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DOTTORATO DI RICERCA IN
"Medicina Molecolare e Farmacologia"

CICLO XXXII

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The role of adenosinergic system in brain ischemia

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Table of contents

Introduction

Adenosine

Adenosine is an endogenous ubiquitous purine nucleoside composed by a molecule of adenine attached to a ribose sugar molecule via a β-N9-glycosidic bond (Figure 1).

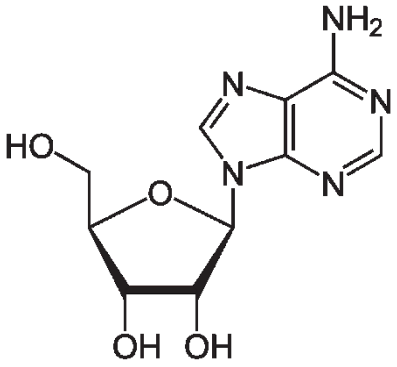


Figure 1. Chemical structure of adenosine.

Owing to its widespread presence, adenosine is one of the most important nucleosides in the human organism, it is the backbone of ATP and it is implicated in the regulation of a large number of physiological and pathological signals (Borea et al., 2015; Cronstein and Stikovsky 2017). Adenosine has also been defined as a "retaliatory metabolite" because it is released by cells in response to different stimuli such as hypoxia, metabolic stress or injury and it is able to promote many processes involved in the response to harmful stimuli (Figure 2). Adenosine is implicated in inflammation where it acts as an endogenous regulator (Cronstein and Stikowsky, 2017).

Adenosine is able to exert its functions through the interaction with four adenosine receptors (ARs), all of them are transmembrane G protein-coupled receptors (GPCRs) named as A_1, A_{2A}, A_{2B}, and A_3. Interestingly, the A_1 and A_3 subtypes are coupled to Gi proteins and have an inhibitory effect on adenylyl cyclase (AC) activity while A_{2A} and A_{2B} stimulate it, through the coupling to Gs proteins, with a consequent modulation of cyclic AMP levels (Fredholm BB. 2014). Although the affinity of adenosine for these receptors may vary, depending on the type of test used to evaluate it, adenosine seems to present a higher affinity for A_1, A_{2A}, and A_3 than for the A_{2B} subtype (Borea et al., 2016).

The production of adenosine occurs through different mechanisms, the principal is by the dephosphorylation of the adenine nucleotides (ATP, ADP, and AMP) to adenosine. Especially ATP serves as the reservoir for the production of adenosine because it is the most abundant molecule in the cell (Figure 3) (Borea et al., 2018). Under resting

conditions, some ATP is dephosphorylated to adenosine but injury, hypoxia or other metabolic insults can trigger increased rates of adenosine formation. More commonly, adenine nucleotides are released into the extracellular space where they are dephosphorylated to adenosine by ectoenzymes at the cell surface (ecto-5'nucleotidase [CD73] and ecto-nucleoside triphosphate phosphohydrolase [CD39]) and by enzymes in blood or other extracellular fluids. Once formed or released into the extracellular space adenosine can be deaminated to inosine and, in humans, ultimately to uric acid or taken up directly by cells by specific nucleoside transporters (ENT1 and ENT2) and re-phosphorylated to ATP (Rosenthal et al., 2013; Beckel et al., 2014).

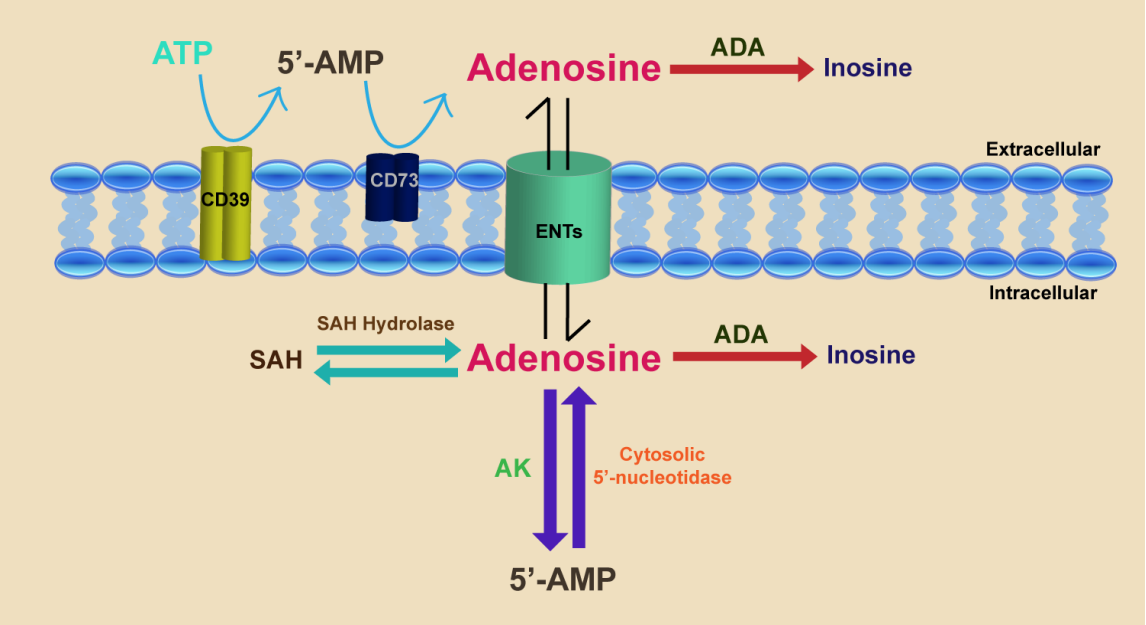


Figure 2. Adenosine metabolism and transport (modified from Borea et al., Physiol Rev 2018, 98: 1591-1625).

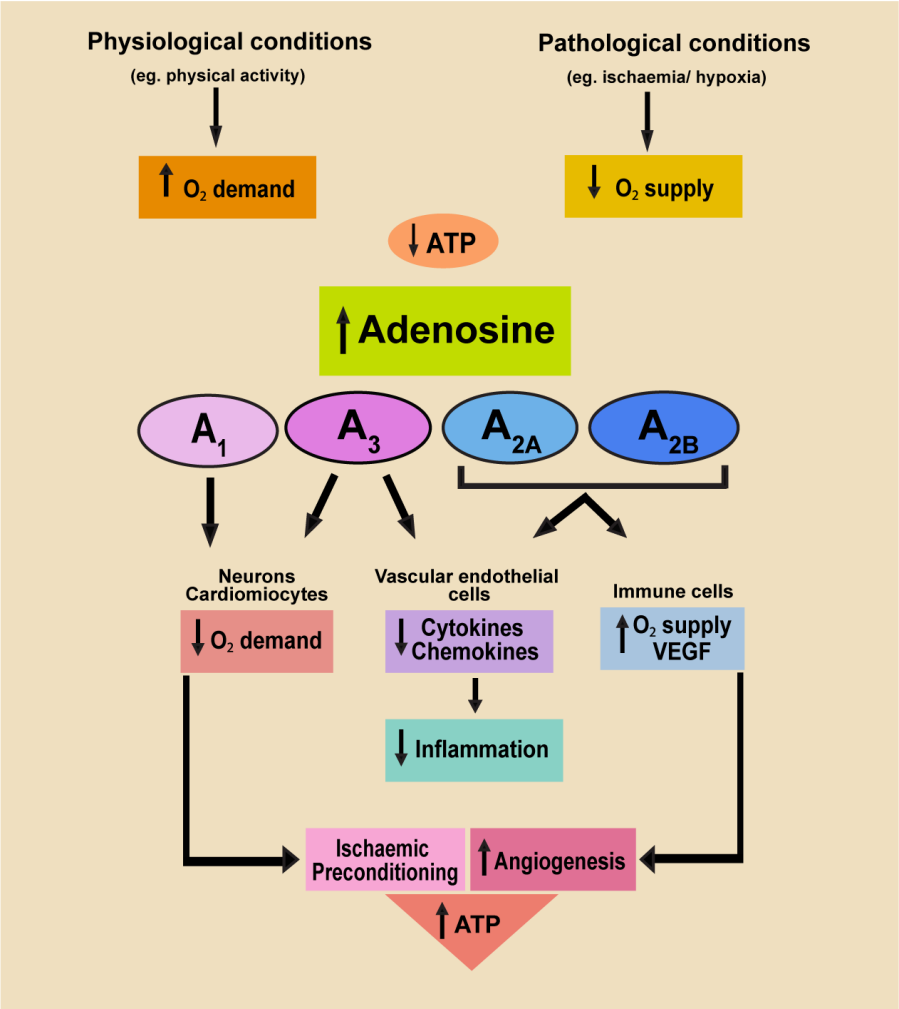


Figure 3. Scheme representing the physiological role of adenosine and its receptors (modified from Borea et al., Physiol Rev 2018, 98: 1591-1625).

Adenosine Receptors (ARs)

The many and various physiological effects of adenosine are elicited by the interaction of this purine with its receptors. ARs are present in many tissues (bone, joints, eyes and, skin) and systems, such as nervous, cardiovascular, respiratory, urogenital, gastrointestinal and immune systems (Peleli et al., 2017). The distribution in different tissues and the different effector coupling distinguish the ARs subtypes, these can also be divided on the basis of their affinity for adenosine: there are high-affinity receptors such as A_1, A_{2A} and A_3ARs, and low-affinity ones such as A_{2B}ARs. All four ARs have been well identified, cloned and pharmacologically studied. They share a common structure with a transmembrane domain which crosses the plasmatic membrane seven times, every domain is composed by a helix of 20-27 amino acids and is linked to other domains by three intracellular and three extracellular loops (Fredholm et al., 2001). The extracellular NH_2 terminus contains one or

more glycosylation sites, while the intracellular COOH terminus comprehends sites for phosphorylation and palmitoylation, so it plays a role in the desensitization and internalization mechanisms of the receptor. The AR subtypes differ in the amino acids number: A_{2A}ARs have longer COOH terminus, with 122 amino acids, whereas A_1AR, A_{2B}AR, and A_3AR present shorter COOH-terminal tails of ~30–40 amino acids (Lebon et al., 2011). Crystallization studies helped discover more details about structures of human A_1AR and A_{2A}ARs, which is very useful in order to design of novel ligands based on the structure of these receptor subtypes (Carpenter et al., 2016; Eddy et al., 2018; Glukhova et al., 2017; Sun et al., 2017). ARs show an amino acids sequence homology of 80–95% (with a 70% homology between human and rat), only A_3AR is significantly different among species, while the A_1AR sequence is the most conserved. ARs have been cloned from several species, with A_3AR being the only subtype isolated before its pharmacological characterization. It is noteworthy that a comparison between hA_1AR/hA_3AR and hA_{2A}R/hA_{2B}R shows overall amino acid sequence identities of 46.5% and 46.6%, respectively. Many studies demonstrate the presence of several GPCRs, including ARs, in homomer, oligomer, and heteromer forms (Brugarolas et al., 2014; Ferrè et al., 2010; Navarro et al., 2010a, b; Navarro et al., 2014). The GPCR heteromers seem to be distinct signaling units with different functional features in comparison to homomers. In this sphere, the first macromolecular structure of two different receptors coupled with two different G protein is represented by the adenosine A_1AR-A_{2A}AR unit (Brugarolas et al., 2014). Since A_1AR is coupled to Gi and A_{2A}AR to Gs proteins, the activation of this heteromer leads to opposite cAMP-dependent intracellular signals. In particular, this complex is considered an adenosine concentration cell surface sensor able to distinguish between high and low concentrations of the nucleoside (Navarro et al., 2016a). In particular, with low levels of adenosine is preferentially activated the A_1AR promoter of the heteromer, this triggers the Gi/o protein pathway inhibiting AC, cAMP production and protein kinase A (PKA). Conversely, higher adenosine levels activate the Gs protein pathway and the subsequent AC/cAMP/PKA cascade (Cristovao-Ferreira et al., 2013). The heteromerization is a common phenomenon occurring even for A_3ARs, which form homodimers and A_1AR-A_3AR heterodimers (Hill et al., 2014). This brings in new expectations in drug development; especially, A_{2A}AR-D_2 dopamine receptor heterodimers have been found in the striatum and may be a promising therapeutic target in the treatment of Parkinson's disease (PD) (Navarro et al., 2016b).

A_1ARs

The A_1AR subtype is expressed in the central nervous system (CNS), mainly in the brain cortex, cerebellum, hippocampus, autonomic nerve terminals, spinal cord, and glial cells (Chen et al., 2013). This large distribution mirrors the variety of physiological functions regulated by these receptors such as decreasing neuronal excitability, control of sleep/wakefulness, pain reduction, as well as sedative, anticonvulsant, anxiolytic, and locomotor depressant effects (Gessi et al., 2011 a; Sawynok J. 2016). A_1ARs have also been found at high levels in the heart atria, kidney, adipose tissue, and pancreas, where it induces negative chronotropic, inotropic, and dromotropic effects, reduces renal blood flow and renin release, and inhibits lipolysis and insulin secretion, respectively (Merighi et al., 2015a; Rabadi et al., 2015; Vincenzi et al., 2012). This ARs subtype is present on airway epithelial and smooth muscle cells too, where it stimulates a bronchoconstriction effect, and in immune cells such as neutrophils, eosinophils, macrophages, and monocytes, where it supports proinflammatory effects (Ponnoth et al., 2010; Wilson et al., 2009). A_1AR activates the pathway of phospholipase C (PLC)-β, increasing inositol 1,4,5-trisphosphate (IP_3) and intracellular Ca^{2+} levels, which stimulate calcium-dependent protein kinases (PKC) and/or other calcium-binding proteins. In neurons and myocardium, A_1ARs are able to excite potassium (K) pertussis toxin-sensitive and K_{ATP} channels, while reducing Q-, P-, and N-type Ca^{2+} channels. In addition, this receptor subtype is implicated in the intracellular phosphorylative cascade of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase (ERK), Jun NH2-terminal kinase (JNK), and p38 (Figure 4) (Borea et al., 2018). On one hand, compounds able to further activate A_1ARs in response to adenosine would be very handy in the treatment of CNS, cardiovascular, and inflammatory pathologies. On the other hand, A_1AR activation presents bad side effects, owing to the wide receptor distribution, a large number of physiological effects, and unselective signaling pathway transduction. These effects can be alleviated using allosteric enhancers, molecules able to stabilize the ternary complex formed by agonist, A_1AR and G protein. This enhances the agonist action only at the injured site, where there are higher adenosine concentrations but not in the other sites where adenosine concentrations are ordinary (Romagnoli et al., 2010).

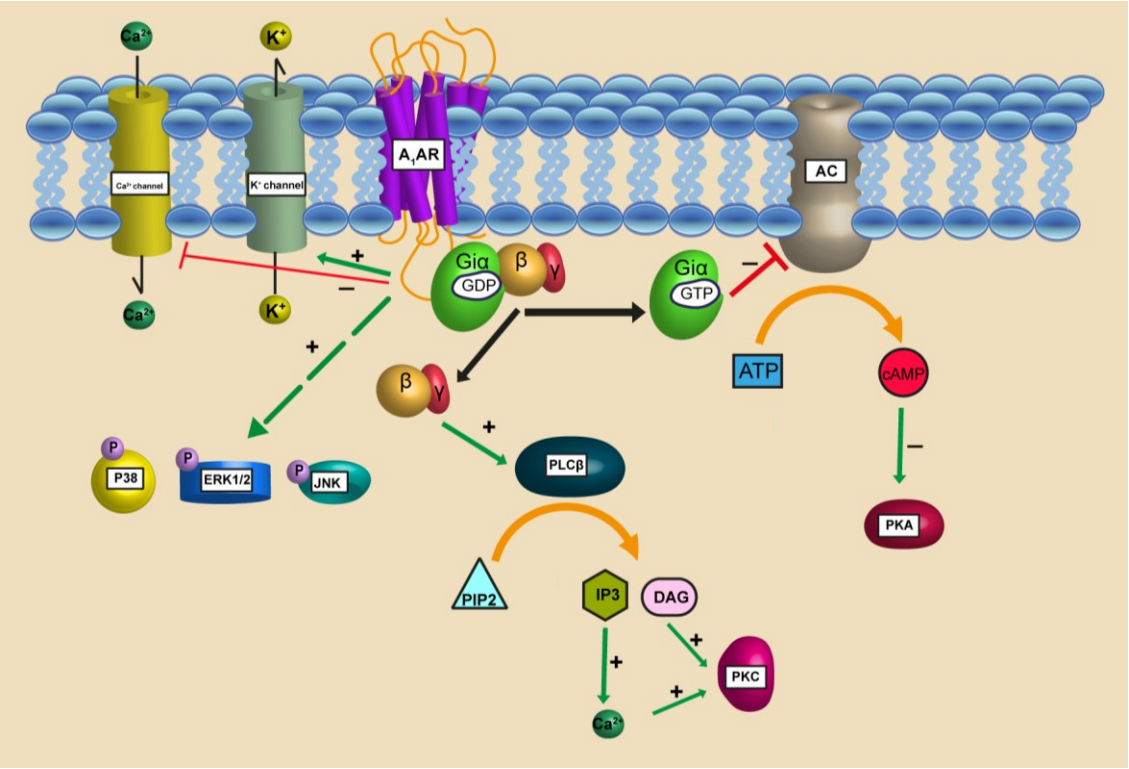


Figure 4. Overview of A_1ARs intracellular signaling pathways. A_1ARs stimulation decreases AC activity and cAMP production, inhibiting PKA, activates PLCβ and Ca^{2+}. K^+ and Ca^{2+} channels are opened and closed, respectively, by A_1AR enrollement. A_1AR activation induces p38, ERK1/2, and JNK1/2 phosphorylation. (modified from Borea et al., Physiol Rev 2018, 98: 1591-1625).

A_{2A}ARs

The A_{2A}AR subtype is located both centrally and peripherally, the largest expression is in the striatum, the olfactory tubercle, and the immune system, while lower levels are found in the cerebral cortex, hippocampus, heart, lung, and blood vessels. Moreover, A_{2A}AR is expressed on both pre and postsynaptic neurons, astrocytes, microglia, and oligodendrocytes, where it is implicated in many processes, such as excitotoxicity, neuronal glutamate release, glial reactivity, Blood-Brain Barrier (BBB) permeability, and peripheral immune cell migration. In the peripheral immune system, A_{2A}ARs are predominant in leukocytes, platelets, and in the vasculature, where they are able to mediate anti-inflammatory, anti-aggregatory, and vasodilatory effects (De Lera Ruiz et al., 2014). The principal effector pathway stimulated by A_{2A}ARs is the cAMP-dependent PKA, which acts through the phosphorylation and activation of many proteins, including receptors, phosphodiesterases, cAMP-responsive element-binding protein (CREB), and dopamine- and cAMP-regulated phosphoprotein (DARPP-32) (Preti et al., 2015). A_{2A}Rs have been

associated with activation of the cAMP-PKA pathway as well as of p38 MAPK and p42/44 MAPK, all of which can activate the CREB transcription factor system (Figure 5) (Acevedo et al., 2016; Fredholm et al., 2007). A_{2A}ARs activation effects could be difficult to study because, in some cases, the receptors can form oligomeric, dimeric or multimeric structures with other receptors. For instance, A_{2A}ARs can associate with other A_{2A}ARs to create homo-oligomers A_{2A}-A_{2A}, or with other receptors, such as dopamine D2 or D3 receptors, to create A_{2A}-D2 or A_{2A}-D3 hetero-dimers (Fredholm et al., 2011). A_{2A}ARs can form other heterodimers with cannabinoid CB_1 (A_{2A}-CB_1) and glutamate mGlu5 receptors (A_{2A}-mGlu5), as well as hetero-trimer A_{2A}-D2-CB_1 (Guerrero A, 2018). Some evidence suggests that the pharmacological differences in A_{2A}AR antagonists tested in PD *in vivo* models could be due to differences in hetero-dimerization between A_{2A}-D2 or A_{2A}-A_1 receptors at postsynaptic and presynaptic sites (Armentero et al., 2011; Orru et al., 2011). A_{2A}ARs seem to be important for the proper targeting of A_{2B}ARs to cell membranes thanks to the A_{2A}-A_{2B} heterodimer formation (Moriyama et al., 2010). Since these results have not yet been confirmed in *in vivo* experiments and there are many studies indicating that monomeric receptors are sufficient to trigger signaling pathways, this additional potential complexity has not yet been taken into account for drug development (Chen et al., 2013).

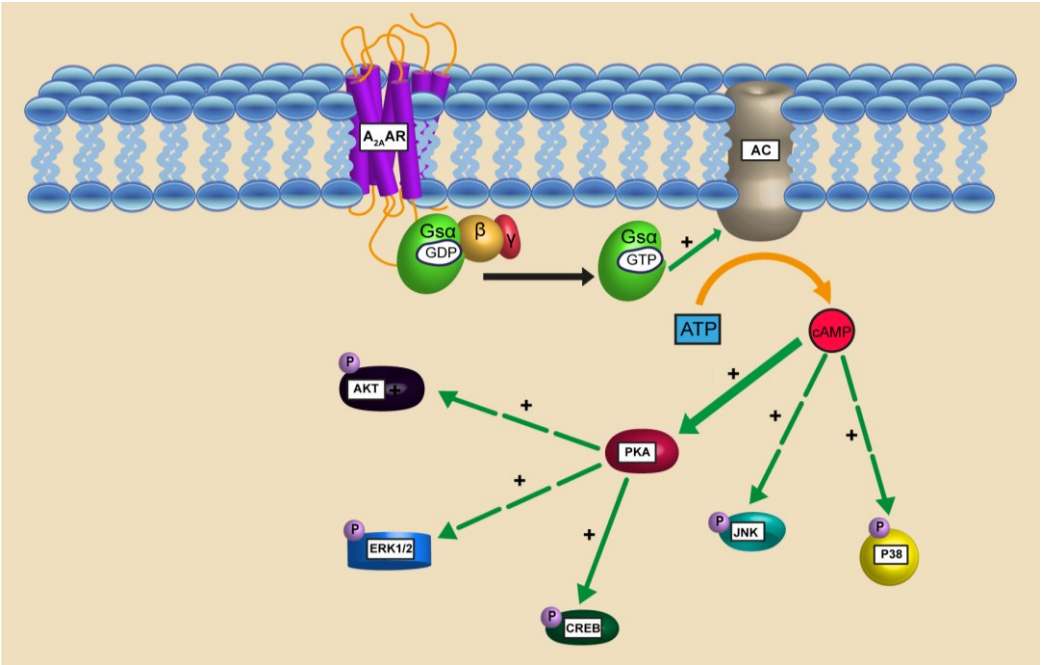


Figure 4. Overview of A_{2A}ARs intracellular signaling pathways. A_{2A}ARs stimulation increases AC activity, cAMP production, PKA, and CREB phosphorylation. AKT and p38, ERK1/2 and JNK1/2 are activated following by A_{2A}ARs recruitment. (modified from Borea et al., Physiol Rev 2018, 98: 1591-1625).

A_{2B}ARs

A_{2B}ARs are markedly expressed in the periphery, in fact they are present in the bowel, bladder, lung, vas deferens, and in various cell types such as fibroblasts, smooth muscle, endothelial, immune, alveolar epithelial, chromaffin, taste cells, and platelets. Regarding the CNS, this subtype is found in astrocytes, neurons, and microglia (Koupenova et al., 2012; Pedata et al., 2016); an increasing body of literature suggests that A_{2B}ARs could have a role in pathologies on the order of cancer, diabetes, renal, vascular and lung diseases through the inflammatory and immune modulation. This sheds new light on the physiological and pathological role of A_{2B}ARs that has been poorly considered until now owing to the low affinity of these receptors for adenosine (Sun et al., 2016). The expression of A_{2B}AR is up-regulated in many harmful conditions such as hypoxia, inflammation, and cell stress; this supports a pathological role for this receptor subtype. In fact, it has been found in the A_{2B}AR promoter a region responsive to hypoxia, in which there is a functional binding site for hypoxia-inducible factor (HIF), explaining its transcriptional regulation from HIF-1, the master regulator of cellular responses to hypoxia (Eckle et al., 2014). A_{2B}ARs signaling pathways involve AC activation through Gs proteins, leading to PKA phosphorylation and recruitment of different cAMP-dependent effectors such as exchange proteins, directly activated by cAMP (Epac). Moreover, A_{2B}ARs can trigger the PLC pathway through the Gq protein, causing Ca^{2+} mobilization, and can regulate ion channels through their βγ subunits (Figure 5). Moreover, this subtype acts as a stimulator of MAPK activation in several cell models, both in central and peripheral systems (Sun et al., 2016). Many studies report that A_{2B}ARs have different binding partners that modulate A_{2B}AR responses and functions; these include netrin-1, E3KARPP-EZRIN-PKA, SNARE, NF-κB1/P105, and α-actinin-1. Netrin-1 is an important molecule increased during hypoxia, it decreases inflammatory response trough the A_{2B}ARs activation and the subsequent inhibition of neutrophils migration (Rosenburger et al., 2009). The interaction between A_{2B}ARs and SNARE protein leads to the recruitment of receptors to the plasma membrane following agonist binding. After this interaction, a protein complex formed by E3KARP (NHERF2) and ezrin stabilizes the receptor in the plasma membrane. Interestingly, A_{2B}AR binding to P105 inhibits NF-kB activity, this explains its anti-inflammatory effects (Sun et al., 2012). Furthermore, α-actinin-1 might favor A_{2A}AR and A_{2B}AR dimerization, thus inducing A_{2B}AR expression on the cell surface (Moriyama et al, 2010).

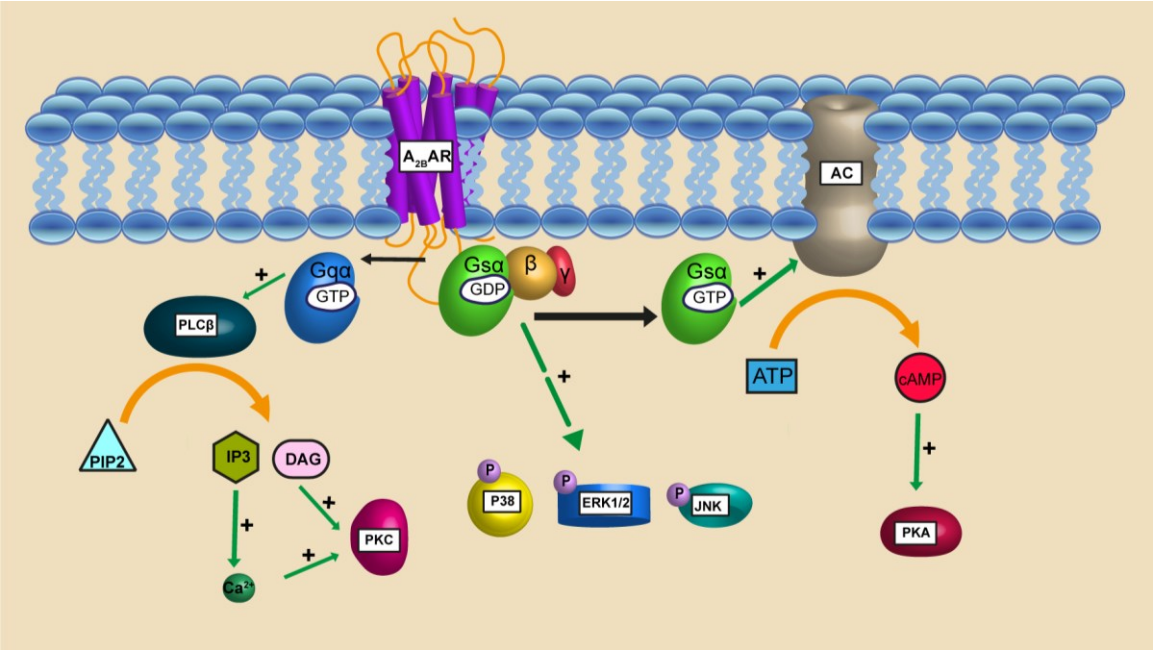


Figure 5. Overview of A_{2B}ARs intracellular signaling pathways. A_{2B}ARs stimulation increases AC activity, cAMP production, and PKA phosphorylation. A_{2B}ARs enrollement activates PLCβ and increases Ca^{2+}. p38, ERK1/2 and JNK1/2 phosphorylation are induced by A_{2B}ARs activation. (modified from Borea et al., Physiol Rev 2018, 98: 1591-1625).

A_3ARs

The A_3AR subtype is largely present in a variety of primary cells, tissues, and cell lines. Low levels have been found in the brain, where it is located in the thalamus, hypothalamus, hippocampus, cortex, and retinal ganglion cells, as well as at motor nerve terminals and the pial and inter-cerebral arteries. A_3ARs are also expressed in microglia and astrocytes, here this receptor subtype is able to inhibit the neuro-inflammatory response and seem to have an analgesic effect (Janes et al., 2014). Although A_{3A}Rs are known to have cardio-protective effects and to be widely expressed in the coronary and carotid artery, their precise location in the heart has not yet been reported. In the periphery, A_{3A}Rs have been found in enteric neurons, epithelial cells, colonic mucosa, lung parenchyma, and bronchi. Moreover, A_3ARs show a large distribution in inflammatory cells such as mast cells, eosinophils, neutrophils, monocytes, macrophages, foam cells, dendritic cells, lymphocytes, splenocytes, bone marrow cells, lymph node cells, synoviocytes, chondrocytes, and osteoblasts, where they promote anti-inflammatory effects (Borea et al., 2016). Of great interest is the over-expression of A_3ARs in many cancer cells and tissues and thus they are likely to have an important anti-tumoral role (Borea et al., 2015). These receptors are responsible for the triggering of various intracellular signaling by

preferentially coupling to Gi proteins, reducing cAMP levels, and, at high concentrations of A_3AR agonists, to Gq proteins or Gβγ subunits, thereby increasing both PLC and calcium. A reduction in cAMP results in PKA inhibition, leading to an increase in glycogen synthase kinase-3β (GSK-3β), down-regulation of beta-catenin, cyclin D1, and c-Myc, and finally a reduction of nuclear factor (NF)-κB DNA-binding ability (figure 6) (Fishman et al., 2016). A_3ARs can exploit their effects through a different signaling pathway, which involves monomeric G protein RhoA and phospholipase D, important for A_3AR-mediated neuro- and cardio-protection. Finally, acting on the regulation of MAPK and by PI3K/Akt, NF-κB signaling pathways, A_3ARs exert anti-inflammatory effects (Borea et al., 2015).

