist activity, substitutions at different positions of the purine scaffold were evaluated. Introduction of a triazolyl moiety at the 8-position of the scaffold led to the water-soluble compounds such as 2-*n*-butyladenine derivative ST1535 (**76a**, Fig. 5.9). The most representative lead compound of the series was 2-phenylethyl analog **77**, which exhibited significantly higher selectivity versus the A_{2B}AR subtype. Other adenine derivatives have been under development by Pinna et al. (2010) (ANR94, **76b**) and by Forest Labs (ATL-444, **76c**).

Subsequently, structural modifications at 2-, 6-, and 9-positions of the adenine scaffold resulted in the 2-amino-6-furyl-9-benzyl purines **78–79**, which were developed as A_{2A}AR antagonists by Schering-Plough (Kiselgof et al. 2005). The purine-based A_{2A}AR antagonists with a 9-carboxamide function, e.g., VER6947 (**80**) and VER7835 (**81**, named after Vernalis), were later developed by Biogen for PD/cancer (Weiss et al. 2003; Gillespie et al. 2002a, 2008b). From this study, it emerged that the 9-benzyl substitution of **78** and **79** was the reason for their A_{2A}/A_1 selectivity, compared to the urea moiety of VER6947 **(80)** and VER7835 **(81)** in the same position. Gillespie et al. has reported the triazolo[4,5-*d*]pyrimidine **82** (vipadenant) as an A_{2A}AR antagonist in which the benzyl group was maintained as in compounds **78** and **79** (Gillespie et al. 2002b, 2009a; Guckian and Kumaravel 2011; Bamford et al. 2009). Vipadenant showed clinical efficacy in Phase 1/2 trials, both alone and in combination with L-DOPA (Pinna 2014).

5.3.2.3 Triazolotriazines/Triazolopyrimidines/Triazolopyridazines

Biaryl cores structurally related to the A_{2A}AR antagonist ZM241385 (**84,** Fig. 5.10), a simplified bicyclic analog of the PTPs, were extensively investigated by Biogen with the aim to improve oral bioavailability, metabolic stability, blood-brain barrier penetration, and in vivo efficacy of the parent compound (Vu et al. 2004a). Indeed, in view of the positive effects upon introducing a piperazine moiety in tricyclic A_{2A}AR antagonists (see preladenant, Fig. 5.6), the company developed [1,2,4]triazolo[1,5-*a*][1,3,5]triazines bearing the benzylpiperazine chain, such as compound **85a** (Fig. 5.10) (Vu et al. 2004b). Within this series, the SAR analysis disclosed that a methylene spacer between the distal (hetero)aryl ring and piperazine was favored over direct substitution or longer chains in the binding site. Moreover, substitution of the (hetero)aryl nucleus with electron-withdrawing groups, mainly fluorine, enhanced both affinity and selectivity against the A_{2B}AR subtype. Replacement of the furan moiety with substituted-phenyl rings or aza-heterocycles led to derivatives that, although well tolerated in terms of in vitro binding profile, resulted in lower in vivo activity in most cases.

The subsequent introduction of a flexible alkylamino spacer between triazolotriazine core and a piperazine ring resulted in compound **85b** with enhanced in vivo potency (Vu et al. 2004c). Constriction of the piperazine moiety into a rigid bicyclic structure was performed to improve the absorption, distribution, metabolism, and excretion (ADME) profile of the synthesized compounds (Zúñiga-Ramírez and Micheli 2013). Applying this strategy, some compounds with subnanomolar potency and outstanding A_{2A} vs. A_1AR selectivity were identified, for example, the octahydropyridopyrazine **86a.** However, a discrepancy between affinity, in vivo potency, and metabolic stability was still observed.

In the triazolo[1,5-*a*]-1,3,5-triazine family, the 5-aminomethylcyclohexylmethan aminium derivative **86b** (K_i = 16.9 nM) is a potent and water-soluble A_{2A}AR antagonist whose A_{2A}AR selectivity was evaluated against the full panel of the other AR subtypes (Federico et al. 2011).

The bicyclic bioisosteres of the triazolotriazine template, such as triazolo[1,5-*a*]pyrimidines (Peng et al. 2004; Vu et al. 2004a), 1,2,4-triazolo[1,5-*a*]pyrazines (Yao et al. 2005), pyrazolo[3,4-*d*]pyrimidines (Chebib et al. 2000; Gillespie et al. 2008b), pyrazolo[4,3-*d*]pyrimidines (Squarcialupi et al. 2014), and pyrrolo[2,3-*d*]pyrimidines (Gillespie et al. 2008b), were evaluated as A_{2A}AR antagonists. They showed a general decrease in binding affinity and/or selectivity in comparison with the parent scaffold, while triazolo[1,5-*c*]pyrimidine **87** developed by Schering-Plough showed an acceptable in vitro profile (Neustadt et al. 2009).

More recently, Falsini and co-workers applied a molecular simplification approach to the previously reported 1,2,4-triazolo[4,3-*a*]quinoxalin-1-one series to identify the 1,2,4-triazolo[4,3-*a*]pyrazin-3-one bicyclic nucleus for A_{2A}AR antagonists (Falsini et al. 2017). The new chemotype resulted from different substituents on the 2-phenyl ring and small para-alkoxy substituents on the 6-phenyl nucleus. Several synthesized compounds showed nanomolar affinities (K_i = 2.9 − 10 nM) and interesting selectivities for the target. Among these, compound **88** demonstrated

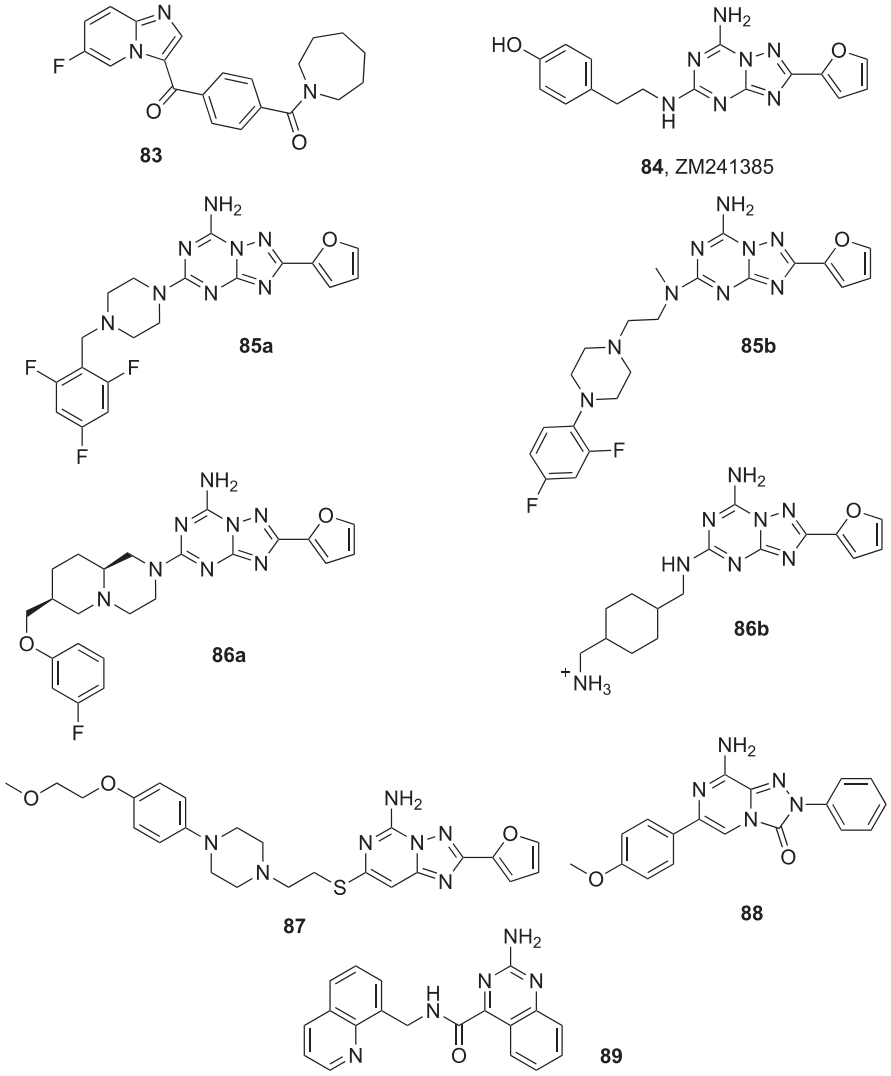


Fig. 5.10 Imidazopyridines and triazolotriazines/triazolopyrimidines/triazolopyridazines and aminoquinazolines as A_{2A}AR antagonists

an ability to counteract MPP^+-induced neurotoxicity in a cultured human neuroblastoma SH-SY5Y cell based on an in vitro Parkinson's disease model (Falsini et al. 2017).

Aminoquinazoline derivatives, e.g., **89**, identified through library screening and subsequent structural optimization based on a pharmacophore model, were found to be potent A_{2A}AR antagonists that were active in the rat catalepsy model (Zhou et al. 2016).

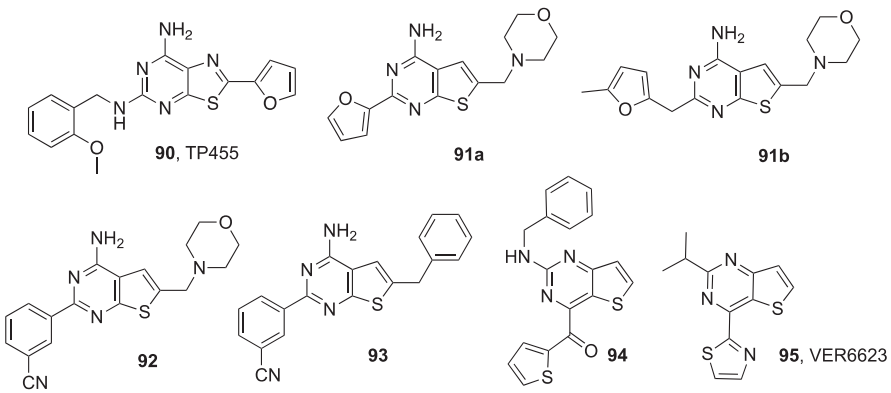


Fig. 5.11 Thiazolo[5,4-d]pyrimidines and thienopyrimidines as A_{2A}AR antagonists

5.3.2.4 Thiazolopyrimidines and Thienopyrimidines

A [1,3]thiazolo[5,4-*d*]pyrimidine-5,7-diamine, TP455 **90** (Fig. 5.11), was reported as a selective A_{2A}AR antagonist for application to cancer (Gessi et al. 2017).

A novel series of aminomethyl-substituted thieno[2,3-*d*]pyrimidines as A_{2A}AR antagonists, represented by compound **91a**, was developed in J&J laboratories (Shook et al. 2011; Barbay et al. 2010a, b; Chakravarty and Shook 2010). This compound was functionalized with a free amino group and a furan ring at the 4- and 2-positions, respectively, sharing structural similarities with some purines/triazolotriazines. In this series, bulky cycloalkyl amines with a relatively low basicity at the 6-position of the thienopyrimidine nucleus were associated to a higher capability to cross the blood-brain barrier.

In a related effort, several substitutions at the 2- and 6-positions of thieno[2,3-*d*] pyrimidine scaffolds maintaining free amino group at 5-position were evaluated. The 2-substituted-5-methylfuran derivative **91b** is an ideal A_{2A}AR antagonist, with optimal in vitro and in vivo potency (Shook et al. 2011).

The replacement of the furan ring with different aryl moieties resulted in the identification of 6-phenyl derivatives A_{2A}AR antagonists, such as **92**, in which a *m*-CN group was essential for the maintenance of in vitro affinity and in vivo potency (Shook et al. 2013; Barbay et al. 2010b). More recently, the effect of alternative 6-arylalkyl substitutions has been explored, such as in **93**. Although active in vivo after a single oral dose of 3 mg/kg (ED_{50} < 1 mg/kg), this benzyl derivative suffered from poor selectivity versus the A_1AR subtype and a short duration of action.

The Vernalis group explored the potential of thieno[3,2-*d*]pyrimidine isomers as A_{2A}AR antagonists, as represented by **94** (Fig. 5.11) (Gillespie et al. 2002c, 2008b). The subsequent optimization resulted in the identification of VER6623 (**95**), characterized by notable A_{2A}AR binding affinity with moderate selectivity over A_1AR and poor oral bioavailability (Yang et al. 2007).

5.3.2.5 Benzofurans

The identification of the benzofuran hit **96** (Fig. 5.12), characterized by micromolar affinity for the A_{2A}AR and about 50% inhibition of CGS21680-mediated catalepsy in vivo at 10 mg/kg po, was described by the Kyowa Hakko group following a high-throughput screen of their chemical library (Saku et al. 2010a).

The SAR studies in the benzofuran series uncovered some important factors for the design of potent A_{2A}AR antagonists, by identifying **97**–**99** as interesting compounds with in vivo efficacy. The replacement of the methoxycarbonyl functionality in **96** with a phenyl group at the 4-position of the benzofuran core, such as in **97**, enhanced A_{2A}AR binding affinity, as well as in vivo potency. However, the inversion of the amide function with arylcarbamate or arylurea moieties resulted in a general decrease of potency. In addition, the replacement of the 4-phenyl group of **98** with a heterocyclic ring improved the PK profile and aqueous solubility. This approach was also applied to compound **99**, which has both good oral bioavailability and in vivo efficacy on motor disability in MPTP-treated common marmosets (Saku et al. 2010b). Furthermore, patent literature claimed that introduction of an additional amide function at the 3-position of the benzofuran core could be beneficial in terms of A_{2A}AR affinity as in compound **100** with subnanomolar potency (Nakamura et al. 2005). In the same structural series, compound **101** (Fig. 5.12) was claimed as an example of furo[2,3-*b*]pyridine series by Kissei Pharmaceutical (Shiohara et al. 2006).

5.3.2.6 Benzothiazoles

Roche company has claimed the benzo[*d*]thiazole skeleton as potent A_{2A}AR antagonist in several patents (Alanine et al. 2001; Flohr et al. 2003, 2005; Norcross 2005). The combination of the 4-methoxy, 7-phenyl or 7-morpholino, and 2-phenylcarboxamide or 2-urea substitutions of **102**–**104** (Fig. 5.13) undoubtedly shows structural analogy to the benzofuran derivatives **98** and **99** (Fig. 5.12, Norcross 2004).

The morpholine derivative **104**, also known as SYN115 and tozadenant (Norcross 2005), is the most investigated member of this class of A_{2A}AR antagonists for its in vitro/in vivo pharmacological and pharmacokinetic properties (de Lera et al. 2014; Pinna 2014; Perez-Lloret and Merello 2014). The bioisosteric replacement of the phenyl ring with six-membered heterocycles resulted in the thiazolo[5,4-*c*]pyridine **105** and the thiazolo[4,5-*d*]pyrimidine **106** with picomolar A_{2A}AR affinity and functional potency (Luthra et al. 2010).

5.3.2.7 Chromones

The virtual screening approach in the search for innovative 6,6-bicyclic templates for the development of AR ligands resulted in the identification of chromone-based hit compounds, whose SAR optimization led to potent and selective A_{2A}AR antagonists, such as derivative **107** (Fig. 5.13, Langmead et al. 2012). Despite the presence

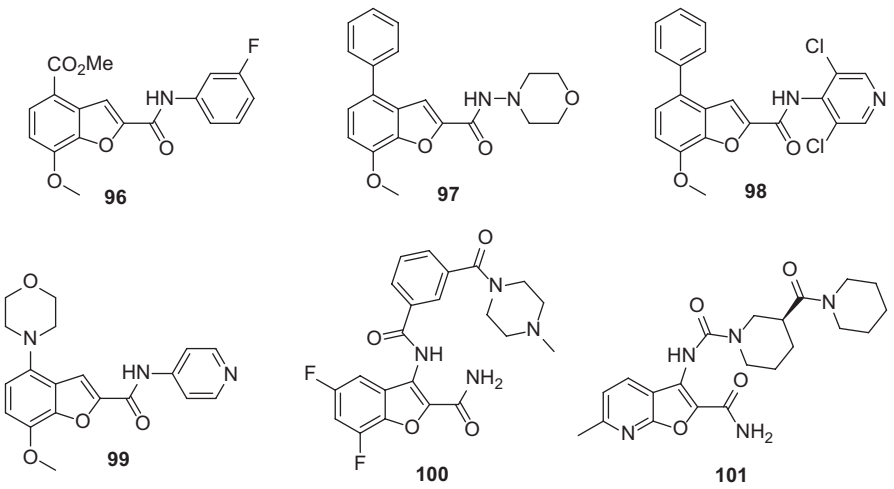


Fig. 5.12 Benzofurans as A_{2A}AR antagonists

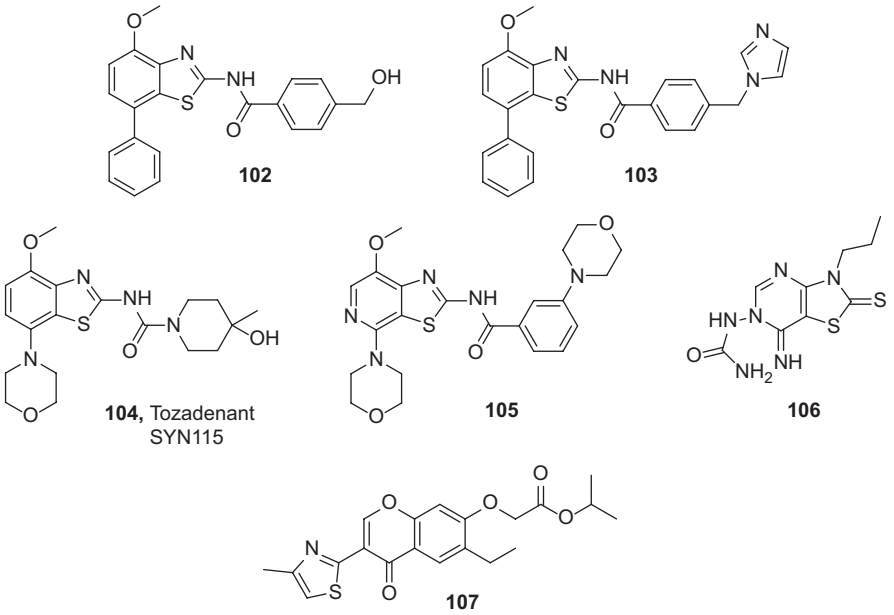


Fig. 5.13 Benzothiazoles and chromones as A_{2A}AR antagonists

of a thiazole ring that has been suggested as a possible source of reactive metabolites, the chromone 6,6-bicycle represents a novel and unexplored chemotype for the design of A_{2A}AR antagonists.

5.3.3 Monocyclic Systems

5.3.3.1 Pyridines and Pyrimidines

A series of 2-amino-6-(furan-2-yl)-4-substituted nicotinonitriles was presented by the group of IJzerman from Leiden University (compounds **108** and **109**, Fig. 5.14) (Mantri et al. 2008). In particular, these compounds were designed using a pharmacophore model based on molecular superimposition of previously known A_{2A}AR nonxanthine antagonists. Several compounds with low nanomolar affinity for the A_{2A}AR were identified by the introduction of combination of (hetero)aromatic ring systems at the 4- and 6-positions of the central monocyclic core. SAR studies indicated that a five-membered heterocycle with an H-bond accepting heteroatom (i.e., furan) is preferred over a phenyl ring at the 6-position.

Compound **108** bearing two furan rings exhibited the highest affinity for the A_{2A}AR subtype (K_i = 1.0 nM) with 12- and 34-fold selectivity over the A_1AR and A_{2B}AR, respectively. The introduction of more stable moieties instead of the metabolically reactive furan ring led to decreased A_{2A}AR affinity. Remarkably, some derivatives, such as **109** (Fig. 5.14), with higher affinity toward A_1AR than A_{2A}AR have been identified. Meanwhile, a series of water-soluble pyrimidine-acetamide derivatives as A_{2A}AR antagonists was investigated by researchers from Almirall and Neurocrine Biosciences (Slee et al. 2008c; Chen et al. 2008; Lanier et al. 2008). The original substitution pattern of the pyrimidine nucleus was represented by compound **110** in which the acylation of the amino group at the 4-position of pyrimidine nucleus demonstrated to improve A_{2A} vs. A_1AR selectivity and also the water solubility by induction of a piperazine moiety. Dimethylation of the pyrazole ring (compound **111**, Fig. 5.14) significantly favored the selectivity profile. Because of poor metabolic stability, the monosubstituted furyl moiety at 2-position was initially replaced with different heterocycles (pyridine, thiazole, oxazole, 5-methylfuran) (Slee et al. 2008a). In particular, the pharmacokinetic profile was improved when pyrimidine was substituted at the 2-position with a 5-methylfuran (**112**, **113,** Fig. 5.14) or with a 2-thiazolyl moiety (**114**, **115,** Fig. 5.14). The best level of affinity and selectivity for the hA_{2A}AR was achieved with a dimethylpyrazole at the 6-position combined with a 5-methylfuran, such as in compound **115** (Slee et al. 2008a).

Consequently, introduction of a 4-acetylamino function combined with several (hetero)aryl substitutions at the 2- and 6-positions, such as TC-G 1004 **116** (Fig.5.14), showed an equipotent activity in binding at h and rA_{2A}ARs and displayed good efficacy in different animal models of PD (Zhang et al. 2008). Further attempts to improve drug-like properties included the replacement of pyridine with aliphatic

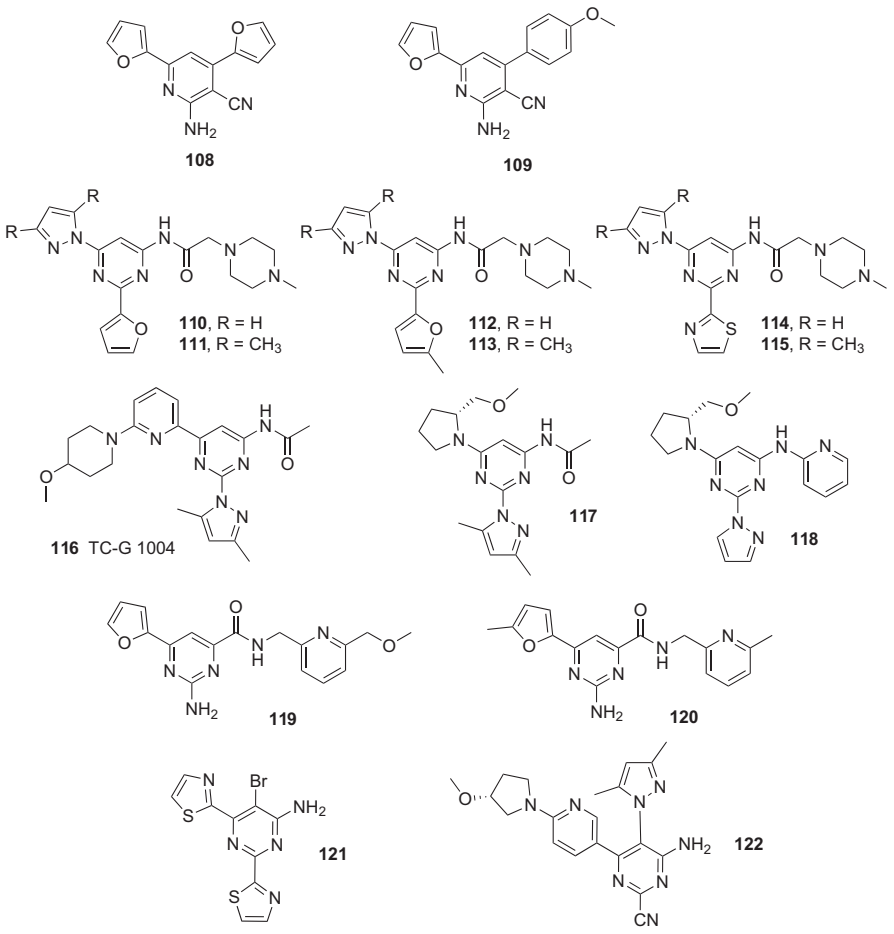


Fig. 5.14 Pyridines and pyrimidines as A_{2A}AR antagonists

(cyclo)amines, such as in the pyrrolidine derivative **117** (Fig. 5.14) (Lanier et al. 2009), which displayed an acceptable pharmacokinetic profile and good in vitro/in vivo potency but low selectivity versus hA_3AR.

Subsequently, the bioisosteric replacement of the 4-acetamide function in order to improve the chemical and metabolic instability (Zheng et al. 2014; Yang et al. 2014) was investigated by the same authors. The pyridine derivative **118** was the most effective in enhancing hA_{2A}AR binding affinity displaying a subnanomolar K_i value (Zheng et al. 2014).

The pyrimidine nucleus as a promising source of A_{2A}AR antagonists was confirmed through research performed parallel by the Vernalis group (Gillespie et al. 2009c, 2009b). From this study, compounds **119** and **120** (Fig. 5.14), bearing a carboxamide function at the 4-position, a 2-furyl ring (**119**) or the more stable 5-methyl-2-furyl group (**120**) at the 6-position, were prepared. A free amino group at the

2-position also improved the physicochemical and pharmacokinetic properties of carboxamides **119** and **120**, resulting in very promising in vivo activity (0.1 and 1 mg/kg, respectively). Subsequently, the 5-bromo-2,6-di(thiazol-2-yl)pyrimidine-4-amine **121** was claimed by Palobiofarma (Spain) as an A_{2A}AR antagonist with a K_i value of 1 nM in an A_{2A}AR binding assay and a K_i value of 12 nM in a cAMP assay (Camacho Gomez and Castro-Palomino Laria 2014).

More recently, a new series of A_{2A}AR antagonists based on a 4-amino-5-carbonitrile pyrimidine template was reported by Zheng and coworkers (Yang et al. 2016). Several compounds from this series exhibited good potency and ligand efficiency with low cytochrome P450 inhibition. One example is compound **122** bearing a pyrimidine core decorated with a 3,5-dimethylpyrazole and a free amino group at the 5- and 4-positions, respectively, combined with a carbonitrile and a substituted pyridine ring at the 2- and 6-positions, respectively (Yang et al. 2016).

5.3.3.2 Triazines

A new series of A_{2A}AR antagonists characterized by an atypical triazine scaffold was developed by Heptares Therapeutics (Langmead et al. 2012). Initial study focused attention on the 1,3,5-isomers **123** and **124** (Fig.5.15) endowed with high affinity for the target but only moderate selectivity over the A_1AR subtype. An independent, receptor-based approach confirmed the potential of this cluster (Carlsson et al. 2012). The proposed binding mode guided the optimization to 1,2,4-triazine isomers to be better accommodated by the receptor domain that is involved in the interaction with the ribose moiety of the endogenous ligand. Compounds **125a–125c** resulted from an innovative X-ray structure-directed optimization based on

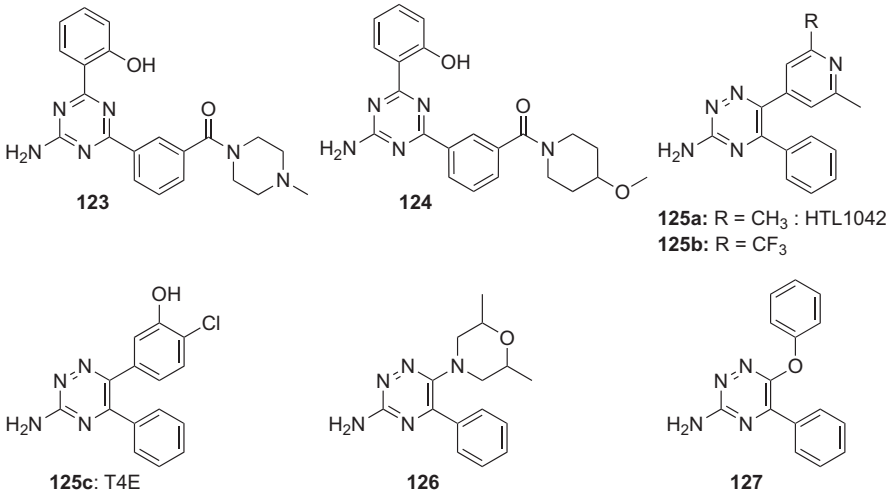


Fig. 5.15 Triazines as A_{2A}AR antagonists

co-crystallization of representative molecules of this class in the receptor (Congreve et al. 2012).

The SAR profile of triazines, also claimed in the related patent (Congreve et al. 2011), indicated that an unsubstituted 5-phenyl ring would be preferred to increase A_{2A}AR affinity, while the replacement of the 6-aryl nucleus with a substituted morpholino (**126**) or a phenoxy group (**127**) significantly improved selectivity, although with decreased A_{2A}AR affinity.

5.3.3.3 Five-Membered Heterocycles

A virtual screening based on the crystal structure of the hA_{2A}AR suggested the triazole-based **128** and **129** (Fig. 5.16) as unusual chemotypes for the development of selective antagonists with high ligand efficacy (Carlsson et al. 2012). Moreover, thiazoles and oxazoles were claimed as A_{2A}AR antagonists (Kase and Kanda 2011; Nell et al. 2009), and compound **130** showed the best pharmacological profile both in terms of affinity for A_{2A}AR and selectivity against the other AR subtypes.

Prodrugs of A_{2A}AR antagonists were designed in the five-membered heterocycle series to overcome the problem of water solubility. The corresponding phosphono-oxymethylene prodrug **131** (LuAA47070) was fully converted to **130** in vivo to liberate the free drug, which efficiently reversed the motor and motivational effects of the D2 receptor antagonists pimozide and haloperidol in in vivo rodent models of PD.

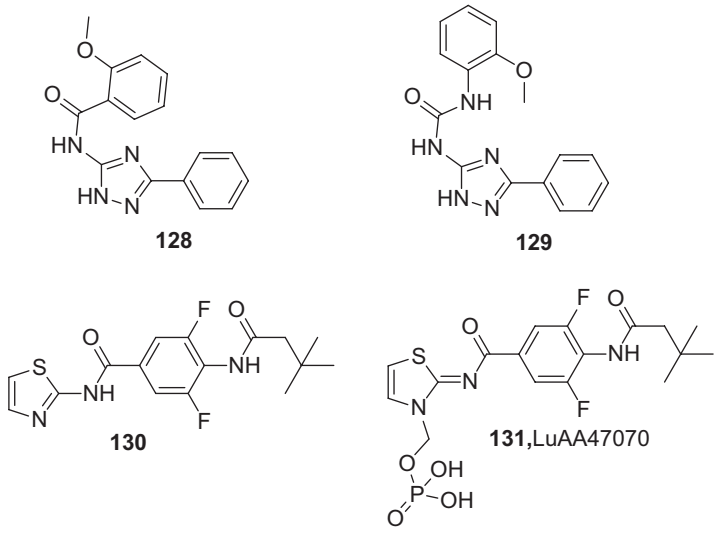


Fig. 5.16 Five-membered heterocycles as A_{2A}AR antagonists

5.4 Clinical Applications of A_{2A}AR Ligands

Adenosine (iv. as Adenoscan) **1** itself and Lexiscan **13** (Regadenoson, CVT-3146, Clinicaltrial.gov identifier NCT01710254) are approved for myocardial perfusion imaging (Al Jaroudi and Iskandrian 2009), by inducing coronary vasodilation through A_{2A}AR activation. A_{2A} agonist apadenoson (**24**, Stedivaze, BMS068645, ATL146e, Rieger et al. 2001) has been in several (e.g., Phase 3, Forest Laboratories, NCT00162084 and NCT00990327) clinical trials for myocardial perfusion imaging using SPECT imaging. Pfizer (formerly King Pharmaceuticals) also completed two Phase 3 trials of binodenoson (**15**, WRC-0470, MRE-0470, NCT00944294 and NCT00944970) in assessing cardiac ischemia using myocardial perfusion imaging. However, the clinical development of these agent for use in myocardial perfusion imaging was discontinued.

A_{2A}AR agonist BVT.115959 **9** has been in Phase 2 testing by Biovitrum to treat diabetic neuropathic pain depending on a low pH near the injured tissue (NCT00452777). Related to the use of A_{2A}AR agonists in COPD, UK-432097 **28** failed to show efficacy in a clinical trial (Mantell et al. 2009; NCT00430300), and GW-328267X **29b** has been tested for tolerability upon infusion in healthy subjects (NCT01640990). Regadenoson **13** has been tested in sickle cell anemia (Field et al. 2013; Phase 2, NCT01788631 and NCT01788631). A_{2A}AR agonist ATL-370 **23b** might be useful in treating sepsis from *Clostridium difficile* infection (Li et al. 2012). A_{2A}AR agonists may be useful in promoting wound healing (Montesinos et al. 2015) and could help treat Niemann-Pick disease type C1 (Visentin et al. 2013). A_{2A}AR activation at the blood-brain barrier might be useful in delivery of drugs to the brain (Kim and Bynoe 2016).

[^{11}C]SCH442416 **44** and other A_{2A}AR antagonists containing positron emitting isotopes have been used in PET imaging, particularly to determine striatal receptor occupancy by A_{2A}AR antagonists (van Waarde et al. 2018). In the striatum, dopamine D_2 receptors and the A_{2A}AR have opposing effects, which has led to the concept of modulating this receptor for treating movement disorders. Clinical trials of A_{2A} antagonists performed to test their efficacy in Parkinson's disease (PD) include istradefylline (**70**, KW6002, Kyowa Hakko, Phase 3, and other trials) and preladenant (**51a**, SCH420814, Schering-Plough, Phase 3, NCT01155479). Also, tozadenant **104** (SYN115, NCT02453386) and vipadenant (BIIB014, NCT00438607) **82** were tested in PD in combination with L-dopa. In addition to Parkinson's disease, other neurodegenerative conditions might benefit from A_{2A}AR antagonists (Orr et al. 2018). However, one recent study found that lack of the receptor was associated with cognitive impairment from a reduction of neurogenesis (Moscoso-Castro et al. 2017).

A_{2A} antagonists, or alternatively CD73 inhibitors, have recently become a target for oncology (Hatfield and Sitkovsky 2016; Young et al. 2014). Levels of adenosine in the tumor microenvironment are greatly elevated, which act on T cells to reduce their ability to attack a tumor (Ohta et al. 2006; Mediavilla-Varela et al. 2017). Clinical trials that combine A_{2A} antagonists with cancer immunotherapy in non-small cell lung cancer include PBF 509 (Palobiofarma/Novartis, structure not dis-

closed, NCT02403193), in combination with PDR001 (Novartis), and AZD4635/HTL-1071 (Astra-Zeneca/Sosei, structure not disclosed, NCT02740985), in combination with durvalumab (Astra-Zeneca). Also, CPI-444 (Corvus Pharmaceuticals, structure not disclosed, NCT02655822) has been in a trial for various cancers, in combination with Tecentriq (Roche). NIR178 (Novartis, structure not disclosed, NCT03207867) has been in a trial for solid tumors and non-Hodgkin lymphoma.

5.5 Conclusions

In conclusion, many agonists and antagonists of the A_{2A}AR have been reported, while allosteric modulators of this receptor are still needed. Many heterocyclic chemotypes have been discovered as A_{2A}AR antagonists, while most of the known agonists are nucleosides or dicyanopyridine derivatives. A few A_{2A}AR ligands have been in clinical trials as antihypertensives, anti-inflammatory or diagnostic compounds (agonists), and drugs for treating PD and cancer (antagonists). The A_{2A}AR has become one of the most widely investigated GPCR structures using biophysical techniques. Thus, the design of agonists, antagonists, and allosteric modulators has become structure-based, with numerous examples of in silico approaches (VLS) to the discovery of novel ligands.

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Chapter 6
Medicinal Chemistry of A_{2B} Adenosine Receptors

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Abstract A_{2B} adenosine receptors (A_{2B}ARs) are in the focus of interest as drug targets in (immuno)oncology since antagonists show anti-proliferative, anti-angiogenic, anti-metastatic, and immunostimulatory properties. Additional (potential) indications for A_{2B}AR antagonists include inflammatory (pulmonary, colon) and autoimmune diseases, pain, fibrosis, infectious diseases, diabetes, and more. Agonists were found to exhibit cardioprotective properties. The A_{2B}AR is most closely related to the A_{2A}AR subtype. Both are G_s protein-coupled receptors, but the A_{2B}AR is additionally coupled to G_q proteins. A_{2B}AR expression is upregulated under pathological conditions (hypoxia, inflammation, ischemia) and on many cancer cells. A_{2B}ARs form stable heteromeric complexes with A_{2A}ARs when co-expressed, and thereby completely block A_{2A}AR signaling. There is still a lack of potent, selective, and fully efficacious A_{2B}AR agonists, while structurally diverse potent and selective competitive antagonists for A_{2B}ARs have become available. The first positive and negative allosteric modulators for A_{2B}ARs were recently described. For the labeling of A_{2B}ARs, antagonist radioligands have been developed, and recently the first potent and selective fluorescent ligands were reported.

Keywords A_{2B} adenosine receptor · Agonist · Antagonist · Cancer · Inflammation · Structure

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6.1 Introduction

The G protein-coupled adenosine receptors (ARs) were initially divided into two subtypes, one that inhibited adenylate cyclase (AC), later termed A_1AR, and the other one that activated AC, designated A_2AR. Based on studies in brain slices, John Daly proposed the existence of two distinct A_2AR subtypes, a low-affinity receptor, which he named A_{2B}, besides the high-affinity receptor, designated A_{2A} (Daly et al. 1983). The existence of the A_{2B}AR was unambiguously confirmed a decade later by the cloning of the receptor (Pierce et al. 1992; Stehle et al. 1992). The A_3AR subtype, a second G_i protein-coupled subtype besides A_1, was the latest AR subtype to be discovered (Ali et al. 1990; Zhou et al. 1992).

The A_{2B}AR is ubiquitously expressed, but under normal conditions it is found mostly in low density. Higher expression levels are observed in the large intestine, in mast cells, and in hematopoietic cells (myeloid, dendritic cells). A_{2B} expression is highly regulated by transcription factors, in particular by hypoxia-inducible factor (HIF) 1α, and is upregulated under hypoxic, ischemic, and inflammatory conditions, as well as on many cancer cells. While the closely related, well-investigated A_{2A}AR subtype is activated by nanomolar concentrations of extracellular adenosine, the A_{2B}AR is only activated by high, typically micromolar concentrations of adenosine (see Fig. 6.1). Such high concentrations can be observed under pathological conditions, e.g., after cell death or under hypoxic conditions, where an up to 100-fold increase in extracellular adenosine levels has been described – from basal levels of around 100 nM reaching concentrations of up to 10 μM (Fredholm et al. 2001, 2011; Müller and Stein 1996).

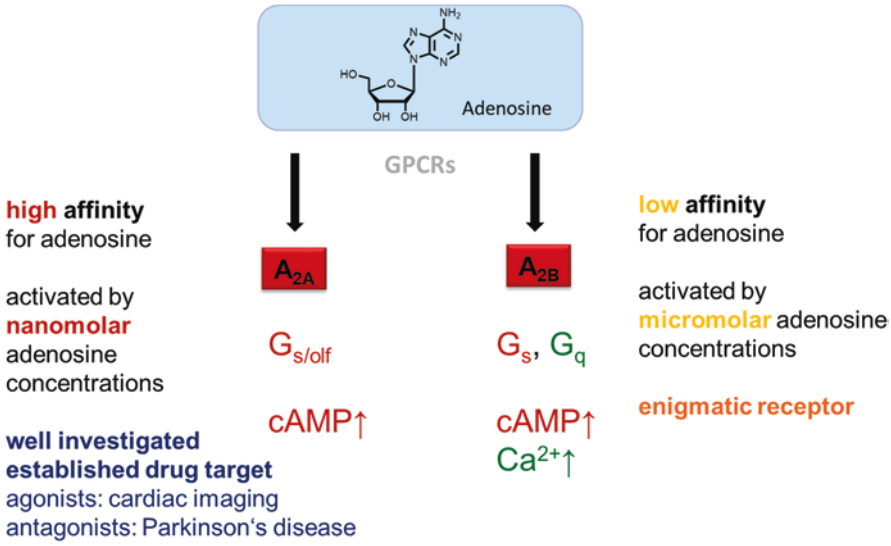

Fig. 6.1 A_{2A} and A_{2B} adenosine receptors

Adenosine is released from cells, e.g., by adenosine transporters (Köse and Schiedel 2009), or produced extracellularly by ectonucleotidases from nucleotides such as ATP, ADP, and AMP (Zimmermann et al. 2012). The concerted upregulation of A_{2B}ARs and an increase in adenosine levels under pathological conditions can lead to robust activation of A_{2B}ARs. In a healthy state, however, A_{2B}ARs may be mostly silent (Beukers et al. 2004a). Therefore, A_{2B}ARs should be ideal drug targets.

Like the A_{2A}AR subtype, the A_{2B}AR is preferentially coupled to G_s proteins, mediating a stimulation of adenylate cyclase and an increase in intracellular cAMP levels (Fredholm et al. 2011). The produced cAMP can be released from the cells and hydrolyzed by nucleotide pyrophosphatases/phosphodiesterases (NPPs) producing AMP, which is further dephosphorylated by ecto-5′-nucleotidase (CD73) yielding adenosine that can again activate ARs. This is a self-reinforcing mechanism as described (Pleli et al. 2018; Sassi et al. 2014).

In contrast to the A_{2A}AR, the A_{2B}AR can additionally couple to G_q proteins in different tissues and cancer cells, which results in phospholipase C activation leading to mobilization of intracellular calcium (see Fig. 6.1) (Linden et al. 1999; Panjehpour et al. 2005; Gao et al. 2017). G_i protein coupling of the A_{2B}AR under certain conditions has also been described to occur in a human urinary bladder epithelial cancer cell line (T24) (Gao et al. 2017). A_{2B}-mediated ERK1/ERK2 phosphorylation has been observed in several cell types, presumably mediated via G proteins. Coupling of the A_{2B}AR can be different depending on the cellular context (Gao et al. 2017).

6.2 A_{2B} Adenosine Receptor Structure

6.2.1 Homology Modeling Based on A_{2A} Adenosine X-Ray Structures

Crystal structures for the human A_1- and A_{2A}AR subtypes have recently become available (for an overview, see Carpenter and Lebon 2017); however, a crystal structure of the A_{2B}AR is still lacking. The A_{2A}AR is most closely related to the A_{2B}AR, both A_2 receptor subtypes sharing an overall sequence identity of 58% and a similarity of 73% (see Fig. 6.2. for receptor alignment) (De Filippo et al. 2016). Several crystal structures of the A_{2A}AR in the agonist-bound active receptor state (in complex with adenosine (**1**), NECA (**3**), and other adenosine derivatives) and in the antagonist-bound inactive receptor state (in complex with caffeine (**8**), and theophylline (**9**), and ZM241385 (**27**), respectively) (Lebon et al. 2011; Lebon et al. 2015; Xu et al. 2011; Doré et al. 2011; Jaakola et al. 2008) have been published by different laboratories, including one structure with a very high resolution of 1.8 Å (Liu et al. 2012).

The orthosteric binding site is located within the helical core of the receptor, but it has been shown that the topology of the extracellular loops, especially the

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SP|P30542|AA1R_HUMAN  ---MPPSISAFQAAYIGIEVLIALVSVPGNVLVIWAVKVNQALRDATFCFIVSLAVADVA 57
SP|P29274|AA2AR_HUMAN ------MPIMGSSVYITVELAIAVLAILGNVLVCWAVWLNSNLQNVTNYFVVSLAAADIA 54
SP|P29275|AA2BR_HUMAN -----MLLETQDALYVALELVIAALSVAGNVLVCAAVGTANTLQTPTNYFLVSLAAADVA 55
SP|P0DMS8|AA3R_HUMAN  MPNNSTALSLANVTYITMEIFIGLCAIVGNVLVICVVKLNPSLQTTTFYFIVSLALADIA 60

SP|P30542|AA1R_HUMAN  VGALVIPLAILINIGPQTYFHTCLMVACPVLILTQSSILALLAIAVDRYLRVKIPLRYKM 117
SP|P29274|AA2AR_HUMAN VGVLAIPFAITISTGFCAACHGCLFIACFVLVLTQSSIFSLLAIAIDRYIAIRIPLRYNG 114
SP|P29275|AA2BR_HUMAN VGLFAIPFAITISLGFCTDFYGCLFLACFVLVLTQSSIFSLLAVAVDRYLAICVPLRYKS 115
SP|P0DMS8|AA3R_HUMAN  VGVLVMPLAIVVSLGITIHFYSCLFMTCLLLIFTHASIMSLLAIAVDRYLRVKLTVRYKR 120

SP|P30542|AA1R_HUMAN  VVTPRRAAVAIAGCWILSFVVGLTPMFGWNNLSAVER----AW---AANGSMGEPVIKCE 170
SP|P29274|AA2AR_HUMAN LVTGTRAKGIIAICWVLSFAIGLTPMLGWNNC-------GQPKEGKNHSQGCGEGQVACL 167
SP|P29275|AA2BR_HUMAN LVTGTRARGVIAVLWVLAFGIGLTPFLGWNSKDSATNNCTEPWDGTTNESC---CLVKCL 172
SP|P0DMS8|AA3R_HUMAN  VTTHRRIWLALGLCWLVSFLVGLTPMFGWNMKLTSEY-------------HRNVTFLSCQ 167

SP|P30542|AA1R_HUMAN  FEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFYLIRKQLNKKVSAS--SGDPQKYYGK 228
SP|P29274|AA2AR_HUMAN FEDVVPMNYMVYFNFFACVLVPLLLMLGVYLRIFLAARRQLKQMESQPLPGERARSTLQK 227
SP|P29275|AA2BR_HUMAN FENVVPMSYMVYFNFFGCVLPPLLIMLVIYIKIFLVACRQLQRTEL----MDHSRTTLQR 228
SP|P0DMS8|AA3R_HUMAN  FVSVMRMDYMVYFSFLTWIFIPLVVMCAIYLDIFYIIRNKLSLNLSN---SKETGAFYGR 224

SP|P30542|AA1R_HUMAN  ELKIAKSLALILFLFALSWLPLHILNCITLFCPSC--HKPSILTYIAIFLTHGNSAMNPI 286
SP|P29274|AA2AR_HUMAN EVHAAKSLAIIVGLFALCWLPLHIINCFTFFCPDC-SHAPLWLMYLAIVLSHTNSVVNPF 286
SP|P29275|AA2BR_HUMAN EIHAAKSLAMIVGIFALCWLPVHAVNCVTLFQPAQGKNKPKWAMNMAILLSHANSVVNPI 288
SP|P0DMS8|AA3R_HUMAN  EFKTAKSLFLVLFLFALSWLPLSIINCIIYFNG----EVPQLVLYMGILLSHANSMMNPI 280

SP|P30542|AA1R_HUMAN  VYAFRIQKFRVTFLKIWNDHFRCQPAPPIDEDLPEER---------------------- 323
SP|P29274|AA2AR_HUMAN IYAYRIREFRQTFRKIIRSHVLRQQEPFKAAGTSARVLAAHGSDGEQVSLRLNGHPPGVW 346
SP|P29275|AA2BR_HUMAN VYAYRNRDFRYTFHKIISRYLLCQADVKSGNGQA----------GVQPALGVGL------ 332
SP|P0DMS8|AA3R_HUMAN  VYAYKIKKFKETYLLILKACVVCHPSDSLDTSIEKNSE---------------------- 318

SP|P30542|AA1R_HUMAN  -----------PDD---------------------------------------------- 326
SP|P29274|AA2AR_HUMAN ANGSAPHPERRPNGYALGLVSGGSAQESQGNTGLPDVELLSHELKGVCPEPPGLDDPLAQ 406
SP|P29275|AA2BR_HUMAN ------------------------------------------------------------
SP|P0DMS8|AA3R_HUMAN  ------------------------------------------------------------

SP|P30542|AA1R_HUMAN  ---------------
SP|P29274|AA2AR_HUMAN DGAGVS---------412
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Fig. 6.2 Sequence alignment of amino acid sequences of the human AR subtypes. Multiple sequence alignment of the AR subtypes obtained using ClustalO. For each sequence, the Uniprot accession number and the organism name is given. The amino acid residues within 6 Å from the ligand PSB-603 (**26**) which was docked into the orthosteric binding site (see below, Fig. 6.7) are highlighted in cyan, and among these residues, those that are unique for the A_{2B}AR are highlighted in yellow

extracellular loop 2, also plays an important role for ligand affinity and receptor activation (De Filippo et al. 2016; Jaakola et al. 2008). The binding site for the physiological agonist adenosine is virtually identical in both A_{2A} and A_{2B} receptor subtypes, as shown by homology modeling of the A_{2B}AR based on the A_{2A}AR crystal structures as templates (Sherbiny et al. 2009; Köse et al. 2018, and unpublished results) (Fig. 6.3). There is only a single, homologous, amino acid exchange, leucine L249 in the A_{2A}AR, for valine (V250) in the A_{2B}AR (see Fig. 6.2). This cannot explain the large affinity difference of adenosine for the receptors. Therefore, other parts of the receptors are probably involved. These could, for example, lead to conformational changes of the binding sites. Another explanation could be that adenosine interacts with an initial recognition site of the receptor proteins close to the extracellular layer of the cell membrane, for which Moro et al. (1999) had coined the term "*meta*-binding site," followed by sliding down into the orthosteric binding pocket. This *meta*-binding site and the channel leading to the orthosteric site would differ more substantially in both receptor subtypes than the actual orthosteric bind-

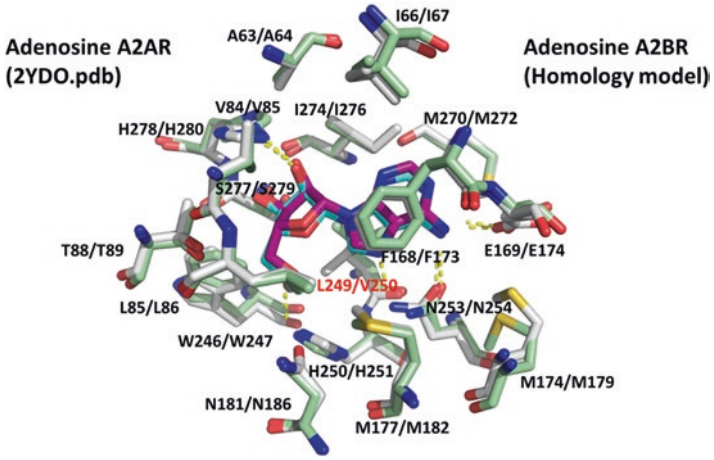

Fig. 6.3 Overlay of the adenosine binding site of the A_{2A} and the A_{2B}AR in complex with adenosine. A_{2A}- and A_{2B}AR have virtually identical binding sites for their cognate agonist adenosine differing only in a single, homologous amino acid exchange: leucine (L249) in the A_{2A}AR is exchanged for valine (V250) in the A_{2B}AR

ing site for adenosine. A third explanation could be that the A_{2B}AR is allosterically modulated by (an)other protein(s). The structural information that has recently become available will allow rational structure-based design of novel ligands (Floris et al. 2013; Jespers et al. 2017, 2018).

6.2.2 *Mutagenesis of the A_{2B} Adenosine Receptor*

A review on mutagenesis data of all AR subtypes and their analysis has been published recently (Jespers et al. 2017). The group of IJzerman pursued random and focused mutagenesis studies of the A_{2B}AR and used a *Saccharomyces cerevisiae* strain as a read-out system (Peeters et al. 2011, 2014; Liu et al. 2015, 2014; Beukers et al. 2004a, b) with the aim to identify amino acid residues and regions involved in ligand binding, receptor activation, and G protein coupling. They found that amino acids in the extracellular loop 1 (ECL1) were involved in A_{2B}AR activation presumably by stabilizing the tertiary structure of the receptor.

Both the A_{2B}- and the A_{2A}AR have a particularly cysteine-rich extracellular loop 2 (ECL2). While in the A_{2A}AR, all possible disulfide bonds in the ECL2 were formed according to X-ray crystallography and found to be required for high affinity and potency of adenosine (De Filippo et al. 2016; Goddard et al. 2010), the A_{2B}AR behaved differently. Only one disulfide bond (C3.25-C45.50, Ballesteros-Weinstein nomenclature), the disulfide bond that is conserved in most class A GPCRs, was found to be essential for high-affinity binding and receptor activation (Schiedel et al. 2011). This indicates that the nonessential cysteine residues in the

ECL2 of the A_{2B}AR may have different functions, e.g., interaction with other proteins. The largest differences between the A_{2A}- and the A_{2B}AR are found in the ECL2, which is much longer in the A_{2B}- as compared to the A_{2A}AR. Exchange of the whole ECL2 of the human A_{2B}AR for that of the A_{2A}AR revealed that the ECL2 determines subtype selectivity of ligands and controls receptor conformation and signaling efficacy (Seibt et al. 2013). Agonists were significantly more efficacious in the chimeric A_{2B}AR which contained the shorter ECL2 of the A_{2A}AR subtype, and an A_{2A}-selective agonist (CGS-21680) was able to activate the chimeric A_{2B}AR (Seibt et al. 2013). Deletion of the intracellular C-terminus of the A_{2B}AR prevented arrestin- and clathrin-dependent receptor internalization and recycling to the cell membrane, but dynamin-independent internalization was still possible (Mundell et al. 2010). A serine residue close to the C-terminus was identified to mediate rapid agonist-induced arrestin-dependent receptor desensitization and internalization (Matharu et al. 2001).

6.2.3 *Formation of Homo- and Heteromeric A_{2B} Adenosine Receptor Complexes*

It is now widely accepted that GPCRs can form homo- or heteromeric assemblies (Guidolin et al. 2015; Franco et al. 2016). Such receptor complexes may be formed transiently and modulated by receptor ligands, or they may constitute stable, long-lasting units. The formation of heteromeric complexes can have a significant impact on receptor pharmacology. The formation of homo- and heteromeric assemblies of A_{2A}ARs (e.g., with dopamine D_2 receptors) has been known for a long time (Borroto-Escuela et al. 2018), and A_{2A}/D_2 heteromers were shown to play a significant role in Parkinson's disease (Fuxe et al. 2003). Like all other AR subtypes, the A_{2B}AR was demonstrated to form homomeric complexes (Hinz, Müller et al., unpublished results). Furthermore, it was demonstrated by a variety of different techniques, including Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), bimolecular fluorescence complementation (BiFC), and proximity ligation (PLA) assays, that stable A_{2A}-A_{2B}AR heteromeric complexes are formed in cells and tissues that co-express both receptor subtypes (Hinz et al. 2018a). The formation of the heteromers was not dependent on the long C-terminal tail of the A_{2A}AR since truncation did not affect heteromerization and was also found to be independent of the presence of receptor agonists or antagonists. Co-expression of A_{2A} and A_{2B}ARs is observed in many different cell types and in various organs and tissues, e.g., in the heart (Chandrasekera et al. 2010), myeloid cells (Morello et al. 2016), T cells (Schiedel et al. 2013), blood platelets (Johnston-Cox and Ravid 2011), and brown and white adipocytes (Gnad et al. 2014), and in many tumors, e.g., prostate cancer (Allard et al. 2017; Sepúlveda et al. 2016). Surprisingly, a dramatically altered pharmacology of the A_{2A}AR was observed when co-expressed with the A_{2B}AR in recombinant as well as in native cells that expressed

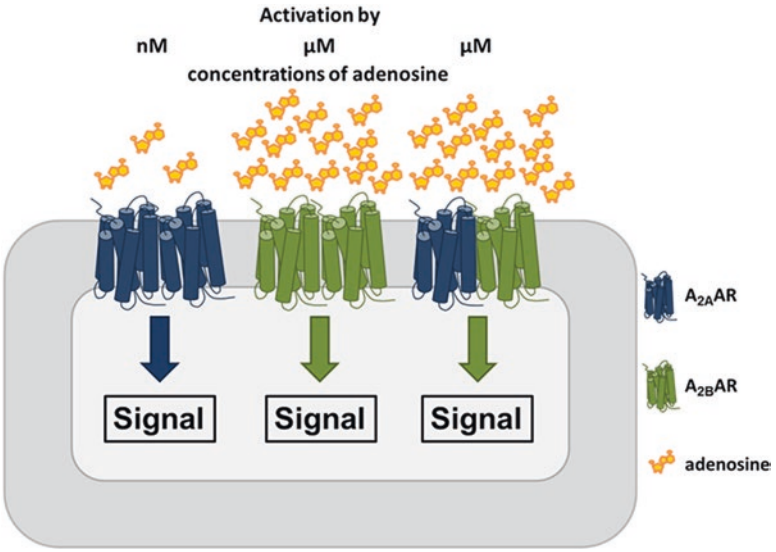

Fig. 6.4 A_{2A}- and A_{2B}AR homomeric and heteromeric receptors and their activation by adenosine. The A_{2A}-A_{2B} heteromeric receptors lose their high affinity for adenosine and behave similarly to the homomeric A_{2B}AR. (Hinz et al. 2018a)

more A_{2B} than A_{2A}ARs. In the presence of A_{2B}ARs, A_{2A}-selective ligands lost high-affinity binding to A_{2A}ARs and displayed strongly reduced potency in cAMP accumulation and dynamic mass redistribution (DMR) assays (Hinz et al. 2018a). A_{2B}ARs were thereby demonstrated to have a novel function – the blockade of A_{2A}ARs – and A_{2A}-A_{2B} heteromers may therefore represent novel pharmacological targets (see Fig. 6.4).

6.2.4 Modulation of A_{2B} Adenosine Receptors by Other Proteins

The A_{2B}AR can interact with other intra- or extracellular proteins, which may modulate receptor function. A close functional link between surface A_{2B}ARs and the adenosine-metabolizing enzyme adenosine deaminase (ADA) was found on cells of the immune system and in the gastrointestinal tract (Arin et al. 2015). Binding of ADA to A_{2B}ARs increased the affinity of the agonist NECA (**3**) as well as cAMP production induced by NECA (Herrera et al. 2001). ADA was reported to also interact with other AR subtypes (Gracia et al. 2013). Corset et al. (2000) reported that netrin-1-mediated axon outgrowth and cAMP production require its interaction with A_{2B}ARs. Moreover, actinin-1 was found to bind to the C-terminus of the A_{2B}AR and to thereby enhance its cell-surface expression (Sun et al. 2016).

6.3 A_{2B} Adenosine Receptor Ligands

Review articles have appeared which summarize previous developments in the field (Kalla et al. 2009; Ortore and Martinelli 2010; Müller and Jacobson 2011a, b). The current review will focus on the most important ligands for A_{2B}ARs and highlight recent developments.

6.3.1 A_{2B} Adenosine Receptor Agonists

The physiological agonist adenosine (**1**, Fig. 6.5) is moderately potent at the A_{2B}AR with EC_{50} values typically in the micromolar range (Table 6.1). Inosine (**2**), a metabolite of adenosine, has been reported to activate A_{2B}ARs at high concentrations (Doyle et al. 2017). It was also described to activate A_1- (Nascimento et al. 2015), A_{2A}- (Welihinda et al. 2018), and A_3ARs (Cinalli et al. 2013). The adenosine derivative NECA (**3**), which is metabolically more stable than adenosine, is a more potent, full A_{2B}AR agonist. However, it activates the three other AR subtypes with even higher potency and is therefore nonselective (see Table 6.1). An N^6-substituted NECA derivative, compound **4** (Fig. 6.5), developed by Baraldi et al. (2007) appears to display some A_{2B}-selectivity, but its true selectivity is difficult to assess since K_i values from binding studies at A_1, A_{2A}, and A_3ARs were compared with EC_{50} values

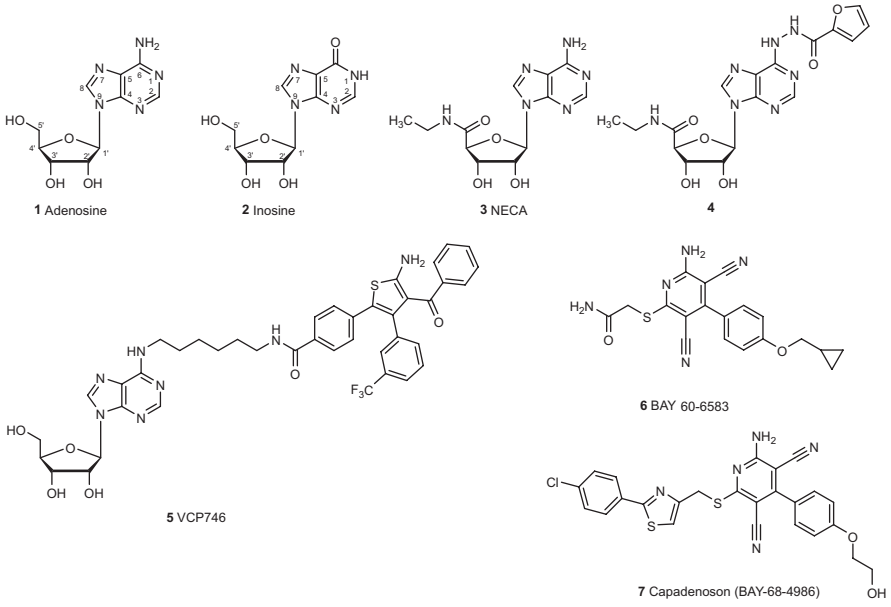

Fig. 6.5 Structures of nonselective and selective A_{2B}AR agonists

Table 6.1 Adenosine receptor affinities of nonselective and A_{2B}-selective agonists

		K_i (nM) from radioligand binding studies or EC_{50} values from functional assays (h = human, m = mouse)				
		A_1	A_{2A}	A_{2B}	A_3	References
1	Adenosine	310 (h)	700 (h)	24,000 (h)	290 (h)	Fredholm et al. (2001)
2	NECA	14 (h) 2.5 (m)	20 (h) 43 (m)	1900 (h) 660 (m)	25 (h) 13 (m)	Alnouri et al. (2015)
4	NECA derivative (furanyl-hydrazine)	1050 (h)	1550 (h)	82 (h)	>5000 (h)	Baraldi et al. (2007)
5	VCP746	nd[a]	nd	55 (h)	nd	Vecchio et al. (2016b)
6	BAY 60–6583	390 (h) 350 (m) (antagonist)	>10,000 (h) >10,000 (m)	110 (h) 140 (m)	220 (h) 3900 (m)	Alnouri et al. (2015)
7	Capadenoson (BAY68–4986)	0.66 (h)	1400 (h)	1.2 (h) (partial agonist)	240 (h) (partial agonist)	Baltos et al. (2017)

[a]nd = no data available

obtained in cAMP accumulation studies at A_{2B}ARs. Functional data are dependent on receptor expression levels and receptor reserve. Compound **4** was twofold more potent than NECA in that study (Baraldi et al. 2007). Only few other A_{2B}AR agonists, derivatives of the nucleoside adenosine, were reported by the group of Baraldi et al. (2007, 2009). Vecchio et al. (2016a, b) synthesized a hybrid molecule consisting of adenosine and an allosteric enhancer moiety for the A_1AR (**5**, VCP746) connected via an alkyl chain to the N^6-position of adenosine. The authors found that the compound activated A_{2B}ARs. In cAMP accumulation assays, it showed a similar A_{2B} affinity as NECA (**3**) and BAY 60–6583 (**6**), but while **3** and **6** were biased toward G_s signaling being weaker in activating G_q proteins, **5** appeared to be unbiased in that study performed in recombinant CHO cells. However, the selectivity of VCP746 versus the other AR subtypes is unknown. It may be suspected that **5** is a potent A_1AR agonist as well, since it had been designed for that purpose.

At present, the most selective A_{2B}AR agonist is the non-nucleoside-derived 2-aminopyridine-3,5-dicarbonitrile derivative BAY 60–6583 (**6**) which was characterized as a partial A_{2B} agonist (Müller and Jacobson 2011a, b; Goulding et al. 2018). It is similarly potent as NECA (**3**), but less efficacious (Hinz et al. 2014). At high levels of adenosine and low A_{2B}AR expression, **6** may therefore act as an antagonist and block the effects of adenosine (Hinz et al. 2014; Goulding et al. 2018). BAY 60–6583 did not activate the other AR subtypes; however, it was recently found to block A_1- (K_i 3.19 μM) and A_3ARs (K_i 5.63 μM) (Alnouri et al. 2015). In another study, **6** was reported to have shown off-target effects unrelated to A_{2B}AR activation (Borg et al. 2017). Although BAY 60–6583 is currently still the best available A_{2B}AR agonist, it has to be used with caution, and additional A_{2B} agonists as

well as selective antagonists should always be employed to confirm that the observed effects are due to A_{2B}AR activation. Beukers et al. (2004a, b) investigated the SARs of 2-aminopyridine-3,5-dicarbonitrile derivatives related to **6**; however, no selective A_{2B}AR agonists were identified. Betti et al. (2018) also synthesized analogs of **6** with the goal to improve A_{2B}AR affinity. They obtained a derivative which was threefold more potent in cAMP assays, but less efficacious and less selective versus the other AR subtypes than **6**. Recently, a related 2-aminopyridine-3,5-dicarbonitrile derivative which preferably activates the A_1AR, capadenoson (**7**), was reported to additionally activate A_{2B}ARs (Baltos et al. 2017) with a preference for G_s-coupled AC activation over G_q-coupled signaling. Capadenoson had been evaluated in a phase II clinical trial for the treatment of atrial fibrillation, but appears not to be further developed.

6.3.2 A_{2B} Receptor Antagonists

The natural alkaloids caffeine (**8**) and theophylline (**9**) are nonselective AR antagonists which display similar inhibitory potency at all four human AR subtypes (see Fig. 6.6 and Table 6.2). However, they are less potent at rodent (mouse and rat) A_3ARs (Müller and Jacobson 2011a, b). The 1,3-dipropyl-8-phenyl-substituted xanthine derivative xanthine amine congener (XAC, **10**) was an early derivative that exhibited very high affinity for all four subtypes of human ARs; it can therefore be envisaged as a potent pan-AR antagonist.

6.3.2.1 Selective A_{2B} Adenosine Receptor Antagonists

Reviews on A_{2B}AR antagonists have previously been published (Baraldi et al. 2009; Ortore and Martinelli 2010). A_{2B}AR antagonists can be subdivided into two classes, xanthines and non-xanthine-derived heterocyclic compounds (see Table 6.2).

6.3.2.2 Xanthine Derivatives

Broad modifications of the substituent in position 8 of the xanthine core structure have resulted in high potency and selectivity for the A_{2B}AR (see Fig. 6.6 and Table 6.2). Phenyl or heteroaromatic residues (e.g., pyridine, pyrazole (Kalla et al. 2008)) were directly connected to the C8 of the xanthine scaffold (compounds **11**–**26**), and the N7 position remained unsubstituted to provide a hydrogen bond donor and to allow the 8-phenyl or 8-heteroaromatic ring to be coplanar with the xanthine core structure. The ideal substituent on N1 of the xanthine is a propyl residue (or a cyclopropyl as in **14**), while polar substituents are not tolerated (Kim et al. 2002; Daly et al. 1991). In the N3-position, ethyl or propyl is present in many cases. A free N3-H providing a hydrogen bond donor increases potency as well as selectivity of

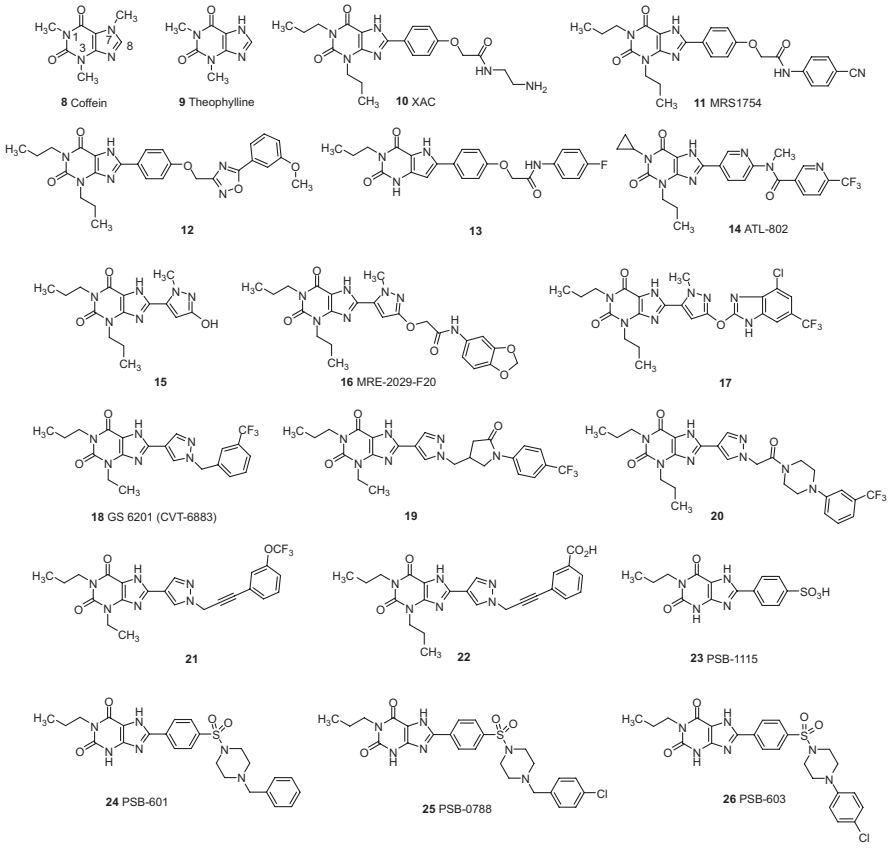

Fig. 6.6 Xanthine-derived nonselective and selective A_{2B} adenosine receptor antagonists

the xanthines (Hayallah et al. 2002). 9-Deazaxanthines first described by the group of Müller to be well tolerated by the A_{2B}AR (Hayallah et al. 2002) were later on taken up by other groups (e.g., compound **13**, Nieto et al. 2010; Carotti et al. 2004, 2006; Stefanachi et al. 2008).

The first potent, A_{2B}-selective antagonist to be reported was MRS1754 (**11**) (Fig. 6.6). Later on, several groups improved the potency, selectivity, and/or pharmacokinetic properties of this class of compounds (see Fig. 6.6). Sulfonamide derivatives (**24**–**26**), such as PSB-603 (**26**) (Yan et al. 2006, Borrmann et al. 2009), show a particularly high affinity and selectivity, not only in humans but also in rodents. The sulfonate PSB-1115 (**23**) exhibits high water solubility and is therefore useful for in vivo studies; however its A_{2B} affinity and selectivity are lower than that for other A_{2B} antagonists, especially in mice (Hayallah et al. 2002; Yan and Müller 2004; Alnouri et al. 2015). Sulfonates are too polar to be orally bioavailable, and therefore a prodrug concept was developed (Yan and Müller 2004). Another polar compound with good water solubility is the carboxylate **22** (Basu et al. 2017).

Table 6.2 Adenosine receptor affinities of nonselective and A_{2B}-selective antagonists

		K_i (nM)[a]				
		A_1	A_{2A}	A_{2B}	A_3	References
Xanthine derivatives						
8	Caffeine	10,700 (h) 50,700 (m)	9560 (h) 11,100 (m)	10,400 (h) 13,000 (m)	13,300 (h) >100,000 (m)	Alnouri et al. (2015)
9	Theophylline	6200 (h) 14,200 (m)	9560 (h) 5770 (m)	7850 (h) 24,300 (m)	22,300 (h) >100,000 (m)	Alnouri et al. (2015)
10	XAC	6.8 (h)	18 (h)	7.8 (h) 4.51 (m)	91.9 (h)	Müller and Jacobson (2011a, b)
11	MRS1754	403 (h) 1.45 (m)	503 (h) >10,000 (m)	1.97 (h) 3.12 (m)	570 (h) >10,000 (m)	Alnouri et al. (2015)
12	CVT-5440	>10,000 (h)	5000 (h)	50 (h)	>9000 (h)	Zablocki et al. (2005)
13	Deazaxanthine derivative	76 (h)	6500 (h)	2.9 (h)	>1000 (h)	Nieto et al. (2010)
14	ATL 802	369 (h) 9583 (m)	654 (h) 8393 (m)	2.36 (h) 8.58 (m)	>1000 (h) >10,000 (m)	Cagnina et al. (2009)
15	Hydroxypyrazole derivative	733 (h)	>1000 (h)	4.0 (h)	>1000 (h)	Baraldi et al. (2012)
16	MRE-2029-F20	200 (h)	>1000 (h)	5.5 (h)	>1000 (h)	Baraldi et al. (2004a, b)
17	Pyrazole derivative[6]	2530 (h)	>1000 (h)	9.4 (h)	>1000 (h)	Baraldi et al. (2012)
18	GS 6201 (CVT-6883)	1940 (h)	3280 (h)	22 (h)	1070 (h)	Elzein et al. (2008)
19	Pyrrolidone derivative	Ca. 100 (h)	Ca. 100 (h)	1.0 (h)	>1000 (h)	Basu et al. (2017a)
20	Piperazine derivative	>100 (h)	>100 (h)	1.5 (h)	>1000 (h)	Basu et al. (2017a)
21	Phenylethynyl dervivative	>100 (h)	≥100 (h)	13 (h)	>1000 (h)	Basu et al. (2017b)
22	Carboxyphenylethynyl derivative	>100 (h)	>100 (h)	5.3 (h)	>1000 (h)	Basu et al. (2017b)
23	PSB-1115	>10,000 (h) 591 (m)	3790 (h) >10,000 (m)	53.4 (h) 1940 (m)	>10,000 (h) >10,000 (m)	Alnouri et al. (2015)
24	PSB-601	2070 (h) 123 (m)	484 (h) 598 (m)	3.6 (h) 2.48 (m)	>1000 (h) >10,000 (m)	Alnouri et al. (2015)

(continued)

Table 6.2 (continued)

		K_i (nM)[a]				
		A_1	A_{2A}	A_{2B}	A_3	References
25	PSB-0788	2240 (h) 118 (m)	333 (h) 235 (m)	0.393 (h) 1.90 (m)	>1000 (h) >10,000 (m)	Alnouri et al. (2015)
26	PSB-603	>10,000 (h) 42.4 (m)	>10,000 (h) >10,000 (m)	0.553 (h) 0.265 (m)	>10,000 (h) >10,000 (m)	Alnouri et al. (2015)
Non-xanthine heterocyclic compounds						
27	CGS15943	3.5 (h) 1.15 (m)	1.2 (h) 0.177 (m)	32.4 (h) 15.0 (m)	35 (h) 2970 (m)	Alnouri et al. (2015)
28	ZM-241385	774 (h) 236 (m)	1.6 (h) 0.554 (m)	75 (h) 31.3 (m)	743 (h) >10,000 (m)	Alnouri et al. (2015)
29	Tricyclic compound	1 (h)	0.34 (h)	5.1 (h)	280 (h)	Cheong et al. (2011)
30	Amino-substituted tricyclic compound	1.6 (h)	54 (h)	27 (h)	65 (h)	Cheong et al. (2011)
31	Tricyclic compound	2 (h)	0.8 (h)	9 (h)	700 (h)	Cheong et al. (2011)
32	Tricyclic compound	1100 (h)	800 (h)	20 (h)	300 (h)	Cheong et al. (2011)
33	Triazinobenzimidazole	>10,000 (h)	>10,000 (h)	3.10 (h)	>10,00 (h)	Taliani et al. (2012)
34	Benzothiazole	690 (h)	530 (h)	20 (h)	nd	Firooznia et al. (2011)
35	Aminobenzothiazole	100 (h)	51 (h)	8 (h)	nd	Cheung et al. (2010)
36	OSIP	37 (h) [176]	328 (h) [176]	0.41 (h) [176]	450 (h) [176]	Stewart et al. (2004)
37	Thienopyrimidine	nd	965 (h)	3.5 (h)	nd	Bedford et al. (2009)
38	Quinolone derivative	19 (h)	>10,000 (h)	28 (h)	nd	McGuinness et al. (2010)
39	LAS101057	Ca. 10,000 (h)	>2500 (h)	24	>10,000 (h)	Eastwood et al. (2010a)
40	LAS38096	2821 (h)	>1000 (h)	17 (h)	1043 (h)	Eastwood et al. (2010c)
41	Pyridine derivative	931 (h)	239 (h)	4 (h)	3754 (h)	Cheung et al. (2010)

(continued)

Table 6.2 (continued)

		K_i (nM)[a]				
		A_1	A_{2A}	A_{2B}	A_3	References
42	QAF805	186 (h)	1775 (h)	3.4 (h)	10.2 (h)	Müller and Jacobson (2011a, b)
43	Imidazopyridine derivative	2444 (h)	2126 (h)	11 (h)	>1000 (h)	Eastwood et al. (2010b)
44	Dihydropyrimidinone derivative	>10,000 (h)	>10,000 (h)	10.2 (h)	>10,000 (h)	Crespo et al. (2013)
45	Dihydropyrimidinone derivative	>10,000 (h)	>10,000 (h)	23.6 (h)	>10,000 (h)	Crespo et al. (2013)
46	Pyrimidine, *R*-enantiomer	>1000 (h)	>1000 (h)	>1000 (h)	>1000 (h)	Carbajales et al. (2017)
47	Pyrimidine, *S*-enantiomer	>1000 (h)	>1000 (h)	15.1 (h)	>1000 (h)	Carbajales et al. (2017)
48	ISAM140	>10,000 (h)	>10,000 (h)	3.49 (h)	>10,000 (h)	El Maatougui et al. (2016)

[a]nd = no data available

The Potent and Selective A_{2B}AR Antagonist PSB-603

PSB-603 (**26**) is among the most widely used A_{2B}AR antagonists due to its subnanomolar potency across species (human, rat, mouse), its extraordinarily high selectivity versus all other AR subtypes, and its commercial availability. The main limitation of **26** is its low water solubility, which is also a problem with many other potent A_{2B}AR antagonists.

Figure 6.7 shows the binding mode of **26** determined by docking studies to a homology model of the A_{2B}AR based on A_{2A}AR X-ray structures. The xanthine core likely binds to the same site as the adenine nucleobase of adenosine, while the 8-substituent of **26** extends to the extracellular domain of the receptor. Interactions with amino acid residues distant from the orthosteric binding site that differ in the various AR subtypes (e.g., K269, D7 in the A_{2B}AR) are likely responsible for the high selectivity of **26** (see Fig. 6.7).

PSB-603 as well as the other xanthine derivatives depicted in Fig. 6.6 most likely display a competitive mechanism of A_{2B}AR blockade based on (i) the observed SARs, (ii) the X-ray co-crystal structure of the A_{2A}AR with XAC (**10**) (Doré et al. 2011) and the high similarity of the orthosteric binding sites of the A_{2A}- to that of the A_{2B}AR, (iii) docking studies, and (iv) mutagenesis studies indicating, for example, that PSB-603 interacts with His280, which is located on the bottom of the orthosteric binding pocket and also interacts with the agonists (Thimm et al. 2013). In a recent publication, PSB-603 was proposed to act as an allosteric A_{2B}AR antagonist (Goulding et al. 2018). PSB-603 showed incomplete inhibition of A_{2B}-induced increase in cAMP production at high concentrations in that particular study. However, this may be an artifact which might be explained by the low water solubility of the compound, which tends to form aggregates with other compounds or precipitate at higher concentrations.

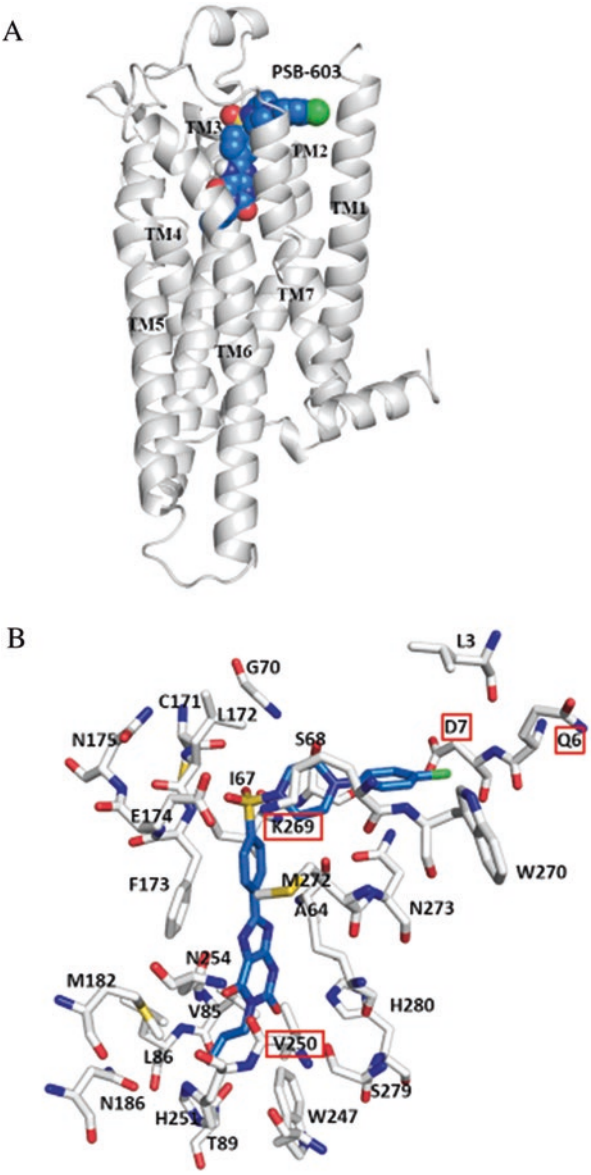

Fig. 6.7 Putative binding mode of PSB-603 (**26**, marine blue) in a homology model of the human A_{2B}AR. The oxygen atoms are colored in red, nitrogen atoms in blue, sulfur atoms in yellow, and chlorine in green. (**A**) The human A_{2B}AR is represented as cartoon model, and atoms of the docked ligand PSB-603 are depicted as spheres. (**B**) The atoms of the docked ligand PSB-603 are depicted as sticks. The amino acids which are unique for the human A_{2B}AR are framed in red

6.3.2.3 Non-xanthine A_{2B} Adenosine Receptor Antagonists

Heterobi- and tricyclic compounds related to adenine that had been initially developed for the A_{2A}AR (Gatta et al. 1993), but CGS15943 and ZM-241385, also show significant affinity for the A_{2B}AR subtype. They have been used as starting points for optimization toward A_{2B}-selective antagonists, e.g., **32**. Another adenine-derived

very potent A_{2B}AR antagonist is OSIP339391 (**36**), which was also prepared in tritiated form as a radioligand (Stewart et al. 2004).

Later on, a variety of tri-, bi-, and mono-heterocyclic A_{2B}-selective antagonists with different scaffolds, mostly based on screening hits, have been developed, some of which showed high potency, selectivity, and good pharmacokinetic properties (see Table 6.2). Recently, a series of partly unsaturated pyrimidine derivatives (**44**–**48**) was synthesized by the Biginelli multicomponent reaction and optimized for interaction with A_{2B}ARs. They feature an ester function which might be metabolically unstable. Despite their relatively small size, these compounds display high A_{2B} affinity and extraordinarily high subtype selectivity. The selectivity of the hydropyrimidines (**44**–**48**) has been explained by docking studies which were supported by the observation of enantiospecific binding (**46**, *R*-entantiomer, inactive; **47**, S-enantiomer, nanomolar potency) (Carbajales et al. 2017) (Fig. 6.8).

6.3.2.4 Allosteric Modulators of A_{2B} Adenosine Receptors

Besides orthosteric ligands, a series of allosteric modulators of the A_{2B}AR has recently been identified: 1-benzyl-3-ketoindole derivatives, such as **49**–**51** (Fig. 6.9) (Taliani et al. 2013; Trincavelli et al. 2014). Indoles **49** and **50** were found to act as positive allosteric modulators (PAMs) increasing NECA-induced cAMP production with EC_{50} values of 250 and 2390 nM, respectively. A maximal increase from 100% to 237% was observed for **49** and to 135% for **50**. The compounds did not activate the receptor in the absence of an agonist. Compound **50** was also shown to increase the maximal effect of other agonists, BAY 60–6583 and, importantly, adenosine. The PAM **50** was shown to potentiate osteoblast differentiation induced by A_{2B}AR agonists in vitro (Trincavelli et al. 2014).

The structurally related indole derivative **52** acted as a negative allosteric modulator (NAM) and completely blocked NECA-induced cAMP accumulation in A_{2B}AR-expressing CHO cells. The compound displayed biphasic inhibition of NECA-induced cAMP accumulation with IC_{50} values of 0.20 nM and 1050 nM, respectively. These compounds did not significantly interfere with the other AR subtypes and were thus reported to be selective for the A_{2B}AR (Taliani et al. 2013). In particular, PAMs of the A_{2B}AR will be very useful since potent and selective full A_{2B}AR agonists are still lacking. It will be interesting to see more biological data on A_{2B}AR-PAMs obtained in different systems and settings to assess their value.

6.3.2.5 Radioligands and Fluorescent Ligands

A selective agonist radioligand for A_{2B}ARs is currently not available (Hinz et al. 2018b). BAY 60–6583 (**6**) was recently prepared in a tritiated form but found to be not suitable for the labeling of A_{2B}ARs due to its only moderate affinity and its

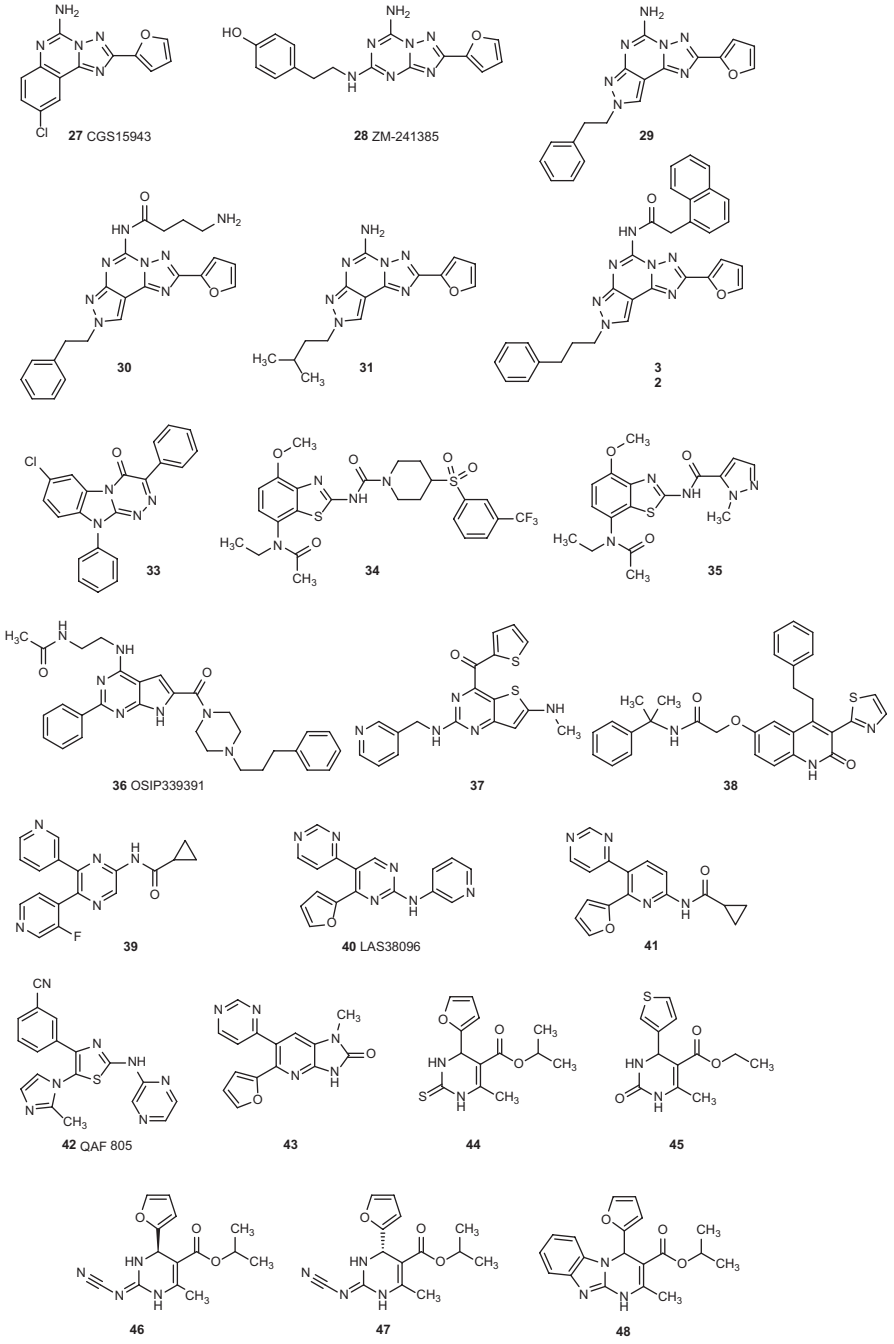

Fig. 6.8 Non-xanthine based nonselective and selective A_{2B} adenosine receptor antagonists

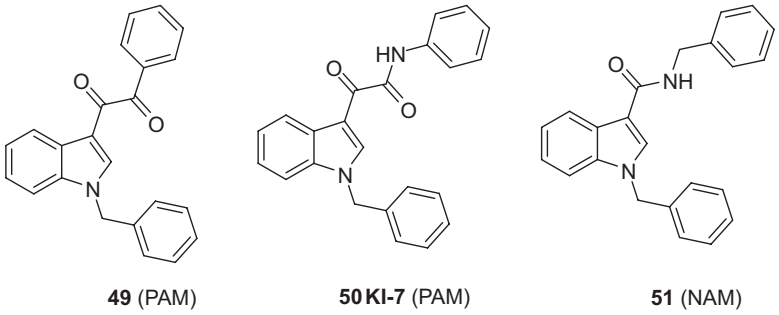

Fig. 6.9 Allosteric modulators of A_{2B} adenosine receptors

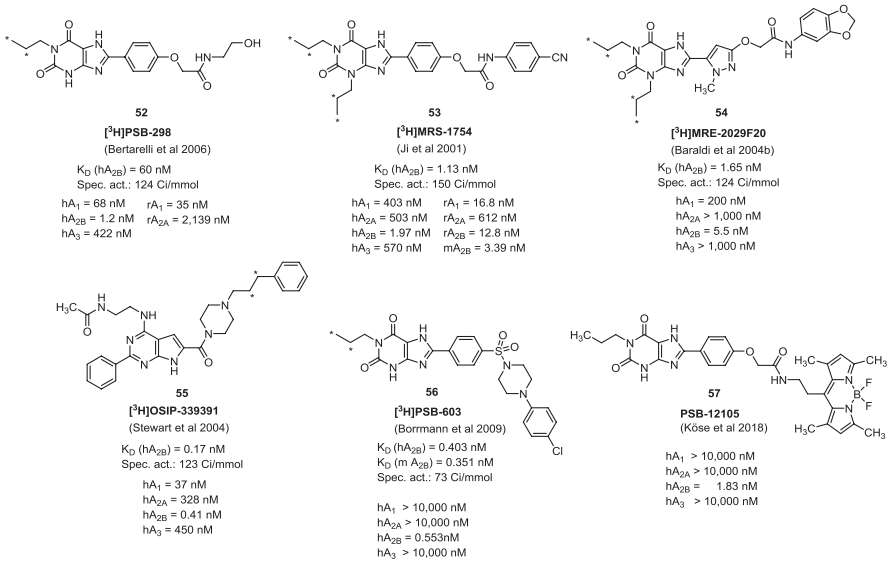

Fig. 6.10 Radioactive and fluorescent ligands for the labeling of A_{2B}ARs

high nonspecific protein binding (Hinz et al. 2018b). Despite its also moderate affinity, the nonselective agonist radioligand [^{3}H]NECA can be used for binding assays if fast dissociation is avoided (e.g., at 4 °C, quick filtration) (Casadó et al. 1992; Hinz et al. 2018b). The compound is hydrophilic due to its ribose moiety and therefore shows low nonspecific binding. However, it can bind with similar affinity to adenotin, recently identified as heat shock paralog Grp94 (Gewirth 2016), a 98-kDa protein that is present in platelets and many other tissues, which shows close homology to heat shock proteins (Hutchison et al. 1990; Müller and Scior 1993).

Several potent and selective antagonist radioligands have been developed for A_{2B}ARs (see Fig. 6.10) (Ji et al. 2001, Stewart et al. 2004, Baraldi et al. 2004b, Bertarelli et al. 2006, Borrmann et al. 2009). The currently most widely used ones are [^{3}H]MRS-1754 (**53**) and [^{3}H]PSB-603 (**56**), both of which display high affinity and selectivity. Efforts have been made to develop ^{11}C-labeled and ^{18}F-labeled A_{2B} antagonists as positron emission tomography (PET) ligands for imaging (Petroni et al. 2016), but so far a suitable candidate has not been obtained.

Very recently, the first fluorescent A_{2B}AR ligands have been developed (Köse et al. 2018). PSB-12105 (**57**) was found to selectively label A_{2B}ARs in confocal imaging and in flow cytometry experiments and was utilized to establish a flow cytometry-based competition assay (Köse et al. 2018).

6.3.2.6 Multi-target Ligands

The concept of polypharmacology or multi-target drugs interacting simultaneously with two or more targets represents a new strategy, in particular for the treatment of complex diseases including cancer or brain diseases (Raghavendra et al. 2018; Geldenhuys and Van der Schyf 2012). Such drugs may exhibit synergistic effects, show a reduced risk of side effects, and result in improved compliance, especially in multi-morbid and elderly patients, as compared to combination therapies. Blockade of A_{2A}- and A_{2B}ARs may be a meaningful combination for the (immuno) therapy of cancer (Allard et al. 2016, 2017). Regarding the fact that the nonselective AR antagonist caffeine is not only a widely consumed psychostimulant but also protects from neurodegenerative diseases (Flaten et al. 2014) and is a safe drug may indicate that compounds that block all four AR subtypes could be beneficial. Recent efforts have been made to obtain such multi-target compounds (e.g., Burbiel et al. 2016).