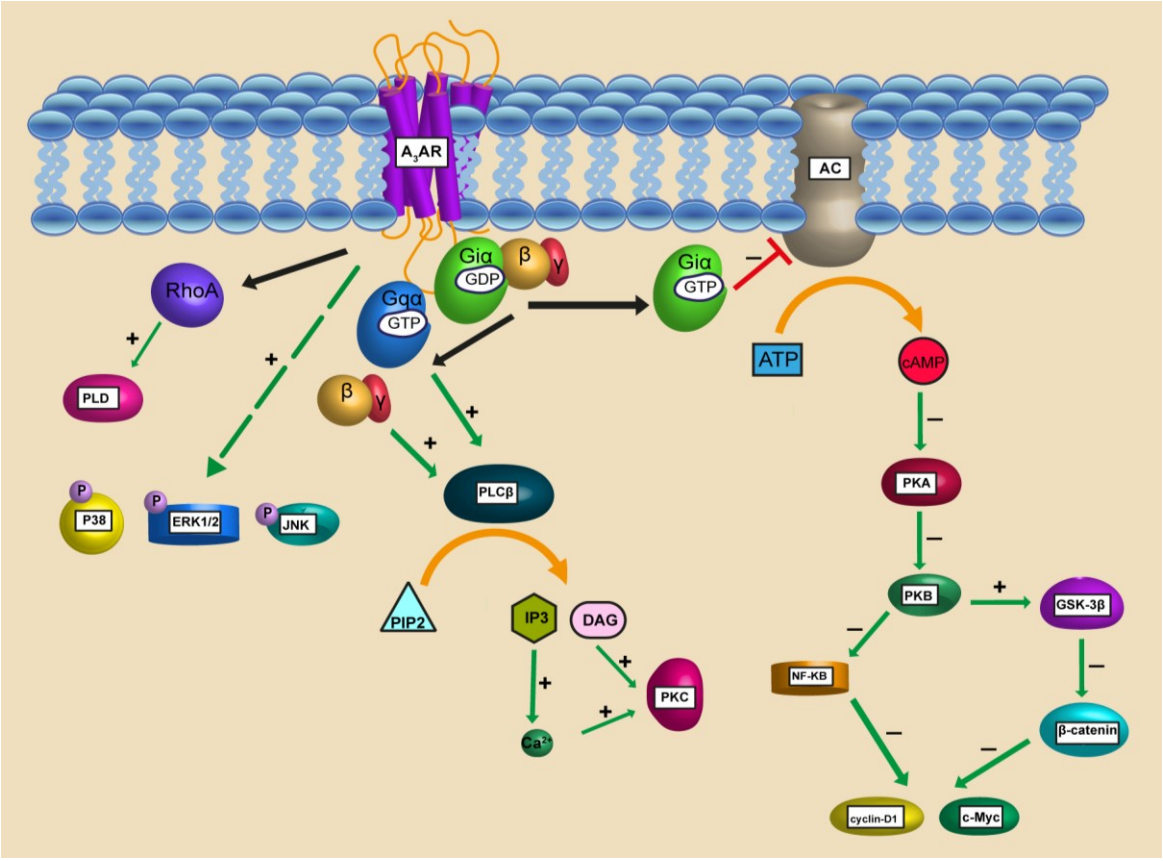


Figure 6. Overview of A_3ARs intracellular signaling pathways. A_3ARs stimulation triggers decrease of adenylate AC activity and cAMP production, activation of GSK-3β, and consequent decrease of β-catenin, cyclin D1, and c-Myc. A_3ARs activation induces a an increase of PLCβ and $Ca^{2+,}$ as well as of RhoA and PLD. p38, ERK1/2, and JNK1/2 phosphorylation are induced by A_3ARs activation. (modified from Borea et al., Physiol Rev 2018, 98: 1591-1625).

ARs and Pathological Aspects

Neurological Diseases

The role of adenosine in diseases affecting the nervous system is related to its influence on a range of mediators including channels, receptors, second messengers, and neurotransmitters, through activation of ARs. While all the four ARs subtypes are present in the brain, the cerebral effects of adenosine are mainly mediated by A_1AR and A_{2A}AR, the subtypes predominantly expressed in the brain.

As above mentioned, the A_1AR subtype is widely and homogeneously distributed in the brain, mainly in excitatory synapses, and plays an important role in the control of the physiological synaptic transmission. In particular, A_1AR activation depresses excitatory transmission through N-type calcium-channel inhibition and neuronal hyperpolarization by regulation of potassium current (Boison D, 2015; Sawynok J, 2016). The subsequent reduction of glutamate release and inhibition of N-methyl-d-aspartate (NMDA) effects maintains A_1ARs-dependent inhibitory tonus in the brain, a helpful effect in several central disease states, including epilepsy, pain, and cerebral ischemia (Borea et al., 2016). For this reason, adenosine is identified as an endogenous anticonvulsant molecule, capable of reducing the frequency of action potentials induced by electrical stimulation by the recruitment of over-expressed A_1ARs (Hargus et al., 2012). Many studies reported protection against seizures resulting from an increase in adenosine levels produced by a ketogenic diet, which apparently inhibits adenosine kinase (ADK) (Masino et al., 2011). This effect may also be related to adenosine impeding with the *S*-adenosylmethionine (SAM) induced DNA methylation pathway involved in epileptogenesis, as a result of ADK reduction, adenosine increase, SAH accumulation and SAM inhibition (Lusardi et al., 2015). These studies support ADK inhibitor's use as therapeutic agents; however, although they have benefic effects increasing adenosine and reversing these epigenetic changes, their toxic side effects have not yet been overcome (Boison D. 2013). An alternative could be represented by adenosine-based treatments. For instance, adenosine delivery might find use either as a preventative treatment or following surgical resection of an epileptogenic focus (Williams-Karnesky et al., 2013). The neuroprotective effects of A_1ARs have been studied in several models of inflammatory and neuropathic pain, in which A_1AR agonists exhibited anti-nociceptive and/or anti-hyperalgesic properties. In fact, the stimulation of these receptor subtype causes a reduction in pain acting on spinal, supra-spinal, and peripheral neurons as well as in glial cells. The analgesic effect involves different

molecular pathways, from the classical signaling mechanisms described above for A_1AR (AC and PKA reduction) to PLC induction, but also Ca^{2+} and K^+ channel regulation, ERK, CREB, calmodulin kinase inhibition, as well as reduction of excitatory amino acid release (Sawynok J, 2016). Moreover, the pathway involving the nitric oxide/cGMP/protein kinase G/KATP channel has been demonstrated to be a molecular effector of A_1ARs mediated pain suppression, via the induction of nociceptive neuron hyperpolarization and inhibition of microglia hyper-activation (Kashfi et al., 2017). Nevertheless, many clinical trials on the systemic administration of A_1AR agonists failed due to the central and cardiovascular side effects of the compounds. However, partial agonists or allosteric modulators could represent a better way; indeed, allosteric enhancers, acting only on the ternary complex, constituted by agonist, A_1AR and G protein, have been shown to minimize side effects in sites expressing A_1ARs, but not in those involved in the injury. Unfortunately, a trial of an allosteric modulator (2-amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)-(4-chlorophenyl) methanone (T62) in post-herpetic neuralgia was terminated because some patients experienced asymptomatic, transient elevations in liver transaminases (Romagonli et al., 2010). Recently, a potent derivative of T62, 2-amino-4-[(4-(phenyl) piperazin-1-yl) methyl]-5- (4-fluorophenyl) thiophen-3-yl- (4-chlorophenyl) methanone (TRR 469), resulted in anti-nociception without motor effects in models of acute and neuropathic pain under chronic treatment (Vincenzi et al., 2014). Intriguingly, the administration of an A_1AR agonist with endomorphin decreases mechanical and thermal hyperalgesia, and A_1AR/opioid blockade counteracts the analgesic effects of electro-acupuncture, a popular Chinese clinical practice used for pain relief (Liao et al., 2017). Encouraged by the positive results of TRR 469 in pain models, its anxiolytic activity has been analysed. Specifically, this compound displayed an anxiolytic behavior similar to diazepam, without sedative drawbacks and ethanol interaction (Vincenzi et al., 2016; Vincenzi et al., 2017). Considering their effect on glutamate release, A_1AR selective agonists and/or allosteric modulators have been suggested as anti-neurodegenerative agents (Gomes et al., 2011). The inhibition of glutamate release caused by A_1ARs is crucial for the prevention and protection against ischemic damage. Certainly, A_1ARs have a role during ischemic preconditioning, a state of tissue protection obtained by exposure to sublethal insults, probably occurring through modulation of NMDA preconditioning-mediated increase of glutamate uptake (Constantino et al., 2015). In addition, some studies report that the activation of A_1ARs reduces intraocular pressure (IOP) by increasing metalloproteinase-2 (MMP-2) secretion. This causes the digestion of collagen type IV, one of the main elements of extracellular matrix in the trabecular meshwork (TM), raising the outflow

facility at the TM and IOP reduction. Of great interest is that patients with ocular hypertension present higher levels of adenosine in the humor aqueous if compared to normotensive patients, thus suggesting a potential role of adenosine in IOP control. In regard to this matter, Trabodenoson (INO-8875), a very selective A_1ARs agonist entered in phase I/II clinical trial, showed a good efficacy in IOP-lowering in patients with ocular hypertension or primary open-angle glaucoma and, at the highest dose tested, it displayed a good profile of safety and tolerability (Laties et al., 2016; Myers et al., 2016). Now the compound is under analysis in a higher range of doses in phase III clinical trials (Lu et al., 2017). The most common side effect showed was moderate hyperemia suggesting an encouraging pharmacological profile. Considering all, the therapeutic potential of A_1AR mimetics has been afflicted by different troubles, for instance side effects linked to atrio-ventricular block, that need to be overwhelmed. Regarding this, another critical point is the desensitization of the receptors, which leads to a loss in the neuroprotective activity of A_1AR agonists (Jajoo et al., 2009). This reduces the time window for the potential neuroprotection of A_1AR-activating agents in ischemia, inflammation, excitotoxicity, and neurodegenerative diseases, as the increase in adenosine concentrations occurring in these pathological conditions can cause AR desensitization and down-regulation.

Regarding A_{2A}ARs, they are recognized as the main AR subtype located in the striatum, where they colocalize with dopamine D2 receptors (D2R). This results in A_{2A}AR/D2R heteromers with a crucial role in the modulation of motor function (Borroto-Escuela et al., 2010). In fact, the observation that A_{2A}AR activation decreases the agonist's binding affinity of D2R was the first experimental proof for the use of A_{2A}AR antagonists as novel therapeutic agents in PD (Ferrè et al., 1991). Certainly, these compounds have proved to increase motor function in various PD animal models by reducing the inhibition of D2R activity provoked by A_{2A}ARs in GABAergic neurons of the striato-pallidal (Fuxe et al., 2015). High concentrations of A_{2A}AR antagonists reduce D2R agonist affinity and function, but D2R antagonist affinity, but these allosteric modulations disappear following agonist and antagonist co-administration. This behavior is justified by the presence of A_{2A}AR/D2R heterotetramers, formed by A_{2A}AR and D2R homodimers, in which allosteric interactions between an agonist or antagonist of A_{2A}AR and an agonist of D2R occur, depending on the quaternary structure of the A_{2A}AR/D2R heteromer. This model has clinical importance, as regards adaptation of the application of A_{2A}AR antagonists in the treatment of PD (Bonaventura et al., 2015). Still, many compounds that block A_{2A}ARs have been developed and brought to the clinical field; till now, Istradefylline is the only such drug approved, in combination with levodopa (L-DOPA)/carbidopa. In adult patients

with PD, Istradefylline was associated with a decrease in OFF Time and an increase in ON Time without troublesome dyskinesia. Another A_{2A}AR antagonist, Preladenant, did not significantly decrease off-time in comparison with a placebo. However, it has been suggested that this trial may have been compromised by study design or execution issues, as the positive controls failed (Hauser et al., 2015; Navarro et al., 2016). Another A_{2A}ARs antagonist, Tozadenant, on the other hand, appears more promising, in fact following positive results from phase IIb trials, a phase III clinical study has begun (Hauser et al., 2014). Recently, a functional association between A_{2A}AR and α-synuclein (α -Syn) has been recorded. Undeniably, in A_{2A}AR knockout (KO) mice there is a block of α -Syn-induced toxicity, and α-Syn aggregation and associated toxicity are reduced by A_{2A}AR blockade, suggesting a robust link between these two harmful proteins in PD (Kachroo et al., 2012; Ferreira et al., 2017). In detail, in the pathogenesis of synucleinopathy is involved the abnormal A_{2A}AR signaling since its genetic deletion decreases hippocampal pathological α-Syn aggregation (Hu et al., 2016). As above mentioned A_{2A}ARs are largely distributed in synapses, where they have a crucial role in synaptic plasticity, promoting glutamate release and potentiating NMDA receptor effects. In presynaptic A_{2A}/A_1AR heteromers, A_{2A}AR monitors the A_1-induced suppression of glutamate transmission, adjusting cortico-striatal glutamate levels in a time-dependent manner. Moreover, A_{2A}ARs inhibit the glutamate uptake transporter GLT-1 and trigger glutamate release in astrocytes. Thus, A_{2A}ARs in neurons and glia are involved in a significant manner in the pathogenesis of neuropsychiatric illnesses such as major depression and schizophrenia (Krügel et al., 2016; Yamada et al., 2014a). For example, in rodent depression models of learned helplessness (LH), A_{2A}AR antagonists enhanced escape deficit in LH rats with an efficacy comparable to tricyclic antidepressants and a selective serotonin reuptake inhibitors, such as desipramine or fluoxetine (Yamada et al., 2014b). In addition, A_{2A}ARs may be a therapeutic target in other neuronal diseases such as Alzheimer's disease (AD), Huntington's disease (HD), epilepsy, acute and chronic stress, and memory fear (Cunha et al., 2016; Laurent et al., 2016; Simoes et al., 2016; Tyebji et al., 2015). Intriguing studies show that A_{2A}AR expression is increased in both AD patients and aging mice expressing human amyloid-precursor protein (hAPP) astrocytes. It has also been shown that young and aging transgenic mice knock-out for A_{2A}ARs in astrocytes have increased long-term memory, as detected in aging mice expressing hAPP (Orr et al., 2015). The inhibition of glutamate uptake caused by A_{2A}ARs leads to the synaptic dysfunction and excitotoxic cell death which governs many neurodegenerative diseases; A_{2A}ARs up-regulation causes a reduction of amyloid-β Aβ (1–42) glutamate transporters and uptake (Matos et al., 2012a,

b). A_{2A}AR silencing improves spatial memory deficits and long-term hippocampal depression induced by Tau pathology, restore to normal values the glutamate/GABA ratio in the hippocampus, and provides a neuro-inflammatory markers decrease and Tau hyper-phosphorylation (Laurent et al., 2016). In a mouse model of AD the genetic silencing, or in the same way the antagonism, renewed long-term synaptic potentiation (LTP) in CA3 pyramidal cells which were blocked by neuronal up-regulated A_{2A}ARs activation (Viana da Silva et al., 2016). Likewise, in animal models of HD, it has been found an overexpression of A_{2A}ARs, and A_{2A}AR antagonists were able to reverse cognitive deficits in HD mice, probably through the control of long-term depression deregulation (Li et al., 2015; Varani et al., 2001). A_{2A} adenosine receptors stimulate proinflammatory functions in the CNS (Borea et al., 2016; Koizumi et al., 2013). Specifically, they are involved in process retraction by the microglia during neurodegeneration and neuroinflammation, playing a role in the functional change of microglia into an activated proinflammatory phenotype. According to this, A_{2A}ARs stimulate microglia proliferation, while A_{2A}ARS antagonism hamper hippocampal neuroinflammation, interleukin (IL)-1β-induced exacerbation of neuronal toxicity and retinal microglia reactivity, granting protection to retinal neuronal cells (George et al., 2015; Gomes et al., 2013; Simoes et al., 2012 Madeira et al., 2016;). In agreement with the role of these receptors in neurotoxicity and in favoring inflammation, it is possible that caffeine, the most widely used drug in the world, exerts its effects, at least in part, through antagonism of A_{2A}AR. This interaction could be responsible for the numerous beneficial prophylactic effects of caffeine against PD, AD, amyotrophic lateral sclerosis (ALS), attention deficit hyperactivity disorder (ADHD), brain injury, incidence of suicide, depression, and stroke (Cunha et al., 2016; Ding et al., 2015; Laurent et al., 2014; Lusardi et al., 2012; Xu et al., 2011). Epidemiological studies showed that caffeine protects against different neurodegenerative diseases, an effect that has been attributed to A_{2A}AR antagonism in *in vivo* models of PD (Xu et al., 2016). Moreover, other studies have demonstrated a protective effect of caffeine intake against cognitive impairment in both humans and animals (Daniele et al., 2014). Caffeine intake has been linked to a decrease in the mood and memory dysfunction caused by chronic stress, by modulation of neuronal A_{2A}ARs; it reverts performance deficits in rats after treatment with reserpine (Kaster et al., 2015; Minor et al., 2015). Caffeine administration to helpless mice (HM), an animal model of depression, seems to revert memory deficits by upregulating functional hippocampal A_{2A}ARs. Through the modulation of synaptic glutamate release, caffeine is able to revert the depletion of synaptic markers in the hippocampus, without affecting helpless or anxiety behavior (Machado et al., 2017).

Even if there are fewer A_{2B}ARs expressed in the CNS, A_{2B}ARs expression is up-regulated following lipopolysaccharide (LPS) and hypoxic stimulation (Gessi et al., 2013). In human astrocytes, A_{2B}ARs provoke astrogliosis, and after short-term tumor necrosis factor (TNF)-α treatment, undergo to desensitization in order to defense cells. A_{2B}ARs present in the brain have a role in ischemia as will be described further. Other studies proposed a pronociceptive and proinflammatory role for A_{2B}ARs in the periphery (Sawynok et al., 2016). Recently, it has been reported in two chronic pain models that A_{2B}ARs on myeloid cells are implicated in pain perception through the stimulation of IL-6 receptor signaling and the promotion of immune-neuronal interactions (Hu et al., 2016). Furthermore, A_{2B}ARs seem to have a proinflammatory function since it has been found that this subtype mediates the secretion of IL-6 and a consequent increase of cell proliferation through p38 pathway in microglia (Merighi et al., 2017a). A_{2B}ARs stimulation has an anti-inflammatory effect correlated to IL-10 production and TNF-α inhibition (Merighi et al., 2015b; Koscsò et al., 2012).

Although A_3ARs expression in the brain is not as copious as in the periphery, these receptors are prominent in different central diseases including ischemic stroke. The role of A_3ARs has been analysed in pain, even though with contradictory results. In some studies, conducted with nonselective ligands or KO mice, A_3ARs showed a pronociceptive function, while other studies proposed A_3ARs as an anti-nociceptive target (Janes et al., 2016). Certainly, A_3AR agonists demonstrate to elicit positive effects in neuropathic pain models through the inhibition of mechano-allodynia onset after chronic constriction injury and by rising classical analgesic drugs potency, including morphine and gabapentin (Little et al., 2015; Chen et al., 2012). Noteworthy, the analgesic effects of these compounds have been confirmed in neuropathic pain induced by chemotherapy in animal models of bone metastasis associated with breast cancer (Gessi et al., 2011 a; Janes et al., 2014; Janes et al., 2015; Varani et al., 2013). Current A_3ARs agonist clinical trials in other pathologies are showing no bad side effects after administration, so the analgesic potential of these compounds is further bolstering the development of A_3AR ligands.

Cerebral Ischemia

Ischemia is a multifactorial pathology in which many events evolve in time. After the ischemic insult, primary damage caused by the early massive increase of extracellular glutamate is followed by activation of resident immune cells, such as microglia, and production or activation of inflammation mediators. Adenosine extracellular concentration drastically increases during ischemia and reaching values that can stimulate ARs. The increase of adenosine extracellular level after ischemia is caused by different mechanisms. As estimated *in vivo*, in the first 20 min after ischemia, adenosine extracellular concentration is in the range of 1000 nM (Figure 7). Still, the principal mechanism is represented by the hydrolysis to adenosine of extracellularly released ATP by ectonucleotidases. Then, in the hours after ischemia, adenosine is mainly released from cells. Extracellular adenosine concentrations return to basal values after about 4 hours (Melani et al., 2015). Adenosine seems to have a role as an endogenous mediator of neuroprotection in the homeostatic response to changes occurring during ischemia. Of course, through A_1ARs activation, it hampers Ca^{2+} influx inducing presynaptic inhibition and reducing excitatory neurotransmitters release. Adenosine increases the conductance of K^+ and Cl^- ions, causing a rapid decrease in neuronal excitability and having a key role in ischemic preconditioning (Boison and Shen, 2010; Ciruela et al., 2012). Many studies have demonstrated that the selective A_1AR antagonist, DPCPX, increases aspartate and glutamate efflux, caused by oxygen and glucose deprivation (OGD), in rat cerebro-cortical slices. Accordingly, A_1ARs-mediated OGD-induced depression of synaptic transmission is counteracted by the administration of selective A_1AR antagonists to rat hippocampal slices (Batti and O'Connor, 2010). Moreover, the synaptic depression in hypoxic hippocampal brain slices caused by A_1ARs involves clathrin-mediated GluA2 and GluA1 containing AMPA receptor internalization according to data showing AMPA receptor down-regulation (Chen et al., 2014). The tonic activation of A_1ARs might help the enhancement of inhibitory synaptic transmission in CA1 neurons. A transient enhancement of GABA-mediated inhibitory neurotransmission by endogenous adenosine has been demonstrated in CA1 hippocampal slices from rats subjected to ischemia (Liang et al., 2009). This effect may contribute to the protection of CA1 pyramidal neurons, and hamper the neuronal death after ischemia. According to A_1ARs protective role, it has been displayed that the acute administration of A_1AR antagonists worsened ischemia damage. As said above, A_1AR agonists ameliorate ischemic or excitotoxic neuronal damage after ischemia. The neuroprotective role of these receptors is well accepted, but the use of selective A_1AR

agonists presents limitations due to the bad side effects such as sedation, bradycardia, and hypotension (Melani et al., 2014a).

Regarding A_{2A}ARs, they emerged not a long time ago as a potential therapeutic target in ischemia. Studies suggest that this receptor subtype has a dual role: in the first phase of ischemia, it potentiates excitotoxicity, while hours and days after ischemia, A_{2A}ARs on immune blood cells potentiate cell adhesion mechanisms and infiltration in the ischemic parenchyma. Accordingly, A_{2A}AR agonists/antagonists (at doses that do not modify blood pressure and heart rate) should be carefully evaluated in function of time after ischemia (Melani et al., 2014b). A_{2A}ARs can even give beneficial effects on neurogenesis, especially in the CNS, where they are involved in neuroprotection against brain ischemia by increasing neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which are able to recover brain activities (Gomes et al., 2013). It was firstly demonstrated in 1994 by Gao and Phillis that the non-selective A_{2A}ARs antagonist, 9-chloro-2-(2-furanyl)-[1,2,4] triazolo[1,5-c]quinazolin-5-amine (CGS15943), decreased damage in the gerbil following global forebrain ischemia. From that time forward many studies have confirmed the neuroprotective role of A_{2A}AR antagonists in different models of ischemia. The selective A_{2A}ARs antagonist, 8-(3-chlorostyryl) caffeine (CSC), and even CGS15943 and 4-amino [1,2,4] triazolo [4,3a] quinoxalines (CP66713), both administered before the ischemic insult ischemia, protected against hippocampal cell injury during global forebrain ischemia in gerbils. The same antagonist, administered sub-chronically after focal ischemia, was protective against brain damage, neurological deficit and disorganization of myelin 24 hours after focal cerebral ischemia in the adult rat (Melani et al., 2009). In the model of global ischemia in newborn piglets, post-treatment with SCH58261 improved neurologic recovery and protected striatopallidal neurons 4 days after ischemia. SCH58261 results in a protective agent at a dose that does not cause cardiovascular effects (Yang et al., 2013). Even the selective A_{2A}AR antagonists, ZM241385 and SCH58261, delay anoxic depolarization, a phenomenon strictly related to cell damage and death, protect from the synaptic activity depression caused by OGD and protect CA1 neuron and astrocyte from injury (Pugliese et al., 2009). ZM241385 administration displayed the same effects after a severe 9 min OGD period in the gyrus dentatus of the hippocampus (Maraula et al., 2013). Another way for A_{2A}AR antagonists to induce protection maybe by increasing GABA extracellular concentration during ischemia. The majority of excitatory glutamatergic innervation is modulated by inhibitory GABA-releasing interneurons. Enhancing GABAergic synaptic transmission results in neuroprotection in several experimental models of cerebral

ischemia. GABA is strongly increased in the cortex and striatum during ischemia and experimental evidence shows that selective A_{2A}ARs activation decreases ischemia-evoked GABA outflow and enhances GABA transport into nerve terminals by restraining PKC inhibition of GABA transporter-1 (Cristóvão-Ferreira et al., 2009).

As long as, A_{2A}AR antagonists resulted in protective after ischemia, in an apparent paradoxical manner, even A_{2A}AR agonists were found protective under hypoxia/ischemia. In fact, recent studies have showed that the A_{2A}ARs agonist, CGS21680, administered twice a day for 7 days at dose of 0.01 and 0.1 mg/kg, starting 4 hours after transient ischemia, protected from neurological deficit, weight loss, cortical infarct volume, myelin disorganization and glial activation evaluated 7 days after ischemia (Melani et al., 2015).

Other studies report that also acute exposure to CGS21680 decreases glutamate uptake while, the protracted exposure to the agonist inhibits GLT-1 and glutamate-aspartate transporter mRNA and protein levels in astrocytes (Matos et al., 2012). This inhibition occurs by modulating Na+/K+-ATPase (Matos et al., 2013).

Some of the A_{2A}AR agonists protective effects could be attributed to central effects, but a great body of literature suggests that peripheral effects on A_{2A}ARs located on blood cells are responsible for protective effects of A_{2A}AR agonists after ischemia. A_{2A}ARs are expressed in fact both on cells of innate (microglia, macrophages, mast cells, monocytes, dendritic cells, neutrophils) and on adaptive immunity (lymphocytes) (Antonioli et al., 2014a). Ischemia causes an alteration in BBB permeability allowing infiltration of leukocytes (neutrophils, lymphocytes, and monocytes) which aggravate ischemic damage (Iadecola and Anrather, 2011a).

In CNS, A_{2B}ARs are scarcely but are uniformly expressed including in the hippocampus but their function under ischemic conditions is still to be clarified (Pedata et al., 2018). It is known that A_{2B}ARs have a low affinity for adenosine, however they can be activated during hypoxic conditions when the extracellular adenosine levels are high (Koeppen et al., 2011; Popoli and Pepponi, 2012). In the stratum radiatum of CA1 hippocampal slices, the number and density of A_{2B}ARs on astrocytes are increased after ischemic preconditioning. In primary murine astrocytes, the expression of A_{2B}ARs is strongly stimulated by LPS in concert with hypoxia and inhibited by adenosine acting through A_1ARs and A_3ARs (Gessi et al., 2013). A_{2B}ARs have important functions in the rapid activation of p38 and in the subsequent inflammatory process (Koscsó et al., 2012; Wei et al., 2013). Altogether these data suggest that, after ischemia, similarly to A_{2A}ARs, A_{2B}ARs located on brain cells have a damaging role while A_{2B}ARs on endothelial and blood immune cells are implicated in dampening vascular adhesion signals and hypoxia-induced inflammation. Thus far,

blockade of A_{2B}ARs with antagonists could protect neuronal cells from ischemic brain damage, while stimulation could be useful to counteract the inflammatory status (Koeppen et al., 2011).

Data in the literature about A_3ARs role in the pathophysiology of cerebral ischemia are rather contradictory. It is known that A_3ARs play an initial protective role acting with A_1ARs by inhibiting excitatory synaptic transmission. While prolonged activation increases excitotoxicity and the risk of damage, possibly via the activation of PKC and subsequent calcium increase. Thus, the protective or deleterious role of A_3ARs depends on the severity and duration of ischemia (Melani et al., 2014a). This could be due to a fast A_3ARs desensitization after sustained receptor activation by an exogenous A_3ARs agonist and concomitant endogenous adenosine, which is increased during ischemia (Pedata et al., 2015). Other studies indicate an A_3ARs role in brain ischemia through immunomodulation. In fact, A_3ARs are involved in glial functions through the modulation of cell migration and TNF-α production in microglia (Ohawa et al., 2012). In astrocytes, it has been shown that A_3ARs decrease HIF-1 expression in both normoxic and hypoxic conditions, causing an inhibition of proinflammatory genes including those for inducible nitric oxide synthase and A_{2B}ARs. This suggests an anti-inflammatory role of this AR subtype in the CNS (Gessi et al., 2013). Moreover, by A_3ARs and A_{2B}ARs stimulation, adenosine may decrease hypoxia-triggered inflammation and vascular damage in ischemia slowing neutrophil infiltration, promoting angiogenesis, and inhibiting migration of the microglia and monocytes in ischemic areas (Pedata et al., 2014).

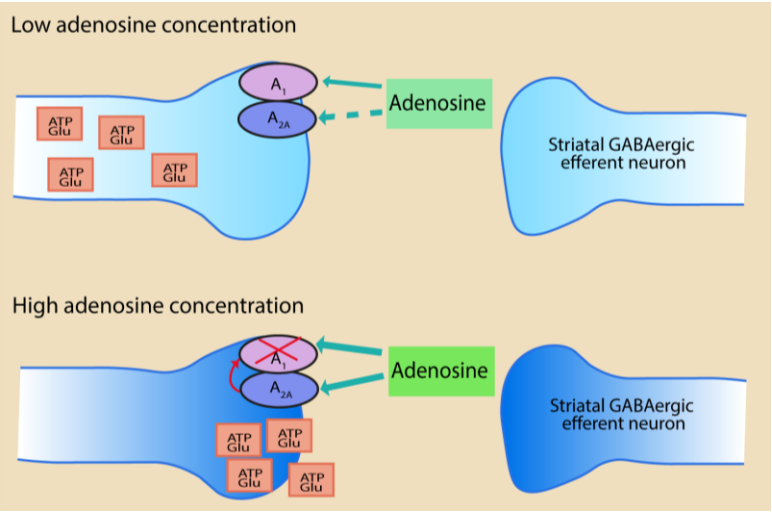


Figure 7. Schematic representation of A_1AR–A_{2A}AR heteromer as adenosine sensor. Low adenosine concentration preferentially stimulates the A_1ARs protomer of the heteromer, which would inhibit glutamatergic transmission. While, high adenosine concentration activates adenosine A_{2A}ARs that block adenosine A_1ARs-mediated effects and results in potentiation of glutamate release. (modified from Borea et al., Physiol Rev 2018, 98: 1591-1625).

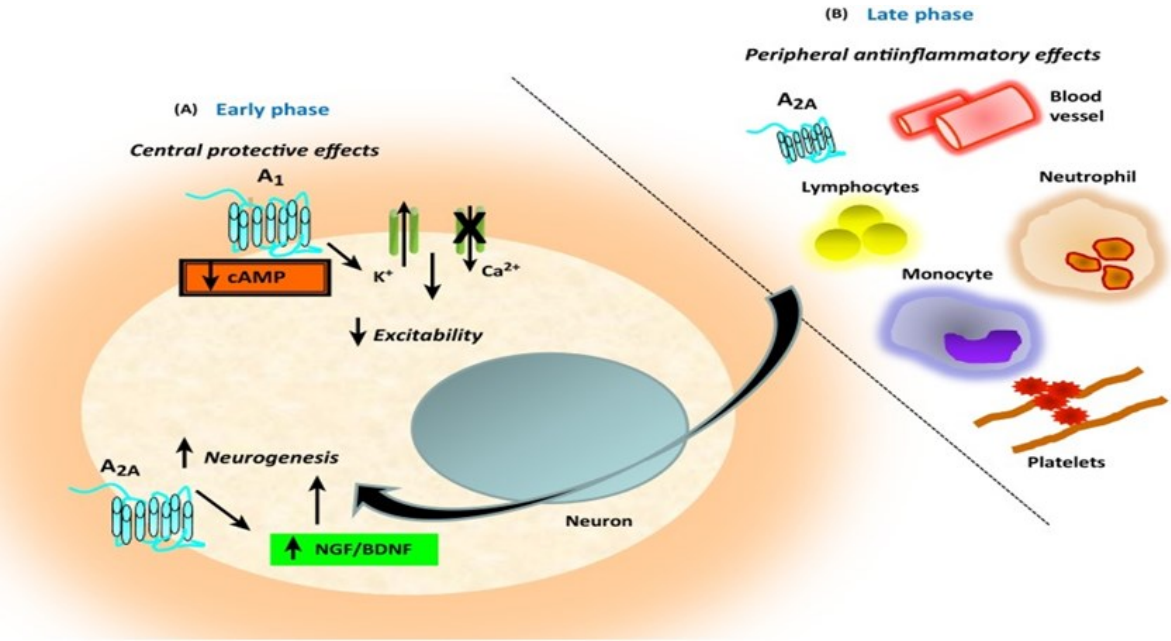


Figure 8. Schematic view of the main adenosine-mediated effects in ischemia. (**A**) A_1ARs activation inhibits Ca^{2+} influx while increasing K^+ conductance, thus reducing excitability. Adenosine is able to reduce cellular metabolism and energy consumption in ischemia within a few hours (early phase). Recruitment of A_{2A}ARs increases NGF and BDNF. (**B**) Hours and days after the insult (late phase), A_{2A}ARs activation inhibits platelet aggregation, mediates vasodilation, reduces leukocyte infiltration, and suppresses the inflammatory response, thereby attenuating neuroinflammation after ischemia. (from Borea et al., TIPS 2016, 37(5): 419-434).

Cardiovascular Diseases

In the cardiovascular system, especially in the heart, adenosine is linked to regulatory functions, including control of cardiac contractility, adrenergic responsiveness, impulse generation and conduction, coronary vascular tone and cardiac substrate utilization (Headrick et al., 2011). In particular, adenosine indirectly modifies cardiac contractility via the modulation of adrenergic responses and the inhibition of norepinephrine release from cardiac nerves. It is well accepted that adenosine decreases heart rate and impulse generation in supraventricular tissues and the His-Purkinje system, yet it adjusts vascular tone and regulates vasculogenesis and angiogenesis by fine-tuning vascular cell growth. Adenosine is able to inflect glucose metabolism and fatty acid availability, with great results on the metabolism of myocardium and responses to hypoxic or ischemic stress (Headrick et al., 2013).

A_1ARs expressed in smooth muscle cells and cardiomyocytes in atria and ventricular tissues may be useful in the treatment of different cardiovascular pathologies such as angina pectoris, ischemic injury during acute coronary syndrome or heart failure and in the cardiac rhythm control (Burnstock et al., 2015). Surely the activation of the A_1AR subtype regulates tissue transglutaminase activity in cytoprotection, and in cardiomyocyte-like cell survival during hypoxia-induced cell death (Vyas et al., 2016). A great body of evidence proposes that A_1ARs mediate antiadrenergic effects via the inhibition of β-adrenoceptor-stimulated PKA activation and Gs cycling. It has been reported that A_1ARs may inhibit β-adrenergic signaling through PKC and PLC activation, leading to the modulation of p38-MAPK and HSP27 (Fenton et al., 2010). When an ischemic insult occurs in the heart, A_1ARs mediate a positive inotropic response. A_1ARs activation has negative chronotropic effects through the inhibition of K^+ and Ca^{2+} currents, and by the hyperpolarization-activated "funny" current (Belardinelli et al., 1995). Moreover, it is well known that A_1ARs increase smooth muscle proliferation and participate in the promotion of stenosis; so, they play a role in atherosclerosis and vascular remodeling (Edwards et al., 2008). Moreover, the association between A_1ARs and atrial fibrillation in infarct and coronary artery bypass graft patients is reported by many studies (Yavuz et al., 2004). Given the electrophysiological action of A_1ARs and their involvement in arrhythmo-genesis adenosine (Adenocard) is used as a therapeutic agent for supra-ventricular tachycardia, and in electrophysiological diagnostics (Bibas et al., 2016).

Many clinical trials have been performed on more selective A_1AR agonists, they resulted inefficacious as type IV anti-arrhythmics for supra-ventricular tachycardia and atrial fibrillation (Peterman et al., 2005). Nevertheless, A_1ARs cardiovascular effects may be linked with several bad side effects and receptor desensitization which is considered the major obstacle for the chronic use of full agonists (Roman et al., 2008). For this reason, the development of partial A_1AR agonists, low efficacy compounds that give only a submaximal response, could be used as a valid alternative to induce just some of the physiological receptor responses with less desensitization than full agonists, making them ideal for chronic treatment with a wider range of doses (Albrecht-Küpper et al., 2012). In this regard, neladenoson, a partial A_1AR agonist prodrug, reveals potential cardio-protective effects without the negative ones on heart rate, atrioventricular conduction, or blood pressure in clinical trials (Meibom et al., 2017). A_1ARs mediate tissue protection in the myocardium during ischemia-reperfusion through an increase in protein *S*-nitrosylation (Williams-Pritchard et al., 2011; Shao et al., 2017). It is interesting notice that the infarct size reduction depending on the post-conditioning is modulated by the activation of

A_1ARs, this is demonstrated by the loss of the cardioprotective effect caused by the deletion of this receptor subtype (Xia et al., 2015). In the ischemic myocardium, A_1ARs activation results in a lag conduction via Gi protein pathway, A_1AR stimulation mitigates cardiac hypertrophy and prevents heart failure following adrenergic stimulation in both a rat and mice model (Puhl et al., 2016; Chuo et al., 2016). Latest evidence showed that there is a higher expression of A_1ARs in the right atrium in comparison to the left suggesting that the right atrium is more sensitive to repolarization in response to adenosine (Li et al., 2016).

Some studies demonstrate that even A_{2A}ARs are able to cause a direct inotropic effect and to counterbalance the antiadrenergic action of A_1AR activation (Tikh et al., 2006). Nevertheless, A_{2A}ARs are primarily involved in coronary vascular control through their expression in the smooth muscle and endothelium, where they induce vasodilation. The coronary feedback mediated by A_{2A}AR appears to work by PKA activation, and some studies have proposed the cooperation of p38 MAPK and IP_3 signaling. Adenosine stimulates nitric oxide production, which has vasodilatory effects, activating endothelial nitric oxide synthase by A_{2A}ARs (Ray et al., 2006). An over-expression of A_{2A}AR has been found in a streptozotocin mouse model of type 1 diabetes, resulting in augmented coronary flow in the heart (Labazi et al., 2016). It was previously demonstrated that A_{2A}ARs activation mediates an increase in coronary flow in isolated mouse hearts, via a mechanism that is partially mediated by Nox2-derived H_2O_2 (Zhou et al., 2015). Probably the mechanism by which A_{2A}ARs induce cardio-protective effects are due to a decrease in neutrophil accumulation, so these effects are linked to the great anti-inflammatory effects typical of A_{2A}ARs activation. In fact, the cardioprotection is abolished in mice with $CD4^+$ T cells lacking A_{2A}ARs, while A_{2A}AR activation furnished protection against infarction in isolated myocardium through the inhibition of mast cell degranulation (Rork et al., 2008). Moreover, an A_{2A}AR agonist is able to prevent, in a dose-dependent manner, the development of cardiac dysfunction and cardiac remodeling due to myocardial infarction in spontaneously hypertensive rats (Da Silva et al., 2017). On the contrary, the overexpression of A_{2A}ARs seems to be associated with spontaneous calcium release from the sarcoplasmic reticulum in atrial fibrillation patients (Llach et al., 2011). A_{2A}ARs have a pivotal role in atherosclerosis onset and treatment, as long as they are able to hinder foam cell formation. This effect seems to be linked to A_{2A}ARs capability to stimulate the expression of proteins involved in reverse cholesterol transport. In particular, it has been reported that A_{2A}ARs activation increases the expression and function of cholesterol 27-hydroxylase (Bingham et al., 2012; Zhou et al., 2015).

Concerning A_{2B}ARs, it is well accepted that the activation of this subtype inhibits cardiac fibroblast proliferation, vascular smooth muscle cell growth and collagen synthesis (Borea et al., 2018). Recently, an A_{2B}AR agonist has been proved to decrease transforming growth factor (TGF)-β1- and angiotensin II-mediated collagen synthesis in isolated neonatal rat cardiac fibroblasts, advocating that A_{2B}ARs activation has an antifibrotic effect (Vecchio et al., 2016). A_{2B}ARs role has been found in the inhibition of post-infarct remodeling, an action that seems to involve modulation of caspase-1 activity (Toldo et al., 2012). Indeed, there are studies respecting the cardio-protective effect of A_{2B}ARs. In particular, the A_{2B}AR- mediated cardio-protection has been linked to the inhibition of GSK-3β and the permeability transition pore, other evidence suggested that A_{2B}ARs cause myocardial metabolic adaptations through the stabilization of the circadian rhythm protein period 2 (Per2) (Eckle et al., 2012). In addition, it seems that A_{2B}ARs cardioprotection could be related to the modulation of TNF-α and neutrophil function (Koeppen et al., 2012). A_{2B}ARs have a crucial role in ischemic preconditioning mediated cardioprotection in vascular endothelial cells and cardiac myocytes, whereas A_{2B}AR signaling is critical in inflammatory cells during ischemia/reperfusion (Seo et al., 2015). Other studies suggest a beneficial role of A_{2B}ARs in atherosclerosis, due to their capability in reducing vascular injury. More recent studies reported that A_{2B}ARs signaling suppresses MHC II transactivator expression in human aortic smooth muscle cells by handling the interaction between STAT1 and the epigenetic machinery (Xia et al., 2015). In addition, A_{2B}ARs activation under hypoxic conditions bolsters foam cell formation and induces an increase in IL-8 secretion in an ERK 1/2, p38, and Akt kinase ì-dependent manner (Merighi et al., 2017b).

An extensive body of literature shows that A_3ARs decrease injury processes within myocardial tissue and have beneficial anti-inflammatory actions during reperfusion (Headrick et al., 2013). Concerning this, A_3AR agonists could protect against post-ischemic neutrophil-mediated injury and may be involved in the regulation of bone marrow-derived cells (Ge et al., 2010). In particular, A_3ARs activation induce a biphasic hemodynamic response, which is in part A_{2A}ARs mediated. The cardio-protective effect of IB-MECA, a well-known A_3ARs agonist, has been ascribed to the initial activation of A_3ARs followed by A_{2A}ARs stimulation in bone marrow-derived cells (Tian et al., 2015). Moreover, Cl-IB-MECA gives protection against doxorubicin cardiotoxicity by restoring the oxidant/antioxidant status and consequential reduction of inflammatory responses and the resultant apoptotic signals (Galal et al., 2016). A_3ARs activation by an agonist significantly reduces infarct size in both isolated perfused rat hearts and primary rat cardiac

myocytes exposed to ischemia/hypoxia and reperfusion/reoxygenation by up-regulating the p-ERK1/2 and p-AKT. During the re-oxygenation phase, A_3ARs stimulation significantly reduces apoptosis and necrosis, indicating a role for the prosurvival signaling pathways that decrease caspase-3 activity (Hussain et al., 2014). It has been reported that A_3ARs prompt human coronary smooth cell proliferation activating PLC and the induction of the transcriptions factors EGR2 and EGR3; other studies revealed that A_3ARs activation induces coronary vasodilation, and that A_3ARs expression of in cardiovascular tissues is altered in hypertension. Specifically, there is a reduction of A_3ARs in hypertensive hearts, presumably associated with the limited vasodilator responses to A_3AR agonists observed in coronary vessels (Hinze et al., 2012; Ho et al., 2016).

Inflammatory and Autoimmune Diseases

A_1ARs role on immune cells is not clear-cut since they show both pro and anti-inflammatory effects, it depends on the cell type and the pathological state considered. In multiple sclerosis (MS), for instance, A_1ARs activation seems to be protective, this is confirmed by *in vivo* models of A_1AR-deficient mice that show worsen demyelination, axonal injury, and augmented reactivity of microglia/macrophages in comparison to wild-type (WT) animals. Many studies report an A_1ARs protective function in renal and hepatic ischemia/reperfusion (I/R) injury (Rabadi et al., 2015). Moreover, a model of A_1AR-KO mouse displayed high creatinine levels and aggravated renal histology, whereas A_1ARs pre-stimulation in WT mice reduced many renal inflammatory markers, including myelo-peroxidase activity, renal tubular neutrophil infiltration, ICAM-1, IL-1β, and TNF-α. This proposes that A_1ARs pre-ischemic stimulation has protective and beneficial effects versus renal I/R injury. It is interesting to notice that an A_1AR allosteric enhancer is able to elicit strong protection against I/R damage reducing inflammation, necrosis, and apoptosis (Park et al., 2012). In opposition to the protective effects mentioned, different studies reveal that A_1ARs activation in leukocytes boosts neutrophil chemotaxis and endothelial adhesion (Alsharif et al., 2015). Another field where A_1ARs effects have been deeply studied is airway inflammation, in particular in preclinical models of asthma (Polosa et a., 2009). Nevertheless, first data showing broncho-constriction decrease using an antisense oligonucleotide or A_1AR antagonist have not been confirmed in clinical trials in asthmatic patients (Carpenter et al., 2016; Rajalingam et al., 2019). Furthermore, A_1AR antagonists may modulate glucose homeostasis by affecting oxidative stress and immune cell effects (Peleli et al., 2017). In the A_1AR-deficient mice model has been found a decreased

oxidative stress, IL-1β, IL-6, TNF- α, and IL-12, and diminished infiltration of T cells in visceral adipose tissue. Therefore, it may offer protection against age-dependent metabolic disorders such as glucose intolerance, insulin resistance, and obesity (Yang et al., 2015).

The role of A_{2A}ARs in inflammation is very contradictory; in fact they have it is proinflammatory functions in the CNS while managing many anti-inflammatory signaling pathways in the periphery (Borea et al., 2016). Principally, A_{2A}ARs activation decreases neutrophils' inflammatory functions and inhibits cytokine production, T cell activation, eosinophil and monocyte secretion, and mast cell migration (Jazayeri et al., 2017). Surely, A_{2A}ARs KO mice display a major inflammatory response, suggesting that this receptor subtype has an important function in the immune response regulation. The stimulation of A_{2A}ARs is implicated in many inflammatory diseases affecting brain, joints, bone, lung, kidney, and bowel (Allard et al., 2016 a).

Regarding rheumatoid arthritis (RA) for instance, Methotrexate (MTX), which is the gold standard therapy for this pathology, causes an increase in adenosine production. Moreover, the capability of T regulatory (Treg) cells in producing adenosine foresees its efficacy (Cronstein et al., 2017; Haskò et al., 2013; Peres et al., 2015). In mice with collagen-induced RA, an animal model of RA, it has been found an A_{2A}ARs up-regulation both in neutrophils and monocytes at the arthritic knee joint, this increase is reflected by the augment of CD73 in the macrophages, neutrophils, and monocytes of the synovial fluid. This has been the starting point for the development of a new class of selective A_{2A}AR prodrugs, which need CD73 presence to be activated. These compounds are able to limit joint inflammation interacting with A_{2A}ARs on immune cells, without the cardiovascular side effects typical of systemic A_{2A}ARs agonist administration (Flögel et al., 2012). It is known from the literature that adenosine plays a role in the suppression of inflammatory bone resorption. MTX reduces bone degradation in RA patients and mediates anti-inflammatory effects through A_{2A}ARs, which inhibit osteoclast differentiation and modulate bone regeneration by reducing NF-κB activation (Mediero et al., 2012; Mediero et al., 2013; Mediero et al., 2015). Adenosine is able to elicit anti-inflammatory effects acting on A_{2A}ARs in airways (Friebe et al., 2014). As said above, A_{2A}ARs have a pivotal role in the suppressive mechanism of Tregs. According to this, airway inflammation was significantly higher in CD39(-/-) mice in comparison to WT animals, which possess Tregs with stronger A_{2A}ARs (Li et al., 2015). Although many studies have attributed the anti-inflammatory effect of adenosine, through A_{2A}ARs, to NF-κB inhibition, novel findings suggest that the pathway involved is instead the inhibition of MAPKs, by A_{2A}ARs regulation of dual specific phosphatase 1, in macrophages (Köröskenyi et al., 2016). This

supports the idea that A_{2A}ARs targeted therapy could be a promising treatment for human inflammatory lung diseases, especially in those with a strong inflammatory component.

A_{2A}ARs present on macrophages are significant in kidney inflammation, as demonstrated in A_{2A}ARs KO mice, where it has been observed an increased inflammation; this led to glomerular damage, suggesting that endogenous A_{2A}ARs on macrophages are crucial in preventing progressive kidney fibrosis (Truong et al., 2016). Moreover, there is a growing interest about adenosine and the modulation of gut functions, besides its anti-inflammatory effects, in the pathogenesis of intestinal disorders such as inflammatory intestinal ischemia, irritable bowel diseases (IBDs), postoperative ileus, diarrhea, dysmotility, and abdominal pain (Antonioli et al., 2008). It has been reported that A_{2A}ARs decreased colonic motility in a rat model of experimental colitis, and adenosine deaminase inhibitors elicit anti-inflammatory effects in chronic colitis by activating A_{2A}ARs and A_3ARs, these effects are due to both lymphoid and non-lymphoid cell recruitment (Antonioli et al., 2010a; Kurtz et al., 2014). It has been proved that an A_{2A}AR agonist can replace the structural integrity of tissue in two experimental animal models of colitis, so the activation of this receptor subtype may be exploited in the development of new drugs for treating IBD (Pallio et al., 2016). Adenosine participates in several events occurring in wound healing, through A_{2A}ARs activation, from vasodilatation, to angiogenesis, matrix production, and inflammation (Haskò et al., 2013). In fact, topical selective A_{2A}AR agonists inhibit the inflammatory response, with a great decrease in inflammatory cell infiltrate and in LTB4, CXCL-1 levels and TNF-α, while improving dermal fibroblasts growth (Gessi et al., 2014). On the contrary, using A_{2A}AR antagonist prevents dermal changes induced by irradiation like fibrosis and atrophy (Perez-Aso et al., 2016). Surely, A_{2A}ARs stimulation promotes the synthesis of collagen type I and type III, essential mediators of fibrosis and scarring, through pathways involving cAMP/PKA/p38-MAPK/Akt and in the case of collagen III involving beta-catenin (Shaikh et al., 2016). Noteworthy, blockade of A_{2A}ARs inhibits the WNT/β-catenin signaling pathway, reducing dermal fibrosis in diseases such as scleroderma, hypertrophic scarring and keloid (Zhang et al., 2017). Literature evidence reports that A_{2A}ARs and A_{2B}ARs subtypes are, respectively, up- and down-regulated in the psoriatic epidermis; leading to opposite effects in keratinocyte proliferation, which is stimulated by A_{2A}ARs and inhibited by A_{2B}ARs through the modulation of intracellular calcium increase and p38 phosphorylation, respectively (Andrés et al., 2017). Agonists of A_{2A}AR/A_{2B}AR subtypes showed to induce anti-inflammatory effects in this condition, but these effects seem not to be due to AR-mediated interaction. Thus, more studies are needed in order to better define the relevance of A_{2A}AR agonists as anti-inflammatory and/or

A_{2A}AR antagonists as anti-proliferative agents (Merighi et al., 2017a). Another possibility to take advantage of the A_{2A}ARs activation anti-inflammatory effect is by pulsed electromagnetic fields (PEMFs) exposure. Certainly, many studies suggest that PEMFs are able to up-regulate A_{2A}ARs in different cells and tissues (Varani et al., 2008; Varani et al., 2017; Vincenzi et al., 2013a). In particular, increased A_{2A}ARs density and functionality could explain the PEMFs-mediated reduction of proinflammatory cytokines, inhibition of osteolysis and cartilage damage, and chondro-protective effects (Fini et al., 2013).

By A_{2B}ARs, adenosine has a complex role in immune cells, producing either pro or anti-inflammatory effects depending on the organ and the signaling involved. A_{2B}ARs, are expressed in almost all immune cells, hence they influence many inflammatory diseases, from MS, wound healing, fibrosis, asthma and COPD to colitis and diabetes (Borea et al., 2017). For example, A_{2B}AR antagonists can be used in the treatment of MS, studies reported that pharmacological A_{2B}AR blockade improved symptoms and decreased CNS damage. An A_{2B}ARs over-expression has been found in the peripheral leukocytes of MS patients and in mice with EAE (experimental autoimmune encephalomyelitis) lymphoid tissues (Safarzadeh et al., 2016; Wei et al., 2013). Similarly to A_{2A}ARs, A_{2B}ARs have a prominent role in wound healing and remodeling processes. In fact, they are able to limit potential infections and replace tissue integrity by inflammation, neo-vascularization, neo-epithelialization, scar formation, and remodeling. A_{2B}ARs increment angiogenesis and remodeling in cardiac mesenchymal stromal cells after myocardial injury by turning them into myo-fibroblasts (Ryzhov et al., 2014 a). Principally, A_{2B}ARs have been associated with an increase of VEGF synthesis and angiogenesis in many cell types, including cardiac mesenchymal stem-like cells, retinal and skin endothelial cells, mast cells, tumor-infiltrating hematopoietic cells, cancer cells, by involving transcription factors like HIF-1 and JUN-B (Ryzhov et al., 2014 b; Ryzhov et al., 2012; Merighi et al., 2015a). Moreover, studies report a role of A_{2B}ARs in atherosclerosis induced by a high-fat diet: atherosclerosis was higher in the absence of A_{2B}ARs in apolipoprotein E-deficient mice (Koupenova et al., 2012). Interestingly, comparing animals A_{2B}ARs KO in either the myeloid lineage, endothelial cells, or alveolar epithelial cells revealed that alveolar epithelial A_{2B}ARs signaling is consistent for lung protection and that aerosolized A_{2B}AR agonist mitigated lung inflammation (Hoegl et al., 2015). According to these data, A_{2B}ARs have been linked to the decrease of cell migration and microvascular permeability in an *in vivo* model of acute pulmonary inflammation (Konrad et al., 2017). The activation of A_{2B}ARs occurs in pathologies with chronic inflammation and fibrosis including, asthma and COPD, where a role for antagonists has been proposed (Cekic et al., 2016). In fact,

A_{2B}ARs cause an augmented Th-17 differentiation in chronic lung injury and assist the differentiation of activated macrophages, thereby contributing to pulmonary fibrosis (Wilson et al., 2011). A_{2B}ARs result to be up-regulated in the lung tissues of patients affected by fibrosis (Shaikh and Cronstein, 2016). Differently, in asthma and COPD A_{2B}ARs increase cytokine production, eosinophil degranulation, and modulate human mast cells IL-4 secretion, thereby increasing allergic inflammation (Ryzhov et al., 2008). A profibrotic role has been also detected in the kidney where A_{2B}ARs inhibition decreases renal hypoxic fibroblast growth, and the release of profibrotic cytokine, so hindering renal fibrosis development (Tang et al., 2015).

In the colon, A_{2B}ARs is the most copious adenosine receptor subtype. They are involved in modulation of chloride secretion, fibronectin, and IL-6 production in intestinal epithelial cells, and interestingly, A_{2B}ARs resulted to be up-regulated in colitis, where there are contradictory data about the function of these receptors. For instance, is well accepted that A_{2B}ARs have an important anti-inflammatory role in colonic mucosa, as proved in A_{2B}ARs KO animal models, where the lack of the receptors causes major severity of colitis because of deficiency in intestinal epithelial barrier function. In fact, A_{2B}ARs signaling in epithelial cells is pivotal for reducing colonic inflammation through phosphorylation of a vasodilator-stimulated phosphoprotein (Aherne et al., 2015). In opposition to these findings, it has been observed that A_{2B}ARs deficient mice with colitis had clinical aspects, histological outcomes, and myeloperoxidase activity less marked, it has been demonstrated by other studies that the presence of A_{2B}ARs on non-immune cells is decisive for colitis insurgence (Ingersoll et al., 2012). Even A_{2B}ARs function in glucose homeostasis is questionable. According to previous studies A_{2B}AR antagonists have hypoglycemic effects in adenosine-mediated hepatic glucose production *in vivo* models. In fact, A_{2B}AR activation arises rat liver glucose levels acting on glycogenolysis and gluconeogenesis. Other studies showed that the pharmacological blockade of A_{2B}ARs increased insulin resistance through the reduction of IL-6 and other cytokines involved in glucose and fat metabolism in diabetic mice, and reduced caspase-1 activation in rat retinal cells (Vindeirinho et al., 2016; Trueblood et al., 2011; Figler et al., 2011). Nonetheless, agonists of A_{2B}ARs are proposed as potential therapeutic agents for diabetes, this is due to the presence of a connection between this receptor subtype, insulin receptor substrate 2 (IRS-2), insulin pathways and Akt phosphorylation (Johnston-Cox et al., 2012).

Even A_3ARs are considered very important in inflammation because of their wide distribution in immune cells (Antonioli et al., 2010b

; Gessi et al., 2011 a; Haskò et al., 2013; Jacobson et al., 2017). In particular, A_3ARs are preferentially localized on the leading edge of neutrophil cell membranes, by which they are able to induce chemotaxis and migration. ATP and adenosine work together to provoke and accelerate chemotaxis induced by pathogen agents and migration activating P_2Y_2 and A_3AR activation (Butler et al., 2012; Ledderose et al., 2016). Moreover, it has been documented that A_3ARs activation induces hypothermia; leading to a fall in total energy expenditure, physical inactivity, and preference for cooler environmental temperatures through the stimulation of histamine release acting on central H1 receptors and on peripheral mast cells. This is notably important because hypothermia can help in reducing inflammation and in particular the cytokine increase caused by sepsis (Carlin et al., 2016; Carlin et al., 2017). Interestingly, in autoimmune inflammatory diseases A_3ARs can serve as new biological predictive markers. In fact, it has been demonstrated that A_3ARs are up-regulated in the peripheral blood mononuclear cells (PBMCs) of patients with RA, Crohn's disease, and psoriasis. In lymphocytes obtained from RA patients, A_3ARs reduce NF-κB signaling, inflammatory cytokines levels, and matrix metalloproteinases. Of interest, A_3ARs expression level was inversely correlated to the DAS28 and DAS scores used to judge disease activity in RA (Varani et al., 2011a). The signaling pathway involved in the anti-inflammatory effect of A_3ARs in RA patients comprises NF-κB and TNF-α in the synoviocytes. *In vitro* and *in vivo* studies results proposed A_3AR agonists in clinical trials for the therapy of different inflammatory diseases. It has been demonstrated that these compounds are safe and well-tolerated in preclinical and human studies, and specifically, the agonist IB-MECA (Piclidenoson, CF101) has been tested in phase II trials on RA patients where it showed a significant anti-rheumatic action. Interestingly, basal receptor expression correlated with the patients' reaction to the drug, suggesting that A_3ARs may be a biological prognostic marker the response to CF101 (Fishman et al., 2016). In addition, CF101 was efficacious in clinical trials on plaque psoriasis, in which it demonstrated to have a better profile than the PDE4 inhibitor apremilast (David et al., 2012; David et al., 2016). Its great safety profile makes it a promising drug for chronic psoriasis therapy. On the other hand, CF101 was not efficacious in trials for ocular hypertension and dry eye disease and, in combination with methotrexate, for RA. Many studies suggest a role for A_3ARs in asthma due to its expression in mast cells. A_3ARs are thought to have a pivotal function in rodent mast cell activation and degranulation, this effect has been demonstrated in both primary human and LAD2 mast cells (Gomes et al., 2011; Leung et al., 2014). A_3ARs are implicated in adenosine-induced broncho-constriction in asthmatics. In it well known that, in asthma, A_3ARs activation in human mast cells increases proinflammatory

mediators, such as IL-8, IL-6, VEGF, amphiregulin and osteopontin (Zhou et al., 2012). Moreover, A_3ARs stimulation decreases the expression of the receptors themselves, thus suppressing its basal inhibition on cytokine production (Rudich et al., 2015). Adenosine, by stimulating A_3ARs, modulates monocyte-macrophage functions, which causes inflammatory mediator production and healing. In addition, A_3ARs stimulation increases TNF-α production in activated macrophages (Forte et al., 2011). Functional A_3ARs are expressed in dendritic cells, antigen-presenting complexes which activate naive T lymphocytes and start primary immune responses (Gessi et al., 2010; Koscsò et al., 2011).