6.4 Species Differences

Human, rat, and mouse A_{2B}ARs show high sequence identity (see Fig. 6.11). Human and rat A_{2B}AR are 86% identical, while the human and mouse A_{2B}ARs display 88% sequence identity. Mouse and rat share 96% of the amino acids. Therefore, species differences for A_{2B}AR ligands are in most cases moderate (Alnouri et al. 2015). However, there are exceptions, e.g., PSB-1115 (**23**) is significantly less potent at mouse and rat A_{2B}ARs as compared to the human subtype. Even moderate changes at the different AR subtypes can in sum result in a significantly altered selectivity profile (Alnouri et al. 2015). Unfortunately, for most compounds, only data at human ARs are available. Before using these compounds in animal studies, they should be tested at the AR subtypes of the respective species.

```
SP|Q60614|AA2BR_MOUSE MQLETQDALYVALELVIAALAVAGNVLVCAAVGASSALQTPTNYFLVSLATADVAVGLFA 60
SP|P29276|AA2BR_RAT   MQLETQDALYVALELVIAALAVAGNVLVCAAVGASSALQTPTNYFLVSLATADVAVGLFA 60
SP|P29275|AA2BR_HUMAN MLLETQDALYVALELVIAALSVAGNVLVCAAVGTANTLQTPTNYFLVSLAAADVAVGLFA 60
                      * ******************:************:::.:**************:*********

SP|Q60614|AA2BR_MOUSE IPFAITISLGFCTDFHGCLFLACFVLVLTQSSIFSLLAVAVDRYLAIRVPLRYKGLVTGT 120
SP|P29276|AA2BR_RAT   IPFAITISLGFCTDFHSCLFLACFVLVLTQSSIFSLLAVAVDRYLAIRVPLRYKGLVTGT 120
SP|P29275|AA2BR_HUMAN IPFAITISLGFCTDFYGCLFLACFVLVLTQSSIFSLLAVAVDRYLAICVPLRYKSLVTGT 120
                      ***************:.*****************************  ******.*****

SP|Q60614|AA2BR_MOUSE RARGIIAVLWVLAFGIGLTPFLGWNSKDSATSNCTELGDGIANKSCCPVTCLFENVVPMS 180
SP|P29276|AA2BR_RAT   RARGIIAVLWVLAFGIGLTPFLGWNSKDRATSNCTEPGDGITNKSCCPVKCLFENVVPMS 180
SP|P29275|AA2BR_HUMAN RARGVIAVLWVLAFGIGLTPFLGWNSKDSATNNCTEPWDGTTNESCCLVKCLFENVVPMS 180
                      ****:*********************** **.****  ** :*:*** *.**********

SP|Q60614|AA2BR_MOUSE YMVYFNFFGCVLPPLLIMLVIYIKIFMVACKQLQRMELMDHSRTTLQREIHAAKSLAMIV 240
SP|P29276|AA2BR_RAT   YMVYFNFFGCVLPPLLIMMVIYIKIFMVACKQLQHMELMEHSRTTLQREIHAAKSLAMIV 240
SP|P29275|AA2BR_HUMAN YMVYFNFFGCVLPPLLIMLVIYIKIFLVACRQLQRTELMDHSRTTLQREIHAAKSLAMIV 240
                      ******************:*******:***:***: ***:*********************

SP|Q60614|AA2BR_MOUSE GIFALCWLPVHAINCITLFHPALAKDKPKWVMNVAILLSHANSVVNPIVYAYRNRDFRYS 300
SP|P29276|AA2BR_RAT   GIFALCWLPVHAINCITLFHPALAKDKPKWVMNVAILLSHANSVVNPIVYAYRNRDFRYS 300
SP|P29275|AA2BR_HUMAN GIFALCWLPVHAVNCVTLFQPAQGKNKPKWAMNMAILLSHANSVVNPIVYAYRNRDFRYT 300
                      ************:**:***:** .*:****.**:*************************:

SP|Q60614|AA2BR_MOUSE FHKIISRYVLCQAETKGGSGQAGAQSTLSLGL 332
SP|P29276|AA2BR_RAT   FHRIISRYVLCQTDTKGGSGQAGGQSTFSLSL 332
SP|P29275|AA2BR_HUMAN FHKIISRYLLCQADVKSGNGQAGVQPALGVGL 332
                      **:*****:***::.*.*.**** * ::.:.*
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Fig. 6.11 Multiple sequence alignment of mouse, rat, and human A_{2B} adenosine receptors

6.5 Potential Therapeutic Applications of A_{2B} Receptor Ligands

A recent review article on the potential of the A_{2B}AR as a drug target has appeared (Sun and Huang 2016). A selection of proposed therapeutic applications for A_{2B}AR agonists and antagonists is collected in Table 6.3. In some cases, activation or blockade of A_{2B}ARs has been proposed for the same indication based on different studies. These conflicting results may be partly due to the lacking of a suitable A_{2B}AR agonist which would be required for target validation studies. In most studies, BAY 60–6583 was employed, which is a partial agonist. Another explanation could be that A_{2B}AR signaling can vary based on the cellular background, e.g., G_s versus G_q signaling (Gao et al. 2017).

6.5.1 Agonists

There is evidence for cardioprotective effects of A_{2B}AR agonists (see Table 6.3). Effects on other organs and conditions have been described but require confirmation.

Table 6.3 Proposed therapeutic applications of A_{2B} adenosine receptor ligands based on in vitro studies and animal models (selection)

Indication	Compounds used and additional information	References
Agonists		
Brain inflammation (microglia); excitotoxicity (cortical neurons); ischemic stroke		Koscsó et al. (2012), Moidunny et al. (2012), Li et al. (2017)
Cardioprotection, heart infarction, fibrosis	BAY 60–6583 (**6**), NECA (**2**), VCP-746 (**5**)	Phosri et al. (2017), Vecchio et al. (2016a, b), Methner et al. (2010)
COPD	BAY 60–6583 (**6**)	Greer et al. (2013)
Liver ischemic/ reperfusion injury		Choukèr et al. (2012)
Wound healing	Upregulation of VEGF production	Ryzhov et al. (2014)
Kidney protection	NECA (**2**)	Patel and Thaker (2015)
Bladder overactivity in elder patients	BAY 60–6583 (**6**)	Weller et al. (2015)
Obesity-induced diabetes, hyperlipidemia, and atherosclerosis	BAY 60–6583 (**6**)	Johnston-Cox et al. (2012), Koupenova et al. (2012)
Cancer (proliferation)	BAY 60–6583 (**6**)	Jafari et al. (2018)
Acute colitis	BAY 60–6583 (**6**)	Aherne et al. (2015)
Obesity-induced diabetes, hyperlipidemia, and atherosclerosis	BAY 60–6583 (**6**)	Johnston-Cox et al. (2012), Koupenova et al. (2012)
Erectile dysfunction	BAY 60–6583 (**6**)	Wen et al. (2015)
Antagonists		
Bronchial inflammation, acute pulmonary inflammation, COPD, (allergic) asthma		Pejman et al. (2014), Konrad et al. (2017), Basu et al. (2017), Chugh & Mookhtiar (2017), Eckle et al. (2014), Karmouty-Quintana et al. (2015),
Pulmonary fibrosis		Giacomelli et al. (2018), Philip et al. (2017)
Pulmonary hypertension	GS-6201 (**18**)	Karmouty-Quintana et al. (2012)
Cancer: Anti-proliferative, anti-angiogenic, anti-metastatic, immunostimulatory	PSB-1115 (**23**), PSB-603 (**26**), MRS-1754 (**11**) PBF-1129 (structure undisclosed), phase I clinical trial will be started, indication: Non-small cell lung cancer (NSCLC)	Sepulveda et al. (2016), Iannone et al. (2013), Zhou et al. (2017), Sorrentino et al. (2015), Wei et al. (2013a), Kalhan et al. (2012), Ma et al. (2010), Du et al. (2015), Vecchio et al. (2016a, b), Allard et al. (2016, 2017), Kaji et al. (2014) Molck et al. (2016)

(continued)

Table 6.3 (continued)

Indication	Compounds used and additional information	References
Infectious diseases (prevention of immune escape; brain infection)	PSB-1115 (**23**) (leishmania; dendritic cell activation); PSB-603 (**26**) (haemophilus influenza; prevent disruption of blood-brain barrier)	Figueiredo et al. (2017), Caporarello et al. (2017)
Pain	PSB-1115 (**23**) and others	Abo-Salem et al. (2004), Bilkei-Gorzo et al. (2008)
Colon inflammation, hypoxic/ischemic conditions, reperfusion injury	PSB-1115 (**23**) improves intestinal barrier function	Yang et al. 2014
Inflammatory bowel disease	PSB-601 (**24**), synergism with A_{2A}AR agonist	El-Tayeb et al. (2011), Michael et al. (2010)
Irritable bowel disease	A_{2B} is involved in visceral hypersensitivity	Asano and Takenaga (2017)
Colonic motor dysfunction in obesity		Antonioli et al. (2017)
Heart: Myocardial infarction	GS-6201 (**18**), blockade of cardiac remodeling; reduction of ventricular dysfunction and arrhythmias	Toldo et al. (2012), Zhang et al. (2014)
Sickle cell disease		Field et al. (2014)
Diabetes: Increased insulin sensitivity, decreased glucose production		Rüsing et al. (2006)
Autoimmune disease	MRS1754 (**11**)	Chen et al. (2015)
Multiple sclerosis		Wei et al. (2013b)
Preservation of lungs for transplantation	ATL802 (**14**)	Charles et al. (2017)

6.5.2 Antagonists

The nonselective AR antagonist caffeine (**1**) is broadly used as a central stimulant and as a painkiller in combination with nonsteroidal anti-inflammatory drugs and/or paracetamol (acetaminophen). While the central stimulatory effect is probably due to A_1AR (and A_{2A}) AR blockade (Elmenhorst et al. 2007, 2017; Lazarus et al. 2017), the analgesic effects of caffeine are dependent on A_{2B}AR antagonism (Abo-Salem et al. 2004; Bilkei-Gorzo et al. 2008). A more recent application is the use of caffeine infusions for preterm infants to support breathing function (Orozco-Gregorio et al. 2011). Theophylline (**2**) has mainly been used for the chronic treatment of asthma, but it is rarely used nowadays due to its narrow therapeutic window. There is a growing body of evidence that A_{2B}-selective antagonists could be useful therapeutics for various indications (see Table 6.3).

The effectiveness of A_{2B}AR antagonists in preclinical cancer models has recently attracted much attention since they do not only prevent suppression of immune cells by adenosine in the microenvironment of cancer tissues, similarly as A_{2A}AR antagonists, but they additionally display direct anti-proliferative effects on cancer cells and inhibit metastasis and angiogenesis (see Table 6.3). A_{2B} antagonists were shown to suppress tumor growth in different cancer cell lines (Wei et al. 2013a, b; Kalhan et al. 2012). A_{2B}ARs are upregulated on many cancer cells, and high A_{2B}AR expression has been reported to result in a worse prognosis in breast cancer (Mittal et al. 2016; Jafari et al. 2018). A phase I clinical trial with the A_{2B}AR antagonist PBF-1129 (structure undisclosed), developed by the company Palobiofarma, Spain, will soon start in patients with locally advanced or metastatic non-small cell lung carcinoma (NSCLC) to evaluate its safety and tolerability.

A_{2B}AR antagonists have been shown to be useful for treating infections due to their immunostimulatory effects (Figueiredo et al. 2017) and because they can prevent A_{2B} receptor-induced opening of the blood-brain barrier (Caporarello et al. 2017).

A_{2B}antagonists have been shown to relieve pain by a peripheral mechanism and to act synergistically with other analgesics including paracetamol, nonsteroidal anti-inflammatory drugs (NSAIDs), and opioids (Abo-Salem et al. 2004; Bilkei-Gorzo et al. 2008; Hu et al. 2016). Since cancer patients often suffer from severe pain, A_{2B} antagonists could be ideal drugs for their treatment by combination therapies.

Further well-documented indications for A_{2B}AR antagonists include bronchial inflammation, fibrosis, and colitis/inflammatory bowel disease. Positive effects in diabetes, myocardial infarction, and autoimmune diseases like multiple sclerosis and sickle cell disease, among others, have also been described (Table 6.3).

6.6 Conclusion

In particular, A_{2B}AR antagonists have a great potential as future drugs, and one of the prominent, most advanced applications will be in cancer immunotherapy.

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Chapter 7
Medicinal Chemistry of the A_3 Adenosine Receptor

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Abstract Numerous structure-activity relationship (SAR) studies of ligands of the A_3 adenosine receptor (AR) have generated selective agonists, antagonists, partial agonists, and allosteric modulators. The efficacy of nucleoside agonists may be reduced, while retaining affinity, by successive structural changes. Subnanomolar affinity and selectivity of >10,000-fold have been achieved for various compound classes, but often with a pronounced species dependence, especially for diverse heterocyclic antagonists. Two prototypical A_3AR agonists, IB-MECA and Cl-IB-MECA, are being evaluated clinically for treating autoimmune inflammatory disorders and liver diseases. The design of A_3AR orthosteric ligands is now largely guided by computational approaches, in which the receptor is modeled by homology to X-ray structures of the A_{2A}AR and other G protein-coupled receptors (GPCRs). Thus, we have amassed a large body of data concerning the relationship of receptor structure and function and have supported many of the hypotheses generated with experimental data.

Keywords A_3 adenosine receptors · A_3 agonists · A_3 antagonists · A_3 allosteric modulators · Structure-activity relationship

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7.1 Introduction

Since its identification as one of the four subtypes of adenosine receptors (ARs) in human (Zhou et al. 1992; Salvatore et al. 1993), the A_3AR has been well studied by medicinal chemists in search of selective agonists, antagonists, and allosteric modulators. The A_3AR has become a target for the design of drugs for treating chronic diseases, including cancer, stroke, glaucoma, chronic neuropathic pain, inflammatory diseases, and cardiovascular diseases (Jacobson et al. 2017; Janes et al. 2016). Initial findings suggested that a selective A_3AR antagonist might have anti-inflammatory or anticancer effects (Gessi et al. 2011; Torres et al. 2016; Borea et al. 2017), but upon further delving into the biology, particularly in vivo, it appears that A_3AR agonists also produce effects that are predictive of their therapeutic potential (Fishman et al. 2001, 2012; Borea et al. 2016). Two of the A_3AR agonists are entering advanced clinical trials for psoriasis, rheumatoid arthritis, and liver diseases (David et al. 2016; Stemmer et al. 2013; Fishman and Cohen 2016; Jacobson et al. 2017).

There is not yet an X-ray crystallographic structure of the A_3AR, but considerable modeling has been performed based on its homology to the human (h) A_{2A}AR, for which both agonist- and antagonist-bound structures have been determined (Jespers et al. 2018). The A_{2A}AR structures can serve as templates for the modeling of the A_3AR, in which many of the key residues involved in ligand recognition are conserved. Thus, ligand design for the A_3AR is increasingly structure-guided, and many of the newer agonists and antagonists reported have been docked in homology models in an effort to understand the structure-activity relationship (SAR). Virtual (in silico) screening to discover both A_3AR agonists and antagonists is now feasible.

The effects on A_3AR affinity and efficacy of structural changes at specific sites to adenosine and diverse antagonists are discussed below. It is noteworthy that there are species differences in the affinities of A_3AR ligands, particularly nonnucleoside antagonists, which often are weak or inactive at the rodent homologues. This is consistent with a low sequence identity among rodent vs. primate A_3ARs, which for mouse (m) A_3AR vs. human (h) A_3AR is only 73% (Paoletta et al. 2013).

7.2 Nucleosides as A_3AR Agonists

The rat (r) A_3AR sequence was first identified in a cDNA library prepared from rat testes (Meyerhof et al. 1991), but only later was identified as a pharmacologically novel AR (Zhou et al. 1992). Soon thereafter, the cloned hA_3AR was validated as an AR (Salvatore et al. 1993), at which [^{125}I]I-ABA **3** (Fig. 7.1) bound with high affinity (10 nM) and functioned as a partial agonist. The order of affinity in agonist binding at the hA_3AR (K_i, nM) was NECA **8** (26) ~ R-PIA **1** (34) > CPA **2** (89). This indicated that nucleosides previously considered to be A_1AR–selective displayed considerable affinity at this new receptor. The levels of expression were highest in

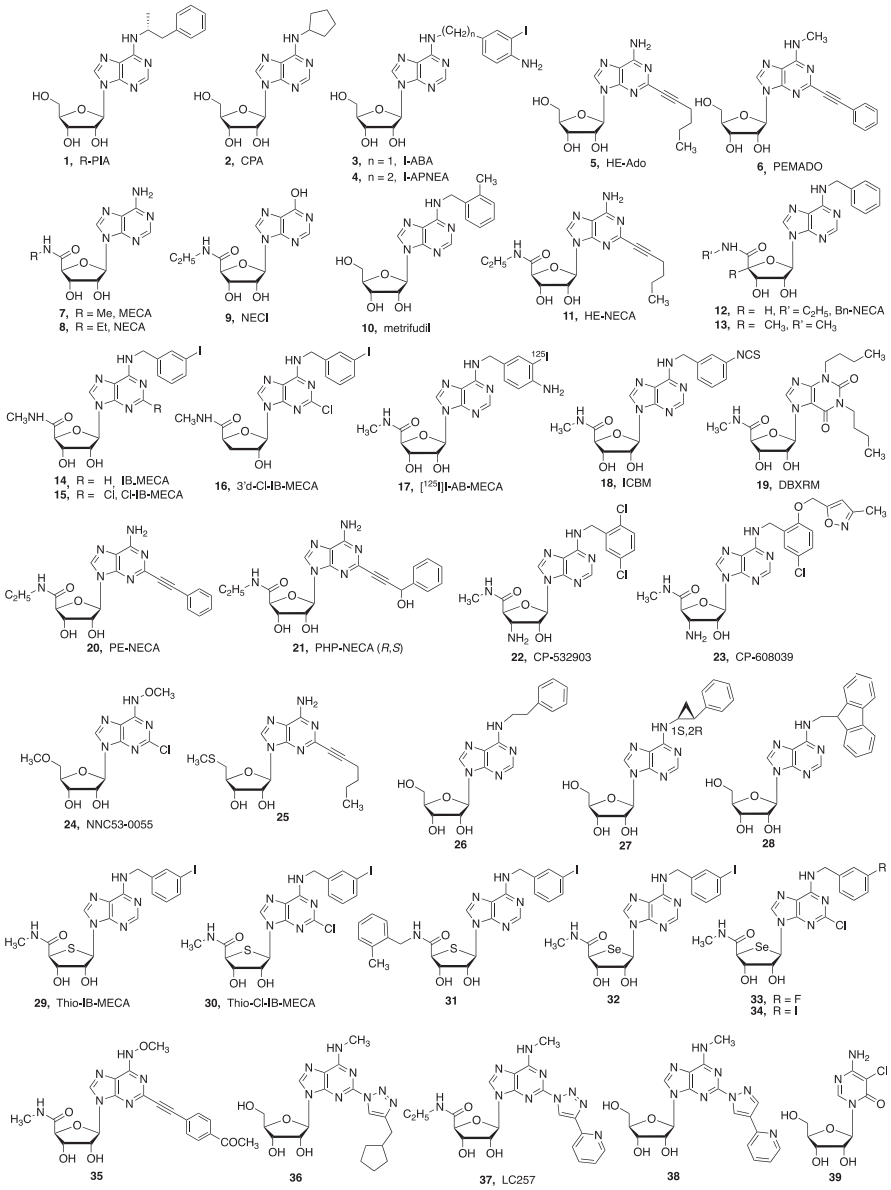

Fig. 7.1 Ribose-containing A_3AR agonists

human lung and liver, which was unlike the distribution of other AR subtypes. Research was initiated at NIH to computationally model this atypical AR and to identify structural features of known AR agonists that increased A_3AR affinity or selectivity (van Galen et al. 1994). Initially, affinity at the rA_3AR was used as a criterion (Gallo-Rodriguez et al. 1994), and only in later SAR studies was screening performed at the human homologue (Gao et al. 2003a).

7.2.1 Nucleobase Substitutions

7.2.1.1 Purine 6-Position Substitutions

The initial reports on radioligand binding at the rA_3AR by Stiles and coworkers utilized [^{125}I]APNEA **4** as a radioligand having a K_d value of 15.5 nM (Zhou et al. 1992). Also, the widely used nonselective 5′-modified AR agonist NECA **8** was a potent activator of the A_3AR with a binding IC_{50} value of 74 nM. Thus, it was evident that both N^6-arylalkyl and 5′-*N*-alkyluronamide modifications were possible. The combination of these two modification sites was reported by Jacobson and coworkers (van Galen et al. 1994; Gallo-Rodriguez et al. 1994), leading to the first slightly selective (7-fold) A_3AR agonist N^6-benzyl-NECA **12** and later to more selective agonists. A comparison of various N^6-arylalkyl modifications of adenosine determined the following rank order of affinity at the rA_3AR: 2-(phenyl)ethyl- **26** = benzyl- > phenyl-adenosine. The choice between N^6–2-(phenyl)ethyl and N^6-benzyl substituents was informed by the selectivity ratios of the corresponding adenosine derivatives. Although both were associated with high affinity at the A_3AR, the latter group was much weaker than the former at A_1 and A_{2A}ARs. Thus, an N^6-benzyl group was deemed optimal in the series to provide A_3AR selectivity. A survey of the affinity of diverse AR ligands and related purines at the rA_3AR, accompanied by molecular modeling of the receptor and its binding site, was also performed.

An N^6-benzyl derivative of adenosine, metrifudil **10** (Table 7.1.), was administered orally in a preliminary clinical trial for glomerulonephritis in the 1970s (Wildbrandt et al. 1972), and it demonstrated a trend to reduce proteinuria. It displays a K_i value of 360 nM at the rA_3AR, although it is roughly an order of magnitude more potent at the rA_1AR and the $rA_{2A}AR$ (Siddiqi et al. 1995). Metrifudil was later shown to be a nonselective, full agonist at the hA_3AR (Gao et al. 2003a). Thus, metrifudil was the first A_3AR agonist with moderate affinity to be administered in humans.

Subsequently, other N^6 modifications were explored for achieving selectivity at the A_3AR. For example, N^6-methyl, e.g., **6** and **36**–**38**, and N^6-ethyl groups were found to be suitable for hA_3AR selectivity (Volpini et al. 2002; Zhu et al. 2006). However, these small N^6-alkyl groups did not maintain the degree of selectivity at the mouse or rA_3AR seen with the N^6-benzyl derivatives, which was considered an important feature for animal model studies. The N^6-methoxy group as in **35** was also reported to be suitable for binding at the A_3AR (Volpini et al. 2007).

N^6-Monoalkyl derivatives are more potent at the A_3AR than corresponding dialkyl derivatives. N^6-Acyl and urea groups were evaluated as modifications of known A_3AR agonists, but these derivatives displayed only moderate affinity (Baraldi et al. 1998).

N^6–2-Phenylcyclopropyl groups were explored at the hA_3AR as sterically constrained analogues of the N^6-phenylethyl group, which is known to afford high affinity. In that series, it was found that the (1*S*,2*R*) stereoisomer, e.g., **27**, provided

Table 7.1 Affinity of selected nucleoside derivatives as A_3AR agonists, partial agonists, and antagonists

Compound	pK_i value			Ref.
	A_1AR	A_{2A}AR	A_3AR	
6	4.48 (h)	4.38 (h)	8.52 (h)	Volpini et al. (2002)
10, metrifudil	7.22 (r)	7.62 (r)	7.33 (h)	Gao et al. (2003a)
11	7.22 (h)	8.19 (h)	8.62 (h)	Volpini et al. (2002)
14, IB-MECA	7.29 (h)	5.50 (h)	8.74 (h)	Melman et al. (2008)
	7.27 (r)	7.25 (r)	8.96 (r)	"
	8.23 (m)	~6 (m)	10.1 (m)	"
15, Cl-IB-MECA	6.66 (h)	5.27 (h)	8.85 (h)	Melman et al. (2008)
	6.09 (r)	6.33 (r)	9.48 (r)	"
	8.14 (m)	5.27 (m)	9.10 (m)	"
21	8.57 (h)	8.51 (h)	9.38 (h)	Volpini et al. (2002)
23	5.14 (h)	<4.3 (h)	8.24 (h)	DeNinno et al. (2003)
29	<5 (h)	<5 (h)	7.81 (h)	Jeong et al. (2006)
30	6.71 (h)	5.36 (h)	9.42 (h)	Jeong et al. (2006)
35	4.27 (h)	4.98 (h)	8.60 (h)	Volpini et al. (2007)
37, LC-257	5.79 (h)	<4 (h)	8.74 (h)	Cosyn et al. (2006)
38	5.42 (h)	<5.3 (h)	8.70 (h)	Cosyn et al. (2006)
43	7.74 (h)	5.49 (h)	8.43 (h)	Jacobson et al. (2005)
46, MRS3558	6.59 (h)	5.64 (h)	9.54 (h)	Tchilibon et al. (2005)
48, MRS3609	5.66 (h)	<5 (h)	8.44 (h)	Tchilibon et al. (2005)
49, MRS3611	6.21 (h)	~5 (h)	8.82 (h)	Tchilibon et al. (2005)
50, MRS5151	4.83 (h)	~5 (h)	8.62 (h)	Tosh et al. (2009)
53, MRS5698	<5 (h)	<5 (h)	8.46 (h)	Tosh et al. (2014)
	<5 (m)	<5 (m)	8.51 (m)	"
54, MRS5679	<5 (h)	<5 (h)	8.51 (h)	Tosh et al. (2014)
55, MRS5980	<5 (h)	<5 (h)	9.15 (h)	Tosh et al. (2014)
58, MRS5841	<5 (h)	<5 (h)	8.72 (h)	Paoletta et al. (2013)
64, MRS5919	<5 (h)	<5 (h)	8.22 (h)	Tosh et al. (2016)
65	<4 (h)	<4 (h)	6.19 (h)	Volpini et al. (2001)
68, MRS1292	ND	ND	7.53 (h)	Gao et al. (2002a)
74	5.60 (h)	6.47 (h)	8.38 (h)	Jeong et al. (2007)
76	<4 (h)	8.14 (h)	7.93 (h)	Hou et al. (2012)
77, MRS5127	5.75 (h)	5.80 (h)	9.14 (h)	Müller and Jacobson (2011)
78, MRS5147[a]	5.52 (h)	5.97 (h)	8.84 (h)	Müller and Jacobson (2011)
79	5.23 (h)	<5 (h)	7.54 (h)	Perreira et al. (2005)
80	<5 (h)	<5 (h)	8.03 (h)	Jeong et al. (2008)
81, MRS5776	<5 (h)	<5 (h)	7.70 (h)	Tosh et al. (2012b)
82	<5 (h)	5.13 (h)	8.31 (h)	Nayak et al. (2014)
85	9.34 (h)	6.48 (h)	9.50 (h)	Petrelli et al. (2017)

h human, *r* rat, *m* mouse, *ND* not determined

[a]as stable Br isotope

38-fold higher hA_3AR affinity than the corresponding (1*R*,2*S*) diastereoisomer (Tchilibon et al. 2004).

In addition to NECA **8**, the corresponding inosine derivative, i.e., NECI **9**, was found to bind to the rA_3AR with a K_i value of 5 μM (van Galen et al. 1994). This was the first indication that inosine (K_i at rA_3AR 45 μM) and its derivatives could serve as A_3AR ligands, although adenosine-like effects of inosine on rat mast cells were previously reported (Marquardt et al. 1978). Inosine was later shown to be a weak partial agonist of the hA_3AR (Jin et al. 1997; Gao et al. 2011), and due to its generation in vivo from the action of ubiquitous adenosine deaminase on adenosine, it could be considered an alternate endogenous A_3AR agonist under stress conditions. Inosine derivatives, such as **42**, were later explored as potential A_3AR agonists (Ravi et al. 2001; Tosh et al. 2016).

7.2.1.2 Alternate Nucleobases

One of the early characteristics of the rA_3AR observed is that the conventional AR antagonists, i.e., alkylxanthines, were much weaker than at the rA_1AR. However, by appending a ribose moiety to the 7-position, they were able to bind to the rA_3AR, in some cases with selectivity. 1,3-Dibutylxanthine-7-ribosides, e.g., **66**, were shown to be the optimal alkyl chain length for binding to the rA_3AR (Park et al. 1998). The corresponding 5′-*N*-methyluronamide DBXRM **19** is a selective agonist, either partial or full, at the rA_3AR. The 7-riboside series was later expanded to the replacement with bicyclic ribose substitutes, e.g., **44**, but the observed A_3AR affinity was reduced compared to ribose analogues.

Virtual screening for AR agonists identified 6-amino-5-chloropyrimidin-4(1H)-one riboside **39** as a novel A_3AR full agonist, although it also activated the A_1AR (Rodriguez et al. 2016). The screening utilized the structure of an agonist-bound $A_{2A}AR$ as a template, but this required a specially devised routine for virtually screening the commercially available nucleobases. These ring NH-containing bases were first converted computationally to their ribosides and then chemically adding the ribose moiety to the hit molecules.

7.2.1.3 Purine C2-Position Substitutions

Another position of substitution was added to the growing list of A_3AR agonist modifications with the observation that elongation of groups at the C2-position was compatible with receptor binding (Kim et al. 1994; Volpini et al. 2002; Gao et al. 2004). Thus, the $A_{2A}AR$ agonist 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680, structure not shown), reported in 1990, was found to be only 2.5-fold less potent at the hA_3AR than at the $hA_{2A}AR$. Within the range of C2 substitutions, the 2-chloro group in Cl-IB-MECA **15** was shown to increase selectivity in binding to the rA_3AR to >1000-fold (Kim et al. 1994). Thus, Cl-IB-MECA **15** became a widely used selective A_3AR agonist tool molecule,

although with less selectivity for the hA_3AR. However, even with moderate selectivity in A_3AR binding, there are examples in the literature that IB-MECA and Cl-IB-MECA might activate the A_1AR, $A_{2A}AR$ or even the $A_{2B}AR$, depending on the model used and the dose range (Murphree et al. 2002; Tian et al. 2015). Thus, agonists with even greater A_3AR selectivity were sought as pharmacological probes. Nevertheless, clinical trials of these two prototypical A_3AR agonists for treating autoimmune inflammatory disorders (**14**, entering Phase III) and liver diseases (**15**, entering Phase II) are continuing and appear encouraging (Jacobson et al. 2017).

Adenosine C2-alkynyl homologues were introduced by the Matsuda (Homma et al. 1992) and Cristalli (Cristalli et al. 1994) groups as $A_{2A}AR$ agonists of increased affinity, but they were later found to be A_3AR agonists as well (reviewed in Dal Ben et al. 2011). In particular, a C2-(2-hexynyl) group in HE-Ado **5** was studied initially at the $A_{2A}AR$ and later shown to be tolerated in potent binding at the A_3AR (Baraldi et al. 1998). The combination of a C2-alkynyl group with a 5′-*N*-ethyluronamide group, i.e., HE-NECA **11**, also resulted in high A_3AR binding affinity, but it lacked selectivity (Jacobson et al. 1995; Volpini et al. 2002). Many adenosine analogues in the riboside series containing C2-phenyl-ethynyl or phenyl-alkylethynyl groups, e.g., **20** and **35**, have been reported to be highly selective agonists (Volpini et al. 2002, 2007, 2009; Dal Ben et al. 2014). Thus, the combination of extended 2-ethynyl groups with other A_3AR-enhancing modifications of adenosine proved to be additive.

Agonists with heterocyclic groups, such as triazoles (Cosyn et al. 2006), attached directly at the C2-position have been introduced as A_3AR agonists. Adenosine derivative **38** containing a C2-pyrazole group was found to be highly selective in binding to the hA_3AR (K_i 2 nM, Elzein et al. 2004), but its functional activity was not presented.

7.2.2 Ribose Group Modifications

7.2.2.1 5′-Position

Optimization of N^6-arylalkyl and 5′-uronamide substitutions was reported by Gallo-Rodriguez et al. (1994). The smaller 5′-*N*-methyluronamide in MECA **7** was more conducive to A_3AR selectivity than the corresponding *N*-ethyl group, and the substitution pattern of the N^6-benzyl group favored *m*-substituted halogens and other groups. Thus, IB-MECA **14** was identified as the first useful A_3AR agonist probe, displaying ~50-fold selectivity for the rA_3AR in comparison to A_1 and $A_{2A}ARs$. Alternative small amides at the 5′-position were explored by Tosh et al. (2012a), and *N*-propyl and *N*-cyclopentyl groups were found to be tolerated at the hA_3AR.

When the cloned hA_3AR became available for compound screening, it was noted that the A_3AR selectivity and nM affinity of IB-MECA and many of its 5′-*N*-alkyluronamide derivatives generalized to this species (Gao et al. 2003a). An alternative to the use of nonselective AR agonist I-APNEA as an A_3AR radioligand

was needed, and the N^6–4-amino-3-iodobenzyl derivative I-AB-MECA **17** with a K_d value at the cloned rA_3AR of 1.48 nM fulfilled this need (Olah et al. 1994). Among other affinity reagents for studying the A_3AR introduced early, a 3-isothiocyanatobenzyl 5′-*N*-methyluronamide derivative **18** was shown to irreversibly label the rA_3AR and was presumed to be covalently binding to the receptor because of the presence of the electrophilic group and the inability to restore A_3AR radioligand binding (Ji et al. 1994).

Knutsen and coworkers modified the 5′-position with ethylene, methyl ether NNC53-0055 **24**, and chloromethyl groups and found significant hA_3AR selectivity (Mogensen et al. 1998). IJzerman and coworkers explored 5′-alkylthioether modifications, such as in **25**, that still allowed A_3AR selectivity (van Tilburg et al. 2002).

As stated above, the 5′-amides with small alkyl groups enhance A_3AR affinity and functional efficacy compared to 5′-CH_2OH. Nevertheless, certain bulky groups present on the amide nitrogen are still compatible with high affinity at the A_3AR. For example, a 5′-*N*-(2-methylbenzyl)-amide group in **31** provided a K_i value of 31 nM at the hA_3AR, and this compound was inactive at A_1AR and $A_{2A}AR$ (Choi et al. 2009).

7.2.2.2 4′-Position

The 4′-methyl derivative **13** of N^6-benzyl-MECA displayed selectivity for the rA_3AR with a K_i value of 604 nM. Thus, steric bulk at this ribose carbon is tolerated at the A_3AR (Siddiqi et al. 1995), although with reduced affinity.

The ribose ring oxygen can be substituted with sulfur or selenium, with retention of A_3AR selectivity. 4′-Thio derivatives **29** and **30** of prototypical A_3AR agonists display high affinity. 4′-Seleno derivatives **32**–**34** were recently reported as potent A_3AR agonists by Yu et al. (2017). The oxo- and thio- analogues were predicted in receptor docking to attain an *anti*-conformation of the glycosidic bond, as was found for adenosine derivatives in the $A_{2A}AR$ X-ray structures. However, an X-ray structure of compound **34** alone (K_i 4.2 nM; maximal efficacy (E_{max}) 94% of 10 μM NECA) indicated a *syn*-conformation; presumably, the energetic stabilization of the A_3AR interaction of this nucleoside converts it to an *anti*-conformation as required to fit the binding site.

7.2.2.3 Ribose 2′ and 3′ Hydroxyl Group Modifications

The 2′ and 3′ hydroxyl groups of adenosine are considered positions that are not tolerant of extensive modification in AR agonists (Siddiqi et al. 1995). We now know the structural explanation for this finding; the ribose resides in a sterically limited sub-pocket of the receptor and is surrounded by hydrophilic residues, which coordinates it through H-bonding (Ciancetta and Jacobson 2017). Nevertheless, there are isolated examples of modification of these two hydroxyl groups that maintain A_3AR

selectivity. For example, 3′-deoxy Cl-IB-MECA **16** displayed an affinity of 33 nM at the rA_3AR, which it fully activated in a measure of cAMP inhibition (Jacobson et al. 1995). Cordycepin (3′-deoxyadenosine, structure not shown) was found to exert an antitumor effect in mouse by activation of the A_3AR (Nakamura et al. 2006). However, the affinity of this compound at the rA_3AR was shown to be weak with 33% binding inhibition at 100 μM (van Galen et al. 1994). Some 3′-amino-3′-deoxy adenosine derivatives are potent hA_3AR agonists, e.g., the anti-ischemic agents **22** and **23** (DeNinno et al. 2006), but the preservation of A_3AR affinity in 3′-amino derivatives does not generalize across the range of adenosine modifications.

7.2.3 Methanocarba Analogues

The rigid methanocarba modification of nucleosides features a rigid bicyclo[3.1.0] hexane ring system replacing the tetrahydrofuryl group of ribose. There are two isomeric methanocarba modifications of ribose that result in locking the conformation as either a North (N)- or South (S)-envelope conformation, i.e., adenosine analogues **40** and **41**, respectively (Fig. 7.2). These modifications were applied in earlier studies of antiviral nucleosides, and Jacobson et al. (2000) first applied this pair of isomeric modifications to nucleosides acting at cell surface receptors. There was a consistent increase of hA_3AR affinity and selectivity, across a variety of adenosine derivatives, associated with the (N)-methanocarba analogue compared to both the (S) analogue and the native riboside. (N)-methanocarba analogues were also more potent at the A_3AR than the simple carbocyclic (cyclopentane) analogues. For the simple adenosine analogues, K_i values in binding to the hA_3AR were determined to be 404 nM (**40**) and 62.5 μM (**41**), respectively. The A_3AR, among all of the ARs, most benefitted from a locked (N)-methanocarba conformation. This suggested that the (N)-methanocarba modification achieve a pre-locking of the A_3AR-preferred conformation of the ribose ring. Although the three other ARs also likely require a (N)-conformation of ribose, as is now known from X-ray crystallographic structures of agonist-bound $A_{2A}AR$, the (N)-methanocarba modification is most suited structurally to binding at the A_3AR.

Functionality that is known to enhance A_3AR affinity and selectivity was combined with the (N)-methanocarba modification, and this combination was shown to be general for the range of SAR at this receptor (Tchilibon et al. 2005). Direct replacement of Cl-IB-MECA and its N^6-(3-halobenzyl) congeners with (N)-methanocarba provided potent and selective A_3AR agonists **45** and **46**. Compound **45** and its bromo analogue **47** were also radiolabeled, and these radiotracers were shown to have low nonspecific binding and to be useful in receptor characterization (Gao et al. 2009; Kiesewetter et al. 2009). Alternative functionality at the 2-position was allowed, e.g., 2-iodo **48** and 2-methylthio **49** analogues. The enhancement of A_3AR selectivity by this modification is so robust that even combination with the A_1AR-enhancing N^6-cyclopentyl group led to a balanced

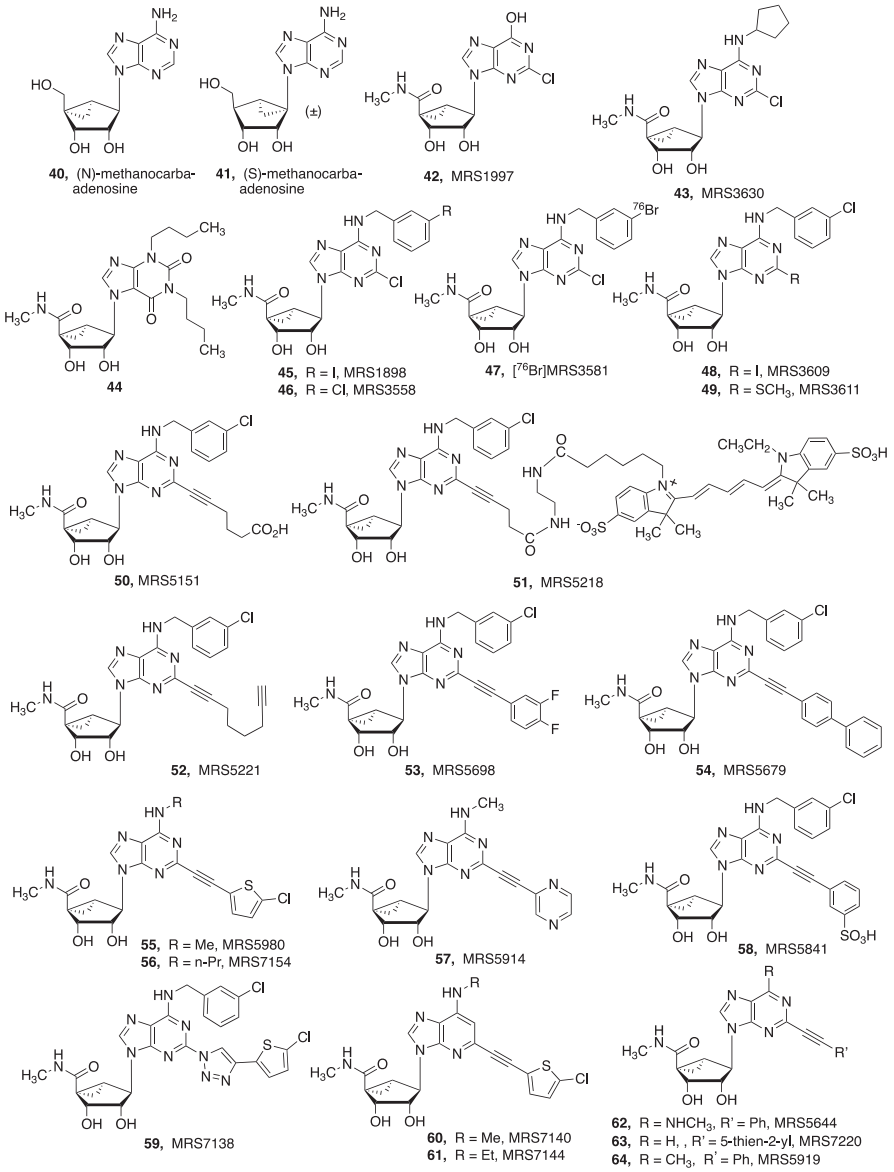

Fig. 7.2 Methanocarba analogues of A_3AR agonists

A_1AR/A_3AR mixed agonist **43**, which was shown to have anti-ischemic properties in the isolated mouse heart (Jacobson et al. 2005).

The N^6 group can be eliminated entirely, but this applies only when other affinity-enhancing groups, such as C2-extended substituents (Tosh et al. 2016), are present on the molecule, e.g., in 6-H derivative MRS7220 **63** (K_i hA_3AR, 60 nM) and 6-methylpurine derivative MRS5919 **64** (6.0 nM). Nevertheless, **64** was

sevenfold less potent in binding to the A_3AR than the corresponding 6-methylamino analogue **62**.

The (N)-methanocarba modification was suitable for functionalized congeners of A_3AR agonists (Tosh et al. 2009), such as an affinity-optimized carboxylic acid congener **50** containing a three-methylene spacer. The shorter two-methylene carboxylic acid homologue could be labeled by coupling to an amine-functionalized cyanine5 (Cy5) fluorophore to provide the high affinity fluorescent A_3AR agonist MRS5218 **51** which was shown to be a useful tracer for characterizing the receptor on whole cells or for use in drug screening (Kozma et al. 2013). For coupling to reporter groups or polymeric carriers, terminal alkyne **52** served as an intermediate for efficient click reactions rather than coupling by amide bond formation (Tosh et al. 2009). Conjugates of both the carboxylic acid and terminal alkyne functionalized congeners tended to retain A_3AR affinity.

C2-arylalkynyl (N)-methanocarba derivatives demonstrated that the A_3AR is highly permissive of bulky aryl groups on the alkyne, e.g., N^6-(3-chlorobenzyl) derivatives **53** and **54**, which was also confirmed in the case of N^6-methyl analogues, such as **62** (Tosh et al. 2014). A sulfonated agonist that would not diffuse across biological membranes was desired for in vivo studies; compound **58** was predicted computationally and proved to be highly potent and selective at both the mA_3AR and hA_3AR (Paoletta et al. 2013). An in vivo phenotypic screen allowed the comparison of C2-arylalkynyl (N)-methanocarba analogues based on efficacy and duration of action in a model of chronic neuropathic pain (Tosh et al. 2014; Janes et al. 2016). In this screen, a 5-chlorothienylethynyl group was particularly conducive to in vivo activity and therefore was incorporated in adenine derivatives MRS5980 **55**, MRS7154 **56**, and MRS5914 **57** and in 1-deazaadenine derivatives MRS7140 **60** and MRS7144 **61** and other analogues (Tosh et al. 2015, 2016).

A C2-triazole group, as in (N)-methanocarba analogue **59**, was found to be a suitable bioisosteric replacement for the diarylalkyne of MRS5980 and its congeners (Tosh et al. 2015). In the ribose series, C2-triazoles were similarly shown to promote A_3AR affinity in compounds **36** and **37** (Cosyn et al. 2006).

7.3 Nucleosides as A_3AR Antagonists and Partial Agonists

The conversion of selective A_3AR agonists into selective A_3AR antagonists was found to be relatively facile compared to comparable attempts at other AR subtypes. Modifications of the ribose moiety, particularly around the 5′-position, were found to be effective in reducing the relative efficacy of the nucleosides in functional assays, i.e., inhibition of the formation of cyclic AMP (cAMP, Gao et al. 2002a). Steric constraint, truncation, and reducing the H-bond donor ability of the ribose ring moiety all had the effect of reducing A_3AR efficacy resulting in partial agonists or antagonists (Gao et al. 2006).

Several issues in determining the E_{max} (as % of a full agonist effect, typically at 10 μM) of a given nucleoside derivative are (1) the reference full agonist used for comparison and (2) the dependence of E_{max} on the pathway measured. Both NECA **8**

and Cl-IB-MECA **15** are full agonists in inhibition of cAMP accumulation. However, the E_{max} of Cl-IB-MECA is only ~50% of NECA in some signaling events, such as A_3AR-induced GTP-γ-S binding and mobilization of Ca^{2+} (Gao et al. 2008; Gao et al. 2011). Therefore, even for the same readout, which reference compound is used is important in classifying the nucleoside as a low- or high-efficacy partial agonist.

Introduction of an 8-(hexyn-1-yl) group reduced the A_3AR efficacy of adenosine in antagonist **65** (Volpini et al. 2001, Fig. 7.3). However, most nucleoside-based antagonists reported are modified at other sites on the adenine or ribose moieties. Commonly used A_1AR agonist **67** proved to be an antagonist at the hA_3AR, while substitution of the N^6 group with a 3-iodobenzyl moiety in **71** produced a low-efficacy agonist (Gao et al. 2002a). Steric constraint of the 5′-amide in the form of a spirolactam reduces the efficacy such that compound **68** is a potent A_3AR antagonist (K_i 29 nM) that likely retains binding selectivity, by analogy to an earlier acyclic 4′-methyl-5′-amide derivative (structure not shown, Gao et al. 2002a; Siddiqi et al. 1995). Furthermore, in a limited number of cases, modifications of the N^6 and C2 substituents also were found to reduce efficacy. For example, although the sterically bulky fluorenylmethyl derivative **28** is a full agonist at the hA_3AR, its more flexible analogue **69** is an A_3AR antagonist. Thus, introducing rigidity at various nucleoside positions may either reduce or increase E_{max}.

4′-Truncation of adenosine derivatives in both ribo, e.g., **74**–**76**, and (N)-methanocarba series, e.g., **77**, **78**, and **82**, were A_3AR antagonists or low-efficacy agonists, although truncation tends to lower their affinity at r and mA_3ARs. However, some truncated derivatives, e.g., **81**, were noted to bind appreciably at the mA_3AR, with moderate selectivity as an antagonist (Tosh et al. 2012b). 4′-Truncated 4′-thionucleoside **76** both activated the A_{2A}AR and antagonized A_3AR (Hou et al. 2012). *N,N*-Dimethyl oxo-nucleoside **79** and thionucleoside **80** were pure antagonists at the A_3AR, with selectivity in binding and K_i values of 29 and 9 nM, respectively (Jeong et al. 2008). 4′-Ester derivatives of adenosine in the ribo, e.g., **70**, and (N)-methanocarba series, e.g., **83** (K_i, 5.4 nM, E_{max} 12% of NECA in forskolin-stimulated cAMP production in CHO cells) and **84**, also tend to be partial hA_3AR agonists (Tosh et al. 2017). 4′-Tetrazole derivative **85** of adenosine was recently reported to potently activate A_1AR and antagonize the A_3AR, while other N^6 substitutions produced mixed A_1AR/A_3AR agonists (Petrelli et al. 2017).

2-Substituted adenosine analogues display a range of A_3AR efficacies (relative to NECA **8**, cAMP), e.g., 2-(2-(3-chlorophenyl)ethyl)-adenosine (K_i, 41 nM, E_{max} 31%) and 2-(3-chlorobenzyl)-adenosine (K_i, 72 nM, E_{max} 16%) (structures not shown, Gao et al. 2004).

7.4 Nonnucleoside Heterocycles as A_3AR Antagonists

In addition to the nucleoside antagonists of the A_3AR, diverse classes of heterocycles have been identified as scaffolds for hA_3AR antagonists. Broad screening of various heterocyclic libraries, including known pharmacological agents and

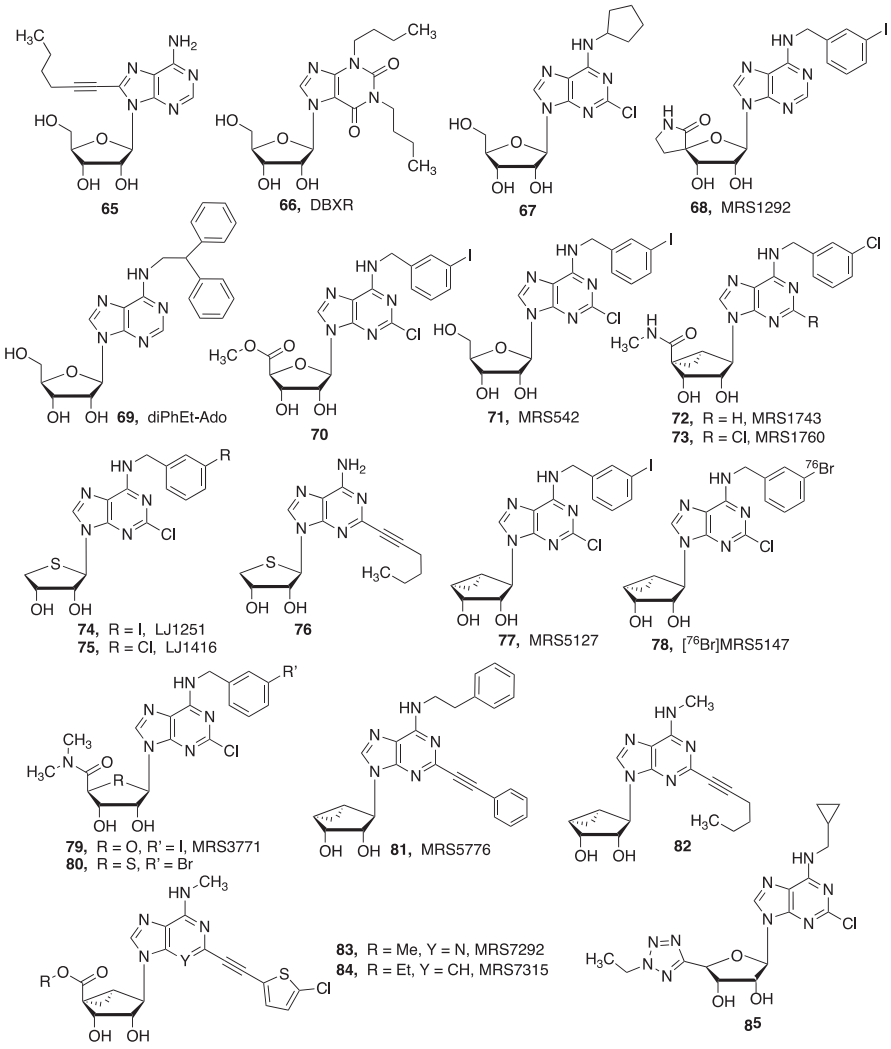

Fig. 7.3 Nucleoside-derived A_3AR antagonists and partial agonists

phytochemicals, has been performed in order to obtain new leads for potent and highly selective A_3AR antagonists. Xanthine or purine analogues were examined first, but none of the tested compounds showed significant affinity or selectivity at rA_3AR (Jacobson et al. 2009). Inhibition of rA_3AR binding by diverse structures identified novel ligands, e.g., sulfonylpiperazines, a pyridazinone, imidazopyrimidines, pteridines, and a carbazolenine, as weak ligands (Siddiqi et al. 1996). Currently, virtual screening for AR antagonists is based on either antagonist-bound A_{2A}AR X-ray structures or homology models of the other AR subtypes. Often, new chemotypes are found for other ARs, including the A_3AR when docking chemical libraries to an A_{2A}AR structure (Rodriguez et al. 2015).

Subsequent to early broad library screening, a large number of compounds with high potency and selectivity as hA_3AR antagonists were documented that are generally characterized as structurally diverse nitrogen-containing aromatic monocyclic/bicyclic/tricyclic systems. Nonnucleoside A_3AR antagonists can be grouped into two broad categories: (1) xanthine analogues and (2) other aromatic monocyclic/bicyclic/tricyclic systems.

7.4.1 Xanthine Analogues (Table 7.2)

The natural products 1,3-dimethylxanthine (theophylline) and 1,3,7-trimethylxanthine (caffeine) showed negligible affinity at the rA_3AR (Müller 2001). Structural modifications at different positions of the xanthine core aimed at improving A_3AR affinity led to a series of tricyclic analogues of xanthine such as 1-benzyl-3-propyl-1*H*,3*H*-pyrido[2,1-*f*]purine-2,4-dione **86** (Fig. 7.4), which showed good affinity (K_i 4.0 nM) but low selectivity over the other ARs (Priego et al. 2002). Introduction of a cyclopropylmethyl group at the N^3-position in combination with a 4-methylbenzyl group at the 1-position led to compound **87**, which preserved affinity at the A_3AR with a significant enhancement of selectivity (Priego et al. 2008). The strictly correlated imidazo[2,1-*i*]purinones were found to be potent and selective A_3AR antagonists. The most important compound of this series is PSB-11 (**88**) that showed a K_i value of 2.3 nM at the hA_3AR and good selectivity versus the other AR subtypes (Müller et al. 2002b). The radiolabeled derivative of this compound exhibited a K_d value of 4.9 nM (Müller et al. 2002a). Another similar compound KF-26777 (**89**, 2-(4-bromophenyl)-7,8-dihydro-4-propyl-1*H*-imidazo[2,1-*i*]purin-5(4*H*)-one) offered high affinity and selectivity to the hA_3AR (K_i 0.20 nM) (Ozola 2003).

Subsequently, substitution of the 2-phenyl ring of **88** and congeners with five-membered heterocycles, in particular 1,5-disubstituted (not shown) and 1,3-disubstituted pyrazoles or 3-substituted isoxazoles, led to the tricyclic xanthine derivatives such as compounds **90** and **91**, respectively. These antagonists were endowed with high affinity and selectivity for hA_3AR. The hypothetical binding mode of these A_3AR antagonists was determined in docking studies to an A_3AR homology model (Baraldi et al. 2011).

In this class of compounds, triazolopurine derivatives in which a simple xanthine structure is elaborated with an additional pyrimidine-fused ring are also reported. One example is OT-7999 (**92**), which proved to be a potent and selective hA_3AR ligand (K_i 0.95 nM) and > 10,000-fold selectivity compared to other AR subtypes (Okamura et al. 2002).

Table 7.2 Affinity of selected A_3AR antagonists

Compound	pK_i value or % inhibition at 10 μM			Ref.
	A_1AR	A_{2A}AR	A_3AR	
86	7.30 (h)	6.92 (h)	8.40 (h)	Priego et al. (2002)
87	*24%*	*0%*	8.66 (h)	Priego et al. (2008)
88, PSB-11	5.79 (h)	5.89 (h)	8.63 (h)	Müller et al. (2002b)
89, KF26777	5.74 (h)	6.33 (h)	9.70 (h)	Ozola (2003)
90	5.60 (h)	<5.3 (h)	8.84 (h)	Baraldi et al. (2011)
91	5.52 (h)	5.82 (h)	8.71 (h)	Baraldi et al. (2011)
92, OT-7999	*4%* (h)	*31%* (h)	9.02 (h)	Hou et al. (2012)
93, MRS1523	<5 (h)	5.44 (h)	7.72 (h)	Li et al. (1998)
	4.81 (r)	5.69 (r)	6.95 (r)	Müller and Jacobson (2011)
94, MRS1097	5.23 (r)	5.32 (r)	6.97 (h)	Jiang et al. (1996)
95, MRS1191	3.40 (r)	*<10%* (r)	7.50 (h)	Jiang et al. (1997)
96, ISVY130	*1%* (h)	*10%* (h)	8.44 (h)	Cosimelli et al. (2008)
97	<6.18 (h)	<6.08 (h)	9.44 (h)	Jung et al. (2004)
98	*24%* (h)	*28%* (h)	9.10 (h)	Huffman et al. (2005)
99, VUF5574	*52%* (r)	*43%* (r)	8.39 (h)	Van Muijlwijk-Koezen et al. (2000)
100	6.37 (h)	5.09 (h)	8.22 (h)	Biagi et al. (2005)
101, MRS3777	*26%* (h)	*16%* (h)	7.33 (h)	Perreira et al. (2005)
102, MRS1067	*36%* (r)	*19%* (r)	6.25 (h)	Karton et al. (1996)
103	*0%* (h)	*19%* (h)	10.11 (h)	Poli et al. (2011)
104	5.98 (h)	5.50 (h)	10.74 (h)	Taliani et al. (2010)
105	>5.0 (h)	>5.0 (h)	10.11 (h)	Taliani et al. (2010)
106	8.92 (h)	*5%* (h)	*1%* (h)	Lenzi et al. (2009)
107	*1%* (h)	*1%* (h)	11.57 (h)	Squarcialupi et al. (2016)
108, CGS15943	7.68 (r)	8.49 (r)	7.86 (h)	Kim et al. (1998)
109, MRS1220	7.28 (r)	8.00 (r)	9.19 (h)	Jacobson et al. (1997)
	7.09 (m)	8.04 (m)	~4[a] (m)	Wan et al. (2004)
110, MRE3008-F20	<5 (r)	5.70 (r)	9.54 (h)	Baraldi et al. (2000)
111, MRE3005-F20	6.60 (h)	7.22 (h)	10.40 (h)	Maconi et al. (2002)
112	5.47 (h)	<5.3 (h)	8.01 (h)	Baraldi et al. (2012)
113	*0%* (h)	*21%* (h)	8.05 (h)	Colotta et al. (2007)
114	>6 (h)	>6 (h)	8.05 (h)	Baraldi et al. (2005)
115	>5 (h)	>5 (h)	10.10 (h)	Jacobson et al. (2009)
116	5.57 (h)	>5 (h)	8.80 (h)	Da Settimo et al. (2007)

h human, *r* rat

[a]31% inhibition at 100 μM

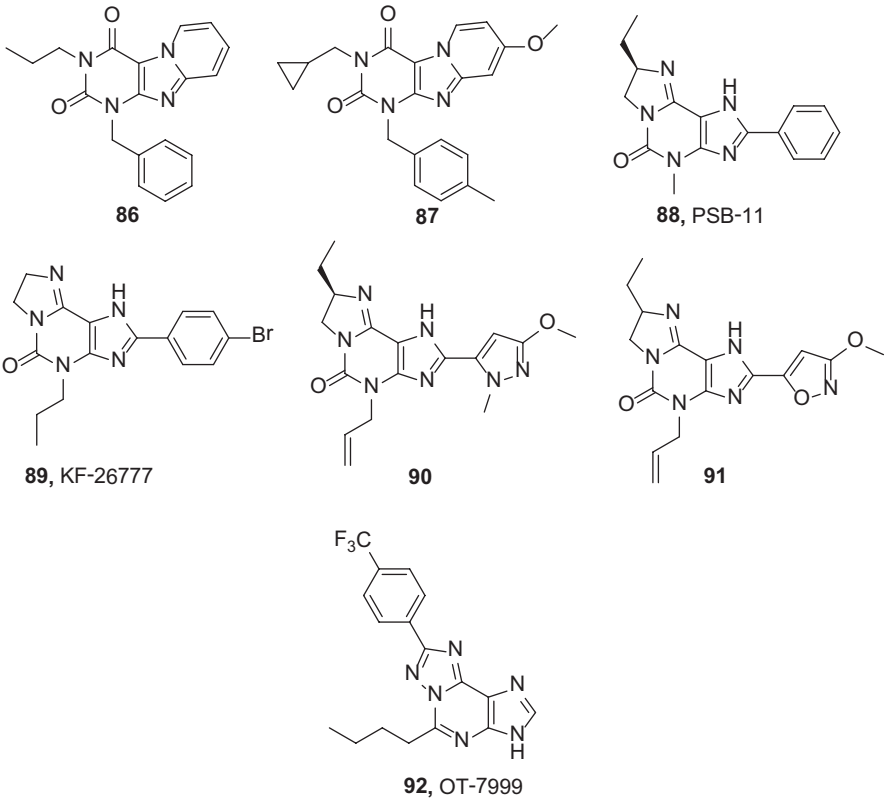

Fig. 7.4 Xanthine analogues as A_3AR antagonists

7.4.2 Aromatic Monocyclic/Bicyclic/Tricyclic Systems (Table 7.2)

Jacobson and coworkers investigated the SAR profile of the pyridine and the 1,4-dihydropyridine nucleus as A_3AR antagonists (van Rhee et al. 1996). Introduction of sterically bulky groups at the 6-position of pyridine led to one of the first heterocyclic, selective, and competitive A_3AR antagonist MRS1523 (**93**, Fig. 7.5). This compound showed good potency in both humans and rodents, with K_i values of 18.9 nM for hA_3AR and 113 nM for rA_3AR. A later study comparing the species dependence of common AR antagonists showed MRS1523 **93** to be only moderately selective for the rA_3AR (Alnouri et al. 2015).

The A_3 antagonists related to the 1,4-dihyropyridine nucleus with sterically bulky groups at the 4-, 5-, and 6-positions, such as 2-methyl-6-phenyl-4-styryl-1,4-dihydro-pyridine-3,5-dicarboxylic acid diethyl ester and 2-methyl-6-phenyl-4-phenylethynyl-1,4-dihydropyridine-3,5-dicarboxylic acid 5-benzyl ester, named MRS1097 and MRS1191, respectively (**94** and **95**), were also reported (Jiang et al. 1996; Jiang et al. 1997).

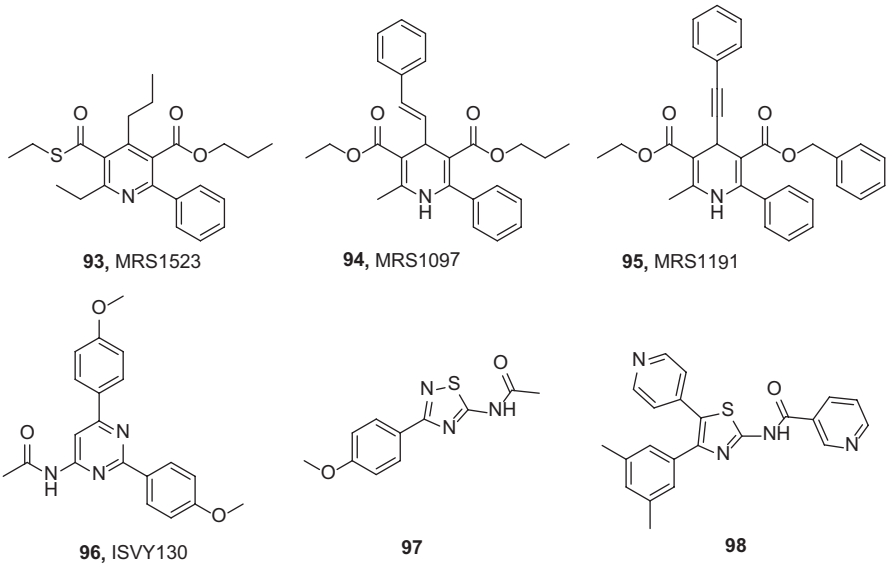

Fig. 7.5 Aromatic monocyclic systems: pyridine, dihydropyridine, pyrimidine, thiazole, and thiadiazole derivatives as A_3AR antagonists

Among monocyclic compounds, the diaryl 2- or 4-amidopyrimidines have been reported as A_3AR antagonists. In particular, *N*-(2,6-bis(4-methoxyphenyl) pyrimidin-4-yl)acetamide derivative **96** named ISVY130 showed favorable affinity at the hA_3AR (K_i 3.6 nM) (Cosimelli et al. 2008).

Thiazole and thiadiazole analogues were initially identified by simplifying the bicyclic ring system of isoquinolines and quinazolines with several monocyclic rings as a promising class of adenosine A_3AR antagonists (Jung et al. 2004). In this group, *N*-[3-(4-methoxyphenyl)-[1,2,4]thiadiazol-5-yl]acetamide (**97**) was reported as a potent hA_3AR antagonist with a K_i value of 0.79 nM (Jung et al. 2004). Subsequently, a series of 4-phenyl-5-pyridyl-1,3-thiazole derivatives with hA_3AR affinity was identified (Miwatashi et al. 2008). As a result, the SAR study identified a potent A_3AR antagonist **98** with K_i values of 0.36 nM for hA_3AR and 1.6 nM for rA_3AR, although no further studies have been published using this compound.

A class of hA_3AR antagonists, structurally related to the bicyclic isoquinoline and quinazoline urea derivatives, has been reported. The combination of the optimal substituents in the two series led to the potent hA_3AR antagonist *N*-(2-methoxyphenyl)-N^9-(2-(3-pyridyl)quinazolin-4-yl)urea **99** (VUF5574, Fig. 7.6) with a K_i value of 4.0 nM and > 2400-fold selectivity versus A_1 and A_{2A}ARs (Van Muijlwijk-Koezen et al. 2000).

The first class of A_3AR antagonists with a bicyclic structure, rigorously related to the adenine nucleus, was described within a series of N^6-ureido-substituted 2-phenyl-9-benzyl-8-azaadenines. In this family, the adenine-like structure was responsible for the antagonist activity, while the phenylcarbamoyl group was

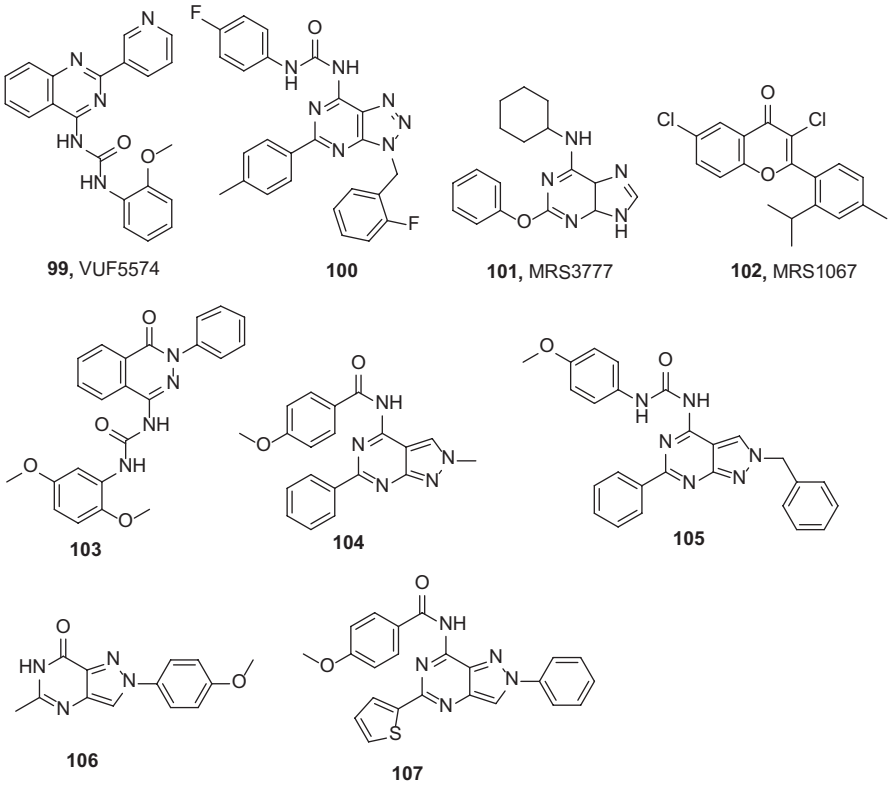

Fig. 7.6 Aromatic bicyclic systems: quinazoline, (aza) adenine, flavone, 2-phenylphthalazine, pyrazolo[3,4-d]pyrimidine, and pyrazolo[4,3-d]pyrimidin derivatives as A_3AR antagonists

important for selectivity at the A_3AR (**100**) (Biagi et al. 2005). A series of adenine-based derivatives was also synthesized using "reversine" (2-(4-morpholinoanilino)-N^6-cyclohexyladenine) as a template. One of the most interesting compounds in terms of hA_3AR affinity and selectivity was MRS3777 (**101**, K_i hA_3AR = 47 nM), which was derived from substitution of the N^6-cyclohexyl moiety of reversine with a 2-phenyloxy group. In rA_3AR binding assays, these adenine derivatives reflected the species dependence of affinity that is typical of most known nonnucleoside A_3AR antagonists, i.e., they were inactive at 10 μM (Jacobson et al. 2009).

The SAR optimization of the bicyclic flavone nucleus led to the MRS1067 (**102**) as the most potent and selective hA_3AR compound of this series (K_i hA_3AR = 591 nM) (Jacobson et al. 1997). At the rA_3AR, MRS1067 (30 μM) completely antagonized agonist effects in RBL-2H3 rat basophilic cells (Shin et al. 1996).

Among bicyclic systems, the 2-phenylphthalazin-1(2*H*)-one scaffold was identified for the design of hA_3AR antagonists. Introduction of different amide and ureido moieties led to the 2,5-dimethoxyphenylphthalazin-1(2*H*)-one **103** being the most potent and selective A_3 antagonist among this series (K_i hA_3AR = 0.77 nM) (Poli et al. 2011).

The pyrazolo[3,4-*d*]pyrimidine nucleus structurally related to the adenine nucleus has been also reported (Taliani et al. 2010). The SAR profile of this series highlighted the importance of amide or ureide functions at the 4-position along with a phenyl ring at the 6-position for A_3AR affinity and selectivity, such as in compounds **104** and **105**, respectively. In a related work, the 2-arylpyrazolo[4,3-*d*] pyrimidin-7-one derivatives were also examined, in which the new derivatives showed high affinity for the hA_3AR and increasing selectivity versus the other AR subtypes in comparison with the pyrazolo[3,4-*d*]pyrimidine isomers. Aryl/arylalkyl substitution at the 5-position of such derivatives was poorly tolerated for A_3AR binding affinity, while small groups at the same position were shown to increase ligand−receptor interaction. In addition, the introduction of a methoxy group on the 2-phenyl ring led to the most potent compound of the series (**106**) (Lenzi et al. 2009) Furthermore, a large number of 2-arylpyrazolo[4,3-*d*]pyrimidin-7-amine or 7-acylamine derivatives have been reported as potent A_3AR antagonists (Squarcialupi et al. 2013, 2016). In particular, the 2-phenyl-5-(2-thienyl)-pyrazolo[4,3-*d*] pyrimidin-7-(4-methoxybenzoyl)amine **107** was a potent hA_3AR antagonist in this series with a K_i value of 0.02 nM (Squarcialupi et al. 2016).

The tricyclic triazoloquinazoline scaffold represented by compound CGS15943 (**108**, Fig. 7.7) was one of the first nonxanthine hA_3AR antagonists. CGS15943 displayed a K_i value of 514 nM for hA_3AR and thus was a nonselective AR antagonist. This heterocycle proved to be a suitable starting template for the design of potent and selective hA_3AR antagonists (Kim et al. 1998). Acylation of the free amino group at the N^5-position of CGS15943 with aryl or arylalkyl moieties has enhanced both hA_3AR affinity and selectivity. This finding was exemplified by MRS1220 (**109**) that showed subnanomolar affinity at the hA_3AR with ~400- and ~40-fold selectivity vs. rA_1AR and rA_{2A}AR subtypes, respectively (Kim et al. 1996, 1998). However, the selectivity in human was not maintained in rat and mouse. In particular, MRS1220 is A_{2A}AR-selective in those species, with a K_i values >10 μM at the r and mA_3ARs (Wan et al. 2004; Gao et al. 2009). The structurally related pyrazolo-triazolo-pyrimidines for the development of AR antagonists have been broadly reviewed (Baraldi et al. 2008; Cheong et al. 2013). Bioisosteric replacement of the phenyl ring of CGS15943 with a heterocyclic pyrazole ring led to the first example of an A_{2A}AR antagonist named 8FBPTP, featuring an 8-substituted pyrazolo-triazolo-pyrimidine core (Gatta et al. 1993; Dionisotti et al. 1994). Subsequently, a large number of tricyclic compounds (MRE series) were prepared during SAR optimization studies based on facile synthetic chemistry leading to substitutions at the C^2-, C^5-, C^9-, N^7-, and N^8-positions of the pyrazolo-triazolo-pyrimidine nucleus (Baraldi et al. 2008). Attention was focused on the N^8 substitution patterns, due to the complete inactivity of the N^7-substituted derivatives at the hA_3AR. The most potent and selective compounds at the hA_3AR subtype emerged from the combination of a small alkyl chain at the N^8-pyrazole position with a (substituted)phenylcarbamoyl residue at the N^5-position (Baraldi et al. 2000). Compound **110** is one of the most favorable examples representing this class, with high affinity (K_i hA_3AR = 0.29 nM) and selectivity over both rat and hA_1ARs and A_{2A}ARs (Varani et al. 2000). Another important compound of this series is the 4-pyridyl-carbamoyl derivative **111** that showed high affinity with a K_i value of 10 pM at hA_3AR (Maconi et al. 2002).

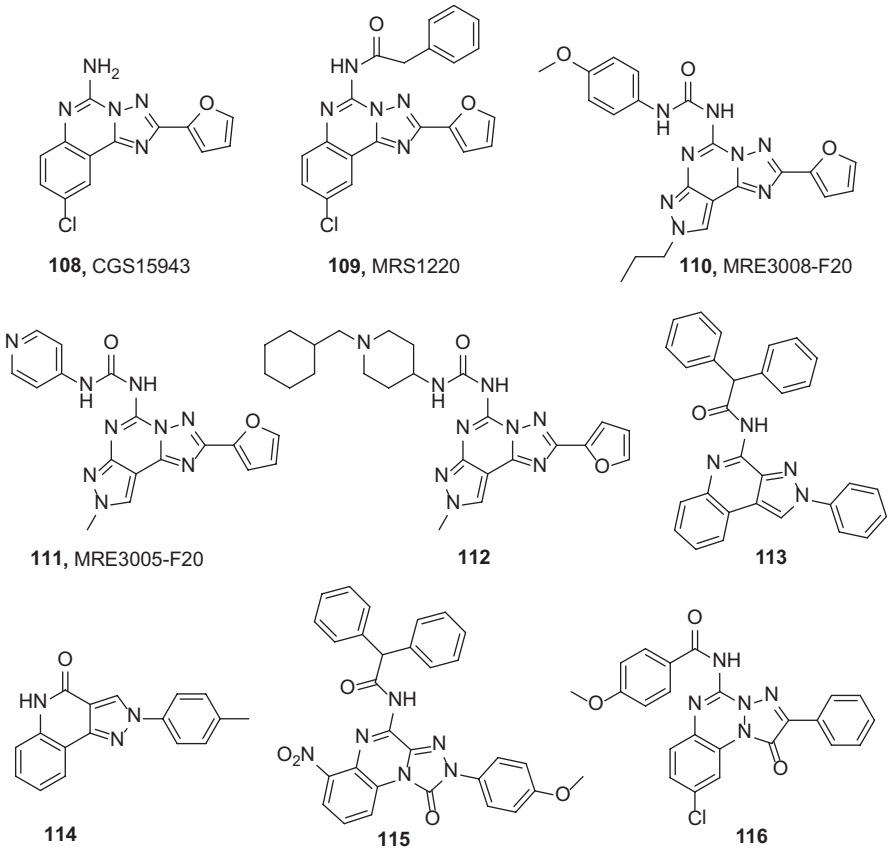

Fig. 7.7 Aromatic tricyclic systems: triazoloquinazoline, pyrazolo-triazolo-pyrimidine, pyrazoloquinolines, triazoloquinoxaline, and aminophenyltriazolobenzotriazinone derivatives as A_3AR antagonists

Consequently, replacement of pyridin-4-yl moiety of MRE3005-F20 **111** with a substituted piperidine ring led to the hydrochloride salt of 1-(1-(cyclohexylmethyl) piperidin-4-yl)-3-(2-(furan-2-yl)-8-methyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl)urea **112**. This compound was the most active of the series showing high hA_3AR affinity and selectivity against the other subtypes, with aqueous solubility of 8 mg/mL at physiological pH (Baraldi et al. 2012).

The tricyclic pyrazolo[3,4-*c*]/[4,3-*c*]quinolines have been reported as A_3AR antagonists. Several 4-benzoylamido derivatives were prepared by introduction of bulky and lipophilic (hetero)aroylamino groups or a benzylcarbamoyl residue at the 4-position of pyrazolo[3,4-*c*]quinoline. An example of these derivatives is compound **113,** shown in Fig. 7.7, that exhibited a K_i value of 8.9 nM in binding experiments (Colotta et al. 2007). In a related effort, further pyrazoloquinolines as structural isomers of the parent 2-arylpyrazolo[3,4-*c*]quinoline derivatives have also

been reported. Among them, the 2-(*p*-tolyl)-2*H*-pyrazolo[4,3-*c*]quinolin-4(5*H*)-one derivative **114** showed high affinity and selectivity (K_i hA_3AR = 9 nM) as evaluated in radioligand binding assays (Baraldi et al. 2005).

Triazolo[4,3-*a*]quinoxaline was also identified as a suitable scaffold for A_3AR antagonists (Colotta et al. 2004; Lenzi et al. 2006). Efficient substitution of the 2-, 4-, and 6-positions of the tricyclic template, with molecular modeling investigations, led to the identification of optimal structural requirements for A_3AR affinity and selectivity. In particular, sterically hindered and lipophilic acylamino moieties at the 4-position enhanced A_3AR affinity and selectivity (**115**, K_i hA_3AR = 0.8 nM, Fig. 7.7) (Jacobson et al. 2009).

The aminophenyltriazolobenzotriazinone A_3AR antagonists have been reported. In this series, the structural modifications by introduction of appropriate moieties on the 5-amino function and in the 4′-and/or 9-positions led to compound **116** (Fig. 7.7) which showed a K_i value of 1.6 nM at the A_3AR and no significant affinity at the other ARs (Da Settimo et al. 2007).

7.5 Allosteric Modulators of the A_3AR

The SAR of three major heterocyclic classes of positive allosteric modulators (PAMs) have been explored: 3-(2-pyridinyl)isoquinolines (e.g., **117**, Fig. 7.8), 1*H*-imidazo-[4,5-*c*]quinolin-4-amines, and 2,4-disubstituted quinolines (Göblyös et al. 2006; Kim et al. 2009; Heitman et al. 2009). The imidazo-[4,5-*c*]quinolin-4-amines (**118**–**121**) have been most extensively explored, and a key PAM in this series is LUF6000 **119**. The closely related series of 2,4-disubstituted quinolines is represented by amide derivative LUF6096 **122**, which was shown to be a potent PAM, but with a short half-life in vivo (Du et al. 2012). Species differences are evident in the A_3AR PAMs, and a potent PAM at the r or mA_3ARs is still lacking (Du et al. 2018). However, **119** was reported to alleviate erectile dysfunction in rats treated with streptozotocin to induce diabetes (Cohen and Fishman 2016).

A functional bias in the allosteric actions of imidazo-[4,5-*c*]quinolin-4-amines has been characterized (Gao et al. 2011). LUF6000 was found to be more efficacious in enhancing agonist E_{max} of low-efficacy partial agonists than high-efficacy agonists, suggesting flexibility in modulating E_{max}.

7.6 Modeling and Structural Probing of the A_3AR

The facility of having a consistent model of ligand recognition at the A_3AR has guided the design of novel orthosteric ligands. Extensive site-directed mutagenesis (SDM) of the hA_3AR has been performed to locate the residues involved in ligand recognition (Gao et al. 2002b; Duong et al. 2005). Constitutively active mutations

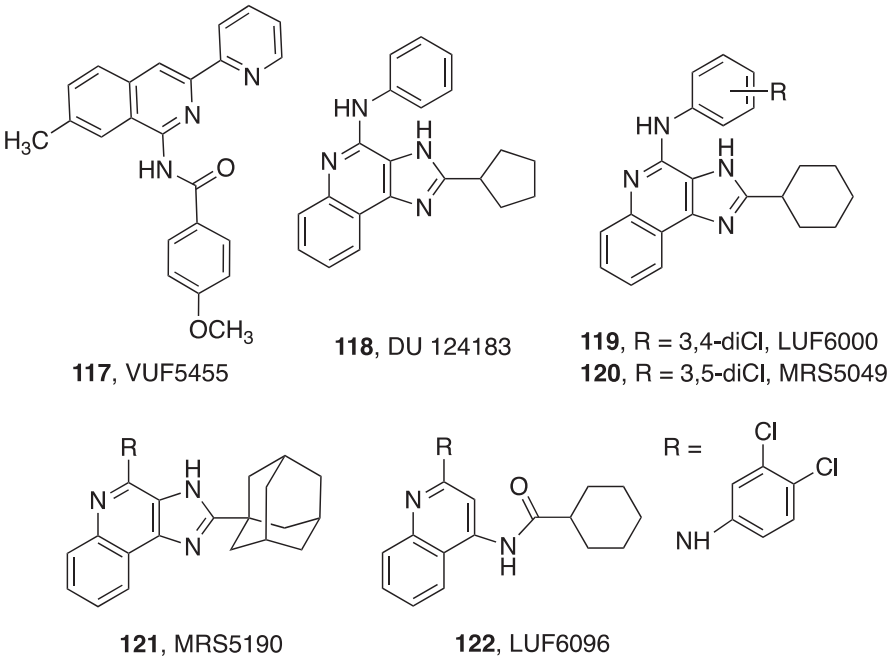

Fig. 7.8 Representative positive allosteric modulators (PAMs) of the A_3AR

of the A_3AR were reported (Chen et al. 2001). Homology modeling of the hA_3AR based on several successive templates (rhodopsin and the $hA_{2A}AR$) has identified conserved residues in the putative binding site that recognize the ribose moiety and the adenine moiety (Cheong et al. 2013; Ciancetta and Jacobson 2017; Dal Ben et al. 2014). Both docking and molecular dynamics simulations have been performed to predict ligand complexes of the A_3AR. In addition, a neoceptor approach to identifying complementarity between the receptor protein and a bound agonist analogue has been applied to the A_3AR (Jespers et al. 2018), and its prediction of proximity of the ribose moiety to hydrophilic side chains in TM3 and TM7 has been supported by experimental and computational methods. A hybrid model of the agonist-bound hA_3AR has been proposed in order to accommodate the bulky C2-arylethynyl groups when combined with the (N)-methanocarba modification. An outward movement of TM2 (second transmembrane helix), similar to its position in active states of opsin and the α_2-adrenergic receptor, is needed to prevent steric clash of the receptor protein with the C2 substituent. A functional bias in the efficacy of orthosteric agonists to favor the cAMP pathway has been found to correlate with the length of the rigid C2-substituent (Baltos et al. 2016); compound **54** was the most elongated analogue tested.

The amino acid residues that are associated with the allosteric action of 3-(2-pyridinyl)isoquinolines and imidazo-[4,5-*c*]quinolin-4-amines have been probed through mutagenesis (Gao et al. 2003b) and molecular modeling (Deganutti et al. 2015). However, the precise binding site of the A_3AR PAMs has not been established.

7.7 Conclusions

The clinical studies with two A_3AR agonists (**14** and **15**) for treating autoimmune inflammatory disorders and liver diseases are continuing and appear encouraging. The interest in both agonists and antagonists of the A_3AR for therapeutic application has motivated numerous SAR studies of selective agonists, antagonists, partial agonists, and allosteric modulators. Subnanomolar affinity and selectivity of >10,000-fold have been achieved for various compound classes. The issue of species dependence of the A_3AR affinity has to be addressed in each medicinal chemistry study, especially considering that most antagonist classes greatly favor the hA_3AR over the rat and mouse homologues. The design of A_3AR orthosteric ligands is now largely guided by computational approaches. We have amassed a large body of data concerning the relationship of receptor structure and function and have supported many of the hypotheses generated with experimental data.

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Chapter 8
Binding Thermodynamic Characteristics of Adenosine Receptor Ligands

Fabrizio Vincenzi, Katia Varani, and Pier Andrea Borea

Abstract Receptor binding thermodynamics is a powerful tool to gain deep insight, at the molecular level, of the events that occur during drug-receptor interactions. This chapter focuses on the determination of thermodynamic parameters based on the van't Hoff analysis as a traditional method to discover the enthalpic and entropic contributions during drug-receptor binding. Thermodynamic parameters of adenosine receptor ligands such as standard free energy (ΔG°), standard enthalpy (ΔH°), and standard entropy (ΔS°) are reported, discussed, and compared with those observed for other membrane receptors investigated from a thermodynamic point of view. The available thermodynamic data are evaluated in terms of two important physical phenomena, the thermodynamic discrimination and enthalpy-entropy compensation. Thermodynamic parameters obtained by means of radioligand binding studies for adenosine receptor ligands, as well as for other classes of receptors, represent relevant information to the drug design and optimization providing a benefit to the drug discovery process.

Keywords Thermodynamics · Enthalpy · Entropy · Free energy · van't Hoff equation · Adenosine receptors

8.1 Introduction

Thermodynamic analysis offers invaluable information on drug-receptor interactions potentially unavailable by other means. With regard to the binding of agonist or antagonists, drug-receptor interactions are usually characterized by a single measure of affinity that is quantified by the use of the equilibrium association constant (K_A) or, more commonly, its reciprocal the dissociation constant (K_D). The typical receptor binding assays performed at a single temperature provide little information about the molecular mechanisms underlying the interaction of a drug with a given receptor. In fact, the simple determination of a ligand affinity makes it possible to

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calculate the standard free energy ΔG° ($\Delta G^\circ = -RT\ln K_A$) but not its two components, the equilibrium standard enthalpy (ΔH°) and entropy (ΔS°), as defined by the Gibbs equation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$. Standard enthalpy can be employed as a quantitative indicator of the changes in intermolecular bond energies which develop during binding (Borea et al. 2000; Holdgate and Ward 2005). Standard entropy can be considered an indicator of the rearrangements undergone by the solvent molecules during the same process (Gilli et al. 1994). Similar to most other biochemical reactions, the forces typically involved in drug-receptor interactions are not covalent, but rather a combination of non-covalent bonds such as hydrogen bonds, van der Waals forces, and hydrophobic interactions. When a drug molecule interacts with a receptor, it triggers a rearrangement of not only the receptor molecule with which it couples but also of the solvent molecules from which it uncouples.

The simultaneous optimization of enthalpy and entropy is complicated by several factors. First is the difficulty to optimize the forces that contribute to the binding enthalpy, and, second, the enthalpy gain is often compensated by an entropy loss. On the contrary, the binding entropy is easier to optimize because it is dependent primarily on the hydrophobic effect and is less affected by enthalpy compensation. Consequently, the recent trend has been toward increasingly hydrophobic, poorly soluble, entropically optimized drug candidates. However, it appears that a better binding enthalpy is critical for the development of improved drugs (Freire 2008). A favorable interaction enthalpy indicates that the drug establishes good and strong interactions with the target compensating the unfavorable enthalpy associated with desolvation. Conversely, an unfavorable binding enthalpy usually is an indication that polar groups are not forming strong bonds with the target and that the desolvation penalty dominates (Freire 2008). The most effective drug design and development platform comes from an integrated process. The understanding of the energetic basis of molecular interactions utilizing all available information from structural, thermodynamic, and biological studies is essential to realize an effective drug design (Garbett and Chaires 2012).

8.2 The van't Hoff Equation

The forces typically involved in drug-receptor interactions are not covalent, but rather are one or more of the following types: hydrogen bonds (of various strengths), van der Waals (and London) forces, hydrophobic interactions, and other similar phenomena. Because drug-receptor interactions are typically reversible, they are generally ascribable to standard equilibrium thermodynamic analysis. It is increasingly acknowledged that, to fully appreciate relevant molecular properties of potential drug candidates in a drug-design process, there is a need for thermodynamic studies. Traditionally, van't Hoff analysis has been used for thermodynamic studies.

There are two major ways of measuring thermodynamic parameters. One way has been proposed by the Dutch chemist J.H. van't Hoff in 1884. The van't Hoff equation provides information about the temperature dependence of the equilibrium constant. The van't Hoff equation may be derived from the Gibbs-Helmholtz equation, which gives the temperature dependence of the Gibbs free energy as

$$\Delta G^{\circ} = -RT \ln K_{\mathrm{A}}$$

(where T is the temperature in Kelvin and R is the ideal gas constant = 8.314 J/K/mol)

Because ΔG is related to the change in enthalpy (ΔH°) and entropy (ΔS°) by the equation $\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ}$, the former equation can be rearranged to

$$\ln\left(K_{\mathrm{A}}\right) = \left(-\Delta H^{\circ} / R\right)\left(1/T\right) + \Delta S^{\circ} / R$$

which is the integrated form of the van't Hoff equation. It actually follows from the van't Hoff equation $\mathrm{d}(\ln Keq)/\mathrm{dT} = \Delta H^{\circ}/RT^2$ and is an approximation that is valid when ΔH° and ΔS° are not temperature dependent. It is worth noting that this equation represents a linear relationship between $\ln(K_{\mathrm{A}})$ and $1/T$ with slope = $-\Delta H^{\circ}/R$ and y-intercept = $\Delta S^{\circ}/R$. It is a common practice in thermodynamic analysis of pharmacological interactions to determine K_{A} at several different temperatures and then construct a van't Hoff plot from which ΔH° and ΔS° are determined from the slope and the y-intercept of the resultant data plotted as $\ln(K_{\mathrm{A}})$ against $1/T$ (which is a line if the heat capacity is independent of temperature) (Fig. 8.1a). For an endothermic reaction, the slope is negative, and so as the temperature increases, the equilibrium constant increases, as shown in Fig. 8.1b. For an exothermic reaction, the slope is positive, and so as temperature increases, the equilibrium constant decreases, as illustrated in Fig. 8.1c.

The terms ΔG°, ΔH°, and ΔS° indicate the measurements made under standard state conditions of 1 atmosphere, unit activity (1 M concentration), and at 1 M hydrogen ion concentration (pH 0). A smaller error in ΔH° is obtained if ΔS° is determined first from the van't Hoff plot and then ΔH° from $\Delta H^{\circ} = \Delta G^{\circ} + T\Delta S^{\circ}$. Therefore, a method based on K_{D} measurements over a range of temperatures combined with van't Hoff plot analysis has been successfully applied to different receptor systems to obtain the thermodynamic terms of Gibbs equation (Borea et al. 2000).

Different receptorial systems have been so far studied in greater detail from a thermodynamic point of view, most of which concern membrane receptors: (i) G-protein-coupled receptors such as adenosine A_1 (Borea et al. 1994, 1996a, 1996b, Dalpiaz et al. 1998, 1999, 2000, 2002), A_{2A} (Borea et al. 1995; Baraldi et al. 1998), A_{2B} (Gessi et al. 2008), and A_3 (Merighi et al. 2002); β-adrenergic (Weiland et al. 1979; Contreras et al. 1986); dopamine D_2 (Kilpatrick et al. 1986; Agui et al. 1988; Duarte et al. 1988); and serotonin 5-HT_{1A} (Dalpiaz et al. 1995, 1996); (ii) ligand-gated ion channel

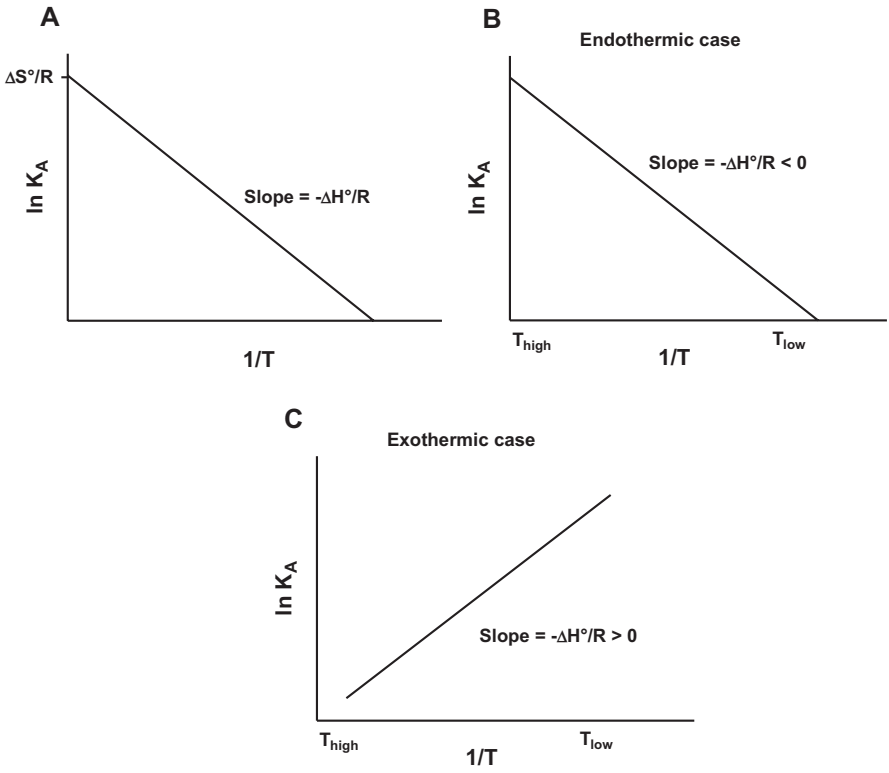

Fig. 8.1 The slope and intercept of a van't Hoff plot (**a**) and van't Hoff plot in endothermic (**b**) or exothermic (**c**) case

receptors such as glycine (Ruiz-Gómez et al. 1989), $GABA_A$ (Ruiz-Gómez et al. 1989), 5-HT_3 (Borea et al. 1996a; Maksay 1996), and nicotinic (Banerjee and Ganguly 1995, 1996; Borea et al. 1998, 2004); and (iii) the receptor for glucocorticoid hormones (Eliard and Rousseau 1984).

ΔG°, ΔH°, ΔS°, and $\Delta C_p{}^\circ$ (standard heat capacity) values have been collected for a remarkable number of ligands, including agonists, partial agonists, inverse agonists, or antagonists, both in the absence and in the presence of suitable modulators. The information provided by these data could be very useful from a pharmacological and pharmaceutical point of view, allowing us to discover new thermodynamic relationships related to drug−receptor interactions and their molecular mechanisms. As an example, ΔH° and ΔS° values can be used, in some membrane receptors, as indicators of the agonist or antagonist behavior of the ligands, the agonist and antagonist binding being, respectively, entropy-driven ($\Delta S^\circ \gg 0$; $\Delta H^\circ \geq 0$) and enthalpy-driven ($\Delta H^\circ \ll 0$; $\Delta S^\circ \leq 0$ or >0) or vice versa. This phenomenon, called thermodynamic discrimination, has been monitored for β-adrenergic, adenosine, glycine, $GABA_A$, serotonin 5-HT_3, and nicotinic membrane receptors.

Thermodynamic discrimination would hold even if the antagonists are to be classified in a different way, in agreement with the fact that a large number of antagonists of several membrane receptors have been recognized as inverse agonists, in touch with theoretical predictions indicating neutral antagonists as minority species in pharmacological space (Kenakin 2004). Another thermodynamic aspect, which characterizes all membrane receptors, is the ΔC_p° value nearly zero, a phenomenon which is not completely understood and is not usual in reactions involving biomacromolecules in solution (Sturtevant 1977).

8.3 Affinity Constant and Thermodynamic Parameters Determination

8.3.1 Affinity Constant Determination

Binding assays are usually performed in the temperature range 0–35 °C. Affinity constants are determined by means of two experimental procedures: saturation and inhibition experiments. The former are accomplished by incubating at equilibrium fractions of tissue homogenates with increasing concentrations of radiolabeled ligand. For a generic binding equilibrium

$$L + R \rightleftarrows LR$$

(where L = ligand, R = receptor), affinity constants are calculated as

$$K_A = [LR] / ([L][R]) = [LR] / [L_{MAX} - LR][B_{MAX} - LR] = 1 / K_D$$

where $[L_{MAX}]$ = total concentration of the ligand added, $[B_{MAX}]$ = total concentration of the binding sites, and K_D = dissociation constant.

Since

$$[LR] / [L_{MAX} - LR] = [\text{Bound} / \text{Free}] = [B_{MAX}] K_A - K_A [\text{Bound}]$$

the K_A and the B_{MAX} values can be obtained from the slope and the intercept of the plot [Bound/Free] versus [Bound] (Scatchard plot).

Inhibition experiments are performed by displacing a fixed concentration of radiolabelled ligand [C*] from the receptor preparation with increasing concentration of the unlabelled ligand under investigation with the aim of determining its IC_{50} value, that is, the inhibitor concentration displacing 50% of the labelled ligand. The affinity constant of the unlabelled drug, K_i, is subsequently calculated from the Cheng and Prusoff equation, $K_i = IC_{50}/1+[C^*]/K_D^*$, where K_D^* is the radioligand dissociation constant (Cheng and Prusoff 1973); under controlled conditions $K_i = K_D = 1/K_A$.

8.3.2 Thermodynamic Parameters Determination

Measurements of K_A values at different temperatures allow the equilibrium thermodynamic parameters $\Delta G° = -RT \ln K_A$ and $\Delta H°$ and $\Delta S°$ to be obtained.

Two cases can be distinguished:

(a) The standard specific heat difference of the equilibrium ($\Delta C_p°$) is nearly zero. In this case the van't Hoff equation $\ln K_A = -\Delta H°/RT + \Delta S°/R$ gives a linear plot $\ln K_A$ versus $1/T$ and the standard enthalpy can be calculated from the slope, $-\Delta H°/R$, and the standard entropy from the intercept, $\Delta S°/R$, or as $(\Delta H° - \Delta G°)/T$, with $T = 298.15$ K and $R = 8.314$ J K^{-1} mol^{-1}.

(b) $\Delta C_p°$ is different from zero. In this case the van't Hoff plot is often parabolic and other mathematical methods are available for the analysis (Borea et al. 2000).

8.4 Binding Thermodynamics of Adenosine Receptor Ligands

In the field of adenosine receptors, binding thermodynamic analysis has been performed at A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors and has added important findings such as the thermodynamic discrimination of agonists from antagonists and the recurrent phenomenon of entropy-enthalpy compensation (Gilli et al. 1994; Borea et al. 1994, 1995; Merighi et al. 2002; Gessi et al. 2008). All the examined compounds display essentially linear van't Hoff plots (Fig. 8.2). This behavior indicates that $\Delta Cp°$ (standard specific heat difference of the equilibrium) values of the drug-receptor binding equilibrium are nearly zero or in other words that $\Delta H°$ values are not significantly affected by temperature in the range investigated (0–30 °C). This phenomenon suggests that the conformational changes needed to produce the pharmacological effect are relatively small in this class of molecules most probably because larger modifications would make the association of the receptor with the cell membrane unstable. In addition, such linearity appears to be a typical property of the drug-membrane receptor binding at variance with the most binding processes between molecules and biomacromolecules occurring in solution (Sturtevant 1977; Tomlinson 1983; Grunwald and Steel 1995). Table 8.1 summarizes the thermodynamic parameters of adenosine receptor ligands where the ranges of $\Delta G°$, $\Delta H°$, and $\Delta S°$ for both agonist and antagonist binding ($n = 85$) are given together with a qualitative classification of the equilibrium driving force. Agonist binding at the A_1 adenosine receptors can be classified as totally entropy-driven ($9 \leq \Delta H° \leq 50$ kJ/mol; $-106 \leq -T\Delta S° \leq -61$ kJ/mol), while antagonist binding is enthalpy- and entropy-driven ($-44 \leq \Delta H° \leq -12$ kJ/mol; $-18 \leq -T\Delta S° \leq 7$ kJ/mol/K) (Borea et al. 1994; Lorenzen et al. 2000). As for the A_{2A} adenosine receptors, the agonist binding is totally entropy-driven ($7 \leq \Delta H° \leq 50$ kJ/mol; $-83 \leq -T\Delta S° \leq -53$ kJ/mol/K), and the antagonist is enthalpy- and entropy-driven ($-60 \leq \Delta H° \leq -7$ kJ/mol; $-28 \leq -T\Delta S° \leq 10$ kJ/mol/K) (Borea et al. 1995). In a similar way, agonists at

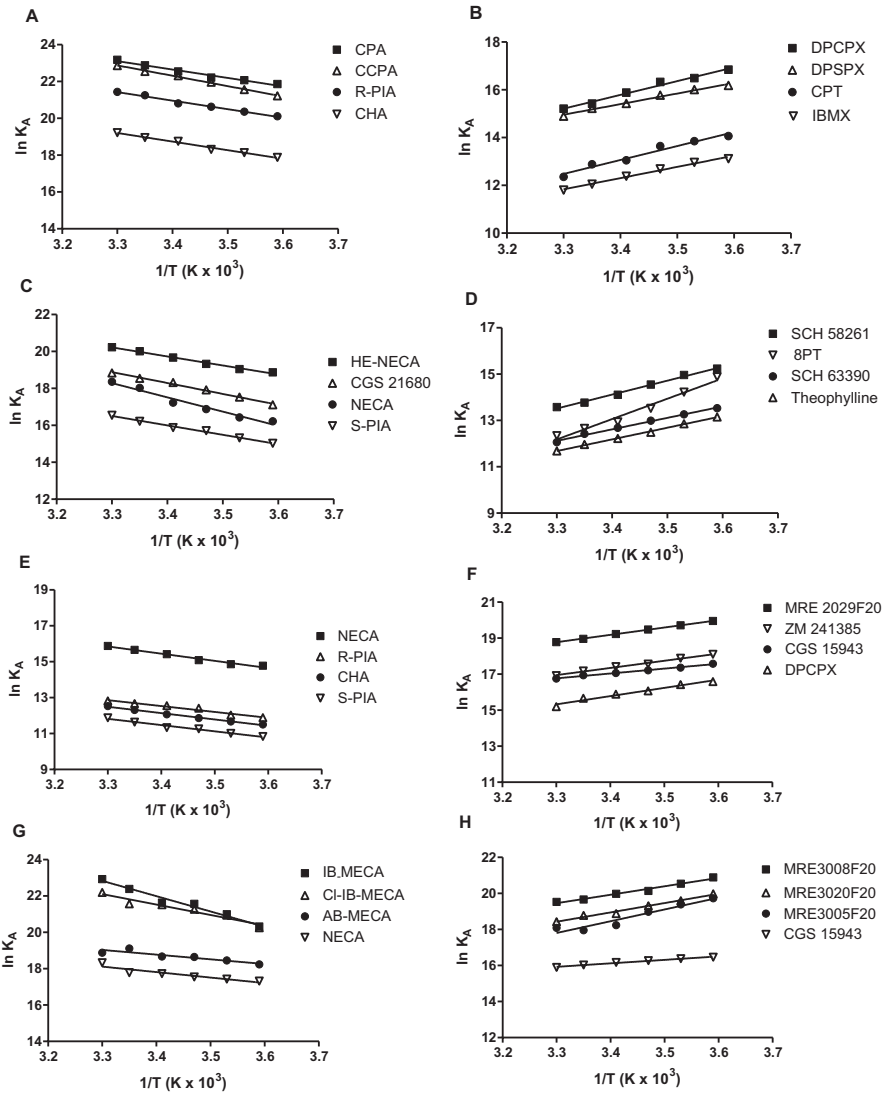

Fig. 8.2 Representative van't Hoff plots showing the effect of temperature on the equilibrium association constants of selected adenosine agonists or antagonists for A_1ARs (**a**, **b**), A_{2A}ARs (**c**, **d**), A_{2B}ARs (**e**, **f**), and A_3ARs (**g**, **h**)

A_{2B} adenosine receptors show a totally entropy-driven binding ($7 \leq \Delta H^\circ \leq 23$ kJ/mol; $-65 \leq -T\Delta S^\circ \leq -37$ kJ/mol), while antagonist binding is enthalpy- and entropy-driven ($-20 \leq \Delta H^\circ \leq -40$ kJ/mol; $-27 \leq -T\Delta S^\circ \leq -3$ kJ/mol) (Gessi et al. 2008). Similarly for A_3 adenosine receptors, the thermodynamic parameters fall in the ranges $21 \leq \Delta H^\circ \leq 67$ kJ/mol; $-122 \leq -T\Delta S^\circ \leq -67$ kJ/mol for agonists and $-52 \leq \Delta H^\circ \leq -9$ kJ/mol; $-24 \leq -T\Delta S^\circ \leq -5$ kJ/mol for antagonists showing that agonist binding is always totally entropy-driven while antagonist binding is

Table 8.1 Thermodynamic parameters, $\Delta G°$, $\Delta H°$, and $\Delta S°$, of a series of typical adenosine receptor ligands

Adenosine receptor subtypes	*N*	$\Delta G°$ kJ/mol	$\Delta H°$ kJ/mol	$\Delta S°$ J/mol/K	EDF	References
Adenosine A_1						Borea et al. (1994)
Agonists	23	–60 to –25	9 to 50	205 to 356	S-driven	
Antagonists	16	–49 to –24	–44 to –12	–23 to 60	H&S-driven	
Adenosine A_{2A}						Borea et al. (1995)
Agonists	7	–50 to –27	7 to 50	178 to 278	S-driven	
Antagonists	16	–50 to –26	–60 to –7	–34 to 94	H&S-driven	
Adenosine A_{2B}						Gessi et al. (2008)
Agonists	6	–43 to –29	7 to 23	123 to 219	S-driven	
Antagonists	6	–47 to 40	–40 to –20	10 to 91	H&S-driven	
Adenosine A_3						Merighi et al. (2002)
Agonists	6	–54 to –41	21 to 67	225 to 410	S-driven	
Antagonists	5	–49 to –33	–52 to –9	16 to 81	H&S-driven	

Note: Temperature used = 298.15 K; *N* = number of ligands; $\Delta G°$ = standard free energy; $\Delta H°$ = standard free enthalpy; $\Delta S°$ = standard free entropy; EDF = equilibrium driving force.

enthalpy- and entropy-driven (Varani et al. 2000; Merighi et al. 2002). The overall analysis of the thermodynamic data indicates that the variability of $\Delta H°$ (–224 to 90 kJ/mol) and $\Delta S°$ (–590 to 456 J/mol/K) values is again much greater than that of the $\Delta G°$ values (–63 to –24 kJ/mol), suggesting the possibility that enthalpy and entropy could be proposed as indicators of the pharmacological profile of adenosine ligands. In agreement with the idea that while $\Delta H°$ values are determined by the features of the ligand-receptor binding process, $\Delta S°$ values are determined by the rearrangements occurring during the binding in the solvent-drug and solvent-receptor interfaces. As a matter of fact, in the adenosine agonist-receptor interaction, the insertion of the ribose moiety and the depletion of the water network induce conformation changes in the receptor site able to mediate the final biological effect. Consequently, a high degree of correlation between intrinsic activity and $\Delta S°$ values was reported for adenosine ligands acting as full or partial agonists and as antagonists (Borea et al. 1994).

As for all adenosine receptor subtypes, it appears clearly apparent the thermodynamic interdependence of $\Delta H°$ and $-T\Delta S°$ where all the experimental points appear to be arranged along the same diagonal line, according to the equation: $\Delta H°$ (kJ/mol) = –41 (±2) + 288 (±3) $\Delta S°$ kJ/mol/K ($n = 85$, $r = 0.981$, $p < 0.001$) (Fig. 8.3).

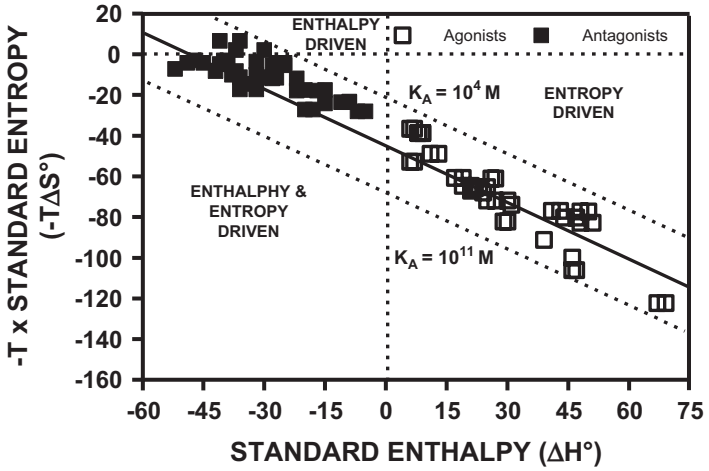

Fig. 8.3 Scatter plot of $-T\Delta S°$ against $\Delta H°$ for adenosine compounds. Full and open symbols indicate antagonists and agonists, respectively. All points lie on the same regression line. The two dashed lines indicate the loci of the points representing possible combinations of $\Delta H°$ and $-T\Delta S°$ values giving rise to the two different equilibrium constants indicated ($K_A = 10^4$ M^{-1} and $K_A = 10^{11}$ M^{-1})

8.5 Binding Thermodynamics of G-Protein-Coupled Receptor Ligands

Table 8.2 and Fig. 8.4a summarize the thermodynamic parameters of G-protein-coupled receptors (GPCRs) so far studied where the ranges of $\Delta G°$, $\Delta H°$, and $\Delta S°$ for both agonist and antagonist binding ($n = 203$) are given together with a qualitative classification of the equilibrium driving force. The analysis of the data revealed that six out of the ten GPCRs reported are discriminated. For dopamine D_2 receptor ligands, thermodynamic values for antagonist ($-89 \leq \Delta H° \leq 59$ kJ/mol; $-105 \leq -T\Delta S° \leq 107$ kJ/mol/K) and agonist binding ($-224 \leq \Delta H° \leq 90$ kJ/mol; $-136 \leq -T\Delta S° \leq 176$ kJ/mol/K) are scattered over their complete range. Therefore, agonists and antagonists do not show thermodynamic discrimination (Duarte et al. 1988). A similar behavior is shown by the 5-HT_{1A} receptors where antagonist ($15 \leq \Delta H° \leq 80$ kJ/mol; $-109 \leq -T\Delta S° \leq -47$ kJ/mol/K) and agonist binding ($-65 \leq \Delta H° \leq 58$ kJ/mol; $-109 \leq -T\Delta S° \leq 20$ kJ/mol/K) do not suggest any agonist-antagonist discrimination (Dalpiaz et al. 1996). As for opioid receptors, antagonists ($-52 \leq \Delta H° \leq 5$ kJ/mol; $-15 \leq -T\Delta S° \leq -2$ kJ/mol/K) and agonists ($-42 \leq \Delta H° \leq 12$ kJ/mol; $-19 \leq -T\Delta S° \leq -4$ kJ/mol/K) are not thermodynamically discriminated (Borea et al. 1988; Li et al. 1998). This result is in qualitative agreement with that reported for the binding of nociceptin receptors where the agonist binding was entropy-driven (Varani et al. 1998). The cholecystokinin CCK_2 receptor ligands have been also investigated to verify the discrimination of agonists and antagonists. The finding of a lack of thermodynamic discrimination between

Table 8.2 Thermodynamic parameters, $\Delta G°$, $\Delta H°$, and $\Delta S°$, of different G-protein-coupled receptor ligands

GPCRs	*N*	$\Delta G°$ kJ/mol	$\Delta H°$ kJ/mol	$\Delta S°$ J/mol/K	EDF	References
Dopamine D_2						Duarte et al. (1988)
Agonists	11	−53 to −34	−224 to 90	−590 to 456	ND	
Antagonists	22	−59 to −24	−89 to 59	−359 to 352	ND	
Serotonin 5-HT_{1A}						Dalpiaz et al. (1996)
Agonists	8	−58 to −36	−65 to 58	−67 to 366	ND	
Antagonists	7	−49 to −29	15 to 80	158 to 366	ND	
Opioid						Borea et al. (1988)
Agonists	9	−63 to −47	−42 to 12	13 to 64	ND	
Antagonists	6	−59 to-50	−52 to 5	5 to 49	ND	
Cholecystokinin CCK_2						Harper et al. (2007a)
Agonists	2	−47 to −49	−71 to −64	−74 to −58	ND	
Antagonists	6	−51 to −36	−65 to −3.5	−67 to 152	ND	
β-Adrenoceptors						Weiland et al. (1979)
Agonists	14	−51 to −26	−143 to −17	−312 to 27	H-driven	
Antagonists	23	−61 to −31	−21 to 16	54 to 178	H&S-driven	
Histamine H_3						Harper et al. (2007b)
Agonists	7	−58 to −48	−31 to −23	198 to 311	S-driven	
Antagonists	3	−55 to −47	6 to 45	57 to 120	H&S-driven	
Cannabinoid CB_1						Merighi et al. (2010)
Agonists	5	−51 to −36	17 to 59	213 to 361	S-driven	
Antagonists	3	−49 to −33	−52 to −26	−12 to 38	H&S-driven	
Cannabinoid CB_2						Merighi et al. (2010)
Agonists	5	−48 to −40	27 to 48	234 to 300	S-driven	
Antagonists	3	−41 to −32	−19 to −17	43 to 74	H&S-driven	

Note: Temperature used = 298.15 K; *N* = number of ligands; $\Delta G°$ = standard free energy; $\Delta H°$ = standard free enthalpy; $\Delta S°$ = standard free entropy; EDF = equilibrium driving force.

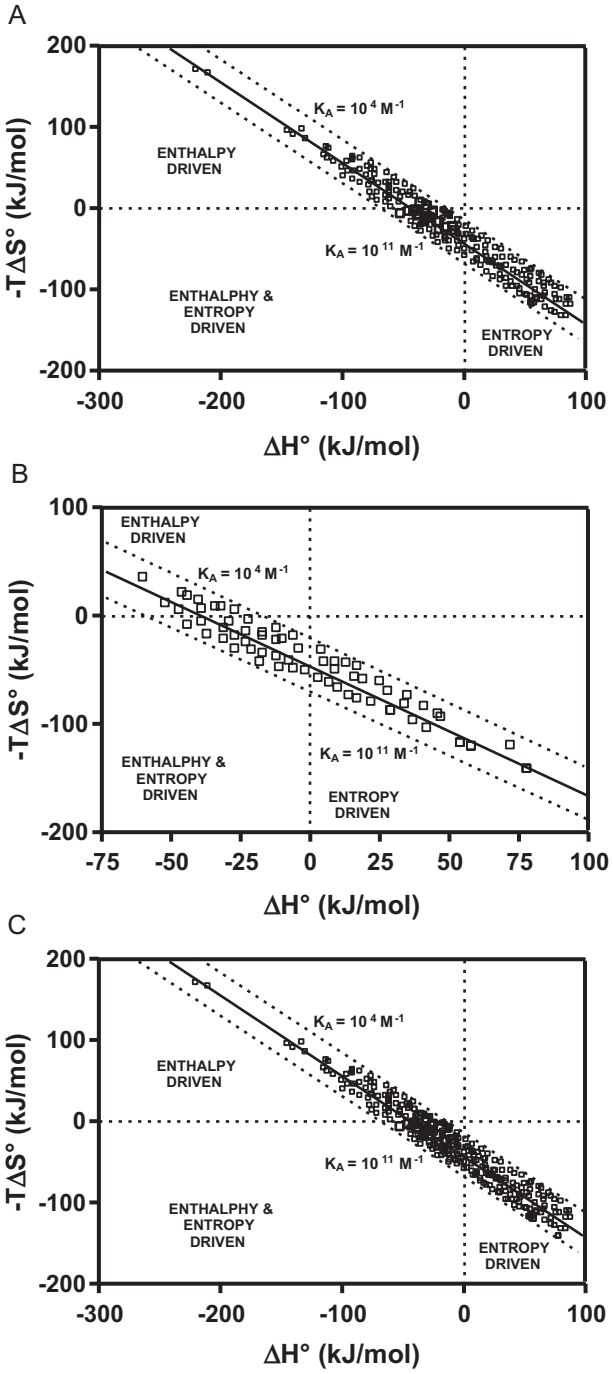

Fig. 8.4 Scatter plot of $-T\Delta S°$ versus $\Delta H°$ values for the GPCR (**a**, $n = 203$), LGICR (**b**, $n = 68$), and GPCR and LGICR (**c**, $n = 271$) agonists and antagonists. All points lie on the same regression line. The two dashed lines indicate the loci of the points representing possible combinations of $\Delta H°$ and $-T\Delta S°$ values giving rise to the two different equilibrium constants indicated ($K_A = 10^4\ M^{-1}$ and $K_A = 10^{11}\ M^{-1}$)

agonists and antagonists at the CCK_2 receptors has been explained by suggesting that small molecules may each have a unique combination of individual interactions with the receptors (Harper et al. 2007a, 2008). As for the β-adrenergic receptor, agonist cluster is in the exothermic region ($-143 \leq \Delta H^\circ \leq -17$ kJ/mol) with negative or weakly positive standard entropy values ($-8 \leq -T\Delta S^\circ \leq 93$ kJ/mol/K). Agonist binding has therefore to be classified as enthalpy-driven. Conversely, the antagonist binding is mostly or totally entropy-driven ($-21 \leq \Delta H^\circ \leq 16$ kJ/mol; $-53 \leq -T\Delta S^\circ \leq -16$ kJ/mol/K) (Weiland et al. 1979). The thermodynamic parameters for CB1 receptors fall in the ranges $17 \leq \Delta H^\circ \leq 59$ kJ/mol and $213 \leq \Delta S^\circ \leq 361$ kJ/mol for agonists and $-52 \leq \Delta H^\circ \leq -26$ kJ/mol and $-12 \leq \Delta S^\circ \leq 38$ kJ/mol for antagonists. The thermodynamic parameters for CB2 receptors fall in the ranges $27 \leq \Delta H^\circ \leq 48$ kJ/mol and $234 \leq \Delta S^\circ \leq 300$ kJ/mol for agonists and $-19 \leq \Delta H^\circ \leq -17$ kJ/mol and $43 \leq \Delta S^\circ \leq 74$ kJ/mol for antagonists. Collectively, these data show that agonist binding is always totally entropy-driven while antagonist binding is enthalpy- and entropy-driven, indicating that CB1 and CB2 receptors are thermodynamically discriminated (Merighi et al. 2010). Finally, the finding that histamine H_3-receptor agonist binding was entropy-driven was explained by the disorganization of a solvation sphere around the ligands as they bind to the receptor (Harper et al. 2007b; Harper and Black 2007). Another possible explanation suggested was that the agonist binding at histamine H_3-receptors induces ternary complex formation and this brings to the large increase in entropy. Interestingly, the presence of salts such as $CaCl_2$ in the buffer solution changes the thermodynamic behavior of histamine ligands. In these experimental conditions, agonists and antagonists showed similar thermodynamic parameters. This may be a consequence of the capability of buffer salts to increase the hydration of the ligands so that more water has to be removed during the receptor binding interaction (Harper and Black 2007).

8.6 Binding Thermodynamics of Ligand-Gated Ion Channel Receptor Ligands

Analysis of thermodynamic parameters of ligand-gated ion channel receptor ligands (LGICR) has revealed that five out of six receptors are thermodynamically discriminated (Table 8.3, Fig. 8.4b). As for the glycine receptor, the agonist binding has to be classified as entropy-driven ($2 \leq \Delta H^\circ \leq 20$ kJ/mol; $-56 \leq -T\Delta S^\circ \leq -25$ kJ/mol), whereas the antagonist binding is mostly enthalpy-driven ($-58 \leq \Delta H^\circ \leq -15$ kJ/mol; $-15 \leq -T\Delta S^\circ \leq 29$ kJ/mol) (Ruiz-Gómez et al. 1989). Agonist binding to the $GABA_A$ receptor is entropy-driven ($-1 \leq \Delta H^\circ \leq 14$ kJ/mol; $-48 \leq -T\Delta S^\circ \leq -28$ kJ/mol), while antagonist binding is enthalpy- and entropy-driven ($-23 \leq \Delta H^\circ \leq -12$ kJ/mol; $-31 \leq -T\Delta S^\circ \leq -15$ kJ/mol) (Maksay 1994). A similar result is also obtained for the serotonin 5-HT_3 receptor where the agonist binding is totally entropy-driven ($18 \leq \Delta H^\circ \leq 53$ kJ/mol; $-95 \leq -T\Delta S^\circ \leq -60$ kJ/mol) and antagonist binding is both enthalpy- and entropy-driven ($-16 \leq \Delta H^\circ \leq 0$ kJ/mol; $-53 \leq -T\Delta S^\circ \leq -21$ kJ/mol)

Table 8.3 Thermodynamic parameters, $\Delta G°$, $\Delta H°$, and $\Delta S°$, of different ligand-gated ion channel receptor ligands

LGICRs	N	$\Delta G°$ kJ/mol	$\Delta H°$ kJ/mol	$\Delta S°$ J/mol/K	EDF	References
Glycine						Ruiz-Gómez et al. (1989)
Agonists	4	−48 to −24	2–20	94 to 188	S-driven	
Antagonists	7	−44 to −23	−58 to −15	−45 to 97	H&S-driven	
$GABA_A$						Maksay (1994)
Agonists	6	−40 to −30	−1 to 14	94 to 161	S-driven	
Antagonists	5	−48 to −30	−23 to −12	50 to 104	H&S-driven	
Serotonin $5\text{-}HT_3$						Borea et al. (1996a)
Agonists	7	−52 to −28	18 to 53	201 to 319	S-driven	
Antagonists	4	−53 to-37	−16 to 0	70 to 178	H&S-driven	
Nicotinic						Borea et al. (2004)
Agonists	7	−51 to −25	−58 to −29	−114 to 70	H&S-driven	
Antagonists	6	−37 to −21	9 to 82	97 to 409	S-driven	
$P2X_3$ purinergic						Varani et al. (2008)
Agonists	5	−46 to −41	−26 to −18	59 to 73	H&S-driven	
Antagonists	6	−40 to −30	14 to 36	149 to 249	S-driven	
$P2X_1$ purinergic						Varani et al. (2008)
Agonists	5	−46 to −37	−31 to −23	41 to 50	ND	
Antagonists	6	−30 to −25	−22 to −19	17 to 34	ND	

Note: Temperature used = 298.15 K; N = number of ligands; $\Delta G°$ = standard free energy; $\Delta H°$ = standard free enthalpy; $\Delta S°$ = standard free entropy; EDF = equilibrium driving force.

(Borea et al. 1996a). At variance with the other ion channel receptors, agonist binding to the nicotinic receptor is essentially enthalpy-driven ($-58 \leq \Delta H° \leq -29$ kJ/mol; $-21 \leq -T\Delta S° \leq 34$ kJ/mol), whereas antagonist binding is totally entropy-driven ($9 \leq \Delta H° \leq 82$ kJ/mol; $-122 \leq -T\Delta S° \leq -29$ kJ/mol) (Borea et al. 1998, 2004). $P2X_1$ and $P2X_3$ purinergic receptors have been also characterized from a thermodynamic point of view with the following parameters: $-31 \leq \Delta H° \leq -19$ kJ/

mol; $-15 \leq -T\Delta S^\circ \leq -5$ kJ/mol and $-26 \leq \Delta H^\circ \leq 36$ kJ/mol; $-74 \leq -T\Delta S^\circ \leq -18$ kJ/mol, respectively. Interestingly, $P2X_1$ and $P2X_3$ purinergic receptors have a different thermodynamic behavior as demonstrated by the fact that agonists and antagonists for $P2X_1$ receptors show similar enthalpy and entropy values. On the contrary $P2X_3$ receptors can be considered thermodynamically discriminated because agonist binding is enthalpy- and entropy-driven and antagonist binding is totally entropy-driven (Varani et al. 2008). The overall $-T\Delta S^\circ$ versus ΔH° scatter plot of the data for GPCRs and LGICRs is reported in Fig. 8.4c.

8.7 Conclusions

The adenosine receptor ligand so far investigated displays essentially linear van't Hoff plots indicating that ΔCp° (standard specific heat difference of the equilibrium) values of the drug-receptor binding equilibrium are nearly zero or in other words that ΔH° values are not significantly affected by temperature in the range investigated (0–30 °C). This phenomenon seems to indicate that the conformational changes needed to produce the pharmacological effect are relatively small in this class of molecules most probably because larger modifications would make the association of the receptor with the cell membrane unstable. In addition, such linearity appears to be a typical property of the drug-membrane receptor binding at variance with the most binding processes between molecules and biomacromolecules occurring in solution (Sturtevant 1977; Tomlinson 1983; Grunwald and Steel 1995). As for all adenosine receptor subtypes, it appears clearly apparent the thermodynamic interdependence of ΔH° and $-T\Delta S^\circ$ where all the experimental points appear to be arranged along the same diagonal line, according to the equation:

$$\Delta H^\circ\left(\text{kJ/mol}\right) = -41\left(\pm 2\right) + 288\left(\pm 3\right)\Delta S^\circ \text{kJ/mol/K}\left(n = 85,,r = 0.981,,p < 0.001\right)$$

For the overall GPCR agonists and antagonists investigated, the equation was

$$\Delta H^\circ\left(\text{kJ/mol}\right) = -41\left(\pm 2\right) + 304\left(\pm 4\right)\Delta S^\circ \text{kJ/mol/K}\left(n = 203,,r = 0.975,,p < 0.001\right)$$

while for the 68 LGICR ligands was

$$\Delta H^\circ\left(\text{kJ/mol}\right) = -37\left(\pm 2\right) + 250\left(\pm 3\right)\Delta S^\circ \text{kJ/mol/K}\left(n = 68,,r = 0.965,,p < 0.001\right)$$

The regression equation obtained by plotting standard enthalpy and entropy data of 271 ligands performed on 16 different membrane receptor systems belonging to the GPCR and LGICR families was

$$\Delta H^{\circ}\left(\text{kJ / mol}\right) = -41\left(\pm 2\right) + 297\left(\pm 3\right)\Delta S^{\circ}\text{kJ / mol / K}\left(n = 271,,r = 0.971,,p < 0.001\right)$$

These equations could be rewritten as $\Delta H° = \beta \Delta S°$, which is the form for a case of enthalpy-entropy compensation with a compensation temperature of 302 K. It is generally accepted that entropy and enthalpy values in a scatter plot are arranged on the same diagonal band encompassed between the two dashed lines which represent the loci points defined by the limiting K_D values of 100 μM and 10 pM. This phenomenon seems to be a common feature in all cases of drug-receptor binding. The enthalpy-entropy compensation phenomenon has been attributed for drug-receptor interactions to the solvent reorganization that accompanies the receptor binding process in diluted solutions (Tomlinson 1983; Grunwald and Steel 1995). According to this point of view, while the features of the ligand-receptor binding process most probably determine $\Delta H°$ values, $\Delta S°$ values appear strongly affected by the rearrangements occurring in the solvent. It seems reasonable to assume that solvent effects might be responsible for the in vitro thermodynamic discrimination between agonists and antagonists observed for the majority of LGICRs and some of the GPCRs studied. The finding that the binding of adenosine receptor agonists is entropy-driven can be explained by the disorganization of a solvation area around the ligand-receptor interaction. Another possible explanation is that the agonists induce a change in receptor conformation perhaps into a less-constrained state, which, in turn, leads to the formation of a ternary complex with a G-protein, and this consequently results in a decrease in the solvation of the cytosolic side of the receptor. The finding of the increase in enthalpy associated with antagonist binding may be explained by hydrogen bond formation and van der Waals interactions occurring between the ligands and the binding pocket which cannot be compensated for by changes in entropy that result from agonist-induced conformational changes in the receptor.

In conclusion, the thermodynamic data represent relevant information to the drug design and development (Holdgate and Ward 2005). In particular, when compounds have similar affinities, their enthalpy values can be used to select one as the preferred lead compound for optimization. A favorable enthalpy values implies better complementarity of the binding interfaces because enthalpy corresponds to the energy associated with the net change in non-covalent bonds. The knowledge of the thermodynamic parameters could help the discovery and characterization of novel selective receptor agonists or antagonists.

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Chapter 9
Adenosine Receptors and Neuroinflammation

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Abstract Neuroinflammation, mainly sustained by microglial activation, is one of the hallmarks of many neurodegenerative diseases, including Parkinson's disease, Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis. A broad spectrum of functionally distinct microglial phenotypes has been described, differently affecting the central nervous system (CNS) homeostasis. Manipulating the activation state of microglia toward neuroprotective functions can thus be of therapeutic benefit in a number of CNS diseases.

Adenosine is an endogenous neuromodulator acting through the stimulation of four receptor subtypes, namely, A_1, A_{2A}, A_{2B}, and A_3 receptors (Rs). Among its numerous effects, adenosine plays an important immunoregulatory role in the CNS. A_{2A}R activation, in particular, appears to play a crucial role mainly by regulating microglial function. Emerging evidence indicates that such receptors may mediate different and even opposite effects on brain inflammation according to the stage of the pathological condition and to the different inflammatory cell types involved in that particular stage. The complex role of A_{2A}Rs in controlling neuroinflammation is strongly dependent also on the interplay with other neurotransmitters.

In this chapter, we will critically discuss the role of adenosine receptors in neuroinflammation (with particular emphasis on the A_{2A}R subtype), and its possible relevance to neurodegeneration.

Keywords Adenosine A_{2A} receptors · Central nervous system · Neurodegenerative diseases · Microglia · Astrocytes

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9.1 Neuroinflammation

Inflammation is in the first instance a self-defensive reaction that may, under specific circumstances, develop into a chronic state and become a causative factor in the pathogenesis of a broad range of disabling diseases. For many of these pathologies, regardless of the nature of the primary pathogenic event, inflammation remains the best therapeutic target, and the development of novel strategies to treat inflammation is a primary task for medical research and pharmaceutical design.

This holds true also for neuroinflammation, defined as the response of brain cells toward any alterations of CNS homeostasis. Indeed, neuroinflammation is increasingly believed to contribute to the pathogenesis of a broad spectrum of brain disorders, not only in classical infectious and immune-mediated disorders but also in acute and chronic neurodegenerative diseases that were not originally considered to be inflammatory (Minghetti et al. 2005; Amor and Woodroofe 2014; Ransohoff 2016).

Chronic neurodegenerative diseases comprise high social impact disorders – among which AD, Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) – characterized by different etiologies, clinical signs, and incidences but sharing some common features. The most striking one is the aggregation of misfolded proteins, which accumulate in the central nervous system in a disease- and protein-specific way, leading to progressive cellular dysfunction and loss of specific neuronal populations. Despite the specific cellular and molecular alterations responsible for the peculiar clinical picture of a given disease, gliosis (i.e., the activation of microglia and astrocytes and the production of inflammatory mediators in the brain) is a common mechanism contributing to neurodegeneration. Microglia cells are the resident macrophages of brain parenchyma and are generally viewed as a major source of proinflammatory and potentially neurotoxic molecules in the damaged brain. A direct link between activated microglia and tissue damage has not been univocally demonstrated in vivo, and recent studies have rather documented an exacerbation of injury following selective microglial ablation or anti-inflammatory treatments. In recent years, thanks to the development of in vitro, ex vivo, and in vivo models more closely mimicking specific aspects of neurodegenerative diseases, the complexity of microglial activation has begun to be unraveled. In fact, in opposition to a "linear model" of activation, which call for microglia to proceed through a graded transformation from a resting status into a potentially cytotoxic one, a more complex "plasticity model" proposes that in different forms of injury or disease, activated microglia might synthesize a range of different molecules, including neurotrophic factors, whose typical profile will determine the outcome of microglial activation in terms of repair or injury. Importantly, the different states of activation can be switched between each other during the course of the disease in response to signals from the periphery (Perry et al. 2007). According to this view, activated microglia are likely to play a complex and multifaceted role, which needs to be defined within each disease.

Besides microglia, astrocytes have also an important role in neuroinflammation; in fact, they represent the most abundant subtype in the brain and are necessary for the maintaining of ion balance and for supplying nutrients for neurons; in addition, considering that, as microglia, they can recognize invading pathogens as well as endogenous "danger signals" through the action of several membrane pattern recognition receptors (PRRs), they can contribute to neuroinflammation by releasing cytokines and chemokines (Farina et al. 2007; Bellaver et al. 2017).

In conclusion, it is evident that many factors drive and modulate the CNS inflammatory response, and a better comprehension of their interrelation will help to develop effective therapeutic strategies. To this aim, the following sections will be dedicated to the discussion of the role played by neuroinflammation in some neurodegenerative diseases.

9.2 Neuroinflammation in Neurodegenerative Diseases

Alzheimer's disease (AD) is one of the most studied neurodegenerative disorders, characterized by the progressive loss of neurons of basal forebrain cholinergic system, which results in memory and cognitive decline and, ultimately, in dementia. The two major hallmarks of disease, which mainly affects the hippocampus, the amygdala, and several cortical areas, are the extracellular deposits of β-amyloid (Aβ) in the brain parenchyma (senile plaques) and the neurofibrillary tangles, consisting of intracellular aggregates of aberrantly phosphorylated *tau* protein. Whether the pathogenic processes involve cell-autonomous or non cell-autonomous mechanisms remains an open question, as well as the temporal and mechanistic connection between Aβ and tangle pathology. Despite these doubts, the presence of activated microglia surrounding the senile plaques and the increased levels of elements of the complement system, cytokines, chemokines, and free radicals in the affected areas originated the "neuroinflammatory hypothesis" of AD (McGeer and McGeer 2001; Eikelenboom et al. 2006; Selkoe and Hardy 2016). In this view, neuronal injury and Aβ deposition would be the primary events, responsible for glial activation and secretion of harmful substances that may drive a self-propagating toxic cycle, exacerbating neurodegeneration, and Aβ deposition (Mrak and Griffin 2005).

This "autotoxic" hypothesis is supported by a large body of in vitro evidence showing that Aβ peptides are proinflammatory and activate microglia to release potentially neurotoxic factors such as cytokines (IL1-β, TNF-α) and free radicals, such as nitric oxide (NO) and superoxide (Akiyama et al. 2000; Eikelenboom et al. 2006). Nonetheless, substantial in vitro and in vivo evidence indicates that microglia persistently exposed to inflammatory agents, such as bacterial endotoxin or specific cytokines, or interacting with apoptotic neurons, undergo a process of molecular reprogramming. During this process, some anti-inflammatory functions are gained, and others, such as the expression of many proinflammatory products, are lost (De Simone et al. 2004, 2010; Schwartz et al. 2006; Ajmone-Cat et al. 2013, 2016).

Interestingly, recent evidence suggests that plaque-associated microglia in vivo are in a suppressed phagocytic state due to the overproduction of IL-10, prostaglandin E_2 (PGE_2), and arginase-1 (Spangenberg and Green 2017 and refs therein). The phagocytic ability of microglia seems to be restored by the opportune stimulation with inflammatory agents, as shown in several experimental models (Carnevale et al. 2012; Spangenberg and Green 2017 and refs therein). In this view, the microglial barriers that develop around Aβ deposits early in the disease could represent an attempt to limit their outward expansion and shield neurons from toxic species of Aβ, as also suggested by experimental evidence indicating beneficial roles of microglial activation in early AD (Condello et al. 2015; Hamelin et al. 2016). In the long term, this attempt of plaque restriction could lose its effectiveness, due to the phenotypic switching of chronically challenged microglia.

Astrocytes may also contribute to the β-amyloid pathology through either failure of β-amyloid clearance or even through additional β-amyloid production, by modulating microglial Aβ phagocytosis, or by contributing to the neuroinflammatory response (Thal 2012).

The neuroinflammatory perspective has been recently challenged by genome-wide association studies (GWAS) that identified several single nucleotide polymorphisms (SNPs) – associated with or related to microglial function – which convey the risk of developing AD (Sun et al. 2017 and refs therein). These and other discoveries, including the reported dysfunction of microglial stripping of synapses in the AD brain (Hong et al. 2016), are shifting the focus from neuroinflammation to compromised microglial noninflammatory functions as a contributor to AD pathogenesis (Salter and Stevens 2017). Similarly, alterations of the homeostatic and neuroprotective functions of astrocytes could be an important component of pathogenesis of neurodegenerative diseases, including AD.

Collectively, developing ways to harness and mitigate glia-mediated functions may provide a therapeutic option for identifying effective therapies for AD.

Huntington's disease (HD) is an autosomal-dominant monogenic disease caused by an abnormal CAG repeat expansion in the IT-15 gene encoding for the protein huntingtin (HTT) which becomes prone to misfolding – due to the expanded polyglutamine (polyQ) tract – and undergoes subsequent aberrant accumulation and aggregate formation. The classical neurological symptoms of HD (motor abnormalities, psychiatric, and cognitive alterations) are accompanied by widespread peripheral manifestations, such as weight loss, skeletal muscle wasting, and cardiac failure (van der Burg et al. 2009). Although mutant huntingtin (mHTT) is considered as the trigger of the disease, the cellular mechanisms leading to the pathology remain elusive. Among the major recognized pathogenic mechanisms are excitotoxicity, mitochondrial dysfunctions, and an impairment of neurotrophic factor signaling (Bartlett et al. 2016).

Striatal medium spiny neurons (MSNs) are the neuronal population preferentially lost in this disease, despite mutant huntingtin (mHTT) is ubiquitously expressed in the brain, in both neuronal and glial cells, and throughout the body (Jansen et al. 2017; van der Burg et al. 2009).

Microglia activation, detected in the brain from presymptomatic HD carriers to postmortem HD patients, correlates with disease progression (Crotti and Glass 2015) and the density correlated to the degree of neuronal loss (Sapp et al. 2001). Elevated levels of inflammatory cytokines were found in both the CNS and plasma from HD patients or in premanifest HD gene carriers (Bjorkqvist et al. 2008; Silvestroni et al. 2009; Politis et al. 2015), indicating that neuroinflammation may play a crucial role also in HD. Neuronal mHTT-mediated excitotoxicity and/or mHTT expression in microglia could trigger microglial activation and provoke phagocytic or immunoregulatory dysfunctions. In postmortem human HD tissue, some inflammatory mediators such as IL-1β and TNF-α were found specifically increased in the striatum, while IL-6, IL-8, and MMP-9 were also upregulated in cortex and, surprisingly, the cerebellum, a CNS region commonly thought to be spared in HD (Silvestroni et al. 2009), suggesting distinctive feature of neuroinflammation in HD compared to other neurodegenerative disease such as AD or PD, which shows an upregulation of a wide range of inflammatory mediators (Wyss-Coray 2006; Przedborski 2007).

Marked signs of astrocytosis can also be detected in proximity to degenerated neurons in HD brain, albeit the primary cause of activation (if cell autonomous or non-cell autonomous or both) is unknown (Khakh et al. 2017). mHTT expressing astrocytes show reduced glutamate transporters expression, and it has been suggested that this causes a diminished protection of medium-sized spiny neurons against glutamate neurotoxicity (Zeron et al. 2002; Shin et al. 2005). Moreover, selective mHTT expression in astrocytes has been shown to cause age-dependent neurological symptoms and exacerbate neuronal loss in vivo (Bradford et al. 2009, 2010). A recent study described an activation loop in which neurotoxic reactive astrocytes are induced by activated microglia and contribute to exacerbate neurons and oligodendrocytes damage in neurodegenerative disorders (Liddelow et al. 2017), suggesting that a complex interplay among astrocytes and microglia can contribute to HD pathogenesis as well.

Accumulating evidence indicates that a direct effect of mHTT occurs also within peripheral immune cells and tissues and contributes to HD pathology (van der Burg et al. 2009; Mina et al. 2016). Human monocytes isolated from HD gene carriers, which express mHTT, are pathologically hyperactive in response to lipopolysaccharide (LPS) stimulation, and murine mHTT macrophages and microglia express higher levels of proinflammatory cytokines, likely reflecting the widespread effect of mHTT on immune cells (Bjorkqvist et al. 2008; Dobson et al. 2016). In line with these observations, mouse studies suggested that HD central pathology can be ameliorated by targeting peripheral manifestations (see Carroll et al. 2015). In a more recent study, a microarray gene expression profile analysis on HD brain and blood samples identified two common signatures (i.e., immune response and spinocerebellar ataxias) that are likely an indication of disease changes occurring in parallel between these two tissues (Mina et al. 2016). Collectively these studies argue that targeting the whole body in HD may be of therapeutic relevance.

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by rapidly progressive degeneration of motor neurons in the cerebral cortex brainstem and spinal cord (Cleveland et al. 2001). ALS is in the main a sporadic disease, but about 10% of ALS cases are familial. SOD1 was the first gene to be discovered about two decades ago, but in the last 15 years, about 150 genetic mutations and more than 20 different genes have been identified (Renton et al. 2014). Unraveling the genetic etiology of ALS has led to the development of ALS animal models which have provided critical insights into the cellular and molecular mechanisms underlying neuron degeneration.

A growing body of evidence suggests that, besides many other mechanisms, such as excitotoxicity, oxidative stress, toxicity by protein misfolding, and mitochondrial dysfunction, (see Boillée et al. 2006) neuroinflammation and immune reaction are pivotal features both in ALS patients and in animal models (Zhao et al. 2013; Frakes et al. 2014; Murdock et al. 2015).

Malfunction of both innate and adaptive immune systems can actively influence disease progression in animal models and in familial and sporadic ALS patients (Zhang et al. 2005; Hovden et al. 2013). Substantial numbers of infiltrating T cells and macrophages are found in the spinal cord of ALS patients (Troost et al. 1989; Hickey et al. 1991); the majority of these migrating cells are described as T-helper and T-suppressor/cytotoxic cells (Engelhardt et al. 1993), whereas a decreased number of regulatory T cells (Tregs) was observed in mice and ALS patients during the rapidly progressing phase of the disease (Henkel et al. 2013). The presence of an overt neuroinflammatory response as activated microglial cells in regions of motoneuron damage was reported both in ALS patients and in mouse models of ALS where microgliosis is already present prior to disease onset (Hall et al. 1998; Turner et al. 2004; Potenza et al. 2016).

Evidence of a dual nature of inflammation during disease progression in ALS comes from both human and studies on animal models. Initially in the disease course of ALS, there is an early anti-inflammatory and neuroprotective response, governed mainly by Tregs, astrocytes secreting neurotrophic factors, and protective microglial cells. Late in the course of the disease, as the motor neurons become damaged, a switch from a neuroprotective response to a neurotoxic type response by glial cells and Th1 cells takes place. Hence, microglia and lymphocytes, depending on their phenotype and activation status and according to the stage of the disease, can have both neurotoxic and neuroprotective functions in ALS.

Parkinson's disease (PD) is a chronic, progressive age-related neurodegenerative disorder and the second most common neurodegenerative disease after Alzheimer's disease. The neuropathology has long been characterized by the intraneuronal aggregates of the presynaptic protein α-synuclein and the loss of dopaminergic neurons in the substantia nigra with a concomitant decrease of dopaminergic innervation in the basal ganglia. Though PD is classically diagnosed according to motor symptoms, it is now well recognized that motor symptoms are only one aspect of a multifaceted and complex disorder. Extrapyramidal motor disorder with bradykinesia, resting tremor, rigidity, and postural instability are the classical clinical signs of

the disease. The involvement of other regions of the CNS contributes to series of significant clinical non-motor symptoms such as autonomic dysfunction, depression, and cognitive decline. (Kalia and Lang 2015; Jiang and Dickson 2017).

Among the potential mechanisms underlying dopaminergic neuronal degeneration, mitochondrial dysfunction, oxidative stress, apoptosis, and proteasomal dysfunction have been considered. Neuroinflammation is another crucial feature of Parkinson's disease pathology (Tansey et al. 2010; Calabrese et al. 2017), as demonstrated by increasing evidence from in vitro studies, animal models, and postmortem analyses of human PD brains. Histological analysis of PD brains and animal models revealed striking reactive astrocytes and microglial activation within areas of neurodegeneration in Parkinson's disease (McGeer and McGeer 2008) as well as the presence of infiltrating CD4+ lymphocytes (Brochard et al. 2009).

The upstream events responsible for triggering neuroinflammation in PD are still uncertain. Among the hypothesized factors, misfolded and oxidized forms of α-synuclein, and neuromelanin (a dopamine-oxidized product release by dying dopaminergic neurons) have been considered. Indeed, they behave as potent inflammatory agents in many assays and even can stimulate the activation of brain microglia (Zucca et al. 2017; Zhang et al. 2018).

Activated microglia may also contribute to the removal of damaged dopaminergic neurons. The C1q, an important component for microglial clearance, has been found to be upregulated in the substantia nigra of PD human brains (Depboylu et al. 2011).

The clearance of degenerating neuron debris is an important task of the innate immune system to limit bystander tissue damage and inflammation suggesting that, rather than global suppression of microglial activation, a therapeutic strategy in PD might be the modulation of specific microglial function such as phagocytosis.

Chronic microglial activation can also drive adaptive immune responses, including T cell infiltration and production of antibodies to neuronal antigens (Obeso et al. 2017). Consistently, RANTES and eotaxin, chemokines that are involved in T cell trafficking, were found upregulated in the substantia nigra of postmortem PD brains as compared with age-matched controls. In addition, functional blocking antibodies against RANTES and eotaxin protected against nigrostriatal degeneration in a PD mouse model suggesting that neutralization of RANTES and eotaxin may be beneficial for PD patients (Chandra et al. 2016).

Meta-analysis of GWAS has identified a single nucleotide polymorphism within the human leucocyte antigen region that affects the risk of developing Parkinson's disease, suggesting an immune-related genetic susceptibility to Parkinson's disease (Nalls et al. 2014).

Collectively, strategies aimed to modulate the inflammatory response can halt the progress of neurodegenerative diseases and prevent neuron death. Epidemiological observations that suggest that some nonsteroidal anti-inflammatory treatments may reduce the incidence of clinically manifest PD also support this idea.

9.3 Role of Adenosine and Adenosine Receptors in the Modulation of Neuroinflammation

As previously described, neuroinflammation is emerging as a key mechanism responsible for the progression of neuronal degeneration and death; thus, the understanding of the signaling pathways involved in its modulation would be crucial for the identification of new therapeutic targets for neurodegenerative diseases.

Adenosine is an endogenous nucleoside widely distributed in mammalian tissues where it is involved in the regulation of several functions. In the CNS it acts as a neuromodulator able to control neuron excitability and to modulate the activity of non-neuronal cells, such as astrocytes and microglia (Haskó et al. 2005). Unlike neurotransmitters, adenosine is not stored in vesicles, and its extracellular concentration is regulated by different mechanisms: direct release from cells (Melani et al. 2012), extracellular hydrolysis of ATP (Zimmermann 2000), and reuptake into cells (Bender and Hertz 1986), where it is metabolized by the activity of adenosine kinase (Studer et al. 2006). In physiological conditions, extracellular adenosine levels are kept in the range of nanomolar. However, following brain injury, adenosine levels are dramatically increased (von Lubitz 1999) so that, together with ATP, it can function as an alarm molecule able to either inhibit or further promote damage spreading, depending on its action on different cell types (neurons, glia, peripheral inflammatory cells) and on its interplay with other neurotransmitters (e.g., glutamate; Dai et al. 2010). Such a complex and apparently paradoxical role of adenosine in the neurodegenerative process is exemplified by its modulation of neuroinflammation. In fact, the increased extracellular adenosine level can both inhibit or promote neuroinflammation depending on a host of complex factors, which are only beginning to be understood (Beamer et al. 2016). The main factor influencing the modulation of the neuroinflammatory events is the possibility of interaction with different receptor subtypes expressed on different cell types. In fact, cellular response to extracellular adenosine is managed by four different metabotropic receptors: A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al. 2001), all of which are expressed on immunocompetent cells both resident in the CNS (e.g., astrocytes and microglia; Boison 2012) and circulating in periphery (e.g., lymphocytes and granulocytes; da Rocha Lapa et al. 2014). In the following sections, the role of each subtype in the modulation of neuroinflammation will be critically discussed with a particular attention to A_{2A}Rs for their particularly intriguing and attracting function in neuroinflammation.

9.3.1 A_1R in Neuroinflammation

A_1Rs are expressed in microglia where they play an important role in regulating its activity in pathological conditions (Luongo et al. 2014). Si et al. (1996) reported that A_1R agonists attenuated proliferation of rat microglia stimulated by phorbol.

More recent studies demonstrated enhanced neuroinflammation and microglial activity in A_1 receptor knockout mice (A_1AR -/-; Synowitz et al. 2006), supporting the hypothesis that A_1R activation could be neuroprotective in pathological conditions. Indeed, it was demonstrated that A_1R serves as an important brake on microglial activation both in traumatic brain injury (TBI) and in experimental allergic encephalomyelitis (EAE) models (Haselkorn et al. 2010; Tsutsui et al. 2004). In particular, A_1AR-/- mice developed a more severe progressive form of EAE characterized by a marked demyelination and a greater microglial activation with respect to wild-type mice (Tsutsui et al. 2004); an increased expression of Iba-1-positive microglial cells was revealed also after induction of cortical TBI in several brain regions of A_1AR-/- mice (Haselkorn et al. 2010).

Although microglial cells are key regulators of neuroinflammation, as already mentioned, astrocytes can partake in the inflammatory process and influence neuronal survival and growth by the release of neurotrophic factors and the production of cytokines. Adenosine, acting on A_1Rs, is an important modulator of astrocytic proliferation and functions in either physiological or pathological conditions (Hascó et al. 2005). In fact, adenosine exerts a tonic, inhibitory action on astrocytic proliferation by stimulation of A_1Rs (Rathbone et al. 1991), which also induce the release of nerve growth factor (NGF) (Ciccarelli et al. 1999), important for neuronal survival. Collectively, these results seem to indicate that A_1Rs could have an anti-inflammatory function in the brain.

9.3.2 A_{2A}R in Neuroinflammation

The A_{2A}R is increasingly recognized as an important regulator of neuroinflammation during brain injury. Despite it unequivocally represents the main OFF signal of peripheral inflammation (Blackburn et al. 2009), its modulation of the inflammatory process occurring in the CNS may differentially affect the outcome of brain injury depending on many factors. During inflammation, cytokines released at the site of insult induce an upregulation of A_{2A}Rs in either microglia and astrocytes (see Chen and Pedata 2008 for a review), and their activation deeply influence the functional status of both cell types. In particular, it was demonstrated that while the activation of A_{2A}Rs was not sufficient to induce astrogliosis, pharmacological blockade of A_{2A}Rs inhibited astrogliosis induced by the basic fibroblast growth factor (bFGF; Brambilla et al. 2003). On the contrary, in glioma cells A_{2A}R stimulation inhibited the expression of inducible NO synthase (iNOS) and the production of NO following combined stimulation with LPS and interferon-γ (IFN-γ; Brodie et al. 1998). The finding that in mixed microglia-astrocyte cultures the stimulation of A_{2A}R potentiated LPS-induced NO production (Saura et al. 2005) further complicates the picture. The different effect of A_{2A}R activation on astrocytic function could be related to the different cell cultures examined and to the different inflammatory stimulus used. As for in vivo studies, in different animal models of brain injury (e.g., ischemia, TBI), the genetic deletion of A_{2A}Rs induced a less severe brain

inflammatory response resulting in a less severe neuronal damage (see Wei et al. 2011 for a review). In a very interesting paper Yu et al. (2008) demonstrated that the anti-inflammatory effect induced by $A_{2A}R$ blockade in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease could be attributed to receptors expressed by glial cells (both microglia and astrocytes); in particular, $A_{2A}R$ antagonism largely attenuated the progression of microglial cells to a fully activated phenotype (rather than the initiation), while it was able to reduce astroglial function in different stages of activity. These pieces of evidence suggested that $A_{2A}Rs$ on microglia could have an important role in regulating the over-activation of these cells during the neuroinflammatory process and, as a consequence, the detrimental effect of inflammation on neuronal cells. In addition, the blockade of $A_{2A}Rs$ was also able to prevent the recruitment of microglia to the hippocampal CA3 region of rats injected with kainic acid (KA; Lee et al. 2004), to blunt the toxic effect of IL-1β (a master regulator of neuroinflammation) on hippocampal neurons (Stone and Behan 2007) and to prevent LPS-induced neuroinflammation and synaptic transmission impairment in rat hippocampus in vivo (Rebola et al. 2011). Lastly, $A_{2A}R$ activation differentially affects microglial response in intact versus damaged tissue. In fact, $A_{2A}R$ activation increased microglial NO production only if in an activated status; as a further example, administration of the $A_{2A}R$ antagonist SCH58261 before intrastriatal quinolinic acid (QA) injection reduced the expression of cyclooxygenase-2 and microglial activation in the core of striatal lesion, but it enhanced both of them in the contralateral hemisphere (Minghetti et al. 2007). Differential regulation of microglia by $A_{2A}Rs$ may be related to several factors such as the interplay of adenosine with other neurotransmitters, like glutamate (Dai et al. 2010). In their elegant paper, Dai and coworkers demonstrated that glutamate levels are able to control the switch of $A_{2A}Rs$ from a proinflammatory action to an anti-inflammatory one, both in vitro and in vivo. In particular, in the presence of low levels of glutamate, $A_{2A}R$ activation reduces the LPS-induced inflammation in primary microglial cells, while at high concentrations of glutamate, $A_{2A}R$ activation increases the inflammatory response. The same influence of glutamate on $A_{2A}R$ inflammatory functions was found also in an in vivo model of traumatic brain injury (TBI).

To further complicate the story, peripheral $A_{2A}Rs$ could also contribute to neuroinflammation and, more in general, to neurodegeneration. In fact, during chronic neuroinflammation released cytokines increase the permeability of BBB and the infiltration into the CNS of systemic immune effector cells (Lyons et al. 2000), which have a strong impact on in situ inflammation. As mentioned above, $A_{2A}Rs$ have a strong anti-inflammatory effect (Blackburn et al. 2009) but also a pro-migratory action on peripheral lymphocytes (Mills et al. 2008). The importance of peripheral $A_{2A}Rs$ in controlling neuroinflammation was demonstrated in an elegant study by the group of Jiang-Fan Chen (Yu et al. 2004). By using an animal model of focal ischemia, they demonstrated that the ischemic area in the cerebral cortex of γ-irradiated wild-type mice receiving a bone marrow transplant from $A_{2A}R$ knockout mice (A_{2A}-/-) was increased with respect to that observed in γ-irradiated A_{2A}-/- mice receiving bone marrow from wild-type mice. In other words, neuroprotection

was superior in mice expressing A_{2A}Rs in myeloid cells but not in the brain. More recently, similar results were obtained in mouse models of multiple sclerosis (Mills et al. 2012), spinal cord injury (Genovese et al. 2009), and cerebral ischemia (Melani et al. 2014), indicating that A_{2A}Rs on peripheral immune cells play a neuroprotective role by downregulating inflammatory cascade inside the CNS.

On the whole, it may be proposed that participation of A_{2A}Rs to neuroinflammation depends on the different cell types involved, and glial receptors play an important role. Moreover, when the damage becomes so severe to increase the permeability of BBB, those receptors expressed on inflammatory cells invading the brain parenchyma could become relevant. Thus, from a therapeutic point of view, the possibility to delineate precise time windows for a beneficial or a detrimental effect of A_{2A}R stimulation during the neuroinflammatory process has to be taken into account.

9.3.3 A_{2B}R in Neuroinflammation

The expression of A_{2B}R on glial cells is low, as its affinity for adenosine is (see Popoli and Pepponi 2012 for a review). However, because of the increased extracellular adenosine levels induced by brain injury, A_{2B}R could be activated during pathological conditions. Conflicting conclusions have been reported on the role of A_{2B}Rs in inflammation, with some papers indicating a proinflammatory effect and others suggesting the opposite (see Feoktistov and Biaggioni 2011, for a review). For example, A_{2B}R genetic deletion in mice induced higher levels of proinflammatory cytokines (e.g., TNF-α and IL-6) with respect to WT (Wei et al. 2011), increased inflammation and decreased survival in a mouse model of experimental sepsis (Csóka et al. 2010). These results indicate an anti-inflammatory role for the receptor, and, in line with this hypothesis, Koscsó and coworkers found an increased production of IL-10 upon microglial stimulation of A_{2B}R (Koscsó et al. 2012). In addition, A_{2B}R was demonstrated to be the main adenosine receptor subtype involved in the inhibition of TNF-α production induced by LPS in microglia (Merighi et al. 2015). On the contrary, evidence that A_{2B}R stimulation induced cell proliferation and IL-6 production in primary microglial cells (Merighi et al. 2017) and in mouse striatum (Vazquez et al. 2008) points to a probable proinflammatory role of this adenosine receptor subtype and highlights its role in regulating early steps of microglial activation (Merighi et al. 2017). All together, these results indicate a similarity between the roles played by A_{2A}Rs and A_{2B}Rs in the neuroinflammatory process: both of them could be detrimental or beneficial for tissue inflammation depending on many factors which are not still completely understood.

Thus, the role of A_{2B}R in the CNS inflammation remains largely unknown. However, its low affinity for adenosine (it becomes activated only under pathological conditions) renders this receptor an interesting therapeutic target, and more dedicated studies aimed at better delineating its role in neuroinflammation are warranted.

9.3.4 A_3R in Neuroinflammation

The role of A_3R in regulating neuroinflammation is still controversial. On the one hand, some data indicate that such receptors mediate anti-inflammatory effects: in BV2 microglial cells, A_3R stimulation inhibited LPS-induced TNF-α production (Lee et al. 2006); in cultured murine astrocytes, adenosine, through A_3R activation, led to the inhibition of genes involved in inflammation (e.g., iNOS) (Gessi et al. 2013); in vivo, the administration of the A_3R agonist IB-MECA after ischemia reduced both astrogliosis and microgliosis (von Lubitz et al. 2001). Moreover, Choi et al. (2011) demonstrated that postischemic administration of the A_3R agonist LJ529 prevented microglia and monocyte migration, thus demonstrating a protective effect due to an anti-inflammatory action. On the other hand, it has been demonstrated that A_3R stimulation enhanced microglial chemotactic process extension in primary cultures from rat cortex (Ohsawa et al. 2012). To add further complexity, van der Putten (2009) demonstrated that upon TLR-mediated activation, microglia from rhesus monkey simultaneously upregulated $A_{2A}R$ and downregulated A_3R expression levels; the decreased contribution of A_3R-mediated signaling in the response to adenosine caused the extrication of $A_{2A}R$ inhibitory capacity on proinflammatory cytokine levels. Thus, it could be speculated that during neuroinflammation, the desensitization of A_3 receptor could be seen as a strategy to trigger protective mechanisms and avoid the onset of deleterious effects mediated by the prolonged activation of this subtype. This view is not at odds with the protective effect observed after A_3R stimulation in some injury conditions such as ischemia, which could be ascribed to those receptors located on blood cells which are able to prevent monocyte infiltration in the brain (Pedata et al. 2016). As for $A_{2A}R$, it could be possible that the paradoxical effect of A_3Rs on the neuroinflammatory process could be due to a dynamic change of the receptor expression during the different stages of the process itself, underlying the possibility of specific time window of effectiveness for both agonists and antagonists.

9.4 Therapeutic Implications and Concluding Remarks

Considering the important role played by neuroinflammation in the progression of neurodegenerative pathologies and the fine modulation exerted by adenosine on this process, addressing adenosine receptors could be considered a valid therapeutic approach for the treatment of neurodegenerative diseases.

Indeed, the adenosine system has been implicated in the pathogenesis of many neurodegenerative diseases, and, in particular, its role in AD, PD, and HD has been extensively studied (see Geiger et al. 2007; Rahman 2009 for reviews). Among the different receptor subtypes, $A_{2A}R$ has attracted much attention as indicated by many studies reporting neuroprotective action through its genetic or pharmacological blockade (see Cunha 2016 for a review).

Animal and epidemiological studies reported beneficial effects of caffeine in AD (see Gomes et al. 2011 for a review); although caffeine is a nonselective adenosine antagonist, its neuroprotective action against Aβ-mediated toxicity has been related to the specific blockade of the A_{2A} subtype, both in in vitro (Dall'Igna et al. 2003) and in in vivo studies (Dall'Igna et al. 2007; Orr et al. 2017). The ability of $A_{2A}R$ antagonist to modulate the neuroinflammatory process could play an important role in determining its neuroprotective action in AD. In fact, the observation that $A_{2A}Rs$ are overexpressed in astrocytes from animal models and in AD patients (Orr et al. 2015) and the finding that they control hippocampal neuronal dysfunction through neuroinflammation (Rebola et al. 2011) seem to support the hypothesis that the control of neuroinflammation could be the common mechanism responsible for the robust neuroprotection exerted by $A_{2A}R$ blockade in a diversity of neurodegenerative conditions. A beneficial effect of $A_{2A}R$ blockade in memory dysfunction has been also demonstrated in animal models of AD (Cunha and Agostinho 2010), and, more importantly, astrocytic $A_{2A}Rs$ have been implicated in this effect (Orr et al. 2015). In that interesting paper, the authors described how the conditional ablation of $A_{2A}Rs$ in astrocytes was sufficient to enhance memory in old hAPP mice, a mouse model of AD expressing the human APP minigene and characterized by many amyloid plaques and increased levels of astrocytic $A_{2A}Rs$. On the contrary, the receptor deletion was devoid of any effects in young hAPP mice, with minimal plaques and unaltered $A_{2A}R$ expression. The authors concluded that alterations in astrocytic $A_{2A}Rs$ could contribute to memory deficit in aging hAPP mice and that different pathogenic mechanisms may control different stages of AD.

In conclusion, the role of $A_{2A}R$ activation in the pathophysiology of AD deserves more focused studies aimed to discern the specific role played by the different cell types involved in the pathology.

Adenosine receptors and, in particular, A_{2A} subtypes have been extensively studied in HD, and a very complex profile has emerged, demonstrating that both $A_{2A}R$ agonists and antagonists have beneficial effects depending on the model used, the pharmacological treatment, and the time window of drug administration (see Popoli et al. 2007 for a review). However, although inflammation has been demonstrated to contribute to the pathogenesis of HD in patients and animal models (Tai et al. 2007; Franciosi et al. 2011), very few studies have evaluated if the anti-inflammatory effect of AR activation/blockade could be responsible for the observed protective action on the pathology. Indeed, in a QA-lesioned model of HD, the administration of the $A_{2A}R$ selective antagonist SCH58261 significantly reduced the astrocytic hyperplasia induced by the lesion (Popoli et al. 2002). On the contrary, the $A_{2A}R$ selective agonist CGS21680 was not able to counteract the increase in the mRNA for the macrophage antigen complex 1 induced by 3-nitropropionic acid treatment (3-NP) of animals (Blum et al. 2003), demonstrating that, at least in the 3-NP model of HD, inflammatory mechanisms are not involved in the effects of $A_{2A}R$ ligands. In conclusion, also for HD the role of adenosine receptors toward neuroinflammation remains unclear and deserves further study.

As concerning ALS, several studies have been conducted to evaluate if adenosine receptors and, in particular A_{2A}Rs, could be involved in the disease; once more, conflicting results were obtained: on the one hand, a beneficial effect exerted by A_{2A}R agonists was demonstrated in SOD1-G93A mouse and rat models of ALS (Wiese et al. 2007; Golder et al. 2008; Yanpallewar et al. 2012), and, in agreement with these, chronic administration of caffeine (whose chronic effects are mainly ascribed to A_{2A}R blockade) dramatically reduced the survival of mice (Potenza et al. 2013); on the other hand, a recent study showed that the selective A_{2A}R antagonist KW6002 significantly delayed disease progression of SOD1-G93A mice (Ng et al. 2015). Such a discrepancy could be explained in part by the use of a nonselective vs. a selective antagonist and by the fact that the treatments with caffeine and KW6002 were started at different time points. However, none of these studies addressed the possible involvement of the neuromodulatory role of A_{2A}R in observed effects. The possibility that A_{2A}Rs could influence the peripheral inflammatory component of ALS was recently investigated (Vincenzi et al. 2013): an upregulation of A_{2A}Rs was found in ALS lymphocytes, and the production of cAMP after receptor stimulation by its agonist CGS21680 was found increased; moreover, a positive correlation was found between A_{2A}R density and the ALS functional rating scale relative to patients' quality of life. Such results seem to suggest that A_{2A}R activation could have a protective role in ALS, at least at peripheral level. However, much effort should be made to shed light on this topic.

A_{2A}R has attracted much interest also in PD; in fact, the observation of an antagonistic interaction between A_{2A}R and D_2R at molecular and functional level paved the way for experimental studies aimed to evaluate if A_{2A}R antagonists could attenuate dopaminergic neurodegeneration. Indeed, convincing evidence suggests a neuroprotective role of A_{2A}R inhibition in PD: epidemiological studies demonstrated an inverse correlation between caffeine consumption and the risk to develop PD (Ascherio et al. 2001); A_{2A}R antagonists were found neuroprotective in mouse models of the disease (Morelli et al. 2010). Although the A_{2A}R-mediated mechanisms responsible for such a protection remain unknown, glial A_{2A}Rs could play a role. In the MPTP model of PD, it was demonstrated that A_{2A}R expression is upregulated in microglial cells and that the selective A_{2A}R antagonist KW-6002 attenuated microglial activation (Yu et al. 2008) and astrocytic activity (Pierri et al. 2005). In addition, caffeine reduced damage of striatal neurons through the inhibition of microglial activation and cytokine release by blocking A_{2A}R (Morelli et al. 2009). More recently, the MPTP mouse model was used by Gyoneva and coworkers to study microglial motility toward a mechanically induced tissue injury in a PD-related context. They demonstrated that microglia (found activated in the striatum and in the substantia nigra after MPTP treatment) displayed reduced process extension toward the site of damage suggesting that it might be less efficient in counteract neuronal damage than healthy microglia that was exposed to the same mechanic damage; more importantly, the A_{2A}R antagonist preladenant restored the ability of microglia to extend its processes, suggesting that at least part of the neuroprotective properties of A_{2A}R blockade could be due to the modulation of microglial motility (Gyoneva et al. 2014).

In conclusion, the ability of A_{2A}Rs to modulate the inflammatory process could mediate neuroprotective and symptomatic effects in PD (see Pinna 2014 for a review).

In summary, the role of adenosine in modulating neuroinflammation is multifactorial, involving the regulation and recruitment of astrocytes and microglia. The net effect of increased extracellular adenosine depends on several factors such as pathology, adenosine concentrations, receptor expression, and cross talk with other signaling molecules. Conflicting reports of whether adenosine is pro- or anti-inflammatory underscore the complexity of this molecule's role in mediating neuroinflammatory cascades (Liang et al. 2014). Thus, when designing treatment strategies by using AR agonists or antagonists, many factors have to be taken into account: dosage, drug delivery method, state of disease progression, and extracellular concentrations of potential excitotoxic transmitters. Therefore, it is important to further study the anti-inflammatory actions of adenosine with the goal of better defining mechanisms involved and of identifying possible therapeutic agents for chronic neurodegenerative disorders.

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Chapter 10
Adenosine Receptors as a Paradigm to Identify Dimer/Oligomers of G-Protein-Coupled Receptors and as Targets in Parkinson's Disease and Schizophrenia

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Abstract While adrenergic receptors were instrumental to start to understand the role of GPCRs, other receptors are taking the lead to understand why GPCR homo−/heteromers are needed and to address their physiological consequences in both healthy/homeostatic conditions and disease. Adenosine and dopamine receptors in the CNS are instrumental to understand pathogenic mechanisms in Parkinson's disease and to know the role of receptor heteromers. We here provide the account of the heteroreceptor complexes formed by adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3), and their potential as therapeutic targets. Both adenosine (A_1 or A_{2A})-dopamine (D_1 or D_2) and adenosine A_1A_{2A} heteroreceptor complexes are therapeutic targets in Parkinson's disease and may be altered after chronic levodopa treatment. A short account on the potential of adenosine receptors as targets in schizophrenia is also provided. Apart from potential in combating symptoms, adenosine receptors have potential as targets for neuroprotection. However, the design of neuroprotective drugs requires to understand how adenosine affects microglia and which adenosine-receptor-containing heteromers may be targeted.

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10.1 Introduction

Adenosine and dopamine receptors have been instrumental in identifying complexes with other members of the Class A G-protein-coupled receptor (GPCR) superfamily. For review on dopamine receptor homo−/heteromerization and its relevance, see Rashid et al. (2007), Fuxe et al. (2014a, b), George et al. (2014), Perreault et al. (2014), Borroto-Escuela et al. (2016), Borroto-Escuela and Fuxe (2017) and references therein. Dimers were first identified using coimmunoprecipitation and other biochemical approaches. Later, biophysical techniques were implemented to detect dimers (even trimers) in heterologous expression systems. The existence of receptor-receptor interactions between different GPCRs in the plasma membrane in brain tissue was first indicated in biochemical binding studies on neuropeptide modulation of the affinity and density of monoamine receptor subtypes using monoamine radioligands and membrane preparations from different brain regions (Fuxe et al. 1981, 1983, 1987; Agnati et al. 1982; Fuxe and Agnati 1985). The results gave rise to the concept of direct interactions in the plasma membrane of subtype-specific neuropeptide receptor and monoamine receptors. In 1993, it was proposed that the molecular mechanism for these GPCR receptor-receptor interactions was represented by the formation of a heterodimer in balance with the corresponding homodimers/monomers (Zoli et al. 1993).

Franco et al. (2016) reviewed the strategies that may lead to demonstrate that heteroreceptor complexes formed by GPCR are present in natural sources; in particular, the two that have provided more benefit in our experience are (i) the heteromer print (something that is particular to the complex and does not happen in individually expressed receptors) and (ii) in situ proximity ligation assays, a technique developed for assessing cancer types in samples from patients and that allows to detect GPCR clusters in cells, in samples from animal models, in samples from patients, or in samples from necropsies. The central nervous system (CNS) has been by far the substrate for identifying the complexes formed by adenosine receptors. Actually, the periphery lacks behind the CNS in identifying and addressing the physiological role of GPCRs. Exceptions do occur, and the most straightforward example in the periphery is likely provided by chemokine receptors, which may form homo- and heterodimers that provide pharmacological and signaling diversity to cells of the immunological system (see (Springael et al. 2005; Muñoz et al. 2009, 2011, 2012) and references therein). Indeed, there is consensus in that a receptor heteromer (Het) cannot be considered as such in the absence of any particular property, i.e., a given complex in a natural context should display a particular heteromer print (Het) (Ferré et al. 2009a).

10.2 Adenosine Receptors in the Formation of Heteromers with Non-purinergic GPCRs

Except for error, omission, or very recent discovery, the direct interactions reported for adenosine receptors with other members of the GPCR superfamily are those described below.

The first Het identified for receptors having different endogenous agonists was that constituted by adenosine A_1 (A_1R) and dopamine D_1 (Gines et al. 2000; Torvinen et al. 2002; Cao et al. 2006). In parallel, the Het for two different subtypes of receptors for the same endogenous agonist (mu and delta opioid receptors) was discovered by Gomes et al. (2000). In brain regions related to motor control, functional adenosine-dopamine receptor interactions were known. Also known was the segregation of striatal D_1 and D_2 receptors in, respectively, the so-called direct and indirect pathways of motor control. It turns out that whereas D_1 and A_1 colocalize in striatonigral GABAergic neurons, adenosine A_{2A} ($A_{2A}R$) and dopamine D_2 receptors colocalize in striatopallidal GABAergic neurons. Accordingly, we hypothesized, and later demonstrated, that A_{2A}-D_2 heteromerization in the indirect pathway paralleled the A_1-D_1 heteromerization in the direct pathway (Hillion et al. 2002; Canals et al. 2003, 2004; Fuxe et al. 2003, 2007; Ciruela et al. 2004). The highest $A_{2A}R$ expression in a mammalian body is found in the striatum, a fact whose extent is not fully known. Hence, interactions with other dopamine receptors, which are also expressed in motor control brain areas or with receptors widely distributed in the CNS, have been reported. On the one hand, the effect of activation of $A_{2A}R$ on in vivo actions mediated by dopamine D_3 (Hillefors et al. 1999) prompted us to investigate and identify A_{2A}-D_3 Hets (Torvinen et al. 2005). In vivo activation of A_{2A}Rs in the basal ganglia causes alterations in the pharmacological characteristics of dopamine D_3 receptors that may underlie the atypical neuroleptic-like effect of $A_{2A}R$ receptor agonists (Rimondini et al. 1997; Hillefors et al. 1999); as a matter of speculation, those in vivo effects may be a consequence of the particular pharmacological and functional properties of A_{2A}-D_3Hets. On the other hand, striatal adenosine A_{2A}Rs form functional heteromeric complexes with cannabinoid CB_1 receptors (Carriba et al. 2007) or with histamine H_3 (Márquez-Gómez et al. 2018) receptors; these Hets may, respectively, mediate the motor effects of cannabinoids and deserve attention on assessing the potential of antihistamines in the therapy of CNS diseases. Due to the intrinsic structural and conformational properties of the Class C GPCR subfamily, they can form a myriad of homo- and heteroreceptor complexes (Doumazane et al. 2011; Borroto-Escuela et al. 2014). Interestingly, the $A_{2A}R$ may form functional but also molecular complexes with Class C metabotropic $mGlu_5$ receptors (Ferré et al. 2002, 2003; Nishi et al. 2003; Kachroo 2005; Borroto-Escuela et al. 2017b). Ultrastructural studies have shown that the two receptors colocalize in the nonhuman primate striatum (Bogenpohl et al. 2012). Finally, the adenosine receptor is also able to interact with the orphan GPR37 receptor (Dunham et al. 2009). Pioneering evidence on functional interactions in rat caudate putamen

suggests that the adenosine receptors may also interact with some of the opioid receptor subtypes (Noble and Cox 1995; Borroto-Escuela et al. 2014).

$A_{2A}R$ may form homodimers (Canals et al. 2004) that likely interact with other GPCRs to form high-order heteroreceptor complexes. One example is the Het formed by A_{2A}, cannabinoid CB_1, and dopamine D_2 (Carriba et al. 2007; Navarro et al. 2008; Bonaventura et al. 2014; Pinna et al. 2014a, b). Another is the complex formed by A_{2A}, D_2, and $mGlu_5$ receptors (Cabello et al. 2009).

Consistent with the intense research on potential heteromerization of adenosine receptors, it has been shown that β_1- and β_2-adrenergic receptors may directly interact with the A_1R and that the resulting Het displays particular properties in terms of differential pharmacology and coupling to the signaling machinery (Chandrasekera et al. 2013). Finally, it has been confirmed that prostanoid receptors, namely, the thromboxane A_2 TP receptor, may form hetero-oligomers with the A_1R whose functional properties are conditioned by the presence and concentration of the endogenous agonist of the two receptors (Mizuno et al. 2012, 2013a). Heteromerization has been also reported for A_1R and class C metabotropic glutamate 1 alpha (Ciruela et al. 2001; Franco et al. 2001).

For reasons that are out of the scope of the present chapter, the two most studied adenosine receptors, in terms of receptor-receptor interaction research, are the A_1 and the A_{2A}. The other two types of adenosine receptors (A_3 and A_{2B}) are lacking behind, but, interestingly, the first identified Hets containing A_3 or A_{2B} are between adenosine receptors themselves (see next Sect. 10.3).

10.3 Adenosine Receptors May Interact with Other P1 (to Form Adenosine Isoreceptor Complexes) and with P2 Purinergic Receptors

Soon after the experimental confirmation of GPCR heteromerization and the extensive work made with A_1 and A_{2A} receptors, it was tempting to search for interaction between adenosine, i.e., P1 purinergic receptors, and "ATP" P2 purinergic receptors that are also GPCR members (metabotropic P2Y receptors). Pioneering studies to prove the hypothesis led to the discovery of interactions between A_1 and $P2Y_1$ receptors to form a functional unit with a particular pharmacological print (Yoshioka et al. 2001). Interestingly, A_1 and D_2 receptors were used as negative controls thus confirming previous results and the specificity of the interactions. Discovery of more P1-P2 receptor complexes (e.g., A_1-$P2Y_2$Hets), and/or their physiological roles (especially in the brain), were further reported (Yoshioka et al. 2001, 2002a, b; Suzuki et al. 2006; Tonazzini et al. 2007). The interplay between P1 and P2 receptors opens interesting avenues due, *inter alia,* to the fact that extracellular ATP acting on P2 receptors is degraded into adenosine, which activates P1 receptors (homoreceptors/monomers or forming Hets).

The interest of the P1/P2 receptor interplay prompted (Schicker et al. 2009) the performance of an ambitious project to discover mixed P1/P2 receptor-receptor interactions. The authors tested A_1, A_{2A}, $P2Y_1$, $P2Y_2$, $P2Y_{12}$, and $P2Y_{13}$ receptors and the $P2X_2$ (ligand-gated ion channel) ionotropic receptors. They provided evidence for the formation of *heterooligomers among each other*. $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$, A_1, A_{2A}, and $P2X_2$ receptors are also able to exist as homomers (Schicker et al. 2009). Reviews on the role of P1/P2 receptor-receptor interactions may be found in Nakata et al. (2010) and Suzuki et al. (2013). Of further interest for the present article, these results confirmed the occurrence of A_1R homodimers (Ciruela et al. 1995), $A_{2A}R$ homodimers (Canals et al. 2004), and of A_1A_{2A}Hets for which a structural basis has recently been provided (see next Sect. 10.4).

After prediction by computational means of homodimerization of A_3 receptors (Kim and Jacobson 2006), Hill and colleagues detected both A_3homodimers and heterodimers with A_1 receptors (May et al. 2011; Hill et al. 2014). We also have evidence of A_1A_3 Het expression in the CNS (data in preparation). It is likely that more A_3-receptor-containing Hets exist, but they have not yet (to our knowledge) been identified.

Although some indirect evidence suggested that A_{2B} receptors ($A_{2B}R$) could be interacting with other GPCRs (Moriyama and Sitkovsky 2010), the direct proof is given in a recent publication (Hinz et al. 2018). As a matter of fact, the A_{2B} is an atypical receptor as the affinity for adenosine is very low, but its activation in lymphocytes may lead to calcium mobilization (Mirabet et al. 1997). In summary, it is assumed that A_{2B} receptors are activated is reservoirs with elevated adenosine levels or when hypoxic conditions lead to very high concentrations of the nucleoside. Also, there has been a lack of pharmacological tools that has been progressively solved. Intriguingly the A_{2B} protein has also been described in the CNS as a receptor for netrin-1, involved in axon guidance (Corset et al. 2000; Shewan et al. 2002). The discovery of heteromers formed by A_{2B} and A_{2A} receptors has led to a significant finding, namely, that the activation of the first alters the pharmacology and signaling of the latter. In a heteromeric context, the affinity of $A_{2A}R$ selective ligands is markedly reduced, i.e., the activation of the receptor demands higher concentrations of A_{2A} receptor agonists. Accordingly, the efficacy of ligands targeting the $A_{2A}R$ would be dependent on the heteromeric context, especially in the case of $A_{2A}A_{2B}$Het occurrence (Hinz et al. 2018).

10.4 The A_1-A_{2A} Receptor Heteromer (A_1A_{2A} Het): A Unique Functional Unit

A_1A_{2A} Het is in itself a paradigm to understand a fact that was inscrutable for decades, namely, the co-expression of one receptor for adenosine coupled to G_s and another receptor for adenosine coupled to G_i. In such cells, adenosine would lead to a "contradictory" output as, on the one hand, it would increase adenylate cyclase

activity (via G_s), and, on the other hand, it would decrease adenylate cyclase activity (via G_i). Co-expression of different receptors for a given neurotransmitter in the same cell is quite common, for instance for serotonin receptors (Santana et al. 2004). One of the possibilities (especially in neurons) was to assume that one of the receptors was expressed in a specific location of the cell, whereas the second receptor was located in a different location (always in the cell membrane but far away in spatial terms). In the case of the A_1 and A_{2A} receptors, the explanation is totally different, and, furthermore, it constitutes a clear paradigm of the need of GPCR Hets. In brief, the A_1A_{2A}Het is a device to sense the adenosine concentration to act accordingly, i.e., decreasing cAMP levels when [adenosine] is low and to increase cAMP levels when [adenosine] is high. Adenosine not only increases in hypoxia but its level varies with the metabolic status. Again, this is especially important in regions where neurons are very active and the adenosine/ATP ratio is high. The A_1A_{2A}Het was discovered by Ciruela et al. (2006), and the results on heteromerization of those receptors were later validated by Schicker et al. (2009).

There are different cell types in which the two receptors are co-expressed and where they may likely form A_1A_{2A}Hets. The physiological role of the A_1A_{2A}Het has however shown in the CNS and in relationship to control of neurotransmitter transport by adenosine and, importantly, in both neurons and glial cells. Our results centered in the striatum showed that the levels of co-expression of the two receptors in glutamatergic terminals reaching the striatum were markedly high and that low or high concentrations of adenosine led to opposite effects on glutamate release (Ciruela et al. 2006). This finding in 2006 did not provide any molecular mechanism but suggested that the coupling was different, to either G_s or G_i, depending on the concentration of the nucleoside. Furthermore, it seemed that the heteromeric context was the substrate to block A_1R-mediated signaling when the A_{2A}R was activated. In summary at relatively low adenosine concentrations, the Het was providing A_1R-dependent signaling, whereas at relatively high concentrations it was providing A_{2A}R-dependent signaling.

Fairly similar results were obtained in astrocytes and the control of the transport of one of the main inhibitory neurotransmitters in the CNS, gamma-aminobutyric acid (GABA). First, colocalization of the two receptors and occurrence of A_1A_{2A}Het was demonstrated. Second, the regulation of GABA uptake by cultures of astroglia depended on the concentration of adenosine. Indeed, the regulation of GAT-1 and GAT-3 transporters was via G_i or via G_s depending on whether the receptor activated within the A_1A_{2A}Het was, respectively, A_1R or A_{2A}R (Cristóvão-Ferreira et al. 2013). The molecular basis of such a phenomenon was recently elucidated and described in the next Sect. 10.5.

10.5 The A_1-A_{2A} Receptor Heterotetramer: A Reliable Structural Model

On the one hand, the quaternary structure is crucial for Het function (Navarro et al. 2010). On the other hand, three-dimensional structures of GPCRs are difficult to decipher due to the technical difficulties in obtaining crystals of membrane proteins. Protein engineering and complementary technological advances have led to the elucidation of several GPCR structures and, also, to key structural elements of the GPCR-G protein interactions (see (Cordomí et al. 2015) and references therein). Those advances have served to understand that the most abundant G-protein-coupled signaling unit in the plasma membrane is a GPCR dimer. Exceptions may occur, i.e., a monomer GPCR may eventually couple to a G protein and be able to convey signal toward the inside of the cell. Indeed, this is not the case of the A_1A_{2A}Het whose minimal structure is likely constituted by one A_1R homodimer and one A_{2A}R homodimer, i.e., a heterotetramer. Identification of GPCR oligomers combined with structural data and with modeling and other in silico approaches has provided relevant information concerning the structure of heteroreceptor complexes and their coupled G proteins. Also relevant is the fact that a substantial movement occurs within a GPCR and a G protein when the receptor becomes activated by agonists. Overall, membrane-attached GPCRs, which contain seven transmembrane domains and a tightly coupled alpha G protein subunit, likely form homodimers in a head-to-head fashion. Even allowing to increase the size of the heteroreceptor complex by considering four GPCRs and two coupled G proteins, the number of possible structures for the macromolecular complex is very few, as reported in the quite revealing work by Cordomí et al. (2015)

Using such in silico information, interfering peptides containing transmembrane sequences and, also, data from resonance energy transfer (using both receptors and G protein subunits as probes) and complementation assays, the first reliable structure for a GPCR Hets in complex with one G_s and one G_i protein was provided (Navarro et al. 2016b). The rhombus-shaped structure that contains alpha subunits of G_s or G_i bound to the outer protomers (in both A_1R and A_{2A}R homodimers) would allow signaling via A_1R and via A_{2A}R. We mean that such a symmetrical structure cannot provide an asymmetrical signaling as that involved in the control (by adenosine) of glutamate or GABA transport regulation. The clue that explains the uniqueness and the functional properties of the A_1A_{2A}Het is a recent finding involving the C-terminal tail of the A_{2A}R (Navarro et al. 2018). Different GPCRs display a wide range of lengths in their C-terminal domain. Whereas A_1R has a short C-terminal end, the tail of the A_{2A}R is quite long. Then we hypothesized that a long C-terminal tail would, upon activation of the receptor, block the G-protein-mediated signaling arising from a closely located receptor. Exhaustive experimental and in silico work has provided reliable data showing that removal of the C-terminal domain of the A_{2A}R leads to the disappearance of the Het fingerprint, i.e., activation of a truncated A_{2A}R does not result in impairment of A_1R activation and G_i-mediated signaling. The huge diversity

in the length and structure of C-terminal domains deserves a closer look and is a challenge in future work in the GPCR field.

10.6 Adenosine-Receptor-Containing Heteromers and Schizophrenia

Although the evidence is higher in Parkinson's disease and the success is already evident by the approval of an $A_{2A}R$ antagonist in the therapy of Parkinson's (see below), we would like to make a brief account of data showing that adenosine receptors have also potential in the therapy of schizophrenia. Fuxe et al. (2005) reviewed possibilities of the heteromer as target for schizophrenia. Moreover, the dopamine D_3 receptor is one of the proposed therapeutic targets for treatment of the disease and, accordingly, the discovery of the $A_{2A}D_3$Het receptor (Torvinen et al. 2005) place $A_{2A}R$ ligands as potential therapeutic drugs. Also along this line of reasoning is the above-described occurrence of occurrence of $A_{2A}mGlu_5$Hets. Reviews on the cumulative data that, based on adenosine-receptor-containing heteromers, open new perspectives in antischizophrenia therapy were provided by Fuxe et al. (2008, 2010) and Wardas (2008).

10.7 Adenosine-Receptor-Containing Heteromers and Parkinson's Disease (PD) and Levodopa-Induced Dyskinesia

In this section, we will first focus on the antiparkinsonian efficacy of adenosine receptor ligands to then take into consideration that any drug used by patients is – mostly – targeting Hets. Afterward, we will focus on the adenosine-receptor-containing heteromers that have been studied in both healthy and parkinsonian conditions. Hets constituted by adenosine receptor themselves and by adenosine and dopamine receptors fulfill these rules. The main objective in translational research is to identify suitable targets and efficacious drugs. To this respect dual adenosine-dopamine receptor ligands and bivalent compounds have been developed. The former (dual compounds) (Vendrell et al. 2007) may constitute the basis for the development of novel antiparkinsonian drugs. Instead, the latter (bivalent ligands), being unable to cross the blood-brain barrier and susceptible of being hydrolyzed soon after intake, have been instrumental to confirm the occurrence of adenosine-dopamine Hets in the striatum (A_{2A}-D_2 bivalents in Soriano et al. (2009) and A_1-D_1 bivalents in Shen et al. (2013). Hence, such heteromers are demonstrable targets of antiparkinsonian drugs.

10.7.1 Efficacious Antiparkinsonian $A_{2A}R$ Antagonists

Levodopa-based dopamine replacement therapy started decades ago and is still regarded as being of highest benefit for today's patients (Birkmayer and Hornykiewicz 1962, 1964; Olanow et al. 2004; Hornykiewicz 2006). Based on the early work of Fuxe and Ungerstedt (1974), on translational research and on data from clinical trials (Mizuno et al. 2013b; Saki et al. 2013; Kondo et al. 2015), a selective $A_{2A}R$ antagonist, istradefylline (Nouriast™), was approved in Japan for adjunctive antiparkinsonian therapy. The underlying idea was to reduce the dose of levodopa (or the dopamine-receptor-related medication) to diminish the side effects. In fact, long-term treatment with levodopa may lead to uncontrolled movements.

Cumulative evidence along decades, in different laboratories and under a variety of experimental setups, led to find a dopamine-adenosine antagonism in striatum. Even assuming that receptors are expressed individually (and not as heteromers), activation of adenosine A_1 and dopamine D_1 receptors in the direct pathway (or A_{2A} and D_2 in the indirect pathway) would lead to opposite effects as one of the receptors is coupled to G_i and another to G_s. Solid reviews describing the molecular basis of the antagonism may be found in the literature. As the complete list of reviews is quite notable, we here suggest the following ones that arise from different laboratories and/or present different but complementary perspectives (Bibbiani et al. 2003; Tanganelli et al. 2004; Schwarzschild et al. 2006; Ferré et al. 2007a, 2009b, 2010a, Fuxe et al. 2007, 2010, 2015; Simola et al. 2008; Armentero et al. 2011; Beggiato et al. 2014; Navarro et al. 2016a; Borroto-Escuela et al. 2017b).

In vivo experimental data on the potential of $A_{2A}R$ ligands to i) affect striatal dopaminergic neurotransmission and striatal plasticity and ii) to be efficacious in the unilateral 6-hydroxydopamine rat model of Parkinson's disease were provided by *inter alia* Pinna et al. (1997, 2007), Strömberg et al. (2000), Agnati et al. (2004). $A_{2A}R$ knockout (KO) mice have been used to ensure that a lack of $A_{2A}R$-mediated signaling (and of any A_{2A}Het-mediated signaling) provides data that reinforces the antiparkinsonian potential of receptor blockade (Kachroo 2005).

Reviews on the role of Hets in the pathogenesis of Parkinson's disease and their potential as therapeutic targets of the disease appeared soon after the discovery of GPCR heteromers. Reviews with titles reflecting the relevance of purinergic signaling and/or receptor heteromerization were provided by Maggio et al. (2010), Navarro et al. (2016a, 2017), Borroto-Escuela et al. (2017a). However, the list of relevant reviews on the subject is quite broad. From such list we would recommend the following reviews (and references therein): (Schwarzschild et al. 2002; Morelli et al. 2007; Ferre et al. 2008; Fuxe et al. 2008, 2015; Ferré et al. 2009b).

We also believe that the paper by Short et al. (2006) provides a solid account on an interdependence between dopamine and adenosine receptors disclosed from characterizing receptor expression in adenosine and dopamine receptor KO mice (single KOs, i.e., only one receptor gene knocked out in each of the transgenic lines). This supports the early work of Fuxe and Ungerstedt (1974). Authors concluded that "*the existence of functional interactions between dopaminergic and*

purinergic systems in these reward and motor-related brain regions" (Short et al. 2006).

Therefore, antagonists of adenosine receptors were soon proposed to increase dopamine action in Parkinson's disease, which consists of the depletion of dopamine in striatum due to nigral neurodegeneration. In summary, the conceptual approach was to use adenosine receptor antagonists to increase the dopaminergic action in striatal GABAergic neurons.

10.7.2 Heteromers as Targets of Antiparkinsonian Drugs

Soon after identification of A_1D_1Het and of $A_{2A}D_2$Het, these heteromers were proposed as targets or Parkinson's disease (Fuxe et al. 2003). Despite forgotten due to the usual way to develop novel drugs, i.e., by screening cells expressing individual receptors, it is evident that any antiparkinsonian medication is acting on receptors in heteromeric contexts. Thus, levodopa does not act on isolated dopaminergic receptors but on receptors forming Hets. In the case of Nouriast™, the drug is acting on those Hets identified as of today, namely, $A_{2A}D_2$Hets with or without CB_1 or $mGlu_5$ receptors. The potential of cannabinoids or mGlu5 receptor ligands has been suggested (Ferré et al. 2009b, 2010a), but the underlying reasons are out of the scope of the present article. In terms of adenosine receptors, it was suggested that A_1 receptor agonists acting on A_1R, which are expressed in the direct pathway, reduce D_1 receptor and levodopa-induced dyskinesia (see (Ferré et al. 1994; Florán et al. 2002; Franco et al. 2005; Mango et al. 2014) and references therein). It should be noted that in dyskinesia the level of the D_3 receptor and of D_1D_3Hets increase (Marcellino et al. 2008; Farré et al. 2015). These results suggest that also D_3 receptor ligands may be useful in the therapy of dyskinesia and that Hets may be considered targets for drugs able to counteract this side effect of chronic medication of levodopa and dopamine receptor agonists.

In what concerns the A_1A_{2A}Het, which is presynaptic (unlike A_1D_1 or $A_{2A}D_2$ Hets that are postsynaptic), it is not known how istradefylline (Nouriast™) is affecting its function in striatal glutamatergic terminals of patients. In healthy conditions, blockade of A_{2A}R does not seem to produce any evident effect via those heteromers. In fact, A_{2A}R antagonists are very safe, and this fits with a general rule (surely with exceptions) that receptors antagonists may be taken in chronic regimes by patients of diverse illnesses. A deeper look into the differential pharmacology of Hets has led to find that different drugs may have different "potencies" for the same receptor but in different heteromeric contexts. In brief, there is data showing that the affinity of an antagonist for A_{2A}R, or of caffeine for A_1R or A_{2A}R, is different when tested in different Hets. With data using different (pre- and postsynaptic) Hets and different antagonists, Orru et al. (2011) have suggested that "*on the basis of their preferential pre- versus postsynaptic actions, SCH-442416 and KW-6002 may be used as lead compounds to obtain more effective antidyskinetic and antiparkinsonian compounds,*

respectively." SCH-442416 is a broadly studied $A_{2A}R$ selective antagonist, whereas KW-6002 is another one (also known as istradefylline).

Interestingly, a recent report has linked early-onset Parkinson's disease cases to a point mutation in the gene of the A_1R (ADORA1). The mutation leads to the substitution of a conserved amino acid in transmembrane 7 (Jaberi et al. 2016). Based on current data and in the proposed models for receptor Hets, this mutation would not affect interacting interfaces of homo- or heteromers; then alternative explanations include altered binding of adenosine or altered signaling.

Taking into account the successful case of Nouriast™, one wonders why it is relevant to consider A_{2A}Hets as targets. On the one hand, the adenosine-dopamine antagonism is evidenced at the Het level, i.e., it is a significant print of the adenosine-dopamine Hets. Therefore, the "intracellular" antagonism due to counterbalancing second messenger cAMP levels is complemented with antagonism at the receptor level within the A_{2A}-D_2Het context. The added value of having those Hets in a very precise location, the striatal spine module, also plays a role, as pointed out by Fuxe et al. (1998, 2007), Tanganelli et al. (2004), Ferré et al. (2007a, 2009b, 2010a), Beggiato et al. (2014) and as deduced by its role in controlling striatal glutamatergic neurotransmission (Ferré et al. 2007b).

Unlike for the A_1-A_{2A}Het, no detailed structural model exists for adenosine-dopamine Hets. Allosteric interactions within the quaternary structure are essential for Het function, i.e., for integrating the dopamine and adenosine inputs (Fuxe et al. 2010). Remarkably, the $A_{2A}D_2$Het has been a paradigm to detect electrostatic interactions that are key for the functional activity of the signaling unit. Apart from the consensus on the involvement of transmembrane domains in Het formation, it was demonstrated that strings of amino acid residues with opposite charges do interact, do it tightly, and are important for quaternary structure and function (Borroto-Escuela et al. 2010). One example is provided by the epitope-epitope interactions involving arginine residues in the N-terminal part of the third intracellular loop of the D_2R and acidic residues in the C-terminal end of the $A_{2A}R$ (Ciruela et al. 2004). Complexes formed by synthetic peptides mimicking the interaction are even resistant to mass spectrometry processing thus demonstrating the strength of the epitope-epitope intraction. Finally, it should be noted that structure may be affected by phosphorylation, i.e., whereas serine would not participate on epitope-epitope interactions, a negatively charged phosphorylated serine would. One of the properties of Hets is a differential traffic respect to individually expressed receptors; apart from co-internalization and particular processing of internalized receptors that may be target to degradation or recycled back to the cell surface, there is involvement of both ß-arrestin/clathrin- and caveolin-dependent pathways (Escriche et al. 2003; Genedani et al. 2005; Franco et al. 2007; Borroto-Escuela et al. 2011)

Finally, it is worth mentioning the role of Ca^{2+} in heteromer-mediated signaling. It is likely that changes in the concentration of the ion may alter the quaternary structure of Hets in which electrostatic interactions are relevant. In fact, Ca^{2+} and/or calcium-binding proteins (e.g., calmodulin) modulate structure and function of $A_{2A}D_2$Hets (Ferré et al. 2010b; Woods et al. 2008; Navarro et al. 2009). These results

explain, at least in part, the elusive relationship between dopaminergic transmission and calcium ions.

10.7.3 How Levodopa-Induced Dyskinesia Affects Heteromerization

Parkinson's disease and adenosine-receptor-containing Hets constitute another paradigm due to the fact that their relationships have been investigated in healthy conditions and in the disease before and after chronic medication. On the one hand, the presence of $A_{2A}D_2$Hets and of Hets also including CB_1 receptors was demonstrated in rodent models of the disease (Pinna et al. 2014a, b). In our opinion, these results are important, as they show that these Hets are indeed targets of the dopamine replacement therapy. On the other hand, A_{2A}-CB_1-D_2 receptor heteromerization is disrupted after chronic levodopa administration (Pinna et al. 2014a, b). Remarkably, these results obtained in a rodent model were confirmed in a non-human primate model (Bonaventura et al. 2014), thus pointing to their validity for patients. Also consistent with those findings are the results showing in A_{2A}R knockout animals a reduction in levodopa-induced dyskinesia as reported by Xiao et al. (2011). Interestingly, similar results were obtained upon deletion of the A_1R (Xiao et al. 2011). While it is not known whether heteromer disruption is cause or consequence of chronic medication, these results show that the target of the antiparkinsonian medication changes with time. To our understanding, these results may provide the basis for the design of optimal therapeutic approaches, i.e., varying the medication and/or the dose at different stages of the disease may reduce the side effects that for Parkinson's are not only dyskinesias but cognition deficits.

10.7.4 Adenosine Receptor and Adenosine-Receptor-Containing Heteromers and Neuroprotection

The success of approval of istradefylline for the therapy of Parkinson's disease provides further hopes for other neurodegenerative diseases. In vivo assays in animal models demonstrate the usefulness of A_{2A}R antagonists in acute neural damage, for instance, in hypoxia (Chen and Pedata 2008; Melani et al. 2015; Boia et al. 2017). In the case of (chronic) neurodegenerative diseases (Parkinson's, Alzheimer's, etc.), the real issue is to know whether A_{2A}R antagonists are addressing symptoms or are also affecting disease progression. Animals lacking expression of A_{2A}R are more resistant to neuronal death in an α-synuclein model of Parkinson's disease (see (Kachroo and Schwarzschild 2012) and references therein). Surely one of today's challenges is to demonstrate whether A_{2A}R antagonists are neuroprotective, i.e., they prevent neuronal death (see Franco and Navarra 2018 and references therein).

Apart from the issue of demonstrating whether a given compound is neuroprotective in humans (Kieburtz and Olanow 2015; Olanow et al. 2017), there is evidence of microglia involvement in both promoting neuroinflammation, neuronal death and the release of factors that prevent neuronal death. In fact, after an insult and microglia cell recruitment and activation, there are two possible phenotypes: M1 or proinflammatory and M2 or neuroprotective (see (Franco and Fernández-Suárez 2015) and references therein). Due to the expression of adenosine receptors in resting and reactive microglia, it is suggested that adenosine receptor ligands may be protective (Corriden and Insel 2012; Koizumi et al. 2013; Beamer et al. 2016; Pedata et al. 2016; Woods et al. 2016). However, data is missing on how ligands acting on adenosine receptors may produce M2-skewed cells and on how adenosine-receptor-containing heteromers may contribute to the inflammatory/neuroprotective balance.

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Chapter 11
Adenosine Receptors in Alzheimer's Disease

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Abstract Adenosine operates its effects through adenosine receptors, which have been proposed to be of particular relevance in neuropathological situations, such as Alzheimer's disease (AD). AD is characterized by progressive cognitive impairment, synaptic and neuronal loss, formation of amyloid plaques, mainly composed by amyloid-beta (Aβ) peptides, and neurofibrillary tangles as well as neuroinflammation. Epidemiological studies concluded that the regular consumption of caffeine, a nonselective antagonist of adenosine receptors, is inversely correlated with the incidence of AD. Neurochemical data showed an increased $A_{2A}R$ density in the brain of AD patients, and these $A_{2A}Rs$ interfere with memory, synaptic plasticity, Aβ production and neurofibrillary tangles formation in AD models. Accordingly, pharmacological blockade or genetic inactivation of $A_{2A}R$ prevents cognitive impairment and affords neuroprotection. However, either the mechanisms or the contribution of $A_{2A}R$ in different cell types for the onset and progression of AD are not completely understood. Until now, it was described that neuronal and astrocytic $A_{2A}Rs$ have a role in controlling synaptic plasticity and memory, microglial $A_{2A}R$ modulates neuroinflammation and $A_{2A}R$ in peripheral cells also comes into play in neurodegenerative processes. This chapter will discuss the importance of adenosinergic system in AD patients and experimental models, providing an overview of future adenosine-based therapies.

Keywords Adenosine receptors · Alzheimer's disease · Amyloid-beta · Neuroinflammation · Caffeine

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11.1 Alzheimer's Disease (AD): A Continuum Pathological Process

Increasing evidence support that the pathological process of Alzheimer's disease (AD) initiates years or decades before the diagnosis of dementia, a syndromal state that encompasses memory deficits, problems in language, in planning, and execute familiar tasks and in other cognitive skills that affect the individual's ability to perform daily actions. AD constitutes the most common cause of dementia in the elderly people, accounting for an estimated 70–80% of cases (Braak and Braak 1991; Selkoe et al. 2012). Currently, nearly 44 million people have AD or a related dementia worldwide, and this number is expected to triple by 2050 as the size of elderly population rise (Prince et al. 2016). AD frequently co-occurs with other pathologies that might also contribute to dementia, a situation designated as *multiple etiology dementia*, resulting in AD plus Lewy body dementia (LBD) or AD plus vascular cognitive impairment (VCI)/vascular dementia (VaD), although both LBD and VCI/VaD can lead to dementia in the absence of AD (Montine et al. 2014). This cognitive neurodegenerative landscape is one of the most devastating brain illnesses in terms of handicap, clinical evolution and treatment, thus representing a major socioeconomic concern for the twenty-first century (Prince et al. 2016). Moreover, it is also a challenge for the scientific community, because it remains to be defined the precise biological changes that cause AD, why it progresses more quickly in some individuals than in others, and how the disease can be prevented, slowed or halted.

AD is an age-related progressive disorder that usually begins with a preclinical asymptomatic stage, in which it is claimed to occur an evolving brain damage underlying very subtle deficits in cognition (Sperling et al. 2011; Musiek and Holtzman 2015). This stage may precede the mild cognitive impairment (MCI), a condition in which subjects are only mildly impaired in memory with relative preservation of other cognitive domains and functional activities; thus subjects are cognitively altered, but they do not yet meet the criteria for dementia, and this MCI condition is also designated as prodromal AD state (Petersen et al. 2009; Dubois et al. 2010). Although not all MCI cases progress to a dementia state, epidemiological studies showed that MCI patients, who display significant heterogeneity in their profile of cognitive impairment, progress to AD at rate of 10–15% per year (Weiner et al. 2015). Thus, forecasting who, among a group of MCI patients will be more likely to further decline in cognition and convert into an Alzheimer's dementia, would be crucial to ensure an early intervention and to establish effective therapeutic strategies to treat, halt or prevent AD. Over the last years, neuroimaging techniques have provided information regarding the morphology and the physiology of the brain of patients with MCI or AD, and also the analyses of biomarkers in the cerebrospinal fluid (CSF) and in the plasma have been useful to notice and study the disease pathophysiological process and progression in vivo (Blennow et al. 2010; Sperling et al. 2011). Although the accumulation of amyloid-beta (Aβ) protein is a biomarker and putative trigger of cognitive decline in AD, anatomo-pathological observations of elderly people with normal cognitive function revealed the presence

of extensive Aβ deposition, forming amyloid plaques, in the brain (Serrano-Pozo et al. 2011). These findings were confirmed in more recent in vivo studies using brain imaging techniques and compounds to label Aβ deposits (Elman et al. 2014). Moreover, functional magnetic resonance imaging (fMRI) studies, which were performed during an episodic memory encoding task, showed an increased activation in medial temporal lobe (MTL) regions in both aged cognitively normal individuals with brain Aβ deposition and individuals with MCI as compared with age-mates people without Aβ deposits. This MTL activation was postulated: (i) to be a compensatory mechanism to manage the underlying Aβ deposition and to delay the onset of cognitive decline or (ii) to promote the Aβ deposition in aged people (Sperling et al. 2010; Mormino et al. 2012). These findings certainly contribute to the controversy of whether Aβ protein overproduction and accumulation are beneficial or harmful (Sperling et al. 2010; Elman et al. 2014). Currently, there still exists a great need of better defining biomarkers, imaging markers, and cognitive profiles that predict with greater confidence the progression from preclinical to clinical stages of MCI and AD dementia and to define if Aβ is a causative agent of these pathological conditions.

11.2 Clinical and Neuropathological Features of AD

There are two forms of Alzheimer's: (i) the early-onset (under 65 years of age) familial AD (fAD), which comprises 1–5% of total AD population and has an aggressive and rapid progression with relative short survival time; and (ii) the late-onset (after 65 years of age) sporadic AD (sAD) that represents the majority (>95%) of the cases (Tanzi 2013; Prince et al. 2016).

In both AD forms, the most noticeable clinical symptom is the impairment in formation and retention of new episodic memories. The syndrome of memory decline in AD has been attributed to neuronal loss in the perforant pathway of the MTL (Hyman et al. 1984); however, further functional imaging studies have suggested that memory processes are subserved by a set of distributed, large-scale neural networks (Raichle et al. 2001). In addition to the hippocampus and surrounding MTL cortices, these memory networks are comprised of a set of cortical regions, collectively named as the default network, which typically deactivate during memory encoding and other cognitive demanding tasks focused on external stimuli processing (Sperling et al. 2010). Frontoparietal cortical networks, which support executive function and attentional processes, also likely interact with these memory systems, and thus multiple cognitive domains become dysfunctional as AD progresses (Dickerson and Sperling 2009; Sperling et al. 2010 and references therein).

Characteristic imaging features of sAD and fAD include atrophy of the medial temporal lobe, the precuneus, the ventrolateral temporal, lateral parietal, and posterior cingulate cortices, the amygdala and the anterior hippocampus. Furthermore, hypometabolism and amyloid deposition in these regions can be detected using fluorodeoxyglucose and Pittsburgh compound B positron emission tomography

(FDG-PET and PiB-PET), respectively (Tentolouris-Piperas et al. 2017 and references therein).

Neuropathologically, both forms of AD are characterized by the presence of extracellular amyloid plaques and of intracellular neurofibrillary tangles (NFT), which are deposits of abnormal proteins that progressively grow and spread in the brain parenchyma (Blennow et al. 2006). The amyloid plaques are mainly composed by the amyloid-β (Aβ) protein with 42 or 40 amino acids (Aβ40 and Aβ42), being the first form more abundant than Aβ40 within the plaques (Citron et al. 1996). The intracellular NFTs are primarily composed of paired helical filaments (PHF) consisting of hyperphosphorylated tau, a microtubule-binding protein of cytoskeleton (Bramblett et al. 1993), although more recent studies reported that acetylated tau was present throughout all stages of AD and seems to precede the tau hyperphosphorylation and, eventually, later truncation (Irwin et al. 2012). *Postmortem* studies were crucial to categorize the progression of both amyloid and tangles pathologies, which contributed to the development of AD diagnostic criteria that are currently used (Braak and Braak 1991; Gómez-Isla et al. 1997). The amyloid plaques occur initially and most severely in the precuneus and frontal lobes, whereas neuronal death begins and arises most readily in the entorhinal cortex and hippocampus, regions with relatively few Aβ deposits. In contrast, the NFT correlate more closely with neuronal loss, both spatially and temporally. That is, amyloid pathology appears to begin in the cortex and spreads inward, while tau pathology exhibits an opposite progression. Therefore, brain regions with neuritic plaques, which are amyloid plaques associated to NFT, display an extensive neuronal death (Musiek and Holtzman 2015).

Amyloid plaques are usually surrounded by dystrophic neurites and reactive glial cells (astrocytes and microglia), forming structures known as senile plaques. The activation of glial cells has been considered a double-edge event: it can be seen as an endogenous defensive mechanism against amyloid deposition and neuronal damage, while on the other hand, the persistent activation of glia might trigger a neuroinflammatory process, with increased production of pro-inflammatory cytokines and other neurotoxic factors, such as reactive oxygen species (Akiyama et al. 2000; Agostinho et al. 2010, and references therein). Neuroinflammation is largely driven by glial cells but also by mononuclear phagocyte (10% of cell population in CNS) and by dying neurons; however, the exact role of this process during AD onset and progression is still not well established (Agostinho et al. 2010; De Strooper and Karran 2016).

In the last decades, it was recognized that the reduction of synapse number is perhaps the strongest quantitative neuropathological correlate of dementia in AD (Terry et al. 1991; Selkoe 2002). Indeed, both APP and α- and β-secretases are more abundantly localized in cortical synapses (Pliássova et al. 2016a, b), and synaptic activity is tightly linked to APP and the formation of Aβ (Agostinho et al. 2015; Müller et al. 2017). Moreover, it was shown that soluble Aβ oligomers, but not amyloid plaques cores, collected directly from the cerebral cortex of subjects with AD, impair both synaptic function (e.g., long-term potentiation) and synaptic structure (e.g., dendritic spines) and, consequently, the memory of a learned behavior in healthy adult rats (Shankar et al. 2008; Selkoe and Hardy 2016). These data are

consistent with observations made in transgenic AD mice (with human mutant APP), which showed that amyloid plaques have a penumbra of soluble Aβ oligomers in which the synaptic density is low (Koffie et al. 2009). There are also evidences in humans that Aβ oligomers predict the presence of dementia more accurately than amyloid plaques burden and that these Aβ species are more closely related with tau pathology (Lesné et al. 2013; Musiek and Holtzman 2015). Indeed, these findings have contributed to (i) shift the assumption that amyloid plaques are the likely causative agent of pathology into the concept that soluble Aβ oligomers are more potent in causing synaptic dysfunction and loss and (ii) explain why the amyloid plaques distribution does not match with neuronal damage nor with tau pathology. Hence the prevalent view is that amyloid plaques sequester Aβ oligomers and, thus, protect neurons from their synaptotoxicity. Noteworthy, increasing evidences in the last years also support that astrocytes, a major type of glia cells, reciprocally regulate synaptic connectivity and transmission, forming a "tripartite synapses" (Perea et al. 2009). Aβ peptides can trigger astrocyte activation or astrocytic dysfunction, leading to metabolic and synaptic alterations that might underlie several pathological states, such as AD (Matos et al. 2012; Rial et al. 2016; De Strooper and Karran 2016).

11.3 Putative Initiators and Risk Factors for AD

The "amyloid cascade hypothesis" was proposed in the early 1990s and posits that Aβ deposition initiates a sequence of events leading to progressive tau pathology, synaptic dysfunction, neuroinflammation, vascular damage, neuronal loss, and ultimately impairment of higher cortical activity, such as memory and cognition (Hardy and Higgins 1992). Although, this hypothesis has been the source of considerable controversy, mainly because the clinical trials targeting Aβ failed and there are evidences supporting that tau might by an initiator of neurodegeneration (Selkoe et al. 2012; Musiek and Holtzman 2015), it still is the main theoretical construct for AD. Moreover, this premise has contributed undoubtedly to the replacement of the earlier descriptive studies by more mechanistic and functional studies, and this has contributed to the development of diagnostic and therapeutic strategies for a disease believed before to be either incurable or an inevitable consequence of aging (Hardy and Selkoe 2002).

Human genetics studies strongly support the role of Aβ as disease initiator (see Sect. 11.2). The autosomal dominant familial AD (fAD) is due to mutations in three genes, which are all integrally involved in Aβ production, the (i) amyloid precursor protein (APP) gene, (ii) presenilin 1 (PSEN1) gene, and (iii) presenilin 2 (PSEN2) gene. The APP is a ubiquitous transmembrane protein that can be proteolytically cleaved via amyloidogenic pathways, involving β-and γ-secretases that give rise to Aβ40 or Aβ42; PSEN1 and PSEN2 are catalytic subunits of the γ-secretase complex that cleaves APP to generate Aβ (Hardy and Selkoe 2002; Agostinho et al. 2015). Notably, some APP mutations in the middle of the Aβ coding region are also associated with fAD by promoting not Aβ production but fibrillation and aggregation or

by inhibiting Aβ degradation or clearance (Tomiyama et al. 2008). These forms of AD provide an opportunity to examine a pure Aβ-driven disease in relatively young, often healthy, individuals (Musiek and Holtzman 2015).

The majority of AD cases is considered sporadic (sAD) and can be instigated by aging along with a complex interaction of genetic, metabolic, and environmental risk factors still not understood. The genetics of sAD is also multifactorial, being the strongest genetic risk factor the ε4 allele of apolipoprotein E gene (APOE). This polymorphic gene has three common alleles, ε2, ε3, and ε4, resulting in single amino changes in the apolipoprotein E that is involved in the transport of lipids in the blood. The APOE ε4 allele increases the risk for AD, and the ε2 allele decreases the risk for AD relative to the most frequent ε3 allele. About 20–25% of the population carries at least one copy of ApoE4, which quadruplicates the risk of AD, whereas 2% of the population carries two E4 alleles, having an increased risk of around 12-fold, compared to individuals with the more common ApoE3/E3 genotype (Verghese et al. 2011). Numerous studies have shown accelerated amyloid plaque accumulation in human ApoE4 carriers, and it was also reported that ApoE influences Aβ metabolism, and, in particular, ApoE4 protein promotes amyloid aggregation and deposition (Liu et al. 2013). Noteworthy, there are other genes that might contribute to sAD, such as genes related with proteins involved in the metabolism of cholesterol and lipids, in the immune response, and in synaptic structure and function (Bertram and Tanzi 2004; Tanzi 2013). A recent study had also reported that epigenetic factors (differences in DNA methylation) are associated with sAD in monozygotic twins (Mastroeni et al. 2009).

Two other risk factors chiefly contributing for sAD are age (the probability of developing AD increases from 10% under the age of 65 to 50% over 85 years old) and gender (sex); the prevalence of AD has been greater in women. In fact, the incidence for AD is comparable in women and men at younger ages, but at a later age the incidence becomes greater in women probably because women have a greater longevity of 4.5 years on average (Solomon et al. 2013). Epidemiological studies have also identified several risk factors for cognitive decline and AD more associated with metabolic alterations or lifestyle habits, such as diabetes mellitus, obesity, atherosclerosis, cardiovascular disease, and hypercholesterolemia at midlife, as well as physical and mental inactivity, low educational attainment, poor diet, sleep deprivation, and smoking (Crous-Bou et al. 2017; Tariq and Barber 2017). Thus, sAD can be considered a multifactorial, genetically complex, and heterogeneous disorder formatted by several non-genetic factors (Tariq and Barber 2017).

11.4 Therapeutic and Preventive Strategies for AD

Currently, drugs approved by the FDA (Food and Drug Administration) for the treatment of cognitive decline and AD, such acetylcholinesterase inhibitors (that avert the catabolism of the neurotransmitter acetylcholine to improve cognition) and NMDA receptor antagonists (that reduce glutamate-induced neuronal

excitotoxicity), have limited effects and none of them prevent or reverse the disease pathology (Huang and Mucke 2012). Thus, the establishment of strategies to delay the onset of AD or slow its progression would have a significant impact on public health (Sperling et al. 2011; Prince et al. 2016). In the last years, several intervention studies have moved their focus toward cognitively healthy people at risk of developing AD, which are likely to have not yet substantial irreversible neuronal network dysfunction and loss, as the best strategy to reduce the incidence and prevalence of AD. Moreover, the estimate that a third of AD cases are potentially attributable to modifiable risk factors related with metabolic problems or lifestyle habits has prompted the development of several multi-domain intervention programs to prevent cognitive decline among elderly people. The data from large, long-term, randomized controlled trial have demonstrated that a multi-domain intervention, including equilibrated and healthy diet, exercise, cognitive training and monitoring vascular risk can ameliorate or preserve cognitive functioning in aged individuals (60–77 years old) compared to the general population at risk of dementia (Ngandu et al. 2015; Crous-Bou et al. 2017). Recent prospective studies reported that the adherence to a *Mediterranean-style diet*, which includes proportionally larger consumption of olive oil, legumes, unrefined cereals, fruits and vegetables, moderate consumption of fish, dairy products and wine, and low consumption meat products, might be related with slower cognitive decline and a reduced risk of progression from MCI to AD (Solfrizzi et al. 2011; Panza et al. 2015). Another lifestyle measure shown to attenuate the incidence of cognitive deterioration with aging is a diet rich in nuts (Rajaram et al. 2017), with a regular consumption of ω-3 unsaturated fatty acids (Burckhardt et al. 2016), which has also been confirmed to afford benefits in controlled studies using animal models of AD (Hooijmans et al. 2012; Muthaiyah et al. 2014).

Notably, several epidemiological studies in humans have demonstrated beneficial effects on cognition upon caffeine consumption, which has been associated with a decreased risk of developing AD and other neurodegenerative disorders, as well as age-related cognitive decline (Maia and de Mendonça 2002; van Gelder et al. 2007; Simonin et al. 2013; Flaten et al. 2014; Panza et al. 2015). Caffeine is a methylxanthine with psychostimulant properties, which exists in coffee, tea and chocolate, which are regularly consumed by millions of people around the world (Fredholm et al. 1999). The psychostimulant properties of caffeine are due to its capacity to interact with adenosine receptors in different brain regions (Fredholm et al. 1999), thereby enhancing vigilance and attention, stabilizing mood and improving cognition, mainly memory performance when it is perturbed by pathological conditions, either in human or animal studies (Nehlig et al. 1992; Yu et al. 2017). Apart from these acute effects subjective of role of caffeine as a cognitive enhancer, the regular consumption of caffeine is instead considered as a cognitive normalizer (Cunha and Agostinho 2010). Indeed, some case-control and cross-sectional and longitudinal population-based studies evaluated the long-term effects of caffeine on brain function and provided evidence that its consumption or higher plasma caffeine levels might be protective against cognitive decline and dementia (Santos et al. 2010; Panza et al. 2015). Interestingly, although epidemiological studies support that caffeine consumption can slow down cognitive decline in the elderly

and reduces the risk to develop AD or Parkinson's disease, it has been reported that this methylxanthine is harmful for Huntington's disease, suggesting that caffeine is not beneficial for all neurodegenerative conditions and its effects depend on pathogenic mechanisms (Simonin et al. 2013; Flaten et al. 2014).

11.5 Caffeine and Adenosine Receptors: Impact in AD

Caffeine, at a concentration of 1–30 μM in the body (equivalent to ingestion of 1–5 cups of coffee), exerts its primary effect in the central nervous system through the inhibition of adenosine receptors, mainly A_1 and A_{2A} receptors, and subsequent modulation of neurotransmitter release (Fredholm et al. 1999; Kerkhofs et al. 2018). However, higher concentration of caffeine (millimolar, not corresponding to normal coffee consumption) can affect the release of calcium from intracellular stores, interfere with $GABA_A$ receptors, and inhibit 5'-nucleotidases and alkaline phosphatase (Fredholm et al. 1999, 2005; Cunha and Agostinho 2010). The rapid metabolization (within 1 h) of caffeine in the liver (Nehlig 2018) introduces an additional difficulty in the understanding of caffeine molecular mechanisms in the brain. The main metabolites of caffeine are paraxanthine, theophylline, and theobromine, which are also adenosine receptor antagonists; however, these metabolites can cause distinct, although sometimes also, overlapping responses (Yu et al. 2017). A case-control study provided initial evidence that the plasma levels of caffeine upon coffee intake were associated with a reduced risk of dementia, particularly for those who already have MCI (Cao et al. 2012). However, a subsequent study reported that it was the CSF levels of theobromine rather than levels of caffeine that correlated with a more favorable profile of AD biomarkers in the CSF, mainly $A\beta_{1–42}$ and tau protein (total and phosphorylated), in patients with MCI or AD (Travassos et al. 2015), in agreement with the protection afforded by the consumption of chocolate (rich in theobromine) in AD (Moreira et al. 2016).

The receptors for adenosine (an endogenous neuromodulator) regulate both synaptic transmission and plasticity either by directly modulating synaptic responses or by interfering with other receptors (Cunha 2016). Adenosine receptors are coupled to G proteins and can be antagonized by methylxanthines, such as theophylline and caffeine, which have greater affinities for human than rodent brain adenosine receptors (Kerkhofs et al. 2018). These receptors are classified into A_1 (A_1R), A_{2A} ($A_{2A}R$), A_{2B} ($A_{2B}R$) and A_3 (A_3R) receptors. The A_1R and A_3R inhibit adenylyl cyclase through Gi/o (inhibitory) proteins, while $A_{2A}R$ and $A_{2B}R$ stimulate adenylyl cyclase through Gs/olf (stimulatory) proteins. A_1R inhibits calcium channels and stimulates potassium channels, decreasing stimulus-evoked release of neurotransmitters, in particular of glutamate, as well as postsynaptic responsiveness to glutamate; in contrast, $A_{2A}R$ facilitates the evoked release of glutamate and promotes synaptic plasticity phenomena (Fredholm et al. 2005; Burnstock et al. 2011). A_1R and $A_{2A}R$ are widely located throughout the brain, being present in both neuronal and glial cells, which is in agreement with the predominant effects of caffeine on brain-related

functions (Fredholm et al. 2005; Cunha and Agostinho 2010; Cunha 2016; and references therein). In pathological conditions, A_1R mostly acts as a hurdle that needs to be overtaken to begin neurodegeneration; thus, A_1R only controls neurodegeneration if activated in the temporal vicinity of brain insults (de Mendonça et al. 2000). In contrast, the blockade of $A_{2A}R$ alleviates the long-term burden of brain injuries in different neurodegenerative conditions, such as AD and Parkinson's disease (Gomes et al. 2011).

The setup of adenosine receptors in the brain is altered in AD and other dementia. This was first observed in the early 1990s, in studies comparing brain samples from AD patients and age-matched non-dementia individuals (control) that reported a reduction in A_1R levels in the dentate gyrus and CA3 regions of the hippocampus of AD patients, which are regions of NFT spread and of neuronal loss (Kalaria et al. 1990; Ułas et al. 1993). Deckert et al. (1998) proposed that the reduction of A_1R in CA1 region of hippocampus might not be specific for AD cases, since it also occurred in other types of dementia. Positron emission tomography (PET) in vivo studies using a radioligand for A_1R (^{11}C-MPDX, 8-dicyclopropylmethyl-1-[^{11}C] methyl-3-propylxanthine) revealed a decreased binding of [^{11}C]MPDX in the medial temporal cortex of AD patients when compared with normal elderly individuals, which is consistent with postmortem autoradiographic studies showing a reduction of A_1R levels in patients with AD (Fukumitsu et al. 2008). In contrast, Angulo et al. (2003) showed that the protein levels of A_1R are slightly augmented in the hippocampus of AD patients, mainly in degenerating neurons with NFT and in dystrophic neurites of amyloid plaques, although no significant changes were observed in A_1R mRNA expression. This study also described that $A_{2A}R$, which are located mainly in the striatal neurons in control individuals, appeared in microglia cells in the hippocampus and cerebral cortex of AD patients (Angulo et al. 2003). In the frontal cortex of AD patients, it was also described as an upregulation of A_1R and $A_{2A}R$, as compared with age-matched non-dementia (control) individuals (Albasanz et al. 2008). The levels of A_1R and $A_{2A}R$ were considerably increased in AD patients in early and advanced stages, without differences with disease progression, being the upregulation of these receptors associated with sensitization of the corresponding transduction pathways (Albasanz et al. 2008). Interestingly, it also reported increased levels of $A_{2A}R$ in astrocytes, but not in microglia cells, in the hippocampus of AD patients, and it was postulated that increases in astrocytic $A_{2A}R$ levels contribute to memory loss in AD conditions (Orr et al. 2015). Interestingly, a study performed in peripheral blood mononuclear cells (PBMCs) of MCI and AD patients and age-matched healthy people reported increased $A_{2A}R$ levels in MCI patients, which might indicate the involvement of $A_{2A}R$ in early AD stages (Gussago et al. 2014). Since this increase in $A_{2A}R$ levels was not observed in peripheral cells of individuals with vascular dementia, it was postulated that $A_{2A}R$ could be a biomarker to distinguish these two types of dementia (Gussago et al. 2014).

Since most of the available studies in human tissue samples are from deceased patients with advanced stages of AD, it still remains to be investigated how the adenosine receptors change in terms of density, localization, and function and the onset and progression of cognitive decline and AD; however, this is difficult to

accomplish in human patients. Thus, the use of experimental models to mimic the disease is useful to better understand the initiating mechanisms and to test and validate novel therapeutic strategies for dementia-associated diseases.

11.6 Evidences for the Involvement of Adenosine Receptors in Experimental Model of AD

11.6.1 Caffeine Studies

Numerous epidemiological studies showed that caffeine can decrease cognitive deficits and AD (Chen 2014). Caffeine and its metabolites, at low to moderate doses, act mainly on adenosine receptors (Fredholm et al. 1999); however, the neuroprotective mechanisms of these methylxanthines are not completely defined.

The first study showing a protection by caffeine in AD-like conditions was performed in cultured neurons exposed to the toxic synthetic fragment – $A\beta_{25–35}$ – to mimic AD-like conditions. This study showed that caffeine prevented neuronal cell death, and this neuroprotective effect was mimicked by a selective antagonist of $A_{2A}R$ (ZM 241385), but not when an A_1R antagonist was used, thus indicating that the neuroprotection afforded by caffeine against Aβ-toxicity was mainly mediated by the blockade of $A_{2A}R$ (Dall'Igna et al. 2003). This pioneering in vitro study prompted follow-up studies in animal models of AD to test the effect of caffeine on memory impairment and neurochemical alterations that occur in AD. Arendash and collaborators (2006) treated a transgenic AD mice model (TgAD-APPsw, with a Swedish mutation in APP gene) with caffeine orally for 5 months (1.5 mg consumption daily per mouse, which is equivalent to human intake of five cups a day) and reported an amelioration of reference, working, and recognition memory. This same study indicated that this caffeine protection on memory performance involved a reduction of the hippocampal levels of β- and γ-secretases and consequently in Aβ production (Arendash et al. 2006). The same group further showed that caffeine (oral administration) prevents memory impairment and plaque deposition in aged TgAD-APPsw mice (18 months old) relatively to age-matched Tg mice that were not consuming caffeine (Arendash et al. 2009). This ability of caffeine to prevent Aβ-induced memory deterioration was also observed in an animal model of sAD, consisting in the intracerebroventricular $A\beta_{25–35}$ injection (icv-$A\beta_{25–35}$ that cause memory deficits 1-week later). In fact, the acute or sub-chronical intraperitoneal (ip) caffeine administration prevented memory deficits induced by icv-$A\beta_{25–35}$ and effect mimicked by the $A_{2A}R$ antagonist (SCH58261), whereas selective A_1R antagonists were devoid of effects (Dall'Igna et al. 2007). These studies strongly support a neuroprotective role of caffeine, mainly mediated by $A_{2A}R$ the antagonism, in neurodegeneration triggered by Aβ overload. In a similar way, it was reported that caffeine or the selective blockade of $A_{2A}R$ by SCH58261, both administered by oral gavage, was able to ameliorate age-related memory impairment (Prediger et al. 2005; Leite et al. 2011).

Several studies attempted to further grasp the mechanism underlying the ability of caffeine to attenuate different features pertinent to AD, besides the control of the amyloidogenic processing of APP. In fact, caffeine intake through drinking water at an early pathologic stage in a THY-Tau22 transgenic mouse modelling progressive AD-like tau pathology prevented the development of spatial memory deficits, reduced hippocampal tau phosphorylation and proteolytic fragments, and dampened several upregulated proinflammatory and oxidative stress markers found in the hippocampus (Laurent et al. 2014). Accordingly, Cao and collaborators (2009) reported that high levels of caffeine and their metabolites correlate with the decrease of cytokines in the hippocampus of TgAD-APPsw mice. In this AD mice model, it also described alterations in the intracellular signalling pathways associated with adenosine receptors, such as in protein kinase A (PKA), phospho-AMP responsive element-binding protein (CREB), phospho-c-Jun N-terminal kinase (JNK) and in phospho-extracellular signal-regulated kinase (ERK), which are crucial for synaptic plasticity and oxidative stress (Zeitlin et al. 2011). Furthermore, treatment of TgAD-APPsw mice with caffeine prevented the decrease of mitochondrial membrane potential and respiratory rate and, consequently, the reduction of ATP levels and the reactive oxygen species (ROS) overproduction in the hippocampal and cortical mitochondria (Dragicevic et al. 2012). Also, in rabbits fed with 2% cholesterol-enriched diet, which was considered a model of AD-like conditions, it was reported that the oral administration of caffeine prevented the downregulation of A_1R and the increase in Aβ levels and tau phosphorylation, as well as the endoplasmic reticulum (ER) stress (Prasanthi et al. 2010). Interestingly, it was reported that caffeine was able to decrease low-density lipoprotein (LDL) cholesterol internalization, which impairs the APP internalization into lysosomes and consequently reduces the production of Aβ, in neuronal cultures (Li et al. 2015b). A final group of studies has exploited a model of sporadic AD, based on an intracerebroventricular intoxication with streptozotocin, to show an ability of caffeine to prevent memory deterioration as well as the associated loss of synaptic markers (Espinosa et al. 2013). In keeping with the evidence that the loss of synaptic markers is the current best morphological correlate of AD-related memory impairment (Terry et al. 1991; Selkoe, 2002; Scheff et al. 2007), caffeine and $A_{2A}R$ blockade prevented hippocampal synaptotoxicity and memory deficits in a variety of animal models (Cognato et al. 2010; Duarte et al. 2012; Machado et al. 2017).

All these studies performed in experimental models confirmed the neuroprotective role of caffeine in AD pathology and strength the contention that caffeine reduced the incidence of AD in humans.

11.6.2 Inhibitory Adenosine Receptor: A_1R and A_3R

In aged animals, there is a decrease of A_1R levels and a decreased synaptic transmission in the hippocampus that are mediated by A_1R (Pagonopoulou and Angelatou 1992; Cunha et al. 1995; Sperlágh et al. 1997; Lopes et al. 1999a; Cheng et al. 2000;

Sebastião et al. 2000; Meerlo et al. 2004; Canas et al. 2009a). Confirming previous results, in a mice model of accelerated senescence with short life span and memory deficits, there is also a decrease in the levels of A_1R, an abnormal accumulation of Aβ, and later on, formation of amyloid plaques when compared with a resistant senescence mice strain (Castillo et al. 2009).

The activation of A_1R (with R-PIA) increased the levels of soluble APP in a dose-dependent manner, through a protein kinase C (PKC) signalling pathway, in a neuronal cell line (SH-SY5Y, Angulo et al. 2003). Moreover, in this cell line the activation of A_1R, via ERK-signalling pathway, leads to tau translocation, from cytosol to the cytoskeleton, increasing tau phosphorylation, which supports a possible role of A_1R in key AD events (Angulo et al. 2003). On the contrary, in mixed (glia and neurons) cultures of rat cerebellum, the antagonism of A_1R (with DPCPX) did not prevent $A\beta_{25–35}$-induced neurotoxicity (Mitchell et al. 2009). This finding was later confirmed by the chronic (60 days) administration of DPCPX in a TgAPP-PS1 model, in which no protective effect on memory impairment was granted by this A_1R antagonist; notoriously it was shown that DPCPX deteriorated memory performance in nontransgenic mice (Vollert et al. 2013). However, in an ex vivo model of cultured organotypic hippocampal slice of mice expressing pro-aggregant tau, the blockade of A_1R (by rolofylline) was able to restore synaptic function and morphology (Dennissen et al. 2016). This study also demonstrated that the chronic treatment by rolofylline of pro-aggregant tau transgenic mice prevented memory impairment evaluated by different behavioral tests (y-maze, novel object recognition, and fear conditioning), suggesting that the A_1R antagonism could be a potential therapeutic strategy for memory deficits and neurochemical alterations triggered by tau pathology (Dennissen et al. 2016). However, all the evaluation of the role of A_1R in AD should take into account the known opposite impact of acute and chronic manipulation of A_1R on brain function (see Jacobson et al. 1996) and the fact that there is a tight interaction between A_1R and $A_{2A}R$ (Lopes et al. 1999a; Ciruela et al. 2006).

Although, it was described that activation of A_3R is beneficial in ischemic brain injury (Chen et al. 2006) and retinal degeneration (Galvão et al. 2015); the role of this type of receptors in AD was not yet described. There is only a suggestion that A_3R might control APP internalization into lysosomes and consequently reduce the production of Aβ in neuronal cultures (Li et al. 2015b).

11.6.3 Facilitatory Adenosine Receptor: $A_{2A}R$ and $A_{2B}R$

Several studies reported an increase in $A_{2A}R$ levels and binding sites in the hippocampus and cortex of aged animals, evaluated by different techniques (Cunha et al. 1995; Lopes et al. 1999a, b; Cheng et al. 2000; Canas et al. 2009a). Moreover, cortical $A_{2A}R$ also undergoes a gain of function in aged animals, confirmed through different measures such as facilitation of synaptic transmission, increase of acetylcholine release, and increase in long-term potentiation in hippocampus of aged animals when compared with young adults (Lopes et al. 1999b; Rebola et al. 2003; Rodrigues et al. 2008; Costenla et al. 2011).

As previously mentioned, several studies reported that $A_{2A}R$ blockade mimicked the neuroprotective role of caffeine in different AD experimental models. In a sAD model ($A\beta_{1–42}$ icv administration), the blockade (by SCH58261) or genetic inactivation of $A_{2A}R$ was able to prevent memory impairment (evaluated by Y-maze and novel object recognition tests) and synaptotoxicity (decrease of SNAP-25 and synaptophysin synaptic levels) (Canas et al. 2009b). This study also demonstrated that the blockade of $A_{2A}R$ was able to prevent synaptotoxicity and subsequent neuronal loss, through a p38 mitogen-activated protein kinase (MAPK) signalling rather than by controlling cAMP/protein kinase A in hippocampal cultured neurons (Canas et al. 2009b). Moreover, this neuroprotective effect of $A_{2A}R$ blockade may be explained by predominant localization of $A_{2A}R$ at the synapse (Rebola et al. 2005), since in a synaptosomal preparation, the blockade of $A_{2A}R$ was able to prevent mitochondrial dysfunction triggered by $A\beta_{1–42}$ (Canas et al. 2009b). $A_{2A}R$ blockade also prevented or reverted memory deficits associated with other chronic brain diseases where synapse deterioration is present, such as diabetic neuropathy (Duarte et al. 2012), convulsions in early life (Cognato et al. 2010) or chronic stress (Kaster et al. 2015). In contrast, this neuroprotective effect afforded by $A_{2A}R$ blockade does not occur in other pharmacological models which comprise an acute deterioration of memory and that do not involve synaptotoxicity, such as in rats administrated with scopolamine (antagonist of muscarinic receptors) or MK-801 (antagonist of glutamate receptor) (Cunha et al. 2008).

The $A_{2A}R$ seems also to be involved in the production of Aβ, since the activation of these receptors by HENECA enhanced the activity of γ-secretase and, subsequent, $A\beta_{1–42}$ formation in a human neuroblastoma cell line (Nagpure and Bian 2014). In addition, Lu et al. (2016) demonstrated that $A_{2A}R$ controls Aβ levels by a mechanism that involves a physical interaction of $A_{2A}R$ with γ-secretase, in particular with the catalytic subunit PS1. The $A_{2A}R$ activation, through G_s protein and subsequent cAMP/PKA signal pathway, could cause Aβ overproduction by increasing APP levels and γ-secretase activity; in contrast, the blockade of $A_{2A}R$ decreased the interaction of $A_{2A}R$ with PS1, which promotes in Aβ production (Lu et al. 2016). There are also evidences that $A_{2A}R$ could have a role in tau pathology, similar to that described in models based on Aβ-induced pathology. Laurent et al. (2016) developed a tau pathology animal model with a genetic deletion of $A_{2A}R$, by crossing $A_{2A}R$ global KO mice with THY-Tau22 mice, where they showed that the genetic silencing of $A_{2A}R$ prevented spatial memory impairment, the decrease of hippocampal long-term depression (LTD), the imbalance of glutamate and GABA, and also attenuated neuroinflammation and tau hyperphosphorylation. This study also showed that an antagonist of $A_{2A}R$ (MSX-3, oral administration) was able to improve memory and reduced tau phosphorylation in THY-Tau22 mice (Laurent et al. 2016). Another important link between $A_{2A}R$ and memory deterioration was provided by the conclusion that $A_{2A}R$ controls the expression and function of glucocorticoid signalling in the hippocampus (Batalha et al. 2016), which has a strong impact on memory performance (Lupien et al. 1999) and is disrupted in AD (Popp et al. 2015).

So far, the question arises if the prevention afforded by $A_{2A}R$ in AD animal models is a general effect or is cell or brain region-specific. Viana da Silva et al. (2016) demonstrated that there is an increase of $A_{2A}R$ in synaptic membranes of CA3 hippocampal region of TgAPP-PS1 mice model (6 months old). Moreover, the blockade

of $A_{2A}R$ (SCH58261 or ZM241385) or the neuronal genetic silencing of these receptors in these transgenic AD mice prevented the suppression of long-term potentiation in hippocampal CA3 pyramidal cells and ameliorated memory impairment (Viana da Silva et al. 2016). This study and other studies of our group strongly suggest that in early phases of AD, the synaptic loss and dysfunction could be prevented by $A_{2A}R$ blockade/silencing in neurons (Canas et al. 2009b; Viana da Silva et al. 2016). Furthermore, Li et al. (2015a) also validated the important role of neuronal $A_{2A}R$ in memory, since the optogenetic activation of neuronal $A_{2A}R$ intracellular signalling in hippocampus is sufficient to impair memory, through the control of CREB phosphorylation and long-term potentiation (LTP), which are crucial events involved in memory process (Li et al. 2015a). This role of $A_{2A}R$ in memory was also confirmed by the activation of $A_{2A}R$, achieved by icv administration of CGS21680, which led to memory impairment (Pagnussat et al. 2015).

Remarkably, astrocytic $A_{2A}R$ may also have a role in AD pathology. Matos et al. (2012) observed that the activation of $A_{2A}R$ triggers astrogliosis in cultured astrocytes similar to $A\beta_{1–42}$, an effect prevented by the blockade of $A_{2A}R$. This study showed that $A\beta_{1–42}$ increased the $A_{2A}R$ in astrocytes and caused astrocytic dysfunction, decreasing the glutamate uptake capacity and the levels of glutamate transporters, GLAST, and GLT-1, and these alterations were prevented by the genetic silencing or pharmacological blockade of $A_{2A}R$ (Matos et al. 2012). Moreover, TgAPP-PS1 mice also display increased levels of astrocytic $A_{2A}R$, and the conditional genetic silencing of astrocytic $A_{2A}R$ increases memory performance in this transgenic model (Orr et al. 2015). In another study performed by the same group, the authors also showed an increase in astrocytic $A_{2A}R$ in a transgenic AD mice models (with several familial mutations in human APP) that exhibit amyloid plaques; however, this upregulation of $A_{2A}R$ was not observed in animals that overexpress human APP (wild-type) but never form amyloid plaques (Orr et al. 2017). In these animal models the antagonist of $A_{2A}R$, KW-6002, at low doses (4–10 mg/kg per day) was able to prevent memory deficits (Orr et al. 2017). These studies point to a role of astrocytic $A_{2A}R$ in a late phase of AD, when there is already amyloid plaques.

So far, there is scarce information about the role of $A_{2A}R$ in microglia. In transgenic mice of AD, 5 × FAD mice, with increased number of microglia, it was observed that blockade of $A_{2A}R$ with preladenant decreased the hypermobility of microglia associated with amyloid plaques in hippocampal slices but did not reestablish microglia motility toward tissue damage (Gyoneva et al. 2016). In addition, in an animal model of neuroinflammation, triggered by lipopolysaccharide (LPS) administration, the blockade of $A_{2A}R$ with SCH58261 prevented the decrease of hippocampal LTP, as well the recruitment of activated microglia cells and the overproduction in interleukin-1β (Rebola et al. 2011). Altogether, these findings suggest an involvement of microglial $A_{2A}R$ in controlling memory impairment and pathology of neurodegenerative diseases. Nevertheless, more studies are necessary to understand the role of microglia $A_{2A}R$ in AD.

When designing a therapeutic strategy, it should be taken into account that adenosine receptors (A_1R and $A_{2A}R$) can control the permeability of the blood-brain barrier (BBB), which defines the accessibility of molecules to the brain (Carman et al. 2011). The activation of A_1R or $A_{2A}R$ increased BBB permeability, and this information was later confirmed by the opposite effect displayed by A_1R and $A_{2A}R$

KO mice (Carman et al. 2011). Moreover, administration of NECA (an agonist of adenosine receptors) in Tg-AD animal model (double mutation in APP/PSEN1) allowed the entry of anti-Aβ antibodies and posterior label of β-amyloid plaques (Carman et al. 2011).

In the literature there is limited information about the role of $A_{2B}R$ in AD experimental models, probably due to the lack of good selective drugs for $A_{2B}R$. However, it is worth noting that $A_{2B}R$ controls glucose uptake and availability in astrocytes (Magistretti et al. 1986; Allaman et al. 2003; Lemos et al. 2015), an observation of particular relevance in view of the characteristic cortical hypometabolism used to diagnose AD (Chen and Zhong 2013).

11.7 Conclusions

AD pathology begins several years before clinical symptoms. The complex and multifactorial nature of this pathology implies the use of multimodal interventions to manipulate possible initiators and risk factors of disease. Lifestyle modifications, mainly the mental and physical exercise and the practice of a Mediterranean diet, have been shown to be beneficial for AD, in particular the consumption of coffee, tea, and chocolate that contain caffeine, which affords a general benefit to bolster the quality of life on aging (Freedman et al. 2012). Numerous studies showed that caffeine, the most widely consumed psychoactive drug worldwide, and its metabolites, by acting through adenosine receptors, are a cognitive normalizer and also restrain Aβ overproduction. In fact, alterations of the setup of adenosine receptors, mainly of $A_{2A}R$, can be viewed as a potential biomarker and a target of therapeutic interventions for cognitive decline and AD (Fig. 11.1). Nevertheless, the role of

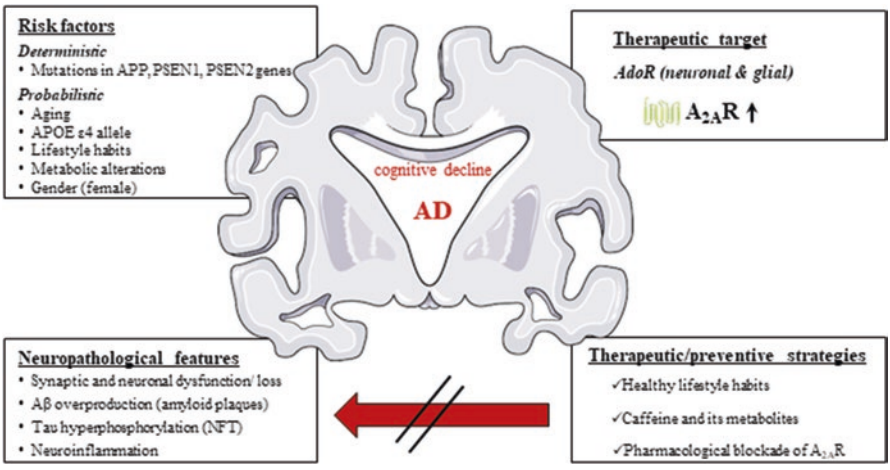

Fig. 11.1 Adenosine receptors might be a key target to prevent, slow and treat AD. The multifactorial nature of AD implies the use of multiple interventions to manipulate possible initiators and risk factors of disease. The manipulation (genetic or pharmacological) of adenosine receptors (AdoR), in particular $A_{2A}R$ that has been reported to be upregulated in AD, can be can be viewed as a potential preventive and therapeutic strategy for cognitive decline and AD

adenosine receptors in early phases of AD and in brain regions affected in AD remains to be further clarified. Although the research in experimental models regarding the impact of adenosine receptors on AD have been an added value, it is mandatory to hustle the translation of basic scientific findings into potential treatments for this disease.

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Chapter 12
What Is the Role of Adenosine Tone and Adenosine Receptors in Huntington's Disease?

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Abstract Huntington's disease (HD) is a devastating hereditary neurodegenerative disorder caused by a CAG mutation within the IT15 gene encoding huntingtin protein. Even though mutant and normal huntingtin are ubiquitously expressed, the degenerative processes primarily occur within the striatum and particularly hit the striatopallidal neurons, particularly enriched with adenosine A_{2A} receptors ($A_{2A}R$), suggesting that the latter might play a role in HD. In agreement, variants in the *ADORA2A* gene influence the age at onset in HD, and $A_{2A}R$ dynamics is largely altered by mutated huntingtin. More generally, adenosine tone and adenosine receptors are involved in a number of processes critical for neuronal function and homeostasis, such as the modulation of synaptic activity and excitotoxicity, the control of neurotrophin levels and functions, and the regulation of protein degradation mechanisms. In the present review, we critically reviewed the current knowledge involving alterations of adenosine tone and adenosine receptors in HD and discussed whether they represent suitable therapeutic targets.

Keywords Adenosine receptors · Huntington's disease · Neurotransmission · A_{2A} heteromers

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12.1 Pathogenetic Mechanisms of Huntington's Disease

12.1.1 Overview

Huntington's disease (HD) is a monogenic autosomal dominantly inherited neurodegenerative disorder generally affecting young adults and characterized by involuntary abnormal movements and postures (chorea, dyskinesia, dystonia), psychiatric disturbances, and cognitive alterations (for review, see Ross and Tabrizi 2011; McColgana and Tabrizi 2018). Prevalence is 4–10/100.000. This disorder is fatal within 15–20 years after onset of symptoms. Although several cerebral regions (cerebral cortex, layers III, V, and VI; pallidum, subthalamic nucleus, cerebellum) show signs of neurodegeneration, primary and prominent neuronal loss is found in the caudate and putamen (Vonsattel and DiFiglia 1998). Within the striatum, the striatopallidal neurons, a subpopulation of medium spiny neurons (MSNs, see 2.1.) that express enkephalin, dopamine D_2 receptors (D2Rs), and adenosine A_{2A} receptors (A_{2A}Rs) (Canals et al. 2003), appear more vulnerable (see Reiner et al. 1998; Schiffmann and Vanderhaeghen 1993).

HD is caused by a mutation in the gene IT15 encoding the protein huntingtin (Htt; The Huntington's Disease Collaborative Research Group 1993). The mutation consists in a CAG triplet repeat expansion translated into an abnormal polyglutamine (polyQ) tract within the N-terminal region of the protein. Penetrance is full, with a CAG length above 40 repeats and longer CAG repeats associated with earlier onset. It is important to stress that although the number of CAG repetitions is the primary determinant of disease onset, it accounts for only ~60% of the variation in age of onset (see Walker 2007 for review). This supports that other genetic and environmental factors are to take into account as disease modifier components.

Htt is a ubiquitous and large protein of about 350 kD involved in an important number of cellular functions (for reviews, see Bantubungi and Blum 2007a, b; Popoli et al. 2008; Ross and Tabrizi 2011; Zuccato et al. 2010). In the central nervous system, Htt has a large distribution, being prominent in neurons, particularly cortical pyramidal neurons, Purkinje cells, and striatal interneurons (Gourfinkel-An et al. 1997; Trottier et al. 1995), as well as glial cells (Shin et al. 2005). The cellular functions of Htt remain incompletely understood (see Ross and Tabrizi 2011; Saudou and Humbert 2016). Among others, physiological Htt function is involved in early embryonic development (Dragatsis et al. 1998), fate of cortical progenitors (Godin et al. 2010; Barnat et al. 2017), axonal transport (Colin et al. 2008; Gauthier et al. 2004), and brain-derived neurotrophic factor (BDNF) expression/transport (Zuccato and Cattaneo 2009). Mutated Htt (mHtt) is thus prone to impair several mechanisms important for neuronal activity and survival by promoting a toxic-gain-of-function but also impairing the normal Htt function through loss-of-function mechanisms (Zuccato et al. 2010). It is not clear yet to which extent HD can be considered as a prion-like disorder, like Alzheimer's or Parkinson's diseases, but transcellular propagation of protein aggregation could underlie the pathological progression in HD (Stopschinski and Diamond 2017). Experimental evidence