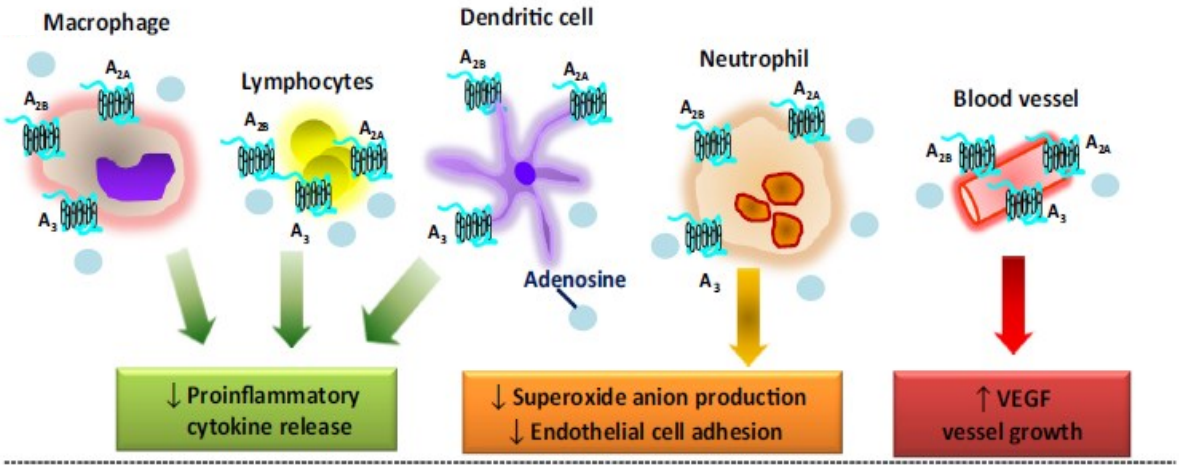


Figure 9. Schematic view of the main adenosine-mediated effects in inflammation. (A) Adenosine exerts anti-inflammatory effects through the modulation of the response of immune cells by of A_{2A}, A_{2B} and A_3ARs activation. (modified from Borea et al., TIPS 2016, 37(5): 419-434).

Cancer

Many studies try to analyse the effects of A_1ARs activation in cancer, but its role is still unclear. The majority of data comes from old studies, often performed with nonselective ligands, and both pro and anti-tumoral effects have been reported (Gessi et al., 2011b; Kazemi et al., 2018). Particularly, anti-proliferative effects have been found in colon cancer, breast cancer, glioblastoma, and leukemia cells. In addition, in astrocytoma and colon cancer cells have been found proapoptotic effects, by an augmented caspase activity. According to this data, A_1ARs display an important role in reducing glioblastoma proliferation and increasing chemotherapy sensitization through cell apoptosis stimulation (Daniele et al., 2014). Despite this, A_1ARs show protumoral effects by the increase in melanoma chemotaxis and breast cancer cell proliferation, or P27 reduction in cervical carcinoma cells. Latest data have demonstrated significantly raised VEGF R2-dependent angiogenesis through stimulation of A_1ARs in an animal model of melanoma (Koszałka et al., 2016).

Certainly, adenosine is an important regulator of several aspects of tumorigenesis. It is implicated in angiogenesis, tumor cell growth, and metastasis affecting immune system cells, like T and natural killer, myeloid-derived suppressor and dendritic cells, as well as macrophages, tumor and endothelial cells, where A_{2A}ARs are involved (Allard et al., 2016 b; Antonioli et al., 2014b; Ohta et al., 2016). Adenosine concentration is increased in hypoxic tumors due to hypoxia-dependent CD73 over-expression and AK down-regulation (Ohta et al., 2016). It is important to note that CD73 expression is linked with poor prognosis in leukemia, brain, breast, ovarian, and prostate tumors (Antonioli et al., 2016; Antonioli et al., 2010; Borea et al., 2017; Leclerc et al., 2016; Loi et al., 2013; Turcotte et al., 2015). In the specific, CD 73 genetic silencing or inhibition decrease cell growth of melanoma, breast, prostate, and fibrosarcoma tumors (Stagg et al., 2011; Stagg et al., 2012; Terp et al., 2013). It is well recognised that immune cells are important against cancer and that adenosine, which is increased in hypoxic tumors, is able to impair cytolytic effector immune cell recognition of cancer cells, suppress α4β7 integrin-dependent adhesion of T lymphocytes to colon adenocarcinoma cells, and reduce the expression of CD2 and CD28 on T cells (Butler et al., 2012). Many studies aimed to characterise which adenosine receptor subtype participates in these effects. However, a huge body of evidence suggested that adenosine, via A_{2A}ARs, is involved in the stimulation of Treg responses and induction of T cell anergy, inhibition of natural killer (NK) activity, thereby promoting tumor escape from the immune system and metastasis (Mandapathil et al., 2010). Accordingly, A_{2A}AR

antagonists may be used as new therapeutic tools to increase the immune response against cancer, by interfering with adenosine-mediated immune-suppression in tumors (Beavis et al., 2017). Although these compounds did not show toxicity, there is a need for new compounds targeting A_{2A}ARs not BBB permeable in order to counteract neurological side effects (Hatfield et al., 2016).

Even if A_{2B}ARs are involved in tumor development, this receptor subtype has not received enough attention due to its low adenosine affinity. In general, through the stimulation of cAMP, A_{2B}ARs, like A_{2A}ARs, cause depression of immune responses, promoting immune-escape. This protumoral effect has been observed in the stimulation of myeloid-derived suppressor cells, but in the activation of M2 macrophages, important for angiogenesis, proliferation, and metastasis, but not on NK cell functions (Beavis et al., 2013; Csòka et al., 2012; Ryzhov et al., 2011). Moreover, A_{2B}ARs activation provoke development of an anomalous phenotype of proangiogenic dendritic cells, suppresses RAS-related protein1 (RAP1) prenylation, important in cell-cell adhesion, and increases the Fra-1 component of activator protein 1 (AP-1) transcription factor, relevant for cell proliferation, motility, and invasiveness, thus promoting cell scattering (Ntantie et al., 2013; Desmet et al., 2013). According to this, A_{2B}ARs are able to augment experimental and spontaneous metastasis in cancer mouse models, and to impair the efficacy of classical chemotherapy drugs. In this mechanism, NK or the myeloid-dependent pathway seem not to be involved, but A_{2B}ARs inhibition causes a decrease in cell adhesion and MAPK-dependent signaling activation, leading to a minor metastatic capability (Mittal et al., 2016). Still, stimulation of metastasis through A_{2B}ARs has been reported in melanoma, ovarian, blood, and breast carcinomas (Beavis et al., 2013; Cekic et al., 2012). It has been recently found in bladder urothelial carcinoma (BUC) that there is an over-expression of A_{2B}ARs, which is associated with poor prognosis of patients. In fact, A_{2B}ARs inhibition diminished proliferation, migration, and invasion of BUC cells and blocked the cell cycle at the G1 phase (Zhou et al., 2017).

Adenosine is able to arise anti-tumoral effects through its action on neoplastic cells, typically through A_3ARs. These receptors are widely expressed in several tumors such as lymphoma, astrocytoma, glioblastoma, melanoma, and sarcoma, as well as thyroid, lung, breast, colon, liver, pancreas, prostate, and renal carcinomas (Gessi et al., 2010; Hofer et al., 2011; Kamiya et al., 2012; Kanno et al., 2012; Madi et al., 2013; Mousavi et al., 2015; Nagaya et al., 2013; Nogi et al., 2012; Otsuki et al., 2012; Sakowicz-Burkiewicz et al., 2013; Varani et al., 2011b; Vincenzi et al., 2012). Notably, the A_3ARs up-regulation in human colorectal and hepato-cellular carcinomas is mirrored by the PBMCs. This reflection of receptor status in remote tumor tissue may make A_3ARs useful tumor

markers. A_3ARs function has been analysed in different types of cancer cells, obtaining contradictory results showing both pro and anti-proliferative effects, but cell migration and apoptosis modulation (Aghaei et al., 2011; D'Alimonte et al., 2015; Gessi et al., 2011 b; Kim et al., 2010 Tliani et al., 2010; Varani et al., 2011b). In addition, A_3ARs are able to decrease telomerase activity and produce cytostatic effects in tumor cells (Fishman et al., 2012). In animal models of cancer, such as syngeneic, xenograft, orthotopic, and metastatic models of colon, prostate, melanoma, and hepato-cellular carcinomas, has been evaluated the efficacy of orally administered A_3AR agonists like IB-MECA and Cl-IB-MECA. These drugs decrease cell proliferation and enhanced the effect of cyclophosphamide in syngeneic and lung metastatic models of murine melanoma (Fishman et al., 2012). Noteworthy, A_3AR agonists reduced *in vivo* proliferation of melanoma cells through the augment of IL-12 and the cytotoxic effects of mouse NK cells. The ex vivo A_3ARs stimulation in $CD8^+$ T cells mitigated melanoma immunotherapy (Montinaro et al., 2012). Furthermore, IB-MECA has the capability to limit cancer growth and to enhance 5-fluorouracil and taxol chemotherapeutic effect in colon and prostate xenograft models, while Cl-IB-MECA inhibits the development of hepato-cellular cancer, liver inflammation, and pain in breast tumor-derived bone metastases (Cohen et al., 2011; Fishman et al., 2012; Varani et al., 2013). Nevertheless, there are contradictory results on A_3ARs behavior in tumor development because some data support the use of A_3AR antagonists in cancer treatment. The reason lies in the ability of A_3ARs to promote HIF-1α accumulation in melanoma, glioblastoma, and colon carcinoma cell lines, thus increasing angiogenic factors. This effect is corroborated by animal models of melanoma, where A_3ARs stimulation increases microvessel density, proangiogenic molecules, and macrophage tumor infiltration (Koszałka et al., 2016). A_3ARs increase MMP-9 production and activity, causing an enhanced cell invasion in glioblastoma, as previously shown in macrophages (Gessi et al., 2010). Although both agonists and antagonists have been analysed at the preclinical level, only A_3AR agonists, in particular Cl-IB-MECA (Namodenoson, CF102), have continued to clinical trials for advanced hepatocellular carcinoma treatment. In fact, phase I and phase II clinical trials have shown until now that the agonist is safe, well tolerated, and efficacious in increasing a median overall survival in patients, a subset of whom were given CF102 as second-line therapy, due to disease progression under sorafenib (Stemmer et al., 2013). A global phase II trial in these patients is in progress, and other trials are planned for CF102 in hepato-cellular carcinoma treatment.

Pathophysiological features of Cerebral Ischemia

The vast majority of cerebral stroke cases are caused by transient or permanent occlusion of a cerebral blood vessel, defined as ischemic stroke, eventually leading to brain infarction. The final infarct size and the neurological outcome depend on a wide range of factors such as the duration and severity of ischemic insult, the existence of collateral systems and an adequate systemic blood pressure, etiology and localization of the infarct, but on age, sex, comorbidities and genetic background (Sommer et al., 2017). For these reasons, stroke is considered a devastating neurological condition and a leading cause of morbidity and mortality worldwide. Almost half of all stroke survivors require long-term care (Sturm et al., 2004). The functional and cognitive disabilities of stroke survivors result in significant long-term health care costs (Noorian et al., 2014). In humans, there are three different major causes of ischemic stroke. About 50% of cases are due to large vessel atherosclerosis and rupture of an atherosclerotic plaque, while about 20% are caused by cardio-embolism. About 25% manifest as lacunar infarcts due to small vessel disease and probably occlusion of deep perforating arteries (Bailey et al., 2012). Some additional rare causes such as vasculitis or extracranial artery dissection account for the remaining 5% (Warlow et al., 2003). These percentages represent mean values over all age groups but change depending on the age of stroke. Cardio-embolic stroke becomes the most frequent subtype with increasing age, while small vessel disease is rarely responsible in young people (Starby et al., 2014). Moreover, it has to be noticed that exist a large number of cases of undetermined cause, named as cryptogenic stroke (Scullen et al., 2014). From a clinical point of view, acute onset of symptoms must be distinguished from transient ischemic attacks (TIAs) where neurological symptoms disappear within 24 h. In contrast to stroke, TIAs are thought to leave no damage to brain tissue, an opinion that may work conceptually but does not fully reflect reality (Sommer et al., 2009). In addition to acute neurological deficits, which can be directly attributed to the injured brain areas, additional cognitive and psychiatric long-term consequences may emerge. Cognitive decline is a major problem that is often present, in particular after lacunar stroke, and is probably superimposed to the underlying small vessel disease (Makin et al., 2013). Even post-stroke depression is a serious problem that develops in about 33% of patients with chronic stroke. The biological basis remains uncertain, which is due to a paucity of appropriate animal models (Kronenberg et al., 2014). Apart from neurological and neuropsychiatric consequences, stroke induces an immediate immune depression which is reflected by the development of fever, within 3 days after stroke onset, in about the 60% of stroke patients

(Haeusler et al., 2008). One important component of ischemic stroke is the evolving brain damage which is explained by the concept of the penumbra. Within minutes, reduction of the blood supply under 15–20% of baseline levels leads to an irreversibly damaged infarct core with rapidly evolving necrotic cell death. In the surrounding brain tissue, blood flow is less reduced leading to loss of neuronal function while structural integrity is maintained. If there is no restoration of blood flow, this so-called tissue at risk will be incorporated into the infarct zone. The range of cerebral perfusion between loss of electrical activity and irreversible neuronal depolarization has been termed penumbra (Sommer et al., 2017). This concept of the penumbra has become a milestone in stroke research since the penumbra can principally be rescued. In fact, through perfusion-weighted and diffusion-weighted MRI (Magnetic Resonance Imaging) methods, tissue at risk can be visualized by determining the perfusion-diffusion mismatch allowing an estimation of savable brain parenchyma (Ebinger et al., 2009).

The lack of blood flow during an ischemic stroke results in an intricate pathophysiological response causing neural injury (Hossmann, 2006). Multiple mechanisms, including excitotoxicity, mitochondrial response, free radical release, protein misfolding, and inflammatory changes, lead to neural cell loss, but many of these pathways ultimately pave the way for recovery. Injury and death of astrocytes, as well as white matter injury, contribute to cerebral damage. The delicate balance between detrimental or beneficial effects often relies on the timing and the magnitude of the factors involved. The inflammatory response is a typical example of a system that both spreads ischemic injury and helps promote recovery. Inflammation initially contributes to cellular injury through the release of cytokines and harmful radicals but secondarily helps to remove damaged tissue, permitting synaptic remodeling. , glial cells present a dual role, helping to regulate the blood-brain barrier, promoting angiogenesis and synaptogenesis, but conversely forming the glial scar that may prevent further plasticity (Gleichman and Carmichael, 2014).

The deficiency of glucose and oxygen, which causes cerebral ischemia, leads to the inability of neuronal cells to maintain normal ionic gradients. Depolarization of these neurons provokes excessive glutamate release resulting in the intracellular influx of Ca^{2+}, triggering cell death pathways such as apoptosis, autophagocytosis, and necrotic pathways (George and Steinberg, 2015). This process has been termed excitotoxicity and is mediated by the glutamatergic pathways involving NMDA receptors, AMPA receptors, and kainate receptors (Moskowitz et al., 2010). The role of Ca^{2+} in excitotoxicity remains complex and has numerous effects in the ischemic environment. The intracellular increase in Ca^{2+} leads

to mitochondrial dysfunction and activation of free radicals, phospholipases, and proteases, which lead to cell death or injury (Szydlowska and Tymianski, 2010). Interestingly, the interplay between the cells is critical to the spread of injury after ischemic insults. The blockage of the gap junctions between cells in the adult brain reduces neuronal death, this indicates the important interactions between cells during neuronal damage (Wang et al., 2010). These processes promote cerebral edema, which has clinical relevance in the first few days after stroke. Numerous therapeutic approaches have centered on interrupting pathways triggered by excitotoxicity to improve stroke recovery, and while often successful in animal models, the translation of these findings into the clinic is still challenging (Namura et al., 2013).

As above mentioned, mitochondria play a critical role in cell energy homeostasis and are thus prominently involved during ischemia when the energy balance is disrupted and ATP synthesis is altered. The fast influx of Ca^{2+}, due to excitotoxicity processes, leads to abnormal accumulation in the mitochondria causing mitochondrial permeability transition pore (mtPTP) opening and cytochrome c release (George and Steinberg, 2015). These events create mitochondrial swelling and membrane collapse, activating caspases-dependent cellular death pathways. The reactive oxygen species (ROS) created by the mitochondria have an important role in reperfusion injury and cell death in the ischemic environment (Kalogeris et al., 2014). Maintaining mitochondrial integrity and limiting their induction of apoptotic and oxidative stress pathways in the cell are important avenues to prevent widespread cell toxicity following ischemic injury.

Cerebral ischemia triggers free radicals, which contribute to the oxidative stresses on neural tissue. The influx of Ca^{2+} triggers nitric oxide (NO) production by nitric oxide synthase (NOS) that leads to injury through the formation of oxygen free radicals and the production of peroxynitrite. The mitochondria undergo dysfunction during ischemia, leading to further oxidative stress (Kalogeris et al., 2014). NADPH oxidase plays a critical role in ROS production in the setting of excitotoxicity and ischemia (Moskowitz et al., 2010). Free radicals activate the PI3-kinase/Akt pathway as well as up-regulate the transcription factor NF-κB. Other pathways of interest are the transient receptor potential (TRP) channels. TRP channels, TRPM7 specifically, are linked to free radicals in ischemia and likely contribute to increasing the influx of Ca^{2+} and cellular toxicity experienced during decreased oxygenation (Sun et al., 2009). Free radicals do not only contribute to initial toxicity, but the prevent recovery, which makes them an important post-stroke therapeutic target (Miyamoto et al., 2013).

The largest stores of intracellular Ca^{2+} reside in the endoplasmic reticulum (ER), an organelle that regulates protein synthesis and responds to protein misfolding (Zhang et al., 2014). Protein misfolding processes are largely affected by ER stress induced by ischemia (Roussel et al., 2013). As excitotoxic changes occur in neural cells, the sarcoplasmic/ER calcium ATPase (SERCA) pump fails due to energy depletion and adds to the occurrence of cell death (Szydlowska and Tymianski, 2010). The increased accumulation of misfolded proteins triggers the protein kinase-like ER kinase (PERK) pathway regulating eIF2α kinase activation, which blocks new protein synthesis. The phosphorylation of eIF2α has been explored as a means to alter damage in cerebral ischemia. Inositol requiring enzyme 1 (IRE1) is another protein involved in the misfolding of proteins that has been shown to induce apoptotic pathways during periods of ER stress (Morimoto et al., 2007). Chaperone proteins, such as oxygen-regulated protein 150 kDa and binding immunoglobulin protein, which normally guide protein synthesis, are altered in ischemia, and up-regulation of these chaperones may reduce apoptosis and limit ischemic induced damage (Roussel et al., 2013). The cumulative effect of SERCA pump failure and chaperone misfunctioning make ER stress and its role in protein misfolding important targets for acute stroke therapies.

After the acute response to hypoxic conditions, the glia helps to modulate inflammation and recovery. Although the glial scar has been shown to prevent new growth, it exhibits positive effects in helping to restore the integrity of the BBB. Additionally, reactive astrocytes, associated with the formation of the glial scar, modulate trophic factors, which enhance recovery (Rolls et al., 2009). Thus, glia plays a prominent role in modulating the injury cascade and eventual recovery after stroke. The glial cells (astrocytes and oligodendrocytes) surrounding neurons and their connections play an integral role in the brain's response to ischemia and recovery. Axons and glial cells are intimately interwoven, forming the connections and signals that compose neural activity and are recognised as key therapeutic targets to improve recovery mechanisms and reduce injurious ones. At baseline, white matter receives less blood supply than gray matter, and this may predispose white matter to ischemic damage with milder variations in blood flow. During an ischemic injury, glial cells are damaged by similar injury pathways to neurons including glutamate toxicity (Sánchez-Gómez et al., 2011). Ischemia triggers P2X7 receptors on oligodendrocytes, which contribute to calcium overload and mitochondrial depolarization (Wang et al., 2009). One of the key differences between the effects of ischemia on white matter compared with gray matter is the reliance on oligodendrocytes for functional deficits as well as the reduced influence of NMDA receptors on white matter injury (Matute et al., 2013).

The immune system plays a vital role in the CNS response to ischemia and to the eventual recovery of function. An intricate cascade of immune cells and inflammatory factors cause BBB breakdown, remodeling of the post-stroke tissue, and offer a margin of neuroprotection from an excitotoxic post-stroke environment characterized by increased free radicals and enzymes (Iadecola and Anrather, 2011b). Initially, microglia reacts to the ischemic insult followed by an increase of dendritic cells, macrophages, and lymphocytes, and as blood-brain barrier breakdown occurs, an influx of neutrophil permeates the infarct and peri-infarct region (Gelderblom et al., 2009). Proinflammatory cytokines (TNF-α and IL-1β) are released, as well as free radicals, by the immune cells in the post-stroke tissue, which increase the inflammatory response and up-regulate cell adhesion molecule expression, further spreading the immune response. In addition, immune response causes an increase in the production of matrix metalloproteins (MMPs) and myeloperoxidase (MPO), which are two major factors involved in BBB breakdown (Bao Dang et al., 2013). The inhibition of the acute inflammatory response after stroke has been shown to decrease injury and improve neurologic outcome in rodent stroke models, but has not yet been translated into the clinic (Arac et al., 2011).

Even components of the complement cascade play a role in ischemic injury and recovery. Since the amount of complement proteins increases after ischemia, evidence suggests that complement proteins are involved in synaptic remodeling (Stephan et al., 2012). Another role of complement proteins, C3a and C5a, in particular, is protecting neurons from the NMDA excitotoxicity that occurs post-stroke (Mukherjee et al., 2008). Immune cells such as eosinophils produce trophic factors such as NGF and neurotrophin-3 that promote neuronal outgrowth and may have a significant impact on post-infarct plasticity (Foster et al., 2011). Microglia play a prominent role through the production of glial cell-derived neurotrophic factor (GDNF) and BDNF, which promote neural growth and healing (Wang et al., 2013, Yang et al., 2012). Insulin-like growth factor (IGF-1), another molecule modulated by microglia, enhances axonal growth as well as neurogenesis in the sub-ventricular zone to improve stroke recovery.

The multifaceted immune response has both a beneficial and deleterious effect on the surviving tissue. The timing and levels of inflammatory factors and cells contribute to the balance of post-stroke injury and the restorative process (Peruzzotti-Jametti et al., 2014). The immune response has a positive role in recovery by pruning unwanted synapses and allowing the formation of new connections. However, there is a negative effect of the inflammatory response as demonstrated by immune-deficient rodent models that show decreased stroke volume and infarct size (Hurn et al., 2007). While neutrophils release

cytokines and radicals that worsen the inflammatory response, inflammatory cells help remove debris and damaged tissue to facilitate recovery. The balance of the inflammatory response after stroke is critical for recovery.

The complex injury pathways described above often disrupt the cortical neuronal maps. In fact, in weeks after ischemia, an increased spine formation and axonal sprouting have been observed. This suggests that there is an enhanced neural plasticity in the peri-infarct area and contra-lesional hemisphere reorganization occurs, in order to restore function. Alterations in synaptic function and vasculature have been shown to correlate with behavioral improvement after stroke as the brain remaps to compensate for damaged networks (Winship and Murphy, 2009). Because of the complexity of the restorative processes occurring after the initial ischemic damage, a single mechanistic pathway will likely not be sufficient to greatly improve functional outcomes. Strategies such as cell therapies, or a combination of different therapeutic approaches, may prove to be the most promising for clinical translation. Currently, the mainstay of acute stroke therapy is intravenous administration of tissue plasminogen activator (tPA), which has been FDA approved within a narrow time window from the ischemic insult. Less than 10% of stroke patients can benefit from such treatments due, in large part, to late referral to the hospital and an inability to meet other eligibility criteria (Azad et al., 2016). Endovascular therapies utilizing intra-arterial mechanical or chemical thrombolysis improve outcomes. After the acute time period, focused physical rehabilitation of the injured area is the primary current therapy that is proven to be effective (Veerbeek et al., 2014). Re-organization of the cortex has been observed with rehabilitation in pre-clinical models as well as in humans. While rehabilitation can be effective, and encouraging results have been demonstrated with constraint-induced movement therapy and other techniques, the extent of neurologic recovery is still limited and novel approaches to augment or enhance the body's endogenous regenerative abilities are required.

Aim of the Thesis

Cerebral ischemic stroke represents a life-threatening neurological disorder characterised by different events evolving over time. It is considered the second leading cause of death and the most common cause of adult long-term disability in developed countries. According to the World Health Organization, 15 million people suffer strokes worldwide each year. Of these, 5 million dies and another 5 million are permanently disabled. Europe averages approximately 650,000 stroke deaths each year. Until now, there is no promising pharmacotherapy for acute ischemic stroke. ARs could be interesting targets for therapeutic implementation in the treatment of stroke because extracellular adenosine concentration increases drastically after ischemia. ARs located both on central nervous system cells and on immune blood cells exert important roles during ischemia. As above mentioned, adenosine mediates clear neuroprotective effects through A_1ARs during ischemia, nevertheless, the use of selective A_1AR agonists is hampered by their bad peripheral side effects. Literature data indicate that A_{2A}AR antagonists give protection centrally by reducing excitotoxicity, while A_{2A}AR agonists, and possibly A_{2B}ARs, and A_3ARs provide protection by controlling massive infiltration and neuroinflammation in the hours and days after brain ischemia (Pedata et al., 2016).

The acute brain injury following ischemia is mostly due to the lack of oxygen and glucose. These conditions cause a fast depolarization of neurons, and an excessive release of glutamate, which provokes excitotoxic cell death, largely due to overactivation of glutamatergic NMDA receptors. In particular, NMDA receptors are highly permeable to Ca^{2+} and are responsible for neurotoxic intracellular Ca^{2+} levels that, activating cell lipases, endonucleases, proteases, and phosphatases, ultimately bring to acute excitotoxic cell death. Many studies indicate that A_1ARs have an inhibitory tone on synaptic transmission. Unfortunately, the development of A_1ARs selective agonists as possible anti-ischemic drugs has been stalled by their sedative and cardiovascular side effects, including bradycardia and hypotension (Borea et al., 2018). From this background, the aims of the present study were to characterise the expression and the density of ARs in ischemic stroke patients in comparison to healthy control subjects. In order to achieve this goal peripheral blood samples were collected both from ischemic stroke patients and from healthy subjects and were analised by RT-PCR, Western blot and saturation binding assays. The other aim of the study was to evaluate the role of adenosine and its receptors, in an *in vitro* model of cytotoxic damage, induced by glutamate, in neuronal-like cells by flow cytometry analysis.

Materials and Methods

Ischemic stroke patients and healthy subjects

All patients enrolled in the present study were recruited from the Neurology Unit of S. Anna Hospital, University of Ferrara, Italy. A total of 50 ischemic stroke patients and 45 control subjects were included. The demographic and clinical features of the subjects are listed in Table 1. Patients were classified on the basis of the kind of ischemic insult, according to OCSP classification (Oxfordshire Community Stroke Project). This classification recognises 4 different types of ischemic stroke named as follows: TACI (total anterior circulation infarcts), PACI (partial anterior circulation infarcts), LACI (lacunar circulation infarcts) and POCI (posterior circulation infarcts). The severity of the stroke consequences was assessed according to NIHSS (National Institutes of Health Stroke Scale or NIH Stroke Scale), a tool used by healthcare providers to objectively quantify the impairment caused by a stroke. The scores are: from 1 to 5 is slight impairment, 6-14 moderate impairment, 15-25 severe impairment, > 25 very severe impairment. Patients were also categorised by subtypes of ischemic stroke mainly based on etiology determined following TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification, which denotes five subtypes of ischemic stroke: LAA (large-artery atherosclerosis), CE (cardioembolism), SVA (small-vessel occlusion), Other (stroke of other determined etiology), and Undetermined (stroke of undetermined etiology).

Healthy control subjects (n=45), matched for similar age and sex rate to the cohort of the examined patients, were volunteers from Ferrara University Hospital Blood Bank. The study was approved by the local Ethics Committee of the University Hospital of Ferrara (Italy) and informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

Sample collection and human lymphocyte preparation

Lymphocytes were isolated and prepared from the peripheral blood of control subjects and ischemic stroke patients. The isolation of blood cells started no later than 3–4 h after the samples had been taken. The blood was supplemented with 6% (by weight) Dextran T500 solution (Sigma-Aldrich) and erythrocytes were allowed to settle down for 60 min. Leukocytes were centrifuged for 15 min at 100 x g and remaining erythrocytes were lysed in distilled water at 4°C. Cells were pelleted by centrifugation for 5 min at 250 x g, suspended in Krebs-Ringer phosphate buffer and layered onto 10 ml of Fycoll-Hypaque (GE Healthcare). After centrifugation, mononuclear cells were washed in 0.02 M phosphate-buffered saline at pH 7.2 containing 5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. To

obtain membrane suspensions, cell fractions were centrifuged in hypotonic buffer at 20000 x g for 10 min. The resulting pellet was resuspended in Tris HCl 50 mM buffer pH 7.4 containing 2 UI/ml adenosine deaminase (Sigma-Aldrich) and incubated for 30 min at 37°C. The suspension was then centrifuged again at 40000 x g for 10 min and the final pellet was used for radioligand binding experiments. The protein concentration was determined by a Bio-Rad method with bovine albumin as reference standard (Varani et al., 2010).

Real-Time Quantitative Polymerase Chain Reaction assays

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method from patients' and control subjects' lymphocytes. Quantitative RT-PCR assays of A_1, A_{2A}, A_{2B} and, A_3AR mRNAs were carried out using gene-specific fluorescently labeled TaqMan MGB probe (minor groove binder) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). For the RT-PCR of A_1, A_{2A}, A_{2B} and A_3ARs the Assays-on-Demand TM Gene Expression Products NM 000674, NM 000675, NM 000676 and NM 000677 were used, respectively. For the RT-PCR of the reference gene, the endogenous control human β-actin kit was used, and the probe was fluorescent-labeled with VIC^{TM} dye (Applied Biosystems) (Varani et al., 2010).

Western Blot analysis

Human lymphocytes were washed with ice-cold PBS and lysed in radio-immunoprecipitation assay buffer (Sigma-Aldrich) containing protease inhibitors and 1 mM sodium orthovanadate. Proteins were eluted in Laemmli buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. Next, the membranes were incubated with specific antibodies for ARs (Alpha Diagnostic International), followed by washing and incubation with HRP-conjugated secondary antibodies. After a stripping step, the blots were reprobed with anti-β-actin antibody (clone EPR1123Y; EMD Millipore).

Saturation binding assays to A_1, A_{2A}, A_{2B} and A_3ARs

Saturation binding experiments to A_1ARs were carried out with the use of [^{3}H]-DPCPX ([^{3}H]-1,3-dipropyl-8-cyclopentyl-xanthine, specific activity 120 Ci/mmol, (Perkin Elmer Life and Analytical Sciences) as radioligand. Human lymphocyte membranes (60 μg of

protein/assay) with 8 to 10 concentrations of [^{3}H]-DPCPX (0.01–20 nM) were incubated for 90 min at 25°C. Non-specific binding was determined in the presence of 1 μM DPCPX. Saturation binding to A_{2A}ARs was performed with the use of [^{3}H]-ZM 241385 ([^{3}H]-4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol, specific activity 27 Ci/mmol, (Biotrend), as radioligand. Cell membranes (60 μg of protein/assay) were incubated for 60 min at 4°C with various concentrations (0.01–20 nM) of [^{3}H]-ZM 241385. Non-specific binding was determined in the presence of 1 μM ZM 241385.

Saturation binding experiments to A_{2B}ARs were performed by using [^{3}H]-MRE 2029F20 ([^{3}H]-N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide, specific activity 123 Ci/mmol, GE Healthcare) as radioligand. Cell membranes (80 μg of protein/assay) and [^{3}H]-MRE 2029F20 (0.01–30 nM) were incubated for 60 min at 4°C and non-specific binding was determined in the presence of 1 μM MRE 2029F20.

Saturation binding experiments to A_3ARs were carried out using [^{3}H]-MRE 3008F20 ([^{3}H]-5N-(4-methoxyphenylcarbamoyl) amio-8-propyl-2-(2- furyl) pyrazolo [4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine, specific activity 67 Ci/mmol, (GE Healthcare) as radioligand. The membranes (80 μg of protein/assay) with [^{3}H]-MRE 3008F20 (0.01–30 nM) were incubated at 4°C for 150 min and MRE 3008F20 1 μM was used to evaluate non-specific binding.

Bound and free radioactivity were separated in a Brandel cell harvester (Brandel Inc.) by filtering the assay mixture through Whatman GF/B glass fiber filters (Whatman). The filter-bound radioactivity was counted in a 2810 TR liquid scintillation counter (Perkin Elmer) (Varani et al., 2010).

Evaluation of S100-β levels in serum samples

Ischemic stroke patients' and healthy control subjects' peripheral blood samples were centrifuged for 15 min at 100 × g to separate serum from remaining blood. Then serum was withdrawn and stored at -20°C until used for analysis. S100-β concentration in patients' serum and control subjects samples was detected using human S100-β ELISA sandwich kit (EMD Millipore) following the manufacturer's instruction.

Cell culture and treatments

Rat pheochromocytoma (PC12) cells were purchased from American Type Culture Collection (Manassas) and were maintained in DMEM High Glucose medium (Euroclone) supplemented with 5% FBS (Euroclone), 10% horse serum (Euroclone), L-glutamine (2 mM) (Euroclone), penicillin (100 U/ml) (Euroclone), and streptomycin (100 µg/ml) (Euroclone) in a humidified atmosphere (5% CO_2) at 37°C. Cells were sub-cultured three times a week then plated in 6 wells plates and treated for subsequent analysis. PC12 cells were treated with glutamate at different concentrations (2, 5, 7.5, and 10 mM) (Sigma Aldrich); adenosine deaminase (ADA) (2 IU/ml) (Sigma Aldrich); non selective ARs antagonist, CGS 15943 (9-Chloro-2- (2-furanyl)- [1,2,4] triazolo [1,5-c]quinazolin-5-amine) (10 µM) (Tocris); non selective ARs agonist, NECA (5′- (N-Ethylcarboxamido) adenosine) (10 µM) (Tocris); selective A_1ARs antagonist, DPCPX (1,3-Dipropyl-8-cyclopentylxanthine) (1-10 µM) (Tocris); selective A_1ARs agonist, CCPA (2-Chloro-N6-cyclopentyladenosine) (10 nM, 100 nM, 500 nM, 10 µM) (Tocris); selective A_{2A}ARs antagonist, SCH 442416 (2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo [4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine) (10 µM) (Tocris); selective A_{2B}ARs antagonist, PSB 603 (8-(4-(4-(4-Chlorophenyl)piperazide-1-sulfonyl) phenyl)-1-propylxanthine) (10 µM) (Tocris); selective A_3ARs antagonist, P 413 (2-phenyl-5-(2-thienyl)-pyrazolo[4,3-d]pyrimidin-7-(4-methoxybenzoyl)amine) (10 µM); selective activator of AC, Forskolin (FK) (5 µM) (Sigma Aldrich); selective A_1ARs allosteric enhancer, TRR 469 ((2-Amino-4-[(4-(phenyl)piperazin-1-yl)methyl]-5-(4-fluorophenyl)thiophen-3-yl)-(4-chlorophenyl)methanone) (1 µM, 3 µM, 7.5 µM, 10 µM) synthesized and previously pharmacologically characterised by our research group (Romagnoli et al., 2012). The chemical structures of the compounds used in the experiments are reported in Scheme 1.

Mouse cortex membrane preparation

For membrane preparation mouse cortex was rapidly removed, resuspended in 50 mM Tris-HCl (pH 7.4) and homogenized by means of a Polytron. The homogenate was then centrifuged for 20 min at 40000 x g and the resulting pellet resuspended and incubated for 30 min in 50 mM Tris-HCl (pH 7.4). The suspension was then centrifuged again for 20 min at 40000 x g and the membrane pellet homogenized in 50 mM Tris-HCl (pH 7.4) and used for binding experiments. The protein concentration was determined according to a Bio-Rad method with bovine albumin as standard reference.

[^{3}H]-CCPA and [^{3}H]-DPCPX binding experiments on mouse cortex membranes

Saturation binding experiments of [^{3}H]-CCPA (0.05–20 nM) in mouse cortex membranes were performed in triplicate at 25°C for 90 min in 50 mM Tris-HCl, pH 7.4, in the absence and in the presence of TRR 469. Non-specific binding was defined with 1 μM R-PIA (Vincenzi et al., 2014). Saturation binding experiments of [^{3}H]-1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]-DPCPX, 0.05-20 nM) in mouse cortex membranes were performed in triplicate at 25°C for 90 min in 50 mM Tris-HCl, pH 7.4. Competition experiments were carried out by incubating 1 nM [^{3}H]-DPCPX with membrane suspension (80 μg of protein/100 μl) and different concentrations of adenosine (0.1 nM – 100 μM) or CCPA (0.01 nM-1 μM) at 25°C for 90 min in 50 mM Tris-HCl, pH 7.4. Non-specific binding was defined as binding in the presence of 1 μM DPCPX (Vincenzi et al., 2014).
Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fibre filters using a Brandel cell harvester (Brandel Instruments). The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

Apoptosis evaluation by Flow Cytometry analysis

PC12 cells were seeded in 6 well plates at a density of 2 x 10^5 cells, and incubate in a humidified atmosphere (5% CO_2) at 37°C. The day after, cells were treated with glutamate, ADA and/or specific ligands of ARs for 24 hours. Then, cells were detached using Accutase detaching solution (Invitrogen) and subsequently stained by adding 1 drop of Alexa Fluor 488 Annexin Ready Flow Reagent (Thermo-Fisher Scientific) to 1 x 10^5 cells in 100 μl of Annexin Binding Buffer (Thermo-Fisher Scientific). Cells were then incubated for 5 minutes at 25°C, followed by the addition of 1 μM SYTOX AADvanced Ready Flow Reagent (Thermo-Fisher Scientific). Data were acquired on an Attune NxT Flow Cytometer (Thermo-Fisher Scientific). PC12 cells were gated according to physical parameters and cell aggregates were removed from the analysis.

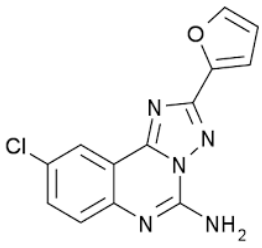
Activation of caspases 3/7 by Flow Cytometry analysis

PC12 cells were seeded in 6 well plates at a density of 2 x 10^5 cells, and incubate in a humidified atmosphere (5% CO_2) at 37°C. The day after cells were treated with glutamate, ADA and/or specific ligands of ARs for 24 hours. Then, cells were detached using

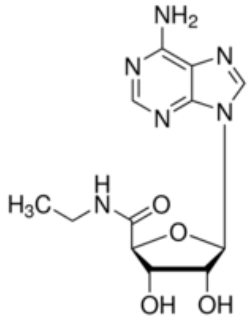
Accutase detaching solution (Invitrogen) and subsequently stained by adding 1 µl of CellEvent™ Caspase-3/7 Green Detection Reagent (Thermo-Fisher Scientific) to 1 ml of sample (1 x 10^5 cells) resuspended in PBS and incubated for 30 minutes at 25°C. Data were acquired on an Attune NxT Flow Cytometer (Thermo-Fisher Scientific). PC12 cells were gated according to physical parameters and cell aggregates were removed from the analysis.

Statistical Analysis

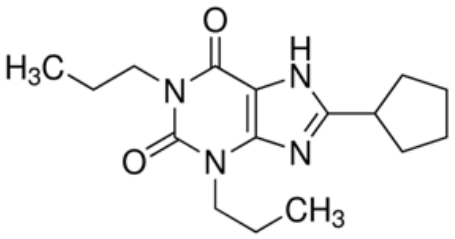
Dissociation equilibrium constants for saturation binding, affinity, or K_D values, as well as the maximum densities of specific binding sites (Bmax), were calculated for a system of one- or two-binding site populations by means of a non-linear curve fitting using GraphPad Prism software version 6.0 (GraphPad Software). All data are reported as mean ± SEM of different independent experiments as indicated in the "results" section or in the figure legends. Analysis of data was performed by one-way analysis of variance (ANOVA). Differences between the groups were analysed with Bonferroni's test and were considered significant at a value of $p < 0.01$.



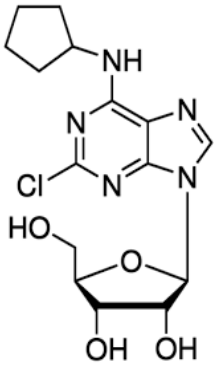
CGS 15943



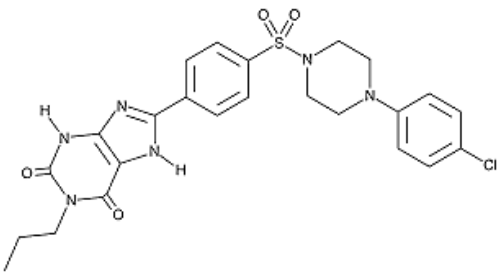
NECA



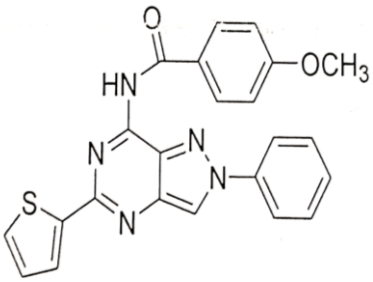
DPCPX



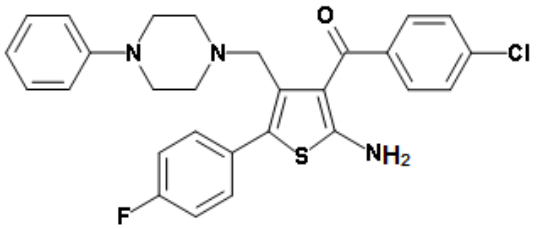
CCPA



PSB 603



P 413



TRR 469

Scheme 1. Chemical structure of ARs ligands used in the study.

Results and Discussion

Part one:

Adenosine receptors in

ischemic stroke patients

A_{2A}ARs mRNA and protein expression are up-regulated in lymphocytes of ischemic stroke patients

ARs mRNA and protein expression were evaluated in lymphocytes of patients affected by ischemic stroke in comparison with those of healthy subjects by quantitative RT-PCR assay and Western blot analysis, respectively. Figure 10A reports the relative A_1, A_{2A}, A_{2B}, and A_3ARs mRNA levels determined by RT-PCR in human lymphocytes of healthy subjects and ischemic stroke patients. Among these receptors, only A_{2A}ARs mRNA expression was significantly increased in patients in comparison to control subjects. Figure 10B and Figure 10C show Western blot and densitometric analysis which indicated a significant increase in A_{2A}ARs protein expression in lymphocytes of patients compared with healthy subjects, while no differences were found in A_1, A_{2A}, A_{2B}, and A_3ARs.

A_{2A}ARs increase in affinity and density in lymphocytes of ischemic stroke patients

The affinity (K_D) and density (Bmax) of ARs in lymphocyte membranes are reported in Table 2. Figure 11 shows the density, expressed as Bmax, of ARs in patients' lymphocytes highlighting a significant increase in A_{2A}ARs density no differences in density values of the other ARs subtypes were found. Saturation curves and Scatchard plots of [^{3}H]-ZM 241385 in human lymphocytes, confirmed the up-regulation of A_{2A}ARs in ischemic stroke patients compared with healthy subjects (Figure 12A, 12B). The affinity of the radioligand [^{3}H]-ZM 241385 for A_{2A}ARs, expressed as K_D (nM) is increased from 1.48 to 0.97 nM, in lymphocytes from patients compared with that of the control group (Table 2). Interestingly, the A_{2A}ARs density was significantly increased in patients reaching a 2.7-fold increment.

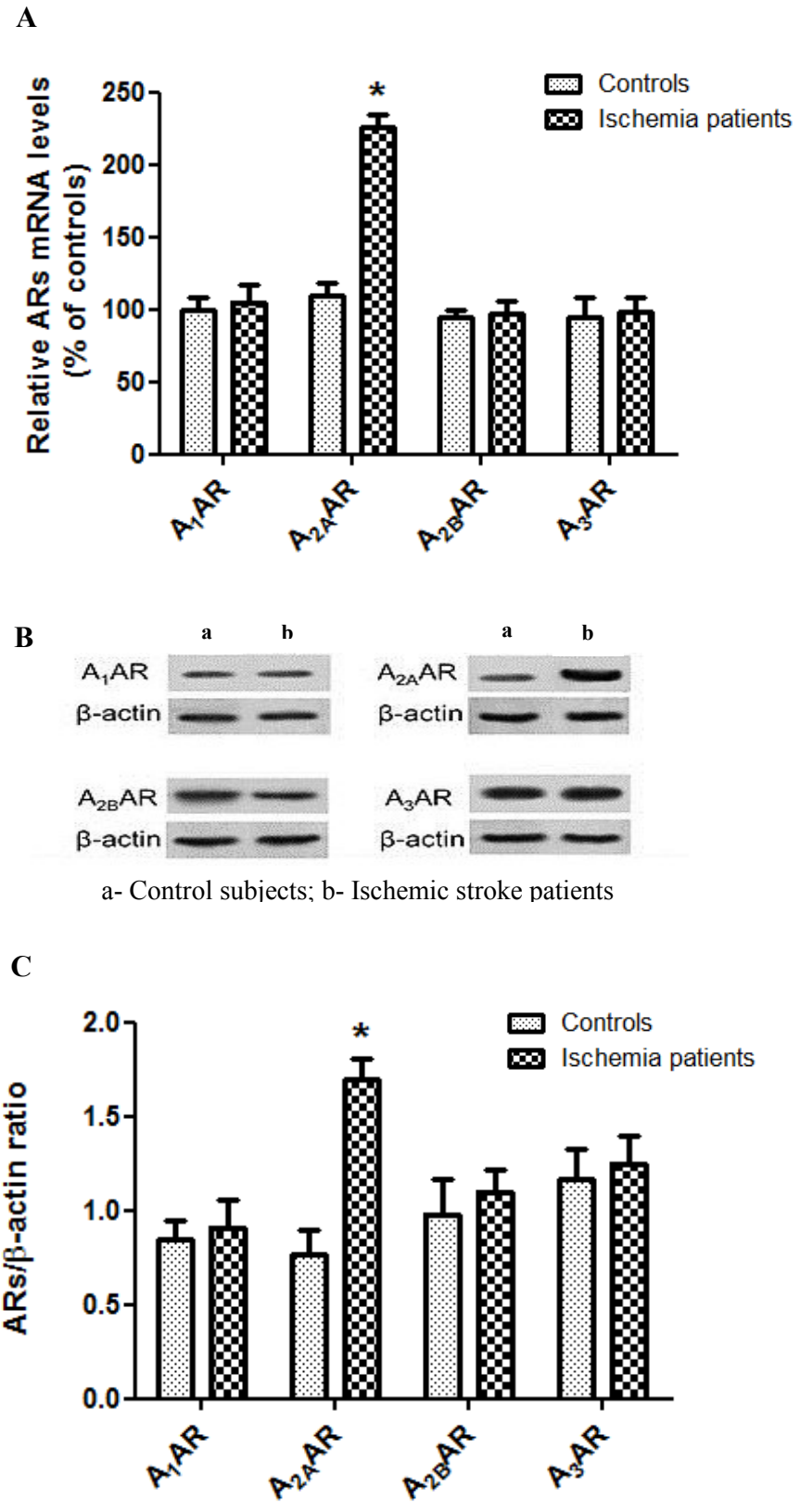
S100-β levels are increased in ischemic stroke patients' serum

S100-β (S-100 calcium-binding protein B) is a protein with neurotrophic activity in the CNS expressed primarily by astrocytes. The S-100β levels are associated with a variety of acute disorders and other chronic diseases (Li et al., 2015). Thus, the presence of S100-β has been evaluated in serum samples of a restricted cohort of ischemic stroke patients (n=10) and healthy subjects (n=10), revealing a significant 11-fold increase of this protein in ischemic stroke patients in comparison to healthy subjects from (Figure 13).

Table 1. Demographic and clinical features of ischemic stroke patients and healthy subjects enrolled in the study.

Ischemic stroke patients	**n=50**
Demographic features	
N° female/male	**22/28**
Age	**76.38 ± 12.02**
Hypertension	**32**
Diabetes	**7**
Smoke	**8**
Atrial fibrillation	**17**
OCSP patients classification	
TACI	**12**
PACI	**27**
POCI	**6**
LACI	**5**
NIHSS	
Slight impairment (1-5)	**21**
Moderate impairment (6-14)	**19**
Severe impairment (15-25)	**10**
Very severe impairment (> 25)	**0**
TOAST Classification	
LAA	**14**
CE	**24**
SVA	**6**
Other	**0**
Undetermined	**6**
Healthy subjects	**n=45**
Demographic features	
N° female/male	**18/27**
Age	**69.24 ± 10.17**

OCSP classification: Oxfordshire Community Stroke Project classification; TACI: total anterior circulation infarcts; PACI: partial anterior circulation infarcts; LACI lacunar circulation infarcts; POCI: posterior circulation infarcts; NIHSS: National Institutes of Health Stroke Scale or NIH Stroke Scale; TOAST classification: Trial of Org 10172 in Acute Stroke Treatment classification; LAA: large-artery atherosclerosis; CE: cardioembolism; SVA: small-vessel occlusion.



a- Control subjects; b- Ischemic stroke patients

Figure 10. Adenosine receptors expression levels in ischemic stroke patients and healthy subjects. Relative ARs mRNA levels determined by RT-PCR (**A**), Western blot analysis (**B**) and densitometric analysis (**C**) in human lymphocytes from ischemic stroke patients (n = 50) and control subjects (n = 45). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control group.

Table 2. Adenosine receptors binding parameters in lymphocytes from patients with ischemic stroke in comparison with healthy subjects. Data are expressed as the mean ± SEM. Differences were considered significant at a value of * $p < 0.01$ vs healthy controls.

	A_1ARs K_D (nM) Bmax (fmol/mg protein)	A_{2A}ARs K_D (nM) Bmax (fmol/mg protein)	A_{2B}ARs K_D (nM) Bmax (fmol/mg protein)	A_3ARs K_D (nM) Bmax (fmol/mg protein)
Healthy subjects (n=45)	1.74 ± 0.11 52 ± 4	1.48 ± 0.10 63 ± 4	2.11 ± 0.20 53 ± 5	1.87 ± 0.14 76 ± 11
Ischemia patients (n=50)	1.58 ± 0.12 45 ± 3	0.97 ± 0.04* 168 ± 4*	2.34 ± 0.16 58 ± 4	1.91 ± 0.11 87 ± 15

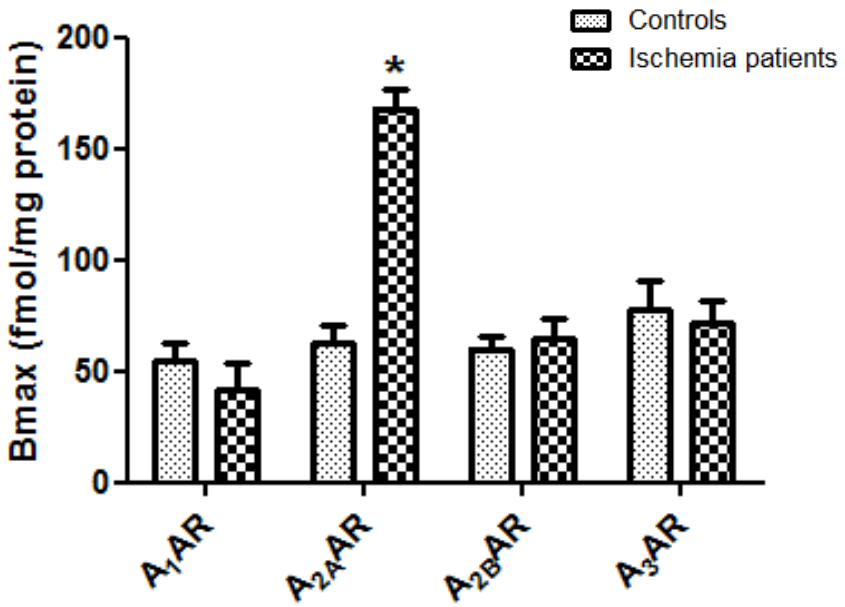


Figure 11. Adenosine receptors density in ischemic stroke patients and control subjects. Density of A_1, A_{2A}, A_{2B}, and A_3ARs, expressed as Bmax, in lymphocytes derived from ischemic stroke patients (n = 50) in comparison to control subjects (n = 45). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control group.

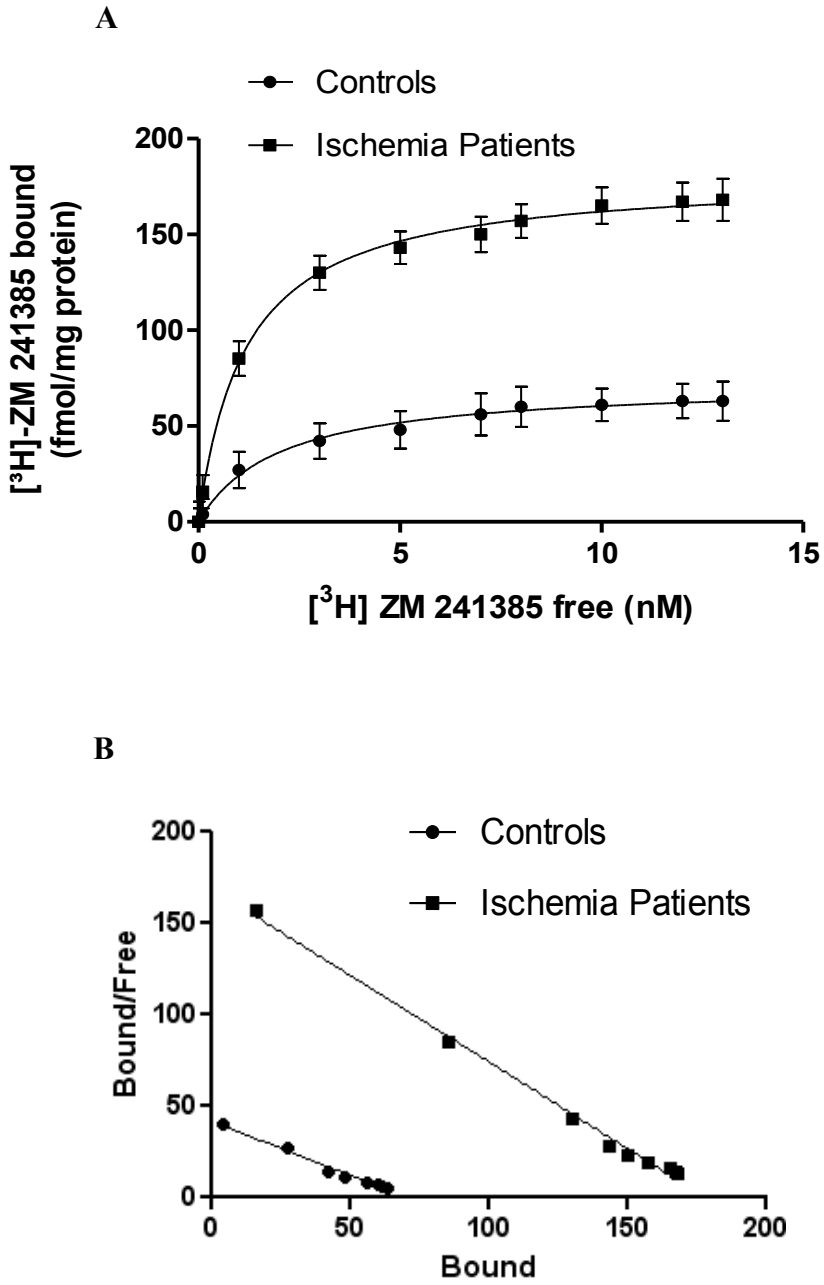


Figure 12. Increased density of A_{2A}ARs in ischemic stroke patients in comparison to control subjects. Saturation curve (**A**) and Scatchard plot (**B**) showing the binding of [^{3}H]-ZM 241385 to A_{2A}ARs in lymphocyte membranes derived from 45 controls, 50 ischemic stroke patients. Data are expressed as the mean ± SEM.

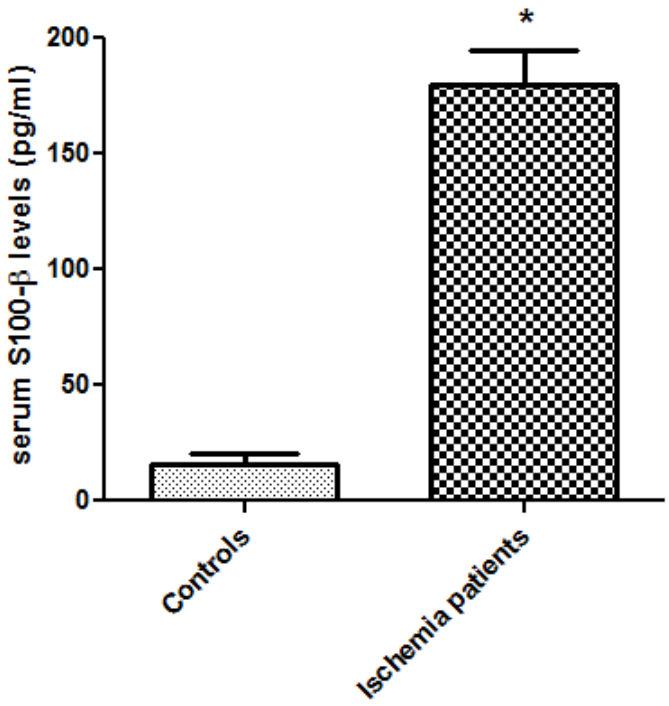


Figure 13. S100-β levels in serum samples derived from 10 controls, 10 ischemic stroke patients. Data are expressed as the mean ± SEM. * $p < 0.01$ vs control group.

Discussion

The aim of this first part of the study was to assess the presence of ARs in lymphocytes from brain ischemic stroke patients compared to healthy subjects. Noteworthy, only A_{2A}ARs were significantly increased in patients while no differences were found for A_1, A_{2B} or A_3AARs. This up-regulation has been found at mRNA level, protein level, in receptors density and even in affinity. The alteration of the above parameters has been found correlated with clinical phenotype in neurodegenerative diseases, such as PD, in which A_{2A}R ligands have shown a therapeutic effect both in experimental models and in clinical trials (Varani et al., 2010; Mizuno et al., 2010). It has been shown that A_{2A}ARs are implicated in other neurodegenerative pathologies such as ALS and HD (Vincenzi et al., 2013b; Borea et al., 2017). Until now, there were no studies in the literature reporting the presence of ARs in lymphocytes from ischemic stroke patients, some information is available on the effect of adenosine and/or ARs in the central nervous system. Studies suggest that A_{2A}ARs have a dual role in cerebral ischemia: A_{2A}AR antagonists provide early protection via centrally mediated control of excessive excitotoxicity, while A_{2A}AR agonists provide protracted protection by controlling massive blood cell infiltration in the hours and days after ischemia (Pedata et al., 2014). Accordingly, A_{2A}AR agonists and/or antagonists (at doses that do not modify blood pressure and heart rate) should be carefully evaluated in function of the time after stroke (Melani et al., 2014a). In the CNS, they are involved in neuroprotection against brain ischemia by increasing NGF and BDNF, important factors involved in the recovery of brain activities after an ischemic insult (Gomes et al., 2013). Moreover, a great body of literature suggests that peripheral effects on A_{2A}ARs located on blood cells are responsible for the protective effects of adenosine A_{2A}AR agonists after ischemia. A_{2A}ARs are expressed both on cells of innate (microglia, macrophages, mast cells, monocytes, dendritic cells, neutrophils) and on adaptive immunity (lymphocytes) (Antonioli et al., 2014a). It has been reported that adenosine, through A_{2A}ARs, reduces glutamate uptake in astrocytes *in vitro*, via GLT-1 transporters, and stimulates glutamate release via PKA pathway (Stockwell et al., 2017). There is great interest, for instance, in the potential role of A_{2A}AR agonists in chronic rheumatic inflammatory diseases, such as RA. These novel data open up the possibility to study a pharmacologic therapy based on the modulation of A_{2A}ARs. Evidence suggests that A_{2A}AR antagonists provide early protection via centrally mediated control of excessive excitotoxicity, while A_{2A}AR agonists provide protracted protection by controlling massive blood cell infiltration in the hours and days after ischemia. Focus on inflammatory

responses provides for adenosine A_{2A}AR agonists a wide therapeutic time-window of hours and even days after stroke (Melani et al., 2014b). These results report, for the first time, the presence of an A_{2A}ARs up-regulation in lymphocytes obtained from ischemic stroke patients suggesting the involvement of A_{2A}ARs in this pathology.