suggests that mHtt triggers misconformation of wild-type Htt. Neuropathological observation in patients who received intracerebral allografts supports the transfer of HD pathology from cell to cell (Stopschinski and Diamond 2017). In the next two paragraphs, we will specifically address pathways impaired by mHtt and prone to be modulated by adenosine receptors, namely, excitotoxicity as well as glial and BDNF functions.

12.1.2 Excitotoxicity and Mitochondrial Dysfunctions

Excitotoxicity is primarily defined as cell death ensuing from the toxic action of glutamate through excessive activation of glutamate receptors. Despite it remains unclear whether glutamate is over-released or not by cortical afferents in HD, there is clear evidence indicating that dysfunctions of the glutamatergic system in the striatum account for the toxic effect of mHtt (Fan and Raymond 2007; Stack et al. 2007). Several neuronal impairments contribute to excitotoxicity, such as deficient glial reuptake of glutamate and/or NMDA receptor hypersensitivity, the latter being itself dependent on mitochondrial function (Brouillet et al. 2005; Fan and Raymond 2007; Jacquard et al. 2006). Interestingly, several previous studies involved abnormal NMDA receptor arrangement/activity in HD. R6/2 transgenic HD mice exhibit a reduced NR2A/NR2B ratio, which is an index of vulnerability to excitotoxic cell death (Ferrante et al. 2010; Martire et al. 2010), and an enhanced response to NMDA (Cepeda et al. 2001). Intraneuronally, mHtt increases the activity of the NR2B subunit of the NMDA receptor – preferentially expressed by the medium spiny neurons within the striatum – possibly through a dysfunction of PSD95, a docking protein whose interaction with NR2B is reduced in the presence of the mutated protein (Chen et al. 1999; Sun et al. 2001; Zeron et al. 2002). These abnormal interactions lead to increased NMDA currents itself accompanied by an altered NMDA receptor trafficking (Fan and Raymond 2007) and neuronal vulnerability to NMDA (Shehadeh et al. 2006; Zeron et al. 2002). Recent data particularly support that the latter is related to an increased expression of extrasynaptic NR2B-contaning NMDA receptors (Milnerwood et al. 2010).

In HD, increased NMDA response is probably favored by environmental modulation of NMDA receptor. Indeed, it has been demonstrated there is an early endogenous increase of quinolinic acid (QA) in the striatum from HD patients and animal models (Guidetti et al. 2006, 2004). QA is an NMDA agonist, derived from the kynurenine pathway, a major route of tryptophan degradation, able to produce lesions reminiscent of HD in animals (Beal et al. 1986) and favoring glutamate release from corticostriatal endings (Blum et al. 2003a; Popoli et al. 2002). In addition, mHtt has been shown to impair expression of glutamate transporters and glutamate handling by astrocytes (Bradford et al. 2009; Faideau et al. 2010; Lievens et al. 2001; Shin et al. 2005), favoring its increase in the synaptic cleft.

Excitotoxicity is favored by mitochondrial alterations underlying HD. Several imaging studies have revealed an early metabolic dysfunction in the striatum of HD

patients (Brouillet et al. 1999, 2005; Liot et al. 2017). Importantly, the severity of metabolic alterations correlates with the size of the CAG expansion (Jenkins et al. 1998). Several postmortem studies point to a significant reduction in the activity of complexes II–III (which includes succinate dehydrogenase) in the caudate nucleus of HD patients (Browne et al. 1997; Gu et al. 1996; Tabrizi et al. 1999). Such alterations could be related to an altered expression of the complex II subunits induced by mHtt (Benchoua et al. 2006) and be favored by dopamine (Benchoua et al. 2008). The instrumental role of complex II inhibition in the striatal degeneration in HD is also suggested by the specific profile of degeneration in animals treated by the irreversible complex II inhibitor 3-nitropropionic acid (3NP) (Brouillet et al. 2005). Accordingly, several other works have reported strong mitochondrial alterations promoted by mHtt. Indeed, the latter, found localized in the neuronal mitochondrial membrane (Panov et al. 2002), has been shown to impair mitochondrial biogenesis, fission (Kim et al. 2010; Weydt et al. 2006), axonal transport (Shirendeb et al. 2011), calcium handling, and membrane potential (Panov et al. 2002) as well as ATP production (Milakovic and Johnson 2005; Seong et al. 2005) and calcium handling (Choo et al. 2004; Panov et al. 2002). With regard to mitochondria impairments, mitophagy has been involved in HD (Liot et al. 2017). In line with the above hypothesis, prevention of mitochondrial fission and cristae remodeling has been shown to delay HD progression (Costa et al. 2010; Guo et al. 2013). Such mitochondrial defects represent one of the events contributing to the emergence of neuronal excitotoxicity in HD (Brouillet et al. 2005; Jacquard et al. 2006). Therefore, rescue of impaired mitochondria (Lee and Chern 2014) and poor energy homeostasis might represent a valuable therapeutic approach to HD (Guo et al. 2013; Ju et al. 2011; Lin et al. 2013).

12.1.3 BDNF

BDNF is an abundant neurotrophin in the mammalian brain involved in a variety of brain processes as development, differentiation, neuronal plasticity, or synaptic activity (Chao 2003). In the striatum, BDNF essentially comes from the cerebral cortex, anterogradely transported to cortical nerve endings to be released in the striatum (Zuccato and Cattaneo 2007). In HD, mHtt alters BDNF transcription (Zuccato et al. 2001), trafficking, and axonal transport (Gauthier et al. 2004). It has been established that mHtt perturbates the negative modulation exerted by wild-type Htt on the silencing activity of the RE1/NRSE silencer, favoring the downregulation of a set of genes, including the one coding BDNF (Zuccato et al. 2003). The mHtt also alters the axonal transport of BDNF vesicles (Dompierre et al. 2007) as well as the post-Golgi trafficking of this factor (del Toro et al. 2006). More recently, alteration of BDNF transport in HD has been suggested to involve abnormal interaction between pro-BDNF and Htt-associated protein 1 (Wu et al. 2010). Importantly, loss of striatal BDNF may preferentially affect the function of the striatopallidal neurons, known to be early impaired in HD. These latter observations support that

BDNF impairment is crucially involved in the early vulnerability of striatopallidal neurons. In accordance with such important role of BDNF in HD, its increase, by gene overexpression and pharmacological or environmental modulation, has been shown to be beneficial in several experimental models of HD (Borrell-Pages et al. 2006; Gharami et al. 2008; Giralt et al. 2010; Lynch et al. 2007; Peng et al. 2008; Simmons et al. 2009; Xie et al. 2010).

12.1.4 Two Major Protein Degradation Systems: Proteasome and Autophagy

In HD, the expansion of polyglutamine (polyQ) in the N-terminal region of Htt results in protein misfolding and aggregation (Goldberg 2003; Gusella and MacDonald 2006; Kopito 2000). The ubiquitin-proteasome system (UPS) plays an important role in the degradation of damaged or misfolded proteins via polyubiquitination targeted by E3 ligases (Demartino and Gillette 2007; Hershko and Ciechanover 1998). Global changes in the ubiquitin system, an indicator of the UPS function, were found in HD patients and in HD animal models (Bennett et al. 2007; Finkbeiner and Mitra 2008; Ortega and Lucas 2014). Suppression of the UPS function by mHtt has been demonstrated in the cells and brains of mice and humans with HD (Seo et al. 2004; Wang et al. 2008; Zheng et al. 2016). Enhancement of UPS activity, which facilitates the degradation of soluble mHtt at its pathological stage, has been shown to improve proteasome function and motor coordination in HD (Jeon et al. 2016; Jia et al. 2012; Kim and Seo 2014; Lin et al. 2013; Liu et al. 2014; Seo et al. 2007; Wong et al. 2008). Macroautophagy, hereafter referred to as autophagy, is also essential for the removal of aggregated proteins by delivering them to the lysosome for degradation (Nixon 2013). Htt has been found to function as an important regulator and substrate for selective autophagy (Gelman et al. 2015; Rui et al. 2015). Impairments of the autophagic process are associated with HD: in fact, a damaged ability of autophagic vacuoles to recognize cytosolic cargo has been demonstrated (Kiriyama and Nochi 2015; Martinez-Vicente et al. 2010). The resultant inferior activity of autophagy causes slower turnover and accumulation of mHtt. In support of the hypothesis that the clearance of mHtt is important, upregulation of autophagy produces beneficial effects (Jia et al. 2012; Koga et al. 2011; Martin et al. 2015; Sarkar et al. 2007; Williams et al. 2008).

12.1.5 Nonneuronal (Glial) and Peripheral Cells

mHtt is found in neurons and glial cells in the brains of HD (Hsiao and Chern 2010; Lee et al. 2013a, b; Shin et al. 2005; Yu et al. 2003). Although neuronal cells are preferentially damaged in HD, expression of mHtt in astrocytes and other glial cells causes age-dependent neurological symptoms and contributes to neuronal

excitotoxicity (Bradford et al. 2009; Crotti et al. 2014; Huang et al. 2015; Shin et al. 2005). mHtt in astrocytes clearly contributes to HD pathogenesis (Bradford et al. 2009, 2010; Chou et al. 2008; Hsiao et al. 2013). Specifically, mHtt alters several major astrocytic functions as follows: impaired glycolysis (Powers et al. 2007), lower expression of EAAT2 (GLT-1) that causes lower glutamate uptake (Chen et al. 2012; Shin et al. 2005), greater glutamate synthesis (Lee et al. 2013a, b), inferior GABA release (Wojtowicz et al. 2013), insufficient production and release of trophic factors (Chou et al. 2008; Wang et al. 2012), decreased expression of Kir4.1 potassium channel that eventually leads to neuronal excitotoxicity (Tong et al. 2014), dysfunctional calcium and glutamate signaling (Jiang et al. 2016), and higher inflammatory responses (Hsiao et al. 2013, 2014, 2015). Similar to the mechanism of other neurodegenerative diseases, microglia also play a critical role in HD pathogenesis. Abnormal functions of microglia have been implicated in overactivation of inflammatory response (Crotti et al. 2014; Hsiao et al. 2013). A recent study indicates that mHtt in glia can impart disease phenotype to normal mice, while normal glia can ameliorate disease phenotype in transgenic HD mice. This study suggests a causal role for glia in HD (Benraiss et al. 2016). mHtt is also expressed in peripheral cells and altered normal physiology. Specifically, mHtt is expressed in hepatocytes, suppresses the urea cycle activity, and causes high blood ammonia (Chiang et al. 2009; Chiu et al. 1975). The immune system is another important peripheral organ that expresses mHtt. It has been noted that enhanced immune activation in HD mice and patients could be detected in the early stage of HD. HD patients have elevated inflammatory cytokines and chemokines levels in plasma (Bjorkqvist et al. 2008). It has been proposed that mHtt levels of monocytes and T cells were significantly associated with disease progression in HD patients. The expression level of mHtt in immune cells might be used as a noninvasive disease biomarker (Weiss et al. 2012).

12.2 Dysfunction of Striatal Adenosine Receptors in HD

12.2.1 Striatal Adenosine Neurotransmission

Adenosine plays a fundamental role in the modulation of dopaminergic and glutamatergic neurotransmission in the striatum. Dopaminergic and glutamatergic afferents constitute the main extrinsic striatal inputs, which converge in the dendritic spines of the MSNs, the predominant striatal neuronal population (Gerfen 2004). Glutamatergic terminals make a tight synaptic contact with the head of the dendritic spines and astrocyte processes wrap the glutamatergic synapse, constituting the well-established tripartite synapse (Araque et al. 1999). On the other hand, dopaminergic terminals make a loose synaptic contact with the neck of the dendritic spine and allow volume transmission of dopamine to influence dopamine receptors located at the vicinity of the synapse (Rice et al. 2011). The dendritic spines, with their contacting glutamatergic and dopaminergic terminals and astrocyte process,

have been labelled as "striatal spine module" (Ferré et al. 2007), with "local module" being defined as an integrative functional unit of the central nervous system, a minimal portion of one or more neurons and/or one or more glial cells that operate as an independent integrative unit (Ferré et al. 2007). Within the striatal spine module, under normal conditions, extracellular adenosine originates predominantly from ATP released by a vesicular process from the astrocyte and rapidly converted to adenosine by ectonucleotidases (Pascual et al. 2005; Cunha 2016). The effects of extracellular adenosine are mediated by adenosine receptors, mostly adenosine A_1R (A_1Rs) and $A_{2A}Rs$, localized in the different elements of the striatal spine module. Both are G protein-coupled receptors, with A_1R and $A_{2A}R$ coupling to inhibitory Gi/o and excitatory Gs/olf proteins, respectively. Both receptors are co-localized in the glutamatergic terminals and astrocytes, where they form A_1R-$A_{2A}R$ heteromers (Ciruela et al. 2006). In the glutamatergic terminal, the A_1R-$A_{2A}R$ heteromers act as a "concentration-dependent switch" (Ciruela et al. 2006). The activation of A_1R and $A_{2A}R$ receptors by adenosine inhibits and stimulates glutamate release, respectively. Adenosine has more affinity for A_1R than $A_{2A}R$ receptors, and under basal conditions it tonically influences only presynaptic A_1Rs. Thus, gene-targeted vesicular release of astrocytic ATP leads to a loss of A_1R-mediated tonic inhibition of presynaptic hippocampal glutamatergic transmission (Pascual et al. 2005). Under physiological conditions, presynaptic $A_{2A}Rs$ are only activated by phasic increases of extracellular adenosine, which normally occurs upon strong glutamatergic input (which is associated to neuronal and glial co-release of ATP and its conversion to adenosine by 5-nucleotidases; Cunha 2016). Under these conditions, activation of $A_{2A}Rs$ negatively modulates A_1R signaling in the heteromer and, conversely, promotes glutamate release (Popoli et al. 1995; Solinas et al. 2002; Borycz et al. 2007; Quiroz et al. 2009, 2016). The same mechanism has also been described in cultured cortical astrocytes, where A_1R-$A_{2A}R$ heteromers modulate GABA uptake (Cristovao-Ferreira et al. 2013). A_1R, but not $A_{2A}R$, is also found in the dopaminergic terminals where it also exerts a tonic inhibitory modulation of dopamine release (Borycz et al. 2007). Finally, A_1R and $A_{2A}R$ are highly expressed postsynaptically, in the dendritic spines and in the rest of the somatodendritic region of the MSNs. Significantly, however, they are not co-localized but are segregated in the two phenotypically different striatal MSNs.

As stated above, two subtypes of MSNs give rise to the two striatal efferent pathways that connect the striatum with the output structures of the basal ganglia, which are the medial segment of the globus pallidus and the substantia nigra pars reticulata (Gerfen 2004). The striatonigral neurons constitutes the direct pathway, since it directly connects the striatum with the output structures and selectively expresses A_1R and D1R and also D3R in the ventral striatum (Ferré et al. 1997; Ferre et al. 1996; Sokoloff and Le Foll 2017). The striatopallidal neurons connects the striatum with the lateral segment of the globus pallidus and the ventral pallidum and selectively expresses $A_{2A}R$ and D2R (Ferré et al. 1993, 1997). A_1R and D1R and $A_{2A}R$ and D2R form specific receptor complexes, the A_1R-D1R and $A_{2A}R$-D2R heteromers (Ferré et al. 1997, 2016; Ginés et al. 2000; Hillion et al. 2002;

Canals et al. 2003), which act as molecular devices by which endogenous adenosine, by acting on the respective adenosine receptor, tonically inhibits the affinity and signaling of the respective dopamine receptor. Thus, differently from the striatal presynaptic $A_{2A}R$, under physiological conditions, postsynaptic $A_{2A}R$ is tonically activated by endogenous adenosine, as demonstrated by the significant behavioral and biochemical effects secondary to its blockade after the administration of $A_{2A}R$ antagonists (see below).

12.2.2 Adenosine Receptor Single Nucleotide Polymorphisms and Caffeine Intake

Considering the preferential vulnerability of the striatopallidal neurons in HD (Glass et al. 2000; Deng et al. 2004), not surprisingly, both D2R and $A_{2A}R$ were reported to be significantly and differentially downregulated, as compared to D1R, in early pathological stages of HD, but also in symptomatic patients with Vonsattel's pathological grade 0 (Glass et al. 2000), indicative of significant selective functional alterations of this MSN subpopulation. Downregulation of $A_{2A}R$ has also been reported in most studies using different HD mice, and several studies have provided possible molecular mechanisms (see below). On the other hand, although there is consensus about the decreased expression of $A_{2A}R$ in HD, as elaborated below, several studies imply the existence of an aberrant $A_{2A}R$ signaling, amplification, induced by mHtt in HD mice. The question is if those changes in $A_{2A}R$ expression and function are just markers of the selective degeneration of the indirect MSN or if they are involved in the pathogenetic process. Genetic studies would initially seem to reinforce the latter possibility, as a single nucleotide polymorphism (SNP) in *ADORA2A*, rs5751876 (C > T substitution in exon 5), has been associated to an earlier age at onset (AAO) of the disease (Dhaenens et al. 2009; Taherzadeh-Fard et al. 2010). Although rs5751876 constitutes a synonymous mutation (it does not change the encoded amino acid), it is linked by nearly complete linkage disequilibrium to other SNPs that could potentially modify $A_{2A}R$ transcription. Those include rs35320474, a T deletion in the 3′ untranslated region that includes U-rich motifs (which provide active sites of interaction with RNA-binding proteins), and rs2298383, a C > T substitution in a potential promoter region with a regulatory element predicted from alignment of human and other mammalian genes (Alsene et al. 2003; Childs et al. 2008; Rogers et al. 2010; Shinohara et al. 2013). Interestingly, the recent study by Shinohara et al. (2013) demonstrated a significant increase in the expression of $A_{2A}R$ in the brain of subjects homozygous for a rs5751876 polymorphic block (including rs35320474 and rs2298383) suggesting that, indeed, transcriptional dysregulation of $A_{2A}R$ is associated with HD. How these data reconcile with previous postmortem binding and expression studies in postmortem human brain and mouse models remains to be elucidated.

Another epidemiological study linking adenosine receptors to HD is the association of habitual consumption of caffeine with earlier AAO of HD (Simonin et al. 2013). Although caffeine is a nonselective $A_1R/A_{2A}R$ antagonist, the authors suggested $A_{2A}R$ blockade as the most probable explanation for the apparent caffeine-mediated increased acceleration of neurodegeneration. This assumption was based on the preferential tolerance to the A_1R versus $A_{2A}R$ blocking effects with chronic caffeine exposure (Karcz-Kubicha et al. 2003) and on the experimental evidence that indicates that high doses of $A_{2A}R$ antagonists or global $A_{2A}R$ blockade worsen disease progression in HD models (Blum et al. 2003a, b; Mievis et al. 2011), while $A_{2A}R$ agonists produce beneficial effects (Chou et al. 2005). However, A_1R blockade was not discarded as alternative mechanism, and an A_1R agonist has also been shown to protect against neurodegeneration in a rat HD model (Blum et al. 2002). A way to reconcile some of these findings could be the recently described evidence of alterations of adenosine metabolism in animal models of HD, a striatal hypoadenosinergic tone (see below), which could be mimicked by chronic caffeine exposure. Interestingly, an association between the *ADORA2A* rs5751876 polymorphism and caffeine intake was reported by Cornelis et al. (2007), which could have established a possible connection between this polymorphism, caffeine intake, and HD progression. However, this association has not been confirmed in a recent genome-wide meta-analysis of polymorphisms and habitual coffee intake (Coffe and Caffeine Genetics Consortium et al. 2015).

12.2.3 Alterations of A_1R Function During HD Progression

The stimulation of A_1R exerts a clear neuroprotective effect in different conditions (Paul et al. 2011; von Lubitz et al. 1988), including HD models. Thus, A_1R activation has been demonstrated to attenuate limb dystonia and striatal degeneration in the 3NP model of HD (Blum et al. 2002). These findings are in line with other data showing that an A_1R agonist prevented 3NP-induced seizures in mice (Zuchora et al. 2001) and that A_1R blockade was deleterious in another metabolic model of HD induced by malonate (Alfinito et al. 2003). Although no changes of A_1R density were observed in an HD rat model (Tg51 HD rats; see below and Bauer et al. 2005), binding studies in frankly symptomatic R6/2 mice, a widely used transgenic model of HD, revealed a decrease in density but not antagonist affinity, of cortical and striatal A1Rs (Ferrante et al. 2014). Interestingly, however, despite the reduced density of A_1Rs, the same authors found an increased effect of the agonist CPA in reducing synaptic transmission and glutamate release in the striatum of R6/2 versus WT mice, a. The decrease density and increased functionality of A_1Rs were further confirmed in a striatal cell line expressing mHtt (Ferrante et al. 2014). These results are in line with a noninvasive PET imaging study in HD patients in which the level of A_1R was found significantly reduced with respect to non-HD subjects in the symptomatic stages of the disease (Matusch et al. 2014; see 2.5).

12.2.4 Alterations of $A_{2A}R$ During HD Progression

Downregulation of the $A_{2A}R$ has been consistently reported in patients and in animal models, even before the onset of motor dysfunctions (Glass et al. 2000), and in animal models that do not show neuronal loss (Cha et al. 1999; Ishiwata et al. 2002; Bauer et al. 2005; Mievis et al. 2011; Orrù et al. 2011). The first evidence of a downregulation of $A_{2A}R$ in HD was obtained by autoradiography in tissue sections of the human brain (Martinez-Mir et al. 1991) and was later confirmed in the basal ganglia of early, intermediate, and advanced grades of HD patients (Glass et al. 2000). A downregulation of $A_{2A}R$ at the protein and transcript levels has been also found in most of the animal and cell models of HD (with the exception of H46, YAC72, and Tg51 transgenic models; Cha et al. 1999; Chan et al. 2002; Chou et al. 2005; Chiang et al. 2005; Tarditi et al. 2006; Villar-Menendez et al. 2013; Guitart et al. 2016), and these models have been fundamental for the identification of the molecular mechanisms through which a mHtt results in reduction of $A_{2A}R$ expression. It is well documented that aggregated mHtt causes aberrant protein-protein interactions with several transcription factors, which result in changes in gene expression profiles (Steffan et al. 2000; Nucifora et al. 2001; Dunah et al. 2002; Li et al. 2002). These changes appear to be specific since no changes in the expression of several important genes (cytoskeleton proteins, enzymes of metabolism, mitochondrial proteins, caspases, and others) have been reported. As for $A_{2A}R$, Chiang and collaborators (2005a, b) found that expression of mHtt significantly reduces the transcript levels of the endogenous $A_{2A}R$ in PC12 cells and striatal neurons in culture. They identified an atypical CRE site located in the core promoter of the $A_{2A}R$ gene that mediates the suppression of the $A_{2A}R$ gene by mHtt, by preventing CREB binding (Chiang et al. 2005). Interestingly, stimulation of the $A_{2A}R$ restored the reduced CREB binding caused by the mutation and reduced mHtt aggregation. The length of poly(Q)-expanded Htt seems to be critical for the downregulation of $A_{2A}R$ transcript: in HD models that express an extended N-terminal fragment or a full-length mHtt as in HD46 and YAC72 mice, respectively (Chan et al. 2002), a reduction in the expression of $A_{2A}R$ and of other mHtt-sensitive genes has not been found, and it is hypothesized that transcriptional dysfunctions only occur in the presence of a short N-terminal fragment (<171 amino acids) of mHtt.

Interestingly, DNA methylation has been proposed as a key mechanism for the reduced striatal $A_{2A}R$ levels observed in the brain from HD patients and from R6/1 and R6/2 mice (Villar-Menendez et al. 2013; Mangiarini et al. 1996; Vonsattel 2008). DNA methylation (5-methylcytosine, 5mC, and 5-hydroxymethylcytosine, 5hmC) is an important mechanism for epigenetic silencing, and it has been demonstrated to regulate basal $A_{2A}R$ level in the human brain (Buira et al. 2010). In their study, Villar-Menendez and collaborators (2013) found an increase in 5mC levels and a reduction in 5hmC levels in the 5′ untranslated region (5'UTR) of $A_{2A}R$ gene, and these findings were closely associated with the downregulation of the $A_{2A}R$ transcript in R6/2 mice and in the putamen of HD patients. This finding appears to be particularly interesting since it could open new approaches to treat HD by modulating $A_{2A}Rs$.

While the expression of the $A_{2A}R$ has been demonstrated to be reduced in the presence of mHtt (although with some exceptions), an amplification of its signaling has also been reported during the progression of HD. Data from Varani et al. (2001) reported an aberrant amplification of $A_{2A}R$-mediated stimulation of adenylyl cyclase in striatal-derived cells engineered to express mHtt, a result confirmed in the striatum of R6/2 mice (Chou et al. 2005; Tarditi et al. 2006). The amplification of the $A_{2A}R$ signaling was also found in peripheral blood cells from HD subjects, where overstimulation of $A_{2A}R$-mediated cAMP production was associated with aberrant increase in $A_{2A}R$ function and density (Varani et al. 2007). Moreover, $A_{2A}R$ density in blood platelets has been found to correlate with age at onset and CAG repeat expansion in HD patients (Maglione et al. 2006). These findings suggested that $A_{2A}R$ in peripheral blood cells could be used as a biomarker for the prediction of HD prognosis and drug efficacy. However, despite an initial enthusiasm, further studies are needed to ultimately validate this receptor as a biomarker and used for the disease prognosis.

12.2.5 Positron Emission Tomography (PET) Imaging for Adenosine Receptor Occupancy in HD

Positron emission tomography (PET) allows in vivo imaging of regional receptor-binding capacity and, together with magnetic resonance, identifies minimal changes in brain activity, greatly helping in the comprehension of the natural history of several diseases, including HD (Roussakis and Piccini 2015). Different radiotracers have been used with PET to measure brain metabolism, dopaminergic function, neuroinflammation, phosphodiesterases, and other targets in HD (Roussakis and Piccini 2015). However, few adenosine analogue radiotracers have been developed and employed with PET in the noninvasive imaging of A_1R and $A_{2A}R$. The A_1R is ubiquitously expressed in the human brain and can be imaged in vivo with [18F] CPFPX-PET (Bauer et al. 2003; Holschbach et al. 2002; Meyer et al. 2007). A cross-sectional study using [18F]CPFPX-PET and MRI was performed to assess differences in A_1R density between controls and HD patients at different stages of the disease (premanifest patients far from predicted symptoms onset, premanifest patients near to predicted symptoms onset, and manifest patients; Matusch et al. 2014). In this study a 25% reduction in [18F]CPFPX binding in the caudate of manifest HD patients was found. Interestingly, in premanifest patients far from symptoms onset, [18F]CPFPX binding in the thalamus was 31% higher than in healthy controls, while in premanifest patients near to symptoms onset, thalamic [18F]CPFPX binding was similar to the levels in healthy controls, suggesting that A1R switch from upregulation to downregulation during HD progression. Thus, A_1Rs seem to be involved in the pathophysiology of HD, and [18F]CPFPX and PET can be considered useful tools to explore these receptors in preclinical and clinical trials (Matusch et al. 2014).

$A_{2A}R$ antagonist PET tracers have been developed and tested with PET imaging. However, xanthine ligands, including [11C]TMSX, [11C]KF17837, [11C]TMSX, [11C]KF21213, [11C]KF19631, and [11C]KW6002, proved to be not very suitable for molecular imaging mainly because of low signal to noise ratio and high degree of non-specific binding (Khanapur et al. 2014). [11C]SCH442416 was the first non-xanthine ligand being suitable for mapping of $A_{2A}R$ using PET (Moresco et al. 2005). In general, radioligands that lack the xanthine structure appear to offer better specificity for the $A_{2A}R$ subtype and allow quantitative imaging of $A_{2A}R$ in the mammalian striatum but not in other areas of the brain (for an updated review, see van Waarde et al. 2018). Recently, [11C]preladenant has been demonstrated to be a suitable PET tracer for the quantification of $A_{2A}R$ binding sites in the rat brain. The tracer displayed high uptake in striatum and low and homogenous uptake in all extra-striatal regions, and the regional distribution of [11C]preladenant is in agreement with the known $A_{2A}R$ expression in the rat brain (Zhou et al. 2017a). The suitability [11C]preladenant for imaging of $A_{2A}Rs$ in the brain has been confirmed in monkey and human brains (Zhou et al. 2017b; Sakata et al. 2017). Very few studies have been conducted for $A_{2A}R$ in PET images and in particular in HD. In a rat model of HD (intrastriatal injection of quinolinic acid resulting in loss of striatopallidal GABAergic enkephalin neurons), the binding potential of [11C]TMSX in the striatum and globus pallidus was reduced by 25%, similar to the loss of D2R ([11C] raclopride) (Ishiwata et al. 2002). Hopefully, the availability of new and more suitable radiotracers will prompt PET studies of $A_{2A}R$ in HD.

12.2.6 Alterations in Striatal Adenosine Tone in HD

In a recent study on the Tg51 transgenic rat model of HD (von Hörsten et al. 2003), we found a clue for an alteration of the adenosinergic system independent of alterations in $A_{2A}R$ expression (Guitart et al. 2016). Tg51 rats offer a model with a slower neurodegenerative progression as compared to other animal models, which in principle allow an easier evaluation of possible biomarkers during initial stages of HD (von Hörsten et al. 2003). Using methods of analysis of the function of striatal pre- and postsynaptic $A_{2A}R$, it was initially suggested that Tg51 rats had a selective functional impairment of striatal postsynaptic $A_{2A}R$ during early pathological stages (Orrú et al. 2011). This was based on the observation of a complete loss of locomotor-activating effects of $A_{2A}R$ antagonists, without changing their efficacy at modulating presynaptic corticostriatal neurotransmission (Orrú et al. 2011). It was then assumed that the most probable mechanism was the previously demonstrated down-regulation of $A_{2A}R$ in both HD and HD animal models. However, a more extensive pharmacological characterization of the Tg51 indicated that postsynaptic striatal $A_{2A}R$ function was not altered after all. Thus, there was no difference in the locomotor depression induced by an $A_{2A}R$ agonist (which depends on the integrity of postsynaptic $A_{2A}R$) in Tg51 rats as compared to WT littermates (Guitart et al. 2016). More convincingly, radioligand-binding experiments showed no differences in the number of striatal $A_{2A}R$ antagonist binding sites or affinity between Tg51 (homo- or

heterozygous) and WT rats (Guitart et al. 2016). Altogether, the pharmacological results (effect with agonist and lack of effect of the antagonist) suggested a low adenosinergic tone, a decrease in the ability of endogenous adenosine to activate postsynaptic $A_{2A}R$. This would also explain the ability of $A_{2A}R$ antagonists to act presynaptically, blocking corticostriatal transmission (Orrú et al. 2011), which depends on phasic increases of extracellular adenosine (see above). In fact, we could demonstrate a significant reduction in the extracellular striatal concentration of adenosine both in Tg51 rats and in zQ175 knock-in mice (Guitart et al. 2016), a more recently obtained animal model of HD (Menalled et al. 2012). Nevertheless, differently from Tg51 rats, $A_{2A}R$ downregulation was also observed in zQ175 mice, with a hypoadenosinergic tone representing the common striatal alteration (Guitart et al. 2016).

The next step was, therefore, to find the alteration in the mechanisms that regulate the extracellular concentrations of adenosine. It is now well accepted that astroglial vesicular release of ATP is the main source of extracellular adenosine under physiological conditions (Pascual et al. 2005). Extracellular ATP is rapidly converted to adenosine by a series of ectonucleotidases; the extracellular levels of adenosine, the adenosinergic tone, is mostly maintained by the ability ofequilibrative transporters and astrocytic adenosine kinase (ADK) to respectively uptake and metabolize adenosine (Boison et al. 2010; Cunha 2016). In mammals, there are two types of nucleoside transporters, equilibrative and concentrative, which mediate a bidirectional equilibrative transport driven by chemical gradient and a unidirectional concentration transport driven by sodium electrochemical gradient, respectively (Parkinson et al. 2011). Adenosine uptake in the brain occurs primarily by facilitated diffusion via equilibrative transporters, which pharmacological blockade is associated with an accumulation of adenosine in the extracellular space (Parkinson et al. 2011; Dulla and Masino 2013; Cunha 2016). From the four types of equilibrative transporters so far identified (ENT1, ENT2, ENT3, and ENT4), ENT1 and ENT2 are the most expressed in the brain, both by neurons and astrocytes (Parkinson et al. 2011). Nevertheless, some studies suggest that ENT1 has a more salient role in determining the concentration of extracellular adenosine in the brain and its dependence on glutamate receptor activation (Alanko et al. 2006; Bicket et al. 2016). Using the ENT1 selective inhibitor [3H]-S-(4-nitrobenzyl)-6-thioinosine ([3H]NBTI), we found a significant upregulation of the transporter in zQ175 mice (Guitart et al. 2016). More importantly, ENT1 gene (SLC29A1) transcript was significantly upregulated in HD disease patients at an early neuropathological severity stage, but not those with a higher severity stage, relative to non-demented controls (Guitart et al. 2016). Furthermore, SLC29A1 transcript was differentially co-expressed (gained correlations) with several other genes in HD disease subjects compared to the control group, demonstrating that ENT1 constitutes a biomarker of the initial stages of neurodegeneration in HD disease (Guitart et al. 2016). It was also postulated that adenosine could constitute another biomarker and, in fact, in a more recent study, CSF adenosine levels were found significantly lower in HD patients (Kao et al. 2017). In addition, the CSF concentration of ATP was inversely correlated with the number of CAG repeats, and the adenosine/ATP ratio was negatively correlated with the disease duration of HD patients (Kao et al. 2017).

12.3 Adenosine Neurotransmission as a Therapeutic Target in HD

12.3.1 Targeting A_1R in Phenotypic HD Models

Modulation of A_1R has not been largely evaluated. We have previously tested the A_1R agonist ADAC in the 3NP model of HD (Blum et al. 2002). We interestingly observed that the acute administration of this compound completely prevented the development of hindlimb dystonia related to striatal degeneration in this particular model and reduced the size of 3NP-induced striatal lesions, as well as the ongoing process of striatal degeneration. The protective effect of A1R activation was in line with other data showing that another A_1R agonist was able to prevent 3NP-induced seizures in mice (Zuchora et al. 2001) and that A_1R blockade was deleterious in another metabolic model of HD induced by malonate (Alfinito et al. 2003). The protective effects of ADAC were ascribed to its presynaptic ability to reduce glutamate release within the striatum (Blum et al. 2002). Although the therapeutic potential of A_1R activation in HD has remained difficult to extrapolate, mostly due to potential cardiovascular side effects associated with A_1R activation (see Blum et al. 2003b for review), the new studies targeting the adenosine tone (see below) might reinvigorate A_1R as a direct or indirect target in HD.

12.3.2 Targeting $A_{2A}R$ in Chemical- and Lesion-Induced HD Models

Compelling evidence suggests that inactivation of $A_{2A}R$ in rodents by pharmacological (e.g., antagonists) or genetic (e.g., knockout) approaches ameliorates the striatal damage evoked by an N-methyl-D-aspartate (NMDA) receptor agonist, quinolinic acid (QA), a mitochondrial toxin 3-nitropropionic acid (3-NP), and a mitochondrial complex II inhibitor (malonate) (Lee and Chern 2014). The intrastriatal injection of QA and systemic administration of 3-NP can mimic the anatomical and behavioral deficits of HD, produce the direct and indirect excitotoxicity of HD, and trigger the selective loss of MSN in the striatum (Alston et al. 1977; Brouillet et al. 1993, 2005; Jacobson et al. 2012; Shear et al. 1998). Malonate is a competitive inhibitor of succinate dehydrogenase. Intrastriatal injection of malonate results in significant lesions in the striatum and has been used to create a HD model (Andreassen et al. 2000; Beal et al. 1993; Messam et al. 1995). Several $A_{2A}R$ antagonists (DMPX, SCH58261, ZM241385, ST1535, MSX-3, and CSC) have been shown to elicit multiple beneficial effects in these chemical- and lesion-induced HD models by reducing the striatal atrophy or degeneration, EEG abnormality, and motor hyperactivity, improving the loss of the GABA content, lowering the glutamate outflow, and increasing the life-span (Alfinito et al. 2003;

Blum et al. 2003a, b; Fink et al. 2004; Galluzzo et al. 2008; Popoli et al. 2002; Reggio et al. 1999; Scattoni et al. 2007; Tebano et al. 2004). On the contrary, an $A_{2A}R$ agonist (CGS21680) was shown to increase the 3-NP-induced striatal lesion size (Blum et al. 2003a, b). In addition, different $A_{2A}R$-null mice models were developed to reveal the cell-type-specific functions of $A_{2A}Rs$ in the 3-NP-evoked striatal damage. Surprisingly, global $A_{2A}R$ knockout mice show opposite effects on the 3-NP-induced neurological deficit behaviors and striatal damage at different disease dosages (Blum et al. 2003a, b; Fink et al. 2004; Huang et al. 2006), suggesting the potential involvement of diverse cell types. The selective depletion of $A_{2A}R$ in forebrain neurons does not contribute to the 3-NP-evoked striatal damage (Huang et al. 2006). However, the selective removal of $A_{2A}R$ in bone marrow-derived cells (BMDCs) recapitulates the enhanced 3-NP-induced striatal damage in global A2AR knockout mice. These findings argue against the importance of $A_{2A}R$-mediated glutamate release in the 3-NP-induced striatal damage (Huang et al. 2006). The possible role of $A_{2A}R$ in controlling nonneuronal cells (e.g., glia) might also contribute to the function of $A_{2A}R$ in the brain, which requires further evaluation. In summary, inactivation of $A_{2A}R$ appears to be beneficial in the chemical- and lesion-induced HD models.

12.3.3 Targeting A2AR in Phenotypic HD Models

The first genetic mouse model of HD was developed and characterized two decades ago (Mangiarini et al. 1996). Since then, multiple genetic mouse models of HD (including transgenic, conditional transgenic, and knock-in mice) have been created for in-depth investigations (Ferrante 2009; Li et al. 2005; Menalled 2005; Menalled and Chesselet, 2002). More than 30 genetic mouse models of HD are available from various sources (Lee et al. 2013a, b; Pouladi et al. 2013). It is of great interest to find that modulation of $A_{2A}R$ in HD mice might result in different effects, as opposed to those in wild-type mice. The role of $A_{2A}R$ in HD had been evaluated in two different mouse models (R6/2 and N171-82Q) of HD. R6/2 mice express the exon 1 of the human huntingtin gene (Mangiarini et al. 1996) and show a speedy progression with many major HD symptoms (e.g., motor impairment, aggregate formation, body weight loss) (Cha et al. 1998, 1999; Luthi-Carter et al. 2000). Chronic treatment with an $A_{2A}R$ agonist (CGS21680) has been shown to have beneficial effects in R6/2 mice by reducing the accumulation of mHtt aggregates, lowering the NMDA toxicity, improving the brain atrophy, increasing the rotarod performance, and enhancing proteasome activity (Cepeda et al. 2010; Chiang et al. 2009; Chou et al. 2005; Ferrante et al. 2010; Huang et al. 2011a, b; Ju et al. 2011; Lin et al. 2013; Martire et al. 2007, 2013). Treatment with another $A_{2A}R$ agonist (T1–11) also produces beneficial effects in R6/2 mice by enhancing the rotarod performance and proteasome activity (Huang et al. 2011a, b).

On the other hand, injection of an $A_{2A}R$ antagonist (SCH58261) was shown to reduce the glutamate and adenosine outflow, normalize the alteration in the emotional response, and reduce the NMDA-induced toxicity (Domenici et al. 2007; Gianfriddo et al. 2004). However, SCH58261 exhibited no effect on motor capability (Cipriani et al. 2008; Domenici et al. 2007). Genetic and pharmacological inactivation of $A_{2A}R$ was also found to reduce working memory deficits in R6/2 mice (Li et al. 2015). Interestingly, combined blockade of D1Rs and A2ARs improved cognitive dysfunction in another HD mouse model (R6/1, a transgenic HD mouse model similar to R6/2) (Tyebji et al. 2015). Taken together, results of these studies suggest that $A_{2A}R$ blockade may be beneficial for the impaired cognitive function in HD mice. Genetic inhibition of $A_{2A}R$ in HD was also tested in another mouse model (N171-82Q) that expresses mHtt only in neurons (Mievis et al. 2011). Removal of $A_{2A}R$ shortens the survival and worsens the motor impairment of N171-82Q mice. Together with the earlier studies showing that activation of $A_{2A}R$ improved motor function of HD mice (R6/2 (Chou et al. 2005)), $A_{2A}R$ blockade might be of concern for HD patients. Given that activation and inactivation of $A_{2A}R$ are beneficial on different symptoms (motor functions and cognitive function, respectively) and apparently depends on the model used, the symptom-specific effects of $A_{2A}R$ need to be further investigated.

12.3.4 Targeting ENT1 in Phenotypic HD Models

The results, demonstrating upregulation of ENT1 and reduced adenosinergic tone in both animal models and HD patients, predict that ENT1 could constitute a new therapeutic target to delay the progression of HD. In complete support, pharmacological blockade with the low-affinity ENT1 inhibitor JMF1907 (Chen et al. 2011) or genetic blockade of ENT (global ENT1 knockout) in HD mice led to a significant increase in the mean survival time in the R6/2 mouse model of HD (Kao et al. 2017). In the same study, evidence could also be obtained for an increased expression and activity of ENT1 and ENT2, and decreased striatal adenosine levels could be demonstrated in R6/2 mice and still another animal model of HD, the knock-in Hdh(CAG)150 mouse (Lin et al. 2001). The expression of ectonucleotidases and ADK was also analyzed in both models, and only ADK transcript was found to be upregulated, but only in R6/2 mice (Kao et al. 2017), indicating that alterations in the equilibrative transporters are more likely to represent a key pathogenetic mechanism in HD. ENT1 and less selective ENT1/ENT2 inhibitors should then be considered as potentially new therapeutic drugs to decrease the progression of the disease. Although blood-brain barrier permeable, JMF1907 is still under preclinical evaluation and belongs to a group of multifunctional adenosine compounds that are both ENT1 inhibitors and $A_{2A}R$ agonists (Chen et al. 2011; Huang et al. 2011a, b). Given that inhibitors of ENT1 such as dipyridamole, ticagrelor, or

dilazep have already been used to treat different pathological conditions related to vascular relaxation and platelet aggregation or the NSAID sulindac sulfide for its anti-inflammatory effects, it has been suggested that they should be clinically studied in HD patients in order to evaluate their ability to delay the progression of the disease or the age of onset (Guitart et al. 2017). The main caveat is their purported low brain penetrability.

Using the classical reserpinized mice model, we recently evaluated the ability of the systemic administration of dipyridamole to decrease locomotor activation by dopamine receptor agonists. This model has been very useful for the discovery of the specific antagonistic interactions between adenosine and dopamine receptor ligands that led to the discovery the A_{2A}R-D2R and A_1R-D1R heteromers (Ferré et al. 1991, 1994). At a minimal dose of 30 mg/kg, dipyridamole significantly decreased the locomotor-activating effect of equipotent doses of selective D1R and D2R agonists, and the depressant effect of dipyridamole was totally counteracted by caffeine (Ferré et al. 2017). The results could then be entirely explained by the ability of systemically administered dipyridamole to promote an increase in the basal extracellular levels of striatal adenosine that normally exert a tonic-activating effect of postsynaptic A_1R and A_{2A}R. Such an increase should lead to the observed ability to depress both D1R and D2R agonist-mediated locomotor activation in reserpinized mice. Also, such an increase should be expected to increase the tonic activation of postsynaptic A_{2A}R and A_1R, but also presynaptic A_1R, leading to a decrease in glutamate release, hopefully promoting a therapeutic effect in HD patients.

12.4 Concluding Remarks

Adenosine receptors, and especially A_{2A}R, are clearly linked to HD pathophysiology as attested by a large number of genetic, epidemiological, and experimental studies. Several aspects concerning its pathophysiological involvement remain however to be further deciphered. The pre−/postsynaptic aspects deserve further investigation using specific ligands as well as genetic murine tools. Also, how A_{2A} receptors interact with glial dysfunctions promoted by mHtt has been largely underestimated. Furthermore, given that A_{2A}R heteromerize with several other GPCRs, such as D2R or A_1R, that play a presumable role in striatal dysfunctions and degeneration in HD, one may consider A_{2A}R heteromers as targets for drug development. Finally, since HD is a chronically progressive disease, the multiple mechanisms involving A_{2A}Rs may play different relative roles along the degenerative process. The role of A_1R in HD pathogenesis has been largely understudied, but it is becoming clear that this field deserves to be reconsidered. This is because of new developments on the role of low adenosine tone in HD, with the upregulation of ENT1, which recent studies indicating it could become a new target for drug development in HD.

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Chapter 13
Role of Adenosine Receptors in Epileptic Seizures

Diogo Miguel Rombo, Joaquim Alexandre Ribeiro, and Ana Maria Sebastião

Abstract Epileptic seizures are caused by an electrical disturbance of brain activity that results in abnormal and excessive synchronization of neurons. Adenosine is a long-known anticonvulsant endogenous substance, exerting its actions through diverse mechanisms of action at different cellular targets. In this review we discuss the main actions of adenosine during acute and chronic phases of epileptic seizure progression and the mechanisms involved. There should be considered three main levels of adenosine actions: (1) neuronal level, where adenosine, mostly through its receptors A_1, A_{2A} and A_3, alters intrinsic neuronal properties and excitatory/inhibitory network balance; (2) non-neuronal level, by affecting astrocytic function; and (3) homeostatic control level, through epigenetic regulatory mechanisms. Together, these actions make adenosine as a sort of "universal modulator or maestro" of desynchronization of epileptic focus, with great therapeutic potential in the treatment of resistant forms of epileptic seizures. Indeed, adenosine augmentation therapies are being considered to tackle epilepsy, which include gene therapy strategies and dietary interventions. Further research on new drugs that specifically target the mechanisms of actions involved in the pathological process of the disease are needed to take full advantage of adenosine anticonvulsant actions in the control of epileptic seizures.

Keywords Epilepsy · Seizure models · Neuroprotection · Adenosine-control mechanisms · Adenosine-based therapies · GABAergic transmission.

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13.1 Introduction

This chapter aims to provide an overview on how adenosine, through its receptors, acts as an anticonvulsant substance to control seizure activity. We will focus our attention on the main modulatory targets of adenosine which include the control of intrinsic neuronal properties, excitatory and inhibitory network balance and the homeostatic and epigenetic regulation. The mechanisms involved in these actions will be addressed, and its impact on in vitro and in vivo models of epilepsy will be analysed. Lastly, we will highlight the most recent and innovative adenosine-based strategies developed for therapeutic intervention in the control of epileptic seizures.

13.2 Epilepsy and Epileptic Seizures

Epilepsy is a brain disease with an heterogenous prevalence among different countries but estimated to be of around 1% worldwide (Sander and Shorvon 1996; Beghi and Hesdorffer 2014; Bell et al. 2014). The disease is predominantly characterized by the recurrent and unpredictable interruption of normal brain function by epileptic seizures, and it comprises all the neurobiological, cognitive, psychological and social consequences that this condition may have on people's lives (Fisher et al. 2005). For practical and clinical purposes, it was recently included in the definition of epilepsy the requirement of having at least two epileptic seizures occurring >24h apart or one epileptic seizure and a very high risk of recurrence over the next 10 years (Fisher et al. 2014). This definition, elaborated by the International League Against Epilepsy (ILAE), points out two important aspects that should be emphasized: first, that epilepsy exists when recurrent epileptic seizures occur, but epileptic seizures may occur without implying the diagnose of epilepsy; second, that epilepsy is not only characterized by its clinical neurological manifestations but also by all the repercussions that the disease may have on the patient and its family (including social implication).

Adenosine has been coined as a putative endogenous anticonvulsant a long time ago (Dunwiddie 1980; Dragunow et al. 1985). Since then, there was a great increase in our knowledge of the mechanisms of action of adenosine receptors that mediate its action and metabolic pathways involved in the homeostatic control of its intra- and extracellular concentration. The understanding of seizures also increased. In this chapter we will mostly focus our attention on adenosine actions that are related with seizure events. These include adenosine action on the mechanism underlying epileptic seizures per se, on the sequence of events that convert a normal neuronal network into a hyperexcitable network (epileptogenesis) and on the control of seizure activity in in vitro and in vivo experimental models of epilepsy. Although some of the epilepsy-associated comorbidities will be briefly addressed, we will leave all the other elements that define this disease out of our discussion.

It is important also to clarify the definition of epileptic seizures (or simply referred as seizures), which are described as a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain (Fisher et al. 2005). Clinically, when an epileptic seizure is identified, it is crucial that the clinician classifies the seizure according to its type and aetiology. This is helpful not only for diagnostic purposes but also for the use and development of antiepileptic therapies, investigation of seizure mechanisms and facilitation of worldwide communication among pairs. Seizures are thus classified according to its type in (1) focal, when originated within a network limited to one hemisphere; (2) generalized, when originated in bilaterally distributed networks; or (3) unclassified, when the local of onset is unknown (Berg and Millichap 2013). Each category can be further classified according to the level of awareness (retained awareness seizure or impaired awareness seizure), motor behaviour (atonic, tonic, clonic, myoclonic, epileptic spasms or a combination of these) and nonmotor behaviour (e.g. absence seizures) (Fisher et al. 2017). A focal seizure can eventually generalize, being classified as focal to bilateral tonic-clonic seizure (Fisher et al. 2017).

The etiologic classification recognizes six groups that have also implications for treatment. These include structural, genetic, infectious, immune, metabolic or unknown aetiology (Scheffer et al. 2017). A structural aetiology implies the presence of neuroimaging abnormalities as the most likely cause of seizure. Such changes may be acquired by stroke, trauma or even infection or developmental malformations. Genetic aetiology is when the epileptic seizures directly result from a known or presumed genetic disorder. Infection is one of the most common aetiologies of epileptic seizures worldwide (Vezzani et al. 2016) and includes viral, bacterial, fungal and parasitic infections of the central nervous system that result in seizures. An immune-mediated seizure is mostly associated with auto-immune diseases affecting the central nervous system (such as anti-NMDA receptor encephalitis) and deserves an isolated category given its specific treatment implications (Lancaster and Dalmau 2012). Metabolic causes refer to well-stablished metabolic defects where seizures are the core symptom (examples include porphyria or pyridoxine-dependent seizure). Unknown cause is defined when the aetiology is not possible to stablish. Unknown aetiology accounts for about one-third of all seizures worldwide, but this number varies depending on the healthcare provider and country (Banerjee et al. 2009). In this classification, of course, the categories are not hierarchical nor mutually exclusive, and a seizure event can have more than one aetiology (e.g. tuberous sclerosis complex, which results from mutations in genes TSC1 and TSC2 causing central nervous system tumours, is considered to have a genetic and a structural cause).

Given the diversity of types and aetiologies of seizures, we cannot consider only one form of epilepsy but rather a group of epilepsy syndromes, each of which characterized by the occurrence of specific seizure properties. Such differences need to be considered when planning and carrying experimental research on epilepsy, namely, in the devise of animal models in vitro and in vivo. These models serve a variety of purposes that include the screening of new antiepileptic compounds for their anticonvulsant or antiepileptogenic properties (in acute or chronic models of

epilepsy), their efficacy against different types of epilepsy and the study of the mechanisms involved in drug resistance in epilepsy or associated comorbid features, such as cognitive and psychiatric comorbidities (Löscher 2011). Animal models are usually grouped in "acute seizure models" and "chronic epilepsy models". The term "acute" refers to models in which seizures are induced by electrical or chemical stimulation in otherwise naïve and healthy (non-epileptic) animals; the term "chronic" is used to refer to models in which animals have been made epileptic by electrical, chemical or genetic means (Löscher 2011) (Table 13.1). Other mechanisms can be used to induce acute or chronic seizures, depending on the epilepsy aetiology to be studied (e.g. traumatic brain injury, hyperthermia, hypoxia, systemic/focal infection, among others).

The in vitro seizure models include neuronal cultures, brain slice cultures (organotypic) or acute slice models (Raimondo et al. 2017). These are particularly important for the study of basic physiology, pharmacology and molecular biology of seizures and epileptogenesis. Acute slices can be obtained from "normal" animals, in which in vitro manipulations are used to generate epileptiform activity or obtained from chronically epileptic animals or even human patients. To induce epileptiform activity in otherwise naïve slices, many different strategies can be used depending on what type of seizure activity is intended to be measured. These include high-frequency electrical stimulation, GABAergic inhibition, glutamatergic (kainic acid) or muscarinic (pilocarpine) activation, potassium channel blockers (4-aminopyridine), low extracellular calcium, low extracellular magnesium or high extracellular potassium models (Ivanov and Bernard 2017).

A plethora of >20 antiepileptic drugs (AEDs) are currently available to use in clinical practice. In fact, up to 70% of newly diagnosed people with epilepsy can be successfully treated with the drugs currently available (Kwan and Brodie 2001). The mechanism of action of most drugs reflects what we know about the underlying abnormalities occurring in seizure generation and propagation. These include targeting hyper-excitation or hypo-inhibition by preventing activation of depolarizing sodium or calcium channels, enhancing the inactivation of sodium channels, facilitating hyperpolarizing potassium channels or affecting neurotransmission by blocking glutamate-mediated actions or promoting GABA-induced inhibition (Bialer and White 2010; Rogawski et al. 2016) (Fig. 13.1). Nonetheless, one-third of patients with epilepsy cannot be controlled with these tools (Granata et al. 2009). Resistance to pharmacotherapy may be already present when the treatment is initiated, as occurs in some infant seizure syndromes (e.g. include Ohtahara syndrome, Dravet syndrome and others), or it may evolve during the course of chronic epilepsy or status epilepticus, after a positive initial response to the drug (Heinemann et al. 2006). When medication fails to control seizures, other options are considered and may include neurosurgical treatment (Engel 1996a), vagus nerve stimulation (Uthman 2000) or dietary therapy (e.g. ketogenic diet) (Bough and Rho 2007). Apart from surgery, where the rational is to isolate or resect the epileptogenic tissue (i.e., the focus of the disease), vagus nerve stimulation and ketogenic diet are alternative neuromodulatory approaches aimed to influence several neuronal targets simultaneously and achieve a greater control of the disease. Identical reasoning can

Table 13.1 Acute and chronic models of epilepsy

Model	Induction	Model of human epilepsy	Seizure type	Use
Acute models				
Electrical stimulation				
Maximal electroshock model (MES)	Whole-brain stimulation via the cornea or ear	Model of tonic-clonic generalized seizure	Generalized onset: clonic, tonic, tonic-clonic seizures	Antiepileptic drug screening Electrophysiological and behavioural changes caused by focal seizures Molecular and physiological changes related to acute epileptiform activity
6 Hz psychomotor seizure	Low-frequency stimulation via the cornea	Model of partial limbic seizure	Motor onset: automatisms; Nonmotor onset: behavioural arrest; Generalized onset: myoclonic seizures	
Local electrical stimulation	Cortical stimulation via implanted electrodes	Model of focal motor type of frontal lobe seizures Model of status epilepticus	Motor onset: automatisms, falls, clonic seizures; Nonmotor onset: behavioural arrest	
Chemical stimulation				
GABA-related drugs	Systemic or intracranial injection of PTZ, bicuculline, picrotoxin, 3-MPA, GAD synthesis inhibitors	Model of generalized myoclonic, tonic-clonic and absence seizures Depend on the drug and dose used	Motor onset: clonic, hyperkinetic and myoclonic seizures; Nonmotor onset: absence seizures; generalized onset, clonic, tonic, tonic-clonic seizures	Antiepileptic drug screening Study of antiepileptic drugs for status epilepticus Molecular and physiological changes related to acute epileptiform activity Electrophysiological and behavioural changes caused by focal seizures
Excitatory amino acid-related drugs	Systemic or intracranial injection of kainic acid, AMPA, NMDA, homocysteine	Model of focal motor seizure with automatisms Model of focal to bilateral tonic-clonic seizures Model of status epilepticus Depend on the drug and dose used	Motor onset: automatisms, falls, clonic, hyperkinetic and myoclonic seizures Nonmotor onset: behavioural arrest, autonomic Focal to bilateral tonic-clonic	

(continued)

Table 13.1 (continued)

Model	Induction	Model of human epilepsy	Seizure type	Use
Acetylcholine-related drugs	Systemic or intracranial injection of pilocarpine, organophosphorus compounds	Model of focal impaired awareness seizures Model of human poisoning Model of status epilepticus Depend on the drug and dose used	Motor onset: automatisms, falls clonic and tonic seizures Nonmotor onset: autonomic Focal to bilateral tonic-clonic	
Other drugs	Systemic of intracranial injection of strychnine, aminophylline, insulin-induced hypoglycaemia, antibiotics (penicillin), tetanus toxin	Models of focal epilepsy Depend on the drug and dose used	Motor onset: automatisms (tetanus toxin), clonic seizures (tetanus toxin, hypoglycaemia), myoclonic seizures (penicillin) Nonmotor onset: behavioural arrest (tetanus toxin) Focal to bilateral tonic-clonic (hypoglycaemia) Generalized onset: clonic and tonic seizures (penicillin), tonic-clonic (penicillin, hypoglycaemia)	

Chronic models				
Acquired epilepsy				
Electrical kindling	Repeated and intermittent intracerebral stimulation via implanted electrodes in specific brain regions	Model of focal impaired awareness seizures Model of focal to bilateral tonic-clonic seizures Chronic models of temporal lobe epilepsy Depend on induction, drug and dose used	Motor onset: automatisms, falls, hyperkinetic, clonic and tonic seizures Nonmotor onset: behavioural arrest Focal to bilateral tonic-clonic seizures Generalized onset	Study of epileptogenesis and long-term consequences of epilepsy Study comorbidities associated with epilepsy Antiepileptic drug screening and pharmacoresistant epilepsies
Chemical kindling	Repeated and intermittent systemic or intracerebral administration of convulsant agents in specific brain region: Excitatory amino acids (glutamate, NMDA, AMPA) GABAergic modulators (PTZ, picrotoxin, bicuculline) Cholinergic agents (carbachol, pilocarpine) Others			
Brain pathology models	Hyperthermia seizures Hypoxia model Posttraumatic epilepsy	Model of febrile seizures Models of stroke and brain injury	Motor onset: automatisms, clonic, myoclonic and tonic-clonic seizures Nonmotor onset: behavioural arrest Generalized onset: tonic-clonic seizures	Study of epileptogenesis, mechanisms and long-term consequences of epilepsy Antiepileptic drug screening
Genetic animal models of epilepsy				
Generalized absence epilepsy rats of Strasbourg (GAERS)	Genetic animals	Models of spontaneous recurrent seizures Models of absence seizures Model of idiopathic epilepsies	Generalized nonmotor onset: absence	Study of epileptogenesis and long-term consequences of epilepsy Study comorbidities associated with epilepsy Electrophysiological and behavioural changes caused by absence seizures Antiepileptic drug screening
Genetic epilepsy prone rats (GEPRs)	Genetic animals	Model of reflex seizures	Generalized onset: tonic and tonic-clonic seizures	
Audiogenic models	Acoustic stimulation in genetically prone animals	Model of reflex epilepsy Model of temporal lobe epilepsy	Generalized motor onset: automatisms, hyperkinetic, tonic-conic seizures	

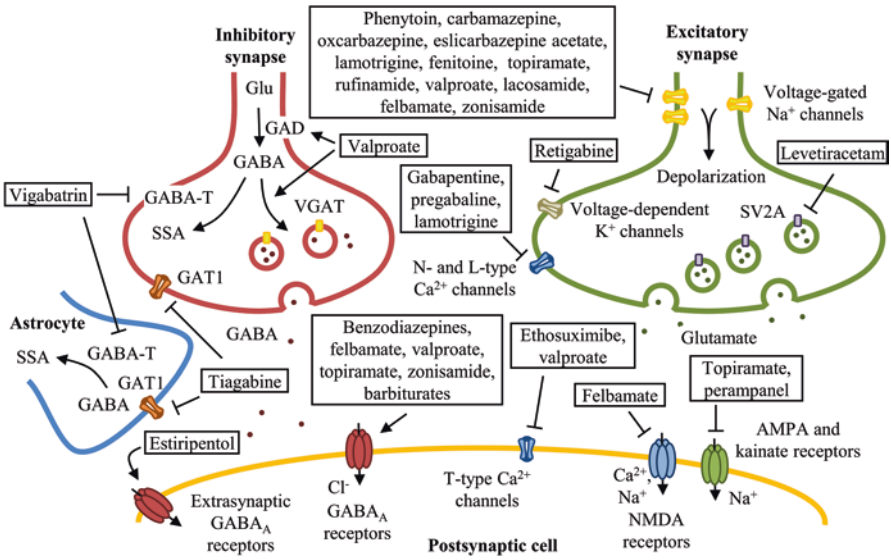

Fig. 13.1 Mechanisms of action of antiepileptic drugs at inhibitory and excitatory synapses and astrocytes. The targets include block activation of sodium channels (phenytoin, carbamazepine, oxcarbazepine, eslicarbazepine acetate, lamotrigine, topiramate, rufinamide, felbamate, zonisamide, valproate) or enhance inactivation of sodium channels (lacosamide, rufinamide), block calcium channels (gabapentin, pregabalin, lamotrigine, ethosuximide, valproate) and open potassium channels (retigabine); enhance $GABA_AR$ actions (benzodiazepines, felbamate, valproate, topiramate, zonisamide, barbiturates) or extrasynaptic $GABA_ARs$ (stiripentol); increase GABA turnover by increasing its synthesis – via GAD – and/or release (valproate), inhibiting GABA reuptake through GABA transporters type 1 (GAT1) (tiagabine) or inhibiting GABA transaminase (GABA-T) (vigabatrin); block AMPA and kainate receptors (topiramate, perampanel) or NMDA receptors (felbamate); and inhibit synaptic vesicle glycoprotein 2 (SV2A) (levetiracetam)

be applied to the use of adenosine-based strategies in the control of seizures and epilepsy. In fact, most of the targets of AEDs mentioned above are, indeed, also targets of adenosine modulation (Sebastião and Ribeiro 2009). Ketogenic diet also involves adenosine-dependent mechanism (Masino and Geiger 2008). Thus, it is not surprising that, apart from all difficulties with the implementation of adenosine in clinical practice (mostly due to the well-known peripheral side effects), adenosine is still highly and enthusiastically studied as an important and promising approach to fight drug-resistant epilepsies.

13.3 Grounds for Anticonvulsant Actions of Adenosine

Since Phillis' first observations that adenosine has a depressive action in the central nervous system (Phillis et al. 1974), many studies have followed showing that adenosine is capable of influencing not only neuronal firing (Kostopoulos et al. 1975;

Phillis and Kostopoulos 1975) but also to directly alter excitatory synaptic responses (Kuroda and Kobayashi 1975; Scholfield 1978; Schubert and Mitzdorf 1979; Dunwiddie and Hoffer 1980). The first evidence relating adenosine and epileptic seizures came from studies showing that right after seizure initiation there was a rapid and dramatic increase in adenosine levels (Pull and McIlwain 1972; Schultz and Lowenstein 1978; Schrader et al. 1980; Winn et al. 1980). Later, adenosine was shown to act as an endogenous anticonvulsant in vitro, in a rat hippocampal preparation of epileptiform discharges (Dunwiddie 1980), and also in vivo, in a rat drug-induced seizure model (Dunwiddie and Worth 1982). These and other findings (Albertson et al. 1983; Barraco et al. 1984; Dragunow et al. 1985; Chin 1989) paved the way for comprehensive studies about the use of adenosine and adenosine-related compounds as putative therapeutic strategies in epilepsy. Today, it is largely accepted that adenosine indeed has a role as an anticonvulsant substance. There are, however, several aspects that should be considered when discussing the influence of adenosine on seizures and epilepsy.

First is that the capacity of adenosine to shape neuronal excitability and arrest seizures depends on the stage of disease progress. This is of particular interest since the adenosinergic system not only influences neuronal excitability during a seizure event but is also dramatically affected by the epileptogenic process per se. This means that adenosine-mediated actions may vary depending on whether they are directed to neuronal circuits that are experiencing a seizure event for the first time (i.e. acute seizures) or circuits suffering from neurobiological modifications after a first neuronal insult (that may also affect the adenosinergic system) as occurs during epileptogenesis and stablished epilepsy (i.e. chronic seizures). This has also important clinical and therapeutic implications. Acute actions of endogenous adenosine in an inaugural seizure event may have significant neuroprotective value but little application for clinical intervention. On the other hand, subsequent adaptive changes that contribute to the development of recurrent seizure events and the establishment of epilepsy, as well as the emergence of resistance to classical EADs, may potentially benefit from adenosine-based therapies.

Another aspect to consider is the targets of adenosine actions. Indeed, adenosine is known to affect excitability far beyond its synaptic effects (Cunha 2001). Thus, in a tentative dissection of the mechanism by which adenosine exerts its anticonvulsant actions, it is relevant to separate the actions occurring at the (1) neuronal level, which include changes in neuronal excitability and synaptic transmission; (2) non-neuronal level, mostly related to regulation of glial function; and (3) homeostatic control level, corresponding to variations in adenosine metabolism and epigenetic control. Nevertheless, all mechanisms are mutually dependent and operate together to generate an orchestrated action to influence network activity and seizures. It is noteworthy to mention that this chapter does not intend exhaustively describe all mechanisms by which adenosine exerts its actions upon excitability. We will only focus on studies where there is direct evidence for the interference of adenosine or adenosine receptors on seizure activity.

13.4 Adenosine as a Seizure Control Substance

13.4.1 A_1Rs in Acute Models of Seizure

As mentioned above, the capacity of adenosine to control seizure events has been proposed a long time ago (Dunwiddie 1980; Dunwiddie and Worth 1982). Studies that followed have confirmed and broaden the actions of adenosine on many different seizure models in vitro and in vivo. We should start by looking to adenosine action on acute seizure models of epilepsy. As first observed by Dunwiddie and Worth, adenosine actions in these models are mostly associated with A_1 receptors (A_1Rs) (Dunwiddie and Worth 1982). When adenosine analogues or selective A_1R agonists were systemically injected in electrical- or chemical-induced seizure models, an anticonvulsant action and arrest of epileptic seizures were observed (Dragunow and Goddard 1984; Turski et al. 1985; Morrisett et al. 1987; Whitcomb et al. 1990; Von Lubitz et al. 1993; Young and Dragunow 1994; Adami et al. 1995; Pourgholami et al. 1997a; Malhotra and Gupta 1997; Sarro et al. 1999; Zgodziński et al. 2001; Huber et al. 2002; Girardi et al. 2007; Li et al. 2013). These actions were observed also in immature animals, suggesting that the A_1R-mediated anticonvulsant effects also occur early in the developing brain (Mareš 2010; Pometlová et al. 2010). Repeated activation of A_1Rs, however, tends to develop tolerance and a gradual loss of their anticonvulsant capacities (Adami et al. 1995). Importantly, there is an endogenous role of adenosine in the suppression of seizures since blocking A_1R activation aggravates epileptic activity (Dragunow and Goddard 1984; Dragunow and Robertson 1987; Morrisett et al. 1987; Whitcomb et al. 1990; Zhang et al. 1993; Von Lubitz et al. 1993; Fukuda et al. 2010) and converts recurrent seizure patterns into status epilepticus (Young and Dragunow 1994). Significant seizure suppression can also be achieved by increasing adenosine levels through inhibitors of adenosine kinase, adenosine deaminase or adenosine transporters blockers (Eldridge et al. 1989; Zhang et al. 1993). Together, these studies highlight three main aspects: first, that adenosine is not only capable of preventing seizure occurrence when administered before the trigger insult but also stop an already stablished seizure event (working as a true anticonvulsant substance); second, adenosine-mediated endogenous mechanisms are crucial to restrain ongoing seizure events, but further anticonvulsant effect is still possible through exogenous activation of A_1R; and, finally, adenosine actions occur in different acute models of epileptic seizures, regardless the mechanism of induction is electrical stimulation (Dragunow and Robertson 1987; Whitcomb et al. 1990; Young and Dragunow 1994; Pometlová et al. 2010) or by manipulation of GABAergic system (Zhang et al. 1993; Adami et al. 1995; Malhotra and Gupta 1997; Girardi et al. 2007; Mareš 2010; Li et al. 2013), of the glutamatergic system (Von Lubitz et al. 1993; Li et al. 2013), or by other chemical manipulation (Turski et al. 1985; Eldridge et al. 1989; Li et al. 2013).

All mentioned studies have in common the control by A_1R via intraperitoneal injections of selective drugs. However, their low permeability through the blood-brain barrier (Brodie et al. 1987) and peripheral actions, mostly related to cardiovas-

cular side effects (Stella et al. 1993; Schindler et al. 2005), has hampered the use of A_1R agonists for the treatment of central nervous system (CNS) diseases. To minimize these effects and potentiate adenosine actions in the brain, other approaches include intracranial activation of A_1R in specific brain regions known to be responsible for the epileptic seizure initiation and/or spreading. In fact, intracranial perfusion of A_1R agonists conferred protection against 3-nitropropionic acid (a mitochondrial toxin) (Zuchora et al. 2001), bicuculline-induced (Franklin et al. 1989) and pilocarpine-induced seizures through changes in glutamate, GABA and dopamine levels at the epileptic focus (Khan et al. 2000, 2001). An alternative, non-invasive possibility is the development of tissue selective adenosine receptor agonists. An A_1R agonist with anticonvulsant activity without causing motor behaviour alterations usually occurring as a consequence of sedation and bradycardia has been identified (Tosh et al. 2012), but the mechanisms subserving such tissue selectivity remain to be identified.

13.4.2 A_1Rs in Chronic Models of Epilepsy

Regarding actions of A_1Rs in chronic models of epilepsy, they are described for the kindling model of limbic seizure propagation, which mostly involves connections between piriform cortex, amygdala, hippocampus and entorhinal cortex (Lopes da Silva et al. 1990). The kindling model is one of the most commonly used chronic models of epilepsy, particularly for the study of temporal lobe (limbic) epilepsy (TLE) (Goddard 1967; Goddard et al. 1969). In kindling animals, seizures initiate and are confined to the focal area of stimulation and progressively propagate through other brain structures that serve as pathways for generalization of subsequent seizures (Sato et al. 1990). Thus, seizure progression may be hypothetically interrupted not only in the original focus of the seizure but also in parts of the brain, other than the epileptic focus, that are responsible for its propagation. This is, indeed, what happens with adenosinergic control of limbic seizures. Intra-hippocampal and intra-amygdala injections of A_1R agonists have, as expected, inhibitory actions on hippocampal- and amygdala-kindling parameters, respectively (Rosen and Berman 1987; Pourgholami et al. 1997b). Importantly, however, activation of A_1Rs in the hippocampus (Pourgholami et al. 1997a; Alasvand Zarasvand et al. 2001), entorhinal cortex (Mohammad-Zadeh et al. 2005), piriform cortex (Rezvani et al. 2007a, b) or perirhinal cortex (Mirnajafi-Zadeh et al. 1999) reduced seizure duration and afterdischarges specifically in amygdala. These effects are pathway dependent, since intra-amygdala A_1R activity had no significant anticonvulsant action neither on hippocampal-kindled seizures (except for secondary afterdischarges) (Mirnajafi-Zadeh et al. 2000), entorhinal cortex-kindled seizures (Mohammad-Zadeh et al. 2005) nor piriform cortex-kindling animals (Shahabi et al. 2006), despite the influence of amygdala in its propagation (Sato et al. 1990). Hippocampal and entorhinal cortex A_1Rs are also involved in supressing entorhinal cortex- and piriform cortex-kindling, respectively (Heidarianpour et al. 2006;

Zeraati et al. 2006; Hosseinmardi et al. 2007). Importantly, blockade of A_1R actions in these models aggravates seizure activity and generalization, pointing to an endogenous action of adenosine, through A_1Rs, in restraining limbic epilepsy. Together, these results indicate that the anticonvulsant actions of A_1Rs (1) are not only relevant in acute seizure control but also in chronic models of epilepsy, (2) are region and pathway specific and (3) are capable of restraining seizure exacerbation not only in the brain region where seizure emerges but also in the surrounding areas responsible for propagation and consequent generalization of seizures.

The translational potential of invasive approaches as intracranial injections of A_1R agonists can only be envisaged for very serious disease conditions. Another potential approach for intracranial activation of A_1Rs is to provide a local and sustained source of adenosine. This was first achieved by intraventricular implantation of an adenosine-releasing synthetic polymer, which led to a reduction of seizure activity in the rat kindling model of partial epilepsy (Boison et al. 1999). Also, ex vivo gene therapy approaches in kindled rats showed profound but transient reduction in seizure activity (Huber et al. 2001; Boison et al. 2002; Güttinger et al. 2005a). Long-term anticonvulsive effects were obtained with local release of adenosine through encapsulated myoblasts implanted in the vicinity of the epileptic focus (Güttinger et al. 2005b). Protection from convulsive seizures lasted for 3 to 8 weeks and caused no desensitization of A_1Rs and no side effects as sedation or changes in locomotor behaviour (Güttinger et al. 2005b).

The capacity of adenosine to keep the epileptic focus localized was also demonstrated in a status epilepticus model using A_1R-knock out (A_1R-KO) mice (Fedele et al. 2006). In fact, A_1R-KO animals submitted to unilateral intra-hippocampal kainic acid injections not only showed severe convulsions and increased mortality when compared to control animals but also displayed greater extend of neuronal loss both in the ipsi- and contralateral hippocampus. Similar results were obtained with A_1R-KO mice subjected to traumatic brain injury-induced seizures (Kochanek et al. 2006).

Despite the neuroprotective and anticonvulsant actions of adenosine A_1Rs in acute and chronic stages of epilepsy, adenosine release and A_1R (but also $A_{2A}R$) activation during seizures have been associated with sudden unexpected death in epilepsy (SUDEP) (Shen et al. 2010; Faingold et al. 2016). Postictal hypoventilation is considered as a major contributor to the cause of death in SUDEP, particularly during the sleeping period (Massey et al. 2014; Richerson et al. 2016). Thus, given the known depressant actions of adenosine on brainstem respiratory network (Vandam et al. 2008; Zwicker et al. 2011) and its sedative effects (Porkka-Heiskanen et al. 1997), seizure-induced elevation of adenosine levels may further contribute to respiratory distress associated with SUDEP. Importantly, caffeine treatment after seizure onset might be beneficial (Shen et al. 2010).

Altogether, data from chronic models of epilepsy reveal that even after the establishment of recurrent seizure events, and besides all the consequent neuronal adaptations that may occur in neuronal circuitry, adenosine A_1Rs are still able to restrain and cease further progression of the disease in a tentative action to re-establish normal network communication. Risk of SUDEP may however be increased.

13.4.3 Adenosine A_{2A}Rs and A_3Rs in Seizure Control

Although the major interest about adenosine control of epileptic activity has been the inhibitory A_1R system, an increasing number of studies are now focusing on facilitatory adenosine A_{2A} receptors (A_{2A}Rs).

The majority of results point to a pro-excitatory role of A_{2A}Rs, although some data is still conflicting. Some studies have shown that A_2R or A_{2A}Rs are not involved in convulsions (Rosen and Berman 1987; Janusz and Berman 1992; Young and Dragunow 1994; Malhotra and Gupta 1997; Uzbay et al. 2007; Rezvani et al. 2007a; Akula and Kulkarni 2014). Surprisingly, studies using acute models of chemical-induced seizures and audiogenic-susceptible seizures show that activation of A_2R or even A_{2A}Rs agonists contribute to seizure suppression (Adami et al. 1995; Jones et al. 1998a, b; Sarro et al. 1999; Boison et al. 2002) and that its blockade has pro-convulsant actions (Vianna et al. 2005). In a kindling model of chronic epilepsy, the effects of NECA (an agonist with slightly more potency to A_2R than A_1R) was compared with that of a prototype A_1R agonist, and data obtained allowed to suggest an A_2R-mediated seizure suppression in the caudate nucleus (Rosen and Berman 1987), but not in the amygdala (Janusz and Berman 1992). Focal injection of CGS21680 (an adenosine A_{2A}R agonist) leads to a reduction in the severity of bicuculline-induced seizures, but these actions were attributed to A_1Rs (Zhang et al. 1994), which highlights the need of careful pharmacological controls before concluding on the action of adenosine receptors, in particular of those expressed at low levels in relevant brain areas, in seizures.

Apart from these initial studies, some of them performed before development of selective adenosine receptor ligands, more recent studies have consistently been showing proconvulsive effects of A_{2A}Rs in chronic models of amygdala (Li et al. 2012b) and piriform cortex (Zeraati et al. 2006; Hosseinmardi et al. 2007) kindling. Genetic ablation of A_{2A}Rs confirms these proconvulsant actions since A_{2A}R-KO animals show attenuated intensity of pentylenetetrazol-induced (El Yacoubi et al. 2008) or ethanol withdrawal-induced (El Yacoubi et al. 2001) seizures. Protection is not only evident during the acute convulsive period but also in preventing proconvulsive epileptogenic changes during evolution of kindling (El Yacoubi et al. 2009). An A_{2A}R antagonist was also shown to reduce synchronous pyramidal cell firing in acute hippocampal slices under hyperexcitable conditions (Rombo et al. 2015).

In a genetic model of absence epilepsy (WAG/Rij), in vivo A_{2A}R activation increases spontaneous discharges and aggravates epileptiform activity of hippocampal slices recorded 1–5 h post-injections (D'Alimonte et al. 2009). It was recently shown that a genetic variation in human adenosine A_{2A}R gene (ADORA2A) associated with increased expression of A_{2A}R and higher levels of cAMP production is a predisposing factor for childhood encephalopathy following severe febrile seizures (Shinohara et al. 2013), a finding that also favours the idea of a proconvulsant action of A_{2A}Rs.

Progressive development of stress-induced seizures and deficits in learning and memory have been reported to occur in mice with genetic deletion of adenosine

kinase (ADK-KO) in the brain (Sandau et al. 2016). This somehow mimics a rare disease in humans, ADK deficiency, which have psychomotor delay and convulsive seizures commencing between the first and third year of life (Bjursell et al. 2011). Intracellular ADK is known to regulate extracellular levels of adenosine, with low ADK activity being associated with protection against seizures (Gouder 2004). Thus, the finding that ADK-KO leads to progressive seizure development was unexpected. A study designed to find out possible mechanisms underlying these findings allowed to conclude that chronically enhanced levels of extracellular adenosine, caused by absence of adenosine kinase in the forebrain, favour $A_{2A}R$ activity, which thus enhances the action of brain-derived neurotrophic factor (BDNF) upon synaptic plasticity (Sandau et al. 2016), which most probably leads to maladaptative circuitry formation. Indeed, blocking $A_{2A}R$ activity in ADK-KO mice, as well as blocking BDNF receptors, attenuated seizure risk and restored cognitive performance. Interestingly, A_1R blockade exacerbates seizure phenotype in this mice model, thus indicating that extracellular adenosine, through A_1R, maintains its anticonvulsant activity (Sandau et al. 2016). This study, together with other studies showing the proconvulsant action of $A_{2A}R$, highlights the need to attenuate the overactivation of $A_{2A}R$ while testing the action of adenosine augmentation therapies against pharmacoresistant forms of epilepsy.

In conclusion, the presently available evidence points towards a proconvulsant action of $A_{2A}R$. Some discrepancies that have been reported may be ascribed to several reasons including pharmacological tools to identify the receptors, the mode of administration and concentrations used (receptor selectivity *vs* access of drugs to the epileptic tissue), differences in the seizure model used (that underlie different mechanisms of seizure induction) and the brain region and the neuronal circuits involved in seizure initiation and propagation (brain region and pathway specificity of $A_{2A}R$ actions). Maladaptive changes of $A_{2A}Rs$ levels in acute and chronic models of epilepsy may also explain part of the inconsistencies (see Chapter 13.5 below).

Regarding A_3Rs, much less information is known, and again, some discrepancies are reported. In an audiogenic seizure model of epilepsy, A_3Rs were ineffective in controlling seizures (Sarro et al. 1999). However, the A_3R-selective agonists, Cl-IB-MECA, were reported to facilitate epileptiform discharges in the CA3 area of the immature hippocampus (Laudadio and Psarropoulou 2004), whereas A_3R antagonists increased the GABAergic current stability in different epileptic tissues (Roseti et al. 2008), in line with the possibility of a proconvulsant action of A_3Rs. In contrast, in acute chemical-induced seizure models, activation of A_3R seemed to be anticonvulsant (Von Lubitz et al. 1995a), and its blockade was proconvulsant (Vianna et al. 2005). In an electroshock seizure model, A_3Rs raised the threshold for electro-convulsions and reduced the severity of seizures, in line with the idea of the anticonvulsant actions of this receptor (Borowicz et al. 2004). The relatively low affinity of adenosine to A_3R (Von Lubitz et al. 1994a; Dunwiddie et al. 1997) makes their activation possible only when relatively high concentrations of extracellular adenosine are reached, as occurs during high-frequency neuronal discharge. Furthermore, the expression of A_3R is much more restricted to some tissues and brain areas than that of A_1R. These two characteristics of A_3R can turn into an advantage and raise the interest to develop A_3R-selective ligands, which may have

less side effects than A_1R agonists to control pharmacoresistant seizures. However, it becomes clear that further steps in this direction require further studies to clarify the role of A_3R in epilepsy.

Overall, it becomes evident that the actions of adenosine in acute and chronic phases of the epileptogenic process are valuable to hamper the progression of epileptic seizures. The long-known strategy of activating A_1Rs as therapeutic approach to suppress seizures may profit from concomitant modulation of $A_{2A}Rs$ and A_3Rs to further promote the anticonvulsant effects of adenosine.

13.4.4 Adenosine and AEDs

Since the early findings that adenosine acts as an anticonvulsant substance, there is interest in the study of its interaction with conventional AED. In fact, there is evidence that some AEDs influence the purinergic transmission and that purine ligands affect AED actions. Early reports suggested that alterations in extracellular levels of adenosine or in the degree of activation of adenosine receptors may be an important component of the anticonvulsant actions of carbamazepine (Lewin and Bleck 1977; Skeritt et al. 1982). The interaction seems much stronger at A_1Rs then $A_{2A}Rs$ (Marangos et al. 1983; Skerritt et al. 1983; Weir et al. 1984; Dodd et al. 1986; Fujiwara et al. 1986). Prolonged treatment with carbamazepine, however, causes a marked upregulation of adenosine receptors in the brain (Marangos et al. 1985). Benzodiazepines such as diazepam and midazolam, as well as diphenylhydantoin, act as potent adenosine uptake inhibitors at therapeutic doses (Hammond et al. 1981; Phillis and Wu 1982; Phillis 1984; Bender and Hertz 1986; Narimatsu and Aoki 1999) causing an accumulation of extracellular endogenously released adenosine and potentiating their anticonvulsant actions (Kaplan et al. 1992; Narimatsu and Aoki 1999).

Conversely, adenosine receptor ligands also interfere with AED actions. Aminophylline and theophylline (non-selective $A_1R/A_{2A}R$ antagonists) have been shown to diminish the efficacy of diazepam and valproate against electrical- and chemical-induced seizures (Czuczwar et al. 1985; Kulkarni et al. 1991; Malhotra et al. 1996; Zuchora et al. 2005), in line with the possibility that some of the anticonvulsant actions of those AEDs are due to enhanced extracellular adenosine levels. This may also apply to the sedative actions of benzodiazepines, since both aminophylline and theophylline showed to reverse the sedative effects of diazepam in postoperative animal models (Arvidsson et al. 1982; Niemand et al. 1984, 1986; Malhotra et al. 1996). Also, adenosine receptor activation potentiated the anticonvulsant actions of diazepam and valproate via A_1Rs (Czuczwar et al. 1990) and phenobarbital, diphenylhydantoin, valproate and carbamazepine via both A_1Rs and A_3Rs (Borowicz et al. 1997, 2000, 2002) in electrical models of epilepsy. Together, these studies show a close interaction between AEDs and adenosine receptors in the control of seizures and highlight the potential concomitant use of conventional and adenosine-based strategies to potentiate their anticonvulsant actions.

13.5 Adenosine Control Mechanisms in Epileptic Seizures

Before discussing the specific mechanisms by which adenosine acts as an anticonvulsant and antiepileptogenic substance, a discussion about how the adenosinergic system per se is affected by acute and chronic seizures should be made (Table 13.2).

Anticonvulsant actions of adenosine result from a rapid increase in its extracellular concentration after seizures initiation, both in animal models (Schrader et al. 1980; Winn et al. 1980; Lewin and Bleck 1981; Berman et al. 2000; Kaku et al. 2001) and humans (During and Spencer 1992; Van Gompel et al. 2014). Adenosine levels increase 6- to 31-folds its basal levels, reaching ~2–3 μM concentration few seconds after the onset of epileptic activity (During and Spencer 1992) and remain high up to seizure termination (Van Gompel et al. 2014). Released adenosine is generated in the cytosol of spiking neurons as a consequence of metabolic exhaustion, reaching extra-

Table 13.2 Acute and long-term adaptive changes of adenosinergic system to epileptic seizures

Adenosine system	Acute adaptations	Long-term adaptations
Adenosine levels	**Increase in adenosine levels**[1–6]	**Moderate increase in adenosine levels**[7] during recurrent seizure events **Low basal levels of adenosine**[7]
A_1Rs	**Increase in A_1R density**[8–13] (hippocampus, cortex and cerebellum) No change in A_1R density (striatum)[9, 10];	**Decrease in A_1R density**[7, 14–18] (rodent and humans) Increase in A_1R protein and mRNA levels[19–23]
A_{2A}Rs	**Increase in A_{2A}R density**[17, 24, 25] (neurons and glia) Decrease in A_{2A}R protein and mRNA level[19]	
ATP and AMP levels	**Increase in ATP and AMP levels**[26, 27]	**Decrease in ATP levels**[7]
Ectonucleotidases	**Increase in density and activity of Ecto-5′-nucleotidase**[7,28, 29] **Decrease in activity of ATPases**[30–32]	
Equilibrative nucleoside transporter (ENT)	**Decrease in density and efficiency of ENT**[7, 33]	
Adenosine kinase (ADK)	**Decrease in ADK expression**[34]	**Increase in ADK expression**[34–37]

In bold are considered the main adaptations. References: (1) Schrader et al. (1980), (2) Winn et al. (1980), (3) Lewin and Bleck (1981), (4) During and Spencer (1992), (5) Kaku et al. (2001), (6) Van Gompel et al. (2014), (7) Rebola et al. (2003), (8) Daval and Sarfati (1987), (9) Angelatou et al. (1990), (10) Angelatou et al. (1991), (11) Daval and Werck (1991), (12) Pagonopoulou et al. (1993), (13) Vanore et al. (2001), (14) Ekonomou et al. (1998), (15) Ekonomou et al. (2000), (16) Ochiishi et al. (1999), (17) Rebola et al. (2005), (18) Glass et al. (1996), (19) Adén et al. (2004), (20) Tchekalarova et al. (2005), (21) Hargus et al. (2012), (22) Angelatou et al. (1993), (23) Luan et al. (2017), (24) Saura et al. (2005), (25) Orr et al. (2015), (26) Wieraszko and Seyfried (1989), (27) Muzzi et al. (2013), (28) Schoen et al. (1999), (29) Lie et al. (1999), (30) Nagy et al. (1990), (31) Bonan et al. (2000a), (32) Bonan et al. (2000b), (33) Pagonopoulou and Angelatou (1998), (34) Gouder (2004), (35) Li et al. (2008), (36) de Groot et al. (2012), (37) Aronica et al. (2011)

cellular space directly through equilibrate nucleoside transporters (ENTs) (Lovatt et al. 2012). Extracellular levels are controlled by the activity of intracellular adenosine kinase (ADK), an enzyme that in the hippocampus, from P14 onwards, is mostly expressed in astrocytes (Studer et al. 2006; Etherington et al. 2009; Kiese et al. 2016). During a seizure event induced by chemical agents, the density of A_1R, or their G-protein coupling, increases after the first minutes to hours after the convulsion (Daval and Sarfati 1987; Angelatou et al. 1990, 1991; Daval and Werck 1991; Pagonopoulou et al. 1993; Psarropoulou et al. 1994; Vanore et al. 2001). An exception to this overall increase seems to be the striatum where no change or a slight decrease in A_1R density was detected, as compared with other brain areas where the expected increase was found (Angelatou et al. 1990, 1991). In electrically evoked seizure models induced with single electroconvulsive shock, no changes were observed in expression of A_1Rs (Newman et al. 1984; Gleiter et al. 1989). However, this may be related with the intensity of stimulus used to induce seizures, since stronger multi-shock stimulation does lead to an increase in A_1R density (Gleiter et al. 1989). It is clear from this set of data that the first adaptation of the adenosinergic system after initiation of seizures is to restrain the abnormal convulsive activity and try to re-establish normal network function. This is mostly achieved by potentiating the neuroprotective actions of adenosine through increase of its extracellular levels and A_1R expression.

On the other hand, long-term adaptations of adenosine and adenosine receptors to recurrent seizures differ from those observed in acute models (Table 13.2). When evaluating adenosine levels and adenosine receptor density in chronic models of seizures, several aspects can be pointed out: (1) the rising concentrations of adenosine during a recurrent convulsion in chronic models do not reach the same values as in an inaugural seizure event (Rebola et al. 2003); (2) outside the convulsive periods, the extracellular concentration of adenosine drops to levels lower then basal concentrations observed in non-epileptic tissue (Rebola et al. 2003); and (3) A_1R density decreases progressively along the epileptogenesis process (Ekonomou et al. 1998, 2000) and remains low several weeks after the first convulsion (Ochiishi et al. 1999; Rebola et al. 2003, 2005). These observations are also supported by a loss of hippocampal A_1Rs in patients suffering from temporal lobe epilepsy (Glass et al. 1996) and are in agreement with the requirement of higher doses of A_1R agonists necessary to produce anticonvulsant effects along status epilepticus progression (Young and Dragunow 1994; Adami et al. 1995). However, part of the reduction in A_1R density may be influenced by significant neuronal loss observed after recurrent seizures events (Engel 1996b). This may explain some of the contrasting results showing increased A_1R protein and mRNA levels in chronic models of seizure (Adén et al. 2004; Tchekalarova et al. 2005; Hargus et al. 2012). Also, in tissue from human temporal lobe epilepsy (Angelatou et al. 1993) and human Rasmussen encephalitis (Luan et al. 2017), it was found an increase in the density of A_1R. The exact reason for discrepancies compared to Glass observations (Glass et al. 1996) is unclear, but one possible explanation may be the differences in control tissue (biopsy *vs* autopsy of non-epileptic brain controls).

Regarding $A_{2A}Rs$, most studies report long-term changes in receptor protein levels. In opposite to A_1Rs, $A_{2A}Rs$ protein levels are increased several weeks after the

establishment of epilepsy (Rebola et al. 2005). Upregulation of A_{2A}Rs does not only occur in neurons but also in glia (Saura et al. 2005; Orr et al. 2015). One study, though, describes a decrease in A_{2A}R protein and mRNA levels using a kindled seizure model of epilepsy (Adén et al. 2004). Curiously, using a genetic model of absence epilepsy, where it is thus possible to evaluate receptor levels before and after seizures onset, it was shown that A_{2A}R levels are only high after the first symptoms appear and not during the presymptomatic period, where A_{2A}R density is even lower than in control animals (D'Alimonte et al. 2009). This increase may thus be a consequence, and not a direct cause, of recurrent seizure activity, although it may contribute to the progression and aggravation of convulsions. Alternatively, one may think that the increase in A_{2A}R levels is itself a gate of the disease, coinciding with the very first event. Pharmacological treatment of these animals with an A_{2A}R antagonist before disease onset would help to clarify this issue.

In parallel with changes in receptor levels, other long-term changes in the adenosinergic system also occur. These include decrease in ATP levels (Rebola et al. 2003) and suppression of recurrent epileptiform discharges through activation of A_1Rs (Avsar and Empson 2004; Klaft et al. 2012; Muzzi et al. 2013); increase in ecto-5´-nucleotidase activity (Schoen et al. 1999; Lie et al. 1999; Rebola et al. 2003), the enzyme responsible for hydrolysing AMP into adenosine (Zimmermann et al. 2012); decrease in ecto-ATPase activity (Nagy et al. 1990; Bonan et al. 2000a, b); decrease in density and efficiency of equilibrative nucleoside transporter (ENT) (Pagonopoulou and Angelatou 1998; Rebola et al. 2003); and upregulation of ADK in rodents and human epileptic tissue (Aronica et al. 2011). Together, these long-term changes will contribute to low, but mostly ATP-derived, extracellular levels of adenosine that will lead to activation of A_{2A}Rs (Cunha et al. 1996) and contribute to propagation of seizure activity (for a resume of the changes, see Table 13.2).

13.5.1 Neuronal Mechanisms of A_1Rs

The in vivo models of seizure are extremely useful to address the consequences of adenosine manipulation for the control of seizures and epileptogenesis, as discussed in the previous section. Nonetheless, when trying to further explore and detail the specific mechanisms by which adenosine and adenosine receptors exert its effects, the use of in vitro models of seizures may prove extremely helpful. Indeed, how A_1Rs exert its anticonvulsant effects was first advanced by Lee and co-workers in vitro by demonstrating that A_1R-mediated suppression of epileptiform activity was chemical synapse independent (Lee et al. 1984). The authors proposed that a hyperpolarizing effect of A_1Rs, through blockage of outward K^+ currents, might be involved in the process. Indeed, some years later, adenosine A_1Rs were shown to change K^+ channels not by inhibiting outward K^+ currents but instead by facilitating G-proteins coupled inwardly rectifying K^+ channels (GIRKs) (Trussell and Jackson 1987), ATP-sensitive K^+ channels (K_{ATP}) (Li and Henry 1992) and small conductance Ca^{2+}-activate K^+ channels (SK) (Clark et al. 2009) and in this way regulate

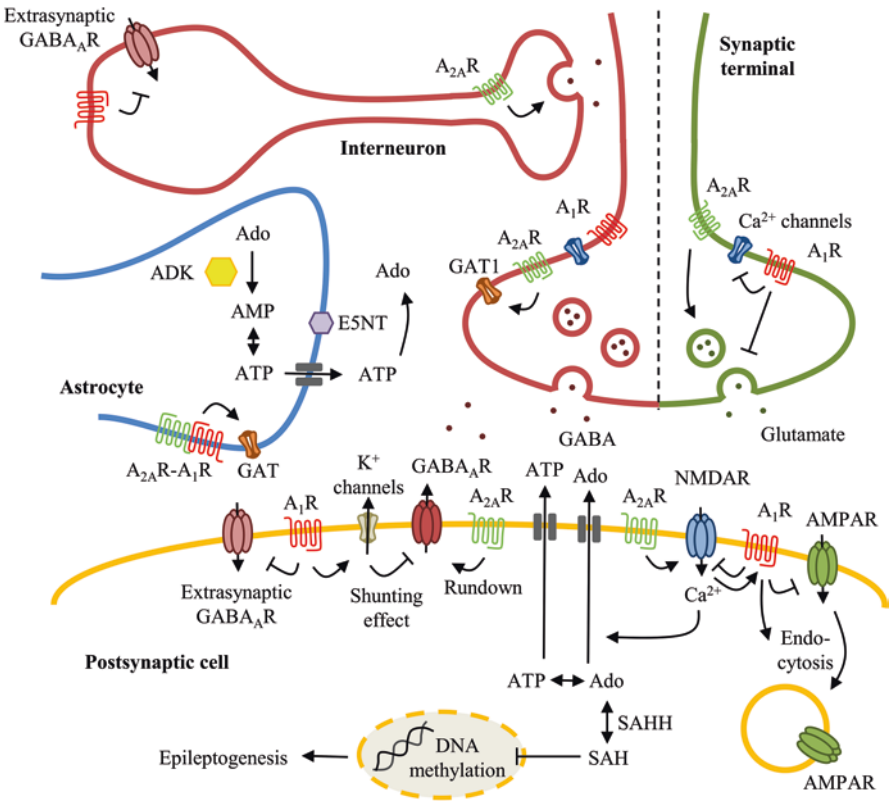

Fig. 13.2 Actions of adenosine during epileptic seizures. The mechanism by which adenosinergic system exerts its anticonvulsant actions is shown. These can be separated in neuronal mechanisms, which include A_1R and $A_{2A}R$ presynaptic actions (on glutamate and GABA release) and postsynaptic actions (through potassium channels, AMPAR, NMDAR and $GABA_AR$s); non-neuronal mechanisms, involving astrocytes and regulation of adenosine levels (through changes in density and activity of ADK, E5NT, GAT or ENT); and homeostatic and epigenetic control, by modulating DNA methylation status and epileptogenesis. See text for further details and references. A_1R, A_1 receptor; $A_{2A}R$, A_{2A} receptor; Ado, adenosine; ADK, adenosine kinase; AMP, adenosine 5'-monophosphate; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ATP, adenosine 5'-triphosphate; E5NT, ecto-5'-nucleotidase; ENT, equilibrative nucleoside transporter, GABA, gamma-aminobutyric acid; $GABA_AR$, GABA type A receptor; GAT, GABA transporter; NMDAR, N-methyl-D-aspartate receptor

local depolarization of neurons through hyperpolarization (Ehrengruber et al. 1997). This is still considered one of the main mechanisms that contribute to A_1R-dependent suppression of seizures.

Many other alternatives and complementary mechanisms have been proposed to explain adenosine actions on the different forms of epileptic seizures (Fig. 13.2). As discussed before, seizures have traditionally been viewed as caused by an imbalance between excitation (too much) and inhibition (too little) that compromise normal network functioning. Hyper-excitation may be caused by ionic (inward sodium or

calcium current) changes or excitatory neurotransmission dysfunction (mostly glutamate); hypo-inhibition may result from dysregulation of potassium or chloride currents or disturbances in GABA-mediated neurotransmission. Most of these systems are also targets of adenosine receptors. In fact, in parallel with the postsynaptic hyperpolarizing effect already mentioned, A_1Rs also control epileptiform activity by altering glutamatergic and GABAergic transmission.

Considering glutamatergic transmission, we can distinguish pre- and postsynaptic actions of adenosine. Presynaptically, A_1Rs reduce the release probability of glutamate through inhibition of voltage-dependent Ca^{2+} channels (VDCCs) (MacDonald et al. 1986; Schubert et al. 1986; Wu and Saggau 1994) or reduction of Ca^{2+}-independent spontaneous release (Scholz and Miller 1992; Scanziani et al. 1992), with demonstrated anticonvulsant consequences in kainic acid-induced and picrotoxin-induced models of seizures (Arvin et al. 1989; Wang et al. 2013). Postsynaptically, actions are mostly related with interactions with AMPARs and NMDARs. Increased neuronal excitability during seizures leads to increased glutamate release and activation of glutamate receptors. Both AMPARs and NMDARs have been implicated in the initiation and establishment of epileptic seizures (Rice and Delorenzo 1998; Kharazia and Prince 2001; Kohl and Dannhardt 2001; Zhang et al. 2003). Suppression of A_1R tonus in vitro causes a sustained synaptic-mediated epileptiform activity that has an AMPAR-dependent (Alzheimer et al. 1993; Moschovos et al. 2012) and NMDAR-dependent component (Thümmler and Dunwiddie 2000). Antiepileptogenic actions through AMPARs may be explained by a recent finding showing that A_1Rs mediate a persistent synaptic depression of glutamatergic transmission through AMPAR endocytosis (Chen et al. 2014). Through NMDARs, A_1R modulation involves the control of synaptic transmission and synaptic plasticity phenomena (de Mendonça et al. 1995; De Mendonça and Ribeiro 2000) that have implications during intense neuronal activity. For example, hampering A_1R activation during burst activity will result in overactivation of NMDARs and consequent induction of maladaptive NMDAR-dependent plasticity phenomena responsible for maintaining and exacerbating disruptive circuits and seizures (Thümmler and Dunwiddie 2000). In a Mg^{2+}-free in vitro model of seizures, the epileptiform activity gated by NMDAR activation (Nowak et al. 1984) is reduced after activation of A_1R (O'Shaughnessy et al. 1988). Also, in a 3-MP model of seizures, A_1Rs prevent the adaptive changes of NMDAR system (decrease in NR2B subunit expression) that occur as a consequence of seizures (Giraldez and Girardi 1998; Girardi et al. 2010). Together, these studies support the idea of a protective role of endogenous adenosine A_1R activation through NMDARs by keeping excessive excitability, thus checking and preventing epileptogenesis, which has been observed both ex vivo (Alzheimer et al. 1989; Hamil et al. 2012) and in vivo (Hamil et al. 2012). Reciprocal modulation has also been observed between A_1Rs and NMDARs. In fact, NMDAR activation during synchronized neuronal activity is another mechanism that explains the rising levels of adenosine during seizures (Hoehn and White 1990a, b; Manzoni et al. 1994). Also, chronic activation of NMDARs (in doses insufficient to cause behaviourally effects) promote a shift in A_1R function towards a high-affinity state (Von Lubitz et al. 1995b) that results in increased potency of protective effects of A_1Rs following a new and intensive insult (Von Lubitz et al. 1994b). The interplay

between A_1R and NMDAR contributes in this way to explain part of the neuroprotective actions of A_1Rs during stablished epileptic activity.

In the case of inhibitory transmission, adenosine A_1Rs have proven to directly influence GABA function in several brain areas (Sebastião et al. 2015). At the hippocampus, however, strong evidence exists about the lack of A_1R effects on phasic GABAergic transmission (Yoon and Rothman 1991; Lambert and Teyler 1991; Prince and Stevens 1992). Alternative mechanisms (Rombo et al. 2016b) include direct influence on GABA type A receptor ($GABA_AR$), the ionotropic receptor for GABA (Petersen 1991; Concas et al. 1993) and control of tonic inhibition (Rombo et al. 2016a). While evaluating control of GABAergic function at the hippocampus, it is important to distinguish between control of GABAergic inputs to inhibitory neurons and control of GABAergic inputs to excitatory neurons. A_1R-mediated decrease in tonic inhibition seems to be less relevant in pyramidal neurons than in interneurons (Rombo et al. 2016a), thus most probably contributing to an overall decrease in pyramidal neuron excitability. Whether the A_1R-mediated inhibition of $GABA_AR$ function reported by Concas et al. (1993) also predominates in interneurons rather than in pyramidal neurons is not known.

A complex interaction seems to exist between adenosine and $GABA_AR$ recognition sites, including sites for allosteric ligands (such as benzodiazepine). There is nevertheless evidence that A_1R-mediated influence over GABA-dependent transmission contributes to the anticonvulsant actions of adenosine (Klitgaard et al. 1993). Some authors document an interference of adenosine analogues on $GABA_AR$ number (Skolnick et al. 1980; Davies 1985) and binding properties (Davies 1985). Others show no effects of adenosine and adenosine receptors on $GABA_AR$ affinity (Williams et al. 1981). Another mechanistic explanation for these actions is that A_1R activation would exert a shunting effect over $GABA_AR$-mediated chloride conductance through facilitation of K^+ channels (Ilie et al. 2012). This effect is particularly relevant during intense neuronal activity, when $GABA_AR$ responses transiently switch from hyperpolarizing to depolarizing and excitatory (Thompson and Gähwiler 1989; Staley et al. 1995; Kaila et al. 1997). In such conditions of increased GABA and adenosine concentrations, inhibition of tonic GABA responses in principal neurons would further suppress network excitability (Ortiz and Gutiérrez 2015; Rombo et al. 2016a). Control of GABA levels by regulating GABA transporter (GAT) function in nerve terminals and astrocytes, in an activity-dependent manner, may also contribute to these actions (Cristóvão-Ferreira et al. 2009, 2013). To our knowledge, besides A_1R-GABA interplay to refrain epileptiform activity during ongoing seizures, there are no studies relating these two systems in the prevention of epileptogenesis and control of mechanisms that lead to stablished epilepsy.

Together, the above-mentioned studies provide experimental evidence to support the general consensus that neuronal anticonvulsant and antiepileptogenic mechanisms of adenosine A_1Rs are mostly mediated by changes in excitatory transmission and hyperpolarization of principal neurons. Other complementary mechanisms affecting GABA-mediated transmission may interfere to further potentiate endogenous A_1R-mediated antiseizure actions and reestablishment of normal network functioning.

13.5.2 Neuronal Mechanisms of A_{2A}Rs and A_3Rs

In opposition to A_1R actions, both A_{2A}Rs and A_3Rs are mostly considered excitatory and proconvulsant. This is supported by in vitro studies where these receptors contribute to the progression of seizures (Fig. 13.2).

Ex vivo, antagonists of A_{2A}Rs restrain epileptiform activity (Etherington and Frenguelli 2004; Rombo et al. 2015), and A_{2A}R agonists show a proconvulsant action (Klitgaard et al. 1993; Longo et al. 1995). The exact mechanisms by which A_{2A}R blockade confers neuroprotection in epilepsy are not completely understood, since most of what is known is from models of brain damage after ischemia (Cunha 2005). It is, however, reasonable to think that similar mechanisms may also be present during excessive neuronal activation generated from seizure events. This would include control of glutamate release, where A_{2A}R blockade protects neurons from excitotoxic glutamate outflow induced by ischemic stimuli (Popoli et al. 2002; Melani et al. 2003; Marcoli et al. 2003). Other possible mechanisms include prevention of neurotoxicity induced by AMPARs (Dias et al. 2012, 2013) and NMDARs (Robledo et al. 1999; Wirkner et al. 2004). However, ex vivo models of seizures show complex modulatory actions of A_{2A}Rs, since while facilitating NMDAR-independent induction of persistent epileptiform activity, A_{2A}Rs also suppress NMDAR-dependent progression of neuronal discharges (Moschovos et al. 2012). A novel mechanism by which brain-wide ADK deficiency (with significantly increased brain adenosine levels) leads to epileptic phenotype includes increased activation of A_{2A}Rs and disruption of synaptic plasticity phenomena, through a mechanism that involves A_{2A}R-mediated exacerbation of BDNF functioning (Sandau et al. 2016). Despite the evidence of direct effect of A_{2A}R activation on excitatory glutamatergic synapses, this may not completely explain the proconvulsive actions of these receptors. In fact, facilitation of epileptiform activity through A_{2A}Rs can also be explained by synergistic disinhibition of pyramidal cells through GABAergic communication between interneurons (Rombo et al. 2015). Direct actions of A_{2A}Rs on GABA function are also observed in human epileptic tissue, where endogenous A_{2A}R activity was shown to increase $GABA_A$R instability and consequent rundown of GABAergic responses (Roseti et al. 2008, 2009). Precluding these actions by preventing A_{2A}R activation during epileptiform activity has significant anticonvulsant effects (Roseti et al. 2008; Rombo et al. 2015).

Much less is known about A_3Rs, and as discussed above there is evidence for anti- as well as proconvulsive actions of A_3R agonists. Their endogenous contribution to the promotion of epileptiform activity is limited, although its blockade is still capable of attenuating seizure intensity (Etherington and Frenguelli 2004). The effects may be direct or indirect, through A_1Rs, influencing presynaptically glutamatergic transmission or postsynaptically K^+ channels function (Dunwiddie et al. 1997) and desensitization of $GABA_A$Rs (Roseti et al. 2009).

Despite scarce information about the operating mechanisms exerted by A_{2A}Rs and A_3Rs during epileptiform activity, it is evident that blockade of A_{2A}R, and most probably also of A_3R, would prevent their synaptic neurotoxic effects responsible for aggravating neuronal excitability and epileptiform activity.

13.5.3 Non-neuronal Mechanisms of Adenosine

Astrocytes play a pivotal role in the generation and propagation of epileptic seizures by controlling synchronization of neuronal firing, ion homeostasis, reuptake of neurotransmitters and neuromodulators and release of gliotransmitters (Seifert et al. 2010). In what concerns adenosine metabolism, astrocytes have been implicated in regulating the levels of endogenous extracellular adenosine by directly participating in the adenosine cycle (Fig. 13.2). This involves (1) neuronal and astrocyte release of ATP (Pascual et al. 2005; Fields and Burnstock 2006), (2) extracellular formation of adenosine via a cascade of ecto-nucleotidases (Zimmermann 2000) and (3) uptake of adenosine back to neurons and astrocytes through ENT (King et al. 2006). The extracellular levels of adenosine are primarily controlled by actions of the astroglial enzyme ADK (Boison 2006). Perturbation of astrocyte homeostasis can affect the adenosinergic system and disrupt its neuroprotective effects. In fact, astrogliosis occurring in the epileptic brain is associated with increased expression of ADK and consequent depletion of adenosine levels, further exacerbating seizures (Gouder 2004; Li et al. 2008; de Groot et al. 2012). This evidence led to the proposal of an "ADK hypothesis of epileptogenesis" (Boison 2008, 2016a) based on biphasic changes in adenosine homeostasis during disease progression. A dysregulation of the normal brain functioning (due to structural, genetic, infectious, immune or metabolic causes) may result in an imbalance between excitatory and inhibitory mechanisms, thus leading to a seizure event. The immediate response to the insult consists of an acute surge in adenosine levels that is potentiated by acute downregulation of ADK (Gouder 2004), resulting in the termination of seizure. However, these seizure control actions may come with a price. Besides neuroprotective A_1R activation, seizure event and adenosine rise will also contribute to adaptive changes that occur in the adenosinergic system including (1) downregulation of inhibitory A_1Rs; (2) upregulation and overactivation of excitatory A_{2A}Rs; (3) switch in ADK expression, from decreased levels to sustained ADK overexpression (Gouder 2004); and (4) exacerbation of astrogliosis (Fiebich et al. 1996; Gebicke-Haerter et al. 1996; Bouilleret et al. 1999). All these adaptive changes contribute for the progression of the epileptogenic process (Li et al. 2007a). In fact, once astrogliosis and ADK overexpression are stablished, recurrent seizure onset is more likely (Li et al. 2007a, 2008, 2012a). Therefore, dysregulation of astrocyte and ADK functioning plays a significant role in the process that turns a normal brain into an epileptic brain (Boison 2016a).