S-100 proteins are localized in the cytoplasm and nucleus of a wide range of cells and are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S-100 genes include at least 13 members, which are located as a cluster on chromosome 1q21; S-100 proteins mainly consist of homo- or hetero-dimers of S-100α and S-100β proteins, the first predominating in neurons and the second in glial cells (Albuerne et al., 1998). A wide number of evidence has demonstrated that altered S-100β expression has been implicated in many diseases besides multiple neurological diseases, such as Alzheimer's disease, frontotemporal dementia, Down's syndrome, cognitive impairment and dementia, epilepsy, sleep apnoea syndrome and melanoma, as well as ischemic and hemorrhagic stroke (Banfalvi et al., 2004; Vos et al., 2006; Sergeeva et al., 2011). Many studies have suggested that S-100β proteins may represent a useful neuro-biochemical marker of brain damage and neurological deficits resulting from acute stroke (Li et al., 2015; Yardan et al., 2011). Compelling studies have evidenced the elevated S-100β levels in cerebrospinal fluid and serum after acute brain ischemia and infarct conditions (Parton et al., 2009). A systematic review showed that elevated levels of serum S-100β after stroke onset implied larger infarction volumes, more severe strokes and worse functional outcome, indicating that serum levels of S-100 β protein during acute stroke are a useful marker of infarct size and clinical outcome (Nash et al., 2008). An increasing number of papers supports the prediction of serum S-100β levels for infarct volume in ischemic stroke (Ahmad et al., 2012). It has been observed that peak serum levels of S-100 proteins were correlated with infarct volume and with neurological outcome. Further, S-100β release in patients with normal flow velocities of basal cerebral arteries at admission was less than that in those with main stem or multiple branch occlusions. The release of S-100β was positively correlated with the severity of the corresponding neurological deficit and the final infarct volume (Wunderlich et al., 2004). In an acute cerebral ischemia/reperfusion rat animal model, the reduction of S-100β serum levels has neuro-protective benefits such as improving neurological scores and lowering infarct volumes (Jiang et al., 2016). S-100β seem to have a good correlation with severity, volume of brain damage and outcome in stroke. Other studies are needed to verify if there is a correlation between S-100β and A_{2A}ARs levels or if the modulation of A_{2A}ARs is linked to changes in S-100β levels.

Results and Discussion

Part two:

Adenosine and adenosine receptors in an *in vitro* model of glutamate excitotoxicity

Adenosine is necessary to glutamate to exert its cytotoxic effect

Glutamate excitotoxicity is a primary mechanism of neuronal injury following stroke. The role of adenosine and its receptors in an *in vitro* model of glutamate excitotoxicity in PC12 cells was investigated. Figure 14A and B show the apoptotic response at different concentrations of glutamate in PC12 cells treated with glutamate for 24 hours. The tested concentrations (2 mM, 5 mM, 7.5 mM and 10 mM) determined 25%, 43%, 75% and 87% of apoptotic cells respectively, indicating a concentration-response effect of glutamate. For the majority of the subsequent experiments, the submaximal concentration of glutamate (7.5 mM) was chosen. To investigate the involvement of adenosine and its receptors in the cytotoxic effect of glutamate, we first evaluate the contribution of endogenous adenosine using its degrading enzyme adenosine deaminase (ADA). As shown in figure 15, treating the cells with ADA reverted glutamate-induced injury at both tested concentrations of 5 mM and 7.5 mM causing a significant reduction in cell apoptosis, from 44% to 14% and from 74% to 12% respectively. The lack of cytotoxicity in the presence of ADA suggests that endogenous adenosine is a requisite for the glutamate effect. To further investigate the mechanism by which adenosine participated in the glutamate excitotoxic damage, cells were treated with forskolin, a specific activator of adenylate cyclase. As reported in figure 16, in the presence of ADA, forskolin re-established the glutamate-induced apoptosis, suggesting that elevated levels of intracellular cAMP are required for the effect of glutamate. This led us to hypothesize that the permissive effect of endogenous adenosine on glutamate cytotoxicity is related to the activation of Gs-coupled AR subtypes.

Adenosine role in glutamate cell damage is via ARs

To investigate if the role of adenosine was receptor-mediated, PC12 cells were treated with the non-selective AR agonist NECA at the 10 μM concentration in the presence of ADA. NECA mimicked the effect of endogenous adenosine as demonstrated by the increase of the apoptotic rate induced by glutamate in comparison to ADA alone, reaching a value similar to that obtained in the absence of ADA (Figure 17). To further corroborate the receptor-mediated contribution of endogenous adenosine to glutamate cytotoxicity, cells were treated with the non-selective AR antagonist CGS 15943 (10 μM). Blocking the four AR subtypes with CGS 15943 resulted in the lack of glutamate-induced apoptosis in a fashion similar to that obtained eliminating endogenous adenosine with ADA (Figure 17). This suggested that the role of adenosine in the glutamate-induce apoptosis is mediated by the activation ARs.

A_1ARs are protective against glutamate damage while A_{2A}ARs and A_{2B}ARs are harmful

PC12 cells were treated with selective AR antagonists to determine which adenosine receptor subtype was responsible for the effect of adenosine on glutamate cytotoxicity. As shown in figure 18, cell treatment with A_1ARs antagonist DPCPX (10 μM), caused a further increase in apoptotic rate exerted by the 7.5 mM glutamate suggesting that the activation of this subtype could be protective. Since the apoptotic rate in the presence of 7.5 mM glutamate was very high (75% of apoptotic cells), the further increase due to DPCPX resulted in difficult quantifying. For this reason, cells were treated with a lower concentration of glutamate (2 mM) in order to better investigate the effect of A_1ARs blockade. As figure 19 shows, from the 25% of apoptotic cells detected in the presence of 2 mM glutamate DPCPX further increased apoptotic rate to 52% and 81%, at the 1 μM and 10 μM, respectively. The selective blockade of A_{2A}ARs with SCH 442416 (10 μM) reduced the percentage of apoptotic cells in a significant way in comparison to glutamate 7.5 mM (Figure 18). An even more pronounced effect was obtained using the selective A_{2B}ARs antagonist PSB 603 (10 μM), which showed a significant reduction of glutamate-induced apoptosis, decreasing the apoptotic rate from 75% to 38%. The selective blockade of A_3ARs with P 413 (10 μM) did not show any effect on apoptosis induced by glutamate (Figure 18). From this data, it is possible to deduce that endogenous adenosine exerts opposite effects on glutamate-induced cytotoxicity, by activating the protective A1ARs and stimulating the detrimental A_{2A}ARs and A_{2B}ARs.

The A_1AR agonist CCPA slightly prevented glutamate cytotoxicity

Since it has been observed a facilitating effect of A_1ARs blockade on glutamate cytotoxicity, the role of A_1ARs activation by using the selective agonist CCPA at different concentrations (from 10 nM to 10 μM) was investigated. Treatment with CCPA did not show any significant difference in apoptotic rate in comparison to glutamate (7.5 mM) at all the tested concentrations, with the exception of the 10 μM concentration, which slightly reduced it (Figure 20). This suggests that the A_1AR agonist alone of the receptor is not enough to give protection against glutamate injury, probably for the concomitant activation of A_{2A}ARs and A_{2B}ARs by endogenous adenosine, which exerted a predominant facilitating action on glutamate cytotoxicity.

Positive allosteric modulation as a strategy to increase the effect of endogenous adenosine on A_1ARs

Given the results obtained with the A_1AR agonist on glutamate-induced apoptotic rate, an alternative strategy to exploit the protective effect of A_1AR activation is represented by allosteric modulation. One of the great advantages of allosteric ligands is their capability to increase the affinity of endogenous ligands exploiting their physiological effects instead of activating the receptors with an exogenous agonist acting on the orthosteric site. Positive allosteric modulators are thought to shift the receptor from the inactive to the active state. Since agonists bind with high affinity the subpopulation of receptors in the active state, we analyzed the effect of TRR 469 on [^{3}H]-CCPA binding in mouse hippocampus, a crucial site for the transmission of pain signals. To evaluate the total binding sites for A_1ARs in mouse cortex membranes, we performed saturation binding experiments with the antagonist radioligand [^{3}H]-DPCPX, that do not discriminate between the active or inactive state of the receptors (Fig. 21A). The lower Bmax value obtained with the agonist radioligand ([^{3}H]-CCPA) is attributable to its preferential binding to the active state of the receptors (Fig. 21B). Interestingly, the presence of the positive allosteric modulator TRR 469 elicited a shift of the receptor population toward the active state, as demonstrated by the increase of [^{3}H]-CCPA Bmax that reached a value similar to that obtained with the antagonist radioligand [^{3}H]-DPCPX (Fig. 21B). The affinity of adenosine in the presence of the positive allosteric modulator TRR 469 was then evaluated. In mouse cortex membranes, adenosine was able to displace [^{3}H]-DPCPX with an affinity (Ki value) of 278 ± 23 nM. Notably, in the presence of TRR 469 (10 μM), competition binding experiments revealed a Ki value for adenosine of 8.3 ± 0.9 nM, corresponding to a 33-fold affinity increase (Fig. 21C). TRR 469 alone was not able to displace the radioligand, confirming its lack of interaction with the orthosteric site. An analogous Ki-shift was observed for CCPA in the absence or in the presence of 10 μM TRR 469 (Fig. 21D).

A_1ARs allosteric enhancer TRR 469 protects cells from cytotoxic effect of glutamate

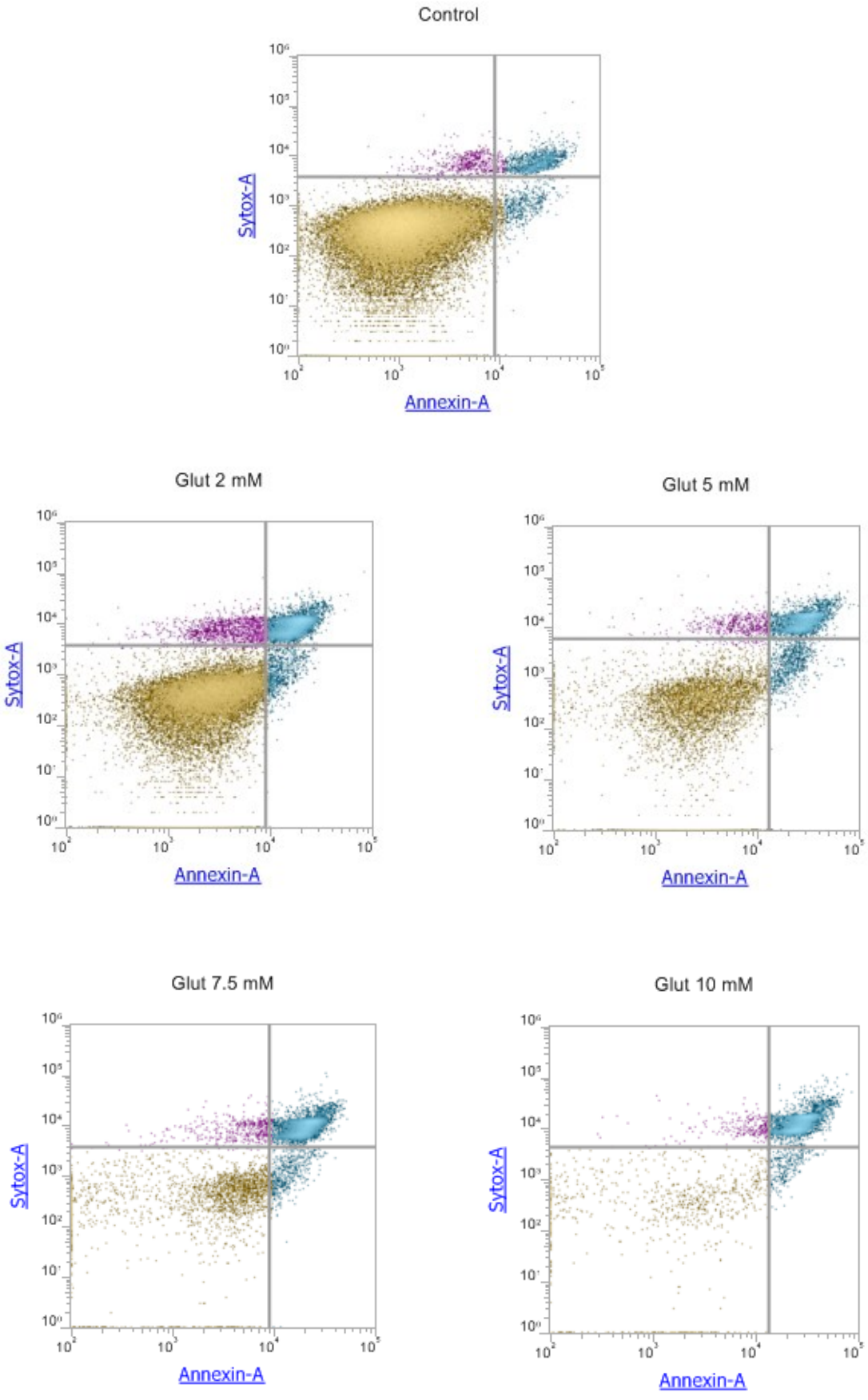
In order to enhance the protective effect of adenosine acting on A_1ARs, cells were subjected to glutamate insult in the absence or in the presence of different concentrations of the A_1AR positive allosteric modulator TRR 469 (Figure 22A and B). TRR 469 treatment showed a significant and concentration-dependent reduction of glutamate-

induced apoptosis. In particular, at the highest concentration tested (10 μM), TRR 469 completely prevented glutamate cytotoxicity. At the 3 μM and 7.5 μM concentrations, TRR 469 reduced glutamate-induced apoptosis by 56% and 85%, respectively, showing a potency of 2.5 μM (Figure 22C). The protective effect of this positive allosteric modulator was almost completely reverted by the selective A_1ARs antagonist, DPCPX 10 μM, suggesting that the observed effect on cytotoxicity caused by glutamate was due to the enhanced activation of A_1ARs by endogenous adenosine (Figure 22A, B).

TRR 469 prevented glutamate-induced caspases 3/7 activation

To better investigate the mechanism by which TRR 469 protected PC12 cells from glutamate cytotoxicity, the activation of caspase 3/7, key effectors of the apoptotic pathway, was measured. Glutamate induced an increase of caspase 3/7 positive cells by 34% and 85% at the 2 mM and 5 mM concentration, respectively. Exposing the cells to the same concentrations of glutamate in the presence of the positive allosteric modulator TRR 469 resulted in significant and complete inhibition of glutamate-induced caspase 3/7 activation (Figure 23).

A


Control
Sytox-A
Annexin-A
Glut 2 mM
Sytox-A
Annexin-A
Glut 5 mM
Sytox-A
Annexin-A
Glut 7.5 mM
Sytox-A
Annexin-A
Glut 10 mM
Sytox-A
Annexin-A

B

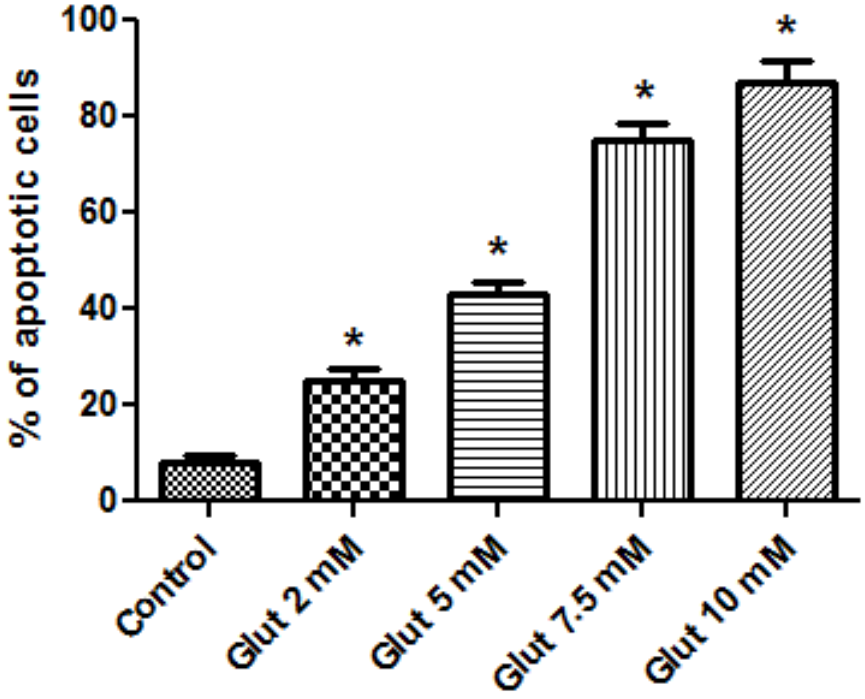
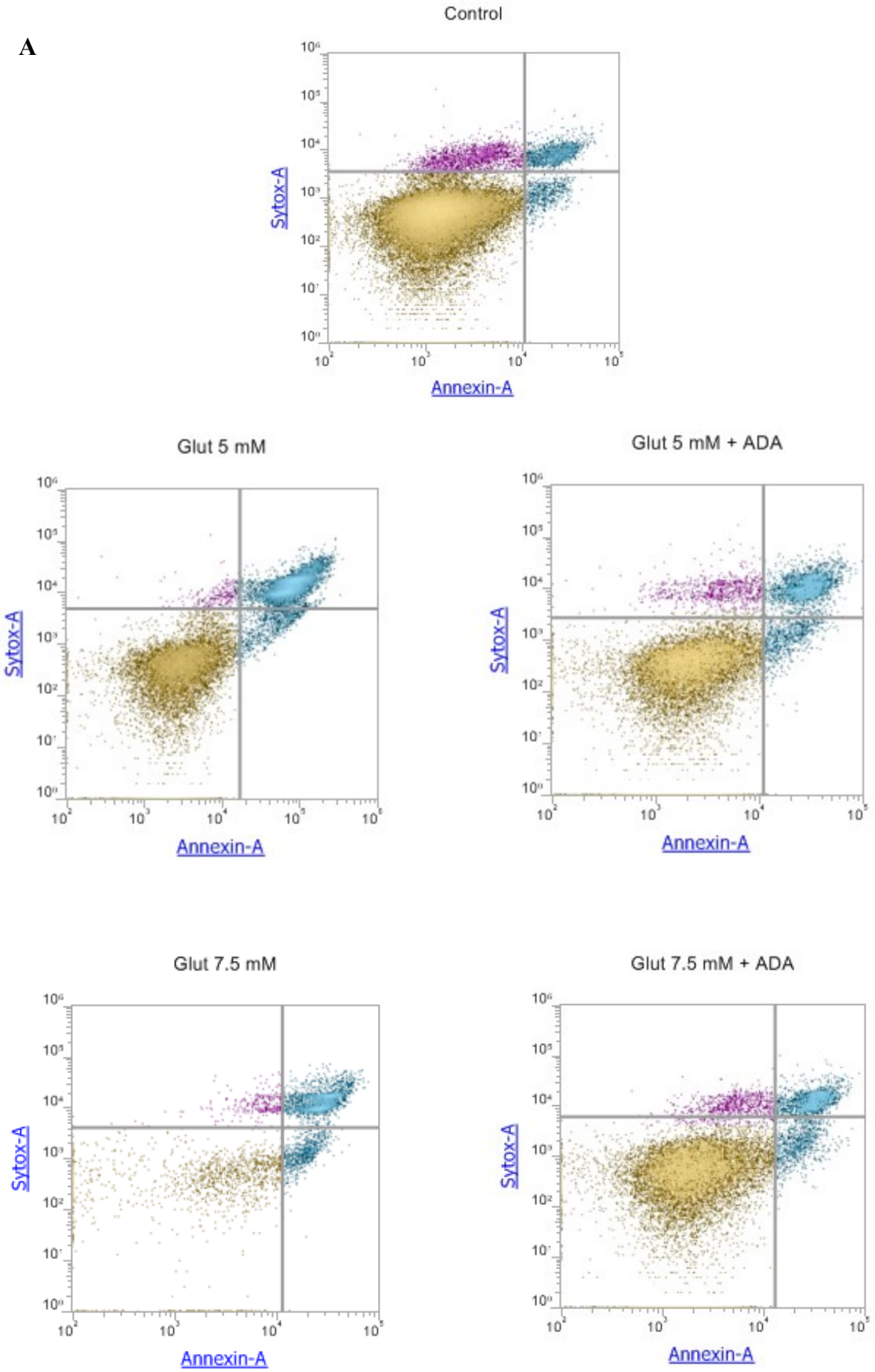


Figure 14. Effect of 24 hours glutamate treatment on PC12 cell viability. Dot plots showing the shift of PC12 cells from living (Annexin -/ Sytox –) to early apoptotic (Annexin +/ Sytox –) and late apoptotic (Annexin +/ Sytox +) in response to different concentrations of glutamate (**A**). Dose-dependent effect of glutamate on cell apoptosis (**B**). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control.

A


Control
Sytox-A
Annexin-A
Glut 5 mM
Sytox-A
Annexin-A
Glut 5 mM + ADA
Sytox-A
Annexin-A
Glut 7.5 mM
Sytox-A
Annexin-A
Glut 7.5 mM + ADA
Sytox-A
Annexin-A

B

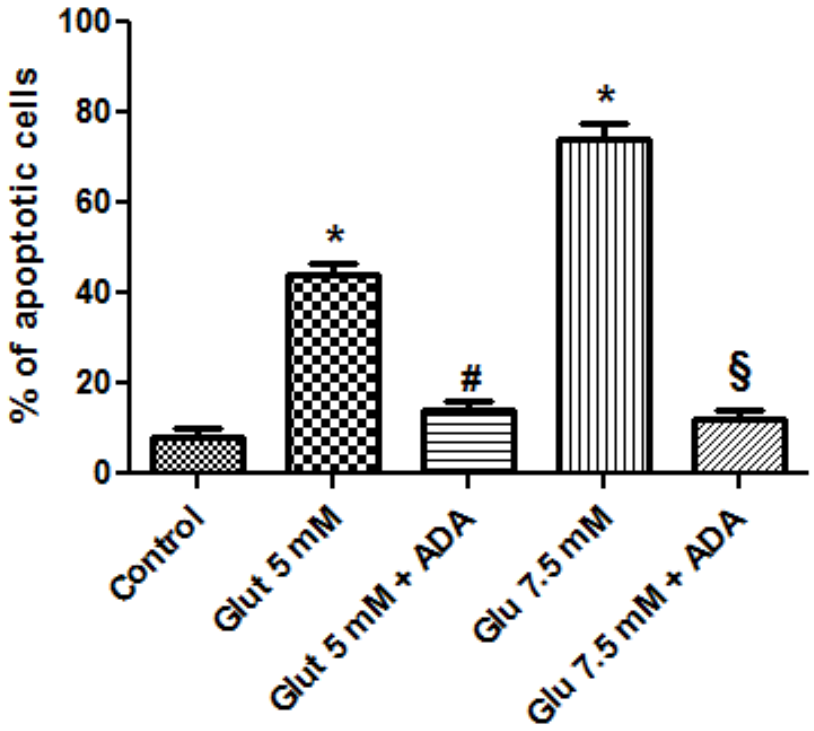
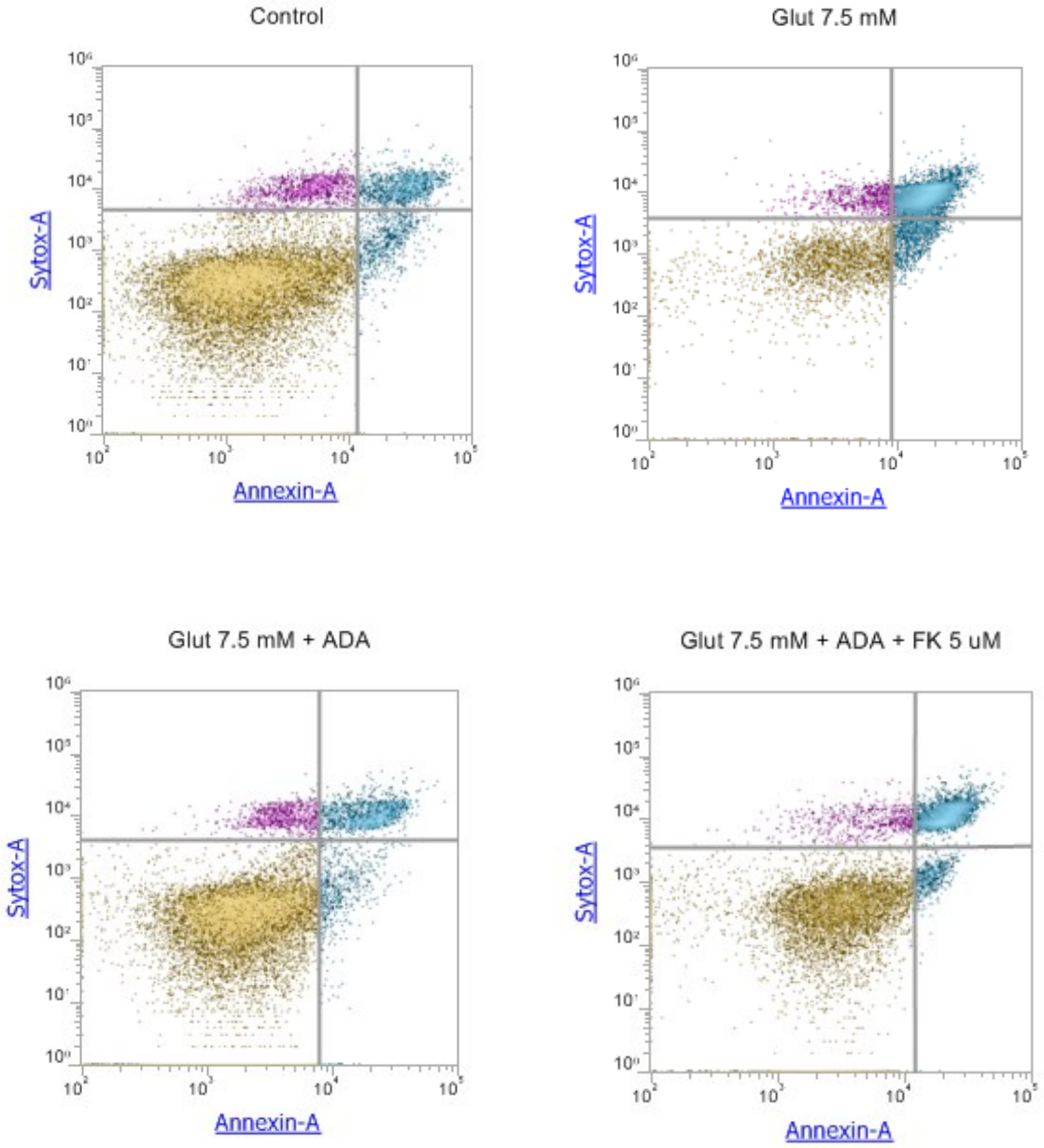


Figure 15. Effect of ADA treatment on glutamate-induced apoptosis. Dot plots showing the shift of PC12 cells from living (Annexin -/ Sytox –) to early apoptotic (Annexin +/ Sytox –) and late apoptotic (Annexin +/ Sytox +) in response to glutamate treatment in absence or presence of ADA (**A**). Histograms showing the percentage of apoptotic cells after glutamate and ADA treatment (**B**). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control; # $p < 0.01$ vs glut 5 mM; § $p < 0.01$ vs glut 7.5 mM.