13.5.4 Homeostatic and Epigenetic Mechanisms

One of the main actions of adenosine in mammalian cells is to control cell metabolism. Adenosine is in a privileged position to do this since minor changes in intracellular ATP concentrations as a result of metabolic challenges will result in disproportional larger changes in extracellular concentrations of adenosine (Cunha 2001). Besides the adenosine receptor-dependent actions discussed in the previous sections, adenosine exerts a more global biochemical regulation of cell function by means of epigenetic control, through DNA methylation (Williams-Karnesky et al. 2013) (Fig. 13.2). The classical source of adenosine is from a hydrolysing cascade reaction from ATP to ADP and to AMP by ectonucleotidases. However, another important source of adenosine is the hydrolysis of S-adenosyl-L-homocysteine (SAH) by SAH hydrolase (SAHH) (Schrader et al. 1981). SAH is involved in the transmethylation pathway responsible for methylation of DNA (James et al. 2002). Alterations in adenosine levels will thus influence SAH levels and DNA methylation. These epigenetic modifications are responsible for altering gene transcription without modifying the underlying DNA sequence. Methylation of the DNA has already been described in cells from the CNS (Ma et al. 2009), and it is responsible for some of the pathological changes observed during epileptogenic process (Henshall and Kobow 2015). When adenosine levels rise (e.g. during a seizure event), there is a shift in the equilibrium of the SAHH reaction towards the formation of SAH. Rises in SAH levels block DNA methyltransferase activity and decrease global DNA methylation levels (Williams-Karnesky et al. 2013). In this phase, adenosine-induced epigenetic mechanisms may be responsible for transcription and expression of epileptogenesis initiating genes (Boison 2016a). In later stages of epileptogenesis, increased ADK expression leads to a decrease in adenosine levels and consequent establishment of an hypermethylated DNA status that will further aggravate the epileptogenic condition (Miller-Delaney et al. 2015; Boison 2016b). These studies highlight a novel modulatory mechanism of adenosine in the control of epileptogenesis involving DNA methylation and epigenetic control.

13.6 Adenosine-Based Therapies

The experimental evidence discussed above about the anticonvulsant, neuroprotective and antiepileptogenic properties of adenosine provides the rational for the use of adenosine augmentation therapies in the treatment of epileptic seizures and epilepsy. This section intends to briefly discuss the most prominent approaches to increase adenosine levels in brain and holt synchronized neuronal activity and seizure progression.

13.6.1 Focal Adenosine Augmentation

One of the main reasons to prefer focal adenosine delivery approaches instead of systemic drug use is to avoid the considerable peripheral side effects of adenosine (mostly cardiovascular). Focal approaches are considered safe and feasible alternatives given the focal nature of many forms of epilepsy (Nilsen and Cock 2004). Tools for focal delivery include polymeric brain implant (Wilz et al. 2008), cell-based therapy and gene therapy (Löscher et al. 2008). To increase the concentrations of adenosine in the epileptic focus and supress seizure, the most effective strategy is by disrupting metabolic adenosine clearance through manipulation of ADK activity.

Starting with gene therapy approaches, two strategies have been used to augment adenosine levels: (1) antisense cDNA to disrupt the endogenous *Adk* gene (Theofilas et al. 2011) and (2) RNA interference (RNAi) to knock down ADK expression (Ren et al. 2007; Boison 2010). Both strategies can effectively be used to engineer focal release of adenosine, but additional studies are needed to evaluate its effectiveness in clinically relevant models of epilepsy (Boison 2016a).

Cell therapy approaches consist of injecting into the brain cell-derived implants that will exert its anticonvulsant actions via paracrine release of adenosine (Nilsen and Cock 2004). This method proved to be extremely efficient in supressing seizures and epileptogenesis (Huber et al. 2001; Li et al. 2007b, 2008, 2009).

Silk-based adenosine delivery strategies have unique therapeutic properties that bring them closer to clinical implementation. These properties include high biocompatibility and slow degradation kinetics (Horan et al. 2005). As occurred with cell-based therapies, polymeric brain implants were effective either when implanted before seizure induction (Wilz et al. 2008) or after full establishment of epilepsy (Szybala et al. 2009).

Together, data briefly discussed above suggests that focal adenosine augmentation therapies are promising strategies for preventing seizure occurrence and hamper epileptogenesis progression.

13.6.2 Dietary Therapies

Dietary therapies are effective, safe, non-pharmacologic treatments for intractable epilepsy, especially in children (Payne et al. 2011). The most used type of diet is the ketogenic diet (KD). This is a high-fat, low-carbohydrate, adequate-protein diet that has been used since the 1920s but resurged in popularity over the past 15 years (Wheless 2008). Despite its long clinical use, the mechanism by which KD suppresses seizure is not completely clarified (Rogawski et al. 2016). The hallmark of KD is the production of ketone bodies by the liver and its use as primary energy source. The anticonvulsant mechanisms include (1) ketone body-mediated inhibition of glutamate release and activation of K_{ATP}; (2) increased GABA synthesis; (3)

increased mitochondrial function and biogenesis (with consequent rise in ATP production); (4) decreased production of reactive oxygen species; and (5) increased adenosine concentration, among others. The adenosine and adenosine receptor-mediated actions are indeed one of the key mechanisms underlying the anticonvulsant actions of KD (Masino et al. 2014). It was shown that reduction of seizure with KD is caused by increased adenosine signalling in the brain (Masino and Geiger 2008, 2009, Masino et al. 2011, 2012), mostly through activation of A_1Rs (Masino et al. 2011). It is now clear the beneficial effects of the KD in the treatment of several forms of epilepsy (Neal et al. 2008; Lambrechts et al. 2017). The success of this strategy probably relies on its strong multifactorial mechanisms, and adenosine A_1R-mediated anticonvulsant actions significantly contribute for this.

13.7 Conclusions and Future Perspectives

Accumulated evidence from the past 50 years of research on adenosine actions to control the initiation and progression of seizure events and epilepsy were briefly reviewed in this chapter. There are three main conclusions we can take from this data:

1. Endogenous adenosine has a true anticonvulsant capacity and antiepileptogenic potential, mostly through A_1R activation. However, recent data have been demonstrating the benefits of concomitantly modulating A_{2A}R and even A_3R to further potentiate its actions. The therapeutic use of adenosine depends on the stage of disease progression (acute *vs* chronic) and the targeting receptor and should always take into consideration its peripheral side effects (mostly cardiovascular-related).
2. There are considerable adaptations of the adenosinergic system during the epileptogenic process that influences the mechanisms of action and disease progression. The anticonvulsant actions are exerted (I) at the synaptic level, mostly through A_1R activation of potassium channels but also by A_1R and A_{2A}R control of glutamate and GABA function at pre-, post- and peri-synaptic compartments; (II) at non-neuronal level, by changing adenosine concentration through ADK expression in astrocytes; and (III) at homeostatic control level, by affecting DNA methylation status and consequently, the epileptogenic process.
3. Several strategies are being developed to take advantage of all the potential of adenosinergic manipulation in the control and treatment of seizure events and prevent epileptogenesis. These include therapeutic adenosine augmentation strategies ranging from gene therapy to dietary intervention (such as ketogenic diet). The concomitant use of adenosine strategies together with conventional AED therapies currently available may potentiate and expand the efficacy of the intervention.

The diversity of adenosine effects and mechanisms of action are, indeed, an advantage for its use in epilepsy. However, this capacity of adenosine to act as a

"universal modulator or maestro" of network functioning may come with a price. In fact, attention should be given to clearly differentiate the anti- and proconvulsant actions of adenosine that result from activation of different receptors at different neuronal and non-neuronal compartments. The mechanisms by which adenosine exerts its influence to regulate excitability in physiological, but mostly during pathophysiological situations, should thus be further understood. The challenge will be to take advantage of the homeostatic role of adenosine without never losing sight on the highly selective and sometimes opposing effects that are exerted by adenosine through its different receptors. Attention should thus be given not only to further develop strategies for increasing adenosine levels in the brain with the least peripheral side effects possible but also in the development of innovative adenosine ligands specifically directed to affect relevant neuronal targets, leaving untouched the ones not involved in the pathology.

As a final remark, and considering epileptogenesis itself, where too little is known on the influence of adenosine, inflammatory pathways have been highlighted as crucial in the underlying molecular mechanisms of epilepsy (van Vliet et al. 2018). Adenosine receptors, in particular $A_{2A}R$ (Chen and Pedata 2008; Dai and Zhou 2011) and A_3R (Jacobson et al. 2017), interfere with the inflammatory cascade in a multiplicity of ways. There is thus time to also explore this avenue to better predict the influence of adenosine-based therapies in epilepsy.

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Chapter 14
Adenosine and Oxygen/Glucose Deprivation in the Brain

Felicita Pedata, Ilaria Dettori, Lisa Gaviano, Elisabetta Coppi, and Anna Maria Pugliese

Abstract Extracellular adenosine concentrations in the brain increase dramatically during ischemia in concentrations that able to stimulate all (A_1, A_{2A}, A_{2B}, and A_3) receptors. Adenosine exerts a clear neuroprotective effect through A_1 receptors during ischemia mainly by reducing precocious excitotoxic phenomena. Unfortunately, the use of selective A_1 agonists is hampered by undesirable peripheral effects. Evidence indicates that A_{2A} receptor antagonists administered early after ischemia provide protection centrally by reducing excitotoxicity. After ischemia, the primary damage due to the early massive increase of extracellular glutamate is followed by activation of resident immune cells, i.e., microglia, and production or activation of inflammation mediators and blood cell infiltration. Evidences are that agonists at $A_{2A,}$ A_{2B}, and A_3 receptors mainly acting on blood and vascular endothelial cells provide protection by controlling neuroinflammation, endothelial leaking, and massive blood cell infiltration in the hours and days after brain ischemia. Since ischemia is a multifactorial pathology characterized by different events evolving in the time and protracted neuroinflammation is recognized as the predominant mechanism of secondary brain injury progression, adenosinergic drugs aimed at dampening damage in the hours/days after ischemia appear promising.

Keywords Brain ischemia · Oxygen/glucose deprivation · Adenosine receptors · Glutamate · Neuroinflammation

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14.1 Introduction

Stroke is today evaluated as the second most common cause of death and a major cause of long-term disability worldwide. Ischemic stroke commonly accounts for approximately 80% of all stroke cases, and is caused from occlusion of a major cerebral artery by a thrombus or an embolism, which leads to loss of cerebral blood flow, a condition of hypoxia and glucose deprivation (oxygen/glucose deprivation: OGD) and subsequently tissue damage in the affected region. The only successful pharmacological treatment approved to date is tissue plasminogen activator (tPA) that aims to decrease ischemia-associated thrombosis risk. Yet, because of the narrow therapeutic time window involved, thrombolytic application is very restricted in clinical settings (Chen et al. 2014). Aspirin, other antiplatelets, and anticoagulants are used as preventive therapy of stroke (Macrez et al. 2011).

After stroke, brain injury results from a complex sequence of pathophysiological events consequent to hypoxia/ischemia that evolve over time (Dirnagl 2012). A primary acute mechanism of excitotoxicity and periinfarct depolarizations is due to increased extracellular concentration of glutamate (see Fig. 14.1). Excitotoxicity brings to activation of resident immune cells, i.e., microglia, and production or activation of inflammation mediators. In the hours and along days after ischemia, protracted neuroinflammation is recognized as the predominant mechanism of secondary brain injury progression (Tuttolomondo et al. 2009). Activated microglial cells proliferate, migrate, and, by production of inflammatory substances and chemokines, trigger an inflammatory response (Dirnagl et al. 1999). Pro-inflammatory mediators and oxidative stress give rise to the endothelial expression of cellular adhesion molecules and to an altered permeability of the blood-brain barrier (BBB) that allows infiltration of leukocytes that on their turn exacerbate neuroinflammation and ischemic damage (Haskò et al. 2008; Iadecola and Anrather 2011). A huge increase of extracellular adenosine concentrations matches the increase of glutamate in the first hours after ischemia (see Fig. 14.1) as demonstrated under OGD conditions in vitro in the hippocampus (Dale et al. 2000; Frenguelli et al. 2007; Latini et al. 1998; Pedata et al. 1993) and in the in vivo models of brain ischemia (Dux et al. 1990; Hagberg et al. 1987; Matsumoto et al. 1992; Melani et al. 1999; Sciotti et al. 1992). In the first minutes after ischemia, the increase of extracellular adenosine concentration is due to the major part of extracellularly released ATP that is hydrolyzed by ectonucleotidases, and then, in the hours after ischemia, adenosine per se is mainly released from cells (Melani et al. 2012). After in vivo ischemia, the extracellular concentrations of adenosine are high enough to stimulate all adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3 receptors) (Melani et al. 2012). All receptor subtypes are expressed at significant levels in neurons and glial cells and in peripheral blood inflammatory cells (Burnstock and Boeynaems 2014) (see Fig. 14.2). The wide distribution is consistent with the multifaceted neurochemical and molecular effects of adenosine and suggests that adenosine role in ischemia is the consequence of an interplay among different receptor activations in neuronal, glial, and inflammatory cells, which varies depending on the time-related development of the

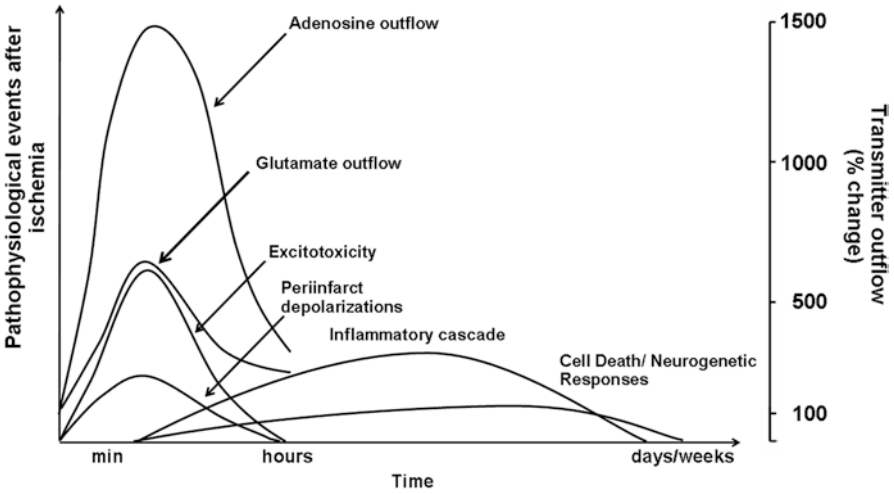

Fig. 14.1 Cascade of pathogenetic mechanisms after ischemia. Primary mechanisms of excitotoxicity lead to acute cell death in the ischemic core. Depolarization spreads in the periinfart areas. Glutamate and extracellular adenosine concentrations increase in the first 4 h after ischemia (Melani et al. 1999, 2003, 2012). The curves of increases of glutamate and adenosine evoked by ischemia and induced by middle cerebral artery occlusion (MCAo) were drawn on the basis of values obtained by striatal microdialysis (Melani et al. 1999). In the following several hours, activation of resident immune cells, i.e., microglia and production of a cascade of inflammation mediators, occurs. Cell death/neurogenetic responses progress along days/weeks after ischemia (figure modified from Dirnagl et al. 1999). Putative therapeutic opportunities with purinergic drugs comprehend strategies aimed at reducing excitotoxicity in the first 4 h after ischemia with adenosine A_{2A} and A_{2B} receptor antagonists. In the hours and days after ischemia, agonists of adenosine A_{2A}, A_{2B}, and A_3 receptors peripherally located on vascular and blood cells may dampen vascular adhesion signals and neuroinflammation

pathological condition. Numerous authors have proposed adenosine and adenosine receptors as important targets for therapeutic implementation in the treatment of stroke.

14.2 Role of Adenosine Receptors in Ischemia

The increase in extracellular adenosine early after ischemia has long been known as an endogenous neuroprotective response (Pedata et al. 2007). In fact, adenosine infusion into the ischemic striatum has been shown to significantly ameliorate neurological outcome and reduce infarct volume after transient focal cerebral ischemia (Kitagawa et al. 2002). Adenosine protection has been attributed to stimulation of the A_1 receptor subtype; however important roles of the other three receptor subtypes have been outlined in the last 20 years.

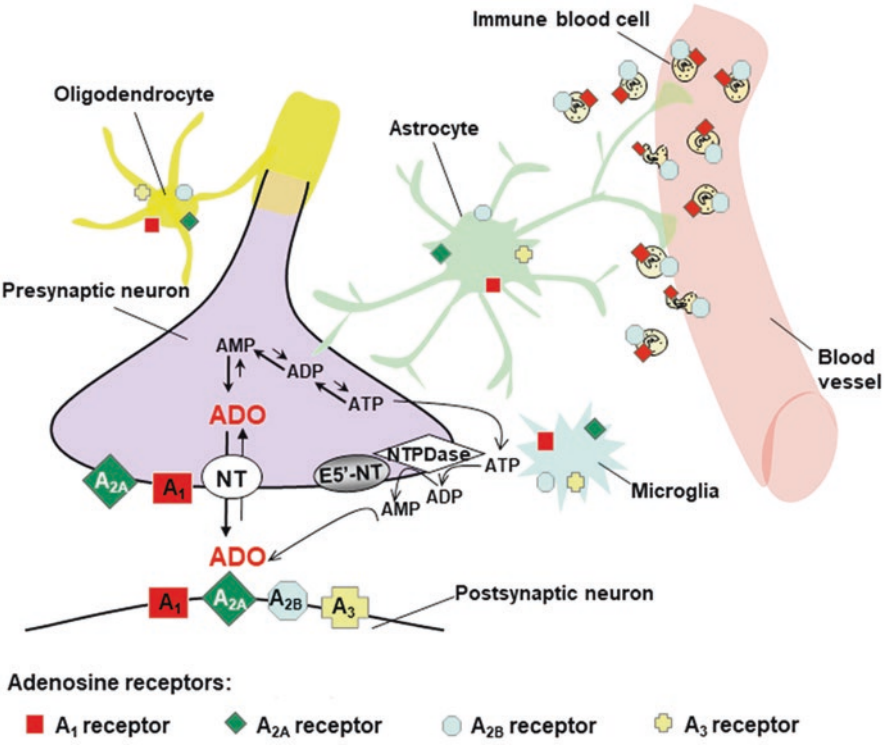

Fig. 14.2 Schematic drawing of adenosine receptors on different cell types. All adenosine receptor subtypes are expressed both at the central level on presynaptic and postsynaptic neurons, on astrocytes, on microglia, and on oligodendrocytes and at the peripheral level on leukocytes and vasculature. After cerebral ischemia, leukocytes infiltrate into ischemic tissue due to increased permeability of BBB. During ischemia, extracellular adenosine levels increase mainly due to (i) extracellular ATP degradation by NTPDase and ecto-5′-nucleotidase enzymes; (ii) release per se from cells likely by the equilibrative nucleoside transporter (ENT) (Melani et al. 2012); and (iii) inhibition of adenosine uptake processes due to downregulation of concentrative nucleoside transporters (CNT) 2 and 3 and of ENT. ADO, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; E5'-NT, ecto-5'-nucleotidase; NT, nucleoside transporter; NTDPase, ectonucleoside triphosphate diphosphohydrolases. The proportions of the various components of the nervous tissue have not been kept

14.2.1 Adenosine A_1 Receptors Are Protective

One of the prime adaptive mechanisms in response to hypoxia-ischemia is the cellular activation of adenosine A_1 receptors that inhibit excitatory synaptic transmission as demonstrated in vitro and in vivo (Latini and Pedata 2001). Adenosine protective effects are greatly attributed to adenosine A_1 receptor activation that due to reduced Ca^{2+} influx, lower presynaptic release of excitatory neurotransmitters (Corradetti et al. 1984; Dunwiddie 1984) and in particular glutamate which exerts an excitotoxic effect during ischemia mainly by overstimulation of NMDA

(Choi 1990) and AMPA receptors (Stockwell et al. 2016). In addition, by directly increasing the K^+ and Cl^- ion conductances, adenosine stabilizes the neuronal membrane potentials, thus reducing neuronal excitability (Choi 1990). Consequent reductions in cellular metabolism and energy consumption (Greene and Haas 1991) and moderate lowering of the body/brain temperature (Tupone et al. 2013; Muzzi et al. 2013) protect against ischemia. A continuous infusion of the adenosine A_1 receptor agonist (6)N-cyclohexyladenosine (CHA) that maintains the body temperature between 29 and 31 °C for 24 h induces better survival and decreases the extent of brain damage in rats subjected to asphyxial cardiac arrest for 8 min (Jinka et al. 2015; Tupone et al. 2016).

Consistent data demonstrate that adenosine acting on adenosine A_1 receptor reduces the ischemia-evoked increase of excitatory transmission. In brain slices, the OGD-induced depression of synaptic transmission is reversed by administration of selective adenosine A_1 receptor antagonists (Pedata et al. 1993) that also increase OGD-evoked aspartate and glutamate efflux (Marcoli et al. 2003), impair the recovery of synaptic potentials (Sebastião et al. 2001), and shorten the onset of anoxic depolarization (AD) induced by hypoxia (Lee and Lowenkopf 1993). Depression of excitatory synaptic transmission brought about by adenosine A_1 receptors during hypoxia/ischemia involves AMPA receptor downregulation (Stockwell et al. 2016) in particular the internalization of GluA1 and GluA2 subunit-containing AMPA receptors (Stockwell et al. 2016). Depression of excitatory synaptic activity and a cross talk with A_{2A} receptor are crucial for the functional recovery of hippocampal circuits upon reoxygenation when adenosine A_{2A} receptors play a critical role by increasing excitatory amino acid efflux (Stockwell et al. 2017). The A_1-mediated depression of excitatory synaptic transmission may also be due to the enhancement of inhibitory synaptic transmission in CA1 neurons (Liang et al. 2009).

In in vitro studies, both adenosine and selective A_1 receptor agonists reduce neuronal damage following hypoxia and/or OGD in primary cortical or hippocampal cell cultures (Daval and Nicolas 1994) and brain slices (Mori et al. 1992). A_1 receptor agonists increase survival in anoxia and anoxia/reoxygenation and decrease reactive oxygen species (ROS) production, while A_1 receptor blockade increases ROS release and cell death in primary neuronal cultures (Milton et al. 2007). Studies in support of the neuroprotective role of adenosine A_1 receptor stimulation demonstrate that hippocampal slices from A_1 receptor knockout (KO) mice showed a markedly reduced and delayed protective response to hypoxia compared to slices from wild-type (WT) mice (Johansson et al. 2001). In astrocytes prepared from A_1 receptor KO mice, more pronounced hypoxic cytotoxicity was observed (Bjorklund et al. 2008). In murine astrocytes exposed to hypoxic injury, adenosine, through activation of A_1 and A_3 receptors, inhibits accumulation of the lipopolysaccharide (LPS)-induced hypoxia-inducible factor-1 (HIF-1), a master regulator of oxygen homeostasis (Gessi et al. 2013).

In in vivo animal models of global cerebral ischemia, it has been demonstrated that local administration of an adenosine analogue, 2-chloroadenosine (CADO), and of a nonselective A_1 receptor agonist, N6-(L-2-phenylisopropyl) adenosine (L-PIA), attenuates neuronal loss in the CA1 region of the rat hippocampus

(Domenici et al. 1996; Evans et al. 1987). The acute systemic or intracerebroventricular (i.c.v.) injection of the A_1 agonists cyclohexyladenosine (CHA) and R-phenylisopropyl-adenosine (R-PIA) improves neurological deficits (Heron et al. 1994; Von Lubitz and Marangos 1990; Zhou et al. 1994), protects the CA1 region of the hippocampus (Von Lubitz et al. 1988), and prevents the reduction of adenosine A_1 receptors (Daval et al. 1989) in rats or gerbils. Similarly, acute administration of the A_1 agonists N6-cyclopentyladenosine (CPA) and 2-chloro-N(6)-cyclopentyladenosine (CCPA) reduces mortality and the loss of neurons after global forebrain ischemia in the gerbil (Von Lubitz et al. 1994a). Systemic administration of the A_1 receptor agonist adenosine amine congener (ADAC) after global ischemia in the gerbil increased survival, preserved neuronal morphology, and maintained spatial memory and learning ability (Phillis and Goshgarian 2001; von Lubitz et al. 1996).

Several intracellular mechanisms might account for adenosine A_1 receptor-mediated neuroprotection in hypoxia/ischemia. Postischemic intraperitoneal (i.p.) administration of adenosine amine congener (ADAC) resulted in preservation of microtubule-associated protein 2 (MAP-2) (von Lubitz et al. 1996). CCPA administered i.c.v. before focal ischemia reduces lipid peroxidation in the cerebral cortex (Sufianova et al. 2014). Chronic coadministration of CCPA and vitamin C i.p. after global ischemia, induced by common carotid arteries ligation, minimized ischemia-reperfusion damage by increasing the expression of antiapoptotic protein Bcl-2 and decreasing the expression of proapoptotic protein Bax in mice (Zamani et al. 2013).

In accordance with a protective role of adenosine A_1 receptors in ischemia, acute administration of adenosine A_1 antagonists exacerbates the damage (Phillis 1995). However, chronic administration of adenosine receptor antagonists administered before an ischemic insult reduced the neuronal injury (Rudolphi et al. 1989), and chronic administration of A_1 agonists worsened survival and increased neuronal loss (Jacobson et al. 1996). It has been suggested these *phenomena* depend on A_1 receptor upregulation and desensitization, respectively.

Plastic changes in A_1 receptors are critical to understand the effects of adenosine A_1 agonists/antagonists but also whether adenosine maintains its neuroprotective efficiency after ischemia. Several studies have shown that short periods of focal or global ischemia produced a long-lasting decrease in the density of A_1 receptors (Lee et al. 1986). In rat hippocampal slices, hypoxia leads to a rapid (<90 min) desensitization of A_1 receptor that is likely due to an internalization of A_1 receptors in nerve terminals (Coelho et al. 2006), a process that may result in hyperexcitability and increased brain damage. In a chronic cerebral ischemic mouse model induced by common carotid artery occlusion, A_1 receptor downregulation, a decreased proteolipid protein (a marker of white matter myelination), inhibition of the anti-inflammatory interleukin-10 (IL-10) production, and cognitive impairment measured by the Morris water maze test have been reported (Cheng et al. 2015). However it has been reported that A_1 receptor KO mice, when exposed to global ischemia, do not show increased neuronal damage in the CA1 region of the hippocampus, in the cortex, or in the striatum (Olsson et al. 2004). These discrepancies may reflect development of compensatory mechanisms after genetic deletion.

In models of hypoxia-ischemia in neonatal rats, it was reported that A_1 receptors contribute to protection of hypoxic brain (Bona et al. 1997). In agreement, most

recently it has been reported that A_1 receptor KO neonatal mice, from 10 to 17 days after brain hypoxia/ischemia, displayed larger infarctions, cognitive impairment, and exaggerated activation of myeloid cells (Winerdal et al. 2016). Since inflammation greatly affects the outcome after neonatal brain injury, activation of myeloid cells is proposed as cause of the increased damage in A_1 receptor KO neonatal mice, (Winerdal et al. 2016). Thus, the decrease of adenosine A_1 receptors (Aden et al. 1994) and increase of adenosine deaminase (Pimentel et al. 2015) that has been described after rat neonatal hypoxia/ischemia would worsen hypoxic brain damage in neonatal period. On the other end, adenosine acting on A_1 receptors appears to mediate hypoxia-induced brain ventriculomegaly during early postnatal development (Turner et al. 2003). It should be remembered that in the formation of the central nervous system (CNS), A_1 receptor activation potently inhibits the development of axons and can lead to leukomalacia (Rivkees et al. 2001). Notably, caffeine, a competitive antagonist of adenosine A_1, A_{2A}, and A_{2B} receptors, that is commonly used in neonates against apnea of prematurity has become a candidate for neuroprotection (Schmidt et al. 2007).

Adenosine by stimulating A_1 receptors plays a crucial role in the "precondition phenomenon" consisting in protection by sublethal anoxic/ischemic insults from subsequent ischemic insults. The A_1 receptor agonist, CADO, markedly enhanced and A_1 receptor antagonists completely prevented the protective effect of ischemic preconditioning in rat hippocampal slices (Pugliese et al. 2003). In accordance with in vivo models of ischemia, the selective A_1 antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), attenuated the neuroprotective effect of ischemic preconditioning (Cui et al. 2013) and CCPA pretreatment-induced ischemic tolerance against cerebral ischemia/reperfusion injury induced by middle cerebral artery occlusion (MCAo) in the rat (Hu et al. 2012). Preconditioning induced also by limb remote ischemia contributes neuroprotective effects against rat focal cerebral ischemic injury induced by transient MCAo, and the selective A_1 antagonist DPCPX abolished the protective effects demonstrating the involvement of A_1 receptors (Hu et al. 2012). Interestingly ischemic preconditioning-induced neuroprotection appears transferable among cells through intervention of A_1 receptors as studied in human neuroblastoma SH-SY5Y cells (Yun et al. 2014). Peculiarly, adenosine A_1 receptors activation is involved in the ischemic tolerance in mice induced by a ketogenic diet (a high-fat, low-carbohydrate diet that increases acetyl-CoA that is involved in ketone body formation that represents an alternative energy source for brain cells under conditions of glucose deprivation) (Yang et al. 2017).

Although data, on the all, demonstrate a neuroprotective effect of adenosine through A_1 receptors during ischemia, the use of selective A_1 agonists is hampered by undesirable peripheral effects such as sedation, bradycardia, and hypotension. Interestingly, nowadays it is proposed that partial agonists at A_1 receptor may be devoid of hemodynamic effects being therefore valuable drugs in ischemia (Baltos et al. 2016). The possibility that new adenosine A_1 receptor partial agonists are protective in ex vivo and in vitro experimental models of ischemia was recently discussed by Martire and coworkers (personal communication 2016).

14.2.2 Adenosine A_{2A} Receptors in Brain Ischemia

14.2.2.1 Brain A_{2A} Receptors Increase Glutamatergic Excitatory Transmission

A_{2A} receptors play an important modulation of synaptic transmission counteracting depression brought about by A_1 receptor (Lopes et al. 2011). In the CA1 area of the rat hippocampus, the selective A_{2A} receptor agonist, 2-p-(2-carboxyethyl) phenethylamino-5′-Nethylcarboxamidoadenosine hydrochloride (CGS21680), clearly reduces the OGD-induced depression of synaptic activity (Latini et al. 1999). In agreement, the selective A_{2A} receptor antagonists, 4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM241385) and 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4,triazolo[1,5-c] pyrimidine (SCH58261), delay the appearance of AD, a phenomenon strictly related to cell damage and death (Somjen 2001), protect from the synaptic activity depression brought about by a severe (7 min) OGD period, and protect CA1 neuron and astrocyte from injury (Pugliese et al. 2009). The same effects of ZM241385 were observed after a severe 9 min OGD period in the *gyrus dentatus* of the hippocampus (Maraula et al. 2013).

Protective effects against OGD by A_{2A} receptor antagonists are greatly attributed to antagonism of excessive excitatory transmission. In fact adenosine A_{2A} receptor regulates glutamatergic excitatory transmission by several mechanisms. Adenosine by stimulating A_{2A} receptors located presynaptically on glutamatergic terminals can directly regulate glutamate outflow under normoxic (Lopes et al. 2002) and ischemic conditions (Marcoli et al. 2003). Moreover A_{2A} receptors modulate glutamate uptake transporter. In particular, A_{2A} receptors located on astrocytes mediate inhibition of glutamate uptake by glutamate transporter-1 (GLT-1) (Pinto-Duarte et al. 2005). An imbalance of A_1/A_{2A} receptor expression might also contribute to inhibition of excitatory synaptic transmission under ischemia. Short periods of global ischemia decrease A_1 adenosine receptor density in the brain likely due to an internalization of A_1 adenosine receptors in nerve terminals (Coelho et al. 2006), thus switching the balance toward A_{2A} receptor-mediated effects. Moreover, adenosine acting on A_{2A} receptor increases AMPA (Dias et al. 2012) and NMDA receptor function (Rebola et al. 2008).

All the above-described modulatory effects of the glutamatergic excitatory transmission by adenosine A_{2A} receptors might be relevant in in vivo ischemia. A definite overexpression of A_{2A} receptors was found in vivo in neurons of the striatum and cortex 24 h after focal ischemia (Trincavelli et al. 2008). The A_{2A} agonist CGS21680 increases excitatory amino acid outflow from the ischemic cortex during in vivo ischemia (O'Regan et al. 1992).

Several studies demonstrated that antagonists of adenosine A_{2A} receptors were protective in in vivo models of global ischemia. Gao and Phillis (1994) demonstrated for the first time that the nonselective A_{2A} receptor antagonist, 9-chloro-2-(2-furanyl)-[1,2,4] triazolo[1,5-c]quinazolin-5-amine (CGS15943),

reduced cerebral ischemic injury in the gerbil following global forebrain ischemia. Thereafter many reports have confirmed the neuroprotective role of A_{2A} receptor antagonists in different models of ischemia. The selective A_{2A} receptor antagonist, 8-(3-chlorostyryl)caffeine (CSC), and the less selective antagonists, CGS15943 and 4-amino [1,2,4] triazolo [4,3a] quinoxalines (CP66713), both administered preischemia and protected against hippocampal cell injury during global forebrain ischemia in gerbils (Phillis 1995; von Lubitz et al. 1995). The selective A_{2A} receptor antagonist, ZM241385, administered preischemia, reduced hippocampal injury, and improved performance in the Morris water maze in hyperglycemic four-vessel occluded rats (Higashi et al. 2002). In all these studies, adenosine A_{2A} receptor antagonists were administered preischemia. However, postischemic administration is more relevant to a possible clinical use of drugs in stroke. The selective A_{2A} receptor antagonist, SCH58261, acutely administered after hypoxia/ischemia in neonatal rats reduced brain damage (Bona et al. 1997) and acutely administered i.p. 5 min after focal ischemia in adult rats was protective from brain damage 24 h thereafter (Melani et al. 2003). The same antagonist, administered subchronically (i.p., 5 min, 6 and 15 h) after focal ischemia, was protective not only against brain damage but also from neurological deficit (Melani et al. 2006, 2009; Pedata et al. 2005) and disorganization of myelin (Melani et al. 2009) 24 h after focal cerebral ischemia in the adult rat. In the model of global ischemia (i.e., 7 min asphyxic cardiac arrest) in newborn piglets, posttreatment infusion with SCH58261 improved neurologic recovery and protected striatopallidal neurons 4 days after ischemia (Yang et al. 2013).

The ability of adenosine A_{2A} receptor antagonists in protecting against ischemic damage in vivo is largely attributed to the control of excessive glutamatergic transmission and of the ensuing acute excitotoxicity after ischemia. The low dose of SCH58261 that 24 h after ischemia has protected against tissue damage induced by MCAo (Melani et al. 2003) or quinolinic acid (QA) excitotoxicity (Popoli et al. 2002), has also reduced, in the first 4 h after ischemia, the increase of extracellular glutamate estimated by microdialysis in the striatum (Melani et al. 2003) and has reduced glutamate content in the hippocampus after occlusion of both carotids in the rat (Svenningsson et al. 1997). In agreement, adenosine A_{2A} receptor KO mice are protected from an excess of striatal glutamate outflow and damage induced by transient MCAo (Gui et al. 2009).

In addition, ZM241385, injected directly to intrahippocampus, is protective against excitotoxicity induced by kainate (Jones et al. 1998), and SCH58261 administered directly in the hippocampus (Mohamed et al. 2016) ameliorates infarct size, memory impairment, and motor incoordination 24 h after occlusion of both carotids in the rat. A further mechanism by which A_{2A} receptor antagonism is protective may be due to the capability of increasing brain GABA extracellular concentration during ischemia (Cristóvão-Ferreira et al. 2009).

SCH58261 behaves as a significant protective agent at a dose (0.01 mg/kg) that does not have cardiovascular effects. This low dose does not affect motor activity in naive animals but decreases controlateral turning behavior after MCAo induced by the monofilament technique (Melani et al. 2003, 2006). At a higher dose, in the

range that is effective in different models of Parkinson's disease (PD), the same drug significantly increases motility and rearing in the rat (Svenningsson et al. 1997).

Control of several intracellular pathways activated by ischemia might account for protection by A_{2A} receptor antagonism. Twenty-four hours after focal ischemia, the A_{2A} receptor antagonist SCH58261 has decreased the ischemia-induced activation of p38 mitogen-activated protein kinase (MAPK) in activated microglia (Melani et al. 2006) and of JNK MAPK that is mainly expressed in mature oligodendrocytes and in oligodendrocyte progenitors (OPCs) (Melani et al. 2009). p38 is considered a death factor in ischemia (Barone et al. 2001), and phospho-JNK is a factor involved in oligodendrocyte death (Jurewicz et al. 2006). JNK MAPK KO mice are in fact protected from damage following cerebral ischemia (Kuan et al. 2003). Reduced activation of JNK might be directly due to A_{2A} receptors located on OPCs (Coppi et al. 2015). In fact in primary OPC culture, selective stimulation of A_{2A} receptors by CGS21680 inhibits maturation of OPCs (Coppi et al. 2013) and inhibits "delayed rectifier" K^+ currents (K_{DR}) (Coppi et al. 2013) that are known to promote proliferation and differentiation of OPC to mature oligodendrocytes, thus preventing myelin deposition.

Direct intrahippocampus administration of SCH58261 after global ischemia, 24 h thereafter, has reduced also phospho-ERK 1/2 bringing to the reduction of different inflammation products and to the increase of the anti-inflammatory cytokine IL-10 (Mohamed et al. 2016).

The reduced MAPK activation by SCH58261 might be due to a direct effect of the A_{2A} receptor antagonists on A_{2A} receptors located on oligodendrocytes or microglia but also to the overall reduction of the excitotoxic cascade that in the initial hours after in vivo ischemia primes microglial activation and MAPK activation. In fact, oligodendroglial cells are extremely sensitive to glutamate receptor overactivation, and ensuing oxidative stress and p38 and ERK1/2 MAPK activation is definitely induced by glutamate receptor stimulation (Kurino et al. 1995).

The recent observation that the A_{2A} receptor antagonist SCH58261 chronically administered after ischemia has not maintained protection 7 days after transient focal ischemia (Melani et al. 2015) supports the idea that the early protection offered by A_{2A} antagonism is overwhelmed on time by the secondary damage due to blood cell infiltration and neuroinflammation.

14.2.2.2 Adenosine A_{2A} Receptor Agonists Are Protective against Ischemic Damage

Considering that A_{2A} receptor antagonists are protective after ischemia, in an apparent paradoxical manner, also adenosine A_{2A} agonists were found protective under hypoxia/ischemia. An early study demonstrated that the adenosine A_{2A} receptor agonist 2-[(2-aminoethylamino)-carbonylethylphenylethylamino]-5'-N-ethylcarboxoamidoadenosine (APEC), administered systemically and chronically for 13 days, before a global 10-min ischemia in the adult gerbil, ameliorated animal

and neuron survival (von Lubitz et al. 1995). Also the selective A_{2A} receptor agonist, CGS21680, administered immediately after 5 min of global ischemia in gerbil at the high dose of 10 mg/kg i.p., exhibited highly significant protection against neuronal loss (Sheardown and Knutsen 1996). In agreement, A_{2A} receptor KO mice subjected to chronic cerebral hypoperfusion by permanent stenosis of bilateral common carotid artery showed impairment in working memory, increased demyelination and proliferation of glia, and increased levels of pro-inflammatory cytokines (Duan et al. 2009). The same transgenic mice, at neonatal age, showed aggravated hypoxic/ischemic injury in comparison to WT littermates (Adén et al. 2003). Most recently, Melani et al. (2014) have demonstrated that the A_{2A} receptor agonist, CGS21680, administered at the low dose of 0.01 mg/kg, twice/day for 7 days i.p. (chronic protocol) starting from 4 h after transient (1 h) MCAo, induced protection from neurological deficit, weight loss, cortical infarct volume, myelin disorganization, and glial activation evaluated 7 days after ischemia.

In considering translation to clinic, a main problem of A_{2A} receptor agonists consists in their cardiovascular effect because adenosine A_{2A} receptors located on vascular smooth muscle and endothelial cells exert a vasodilatory effect. Relevantly, Melani et al. (2014) have demonstrated that the protective dose (0.01 mg/kg) of CGS21680 does not modify either mean blood pressure or heart frequency. Moreover, adenosine by stimulating $A_{2A}R$ G_s-coupled adenylate cyclase in platelets enhances the intracellular cAMP levels, a potent molecule that inhibits platelet activation (Cooper et al. 1995) having thus potential antithrombotic activity. Therapies under study in ischemia (i.e., neuroprotective drugs including hypothermia or antioxidant/anti-inflammatory strategies) need to be associated with thrombolytic drugs since restoration of oxygen and glucose, at the moment, is considered the best therapy to protect against cell death from stroke (Liu et al. 2017), although its efficacy may be limited by the potential hemorrhagic effects. Considering that tPA and/or antiplatelet drugs are also routinely used in prevention of the secondary stroke, administration of a further drug that has antiplatelet activity could potentiate a previous antiplatelet therapy increasing the hemorrhagic potential or on the contrary could be useful in maintaining an antiplatelet effect after the primary stroke. However we found (personal unpublished results) that a chronic treatment with CGS21680, twice/day for 7 days at the dose of 0.01 mg/kg administered i.p. in control rats, does not modify platelet aggregation induced by 10 μM ADP (technique described by Ma et al. 2016; Yang et al. 2015) (50.9% ± 11.5 of ADP induced aggregation in control $n = 3$ versus 49.4% ± 1.3 in treated rats $n = 4$). In agreement, concentrations of CGS21680 that decrease production of free radicals of the oxygen from isolated human neutrophils were calculated three times lower (EC50 300 nM) than those that decrease human platelet aggregation (IC50 1090 nM) (Gessi et al. 2000). Data suggest that adenosine A_{2A} receptors exert antioxidant effects and inhibit granulocyte infiltration at doses /concentrations lower than those necessary to inhibit platelet aggregation.

Protection by CGS21680 after ischemia could be attributable to central effects because it easily crosses the BBB. As a vasodilator agent, adenosine acting on A_{2A} receptors is in fact implicated in cerebral blood flow regulation and might favor

brain reperfusion after ischemia. Recently, importance of adenosine receptors located on vasculature as therapeutic targets in cardiovascular pathologies including stroke was pointed out (Sousa and Diniz 2017). Moreover CGS21680 administered directly into the rat striatum immediately prior to the induction of intracerebral hemorrhage reduces parenchymal neutrophil infiltration and tissue damage: an effect that was related to the inhibition of tumor necrosis factor-α (TNF-α) expression (Mayne et al. 2001). Activation of central A_{2A} receptors is known to increase expression and release of neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) (Sebastião and Ribeiro 2009). The increase in neurotrophic factor expression by adenosine A_{2A} receptor stimulation may contribute to restore neurological functions and cerebral damage after brain ischemia.

A bulk of evidences however indicates that A_{2A} receptors located on blood cells greatly account for protective effects of adenosine A_{2A} agonists after ischemia. The adenosine A_{2A} receptors are expressed in fact both on cells of innate (microglia, macrophages, mast cells, monocytes, dendritic cells, neutrophils) and on adaptive (lymphocytes) immunity. After ischemia altered permeability of BBB allows infiltration of leukocytes (neutrophils, lymphocytes and monocytes) that on their turn exacerbate ischemic damage (Haskó et al. 2008). In the transient MCAo model in the rat, selective immunostaining for granulocytes, by anti-HIS-48 antibody, shows numerous infiltrated cells in ischemic striatal and cortical core, 2 days after transient MCAo (Melani et al. 2014). This is in agreement with observation that after transient MCAo, a peak of neutrophil infiltration occurs at 6 and 48 h thereafter (Zhang et al. 1994). Seven days thereafter, infiltrated blood cells were anymore observed (Melani et al. 2014). Chronic treatment with the A_{2A} adenosine receptor agonist, CGS21680, 2 days after transient MCAo, has definitely reduced the number of infiltrated blood cells in the ischemic areas (Melani et al. 2014). The importance of a protracted treatment with the A_{2A} agonist in order to achieve protection is proved by the observation that the A_{2A} agonist administered subchronically (4 and 20 h after induction of MCAo) did not prove protective 24 h after permanent MCAo nor 7 days after transient MCAo (Pedata et al. 2014).

Many studies have reported that selective activation of A_{2A} receptors directly on blood cells, including platelets, monocytes, some mast cells, neutrophils, and T cells, inhibits pro-inflammatory responses, reduces production of adhesion cell factors, and reduces neutrophil activation, thereby exerting antioxidant and anti-inflammatory effects. A_{2A} receptor activation is known to reduce ischemia-induced rolling, adhesion, and transmigration of various peripheral inflammatory cells (such as lymphocytes, neutrophils) (Haskó et al. 2008). It has been reported that adenosine A_{2A} receptors are sensors of inflammatory disease and increase in number in blood cells in different human peripheral and central inflammation-based pathologies including rheumatoid arthritis multiple sclerosis and amyotrophic lateral sclerosis (Borea et al. 2016). Our (unpublished) results demonstrate that density of adenosine A_{2A} receptor assayed by RT-PCR in leukocytes isolated from sham-operated (mean ± ES; sham-operated, 1.02 ± 0.02) is not modified 48 h after tMCAo (0.95 ± 0.01) but was significantly decreased after 7 days (0.92 ± 0.03*, unpaired

student's t-test, $*p < 0.03$ vs sham-operated rats; results are expressed as fold increase according to the 2^(−ΔΔCt) method, utilizing as target genes ADORA2A) when infiltrated blood cells were anymore observed (Adén et al. 2003).

In support that A_{2A} receptors on blood cells are greatly responsible of the protective effects of A_{2A} agonists, protection of motor deficits by A_{2A} receptor agonists systemically administered after spinal trauma is lost in mice lacking A_{2A} receptors on bone marrow-derived cells (BMDCs) but is restored in A_{2A} receptor KO mice reconstituted with A_{2A} receptors on BMDCs (Li et al. 2006). Moreover, in the spinal cord trauma model in the mouse, CGS21680 protected from damage when injected systemically but not when centrally injected into the injured spinal cord (Paterniti et al. 2011). Consistent with its anti-inflammatory and immunosuppressive role, the protective effect of adenosine A_{2A} receptor stimulation has been observed in different pathologies where inflammatory process has an important role in tissue damage such as ischemia/reperfusion liver injury (Day et al. 2004), spinal cord trauma (Day et al. 2004; Genovese et al. 2010; Paterniti et al. 2011), rheumatoid arthritis (Mazzon et al. 2011), acute lung inflammation (Impellizzeri et al. 2011), intestine ischemia/reperfusion injury (Di Paola et al. 2010; Odashima et al. 2005), and experimental autoimmune encephalomyelitis (Xu et al. 2013).

14.2.2.3 A_{2A} Receptor as Target of Protective Drugs after Ischemia

In conclusion information up to now indicates that stimulation or antagonism of A_{2A} receptors might be a protective strategy secondary to the time-related development of phenomena typical of trauma and ischemia. Protective effects of A_{2A} antagonists, at doses that do not modify hemodynamic parameters and inside a therapeutic window compatible with arrival in a stroke unit, would provide protection by dampening central excitotoxicity, while A_{2A} agonists, at doses that do not modify hemodynamic parameters or platelet activity, provide protection by controlling massive infiltration in the hours after ischemia. Since a major mechanism underlying reperfusion injury is that of poststroke inflammation, targeting anti-inflammatory targets as a combined therapy with pharmacological thrombolysis or mechanical thrombectomy after reperfusion is a potential useful strategy after stroke (Mizuma and Yenari 2017).

14.2.3 Adenosine A_{2B} Receptors in Brain Ischemia

Among adenosine receptors, the adenosine A_{2B} receptor subtype is the least studied and still remains the most enigmatic adenosine receptor subtype because of the relatively low potency of adenosine at this receptor (EC50 value of 24 μM) (Fredholm et al. 2011) and the very few specific agonists that have been described so far. Adenosine A_{2B} receptors, although scarcely, are uniformly expressed throughout the CNS (Dixon et al. 1996) including the hippocampus (Perez-Buira et al. 2007). Their

expression in neurons, glial, and vascular endothelial cells increases after ischemia and mRNA protein expression of A_{2B} receptor increased to a greater extent after ischemia-reperfusion than did expression of the other three adenosine receptors (A_1, A_{2A}, and A_3) 24 h after transient MCAo in the rat (Li et al. 2017). Thus, during conditions of hypoxia or ischemia when the extracellular adenosine levels rise, A_{2B} receptors might be well activated (Xu et al. 2013).

Due to the existence of selective antagonists of A_{2B} receptors, their role under OGD was most recently investigated. Our recent data demonstrate that, in the CA1 area of the rat hippocampus, the selective A_{2B} receptor antagonists, N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) phenoxy]–acetamide (MRS1754) and 8-[4-[4-(4-Chlorophenzyl) piperazide-1-sulfonyl) phenyl]] -1-propylxanthine) (PSB603), prevent the appearance of AD, a phenomenon strictly related to cell damage and death (Pugliese et al. 2006), and protect from the synaptic activity depression, bringing to a significant recovery of an otherwise disrupted neurotransmission induced by 7-min OGD (see Fig. 14.3) (Fusco et al. 2017). The damage to CA1 pyramidal neurons, assessed by the decrease of immunofluorescence density of CA1 $NeuN^+$ neurons, was completely antagonized by treatment with PSB603 (see Fig. 14.3) (Gaviano et al. 2017). A_{2B} receptors are present in mouse hippocampal glutamatergic terminals, where their selective stimulation counteracts the A_1 receptor-mediated inhibition of synaptic transmission (Goncalves et al. 2015). Moreover, in transfected cells, a synergy with A_{2A} receptors has been envisaged because adenosine A_{2A} receptor, when stimulated, facilitates A_{2B} receptor externalization from the endoplasmic reticulum to the plasma membrane, possibly increasing the formation of the A_{2A}-A_{2B} dimer which could regulate glutamate outflow (Moriyama and Sitkovsky 2010).

In primary murine astrocytes, the expression of A_{2B} receptor is strongly stimulated by LPS in concert with hypoxia (Gessi et al. 2013). In human astroglial cells, a selective A_{2B} antagonist, N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) phenoxy]acetamide (MRS1706), completely prevents elongation of astrocytic processes (a morphological hallmark of in vivo reactive astrogliosis) induced by selective stimulation of A_{2B} receptors (Trincavelli et al. 2004). The selective A_{2B} receptor antagonist, MRS1754, administered i.c.v., reduced an early ceramide production from primary astrocytes isolated from the hippocampus of rats subjected to global cerebral ischemia (Gu et al. 2013). Specific secretion of ceramide from astrocytes has been associated with neuroinflammation and is considered a contributing factor to neuronal dysfunction and damage (Wang et al. 2012). Such effect of the A_{2B} antagonist might be due to an early reduction of p38 MAPK activation (Wei et al. 2013) or to reduced expression of the "regulators of G-protein signaling" (RGS) in particular RGS-3 as demonstrated in astrocytoma cells (Eusemann et al. 2015). A_{2B} receptor desensitization described on astroglia might represent a cell defense mechanism in ischemia (Trincavelli et al. 2008). Since A_{2B} receptors are activated only by high adenosine concentrations as can be reached under brain ischemia, they might represent a good selective therapeutic target for antagonists that, by reducing excitotoxicity and neuroinflammation, can subserve a protective mechanism early after ischemia (Popoli and Pepponi 2012).

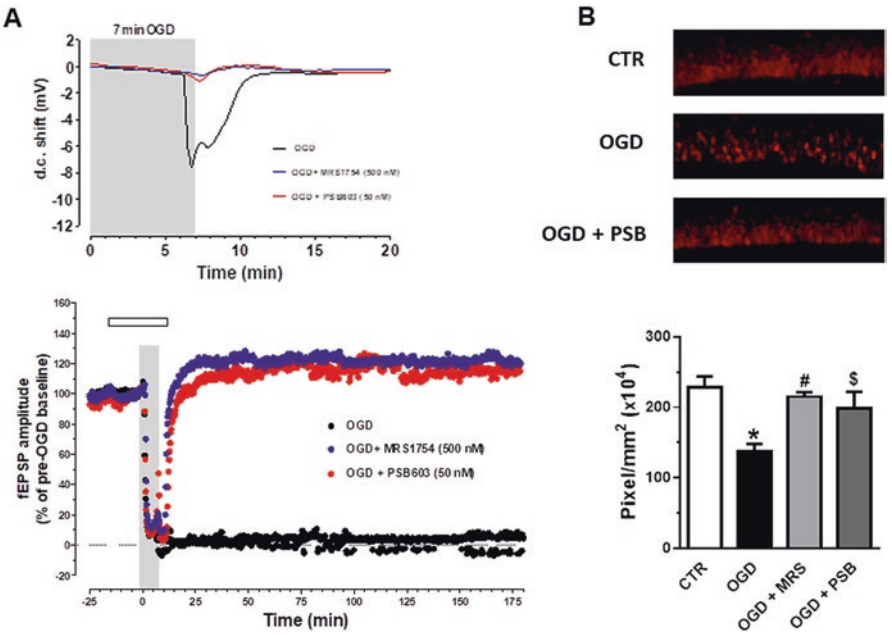

Fig. 14.3 The selective antagonism of adenosine A_{2B} receptors counteracts functional and histological damage induced by severe OGD. (**A**) *Upper panel:* AD was recorded as the negative d.c. shift in response to 7-min OGD in the absence (OGD) or in the presence of 500 nM MRS1754 or 50 nM PSB603. *Lower panel*: the graph shows the time course of 7-min OGD effects on fEPSP amplitude in OGD-untreated slice and in 500 nM MRS1754- or 50 nM PSB-603 treated slices. Amplitude of fEPSPs is expressed as percent of respective pre-OGD baseline. Note that, after reperfusion in oxygenated standard solution, a recovery of fEPSP was found in MRS1754 or PSB603 treated OGD slices. Gray bar, OGD time duration. Open bar, time of drug application. (**B**) Analysis of $NeuN^+$ immunofluorescence in CA1 stratum pyramidale after the OGD insult. *Upper panels*: representative images of $NeuN^+$ immunofluorescence in the region of interest of CA1 of a control slice (CTR), a slice where a 7-min OGD was performed (OGD), and a slice where a 7-min OGD was performed in the presence of 50 nM PSB-603 (OGD + PSB), all collected 3 h after the insult. Scale bar, 75 μm. *Lower panel*: quantitative analyses of $NeuN^+$ immunofluorescence in the four experimental groups. Each column represents the area, expressed in pixels (x 10^6) above a threshold, maintained constant for all slices investigated. Statistical analysis: One-way ANOVA, Newman-Keuls multiple comparison test: $^*P < 0.05$, OGD vs CTR; $^{\#}P < 0.05$, OGD + PSB vs OGD. CTR, $n = 6$; OGD, $n = 5$; OGD + PSB, $n = 3$. All data in the graphs are expressed as mean ± S.E.M

Besides brain cells, A_{2B} receptors are present on blood immune cells, i.e., neutrophils and lymphocytes (Eckle et al. 2008; Gessi et al. 2005), where in most cases they are coexpressed with A_{2A} receptors. They are also expressed at low levels on platelets, where they are upregulated following injury and systemic inflammation in vivo and induce inhibition of platelet aggregation (Yang et al. 2010). Attenuation of hypoxia-associated increases in tissue neutrophil number in different tissues including brain largely depends on hematopoietic cell A_{2B} signaling (Eckle et al. 2008).

Moreover, A_{2B} receptors are expressed on the surface of endothelial cells (Feoktistov et al. 2004) where they are upregulated by the hypoxia-inducible factor (HIF-1α) (Eltzschig et al. 2004). Studies in mice deleted of A_{2B} receptors on bone marrow cells indicate an important contribution of vascular A_{2B} receptors in attenuating vascular leakage during hypoxia (Eckle et al. 2008). The A_{2B} receptor antagonist MRS1754 increases adhesion in human microvascular endothelial cells (HMEC-1 s) exposed to hypoxia (Eltzschig et al. 2004), and adenosine A_{2B} receptor KO mice show increased basal levels of TNF-α and expression of adhesion molecules in lymphoid cells, resulting in increased leukocyte rolling and adhesion (Yang et al. 2006). Evidences indicate that A_{2B} receptors are a valuable target to protect heart (Eltzschig et al. 2013) and kidney from ischemia (Grenz et al. 2008). Recent introduction of new pharmacological tools (Hinz et al. 2014) led to understand a role of A_{2B} receptors in ischemia. The selective A_{2B} receptor agonist BAY60–658380 systemically administered in mice before in vivo normobaric hypoxia exposure decreases vascular leak in the lung, liver, and colon (Eckle et al. 2008). It has also been demonstrated (Li et al. 2017) that treatment with BAY60–6583 (1 mg/kg intravenously), at the start of reperfusion after brain ischemia induced by 2-h transient MCAO, 24-h thereafter, reduced lesion volume and attenuated brain swelling and BBB disruption. In the presence of tPA (administered after ischemic stroke to dissolve intravascular clots), BAY60–6583 also mitigated sensorimotor deficits and reduced tPA-induced hemorrhages at 24 h (Li et al. 2017). The neurovascular protection afforded by BAY60–6583 appears to derive from stimulation of the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) production, inhibition of tPA-induced matrix metalloprotease (MMP) activation, and prevention of tight junction protein degradation. In fact overactivation of MMP leads to increased cerebrovascular permeability after ischemia-reperfusion injury (Mishiro et al. 2012). It is proposed that A_{2B} receptor agonists might be adjuvant to tPA and could be a promising strategy for decreasing the risk of hemorrhages during treatment for ischemic stroke (Li et al. 2017).

All together these studies point toward a role of central A_{2B} receptors, in synergy with A_{2A} receptors in promoting brain excitotoxicity, while A_{2B} receptors located on vascular endothelial cells would play a pivotal role in attenuating hypoxia-induced increases in vascular leak. A_{2B} receptor has been described as implicated in dampening vascular adhesion signals and hypoxia-induced inflammation (Koeppen et al. 2011).

A further possible role of A_{2B} receptors in hypoxia/ischemia might be secondary to promotion of an angiogenic response because activation of A_{2B} receptors by adenosine increases endothelial cell proliferation, chemotaxis, capillary tube formation, and release of vascular endothelial growth factor (VEGF) (Feoktistov et al. 2004).

14.2.4 Adenosine A_3 Receptors in Brain Ischemia

Adenosine A_3 receptor has an affinity of 300 nM in huis widespread in the rat and mouse brain but compared to A_1 and A_{2A} receptors has less affinity for adenosine (10–30 nM versus 1 μM) and is detected at relatively low levels (Gessi et al. 2008). However, since extracellular adenosine concentrations in the first hours after ischemia reach a μM range (Latini and Pedata 2001; Melani et al. 1999), also adenosine A_3 receptor is involved in the tonic adenosine effects in ischemia.

Studies currently in the literature concerning the role of adenosine A_3 receptor in the pathophysiology of cerebral ischemia are rather contradictory (Borea et al. 2009; Pedata et al. 2010). The use of mice with genetic deletion of the A_3 receptors has pointed out a neuroprotective function of adenosine A_3 receptors. Mice lacking A_3 receptors showed in fact increased neurodegeneration in response to repeated episodes of moderate hypoxia (Fedorova et al. 2003) and an increase in cerebral infarction after transient ligation of MCA (Chen et al. 2006). Accordingly, a chronic administration (10-day pre-ischemic) of the A_3 agonist N(6)-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA) reduced ischemic damage after global forebrain ischemia in the gerbil (von Lubitz et al. 1994b), and pretreatment with a selective A_3 agonist, 1-[2-Chloro-6[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-ß-Dribofuranuronamide] (Cl-IB-MECA), intracerebroventricularly or repeatedly intravenously administered before MCA ligation decreased the size of infarction-induced by transient MCAo (Chen et al. 2006).

Under in vitro OGD (5 min), selective activation of adenosine A_3 receptors by a brief (5 min) application of IB-MECA brings about an inhibition of excitatory neurotransmission on cortical neurons (Hentschel et al. 2003), and application of the selective A_3 receptor antagonist, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate (MRS1523), before a brief (2 min) OGD reduces the OGD-induced depression of fEPSP in the CA1 hippocampal area (Pugliese et al. 2007). These findings indicated an inhibitory role of A_3 receptors on synaptic transmission during brief OGD periods and have suggested that A_3 receptors have a synergistic role with A_1 receptors in decreasing synaptic transmission, thus sustaining the neuroprotective effect of A_1 receptors.

On the other hand, when hippocampal slices are submitted to a severe (7-min) OGD, the selective antagonists of adenosine A_3 receptors abolish or delay the occurrence of AD and significantly protect from the irreversible disruption of neurotransmission caused by the severe ischemic episode in the CA1 region of rat hippocampal slices (Colotta et al. 2007, 2008, 2009; Poli et al. 2017; Pugliese et al. 2006, 2007). Depression of synaptic transmission following 15-min OGD was prevented by A_3 receptor antagonists also in the CA3 hippocampal area (Dennis et al. 2011).

To explain results above reported, we should consider that rat cortical neurons exposed to hypoxia in vitro show an increase in activation of protein kinase C (PKC) after selective adenosine A_3 receptor stimulation (Nieber and Hentschel 2006). If

OGD is applied long enough to be considered severe, PKC activation induced by adenosine A_3 receptor could account for an increase in intracellular calcium, which may participate in increasing tissue excitability and thus lead to irreversible synaptic failure. Thus while initially after OGD, massive excitotoxicity may be controlled by adenosine A_3 receptors, later the ensuing cascade of cytotoxic events could be potentiated by prolonged adenosine A_3 receptor stimulation. Moreover ischemia-induced plasticity of A_3 receptors might be relevant to explain the A_3 agonist effects in ischemia. A desensitization of A_3 receptors might account for the effect of a long application (before and during OGD) of Cl-IB-MECA and of new selective A_3 agonists (Volpini et al. 2002, 2007) that like A_3 antagonists protect from the depression of synaptic activity brought about by prolonged OGD and delay the appearance of AD in the CA1 region of rat hippocampal slices (Pugliese et al. 2007).

A_3 receptor mRNA has been identified in mouse astrocytes, in microglia, and in oligodendrocytes. In human D384 astrocytoma cells, Cl-IB-MECA at relatively low concentration (0.8 μM) reduced ATP depletion and apoptosis caused by hypoxic conditions (Bjorklund et al. 2008). Primary astrocytes prepared from adenosine A_3 receptor KO mice were more affected by hypoxia than those prepared from WT mice (Bjorklund et al. 2008). In cultured murine astrocytes, stimulation of A_3 receptors decreases HIF-1 expression induced by LPS under hypoxic conditions (Gessi et al. 2013), leading to inhibition of genes involved in inflammation injury (Gessi et al. 2013). In the in vivo model of transient MCAo, IB-MECA administered after ischemia proved to decrease the intensity of reactive gliosis involving microglia and astrocytes as evaluated 7 days after ischemia (von Lubitz et al. 1996).

Besides being localized on central cells, adenosine A_3 receptors are also localized on blood cells (Gessi et al. 2013). The state of the art about the role of adenosine A_3 receptors in inflammatory responses appears conflicting because exposure of blood peripheral cell lines to selective adenosine A_3 receptor agonists results in both anti- and pro-inflammatory effects (Borea et al. 2009). Choi et al. (2011) have demonstrated that treatment with 2-chloro-N(6)-(3-iodobenzyl)-5'-N-methylcarbamoyl-4′-thioadenosine (LJ529), a selective A_3 agonist administered by intraperitoneal injection 2 and 7 h after transient MCAo, markedly reduced cerebral ischemic injury 24 h thereafter. LJ529 also prevented the infiltration of monocytes and migration of microglia occurring after MCAo. A_3 receptor agonists can mediate their protective effects via anti-inflammatory signaling (inhibition of pro-inflammatory cytokines) and/or concomitant inhibition of innate immune cell trafficking because of A_3 receptor desensitization (Butler et al. 2012).

As adenosine A_{2A} receptor, also A_3 receptors are upregulated in lymphocytes obtained from patients affected by chronic autoimmune inflammatory rheumatic diseases, i.e., rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis (Ravani et al. 2017; Varani et al. 2011) raising the possibility to exploit adenosine A_{2A} and A_3 receptors as therapeutic targets to limit the inflammatory responses.

A_3 agonists, under clinical evaluation for the treatment of inflammatory diseases and cancer, demonstrated excellent safety and efficacy (Fishman et al. 2012).

Overall, results raise the question of the time-related utility of A_3 receptor antagonists/agonists for treatment of ischemia. It may be speculated, that after ischemia,

a prolonged treatment with adenosine A_3 receptor agonists protects first by reducing glutamate-mediated excitotoxicity and later on after ischemia, by desensitizing central A_3 receptors and via anti-inflammatory effects mediated by A_3 receptors on blood cells.

14.3 Conclusions

Information up to now acquired indicate that adenosine receptors located on any cell type of the brain and on vascular and blood cells partake in either salvage or demise of the tissue after a stroke. They thus represent important targets for drugs having different therapeutic time windows after stroke.

One of the prime adaptive mechanisms in response to hypoxia-ischemia is the cellular activation of adenosine A_1 receptors which inhibits excessive excitatory synaptic transmission. At the same time but, on the contrary, adenosine A_{2A} and A_{2B} receptors contribute to excessive excitotoxicity. Unfortunately the use of selective A_1 agonists is hampered by undesirable peripheral effects such as sedation, bradycardia, and hypotension. Early neuroprotective strategies with antagonists of adenosine A_2 receptors would be aimed at targeting the brain parenchima to antagonize excitotoxicity and ensuing production of harmful molecular events responsible for acute brain damage.

In the hours and days after ischemia, adenosine A_{2A}, A_{2B}, and A_3 receptors peripherally located on vascular and blood cells may be the targets of drugs aimed at dampening vascular adhesion signals and neuroinflammation.

Overall, a therapeutic strategy with adenosine receptor antagonists/agonists should be carefully evaluated in terms of time after ischemia due to the balance of central versus peripheral adenosine receptor-mediated effects over time after ischemia. Besides early neuroprotective strategies with A_{2A} and A_{2B} receptor antagonists, strategies aimed at targeting events in a longer time window of days/weeks after ischemia appear promising in antagonizing inflammation and neurovascular protection and promoting neuroplasticity and neurogenesis. Considering that tPA is routinely used after ischemic stroke to dissolve intravascular clots, most recent data indicate that A_{2B} receptor agonists, by providing neurovascular protection, might be a promising strategy against BBB damage and permeability and for decreasing the risk of hemorrhages after stroke.

Compounds active at adenosine receptors are drugs under development and already exist in therapy or in clinical experimentation for other indications; some of them could enter in a reasonable time in clinical trials for stroke. Still there is urgent need of novel compounds to be developed with higher selectivity, oral bioavailability, stability in vivo, longer half-life, and better capability to cross the BBB.

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Chapter 15
The Adenosine Receptor: A Homeostatic Neuromodulator for Fine-Tuning Control of Cognition

Jiang-Fan Chen

Abstract There is a convergence of neurochemical studies showing the dual roles of neuromodulation and homeostatic function by adenosine receptors (AR), with animal studies demonstrating the strong pro-cognitive impact upon AR antagonism in healthy and diseased brains, with the epidemiological evidence in support of caffeine and AR drugs used for the therapeutic modulation of cognition. This perspective led to the proposal that the adenosine and AR may uniquely position to modulate cognitive behaviors in normal and disease conditions. This review first describes the ability of AR to integrate dopamine and glutamate signaling and to modulate synaptic plasticity by acting through the inhibitory A_1 and facilitating A_{2A} receptors ($A_{2A}R$). It is followed by the discussion on the animal studies demonstrating the strong pro-cognitive effects of AR (mainly the A_{2A} receptor) antagonism on a variety of cognitive behaviors. These studies reveal several novel insights into the mechanism underlying AR control of cognition: temporally precise interaction of adenosine with dopamine and glutamate signaling at the striatum, striatopallidal A_{2A}Rs function as a common "break" mechanism to constrain cognition, and selective modulation of distinct phases of working memory information processing. We further describe the evidence for the aberrantly increased adenosine-AR signaling under pathological conditions. Accordingly, blocking the aberrant AR signaling reverses cognitive impairments in animal models of neurodegenerative disorders. AR modification of neurodegenerative proteins (including α-synuclein, β-amyloid, and phosphorylation of Tau) and neuroprotection against synaptic loss are discussed as the potential mechanisms underlying AR control of cognitive deficits. Last, translational potential of $A_{2A}R$ antagonists and caffeine for cognitive improvement is highlighted with non-human primate studies and epidemiological findings. As caffeine is regularly consumed by >50% world population and $A_{2A}R$ antagonists are in phase III clinical trials for Parkinson's disease with noted safety profiles, this convergence of molecular, animal, and epidemiological evidence supporting AR control of cognition will

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stimulate necessary clinical investigations to explore AR-targeting drugs as a novel strategy to ameliorate cognitive deficits in neuropsychiatric disorders.

Keywords Adenosine receptors · Cognition modulation · A_1R antagonism · $A_{2A}R$ antagonism · Neuropsychiatric disorders · Caffeine

15.1 Adenosine Acts as a Dual Controller of Homeostatic Metabolism and Neuromodulatory Function in the Brain

Adenosine has been postulated as a homeostatic regulator of metabolism in cells throughout the body. The basal level of adenosine is driven mainly by metabolic homeostasis and is apparently mostly independent of nerve activity. Under physiological conditions, the constant presence of a finite concentration of adenosine (in the range of ~30–300 nM) inside the cell (Ballarin et al. 1991) is ensured by the bidirectional enzyme activities of adenosine kinase and S-adenosylhomocysteine hydrolase (for generating adenosine by hydrolysis of adenosine monophosphate (AMP) or S-adenosylhomocysteine, respectively) (Fredholm 2007). Because of the presence of the efficient equilibrative purine transporters in all cells, the finite concentration of intracellular adenosine ensures that there is also a substantial extracellular concentration of adenosine (King et al. 2006), which is sufficient to active evolutionarily conserved adenosine receptors that are present on most, if not all, cells. In addition, extracellular adenosine is also formed by a series of ectoenzymes on the cell surface by the conversion of ATP to ADP and then to AMP (via many different ectoenzymes, especially CD39) (Yegutkin 2008) and then from AMP to adenosine (only via ecto-5′ nucleotidase CD73 in the brain) (Resta et al. 1998). Extracellular ATP can be generated not only by controlled co-release from the storage vesicles together with other neurotransmitters from the nerve terminals and uncontrolled leakage from necrotic cells (Eltzschig 2009) but also from the inflammatory cells or vascular endothelium through connexin hemichannels and channels such as P2X7 receptors (Chen et al. 2006; Linden 2006; Faigle et al. 2008) and also from various cells by a “kiss-and-run” mechanism (MacDonald et al. 2006), and lysosome exocytosis (Zhang et al. 2007). Thus, adenosine acts as a dual controller of a homeostatic regulator of metabolic activity by its paracrine signaling ability in all eukaryotic cells and of a specific neuromodulator in the brain by controlling neuronal excitability, the release of various neurotransmitters, and modulation of synaptic plasticity, neuroinflammation and cell death (Sebastiao and Ribeiro 1996). The adenosine control of neuronal function is thus intrinsically linked with its coordinate metabolic activity in the neuron, making it difficult to disentangle the dual roles of adenosine in the brain.

Extracellular adenosine reacts with one of the four adenosine receptors, namely, A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al. 2011). When they are expressed at the same

level (~200,000 receptors/cell), adenosine, under basal physiologic conditions, is sufficient and equally potent at A_1, A_{2A}, and A_3 receptors, whereas A_{2B} receptor is activated at higher levels of adenosine. Brain expression of the A_1 and A_{2A} receptors is significantly higher than the other two receptors (Fredholm et al. 2011), and adenosine mainly acts through inhibitory A_1R and facilitatory $A_{2A}R$ to fine-tune the brain neurotransmission (Fredholm et al. 2005a).

Adenosine A_1 receptor (A_1R): The A_1R is a Gi-protein-coupled receptor (van Calker et al. 1978; Londos et al. 1980) that is widely and abundantly expressed throughout the brain (Reppert et al. 1991; Dixon et al. 1996). The A_1R controls synaptic transmission by the presynaptic inhibition of a variety of neurotransmitters (particularly excitatory neurotransmitters such as glutamate) (Dunwiddie and Fredholm 1997; Dunwiddie and Masino 2001; Ribeiro et al. 2002) and by postsynaptic suppression of N-type calcium channels and NMDA receptors (Dunwiddie and Masino 2001; Ribeiro et al. 2002; Scanziani et al. 1992) and by nonsynaptic activation of inwardly rectifying K^+ channels (GIRKs) (Kim and Johnston 2015) and hyperpolarization of the resting membrane potential (Kirsch et al. 1990). Thus, the neuronal excitability and control of the "basal" synaptic transmission are primarily regulated by the A_1R activation presynaptically and postsynaptically as well as nonsynaptically (Wan et al. 1999).

Adenosine A_{2A} receptor ($A_{2A}R$): $A_{2A}Rs$ are highly enriched in the striatum where the expression is mostly localized to striatopallidal medium spiny neurons of the striatopallidal pathway (Fink et al. 1992; Schiffmann and Vanderhaeghen 1993). In the striatopallidal neurons, $A_{2A}Rs$ co-localize and interact with striatal dopamine D_2 receptors (D_2Rs) (Canals et al. 2003; Hillion et al. 2002; Fuxe et al. 2003) or N-methyl-D-aspartate receptors (NMDARs) (Gerevich et al. 2002; Wirkner et al. 2000) in an *antagonistic* manner, as well as with metabotropic glutamate 5 receptors ($mGlu_5Rs$) (Ferre et al. 2002; Coccurello et al. 2004; Kachroo et al. 2005), or cannabinoid CB_1 receptors (CB_1Rs) (Lerner et al. 2010; Ferre et al. 2010) in a *synergistic* manner. In particular, activation of the striatopallidal $A_{2A}Rs$, likely through the $A_{2A}R$-D_2R heterodimer, inhibits the D_2R binding and antagonizes the D_2R-mediated inhibition of GABA release (Mori and Shindou 2003), DARPP-32 phosphorylation (Shen et al. 2013), and c-Fos expression and inhibits NMDA current in the striatal neurons (Gerevich et al. 2002; Wirkner et al. 2000) as well as D_2R-mediated behaviors (Ferre et al. 1997; Ongini and Fredholm 1996). $A_{2A}Rs$ also modulate brain-derived neurotrophic factor (BDNF) function in the striatum by providing a permissive effect on BDNF release and by the intracellular transactivation of TrkB receptor (Sebastiao and Ribeiro 1996, 2000; Tebano et al. 2008). In CA1 region of the hippocampus, $A_{2A}R$ activity also exerts a permissive effect on the theta burst stimulation (TBS)-induced long-term potentiation with a concurrent increase in ERK1/2 activation, suggesting a possible tripartite A_{2A}, mGlu5, and NMDAR complex (Krania et al. 2018). Cortical $A_{2A}Rs$ located at corticostriatal projections (47, 48) modulate glutamate release (Rosin et al. 2003; Rebola et al. 2005a) to excite this synaptic transmission in the striatal neurons by locally shutting down the A_1R-mediated inhibition (Ciruela et al. 2006; Lopes et al. 1999a). Thus, while A_1R activation plays a prominent inhibitory role in the control of "basal" synaptic

transmission, A_{2A}Rs exert a limited effect on this but may have a facilitating role in controlling local synaptic plasticity (Gomes et al. 2011) (see below).

15.2 Coordinated Glial-Derived Adenosine for A_1R Global Inhibition and Neuronal-Derived Adenosine for Local A_{2A}R Activation

In the brain, extracellular adenosine might originate from neurons (both from nerve terminals and postsynaptic components) and surrounding non-neuronal cells such as glial cells (Halassa et al. 2007, 2009). As a neuromodulator, adenosine generated from different sources may preferentially act at different ARs to exert different control of synaptic plasticity. Indeed, early findings indicate that different sources of adenosine activate A_1R and A_{2A}R (Cunha et al. 1996) and that A_{2A}Rs are selectively activated upon extracellular catabolism by ecto-nucleotidases of ATP (Cunha et al. 1996; Rebola et al. 2008). Several studies have recently demonstrated a selective association of CD73-mediated formation of ATP-derived adenosine with the activation of facilitatory A_{2A}R in the brain (Fredholm et al. 2005a, b). This view is supported by our recent finding that CD73 and A_{2A}R co-localize (Ena et al. 2013) and are physically associated (Augusto et al. 2013) in the striatopallidal neurons and that CD73 provides the particular pool of extracellular adenosine selectively responsible for activating striatal A_{2A}R (Cunha 2001). This functional association between CD73 activity and the activation of striatal A_{2A}R is validated by the abolishment of ex vivo effect (i.e., cAMP formation) as well as in vivo effect (hypolocomotor) of a prodrug for A_{2A}R agonism either by CD73 knockout or by A_{2A}R knockout (Augusto et al. 2013). On the other hand, A_1R activation depends on the tissue workload (Cunha 2001), and the activity-dependent metabolic control of adenosine kinase is postulated to produce a direct outflow of adenosine for the activation of A_1R (Boison 2011; Diogenes et al. 2014; Brundege and Dunwiddie 1998). However, both astrocytes (Halassa et al. 2009; Schmitt et al. 2012) and postsynaptic neuronal components involve the vesicular nucleotide transport (VNUT) (Larsson et al. 2012; Lovatt et al. 2012) and may also be coupled to the activation of A1R.

This selective activation of the A_{2A}R by ATP-derived, CD73-mediated adenosine and activation of the A_1R by the activity-dependent metabolic control of adenosine kinase led to the proposal of a nonsynaptic transmission of adenosine to understand the differential activation of the inhibitory A_1R and facilitatory A_{2A}R according to the functional needs of neuronal circuits (Cunha 2008a). In this proposal, astrocyte-derived adenosine acts at the A_1R to produce global hetero-synaptic inhibition through astrocytic-driven volume transmission, while neuron-derived adenosine – via ATP conversion to adenosine by CD73 – acts at the A_{2A}R to exert local facilitation of plasticity (Gomes et al. 2011), leading to the local increase of a signal to noise ratio for the information processing in the brain (Gomes et al. 2011). As such, adenosine is critical for balancing inhibition and excitation toward homeostasis and in setting the stage for adenosine-mediated meta-plasticity (Dias et al. 2013). The

homeostatic and neuromodulatory control of neuronal processes underlies the ability of adenosine to regulate cognition because adenosine kinase (ADK)-mediated adenosine homeostatic function is necessary and permissive to synaptic actions of adenosine (Diogenes et al. 2014). Hence, mice with a conditional knockout or a brain-specific deletion of Adk ($Adk^{\Delta brain}$) develop seizures and cognitive deficits with increased basal synaptic transmission and enhanced $A_{2A}R$-dependent synaptic plasticity (Sandau et al. 2016).

15.3 A_1 and A_{2A} Receptor Modulation of Synaptic Plasticity Underlies Cognitive Control

Hebbian forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), are fundamental to associated learning and thought to form the cellular correlates of learning and memory. The homeostatic function of adenosine may provide the permissive condition to set the stage for Hebbian forms of plasticity (Dias et al. 2013). By the control of multiple neurotransmitter release and glutamate, dopamine, and BDNF signaling and by controlling neuronal excitability in the brain (Ribeiro 1999), ARs play a critical role in modulation of Hebbian plasticity in various brain regions (de Mendonca and Ribeiro 1997), including thalamocortical project (Blundon et al. 2011), somatosensory cortex (Marquez-Ruiz et al. 2012), hippocampus (CA3-CA1 synapse) (Rebola et al. 2008), corticostriatal projections (Shen et al. 2008b), hypothalamus (Xia et al. 2009), and neuronal muscle junction (Todd et al. 2010) (for review see Dias et al. 2013). Adenosine action at inhibitory A_1Rs and excitatory $A_{2A}Rs$ to modulate synaptic plasticity (e.g., LTP and LTD) in the brain underlies AR control of learning and memory. The precise contribution of A_1Rs and $A_{2A}Rs$ to adenosine regulation of synaptic plasticity in different brain regions, however, remains to be established.

15.3.1 A_1 Receptor Modulation of Synaptic Plasticity in Different Brain Regions

Despite the consistent inhibitory effect of the A_1R on glutamatergic transmission in the brain, studies with pharmacological and genetic manipulations of the A_1R have not produced consistent results on the A_1R control of synaptic plasticity in various brain regions. In the hippocampus, inactivation of A_1Rs can selectively augment mossy fiber basal transmission but attenuate both short-term plasticity (e.g., frequency facilitation and paired pulse facilitation) and LTP at this synapse (Moore et al. 2003). The A_1R activation via G protein-activated inwardly rectifying K(+) (GIRK) current in the hippocampus contributes to depotentiation of the previously potentiated LTP at Schaffer collateral synapses (Chung et al. 2009). However, local activation of A_1Rs impairs paired pulse facilitation but is not critical neither to the basal release probability and plasticity at mossy fiber synapses (Kukley et al. 2005)

nor LTD at the Schaffer collateral-CA1 pathway (Gimenez-Llort et al. 2005). In the striatum, A_1R inactivation has been shown to either abolish NMDAR-triggered LTD (Schotanus et al. 2006) and block short-term depression or have no effect on LTD at these synapses (Lovinger and Choi 1995). In cerebellar Purkinje cells, A_1Rs co-localize and form a heterodimeric complex with type-1 metabotropic glutamate receptor (mGluR1), and activation of the A_1R blocks mGluR1-mediated LTD (*glu-LTD*) (Kamikubo et al. 2013). In developing neocortex, local activation of A_1Rs presynaptically is critical to development shift in the release probability at synapses and potentially in long-term synaptic plasticity (Kerr et al. 2013). Additional studies are required to clarify the exact role of A_1R modulation of synaptic plasticity in various brain regions relevant to cognition.

15.3.2 Brain A_{2A} Receptors Modulate Synaptic Plasticity by Integrating Dopamine and Glutamate Signaling

The $A_{2A}R$, a G protein-coupled receptor, is highly enriched in striatopallidal neurons (Scanziani et al. 1992; Kim and Johnston 2015) where $A_{2A}Rs$ interact (possibly through heterodimerization) antagonistically with D_2Rs (Canals et al. 2003; Hillion et al. 2002; Fuxe et al. 2003) and NMDA receptors (Gerevich et al. 2002; Higley and Sabatini 2010) and synergistically with metabotropic glutamate receptor 5 (mGluR5) (Ferre et al. 2002; Coccurello et al. 2004; Kachroo et al. 2005) and cannabinoid CB_1 receptors (Lerner et al. 2010; Ferre et al. 2010). $A_{2A}Rs$ are also present at corticostriatal projections, mostly located at synapses (Rosin et al. 2003; Rebola et al. 2005a), where they modulate glutamate release that drives striatal neurons (Rebola et al. 2005a; Ciruela et al. 2006). Accordingly, striatal $A_{2A}R$ activation has been documented to promote LTP at the cortico-accumbal synapses (D'Alcantara et al. 2001) and spike-timing-dependent LTP at glutamatergic synapses onto the striatopallidal neurons (Shen et al. 2008a, b) and LTP at the cortico-striatopallidal synapses with FGFR co-activation (Flajolet et al. 2008). Thus $A_{2A}Rs$ at the corticostriatal pathway modulate synaptic plasticity underlying cognition by uniquely integrating dopamine and glutamate signaling in the striatum.

$A_{2A}R$-dopamine interaction: This $A_{2A}R$ facilitation of LTP process by a kinase A (PKA)-dependent mechanism of Ca^{2+} entry through NMDA receptors at the corticostriatal terminal counters the D_2R-mediated inhibitory effect on this synapse (Higley and Sabatini 2010). The intracellular cAMP levels in the striatopallidal neurons dictate bidirectional synaptic plasticity in the striatopallidal neurons in response to the corticostriatal afferent activity (Ferre et al. 2010). Through Gs-coupled $A_{2A}R$ (Mori and Shindou 2003) and Gi-coupled D_2R bidirectional regulation of cAMP signaling, concurrent activation of $A_{2A}Rs$ and D_2Rs in the striatopallidal neurons allow the integration of cAMP signaling and modification of synaptic plasticity in the striatopallidal neurons for behavioral adaptation. The postsynaptic striatal $A_{2A}R$ activation converts striatal LTD, the predominant form of long-term plasticity in the striatum, into LTP by countering D_2R effect (Ferre et al. 1997; Ongini and Fredholm 1996). Because phasic dopamine neuron firing

acts as a "prediction error" signal that causes learning (Kachroo et al. 2005; Lerner et al. 2010), striatopallidal A_{2A}Rs can modify dopamine signal to influence learning and memory through the A_{2A}R-D_2R interaction.

A_{2A}R-glutamate interaction: Glutamate (sensorimotor) signal at the corticostriatal pathway is crucial to striatal synaptic plasticity (such as spike time-dependent plasticity, STDP (Sebastiao and Ribeiro 2000) and the "gain" control of cortical incoming information. A_{2A}Rs may regulate glutamate signaling through its presynaptic control of glutamate release and postsynaptic interaction with NMDA receptors and mGluR5. The A_{2A}R is postulated to selectively engage in the implementation of synaptic changes in this excitatory synapses (Cunha 2008b). This facilitating role of A_{2A}R activation is accomplished by increasing glutamate release (Rodrigues et al. 2005), by facilitating NMDA receptor-mediated responses (Rebola et al. 2008) and by desensitizing presynaptic inhibition of A_1R (Lopes et al. 2002; Ciruela et al. 2006) or cannabinoid CB_1R (Martire et al. 2011).

By those distinct mechanisms, A_{2A}Rs at the corticostriatal pathway are critically important for the integration of incoming information (glutamate sensorimotor signal) and neuronal sensitivity to this incoming information (dopamine reinforcement signal) to control Hebbian synaptic plasticity, learning, and memory (Cunha 2008a, b; Schiffmann et al. 2007; Chen 2014).

Hippocampal A_{2A}Rs are localized postsynaptically at synapses between mossy fibers and CA3 pyramidal cells, and activation of hippocampal A_{2A}Rs modulates synaptic plasticity through multiple mechanisms, involving a postsynaptic NMDA-dependent LTP induced by short bursts of mossy fiber stimulation (Rebola et al. 2008), or AMPA-evoked LTP at the CA3-CA1 synapse by a PKA-dependent GluR1 phosphorylation at the Ser845 (Dias et al. 2012), or the kainate receptor-mediated LTD (*KAR LTD*) induced by high-frequency mossy fiber stimulation, natural spike patterns (Chamberlain et al. 2013), and BDNF-mediated LTP (Fontinha et al. 2008). In a trace eyeblink conditioning paradigm, A_{2A}R blockade inhibits experimentally evoked LTP at the CA3-CA1 synapses in the hippocampus and conditioned response behaviors (Fontinha et al. 2009). In another eyeblink conditioning paradigm from the turtle, in which the cranial nerves are directly stimulated in place of using a tone or air puff, phosphorylated 3-phosphoinositide-dependent kinase-1 (p-PDK1) has been found to increase and decrease, respectively, to paired and unpaired nerve stimulation, with the opposing actions of neurotrophin receptors TrkB and p75 (NTR). Both of these effects are blocked by the A_{2A}R antagonist. It is attributed to unique actions of A_{2A}R to activate Gs signaling and to transactivate TrkB for convergent activation of PDK1 and protein kinase A to initiate classical conditioning during paired stimulation.

15.4 The Tools for Studying Adenosine Receptor Control of Cognition in Behaving Animals

Various pharmacological, genetic, and optogenetic approaches have been used to provide a comprehensive assessment of the impact of each AR subtype in distinct brain regions (e.g., hippocampus, cortex, striatum) on various information processes

(e.g., encoding, storage, consolidation, retrieval) using different behavioral tasks. Earlier studies on the AR control of cognition mostly exploited AR antagonists and agonists to reveal the role of adenosine and its receptor targets in learning and memory. However, these pharmacological studies are limited by their partial specificity of AR drugs. Coupling pharmacological studies with complementary AR knockouts (KO) can overcome this limitation to provide some clarifications of the impact of $A_{2A}R$ and A_1R signaling on various tasks of learning and memory. These global genetic KO studies may, however, be confounded with potential developmental effects. Importantly, using pharmacological tools or even a global AR KO strategy, it is difficult to dissect out the specific contributions of the different AR subtypes in distinct brain regions. To address this issue, conditional KO of $A_{2A}R$ and A_1R genes in defined brain regions (e.g., cerebral cortex versus striatum versus hippocampus) and cell types (e.g., neurons versus astrocytes) has been achieved using the Cre-loxP system (for review see Wei et al. 2011a). Region-specific deletion of $A_{2A}Rs$ has been achieved in the forebrain (i.e., striatum, cerebral cortex, hippocampus) (Bastia et al. 2005; Yu et al. 2008), striatum (Shen et al. 2008a, b), and astrocytes (Matos et al. 2015). In addition, development of adeno-associated virus (AAV) vector carrying short-hairpin RNA targeted to produce site-specific silencing of the $A_{2A}R$ gene (Lazarus et al. 2011; Simoes et al. 2016) and local injection of AAV vectors containing the *cre* transgene into the brains of mice carrying loxP-flanked A_1R or $A_{2A}R$ genes (Scammell et al. 2003; Lazarus et al. 2011) have been used to achieve a temporal and regional specificity. This allow us to the previously uncover underappreciated functions of adenosine receptors in these brain regions, including focal knockdown of the A_1R in hippocampal CA1 or CA3 neurons (Scammell et al. 2003) and $A_{2A}Rs$ in the nucleus accumbens (Lazarus et al. 2011), dorsomedial striatum (Li et al. 2018), dorsolateral striatum (Li et al. 2016), hippocampus (Wei et al. 2014), and amygdala (Simoes et al. 2016). Finally, recent development of optogenetics by light control of neuronal activity with genetically engineered optical proteins (e.g., channelrhodopsin-2 and Arch) (Boyden et al. 2005; Deisseroth 2014; Yizhar et al. 2011) or chemicogenetic control of G-protein signaling by the directed molecular evolution of designer receptors exclusively activated by designer drugs (DREADD) (Farrell et al. 2013; Giguere et al. 2014) has potentiated dissection of specific brain circuits underlying cognition. To study cognitive behaviors such as working memory at the time scale of seconds, we have developed the novel opto-$A_{2A}R$ method to optogenetically control $A_{2A}R$ signaling in defined brain circuits of behaving animals, which enables us to interrogate the causal involvement of $A_{2A}R$ signaling in cognition with unparalleled spatiotemporal resolution (Li et al. 2015a, 2018).

15.5 Adenosine Receptor Modulates Learning and Memory in Normal Animals

Over the last two decades, neurochemical, pharmacological, and genetic knockout studies coupled with diverse sets of behavioral paradigms have begun to reveal the complexities and vastness of AR functions in cognition. Consistent with the ability

of the $A_{2A}R$ to integrate dopamine and glutamate signaling and to modulate synaptic plasticity (LTP in the hippocampus and LTP/LTD in the striatum) (D'Alcantara et al. 2001; Rebola et al. 2008), increasing evidence supports that brain $A_{2A}R$ activity contributes to modulation of learning and memory (Cunha et al. 2008; Cunha 2008b; Shen et al. 2008a, b; Ferre et al. 2008). Under physiological conditions, the $A_{2A}R$ exerts control over a variety of cognitive behaviors: (i) *short-term recognition memory*, as assessed using olfactory discrimination and social recognition memory (Prediger et al. 2005a, b; Prediger and Takahashi 2005), spatial recognition memory, and novelty exploration in Y-maze testing (Wang et al. 2006); (ii) *spatial working memory* (SWM) by radial maze tests (Gimenez-Llort et al. 2007) repeated trials of the Morris water maze and T-maze-based delay-non-match-to-place test (Li et al. 2018; Zhou et al. 2009); (iii) *reversal learning* as assessed by spatial reversal learning paradigm (Wei et al. 2011b); (iv) *goal-directed* vs *habitual behaviors* by satiety-based instrumental paradigm (Li et al. 2016; Hikida et al. 2013); (v) *Pavlovian fear conditioning* by eyeblink conditioning and context and tone fear conditioning (Wei et al. 2014; Hikida et al. 2013); (vi) *aversive learning* by conditioned taste aversion, avoidance behavior using an aversive paradigm, a one-trial inhibitory avoidance task (Pereira et al. 2005; Singer et al. 2013; Kopf et al. 1999); (vii) *effort-related decision-making and effort expenditure* (O'Neill and Brown 2007; Pardo et al. 2012; Pereira et al. 2011; Mott et al. 2009; Mingote et al. 2008); and (viii) *conditional temporal probability* by a task to dissociate the effect of elapsing time in the foreperiod and conditional temporal probability of the imperative stimulus (O'Neill and Brown 2007). Recent studies with refined conditional cell-specific $A_{2A}R$ KO, AAV-based shRNAi interference, and especially optogenetic control of $A_{2A}R$ signaling with unparalleled spatiotemporal resolution have offered several new insights into $A_{2A}R$ ability to fine-tune cognition under physiological conditions. Dissecting the impact of the $A_{2A}R$ on some forms of learning and memory is now leading to the new insights and better understanding of the mechanism underlying the $A_{2A}R$ control of cognition.

15.5.1 Striatopallidal A_{2A} Receptors Function as a Common "Break" Mechanism to Constrain Learning and Memory

Over the last several years, genetic KO studies have shown that the genetic deletion of $A_{2A}R$ or CD73 improves SWM, as gauged from the analysis of repeated acquisition paradigm in the Morris water maze or the 8-arm radial maze (Wei et al. 2011a; Zhou et al. 2009). Moreover, an improved WM is achieved by genetic deletion of $A_{2A}R$ either globally (i.e., global-$A_{2A}R$-KO) or by a selective deletion in the entire forebrain neuron (i.e., cerebral cortex, hippocampus, and striatum; fb-$A_{2A}R$-KO). Genetic deletion of $A_{2A}R$ selectively in the striatal neurons (st-$A_{2A}R$-KO) is sufficient to bolster SWM (Wei et al. 2011a; Zhou et al. 2009), Pavlovian fear conditioning (Wei et al. 2014), reversal learning (Wei et al. 2011b), and goal-directed behavior (Yu et al. 2009). Furthermore, bidirectional manipulations of the striato-

pallidal A_{2A}Rs by optogenetic activation of A_{2A}R signaling and Cre-mediated knockdown of A_{2A}Rs in the DMS unambiguously demonstrated that A_{2A}Rs in the DMS exert an inhibitory control of goal-directed behavior (Li et al. 2016). These findings are consistent with the fact that pharmacological reduction of A_{2A}R-mediated PKA-pCREB signaling in the DMS enhances acquisition of goal-directed ethanol drinking behaviors (Nam et al. 2013) and that A_{2A}R antagonists counter the D_2R antagonist effect and enhance effort-related decision-making in several behavioral paradigms including T-maze cost/benefit procedure and choosing voluntary exercise over sucrose consumption (Pardo et al. 2012; Pereira et al. 2011; Mott et al. 2009; Mingote et al. 2008; Correa et al. 2016). Notably, a recent study has demonstrated that A_{2A}R antagonism promoted impulsive responses during Pavlovian conditioning and the 5-choice serial reaction time task (5-CSRTT), with the reduced ERK1 and ERK2 phosphorylation in the dorsal hippocampus (dHip) (Oliveros et al. 2017). Collectively, these findings from diverse learning paradigms led us to propose that striatopallidal A_{2A}Rs function as a common "break" mechanism to constrain cognition (Chen 2014).

Although the striato-cortical interaction is mostly conceived as supporting the control of actions and procedural memory, there is an increasing recognition that striatal circuits are also actively involved in the control of declarative and episodic memory (Wei et al. 2011a, b; Simpson et al. 2010; Kellendonk et al. 2006; Li et al. 2011; Ito et al. 2008; Ferretti et al. 2010). In fact, the connectivity between the ventral striatum and the hippocampus (van Groen and Wyss 1990; Matthews et al. 2004; MacAskill et al. 2012) is involved in the retrieval of cue contingencies based on spatial locations and in the control of spatial behavior (Ito et al. 2008; Ferretti et al. 2010; Seamans and Phillips 1994; Maldonado-Irizarry and Kelley 1995; Floresco et al. 1997; Gengler et al. 2005; McDonald et al. 2006). With the increasing acceptance that the ventral striatum acts as an integrative unit associated with the adaptive encoding of working memory (Simpson et al. 2010; Scimeca and Badre 2012; Hallock et al. 2013) and reinforcement learning (Johnson et al. 2007; Piray 2011; Pennartz et al. 2011; van der Meer and Redish 2011; Liljeholm and O'Doherty 2012), it is possible to propose the striatopallidal pathway in the ventral striatum as a global inhibitory control system for declarative and episodic memory: this concept is based on the emerging evidence that the activity of the striatopallidal pathway provides inhibitory control for novel object recognition test (Durieux et al. 2012), amphetamine sensitization (Bateup et al. 2010), instrumental learning (Yu et al. 2009; Lobo et al. 2007), addiction (Durieux et al. 2009; Lobo et al. 2010), and probably goal-oriented behavior (Yu et al. 2009) and biases during decision-making (Tai et al. 2012). In this context, the proposed "a common break mechanism" by striatopallidal A_{2A}R activation provides a framework for a pharmacological strategy to improve cognitive deficits in aging and neuropsychiatric disorders by blocking striatopallidal A_{2A}R activity.

Notably, shA_{2A}R-mediated focal knockdown of the A_{2A}R in the brain regions outside the striatum, including the basolateral complex of the amygdala (Simoes et al. 2016), the ventral hippocampus (Wei et al. 2014), and the prefrontal cortex (Li et al. 2016), produced a facilitating effect of the A_{2A}R on Pavlovian fear

conditioning (Simoes et al. 2016; Wei et al. 2014) and SWM (Li et al. 2018). Together, these findings showed the brain-region-specific modulation of cognition by the $A_{2A}R$ activity.

15.5.2 Temporally Precise Integration of $A_{2A}R$ Signaling with Dopamine and Glutamate Signaling on the Striatopallidal Neurons for Cognitive Behavioral Control

The contemporary reinforcement learning theory postulates the "three-factor rule" of striatal plasticity underlying striatum-dependent learning: synaptic strength is regulated by spatiotemporally precise integration of nigra-striatal dopamine signal (the reinforcement signaling from the environment) and corticostriatal glutamate signaling (value coding from the reward history) to converge on the striatopallidal neurons for coding of the action and outcome/reward relationship (Yagishita et al. 2014; Augustin et al. 2014; Aquili et al. 2014). Consistent with this view, neurons in the prefrontal cortex fired selectively to rewarded (but not unrewarded) lever presses and precisely at the time of the reward delivery (Burgos-Robles et al. 2013). Furthermore, time-locked optogenetic stimulation of nigral dopamine and cortical glutamate (within 0.3–2 s) is critical to the modulation of striatal synaptic plasticity (Yagishita et al. 2014). The significance of the temporal relationship of dopamine, glutamate, and striatal signaling is demonstrated by optogenetic control of behaviors (such as stimulus-reward contingency) with the concurrent optogenetic stimulation of the striatal neurons with the onset of cue (within 5 ms but not 150 ms) (Tai et al. 2012) and by optogenetic inhibition of ventral striatal neurons in the time segment (1.5 s) between action selection and outcome (but not other time segments) (Aquili et al. 2014). According to this working hypothesis, concurrent activation of dopamine signal triggered by a motivationally significant event such as reward delivery with a postsynaptic striatal signal such as striatopallidal $A_{2A}R$ activity is critical to the striatum-dependent reinforcement learning (Schultz et al. 1997; Reynolds et al. 2001). Striatopallidal $A_{2A}Rs$ may modulate instrumental learning by acting precisely at the time of the reward to interact with the reward-triggered dopamine and glutamate signaling. Alternatively, striatopallidal $A_{2A}Rs$ may control instrumental learning, by modulating the vigor of actions without affecting the animal's action decision (Desmurget and Turner 2010), by modulating the "off-line" processing of incoming signaling (glutamate) for instrumental behavior (Pomata et al. 2008), or by providing a permissive role in learning association (Brainard and Doupe 2000). In these schemes of the vigor of action, "off-line" coding, or permissive effect, the temporal relationship between the $A_{2A}R$ activity and the reward is not essential. Due to the lack of methods to control $A_{2A}R$ signaling in freely behaving animals with required spatiotemporal resolution, the temporal relationship between $A_{2A}R$ signal and the reward-triggered dopamine and glutamate signaling in the control of instrumental behaviors was unknown until recently. Using our "opto-$A_{2A}R$"

method to optogenetically control the $A_{2A}R$ signaling at the millisecond resolution (Li et al. 2015a), we demonstrated that "time-locked" (but not "random") optogenetic activation of the striatopallidal $A_{2A}R$ signaling at the time of the reward is sufficient to affect instrumental behavioral modes (Li et al. 2015a). These studies define the effective temporal window whereby the striatopallidal neuronal activity (and striatopallidal $A_{2A}R$ activity) modulates learning and memory in the close temporal relationship with dopamine and glutamate signaling associated with cue and reward (Schultz et al. 1997; Reynolds et al. 2001). This integration may affect the intracellular cAMP level by concurrent activation of the D_2 receptor, NMDA receptors, and $A_{2A}R$ in the striatopallidal neurons, dictating bidirectional synaptic plasticity in the striatopallidal neurons for coding of the mode of instrumental learning behavior (Augustin et al. 2014). Interestingly, in the CA1 region of the hippocampus, enhanced NMDAR-dependent neuronal excitability by co-activation of mGluR5 and NMDARs is permitted by the $A_{2A}R$ activation, temporally coinciding with the robust increase in Src kinase-dependent NR2B (Tyr1472) phosphorylation (Sarantis et al. 2015). These studies provide new molecular insights into the temporal integration of adenosine-glutamate signaling in the hippocampus.

15.5.3 Dissecting AR Control of Distinct Information Processing Phases

Cognitive control of SWM involves multiple executive processes including encoding, maintenance, and retrieval of information, but the AR modulation of these SWM processes remains undefined due to lack of the methods to control AR signaling with the temporal resolution of seconds. The recent development of optogenetic control of $A_{2A}R$ signaling has provided a unique opportunity to address this issue. The specificity of opto-$A_{2A}R$ signaling (Li et al. 2015a) and the temporal resolution of the opto-$A_{2A}R$ are validated by the rapid electrophysiological response (within 3–18 s) (Li et al. 2018) and biochemical detection of opto-$A_{2A}R$-induced cAMP accumulation within 30 s (Li et al. 2015a) after opto-$A_{2A}R$ activation by light, which is consistent with the temporal resolution (within seconds) of opto-dopamine D1 receptor and opto-adrenergic α1 and ß2 receptors (Airan et al. 2009; Gunaydin et al. 2014). The opto-$A_{2A}R$ approach allowed us to demonstrate that optogenetic activation of striatopallidal $A_{2A}R$ signaling selectively during *the delay or retrieval* (but not *encoding*) phase impairs SWM performance (Li et al. 2018). Similarly, opto-$A_{2A}R$ activation in mPFC precisely during the *delay* phase (but not the *encoding and retrieval* phase) affects SWM performance (Li et al. 2018). This suggests that the cortico-striatopallidal $A_{2A}R$ signaling is critical to the *maintenance* (striatal and mPFC $A_{2A}Rs$) and *retrieval* (striatal $A_{2A}Rs$) processes of SWM. Lack of the effect of the striatopallidal $A_{2A}R$ activity on the coding of sensory information of SWM is apparently consistent with the previous finding that genetic KO or optogenetic activation of striatopallidal $A_{2A}R$ activity did not affect the acquisition or omission/extinction phases of instrumental learning (Yu et al. 2009; Li et al. 2016). These

findings of the distinct modulation of the three phases of SWM (i.e. encoding, maintenance, and retrieval) by optogenetic $A_{2A}R$ signaling in mPFC and striatum complement the recent ChR2-based optogenetic studies uncovering the vHPC-mPFC projections in the encoding of SWM (Spellman et al. 2015), the mPFC in the maintenance (Liu et al. 2014), and the medial entorhinal cortex (MEC)-hippocampal-thalamus nucleus circuit in the retrieval of SWM (Yamamoto et al. 2014). Collectively, these findings provide the potential circuit framework for passaging SWM information flow from the encoding (vHPC→mPFC projection) to the maintenance (mPFC, striatum, and thalamus) to the retrieval (MEC → HPC → ST → TH loop).

15.5.4 A_1 Receptors and Learning and Memory

For its wide and abundant expression patterns in various brain regions associated with learning and memory, and for its profound effect on neurotransmission, A_1Rs are traditionally thought to execute adenosine's potential modulatory effects on cognition. In line with the evidence of the A_1R control of mainly "basal" synaptic transmission, earlier pharmacological studies support the role of the A_1R control of learning and memory. For example, hippocampal A_1Rs influence working memory (Ohno and Watanabe 1996), prevent scopolamine-induced working memory deficits (Hooper et al. 1996), and prevent morphine-induced impairment in the retrieval of a spatial reference memory (Lu et al. 2010). However, studies from A_1R-KO mice suggest that A_1Rs may not be critical to some mnemonic effects of adenosine because A_1R-KO mice showed normal performance in the water maze, normal acquisition and retention of a spatial reference memory, normal SWM performance, and normal ability to learn the new position of a fixed platform during reversal learning in two different A1R-KO mouse lines (Gimenez-Llort et al. 2002, 2005; Lang et al. 2003). Thus, under physiologic conditions, the A_1R may not be crucial for the expression of normal spatial reference memory or SWM. It should be noted that an altered emotional status (Gimenez-Llort et al. 2002; Johansson et al. 2001) and a possible confounding developmental effect of A_1R KO in mice on A_1R control of cognition cannot be ruled out.

15.6 A_{2A} Receptor Antagonism Reverses Memory Impairments Under Various Pathological Conditions

Cognitive impairment is prevalent on aging and is accelerated in a pathognomonic manner in such neurodegenerative disorders as Alzheimer's disease (AD) and Parkinson's disease (PD), with the greatest socioeconomic impact in the Western world (Murray and Lopez 1997; Olesen et al. 2012; Wimo et al. 2013). Currently, there is no disease-modifying treatment to slow down or hold the disease progression.

The early symptoms associated with mild cognitive impairment (MCI), often evolving to AD (Landau et al. 2010; Ewers et al. 2012; Weintraub et al. 2012), are the emergence of short-term memory (STM) impairments with working memory (WM) deficit at its core (Baddeley et al. 1991; Baddeley 2003; Albert 1996; Grady et al. 2001; Belleville et al. 2008; Sperling et al. 2010; Koppel et al. 2014). Since 1993, FDA has approved three acetylcholinesterase inhibitors and an NMDA receptor antagonist memantine for improving cognition at early-moderate (AChE inhibitors) and moderate-later stage (memantine) of the AD (Aisen et al. 2012). However, these treatments do not have disease-modifying properties, and their use is limited by the poor efficacy (only 25% patients responded to the treatment) (Aisen et al. 2012; Amanzio et al. 2012; Jones 2010; Chaudhuri and Schapira 2009). The use of cholinesterase inhibitors to manage early cognitive impairments in PD patients may worsen their motor deficits (Chaudhuri and Schapira 2009; Richard et al. 2002; van Laar et al. 2011). Thus, identification and intervention at the earliest stage of AD/PD-MCI is a crucial unmet need for the overall care of AD/PD patients. In this context, experimental evidence suggests that pathological brain conditions associated with memory impairment (such as AD, stress, and inflammation) are accompanied by a local increase of the extracellular levels of adenosine (Cunha et al. 2001) and an upregulation and aberrant signaling of the brain $A_{2A}R$ (Chen et al. 2013; Cunha and Agostinho 2010). This led to the demonstration that blocking the "abnormal" activation of $A_{2A}R$ in specific brain regions (e.g., the hippocampus) confers protection against memory impairments under pathological conditions. Accordingly, under various pathological conditions, $A_{2A}R$ blockade prevents or reverses memory impairments caused by Aβ peptides via p38 MAPK pathway (Canas et al. 2009a; Dall'igna et al. 2007) and in transgenic hAPP AD model (Orr et al. 2015), in R6/2 transgenic model of HD (Li et al. 2015b), in the PD model with focal dopamine depletion in the cortex (Kadowaki Horita et al. 2013) or local injection of A53T α-Syn fibrils (Hu et al. 2016), and in the controlled cortical impact model and blast-induced traumatic brain injury (Ning et al. 2013; Zhao et al. 2017a, b) or caused by acute cannabinoid CB1 receptor activation (Mouro et al. 2017) and sporadic dementia (Espinosa et al. 2013). The involvement of the $A_{2A}R$ in pathological cognitive impairment is further supported by targeted neurogenesis gene-based association analysis in cognitively normal and impaired participants, leading to identification of $A_{2A}R$ gene (ADORA2A) as significantly associated with hippocampal volume (Horgusluoglu-Moloch et al. 2017).

15.6.1 The Aberrantly Increased $A_{2A}R$ Signaling in Cognition-Relevant Regions Is Sufficient to Trigger Memory Impairment

Under pathologic conditions, such as trauma and seizure, the activation of postsynaptic neurons can lead to the adenosine release, contributing to adenosine-mediated synaptic depression, an autonomic feedback mechanism to suppress excitatory

transmission during prolonged activity (Lovatt et al. 2012; Klyuch et al. 2012). Noxious brain conditions enhance the extracellular levels of ATP and the extracellular conversion of AMP into adenosine via CD73 enzyme (Zimmermann 2000). Furthermore, the density of hippocampal A_{2A}Rs, localized abundantly in hippocampal synapses (Rebola et al. 2005a), in particular in glutamatergic synapses (Rebola et al. 2005a), increases in aged animals (Canas et al. 2009b; Cunha et al. 1995; Lopes et al. 1999b; Rebola et al. 2003) and human AD (Albasanz et al. 2008), in transgenic mice displaying memory impairments (Espinosa et al. 2013; Cunha et al. 2006; Cognato et al. 2010), in the frontal cortex (mainly A_{2A}Rs in astrocytes) of AD brains (Orr et al. 2015), in the putamen of early (Braak PD stage 1–2) stage of PD (Villar-Menendez et al. 2014), and in the caudate of dyskinetic PD brains (Ramlackhansingh et al. 2011; Mishina et al. 2011). Interestingly, a recent study shows that the upregulation of astrocytic A_{2A}R in the hippocampus and neocortex of aging mice is induced by elevated levels of Aβ, C-terminal fragments of the amyloid precursor protein (APP), or amyloid plaques, but not overexpression of APP per se (Orr et al. 2018). This view of aberrantly increased A_{2A}R signaling is supported by the striking induction of the A_{2A}R in the hippocampus after A53T α-Syn fibril injection (Hu et al. 2016). Thus, the upregulated A_{2A}Rs may serve as a biomarker for PD and AD. Because several positron emission tomography (PET) ligands for the A_{2A}R, such as the A_{2A}R antagonist ligand [^{11}C]-SCH442416 and [^{11}C]-KW6002, have been developed and successfully employed to measure the level of striatal A_{2A}Rs of PD patients (Ramlackhansingh et al. 2011; Mishina et al. 2011; Khanapur et al. 2014), it would be essential to investigate whether these A_{2A}R antagonistic PET ligands can be used as an early diagnostic biomarker for AD and PD.

Is the aberrantly increased adenosine-A_{2A}R signaling a maladaptive consequence of aging, PD and AD pathologies, or a causal factor in the emergence of memory deficits? The finding that light activation of opto-A_{2A}R signaling in hippocampal neurons is sufficient (in the absence of neurodegeneration) to trigger memory impairment (Li et al. 2015a) argues that the marked upregulation of A_{2A}R expression in the hippocampus may be responsible (at least partially) for the development of A53T α-Syn-induced cognitive impairments. Similarly, the activation of A_{2A}Rs with CGS 21680 before the training session is also sufficient to trigger memory impairment in the object recognition task, inhibitory avoidance, and modified Y-maze in naive mice (Pagnussat et al. 2015). Transgenic overexpression of the A_{2A}R in the cortex amplified the synaptic plasticity and memory deficits triggered by GR in the hippocampus, which was reversed by A_{2A}R antagonism (Batalha et al. 2016). This is in line with the "common break" mechanism by activation of the striatopallidal A_{2A}Rs to constrain a variety of cognitive behaviors under physiological conditions (Li et al. 2016). This insight is validated by the reversal of A53T α-Syn fibril-induced working memory deficit by genetic deletion of A_{2A}Rs. In agreement with this view, A_{2A}R blockade can prevent memory dysfunction caused by Aβ peptides via p38 MAPK pathway (Canas et al. 2009a; Dall'igna et al. 2007) and in transgenic hAPP AD model (Orr et al. 2015, 2018) and R6/2 transgenic model of HD (by A2AR antagonists alone or in combination with D1R antagonists) (Li et al. 2015a, b; Tyebji et al. 2015), by the PD model with focal

dopamine depletion in the cortex (Kadowaki Horita et al. 2013), by controlled cortical impact model of traumatic brain injury (Ning et al. 2013, Zhao et al. 2017a, b), by chronic unpredictable stress (Kaster et al. 2015), and by sporadic dementia (Espinosa et al. 2013). Demonstration of the hippocampal $A_{2A}R$ upregulation by A53T α-Syn fibrils and the reversal of α-Syn-induced cognitive impairments, together with the demonstration of the sufficiency of optogenetic activation of $A_{2A}R$ signaling to induce cognitive impairments (Li et al. 2015a), suggest a plausible mechanism linking α-Syn to cognitive impairments in the absence of neurodegeneration. In the stress model induced by maternal separation, the $A_{2A}R$ blockade effectively reverted the behavior and electrophysiological and morphological impairments, with the restoration of the hypothalamic-pituitary-adrenal axis (HPA-axis) activity (Batalha et al. 2016).

On the other hand, the role of astrocytic $A_{2A}Rs$ in the development of cognitive impairment is not clear: selective deletion of astrocytic $A_{2A}Rs$ exhibited enhanced MK-801 psychomotor response and decreased working memory, accompanied by a disruption of glutamate homeostasis characterized by increased GLT-I activity and internalization of AMPA-R (Matos et al. 2015). In a mouse hAPP model of AD, chemogenetic activation of astrocytic Gs-coupled signaling (mimicking upregulation of astrocytic $A_{2A}Rs$ in human AD cortex) impaired long-term memory, while conditional genetic removal of these receptors enhanced memory (Orr et al. 2015). This justifies a need for additional studies to clarify the exact role of astrocytic ARs in cognitive control under normal and pathological conditions.

15.6.2 $A_{2A}R$ Inactivation Reverses Cognitive Impairments in Neurodegenerative Disorders by Modifying Aggregate Protein Processing and Countering Synaptopathy

MCI and early AD and PD are often associated with the changes in the brain levels of different forms of β-amyloid peptides, amyloid plaques, neurofibrillary tangles with phosphorylated Tau proteins for AD (Galasko et al. 1998; Andreasen et al. 2001; Riemenschneider et al. 2002; Mattsson et al. 2009), and α-synuclein aggregates for PD (Brundin and Melki 2017; Goedert et al. 2017; Masuda-Suzukake et al. 2013), argued to be major culprits of AD and PD (Hardy and Selkoe 2002; Walsh and Selkoe 2004). Increasing evidence points to the novel mechanism that $A_{2A}R$ inactivation protects against pathological cognitive impairments by modification of proteins that trigger neurodegeneration, including β-amyloid synaptopathy (Canas et al. 2009a; Cao et al. 2009), α-synuclein (Laurent et al. 2016; Ferreira et al. 2017), and Tau protein (Laurent et al. 2016). (I) Studies of aged AD transgenic (APPsw, Swedish mutation) mice found that caffeine (nonselective adenosine antagonist) treatment (1.5 mg daily dose, equivalent to 500 mg in human) to APPsw mice reduced brain Aβ levels with reduced presenilin 1 (PS1) and beta-secretase (BACE) expression, leading to protection against certain cognitive

impairments (Cao et al. 2009; Arendash et al. 2006, 2009). (II) Three recent studies (including ours) strongly support the $A_{2A}R$ modulation of α-synuclein aggregation by showing decreased α-Syn aggregation in the hippocampal neuron with reduced number of pSer129 α-Syn-rich and p62-positive inclusions in $A_{2A}R$-KO mice (Hu et al. 2016), decreased the percentage of cells displaying α-Syn inclusions in cultured cells after $A_{2A}R$ antagonist treatment (Ferreira et al. 2017), and attenuated toxicity of α-Syn aggregates in vitro and in a yeast proteotoxicity model of PD after caffeine treatment (Kardani and Roy 2015). These findings are in line with the previous study showing that the $A_{2A}R$ KO prevents loss of dopaminergic neurons caused by the transgenic overexpression of intracellular human α-Syn containing both A53T and A30P mutations (Kachroo and Schwarzschild 2012). (III) In a THY-Tau22 model of AD, genetic deletion of the $A_{2A}R$ protects from Tau pathology-induced deficits in terms of spatial memory and hippocampal long-term depression, with a concomitant decrease in Tau hyperphosphorylation, normalization of the hippocampal glutamate/GABA ratio, and a global reduction in neuroinflammatory markers (Laurent et al. 2016). The $A_{2A}R$ antagonist MSX-3 also improved memory and reduced Tau hyperphosphorylation in THY-Tau22 mice (Laurent et al. 2016). In the controlled cortical impact model of traumatic brain injury (TBI), genetic deletion of the $A_{2A}R$ or treatment with the $A_{2A}R$ antagonist ZM241385 or caffeine reduced the level of Tau phosphorylation at Ser404 and alleviated spatial memory dysfunction (Zhao et al. 2017b). Interestingly, 14-month-old proaggregant-Tau-transgenic mice developed neuronal and astrocytic hypoactivity and presynaptic dysfunction, which were reversed by treatment with A_1R rolofylline (KW-3902) (Dennissen et al. 2016). (IV) On the other hand, in HD model, $A_{2A}R$ activation enhanced proteasome activity and reduced mutant huntingtin aggregations through the PKA-dependent pathway (Huang et al. 2011; Chiang et al. 2009). Collectively, these findings support that AR antagonists including caffeine may attenuate PD and PD pathology by a mechanism other than proteasome pathway.

Furthermore, MCI and early AD and PD are also associated with the loss of synapses in defined brain cortical regions, most evident in the hippocampus in MCI and early phases of AD (Scheff et al. 2007; Coleman et al. 2004; Selkoe 2002) and during aging (Burke and Barnes 2010; Morrison and Baxter 2012). In fact, a synapse is the primary target of toxic Aβ oligomers (Hardy and Selkoe 2002), and the loss of synapses in the hippocampus is probably the earliest morphological trait and the best correlated with initial memory impairment in AD (Coleman et al. 2004). Indeed, $A_{2A}R$s are most abundant in hippocampal synapses (Rebola et al. 2005b), in particular in glutamatergic synapses (Rebola et al. 2005b). The density of hippocampal $A_{2A}R$ increases in aged animals (Canas et al. 2009b; Cunha et al. 1995; Lopes et al. 1999b; Rebola et al. 2003) and human AD (Albasanz et al. 2008) as well as in transgenic mice displaying memory impairments (Espinosa et al. 2013; Cunha et al. 2006; Cognato et al. 2010). In AD model with the intracerebral administration of soluble Aβ(1–42) (2 nmol) in rats or mice, memory impairment and a loss of nerve terminal markers without overt neuronal loss, astrogliosis, or microgliosis were observed, whereas the $A_{2A}R$ antagonist SCH58261 (50 nm) prevented

the initial synaptotoxicity (loss of MAP-2, synaptophysin, and SNAP-25 immunoreactivity), through the p38-dependent and cAMP/PKA-independent pathways (Canas et al. 2009a). Similarly, pharmacological and genetic blockade of $A_{2A}R$ and caffeine treatment efficiently prevented chronic unpredictable stress-induced memory deficits and the associated loss of synapses, typified by a decrease in synaptic plasticity and a reduced density of synaptic proteins (synaptosomal-associated protein 25, syntaxin, and vesicular glutamate transporter type 1) (Kaster et al. 2015). Altogether, these evidences indicate that the $A_{2A}R$ plays an effective role in modifying aggregated protein processing and counteracting synaptopathy, both of which contribute to memory function preservation.

15.6.3 $A_{2A}R$ Antagonist Control of Cognition in Nonhuman Primates

Higher cognitive disorders in humans involve the association cortex, which is regulated in a fundamentally different manner from the older sensory-motor cortical and subcortical circuits and thus is not suitable to study in rodent models, whose brains have a very small association cortex (Goldman-Rakic 1987). For the complex nature of higher cognition functions in human, developing the effective pharmacological strategy to improve cognition would require preclinical data from nonhuman primates because higher cognitive functions involve the association cortices, which are evolutionally poorly developed in rodents and thus cannot be adequately addressed by standard pharmacological and genetic studies in rodent models (Goldman-Rakic 1987). In recent clinical trials of $A_{2A}R$ antagonists and caffeine for motor benefits in PD, the possible cognitive effects of $A_{2A}R$ antagonists and caffeine were not evaluated (Aarsland et al. 2010), in part due to the lack of cognitive behavior data from nonhuman primate model of PD. Besides increasing evidence from rodent models of PD supporting that pharmacological and genetic inactivation of $A_{2A}Rs$ can prevent WM dysfunction under multiple pathological conditions (for a review see Chen 2014), two studies have addressed this knowledge gap by testing $A_{2A}R$ antagonists (such as istradefylline in a clinical trial) in nonhuman primate models of PD (Li et al. 2018). In the MPTP-treated macaque model of parkinsonian and dyskinetic motor symptoms, the $A_{2A}R$ antagonist istradefylline reduced the attentional and working memory deficits caused by l-DOPA (Ko et al. 2016). In MPTP-treated cynomolgus monkeys coupled with delay-non-match-to-sample/place (DMTS/DMTP) paradigm, we showed that the $A_{2A}R$ antagonist KW6002 ameliorated spatial working memory deficits (Li et al. 2018). Identification of the proper dose and the treatment paradigm of the $A_{2A}R$ antagonist KW6002 to enhance SWM may provide required preclinical data to facilitate the design of a clinical trial of $A_{2A}R$ antagonists for cognitive benefit in PD patients. Last, in squirrel monkeys trained to self-administer cannabinoids intravenously, the $A_{2A}R$ antagonists SCH-442416 and KW6002 produced a significant shift to the right and left, respectively, of the

cannabinoid self-administration dose-response curves (Justinova et al. 2014), paving the way for the development of $A_{2A}R$-based treatment for drug addiction.

15.7 Epidemiological and Animal Studies Support Pro-cognitive Effects of the Adenosine Receptor Antagonist Caffeine in Aging and Alzheimer's Disease

In the absence of an effective disease-modifying treatment to slow down or stop AD, epidemiological and experimental investigations of the potential risk factors (including dietary factors) that may allow individuals to decrease their risk for AD and improve cognitive symptoms have become compelling. Caffeine is doubtless the most widely consumed psychoactive substance by >50% of the world's adult population, largely for its psychostimulant (and cognitive enhancement) effect. At least seven longitudinal studies support an inverse relationship between caffeine consumption and decreased memory impairments associated with aging as well as a reduced risk of developing AD (for a review see Chen 2014), including the *Maastricht Aging Study* (van Boxtel et al. 2003; Hameleers et al. 2000), the *Canadian Study of Health and Aging (CSHA)* (Lindsay et al. 2002), the *FINE study* (van Gelder et al. 2007), the *French Three-City Study* (Ritchie et al. 2007), the *Cardiovascular Risk Factors, Aging, and Dementia (CAIDE) Study* (Eskelinen et al. 2009), and the *Honolulu-Asia Aging Study* (Gelber et al. 2011). For example, *the Honolulu-Asia Aging Study* involved 3494 men with a mean age 52 at cohort entry in 1965–1968 and found that the men in the highest quartile of caffeine intake were less likely than men in the lowest quartile to have any neuropathologic lesions at death in the 226 men with dementia and the 347 men with cognitive impairment who underwent brain autopsy (Gelber et al. 2011).

In further support of this inverse correlation between caffeine consumption and cognitive decline, animal studies show a causal role of caffeine in neuroprotection in animal models of AD: I) caffeine treatment reduced Aβ peptide-induced aggregation in cultured cerebellum granular cells and protected against loss of learning and memory induced by intracerebroventricular infusion of Aβ peptide (Canas et al. 2009a; Dall'igna et al. 2007; Espinosa et al. 2013) (210, 211, 220). II) Studies with aged AD transgenic (APPsw, Swedish mutation) mice found that long-term administration of a 1.5 mg daily dose of caffeine (equivalent to 500 mg in human) reduced brain Aβ levels and protected against certain cognitive impairments in 4–9-month-old APPsw mice; furthermore, in aged (18–19 months old) APPsw mice, which already exhibit decreased cognitive function, caffeine treatment enhanced working memory compared to non-treated APPsw mice (Cao et al. 2009; Arendash et al. 2006, 2009). III) Long-term oral caffeine treatment not only sustainably reduced plasma Aβ but also decreased both soluble and deposited Aβ in the hippocampus and cortex of aged AD mice (Cao et al. 2009). Intriguingly, caffeine's ability to improve cognitive performance in individual aged AD mice did not correlate with reduced plasma Aβ levels but was closely associated with the

reduced inflammatory cytokine levels in the hippocampus (Cao et al. 2009). In addition, caffeine acts at the neuronal $A_{2A}R$ to reverse cognitive impairments and associated synaptic dysfunction induced by chronic unpredictable stress (Kaster et al. 2015) and by depression-prone, hopeless mice (Machado et al. 2017).

This convergence of the epidemiological and animal evidence led to the proposal that caffeine might be a novel prophylactic agent to alleviate the burden of AD. The recent case-control study involving 124 total individuals provides the first direct evidence that caffeine/coffee intake is associated with a reduced risk of dementia (Cao et al. 2012). The study found that subjects with plasma caffeine levels greater than 1200 ng/ml at study onset were associated with stable MCI → MCI and no conversion to dementia during the 2–4-year follow-up examination (Cao et al. 2012). However, a very recent randomized control clinical trial of caffeine in PD has failed to confirm motor benefits with apparently exacerbated cognitive impairments (Postuma et al. 2017). Additional clinical studies are warranted to clarify this controversy and to test decisively the putative neuroprotective effects of caffeine in clinical trials in patients with AD.

15.8 Translational Potential of the Adenosine Receptor-Based Drugs for Controlling Cognitive Deficits in Neuropsychiatric Disorders

The convergence of clinical, epidemiological, and experimental evidence led to the proposal to translate the cognitive enhancement in rodents and nonhuman primates, and the safety profile of adenosine receptor, the $A_{2A}R$ antagonists, in particular, documented in clinical phase III trials in Parkinson's disease patients, to demonstrate the crucial ability of brain adenosine receptors (such as the $A_{2A}R$) to control cognitive deficits in neuropsychiatric disorders. Over the last 8 years, a total of 25 clinical trials have been conducted (for review see Chen et al. 2013). Six double-blind placebo-controlled clinical phase IIb and III trials of istradefylline (KW-6002) involving >2500 advanced PD patients and one phase IIb trial with preladenant (SCH420814) involving 253 PD patients were reported (Hauser et al. 2011). These clinical IIb and III trials have shown a modest but significant motor benefit: a reduction of the average "OFF" time by ~1.7 h compared to the "optimal" L-dopa dose regimen (Jenner et al. 2009); however, in 2008, the FDA found that efficacy results for motor benefits in these PD clinical trials were not sufficient, considered that this modest motor benefit was not sufficient to support the clinical utility of istradefylline. Additional PD clinical trials with istradefylline in Japan were undertaken to show consistent motor benefits, leading to the approval of istradefylline for treatment of PD in Japan in March 2013 (Dungo and Deeks 2013). Unfortunately, the effects of $A_{2A}R$ antagonists on cognition were not evaluated in these clinical trials. This is mostly due to the insufficient preclinical data on the ability of $A_{2A}R$ to control cognition – a knowledge gap that needs to be filled by future studies. Relevant

to drug discovery for cognitive improvement, these clinical IIb and III trials with the $A_{2A}R$ antagonists showed a very consistent and excellent safety profile in >3000 advanced PD patients (Hauser et al. 2011; Jenner et al. 2009). This safety profile of $A_{2A}R$ antagonists is entirely consistent with the widespread use of the nonselective adenosine receptor antagonist caffeine in 70% human population. Importantly, this provides an opportunity to translate rapidly $A_{2A}R$ antagonists to achieve cognitive improvement in neuropsychiatric disorders.

15.9 Summary

There is a convergence of molecular, animal, and epidemiological evidence suggesting that the $A_{2A}R$ and caffeine represent novel therapeutic strategies to improve cognitive impairments associated with neuropsychiatric disorders. The validity of this novel target is supported by the finding that $A_{2A}R$ antagonists and caffeine not only selectively enhance SWM, recognition memory, reversal learning, goal-directed behavior, Pavlovian conditioning, and effort-related behaviors in normal animals but also reverse SWM impairments in animal models of traumatic brain injury, PD, AD, schizophrenia, and HD. Pharmacological, genetic, and optogenetic studies coupled with well-controlled behavioral paradigms have revealed new insights into the mechanisms underlying AR control of cognition under physiological conditions (e.g., spatiotemporally precise integration of adenosine with dopamine and glutamate signaling, a common "break" mechanism by the striatopallidal $A_{2A}R$ to constrain cognition). Furthermore, $A_{2A}R$ inactivation reverses cognitive impairments in neurodegenerative disorders by blocking aberrantly increased $A_{2A}R$ signaling, by modifying aggregate protein processing, and by countering synaptopathy. Despite the converging animal and epidemiological evidence and the noted safety profiles of $A_{2A}R$ antagonists and caffeine, the therapeutic potential as well as the mechanism of $A_{2A}R$ antagonist effect on cognition in neuropsychiatric disorders remains to be established. Due to the insufficient preclinical data on this aspect, the effect of $A_{2A}R$ antagonists on cognition was not considered in these clinical PD trials. This may justify additional animal studies to better understand the mechanism underlying the $A_{2A}R$-mediated control of cognition in healthy brains (e.g., the permissive effect of AR, the spatiotemporal integration of adenosine/dopamine/glutamate signaling, and the selective control of distinct information processing phase). Further exploration of the molecular pathways whereby the adenosine receptor modifies degenerative proteins (such as phosphorylated Tau, α-synuclein, and β-amyloid) and prevents the early synaptic loss is critically needed. These studies may reveal the cellular and circuit mechanisms underlying the AR control of cognition and provide the required rationale to stimulate the necessary clinical investigation to translate rapidly $A_{2A}R$ antagonists and caffeine as novel strategies to control memory impairment associated with neuropsychiatry disorders.

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Chapter 16
The Adenosine-Receptor Axis in Chronic Pain

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Abstract Chronic pain is a widespread problem that plagues an estimated 10 to 30% of the world's population. The current therapeutic repertoire is inadequate in managing patient pain with narcotic use resulting in a drug overdose epidemic, affirming the need for the development of new therapeutics. Adenosine and its four cognate receptors (A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR) play essential roles in physiological and pathophysiological states, including chronic pain. For decades, preclinical and clinical studies have revealed that adenosine and A_1AR- and to a lesser extent A_{2A}AR-selective agonists have analgesic properties, yet their therapeutic utility has been limited by adverse cardiovascular side effects. There is no evidence that A_{2B}AR plays a role in pain. Recent preclinical studies have demonstrated that selective A_3AR agonists result in antinociception in models of acute and chronic pain while lacking unwanted side effects. These exciting preclinical observations of A_3AR agonists have been bolstered by clinical trials of A_3AR agonists in other disease states including rheumatoid arthritis and psoriasis that suggests a clinical benefit without cardiotoxicity. Our goal herein is to briefly discuss adenosine and its receptors in the context of pathological pain and examine what is known at present regarding A_3AR-mediated antinociception. We will highlight recent findings pertaining to A_3AR in pain and describe possible pathways by which A_3AR may mediate its effects and the current state of selective A_3AR agonists used in pain studies. The adenosine-to-A_3AR pathway represents an important endogenous system that can be targeted to provide safe, effective pain relief in patients suffering with chronic pain.

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16.1 Introduction

Chronic pain afflicts an estimated 10% of the world's adult population (Goldberg and McGee 2011) and approximately 30% of American adults with an estimated societal cost in the billions annually (Institute of Medicine 2011). The current therapeutic approaches for chronic pain include but are not limited to the use of NSAIDs, antidepressants, anticonvulsants, and opioid pain relievers; however, these strategies are frequently inadequate and/or are associated with side effects that reduce quality of life. Escalating doses are needed to produce analgesic efficacy, while side effects and potential addiction often result in the discontinuation of therapy (Goldberg and McGee 2011; Pizzo and Clark 2012). There is a desperate need for novel therapeutics that engage molecular targets in the nociceptive and inflammatory pathways that will not result in unwanted side effects nor result in severe analgesic tolerance. Adenosine and two of its associated adenosine receptor (AR) subtypes, A_1AR and $A_{2A}AR$, have been investigated for their ability to inhibit pain with varying degrees of success but lack a useful therapeutic index due to cardiovascular side effects and are not being currently pursued for pain. However, the A_3 subtype, A_3AR, has resulted in preclinical antinociceptive efficacy in a variety of pain models (Ford et al. 2015; Janes et al. 2014b, 2015; Little et al. 2015; Yoon et al. 2004) and has demonstrated efficacy and safety in trials for non-pain conditions including psoriasis, hepatitis, rheumatoid arthritis, dry eye, and glaucoma. Hence, A_3AR agonists have clinically acceptable therapeutic indices that may be suitable for the treatment of chronic pain (Fishman et al. 2012). Importantly, such compounds lack rewarding behavior removing the potential for addiction and show long-term efficacy after sustained used (Little et al. 2015). The aim of this review is to summarize the existing literature on adenosine and its receptors in the context of pain with a particular emphasis on A_3AR and its prospect as a novel solution to the problem of chronic pain management.

16.2 Adenosine Production and Metabolism

The endogenous purine nucleoside adenosine through its cognate receptors is a potent regulator of a wide variety of physiological processes affecting nervous (Boison 2013, 2016; Gomes et al. 2011; Wei et al. 2011), cardiovascular (Headrick et al. 2011), renal (Vallon and Osswald 2009), immune (Hasko et al. 1998; Hasko et al. 1996), and cell cycle (Fishman et al. 2009) functions. In the central nervous system (CNS), extracellular adenosine provides neuroprotective, anti-inflammatory,

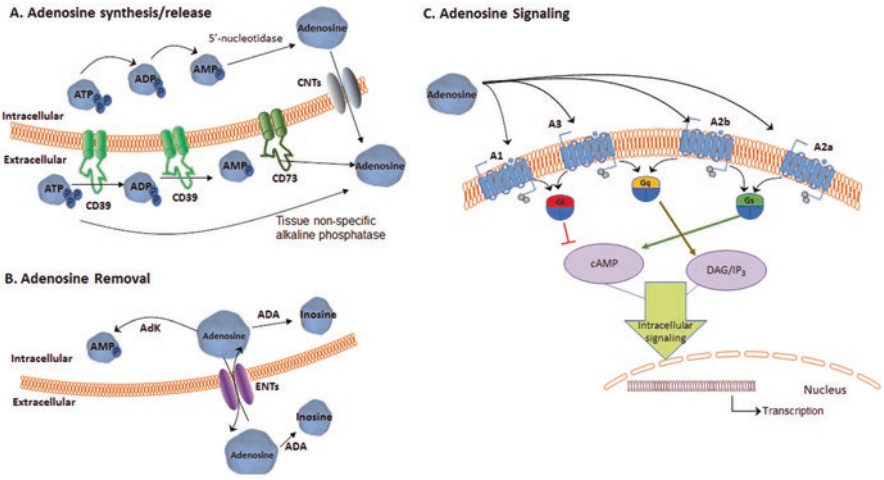

Fig. 16.1 Adenosine synthesis and metabolism. (**a**, **b**) ATP is released from various cell types in response to a number of stimuli. The phosphate groups of ATP can then be sequentially removed giving rise to ADP, AMP, and then adenosine. Ectonucleotidases (CD39, CD73) feed into this pathway by hydrolyzing nucleotides to adenosine for transport back into the cell via equilibrative nucleoside transporters (ENTs) or concentrative nucleoside transporters (CNTs). In the intracellular space, adenosine can be converted to AMP (by adenosine kinase, AdK) which in turn is catalyzed to AMP and then ATP or deaminated to inosine by ADA. Intracellular adenosine can be generated from AMP by 5′-nucleotidase. (**c**) Extracellular adenosine can act on its cognate receptors (ARs: A_1, A_{2A}, A_{2B}, and A_3)

and neuromodulatory effects by regulating glial activity (Cunha 2008; Dias et al. 2013) and glutamatergic, GABAergic, cholinergic, and dopaminergic neurotransmission (Sebastiao and Ribeiro 1996). The extracellular *function* of adenosine is tightly regulated by homeostatic control of the intracellular/extracellular adenosine gradient and the local adenosine receptor profile (Deussen et al. 1999; Zimmermann 2000).

Adenosine is produced by nearly all cells (Zimmermann 2000) through intracellular and extracellular metabolic pathways (Fig. 16.1). Intracellular adenosine is generated either by the dephosphorylation of AMP via soluble 5′-nucleotidases or S-adenosylhomocysteine (SAH) hydrolysis (Latini and Pedata 2001). In the CNS, soluble 5′-nucleotidases activity appears to be the predominate route of intracellular adenosine production (Engler 1991). Extracellular generation of adenosine arises from the dephosphorylation of ATP by ectonucleotidase activity within the extracellular space. Upon its extracellular release during neurotransmission or in response to cellular injury (Ballarin et al. 1991; Engler 1991; Latini and Pedata 2001), ATP is first dephosphorylated by ectonucleoside triphosphate diphosphohydrolases (CD39 family) to AMP and then to adenosine by ecto-5′-nucleotidase (CD73) (Bonan 2012; Robson et al. 2006). Alternatively, extracellular adenosine can be generated by tissue-nonspecific alkaline phosphatase dephosphorylation of any of the adenosine nucleotides (Sebastian-Serrano et al. 2015).

Basal levels of extracellular adenosine within the CNS are maintained around 25–250 nM (Dunwiddie and Masino 2001). This is accomplished by sodium-coupled influx of adenosine through concentrative nucleoside transporters (CNT/Slc28) (Bonan 2012; Choi and Berdis 2012) or by passive influx/efflux of adenosine down its gradient through the ubiquitous equilibrative nucleoside transporters (ENT1/Slc29A1 and ENT2/SLC29A2) (Brundege and Dunwiddie 1998; Peng et al. 2005). The adenosine gradient for ENT function is established by the balance of adenosine production as already described and the depletion of its intracellular stores by adenosine kinase (AdK) phosphorylation of adenosine (Spychala et al. 1996) and catabolism by adenosine deaminase (ADA) to inosine (Blackburn and Kellems 1996). AdK and ADA limit the physiological half-life of adenosine to <1 s (Moser et al. 1989) to establish a normally inward driving adenosine gradient. Studies have revealed AdK is a major driving force for both intracellular and extracellular adenosine (Boison 2016). The expression of AdK in neurons during development of the nervous system is necessary for neurite outgrowth and synaptic formation; but during postnatal development, AdK expression shifts primarily to astrocytes where it is involved in maintaining adenosine homeostasis (Studer et al. 2006). Inhibiting AdK activity significantly increases the intracellular concentrations of adenosine, which reverses its gradient and drives it out through the ENT channels into the extracellular space (Keil and DeLander 1992; Zhang et al. 1993). Consequently, efforts have been made to target AdK activity for the treatment of a number of neuropathologies (Boison 2008b, 2013, 2016; Kowaluk et al. 1999). In pain, pharmacological inhibition of AdK in the CNS results increased the ENT-dependent release of adenosine, which in turn attenuated spinal nociceptive transmission (Otsuguro et al. 2015). Moreover, enhancing endogenous adenosine signaling using AdK inhibitors has been shown to be efficacious in rodent models neuropathic pain (Kowaluk et al. 2000; Little et al. 2015; McGaraughty et al. 2005).

16.3 Adenosine Receptors

Adenosinergic signaling is mediated through four cognate G protein-coupled receptors: A_1, A_{2A}, A_{2B}, and A_3AR. The A_1A and A_3A receptor subtypes couple to Gαi to inhibit adenylate cyclase formation (Boison et al. 2010; Fredholm et al. 2011). However, there is evidence that A_3AR also associates with Gαq/11 to stimulate phospholipase C (Parsons et al. 2000). In contrast, A_{2A} and A_{2B} receptor couple to G_s and stimulate adenylyl cyclase and produce elevations in intracellular cAMP (Fredholm et al. 2001).

In the CNS, A_1AR is widely expressed in the brain and superficial laminae of the spinal cord dorsal horn (Gessi et al. 2011). The expression of A_1AR is highest in neurons (Cunha 2001, 2005) where it is expressed on both the presynaptic and postsynaptic membrane (Cunha 2001, 2005; Gessi et al. 2011) and associated with the modulation of neurotransmission by reducing the presynaptic release of glutamate and increasing the postsynaptic hyperpolarization (Cunha 2005). In glia, A_1AR

expression is downregulated in multiple sclerosis (Johnston et al. 2001) and the dorsal horn of the lumbar spinal cord following plantar incision, a model of postoperative pain (Yamaoka et al. 2013). However, A_1AR has also been shown increase in the dorsal horn following traumatic nerve injury (Yamaoka et al. 2013). These findings suggest receptor expression is differentially regulated depending on the nature of the injury. This is further supported by findings that in primary mouse microglia, A_1AR expression increases in response to ATP but reduced following exposures to endotoxin (Luongo et al. 2014). From a clinical standpoint, it is important to note that A_1AR is also highly expressed in cardiovascular tissue, particularly the atrioventricular node, which is associated with A_1AR-mediated high-grade atrioventricular block mediated following treatments with A_1AR agonists (Kiesman et al. 2009).

A_{2A}AR expression in the brain on striatal postsynaptic neurons, hippocampal and cortical presynaptic neurons, and glial cells (Rebola et al. 2005; Svenningsson et al. 1997). The expression of A_{2A}AR can increase following hypoxia, spinal cord injury, and streptozotocin-induced diabetes (Janes et al. 2014b). In monocytes/microglia, the expression of A_{2A}AR is enhanced by pro-inflammatory mediators such as IL-1β and TNF-α (Morello et al. 2006). In the cardiovascular system, the epithelium of coronary blood vessels express A_{2A}AR and exert vasodilatory effects in response to A_{2A}R agonists (Fredholm et al. 2011; Gao and Jacobson 2007; Jacobson and Gao 2006).

Collectively, A_{1A}R and A_{2A}AR comprise the bulk of the adenosine receptor expression the CNS (Gomes et al. 2011). In contrast, the lower-expressed and lower-affinity A_{2B}AR is found in the neuroimmune cells of the CNS and within the cardiovascular system. A_{2B}AR activity in microglia is associated with IL-6 expression and microglial proliferation (Merighi et al. 2017). However, A_{2B}AR transcript in the cortex of a normal mouse brain has been reported to be expressed mainly in astrocytes and oligodendrocyte progenitor cells (Zhang et al. 2014).

Species-specific differences exist for A_3AR structure and distribution. In rats, A_3AR expression is highest in testis and mast cells, whereas in humans, A_3AR expression is highest in the liver and lung (Borea et al. 2015). High expression of A_3AR has been reported in human coronary and carotid arteries (Grandoch et al. 2013; Hinze et al. 2012) and several studies have found A_3AR signaling is cardioprotective during ischemic injury (Cross et al. 2002; Harrison et al. 2002; Headrick and Peart 2005; Thourani et al. 1999a, b; Tracey et al. 1997) and doxorubicin-induced cardiotoxicity (Shneyvays et al. 1998, 2001). In the CNS, A_3AR is expressed at much lower levels than A_{1A}R and A_{2A}AR. However, A_3AR has higher expression on many immune cell types, including glial cells (Abbracchio et al. 1997; Ochaion et al. 2009; Poulsen and Quinn 1998), and can be found on both peripheral (Ru et al. 2011) and central neurons (Giannaccini et al. 2008; Jacobson et al. 1993; Lopes et al. 2003; Zhang et al. 2010) of the brain and spinal cord (Borea et al. 2015; Haeusler et al. 2015). In pain-processing centers, A_3AR transcript and protein have been identified in the lumbar spinal cord and rostral ventromedial medulla (RVM) (Little et al. 2015).

The expression and distribution of adenosine receptors throughout the CNS and on cells responsible for pathophysiological changes within the CNS during development and maintenance of pain (Cao and Zhang 2008; Nagata et al. 2009; Obata and Noguchi 2008; Watkins et al. 2001) provide unique advantages to targeting these receptors. However, as will be discussed, activation of many of these receptors provides similar effects in models of pain despite differences in the coupling mechanisms of these receptors. These similarities may be due to their tissue distribution, expression regulation under pain conditions, the components of the microdomains in which they associate, and the endogenous ligands to which they respond such as the partial agonism of inosine at A_1AR and A_3AR.

16.4 Adenosine and Pain

The analgesic effects of adenosine have been known for many years now. In the clinic, intrathecal adenosine provided sustained relief for several hours to months of chronic neuropathic pain (Hayashida et al. 2005). Adenosine and its analogues have consistently been shown to inhibit pain behavior in a number of neuropathic and inflammatory pain models arising from various etiologies, such as spinal cord injury, spinal nerve ligation, and exposure to mustard oil, formalin, or carrageenan (Dickenson et al. 2000). The beneficial effects of adenosine have been associated with its regulation of excitatory neurotransmission, persistent neuronal signaling, and glial activation and proliferation (Boison 2008a; Boison et al. 2010; Cunha 2005; Daniele et al. 2014; Studer et al. 2006). Despite the promising data from animal pain models and early clinical chronic pain studies, the effectiveness of adenosine therapy for the prevention of postoperative pain has been mixed. Prophylactic intravenous administration of adenosine prior to surgical procedures conferred persistent pain relief in several studies (Gan and Habib 2007; Hayashida et al. 2005), but not in others (Habib et al. 2008). Moreover, intravenous adenosine therapy is associated with serious adverse cardiac side effects (Zylka 2011) limiting its utility. Thus, evaluating the receptor subtypes involved in order to separate the antinociceptive adenosinergic signaling from cardiovascular adenosinergic effects is important in developing adenosine-based therapeutics in pain.

16.4.1 A_1AR and A_{2A}AR in Pain

Despite demonstrated preclinical efficacy in several pain models, agonists of A_1AR and A_{2A}AR have not been the focus of clinical trials due to their potential cardiotoxicity (Chen et al. 2013; Fredholm et al. 2011; Sawynok 1998; Varani et al. 2017; Zylka 2011). Yet, these receptors have played an important role in evolving our current understanding of adenosine-mediated antinociception. Prior studies attributed the adenosine antinociception to the activation of the A_1 and A_{2A} receptor subtypes

(Sawynok 2013, 2016; Zylka 2011). For example, genetic knockout of A_1ARs elicits thermal hypersensitivity and exacerbates neuropathic behavioral responses to cold and heat (Wu et al. 2005). In contrast, A_1AR activation alleviates nerve injury-induced pain (Cui et al. 1997; Gong et al. 2010), perioperative pain (Gan and Habib 2007), inflammatory pain (Sowa et al. 2010), central pain following spinal cord injury (Sjolund et al. 1998), complex regional pain syndrome type I (CRPS-I) (Martins et al. 2013), and painful diabetic neuropathy (Katz et al. 2015; Vincenzi et al. 2014) in preclinical models. Intrathecal administration of an A_1AR agonist reduced non-evoked spontaneous pain behaviors resulting from a surgical model of pain (Zahn et al. 2007). Repeated sessions of high-intensity swimming exercise increased endogenous adenosine levels, which played a role in the attenuation of mechanical allodynia in an animal model of CRPS-I (Martins et al. 2013). Intervention with the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3nonyl) adenine (EHNA), which limits adenosine degradation, enhanced the pain-relieving effects of swimming through mechanisms involving A_1AR (Martins et al. 2013). Intravenous infusions of adenosine in humans reduced some aspects of neuropathic pain and were shown to decrease postoperative pain mainly through the A_1AR (Gao and Jacobson 2007). The preclinical robustness of A_1AR pain relief resulted in clinical trials for multiple A_1AR agonists and an A_1AR allosteric enhancer; however, these drug trials were discontinued due to limited efficacy, presumably driven by a low therapeutic index (Gessi et al. 2011; Romagnoli et al. 2010). Recently, additional novel allosteric enhancers of the A_1AR, including TRR469, have demonstrated antinociceptive efficacy in two preclinical models of acute pain, writhing and formalin tests, and in chronic streptozotocin-induced diabetic neuropathy (Vincenzi et al. 2014). These data suggest that A_1AR allosteric enhancers may still be promising candidates to treat acute and chronic pain, with the potential advantages of their unique mechanism of action and lack of side effects. TRR469 dramatically increases adenosine affinity in mouse spinal cord membranes, suggesting the possibility of exploiting the antinociceptive effect of endogenous adenosine in a physiological way (Vincenzi et al. 2014).

Controversy surrounds the role of A_{2A}AR in nociception/antinociception. Depressed responses to acute pain stimuli were observed in mice lacking the A_{2A}AR (Ledent et al. 1997). Similarly, an intracerebroventricular injection of an A_{2A}AR-targeted antibody with agonist-like activity produces antinociceptive effects in naïve mice (By et al. 2011). Peripheral administration of an A_{2A}AR agonist is associated with nociceptive behaviors (Taiwo and Levine 1990), whereas very low doses of A_{2A}AR agonists promote reversal of nerve injury-induced pain in rats for weeks after a single spinal injection (Loram et al. 2009). In models of postsurgical pain (Zahn et al. 2007) and inflammatory pain (Poon and Sawynok 1998), intrathecal administration of A_{2A}AR agonists had limited antinociceptive efficacy. At the clinical level, a phase II trial of an oral A_{2A}AR agonist BVT-115959 in the treatment of diabetic neuropathy was completed in 2008 (Gao and Jacobson 2011); no further data has been provided at this time. The differing observations of A_{2A}AR agonists in pain highlight an apparent dichotomy of peripheral versus central A_{2A}ARs in pain signaling.

Unfortunately, a narrow therapeutic focus on only two of the AR subtypes has contributed to a decade of failed preclinical and clinical development efforts. Indeed, a focus on the A_1 and A_{2A} receptors has failed to harness adenosine antinociception effectively and without cardiovascular side effects (Boison 2013; Zylka 2011). In response, we anticipate that a greater emphasis on the A_3 receptor may provide an answer to the question of whether adenosine antinociception can provide safe, clinical pain relief. Recently, the combination of an A_1AR agonist and an A_3AR agonist demonstrated highly potent analgesic activity using a preclinical model of formalin-induced flinching (Petrelli et al. 2017). The combining of both A_1AR and A_3AR agonistic activity in one single molecule may act synergistically reducing the overall dose and therefore reduce the A_1AR-induced cardiotoxicity.

16.4.2 A_3AR and Pain

The A_3AR is a rapidly growing focus in the area of pain. Early literature was confounded by results gleaned from A_3AR-targeted compounds with poor specificity (Sawynok et al. 1997, 1999) or from a single study performed in $A_3AR^{-/-}$ mice (Wu et al. 2002). To inform the progress of A_3AR development, it is important to clarify the findings of these initial studies. In the earliest paper published in 1997 examining the contribution of A_3AR in pain, Sawynok and colleagues reported that subcutaneous administration of N^6-benzyl–NECA into the hindpaw of rodents produces a dose-related increase in nociceptive flinching behavior (Sawynok et al. 1997). It was found that this behavior was blocked by inhibitors of the histamine H_1 receptor and of 5-hydroxytryptamine$_2$ (5-HT_2), but was not modified by A_1AR or A_2AR antagonists. The authors speculated that A_3AR activation was responsible for the pro-nociceptive response, possibly by inducing mast cell degranulation (Sawynok et al. 1997). However, there was no evidence that linked A_3AR to the effects of N^6-benzyl–NECA. Moreover, N^6-benzyl–NECA is not selective for A_3AR (Gallo-Rodriguez et al. 1994). In the follow-up studies in 1999, the effects of N^6-benzyl–NECA were not influenced by an A_3AR antagonist (MRS1191) but rather abrogated by an A_{2B}AR antagonist. These results suggest that the pro-inflammatory, pro-nociceptive effect of N^6-benzyl–NECA was likely due to activation of the A_{2B}AR, a subtype previously implicated in inflammation (Feoktistov and Biaggioni 2011). Unfortunately, the notion of A_3AR-mediated pro-nociceptive effects remained. The erroneous notion that A_3AR activation led to pain and inflammation was further supported by a study in 2002 characterizing the development of carrageenan-induced paw edema and hyperalgesia in the $A_3AR^{-/-}$ mouse. This study reported a minimal increase in thermal hyperalgesia compared to wild-type control animals, but no observable differences in the normal (protective) nociceptive response of $A_3AR^{-/-}$ animals to indicate that A_3AR is not physiologically involved in modulating normal nociception (Wu et al. 2002). However, a year later, another study revealed decreased hot plate but not tail-flick responses of $A_3AR^{-/-}$ mice (Fedorova et al. 2003).

The notion of the A3AR-mediated pro-nociceptive effects was challenged when more selective A_3AR agonists, such as IB-MECA (N^6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide), began to be employed in pain models. IB-MECA is 50-fold more selective for A_3AR over rat A_1AR or A_{2A}AR, whereas N^6-benzyl–NECA only displays 14-fold selectivity (Gallo-Rodriguez et al. 1994; Jacobson 1998). A single investigation in 2005 reported that systemic administration of IB-MECA had no effect on normal nociception nor in the first phase of the formalin test but exerted significant antinociceptive effects on the second phase of the formalin test (Yoon et al. 2005). In a follow-up report, it was noted that intrathecal administration of an A_3AR antagonist (MRS1220) prevented the antinociceptive actions of adenosine in the second phase of the formalin test, supporting a role for spinal A_3ARs in the effect of adenosine (Yoon et al. 2006). No other papers were published between 2006 and 2012 that examined the contribution of A_3AR in pain.

In 2012, we revisited the A_3AR hypothesis and demonstrated that selective activation of A_3AR exerts potent antinociceptive effects in models of neuropathic pain (Chen et al. 2012; Little et al. 2015), validating the observations in models of non-neuropathic pain states (Yoon et al. 2006). Both IB-MECA and Cl-IB-MECA blocked the development of mechano-allodynia following chronic constriction injury (CCI), which was attenuated by an antagonist of A_3AR but not of A_1AR or A_{2A}AR (Chen et al. 2012). Moreover, low doses of IB-MECA that lacked analgesic effects provided profound increases in the analgesic potency of morphine, gabapentin, and amitriptyline when coadministered (Chen et al. 2012). The antinociceptive effects of IB-MECA and Cl-IB-MECA have since been corroborated with even more selective A_3 agonists, such as MRS1898 (>100-fold over A_1AR or A_{2A}AR (Gao et al. 2009)) and more recently MRS5698 (>10,000-fold over A_1AR or A_{2A}AR (Tosh et al. 2012)), in rodent CCI, spared nerve injury, and spinal nerve ligation neuropathic pain models (Chen et al. 2012; Ford et al. 2015; Little et al. 2015). The loss of MRS5698 antinociception in the $A_3AR^{-/-}$ mouse or in the presence of the specific A_3AR antagonist, MRS1523, corroborates the specificity of these newer-generation compounds as A_3AR antinociceptive agents (Little et al. 2015). Indeed, these pharmacological tools have facilitated a better understanding of the levels at which A_3AR functions to attenuate pain: A_3AR agonists administered via intradermal (ipsilateral paw) injection (IB-MECA, 3–60 nmol), intrathecal cannula (MRS5698, 3–60 nmol), or RVM cannula (MRS5698, 0.3–3 nmol) dose-dependently attenuate CCI-induced mechanical allodynia (Little et al. 2015). Systemic administration of a peripherally restricted A_3AR agonist also reverses CCI-induced peak mechanical allodynia, and the inability of an intrathecal A_3AR antagonist to reverse this effect validated its peripheral site of action (Paoletta et al. 2013). Conversely, antinociception conferred via systemic administration of the CNS-permeant MRS5698 is attenuated with intrathecal or intra-RVM delivery of an A_3AR antagonist, highlighting the dual peripheral and central roles of A_3AR in antinociception (Little et al. 2015). Further studies are warranted to explore the relationship between peripheral and central A_3ARs in pain.

The beneficial effects of A_3AR agonists extend to number of cancer-related pain states. In models of neuropathic pain associated with the administration of chemo-

therapeutics (chemotherapy-induced peripheral neuropathy, CIPN), IB-MECA (Chen et al. 2012; Janes et al. 2014b) and MRS5698 (Janes et al. 2015; Little et al. 2015; Wahlman et al. 2018) blocked the development of neuropathic pain. Similar antinociceptive effects were provided by Cl-IB-MECA (Varani et al. 2013) and MRS5698 (Little et al. 2015) in rodent models of pain associated with breast cancer bone metastasis. Interestingly, A_3AR agonists do not interfere with antitumor effects (Chen et al. 2012) but instead are in themselves antitumor agents. High expression of A_3AR is detected on many malignant cell types and accordingly A_3AR agonists have been shown to produce direct anticancer effects on their own and have been documented to enhance the actions of several widely used chemotherapeutics and attenuate the associated myelosuppression (Fishman et al. 2002, 2009, 2012). Cl-IB-MECA was shown to reduce tumor growth in the rat model of breast cancer bone metastasis (Varani et al. 2013). Indeed, Cl-IB-MECA is currently in phase II clinical trials for hepatocellular carcinoma as an anticancer agent. Therefore, the use of A_3AR agonists may provide dual benefits in the treatment of a variety of cancer-related pain states.

The antinociceptive effects of A_3AR agonists persist even with long-term treatment, such as repeated daily injections for 6 days or continuous infusion for 7 days (Little et al. 2015). These findings suggest that there is no development of antinociceptive tolerance to A_3AR agonists, unlike morphine, where tolerance to its antinociceptive effects develops only after 6 days of injections (Muscoli et al. 2010). These findings are curious as all adenosine receptor subtypes exhibit a "desensitization phenomenon" resulting in the diminished response and receptor surface expression after repeated or continuous exposure agonists (Klaasse et al. 2008). However, in animal models of autoimmune disorders and cancer, chronic administration of A_3AR agonists maintains anti-inflammatory/anticancer effects even during A_3AR downregulation (Madi et al. 2003). It is thought that the functionality of A_3AR agonist in inflammation/tumor growth may be dependent on the downregulation of A_3AR to inhibit downstream regulatory proteins (Fishman et al. 2006). Whether this mechanism explains the action of IB-MECA and other A_3AR agonists in pain requires further investigation.

In preclinical animal models, the antinociceptive effects of A_3AR agonists are not dependent upon endogenous opioid or endocannabinoid pathways (Ford et al. 2015; Little et al. 2015), suggesting that A_3AR agonists lack inherent reward properties that would heighten the potential risk of abuse and dependence. Emerging data indicates that A_3AR agonists, such as MRS5698, produce a preference in nerve-injured rats to the particular chamber in which they received the A_3AR agonists termed "conditioned place preference" (CPP) (Little et al. 2015). This suggests that A_3AR agonists provided relief of spontaneous pain in these animals. However, sham rats given A_3AR agonists did not exhibit any CPP, indicating a lack of inherent reward with these compounds (Little et al. 2015). In contrast, opioids and other drugs of abuse elicit CPP from both naïve and injured animals (Prus et al. 2009). Therefore, A_3AR agonists have the potential to selectively modify pathological but not protective pain, while avoiding the tolerance and abuse potential associated with opioid therapy.

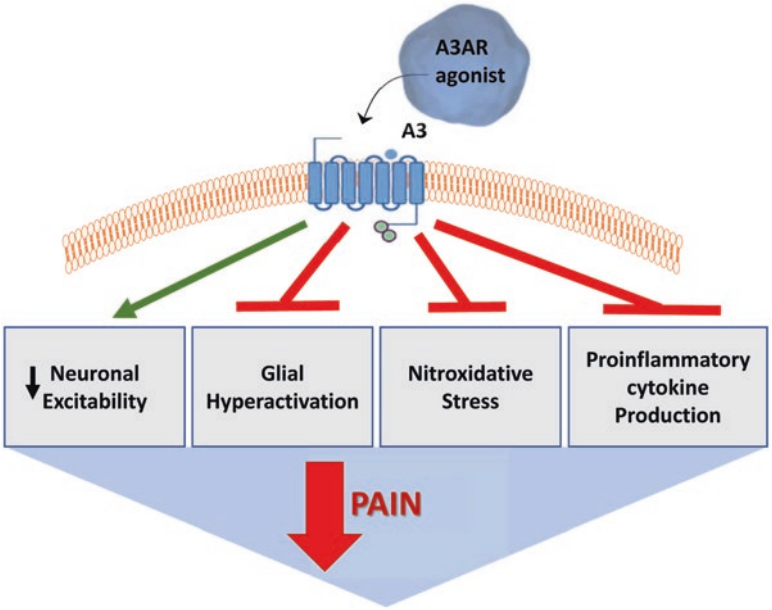

Fig. 16.2 Potential mechanisms of A_3AR-mediated antinociception

16.4.3 Mechanisms of A_3AR Antinociception

The antinociceptive and regulatory mechanisms and pathways modulated by A_3AR agonists in pathological pain states is only now beginning to be explored. However, as already discussed, A_3AR agonists act the level of the peripheral afferent, the spinal cord, and the RVM as selective A_3AR agonists administered via intradermally, intrathecally, or intra-RVM dose-dependently attenuate neuropathic pain behaviors (Little et al. 2015). The actions of A_3AR agonist are independent of opioidergic and cannabinoid systems (Little et al. 2015) and engage serotonergic and noradrenergic bulbospinal circuits in neuropathic pain, suggesting the involvement of A_3AR signaling in summary descending inhibition of wide dynamic range spinal neurons (Little et al. 2015). In other disease states, A_3AR activation has been shown to alter components that are critically involved in the development of central sensitization and pain, including protein kinase activity, glutamatergic neurotransmission, ion conductance, and neuroinflammation. To inform the potential mechanism(s) of A_3AR-mediated antinociception, we have summarized the consequences of A_3AR activation as they are relevant to pain (Fig. 16.2).

The A_3AR agonist MRS5698 has been recently shown to reverse traumatic nerve injury-induced pain by maintaining GABAergic signaling (Ford et al. 2015). The GABAergic system is an important inhibitory regulator of nociceptive transmission. GABA is released from interneurons within the CNS and resulting activation of GABA receptors dampens neuronal excitability to reduce nociceptive signaling

(Zeilhofer et al. 2012). In the pathological pain state, the GABAergic system becomes dysregulated and the balance of nociceptive signaling shifts toward state hyperexcitability (Zeilhofer et al. 2012). GABAergic dysregulation results from reduced GAD65-dependent GABA synthesis (Eaton et al. 1998; Stiller et al. 1996), increased GABA reuptake transporter GAT-1 expression (Eaton et al. 1998; Moore et al. 2002), and reduction in K^+-Cl^- cotransporter (KCC2) activity that results in the loss of the anion gradient necessary to drive Cl^- through $GABA_A$ channels (Coull et al. 2003; Price et al. 2005). In a traumatic nerve injury-induced pain animal model, MRS5698 attenuated the dephosphorylation of GAD65 and GAT-1 and the phosphorylation of KCC2 and maintained appropriate Cl^- flux (Ford et al. 2015). Moreover, A_3AR agonists attenuated brain-derived neurotrophic factor (BDNF) signaling (Ford et al. 2015), which has been shown to inhibit GABAergic signaling (Biggs et al. 2010; Ferrini and De Koninck 2013; Smith 2014).

A_3AR agonists may also exert their effects through the RhoA-phospholipase D (PLD) signaling pathways. In other animal models, A_3AR agonists prevent the decrease in PLD activity in response to reactive oxygen species exposure during cardiomyocyte apoptosis (Asemu et al. 2005; Lee et al. 2001). Proper PLD function is necessary for the production of choline in order to activate α7 nicotinic acetylcholine receptors (Lee et al. 1993). Activation of these receptors is both neuroprotective and antinociceptive during chronic neuropathic pain (Feuerbach et al. 2009).

A_3AR activation is associated with the attenuation of astrocyte reactivity, neuroinflammatory response (Janes et al. 2015), and reactive microglial chemotaxis (Choi et al. 2011), such that A_3AR agonists may reduce BDNF associated with glial hyperactivation and free the GABAergic system to function properly. Glial cells (astrocytes and microglia) are critical to the development and maintenance of many pathological pain states (Cao and Zhang 2008; Nagata et al. 2009; Obata and Noguchi 2008; Watkins et al. 2001). Targeting the glial activity can prevent and attenuate a variety of pain states (Hashizume et al. 2000; Meller et al. 1994; Sweitzer et al. 2001; Watkins et al. 1997, 2001). In pathological pain states, glial cells can release a number of pro-inflammatory cytokines and nitroxidative species that increase neuronal sensitivities in the dorsal horn (Cao and Zhang 2008; Milligan and Watkins 2009) and further increase glial activity to establish an amplification loop that may account for the persistence of hypersensitivities in chronic pain states (Bradesi et al. 2001). Moreover, activation of innate immune receptor toll-like receptor 4 (TLR4) expressed on glial cells has been implicated in the neuroinflammatory response in the development of neuropathic pain (Li et al. 2014; Watkins et al. 2009).

A_3AR agonists are anti-inflammatory in autoimmune and inflammatory diseases (Bar Yehuda et al. 2010). Both in vitro and in vivo studies have revealed that A_3AR attenuates pro-inflammatory cytokines by inhibiting the p38 MAPK and nuclear factor κB (NFκB) signaling pathways (Janes et al. 2014a; Madi et al. 2007; Varani et al. 2010, 2011). IB-MECA has been documented to decrease the TLR4-induced pro-inflammatory mediators, such as tumor necrosis factor (TNF) and macrophage inflammatory protein 1α (MIP-1α) (Hasko et al. 1998; Hasko et al. 1996; Sajjadi et al. 1996; Szabo et al. 1998). A_3AR-mediated suppression of pro-inflammatory

mediators following TLR stimulation is lost in A_3AR knockout mice (Salvatore et al. 2000). In models of CIPN, IB-MECA reduced the level of reactive astrocytes, NFκB and MAPK activation, and level of pro-inflammatory/neuroexcitatory cytokines (Janes et al. 2014a, 2015; Wahlman et al. 2018). In the oxaliplatin-induced neuropathic pain model, administration of oxaliplatin increased NOD-like receptor with pyrin domain subtype 3 (NLRP3) inflammasome activation of IL-1β in the spinal cord and pharmacological inhibition of NLRP3 activity attenuated pain to suggest the involvement of this pathway in the development of mechanohypersensitivities (Wahlman et al. 2018). Attenuation of CIPN with intrathecal MRS5698 was associated with reduced expression and activation of NLRP3 in the spinal cord (Wahlman et al. 2018). Interestingly, A_3AR activation also enhances formation of the anti-inflammatory cytokine IL-10 (Hasko et al. 1996; Janes et al. 2014a, 2015) and glial-derived neuroprotective substances (Wittendorp et al. 2004). Moreover, inhibition of IL-10 with neutralizing antibodies not only attenuated the beneficial effects of A_3AR agonists on pain behavior but also restored the expression and activation of NLRP3 inflammasomes (Wahlman et al. 2018). MRS5698 also lost its beneficial effects on CIPN in IL-$10^{-/-}$ mice (Wahlman et al. 2018). These findings suggest that this shift in the spinal neuroinflammatory environment may be a major contributor to the effects of A_3AR in pain. More work is necessary to understand at what point A_3AR exerts its effects on neuroinflammation.

In addition to neuroinflammatory mediators, nitroxidative species including superoxide (SO), nitric oxide (NO), and their highly pro-nociceptive reaction product peroxynitrite (PN) (Salvemini and Neumann 2010) are important in the development and maintenance of pain of several etiologies, including acute and chronic inflammation (Ndengele et al. 2008), orofacial pain (Yeo et al. 2008), and opiate-induced hyperalgesia and antinociceptive tolerance (Muscoli et al. 2007), nerve injury-induced pain (Rausaria et al. 2011), and CIPN (Doyle et al. 2012; Janes et al. 2013). In CIPN, IB-MECA attenuated the activation NADPH oxidase, a source of SO as a precursor to PN formation (Janes et al. 2014a; Poderoso et al. 1996), in the spinal cord. Inhibition of NADPH oxidase in prostate cells following IB-MECA is linked to the inhibition of intracellular cyclic AMP/PKA (Jajoo et al. 2009) and reduced expression of NADPH oxidase subunits (Rac1 and $p47^{phox}$) through inhibition of ERK1/2 activity (Jajoo et al. 2009).

Activation of A_3AR may play a critical role in the inhibitory actions of adenosine on excitatory neurotransmission and its neuroprotective effects. A_3AR activation in vitro protects against the neurotoxic rises in intracellular Ca2+ and neuronal excitability mediated by P2X7R (Zhang et al. 2006) and NMDAR (Zhang et al. 2010). Dysregulated glutamatergic neurotransmission and increased neuronal excitability are hallmarks of chronic pain (Amadesi et al. 2006; Chen et al. 2010; Doyle et al. 2012; Elliott et al. 1994; Mayer et al. 1999; Muscoli et al. 2007; Xu et al. 2010; Zhang et al. 2012). Treatment with A_3AR agonists attenuates posttranslational nitration of glutamate transporter GLT-1 and glutamate synthase (Janes et al. 2014a) in the spinal cord. Nitration of these proteins leads to a loss in their activity that consequently reduces the capacity to remove glutamate from the synapse and terminate glutamatergic signaling (Mao et al. 2002).

16.4.4 Pharmacological Probes for the Study of A_3AR in Pain

A toolbox of selective A_3AR modulators is now accessible, which includes high affinity directly acting agonists **1**-**8** (Table 16.1, Fig. 16.3) and antagonists **9**-**12**, as well as indirect modulators of A_3AR activity. Indirect modulators include inhibitors of adenosine degrading enzymes, adenosine deaminase (ADA; **13**), and adenosine kinase (ADK; **14**, **15**). Furthermore, there are selective allosteric enhancers of the action of endogenous adenosine at the A_3AR (**16**, **17**). Although these positive allosteric modulators are selective for the human A_3AR and do not act at other AR subtypes, there is a large species dependence such that their activity is only subtle in rodent species.

At the 5′ position, an amide in place of the CH_2OH, as for all agonists shown in Fig. 16.3, favors affinity and efficacy at the A_3AR. At the N^6 position, either small hydrophobic groups, e.g., methyl **7** and ethyl **8**, or large hydrophobic groups, e.g., *m*-substituted benzyl rings in **5** and **6**, are tolerated when bound to the receptor. The A_3AR affinity of N^6-benzyl analogues is often better preserved in rodent species than in compounds with small N^6 groups. For example, MRS5698 (**5**) is of the same affinity (K_i ~3 nM) at human and mouse A_3ARs with high selectivity. At 10 μM (close to its solubility limit), **5** displays a low percent inhibition of binding (less than 50%) at the A_1AR and A_{2A}AR.

Among widely used A_3AR agonists, IB-MECA (**1**) and Cl-IB-MECA (**2**) have varying degrees of AR subtype selectivity, as shown in a comparison of affinities at human, mouse, and rat ARs (Table 16.1). The affinity (Ki value) of IB-MECA at the mouse A_3AR is an impressive 87 pM, and its Ki value at the human A_3AR is 20-fold higher. These two agonists are moderately selective for the A_3AR, which is often sufficient to achieve a dose window of selectivity depending on the pharmacological model and species being studied. Compounds **3** and **4** contain a ring constraint in the ribose-like moiety, known as the (North)-methancarba modification of nucleosides, which maintains a conformation preferred at the A_3AR. Native ribose can freely twist to achieve a range of conformations, but if a favored conformation is pre-installed in the nucleoside, there is an advantage for binding to that subtype. In general, this ribose modification tends to increase affinity and selectivity, because the other AR subtypes are either adversely affected by this ribose substitution (A_{2A}AR) or favor the substitution (A_1AR) to a lesser degree than the A_3AR. The more highly derivatized agonists containing a rigid C2 extension consisting of an arylethynyl group, in addition to the (North)-methancarba modification, are even more A_3AR selective. Thus, the combination of these two substituents, as present in compounds **5**-**8**, achieves 10,000-fold selectivity or greater for the A_3AR. These particularly potent and specific A_3AR agonists are especially useful in pharmacological studies of this receptor in pain models (Tosh et al. 2012, 2014, 2015). Compounds **5**, **7**, and **8** have been shown to be orally active in a dose-dependent manner in reducing or completely suppressing mechanoallodynia in the CCI model. The terminal C2 aryl group in this series of agonists may be substituted with a wide range of chemical functionality and still retain A_3AR selectivity. Compound **5**

Table 16.1 A_3AR-selective agents for use in the study of A_3-mediated antinociception

	Ki or Kd, nM (or *% inhibition at 10 μM*)								
	Human			Mouse			Rat		
Compound	A_1AR	A_{2A}AR	A_3R	A_1AR	A_{2A}AR	A_3R	A_1AR	A_{2A}AR	A_3R
Agonists									
1 IB-MECA	700 ± 270	6200 ± 100	**2.4 ± 0.5**	5.9	~700	**0.087**	54	56	**1.1**
2 Cl-IB-MECA	220 ± 20	5400 ± 2500	**1.5 ± 0.2**	35	~10,000	**0.18**	820	470	**0.33**
3 MRS1898	136 ± 22	784 ± 97	**1.51 ± 0.23**	7.32 ± 1.5	5350 ± 860	**0.80 ± 0.14**	83.9 ± 10.3	1660 ± 260	**1.1**
4 MRS3558	260 ± 60	2300 ± 100	**0.29 ± 0.04**	15.3 ± 5.8	10,400 ± 1700	**1.59 ± 0.46**			**1.0 ± 0.10**
5 MRS5698	*6%*	*41%*	**3.49 ± 1.84**	*16%*	*27%*	**3.08 ± 0.23**			
6 MRS5841	*16%*	*7%*	**1.90 ± 0.03**	*15%*	*1%*	**11.3 ± 1.9**			
7 MRS5980	*6%*	*24%*	**0.70 ± 0.11**	*38%*	*7%*	**36.1 ± 4.7**			
8 MRS7144	*10%*	*0%*	**1.7 ± 0.4**			**16 ± 3**			
Antagonists									
9 MRS1191	40,100 ± 7500	>10,000	**31.4 ± 2.8**	*0%*	*0%*	*32 ± 3%*	>10,000	>10,000	**1850**
10 MRS1334			**2.69**				>10,000	>10,000	
11 MRS1523	>10,000	3660 ± 930	**18.9**	8000	>10,000	**731**	15,600	2050	**113**
12 MRS5776	*29%*	*24%*	**20.0 ± 6.0**	*39%*	*13%*	**480 ± 90**			

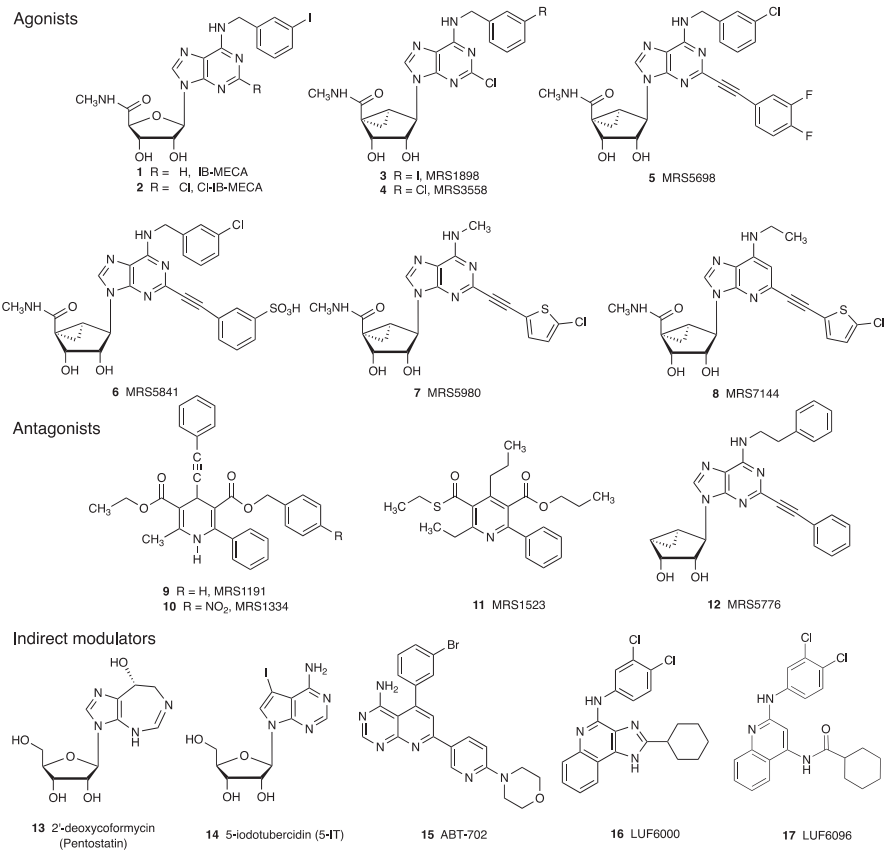

Fig. 16.3 Pharmacological agents useful for the study of A_3AR-mediated antinociception

contains a 3,4-difluorophenyl ring at the terminal position, while compounds **7** and **8** contain a 5-chlorothienyl ring that is associated with long duration of action (3 h or greater) in the mouse CCI model following oral administration. Compound **6** is not intended for oral administration, because it contains a fully negatively charged aryl sulfonate group that prevents its diffusion across biological membranes. It displayed no permeability in the PAMPA model of membrane permeability, indicating that it likely does not diffuse across the blood brain barrier. Due to this property, compound **6** was used to separate central from peripheral effects of A_3AR agonists in the mouse CCI model, depending on the site of administration.

The use of selective A_3AR antagonists or mice in which A_3AR is genetically knocked out in conjunction with agonists or enhancers is important to delineate A_3AR-mediated effects, especially with agonists that are only moderately selective. Nonnucleoside A_3AR antagonists (e.g., 1,4-dihydropyridines **9** and **10** and pyridine **11**) have varying degrees of AR subtype selectivity, depending on species. By progressively truncating the structure of nucleosides that are selective for the A_3AR, it

is possible to shift the activity from full agonist to partial agonist to antagonists. Thus, a truncated nucleoside **12** was shown to have considerable affinity at both human and mouse A_3AR with selectivity, but the efficacy of this compound in A_3AR activation was greatly diminished, such that it can serve as an antagonist of a full A_3AR agonist. Thus, more potent and selective A_3AR antagonists for application to a range of species are still needed.

An indirect means of pharmacologically enhancing activation of the A_3AR, and potentially other ARs, is to enhance levels of extracellular adenosine by inhibiting ADA (e.g., **13** Pentostatin) or ADK (e.g., **14** 5-iodotubercidin and **15** ABT-702). ADK inhibitor **15** was found to reduce both chronic and acute pain through action at both peripheral or central sites (Kowaluk et al. 2000). Recently, Little et al. used **15** to reveal an effect of endogenous adenosine acting through the A_3AR to reduce chronic neuropathic pain.

16.5 Concluding Remarks

The development of selective pharmacological tools targeting A_3AR has uncovered the exciting, robust antinociceptive properties of A_3ARs agonists in a variety of pathological pain states. Emerging evidence suggests that harnessing the endogenous antinociceptive A_3AR pathway yields effective pain relief without altering normal protective nociception and without producing inherent reward that is associated with abuse potential. As selective A_3AR agonists in ongoing phase II/III clinical trials for non-pain conditions display good safety profile, we propose that A_3AR agonists may be a safe and successful strategy for exploiting the potent analgesic actions of adenosine to provide a breakthrough non-opioid treatment for patients suffering from chronic pain.

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Chapter 17
Adenosine Signalling in the Injured Heart

Julia Hesse, Christina Alter, and Jürgen Schrader

Abstract Adenosine plays a prominent role in the cardiovascular system and has been extensively studied for both its therapeutic and diagnostic abilities. This chapter reviews the various sources and metabolic pathways of adenosine formation in the heart. Depending on the individual cell type involved (cardiomyocyte, vascular smooth muscle cell, coronary endothelium, pericyte, fibroblast and cells of cardiac impulse generation and propagation) together with the cell-specific expression pattern of the four adenosine receptors, adenosine importantly regulates key parameters of cardiac function and energy supply including contractility, heart rate, coronary flow and substrate utilization. In the infarcted heart, recent evidence indicates that adenosine formed by CD73 on T cells and epicardial mesenchymal cells critically modulates central processes of cardiac inflammation, post-MI remodelling/fibrosis, regeneration and tissue protection. Since the $A_{2B}R$ appears to be linked to IL6 formation, the adenosine-IL6 axis may be a promising target for the therapy of post-MI inflammation, remodelling and fibrosis.

Keywords Adenosine receptors · Cardiovascular system · Cardiac adenosine · Coronary blood flow regulation · Injured heart

17.1 The History of Adenosine in the Heart

Adenosine is a ubiquitous extracellular signalling molecule with essential functions in human physiology and pathophysiology. Adenosine is an ancient molecule that regulates various biological functions via activating four G protein-coupled receptors, A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R (Chen et al., 2013). Due to the widespread expression of adenosine receptors, it has far-reaching effects across many different organ systems. Adenosine plays a prominent role in the cardiovascular system and has been extensively studied for both its therapeutic and diagnostic abilities. There are

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several well-written reviews on the cardiovascular physiology/pharmacology/clinic of adenosine (Headrick et al., 2013; Layland et al., 2014; Burnstock, 2017; Geldenhuys et al., 2017). Historically, Drury and Szent-Györgyi 1929 were the first to recognize the coronary dilating effects of adenosine in 1929. These authors were also the first to describe two additional effects of adenosine: inhibition of impulse generation in the sinus and atrioventricular (AV) node and the depression of contractile force in atrial muscle. Interest in the biological role of adenosine resumed only in 1963, when two groups – Gerlach and co-workers in Freiburg, Germany, and Berne in Charlottesville, USA – independently reported that the hypoxic heart can form adenosine Gerlach et al., 1963; Berne, 1963. These observations formed the basis for the so-called adenosine hypothesis for the metabolic regulation of coronary blood flow (Berne, 1980), which was intensively studied over the following two to three decades. The history of cardiac adenosine research has been reviewed by Olsson et al, 2003. After many years of research, we now know that cardiac adenosine is most likely not the main mediator of coronary blood flow regulation (Deussen et al, 2012) but is mainly formed whenever local PO_2 decreases as a result of a mismatch between oxygen supply via the coronary vasculature and oxygen consumption by the heart (Bardenheuer and Schrader, 1986).

17.2 Sources and Metabolism of Adenosine

As shown in Fig. 17.1, adenosine is formed by three different enzymatic reactions: 5′-Nucleotidase (5′-NT) and alkaline phosphatase catalyse the dephosphorylation of AMP to adenosine. S-Adenosylhomocysteine (SAH) hydrolase (SAHH) promotes the hydrolytic cleavage of SAH to adenosine and l-homocysteine. The latter reaction is particularly interesting since the equilibrium constant of the SAHH reaction favours synthesis of SAH. Only at very low concentrations of adenosine and l-homocysteine, as is normally the case in the cytoplasm, does hydrolysis of SAH take place. The kinetic features of SAHH can be exploited to measure the intracellular concentration of adenosine: In the presence of saturating concentrations of homocysteine, the rate of SAH formation is directly dependent on the free intracellular concentration of adenosine (Deussen et al. 1988a, b). The same principles can be used for the noninvasive assessment of regional cardiac adenosine using positron emission tomography (PET). This requires ^{11}C-labelled homocysteine which after intravenous infusion traps hypoxia-induced local adenosine by conversion into ^{11}C-SAH which can be visualized by PET (Deussen et al. 1992). The so-called SAH technique for measuring intracellular adenosine is an indirect technique; however, it permits quantification and can differentiate between protein-bound and free adenosine.

There are two principle sites of adenosine production in almost every cell: intracellular and extracellular. Intracellular adenosine formation involves cytosolic 5′-NT and SAHH. In addition, cytosolic adenosine kinase (AK) phosphorylates adenosine back to AMP (salvage pathway). The physiological function of this high turnover of AMP-adenosine metabolic cycle is that tissue hypoxia inhibits AK activity, whereby cellular adenosine becomes elevated. Thus, hypoxia-induced inhibition of AK

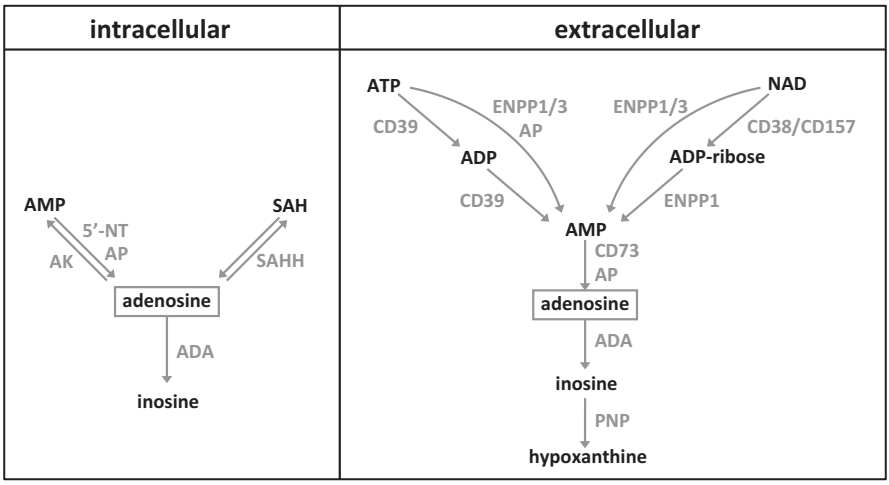

Fig. 17.1 Intracellular and extracellular adenosine metabolism. Intracellular adenosine can be generated from AMP by 5′-nucleotidase and alkaline phosphatase (AP). S-Adenosylhomocysteine (SAH) hydrolase (SAHH) converts SAH to adenosine or synthesizes SAH from adenosine, depending on substrate concentrations. Intracellular adenosine can be degraded to inosine via adenosine deaminase (ADA) or used for AMP synthesis via adenosine kinase (AK). Extracellular ATP is converted to AMP by ecto-nucleoside triphosphate diphosphohydrolase-1 CD39 (via ADP), AP or ecto-nucleotide pyrophosphatases ENPP1 and ENPP3. Extracellular NAD is metabolized to ADP-ribose by NAD glycohydrolases CD38 and CD157 and subsequently to AMP by ecto-nucleotide pyrophosphatase ENPP1. NAD can also be directly degraded to AMP by ecto-nucleotide pyrophosphatases ENPP1 and ENPP3. The ecto-5′-NT (CD73) or AP converts extracellular AMP to adenosine, which is further degraded via ADA and purine nucleoside phosphorylase (PNP)

causes the amplification of small changes in free AMP into a major rise in adenosine (Decking et al. 1997). This mechanism is likely to play an important role in the high sensitivity of the cardiac adenosine system to impaired oxygenation.

While intracellular adenosine formation is strictly oxygen-dependent, the extracellular production of adenosine by ecto-5′-NT (CD73) is not. CD73 hydrolyses extracellular AMP to adenosine, which then can directly act on the various adenosine receptors. The substrate for the formation of AMP is extracellular ATP which is released from dying cells but also upon activation by endothelial cells (Gödecke et al. 2012), immune cells (Borg et al. 2017) and epicardial cells (Hesse et al. 2017). Only recently it was recognized that aside from ATP, also extracellular NAD can be efficiently degraded to AMP serving as substrate for CD73 in the infarcted heart (Hesse et al. 2017; Borg et al. 2017).

17.3 Adenosine as Regulator of Heart Function

The uninjured heart is composed of many cell types: cardiomyocytes, vascular smooth muscle cells, coronary endothelium, pericytes, fibroblasts and cells of cardiac impulse generation and propagation. While cardiomyocytes by cell volume

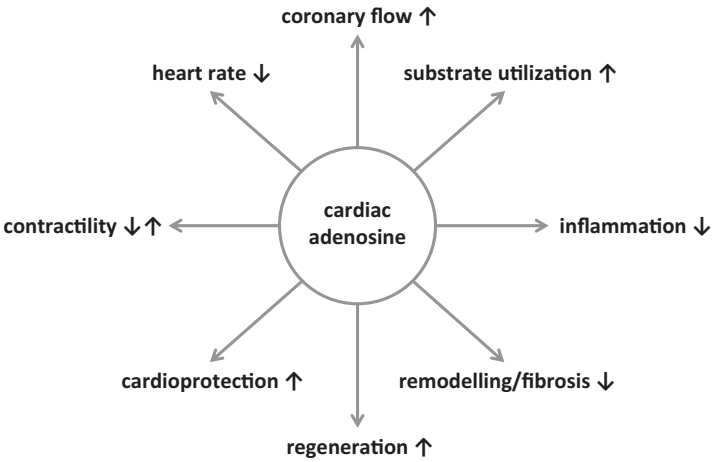

Fig. 17.2 Regulatory role of adenosine in the heart. Adenosine regulates central parameters of cardiac function and energy supply such as contractility, heart rate, coronary flow and substrate utilization. In the response to cardiac injury, adenosine plays a critical role in the regulation of inflammation, remodelling/fibrosis, regeneration and tissue protection

constitute the highest fraction within the heart, endothelial cells constitute the majority of non-cardiomyocytes and are likely to play a greater role in physiological function and response to injury than previously appreciated (Pinto et al. 2016).

As summarized in Fig. 17.2, adenosine formed within the heart may serve many biological functions. This involves the different cell types of the heart, each of which expresses cell-specific densities of the four adenosine receptors.

17.3.1 Coronary Blood Flow

All four adenosine receptor subtypes are found in coronary smooth muscle cells, but only the $A_{2A}R$ and $A_{2B}R$ were shown to be present on coronary endothelial cells (Mustafa et al. 2009). The $A_{2A}R$ plays a pivotal role in controlling vasodilation, and specific $A_{2A}R$ agonists, such as regadenoson, are used as coronary stress agent (Townsend et al. 2017). Also the $A_{2B}R$ mediates coronary vasodilation; however, the role of this receptor in coronary blood flow regulation remains to be explored. That ATP might play a role in coronary flow regulation was pioneered by Forrester (Forrester 1990). However, a major problem in the literature when correlating ATP or adenosine release to organ function is of analytical nature. Almost every cell contains ATP in the millimolar range, while extracellular adenosine normally is only micromolar. It is therefore quite possible that necrosis of just a few cells releases sufficient ATP, which is subsequently degraded extracellularly to adenosine. Only recently, strong evidence was provided using refined analytical techniques that adenine nucleotides and not adenosine control coronary blood flow in chronically instrumented dogs (Gorman et al. 2010).

17.3.2 Heart Rate

Adenosine is long known to reduce heart rate (bradycardia) and to block the conduction in the AV node (AV block). This effect is mainly mediated by the A_1R. However, A_3R deletion has also been shown to increase heart rate. In addition, $A_{2A}R$ deletion was reported to reduce heart rate. These data support a role for the A_3R in contributing to A_1R-induced bradycardia (Headrick et al. 2013). The basis of these effects awaits more detailed investigations. Intravenously applied adenosine is clinically useful to block supraventricular tachycardia in patients (Tebbenjohanns et al. 1999). Since the plasma half-life of adenosine is only very short (Möser et al. 1989), the effect of adenosine on heart rate is only transient but sufficient to convert supraventricular tachycardia into a normal heart rate. Aside from adenosine also infusion of ATP effectively blocks supraventricular tachycardia (Stark et al. 1994).

17.3.3 Cardiac Contractility

In atrial tissue adenosine decreases contractile force which is brought about by inhibition of calcium transients (Schrader et al. 1975) via A_1R activation (Pak et al. 2015). In the ventricular myocardium, A_1R is well characterized including radioligand binding. Evidence indicates that A_1R exerts little, if any, direct effects on contractility, but its activation does attenuate the positive inotropic effects of β-adrenergic stimulation at the whole heart and myocyte level (Schrader et al. 1977; Dobson et al. 2003). It was proposed that endogenously formed adenosine, e.g. after myocardial ischaemia, limits the responsiveness of the heart to β-adrenergic stimulation thereby balancing energy supply to energy demand. Interestingly, the $A_{2A}R$ directly enhances cardiac contractility by modulating A_1R anti-adrenergic effects (Chandrasekera et al. 2010).

17.3.4 Substrate Utilization

Independent of its vasoactive properties, endogenous adenosine alters myocardial glucose utilization to support myocardial contractile function (Fang et al. 1997). Activation of A_1R inhibits lipolysis and lowers plasma free fatty acids (FFA) concentrations by inhibiting adenylyl cyclase and downstream cAMP formation (Dhalla et al. 2003). Since full A_1R agonists also have significant cardiovascular effects, selective but partial A_1R agonists have been developed, which can lower circulating FFA, improve insulin sensitivity and potentiate insulin action (Dhalla et al. 2007).

17.3.5 Immune Cells

$A_{2A}R$ generally have a strong suppressive effect on the activation of immune cells (Linden and Cekic 2012). Their transcription is strongly induced by signals that activate macrophages or dendritic cells (DCs) through toll-like receptors or T cells through T-cell receptors (TCR). The $A_{2A}R$ is therefore considered to be responsible for producing a gradual dissipation of inflammatory responses. $A_{2A}R$ activation is particularly effective in limiting the activation of invariant natural killer T (iNKT) cells that play a central role in acute reperfusion injury (Linden and Cekic 2012).

Despite $A_{2A}R$ agonists being highly effective anti-inflammatory agents, they are also potent vasodilators, which preclude a broad clinical application. To separate immunosuppression from vasodilation, phosphorylated $A_{2A}R$ agonists (prodrugs) were synthesized that require the presence of CD73 to become activated (El-Tayeb et al. 2009). In the model of collagen-induced arthritis, 2-(cyclohexylethylthio) adenosine 5′-monophosphate (chet-AMP = prodrug) potently reduced inflammation as assessed by ^{19}F magnetic resonance imaging and by histology (Flögel et al. 2012). The prodrug effect was blunted by inhibition of CD73 and $A_{2A}R$. Thus, chet-AMP is a potent immunosuppressant with negligible vasodilatory activity.

As shown in Fig. 17.3, CD39 on cardiac resident immune cells is mainly expressed on myeloid cells, while CD73 is dominant on T cells (Bönner et al. 2012). Cardiomyocytes and erythrocytes do not measurably express CD39/CD73, and CD39 dominates on the coronary endothelium of the unstressed heart. CD73

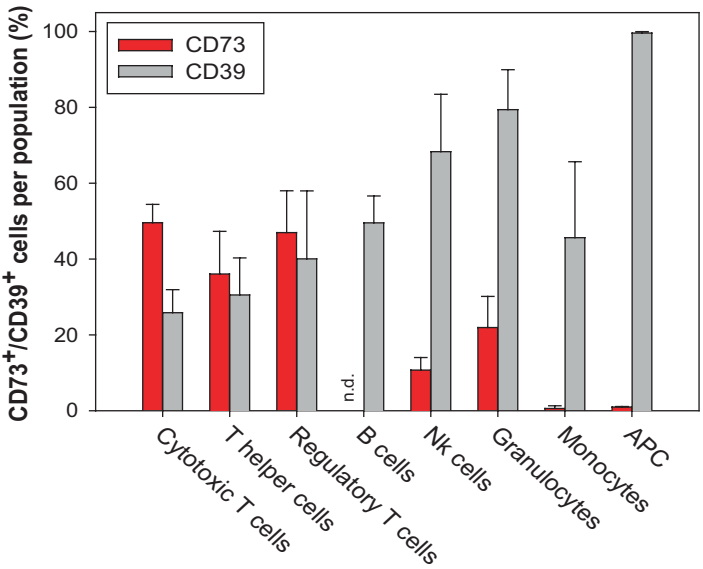

Fig. 17.3 Abundance of CD73 and CD39 on leukocytes in the heart under basal conditions. $CD73^+$ and $CD39^+$ cells in the leukocyte populations isolated from murine cardiac tissue. Values are means ± SD of $n = 5$ experiments. (With permission from (Bönner et al. 2012))

becomes significantly upregulated on invading immune cells in the infarcted heart and comprises 2/3 of total cardiac CD73 when compared with coronary endothelial cells (Bönner et al. 2012).

Mice globally lacking CD73 were reported to show (i) a severe decline in contractile function, (ii) impaired healing involving M1-driven immune response with increased tumour necrosis factor (TNF)α and IL17 and (iii) infarct expansion accompanied by an immature replacement scar and diffuse ventricular fibrosis (Bönner et al. 2013). More recently, a T-cell-specific CD73 knockout fully mimicked the cardiovascular phenotype of the global CD73 mutant demonstrating that CD73 on T cells orchestrates cardiac wound healing after myocardial infarction including purinergic metabolic reprogramming (Borg et al. 2017). For further information on immune cells and the inflammatory response in the infarcted heart, see 17.3.1.

17.3.6 Remodelling/Fibrosis

Cardiac fibroblasts have an essential role in the regulation of the extracellular matrix, which is crucial for maintaining the structural integrity of the heart. Collagen and matrix deposition by fibroblasts is an important part of wound healing but also contributes to pathologic remodelling of organs via $A_{2A}R$ and $A_{2B}R$ (Shaikh and Cronstein 2016), leading to generation of cAMP and activation of downstream targets such as PKA and Epac. In vivo findings with the stable 2-chloroadenosine suggest a salutary effect of $A_{2B}R$ activation on cardiac fibrosis (Wakeno et al. 2006). In contrast, a selective $A_{2B}R$ antagonist (GS-6201) was reported to improve ejection fraction and decreased fibrosis in the non-infarct and border zone (Zhang et al. 2014). The reasons for the anti- and pro-fibrotic effect of cardiac $A_{2B}R$ remain to be explored. For additional information on the role of adenosine signalling during the phases of myocardial remodelling after infarction, see 17.3.5.

17.3.7 Cardioprotection

Adenosine is considered both an important trigger and mediator of cardioprotection elicited by ischaemic preconditioning (IPC). It is the A_1R, and to some extent the A_3R, which participates in the intracellular signalling that triggers cardioprotection by activating phospholipase C (PLC) and/or protein kinase C (PKC) (Cohen and Downey 2008). Another signalling cascade at reperfusion involves activated PKC by stimulation of $A_{2B}R$ (Cohen and Downey 2008). More recently it was found that remote ischaemic preconditioning (RIPC) induced by alternate cycles of preconditioning ischaemia and reperfusion protects the heart against sustained ischaemia-reperfusion-induced injury. This technique has been translated to clinical levels in patients undergoing various surgical interventions including coronary artery bypass graft surgery, abdominal aortic aneurysm repair, percutaneous coronary

intervention and heart valve surgery (Randhawa and Jaggi 2016). Adenosine may be a critical trigger as well as a mediator in RIPC-induced cardioprotection. For more details on adenosine and cardioprotection, see 17.3.7. The regenerative aspects of adenosine post-infarction are dealt with in 17.3.6.

17.4 Adenosine After Myocardial Infarction (MI)

Extracellular adenosine is markedly increased in the infarcted heart (Van Wylen 1994; Martin et al. 1997). Under hypoxic conditions, extracellular adenosine is derived from intracellular adenine nucleotides as well as from extracellular ATP, which is rapidly metabolized to adenosine via the so-called purinergic ectoenzyme cascade (Yegutkin 2014; Burnstock and Pelleg 2015). ATP can either be passively released from lytic cells as direct consequence of the myocardial damage or actively released from cells, e.g. in response to mechanical or hypoxic stimuli (Antonioli et al. 2013; Burnstock and Pelleg 2015).

In general, there is a close association of tissue adenosine and hypoxia and inflammation (Antonioli et al. 2013; Bowser et al. 2017). Hypoxic conditions promote the expression of ATP-degrading ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39) (Eltzschig et al. 2009) as well as adenosine-generating ecto-5′-NT (CD73) (Synnestvedt et al. 2002) and repress the expression of the transporters ENT1 and ENT2, responsible for cellular adenosine uptake (Eltzschig et al. 2005; Morote–Garcia et al. 2009). Functional binding sites for hypoxia-inducible factors have been identified in the promotor regions of $A_{2A}R$ (Ahmad et al. 2009) as well as $A_{2B}R$ (Kong et al. 2006). Putative binding sites have also been identified in the promotor regions of A_1R and A_3R (St. Hilaire et al. 2009). The $A_{2B}R$, which requires higher adenosine concentrations (> 10 μM) compared to the other adenosine receptors, may be a therapeutic target in hypoxia and inflammation, because of the ability to attenuate the consequences of cardiac ischaemia (Haskó et al. 2008; Aherne et al. 2011; Eltzschig et al. 2013).

17.4.1 Inflammation

The injured myocardium is a source of factors that trigger an immune response leading to the activation and infiltration of neutrophils, macrophages and lymphocytes. All of these processes can be modulated and regulated by adenosine. In general, the A_1R and A_3R are considered to promote inflammation, while $A_{2A}R$ and $A_{2B}R$ are mainly anti-inflammatory and reparative (Boros et al. 2016). This is explained by the intracellular coupling to G proteins: A_1R and A_3R are coupled to G_i that decreases cAMP thus promoting the cellular activity, while $A_{2A}R$ and $A_{2B}R$ are coupled to the

cAMP-increasing G_s that inhibits immune cell activity. Confusingly, $A_{2B}R$ is additionally coupled to G_q, which activates PKC that plays a critical role in T-cell activation (Isakov and Altman 2013). However, in line with the general interpretation of A_1R and A_3R being pro-inflammatory and $A_{2A}R$ and $A_{2B}R$ acting immunoregulatory, chemotaxis of neutrophils was reported to be stimulated by A_1R (Cronstein et al. 1990) and A_3R (Chen et al. 2006), while $A_{2B}R$ was suggested to inhibit chemotaxis (Aherne et al. 2012). Similarly, the A_1R had a stimulatory effect on neutrophil adhesion, while the $A_{2A}R$ prevented neutrophil adhesion to the endothelium (Barletta et al. 2012). The $A_{2A}R$ and $A_{2B}R$ were also linked to a reduction in free radical production (Zhao et al. 1996; van der Hoeven et al. 2011), and $A_{2A}R$ prevented the TNFα release after neutrophil activation with lipopolysaccharide (LPS) (McColl et al. 2006). Likewise, the $A_{2A}R$ and $A_{2B}R$ are associated with a reduction of TNFα, IL12 and nitric oxide (NO) production in macrophages thus preventing the classical (pro-inflammatory) activation of these cells. Furthermore, $A_{2A}R$ and, to a minor extent, $A_{2B}R$ activation was shown to increase the secretion of anti-inflammatory IL10 (Boros et al. 2016). Finally, the $A_{2B}R$ may play a role in tissue remodelling and fibrosis via promoting alternative macrophage activation (Csóka et al. 2011).

On lymphocytes, the influence of adenosine is mainly anti-inflammatory: the main adenosine receptor expressed is the $A_{2A}R$, and after lymphocyte activation, it is shifted to the $A_{2B}R$ (Boros et al. 2016; Borg et al. 2017). In the heart, the $A_{2B}R$ was reported to reduce infarct size in several publications when analysing short-term effects. For instance, transplantation of the bone marrow from $A_{2B}R$-deficient mice into wild-type (WT) mice increased infarct sizes and enhanced troponin I levels (Koeppen et al. 2012). Furthermore, the depletion of $A_{2B}R$ on monocytes and macrophages also increased infarct size, cardiac troponin serum levels as well as IL6 and TNFα after 60-min ischaemia and 120-min reperfusion (Seo et al. 2015). In line with this observation, activation of the $A_{2B}R$ with BAY60-6583 reduced infarct size after MI particularly after cardiac preconditioning and was associated with an increase of anti-inflammatory macrophages and a decrease of classically activated macrophages and neutrophils (Tian et al. 2015). In contrast, blockade of $A_{2B}R$ by GS-6201 in a murine model of total coronary occlusion was reported to attenuate cardiac remodelling (28 days post-MI), including the reduction in IL6 and TNFα levels as well as a decrease in caspase-1 activity (Toldo et al. 2012). Whether these divergent findings relate to differences in specificity and/or bioavailability of the applied agonist or antagonist in the in vivo situation is presently not know but clearly needs further study.

It finally should be noted that the impact adenosine may have on different immune cell subpopulations is dependent on receptor expression/density on the respective cell type but also on the concentration of adenosine which is determined by the rate of production (CD73) and rate of removal (adenosine deaminase, cellular reuptake) of adenosine. Low extracellular adenosine favours activation of A_1R, $A_{2A}R$ and A_3R, while high adenosine concentrations are required for $A_{2B}R$ activation.

17.4.2 Cellular Distribution of CD73

CD73 is expressed in several tissues such as the brain, kidney, lung and heart and is mainly associated with leukocytes and endothelial cells (Antonioli et al. 2013). CD73 can be induced under hypoxic (Synnestvedt et al. 2002) and inflammatory conditions induced by interferons (IFNs), TNFα, IL1β or prostaglandin E2 (Beavis et al. 2012). Also tumour growth factor (TGF)β induces CD73 expression, and this effect can be modulated by pro-inflammatory cytokines (Regateiro et al. 2011). On cardiac infiltrating immune cells, CD73 is mainly expressed on T cells, natural killer (NK) cells and granulocytes but not on B cells, monocytes and antigen-presenting cells (APC) 3 days after MI (Bönner et al. 2012). Interestingly, on cardiac resident immune cells, CD39 is mainly expressed on myeloid cells, while CD73 is dominant on T cells (Bönner et al. 2012), as shown in Fig. 17.3. Among lymphocytes, CD73 is mainly expressed on regulatory T cells (Tregs) and contributes to their immunosuppressive activity (Deaglio et al. 2007). Both CD39 and CD73, however, are not exclusively expressed on Tregs but also on effector T cells (Teffs) after T -cell activation (Chalmin et al. 2012).

17.4.3 Purinergic Signalling on T Cells

Purinergic signalling on T cells is modulated by TCR responses. Already during T-cell maturation in the thymus, T-cell selection is controlled by ATP which involves $P2X_7R$ (Lépine et al. 2006) and adenosine acting via $A_{2A}R$ (Cekic et al. 2013). In general, ATP mediates excitatory effects thereby supporting TCR response, while adenosine is inhibitory to the TCR signalling. Signalling of ATP is likely to involve a variety of ATP receptors, P2XRs and P2YRs, since transcripts of most of the ATP receptors are detectable in human lymphocytes (Wang et al. 2004). Because of the close interaction of T cells and DCs during T-cell activation, ATP may serve as a positive feedback molecule at the immunological synapse. ATP is released by the T cell in a Pannexin-1-, $P2X_1R$- and $P2X_4R$-dependent fashion (Woehrle et al. 2010). Adenosine has opposite effects in that it inhibits the secretion of cytokines when present during or after activation. The secretion of IFNγ from Teffs after in vitro stimulation can be effectively blocked by $A_{2A}R$ (Romio et al. 2011) and to a minor extent also by $A_{2B}R$ (Borg et al. 2017). Both $A_{2A}R$ and $A_{2B}R$ couple to G_s proteins and thus induce cAMP accumulation together with PKA activation that was shown to counteract TCR activation (Vang et al. 2001). In contrast to $A_{2A}R$, $A_{2B}R$ in addition couples to G_q that activates the PLC-Ca^{2+} signalling pathway which curtails responses of TCR activation such as IL2 secretion (Desai et al. 1990). However, the affinity of $A_{2B}R$ to adenosine is much lower than that of $A_{2A}R$, and $A_{2B}R$ expression – absent in naïve T cells – is upregulated only after T-cell stimulation (Borg et al. 2017). The role of adenosine in the crosstalk between developing thymocytes and macrophages in the thymus has been recently reviewed (Köröskényi et al. 2017).

It is generally assumed that the key enzymes degrading pro-inflammatory ATP to anti-inflammatory adenosine are CD39 and CD73. Both enzymes are highly expressed on T cells. This view, however, neglects that aside from CD39 also ectonucleotide pyrophosphatases (ENPPs) can effectively degrade ATP to AMP (see Fig. 17.1). Recent studies have shown that T cells express ENPP1 and ENPP3, which are functionally active (Borg et al. 2017). While naïve T cells lacking CD39 degrade ATP to a significant lesser extent compared to WT T cells, degradation of ATP in T cells after stimulation was not altered by the lack of CD39. This indicates that under certain conditions, ENPPs can fully compensate for the loss of CD39. That ENPPs are functional is also supported by the observation that activated T cells can break down extracellular NAD via AMP to adenosine (Fig. 17.1) (Borg et al. 2017). Similar to ATP, activated cells can release NAD, which stimulates immune responses by hindering Tregs and may thereby be the prototype of a new category of danger signals (Adriouch et al. 2012). NAD-dependent protein modifications such as ADP-ribosylation have a decisive impact on vital cellular processes (Nikiforov et al. 2015). Whether and to what extent NAD can influence purinergic signalling and extracellular purine metabolism remains an interesting topic of future research.

Within the T-cell population, Tregs show high expression of CD39 and CD73 that participate in their suppressive function (Deaglio et al. 2007). However, CD39 is only fully active after Treg stimulation (Borsellino et al. 2007). Treg-derived adenosine mediates its suppressive effect on Teffs via $A_{2A}R$ by the downregulation of NFκB signalling (Romio et al. 2011). Tregs are regulated by adenosine and ATP: $A_{2A}R$ promotes their expansion (Ohta et al. 2012), while $P2X_7R$ inhibits their suppressive activity. Treg plasticity is negatively influenced by ATP which prevents Treg differentiation and favours Th17 polarization: IL6, a Th17-polarizing cytokine, triggers mitochondrial ATP synthesis and thereby induces Th17 differentiation via $P2X_7R$ activation, whereas $P2X_7R$ blockade favours Treg polarization (Schenk et al. 2011). Depletion of CD73 on Tregs leads to diminished suppressive activity (Kinsey et al. 2012), showing that adenosine supports the regulatory action of Tregs whereas ATP works in the opposite direction.

17.4.4 CD73-Derived Adenosine in the Healing Process

While T cells are a source of adenosine in an inflammatory microenvironment, the functional relevance of the pathway involving CD73 was only recently explored. T-cell-specific depletion of CD73 in a transgenic mouse model resulted in a pronounced reduction in cardiac function after ischaemia-reperfusion, which was associated with an increase in cardiac fibrosis when studied over 28 days (Borg et al. 2017). Interestingly, $A_{2B}R$ expression was significantly upregulated in $CD4^+$ and $CD8^+$ T cells as well as in APCs and cardiomyocytes (Borg et al. 2017), suggesting a functional role of this receptor in the healing phase after ischaemia. Similarly, in transverse aortic constriction (TAC), a model which mimics the failing heart,

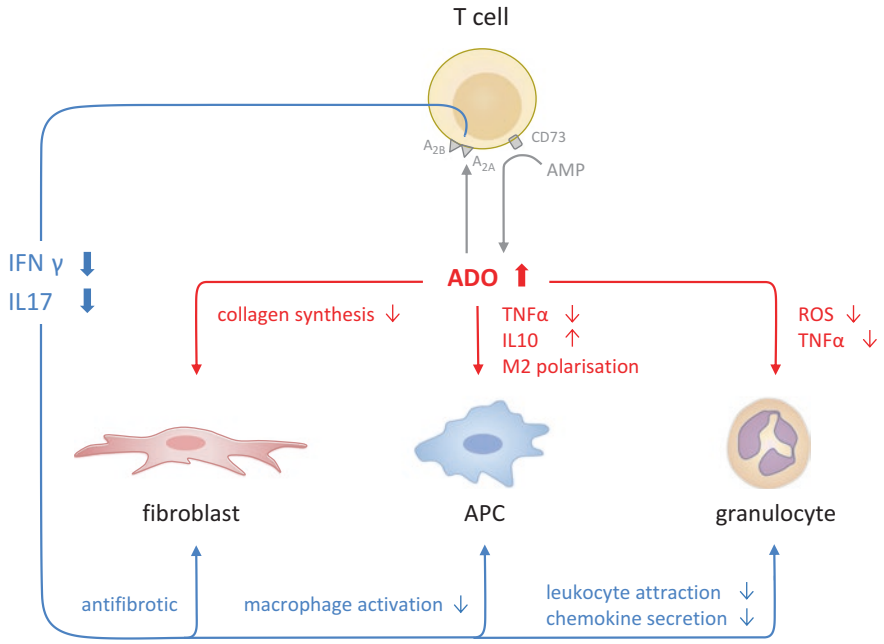

Fig. 17.4 Adenosine formed by T cells orchestrates wound healing after myocardial injury through autocrine and paracrine mechanisms. Adenosine (ADO) generated by CD73 on T cells can act in an autocrine manner, inhibiting production of pro-inflammatory (IFNγ) and pro-fibrotic (IL17) cytokines via $A_{2A}R$ and $A_{2B}R$. In a paracrine manner, ADO can decrease collagen synthesis by fibroblasts and has various anti-inflammatory effects, e.g. reduction of TNFα secretion from APCs and granulocytes. (Permitted reproduction from Borg et al. (2017))

contractile function of the heart was significantly depressed when CD73 was lacking on T cells (Quast et al. 2017). Why in the TAC model the $A_{2A}R$ was found to be upregulated on T cells, while it was the $A_{2B}R$ in the ischaemia-reperfusion model, remains to be explored. In both models, the enzymes involved in ATP and also NAD degradation (CD38 and CD157) were upregulated on infiltrating T cells. Together these data demonstrate that CD73 on T cells orchestrates cardiac wound healing which includes purinergic metabolic reprogramming. This may be a general mechanism by which local production of adenosine modulates T-cell activity.

Figure 17.4 summarizes our present understanding on the mechanisms by which CD73-derived adenosine on T cells promotes healing of the injured heart and limits cardiac fibrosis. Adenosine formed by T cells acts through autocrine and paracrine mechanisms. The autocrine mechanism includes $A_{2A}R$-/$A_{2B}R$-mediated inhibition of IFNγ and IL17 production, which are central pro-inflammatory and pro-fibrotic cytokines. The paracrine action of adenosine includes the well-known inhibition of collagen synthesis by fibroblasts and a variety of anti-inflammatory actions on APCs and granulocytes.

The presence of T cells in the early phase after ischaemia-reperfusion was reported to be harmful (mediated by IFNγ derived from $CD4^+$ T cells) and could be antagonized by activation of $A_{2A}R$ (Yang et al. 2006). Activation of $A_{2A}R$ right before the onset of reperfusion diminished the accumulation of T cells and reduced the extent of tissue damage (Yang et al. 2006). The role of Tregs after myocardial infarction is still not fully clear. On the one hand, infused Tregs were reported to decrease the infarct size after in vitro activation (Xia et al. 2015). On the other hand, infusion of the complete CD4 T-cell subset (including $FoxP3^+$ Tregs) was reported to increase infarct size rather than the infusion of the CD4 T-cell subset lacking Tregs (Mathes et al. 2016). Whether the difference in the results is related to the Treg contribution to adenosine formation needs to be explored.

17.4.5 Remodelling/Fibrosis

The initial inflammatory response in the first hours to days after MI ("inflammatory phase") is followed by a phase of cell proliferation and tissue remodelling ("proliferative phase"), eventually leading to the formation of a stable, mature scar ("maturation phase"). Cardiac fibroblasts play an important role in every phase of the post-MI myocardial repair and are therefore considered as promising target for novel treatment strategies (Ma et al. 2017). Cardiac fibroblasts are a heterogeneous cell population (Doppler et al. 2017) and are characterized by a high phenotypic/functional plasticity (Chistiakov et al. 2016). During the initial inflammatory phase, cardiac fibroblasts are activated and acquire an inflammatory phenotype (Chen and Frangogiannis 2013): While they lose their capability to produce collagen (Siwik et al. 2000), they now secrete matrix-degrading metalloproteinases (Fan et al. 2012) and multiple pro-inflammatory mediators such as IL1β, IL6 and TNFα (Turner et al. 2009). In the following proliferative phase, cardiac fibroblasts adopt a proliferative, migratory and secretory myofibroblast phenotype (Talman and Ruskoaho 2016). The myofibroblasts, characterized by expression of α-smooth muscle actin (SMA), infiltrate the infarction area and produce large amounts of interstitial collagens, initially collagen type III. This is replaced later on by collagen type I, which is cross-linked in the maturation phase to form a stable scar (Talman and Ruskoaho 2016). In this late phase, myofibroblasts become quiescent, reducing both proliferation and collagen synthesis, and may undergo apoptosis. The responsible inhibitory signalling is only poorly understood, although it is essential to prevent overactive fibrosis and dysfunction of the remodelling myocardium (Shinde and Frangogiannis 2017).

Adenosine has been shown to modulate the pivotal secretory activity of cardiac fibroblasts. Of the four adenosine receptors, $A_{2B}R$ has the highest expression level in cardiac fibroblasts, followed by $A_{2A}R$ (Epperson et al. 2009). $A_{2B}R$ activation on cardiac fibroblasts inhibits collagen and total protein synthesis (Dubey et al. 1998; Chen et al. 2004) as well as cell proliferation (Dubey et al. 1997, 2001), suggesting an anti-fibrotic activity of adenosine. The inhibition of collagen was shown to be mediated via a G_s-adenylyl cyclase-cAMP-Epac-PI_3K-dependent, PKA-independent

pathway (Villarreal et al. 2009). The same pathway was recently identified to be responsible for the A_{2B}R-mediated inhibition of endothelin-1-induced proliferation and α-SMA expression of rat cardiac fibroblasts, which may attenuate adverse remodelling after MI (Phosri et al. 2017). In contrast, the A_{2B}R-mediated induction of IL6 released from mouse cardiac fibroblasts was reported to be mediated by a G_s-cAMP-independent, G_q-PLC-PKC-dependent pathway (Feng et al. 2010). However, the coupling of A_{2B}R in cardiac fibroblasts might be species- and/or cell preparation-specific and needs further clarification, since Epperson et al. previously found only a cAMP-dependent, PLC-independent signalling pathway coupled to A_{2B}R in rat cardiac fibroblasts (Epperson et al. 2009). In line with the collagen-reducing and anti-proliferative effects of adenosine on cardiac fibroblasts in vitro, there is evidence that long-term A_{2B}R stimulation in vivo reduces collagen deposition, prevents adverse cardiac remodelling and improves cardiac function after MI (Wakeno et al. 2006). However, contradictory observations have been made with A_{2B}R inhibition after MI, where an A_{2B}R antagonist reduced caspasce-1 activity in the heart and attenuated cardiac remodelling (Toldo et al. 2012). To which extent A_{2B}R activation/inhibition specifically on cardiac fibroblasts is involved in these opposing findings is not clear, and the role of adenosine signalling in cardiac fibrosis is still controversial (Novitskaya et al. 2016; Vecchio et al. 2017).

17.4.6 Regeneration

In the proliferative phase after MI, angiogenic signalling stimulates the formation of a microvascular network, which restores blood flow to the infarcted area (Headrick et al. 2013; Jivraj et al. 2014). Adenosine may mediate 50–70% of hypoxia-induced angiogenesis and promote endothelial proliferation and migration via A_{2A}R and A_{2B}R (Adair 2005). Adenosine may further support endothelial growth by promoting the adhesion of endothelial progenitor cells to coronary endothelium via A_1R and A_{2B}R activation (Ryzhov et al. 2008). In contrast to endothelial cells, A_{2B}R activation on coronary smooth muscle cells inhibits proliferation (Jackson et al. 2011), while A_1R activation stimulates the proliferation of these cells (Shen et al. 2005).

A major mechanism by which adenosine promotes angiogenesis appears to be regulation of the expression of pro- and anti-angiogenic molecules in vascular and immune cells via all four adenosine receptor subtypes (Headrick et al. 2013). Emerging sources for angiogenic signalling in the post-MI heart are the interstitial and epicardial populations of resident cardiac progenitor cells. Interstitial Sca1-positive cardiac progenitor cells in the adult heart have been characterized as self-renewing, multipotent and clonogenic cardiac cells with the capability to differentiate into cardiomyocytes as well as endothelial cells and smooth muscle cells (Valente et al. 2014). When injected into the infarcted heart, Sca-1-positive cardiac progenitor cells promote neovascularization, differentiate into cardiomyocytes and endothelial cells and attenuate the functional decline and the adverse remodelling after MI (Wang et al. 2006; Matsuura et al. 2009). Sca-1-positive cardiac progenitor cells

preferentially express $A_{2B}R$, and its activation increases the secretion of proangiogenic factors such as IL6, CXCL1/IL8 and VEGF from these cells (Ryzhov et al. 2012). In line with this finding, $A_{2B}R$ signalling proved to be essential for the Sca-1-positive cardiac progenitor cell-mediated cardiac recovery after MI (Ryzhov et al. 2013).

During embryonic cardiac development, epicardium-derived cells (EPDC) contribute to the coronary vasculature (Carmona et al. 2010) and give rise to a population of cardiomyocytes (Zhou et al. 2008) and the majority of interstitial fibroblasts (Fang et al. 2016). In the adult post-MI heart, cells of the previously dormant epicardium undergo EMT and proliferate, thereby forming a layer of EPDC at the heart surface (Smits et al. 2018). Adult EPDC migrate into the damaged myocardium and have been reported to differentiate into cardiovascular cells and fibroblasts as well as – though to a very small extent – to cardiomyocytes (Smart et al. 2011; Wijk et al. 2012; Ruiz-Villalba et al. 2015). EPDC secrete proangiogenic factors, such as FGF2, IL6 and VEGF, and EPDC-conditioned medium injected into the infarcted heart reduces infarct size and improves heart function (Zhou et al. 2011). $A_{2A}R$ and $A_{2B}R$ are the two most highly expressed adenosine receptors in EPDC, and $A_{2B}R$ activation strongly promotes the secretion of IL6 and VEGF by EPDC and induces the release of ATP (Hesse et al. 2017). Extracellular ATP can be rapidly degraded to adenosine by the purinergic ectoenzyme cascade (see Fig. 17.1) on the EPDC surface, thus sustaining proangiogenic adenosine signalling (Hesse et al. 2017). Furthermore, ectoenzymes on the EPDC surface metabolize NAD to adenosine to the same extent as ATP (Hesse et al. 2017), suggesting that NAD is a second source of adenosine after MI. Together these studies indicate that adenosine exerts beneficial effects on cardiac remodelling/regeneration after MI by modulating the pro-angiogenic paracrine activity of interstitial and epicardial progenitor cells. These cells are likely to generate adenosine by themselves for auto-and paracrine signalling. Whether adenosine plays a role in the differentiation processes of these resident progenitor cell types in the infarcted heart is presently unknown.

17.4.7 Cardioprotection

Each of the four adenosine receptor subtypes has been shown to be cardioprotective; however, the underlying mechanisms are not fully understood (Peart and Headrick 2007; McIntosh and Lasley 2012; Burnstock and Pelleg 2015). While there is controversy about the role of endogenous adenosine in inherent cardioprotection mechanisms (Headrick et al. 2013), the importance of endogenous adenosine in cardioprotection by ischaemic pre- and postconditioning is well established (Burnstock and Pelleg 2015; Gile and Eckle 2016). Inducing brief episodes of nonlethal ischaemia in the heart prior to, during or after an episode of sustained lethal myocardial ischaemia can dramatically reduce myocardial damage (Hausenloy and Yellon 2009). Stimulation of adenosine receptors can mimic this cardioprotective effect (Burnstock and Pelleg 2015). Activation of A_1R and A_3R prior to or during

ischaemia proved to be highly protective, whereas $A_{2A}R$ and $A_{2B}R$ activation seems to be important in post-ischaemic events (Headrick et al. 2011; McIntosh and Lasley 2012; Gile and Eckle 2016). However, the cardioprotective effect of ischaemic preconditioning was completely abolished in $A_{2B}R$-deficient mice, while there was still some protection in A_1R-, $A_{2A}R$- or A_3R-deficient mice in a head-to-head comparison (Eckle et al. 2007).

17.4.8 Clinical Implications

Recent therapeutic developments in purinergic signalling have been reviewed (Burnstock 2017). With respect to the clinical setting, adenosine used with reperfusion after MI is considered as one of the most promising therapeutic strategies to enhance cardioprotection (Kloner et al. 2017). High-dose adenosine infusions as adjunct to reperfusion significantly reduced anterior wall myocardial infarct size in two clinical trials, AMISTAD 1 (Mahaffey et al. 1999) and AMISTAD 2 (Ross et al. 2005). It is likely that these cardioprotective effects are mainly mediated by $A_{2A}R$ and $A_{2B}R$. However, the A_3R has also been proposed as promising therapeutic target of cardiovascular disease (Nishat et al. 2016).

Because of their vasodilatory properties, adenosine and $A_{2A}R$ agonists cannot be systemically applied at high doses without disadvantageous side effects. This problem, however, can be circumvented by using prodrugs of the $A_{2A}R$ which show only little vasodilatory but high anti-inflammatory activities (Flögel et al. 2012). Recently the $A_{2B}R$ has attracted attention, because this receptor becomes selectively upregulated on immune cells after MI (Borg et al. 2017) and novel $A_{2B}R$ agonists have been reported to stimulate anti-fibrotic signalling (Vecchio et al. 2016). The wildly used $A_{2B}R$ agonist BAY60-6583 may not be sufficiently specific since off-target effects have been reported (van der Hoeven et al. 2011; Borg et al. 2017). Thus, novel $A_{2B}R$-specific agonists are required and may be promising drugs for the treatment of post-MI inflammation, remodelling and fibrosis.

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Chapter 18
Adenosine Receptors in the Lungs

Amir Pelleg and Riccardo Polosa

Abstract The ubiquitous adenine nucleoside adenosine (Ado), which plays an important role in cellular energetics, is released from cells under physiologic and pathophysiologic conditions. Another source of extracellular Ado is rapid degradation of extracellular adenosine 5′-triphosphate (ATP) by ectoenzymes. Extracellular Ado acts as an autocrine and paracrine agent by the activation of G protein-coupled cell surface receptors (GPCRs), designated as A_1, A_{2A}, A_{2B}, and A_3. Almost four decades ago, published data have indicated that Ado could play a role in immune-mediated histamine release from pulmonary mast cells. Since then, numerous studies have indicated that Ado's signal transductions are involved in various pulmonary pathologies including asthma and COPD. This chapter is a succinct review of recent studies in this field.

Keywords Adenosine · Adenosine receptors · Lungs · Pulmonary mast cells · Asthma · COPD

18.1 Introduction

The ubiquitous adenine nucleoside adenosine (Ado) plays a major role in cellular metabolism and energetics. The levels of extracellular Ado are determined by its release from cells under physiologic and pathophysiologic conditions as well as the degradation of extracellular adenosine 5′-triphosphate (ATP) by ectoenzymes, CD39 and CD73 in particular (Zimmermann 2000; Zimmermann et al. 2012). Extracellular Ado is eliminated from the extracellular space by ecto-adenosine deaminase (ADA) (Franco et al. 1997) and active Ado transporters that transport Ado into cells (Thorn and Jarvis 1996). Extracellular Ado acts as an autocrine and

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paracrine agent, the effects of which are mediated by four different G protein-coupled receptors: A_1, A_{2a}, A_{2b}, and A_3 (A_1AdoR, A_{2a}AdoR, A_{2b}AdoR, and A_3AdoR, respectively). These receptors, which are expressed to various degrees by different cell types throughout the body including the lungs (Burnstock et al. 2012; Zhou et al. 2009), manifest variable affinity to Ado (Fredholm et al. 2001). AdoR are coupled to cascades of intracellular cellular-response pathways, mainly through the activation or inhibition of adenylate cyclase and the subsequent alteration of intracellular cyclic adenosine monophosphate (cAMP) levels (Karmouty-Quintana et al. 2013). The activation of AdoR can lead to both pro-inflammatory and anti-inflammatory effects, depending on the type of receptor and the experimental setting (Schepp and Reutershan 2008).

In the early 1980s, Holgate and his colleagues have shown for the first time that inhaled Ado induces bronchoconstriction in asthmatic but not healthy human subjects and that this action was mediated mainly by histamine released from lung mast cells and not by a pulmonary-pulmonary central vagal reflex triggered by Ado (Cushley et al. 1983; Mann et al. 1985; Church and Holgate 1993). Since then, data obtained in numerous studies have shown that Ado plays important roles in pulmonary physiology and pathophysiology (Caruso et al. 2006; Polosa and Blackburn 2009; Caruso et al. 2009). One characteristic of acute lung injury (ALI) is elevated Ado levels in the lung, which activate anti-inflammatory and tissue-protective mechanisms (Zhou et al. 2009; Karmouty-Quintana et al. 2013; Gonzales et al. 2014). Specifically, initial activation of AdoR blunts the production of multiple cytokines, decreases inflammatory cell infiltration, and preserves pulmonary vascular barrier function (Eckle et al. 2009). Similarly, Ado exerted a protective anti-inflammatory effect in an in vitro model of lung transplantation/ischemia-reperfusion model (Smail et al. 2016). In contrast, sustained AdoR activation results in pro-inflammatory effects, e.g., increased cytokine production and inflammatory cell infiltration (Zhou et al. 2009). This conundrum of the Ado's dual nature has stymied the development of new drugs aimed at the modulation of AdoR signal transductions.

This chapter is a succinct review of recent studies in this field. The large number of relevant publications and the limited scope of this review inevitably impose selective articles' citation. We thus apologize if our selection of articles does not include article(s) that others may feel should have been cited.

18.2 A_1AdoR