A


Control
Glut 7.5 mM
Glut 7.5 mM + ADA
Glut 7.5 mM + ADA + FK 5 uM
Sytox-A
Annexin-A

B

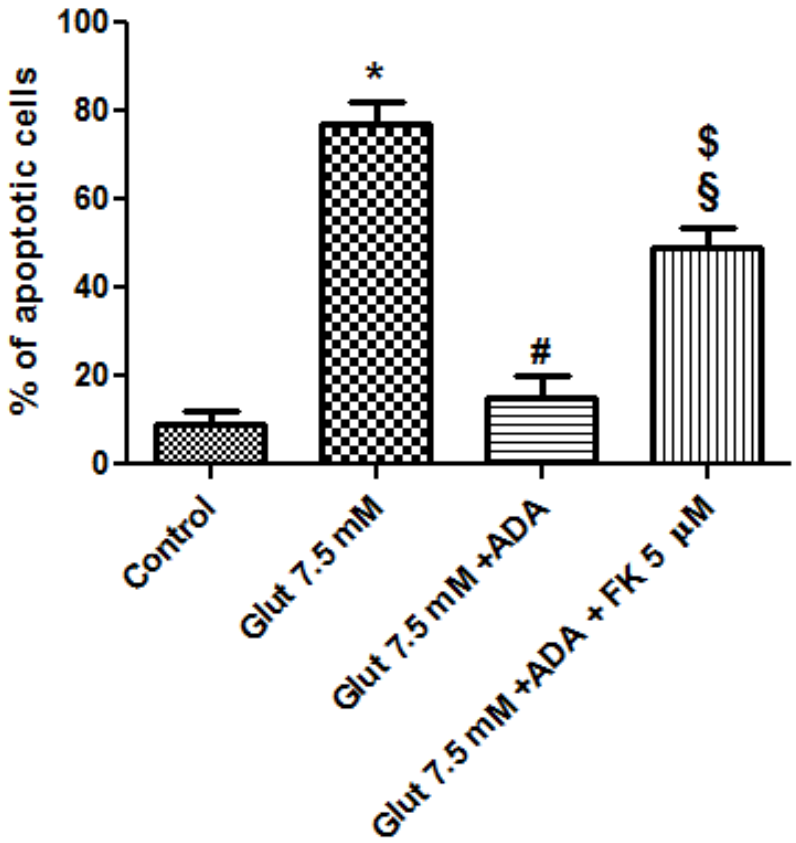
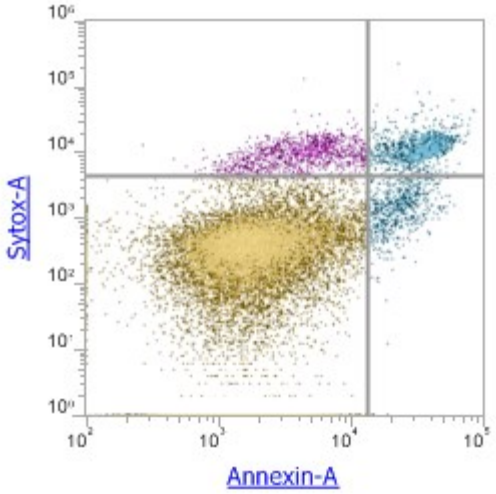


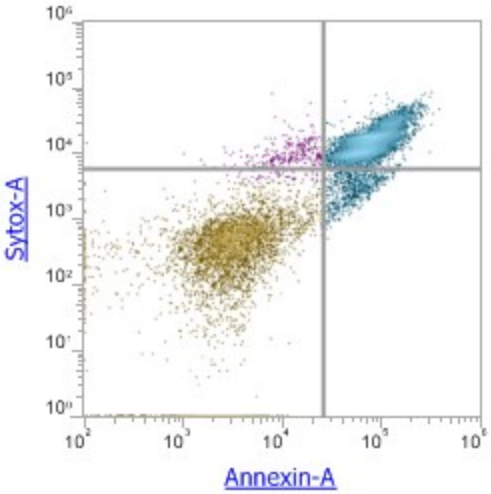
Figure 16. Effect of adenylate cyclase activation, with FK, on cytotoxicity induced by glutamate. Dot plots showing PC12 cells shifting from living (Annexin -/ Sytox –) to early apoptotic (Annexin +/ Sytox –) and late apoptotic (Annexin +/ Sytox +) (**A**) and percentage of apoptotic cells (**B**) after treatment with glutamate (7.5 mM), ADA and FK (5 µM). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control; # $p< 0.01$ vs glut 7.5 mM; § $p < 0.01$ vs glut 7.5 mM + ADA; $ $p < 0.01$ vs glut 7.5 mM.

A

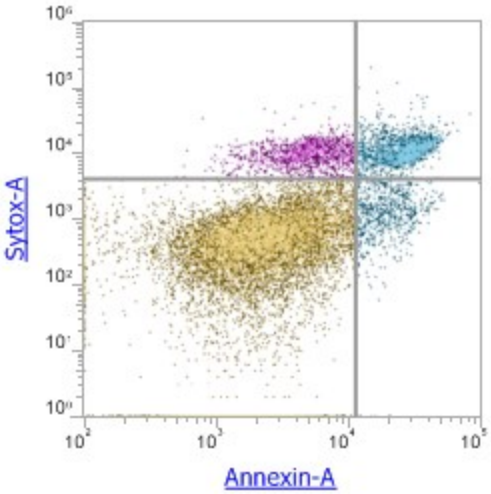
Control



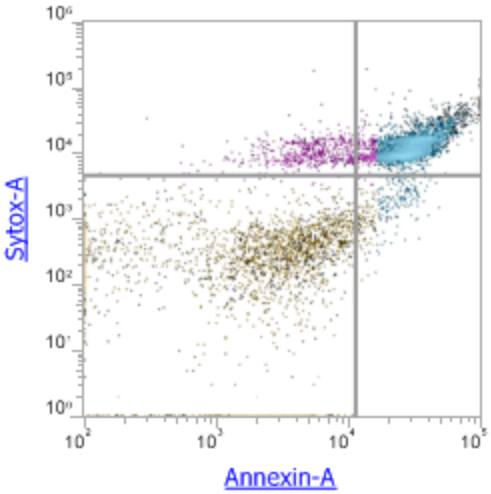
Glut 7.5 mM



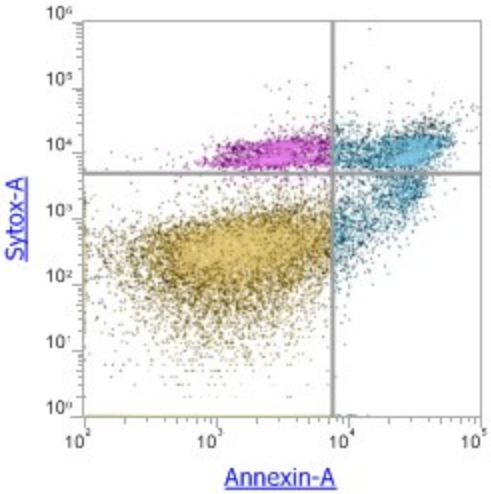
Glut 7.5 mM + ADA



Glu 7.5 mM + NECA 10 uM



Glu 7.5 mM + CGS 15943 10 uM



B

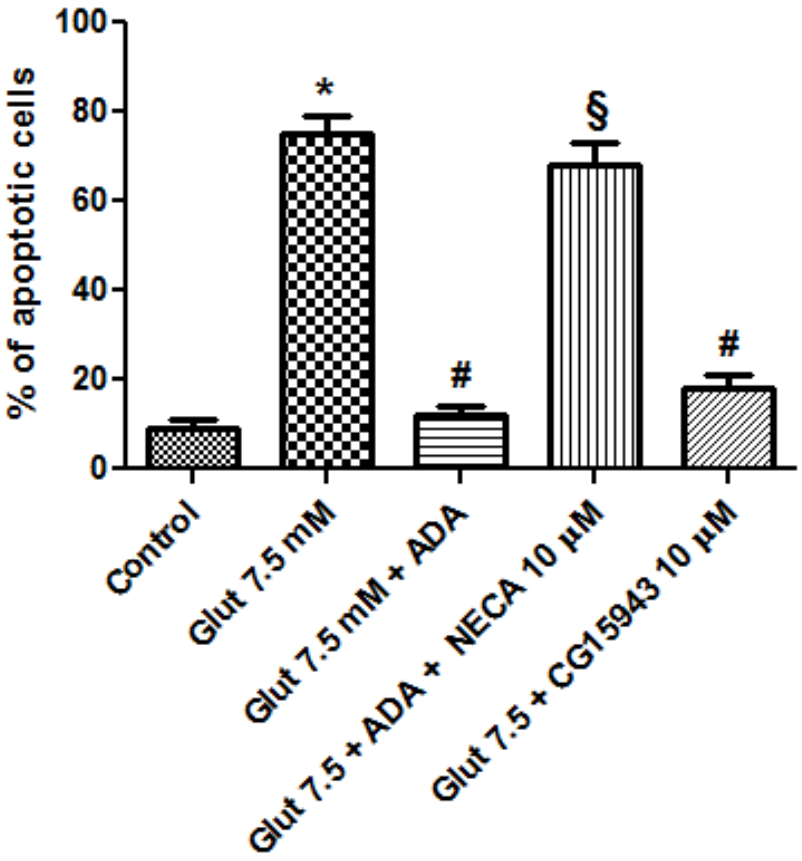
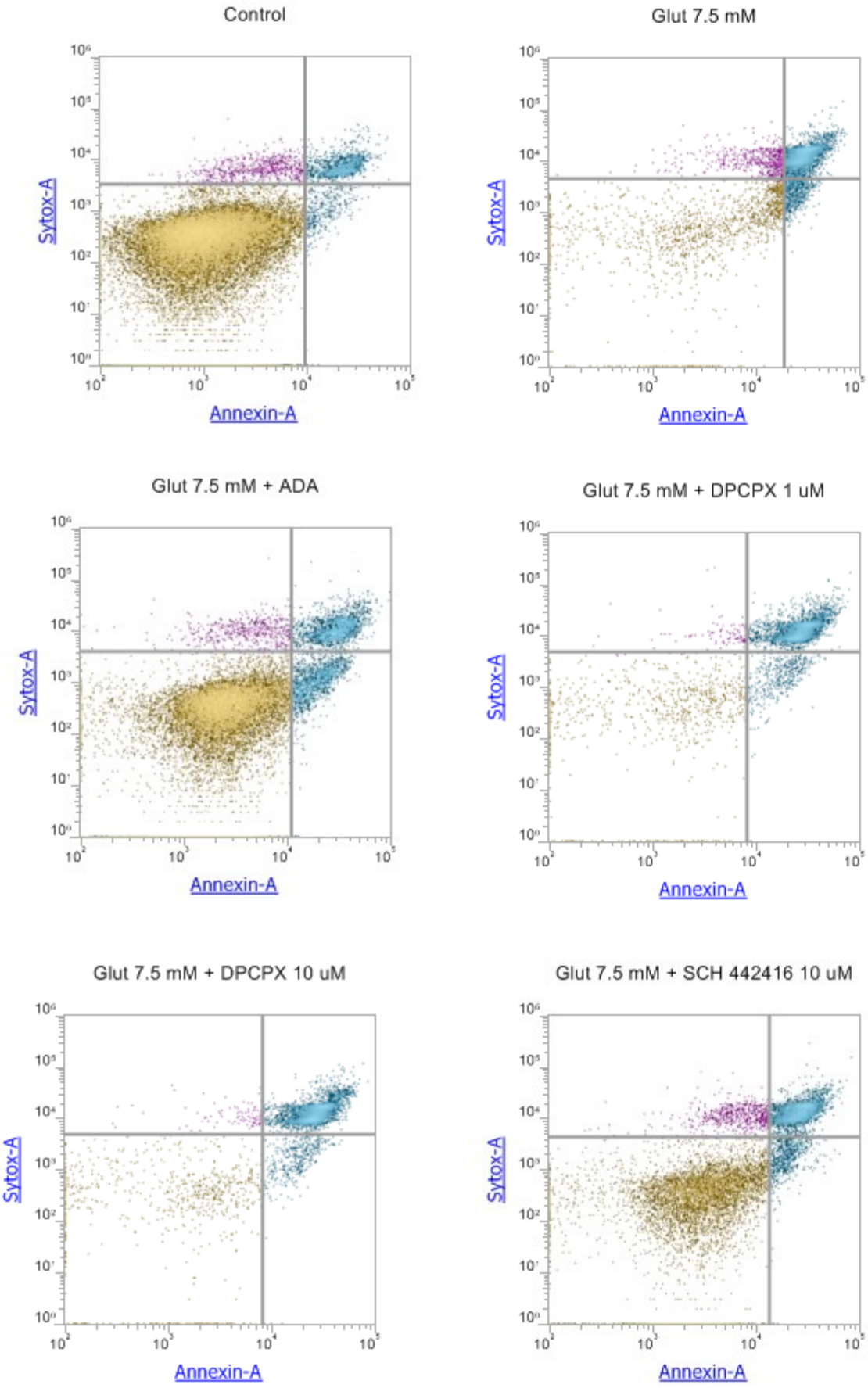


Figure 17. Effect of treatment with non selective ARs agonist (NECA) and antagonist (CGS 15943) on glutamate induced damage. Dot plots showing PC12 cells shifting from living (Annexin -/ Sytox –) to early apoptotic (Annexin +/ Sytox –) and late apoptotic (Annexin +/ Sytox +) (**A**) and percentage of apoptotic cells (**B**) after treatment with glutamate (7.5 mM), ADA, NECA (10 µM) and CGS 15943 (10 µM). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control; # $p < 0.01$ vs glut 7.5 mM: § $p < 0.01$ vs glut 7.5 mM + ADA.

A


Control
Glut 7.5 mM
Glut 7.5 mM + ADA
Glut 7.5 mM + DPCPX 1 uM
Glut 7.5 mM + DPCPX 10 uM
Glut 7.5 mM + SCH 442416 10 uM
Sytox-A
Annexin-A

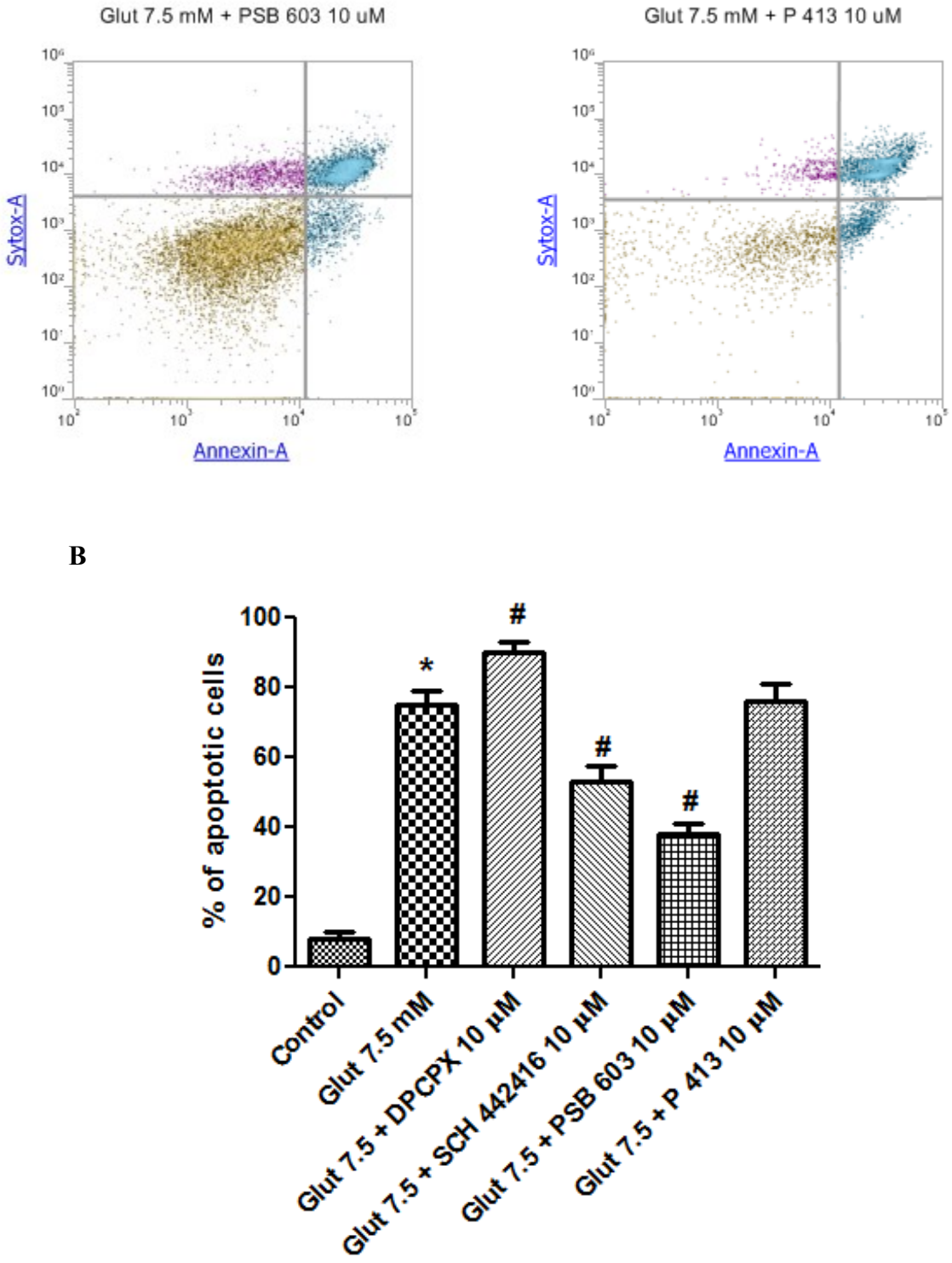
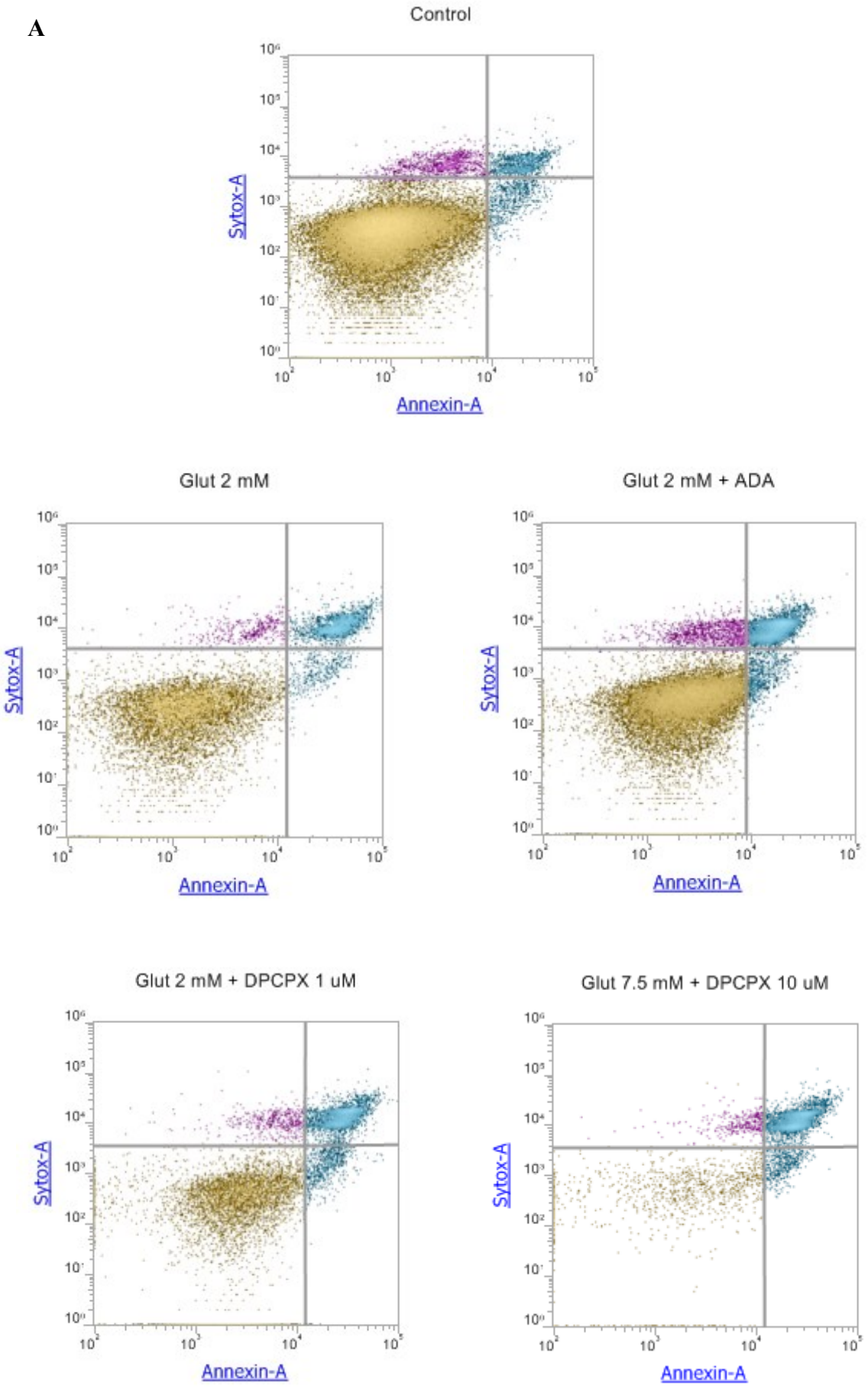


Figure 18. Effect of the selective blockade of adenosine receptors, with selective anatgonists for each receptor subtype, on glutamate cytotoxicity. Dot plots reporting PC12 cell changes from living (Annexin -/ Sytox –) to early apoptotic (Annexin +/ Sytox -) and late apoptotic (Annexin +/ Sytox +) (**A**) and percentage of apoptotic cells (**B**) after treatment with glutamate (7.5 mM), ADA, DPCPX (10 µM), SCH 442416 (10 µM), PSB 603 (10 µM) and P 413 (10 µM). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control; # $p < 0.01$ vs glut 7.5 mM.

A


Control
Sytox-A
Annexin-A
Glut 2 mM
Sytox-A
Annexin-A
Glut 2 mM + ADA
Sytox-A
Annexin-A
Glut 2 mM + DPCPX 1 uM
Sytox-A
Annexin-A
Glut 7.5 mM + DPCPX 10 uM
Sytox-A
Annexin-A

B

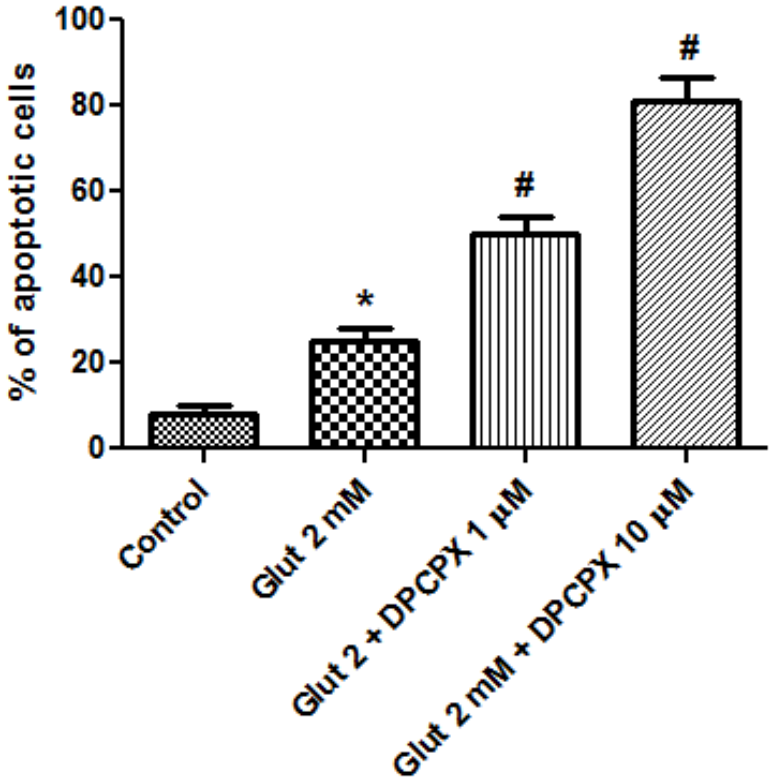
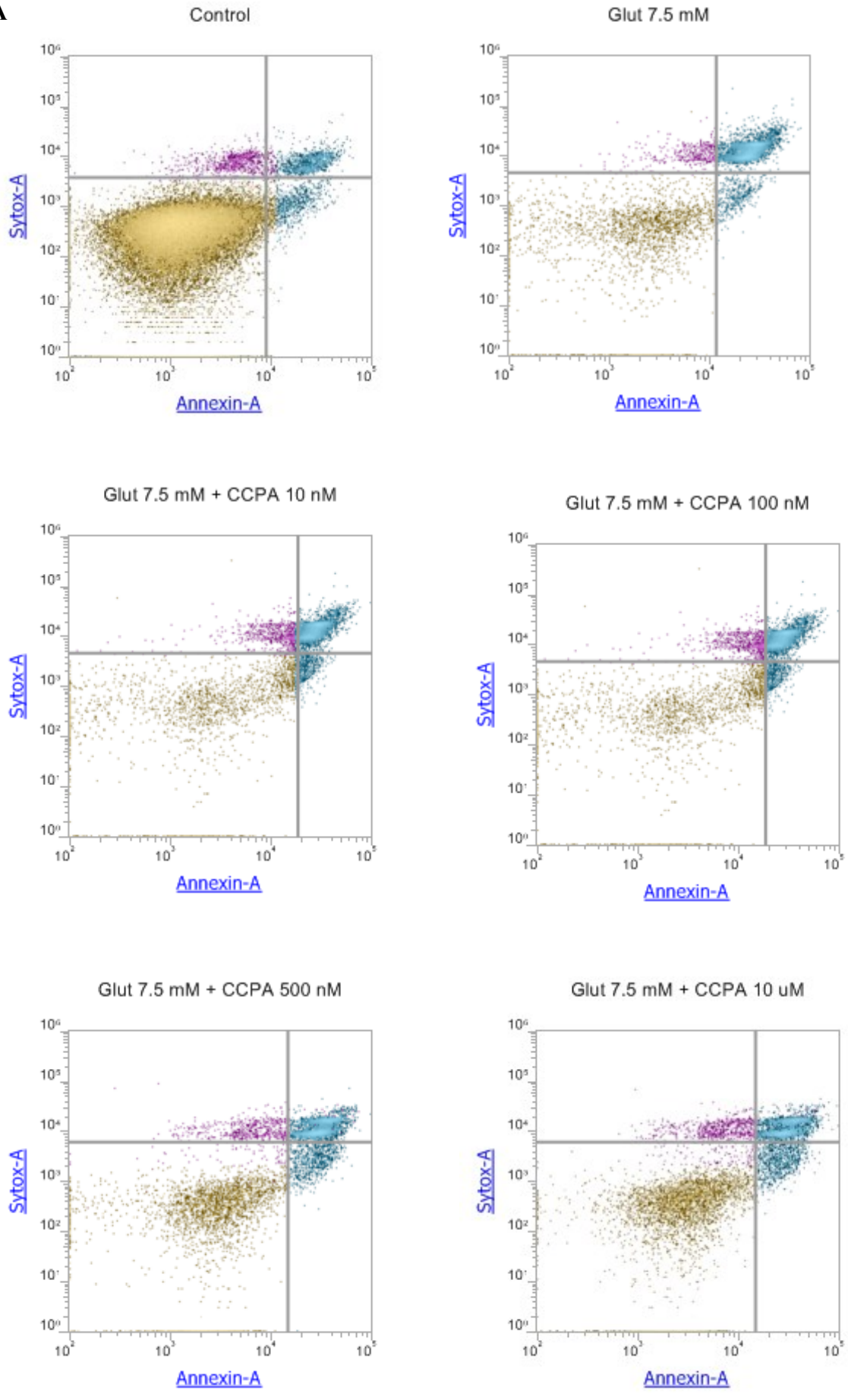


Figure 19. Effect of selective blockade of A_1ARs on glutamate induced apoptosis. Dot plots reporting PC12 cell shifting from living (Annexin -/ Sytox –) to early apoptotic (Annexin +/ Sytox -) and late apoptotic (Annexin +/ Sytox +) (**A**) and percentage of apoptotic cells (**B**) after treatment with glutamate (2 mM), ADA, DPCPX (1 µM), DPCPX (10 µM). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control; # $p < 0.01$ vs glut 2 mM.

A


Control
Glut 7.5 mM
Glut 7.5 mM + CCPA 10 nM
Glut 7.5 mM + CCPA 100 nM
Glut 7.5 mM + CCPA 500 nM
Glut 7.5 mM + CCPA 10 uM
Sytox-A
Annexin-A

B

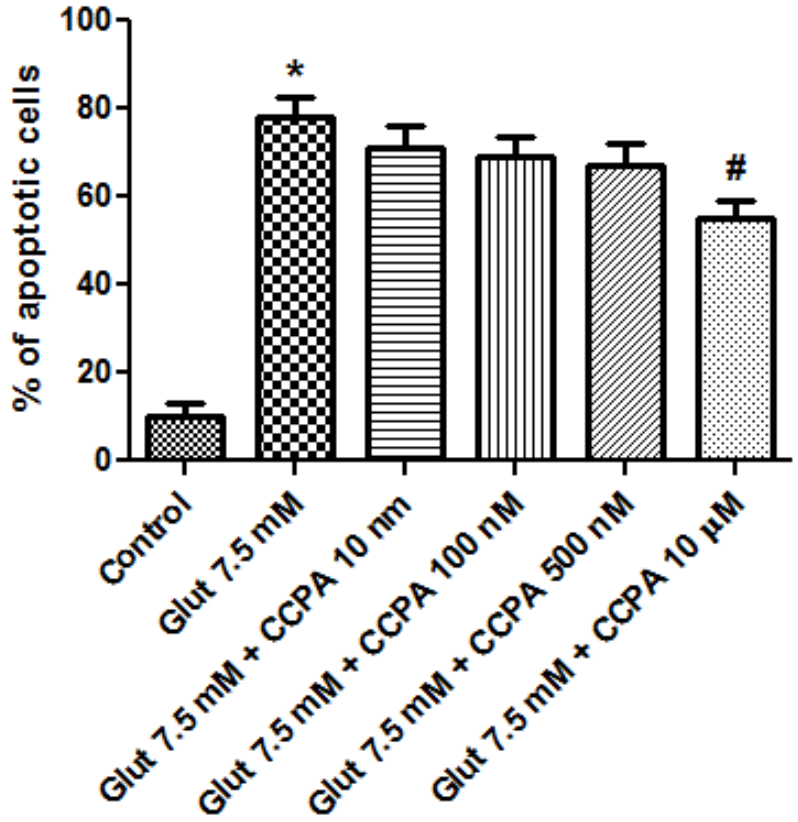


Figure 20. Effect, on glutamate damage, of selective activation of A_1ARs. Dot plots reporting PC12 cell changes from living (Annexin -/ Sytox –) to early apoptotic (Annexin +/ Sytox -) and late apoptotic (Annexin +/ Sytox +) (**A**) and percentage of apoptotic cells (**B**) after treatment with glutamate (7.5 mM), CCPA (10 nM – 10 µM). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control; # $p < 0.01$ vs glut 7.5 mM.