A_1AdoR, which has the highest affinity to Ado, is expressed at low levels in the lung; relatively higher levels were detected in the newborn mice (Metsola et al. 2014). The activation of A_1AdoR reduces adenylate cyclase's activity via the activation of pertussis toxin-sensitive Gi and Go proteins, stimulates phospholipase C via $G_{\beta\gamma}$ subunits, and activates pertussis toxin-sensitive K^+ channels (Pelleg et al. 1996)

and K_{ATP} channels (Schepp and Reutershan 2008). Activation of the A_1AdoR could be pro- or anti-inflammatory depending on the specific pathophysiologic conditions and the levels of extracellular Ado.

Early studies have shown that ischemia-induced constriction of the pulmonary vasculature is mediated by the activation of A_1AdoR, which results in thromboxane release (Neely et al. 1991) as well as pulmonary ischemia-reperfusion injury (Neely and Keith 1995). Subsequent in vivo studies using a mouse model have shown that physiologic doses of Ado decrease alveolar fluid clearance (AFC), probably by means of an A_1AdoR-dependent mechanism that causes Cl^- efflux through CFTR, whereas lower doses increased AFC via the A_2aR and/or A_3R (Factor et al. 2007). Similarly, alcohol decreases alveolar fluid clearance and impairs survival from acute lung injury. Alcohol induced increases Ado levels in the lung, which may be responsible for reduction in AFC and associated worsening of lung injury (Dada et al. 2012).

A_1AdoR expression is increased significantly during LPS-induced injury thereby suggesting that extracellular Ado levels and Ado receptors may play a role in LPS-induced toxicity (Metsola et al. 2014). Indeed, A_1AdoR has been implicated in polymorphonuclear cell trafficking and alterations in microvascular permeability in lipopolysaccharide (LPS)-induced lung injury model (Ngamsri et al. 2010). A more recent study in a mouse model has shown that A_1AdoR activation improves lung function and decreases inflammation, edema, and neutrophil chemotaxis after ischemia-reperfusion (Fernandez et al. 2013a). This protective effect of A_1AdoR's activation agrees with the finding that pulmonary injury was exacerbated in ADA double-knockout mice, which is also deficient in the expression of A_1AdoR (Sun et al. 2005). Similarly, an A_1AdoR agonist significantly increased lung compliance and oxygenation and decreased pulmonary artery pressure, decreased neutrophil infiltration by myeloperoxidase activity and edema, and reduced tumor necrosis factor-alpha production in an isolated, ventilated, blood-perfused rabbit lung model of ischemia-reperfusion (Gazoni et al. 2010).

The expression of A_1AdoR in the airways of asthmatic subjects is upregulated (Wilson et al. 2009). Ado-induced bronchoconstriction in human subjects is indirect (see above); however, in rodents this action is mediated by A_1AdoR and a central vagal reflex (Wilson et al. 2009). Although A_1AdoR activation affects "different cell types to produce bronchoconstriction, inflammation, mucous gland hyperplasia, angiogenesis, and fibrosis, all of which are important in the pathophysiology of human asthma" (Wilson et al. 2009), the exact role of Ado and A_1AdoR in asthma has not been fully delineated, and accordingly none of the drug candidates targeting A_1AdoR signal transduction has been approved by the FDA (see, e.g., Gottlieb et al. 2011).

Adenosine activation of leukocyte A_1AdoR plays a significant role in their recruitment to lungs infected with an influenza virus and thereby contributes to influenza pathogenesis (Aeffner et al. 2014).

18.3 A_{2A}AdoR

A_{2A}AdoR is expressed by various inflammatory cell types, and its activation results in broad anti-inflammatory effects (Hasko and Pacher 2008). A_{2A}AdoR signaling attenuates acute lung injury by enhancing alveolar fluid clearance in mice (Eckle et al. 2008). The expression of A_{2A}AdoR was decreased significantly by an allergen challenge in a mouse model of asthma suggesting that A_{2A}AdoR deficiency leads to airway inflammation and airway hyperresponsiveness in this setting (Nadeem et al. 2007).

A_{2A}AdoR activation stimulates the formation of cAMP (Ongini and Fredholm 1996); the latter can significantly upregulate SOCS-3 protein expression (Sands et al. 2006). *In* vitro and *in vivo* studies have shown that hypoxia can induce pulmonary artery smooth muscle cell A_{2A}AdoR expression (Qian et al. 2013; Fan et al. 2016). A_{2A}AdoR upregulates SOCS-3 protein, which inhibits pulmonary vascular remodeling in lung tissue of hypoxic pulmonary hypertension rats (Fan et al. 2016). In addition, a recent study utilizing a hypoxia-induced pulmonary hypertension (HPH) mouse model has shown that hypoxia enhanced the expression of A_{2A}AdoR, the activation of which attenuated the release of specific inflammatory cytokines in the lung (Huang et al. 2017). The latter was associated with reduced thickening of pulmonary arterioles and improved hypoxemia (Huang et al. 2017).

An A_{2A}AdoR agonist significantly increased lung compliance and oxygenation and decreased pulmonary artery pressure, decreased neutrophil infiltration by myeloperoxidase activity and edema, and reduced tumor necrosis factor-alpha production in an isolated, ventilated, blood-perfused rabbit lung model of ischemia-reperfusion (Gazoni et al. 2010).

In contrast, it was recently shown that the activation of A_{2A}AdoR modulated the activation of fresh human alveolar inflammatory cells in patients with interstitial lung disease (Alfaro et al. 2017). A_{2A}AdoR plays a role in the regulation of different stages of immune responses, including antigen presentation, T-cell activation, expansion, survival, and memory (Ohta and Sitkovsky 2001). Specifically, Ado suppresses the immune response by activation of A_{2A}AdoR expressed by T-cells. Indeed, a selective antagonist of A_{2A}AdoR reduced the tumor burden in a mouse lung cancer in vivo model and restored immune responsiveness ex vivo (Mediavilla-Varela et al. 2017).

Chronic lung inflammation is associated with fibroblasts proliferation, beginning to proliferate, the formation of new blood vessels, and the increase in extracellular matrix resulting in the development of pulmonary fibrosis (Della Latta et al. 2013). Genetic knockout of A_{2A}AdoR in mice significantly exacerbates, while activation of A_{2A}AdoR attenuates the progression of pulmonary fibrosis induced by bleomycin (a chemotherapeutic agent used to treat several neoplastic diseases and widely used ones for the induction of lung fibrosis (Della Latta et al. 2015; Chen et al. 2017). The beneficial effects of A_{2A}AdoR activation in this setting are mediated at least partially via the stromal cell-derived factor-1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4) pathway, the inhibition of which protects the lungs from fibrogenesis in BLM-exposed mice (Chen et al. 2017).

18.4 A_{2B}AdoR

Ado and its cell surface receptors play an important role in the regulation of inflammation following acute lung injury (Karmouty-Quintana et al. 2013). A_{2B}AdoR are expressed by mast cells, bronchial smooth muscle cells, and lung fibroblasts; their activation increases the release of various inflammatory cytokines and promotes differentiation of lung fibroblasts into myofibroblasts, typical of the fibrotic events (Della Latta et al. 2013).

A_{2B}AdoR mediates the relaxing effects of Ado on guinea pig airways (Breschi et al. 2007). Hypoxia is associated with increased soluble CD73 activity, which contributed to hypoxia-induced increase in Ado's plasma level and Ado-mediated erythrocyte A_{2B}AdoR activation inducing 2,3-BPG production and triggering O_2 release that prevents multiple tissue hypoxia, inflammation, and pulmonary vascular leakage (Liu et al. 2016). Extracellular Ado promote dermal fibrosis (Fernandez et al. 2013b), and the activation of A_{2B}AdoR promoted renal fibrosis in both mice infused with angiotensin II and mice subjected to unilateral ureteral obstruction (Dai et al. 2011). A study in a mouse model of bleomycin-induced injury and whole-lung lysate from patients without and with idiopathic pulmonary fibrosis has shown that inhibition of hypoxia-inducible factor 1-α (HIF1α) attenuated pulmonary fibrosis in association with reductions in A_{2B}AdoR expression in alternatively activated macrophages (Philip et al. 2017). These data were interpreted to suggest that hypoxia, through HIF1α, contributes to the development and progression of pulmonary fibrosis through its regulation of A_{2B}AdoR expression on alternatively activated macrophages (AAMs), cell differentiation, and production of profibrotic mediators (Philip et al. 2017).

In contrast, oxygenation may weaken local tissue hypoxia-dependent Ado and A_{2A}AdoR-mediated anti-inflammatory mechanism and thereby further exacerbating lung injury in the setting of acute respiratory distress syndrome (ARDS) (Thiel et al. 2005; Aggarwal et al. 2013). That notwithstanding, exposure to a hyperoxic environment causes lung injury associated with an increase of Ado levels, which protects vascular barrier function in hyperoxic lung injury through the A_{2B}AdoR-dependent preservation of the endothelial cellular adhesion protein occluding (Davies et al. 2014). In addition, the activation of A_{2B}AdoR reduced endotoxin-induced acute lung injury in a murine model (Schingnitz et al. 2010).

A_{2B}AdoR was found to play an important role in mediating lung inflammation after an ischemia-reperfusion challenge by stimulating cytokine production and neutrophil chemotaxis in a mouse model in vivo (Anvari et al. 2010). In contrast, several studies have shown that Ado and A_{2B}AdoR play an important role in the resolution of pulmonary edema and inflammation during ALI (Eckle et al. 2009). For example, in a mouse model of ALI (intratracheal LPS treatment followed by injurious mechanical ventilation), it was found that alveolar epithelial A_{2b}AdoR signaling contributes to lung protection (Hoegl et al. 2015).

Stromal cell-derived factor (SDF)-1 is a chemokine that regulates the release of neutrophils from the bone marrow into the circulation; during inflammation, the

concentration of SDF-1 in the bone marrow decreases, and polymorphonuclear neutrophils enter the circulation from where they can migrate to the lungs during ALI (Konrad et al. 2017). SDF-1 is expressed in the human lung during acute lung injury (Petty et al. 2007). The effects of SDF-1 are mediated by two receptors: CXCR4 and CXCR7 (Rath et al. 2014), the inhibition of which results in an anti-inflammatory effect during ALI (Konrad et al. 2017). Using a mouse model of ALI and an in vitro preparation of human epithelium/endothelium, it was found that the anti-inflammatory effects of CXCR4 and CXCR7 antagonism in terms of PMN migration, chemokine release, and microvascular permeability are linked to adenosine A_{2B}AdoR signaling in hematopoietic cells (Konrad et al. 2017).

18.5 A_3AdoR

Significant amounts of A_3AdoR are expressed in the rat lung (Haeusler et al. 2015). A_3AdoR levels have been found to be elevated in subjects with chronic lung disease and in different pulmonary pathological conditions (Della Latta et al. 2013). The selective Ado A_3AdoR agonist 2-chloro-N6-(3-iodobenzyl)adenosine-5′-N-methylcarboxamide (2-Cl-IB-MECA) significantly increased lung compliance and oxygenation and decreased pulmonary artery pressure, decreased neutrophil infiltration by myeloperoxidase activity and edema, and reduced tumor necrosis factor-alpha production in an isolated, ventilated, blood-perfused rabbit lung model of ischemia-reperfusion (Gazoni et al. 2010). Similarly, 2-Cl-IB-MECA attenuated lung dysfunction, inflammation, and neutrophil infiltration in a mouse model of lung ischemia-reperfusion (Mulloy et al. 2013).

Previous studies have shown that extracellular purine nucleosides and nucleotides are potent modulators of human lung mast cell (HLMC) degranulation and histamine release (Pelleg and Schulman 2002; Polosa et al. 1995). Low and high concentrations of Ado enhanced and suppressed, respectively, the release of histamine from FcεRI-stimulated HLMC; this action was mediated by A_3AdoR (Gomez et al. 2011). The dose-dependent contrasting effects of Ado on histamine release associated with an allergic reaction were confirmed in a subsequent study using cultured HLMC (Nishi et al. 2016). Rudich et al. examined whether the activation of A_3AdoR modulates rather than mediates the effects of Ado in human mast cells, presumably at a transcriptional level (Rudich et al. 2015). Using the HMC-1 cell line, Rudich et al. found that A_3AdoR activation represses the expression of genes involved in tissue remodeling and that is coupled to downregulation of the receptor expression, both at protein and mRNA levels (Rudich et al. 2015). Furthermore, since in this study, dexamethasone, a commonly prescribed asthma medication, exerted a synergistic signal that increased Cl-IB-MECA induced gene upregulation and facilitated cytokine secretion, it was speculated that this interaction might underlie the resistance to corticosteroids that is experienced in severe refractory asthma (Rudich et al. 2015).

18.6 Conclusions

Although the prevailing school of thought is that the production of Ado and its activation of AdoR play largely beneficial roles in acute pathophysiologic condition and sustained elevated Ado levels can become detrimental by activating pathways that promote tissue injury and fibrosis (Karmouty-Quintana et al. 2013), many studies challenge this concept. Specifically, it seems that even in the acute phase of injury, the resulting effects of elevated extracellular Ado levels critically depend on the targeted cell type and the localized Ado level at the receptors' sites. This could explain why, until now, neither selective agonists nor antagonists of AdoR subtypes have been approved by the FDA for the treatment of either acute or chronic lung injury and inflammation (Borea et al. 2016).

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Chapter 19
Renal Adenosine in Health and Disease

H. Thomas Lee and Jurgen Schnermann

Abstract Adenosine-dependent regulation of renal function in healthy and diseased kidney is mediated by activation of the four types of P1 purinergic adenosine receptors (A_1AR, A_{2A}AR, A_{2B}AR, A_3AR). The dominant effect of an elevation of plasma adenosine in the renal vasculature is an A_{2A}AR- and A_{2B}AR-mediated vasodilatation that increases global as well as medullary renal blood flow and is in part endothelium-dependent. In addition, a high expression of A_1AR in afferent glomerular arterioles can cause a localized vasoconstriction, especially when accessed from the vessel outside, a reaction most evident in the tubuloglomerular feedback response. Effects of adenosine on tubular transport are most pronounced in the proximal tubule where the nucleoside stimulates NaCl reabsorption in the subnormal concentration range while inhibiting transport at elevated levels. Because adenosine production increases in hypoxia, the issue of a role of the nucleoside in the renal injury following ischemia reperfusion has been studied extensively. Experimental evidence supports the notion that adenosine protects against ischemia-induced acute kidney injury by directly acting on renal endothelial and tubular A_1AR. Moreover, adenosine protects against renal ischemic reperfusion injury by the anti-inflammatory effect of enhancing the activity of regulatory T cell and by attenuating the inflammatory injury produced by neutrophils via A_2AR activation.

Keywords Renal adenosine · Adenosine receptors · Kidney · Renal blood flow regulation · Ischemia-induced acute kidney injury

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19.1 Introduction

Adenosine, a metabolic breakdown product of adenosine triphosphate (ATP), generated by highly efficient nucleotidases such as CD39 and CD73, is a well-recognized paracrine regulator of physiological as well as pathophysiological events in multiple organ systems and cell types (Jacobson and Muller 2016; Menzies et al. 2017). In the kidney, adenosine participates in the regulation of fundamental physiological processes including renal blood flow, glomerular filtration rate, renin release, and tubular electrolyte transport (Osswald et al. 1997). In addition and perhaps more importantly, studies over the past two decades have made adenosine highly clinically relevant by revealing that the nucleoside is also a powerful modulator of ischemic AKI (Bauerle et al. 2011; Yap and Lee 2012). Indeed, during ischemia, preferential breakdown of ATP to adenosine results in substantially increased interstitial adenosine concentrations. In addition, the possible contribution of adenosine to the renal pathology associated with diabetes mellitus has been the topic of a considerable experimental effort.

Extracellular adenosine stimulates four G protein-coupled P_1 purinergic adenosine receptors (ARs) classified as A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR (Bauerle et al. 2011; Yap and Lee 2012). Each AR subtype appears to produce distinct and partially overlapping cellular signaling cascades to modulate kidney function in the basal state and especially in clinical conditions such as ischemic AKI, diabetes mellitus, and others. Local production of adenosine at the glomerular vascular pole modulates A_1AR-dependent constrictor tone and GFR through the tubuloglomerular feedback mechanism and inhibits renin release from juxtaglomerular granular cells. A_1AR activation protects against ischemic insults by reducing renal tubular electrolyte transport, apoptosis, necrosis, and inflammation. Activation of A_{2A}AR lessens renal injury by targeting leukocyte-mediated inflammation as well as directly reducing renal tubular inflammation. A_{2B}AR appears to be a critical mediator of renal preconditioning. Finally, unlike all other subtypes of AR, A_3AR activation exacerbates renal IR injury, while A_3AR antagonism protects against ischemic AKI (Yap and Lee 2012).

19.2 Renal Adenosine in Health

19.2.1 Hemodynamics

Expression of all four adenosine receptor subtypes (A_1AR, A_{2A}AR, A_{2B}AR, A_3AR) has been found along the renal vasculature. A_1AR protein and mRNA is expressed in preglomerular preparations of larger vessels including arcuate and interlobular arteries (Jackson et al. 2002), but the most consistent location of A_1AR expression is the afferent glomerular arteriole (Weaver and Reppert 1992; Yamaguchi et al. 1995; Smith et al. 2001). Expression of A_2-type receptors in renal vessels is

widespread, and expression sites include large vessels, glomerular arterioles, and descending vasa recta in the outer medulla (Kreisberg et al. 1997; Jackson et al. 2002; Al-Mashhadi et al. 2009). A_3 receptor expression is comparatively low, requiring RT-PCR to demonstrate A_3AR mRNA in pre- and postglomerular arterioles (Al-Mashhadi et al. 2009; Lu et al. 2015).

The effects of AR inhibition or activation in modulating renal blood flow are complex because of the extensive presence of vascular ARs and their opposing vasoactive actions, i.e., vasoconstriction with A_1AR activation and vasodilatation with activation of A_{2A}AR and A_{2B}AR. Nevertheless, general AR inhibition with methylxanthines such as caffeine or theophylline in anesthetized dogs did not affect renal blood flow significantly although renal vascular tone may decrease slightly because of small blood pressure reductions (Ibarrola et al. 1991; Osswald 1975; Premen et al. 1985). Theophylline or caffeine also does not alter renal plasma flow in humans to a measurable extent (Beutler et al. 1990; Brater et al. 1983; Brown et al. 1993; Passmore et al. 1987). Glomerular filtration rate (GFR) sometimes increases in response to methylxanthines leading to increases of filtration fraction (Fulgraff 1969). The absence of major effects of global inhibition of adenosine receptors by methylxanthines on renal hemodynamics suggests that renal vascular tone under basal conditions may represent a state of balanced activation of vasoconstrictor A_1AR and vasodilator A_2AR.

19.2.1.1 Renal Vasoconstriction Through A_1AR

Afferent Arterioles

Increased levels of adenosine supplied by bolus injections or constant infusion cause a short-lasting reduction in renal blood flow (Thurau 1964; Hashimoto and Kumakura 1965; Tagawa and Vander 1970; Osswald et al. 1975). Since this blood flow response was seen when adenosine was injected into the renal artery, it is not mediated by systemic consequences of adenosine such as a reduction in blood pressure (Hashimoto and Kumakura 1965; Osswald 1975). The transient vasoconstriction was prevented by A_1AR-specific antagonists, and it was absent in A_1AR knockout mice indicating that it is mediated by activation of A_1AR (Osswald 1975; Aki et al. 1997; Hansen et al. 2005). This is supported by the persistent reduction in renal blood flow and GFR induced by the infusion of the A_1AR-specific agonist cyclohexyladenosine (CHA) (Cook and Churchill 1984). Hemodynamic modeling indicates that the reduction in renal blood flow is attributable to a preglomerular, presumably afferent arteriolar vasoconstriction (Tagawa and Vander 1970; Murray and Churchill 1985).

Vasoconstriction of afferent arterioles by A_1AR activation has been observed directly in preparations that permit visualization of vessel diameters. In these in vitro preparations, the testable adenosine concentrations include the subnormal range, thereby facilitating the detection of A_1AR-mediated effects. In afferent arterioles of neonatal hamster kidneys transplanted into the cheek pouch of adult

animals, adenosine, topically applied through micropipettes, caused dose-dependent vasoconstriction of afferent arterioles while it dilated the arterioles of the cheek pouch itself (Joyner et al. 1988). In isolated perfused afferent arterioles from the rabbit, addition of adenosine to the bath caused a 30% reduction in vessel diameter in proximal parts of the arteriole with maximum effects being reached at 10^{-6} M (Weihprecht et al. 1992). The constrictor effect waned at higher concentrations indicating that in this part of the arteriole A_1AR-mediated vasoconstriction is counteracted by A_2AR-dependent vasodilatation as adenosine concentrations increase (Lai et al. 2006; Li et al. 2012a). The vasoconstriction caused by the A_1AR agonist CHA was slightly greater than that caused by adenosine over the entire concentration range (10^{-9}–10^{-4} M) suggesting that both high-affinity A_{2A}AR and A_{2B}AR oppose A_1AR-mediated constriction in afferent arterioles (Weihprecht et al. 1992; Lai et al. 2006). The recent observation that IB-MECA also antagonizes A_1AR-induced vasoconstriction in perfused afferent arterioles suggests that activation of A_3AR may provide another vasodilator input (Lu et al. 2015). Afferent arterioles from mice with transgenic overexpression of A_1AR have an augmented constrictor response to CHA indicating that expression levels of the receptor can affect the magnitude of the constrictor response (Oppermann et al. 2009). Conversely, A_1AR activation was without effect in afferent arterioles from mice with vessel-specific deletion of A_1AR (Li et al. 2012a). Vascular perfusion studies have revealed an important longitudinal difference in the responsiveness to adenosine. In the afferent arteriole in the immediate vicinity of the glomerulus, adenosine caused a monotonic vasoconstriction consisting of a 45% reduction in vessel diameter at 10^{-4} M, the highest concentration tested. The absence of a discernible vasodilator effect at concentrations at which A_{2B}AR should be activated indicates that the short section of the afferent arteriole close to and inside the glomerulus is unique in that A_1AR-induced constriction does not appear to be opposed by A_2AR to a detectable extent. In the hydronephrotic kidney preparation, another technique permitting direct observation of arteriolar responses, the abluminal administration of CHA caused a stable diameter reduction that was dose-dependent in the range between 10^{-8} and 10^{-6} M, and that was most pronounced in the distal part of the arteriole where it exerted a maximum diameter reduction by about 50%. The reduction in vascular diameter was accompanied by a reduction in glomerular blood flow by 30–40% at 10^{-7} M and by more than 50% at 10^{-5} M (Holz and Steinhausen 1987; Dietrich et al. 1991). In contrast to CHA, adenosine caused only a transient vasoconstriction over the 10^{-6}–10^{-4} M dose range, and a similar effect was seen in an in vitro perfused hydronephrotic kidney with luminal application (Tang et al. 1999; Gabriels et al. 2000). In view of the normal actions of CHA, the waning effect of adenosine in this preparation may reflect an increased expression of dilatory A_2AR.

Afferent arterioles of juxtamedullary nephrons may be less responsive to adenosine than arterioles from superficial or mid-cortical nephrons, but they appear to respond in a qualitatively similar way. For example, juxtamedullary afferent arterioles, studied in a blood-perfused preparation, respond to abluminal application of adenosine at 10^{-6} and 10^{-5} M with a marked transient as well as a smaller steady-state reduction of vessel diameter that was prevented by the A_1AR antagonist

KW-3902 and magnified by A_{2A}AR inhibition (Inscho et al. 1992; Carmines and Inscho 1994; Nishiyama et al. 2001a). Diameter evaluations in these studies were made at a distance of 100 μm from the glomerulus and did not distinguish between proximal and distal regions of afferent arterioles. The response of afferent arterioles of juxtamedullary nephrons to CHA in the hydronephrotic kidney preparation consisted of a dose-dependent diameter reduction in the tested concentration range of 10^{-8}–10^{-6} M that was about half that seen in more superficial arterioles (Dietrich and Steinhausen 1993). Glomerular blood flow was reduced by about 40% at 10^{-6} M, a response that was smaller than seen in superficial nephrons. Afferent arterioles of juxtamedullary nephrons appear to have a lower expression level of functional A_1AR and a higher level of A_2AR compared to superficial arterioles (Dietrich and Steinhausen 1993). This is in agreement with the observation in the blood-perfused juxtamedullary nephron preparation that the modest constrictor effect of adenosine can be markedly enhanced by A_2AR blockade, most markedly by A_{2B}AR blockade (Feng and Navar 2010). Nevertheless, the dilator action of A_1AR inhibition indicates that in juxtamedullary afferent arterioles, the dominant effect of adenosine up to a concentration of 10 μM is vasoconstriction.

Cellular Mechanisms of A_1AR Vasoconstriction

A defining characteristic of AR has been their interaction with G proteins directing activation or inhibition of adenylate cyclase (AC). A_1AR are directly coupled to Gi/Go resulting in inhibition of AC, but the contribution of this signaling pathway to smooth muscle cell activation is unclear. In perfused afferent arterioles from the mouse, inhibition of the protein kinase A pathway with the PKA inhibitor SQ 22536 did not induce significant vasoconstriction of the afferent arteriole arguing against a direct role of Gαi in adenosine-induced constriction (Hansen et al. 2003). Vasoconstriction induced by adenosine or CHA was blocked by pretreatment with pertussis toxin indicating that interaction with a Gi protein is in fact involved in the intracellular signaling pathway (Hansen et al. 2003). RT-PCR corroborated the presence of Gi, but not Go mRNA, in the kidney cortex and in microdissected preglomerular vessels. The constrictor response to both adenosine and angiotensin II was blocked by the phospholipase C (PLC) inhibitor U73122 suggesting activation of PLC presumably by the βγ subunits released from Gαi. Adenosine at 10^{-7} M has been shown to increase intracellular calcium concentration in mouse isolated afferent arterioles measured by fura-2 fluorescence (Hansen et al. 2007). Thapsigargin sensitivity of adenosine-induced vasoconstrictor is indicative of release of calcium from the sarcoplasmic reticulum (SR), stimulated presumably by IP3. In agreement with this notion is the observation that 2-aminoethoxydiphenyl borate (100 μM) blocked the adenosine-induced constriction, whereas the protein kinase C inhibitor calphostin C had no effect (Hansen et al. 2007). The calcium-activated chloride channel inhibitor IAA-94 inhibited the adenosine-mediated constriction, and patch clamping of preglomerular smooth muscle cells showed that Cl channel blockade abolished the depolarizing current induced by adenosine. Finally, the

vasoconstriction caused by adenosine was significantly inhibited by nifedipine suggesting involvement of voltage-dependent calcium channels. Overall, the constrictor response to adenosine in the afferent arteriole is mediated by A_1AR coupled to a PTX-sensitive Gi protein, and subsequent activation of PLC, presumably through βγ subunits released from Gαi. This results in an increase of intracellular calcium concentration by calcium release from the SR followed by activation of Ca^{++}-activated Cl channels leading to depolarization and influx of calcium through voltage-dependent calcium channels.

Sidedness of A_1AR Vasoconstriction

Since the studies examining the effect of adenosine on renal blood flow in the whole kidney have been performed during systemic administration of adenosine whereas in vitro experiments are typically done during abluminal adenosine application, the possibility exists that the strength and direction of the vasomotor response varies with the route of administration. Measurements of regional blood flow in mice with laser Doppler flow probes have shown that adenosine given i.v. caused an increase in superficial renal blood flow, whereas the infusion of adenosine into the interstitial region below the flow probe caused a reduction in blood flow (Hansen et al. 2005). Furthermore, the vasoconstriction of isolated perfused afferent arterioles from the mouse caused by the bath addition of adenosine was not seen when adenosine was added to the luminal perfusate (Hansen et al. 2005). A comparison of the effects of i.v. infusion of high and low molecular weight polyadenylic acids on renal blood flow in dogs has shown that the low molecular weight compound (MW 5000) induced transient vasoconstriction like adenosine, while the high molecular weight compound (MW 100,000) caused an exclusive and long-lasting vasodilator response that was inhibited by theophylline (Thompson and Spielman 1992). The authors concluded that adenosine activates A_2AR through an intravascular, probably endothelial, site, whereas A_1AR causing vasoconstriction is normally accessed from the interstitial aspect of the vessel. A possible explanation of the sidedness of the effect of adenosine might be that A_1AR are present in endothelial cells along the renal vasculature and that adenosine causes the release of nitric oxide and perhaps other endothelial vasodilators when administered from the vascular, but not from the interstitial aspect of the vessel. The resulting A_1AR-induced constriction would therefore be blunted by endothelial factors only when adenosine is given intravascularly. In a study in dogs, the administration of NOS inhibitors caused a marked augmentation in the constrictor response of renal blood flow to bolus injections of adenosine, while the dilator effect of the A_2 agonist CGS 21680 was unaffected indicating that adenosine may cause NOS activation through an A_1AR-mediated mechanism (Okumura et al. 1992). Enhancement of A_1AR agonist-induced vasoconstriction by L-NAME and a marked left shift of the dose-response relationship between adenosine concentration and vasoconstrictor response have also been observed in the rat (Barrett and Droppleman 1993; Pflueger et al. 1999a). Studies showing a similar left shift in the adenosine dose-response curve during application

of indomethacin suggest that a vasodilator prostaglandin may be another endothelial factor opposing A_1AR-mediated constriction (Pflueger et al. 1999b). The results of these studies do not establish that A_1AR activation is directly coupled to the release of NO or prostaglandins since they also are compatible with the possibility that the constrictor effect of adenosine is merely enhanced by the removal of a constitutive vasodilator influence. It is also of note that adenosine administered into the vascular space must cross the endothelial cell layer to interact with smooth muscle cells. In addition to being a potential physical barrier to the movement of adenosine, endothelial cells from coronary cells have been shown to rapidly metabolize adenosine with incorporation into various nucleotide pools (Nees et al. 1985). These authors suggest that in coronary vessels, transvascular adenosine movement may be impeded more by this metabolic barrier function of the endothelium than by its physical properties.

Tubuloglomerular Feedback

Changes in NaCl concentration in the tubular lumen near the tubulo-vascular contact point at the distal end of the ascending loop of Henle elicit adjustments in glomerular arteriolar resistance, a phenomenon referred to as tubuloglomerular feedback (TGF) (Schnermann and Castrop 2013). The TGF response consists of a reduction of glomerular capillary pressure and GFR when NaCl concentration at the sensor site is increased within the physiological range of approximately 15–60 mM (Schnermann and Castrop 2013). The primary target of the trans-JGA signaling cascade is the glomerular afferent arteriole. It responds to an increase of luminal NaCl with graded vasoconstriction resulting in maximum reductions of glomerular capillary pressure (P_{GC}) by about 20% and of glomerular filtration rate by about 45%. Afferent arteriolar resistance increases by 50% or less, consistent with an arteriolar radius reduction of about 10%. TGF is a local mechanism controlled by a vascular mediator originating in the JGA interstitium and therefore reaching its target vessel from the vessel outside. Considerable evidence indicates that adenosine acting through A_1AR is an important component of the changes in the juxtaglomerular environment which are elicited by luminal NaCl and lead to afferent vasoconstriction (Schnermann 2015). For example, specific A_1AR antagonists such as 8–cyclopentyl-1,3-dipropylxanthine (DPCPX) inhibit TGF responses when added to the tubular lumen or the peritubular blood (Schnermann et al. 1990). A similar effect has been seen earlier with nonspecific blockers such as theophylline or IBMX (Schnermann et al. 1977; Osswald et al. 1980; Bell 1985; Franco et al. 1989; Ren et al. 2002). Addition of the non-xanthine A_1AR receptor antagonist FK838 to the perfusate or the bath also eliminated TGF responses in an isolated tubule preparation (Ren et al. 2002). Furthermore, TGF responses of stop-flow pressure or single-nephron GFR are abolished in mice with targeted deletion of A_1AR (Brown et al. 2001; Sun et al. 2001). While the net effect of adenosine in the juxtaglomerular interstitium is vasoconstriction, simultaneous activation of A_2AR has been found to attenuate the constrictor effect of A_1AR during the TGF response, perhaps by

stimulating NO production through eNOS (Carlstrom et al. 2010; Carlstrom et al. 2011). Adenosine acting in the TGF mechanism appears to be locally generated since TGF responses are markedly compromised in mice with deletion of NTPDase1 or ecto-5′-nucleotidase, the enzymes generating AMP and adenosine by ATP dephosphorylation (Castrop et al. 2004; Huang et al. 2006; Oppermann et al. 2008). In addition, in an in vitro JGA preparation in which both the macula densa segment and the attached afferent arteriole were perfused, bath addition of the ecto-ATPase apyrase enhanced, and of the e-5NT-inhibitor α,β-methylene adenosine 5-diphosphate (MADP) abolished, TGF responses suggesting that extracellular generation of adenosine from ATP is critical for JGA signaling (Ren et al. 2004).

19.2.1.2 Renal Vasodilatation Through A2AR

Adenosine Administration

In contrast to bolus injections, adenosine administered by constant infusion usually leads to a reduction of renal vascular resistance (Tagawa and Vander 1970; Osswald 1975; Osswald et al. 1978; Hall et al. 1985). The causes for the steady-state vasodilatation have been ascribed to preferential relaxation of the efferent arteriolar or medullary vascular beds, but a convincing argument for either explanation cannot be made on the basis of studies at the organ level. Nevertheless, the selective A_2AR agonist CGS 21680A elicits a monophasic reduction in renal vascular resistance, clearly indicating that activation of A_2AR is the cause for the transient nature of the renal constrictor response to adenosine (Levens et al. 1991). Even though obvious, it is relevant to point out that the infusion studies discussed above examine the effect of an addition of adenosine to the existing endogenous levels and therefore limit the analysis to the supranormal concentration range. A consideration of the baseline adenosine concentrations in plasma and in the renal interstitial fluid may therefore be helpful to predict the expected changes in receptor engagement with adenosine infusions taking into account the known affinity and dissociation constants of the different adenosine receptors. Plasma adenosine levels have been reported to be somewhere between 100 nM and 1 μM, i.e., in the 10^{-7}–10^{-6} M range (Kost Jr and Jackson 1991; Zhang et al. 1994; Franco et al. 1996; Yoneyama et al. 2000; Chen et al. 2002). Renal interstitial concentrations of adenosine as determined by microdialysis are in the same order of magnitude, between 50 and 200 nM in the cortex and between 160 and 210 nM in the medulla (Baranowski and Westenfelder 1994; Siragy and Linden 1996; Nishiyama et al. 1999b; Zou et al. 1999; Nishiyama et al. 2001b). Analysis of ligand-binding kinetics has established that A_1AR as well as A_{2A}AR have affinity constants for adenosine in the order of 10^{-8} M, whereas the affinity of A_{2B}AR is much lower, around 10^{-5} M (Van Calker et al. 1979; Londos et al. 1980; Daly et al. 1983; Fredholm et al. 1994). Thus, at the prevailing extracellular adenosine concentrations of about 10^{-7} M, one would expect A_1AR as well as the high-affinity A_{2A}AR to be partly occupied, whereas A_{2B}AR are probably not. Thus, the increments in adenosine concentration resulting from the infusion should

mostly be targeted to the A_{2B}AR receptor pool. For this simple reason, it is perhaps not surprising that adenosine infusions result in relaxation of all vessels expressing A_{2B}AR, the majority of the renal vasculature, and therefore cause global renal vasodilatation. In view of the evidence discussed above that the afferent arteriole near the glomerulus may not vasodilate even at elevated levels of adenosine, at least when adenosine is administered from the interstitial side, it is relevant to point out that the afferent arteriole is not the only resistance vessel in the kidney. Aside from the significant contribution of the efferent arterioles, interlobular arteries in the rat kidney have been estimated to represent as much as 50% of renal preglomerular resistance (Heyeraas Tonder and Aukland 1979; 1980; Boknam et al. 1981) and have also been shown to contribute importantly to autoregulatory adjustments of renal vascular resistance (Heyeraas and Aukland 1987). Furthermore, the renal artery has been shown to regulate renal vascular resistance by the release and downstream action of endothelium-derived vasodilators (Kon et al. 1990). Therefore, global renal vasodilatation may well occur in the absence of overt vasodilatation in afferent arterioles.

Endothelium Dependence

The majority of vasodilator agents act by binding to their receptors on endothelial cells and by eliciting the generation and release of endothelial relaxing factors, most notably nitric oxide, EDHF, and prostaglandins. The presence of A_2AR in endothelial cells of the renal vasculature has not been established directly, but a number of studies in various excised vessel preparations indicate that adenosine-induced vasodilatation is probably to some extent endothelium-dependent. In the majority of these studies, adenosine appears to augment NOS activity and NO release through an A_2AR-mediated process, an action that would enhance the dilator component rather than diminish the constrictor component of the adenosine actions (Zanzinger and Bassenge 1993; Martin and Potts 1994; Steinhorn et al. 1994; Abebe et al. 1995; Grbovic et al. 2000). In addition, adenosine has also been reported to dilate rabbit renal arteries through an endothelial relaxing factor that does not appear to be NO (Rump et al. 1999). Finally, adenosine has been shown to consistently stimulate the production of NO in cultured endothelial cells, usually through an A_2AR-dependent mechanism (Li et al. 1998; Olanrewaju and Mustafa 2000; Wyatt et al. 2002). Thus, in addition to the possible blunting of A_1AR-induced vasoconstriction as discussed above, endothelial dilator factors generated in response to A_2AR activation may enhance renal vasodilatation thereby contributing to the waning renal constriction in the kidney during intravenous administration. The overall conclusion from these studies at the organ level would be that the intravenous administration of exogenous adenosine, i.e., an elevation of plasma adenosine concentrations above normal, causes a short-lasting net vasoconstriction mediated by high-affinity A_1AR. However, this effect is overcome, at the elevated plasma adenosine levels resulting from the addition of exogenous nucleoside, by the simultaneous activation of lower-affinity A_{2B}AR, so that the dominating and lasting effect is net vasodilatation in most cases.

Effect of Adenosine in Efferent Arterioles

Although efferent arterioles express the mRNAs of all AR subtypes as assessed by RT-PCR, the dominant effect of adenosine in efferent arterioles appears to be vasodilatation. Bath addition of adenosine caused relaxation of perfused efferent arterioles pre-constricted with a thromboxane agonist in the 10^{-7}–10^{-5} M concentration range suggesting that it was probably mediated by A_{2B}AR (Al-Mashhadi et al. 2009). Furthermore, in a perfused vessel/tubule preparation, the TGF response to elevated NaCl includes a component of efferent vasodilatation that could be blocked with an A_2AR antagonist (Ren et al. 2001). In intact rats, administration of the A_{2A}AR antagonist ZM241385 caused an increase in GFR without a change of renal blood flow consistent with the removal of a tonic efferent vasodilator influence of endogenous adenosine (Persson et al. 2015a). Results in efferent arterioles of juxtamedullary nephrons are less supportive of a dominant vasodilatory effect of adenosine. In the blood-perfused juxtamedullary nephron preparation, the effect of adenosine on the diameter of efferent arterioles was qualitatively similar to that seen in afferent arterioles consisting of a stable diameter reduction by about 6% at a concentration of 10^{-5} M, a constrictor effect that was smaller than that seen in afferent arterioles (Carmines and Inscho 1994; Nishiyama et al. 2001a). Vasodilatation in the presence of an A_1AR blocker and enhanced constriction in the presence of an A_{2A}AR blocker resembled the effects noted in afferent arterioles. On the other hand, in the hydronephrotic kidney, adenosine at 10^{-5} M caused a steady-state diameter increase of about 14% that was not changed much by the A_1AR antagonist DPCPX but abolished by the A_2AR antagonist DMPX (Gabriels et al. 2000). These results suggest the absence of A_1AR in efferent arterioles in this preparation, a notion supported by previous reports using the same preparation in which the A_1AR agonist CHA caused only small or no diameter reductions in efferent arterioles up to a concentration of 10^{-5} M (Holz and Steinhausen 1987; Dietrich and Steinhausen 1993).

Adenosine and Medullary Blood Flow

Vasodilatation of the vessels controlling renal medullary blood flow has been proposed as being responsible for the net vasodilatation of the kidney in response to continuous i.v. infusion of adenosine. Renal blood distribution measured with microspheres showed an increase in inner cortical blood flow while outer cortical blood flow was unchanged (Spielman et al. 1980). The magnitude of this increase varied between 23 and 94% depending on the renin status of the dogs. Interstitial infusion of adenosine induced an increase in medullary blood flow measured with laser Doppler flowmetry by about 40% (Agmon et al. 1993). Infusion of adenosine into the renal medulla caused an about 25–30% increase in both outer and inner medullary blood flows (Zou et al. 1999). Direct assessment of blood flow in single inner medullary vasa recta by videomicroscopy showed an increase in red cell velocity without a diameter change only during intrarenal adenosine infusion at the

highest dose tested (Miyamoto et al. 1988). The infused amounts did not induce significant changes in inulin or PAH clearances. In isolated perfused outer medullary vasa recta, the administration of increasing concentrations of adenosine induced a biphasic response, consisting of a vasoconstriction in the dose range between 10^{-11} and 10^{-7} M and a vasodilatation at concentrations of and above 10^{-6} M (Silldorff et al. 1996). In contrast to cortical resistance, vessel administration of adenosine to vasa recta pre-constricted by Ang II leads to vasodilatation (Silldorff et al. 1996; Silldorff and Pallone 2001). The concentration of adenosine in the interstitial fluid of the medulla is between 10^{-7} and 10^{-6} M, a level where one may expect not much impact on resting tone but where an increase of adenosine concentration should cause vasodilatation (Siragy and Linden 1996; Zou et al. 1999). In summary, most studies agree that the administration of adenosine causes an increase in medullary blood flow by relaxing both juxtamedullary afferent and perhaps efferent arterioles and outer medullary vasa recta pericytes. It is not entirely clear whether this increase in medullary blood flow can account for the overall increase in total renal blood flow seen with constant infusions of adenosine. Medullary blood flow represents only about 10% of total renal blood flow. Thus, a reduction in cortical blood flow by 50% would require a more than fivefold increase in medullary flow for compensation. The magnitude of the observed increase in medullary blood flow, variable as it may be, is not even close to this expectation. Thus, much of the compensatory increase in total renal blood flow in response to continuous adenosine infusions must take place in the renal cortex.

19.2.2 Tubular Transport

Transcriptome analysis by deep sequencing of segment-specific cDNA libraries did not show the presence of A_{2A}AR or A_3AR in any of the examined 15 tubular segments, while A_1AR sequence was only found in thin descending limbs and inner medullary collecting ducts (Lee et al. 2015). With RT-PCR, mRNA expression of A_1AR was found in proximal tubules, thin limbs of Henle's loop, thick ascending limbs, and medullary collecting ducts (Yamaguchi et al. 1995; Vitzthum et al. 2004). A_{2B}AR mRNA was found in cortical thick ascending limbs and distal convoluted tubules (Vitzthum et al. 2004). Thus, tubular segments express A_1AR and A_{2B}AR at the low levels typical for G protein-coupled receptors, while there is no evidence for mRNA expression of A_{2A}AR and A_3AR in tubular epithelium.

19.2.2.1 Proximal Nephron

General inhibition of adenosine receptors with methylxanthines is accompanied by increased urine flow and increased excretion of sodium, chloride, calcium, phosphate, magnesium, and other urinary solutes. Although methylxanthines have in some studies been found to increase the tubular Na load, significant natriuresis can

occur without changes in GFR or renal blood flow indicating that the natriuresis caused by adenosine receptor inhibition is predominantly the result of reductions of tubular salt transport (Davis and Shock 1949; Ludens et al. 1970; Shirley et al. 2002). Strong experimental evidence suggests that the inhibition of salt reabsorption by adenosine is mediated by A_1AR. The diuretic and natriuretic effect of caffeine (45 mg/kg) or theophylline (45 mg/kg) was entirely absent in A_1AR-deficient mice (Rieg et al. 2005). Infusion of the A_1AR antagonist DPCPX caused a marked increase in urinary NaCl excretion without significantly changing GFR or renal plasma flow (Munger and Jackson 1994; Patinha et al. 2013).

The natriuresis caused by general and A_1AR-specific AR inhibitors is mainly a consequence of inhibition of salt transport along the proximal convoluted tubule. Administration of 400 mg of caffeine to healthy human subjects caused an about 1.5-fold increase in Na excretion, and this was associated with an increase in the clearance of lithium (Shirley et al. 2002). A reduction of proximal solute reabsorption in humans as assessed by lithium clearance was also caused by theophylline and aminophylline (Brater et al. 1983; Beutler et al. 1990). At the level of the single tubule, systemic administration of theophylline (20 mg/kg s.c.) caused an about 20% reduction in proximal tubular reabsorptive capacity as determined with the split-droplet technique in the rat (Fulgraff 1969). The natriuresis caused by systemic administration of A_1AR-selective inhibitors such as CVT-124, DPCPX, or KW-3902 has been shown by lithium clearance and renal tubular micropuncture to be associated with and presumably caused by inhibition of proximal tubular fluid reabsorption (Knight et al. 1993; Mizumoto and Karasawa 1993; Wilcox et al. 1999; Kost Jr et al. 2000). DPCPX-induced natriuresis can be prevented by pertussis toxin, consistent with an involvement of the Gi-coupled A_1AR (Kost Jr et al. 2000). The conclusion would be that proximal NaCl transport increases when adenosine-mediated activation of A_1AR extends from the fully blocked to the normally activated state.

In view of this conclusion, it seems paradoxical that further increasing adenosine levels by intrarenal infusion do not further stimulate NaCl transport. In fact, several studies have documented that intrarenal infusion of adenosine elicits a clear increase in NaCl excretion without consistent increments of renal blood flow suggesting inhibition of NaCl transport (Miyamoto et al. 1988; Yagil 1994; Fransen and Koomans 1995; Kuczeriszka et al. 2013). Thus, the relationship between adenosine concentration and NaCl transport in the proximal tubule appears to follow a bimodal relationship with stimulation of transport at subnormal and inhibition of transport at supranormal concentrations. This possibility is supported by studies in opossum kidney cells in which low concentrations of an A_1AR agonist ($<10^{-8}$ M) activate NHE_3, while high concentrations inhibit it (Di Sole et al. 2003; Di Sole 2008). These observations are paralleled by the findings that O_2 consumption of renal cortical tissue decreased both when A_1AR were inhibited with DPCPX and when they were activated with CPA (Babich et al. 2015). There is no evidence for transport stimulation by adenosine in the renal medulla where O_2 partial pressures are constitutively low. In fact, nanomolar concentrations of adenosine added to the perfusate of isolated perfused mTAL segments reduce net Cl flux by about 50% (Beach and

Good 1992). Suspensions of medullary thick ascending limbs (mTAL) generate adenosine, and this is further enhanced by reductions of O_2 tension (Beach et al. 1991). In A_1AR-deficient mice, Na reabsorption was found to be elevated in a water-impermeable segment of the loop, presumably the TAL supporting tonic suppression of TAL absorption by adenosine acting on A_1AR (Vallon et al. 2004). In contrast to proximal tubules, inhibition of adenosine receptors by theophylline or IBMX did not significantly reduce Cl reabsorption in microperfused loops of Henle indicating absence of adenosine-mediated transport stimulation (Schnermann et al. 1977). Overall, one might conclude that elevations of adenosine levels reduce NaCl transport in proximal tubules and TAL. Since elevated adenosine levels are usually the result of compromised O_2 delivery, a reduction of energy-consuming transport processes would seem to be a useful adaptation. In contrast, the reduction of NaCl transport during full A_1AR blockade might be of greater pharmacological than physiological interest.

The cellular mechanisms underlying transport modification by adenosine have not been fully clarified. An A_1AR agonist has been found to activate NHE_3 in opossum kidney cells, an effect mediated by inactivation of adenylyl cyclase (Di Sole et al. 2003). Downregulation of NHE_3 and of α1/β1-NaKATPase protein expression was observed following a 1-day treatment with caffeine in rats (Lee et al. 2002). On the other hand, theophylline (1 mM) did not affect HCO_3 flux as assessed from the pH recovery in stationary microperfusion studies in the rat (Bailey 2004). Furthermore, in mice with tubule-specific deletion of NHE_3, caffeine administration caused diuresis and natriuresis that were not distinguishable from wild-type mice, data not consistent with a critical role of NHE_3 in caffeine-induced inhibition of Na transport (Fenton et al. 2015). Since in these studies caffeine-induced natriuresis could be blocked by DIDS, it seems possible that the transport effect of adenosine may be mediated by the basolateral Na/HCO_3 cotransporter. In fact, theophylline and A_1AR-selective xanthine derivatives inhibit a basolateral HCO_3 conductance in microperfused rabbit proximal convoluted tubules, and this effect was mimicked by forskolin and chlorophenylthio-cAMP supporting the notion that A_1AR activation in the subnormal concentration range stimulates Na/HCO_3 cotransporter activity by increasing intracellular cAMP (Takeda et al. 1993).

Na/Pi cotransport may be another proximal transport system regulated by adenosine. In renal proximal tubular cell cultures and opossum kidney cells, A_1 adenosine receptor activation stimulated and inhibition of A_1 adenosine receptors by DPCPX or KW-3902 inhibited apical Na/Pi (Coulson et al. 1991; Cai et al. 1994, 1995) and Na/glucose cotransport (Coulson et al. 1991, 1996). Inhibition of Pi uptake by A_1 adenosine receptor antagonists was associated with a dose-dependent increase of cellular cAMP production as well as an increase in PKC activity (Coulson et al. 1991, 1996; Cai et al. 1995). To the extent that caffeine causes an increase in arterial blood pressure, a potential direct role of blood pressure in inhibiting tubular reabsorption and altering NHE3 distribution needs to be considered (Nussberger et al. 1990; Rachima-Maoz et al. 1998; Rakic et al. 1999).

19.2.2.2 Distal Nephron

The effect of AR activation or inhibition on electrolyte and water transport in tubular segments beyond the proximal tubule and the loop of Henle has not been explored in similar detail although on the basis of indirect evidence an inhibitory action in more distal parts of the tubule has been proposed (Brater et al. 1983; Shirley et al. 2002). In an inner medullary collecting duct cell line (IMCD-K2) grown in an Ussing chamber, addition of adenosine to the basolateral side increased transepithelial resistance and reduced Na uptake, an effect mimicked by the A_1AR agonist CHA and inhibited by the A_1AR antagonist DPCPX (Yagil et al. 1994). Furthermore, vasopressin-stimulated electrogenic Cl secretion was enhanced when the A_1AR antagonist was present on the basolateral side, and this effect was reversed by CHA or by adenosine deaminase inhibition (Moyer et al. 1995). Activation or inhibition of A_1AR did not affect electrogenic Cl secretion under basal conditions. The inhibitory effect of A_1AR activation on Cl secretion and probably vasopressin-stimulated water permeability was mediated by a reduction of intracellular cAMP (Yagil 1990; Moyer et al. 1995). The physiological import of the inhibitory effect of adenosine on Cl secretion is difficult to assess in view of the more recent finding that adenosine added to the apical side of IMCD-K2 cell sheets stimulates rather than inhibits Cl secretion, an effect mediated by A_{2B}AR activation and subsequent enhanced signaling through the cAMP/protein kinase A pathway (Rajagopal and Pao 2010).

19.2.3 Renin Secretion

The evidence in support of a role of adenosine as a paracrine mediator of tubuloglomerular feedback raises the question of a participation of the nucleoside in renin secretion that is under macula densa (MD) control (Thomson et al. 2000; Brown et al. 2001; Sun et al. 2001). At increasing concentrations over a concentration range between 10^{-10} and 10^{-6} M, adenosine causes dose-dependent inhibition of renin secretion in isolated JG cells and kidney slices (Churchill and Churchill 1985; Kurtz et al. 1988). Agonist selectivity indicates that this effect is mediated by A_1AR (Churchill and Churchill 1985; Albinus et al. 1998). A_1AR-mediated tonic inhibition of renin secretion by endogenous adenosine is suggested by the increase of renin secretion caused by administration of inhibitors of A_1AR such DPCPX (Kuan et al. 1989, 1990; Jackson 1991). This pharmacological profile of adenosine is consistent with the notion that the inhibition of renin secretion caused by an elevated MD NaCl concentration may be adenosine-mediated. Renin secretion by nonperfused afferent arterioles was found to be lower when the MD was present. This difference could be abolished by adding adenosine to afferent arterioles without the MD or theophylline to afferent arterioles containing the MD (Itoh et al. 1985). Furthermore, furosemide was noted to stimulate renin release only when the MD was included in the dissected specimen, but not in its absence (Itoh and Carretero 1985). Thus, MD cells under basal conditions appear to generate adenosine, and this

generation can be suppressed by furosemide. It is surprising that furosemide exerts this effect even when the loop of Henle is not perfused and the diuretic may not have direct access to the luminal aspect of the MD cells. In the isolated perfused JGA, the selective A_1AR blocker 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) blunted the fall in renin secretion caused by an elevation in luminal NaCl by about 50%, but did not abolish it (Weihprecht et al. 1990). In contrast, macula densa-dependent renin secretion as tested by the secretory response to bumetanide was not measurably altered in perfused kidneys isolated from A_1AR-deficient compared to wild-type mice (Schweda et al. 2003). These two observations are not necessarily in conflict since bumetanide administration only examines the effect of a reduction in NaCl transport, that is only about half of the NaCl concentration range over which the MD mechanism operates. Thus, it is conceivable that adenosine may contribute to the inhibition of renin secretion at high NaCl concentration, but that disinhibition at low luminal NaCl with reduced adenosine levels is not an important drive for renin secretion. In agreement with this notion are findings in intact animals showing that bolus injections of NaCl reduce renin secretion in wild-type but not in A1AR-deficient mice. In contrast, the stimulatory effect of furosemide on renin secretion was not different between these genotypes (Kim et al. 2006). The cellular mechanism of adenosine-dependent changes in renin secretion is not clear, but it is likely that an increase in [Ca]i may play a critical role (Grunberger et al. 2006; Ortiz-Capisano et al. 2007). In primary cultures of JG cells, the A_1AR agonist CHA reduced basal renin secretion, and this inhibitory effect could be prevented by chelation of extracellular or intracellular Ca by EGTA and BAPTA-AM, respectively. Ca entry in response to CHA may occur through TRPC channels (Ortiz-Capisano et al. 2013).

19.3 Renal Adenosine in Disease

19.3.1 Ischemic Acute Kidney Injury

Acute kidney injury (AKI) is a frequent and major complication during the perioperative period and in hospitalized patients resulting in ~10 billion of dollars in healthcare costs annually in the United States (Chertow et al. 2005). Despite many decades of intense research, there is no effective therapy or prevention for AKI (Jones and Lee 2008). At the same time due to increasing surgical complexity coupled with aging surgical population, the incidence and morbidity from AKI are increasing (Hoste et al. 2010; Srisawat et al. 2010). Renal ischemia reperfusion (IR) injury is a major cause of perioperative AKI (Hoste and Kellum 2007; Hoste et al. 2010; Srisawat et al. 2010). Renal IR injury leading to AKI results in severe renal tubular cell death by necrosis, apoptosis, and inflammation that is orchestrated by complex signaling events generated from renal tubular cells, endothelial cells, and resident and infiltrating leukocytes (Jang and Rabb 2015).

19.3.1.1 A_1AR and Renal IR Injury

In addition to its important role in renal physiology by modulating renal hemodynamics, GFR, and tubuloglomerular feedback, A_1AR activation with selective synthetic A_1AR agonists protects against renal IR injury by decreasing renal tubular necrosis, apoptosis, and the inflammatory response (Joo et al. 2007; Park et al. 2012). Consistent with these findings, genetic deletion of A_1AR exacerbates ischemic AKI (Lee et al. 2004b). Moreover, a selective A_1AR antagonist treatment resulted in markedly worsening of renal IR injury in mice with increased renal tubular necrosis, inflammation, as well as apoptosis (Lee et al. 2004a). Subsequently studies with proximal tubule-specific genetic deletion of A_1AR in vivo potentiated renal IR injury suggesting an endogenous renal protective role for renal proximal tubular A_1AR (Kim et al. 2013). Furthermore, in vivo reconstitution of renal A_1AR with intrarenal injection of A_1AR overexpressing lentivirus was protective against ischemic AKI with improved renal function, and reduced tubular inflammation and apoptosis (Kim et al. 2009). These studies collectively suggest that renal proximal tubular A_1AR plays a major role in mediating the endogenous cytoprotective effects of adenosine in the kidney.

In vitro cell culture studies showed that A_1AR activation directly protects cultured renal tubules against necrosis as well as apoptosis (Lee et al. 2007). Moreover, overexpression of the A_1AR in a pig renal tubule cell line protected against necrosis and apoptosis. These protective effects of A_1AR overexpression was achieved by upregulation of total and phosphorylated heat-shock protein 27 (HSP27) and via enhanced p38 MAPK activation. Additional cytoprotective signaling cascade occurs after renal tubular A_1AR activation to protect against ischemic AKI (Park et al. 2012; Kim et al. 2013). Renal tubular A_1AR activation results in increased interleukin-11 (IL-11) synthesis via ERK MAPK activation and IL-11 to directly protect against ischemic AKI (Lee et al. 2012; Kim et al. 2013). Furthermore, renal proximal tubular A_1AR activation induces sphingosine kinase-1 (SK-1) synthesis and sphingosine 1-phosphate generation to protect against ischemic AKI (Park et al. 2012). IL-11 as well as SK1 synthesis was critical for A_1AR-mediated renal tubular protection, as mice deficient in either IL-11 or SK-1 were not protected against ischemic AKI with A_1AR activation. Subsequent studies suggest that IL-11 receptor activation induces SK-1 synthesis to increase cytoprotective S1P in renal proximal tubules (Kim et al. 2013).

Finally, renal tubular A_1AR activation results in a biphasic protection against ischemic kidney injury – acute (within minutes to hours) protection occurs first and wanes followed by delayed (4–24 h) protection from renal ischemic insult by distinct signaling pathways (Joo et al. 2007). Acute renal protection by A_1AR is mediated via phosphorylation of HSP27, Akt, and ERK MAPK, whereas the delayed phase of renal protection with A_1AR activation that occur hours later is via induction of new HSP27 synthesis.

19.3.1.2 A_{2A}AR and Renal IR Injury

The A_{2A}AR activation attenuates the inflammatory response after renal IR injury (Okusa 2002). In particular, A_{2A}AR activation blunts the cytokine and chemokine expression in renal tubules cells and decreases the leukocyte infiltration including macrophages, lymphocytes, as well as neutrophils after renal IR (Day et al. 2003, 2004; Lange-Sperandio et al. 2005; Day et al. 2006). The A_{2A}AR-mediated stimulation of adenylyl cyclase and PKA resulting in *cAMP response element-binding* protein (CREB)-mediated phosphorylation and gene transcription regulates the anti-inflammatory effects of A_{2A}AR activation (Okusa et al. 2001; Jackson et al. 2006; Linden 2006; Hasko et al. 2008). Furthermore, increased medullary vasorelaxation after IR may also promote tissue recovery after prolonged ischemic period (Okusa 2002; Linden 2006).

Focusing on cell types responsible for A_{2A}AR-mediated protection against AKI, studies suggest that adenosine suppresses renal inflammation via A_{2A}AR-mediated modulation of bone marrow-derived anti-inflammatory regulatory T cells (Tregs) (Kinsey et al. 2013; Kinsey and Okusa 2014). Adoptive transfer of Tregs unable to synthesize adenosine (CD73 deficient Tregs) or Tregs deficient in A_{2A}ARs led to inhibition of Treg function suggesting that A_{2A}AR activation of Tregs is critical for suppressing innate immune responses in renal IR injury. Recent studies also demonstrate that dendritic A_{2A}AR activation plays role in protection against ischemic AKI (Li et al. 2012b). Indeed, mice with dendritic cell A_{2A}AR deficiency showed exacerbated kidney injury after IR. Furthermore, dendritic cell A_{2A}AR-deficient mice were not protected against ischemic AKI with an A_{2A}AR agonist treatment. Finally, exogenous administration of dendritic cells treated ex vivo with A_{2A}AR agonist provided protection against ischemic AKI by suppressing NK T-cell mediated renal inflammation.

19.3.1.3 A_{2B}AR and Renal IR Injury

In mice, A_{2B}ARs mediate the protective effects of kidney ischemic preconditioning defined as brief, multiple periods of intermittent ischemia before prolonged ischemic injury. The renal protective effects of ischemic preconditioning was absent in A_{2B}AR-deficient mice and in mice treated with a selective A_{2B}AR antagonist (PSB-1115) (Grenz et al. 2008). In contrast, kidney ischemic preconditioning was intact in animals lacking A_1AR, A_{2A}AR, or A_3AR. Moreover, mice treated with a selective A_{2b}AR agonist (BAY 60-6586) were markedly protected from ischemic AKI and showed renal tubular necrosis and inflammation. Bone marrow chimera studies conducted in mice suggest that bone marrow-derived leukocyte A_{2B}ARs do not play a role in A_{2B}AR-mediated renal protection against IR injury. Therefore, unlike the A_{2A}ARs that attenuates ischemic AKI by regulating pro-inflammatory leukocytes and modulating Tregs, the A_{2B}ARs involve renal tubular endothelial cells to protect against ischemic AKI.

19.3.1.4 A_3AR and Renal IR Injury

Of four AR subtypes in the kidney, the A_3AR is the least understood AR subtype as the exact physiological role for A_3ARs in the kidney is still unknown (Mozaffari et al. 2000; Guan et al. 2007; Fredholm et al. 2011). Although A_3AR transcripts are detected throughout the kidney, A_3AR activation has no impact on solute excretion, TGF or GFR (Mozaffari et al. 2000; Vallon and Osswald 2009). In nonrenal cell lines, the A_3ARs couple to either G_i or G_q proteins (Fredholm et al. 2011).

A selective A_3AR agonist potentiated ischemic AKI by worsening renal tubular necrosis, apoptosis, and inflammation (Lee et al. 2003). Conversely, mice deficient in A_3ARs or wild-type mice treated with a specific A_3AR antagonist were protected against ischemic AKI. Unfortunately, the mechanisms of A_3AR-mediated modulation of ischemic renal injury have remained unclear. Activation of A_3AR degranulates mast cells and increases the release of several inflammatory mediators including proteolytic enzymes and histamine (Fozard et al. 1996; Reeves et al. 1997). Consistent with the histamine hypothesis, a selective A_3AR agonist increased the plasma histamine levels in mice (Lee et al. 2003). Furthermore, A_3AR activation induces calcium influx as well as apoptosis in several cell types including human proximal tubule cells, cardiomyocytes, and leukocytes (Kohno et al. 1996; Jacobson 1998; Shneyvays et al. 1998). Consistent with these detrimental cellular effects, overexpression of A_3AR results in embryo death with DNA fragmentation and chronic A_3AR activation is detrimental to cell survival (Zhao et al. 2002). Therefore, mast cell activation and subsequent histamine release as well as increased DNA fragmentation may explain the A_3AR-mediated exacerbation of ischemic AKI.

19.3.2 Diabetic Nephropathy

Substantial experimental effort has gone into exploring the role of adenosine and AR in the renal pathology associated with diabetes mellitus. Most evidence supports the notion that adenosine ameliorates the severity of diabetic nephropathy in various animal models of both types of diabetes mellitus although the mechanisms underlying this effect are not clear. Some evidence indicates that renal adenosine levels increase in response to the diabetic condition and that this increase provides some degree of protection. Levels of adenosine in renal tissue and in urine have in fact been found to be elevated in streptozotocin-induced DI in rats and mice (Tak et al. 2014; Oyarzun et al. 2015). Increased extracellular adenosine formation appears to be driven at least in part by about threefold increase of renal expression levels of 5′ nucleotidase (CD73) that has been found both in streptozotocin-induced DI and in the Akita and Db/Db mouse models of diabetes (Tak et al. 2014, Oyarzun et al. 2015). Absence of the adaptive increase of renal adenosine formation in diabetic CD73-/- mice was associated with a more severe nephropathic phenotype as indicated by greater albuminuria, enhanced hyperfiltration, and reduced nephrin expression (Tak et al. 2014). However, in another study, CD73-dependent

upregulation of adenosine in diabetes has been found to be without effect on the development of diabetic nephropathy (Oyarzun et al. 2015). Nevertheless, elevations of extracellular adenosine by administration of adenosine, inhibition of uptake with dipyridamole, and blockade of adenosine kinase all reduced the levels of various biomarkers of the severity of diabetic nephropathy supporting the notion of a protective role of elevated adenosine in diabetic nephropathy (Pye et al. 2014; Elsherbiny et al. 2015; Taskiran et al. 2016).

Both A_{2A}AR and A_{2B}AR mediate adenosine-dependent amelioration of diabetic nephropathy. Administration of the A_{2A}AR agonist by continuous subcutaneous infusion provided protection against the renal functional changes of streptozotocin-induced diabetes in rats, and diabetic nephropathy was more pronounced in A_{2A}AR-/- mice (Awad et al. 2006; Persson et al., 2015b). The mechanisms underlying this effect appear to be related to the well-described anti-inflammatory of adenosine. As to A_{2B}AR, mouse models of both type I and type 2 diabetes have been found to be associated with a selective increase in the expression of this AR subtype, and substantial evidence indicates an important role of A_{2B}AR in the renal protection against diabetic nephropathy. Nephropathy was more severe in A_{2B}AR-/- than wild-type mice, and the A_{2B}AR agonist Bay 60-6583 provided effective protection (Tak et al. 2014). It is unclear how A_{2B}AR exert their protective actions. Since loss of protection was only seen when A_{2B}ARs were conditionally deleted from endothelial cells, but not when they were removed from proximal tubular epithelium, it seems to be the expression in the renal vasculature that is critical in preventing diabetic nephropathy (Tak et al. 2014). On the other hand, activation of A_{2B}AR has been shown to cause overexpression of glomerular VEGF expression in diabetes, a known adverse event in maintaining glomerular functional integrity. Administration of an A_{2B}AR antagonist reduced renal VEGF production and improved renal functional parameters (Cardenas et al. 2013; Patel and Thaker 2014). Reconciliation of the A_{2B}AR-mediated deterioration of function through the VEGF pathway with the beneficial effects of A_{2B}AR via their anti-inflammatory actions will require more work.

Inhibition of the Na/glucose cotransporter SGLT2 has recently been shown to reduce the progression of diabetic nephropathy in humans (Wanner et al. 2016). It has been proposed that SGLT2 inhibitors elevate NaCl concentrations at the macula densa and thereby activate the TGF mechanism resulting in a reduction of GFR and of the causative elevation of glomerular capillary pressure (Thomson et al. 2011). Since adenosine plays an important role as a TGF vasoconstrictor, this might be another mechanism of renal protection provided by adenosine. A recent study in patients with type 1 diabetes during clamped hyperglycemia has shown that SGLT2 inhibition causes an increase in urinary adenosine excretion corrected for creatinine although the increase in the ratio was entirely due to a fall in urinary creatinine concentration (Rajasekeran et al. 2017). While inhibition of SGLTs by phlorizin did indeed attenuate the hyperfiltration found in early diabetes in mice, this effect might not be mediated by TGF since it was also seen in the TGF-deficient A1AR-/- animals (Sallstrom et al. 2014). It would appear that the reduction of GFR in response to SGLT2 inhibition may be multifactorial.

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Chapter 20
Adenosine Regulation of the Immune System

Luca Antonioli, Matteo Fornai, Corrado Blandizzi, and György Haskó

Abstract Adenosine is an endogenous nucleoside, released into the extracellular space in response to metabolic stress and cell damage and critically involved in the maintenance of tissue integrity by modulation of the immune system.

The magnitude and duration of adenosine signaling are dictated by the expression and activity of a plethora of synthetic and catabolic enzymes as well as nucleoside transporters, which calibrate finely the concentration of this nucleoside in the biophase of specific receptors. Indeed, once released into the extracellular space, adenosine governs several aspects of immune cell functions by interaction with four G-protein-coupled cell membrane receptors, designated as A_1, A_{2A}, A_{2B}, and A_3 receptors.

The engagement of such receptors, expressed heterogeneously on the surface of several immune cell populations, including neutrophils, macrophages, dendritic cells, mast cells, and lymphocytes, shapes a broad array of immune cell functions, which include cytokine production, degranulation, chemotaxis, cytotoxicity, apoptosis, and proliferation.

Keywords Adenosine receptors · Immune system · Inflammation · Cytokines · Macrophages and neutrophils · Dendritic cells

20.1 Introduction

The earliest experimental observation describing the biological activity of adenosine in the immune system can be dated back to 1954, when Dubois and Petersen demonstrated that sublethal doses of X-ray produced an increase in adenosine triphosphatases and 5′-nucleotidase activity in hematopoietic tissues of rats and mice

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(Dubois and Petersen 1954). This study was followed by other pioneering investigations aimed at assessing the expression and activity of adenosine deaminase in the regulation of immune cells (Hall 1963; Karker 1965). However, it was in the early 1970s that more specific studies led to the characterization of the enzyme machinery involved in shaping adenosine concentrations, describing its significant impact on the physiology of the immune system (DePierre and Karnovsky 1974; Green and Chan 1973; Harrap and Paine 1976; Hovi et al. 1976; Snyder et al. 1976). An interesting study by Giblett et al. (1972) reported, for the first time, a direct correlation between the lack of adenosine deaminase expression and the presence of a congenital combined immunologic deficiency, thus pointing out a critical role for adenosine in the development of immune cell armamentarium.

The demonstration of the association of a human genetic deficiency of adenosine deaminase with combined immunodeficiency (Cohen 1975; Meuwissen et al. 1975) prompted a series of studies aimed at deepening the effects of adenosine on human lymphocyte maturation and proliferation (Fleit et al. 1975; Hovi et al. 1976; Allison et al. 1977; Ballet et al. 1976; Burridge et al. 1977; Seegmiller et al. 1977a; Snyder et al. 1977) as well as at elucidating the role of adenosine metabolism in the regulation of lymphocyte activity (Cohen et al. 1978; Seegmiller et al. 1977b; Snyder et al. 1976). In 1978, Schwartz et al. published a seminal paper in which they described, for the first time, that T lymphocytes isolated from a patient with x-linked agammaglobulinemia displayed the expression of an adenosine receptor linked to cyclic AMP accumulation. The authors concluded that a persistent and elevated level of cyclic AMP inhibited both lymphocyte proliferation and antibody synthesis (Schwartz et al. 1978).

A step forward was made at the end of the 1970s, when Burnstock proposed a basis for distinguishing two types of purinoceptors, designated as P1 and P2, which were preferentially activated by adenosine and ATP, respectively (Burnstock 1976). Subsequently, the availability of novel and more specific ligands allowed to perform a series of pharmacological studies leading to the distinction of two P1 receptor subtypes, based on their ability to inhibit (A_1 receptor) or stimulate (A_2 receptor) intracellular cAMP accumulation (Londos et al. 1980; van Calker et al. 1979). In 1985, pharmacological criteria for discriminating two types of ATP P2 receptors (P2X and P2Y) were proposed (Burnstock and Kennedy 1985). Subsequently, on the basis of studies on transduction mechanisms and cloning of nucleotide receptors, it was demonstrated that P2X are ligand-gated ion channel receptors, whereas P2Y belong to the G-protein-coupled receptor family (Abbracchio and Burnstock 1994).

The above observations fostered strong interest on the role played by adenosine in the modulation of immune functions. Initially, the presence of adenosine receptors in several immune/inflammatory cell populations (lymphocytes, neutrophils, monocytes, macrophages, dendritic cells, and mast cells) (Dinjens et al. 1986; Marone et al. 1986; Nishida et al. 1986; Samet 1986) and their active involvement in shaping immune cell activity were demonstrated (Birch and Polmar 1986; Cronstein et al. 1985a, b; Samet 1986). In particular, a number of studies indicated adenosine as a prominent player in the physiological mechanisms deputed to downregulate activated immune cells and to protect tissues from inflammatory damage

(Antonioli et al. 2013a, b; Hasko and Cronstein 2013; Hasko et al. 2009; Hasko et al. 2008; Sitkovsky and Lukashev 2005; Sitkovsky and Ohta 2005).

Subsequently, the huge work performed by several research groups around the world has promoted a considerable expansion of our knowledge on the role played by adenosine in the control of immune functions. In this context, consistent evidence supported the involvement of adenosine pathways in the anti-inflammatory and immunomodulating effects exerted by drugs widely employed in the medical management of chronic inflammatory diseases (i.e., methotrexate, salicylates) (Chan and Cronstein 2010, 2013; Cronstein et al. 1994, 1999). These findings spurred the research of novel pharmacological entities suitable for the therapeutic management of several inflammatory disorders through the pharmacological modulation of adenosine pathways (Antonioli et al. 2012, 2014). At present, some of these compounds are being tested on several preclinical models of disease (asthma, chronic obstructive pulmonary disease, diabetes) with encouraging results (Antonioli et al. 2014), while others have already entered the phase of clinical development for treatment of rheumatoid arthritis (Silverman et al. 2008), Parkinson's disease (Hauser et al. 2015; Stocchi et al. 2017) or as novel anticancer immunotherapies (Antonioli et al. 2016, 2017).

20.2 The Adenosine System Between Innate and Adaptive Immunity

Over the years, several lines of evidence have revealed a pivotal role of extracellular adenosine in orchestrating immune/inflammatory responses through the activation of adenosine receptors on the surface of innate and adaptive immune cells (Fig. 20.1) (Hasko et al. 2007, 2008; Cekic and Linden 2016; Hasko and Cronstein 2004; Hasko and Pacher 2012; Linden 2011; Linden and Cekic 2012; Sevigny et al. 2015; Sitkovsky and Lukashev 2005).

20.2.1 Adenosine and Innate Immunity

20.2.1.1 Neutrophils

Neutrophils stem from the bone marrow (55–60% of the bone marrow is dedicated to their production) during hematopoiesis in response to the presence of various cytokines, predominantly the granulocyte colony-stimulating factor (G-CSF) (Mayadas et al. 2014).

Adenosine and its precursors are abundantly released from neutrophils, contributing actively to the regulation of neutrophil activity under resting conditions and in the presence of inflammation (Barletta et al. 2012; Linden 2006). Under inflammatory conditions, in parallel to adenosine, neutrophils release massively

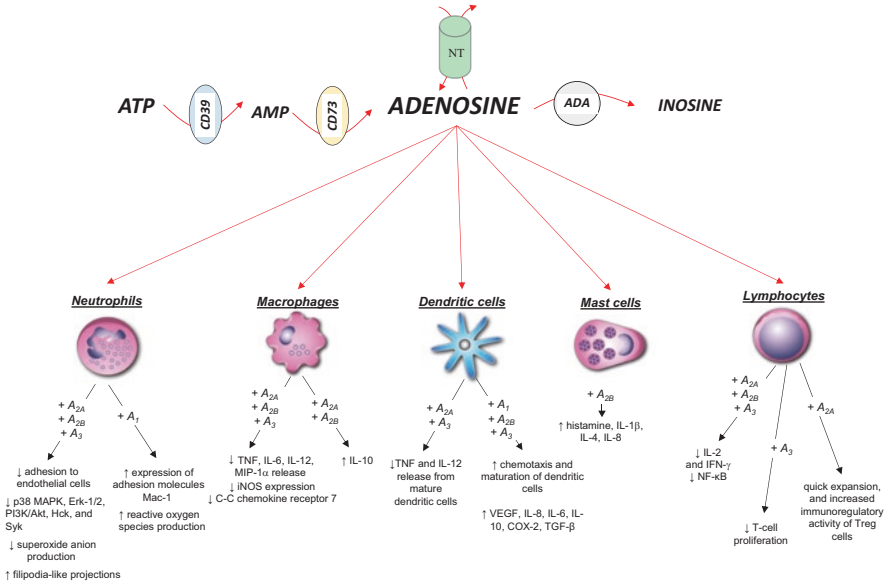

Fig. 20.1 Schematic representation of the main functions mediated by adenosine receptors on immune cells. Abbreviations: ADA adenosine deaminase, COX-2 cyclooxygenase-2, ERK-1/2 extracellular signal-regulated kinases 1/2, IFN interferon, IL interleukin, iNOS inducible nitric oxide synthase, MIP macrophage inflammatory protein, NT nucleoside transporters, p38-MAPK P38 mitogen-activated protein kinases, TGF-β transforming growth factor β, TNF tumor necrosis factor, VEGF vascular endothelial growth factor, ↑ increases, ↓ decreases.

also ATP, via connexin 43 hemichannels, thereby providing a substrate for extracellular generation of adenosine driven by the ectoenzymes CD39 and CD73, expressed on the neutrophil surface (Barletta et al. 2012). It is worth to note that, in the presence of inflammation, high extracellular levels of adenosine in the biophase of neutrophils are maintained in part by inactivation of adenosine deaminase and also by a significant reduction of the equilibrative nucleoside transporter expression (Barletta et al. 2012).

Once released into the extracellular space, adenosine, through interaction with own receptors widely expressed on neutrophils, participates actively to the modulation of their functions, eliciting a dual effect, based on the engagement of different receptor subtypes. In particular, the stimulation of A_1 receptors induces an upregulation of the neutrophil adhesion receptor Mac-1 and an increased expression of the complement receptors, responsible for an enhanced adhesion of neutrophils to vascular endothelium (Bours et al. 2006). Since A_1 receptors display a higher affinity for adenosine than the other receptor subtypes, it has been hypothesized, that in the early stages of inflammation, low local concentrations of adenosine can promote neutrophil recruitment via A_1 receptor engagement, whereas, in the later phases, the higher concentrations reached by adenosine in the biophase limit neutrophil recruitment by activation of A_2 receptors. Indeed, both A_{2A} and A_{2B} receptor engage-

ment was found to mediate the inhibition of neutrophil adhesion to endothelial cells (Eltzschig et al. 2004). Recently, Yago et al. (2015) provided novel evidence about the mechanism through which A_{2A} receptor signaling affects the human and murine neutrophil adhesion. In particular, the authors observed that the incubation of neutrophils with the selective A_{2A} receptor agonist, ATL313, inhibited the selectin-induced, β2 integrin-dependent slow rolling and the chemokine-induced β2 integrin-dependent arrest on ICAM-1 (Yago et al. 2015). Furthermore, ATL313 suppressed the selectin-triggered activation of Src family kinases (SFKs) and p38 MAPK, the chemokine-triggered activation of Ras-related protein 1, and the β2 integrin-triggered activation of SFKs and Vav cytoskeletal regulatory proteins (Yago et al. 2015). A recent study by Giambelluca and Pouliot (Giambelluca and Pouliot 2017) provided further evidence about the molecular mechanisms underlying the anti-inflammatory role of A_{2A} receptors expressed on neutrophil surface. The incubation of neutrophils with the A_{2A} receptor agonist CGS 21680 markedly decreased phosphorylation of several key signaling protein kinases involved in the modulation of neutrophil function, such as p38 MAPK, Erk-1/2, PI3K/Akt, Hck, and Syk.

Adenosine exerts a protective action on host tissues through modulation of neutrophil bactericidal functions. A dual regulatory effect has been reported for adenosine on phagocytosis. Indeed, the activation of A_1 receptors augments this process, while the stimulation of A_{2A} receptors was found to reduce the phagocytic activity of neutrophils (Zalavary and Bengtsson 1998). Adenosine participates also to a differential regulation of reactive oxygen species (ROS) generation in neutrophils, exerting a stimulatory or inhibitory action based on the levels reached in the biophase of different receptors. In particular, it has been reported that adenosine stimulates ROS production from activated neutrophils via A_1 receptor recruitment, whereas it downregulates ROS generation through A_{2A} receptors (Frasson et al. 2017; Sun et al. 2007). The stimulation of A_{2A} receptors was shown also to counteract significantly the release of IL-8, a critical chemokine that facilitates the development of inflammatory reactions by promoting the chemoattraction of leukocytes to the site of inflammation, activation of phagocytosis by neutrophils, and neutrophil degranulation (Frasson et al. 2017).

In addition, also the selective activation of A_{2B} receptors with BAY 60-6583 inhibited the formyl-methionine-leucine-phenylalanine (fMLP)-stimulated superoxide production in murine neutrophils (van der Hoeven et al. 2011). However, BAY 60-6583 failed to influence chemotaxis in response to fMLP, thus indicating a role of A_{2B} receptors in suppressing the oxidase activity, but not chemotaxis of murine neutrophils, and indicating that this low-affinity receptor participates jointly with A_{2A} receptors in regulating the pro-inflammatory responses of neutrophils (van der Hoeven et al. 2011).

At present, despite that molecular studies have demonstrated the expression of A_3 receptors on human neutrophils, their functional characterization remains scarcely investigated. Inoue et al. (Chen et al. 2006) reported that A_3 receptors hold a critical role in the regulation of neutrophil migration at the site of inflammation. In particular, the authors reported that human neutrophils release ATP at the leading edge of cell surface, which undergoes a quick degradation into adenosine by the

ectoenzymes CD39 and CD73, with consequent activation of A_3 receptors that stimulate neutrophil migration (Chen et al. 2006). A critical role for A_3 receptors in orchestrating neutrophil migration has been confirmed by the same research group in a mouse model of sepsis (Inoue et al. 2008). By contrast, Van der Hoeven et al. (2008) reported that A_3 receptor activation reduced neutrophil migration and superoxide production by suppressing the monomeric GTPase Rac.

Besides regulating chemotaxis, A_3 receptors appear to play a critical role also in promoting the formation of filipodia-like projections (Corriden et al. 2013). Indeed, the exposure of neutrophils to the selective A_3 receptor agonist 2-Cl-IB-MECA promoted the formation and rapid extension of these structures, thus improving bacterial phagocytosis (Corriden et al. 2013).

20.2.1.2 Monocytes and Macrophages

Macrophages comprise a heterogeneous population of mononuclear cells distributed ubiquitously throughout the body (Gordon et al. 2014). These cells hold a critical role in orchestrating and implementing most homeostatic, immunological, and inflammatory processes (Gordon et al. 2014). Because of their ubiquitous tissue distribution, these cells are quickly called into play in mounting an effective response against foreign agents prior to the migration of polymorphonuclear neutrophils and thus representing the major actors in the body's first line of immune defense (Gordon et al. 2014). The differentiation, maturation, and proliferation of macrophages are tightly regulated processes that are important in defining the nature and degree of macrophage responsiveness toward adverse stimuli (Gordon et al. 2014; Hasko et al. 2007). In this context, evidence indicates that adenosine can affect the course of macrophage proliferation and differentiation (Hasko et al. 2007).

The expression of all four adenosine receptors has been documented in monocytes and macrophages, while several studies have demonstrated significant changes in their levels and function during the maturation process. Indeed, the expression of A_1, A_{2A}, and A_3 receptors appears to be low in quiescent monocytes, while their density increases during differentiation into macrophages (Thiele et al. 2004). The patterns of receptor expression are influenced by the release of cytokines during the occurrence of inflammatory conditions. In particular, interleukin-1 (IL-1) and tumor necrosis factor (TNF) are known to upregulate A_{2A} receptor expression in human monocytes, while IFN-γ exerts opposite effects (Khoa et al. 2001). In addition, isolated human alveolar macrophages exposed to pro-inflammatory stimuli display a significant increase in A_{2A} receptor expression and subsequent enhancement of anti-inflammatory responses (Alfaro et al. 2017). Interestingly, Cohen et al. (2015) have described a modulation of macrophage A_{2B} receptors expression in response to different pro-inflammatory stimuli. This study showed that the expression of such receptors undergoes upregulation upon by TLR stimulation, leading to the induction of immunoregulatory macrophages with enhanced sensitivity to immunosuppressive extracellular adenosine. On the other hand, A_{2B} induction is prevented by IFN-γ,

which mitigates their sensitivity to adenosine and prevents their transition toward an immunoregulatory phenotype. A regulatory role of adenosine in the recruitment of monocytes at inflammatory sites, through A_{2A} and A_{2B} receptor-mediated inhibition of cell adhesion molecule expression on endothelium, has been also described (Delikouras et al. 2003). Moreover, Williams and Cronstein (Williams and Cronstein 2012) observed that in human THP1 macrophages, the activation of A_{2A} receptors decreased the expression of C-C chemokine receptor 7 (CCR7) involved in chemotactic functions, along with a decrease in cell migration.

Recently it has been described an involvement of adenosine A_3 receptors in macrophage migration toward apoptotic cells has been documented. In particular, Joós and colleagues (Joos et al. 2017) showed that autocrine release of ATP and subsequent conversion into adenosine is essential to maintain velocity and direction of macrophages toward apoptotic thymocytes, while A_3 gene deletion delayed the kinetics of apoptotic cell clearance in vivo.

Several studies have investigated the effects of adenosine on cytokine production in monocytes and macrophages. Most of current data indicate that the production of IL-12, TNF, IL-6, macrophage inflammatory protein (MIP)-1α, and nitric oxide (NO) is reduced by adenosine through the recruitment of A_{2A}, A_{2B}, and A_3 receptors (Bowlin et al. 1997; Hasko et al. 1996, 2000, 2007; Lee et al. 2011; Mabley et al. 2003; McWhinney et al. 1996; Ohta and Sitkovsky 2001; Szabo et al. 1998). On the other hand, extracellular adenosine stimulates the release of the anti-inflammatory cytokine IL-10 by monocytes and macrophages via A_{2A} and A_{2B} receptors, thus promoting the termination of inflammatory responses (Hasko et al. 2009; Khoa et al. 2001; Koscso et al. 2013; Nemeth et al. 2005).

Phenotypically, macrophages can be classified into two broad groups: a) pro-inflammatory M1 macrophages, whose activation occurs in a Th1 cytokine environment and pro-inflammatory nature is instrumental in the protection against pathogenic agents, and b) M2 macrophages, alternatively designated also as activated macrophages, which are essential participants in tissue remodeling and resolution of inflammation, whose development takes place in a Th2 cytokine environment (Csoka et al. 2012). Over the years, a number of experimental findings have highlighted a critical role of adenosine in driving the phenotype switch of macrophages. In particular, the stimulation of A_{2A} and A_{2B} receptors has been found to determine a switch from M1 to M2 phenotype (Csoka et al. 2012; Ferrante et al. 2013).

20.2.1.3 Dendritic Cells

Dendritic cells represent a sort of antigen-presenting cells (APCs), which are primarily involved in the activation of the adaptive immune response, through their actions on antigen interception, processing, and activation of specific lymphocyte-effector mechanisms (Hasko and Cronstein 2004). In this context, adenosine has been shown to regulate dendritic cell functions via interactions with specific receptors, the expression and function of which is strictly related to the maturation status

of these immune cells (Hasko and Cronstein 2004). Immature human dendritic cells express mainly A_1 and A_3 receptor subtypes, which are involved in the control of chemotaxis via an increase in intracellular calcium, thus allowing the recruitment of dendritic cells within inflammatory sites. Activated mature dendritic cells mainly express A_{2A} receptors, which confer sensitivity to the anti-inflammatory effects of adenosine, with a significant reduction of cytokine production and compensatory effects against chronic cell activation that is responsible for tissue damage (Schnurr et al. 2004). On the other hand, most of current data support a pro-inflammatory role of A_{2B} receptors expressed on mature dendritic cells. For instance, Pacheco et al. (2005) have demonstrated the presence of A_{2B} receptors in both immature and mature dendritic cells, where they act as adenosine deaminase anchoring proteins. Adenosine deaminase and A_{2B} receptor form a molecular complex that interacts with CD26 expressed on T cells and evokes a co-stimulatory signal on IFN-γ and TNF production. A role of adenosine A_{2B} receptors in differentiation of dendritic cells toward a pro-angiogenic, pro-inflammatory phenotype has also been described by Novitskiy et al. (2008). In this study, the authors observed that the activation of A_{2B} receptors, by adenosine generated in a hypoxic environment, elicited an increase in vascular endothelial growth factor (VEGF), IL-8, IL-6, IL-10, cyclooxygenase (COX)-2, transforming growth factor (TGF)-β, and indoleamine 2,3 dioxygenase (IDO) expression. More recently, Liang et al. (2015) showed that the activation of A_{2B} receptors in bone marrow dendritic cells stimulated the differentiation of Th17 T cells, thus confirming a pro-inflammatory role of this receptor subtype.

Dickenson et al. (2003) performed studies on the murine dendritic cell line XS-106, demonstrating that A_{2A} and A_3 receptors inhibit the release of TNF and proposed these cells as a useful model to evaluate the role of adenosine in the regulation of dendritic cell functions. Of note, most investigations, aimed at examining the influence of adenosine on APC functions, have been performed on single cell lines or a single source of APCs. Therefore, additional studies, conducted on different cell models, might help to gain further insight into the role played by adenosine in these immune cells.

20.2.1.4 Mast Cells

Mast cells are generally recognized as crucial players in allergic reactions and key initiators of innate immune responses. They can also have a role in the pathogenesis of inflammatory processes and pain perception (Thacker et al. 2007). Interestingly, adenosine can exert differential effects on mast cell activation, depending on the activated receptor subtype and the species under consideration (Rudich et al. 2012). For instance, it is well established that the activation of A_{2B} and A_3 receptors trigger the hyper-responsiveness and activation of murine mast cells. In particular, the recruitment of such receptors stimulates the degranulation of mast cells, which then release histamine, serotonin, chemokines, and proteases (Hasko and Cronstein 2004). On the other hand, data regarding the effects of adenosine receptors in human mast cells are controversial. Indeed, human studies provided evidence that

adenosine A_{2B} receptors are primarily involved in the activation of mast cells, while A_3 receptors are likely to mediate anti-inflammatory effects (Rudich et al. 2012). However, recent evidence suggests that, in human HMC-1 cells, A_3 receptors are likely involved in priming mast cells toward a tissue remodeling activity, by increasing the transcription of different genes involved in fibrosis, while the secretion of pro-inflammatory factors is only modestly affected (Rudich et al. 2012). These data suggest that, in humans, A_3 receptors seem to trigger mast cell hyper-responsiveness to additional factors able to promote the release of cell factors. A similar role has also been described for A_1 receptors in human cultured mast cells, in which the activation of such a receptor did not produce degranulation, although potentiating the activation of mast cells induced by anti-IgE (Yip et al. 2011).

Interestingly, histamine released from mast cells in response to adenosine exerts inhibitory effects on TNF biosynthesis, via interaction with macrophage H_2 histamine receptors (Smith et al. 2002). Nevertheless, the pro-inflammatory actions, resulting from direct adenosine-induced mast cell stimulation, seem to overcome the anti-inflammatory response promoted by adenosine via the histamine-mediated negative feedback on mast cell activation (Hasko and Cronstein 2004).

20.2.2 Adenosine and the Adaptive Immune System

Lymphocyte T can be stimulated by the presentation of antigenic moieties by APCs, such as dendritic cells or macrophages (Linden and Cekic 2012). Antigenic molecules, once exposed on the surface of APCs by the major histocompatibility proteins (MHC), are known to activate T-cell receptors on lymphocytes (Linden and Cekic 2012), thus triggering T-cell differentiation and eliciting cytokine production or cytotoxic activity (Linden and Cekic 2012).

Besides the indirect regulation of lymphocyte function by stimulating adenosine receptors on innate immune cells such as dendritic cells, adenosine can also affect directly lymphocyte responses via stimulation of adenosine receptors on lymphocytes (Hasko et al. 2008; Linden and Cekic 2012). In particular, a number of studies, performed by means of adenosine-receptor-knockout mice, allowed to characterize the effects of adenosine receptors on various lymphocyte functions (Hasko et al. 2008). In this setting, it has been observed that mouse T lymphocytes express A_{2A}, A_{2B}, and A_3 receptors (49–51), with a prevalent presence of the A_{2A} receptor subtype. Scarce is the presence of mRNA encoding for A_1 receptors (Hoskin et al. 2002; Lukashev et al. 2003; Zhang et al. 2004). Likewise, human T lymphocytes express mainly A_{2A}, A_{2B}, and A_3 with a limited expression of the A_1 receptor subtype (Gessi et al. 2004; Koshiba et al. 1999; Mirabet et al. 1999).

The consensus emerging from the above investigations, and pharmacological studies, is that A_{2A} receptors are the leading receptor subtype in dictating lymphocyte responses (Hasko et al. 2008). By contrast, the function of A_1 and A_3 receptors on T cells and their subsets is scarcely known and deserves further investigations (Cronstein and Sitkovsky 2017). In particular, a recent study by Gessi et al. (2001)

provided evidence about the expression of A_3 receptors on Jurkat cells. However, the expression and function of this receptor subtype in primary cells has not been established.

Studies performed on A_{2A}-knockout mice allowed to demonstrate that A_{2A} receptor activation inhibits IL2 secretion (Naganuma et al. 2006) by naive $CD4^+$ T cells, thus blunting their proliferation following T-cell receptor stimulation (Sevigny et al. 2007). In parallel, the engagement of A_{2A} receptors inhibited also the release of both IL4 and IFN-γ by both naive $CD4^+$ T cells and T_h1 and T_h2 cells (Csoka et al. 2008; Lappas et al. 2005; Naganuma et al. 2006). Further line of evidence showed that the pharmacological activation of A_{2A} receptors induced an upregulation of cytotoxic T-lymphocyte antigen 4 (CTLA4) and programmed cell death 1 (PD1), two negative co-stimulatory molecules, as well as a reduction of the positive co-stimulatory molecule CD-40 L (Sevigny et al. 2007).

Along the same line of what reported on $CD4^+$ cells, adenosine, via A_{2A} receptors, reduced IL2 release in polarized type 1 cytotoxic T (TC1) and TC2 $CD8^+$ cells (Erdmann et al. 2005). By contrast, the pharmacological activation of A_{2A} receptors failed to curb TC1 or TC2 cell cytolytic functions (Erdmann et al. 2005). Experimental data, obtained from A_1-, A_{2A}- and A_3-receptor-knockout mice, highlighted a critical role for A_{2A} receptors in counteracting the cytolytic activity of natural killer cells (Raskovalova et al. 2005).

Of note, adenosine can suppress the expression of intercellular adhesion molecule-1 in lymphocytes from enteric Peyer's patches (Johnston et al. 2005), thus limiting the accumulation of lymphocytes at inflammatory sites and reducing their adhesion and migration into extravascular sites (Yang et al. 2005).

A number of studies have demonstrated a pivotal role for adenosine in shaping the immune suppressive activity of regulatory T (T_{Reg}) cells, a subset of T cells that play a key role in controlling the immune system overactivity, thereby preventing excessive tissue injury (Antonioli et al. 2008). In humans, 90% of $Foxp3^+$ Tregs have been found to be $CD39^+$. Although the surface expression of CD73 on Tregs is scarce, CD73 is markedly represented in the cytoplasm of these cells (Antonioli et al. 2013b). Pharmacological studies performed by Deaglio et al. (2007) have shown that the CD39/CD73 enzyme axis converts extracellular nucleotides into pericellular adenosine, which then promotes an immune suppression via engagement of A_{2A} receptors expressed on activated T effector cells. In particular, studies performed by Romio et al. (2011) showed that CD73-derived adenosine produced by Tregs counteracted nuclear factor-κB activation in T effector cells via A_{2A} receptor stimulation, thereby inhibiting the production and release of several pro-inflammatory cytokines and chemokines. Interestingly, Ohta et al. (2012) provided evidence about the occurrence of a self-reinforcing loop in the immunosuppressive activity of Tregs, supported by adenosine production. Indeed, following the activation of A_{2A} receptors on Tregs, these cells underwent a quick expansion, acquiring an increased immunoregulatory activity (Ohta et al. 2012).

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Chapter 21
Adenosine Receptors Regulate Bone Remodeling and Cartilage Physiology

Carmen Corciulo, Natasha Irrera, and Bruce Neil Cronstein

Abstract Bone is a dynamic tissue that undergoes constant remodeling. Many intercellular messengers and cellular mechanisms regulate the rate and efficacy of bone remodeling, and disruption of this process can lead to such pathology as osteopenia and osteoporosis on one hand and osteopetrosis on the other. Results of recent studies indicate a central role for adenosine and its receptors in the control of bone and cartilage metabolism. Many studies using pharmacological and genetic approaches were performed in different laboratories in order to clarify the role, sometimes controversial, of adenosine in the skeletal system.

New bone formation during fractures or during development depends on differentiation and function of osteoblasts. Osteoblast differentiation and mineral deposition are processes stimulated by A_{2A} and A_{2B} adenosine receptors ($A_{2A}R$ and $A_{2B}R$). $A_{2A}R$ and $A_{2B}R$ also block the differentiation and function of osteoclasts, the multinucleated giant cells that mediate bone resorption. In contrast the A_1 adenosine receptor plays a prominent role in osteoclast where it stimulates their activity in bone resorption. Moreover it has been shown recently from our laboratory and previously from others that adenosine receptor, through $A_{2A}R$, exerts an important role in cartilage protection during mechanical stress, inflammation, and osteoarthritis.

Keywords Adenosine · Bone · Cartilage · CD73 · CD39

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21.1 Bone Physiology

Bone is a metabolically active tissue characterized by continuous remodeling. During remodeling there is a very tight balance between bone formation and resorption both in physiological conditions such as growth, changing mechanical needs, and regulation of calcium homeostasis and in pathological conditions to restore possible damage (Raisz 1999).

Different cell types cooperate to regulate bone homeostasis: osteoblasts, responsible for new bone formation and mineralization; osteoclasts, which play a pivotal role in resorbing bone; and osteocytes, which regulate bone formation in response to mechanical and other signals (Klein-Nulend et al. 2013; Olsen et al. 2000). Imbalance among these tightly linked cells can result in a pathological loss of bone, as in osteoporosis, or in abnormal bone density, as in osteopetrosis. Indeed, the most commonly used drugs in the treatment of osteoporosis prevent bone resorption by diminishing osteoclast differentiation and function (bisphosphonates, estrogens, calcitonin) (Henriksen et al. 2011).

Osteoclasts differentiate from myeloid precursors and are characterized by cell polarization after contact with the bone surface and the presence of resorptive organelles associated with the cell membrane (Teitelbaum and Ross 2003). During the processes of bone growth or fracture healing, osteoclast precursors are recruited to the bone remodeling site where differentiated osteoclasts generate a resorption lacuna and osteoblasts deposit new bone.

Differentiation of osteoclasts from bone marrow precursors is tightly regulated by two cytokines: macrophages colony-stimulating factor (M-CSF) and receptor activator of nuclear factor KB ligand (RANKL). M-CSF is responsible for the proliferation, survival, and cytoskeleton rearrangement of osteoclast precursors and RANKL for their full differentiation (Kim and Kim 2016).

Following activation of RANK, TNF receptor-associated factor (TRAF)-6 associates with the receptor leading to the activation of the nuclear factor kB (NF-kB) with consequent transcription of osteoclast-specific genes. During the early phase of osteoclastogenesis, NF-kB activates NFATC1, a transcription factor responsible for the terminal differentiation of osteoclasts (Kim and Kim, 2016). In addition to positive regulation of osteoclastogenesis by RANKL and M-CSF, there is negative regulation of osteoclastogenesis by the decoy receptor for RANKL, osteoprotegerin (OPG), a protein secreted primarily by osteoblasts. Interestingly, more recent studies indicate that downregulation of OPG expression in osteoclasts also enhances osteoclastogenesis and decreases apoptosis (Emery, 2006; Kang et al. 2014).

Mesenchymal stem cells (MSC) are pluripotent cells that can differentiate into many different types of cells including osteoblasts (Garg et al. 2017; Ponte et al. 2007; Uccelli et al. 2008). During bone formation, osteoblast precursors interact with the bone surface through specific intracellular proteins linked to the cytoskeleton, integrins, and cadherins. These molecules modulate cell anchorage and cell

signaling leading to osteoblast differentiation (Marie et al. 2014). Osteoblasts synthesize new collagenous organic matrix and promote mineralization by releasing small matrix vesicles and mineralization inhibitors such as pyrophosphatase and proteoglycan (Anderson 2003). Once in the mineralized matrix, osteoblasts change their morphology, becoming osteocytes, residing in space called lacunae and connecting to each other by cell extensions that pass through the canaliculi. The main function of osteocytes is to sense mechanical signals and to regulate osteoblast and osteoclast function (Hemmatian et al. 2017).

21.2 Purine Metabolism and Bone Metabolism

Among the many molecular mechanisms regulating bone homeostasis, purinergic signaling assumes an important role. Two major purinergic receptor families have been identified: P1 receptors are G protein-coupled receptors activated by adenosine and antagonized by methylxanthines and P2 receptors, which are stimulated by ADP and ATP and are further subdivided into P2Y (G protein coupled receptors) and P2X receptors (Burnstock et al. 1978).

Adenosine is present in the extracellular fluid throughout the body with basal concentrations ranging from 30 to 200 nM, and adenosine concentration can reach 10–100 uM during stress conditions (Ballarin et al. 1991; Fredholm 2007). Adenosine is formed extracellularly from the hydrolysis of adenine nucleotides by cell surface and soluble enzymes and is also exported from cells by transporters. Increase of adenosine concentration regulates cellular and tissue functions via interaction with cell membrane enzymes and transportation through membrane channels, rapidly regulating adenosine concentration in the extracellular space.

There are four P1 receptors, A_1, A_{2A}, A_{2B}, and A_3, and, once activated by adenosine, these receptors trigger a variety of molecular signals (Fredholm et al. 2001). As noted above, these receptors belong to the large family of G protein-coupled receptors: A_{2A} and A_{2B} receptors are coupled to Gs/Golf signal transduction proteins that increase cellular cAMP content followed by activation of protein kinase A; in contrast, A_1 and A_3 receptors inhibit cAMP production (Sheth et al. 2014). The adenosine receptors show high sequence similarity and a similar potency to modulate cAMP, except for $A_{2B}R$ that is 50 times less potent than the other receptors (Jacobson and Gao 2006). Adenosine receptor activation is regulated by mechanisms of desensitization involving their phosphorylation mediated by G protein-coupled receptor kinases. Desensitization is rapid for $A_{2A}R$ and $A_{2B}R$ (few minutes), and it is a relatively slow process for A_3R and A_1R (respectively, 1 h and few hours) (Sheth et al. 2014).

Adenosine receptors are ubiquitously expressed and play a role in the physiology of many tissues and organs. Moreover, forming heterodimers with other G protein-coupled receptors, e.g., dopamine and cannabinoid receptors, can interfere

with and regulate other molecular signaling (Fredholm et al. 2011). In the last few years, adenosine receptors have been studied as a target for treatment of many diseases like cancer, alteration of the immune system, skin fibrosis, neurodegenerative conditions, and disorders of bone metabolism (Burnstock 2016; Cekic and Linden 2016; Di Virgilio and Adinolfi 2016; Perez-Aso et al. 2016; Strazzulla and Cronstein 2016).

As noted above, there is a great deal of evidence supporting a role for purines and their metabolites in regulating bone metabolism (Hoebertz et al. 2003; Orriss et al. 2010). It has recently been appreciated that many cells, including those in the skeletal system, release ATP via specific transporters (pannexin 1 and 3, ank, connexin 43) which both stimulates P2 receptors directly and is a source of extracellular adenosine (Velasquez and Eugenin 2014). Adenosine generation from ATP is mediated by ectonucleotidases including ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD-1) (CD39), ecto-5′-nucleotidase (CD73, EC 3.1.3.5), nucleotide pyrophosphatase phosphodiesterase 1 (NPP-1, EC 3.1.4.1), and tissue-nonspecific alkaline phosphatase (TNAP) (He et al. 2013b). These enzymes are responsible for the hydrolysis of ATP to ADP, AMP and adenosine. Intracellular adenosine is also transported into the extracellular space by equilibrative nucleoside transporters (Young et al. 2013). Finally, adenosine deaminase (ADA) catalyzes the irreversible deamination of adenosine and deoxyadenosine. Hereditary absence of ADA activity is responsible for the accumulation of metabolic substrates such as adenosine, deoxyadenosine, and dATP which lead to severe combined immunodeficiency (Honig et al. 2007). Evidence regarding the role of the purinergic system in bone resorption and cartilage biology comes from patients with ADA deficiency who suffer multiple skeletal alterations (Manson et al. 2013). Moreover loss of ADA is responsible for the reduction of trabecular bone volume (Sauer et al. 2009).

He et al. have identified CD39 and CD73 mRNA both in murine osteoclast precursors and in mature osteoclasts (He et al. 2013b). CD73KO mice have osteopenia and more osteoclast formation and impairment in osteoblast differentiation than WT mice as well as CD39KO (Takedachi et al. 2012). Recently the important role of CD73 in bone remodeling in a model of age-related bone damage. CD73KO mice, 52 weeks old, showed delayed bone regeneration due to reduced number of osteoclasts and reduced osteoblast activity compared to the age-matched WT (Bradaschia-Correa et al. 2017). Interestingly, adenosine availability in the bone is regulated by Lrp4, a transmembrane protein that, in osteoblasts, negatively regulates ATP release and therefore the products of ATP hydrolysis (Xiong et al. 2017). Moreover adenosine is a substrate for the enzyme responsible for adenosine deamination to inosine, ADAR1. This enzyme is responsible for posttranscriptional modifications of mRNA, and it has been shown that its ablation in mice reduces bone mass by decreasing bone marrow proliferation and osteoblast differentiation (Yu et al. 2013).

21.3 The Role of Adenosine Receptors in Regulation of Bone Metabolism

21.3.1 A_1 Adenosine Receptor Promotes Osteoclast Differentiation

A_1RKO mice do not have any apparent skeletal deformities, and histologic analysis does not reveal any difference in number or morphology of osteoblasts. Nonetheless, by 6 months of age A_1RKO mice have more trabecular and cortical bone density than WT animals. Moreover, they exhibit residual cartilage within the bone consistent with osteopetrosis (Kara et al. 2010b).