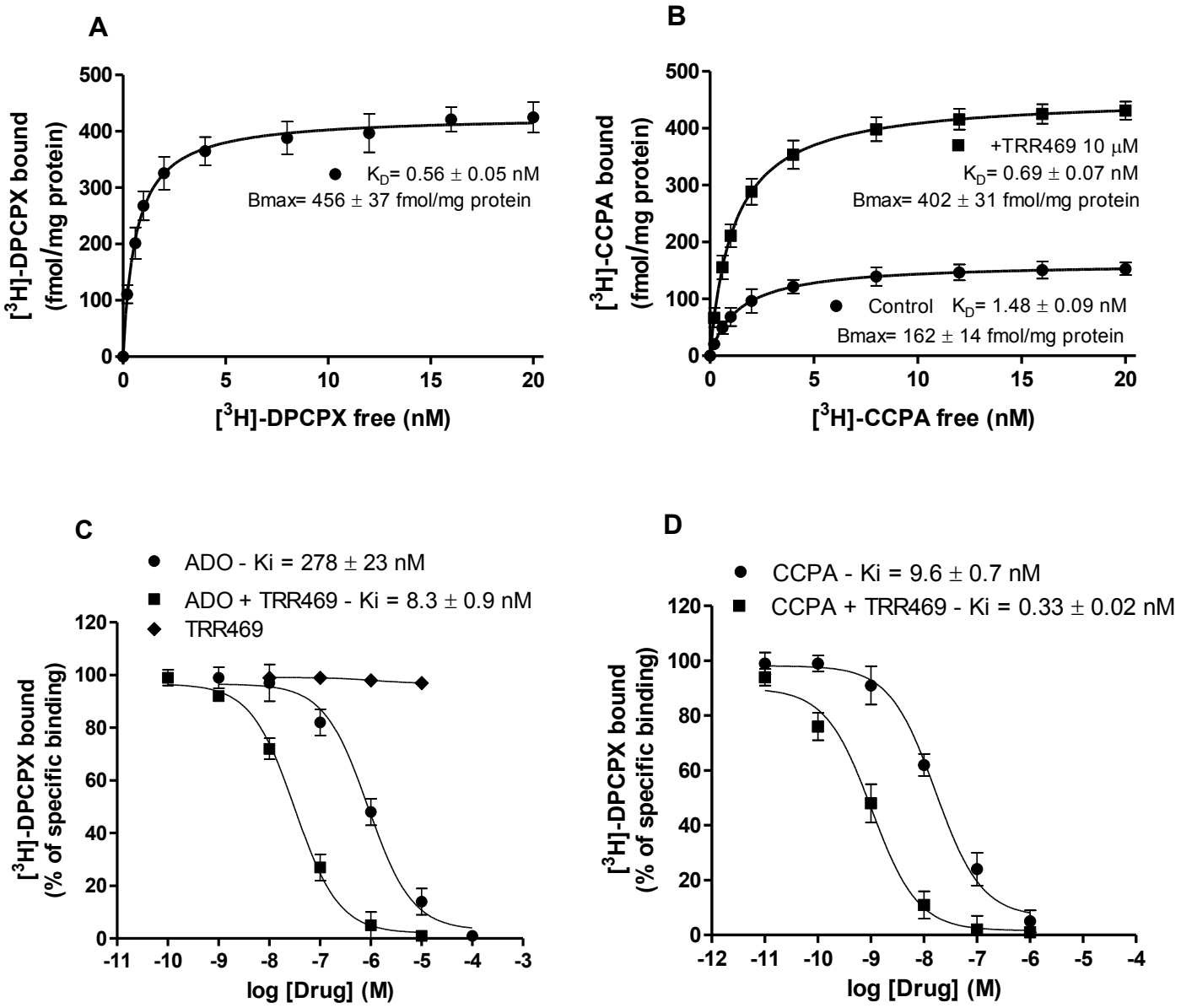
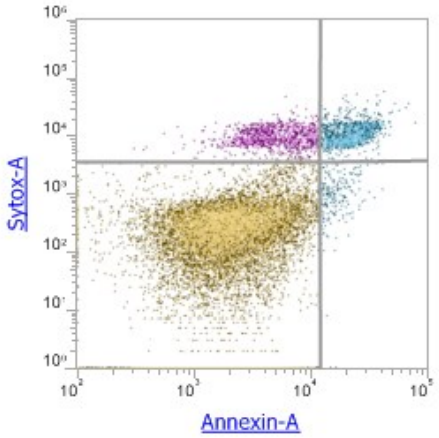


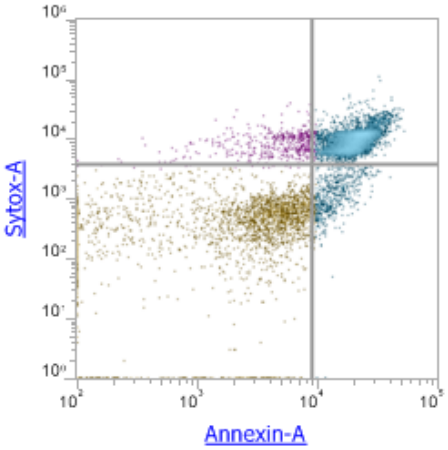
Figure 21 Effect of TRR 469 on agonist binding in mouse cortex membranes. Saturation curve of the A_1AR antagonist radioligand [^{3}H]-DPCPX (0.05–20 nM) in mouse cortex membranes (**A**). Saturation curves of the A_1AR agonist radioligand [^{3}H]-CCPA (0.05–20 nM) in mouse cortex membranes in the absence or presence of 10 μM TRR 469 (**B**). Competition curves of adenosine (ADO), adenosine + TRR 469 (10 μM) and TRR 469 in mouse cortex membranes (**C**). Competition curves of CCPA in the absence or in the presence of TRR 469 10 μM in mouse cortex membranes (**D**). Data are presented as mean ± SEM.

A

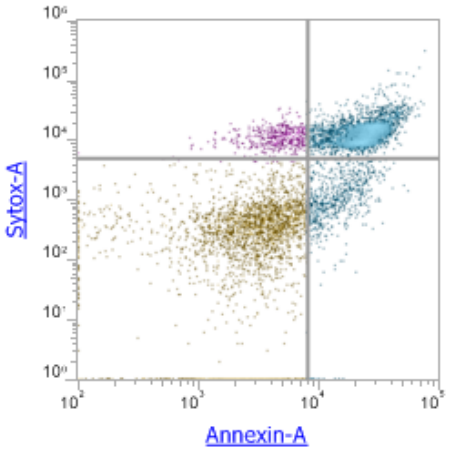
Control



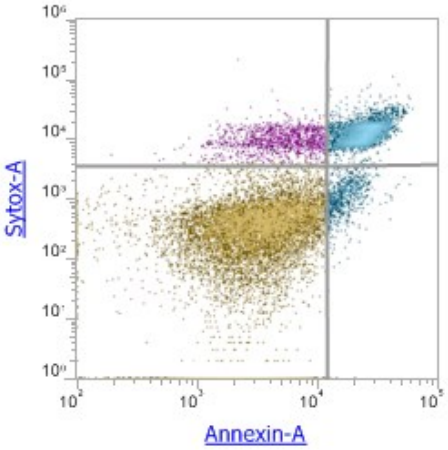
Glut 7.5 mM



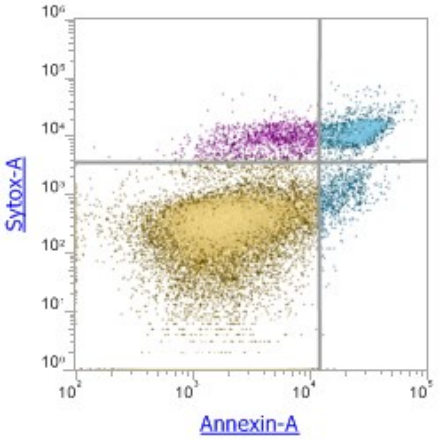
Glut 7.5 mM + TRR 469 1 uM



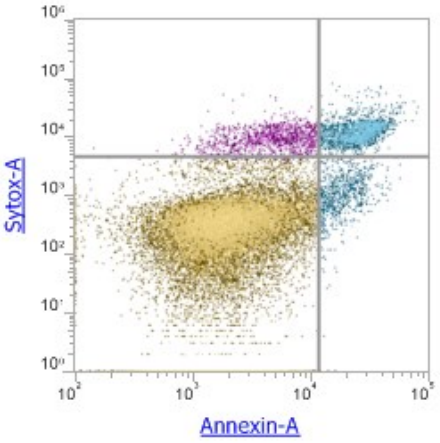
Glut 7.5 mM + TRR 469 3 uM



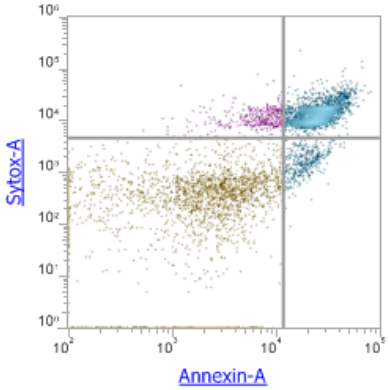
Glut 7.5 mM + TRR 469 7.5 uM



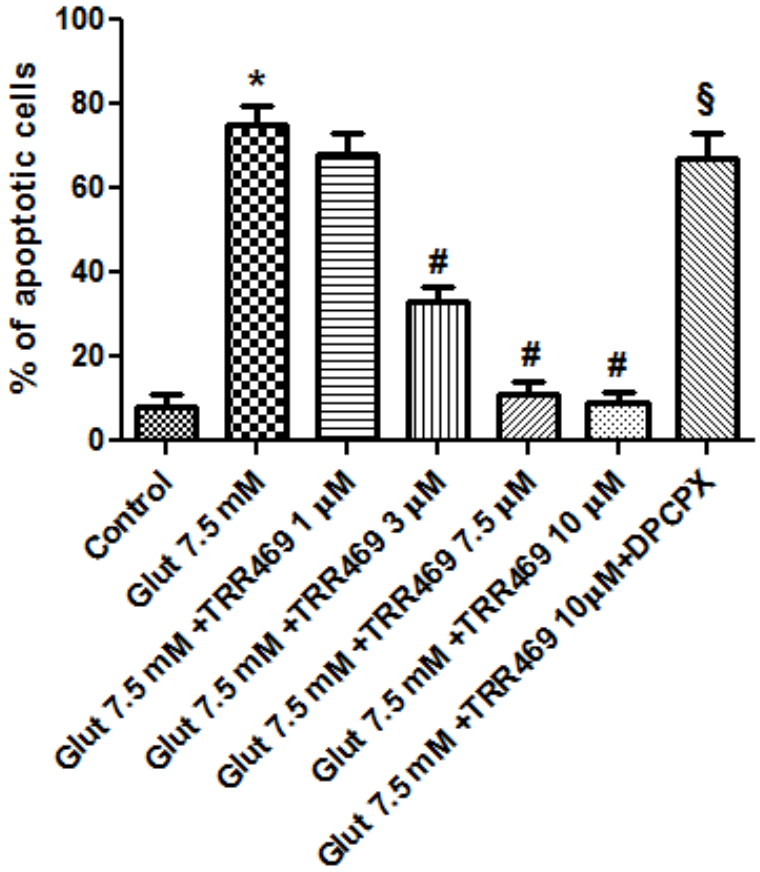
Glut 7.5 mM + TRR 469 10 uM



Glut 7.5 mM + TRR 469 10 uM + DPCPX 10 uM



B



C

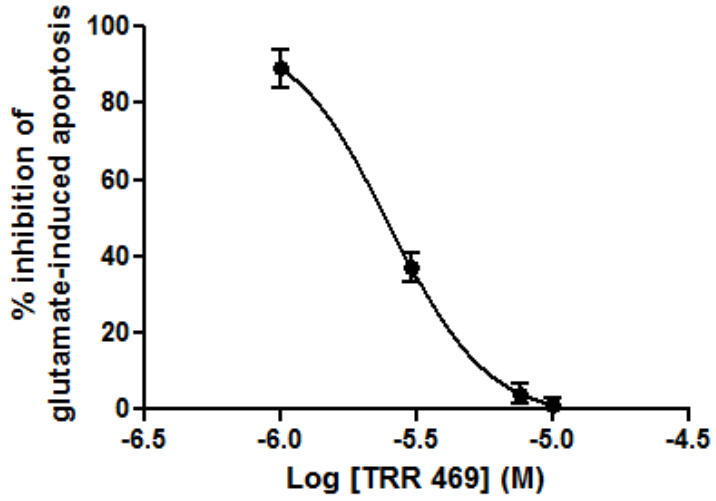
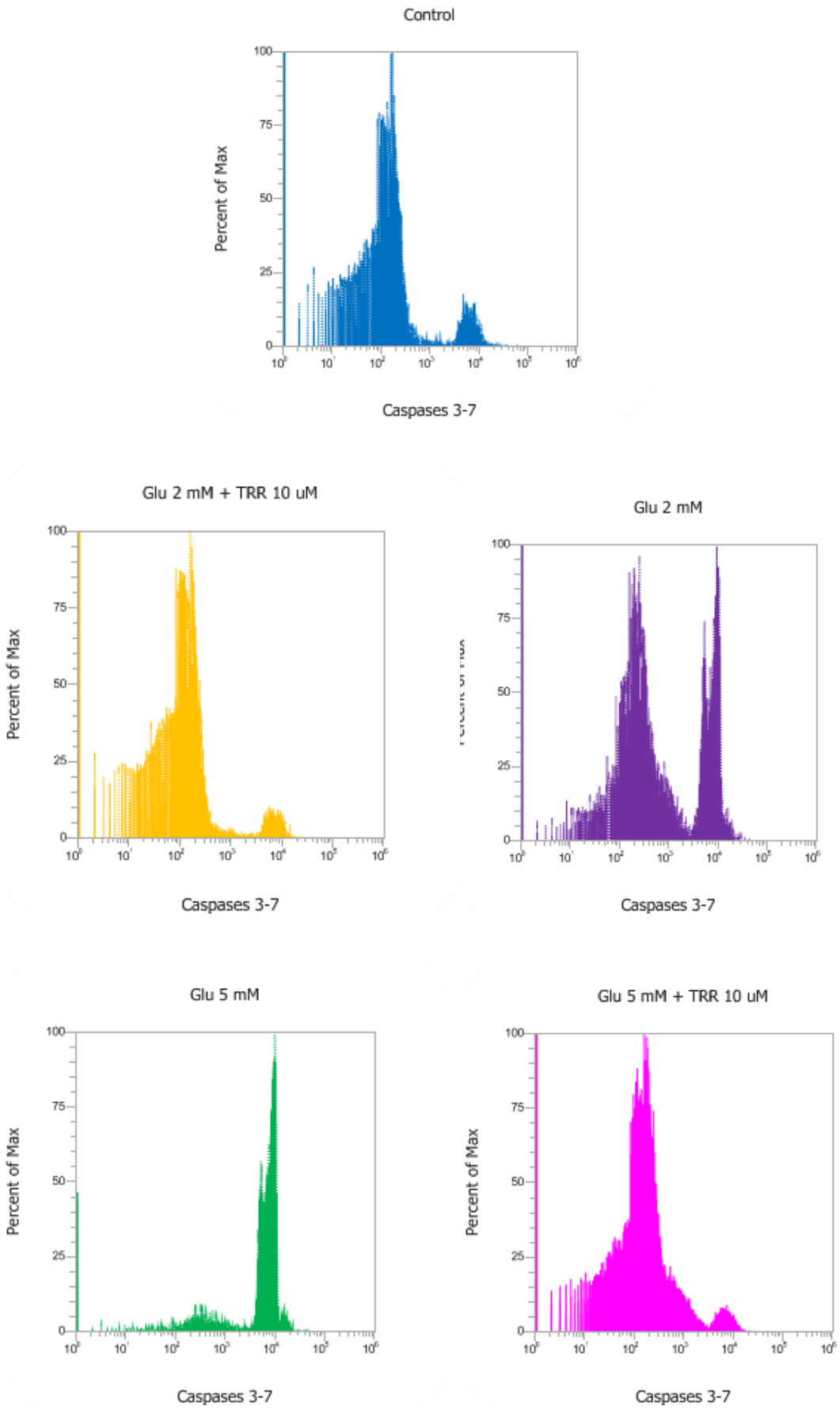
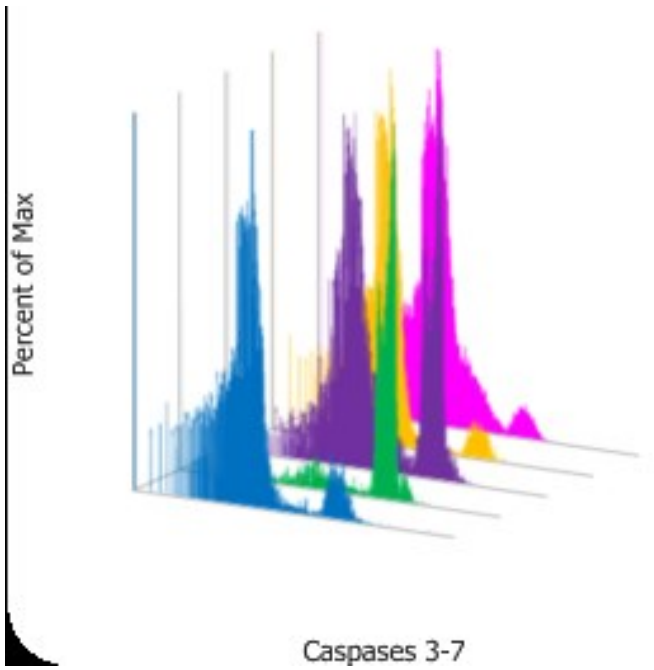


Figure 22. Effect of TRR 469 on glutamate induced apoptosis. Dot plots reporting PC12 cell changes from living (Annexin -/ Sytox –) to early apoptotic (Annexin +/ Sytox -) and late apoptotic (Annexin +/ Sytox +) (**A**) and percentage of apoptotic cells (**B**) after treatment with glutamate (7.5 mM) in absence or presence of TRR 469 (1- 10 µM). Concentration response curve of TRR 469 on 7.5 mM glutamate- induced apoptosis (**C**). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control; # $p < 0.01$ vs glut 7.5 mM; § $p < 0.01$ vs TRR 469 10 µM.

A


Control
Percent of Max
Caspases 3-7
Glu 2 mM + TRR 10 uM
Percent of Max
Caspases 3-7
Glu 2 mM
Caspases 3-7
Glu 5 mM
Percent of Max
Caspases 3-7
Glu 5 mM + TRR 10 uM
Percent of Max
Caspases 3-7

B



C

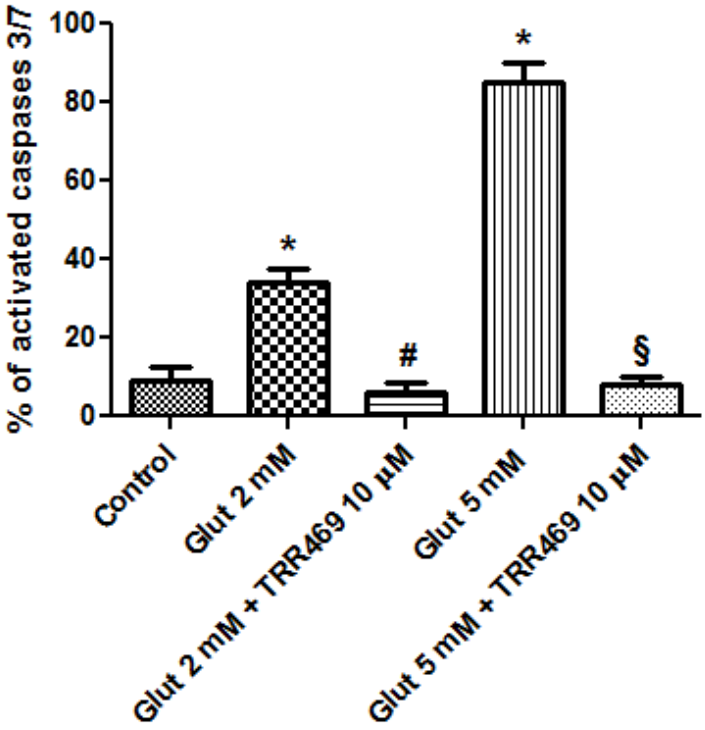


Figure 23. Activation of caspases 3/7 after treatment with glutamate and TRR 469. Histograms showing fluorescence intensity of caspases 3/7 labeling in PC12 cells after tratment with glutamate (2mM and 5 mM) in absence or presence of TRR 649 (10 µM) and overlay of previous histrograms (**A**). Overlay of the fluorescence histograms (**B**). Percentage of caspases 3/7 activation in different treatment conditions (**C**). Data are expressed as the mean ± SEM. * $p < 0.01$ vs control; # $p< 0.01$ vs glut 2 mM; § $p < 0.01$ vs glut 5 mM.

Discussion

The aim of this part of the study was to better clarify the role of adenosine and ARs in ischemia, especially in an *in vitro* model of cytotoxicity induced by glutamate. It is well known that one of the most important consequences of ischemic injury is the rapid and dramatic increase in glutamate concentration. This further compromises neuronal functions causing cell death through the activation of NMDA channels. The results of this study highlight a role of adenosine in glutamate cytotoxicity. In fact, the lack of endogenous adenosine completely prevented glutamate-induced apoptosis, suggesting that adenosine, through a receptors-mediated mechanism, exerts a facilitating effect on glutamate excitotoxicity. In particular, adenosine is involved in glutamate damage through the activation of A_{2A} and A_{2B}ARs, as demonstrated by the decrease of apoptotic levels caused by the selective blockade of these receptor subtypes. In particular, it is known that adenosine, through A_{2A} and A_{2B}ARs activation, stimulates the production of cAMP activating PKA which phosphorylates NMDA channels, thus causing the entrance of Ca^{2+} in the cells leading to cell death (Dias et al., 2012). In the present study, it has been corroborated that the negative effect of adenosine may be due to the increase in cAMP levels, as confirmed by the restoration, in the absence of endogenous adenosine, of glutamate-induced apoptosis caused by the treatment with forskolin, a direct activator of adenylate cyclase. The increase of cAMP levels obtained with forskolin activates PKA pathway provoking cell death as A_{2A}ARs and A_{2B}ARs activation does. On the other hand, the stimulation of the Gi protein-coupled A_1ARs causes opposite effects by decreasing cAMP production and protecting cells from excessive Ca^{2+} influx. The antithetical effect on cAMP/PKA pathway could explain the protective effect of A_1ARs and the facilitating action of A_{2A}ARs and especially A_{2B}ARs on glutamate excitotoxicity. Recently, it has been shown that A_1ARs activation induces the internalization of GluA1 and GluA2 subunit-containing AMPA receptors (Chen et al., 2016; Chen et al., 2014; Stockwell et al., 2016). On the contrary, the activation of A_{2A}ARs has been shown to increase the expression of the calcium-permeable GluA1 subunit of the AMPA receptor through the activation of PKA (Dias et al., 2012). Many papers in the literature demonstrated that after ischemia, excitotoxicity is the first phenomenon occurring in the brain, within the first 4 h from the insult, when A_{2A}ARs are responsible for the increase in glutamate levels, through both the release of glutamate from glutamatergic terminals and the inhibition of the glutamate-1 transporter (GLT-1) in astrocytes. Indeed, it has been shown that A_{2A}ARs blockade protects against glutamate excitotoxicity. Furthermore, A_{2A}ARs activation reduces the affinity of agonists for A_1ARs in A_1–A_{2A} receptor heteromers, which exert a

presynaptic control of striatal glutamate release, resulting in the fine-tuning of the modulatory effect on striatal glutamatergic neurotransmission (Matos et al., 2012b; Matos et al., 2013).

A large body of evidence indicates that adenosine, interacting with A_1ARs, may be an endogenous protective agent in the human body since it prevents the damage caused by various pathological conditions, such as in epileptic seizures, excitotoxic neuronal injury and cardiac arrhythmias in the cardiovascular system (Varani et al., 2017). In many experimental paradigms, it has been demonstrated that the stimulation of A_1ARs reduces ischemic-related neuronal injury and the protective effects are greatly attributed to A_1ARs mediated reduction of Ca^{2+} influx (Pedata et al., 2016). Despite their promising therapeutic potential, the use of A_1AR agonists has been hampered by numerous side effects, poor receptor subtype selectivity and receptor desensitization. Moreover, in the present study, the treatment with the A_1ARs agonist CCPA did not fully protect cells from glutamate damage, probably due to the concomitant activation of Gs-coupled ARs by endogenous adenosine.

To overcome the problems associated with the utilization of A_1AR agonists, in our laboratory, a series of positive allosteric modulators have been developed as a potential alternative for A_1ARs-targeted therapies (Romagnoli et al., 2010; Vincenzi et al., 2014; Vincenzi et al., 2016). Positive allosteric modulators are an attractive concept in drug targeting because of their potential advantages over conventional agonists. In particular, since Positive allosteric modulators are able to enhance the effect of endogenous agonists, they are expected to have a much lower side effect potential than orthosteric agonists, a low propensity for receptor desensitization and a high receptor subtype selectivity. TRR 469 is one of the most potent Positive allosteric modulators for A_1ARs so far synthetized. In our previous work, it has been observed the anti-nociceptive effects of TRR 469 in two models of acute pain and in chronic streptozotocin-induced diabetic neuropathy with effects comparable to those of the reference analgesic morphine. In contrast to the A_1AR agonist CCPA, TRR 469 did not show locomotor or cataleptic side effects (Vincenzi et al., 2014). From these encouraging results, in a subsequent work it has been investigated the potential anxiolytic-like activity of TRR 469 in mice (Vincenzi et al., 2016). In classical behavioral tests, TRR 469 exhibited robust anxiolytic-like effects comparable to those of benzodiazepine diazepam, which was used as a positive control. In membranes obtained from the different mouse brain regions, it has been shown that TRR 469 was able to greatly increase the affinity of the adenosine analogue CCPA (Vincenzi et al., 2016). This result is particularly important because one of the great advantages of positive allosteric modulators

is their ability to increase endogenous agonist affinity, enhancing the activation of the receptor in a more physiological way. On this background, we tested the effect of TRR 469 in the *in vitro* model of glutamate cytotoxicity. Very promising results of the present study revealed that TRR 469 is able to completely protect PC12 cells from the cytotoxic effect of excessive glutamate concentrations. The effect of TRR 469 was concentration-dependent and mediated by the activation of A_1ARs by endogenous adenosine as demonstrated by the use of the selective antagonist DPCPX that abolished TRR 469 cytoprotection. To further corroborate the enhanced activation of A_1ARs by endogenous adenosine in the presence of TRR 469, the affinity of adenosine was investigated in mouse cortex membranes. TRR 469 was able to induce a 33-fold increase of adenosine affinity, suggesting its capability to enhance A_1AR-mediated adenosine protective effects. Since activation of caspase 3 and 7 is a typical hallmark of glutamate-induced cytotoxicity, the reduction of their activation by TRR 469 further confirmed the cytoprotective action of the A_1AR positive allosteric modulators.

Therefore, the present work proposes the A_1AR positive allosteric modulator TRR 469 as a potentially attractive therapeutic tool in the treatment of cerebral ischemia, by reducing glutamate excitotoxicity. This strategy could allow enhancing the protective effects of endogenous adenosine through A_1ARs activation possibly avoiding the issue related to the use of A_1AR agonist.

General conclusions

Cerebral ischemic stroke represents a life-threatening neurological disorder. It is the second leading cause of death and the most common cause of adult long-term disability in developed countries. According to the World Health Organization, 15 million people suffer strokes worldwide each year. Of these, 5 million dies and another 5 million are permanently disabled. Europe averages approximately 650,000 stroke deaths each year. Until now, there is no promising pharmacotherapy for acute ischemic stroke, apart from systemic thrombolysis by using plasminogen activators, acetylsalicylic acid, heparin, warfarin, and mechanic recanalization. ARs could be interesting targets for therapeutic implementation in the treatment of the pathology because extracellular adenosine concentration increases drastically after ischemia. Since ARs are present both on CNS cells and on immune blood cells, they exert important roles during ischemia. Adenosine exerts clear neuroprotective effects through A_1ARs; nevertheless, the use of selective A_1AR agonists is hampered by their peripheral side effects. Different studies suggest that A_{2A}AR antagonists give protection centrally by reducing excitotoxicity, while A_{2A}AR agonists, and possibly also A_{2B}ARs, and A_3ARs provide protection through the control of neuro-inflammation occurring in the hours and days after brain ischemia.

The aim of the first part of the present study was to assess the presence of ARs in lymphocytes from ischemic patients compared to healthy subjects. Noteworthy, only A_{2A}ARs were significantly increased in patients while no differences were found for A_1, A_{2B} or A_3AARs. Until now, there were no studies in the literature reporting the presence of ARs in lymphocytes from ischemic stroke patients. The role of A_{2A}ARs in ischemic stroke is controversial probably due to their dual role: in a first phase of ischemia, it potentiates excitotoxicity, while hours and days after ischemia, A_{2A}ARs provide protracted protection by controlling massive blood cell infiltration.

The present study reports, for the first time, the presence of an A_{2A}ARs up-regulation in lymphocytes obtained from ischemia patients suggesting the involvement of A_{2A}ARs in this pathology. Moreover, it has been observed a significant increase of S100-β serum levels in patients affected by ischemia in comparison to control subjects. Serum S100-β seems to have a good correlation with severity, volume of brain damage and outcome in stroke. Other studies are needed to verify if there is a correlation between S100-β and A_{2A}AR levels or if the modulation of A_{2A}ARs is linked to changes in S100-β levels.

The second part of the study aimed to better clarify the role of adenosine and ARs in ischemia, especially in an *in vitro* model of cytotoxicity induced by glutamate. It is well known that one of the most important consequences of ischemic injury is the rapid and dramatic increase in glutamate concentration. This further compromises neuronal functions causing cell death through the activation of NMDA channels. The results of this study highlight a role of adenosine in glutamate cytotoxicity. In particular, adenosine facilitates glutamate injury through the activation of A_{2A} and A_{2B}ARs, as demonstrated by the decrease of apoptotic levels caused by the selective blockade of these receptor subtypes. A_1ARs selective blockade further increased glutamate-induced apoptosis, suggesting a protective role of A_1ARs. Nonetheless, the well-known A_1ARs agonist, CCPA, was not able to protect cells from glutamate damage. In addition, despite their promising therapeutic potential, the use of A_1AR agonists has been hampered by numerous side effects, poor receptor subtype selectivity, and receptor desensitization.

To overcome these problems, in our laboratory a series of A_1AR positive allosteric modulators have been developed as an alternative strategy to exploit the protective effect of adenosine acting on A_1ARs. The complete prevention of apoptosis in an *in vitro* model of glutamate cytotoxicity in PC12 cells suggests that the positive allosteric modulator TRR 469 could be considered a potential therapeutic agent against excitotoxicity, one of primary causes of brain damage after ischemic stroke.

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