A_1Rs are constitutively active in osteoclast precursors since the A_1R agonist N6-cylopentyladenosine has no effect on osteoclast differentiation. In contrast, blockade of the A_1R inhibits osteoclast differentiation and increases intracellular cAMP concentration in murine bone marrow and human osteoclast precursors (Kara et al. 2010a). Because the A_1R is a Gi-coupled receptor, the increase in cAMP observed in osteoclasts treated with an A_1R antagonist is thought to be consistent with the hypothesis that A_1R antagonists act, in this setting, as inverse agonists. Because A_1R blockade diminishes osteoclast differentiation and function, it was studied as a means to inhibit the development of postmenopausal osteoporosis in a model in which post-ovariectomy bone loss in mice was studied. A_1R blockade prevents bone loss in this model primarily by inhibiting osteoclast differentiation with little effect on osteoblast function since the amount of circulating RANKL does not change in the treated compared to the control mice (Kara et al. 2010b).

The molecular pathway underlying the A_1R-mediated effect on osteoclast differentiation and function involves NF-κB and TRAF6 signaling. Pharmacological blockade of A_1R prevents formation of the TAK1-TRAF6 complex necessary for the activation of NF-κB following RANK activation. A_1R blockade also inhibits cytosolic IκB degradation and the translocation of p65 subunit (activated NF-κB) into the nucleus. Consequently, there is a reduction of RANKL-induced phosphorylation of c-jun and c-fos in A_1R antagonist-treated cells (He and Cronstein 2012).

21.3.2 A_{2A} Adenosine Receptor Stimulation Prevents Bone Resorption by Osteoclasts

As with their actions on cAMP accumulation, activation of A_{2A}R exerts an opposing effect on osteoclast differentiation, compared to A_1R activation. Mediero et al. have reported that A_{2A}R activation reduces both the number of differentiated osteoclasts and their expression of osteoclast markers (cathepsin K and osteopontin). When the A_{2A}R is stimulated in bone marrow cells osteoclast formation is inhibited. These results were confirmed by the observation of increased osteoclasts in bones of mice

lacking A_{2A}Rs (Mediero et al. 2015a). In contrast, Pellegatti and colleagues reported that activation of the A_{2A}R increases osteoclast differentiation both indirectly, by increasing adenosine levels in the supernatant, and directly by promoting osteoclast fusion (Pellegatti et al. 2011). The differences observed in this study most likely arose from the fact that this group studied the effect of adenosine receptor agonists on osteoclast differentiation from peripheral blood mononuclear cells rather than bone marrow-derived precursors.

Stimulation of A_{2A}R may also regulate osteoclast differentiation by a different indirect mechanism. Stimulation of A_{2A} receptor has long been known to exert potent anti-inflammatory effects on numerous cell types resulting in reduction of pro-inflammatory cytokines, such as TNF-α and IL-1β that can promote osteoclast formation (Bitto et al. 2011; Chan and Cronstein 2010; Kim et al. 2005). Together with reduction of pro-inflammatory cytokine levels, A_{2A}R stimulation also increases levels of anti-inflammatory cytokines, such as IL-10, which strongly blocks RANKL-induced expression of NFATcl. In a model of inflammatory osteolysis, A_{2A}R stimulation increases IL-10 levels systemically and locally, and production of anti-inflammatory cytokines likely contributes to the reduction of osteoclast differentiation and activity with resulting inhibition of inflammatory osteolysis (Ivashkiv et al. 2011; Park-Min et al. 2009).

Inhibition of osteoclast formation via adenosine receptors may provide another therapeutic approach to inhibit bone destruction in rheumatoid arthritis and other inflammatory bone diseases. Indeed, low-dose methotrexate, a mainstay in the treatment of rheumatoid arthritis, limits bone destruction in patients, and this effect is most likely mediated by activation of A_{2A}R (Cronstein et al. 1993). In a murine model, treatment with MTX diminishes wear particle-induced osteolysis, another form of inflammatory bone destruction, via ligation of A_{2A}R (Mediero et al. 2015b). In a prior study, this same group demonstrated that activation of A_{2A}R reduced the levels of soluble RANKL and as well as its expression on cells (Mediero et al. 2012a). Since the correlation between RANK/RANKL is critical for bone resorption, the lower concentration of RANKL also contributes to the reduction of osteoclast differentiation. In this paper, the authors also observed a significant A_{2A}R-mediated reduction in the levels of M-CSF which is also required for osteoclast differentiation from bone marrow precursors.

A_{2A}R agonists have also been effective in an animal model of type II collagen-induced arthritis reducing bone resorption and progression of arthritis (Bitto et al. 2011; Mazzon et al. 2011). This effect might be related, in part, to the abrogation of osteoclast differentiation (Mediero et al. 2012b).

Further evidence for the potential therapeutic benefit of A_{2A} receptor-mediated suppression of osteoclast differentiation was provided by experiments in which bone regeneration in a murine trephination model was enhanced by direct application of an A_{2A} agonist or by the application of agents that enhance extracellular adenosine levels by blocking ENT1-mediated adenosine uptake (dipyridamole and ticagrelor) (Ishack et al. 2015; Mediero et al. 2015b; Mediero et al. 2016).

21.3.3 Osteopenic Phenotype in A_{2B}RKO Mice

A_{2B}RKO mice are osteopenic and have delayed fracture healing as compared to wild-type mice. This defect has been ascribed to an inability of bone marrow-derived mesenchymal stem cells from A_{2B}RKO mice to differentiate into osteoblasts (Carroll et al. 2012). In the mouse calvaria cell line MC3T3-E1 the overexpression of CD73 increases osteoblast functionality though adenosine binding to A_{2B}R (Takedachi et al. 2012). In more recent studies, we have observed that there is a reduction in trabecular number in subchondral and distal bone in A_{2B}RKO mice and that since A_{2B}R stimulation also suppresses osteoclast differentiation, enhanced bone resorption in these mice may also contribute to osteopenia (Corciulo et al. 2016). Moreover, in other in vitro experiments A_{2B}R stimulation diminishes osteoclast differentiation by human bone marrow osteoclasts from both normal individuals and patients with multiple myeloma (He et al. 2013a).

21.3.4 The Unclear Role of A_3 Adenosine Receptors in Bone Metabolism

The effect of A_3R in osteoclast differentiation and function or resorption has not been well studied. A_3R agonists have been tested for therapeutic effects in inflammatory arthritis models in which bone destruction is a major component. Thus, Rath-Wolfson and colleagues analyzed the therapeutic effect of IB-MECA (an A_3R agonist) on bone destruction in the adjuvant-induced arthritis model in rats and observed, in addition to a marked reduction in inflammation, that there was a marked reduction of bone destruction associated with a 73% reduction in the number of osteoclasts on the bone surface (Rath-Wolfson et al. 2006). In contrast, He and colleagues reported that neither the same A_3R agonist, IB-MECA, nor the A_3R antagonist MR1191 affected osteoclast differentiation in vitro (He et al. 2013a).

21.3.5 Controversial Interpretations of Adenosine Role in Bone

In a recent article, Hajjawu and colleagues stated that ATP assumes a more important role than adenosine on the signaling of osteoclast differentiation and function. According to this work even if osteoclasts express A_{2A}, $A_{2B,}$ and A_3 receptors, adenosine and its analogue 2-chloroadenosine are not able to exert any effect on mouse osteoclasts in vitro. The authors suggest that the osteopetrosis bone phenotype in A1RKO mice and the decrease of bone content in A_{2B}RKO described in different papers (Carroll et al. 2012; He et al. 2013a) are indirect effects of the lack of adenosine receptors in other tissues and organs (Hajjawi et al. 2016). Furthermore Pellegatti

and colleagues demonstrated the key role of ATP in osteoclasts differentiation but as a coactor in the "purinergic axis": P2X7 receptor activated by ATP and adenosine derived from ATP catabolism-binding $A_{2A}R$ (Pellegatti et al. 2011).

21.4 Adenosine Signaling in Cartilage

The articular cartilage is an important avascular tissue exerting its main function by protecting the subchondral bone beneath from mechanical stress and by actively responding to weight bearing and inflammatory response. These functions can be exerted, thanks to the peculiar architecture of the extracellular matrix, formed mainly by water, collagen fibers, proteoglycans, and glycosaminoglycan that confer elasticity to the cartilaginous tissue (Sophia Fox et al. 2009).

Evidence from different laboratories suggests that adenosine as an important regulator of chondrocytes viability and function and cartilage health (Fig. 21.1).

In experiments performed in equine cartilage, adenosine inhibits LPS-induced NO production in chondrocytes. Similar results were obtained with adenosine, the adenosine deaminase inhibitor EHNA, with the adenosine receptors agonist NECA or in presence of the specific agonist for $A_{2A}R$ DPMA (Benton et al. 2002; Tesch et al. 2002). Exposure to ADA or to a specific $A_{2A}R$ antagonist induce a concentration-dependent increase in glycosaminoglycan, metalloproteinases (MMP-3 and MMP-13), PGE2 and NO (Tesch et al. 2004).

In our laboratory we have demonstrated that, in physiological conditions, release of adenosine increases after mechanical stimulation. Adenosine in this way exerts

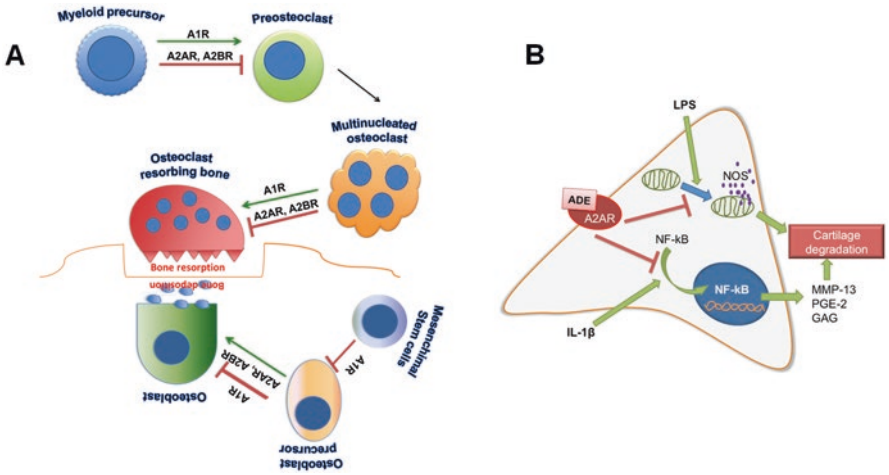

Fig. 21.1 Adenosine modulates bone remodeling (**a**) and protects articular cartilage (**b**) in physiological and pathological conditions

an anti-inflammatory effect, through $A_{2A}R$ and consequent inhibition of the NF-kB pathway blocking metalloproteinase production. Presence of IL-1β decreases CD73, Pannexin-1, ANK, and ENT leading to a drastic reduction in adenosine levels in the extracellular fluid as well as cartilage injury (Corciulo et al. 2017).

In STR/ort old mice, a genetic model of spontaneous OA, 5'NT expression increases close to the OA lesion and the same group showed that high levels of endogenous adenosine induced by inhibition of ADA lead to cell death in MC615 cells in a receptor-independent fashion. The explant of a healthy knee incubated with the inhibitor of ADA, EHNA, reveals no effect on cell death, but adenosine at 5 mM has more dead cells compared to the control (Mistry et al. 2006). The difference in the results are likely due to the different model of osteoarthritis studied (STR/ort mice are 2 years old) and in using concentrations of adenosine three log orders higher than those reported to exist in any cell or tissue.

All adenosine receptors are expressed in bovine, mouse, and human chondrocytes (Varani et al. 2008a; Varani et al. 2008b; Vincenzi et al. 2013). Activation of the A_3R is efficacious in the treatment of experimental osteoarthritis induced by monosodium iodoacetate. The compound CF101 has been shown to prevent cartilage damage and osteophyte formation by a mechanism involving NF-kB deregulation (Bar-Yehuda et al. 2009). In our model of post-traumatic OA in rats, we didn't measure any effect mediated by A_3R. Instead activation of $A_{2A}R$ results in the most effective impact on cartilage protection. Moreover we found that $A_{2A}R$-KO mice develop spontaneous OA (Corciulo et al. 2017).

21.5 Conclusion

In the last few years, a number of efforts have been made to understand how purinergic signaling, and in particular adenosine, affects bone metabolism. Indeed, the therapeutic potential of adenosine receptor stimulation in conditions characterized by inflammatory bone loss has been clarified. Adenosine, whether released as a result of treatment with methotrexate or accumulated extracellularly as a result of ent1 blockade (by dipyridamole or ticagrelor) can diminish bone loss due to inflammation. In addition, drugs already available on the market, such as ticagrelor and dipyridamole, can block osteoclast differentiation by preventing adenosine uptake and thereby prevent osteoclast-mediated bone loss and promote osteoblast-mediated bone formation (Mediero et al. 2016). Deploying this approach in human disease may be useful in promoting bone regeneration and healing as well as diminishing inflammatory bone destruction.

Experimental evidence in chondrocytes and articular cartilage suggest that adenosine replacement in the joint may be a new therapeutic approach to disease involving cartilage damage such as OA.

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Chapter 22
Adenosine Receptors in Gestational Diabetes Mellitus and Maternal Obesity in Pregnancy

Fabián Pardo and Luis Sobrevia

Abstract Regulation of blood flow depends on the systemic and local release of vasoactive molecules including the endogenous nucleoside adenosine. Vasodilation caused by adenosine results from the activation of adenosine receptors (ARs) at the vascular endothelium and smooth muscle. Adenosine receptors are four subtypes, i.e. A_1AR, A_{2A}AR, A_{2B}AR and A_3AR, of which A_{2A}AR and A_{2B}AR activation in the endothelium lead to increased generation of nitric oxide and relaxation of the underlying smooth muscle cell layer. Adenosine also causes vasoconstriction via a mechanism involving A_1AR activation by increasing the release of vasoconstrictors. Adenosine increases the sensitivity of vascular tissues from diseases coursing with insulin resistance, including gestational diabetes mellitus (GDM) and obesity. ARs also play a role in obesity since they modulate D-glucose homeostasis, inflammation and adipogenesis. Agonists and/or antagonists of high selectivity for ARs may result in reversing the obesity state since normalises lipolysis and insulin sensitivity. A considerable fraction of pregnant women with GDM show with pregestational

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obesity and/or supraphysiological gestational weight gain. These conditions associated with reduced vascular responsiveness to adenosine and insulin. However, it is unclear whether GDM plus obesity in pregnancy could worsen these alterations in the foetoplacental vascular function. This chapter summarises available findings that address the potential involvement of ARs to modulate human foetoplacental vasculature in GDM and obesity in pregnancy.

Keywords Adenosine · Diabetes · Obesity · Vascular · Human endothelium · Smooth muscle

22.1 Introduction

Diabetes mellitus and obesity are diseases that associate with altered vascular response to vasodilators and vasoconstrictors (Silva et al. 2017; Villalobos-Labra et al. 2017; Pardo et al. 2017, 2018; Sáez et al. 2018a, b; Subiabre et al. 2018). Circulating or locally released vasoactive molecules are responsible for the vascular endothelial and smooth muscle cells regulation of the vascular tone in health and disease. The endogenous nucleoside adenosine (Antonioli et al. 2015; Headrick et al. 2013; Westermeier et al. 2011) acts on plasma membrane receptors triggering differential or common cell signalling mechanisms depending on the type of receptors that are activated (Fredholm 2010, 2014; Fredholm et al. 2011; Burnstock 2016; Sobrevia and Fredholm 2017; Peleli et al. 2017).

The broad spectrum of actions of adenosine depends on its extracellular concentration, binding kinetics to plasma membrane adenosine receptors (ARs) and subsequent triggered cell signalling (San Martín and Sobrevia 2006; Burnstock 2016; Sobrevia and Fredholm 2017; Silva et al. 2017). ARs include at least four subtypes of G-coupled plasma membrane proteins expressed in the human placenta vasculature (Salsoso et al. 2015, 2017; Silva et al. 2017). Activation of these receptors plays a role in the foetoplacental endothelial dysfunction seen in gestational diabetes mellitus (GDM) (San Martín and Sobrevia 2006; Silva et al. 2017; Sáez et al. 2018a). ARs are also critical in the biological effects of other vasoactive molecules, including the hormone insulin in the human vasculature (Salsoso et al. 2015) and adipocytes (Ciaraldi 1988; Lönnroth et al. 1988). Since different ARs are involved in the regulation of the function of human umbilical vein endothelial cells (HUVECs) from GDM compared with normal pregnancies (Guzmán-Gutiérrez et al. 2012, 2016), a differential role of these membrane receptors is critical in health and disease in this cell type. Equally, obesity in pregnancy and pregestational maternal obesity (PGMO) are conditions where HUVECs also show with altered function. Interestingly, GDM results in increased expression and activity of nitric oxide synthases (NOS); however, obesity in pregnancy and PGMO show with reduced NOS expression and activity in this cell type. Therefore, it seems clearer nowadays that adenosine and ARs play critical and differential roles in GDM and obesity in pregnancy, two abnormal metabolic conditions in pregnancy that may be related or interdependent to cause a potentially different entity referred as diabesity (i.e. diabetes + obesity).

In this review, we summarised the available information regarding ARs and adenosine as potential factors involved in the placental endothelial dysfunction seen in GDM and obesity in pregnancy and potentially in diabesity.

22.2 Adenosine Receptors

Several comprehensive reviews are available addressing the molecular and functional aspects of ARs in mammalian cells (Burnstock 2017; Fredholm et al. 2017; Peleli et al. 2017; Sobrevia and Fredholm 2017). ARs include four subtypes which have been identified in human tissues, i.e. A_1AR, A_{2A}AR, A_{2B}AR and A_3AR. These ARs subtypes contain seven transmembrane domains with an extracellular N-terminal extreme of 7–13 amino acids and an intracellular C-terminal extreme of 32–120 amino acids (Fredholm et al. 2011). Adenylyl cyclase is a common protein that is activated by activation of A_{2A}AR and A_{2B}AR but inactivated following A_1AR and A_3AR activation. Adenosine biological effects are broad but have highly specific involvement of specific ARs subtypes in several pathologies (Fredholm 2014) including GDM (Guzmán-Gutiérrez et al. 2012, 2016), maternal obesity (Pardo et al. 2015, 2017, 2018) and preeclampsia (Salsoso et al. 2015, 2017; Chiarello et al. 2018).

In the vascular system, adenosine acts as a regulator of the blood flow (Westermeier et al. 2011; Silva et al. 2017). In HUVECs, A_{2A}AR, A_{2B}AR and A_3AR expressions are higher than A_1AR (Wyatt et al. 2002; Salsoso et al. 2015). However, in primary cultures of human placental microvascular endothelial cells (hPMECs), only A_{2A}AR and A_{2B}AR have been reported (Escudero et al. 2008). The expression of the different subtypes of ARs in the human placenta endothelium is under modulation of several factors, such as partial pressure of oxygen (von Versen-Höynck et al. 2009; Kurlak et al. 2015) and insulin level (Salsoso et al. 2015; Sobrevia et al. 2016) or pathological conditions including GDM (Westermeier et al. 2011; Silva et al. 2017; Sáez et al. 2018a), obesity in pregnancy (Pardo et al. 2017, 2018) and preeclampsia (Escudero et al. 2008; Salsoso et al. 2015, 2017; Chiarello et al. 2018). Thus, environmental factors and diseases of pregnancy are key in the differential expression of ARs subtypes contributing to a functional and dysfunctional heterogeneity of the human placental vascular endothelium (Vásquez et al. 2004; San Martín and Sobrevia 2006; Sobrevia et al. 2011, 2015, 2016; Sobrevia and Fredholm 2017; Peleli et al. 2017).

22.3 Gestational Diabetes Mellitus (GDM)

GDM is a disease of pregnancy where the mother shows altered the handling of D-glucose leading to hyperglycaemia and the broad associated consequences in the mother and the growing foetus of this abnormal condition (American Diabetes Association 2017). This disease is diagnosed in the second trimester of pregnancy

in women that are with pregestational and gestational normal weight, overweight, or with obesity (Silva et al. 2017; Pardo et al. 2017, 2018). The clinical manifestations of GDM are attributed mainly to the condition of maternal and foetal hyperglycaemia and hyperinsulinaemia (Pandolfi and Di Pietro 2010; Brown et al. 2017a). Recent studies report that treating pregnant women with insulin (i.e. insulin therapy), controlled diet and oral antidiabetic pharmacological drugs (metformin, glibenclamide) or changing their lifestyle restores glycaemia to physiological values (Brown et al. 2017a, b, c; Tieu et al. 2017; Subiabre et al. 2017, 2018; Sáez et al. 2018a, b). However, even restoring the maternal glycaemia a GDM-associated foetoplacental endothelial dysfunction is seen which results in an adverse maternal (v.g. DNA methylation, increased gestational weight gain, high cardiovascular risk) and foetal (v.g. epigenetic modulation, macrosomia, altered birthweight) outcome (Brown et al. 2017a, b, c; Tieu et al. 2017; Subiabre et al. 2017, 2018).

22.3.1 Vascular Dysfunction in GDM

GDM associated with increased nitric oxide (NO) synthesis in human placental veins and arteries and in primary cultures of HUVECs and hPMECs (Westermeier et al. 2011, 2015; Salomón et al. 2012; Subiabre et al. 2017, 2018; Sáez et al., 2018a, b). GDM-associated foetal vascular dysfunction was shown to result from a functional dissociation between the capacity of endothelial cells to generate NO and the uptake of the cationic amino acid L-arginine, i.e. the substrate for NO synthesis via endothelial NO synthase (eNOS). A critical role of adenosine, whose concentration is increased in the umbilical vein blood at delivery, in the control of L-arginine uptake and NO synthesis (i.e. the L-arginine/NO signalling pathway) in HUVECs and hPMECs has been demonstrated in GDM (Vásquez et al. 2004; San Martín and Sobrevia 2006; Westermeier et al. 2011, 2015; Salomón et al. 2012; Guzmán-Gutiérrez et al. 2012, 2016). This phenomenon involves the activation of A_2AR due to increased extracellular adenosine concentration caused by the reduced uptake of this nucleoside in HUVECs from GDM, a signalling pathway referred as ALANO (for adenosine/L-arginine/nitric oxide) signalling pathway (San Martín and Sobrevia 2006). This mechanism is now regarded as an approach to the understanding of foetoplacental endothelial dysfunction triggered by GDM and extracellular hyperglycaemia (Pandolfi and Di Pietro 2010; Sobrevia et al. 2015, 2016).

22.3.2 Role of Adenosine Receptors in GDM

Adenosine causes vasodilation via activation of ARs leading to increased synthesis of NO by the endothelium, a phenomenon that depends on differential expression and function of ARs (Fredholm 2010; Fredholm et al. 2011, 2017; Burnstock 2017). Adenosine vascular effect is relevant in GDM since HUVECs from this disease

show altered reactivity to adenosine and other molecules, such as insulin, compared with cells from normal pregnancies (Guzmán-Gutiérrez et al. 2016; Subiabre et al. 2017). GDM associated with lower transcriptional activity of the gene *SLC7A1* coding for the human equilibrative nucleoside transporter 1 (hENT1) isoform in HUVECs (Farías et al. 2010; Westermeier et al. 2011, 2015) and hPMECs (Salomón et al. 2012). This phenomenon results in lower expression of hENT1 and uptake of adenosine leading to extracellular accumulation of this nucleoside (Vásquez et al. 2004). Increased extracellular adenosine results in activation of A_{2A}AR increasing the activity of human cationic amino acid transporters 1 (hCAT-1) isoform for L-arginine. Since increased L-arginine transport via this membrane transporter isoform is coupled to the activity of eNOS, an increase in the generation of NO is seen. Interestingly, the umbilical whole and vein blood, but not umbilical arterial blood, show elevated adenosine concentration in GDM compared with normal pregnancies (Salomón et al. 2012). The latter is a phenomenon that seems not to be restricted to GDM since this increase in the adenosine concentration is also seen in whole umbilical blood from pregestational diabetes mellitus (Maguire et al. 1998). The increased extracellular concentration of adenosine in GDM could result from an adaptive mechanism of the growing foetus altering the placenta blood flow in this disease (Silva et al. 2017). Interestingly, Doppler approach shows unaltered blood flux velocity in umbilical vessels in GDM (Brown et al. 1990; Pietryga et al. 2006), a finding suggestive of unaffected umbilical vessels reactivity in this disease. However, the optimal diameter of human umbilical vein rings in vitro is lower in GDM compared with normal pregnancies (Westermeier et al. 2011), suggesting that molecules leading to vasodilation (v.g. adenosine or NO) could be permanently active in these vessels in GDM.

Alterations in the ALANO signalling pathway in HUVECs from GDM are restored by insulin in a physiological range of concentrations (up to 1 nmol/L) (Guzmán-Gutiérrez et al. 2016). Insulin is a vasodilator in most vascular beds, and this biological effect is mediated by activation of eNOS leading to NO generation in the human placenta vascular endothelium (Guzmán-Gutiérrez et al. 2016; Villalobos-Labra et al. 2017). Interestingly, insulin beneficial effects on vascular endothelial dysfunction are mediated by insulin receptors A (IR-A) isoform expressed in HUVECs (Westermeier et al. 2011, 2015; Subiabre et al. 2017) and hPMECs (Salomón et al. 2012). Activation of IR-A triggered protein kinase B/Akt signalling cascade leading to activation of eNOS and increased NO synthesis (Fleming 2010; Villalobos-Labra et al. 2017). In HUVECs from GDM pregnancies, insulin restoration of the upregulated ALANO activity to values in cells from normal pregnancies requires the expression and activation of A_1AR (Guzmán-Gutiérrez et al. 2016). Thus, a differential role of ARs is played in response to insulin in HUVECs from normal pregnancies, where A_{2A}AR is required, compared with GDM pregnancies, with A_1AR involvement instead, for L-arginine transport and NO synthesis. Unfortunately, it is not yet documented whether insulin modulation of hENT1- or hENT2-mediated adenosine transport in the human foetoplacental vasculature from GDM requires differential activation of A_1AR and A_{2A}AR or other ARs subtypes (Silva et al. 2017) (Fig. 22.1).

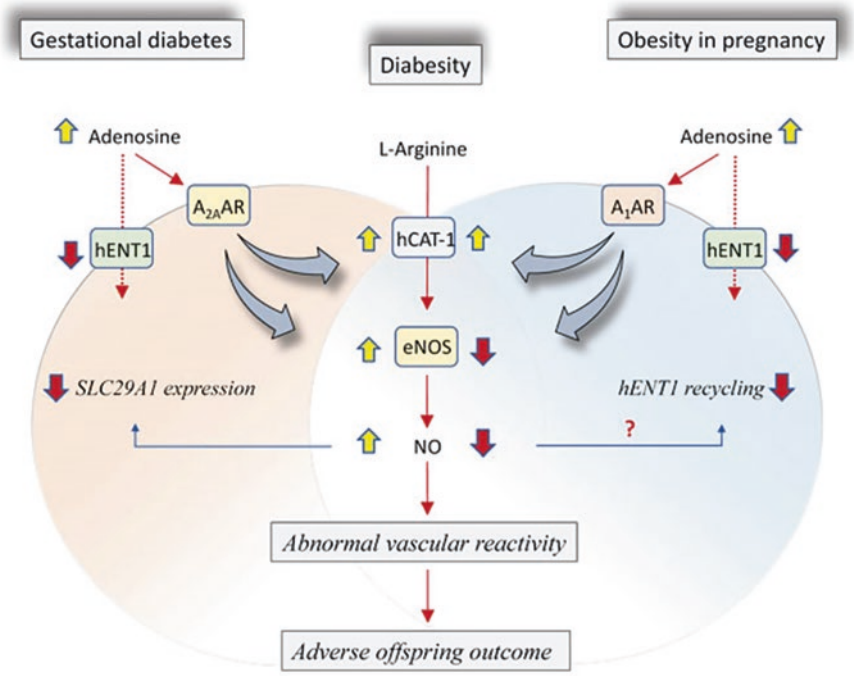

Fig. 22.1 Modulation of foetoplacental endothelial function by adenosine in gestational diabetes mellitus and maternal obesity in pregnancy**.** Gestational diabetes mellitus and maternal obesity in pregnancy reduce (⇩) the bioavailability and activity (dotted red arrows) of the human equilibrative nucleoside transporters 1 (hENT1) in human umbilical vein endothelial cells. A mix of the adverse effects of gestational diabetes mellitus and obesity in pregnancy results in a new condition with common alterations in HUVECs function referred as 'diabesity'. Reduced expression of hENT1 results from lower expression of the *SLC29A1* gene (for hENT1) but a potential (?) reduction in hENT1 recycling accumulating this type of nucleoside transporter in intracellular compartments. Reduced adenosine transport results in increased (⇧) extracellular concentration of adenosine leading to preferential activation of A_{2A} adenosine receptors ($A_{2A}AR$) in gestational diabetes mellitus but A_1AR in obesity in pregnancy. Activation of $A_{2A}AR$ and A_1AR increases L-arginine transport via the human cationic amino acid transporters 1 (hCAT-1). Increased L-arginine transport results in activation of the endothelial nitric oxide synthase (eNOS) (likely due to increased expression and activator phosphorylation of Ser^{1177}) in gestational diabetes mellitus but inactivation (likely due to reduced expression and increased inhibitory phosphorylation at Thr^{495}) in obesity in pregnancy. These phenomena lead to increased or reduced NO synthesis, respectively, thus altering the vascular reactivity (*abnormal vascular reactivity*). NO is crucial in downregulating *SLC29A1* expression and seems required for hENT1 recycling in HUVECs. Altered L-arginine transport and NO synthesis in the foetoplacental endothelium end in altered functional characteristics at delivery (*adverse offspring outcome*) with potential deleterious consequences at young and adulthood

22.4 Obesity

Obesity is epidemic worldwide (The GBD 2015 Obesity Collaborators 2017) and is defined by a body mass index (BMI) ≥30 kg/m^2 (World Health Organization (WHO) 2017). The number of women at reproductive age affected by this disease is large, a phenomenon that could result in long-term consequences for the offspring in its young and adulthood (UN general assembly 2015). This phenomenon is worth when women get pregnant being obese before pregnancy, i.e. pregestational maternal obesity (PGMO), with an adverse outcome including preeclampsia, congenital anomalies, and newborn macrosomia (Trojner Bregar et al. 2017). One of the critical elements that have been raised as determinant factors altering the normal foetus growth is the maternal gestational weight gain (GWG) in pregnancy (Henriksson et al. 2014; Pardo et al. 2015). When this total GWG (tGWG) parameter goes over the physiological range for a woman with normal pregestational BMI (tGWG = 11.5–16 kg and GWG rate (rGWG) ≥0.42 kg per week for the second and third trimester of pregnancy) (Henriksson et al. 2014) or as early as in the first trimester with rGWG ~0.11 kg per week (Pardo et al. 2015), several abnormalities in the placental vascular function are seen (Pardo et al. 2015, 2017). Since increased fat mass in the infants from women with normal prepregnancy BMI that develop supraphysiological GWG (spGWG) is seen (Henriksson et al. 2014), it is likely that abnormal placenta function under this abnormal metabolic condition of the mother will cause alterations in the offspring vasculature. Thus, attention should be put on the pregestational metabolic status of the mother aiming to prevent alterations in the foetus development and growth and adverse outcomes of the offspring and in their young and adulthood.

In the last years, a crucial role for adenosine has been identified in obesity including obesity in pregnancy (Pardo et al. 2015, 2017, 2018). However, few studies had actually measured the plasma concentration of this nucleoside. The adenosine plasma level in women with overweight or obesity in pregnancy is higher than in lean pregnant women (Badillo et al. 2017). The concentration detected in these patients is enough to activate A_1AR, A_{2A}AR and A_3AR receptors (Fredholm et al. 2011; Peleli et al. 2017). Thus, it is likely that activation of these if not all ARs in women with overweight or obesity in pregnancy could result in triggering signalling pathways that may be described as prone to obesity.

22.4.1 Vascular Dysfunction in Obesity in Pregnancy

Maternal obesity leads to alterations in the placental function including altered nutrients transport from the maternal to the foetal circulation (Lewis and Desoye 2017). Thus, a potential link between maternal obesity and offspring metabolic complications may involve an excess of this mechanism. The placenta from pregnant women with obesity or overweight shows altered vascular function

characterised by a lower endothelium-dependent relaxation of human umbilical vein rings to insulin (Pardo et al. 2015). Interestingly, the optimal diameter of these vessels was unaltered in women with spGWG compared with physiological GWG, suggesting a normal vessel tone. Thus, reduced relaxation in response to insulin is likely due to vasoreactivity to this hormone and not due to a dilated tone of these vessels in this abnormal metabolic condition. The reduced reactivity to insulin by human umbilical vein rings is a phenomenon that resulted from a lower NOS activity due to increased inhibitory phosphorylation of Thr^{495} in eNOS but not to a decreased activator phosphorylation of Ser^{1177} at this enzyme. Equally, spGWG associated with reduced eNOS protein abundance in HUVECs. Thus, reduced NO generation results from a combination of these two mechanisms, i.e. reduced expression and increased inhibition of eNOS. Interestingly, even when increased NO is shown to reduce the availability at the plasma membrane of hENT1 and in HUVECs from GDM pregnancies (San Martín and Sobrevia 2006), in this cell type from women with spGWG, inhibition of eNOS also resulted in lower uptake of adenosine leading to extracellular accumulation of this nucleoside (Pardo et al. 2015). This apparently contradictory result may result from two different states of metabolic stress in this cell type from these two conditions of pregnancy.

22.4.2 Role of Adenosine Receptors in Obesity and Obesity in Pregnancy

No studies are addressing whether ARs play a role in obesity in pregnancy in humans. Studies in rat show that A1AR may be involved in heavier offspring when treated in utero with caffeine (Buscariollo et al. 2014). Decreased cardiac output and increased left ventricular wall thickness were detected in these animals. Interestingly, heart global reduced DNA methylation was detected affecting genes associated with cardiac hypertrophy. Thus, A1AR are potentially involved in obesity in this experimental model. Offspring from mothers fed with low-protein during pregnancy/lactation are predisposed to obesity in rats. Under this approach, it was reported that adenosine enhanced the pyruvate dehydrogenase kinase 4 (PDK4) and peroxisome proliferator-activated receptor γ mRNA gene expression in adipocytes (Holness et al. 2012). However, adenosine-increased PDK4 expression was apparently not involved in stimulation of lipolysis in this cell type. Unfortunately, a characterisation of the subtypes of ARs potentially involved in PDK4 activation and lipolysis was not reported in this study. Studies reported in GDM show that women with this disease present with obesity or overweight (WHO 2017). In some of those studies, the group of pregnant was not separated between GDM in lean (normoweight, BMI <20 kg/m^2) (WHO 2017), overweight or obese women. Thus, the potential involvement of A_{2A}AR or A_1AR in HUVECs may reflect a mix of pathological conditions, i.e. GDM + obesity, a condition now referred as diabesity. This

is a phenomenon that was also detected in women with late-onset preeclampsia and obesity (Salsoso et al. 2015). In this case, separating lean pregnant women from those with obesity showed no differences for L-arginine transport and NO synthesis between these two groups. Thus, even when the differentiation is not yet reported for GDM and obesity in pregnancy, this could also be a possibility.

Contrary to the limited studies in pregnancy, several studies describe the role of adenosine receptors in obesity, and efforts have been made to elucidate their involvement in the development or in the establishment of obesity and its associated pathologies (Pardo et al. 2017, 2018). Activation of ARs with the general agonist 5'-N-ethylcarboxamidoadensoine (NECA) associated with glucose intolerance in lean mice (Figler et al. 2011). Meanwhile, systemic inhibition of ARs with 8-phenyltheophylline and BWA1433 increased insulin sensitivity in the muscle and liver but not in the adipose tissue in obese rats with insulin resistance (Budohoski et al. 1984; Challiss et al. 1992; Crist et al. 2001, 1998). In the muscle, the general antagonism of ARs reduces the impaired insulin signalling by lowering the activity of protein tyrosine phosphatase 1 leading to higher insulin-stimulated insulin receptors phosphorylation (Crist et al. 2001). This phenomenon suggests that general activation of ARs, i.e. not specific for each subtype or groups of ARs, results in reduced insulin sensitivity in this tissue. Thus, ARs expression pattern at different tissues could be involved in the development of the alterations associated with obesity. We propose that ARs role has to be studied at a tissue-specific level to evaluate their usefulness as a tool for prevention or as a therapeutic approach in maternal obesity in pregnancy (Pardo et al. 2017) as well as other diseases of pregnancy (Silva et al. 2017; Salsoso et al. 2017; Chiarello et al. 2018).

Activation of A_1AR improves insulin sensitivity in obesity (Dhalla et al. 2007; Schoelch et al. 2004). Even when the systemic A_1AR antagonism demonstrates improvement of D-glucose homeostasis (Xu et al. 1998), a reduced insulin-induced D-glucose uptake in adipose tissue is seen (Crist et al. 1998). It is reported that activation of A_1AR promotes adipogenesis in preadipocytes but activation of A_{2B}AR inhibited adipogenesis (Gharibi et al. 2012). Thus, A_1AR activation results in making an individual prone to obesity, but activation of A_{2B}AR could protect from this disease. It is worth noting that A_1AR is the subtype of ARs most highly expressed in adipose tissue (Kaartinen et al. 1991; Gnad et al. 2014). Furthermore, a higher adenosine level has been described in adipose tissue from obese patients (Kaartinen et al. 1991) and in pregnant women that are obese (Badillo et al. 2017); thus it is a phenomenon that could relate with activation of A_1AR in obesity in pregnancy. Interestingly, a recent study shows increased A_1AR expression in neurons of the paraventricular nucleus in diet-induced obese mouse, and A_1AR antagonists reduced the body weight by appetite suppression and increased energy expenditure (Wu et al. 2017). Thus, this subtype of ARs plays a role at a cellular level as well as controlling systemic neurohumoral responses to insulin in obese patients. Whether this phenomenon is happening in the human foetus in pregnancies where the mother is obese is unknown.

22.5 Concluding Remarks

Diabetes in pregnancy, particularly GDM, and obesity in pregnancy including PGMO are abnormal metabolic conditions that have a deleterious effect on the mother and the growing foetus leading to an adverse offspring outcome. This phenomenon regards with alterations in the placenta function including its vascular reactivity to vasodilators and vasoconstrictors. Adenosine is a vasodilator in the placenta vasculature following activation of ARs in the placenta micro- and macrovascular endothelium. GDM results in foetoplacental endothelial dysfunction involving activation of A_{2A}AR under the pathological foetal hyperinsulinaemia (~70 nmol/L compared with ~40 nmol/L insulin in a non-GDM pregnancy) seen in this disease (Westermeier et al. 2011; Guzmán-Gutiérrez et al. 2016). This phenomenon turns into recruiting a different ARs subtype, i.e. the A_1AR, when levels of insulin are over this pathological foetal hyperinsulinaemia (up to 1 nmol/L insulin) (Guzmán-Gutiérrez et al. 2016). Interestingly, insulin via IR-A activation restores GDM-associated alterations in endothelial function in the human placenta vasculature, a mechanism that required A_1AR expression and activation. Thus, a differential role for ARs is evident in the placental micro- and macrovascular endothelium response to vasodilators such as insulin with different consequences for the offspring.

Obesity in pregnancy and PGMO are abnormal conditions where the vascular reactivity of the human placenta vasculature is also altered. In this pathological state, the release of NO from HUVECs is decreased due to reduced eNOS expression and increased inhibitory phosphorylation of this enzyme. Equally, the response of placental vessels to insulin is reduced, or almost absent, potentially via endothelial dysfunction. Thus, GDM and obesity in pregnancy are abnormal metabolic conditions of the mother that have a deleterious consequence to the foetoplacental vasculature. Even when in GDM the maternal and foetal glycaemia is corrected by either a controlled diet or with the use of insulin (i.e. insulin therapy) or anti-hyperglycaemic pharmacological agents (v.g. metformin, glibenclamide), the alterations in the umbilical vessels are still seen at delivery. Similarly, when PGMO is present in human pregnancies, a controlled gestational weight gain to reach physiological rates of GWG may result in normalising the metabolic state of the mother and the foetus, but similar alterations to those in GDM pregnancies are still evident in the placenta and umbilical vessels at delivery. Involvement of ARs subtypes is unclear in obesity in pregnancy, and some limited evidence is available for GDM.

It is more than determinant to understand whether ARs are a target in GDM and obesity in pregnancy to plan therapeutic protocols in these disturbed pregnancies and even more important whether these receptors are essential in response to insulin in these states where insulin resistance is seen. Clarifying a role for ARs in this phenomenon may help to understand and generate therapeutic approaches to improve the adverse offspring outcome in GDM and obesity in pregnancy facilitating its intrauterine life, the first hours of life and young and adulthood.

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Chapter 23
Adenosine Receptors and Current Opportunities to Treat Cancer

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Abstract Adenosine is an endogenous modulator exerting its physiological effects by activating four A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors. This nucleoside increases in hypoxia that characterizes solid tumors, thus affecting vasculature, immunoescaping, and cancer growth. This chapter offers an updated overview on the current opportunities to treat tumors coming from the adenosinergic field. Several years of research has led to the conclusion that A_{2A} and A_3 subtypes are the most promising for drug development. As for A_3 receptors, consequent to the efficacy of their agonists in numerous animal models of cancer, the lead compound, Namodenoson, has entered in clinical trials for hepatocellular carcinoma. Phase I results proved its optimal safety profile and efficacy, so that phase II studies are in progress. Specifically, A_{2A} receptor is responsible for immunosuppressive effects, reducing antitumor immunity and promoting immunoescaping of cancer. Therefore, A_{2A} receptor antagonists have been proposed to fight cancer by enhancing immunotherapy, supported also by their safety already demonstrated in clinical trials for Parkinson's disease. Overall, from these positive results, it may be expected that A_3 agonists and A_{2A} antagonists may become future anticancer drugs with the ability to save and improve human health also for diseases with very limited treatment options.

Keywords A_{2A} receptors · A_3 receptor · Cell proliferation · Immunoescaping · Immunoprotection · Clinical trials

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23.1 Introduction

Adenosine affects cancer biology by regulating both tumoral and immune cells impacting on proliferation, metastasis, and angiogenesis, as well as producing immunosuppressive effects, respectively (Antonioli et al. 2014; Allard et al. 2016; Ohta 2016; Borea et al. 2016). This nucleoside is produced under physiological conditions at nanomolar levels, while it presents a huge increase, in the micromolar range, under low oxygen concentrations typical of tumors. In particular, CD73 and AK enzymes, affecting adenosine levels, undergo to an upregulation/inhibition, respectively, by hypoxia (Ohta 2016). The role of adenosine in cancer development dated back around 1997 with the seminal paper by Blay and coworkers, describing that extracellular fluid of solid tumors such as lung and colon adenocarcinomas is commonly hypoxic and contains sufficient amount of adenosine levels to inhibit antitumor immune response (Blay et al. 1997). Importantly, in that years, pioneering studies by Fishman's group demonstrated the reason why muscles are resistant to tumor metastases. Interestingly, their work revealed that adenosine and surprisingly natural ligands of its A_3 receptor subtype were released by muscle cells. Specifically, the last ones exerted a cytostatic effect on cancer cell proliferation, without affecting normal cell growth (Fishman et al. 1998; Bar-Yehuda et al. 1999, 2001; Ohana et al. 2001). In the same period, Sitkovsky's group of research on adenosine field focused on the nonpleonastic role of A_{2A} receptors in the reduction of inflammation and later on its role in tumor survival (Ohta and Sitkovsky 2001; Ohta et al. 2006). Interestingly, it was found that tumor cells were protected from immune damage in hypoxic tumor due to the inactivation of antitumor T cells by the simultaneous action of two hypoxia-driven mechanisms, identified in adenosine acting through A_{2A} receptors and the hypoxia-inducible factor 1 (HIF-1α) (Lukashev et al. 2007a). From that years, a lot of research has been conducted by scientists in the adenosine field around the world to identify A_3 agonists and/or A_{2A} antagonists as lead candidates for drug development, exploiting the double role of adenosine as antiproliferative and immunosuppressive molecule.

The aim of this review is to cover the current biological research on the most promising clinical candidates in the fight against cancer coming from A_3 and A_{2A} adenosine receptors field, identified in A_3 agonists and A_{2A} antagonists.

23.2 A_3 Adenosine Receptor Agonists

23.2.1 A_3 Receptor Marker

The involvement of receptor agonists activating the Gi-coupled A_3 adenosine receptor in tumor development has been demonstrated and well documented in the literature by means of both in vitro and in vivo studies (Fishman et al. 1998, 2000b, 2012; Merighi et al. 2003; Gessi et al. 2008, 2011). One of the most important evidence concerning the rationale to study the role of A_3 adenosine receptor agonists in

tumors derived from the evidence of a high expression of this subtype in several variety of cancer cells, spanning from leukemia and lymphoma to melanoma, glioblastoma, prostate, colon, and mesothelioma (Fishman et al. 2000b; Gessi et al. 2001, 2002, 2010a, b; Merighi et al. 2001, 2006; Ohana et al. 2003; Jajoo et al. 2009; Varani et al. 2011). Importantly, A_3 subtype overexpression was observed in colorectal, breast, thyroid cancer tissues as well as in malignant mesothelioma pleura, obtained from patients undergoing surgery in comparison to healthy counterpart (Madi et al. 2004; Gessi et al. 2004; Morello et al. 2008; Varani et al. 2011). This finding was reflected in peripheral neutrophils and lymphocytes, isolated from patients affected by colon and hepatocellular carcinoma (HCC), thus suggesting a role for this adenosine subtype as a possible new tumor marker (Gessi et al. 2004; Bar-Yehuda et al. 2008). Interestingly, these findings lead to the conclusion that A_3 adenosine receptors expressed in peripheral blood mononuclear cells (PBMC) mirror receptor status in tumor tissue (Gessi et al. 2004).

23.2.2 In Vitro Studies

As for the role of A_3 adenosine receptor agonists in cancer cells, antiproliferative effects have been reported (Merighi et al. 2005; Gessi et al. 2007; Varani et al. 2011). One of the first evidence of growth inhibition was attributed to the reduction of telomerase activity resulting in cytostatic effects (Fishman et al. 2000b, 2001). In addition, the A_3 receptor agonist inhibited prostate and malignant mesothelioma cell proliferation and migration while provoked cell cycle block and apoptosis (Jajoo et al. 2009; Morello et al. 2009; Varani et al. 2011; Aghaei et al. 2011). In cultured neural cancer and glioblastoma cells, the antitumor effect of A_3 adenosine receptors is increased by pulsed electromagnetic fields (PEMFs), by reducing NF-kB transcription factor and cell proliferation. Furthermore, PEMFs and A_3 adenosine receptor activation increase p53, cytotoxicity, and apoptosis in tumor cells (Vincenzi et al. 2012). Importantly, the intracellular signaling machinery involved in A_3 adenosine receptor-mediated tumor growth inhibition was established (Fishman et al. 2002b, 2004, 2012; Merimsky et al. 2003). Specifically, the molecular mechanism induced by A_3 adenosine receptor concerns deregulation of the Wnt pathway, stimulating cell cycle progression and cell proliferation in embryogenesis and tumorigenesis. In particular, inhibition of PKA and PKB/Akt increases glycogen synthase kinase 3β (GSK-3β) activity, resulting in phosphorylation and ubiquitination of β-catenin as well as inhibition of cyclin D1 and c-myc level. Also the reduction of NF-kB, a promoter of apoptosis, is involved in the ability of A_3 adenosine receptor agonist to decrease melanoma and hepatocellular carcinoma growth (Fishman et al. 2012) (Fig. 23.1).

The behavior of A_3 adenosine receptor agonists was particularly interesting and peculiar for their double role in white normal versus tumoral cells (Ohana et al. 2001). Indeed, they exerted immunosuppressive effects in solid tumors, through stimulation of the granulocyte colony-stimulating factor (G-CSF) by PBMC, thus increasing murine bone marrow cell proliferation. Accordingly, treatment with

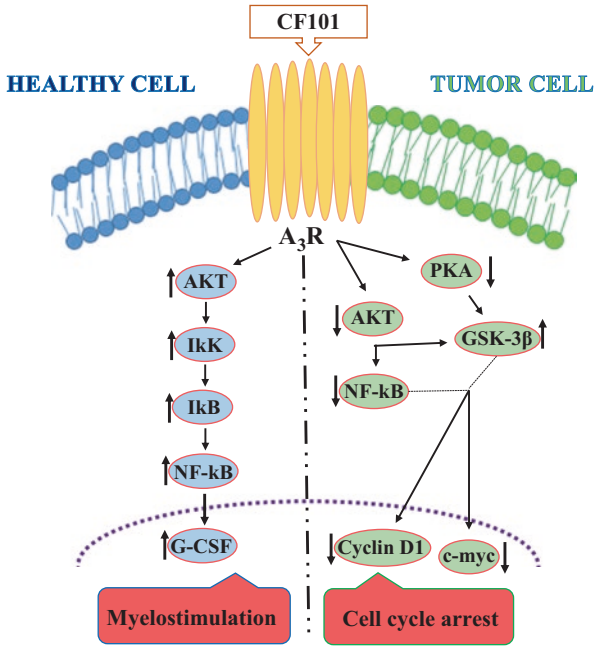

Fig. 23.1 Schematic representation of the principal pathways of A_3AR in healthy and tumor cells. Agonist stimulation of A_3AR leads to myelostimulation, via a signaling involving increase of AKT, NF-kB, and G-CSF, in healthy cells, and cell cycle arrest through a pathway involving inhibition of PKA and AKT, increase of GSK-3β, destabilization of β-catenin, and reduction of cyclin D_1, c-myc, in cancer cells

adenosine before chemotherapy induced an increase in leukocyte and neutrophil numbers (Fishman et al. 2000a, 2001; Hofer et al. 2006). This effect was obtained following activation of PI3K, PKB/Akt, IKK, and NF-kB signaling molecules (Bar-Yehuda et al. 2002; Merimsky et al. 2003). In addition, A_3 receptor enhanced natural killer (NK) cell activity and most likely the NK cell-mediated destruction of tumor cells (Ohana et al. 2003; Harish et al. 2003). Furthermore, ex vivo treatment of CD8+ lymphocytes with the A_3 adenosine receptor agonist increased TNF-α production resulting in anticancer therapeutic efficacy, when these cells were injected to mice (Montinaro et al. 2012).

23.2.3 In Vivo Studies

The effect of A_3 receptor agonists, IB-MECA and Cl-IB-MECA, was investigated in various syngeneic, xenograft, orthotopic, and metastatic experimental animal models of melanoma, colon, prostate, and hepatocellular carcinomas, through oral administration, allowed by their good stability and bioavailability (Van Troostenburg et al. 2004; Jacobson et al. 2017).

Initial studies demonstrated that administration of skeletal muscle cell-conditioned medium to mice inoculated with melanoma or sarcoma cells decreased metastatic lung foci (Bar-Yehuda et al. 1999). Indeed, it was found in a syngeneic

model of melanoma that natural A_3 receptor agonists instead of adenosine were responsible for the cytostatic effect (Bar-Yehuda et al. 2001).

Subsequently, it was shown that Cl-IB-MECA, given to melanoma-bearing mice, blocked the growth of melanoma lung metastases and produced a synergistic antitumor effect with cyclophosphamide, preventing its myelotoxic effect (Fishman et al. 2001, 2002a; Merimsky et al. 2003). Then, IB-MECA demonstrated its efficacy as anticancer molecule in a xenograft model of prostate carcinoma (Fishman et al. 2003). Markedly, it suppressed the development of B16-F10 melanoma tumor growth, in a way blocked by the A_3 receptor antagonist, MRS1523. In tumor lesions derived from these animals, A_3 receptor, c-Myc, and cyclin D1 were decreased, while GSK-3β was increased (Madi et al. 2003). In addition, CF101 (IB-MECA), orally administered, inhibited the expansion of primary tumors in xenograft and syngeneic models of colon carcinoma cells. This compound inhibited colon cancer liver metastases in syngeneic mice by increasing interleukin-12 secretion and enhancing NK cell activity. In the colon xenograft model, its administration with 5-fluorouracyl (5-FU) showed an additive anticancer effect, with prevention of 5-FU-dependent myelotoxicity (Ohana et al. 2003; Bar-Yehuda et al. 2005). The molecular mechanism triggered by CF101 to inhibit colon carcinoma growth in mice was attributed to the modulation of the key proteins GSK-3β and NF-kB, confirming previous studies (Fishman et al. 2004, 2009). Finally, Cl-IB-MECA reduced hepatocellular tumor growth and liver inflammation in a xenograft animal model (Bar-Yehuda et al. 2008; Cohen et al. 2011). Accordingly, it inhibited tumor growth and cancer pain in rat bone-residing breast cancer (Varani et al. 2013).

23.2.4 Human Studies

The A_3AR agonist Cl-IB-MECA (Namodenoson, CF102) has entered to clinical trials for advanced HCC therapy. It resulted safe, well tolerated, efficacious to augment a *median overall survival* (OS) by 7.8 months in phase I/II (NCT00790218) clinical trials (Stemmer et al. 2013). A global phase II trial in this patient population is currently ongoing, and patient enrolment has been completed (phase II, NCT02128958). Data are expected on second half of 2018.

23.3 A_{2A} Adenosine Receptor Antagonists

23.3.1 The Hellstrom Paradox

A different approach to fight cancer recruiting adenosine machinery resides in the exploitment of the role of this nucleoside in the immune system. It is well established that adenosine is fundamental in the reduction of immune response (Ohta and

Sitkovsky 2001). Specifically, the main actor for this process is the A_{2A}AR, expressed on the surface of T cells. This receptor subtype, coupled to Gs proteins by increasing cAMP and decreasing TCR-induced signaling, inhibits the capability of T cells to exert their effector functions, thus avoiding excessive inflammation during tissue injury and allowing adenosine to cover the role of guardian angel (Borea et al. 2016). However, this immunosuppressive behavior should be avoided in the heart of solid cancer where the Hellstrom paradox, which is the coexistence of both tumor cells and antitumor T cells, has been demonstrated (Lukashev et al. 2007b). It is well known that solid tumors present low oxygen concentration resulting in transient or chronic hypoxia, where the hypoxia-inducible factor 1 (HIF-1) is responsible for both inhibition of TCR-mediated T cell response and increase of angiogenesis, thus worsening patient prognosis. Inside this picture, it has to be considered the increased production of adenosine in solid tumors, as a consequence of hypoxic inhibition of adenosine kinase and stimulation of 5′-nucleotidase, helping tumors to evade immune destruction (Borea et al. 2017).

23.3.2 Vitro Studies

Indeed, A_{2A} adenosine receptor molecular pathway on several immune cell types, such as macrophages, dendritic cells, and lymphocytes, is essential to reduce their effector function, by triggering the cAMP/PKA immunosuppressive signal (Lappas et al. 2005b; Bruzzese et al. 2014). There are several immune responses that A_{2A} adenosine receptor inhibits to affect inflammation such as the reduction of pro-inflammatory cytokine production, C2 activation, macrophage-induced phagocytosis, and superoxide anion generation (Huang et al. 1997; Khoa et al. 2001; Schnurr et al. 2003; Lappas et al. 2005a; Naganuma et al. 2006; Serra et al. 2016). Specifically, in lymphocytes the Gs-cAMP pathway that can be triggered by both A_{2A}/A_{2B} adenosine receptors provokes COOH-terminal Src kinase (Csk) phosphorylation with consequent TCR signaling reduction (Torgersen et al. 2002). This event leads to inhibition of CD8+ lymphocyte proliferation (Huang et al. 1997), T cell recognition and consequent destruction of cancer cell through perforin release (Pardoll 2002), overexpression of Fas ligand (Koshiba et al. 1997), and decrease of IFN-γ release (Sitkovsky 2003; Sitkovsky et al. 2004). In particular, IFN-γ produced by CD8+ T cells exerts a relevant antitumor effect through inhibition of tumor angiogenesis (Ohta et al. 2006). Furthermore, A_{2A} adenosine receptor activation on T cells specifically reduces pro-inflammatory cytokine expression leaving unaltered the anti-inflammatory cytokine production (Naganuma et al. 2006). Accordingly, adenosine inhibits both anti-melanoma-specific CD4+ and CD8+ cytotoxic activity and cytokine production, through A_{2A} adenosine receptor activation (Raskovalova et al. 2007).

The contribution of A_{2A} adenosine receptors to the immunoescaping of cancer arises also from their stimulatory effect of regulatory T (Treg) cells that suppress T cell functions allowing tolerance to self and avoiding autoimmune diseases and

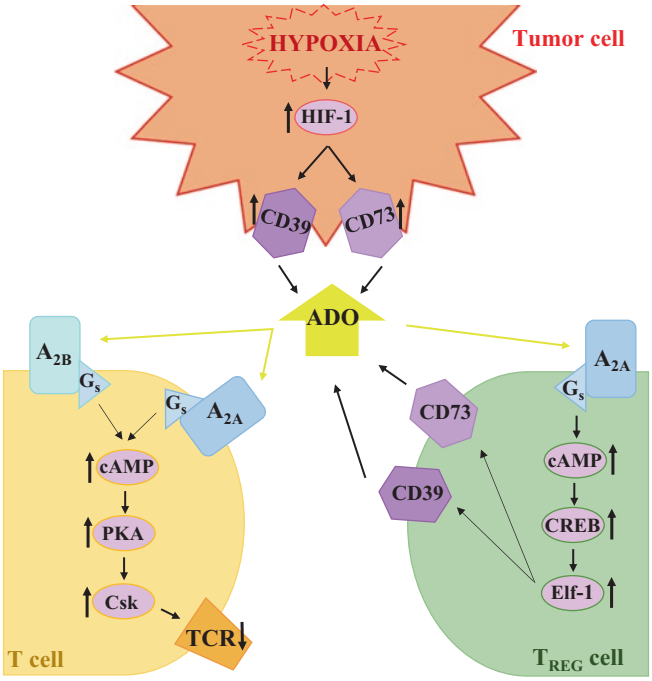

Fig. 23.2 Schematic representation of the adenosine-induced immunoescaping of cancer. During hypoxia, in tumor cells, HIF-1 upregulated CD39 and CD73, thus increasing adenosine concentrations in tumor microenvironment. Adenosine activates A_{2A} receptors, thus raising cAMP, PKA, and CSK that decrease TCR function, in T cells, and stimulates CD39 and CD73, thus further increasing its own release, in Treg

allograft rejections, thus increasing tumor immune evasion (Sitkovsky et al. 2008; Sitkovsky 2009; Bao et al. 2016). Interestingly, in tumors microenvironment apoptotic Treg cells release ATP and through CD39 and CD73 transform it to adenosine that mediates immunosuppression, via the A_{2A} adenosine receptor signaling. This event represents a new mechanism of immunoescaping in which Tregs die to potentiate immunosuppression in the hypoxic tumor (Maj et al. 2017). In the same cells, the expression of CD39 and CD73 was induced by A_{2A} adenosine receptor stimulation by triggering E2F-1 and CREB transcription factors (Bao et al. 2016) (Fig. 23.2).

23.3.3 In Vivo Studies

Importantly, A_{2A} adenosine receptors KO mice exhibited enhanced antitumor immune responses by CD8+ T cells, de-inhibition of IFN-γ release, as well as a survival improvement due to a dramatic reduction in the growth of experimental tumors, such as lung metastasis and melanoma, in comparison to wild-type controls

(Ohta et al. 2006; Beavis et al. 2013; Mediavilla-Varela et al. 2013). This phenomenon was observed also in other cancer types, such as the ovaric and breast ones (Jin et al. 2010; Loi et al. 2013). According to previous data, it has been found a reduction in lung metastasis and an increased responsiveness of human tumor-infiltrating lymphocytes, following treatment with a recently synthesized A_{2A} antagonist (Mediavilla-Varela et al. 2017). The utility of A_{2A} blockers against cancer has been observed in combination with anti-CTLA-4 and anti-PD-1/PD-L1 antibodies (mAbs), emerged as a tumor therapy with high potential, where they enhanced the efficacy of anti-PD-1 mAb. Specifically, they increased IFN-γ and Granzyme B by tumor-infiltrating CD8+ T cells and inhibited tumor growth and survival of mice, suggesting that the efficacy of anti-PD-1 mAb can be raised by A_{2A} antagonists (Beavis et al. 2015). As confirmation that the blockade of this receptor subtype is useful in cancer therapy and supporting previous results, it has been found that A_{2A} antagonist inhibited Tregs and increased the antitumor response of CD8+ T cells in a mouse model of head and neck squamous cell carcinoma (Ma et al. 2017). With this background, promising strategies to increase immunosurveillance of cancer will derive by the research of potent and selective A_{2A} adenosine receptor antagonists that may contrast adenosine in its task to immunosuppress tumors. However, it has to be considered that targeting A_{2A} receptors by both genetic manipulation and pharmacologic antagonists has limitations, due to the complementary role in immunosuppression of unaltered A_{2B} adenosine receptors, which may explain the failure of CD8+ T cell to destroy tumor. In particular, only ∼60% of mice lacking A_{2A} receptors inhibit tumor growth (Ohta et al. 2006). A_{2B} receptors have also been included as regulators of myeloid cells and of tumor progression (Ryzhov et al. 2008; Iannone et al. 2013). This explains why the therapeutic potential of double A_{2A}/A_{2B} receptor antagonism has been suggested for the therapy of tumor metastasis (Beavis et al. 2013; Hatfield and Sitkovsky 2016). Anyway novel interesting A_{2A} receptor antagonists have been recently synthesized and tested for their potential immunotherapeutic activity (Yuan et al. 2017).

23.3.4 Human Studies

From the data reported above and concerning the effects of A_{2A} receptors in immunosuppression, it has been promoted the use of A_{2A} receptors as promising targets for the development of a novel class of antitumoral drugs aimed to contrast the hypoxia-adenosinergic vicious circle. Importantly, existing A_{2A} antagonists in clinical development for Parkinson's disease present a good safety profile. Currently, some of them that have entered clinical studies include preladenant, in phase I for neoplasms (NCT03099161), PBF-509 in phase I/II for non-small cell lung cancer (NCT02403193), and CPI-444 in phase I for non-small cell lung cancer, malignant melanoma, renal cell cancer, triple-negative breast cancer, colorectal cancer, bladder cancer, and metastatic castration-resistant prostate cancer (NCT02655822).

23.4 Conclusions and Perspectives

The A_3 adenosine receptor represents a very attractive target for cancer therapeutic development by exploiting its dual antiproliferative effect toward cancer cells and protective effects toward normal body cells. A_3 adenosine receptor agonists have been shown to enhance NK cell activity and most likely the NK cell-mediated destruction of tumor cells, which presents the opportunity for synergistic combination therapy with cytotoxic agents or checkpoint inhibitor immunotherapy. Importantly, evidence of high or overexpression of A_3 adenosine receptor in serval cancer types, including leukemia, lymphoma, melanoma, glioblastoma, prostate, colorectal, hepatocellular, breast, thyroid, and mesothelioma, cast a wide potential commercial opportunity for A_3 adenosine receptor agonists.

Specifically, deregulation of the Wnt pathway and reduction of NF-kB signaling have particular relevance in HCC where the A_3 adenosine receptor agonist Namodenoson is being investigated in a phase II clinical trial. Encouraging preclinical data and positive overall survival data in earlier-stage clinical studies with A_3 adenosine receptor agonists in this indication provides optimism for targeting HCC, one of the fastest-growing cancers and a disease with very limited treatment options.

Although the field of A_{2A} antagonists appears very promising against cancer, for the future, it will be suggested the development of new receptor inhibitors unable to cross the blood-brain barrier, thus avoiding the occurrence of potential neurological side effects in cancer patients with noncerebral tumors, and characterized by a long in vivo half-life (Hatfield and Sitkovsky 2016). This aim could be reached exploiting the recent improvement on the knowledge of the molecular basis of A_{2A} receptors helping the structure-based design of new A_{2A}AR antagonist molecules (Carpenter et al. 2016; Jazayeri et al. 2017). In addition, due to their efficacy as immunological stimulators, several pharmaceutical groups are projecting clinical trials including immunotherapeutic drugs such as anti-PD-1 monoclonal antibody in combination with A_{2A} adenosine receptor antagonists to combat the hypoxia-A_2-adenosinergic immunosuppressive vicious cycle.

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Chapter 24
Role of Adenosine Receptors in Clinical Biophysics Based on Pulsed Electromagnetic Fields

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Abstract Clinical biophysics studies the effects of physical agents such as the low frequency low energy pulsed electromagnetic fields (PEMFs) utilized for the treatment of different human pathologies. Much research activity has focused on the mechanisms of interaction and the metabolic pathways involved between PEMFs and the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (ARs). In particular, PEMF exposure mediates a significant upregulation of A_{2A} and A_3ARs expressed in various cells and tissues present in both the peripheral and central nervous system involving primarily a significant reduction in some of the most interesting pro-inflammatory cytokines. Of interest is that PEMFs through the increase of ARs enhance the working efficiency of adenosine, producing a more physiological effect than the use of drugs without the side effects, desensitization, and receptor downregulation often related to the use of agonists. This observation suggests the hypothesis that PEMFs may be an interesting approach as a noninvasive treatment with a low impact on daily life mediating a significant increase on the effect of the endogenous modulator. In this chapter, the role of ARs and PEMFs and their relevance in various inflammatory diseases in both peripheral or in central nervous system disorders will be reported.

Keywords A_{2A} adenosine receptors · Pulsed electromagnetic fields
Chondrocytes · Synoviocytes · Osteoblasts · Neuronal and microglial cells

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24.1 Introduction

Adenosine, interacting with A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (ARs), plays an important role in human health with a behavior of guardian angel in various disease states exerting several protective effects against cell damage in both the brain and the periphery (Borea et al. 2016). Literature data suggest that the most promising candidates for clinical application could be agonists or allosteric enhancers of A_1, A_{2A}, and A_3ARs for inflammation, pain, and cancer (Jacobson et al. 2017; Varani et al. 2017a). Another important role of adenosine and ARs is linked to the management of ischemic damage and their potential protective effects (Gessi et al. 2011; Borea et al. 2015).

In clinical biophysics the study of the low frequency low energy pulsed electromagnetic fields (PEMFs) is relevant to better understand the development and the importance of the physical stimuli to control biological activities. Many in vitro or in vivo experiments have aimed to identify the biophysical stimulation induced by PEMFs as potential alternative to the pharmacological treatments in several inflammatory-related pathologies (Fini et al. 2013; Di Lazzaro et al. 2013; Varani et al. 2017b). It has been reported that PEMFs could act modulating cartilage and bone metabolism through the chondrocyte and/or osteoblast cell proliferation and the synthesis of extracellular matrix components showing various positive effects in the joint (Varani et al. 2017b). In particular, PEMFs stimulate proteoglycan synthesis without affecting the degradation suggesting their potential use to preserve the function and the integrity of the cartilage (De Mattei et al. 2003). A beneficial effect in human synoviocytes, chondrocytes, and osteoblasts is represented by a significant reduction of the most relevant pro-inflammatory cytokines and by stimulation of cell proliferation, inducing osteoblastogenesis (Ongaro et al. 2012; Sollazzo et al. 2010).

A specific PEMF exposure modulates the differentiation of human osteoblast function in a dependent manner by the extracellular signal-regulated kinases (ERK1/2) producing nontoxic amounts of reactive oxygen species (ROS) which induces antioxidative defense mechanisms (Ehnert et al. 2017). In a cellular model of Alzheimer's disease, PEMF treatment modulates the expression of specific genes that are dysregulated suggesting their role for tissue regeneration and their ability to stimulate cell proliferation and immune functions via the heat shock protein (HSP70) family (Capelli et al. 2017).

The combination of a biological treatment as bone marrow concentrate with PEMFs in a rabbit with osteochondral lesions of both knees significantly enhances the osteochondral regeneration by an improvement in cartilage cellularity and matrix parameters (Veronesi et al. 2015). PEMF therapy has also a positive effect on rat rotator cuff healing for each PEMF frequency and treatment duration tested with a collagen organization and a type I collagen and fibronectin expression improved in the treated groups (Huegel et al. 2017). Moreover, PEMFs preserve the structural integrity of subchondral bone in knee osteoarthritis rats by promoting the activation of Wnt/β-catenin signaling and osteoprotegerin (OPG)/receptor activator of NF-κB

ligand (RANKL)/receptor activator of NF-κB (RANK) signaling (Yang et al. 2017). It has been observed that in algodystrophy, a complex syndrome characterized by pain, allodynia, and hyperalgesia PEMFs increases osteoclast apoptosis, osteoblast viability, bone protein and matrix calcification, and antioxidant protein, while it decreases the levels of pro-inflammatory cytokines (Pagani et al. 2017).

In a prospective, randomized, and double-blind study, PEMFs used after arthroscopic surgery result in faster and complete functional recovery compared to controls in the short-term and in the follow-up (Zorzi et al. 2007). A systematic analysis of randomized controlled trials has reported that PEMFs significantly shorten time to radiological union and accelerate the time to clinical union for acute fractures. As a consequence, PEMFs are used in treating patients with delayed fracture healing (Hannermann et al. 2014). In the treatment of postmenopausal osteoporosis, PEMFs enhance osteoblastogenesis and inhibit osteoclastogenesis, thus contributing to an increase in bone mass and strength (Zhu et al. 2017). PEMF therapy reduces postoperative pain and narcotic use in the immediate postoperative period by a mechanism that involves endogenous interleukin (IL-1β) in the wound bed (Rohde et al. 2010). A randomized controlled trial in women has shown a positive effect of PEMFs versus ultrasound in the treatment of postnatal carpal tunnel syndrome evaluating, before and after treatment, different clinical parameters such as pain level, sensory and motor distal latencies and conduction velocities of the median nerve, functional status scale, and handgrip strength (Kamel et al. 2017). The efficacy of PEMFs in the reduction of postoperative pain was found in patients undergoing cesarean section with a significant decrease of postsurgical pain, analgesic use, surgical wound exudate, and edema (Khooshideh et al. 2017).

It is well reported that electromagnetic therapy has been extensively used in the clinical setting in the form of transcranial magnetic stimulation, repetitive or high-frequency transcranial magnetic stimulation, and PEMF therapy which can also be used in the domestic setting. From the cellular point of view, a protective effect of PEMFs has been found in a human neuroblastoma cell line, SH-SY5Y, and in rat pheochromocytoma PC12 cells on cell viability and on apoptosis in normoxic or hypoxic conditions (Vincenzi et al. 2012, 2017). In N9 microglial cells, a commonly used model to study inflammatory responses of microglial cells, PEMF exposure mediated a significant reduction of ROS production and of the most relevant pro-inflammatory cytokines (Vincenzi et al. 2017).

A beneficial effect of PEMF exposure has been found by inhibiting of hypoxia/reoxygenation-induced death of human renal proximal tubular cells or protecting the heart against ischemia/reperfusion-induced cardiac contractile dysfunction and heart injury (Lim et al. 2015; Bialy et al. 2015). Moreover, the electromagnetic stimulation may accelerate the healing of tissue damage following ischemia suggesting that PEMF exposure of short duration may have implications for the treatment of acute stroke (Grant et al. 1994). In a distal middle cerebral artery occlusion in mice, PEMFs significantly influence expression profile of pro- and anti-inflammatory factors in the hemisphere ipsilateral to ischemic damage and mediate a significant reduction of infarct size (Pena-Philippides et al. 2014). PEMF treatment

significantly reduces the apoptosis and ROS levels in primary neonatal rat cardiac ventricular myocytes induced by hypoxia/reoxygenation. In particular, PEMFs increase the phosphorylation of protein kinase B (Akt) and endothelial nitric oxide synthase (eNOS), which might be closely related to attenuated cell apoptosis by increasing the releasing of nitric oxide (NO) as a potential candidate for ischemia/reperfusion injury (Ma et al. 2016). In a murine model of hind limb ischemia, PEMF treatment stimulates multiple angiogenic pathways, improves blood perfusion, and reduces the necrosis or skin ulcers. Moreover, PEMFs inhibit the process of hypoxia-induced apoptosis and augment the migration and proliferative capacities of human umbilical vein endothelial cells (HUVEC). Additionally, PEMF exposure increases vascular endothelial growth factor (VEGF) secretion, as well as the eNOS and Akt phosphorylation, and these benefits could be blocked by either phosphoinositide 3-kinase (PI3K) or eNOS inhibitor suggesting their potential involvement as a valuable treatment for the patients with critical limb ischemia (Li et al. 2015).

The effects of PEMFs have been investigated in a randomized controlled trial in older adults with low bone mineral density, showing their potential beneficial effects on gait characteristics (Giusti et al. 2013). PEMF approach has been also studied for the management of several pathological conditions including neurodegenerative diseases such as Parkinson's disease. The mechanisms and therapeutic applications of PEMF therapy to alleviate motor and non-motor deficits have been clarified and no adverse event has been observed (Vadalà et al. 2015). It is noteworthy that in clinical translational studies a beneficial effect of PEMFs has been observed on improving function in osteoarthritis knees (Iwasa and Reddi 2017).

Increasing evidence suggests that the beneficial effects of PEMFs are mediated by the modulation of ARs, specifically by an upregulation of A_{2A} and/or A_3AR subtypes. A significant role of PEMFs in modulating AR activity in bovine or human chondrocytes, synoviocytes, or osteoblast has been well documented (Varani et al. 2017b). In particular, PEMFs interacting with A_{2A} and/or A_3ARs stimulate chondrocyte proliferation, differentiation, and extracellular matrix synthesis by the release of bone morphogenetic proteins and anti-inflammatory cytokines (Varani et al. 2017b).

The treatment with PEMFs induce a transient and significant increase in A_{2A}ARs expressed in rat cortex membranes and in rat cortical neurons dependent by the exposure time and intensity used (Varani et al. 2012). The protective effect of PEMFs on hypoxia damage in neuron-like cells and in anti-inflammatory effect in microglial cells supports that PEMFs could represent a potential approach in cerebral ischemic conditions (Vincenzi et al. 2017).

The beneficial effect of PEMFs has been investigated in various pathological conditions such as in cancer where PEMF treatment reduces tumor growth and proliferation (Jimenez-Garcia et al., 2010; Crocetti et al. 2013). Moreover, a significant effect on MCF-7 breast cells after treatment with PEMFs at the resonant frequencies of various genes such as peroxisome proliferator-activated receptor (PPARG) for specific durations of exposure has been observed (Alcantara et al. 2017).

A potentiated antitumor effect of A_3ARs by PEMFs has been found in different cell lines such as PC12 and human glioblastoma (U87MG) cell lines by the inhibition

of NF-kB and p53 activation together with the reduction of cell proliferation and an increase of cytotoxicity and apoptosis (Vincenzi et al. 2012). These data confirm previous research studies showing that A_3AR activation mediates potent antitumor effects in various in vivo and in vitro models (Gessi et al. 2008; Fishman et al. 2009, 2012; Varani et al. 2011, 2013) suggesting that PEMF treatment associated with potential anticancer drugs could be an example of noninvasive applications associated with cancer therapy.

From the cellular point of view, it could be of interest to note that PEMFs through the AR upregulation enhance the working efficiency of adenosine producing a more physiological effect than the use of drugs without side effects, desensitization, and receptor downregulation associated to the use of agonists. A prolonged stimulation of the membrane receptors with an exogenous agonist can dampen the ability to transduce the signal which is followed by the process of the receptor internalization into specific vesicles inside the membrane. The prolonged use of agonists decreases the receptor density by reducing the effect of the drug itself, while the PEMFs potentiate the effect of endogenous adenosine as an anti-inflammatory agent.

24.2 Biophysical Treatments and PEMFs in Complementary Therapy

The combined therapy including the use of physical agents and the drugs is feasible by the property of biological systems to absorb energy introduced as by other characteristics of the physical agent although the different biological targets could have specific susceptibility and sensitivities (Cadossi et al. 2017). The clinical biophysics studies the specificity of the physical agents, their mechanism of action and metabolic pathways, and their involvement in different pathological conditions. The specificity is defined as the capacity of the physical signal applied to the biological target to trigger a specific biological response that depends on its characteristics such as waveform, frequency, pulse duration, cycle, and amplitude. Another principle linked to the clinical biophysics is the recognition as the capacity of the biological target to identify the presence of the physical agents. The physical signal through the cell membrane modulates a series of intracellular events that mediate the biological response (Cadossi et al. 2017). In particular, different biophysical stimulations activate membrane channels and/or receptors suggesting a transitory increase in ion forces present in the cells (Brighton et al., 2001). Moreover, the effects of biophysical stimulation are local and limited to the site of application without systemic effects following the exposure of the physical energy in the form of electric currents, electromagnetic fields, and PEMFs (Aaron et al. 2004, 2006). An increasing number of studies have shown the biological effects of the PEMFs used for the cells and tissues in the peripheral and/or in the central nervous systems. The analyzed form of the PEMF exposure system (IGEA, Carpi, Italy) with an intensity range from 0.1 to 4.5 mTesla and the frequency range from 10 to 120 Hz

has been widely used in different experimental approach for in vitro experiments (Cadossi et al. 1992; Varani et al. 2008, 2012; De Mattei et al. 2009; Ongaro et al. 2011, 2012; Vincenzi et al. 2012, 2013, 2017).

PEMFs were generated by a pair of rectangular horizontal coils each made of 1400 turns of copper wire placed opposite to each other. The coils were powered by the PEMF generator system which produced a pulsed signal with the following parameters: pulse duration of 1.3 ms and frequency of 75 Hz, yielding a 10% duty cycle. The peak intensity of the magnetic field and peak intensity of the induced electric voltage were detected in air between two coils from one side to the other, at the level of the culture flasks. The peak values measured between two coils in air had a maximum variation of 1% in the whole area in which the culture flasks were placed. The most used peak intensity of the magnetic field was 1.5 mTesla, and it was detected using the Hall probe (HTD61-0608-05-T, F.W. Bell, Sypris Solutions, Louisville, KY) of a gauss meter (DG500, Laboratorio Elettrofisico, Milan, Italy) with a reading sensitivity of 0.2%. The corresponding peak amplitude of the induced electric voltage was 2.0 ± 0.5 mV. It was detected using a standard coil probe (50 turns, 0.5 cm internal diameter of the coil probe, 0.2 mm copper diameter), and the temporal pattern of the signal was displayed using a digital oscilloscope (Le Croy, Chestnut Ridge, NY). The shape of the induced electric voltage and its impulse length were kept constant.

Similar PEMF generator systems have been used for the in vivo assays both in animal models (Fini et al. 2002, 2005a, b, 2008, 2013; Benazzo et al. 2008a; Veronesi et al., 2014, 2015; Tschon et al. 2018) and in patients (Zorzi et al. 2007; Benazzo et al. 2008b; Gobbi et al. 2013; Cadossi et al. 2014; Adravanti et al. 2014; Servodio Iammarrone et al. 2016; Collarile et al. 2017).

Nowadays, several PEMF exposure systems have been developed with the aim to improve their application in in vitro experiments and in in vivo assays such as animal models or human subjects (Fig. 24.1).

24.3 Adenosine Receptors and PEMFs in Human Peripheral Blood Cells

The interaction of adenosine with A_{2A} and A_3ARs modulates the immune processes and controls the inflammatory status (Varani et al. 1997, 1998). On the other hand, PEMFs are able to influence the membrane functions by modulating the passage of ions and/or the distribution of proteins (Bersani et al. 1997; Chiabrera et al. 2000). The saturation binding experiments of A_{2A} or A_3ARs have shown that their affinity is in the nanomolar range and that PEMF treatment does not modify the ligand-receptor interaction mechanism. In contrast the A_{2A} or A_3AR, density is modified by PEMFs in function of the time and applied intensity with a significant increase in the number of A_{2A} or A_3ARs (Varani et al. 2002, 2003). These data suggest that the increase in the receptor density could be due to the translocation of the receptors

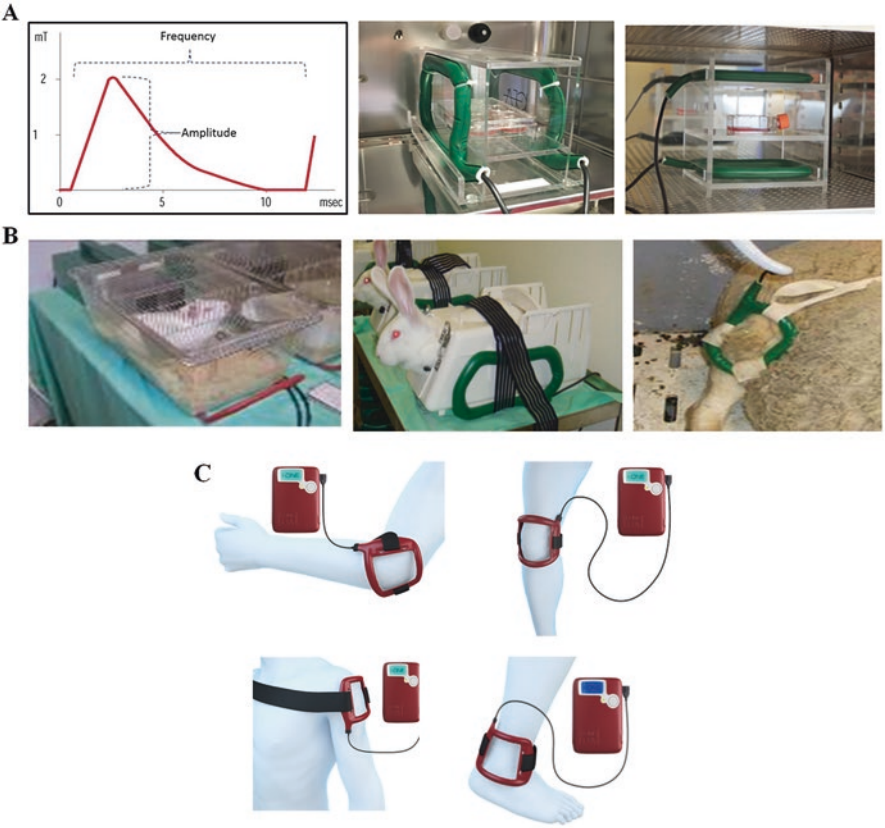

Fig. 24.1 PEMF treatment in various in vitro and in vivo assays. Scheme of the amplitude and frequency of the PEMF exposure system used (mT vs msec) and pictures of PEMF exposure in in vitro cellular models (**a**). Pictures of PEMF treatment in guinea pigs, rabbit, and sheep (**b**). Different pictures of PEMF exposure in human body for peripheral system disorders (**c**)

from cytoplasmic vesicles to the surface of the cell membrane. The effect of PEMFs is associated to A_{2A} and A_3ARs and do not influence the other adenosine subtypes as A_1 or A_{2B}ARs or different membrane receptors coupled to the G proteins such as the adrenergic or opioid receptors (Varani et al. 2002). Furthermore, PEMFs do not modify the adenylate cyclase activity in the absence or in the presence of forskolin, a direct activator of this enzyme, and in the production of cAMP. The potency of two A_{2A} or A_3AR agonists is considerably increased by the presence of PEMFs suggesting that the increase in the receptor density is closely related to the increase of their functionality (Varani et al. 2002, 2003). The cAMP and superoxide anion production is closely associated to A_{2A} or A_3ARs as demonstrated by the selective antagonists that are able to block the stimulatory effect of the agonists (Varani et al. 2002, 2003).

The kinetic binding parameters as the association and dissociation constants are similar in the absence or in the presence of PEMFs suggesting that the exposure to fields do not modify the disorder of the system within the receptor pocket and the ability to form hydrogen bonds involved in the interaction ligand-receptor. In addition, the binding thermodynamic parameters such as the standard free energy ($\Delta G°$), enthalpy ($\Delta H°$), and entropy ($\Delta S°$) are not modified by the presence of PEMFs confirming that the ligand-receptor interaction mechanism is not influenced by the treatment (Varani et al. 2002, 2003). The parameters obtained by the van't Hoff graphs indicate that the binding of A_{2A} and A_3AR antagonists is driven by enthalpic and entropic forces confirming the typical trend of ligands interacting with ARs (Varani et al. 2002, 2003). As a consequence, the thermodynamic parameters in untreated cells have shown values quite similar to those calculated after PEMF exposure (Varani et al. 2002, 2003). The receptor density (Bmax) appears to be closely dependent by PEMFs which mediated a significant upregulation of the examined receptors (Varani et al. 2002, 2003). These results are also quite similar to those obtained by studying the thermodynamic parameters of ARs in different cell lines where it is verified that the affinity of the agonists increases with temperature while the antagonist affinity decreases (Dalpiaz et al. 2000; Borea et al. 2000; Merighi et al. 2002; Gessi et al. 2008).

In the literature, several works propose that PEMFs are able to modify in different ways the blood cells and vascular system. In particular, PEMFs mediate an increase of the blood flow velocity of the smallest vein in patients affected by diabetes respect to untreated subjects (Sun et al. 2016). Moreover, an animal study has demonstrated that PEMFs could enhance angiogenesis in both normal mice and diabetic mice (Callaghan et al. 2008). Platelet-rich plasma (PRP) and PEMFs mediate a beneficial and an effective combination in terms of bone regeneration (Kapi et al. 2015). Interestingly, the effect of PEMFs on PRP mediates a beneficial and effective combination in terms of bone regeneration (Kapi et al. 2015). The effect of another biophysical stimulation such as pulsed electric field on some hematological parameters in rats has been studied suggesting that the exposure group induced a significant increase in the rates of white blood cells, red blood cells, hemoglobin, hematocrit, and platelets in blood when compared with control (Coskun and Comlecki 2013). In fibroblast-like cells derived from mononuclear peripheral blood cells, PEMF irradiation protocol decreases some of the most important pro-inflammatory cytokine secretions such as TNF-α and IL-1β and a significant increase in IL-10, a well-known anti-inflammatory cytokine (Gomez-Ochoa et al. 2011). The lymphocyte proliferation is modulated by in vitro exposure to PEMFs suggesting that the T-cell apoptosis in human tissues could be used to enhance healing by limiting the production of pro-inflammatory cytokines (Johnson et al. 2001).

PEMFs influence the viability of proliferating in vitro peripheral blood mononuclear cells (PBMCs) isolated from Crohn's disease patients as well as acute myeloblastic leukemia (AML) patients. Experiments with lymphoid U937 and monocytic MonoMac6 cell lines have shown a protective effect of PEMF on the death process in cells treated with death inducers. The analysis of expression of apoptosis-related genes reveals changes in mRNA of some genes engaged in the

intrinsic apoptotic pathway belonging to the Bcl-2 family and the pathway with apoptosis-inducing factor (AIF) abundance upon PEMF stimulation of PBMCs (Kaszuba-Zwoinska et al. 2015).

24.4 Adenosine Receptors and PEMFs in Articular and Bone Cells

It is well reported that cartilage lesions represent an important health problem for the highest rate of world disability primarily due to the limited regeneration capability of the cartilage. Several studies have been developed in the last decades to resolve this disability cause including physical stimuli approaches (Table 24.1). From the cellular point of view, various in vitro studies have reported in detail the effect of PEMFs on the articular cells such as chondrocytes and synoviocytes (Fig. 24.1). The ARs are expressed in these cell lines with variable density and only A_{2A} and A_3ARs are increased in the presence of PEMFs while A_1 and A_{2B}ARs show binding parameters similar to control condition (Varani et al. 2008). A_{2A} and A_3AR agonists reveal an effect amplified after PEMF treatment on the production of cAMP that is also blocked by the presence of selective A_{2A} and A_3AR antagonists (Varani et al. 2008).

Table 24.1 Effect of PEMFs and adenosine receptors in articular and bone cells or tissues

Cell lines or tissues	Main results	References
Bovine chondrocytes or fibroblast-like synoviocytes	A_{2A}AR and A_3AR upregulation Modulation of the cell proliferation Decrease of PGE_2 release	Varani et al. (2008), De Mattei et al. (2009)
Bovine articular cartilage explants	Proteoglycan synthesis increase	De Mattei et al. (2003)
Human fibroblast-like synoviocytes	A_{2A}AR and A_3AR upregulation Inhibition of TNF-α, IL-6 release Reduction of PGE_2 release	Varani et al. (2010), Ongaro et al. (2012)
Human cartilage explants	Increase of proteoglycan synthesis Counteract the catabolic activity of IL-1β	Ongaro et al. (2011)
Human osteoblasts or MG-63 cells	Increase of cell proliferation Induce osteoblastogenesis and differentiation	Sollazzo et al. (2010)
Human chondrocytes T/C-28a2 or osteoblasts hFOB 1.19	A_{2A}AR and A_3AR upregulation Modulation of cAMP levels Decrease of NFkB activation, IL-6, IL-8, PGE_2, VEGF production Increase of cell proliferation and OPG levels	Vincenzi et al. (2013)
Human tendon cells	Increase of cell viability and IL-10 production Increase of cell proliferation, VEGF-A mRNA and protein	De Girolamo et al. (2013, 2015)

The cell proliferation has been significantly inhibited by A_3AR stimulation and by the presence of PEMFs, while the copresence of the A_{2A} agonist and PEMFs increases the cell proliferation (Varani et al. 2008). A_{2A} and A_3AR activation after PEMF treatment reduces the release of prostaglandin E2 (PGE2) and the expression of cyclooxygenase type 2 (COX-2) suggesting their involvement in the reduction of inflammation and cartilage degradation associated with joint disease (De Mattei et al. 2009). Moreover, A_{2A} and A_3ARs have been studied and characterized in human synoviocytes where their stimulation reduces the release of TNF-α and IL-8 and inhibits the p38 MAPK and NF-kB activation (Varani et al. 2010). In human synoviocytes, the treatment with PEMFs determines a significant upregulation of A_{2A} and A_3ARs as demonstrated by mRNA experiments, Western blotting, and saturation-binding experiments (Ongaro et al. 2012). In these cells, A_{2A} and A_3AR stimulation mediates a significant reduction in the release of PGE2, IL-6, and IL-8 and the increase in IL-10 release (Ongaro et al. 2012). PEMF exposure to T/C-28a2 cells, a line of human chondrocytes, and hFOB 1.19 cells, a line of human osteoblasts, mediates a statistically significant increase in A_{2A} and A_3ARs also confirmed through RT-PCR assays, Western blotting, and saturation-binding experiments (Vincenzi et al. 2013). A positive anti-inflammatory effect by the A_{2A}AR stimulation in the presence of PEMFs also mediated an increase in chondrocytes and osteoblasts proliferation and a reduction of various pro-inflammatory mediators (Vincenzi et al. 2013). In human chondrocytes the stimulation of A_{2A} and A_3ARs reduces vascular endothelial growth factor (VEGF), an important mediator of angiogenesis. Moreover, the effect of A_{2A} and A_3AR in the presence of PEMFs has been studied on the activation of OPG, a protein capable of blocking the binding of the receptor activator of RANKL to RANK. In particular, PEMFs increase the release of OPG able to inhibit the differentiation and activation of osteoclasts. The activation of NF-kB, which strongly inhibits by A_{2A} and A_3AR stimulation in the presence of PEMFs, is essential for the synthesis and the activation of pro-inflammatory cytokines and of other mediators involved in joint inflammation and bone diseases (Vincenzi et al. 2013). The increase of A_{2A} and A_3AR expression and functionality induced by PEMFs indicate a significant cellular compensatory mechanism to counteract the inflammatory state as suggested by the reduction of the pro-inflammatory cytokines (Fig. 24.2).

Several papers have shown that PEMFs increase chondrocyte proliferation and the synthesis of specific cartilage components including proteoglicans, collagen type II, and IGF-1, the main cartilage anabolic growth factor (De Mattei et al. 2003; Massari et al. 2007; Ongaro et al. 2011). The rationale for using PEMFs in tissue-engineering techniques involved in cartilage repair is based on different findings such as the increase in anabolic activity of chondrocytes and cartilage explants exposed to PEMFs and preventing the catabolic effects of inflammation due to the significant reduction of pro-inflammatory cytokines mediated by AR involvement (Fini et al. 2005a, b).

Moreover it has been reported that PEMFs represent a potential candidate for the prevention and treatment of osteoporosis stimulating osteoblastic differentiation and mineralization making a selective osteogenic effect possible (Xie et al. 2016).

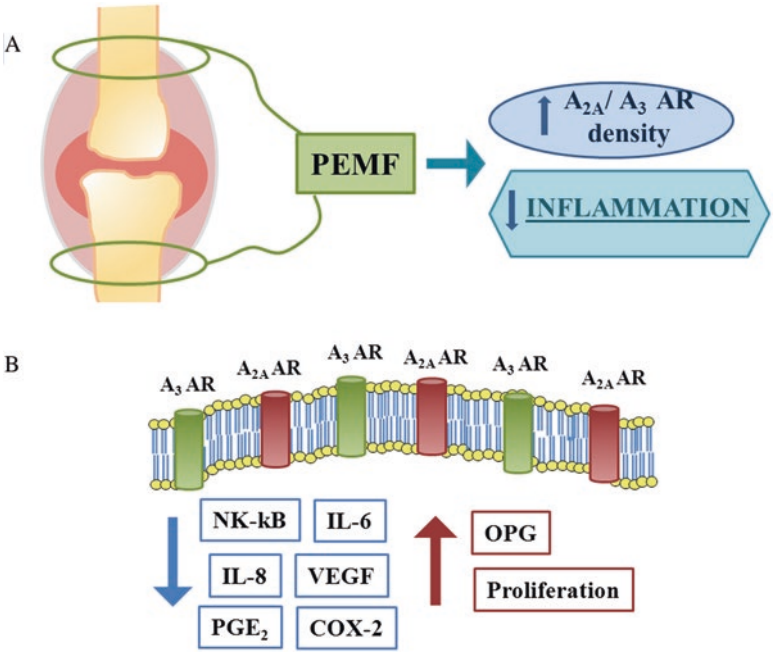

Fig. 24.2 Effect of the biophysical modulation on A_{2A} and A_3ARs in joint cells. Scheme representing the therapeutic potential of PEMFs on ARs (**a**) and on the inflammatory response of the cell (**b**). Nuclear factor kB (NF-kB), vascular endothelial factor (VEGF), prostaglandin E2 (PGE2), cyclooxygenase type 2 (COX-2), interleukin IL-6, IL-8, and osteoprotegerin (OPG)

Biophysical therapies such as PEMFs are used in chronic tendinopathy which is a degenerative process causing pain and disability. The functional in vitro response of human tendon cells to different dosages of PEMF is primarily represented by an increase of cell proliferation, of VEGF-A mRNA transcription, and of its related protein (de Girolamo et al. 2013). None of the different dosages of PEMFs used provokes apoptotic events while mediates a significant increase of cell viability and IL-10 production (de Girolamo et al. 2015).

PEMFs, as a safe noninvasive method, might become a promising biophysical modality for enhancing the repair efficiency and quality of the implants in bone defect increasing cellular attachment and osteoblast proliferation and inducing well-organized cytoskeleton (Jing et al. 2016). PEMFs also affect the osteogenic differentiation as an effective, noninvasive, and safe treatment method for a variety of clinical conditions, especially in settings of recalcitrant healing, and can be considered an appropriate candidate to accelerate repairing process (Ferroni et al. 2016). Substantial evidence indicate that PEMFs enhance fracture healing and bone mass inducing a well-organized cytoskeleton and promote formation of extracellular matrix mineralization nodules stimulating osteoblastic functions through a selective approach on the Wnt/β-catenin signaling-associated mechanism and

regulates downstream osteogenesis-associated gene/protein expressions (Zhai et al. 2016).

The biophysical stimulation of the bone and cartilage by using PEMFs covers many different aspects of bone formation and/or cartilage repair, such as healing of risk fracture, delayed fractures, nonunion, bone necrosis, edema, and osteocartilaginous defects (Fini et al. 2002, 2005a, b, 2013; Cadossi et al. 2015). Several clinical advantages, in terms of early recovery, histological, and histomorphometric parameters in patients suffering of severe osteoarthritis, have been reported (Veronesi et al. 2014). Moreover, PEMF stimulation around hip or knee joint implants could be useful to reduce the bone edema and pain and to reduce excessive bone reabsorption around the femoral stems (Massari et al. 2007, 2015). It has been found that PEMFs counteract the catabolic activity of IL-1β in OA patients and increase proteoglycan synthesis and that PEMFs and IGF-1 increase the cartilage explant anabolic activities and the proteoglycan synthesis (Fini et al. 2008). Therefore, PEMFs have a chondroprotective effect in OA progression in the knee joints of guinea pigs reducing the lesion progression in all examined knee areas and reducing histomorphometric measurements of cartilage thickness, fibrillation index, and subchondral bone thickness (Fini et al. 2008). After the PEMF treatment in the osteochondral autografts in sheep, IL-1β and TNF-α are lower, and TGF-β is significantly increased in the synovial fluid exerting a positive effect on osteochondral graft healing by favoring early subchondral bone integration and by limiting inflammation and cartilage degeneration (Benazzo et al. 2008a). A combined effect of PEMFs and bone marrow concentrate in rabbit was found where they improve the quality of the regenerated tissue and matrix parameters increasing the cartilaginous tissues healing and protecting the cells from the catabolic effects of inflammation (Veronesi et al. 2015). In addition, a combination of PEMFs and PRP increases cell viability over time analyzed and reduces osteoclastogenesis in comparison to PRP alone (Tschon et al. 2018). As a consequence, a double role for PEMFs could be designed as a strategy for construct defense (Fini et al. 2013): (a) in vitro, by stimulating cell proliferation, colonization of the scaffold, and production of tissue matrix and (b) in vivo, after surgical implantation of the construct, by favoring the anabolic activities of the implanted cells and surrounding tissues and protecting the construct from the catabolic effects of inflammation (Fig. 24.1).

Several clinical studies of articular biophysics have been performed from the chondroprotection to the patient's care in different pathologies such as early osteoarthritis, spontaneous osteonecrosis of the knee, osteochondral defects, microfractures, anterior cruciate ligament and meniscectomy, total knee arthroplasty, and arthroscopy surgery (Marcheggiani Muccioli et al. 2013; Cadossi et al. 2015). After PEMF treatment is evident, a better functional recovery after arthroscopy surgery and the treatment aided patient recovery reducing the use of nonsteroidal anti-inflammatory drugs (Zorzi et al. 2007). The use of PEMFs should be considered after anterior cruciate ligament reconstruction to shorten the recovery time, to limit joint inflammatory reaction and its catabolic effects on articular cartilage, and for joint preservation (Benazzo et al. 2008b). One month after total knee arthroplasty, pain, knee swelling, and functional score were better after PEMF-treated patients,

and after a long-term follow-up, the walking limitations were reported in a significantly lower number of treated patients compared with controls (Adravanti et al. 2014). The possible advantage of the combination of low analgesic drug doses with the PEMF therapy might reduce the risk of adverse and/or systemic effects that increase when nonsteroidal anti-inflammatory drugs are administered at high doses (Fig. 24.1).

The effects of the biophysical stimulation are linked to the anabolic on cartilage tissue angiogenic, analgesic and anti-inflammatory effects, osteogenic on bone tissue, and reduction of osteoclastogenesis. In particular, the short-term effects are dependent on analgesic effect, low intake of nonsteroidal anti-inflammatory drugs, best articulation movement, and functional time half-life, while the long-term effects are primarily linked to improved quality of the patient's life.

24.5 Adenosine Receptors and PEMFs in Neuronal and Microglial Cells

Several papers show that PEMFs could be considered an interesting therapeutic approach for the management of various pathological conditions including neurodegenerative diseases and stroke. It has been reported that different biophysical stimuli have been extensively used in the form of transcranial magnetic stimulation, repetitive or high-frequency transcranial magnetic stimulation, and PEMF therapy. Neurophysiological studies report measurable changes in brain electrical activity after PEMF exposure suggesting that they can influence neuronal functions such as motor control, sensory perception, cognitive activities, and sleep and mood (Cook et al. 2002, 2006; Di Lazzaro et al. 2013). Various techniques have been characterized to better investigate PEMF effect such as electroencephalogram (EEG) that permit to correlate the effects of the exposures on the power of the corresponding EEG bands (Cook et al. 2004; Cvetkovic and Cosic 2009). An additional way to investigate the effects of PEMFs on brain activity is represented by the evoked potentials currently used in the clinical practice as useful and noninvasive tools to study the function of the somatosensory, motor, visual, and auditory pathways (Di Lazzaro et al. 2013). Interestingly, PEMF treatment produces a significantly increase of the intracortical facilitation related to cortical glutamatergic activity (Capone et al. 2009). The effects of PEMFs on human pain perception are closely associated to a significant increase in pain sensitivity associated with changes in cardiovascular regulation such as slight increase in blood pressure and abnormal response of heart rate variability (Ghione et al. 2004, 2005). Specific PEMFs produce an improvement of the standing balance and of the normal postural sway increasing the activity of the motor system (Thomas et al. 2001; Prato et al. 2001). PEMFs can modify the postural tremor features facilitating the decrease of tremor intensity in a manner comparable to the relaxation (Legros and Beuter 2005, 2006). In particular, the therapeutic applications of PEMFs are widely used to alleviate Parkinson's disease

motor and non-motor deficits in different phases of the pathology (Vadalà et al. 2015). The positive effect of PEMFs has been studied in two cognitive dimensions such as the visual discrimination and flexibility which analyze the performance of the exposed subjects (Barth et al. 2010). PEMFs also produce an improvement in emotional states by a direct influence on brain emotional circuits (Stevens 2007). PEMF stimulation has been found as a promising strategy for treatment-resistant depression, and this may be specifically attributable to its effects on local brain and connectivity (van Belkum et al. 2016).

According to the World Health Organization, 15 million people suffer stroke worldwide each year, and of these 5 million die, and 5 million are left permanent disabled. Stroke is a leading cause of serious long-term disability and has relevant clinical and socioeconomic impact. Epidemiological studies have identified several risk factors for ischemic stroke, including hypertension, smoking, diabetes mellitus, and hemostatic factors (Arnao et al. 2016). The pathophysiology of the stroke involves different events such as excitotoxicity mechanisms, inflammatory pathways, oxidative damage, ionic imbalances, apoptosis, and angiogenesis (Chen et al. 2011). Therapeutic strategies in stroke have been developed by using the restoration of cerebral flow and the minimization of the deleterious effects of ischemia on neurons (Deb et al. 2010). There is great interest in the development of novel therapies for acute stroke because to date thrombolysis is the only approved treatment to improve long-term outcome. In a rabbit model of stroke obtained from transorbital clip occlusion of the left internal carotid, PEMF exposure is able to attenuate cortical ischemia edema at the most anterior coronal level by 65% and reduce ischemic neuronal shrinkage in the rabbit cortical regions and in the striatum (Grant et al. 1994). These preliminary data have suggested that exposure to PEMFs of short duration may have implications for the treatment of acute stroke.

In cerebral ischemic strokes is often focal the blood flow distribution because in the central core regions of the insult there is almost total cerebral blood flow arrest. This area evolves rapidly toward death within a minute even if functional thresholds transiently lie above the threshold of cell death in a particular zone called the penumbra which permits only cell survival for a certain period of time (Xing et al. 2012). Therefore, neuronal damage represents a crucial event as well as the role of microglial cells that are the immunocompetent cells of the central nervous system able to produce diverse substances such as ROS, nitrogen-like nitric oxide, and several classes of the pro-inflammatory cytokines (Kettenmann et al. 2013). These cytokines up-regulate cell adhesion molecules and exert an important role in promoting blood cell infiltration and accumulation in ischemic tissue (Wang et al. 2007).

To better clarify the PEMF mechanism of action, in vitro assays have been carried out showing a selective effect of PEMFs on the affinity and density of ARs in rat cerebral cortex and in cortical neurons (Varani et al. 2012). Saturation-binding experiments to A_{2A}ARs showed a transient time-dependent effect of PEMFs in the brain in toto and a constant effect in the membrane treatment from 2 to 8 h of exposure. The increase of A_{2A}ARs by PEMFs in primary cultures of rat cortical neurons is quite comparable to those present in the brain and closely dependent from the

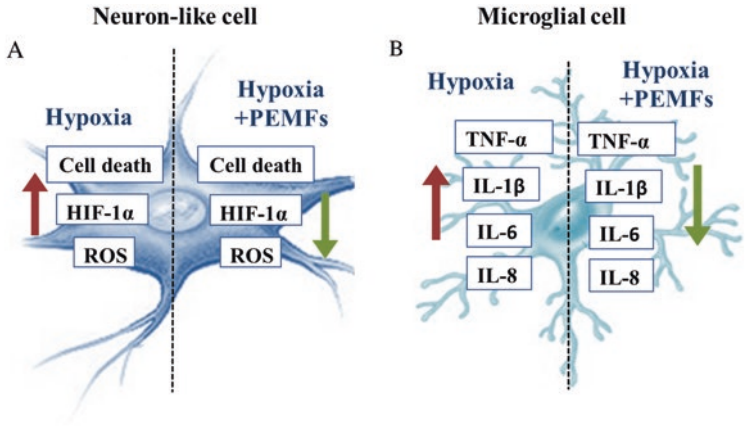

Fig. 24.3 Effect of the biophysical modulation in neuron-like cells (**a**) and in N9 cells (**b**). Pulsed electromagnetic field (PEMFs), tumor necrosis factor-α (TNF-α), hypoxia-inducible factor-1α (HIF-1α), reactive oxygen species (ROS), and interleukin IL- 1β, IL-6, and IL-8

time and intensity of PEMF exposure (Varani et al. 2012). PEMF exposure mediates a significant increase of A_{2A} and A_3AR density without alteration in the affinity values in neural cell lines such as U87MG and PC12 cells suggesting that the receptor upregulation could involve modifications linked to the receptor recycling on the cell membrane associated with a regulatory effect at the transcriptional level (Vincenzi et al. 2012). A direct neuroprotective effect of PEMF exposure in PC 12 and SH-SY5Y cells subjected to hypoxic insult was reported where PEMFs partially restore hypoxia-inducible factor-1α (HIF-1α) activation and inhibit ROS production following hypoxic incubation (Vincenzi et al. 2017). In N9 microglial cells, PEMF exposure reduces ROS generation and pro-inflammatory cytokine release strictly associated to ischemic condition (Vincenzi et al. 2017). These results show a protective effect of PEMFs on hypoxia damage in neuron-like cells and an anti-inflammatory effect in microglial cells suggesting that PEMFs could represent a potential therapeutic approach in cerebral ischemic conditions (Fig. 24.3).

Several approaches have been performed in human patients by using various biophysical treatments such as the transcranial brain stimulation and PEMFs used as signal pulse, with a frequency of 75 ± 2 Hz and a pulse duration of 1.3 ms, a peak intensity of magnetic field of 1.8 ± 0.2 mT, and an amplitude of the induced electric field of 3 ± 1 mV. After 45 min of PEMF exposure, intracortical facilitation produced by paired-pulse brain stimulation was significantly enhanced with an increase of about 20%, while other parameters of cortical excitability remained unchanged (Fig. 24.4). The increase in paired-pulse facilitation, a physiological parameter related to cortical glutamatergic activity, suggests that PEMF exposure may produce an enhancement in cortical excitatory neurotransmission (Capone et al. 2009). The safety and tolerability of the PEMF were evaluated by measuring (a) the incidence of adverse effects and mortality throughout the stimulation period and along the 1-year follow-up; (b) the tolerability of the stimulation has been evaluated by the

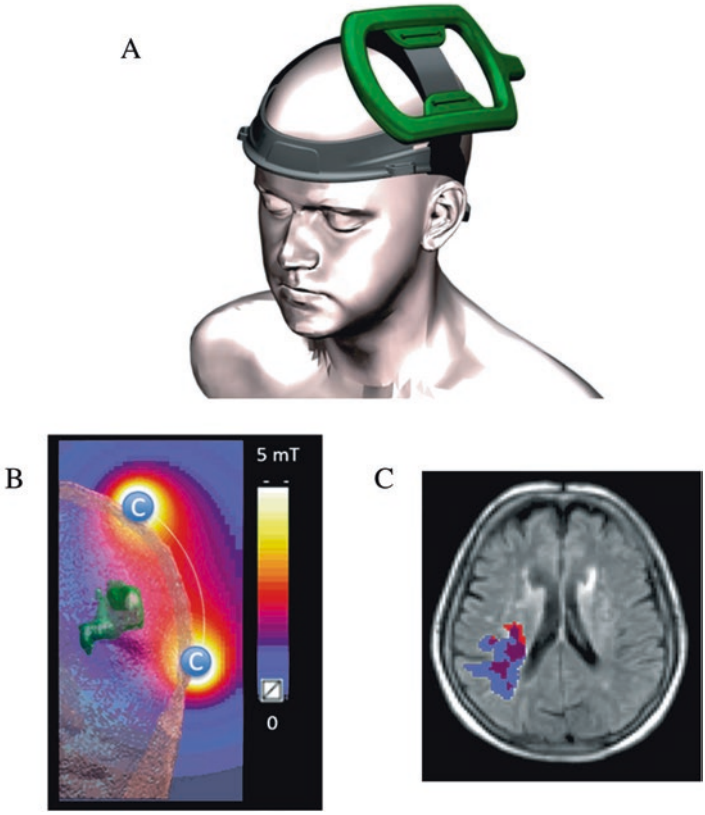

Fig. 24.4 PEMF treatment in central nervous system diseases. Picture of PEMF exposure in human brain (**a**). Ischemic area (green) in the left brain hemisphere exposed by copper wire coil (**c**) to an electromagnetic field. Magnetic field gradient from 0 to 5 mT (**b**). Ischemic lesion size by magnetic resonance imaging evaluation show a reduction of the volume infarct (**c**)

number of subjects requesting to stop treatment; and (c) clinical evaluations have been performed by means of international well-validated scales: National Institute of Health Stroke Scale (NIHSS), modified Rankin Scale (mRS), and Barthel Index (BI) (trial is registered on clinicaltrials.gov, NCT01941147, Capone et al. 2014). The scores obtained at follow-up visits were compared with the baseline scores, and all magnetic resonance imaging (MRI) images were obtained with a 1.5 T scanner (Magnetom Symphony, Siemens Medical System, Erlangen, Germany) (Fig. 24.4). In particular, seven patients were recruited, six patients completed the 5-day treatment period, and five patients completed the 12-month follow-up period suggesting that the PEMF stimulation is safe and tolerable in patients affected by acute stroke. No adverse effects both during the treatment and the follow-up phase were observed. The vital parameters (respiratory rate, heart rate, blood pressure, pulse oximetry, and ECG signal) remained stable during the PEMF stimulation and the clinical

conditions improved in all patients. The effect of PEMF exposure on the evolution of ischemic lesion size reduces the ischemic volume by MRI evaluation toward a reduction of volume infarct (Fig. 24.4). The lesion size was reduced in all the patients stimulated for 120 min and MRI evaluation was carried out before and 30 days after PEMF exposure (Capone et al. 2017). The dosimetric analysis in the 3D model of the lesion (Sim4Life ViP 1.0) was analyzed by using a magnetic field intensity within the ischemic lesion and was in the range 1–2 mT, which represents the minimum value to trigger a biological effect in preclinical studies such as the A_{2A}AR upregulation. Since the presence of the head structures, including ischemia, does not alter the magnetic distribution, all the tissues experience a magnetic field that depends only on the distance from the coil center and the current intensity that feeds the coil suggesting that the higher is the distance from the coil, the lower is the field intensity (Capone et al. 2017). A multicenter, prospective, randomized, and double-blind study is in progress where 124 patients, after a signed written informed consent, have been studied. The patients' age was from 50 to 80 years, with the first-onset, mono-hemispheric ischemic stroke in the middle cerebral artery territory, the onset of symptoms within 48 h, and the NIHSS score between 4 and 25. To validate PEMF stimulation as noninvasive, safe, and effective neuroprotective therapy in patients with acute ischemic stroke, the trial consists of a 5-day treatment phase with a 3-month follow-up phase. The primary outcome will be to evaluate the effect on infarct size/volume measured by MRI, and the clinical efficacy was modified Rankin Scale, Barthel Index, and NIHSS. The safety will be evaluated by the incidence of adverse events and mortality throughout the stimulation period and along 3-months follow-up. Vital parameters and rate of early neurological worsening change, the percentage of hemorrhagic transformation of ischemic lesion, and the tolerability will be investigated. Interestingly, the evaluation of PEMF effect on ischemic stroke as potential effective tool to promote recovery in acute ischemic stroke patients is in progress together different researches with the aim to clarify the PEMF mechanism of action (trial is registered on clinicaltrials.gov, NCT02767778, Di Lazzaro et al. 2016).

24.6 Conclusions

Several areas are addressing evincing interest in the possibilities of employing non-chemical means for intervention on various pathologies. Some applications are still at the initial stages or involved in preliminary in vitro investigations or in vivo studies, for example, the research performed in neurological sector where PEMF treatment could have potential positive effects in the human health. As a consequence the knowledge of the cellular mechanism of PEMF action could suggest the rationale for clinical use with the aim to design clinical trials and relative endpoints in a coherent way.

The inflammatory state represents a complex issue in many pathological conditions at both the peripheral and central nervous systems related to the presence of

elevated levels of pro-inflammatory mediators. It is well known that biophysical stimulation with PEMFs promotes anabolic activity resulting in an increase in chondrocyte proteoglycan synthesis (Aaron et al. 2004, 2006; Lotz et al. 2010). Several experimental results support the hypothesis that treatment with PEMFs is chondroprotective and is accompanied by the control of inflammation (De Mattei et al. 2001; Fini et al. 2013; Varani et al. 2017b). The effectiveness of the treatment has also been shown in patients where the control of joint microenvironment by PEMFs is an important therapeutic approach in the perspective of a new regenerative medicine for musculoskeletal disorders (Moretti et al. 2012). Recently, different research studies both in preclinical and in clinical field is being carried out in order to demonstrate the validity of the PEMFs in the treatment of neurodegenerative disorders or in ischemic stroke (Capone et al. 2009, 2014, 2017; Di Lazzaro et al. 2013, 2016).

The results reported in this chapter highlight that the increase of A_{2A} and A_3ARs induced by PEMFs in different cells involves a reduction of some of the most relevant pro-inflammatory cytokines (Varani et al. 2017b). Of particular interest is the observation that the PEMFs determine an increased functioning of the endogenous agonist adenosine, a potent modulator of various physiological and pathological responses (Borea et al. 2016, Varani et al. 2017a, Jacobson et al. 2017). In fact, PEMFs through the increase of ARs enhance the working efficiency of adenosine, producing a more physiological effect than the use of drugs. Consequently, the anti-inflammatory effect of adenosine enhanced by PEMF may not be accompanied by the side effects, desensitization, and receptor downregulation often related to the use of agonists (Kenakin 2004). In particular, a prolonged stimulation of the membrane receptors with an exogenous agonist can dampen the ability to transduce the signal which is followed by the process of the receptor internalization into specific vesicles inside the membrane (Kenakin 2013). Moreover, the availability of different functional pharmacological assays has revealed that agonists for receptors that are pleiotropically coupled to multiple signaling pathways in the cell can emphasize signals to some pathways over others. This, in turn, opens the possibility that agents can be used to emphasize favorable signals, de-emphasize harmful signals, or selectively block the ability of the natural agonist to produce unfavorable signals (Kenakin 2017).

It could be remarked that the prolonged use of agonists decreases the receptor density by reducing in this way the effect of the drug itself while the PEMFs potentiate the effect of endogenous adenosine as anti-inflammatory agent. This observation suggests the hypothesis that PEMFs may be an interesting approach as a noninvasive treatment with a low impact on daily life mediating a significant increase on the effect of the endogenous modulator. In conclusion, adenosine via the stimulation of ARs and the presence of PEMFs could represent an important approach in the pharmacological field as an example of *soft pharmacology* providing excellent therapeutic results in various inflammatory diseases, in the functional recovery of the damaged cartilage tissues, in pain, or in central nervous system disorders.

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